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Biochemical and Cellular Studies of Human MSH2 ATPase Domain Mutants Support the Molecular Switch Model of Mismatch Repair In Vivo

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Lynch syndrome is the most common form of hereditary colorectal cancer. It is caused by genetic mutations in the DNA mismatch repair (MMR) pathway. The MMR pathway is a cellular mechanism for repairing nucleotide mismatches that arise during DNA replication. Generally, MSH2-MSH6 heterodimers bind mismatches then are themselves bound by a second mismatch repair heterodimer, MLH1-PMS2. This complex regulates an exonuclease, Exo1, to remove the mismatch-containing DNA that is re-synthesized and sealed by DNA polymerase δ and DNA ligase. There are multiple models to explain the specific MMR molecular mechanism including the molecular switch model. In this model, mismatch binding acts as an exchange factor that reduces ADP affinity and increases ATP affinity in MSH2-MSH6. In the molecular switch model, the form of MSH2-MSH6 that is not bound to DNA and ATP is incapable of initiating downstream repair, thus MMR is "off". The DNA and ATP-bound form of MSH2-MSH6 is capable of initiating repair, thus MMR is "on". The MMR pathway also acts as a sensor for some kinds of DNA damage and can activate a cell cycle arrest and apoptosis response. The DNA methylating agent, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), causes O^6-methylguanine (O^6-me-G) lesions that strongly activate the MMR-dependent DNA damage response by causing O^6-me-G:T mismatches. Most work on the MMR pathway has been *in vitro* biochemistry in

lower organisms. Because of this, there is a substantial knowledge gap about the molecular mechanism of MMR *in vivo* in humans. Understanding the cellular MMR mechanism in repair and the damage response will have the greatest impact clinically for Lynch syndrome patients. Using three MSH2 mutants in the ATPase domain of the protein that we hypothesized would impair the molecular switch model of MMR at different steps, we restored expression of MSH2 in an MSH2-null cell line. Using *in vivo* chromatin localization, MMR and cell cycle arrest assays, we generated data that support the molecular switch model of MMR *in vivo* in humans for both repair and activation of the MMR-dependent DNA damage response to MNNG lesions.

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5-FU	5-Fluorouracil
6-TG	6-Thioguanine
ABC	ATP-binding cassette
APC	Adenomatous polyposis coli
ATR	Ataxia telangiectasia and Rad3-related
ATRIP	ATR-interacting protein
CRC	Colorectal cancer
DDR	DNA damage response
DMSO	Dimethyl sulfoxide
FAP	Familial adenomatous polyposis
GFP	Green fluorescent protein
H3	Histone 3
HU	Hydroxyurea
LS	Lynch syndrome
MLH	Mut L homologue
MMR	Mismatch repair
MNNG	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
MSH	Mut S homologue
MSI	Microsatellite instability
O^6-BG	O^6-benzylguanine
O^6-me-G	O^6-methylguanine
PBS	Phosphate-buffered saline

PCNA	Proliferating cell nuclear antigen
PMS	Postmeiotic segregation increased
RFC	Replication factor C
RFP	Red fluorescent protein
SPR	Surface plasmon resonance
ss	Single-stranded
TBP	TATA-binding protein

Chapter I:

Introduction

A. Colorectal Cancer

Colorectal cancer (CRC) is the third most commonly-diagnosed cancer for both men and women in the United States and is the third-leading cause of cancer deaths (American Cancer Society, 2013). Because of its severity, CRC has been a focus of cancer research for years; however, owing to its location, CRC has also presented researchers with a unique opportunity to study the progressive development of solid tumors. Early cancer genetics work resulted in the hypothesis that tumorigenesis required the acquisition of sequential mutations that gave the cells in which they occurred a growth advantage over normal, neighboring cells (Fearon and Vogelstein, 1990). Each ensuing sporadic mutation in those already mutated cells enhanced the growth advantage and progressed the cells from benign early-stage adenoma through to malignant colorectal carcinoma (Fearon and Vogelstein, 1990). Since the median age of CRC diagnosis for the population at large is 68-72 (American Cancer Society, 2011), this multi-step model of CRC tumorigenesis required the gradual acquisition of sporadic mutations taking place over decades. This genetic model can serve as a framework for interpreting sporadic CRC incidences in the general population, but how to explain incidences of CRC with a much earlier age of onset, presumably before so many mutations could accumulate over the decades, as well as cases of the disease that seem to run in families (Kinzler and Vogelstein, 1996)? Using the CRC and retinoblastoma genetic framework developed by Fearon and Vogelstein and Knudson (Knudson, 1971), these hereditary forms of CRC could be explained by one key inherited mutation that fast-tracked an individual towards CRC. There are two main forms of hereditary CRC: Familial adenomatous polyposis (FAP) and Lynch syndrome (LS).

