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In Vitro Characterization of MSH2-MSH6 and MLH1-PMS2 interactions

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Master in Science Thesis

In Vitro Characterization of MSH2-MSH6 and MLH1-PMS2 interactions

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2016

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Finally, I would like to acknowledge my family and friends for their continued belief in me. Thank you for showing interest in my research even when you had no idea what I was saying.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATPase	ATP hydrolysis
bp	basepair
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
DTT	dithiothreitol
IDL	insertion/deletion loop
LS	Lynch syndrome
µL	microliter
mM	millimolar
µM	micromolar
min	minutes
MSI	microsatellite instability
MMR	mismatch repair
MSH	MutS homolog
MLH	MutL homolog
ssDNA	single-stranded DNA
WT	wild-type

ABSTRACT

Through evolution, organisms have developed many mechanisms to maintain genomic integrity. The DNA mismatch repair (MMR) pathway is one of such surveillance mechanisms. MMR acts to repair single base-pair mismatches and insertion/deletion loops. After their recognition by MSH2-MSH6, followed by their removal through the combined action of MSH2-MSH6 and MLH1-PMS2, MMR prevents mutations from becoming permanent and eventually leading to the development of cancer. In the event of MMR deficiency, unrepaired errors might lead to the altered function of oncogenes and deactivation of tumor suppressor genes. Under the right conditions, the resulting mutations can serve as a survival and/or growth advantage leading to tumorigenesis. Deficiencies in MMR are responsible for the most common form of hereditary cancer predisposition syndrome – Lynch syndrome (LS). Patients with this syndrome are predisposed to the development of mostly colorectal cancers at an early age (40s instead of 60s).

Previous studies have provided us with a lot of information regarding the mismatch recognition by MSH2-MSH6, but there is limited understanding of the nature of the interaction between MSH2-MSH6 and MLH1-PMS2 for downstream signaling for mismatch removal. Three interaction models have been proposed to explain how this signaling for excision occurs. The first model states that MSH2-MSH6 recognizes the mismatch and in the presence of ATP forms a sliding clamp that interacts with a single MLH1-PMS2. This quaternary structure functions as a unit to signal excision. The second model proposes that MMR complexes interact in a 1:1 ratio and remain at the mismatch. This causes the DNA to bend which results in stimulation of downstream signaling for excision. A third model states that once MSH2-MSH6 recognizes the mismatch it acts as a beacon that signals for recruitment of multiple MLH1-PMS2 heterodimers that polymerize on DNA and function as the signal for downstream excision.

In this study we aimed to explore the hypothesis that the two protein heterodimers (MSH2-MSH6 and MLH1-PMS2) form a stable quaternary structure that functions as a unit to signal excision. DNA/protein pulldowns followed by Western blot analysis were used as the main experimental approach. Our results suggest that the two protein heterodimers do not function as a unit. Instead we find that MLH1-PMS2, though dependent on MSH2-MSH6 for association with DNA, remains stable for longer on DNA than MSH2-MSH6. This result suggests that these MMR heterodimers may have distinct functions involving MLH1-PMS2 being loaded directly onto the DNA.

Chapter I - Introduction

Lynch Syndrome

On a daily basis, living organisms are exposed to agents that do damage to their genetic material leading to mutations. Under normal circumstances, there is a spontaneous mutation rate of approximately $1x10^{-10}$ base pairs changed per replication event. This mutation rate is in part due to mistakes made by the DNA polymerase. The intrinsic proofreading activity of DNA polymerase allows it to correct some mistakes, and organisms have developed highly conserved surveillance mechanisms that can address errors that evade this fidelity (Lyer et al 2006). A deficiency in any of these surveillance DNA repair mechanisms is associated with the development of many diseases such as cancer.

Lynch syndrome (LS) is one such disease, accounting for 2-7% of all colorectal carcinomas (Walsh 2015). LS is an autosomal dominant disease that predisposes patients to a 80-90% increased risk of developing colonic as well as extracolonic cancers including endometrial, ovarian, pancreatic and gastric (Walsh 2015; Sijmons et al 2016; Goyal et al 2016). Spanning a wide spectrum of phenotypes, these cancers share an increased tumorigenic progression rate when compared to sporadic cancers. Diagnosis relies heavily on tumor immunohistochemistry for MMR protein levels, mutational analysis and microsatellite instability (MSI) testing. MSI, a nucleotide level genomic instability, is considered the hallmark of MMR deficiency. Once a family member is identified with LS, it is recommended that other family members go through frequent and early cancer screenings. Specifically, LS associated cancers develop due to the inheritance of mutations in the DNA mismatch repair (MMR) genes. Deficiency in the MMR pathway leads to MSI and eventual inactivation or altered function of oncogenes and tumor suppressor genes (Walsh 2015).

Patients with LS will inherit one mutated allele of one of the MMR genes, and at some point in their lifetime will lose the remaining WT copy. This may happen through loss of heterozygosity, second hit mutation or methylation (Goyal et al 2016). Unlike sporadic cancers, which have an average onset of 60 years of age, the hereditary nature of LS-associated cancers together with its mutator phenotype give it an early age of onset. In the event that an LS association is identified, the recommended course of action is surgery and cautious selection of a chemotherapeutic agent. Aside from displaying a higher rate of tumorigenesis, LS tumors have been identified as resistant to some chemotherapy; for instance, methylating agents such as *N*-methyl-*N'*-nitro-nitrosoguanidine (MNNG). MNNG relies on the processing of O^6-methylguanine DNA modifications to kill the cancer cells. In MMR proficient cells, MMR processes the resulting O^6-methylguanine lesions generated by the methylating agents leading to the formation of highly toxic double strand breaks. On the other hand, in non-proficient cells (MMR-), these lesions are no longer toxic and become instead very mutagenic. In some situations this resistance may provide MMR defficient cells with a survival advantage (Wojciechowicz et al 2014). Novel therapies that exploit DNA MMR deficiency are being pursued. Examples of such are Immune checkpoint blockade agents such as PD-1 (programmed death-1) inhibitors. These inhibitors prevent the interaction between PD-1 and PD-L1 (programmed death-1 ligand), therefore facilitating the killing of tumor cells by activated T cells (Goyal et al 2016).

Mismatch Repair Mechanism

MMR maintains genomic stability by recognizing and coordinating the repair of single base-pair mismatches and small insertion/deletion loops (IDLs) that occur due to normal

replication (Jiricny 2006). Its ability to repair mistakes that escape proofreading elevates replication fidelity and, therefore, genetic integrity by 50-1000 fold (Lyer et al 2006). There are four main genes responsible for the function of MMR: *MSH2*, *MSH6*, *MLH1* and *PMS2*. The majority of germline mutations found in LS patients are truncations found in the major MMR genes: *MSH2* (34%) and *MLH1* (40%). To a lesser extent, mutations are also found in the minor MMR genes - *MSH6* (18%) and *PMS2* (8%) (Woods et al 2007; Heinen et al 2011; Peltomaki 2016). The lifetime risk of developing cancers is higher for carriers of *MSH2* or *MLH1* mutations than for carriers of *MSH6* or *PMS2* mutations (Peltomaki 2006). Other MMR components include PCNA, RFC, RPA, Exonuclease I (EXO I), POL δ and DNA Ligase I (Zhang et al 2005; Kunkel et al 2005; Dzantiev et al 2004).

The function of the proteins encoded by the MMR genes is highly dependent on their ATPase activity (Alani et al 1997; Gradia et al 1997). The MSH2 and MSH6 proteins form an obligate heterodimer that in its ADP-bound conformation recognizes mismatches as well as small IDLs on DNA (Martín-López et al 2013). The mechanism of strand discrimination for the strand containing the incorrect base is not fully understood in Eukaryotes (Walsh 2015). In Gram-negative bacteria, in which the pathway is better understood, the strand discrimination signal is provided by the transient unmethylated status of the daughter strand. There is a lag in time between replication and methylation of the newly synthesized strand (Schofield et al 2001). For Eukaryotes, this discrimination signal is not conserved. It is instead believed that a strand discontinuity marks the nascent strand (Lyer et al 2006). Once MSH2-MSH6 is bound to DNA, there is an ADP to ATP shift that results in a conformational change. Through this change, MSH2-MSH6 forms a sliding clamp that is free to diffuse along the DNA and away from the mismatch in an ATP-hydrolysis independent manner. At this point the mismatch is once again

free to be recognized by subsequent MSH2-MSH6 heterodimers (Gradia et al 1999; Heinen et al 2002). It is thought that the MSH2-MSH6 sliding clamp recruits a heterodimer of MLH1 and PMS2. Together, they signal the recruitment of EXO I, which can remove the erroneous region on the daughter strand in a 5'→3' manner (Martín-López et al 2013; Constantin et al 2005; Fukui et al, 2010). Despite this 5'→3' activity, processing may occur 5'→3' as well as 3'→5' from the nick. This is possible because of the endonuclease activity of PMS2. By nicking the DNA, PMS2 creates a new point of entry for EXO I which allows it to process 3' nick-directed MMR. This endonuclease activity of MLH1-PMS2 is also important for the restriction of the excision activity of EXO I as it is thought to modulate the distance away from the mismatch that is removed (Kadyrov et al 2007). Once the error containing region has been removed, DNA polymerase re-synthesizes the region and DNA Ligase I seals it shut (Figure I-1).

Figure I-1

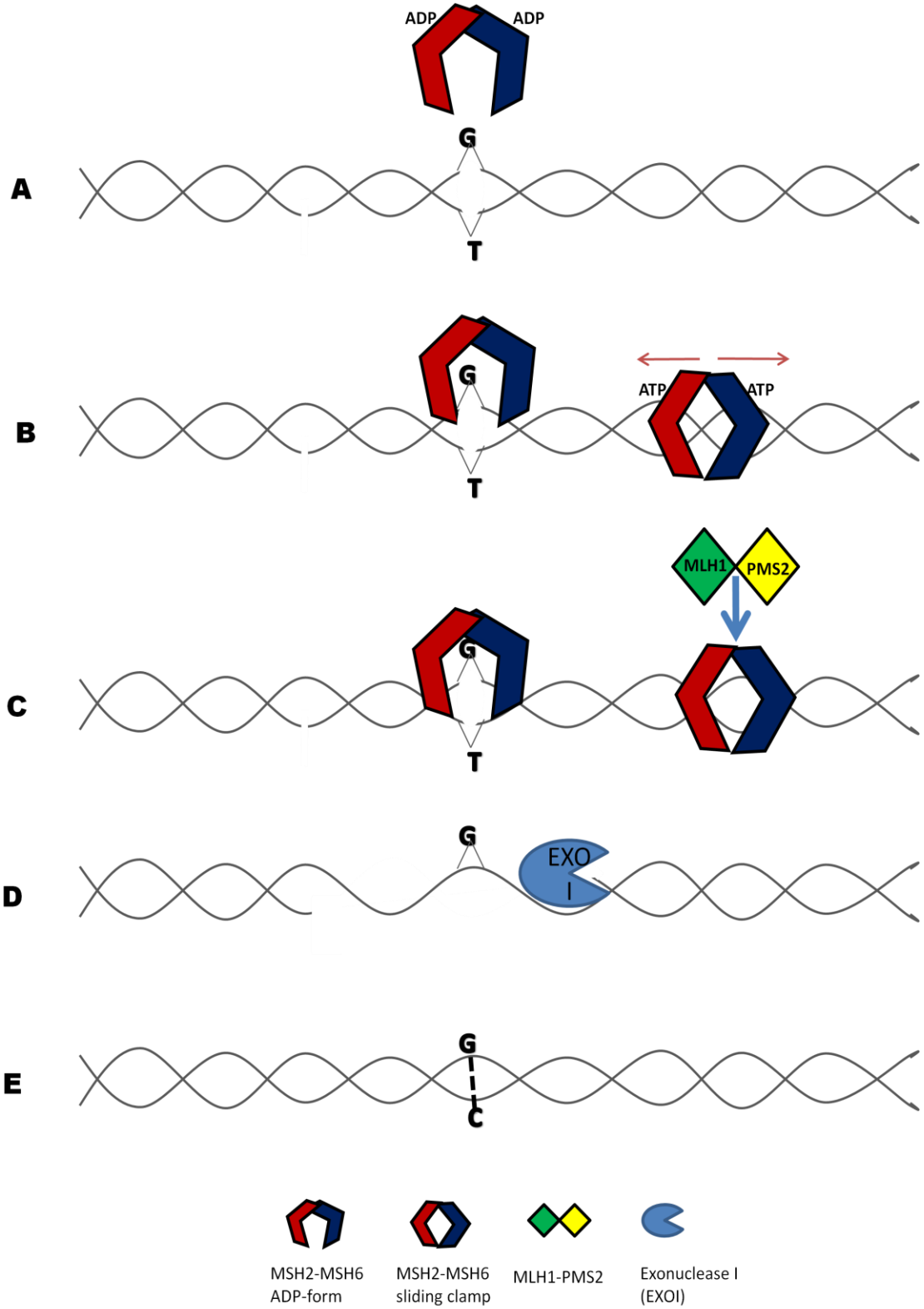

ADP
ADP
G
T
A
G
T
ATP
ATP
B
MLH1
PMS2
G
T
C
G
EXO
I
D
G
C
E
MSH2-MSH6
ADP-form
MSH2-MSH6
sliding clamp
MLH1-PMS2
Exonuclease I
(EXOI)

Figure I-1. MMR Mechanism. A. MSH2-MSH6 recognizes mismatch (G/T) in ADP-bound form. **B.** ADP to ATP shift result in conformational change- sliding clamp. Mismatch is then available to be recognized again by ADP-bound MSH2-MSH6. **C.** MSH2-MSH6 sliding clamp recruits MLH1-PMS2. **D.** Exonuclease I is recruited to remove strand containing incorrect base. **E.** Gap is re-synthesized by DNA Polymerase and ligated by DNA Ligase I.

There is abundant information regarding the recognition of the mismatch by MSH2-MSH6, but the nature of the interaction between MSH2-MSH6 and MLH1-PMS2 to signal for EXO I (excision) and repair remains elusive and controversial. Three main models aim to answer this question.

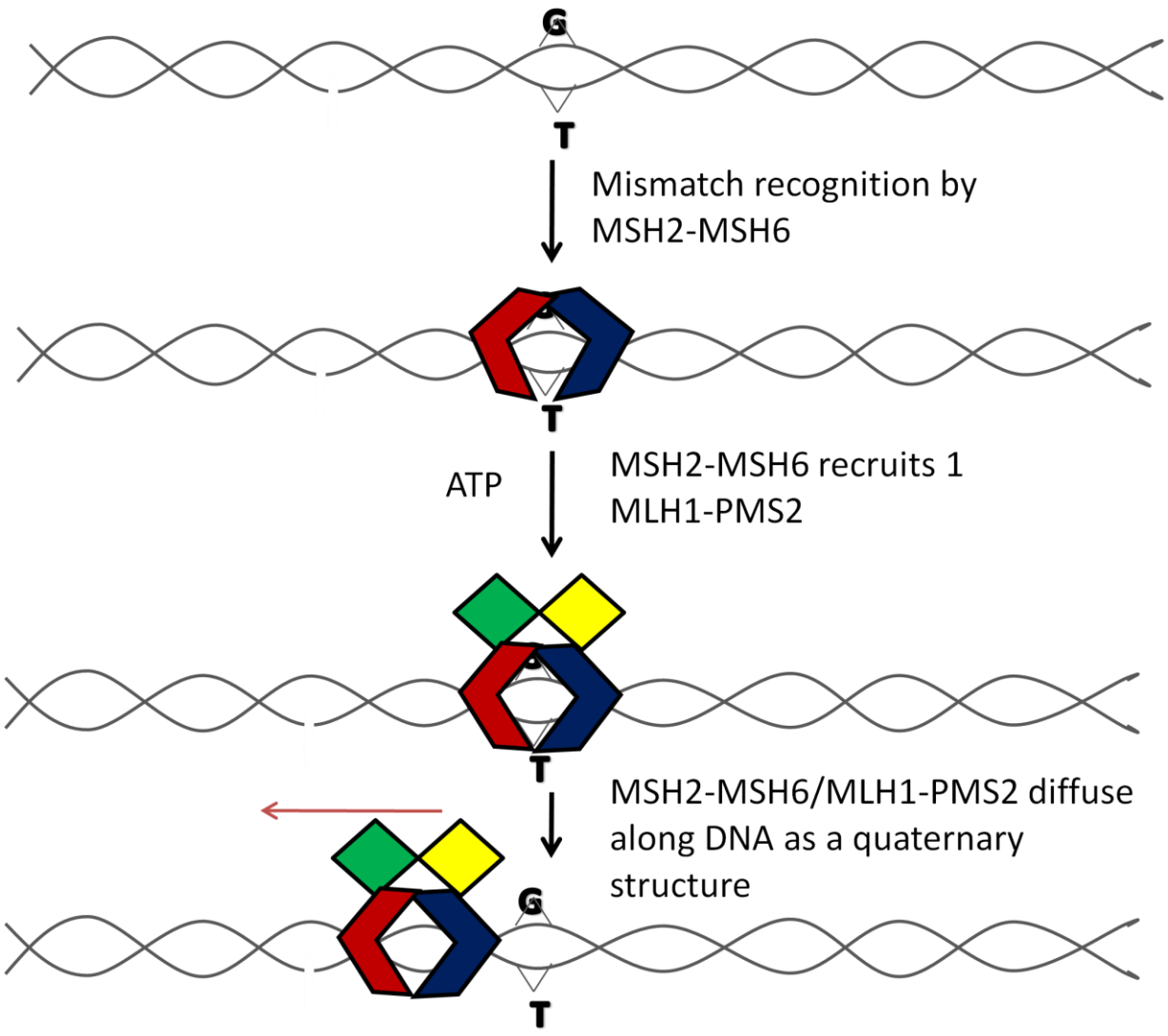
The first model proposes that MSH2-MSH6 interacts with MLH1-PMS2 in 1:1 stoichiometry to form a complex. MSH2-MSH6 recognizes the mismatch and in the presence of ATP recruits and interacts with a single MLH1-PMS2 (Lyer et al 2006) (Figure I-2A). In one study, using single-molecule techniques, the authors support this model by showing that quantum-dot tagged MSH2-MSH6 and MLH1-PMS2 only co-localize on mismatched DNA when MSH2-MSH6 is bound to the mismatch. It has also been shown that once this MSH2-MSH6/MLH-PMS2 complex is formed, ATP allows the complex to diffuse away from the mismatch (Gorman et al 2012; Allen et al 1997). In this manner a quaternary structure forms and functions as a unit to recruit EXO I.

A second model suggests that MSH2-MSH6 and MLH1-PMS2 remain at the mismatch and that DNA bending stimulates downstream signaling for excision (Lyer et al 2006; Qiu et al 2015; Junop et al 2001) (Figure I-2B). This model further proposes that the ATPase activity of MSH2-MSH6 serves as a proofreading tool to verify the mismatch (Junop et al 2001). Support for this model comes in the form of studies identifying that the association between MSH2-MSH6 and MLH1-PMS2 hinders dissociation from the mismatch (Schofield et al 2001; Erie et al 2014). Also, experiments that place a physical barrier (i.e. biotin-streptavidin) have shown that the barrier has no significant effect on excision (Wang et al 2004). The complex remains, then, at the mismatch and bending of DNA brings important players into close proximity so that signal propagation is possible.