a. Familial adenomatous polyposis

FAP is a form of hereditary CRC characterized by the development of hundreds to thousands of adenomatous polyps starting in the second decade of life. Although a rare form of CRC, it affects approximately 1:10,000 people (Church, 2009), the sheer number of adenomas that develop in FAP patients virtually guarantees that some will progress into CRC. Typically the only recourse is to remove the colon.

FAP is an autosomal dominant form of hereditary CRC. Studies of familial genetics demonstrated a linkage between FAP and a marker on chromosome 5q21 (Bodmer et al., 1987) even though the disease was first described in the mid-19th century. The underlying cause was a mutation in the *APC* gene, which produces the adenomatous polyposis coli (APC) protein (Olschwang et al., 1991). APC is a scaffolding protein involved in the Wnt signaling pathway and sequesters β-catenin, a transcription factor also involved in cell adhesion. FAP-associated mutations typically result in truncations of APC that result in a reduced ability to bind and sequester β-catenin thereby causing increased proliferation in colon cells. Inheriting a single mutation in the *APC* gene is sufficient to diagnose FAP individuals even though tumorigenesis requires the loss of the second *APC* allele.

b. Lynch syndrome

Lynch syndrome (LS), also known as hereditary non-polyposis colorectal cancer, is differentiated from FAP by its lack of multiple adenomatous polyps in the colon and greatly increased risk of developing extra-colonic tumors in addition to CRC, most notably endometrial, stomach, ovarian, small intestine and pancreas (Watson and Lynch, 1993; Lynch et al., 2009). Emerging evidence suggests that LS increases the risk for breast, bladder and prostate cancers as well (Engel et al., 2012). LS is much more common than FAP and affects approximately 3% of

total CRC cases (Lynch et al., 2009). Like FAP, familial genetics played a role in helping to define this form of hereditary CRC. In 1966, a pathologist, Henry Lynch, published his findings regarding two large families with high frequencies of specific carcinomas including many cases of CRC (Lynch et al., 1966), which was supported by further studies of familial CRC (Boland and Troncale, 1984).

Since LS lacks a highly noticeable phenotype like FAP, its defining characteristics are important in order to distinguish LS from cases of sporadic CRC. International efforts to characterize LS and aid in the identification of patients led to the development of the Amsterdam II criteria in 1999 (Vasen et al., 1999) and revised Bethesda Guidelines in 2002 (Umar et al., 2004). The defining characteristics of LS are summarized in Table 1-1. One of the primary LS characteristics is microsatellite instability (MSI).

Microsatellites are short, repetitive sequences of DNA. The Bethesda Guidelines recommends examining a panel of five known microsatellite regions for expansion or contraction as almost all LS-associated cases of CRC exhibit significant MSI (Umar et al., 2004). Based on the observation that LS tumors displayed significant MSI (Peltomäki et al., 1993a; Aaltonen et al., 1993; Aaltonen et al., 1994) and given the role of prokaryotic MMR in fixing MSI, researchers hypothesized that prokaryotic MMR homologues in humans might cause LS. Additionally, eukaryotic MutS (Msh) and MutL (Mlh) homologues for bacterial MMR proteins had been described in yeast (Reenan and Kolodner, 1992; Kramer et al., 1989) and mutations within yeast Msh and Mlh caused MSI similar to that seen in human LS patients (Strand et al., 1993). Studies initially associated LS with a locus on chromosome 2p (Peltomäki et al., 1993b) before the discovery of human MutS homologue 2 (*MSH2*) and its association with LS causation in 1993 (Leach et al., 1993; Fishel et al., 1993). Subsequent work discovered additional human

Table 1-1. Diagnostic criteria for Lynch syndrome. Adapted from Vasen et al., 2007.