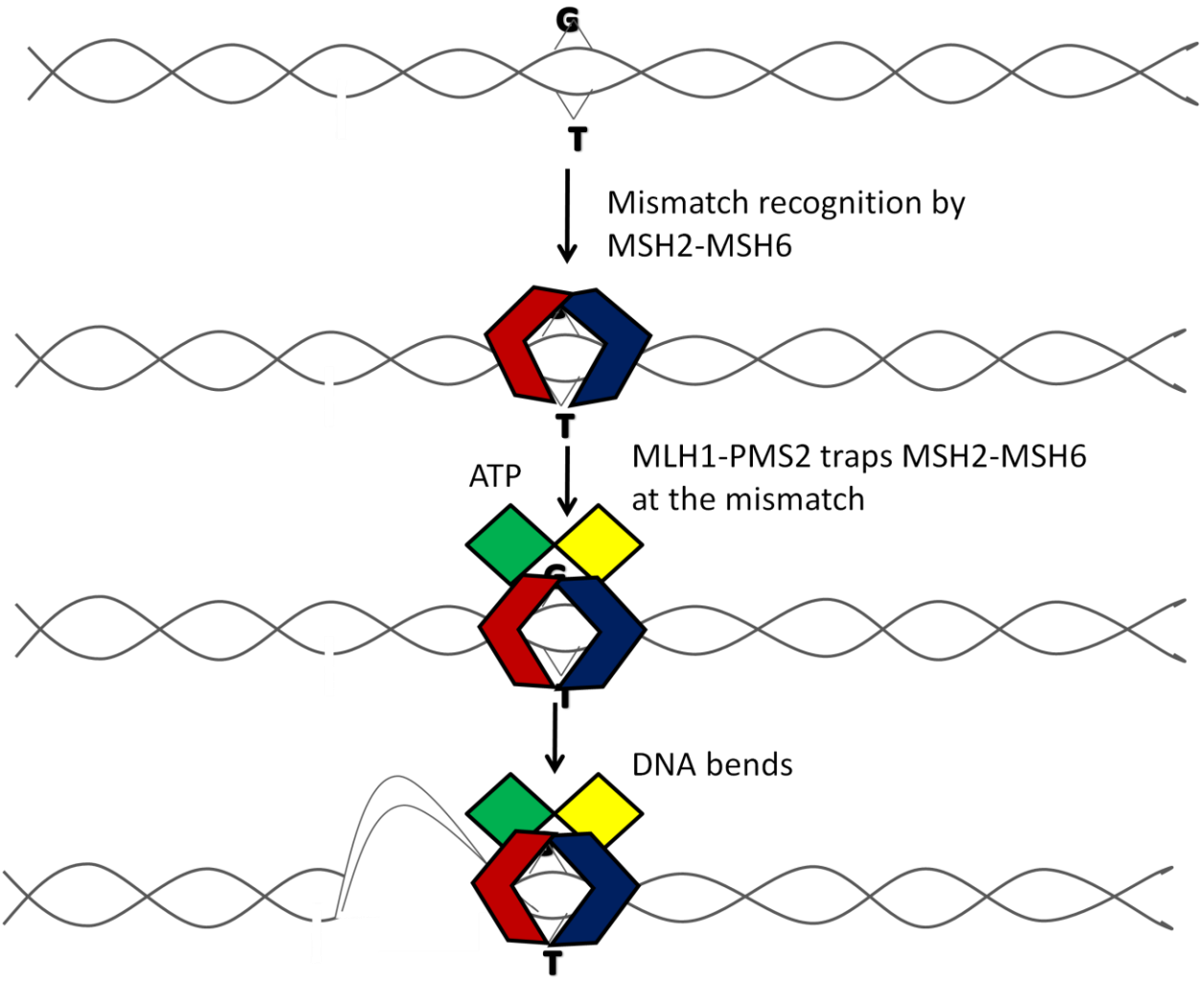
The third model stipulates that once the mismatch is recognized by MSH2-MSH6, it acts as a polymerization signal for MLH1-PMS2 on the DNA (Figure I-2C). This model is supported by findings that MLH1-PMS1 (yeast) foci, though dependent on MSH2-MSH6 for formation, rarely co-localize with MSH2-MSH6 (Hombaeur et al 2011). In addition, it has been found that MLH1-PMS2 is present at much higher stoichiometry (ie. 1 MSH2-MSH6 to 3 MLH1-PMS2) (Qiu et al 2015). Studies have also found that MLH1-PMS2 has DNA binding properties that are independent of MSH2-MSH6 at low salt concentrations (Drotschmann et al 2002; Plys et al 2012). Extensive research has been done to determine the model that best explains how the MMR protein complexes interact for downstream signaling, but there are still weaknesses in all models. Determining the best suited model will require further work and the consistent use of physiologic conditions.

Figure I-2

A


G
T
Mismatch recognition by
MSH2-MSH6
T
ATP
MSH2-MSH6 recruits 1
MLH1-PMS2
T
MSH2-MSH6/MLH1-PMS2 diffuse
along DNA as a quaternary
structure
G
T

B


G
T
Mismatch recognition by
MSH2-MSH6
T
ATP
MLH1-PMS2 traps MSH2-MSH6
at the mismatch
G
T
DNA bends
T

C

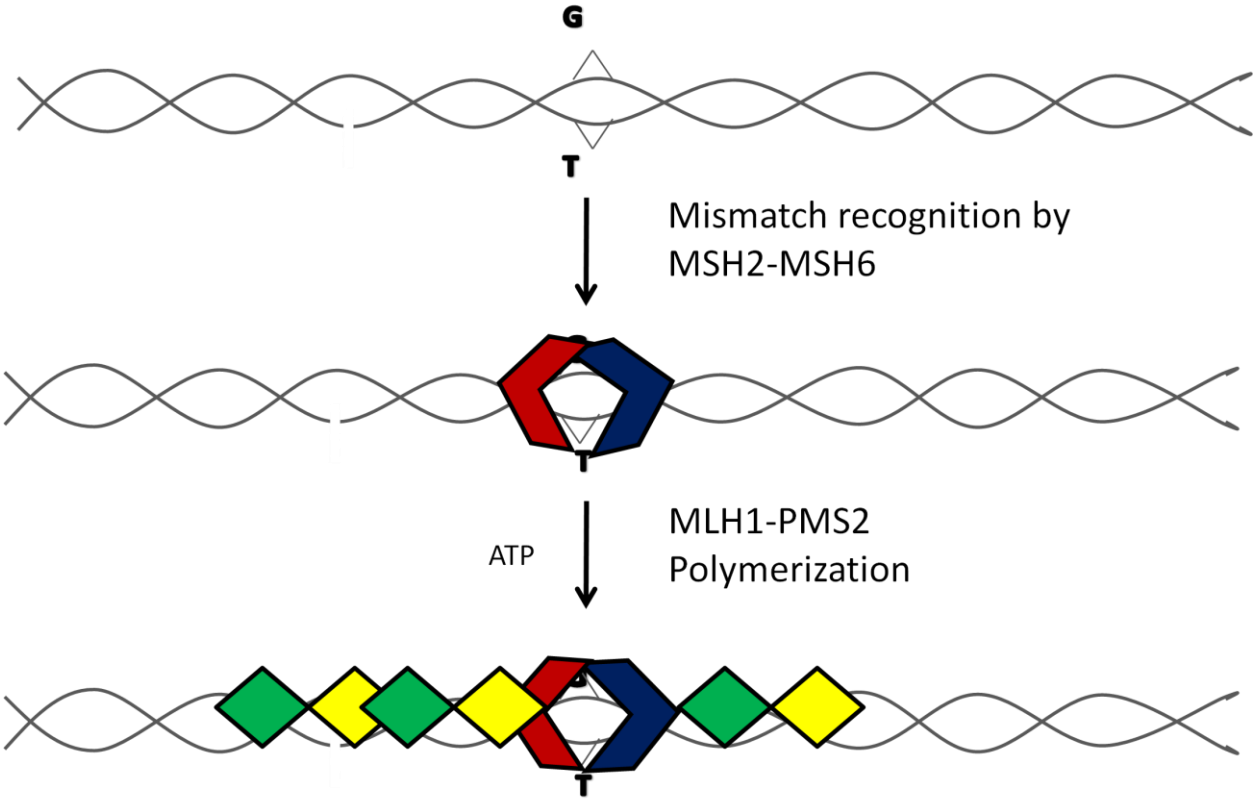

G
T
Mismatch recognition by
MSH2-MSH6
T
ATP
MLH1-PMS2
Polymerization
T

Figure I-2. Models of MMR protein interaction for excision control. A. MSH2-MSH6 recognizes the mismatch and in an ATP dependent manner it recruits a MLH1-PMS2 heterodimer. **B.** MLH1-PMS2 traps MSH2-MSH6 at the mismatch and their interaction results in the bending of the DNA. **C.** MSH2-MSH6 recognizes the mismatch and once bound it serves as a polymerization signal for MLH1-PMS2 heterodimers.

The goal of this project was to better understand the nature of the interaction between MSH2-MSH6 and MLH1-PMS2. More specifically, we aimed to discern between a MMR model that proposes that MSH2-MSH6 and MLH1-PMS2 function as a unit and one that suggests that MSH2-MSH6 interacts with MLH1-PMS2 and loads it directly onto DNA. To achieve this goal we took advantage of protein pulldowns using biotinylated mismatch-containing DNA as a substrate and WT and missense mutants of MSH2-MSH6 (MSH2wt-MSH6G566R).

Chapter 2

Introduction

Organisms are constantly exposed to agents that damage their genetic material leading to mutations. Spontaneously, the normal mutation rate is approximately $1x10^{-10}$ mutations per replication event. This spontaneity is in part due to DNA Polymerase errors. The intrinsic proofreading activity of DNA Polymerase allows it to correct some mistakes and organisms have developed highly conserved surveillance mechanisms that can address errors that evade this fidelity (Lyer et al 2006). A deficiency in any of these surveillance DNA repair mechanisms is associated with the development of diseases such as Lynch syndrome (LS).

LS is an autosomal dominant disease that increases the lifetime risk (80-90%) of developing predominantly colonic cancers at an early age. These cancers develop due to the inheritance of mutations in the DNA mismatch repair (MMR) genes. In addition, the loss of MMR function through somatic mutations is also present in a subset of sporadic cancers (Sijmons et al 2016). MMR maintains genomic stability by recognizing and coordinating the repair of single base-pair mismatches and small insertion/deletion loops (IDLs) that occur due to normal replication (Jiricny 2006). Its ability to repair mistakes that escape proofreading elevates replication fidelity and, therefore, genetic integrity by 50-1000 fold. Four main genes are responsible for the function of MMR: *MSH2*, *MSH6*, *MLH1* and *PMS2*. The majority of mutations that inactivate this pathway can be found in the *MSH2* (40%) and *MLH1* (50%) genes (Walsh 2015; Goyal et al 2016; Lyer et al 2006).

The function of the proteins encoded by the MMR genes is highly dependent on their DNA mismatch stimulated ATPase activity (Alani et al 1997; Gradia e al 1997). The MSH2 and MSH6 proteins form an obligate heterodimer that in its ADP-bound conformation recognizes

mismatches as well as small IDLs on DNA (Martin-Lopez et al 2013). In Eukaryotes, the general consensus is that a strand discontinuity marks the nascent strand (Walsh 2015; Schofield et al 2001). This discontinuity can result from normal DNA processing such as the Okazaki fragments in the lagging strand replication (Lyer et al 2006). Once MSH2-MSH6 is bound to DNA, in the presence of ATP, there is an ADP to ATP exchange that results in a conformational change. Through this change, MSH2-MSH6 forms a sliding clamp that is free to diffuse along the DNA and away from the mismatch in an ATP-hydrolysis independent manner. At this point the mismatch is once again free to be recognized by a subsequent MSH2-MSH6 heterodimer (Gradia et al 1999; Heinen et al 2002). In this ATP bound form, MSH2-MSH6 recruits a heterodimer of MLH1 and PMS2. Together, they signal the recruitment of Exonuclease I (EXO I) which can remove the erroneous region on the daughter strand in a 5'-3' manner (Constantin et al 2005; Fukui et al 2010). In the 3' directed MMR the endonuclease activity of PMS2 becomes essential for excision as it creates a point of entry for EXO I. This endonuclease activity of MLH1-PMS2 is also important for the restriction of the excision activity of EXO I as it is thought to modulate the distance away from the mismatch that is removed (Kadyrov et al 2007). Once the error containing region has been removed, DNA polymerase once again re-synthesizes the regions and DNA Ligase I ligates it.

There is abundant information regarding the recognition of the mismatch by MSH2-MSH6, but the nature of the interaction between MSH2-MSH6 and MLH1-PMS2 to signal for EXO I and repair remains elusive and controversial. Attempts to answer this question have given rise to three main models. The first model states that MSH2-MSH6 recognizes the mismatch and in the presence of ATP recruits and interacts with a single MLH1-PMS2. This quaternary structure functions then as a unit to signal excision (Gorman et al 2012). The second model

proposes that MMR complexes interact in a 1:1 ratio and remain at the mismatch. This causes the DNA to bend which results in stimulation of downstream signaling for excision (Qiu et al 2015; Junop et al 2001). A third model states that once MSH2-MSH6 recognizes the mismatch it acts as a beacon that signals for recruitment of multiple MLH1-PMS2 heterodimers (Hombaeur et al 2011; Qiu et al 2015). This polymerization functions as the signal for downstream excision. (Lyer et al 2016)

Despite the proposed models, it has been difficult to definitely select one as the best fit to explain the MMR protein interaction in Eukaryotes for downstream signal. Here, we used protein pulldowns to examine the nature of the interaction between MSH2-MSH6 and MLH1-PMS2 and determine the validity of the quaternary structure model. To this end, we made recombinant wild-type and mutant MSH2-MSH6 proteins as well as MLH1-PMS2.

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Materials and methods

DNA substrates - 60 base-pair oligonucleotides with 5' Biotin (btn) and 5' Digoxigenin (dig) modifications were synthesized by Integrated DNA Technologies using sequences from the pBSK plasmid. Complementary oligos were then annealed to oligos that differed at one nucleotide in order to make heteroduplex (btnG/digT, btnG/T) dsDNA or were perfectly complementary to generate homoduplex (btnG/digC) dsDNA.

The 255 bp heteroduplex (btnG/digT, btnG/T) and the homoduplex (btnG/digC) were PCR-derived substrates using a pBSK-GC and pBSK-AT plasmid as a template DNA and were prepared as follows. Biotinylated C-strand was prepared through the amplification of a 255bp region using a reverse 5'-btn primer (5'Biosg CGCGAATTTTAACAAAATATTAACGC 3') and a forward 5'-Phosphorylated primer (5'- Phos GCGTCGATTTTTGTGATGCTC 3'). Dig modified G-strand was prepared using 5'Dig forward primer (5'DigN GCGTCGATTTTTGTGATGCTC 3') and 5'-phos reverse primer (5'- Phos GCGTCGATTTTTGTGATGCTC 3'). Unmodified G-strand was made following PCR amplification procedure using a forward primer (5'GCGTCGATTTTTGTGATGCTC 3') and a reverse primer (5'- Phos GCGTCGATTTTTGTGATGCTC 3'). Biotin modified T-strand was prepared using 5'phosphorylated forward primer (5'- Phos GCGTCGATTTTTGTGATGCTC 3') and 5'-biotin reverse primer (5'Biosg CGCGAATTTTAACAAAATATTAACGC 3'). PFU Ultra Polymerase AD was used for all amplification. Following amplification, dsDNA was digested using lambda (λ) exonuclease (New England Biolabs). Desired single- stranded DNAs were mixed together and annealed to make dsDNA. Final product was purified using Centri-Sep spin columns (Princeton Separations).

Protein purification – Recombinant MSH2-MSH6 and MLH1-PMS2 were expressed in insect cells using the baculovirus system as described previously (Geng et al., 2011, Analytical Biochemistry). Purification was performed using a 6 ml Resource Q anion exchange column (GE Healthcare), 1 ml HiTrap Heparin affinity column (GE Healthcare) and HiLoad 16/60 Superdex 200 sizing column (GE Healthcare) as previously described (Geng et al 2011). Wild type and mutant MSH2-MSH6 ($MSH2^{wt}$-$MSH6^{G566R}$) were eluted in buffer A (25Mm Hepes, pH 7.5; 0.1 Mm EDTA; 10% glycerol; 1mM DTT; Pepstatin and Leupeptin; 0.1% PMSF) with 300 mM KCl. MLH1-PMS2 was eluted in buffer A with 200 mM KCl. The concentrations were determined by absorbance at 280 nm.

$MSH2^{wt}$-$MSH6^{G566R}$ was generated by removing the His-tag from a previously described (Cyr et al 2008) HIS-$MSH2^{wt}$-$MSH6^{G566R}$ mutant using restriction enzyme double digestion.

Protein Pulldown - 60bp or 255bp DNA(btnG/digT, btnG/T and btnG/digC) was incubated with Dynabeads M-270 Streptavidin (invitrogen) for 5min followed by 2 washes with reaction buffer (25 mM Hepes pH 8.1, 110 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 2% glycerol, 0.025% Tween, 20 nM anti-Digoxigenin). Dynabeads were washed as per manufacture specifications with 2 M NaCl followed by re-suspension in 1 M Nacl (10 µl/reaction #). 0.1 µM of protein was incubated with specified DNA for 5 min at room temperature. Removal of unbound protein and final wash (2 times) with cold reaction buffer with or without 100 µM ATP was performed on ice. 1X gel loading buffer was added to Dynabeads and sample was boiled. All pulldowns were performed using a 12-slot magnetic rack. Dynabeads were removed by placing reaction tubes in magnetic rack prior to gel loading.

For stability assays, protein complexes were loaded onto DNA as described and then incubated for the indicated time points in a buffer containing a 41 bp competitor heteroduplex DNA lacking Dig and btn modifications (G/T).

Western Blotting -The resulting protein pulldown samples were separated by electrophoresis on a 6% SDS-polyacrylamide gel. Following membrane blocking with 5% milk, the primary antibodies used were: anti-MLH1 (BD Biosciences – #550838), anti-MSH2 (CAI Biochem - # NA27). Membranes were probed for visualization with Mouse 2˚ antibody (BioRad - # 170-6516).