At least three relatives, including one first-degree relative, diagnosed with a LS-associated cancer
CRC diagnosis prior to the age of 50
LS-associated cancer diagnoses in at least two successive generations
Presence of synchronous or metachronous LS-associated tumors
Diagnosis of a family member's LS-associated cancer prior to the age of 50
CRC with high MSI in a patient prior to the age of 60

MutL homologues, *MLH1* and *PMS2* (Lindblom et al., 1993; Nicolaides et al., 1994; Papadopoulos et al., 1994; Bronner et al., 1994), and MutS homologues, *MSH6* (Palombo et al., 1995; Drummond et al., 1995; Miyaki et al., 1997), involved in mismatch-binding and mismatch repair and mutations that were also implicated in causing LS. Mutations in *MLH1*, *MSH2* and *MSH6* account for approximately 95% of all LS-associated mutations (Peltomäki, 2005).

B. DNA Mismatch Repair

a. Prokaryotic MMR

The MMR pathway is a highly-conserved mechanism for repairing single nucleotide mispairs and small insertion/deletion loops that can arise during replication. The components of prokaryotic MMR have been well-characterized, most notably in the bacterium *Escherichia coli*, where it was observed that mutations at certain loci in the *E. coli* genome, now known to correspond to MMR genes, led to significantly higher genomic mutational frequencies (Cox, 1976).

In bacteria, mismatches are recognized by homodimers of MutS proteins with ADP bound by one of the monomers (Su and Modrich, 1986; Lamers et al., 2000; Obmolova et al., 2000). Binding to mismatches drives a MutS conformational change that simultaneously reduces affinity for the mismatch and ADP and increases MutS affinity for ATP binding (Selmane et al., 2003; Junop et al., 2001). This converts the MutS homodimer into a very stable, DNA-bound clamp that diffuses from the mismatch freeing it to be recognized by another MutS homodimer (Acharya et al., 2003). Iterative loading of ATP-bound, MutS clamps follows (Acharya et al., 2003). These ATP-bound MutS clamps bind a second homodimer, MutL (Grilley et al., 1989), which activates an endonuclease, MutH, in an ATP-dependent manner through direct interaction (Welsh et al., 1987; Hall and Matson, 1999). MutH has specificity for unmethylated d(GATC)

sequences (Welsh et al., 1987). Because newly replicated *E. coli* DNA is transiently unmethylated, the methylation status of the d(GATC) sequence serves as the signal to ensure repair strand specificity and direct MMR to the newly-synthesized DNA strand (Au et al., 1992). Following incision of the repair strand by MutH, the DNA is unwound by DNA helicase II (Yamaguchi et al., 1998) and excised by different exonucleases depending on the 5' or 3' orientation of the incision. Excision continues through the mismatch at which point DNA Polymerase III resynthesizes an error-free DNA strand and it is ligated (Lahue et al., 1989; Grilley et al., 1993).

b. Eukaryotic MMR

Because of genetic conservation, the eukaryotic MMR system is similar fundamentally to the classical MMR system worked out in bacteria, but is more complex and has several notable distinctions although the exact molecular mechanism linking mismatch binding to excision is still unclear. Mismatch recognition is accomplished with MSH2-MSH6 heterodimers (Palombo et al., 1995; Acharya et al., 1996), not MutS homodimers as in bacteria. Only the MSH6 subunit directly binds mismatches through a conserved Phe-X-Glu motif like that found in bacterial MutS, a motif that MSH2 lacks (Drotschmann et al., 2001). The result in both eukaryotic and bacterial MMR is asymmetrical binding by the dimer subunits at mismatches. Iterative loading of ATP-bound MSH2-MSH6 clamps on mismatch-containing DNA is similar between prokaryotes and eukaryotes (Gradia et al., 1997; Gradia et al., 1999; Gradia et al., 2000), but downstream licensing of MMR in humans requires binding MutL homologue heterodimers of MLH1-PMS2 in humans or Mlh1-Pms1 in yeast as opposed to MutL homodimers in bacteria (Zhang et al., 2005; Mendillo et al., 2005). Additionally, there is no MutH homologue in human MMR; however one of the MutL homologues, PMS2, possesses endonuclease activity as does Pms1 in