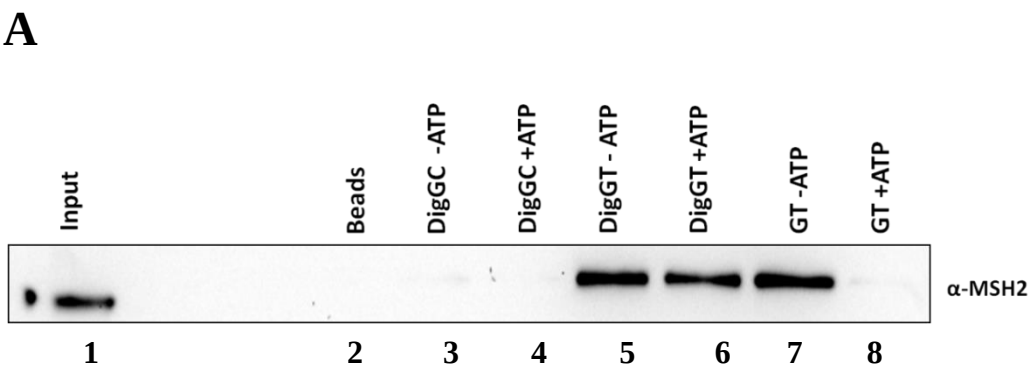
Results

MSH2-MSH6 forms a sliding clamp on mismatched DNA in the presence of ATP

Previous studies have suggested that MSH2-MSH6 undergoes a conformational change after encountering a mismatch in the presence of ATP that results in the formation of a sliding clamp (Gradia et al 1997). In order to begin characterizing the MMR protein (MSH2-MSH6/MLH1-PMS2) interaction we decided to start by looking at the dynamics of the MMR recognition complex (MSH2-MSH6). To this purpose we performed a protein pulldown in which homo- and heteroduplex DNA with or without both ends blocked was incubated with recombinant wild-type MSH2-MSH6 in the presence or absence of ATP. Western blot analysis demonstrated that MSH2-MSH6 could only be captured on unblocked hetroduplex DNA in the absence of ATP (Figure II-1A, lane 7). Once ATP was available, the complex could no longer be pulled-down (Figure II-1A, lane 8). This no longer held true when we blocked the free DNA end using an antibody against the 5' Digoxigenin DNA modification. MSH2-MSH6 could then be captured even in the presence of ATP (Figure II-1A, lane 5 and lane 6). This dynamic was only observed in a mismatch and ATP dependent manner (Figure II-1A, lane 3, 4). To further consolidate this idea of a sliding clamp forming, we next decided to look at the amount of MSH2-MSH6 heterodimer loaded onto DNA in the presence of ATP. To do this we incubated heteroduplex DNA (DigGT) with increasing concentrations of MSH2-MSH6 and looked at the protein levels bound to DNA. Western blot analysis shows more significant increasing amount of bound protein in the presence of ATP. In agreement with our evidence for a sliding clamp that moves away from the mismatch allowing other heterodimers to bind, we found twice the amount of MSH2-MSH6 clamps captured on a blocked-end 60 bp heteroduplex substrate in the presence of ATP compared to in the absence of ATP (Figure II-1B, Figure II-1C). Given that in the absence

of ATP, only one MSH2-MSH6 heterodimer should be stably bound to each mismatch, these results suggest that up to two sliding clamps can load on a 60 bp heterodimer in the presence of ATP. These results are consistent with the previously published DNA binding footprint of MSH2-MSH6 of approximately 27 nucleotides (Gradia et al. 1997). Taken all together, our results show that a MSH2-MSH6 sliding clamp does in fact form on heteroduplex DNA in the presence of ATP.

Figure II-1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Input
Beads
DigGC -ATP
DigGC +ATP
DigGT - ATP
DigGT +ATP
GT -ATP
GT +ATP
α-MSH2
1
2
3
4
5
6
7
8
B
-ATP
+ATP
Input
0μM
0.02μM
0.04μM
0.06μM
0.08μM
0.1μM
0μM
0.02μM
0.04μM
0.06μM
0.08μM
0.1μM
α -MSH2
C
Band Intensity
40000
30000
20000
10000
0
0
0.02
0.04
0.06
0.08
0.1
MSH2-MSH6 (uM)
w/ ATP
w/o ATP

Figure II-1. MSH2-MSH6 forms a sliding clamp on mismatched DNA in the presence of ATP. A. Representative western blot analysis of MSH2-MSH6 dynamics on homoduplex (DigGC) and heteroduplex (DigGT, GT) in the presence or absence of ATP. B. Titration of increasing MSH2-MSH6 concentartions onto blocked-end heteroduplex DNA. C.Band intensity quantification using ImageJ. Values from 2 independent experiments normalized using MSH2-MSH6 inputs.

MLH1-PMS2 recruited to mismatched DNA in a ATP dependent manner

Once the mismatch is recognized by MSH2-MSH6, its recruitment of MLH1-PMS2 becomes essential for regulated excision and mismatch repair (Mendillo et al 2005; Prolla et al 1994). With that in mind, we sought to determine the conditions and requirements for MLH1-PMS2 recruitment by MSH2-MSH6. Previous studies have shown that MSH2-MSH6 recruits and interacts with MLH1-PMS2 in the presence of ATP (Habraken et al 1998). We incubated DigGC and DigGT DNA substrates with both MSH2-MSH6 and MLH1-PMS2 MMR protein complexes in the presence or absence of ATP. As previously reported in the literature, we found that MLH1-PMS2 is only recruited to the DNA in the presence of a mismatch and ATP (Figure II-2, lane 2). In the absence of ATP, MSH2-MSH6 recognized the mismatch but failed to recruit MLH1-PMS2 (Figure II-2, lane 3).The necessity for ATP suggests that MLH1-PMS2 interacts with MSH2-MSH6 in its sliding clamp conformation.

Figure II-2

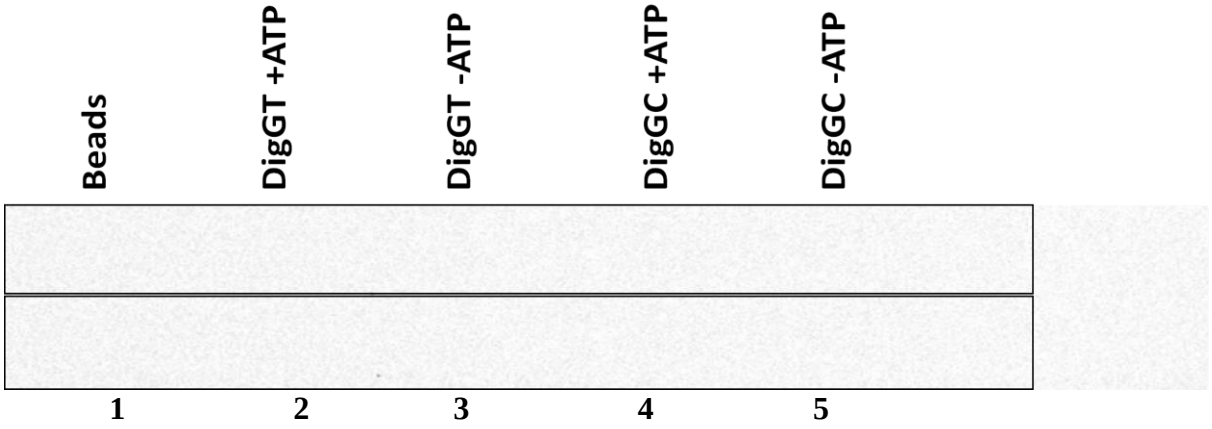


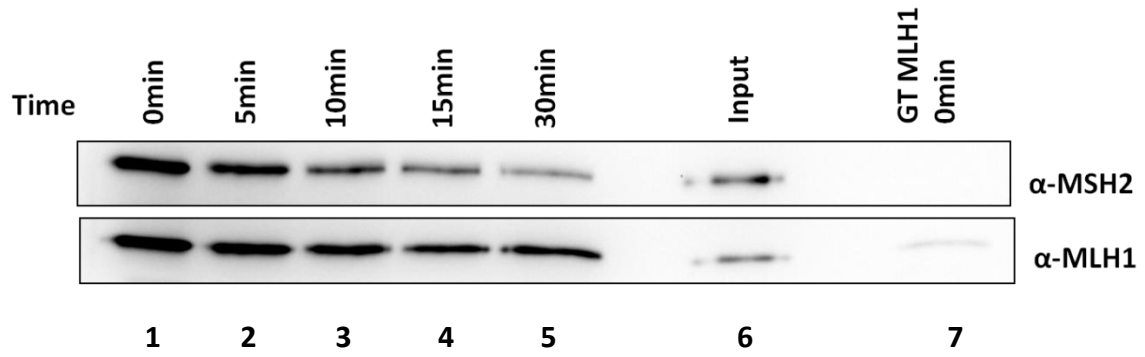
Figure II-2. MLH1-PMS2 recruited to mismatched DNA in a ATP dependent manner. Western blot analysis showing conditions for MLH1-PMS2 recruitment utilizing homoduplex and heteroduplex blocked end substrate.

MLH1-PMS2 remains stable on mismatched DNA in the absence of MSH2-MSH6

We next wanted to look at the stability of the complexes formed on heteroduplex DNA. Previous studies have suggested that a quaternary complex, composed by MSH2-MSH6 and MLH1-PMS2 (Antony et al 2004; Jiricny 2006; Schofield et al 2001), forms on the DNA and as a unit regulates the downstream excision and repair of mismatches. Thus, we predicted that the stability of MSH2-MSH6 and MLH1-PMS2 on the heteroduplex DNA should be the same, i.e., if MSH2-MSH6 dissociated from the DNA, then due to the direct interaction, so too would MLH1-PMS2. To test this, we performed a protein pulldown in which we incubated the DNA concurrently with MSH2-MSH6 and MLH1-PMS2 on blocked-end mismatched DNA in the presence of ATP. We then washed off any excess, unbound proteins and incubated the remaining DNA-protein complexes for increasing lengths of time in a reaction buffer containing ATP and a 41 bp competitor heteroduplex DNA. Western blot data showed that the two protein heterodimers have very different stabilities (Figure II-3). MLH1-PMS2 remains stably bound on the DNA even after MSH2-MSH6 falls off the DNA (Figure II-3A, lane 5 and II-3B). This data suggests that once MLH1-PMS2 is recruited to the mismatch by MSH2-MSH6 that it has an ability to bind directly to DNA. Up to now, there has been controversy regarding this proposed ability of MLH1-PMS2 to directly bind DNA. Studies supporting this idea have mostly demonstrated a mismatch independent loading under low salt conditions (Bende et al 1991; Hall et al 2001; Ban et al 1999; Groothuizen et al 2015).

Figure II-3

A



B

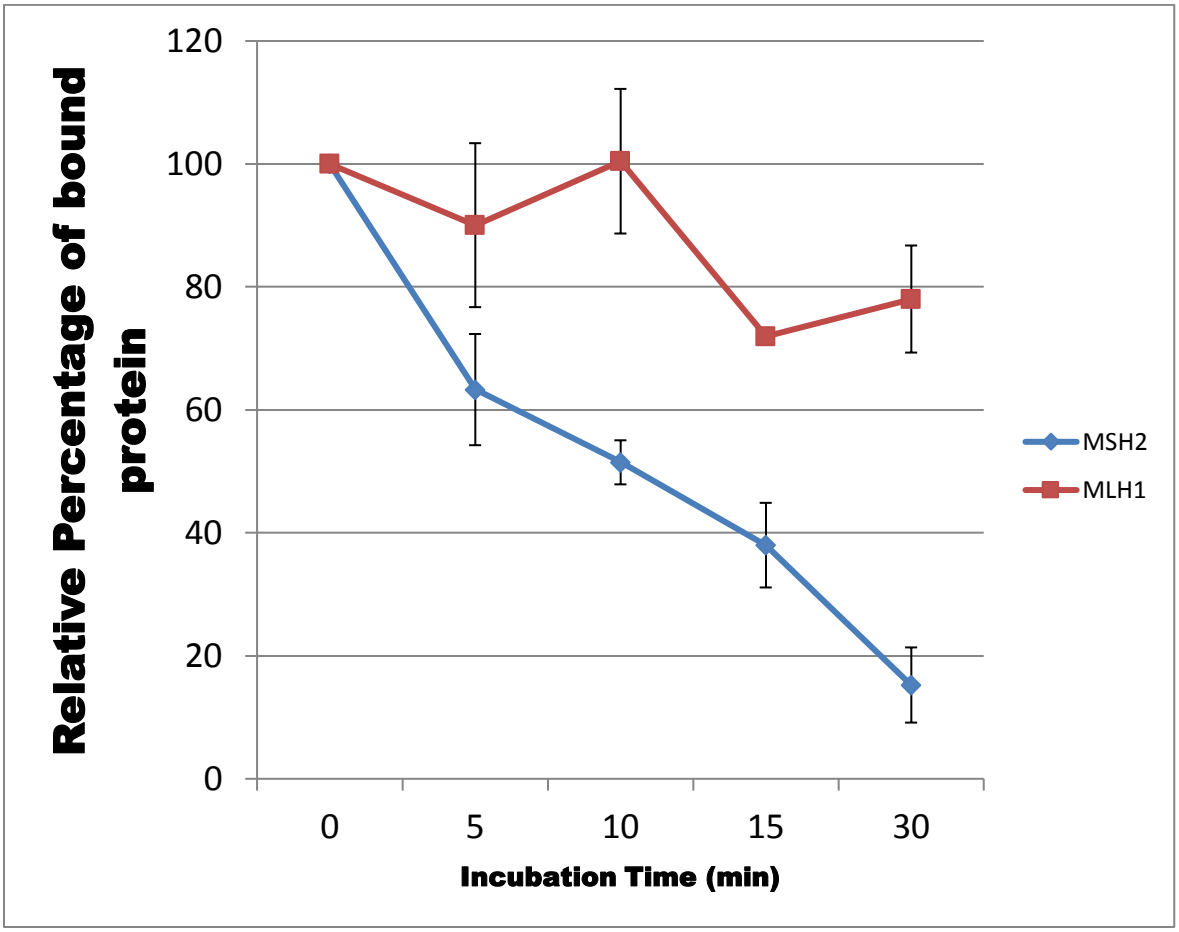


Figure II-3. MLH1-PMS2 remains stable on mismatched DNA in the absence of MSH2-MSH6. A. Western blot analysis of levels of MMR proteins loaded on DigGT DNA after incubation (0, 5, 10, 15 and 30 minutes) with a 41bp competitor DNA in the presence of ATP. B. Relative percentage of MSH2-MSH6 and MLH1-PMS2 bound to DigGT DNA. Values based on ImageJ quantification on 3 independent experiments.

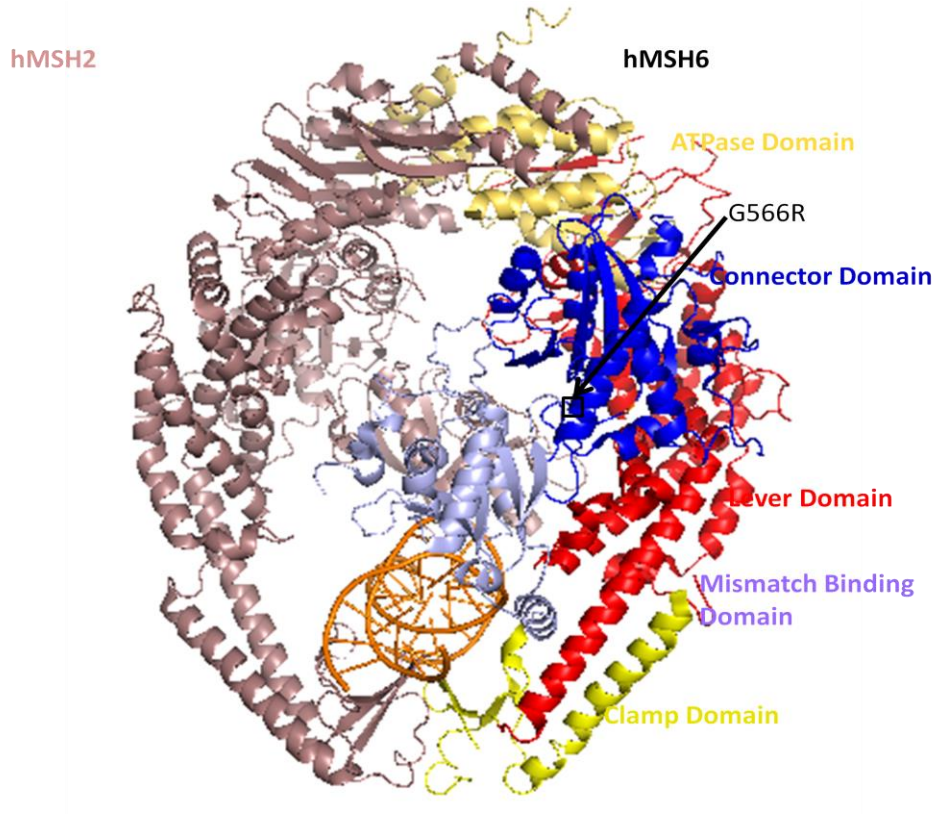
ATP hydrolysis by MSH2-MSH6 is not required for MLH1-PMS2 loading.

We next began testing the biochemical requirements for loading of MLH1-PMS2 onto mismatched DNA via MSH2-MSH6. In the proposed models for MMR downstream signaling for excision there are two existing ideas for the dynamics of the recognition complex (MSH2-MSH6) (Lyer et al 2006). One is the Translocation model and the other the Molecular Switch or sliding clamp model. The Translocation model states that the recognition complex requires ATP hydrolysis for DNA diffusion. In this way the movement away from the mismatch is therefore ATP dependent (Li 2008; Allen et al 1997; Blackwell et al 1998). On the other hand, the second model – Molecular Switch– stipulates that no ATP hydrolysis is required for this diffusion along the DNA (Gradia et al 1999; Honda et al 2013). We asked whether ATP hydrolysis was required for MSH2-MSH6 movement along DNA and/or loading of MLH1-PMS2. To do this we took advantage of a MSH2-MSH6 mutant previously characterized. MSH2WT-MSH6G566R (G566R) contains a mutation in the connector domain of MSH6 (Figure II-4A). Our laboratory has previously shown that G566R has slightly reduced ATP binding and has lost its mismatch-stimulated ATPase activity when compared to the WT MSH2-MSH6 (Cyr et al 2008). Similar pulldown experiments using the G566R mutant incubated with DigGC, DigGT and GT showed that G566R dynamics were similar to wild-type (Figure II-4B). A deficiency in ATP hydrolysis had no effect on the sliding clamp formation and dissociation as G566R is still able to slide off of the open end of the DNA (Figure II-4B, lane 5 and lane 6) as previously shown. Furthermore, this deficiency seems to also have no effect on the ability of MSH2-MSH6 to recruit MLH1-PMS2 to the mismatch (Figure II-4B, lane 4).

Taken together, these results support the notion of the formation of a sliding clamp that diffuses along the DNA independently of ATP hydrolysis and therefore supports the MMR Molecular switch model.

Figure II-4

A



B

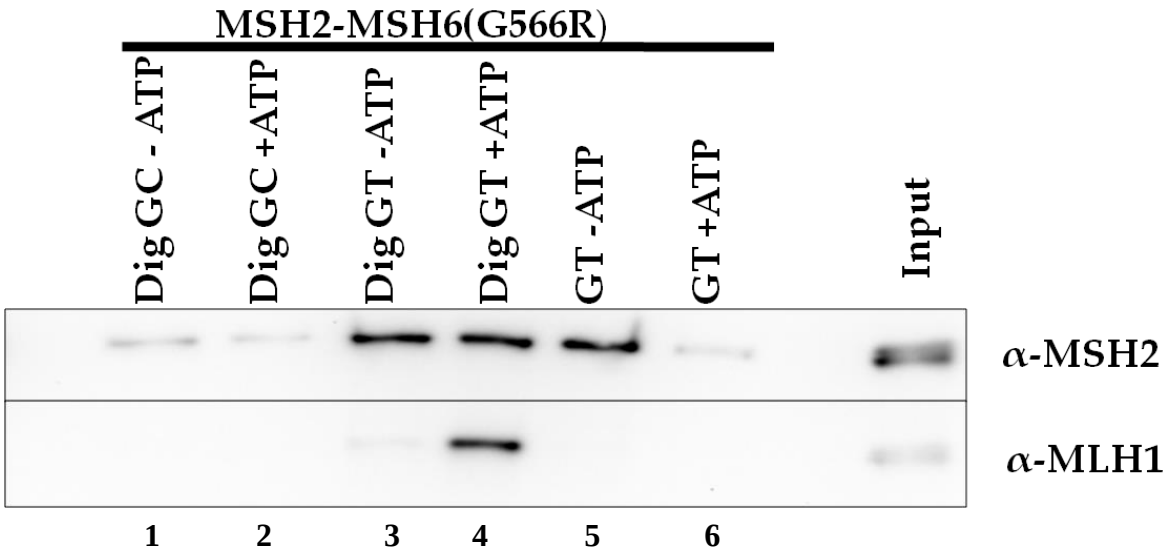


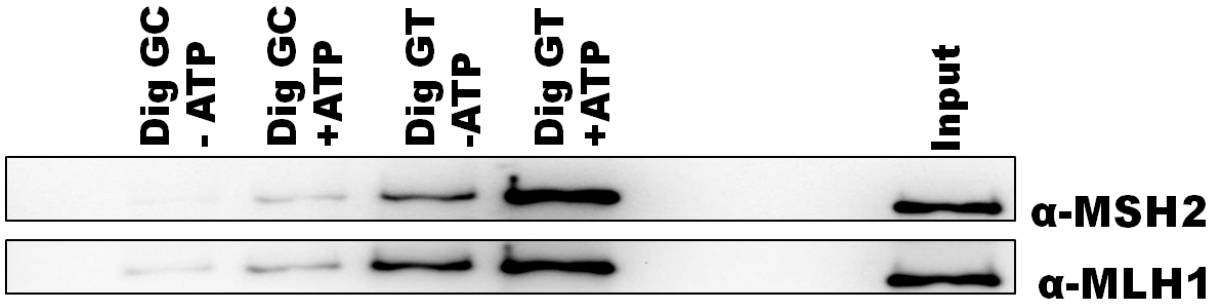
Figure II-4. ATP hydrolysis by MSH2-MSH6 is not required for MLH1-PMS2 loading. A. Pymol generated representation of MSH2-MSH6 heterodimer bound to DNA and location of MSH6-G566R missense mutation in connector domain. **B.** Western blot analysis of $MSH2^{WT}$-$MSH6^{G566R}$ dynamics of on blocked (DigGC, DigGT) and unblocked (GT) substrates. Recruitment of MLH1-PMS2 is also shown.

A single MSH2-MSH6 loads one MLH1-PMS2 heterodimer per mismatch

We next asked whether multiple MLH1-PMS2 heterodimers could be loaded by a single MSH2-MSH6 sliding clamp on mismatch-containing DNA. The presence of multiple MLH1-PMS2 molecules in a greater than 1:1 ratio with MSH2-MSH6 has previously been reported (Hombaeur et al 2011; Qiu et al 2015). To determine the MSH2-MSH6 to MLH1-PMS2 ratio at a mismatch we pre-incubated a larger 255 bp DigGC and DigGT DNA with MSH2-MSH6 in the presence or absence of ATP. Following a wash to remove excess, unbound MSH2-MSH6, MLH1-PMS2 was added in an ATP buffer (Figure II-5A). The resulting data showed that at the concentration tested on the longer DNA substrate, one MSH2-MSH6 loaded a single MLH1-PMS2 heterodimer. When MSH2-MSH6 pre-incubation was performed in the presence of ATP, up to four MSH2-MSH6 sliding clamps could load. These clamps in turn loaded approximately 3 MLH1-PMS2 heterodimers (Figure II-5B).

Figure II-5

A



B

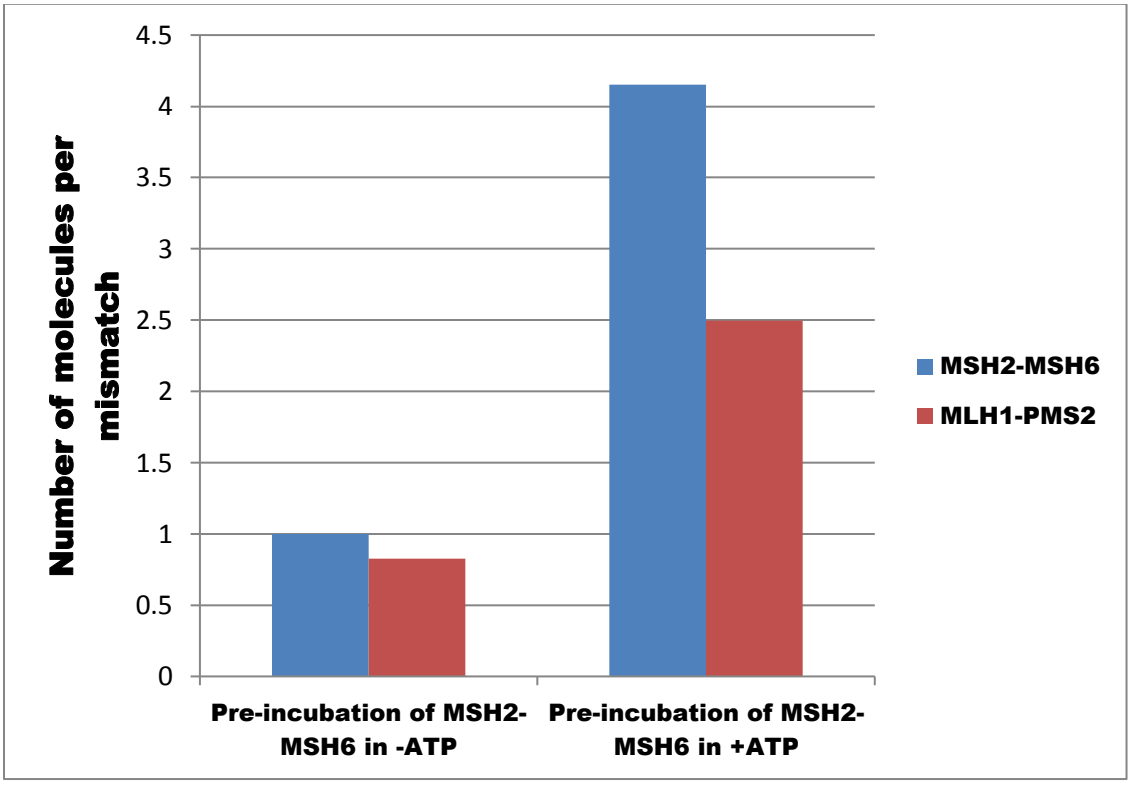


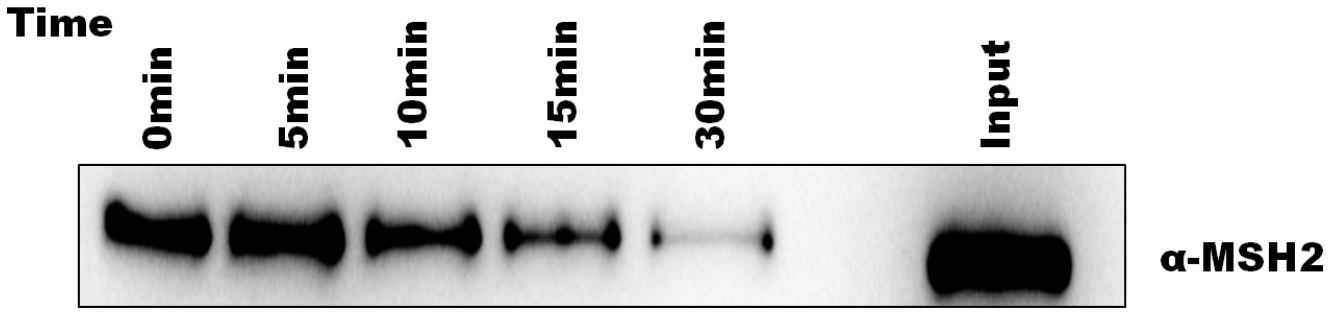
Figure II-5. A single MSH2-MSH6 loads one MLH1-PMS2 heterodimer per mismatch. A. Western blot analysis of MSH2-MSH6 and MLH1-PMS2 levels on DigGC and DigGT DNA after pre-incubation with MSH2-MSH6 in the presence or absence of ATP. B. Number of MSH2-MSH6 and MLH1-PMS2 molecules per mismatch as per ImageJ band intensity quantification.

MLH1-PMS2 enhances the off-rate of MSH2-MSH6 from DNA

MLH1-PMS2 has previously been shown to affect the kinetics of MSH2-MSH6 by promoting its turnover (Acharya et al 2003; Galio et al., 1999). We therefore asked whether this difference in stabilities observed between MSH2-MSH6 and MLH1-PMS2 (Figure II-6) was the result of MLH1-PMS2 recruitment and interaction. To answer this question, we repeated the MSH2-MSH6 stability assay in the absence of MLH1-PMS2. Western blot analysis demonstrated that MSH2-MSH6 dissociated more slowly from mismatched DNA under these conditions (Figure II-6A and II-6B). Together, our data show the difference in stability of the MMR complexes suggesting that MSH2-MSH6 and MLH1-PMS2 do not form a stable quaternary structure.

Figure II-6

A



B

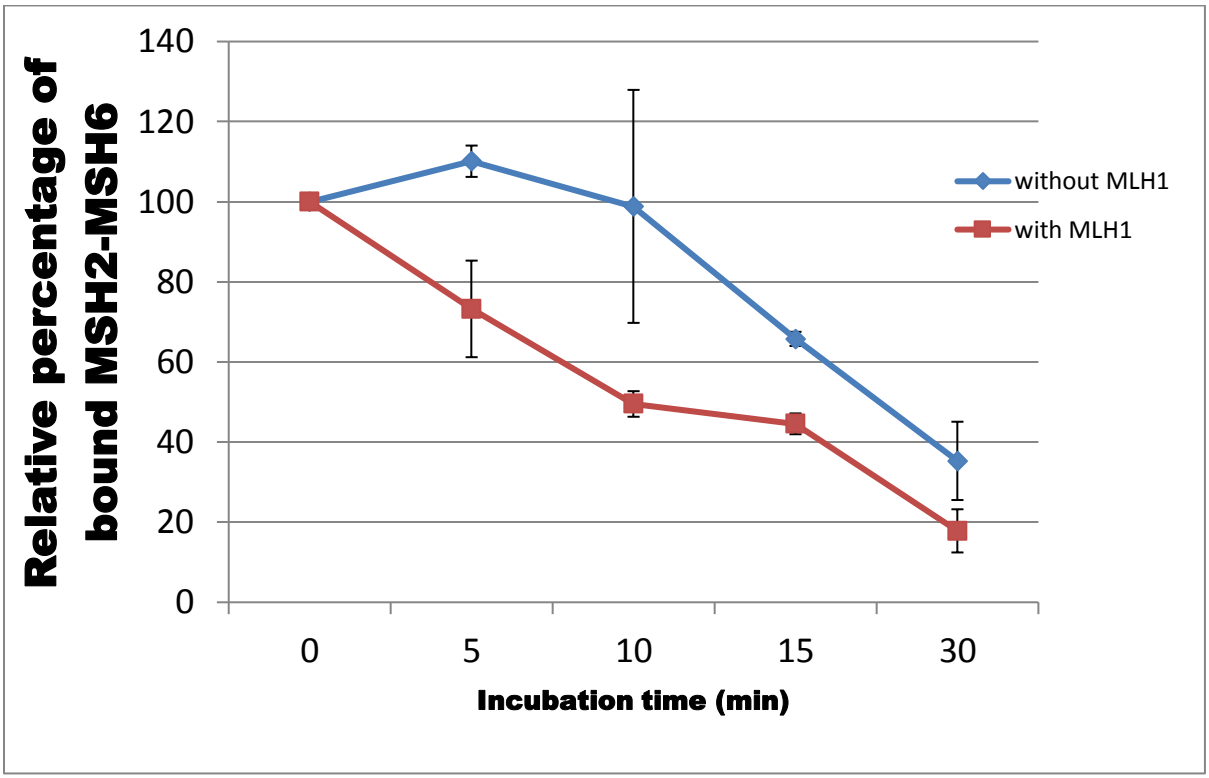


Figure II-4. MLH1-PMS2 enhances the off-rate of MSH2-MSH6 from DNA. A. Western blot analysis of MSH2-MSH6 levels in the absence of MLH1-PMS2 on blocked end heteroduplex (DigGT). B. Relative percentage of MSH2-MSH6 bound to DigGT DNA. Values based on ImageJ quantification of 3 independent experiments.

Discussion

Many Eukaryotic studies of MMR have been focused on improving the understanding of the nature of the interaction between MSH2-MSH6 and MLH1-PMS2 for excision signaling. Nonetheless, many questions still exist such as what happens once MLH1-PMS2 is recruited, what is the stoichiometry of this interaction and how does this interaction signal for excision. Our results show that a MSH2-MSH6 sliding clamp forms on mismatched DNA in the presence of ATP and that this clamp is likely required for the recruitment of MLH1-PMS2. In the absence of ATP no MLH1-PMS2 can be isolated on DNA through our pulldowns. We furthermore demonstrated that MLH1-PMS2 and MSH2-MSH6 have different stability profiles on mismatched DNA and that the loading of MLH1-PMS2 actually increases the dissociation of MSH2-MSH6 from the DNA. Overall, these results suggest that MSH2-MSH6 and MLH1-PMS2 do not function as a unit for downstream signaling. It suggests instead that MSH2-MSH6 may load MLH1-PMS2 onto DNA and that MLH1-PMS2 remains stably bound on mismatched DNA even after most of the MSH2-MSH6 has dissociated from the DNA. MLH1-PMS2 DNA binding potential, therefore, may be consistent with a model in which MLH1-PMS2 polymerizes on DNA near the mismatch for signal amplification. If this model is correct, a MMR protein ratio in which there are more MLH1-PMS2 molecules as compared to MSH2-MSH6 per mismatch would be expected. However, our results suggest that only a single MLH1-PMS2 molecule is loaded per MSH2-MSH6 heterodimer, negating this MLH1-PMS2 polymerization model. As a whole, this novel finding gives rise to a MMR model in which although MSH2-MSH6 is required for the recruitment of MLH1-PMS2 to the DNA, the downstream activities of these proteins, once bound, is different. We speculate that MSH2-MSH6 may be required for loading of ExoI onto DNA whereas MLH1-PMS2 regulates the termination of excision to prevent

excessive DNA degradation beyond the mismatch. Taken all together, we propose a hybrid model we refer to as the Drop Off Model. MSH2-MSH6 recognizes the mismatch and binds to the DNA. The ADP to ATP exchange results in a conformational change to form a sliding clamp which allows for the recognition of the mismatch by other MSH2-MHS6 heterodimers. The MSH2-MSH6 sliding clamp then recruits a MLH1-PMS2 heterodimer which it then "drops off" directly onto the DNA. In this manner there is communication between the mismatch and the nick that serves as the entry point for Exonuclease I (Figure II-7). In the future, further experiments will need to be done to determine the position of the MSH2-MSH6 on the DNA for MLH1-PMS2 recruitment. It will be also important to perform repair assays in which the other components are present to determine at what point the exonuclease comes into play.

Figure II-7

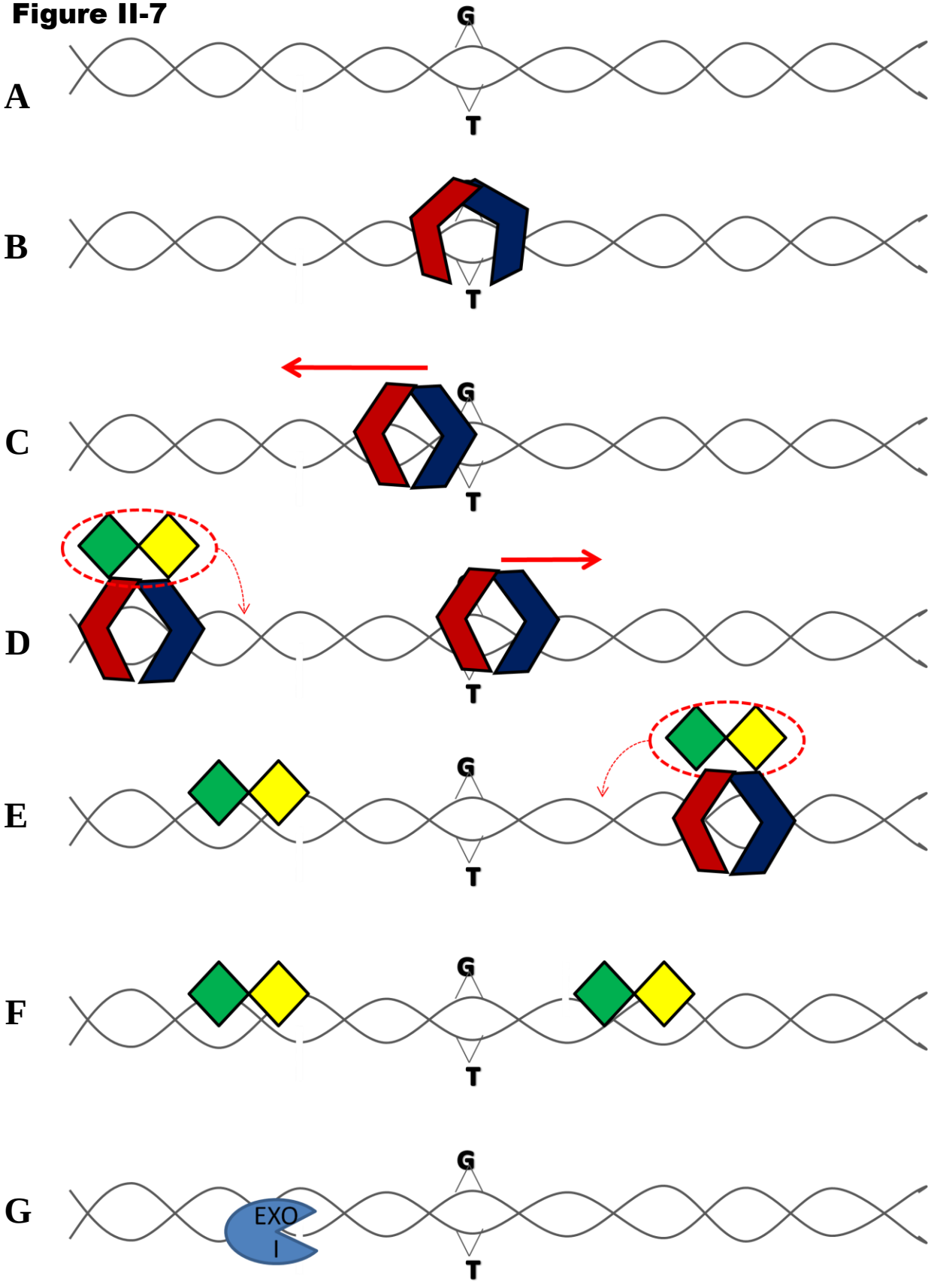


Figure II-7. Proposed "Drop Off" model for MMR excision signaling.. A. Mismatch (GT) generated due to DNA Polymerase error. **B.** MSH2-MSH6 recognizes mismatch (G/T) in ADP-bound form. **C.** ADP to ATP exchange result in conformational change- sliding clamp.MSH2-MSH6 is free to diffuse along DNA leaving the mismatch once again open. **D.** MLH1-PMS2 heterodimer is recruited and dropped off onto the DNA. Mismatch is once again recognized and sliding clamp forms **E.** Initial sliding clamp loses stability on DNA, leaving MLH1-PMS2 behind. Subsequent MLH1-PMS2 is recruited and dropped off onto DNA. **F.** MLH1-PMS2 nicks DNA, creating a restriction for future excision. **E.** Exonuclease I is recruited to remove strand containing incorrect base.

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