yeast (Kadyrov et al., 2007; Kadyrov et al., 2006). In bacteria, MutS, MutL, mismatch-containing DNA and ATP are sufficient to activate the endonuclease activity of MutH (Hall and Matson, 1999), but additional factors are required in human MMR in order to activate PMS2 endonuclease activity and ensure the newly-replicated DNA strand is incised. The eukaryotic DNA polymerase processivity factor, proliferating cell nuclear antigen (PCNA), and its clamp loader, replication factor C (RFC), are required to both activate PSM2 endonuclease activity as well as orient the DNA incision to the nascent strand (Pluciennik et al., 2010). The homotrimeric, clamp-like PCNA accomplishes this because of its asymmetric faces. Loading of PCNA at the 3' ends of DNA via RFC, and PCNA's subsequent diffusion along the DNA in the same orientation, ensures that the incision made by PMS2 is restricted to new strands of DNA. Ensuring MMR is restricted to a newly-replicated DNA strand and not the template strand is vital to prevent the permanent introduction of mutations into the human genome. In humans, MMR strand discrimination does not depend on transient hemimethylation at d(GATC) sequences on the newly-synthesized DNA strand (Drummond and Bellacosa, 2001). Instead, biochemical data suggests that human MMR restricts itself to nascent DNA strands through the recognition of strand discontinuities such as those found between Okazaki fragments on the lagging strand during DNA replication (Dzantiev et al., 2004; Constantin et al., 2005; Pluciennik et al., 2010). Recent evidence also implicates the misincorporation of ribonucleotides into DNA during replication as an additional source of strand discontinuities, and entry sites for MMR, as they are being removed (Ghodgaonkar et al., 2013). Whereas the bacterial MMR system utilizes different exonucleases depending on whether the MutH incision is upstream or downstream of the mismatch, the human MMR system requires only one exonuclease, EXO1, which has 5'-to-3' exonuclease activity. However, the endonuclease activity of PCNA-activated PMS2 can incise

nascent DNA upstream of a mismatch in order to direct EXO1 to the proper strand. Resynthesis and ligation of the repaired DNA strand are done by the replication DNA polymerase δ and DNA ligase.

C. MSH2-MSH6 protein domains

Central to the molecular mechanism of eukaryotic MMR is the conformational change in the mismatch recognition heterodimer that accompanies mismatch binding. This conformational change alters the nucleotide-binding states of the MSH2-MSH6 subunits and licenses the downstream MMR process. MSH2 and MSH6, like MutS, are members of the ATP-binding cassette (ABC) ATPase family (Hollenstein et al., 2007). Members of this family are involved in diverse biological functions, but all contain ATP-binding and ATPase activity when dimerized. The crystal structures of different prokaryotic MutS proteins have been solved (Obmolova et al., 2000; Lamers et al., 2000) as has the MSH2-MSH6 heterodimer bound to mismatched DNA (Warren et al., 2007).

Both MSH2 and MSH6 contain five domains: mismatch-binding, clamps, connector, levers and ATPase (Figure 1-1). The mismatch-binding domain must allosterically communicate a change in state to the ATPase domain through the intervening connector, clamp and lever domains over approximately 100 angstroms (Warren et al., 2007). MSH2 and MSH6 have composite ATPase domains at the carboxy-terminal dimerization interface. Each subunit's ATPase domain contains Walker A/B motifs for ATP-binding and hydrolysis in the presence of magnesium cofactors (Warren et al., 2007). The ATPase domain of MSH2 is slightly more ordered than that of MSH6 (Warren et al., 2007) and has an increased affinity for ADP (Mazur et al., 2006, Heinen et al., 2011). *In vitro* biochemistry suggests that the human mismatch recognition heterodimer binds mismatches in a (ADP)MSH2-MSH6(empty) state; mismatch

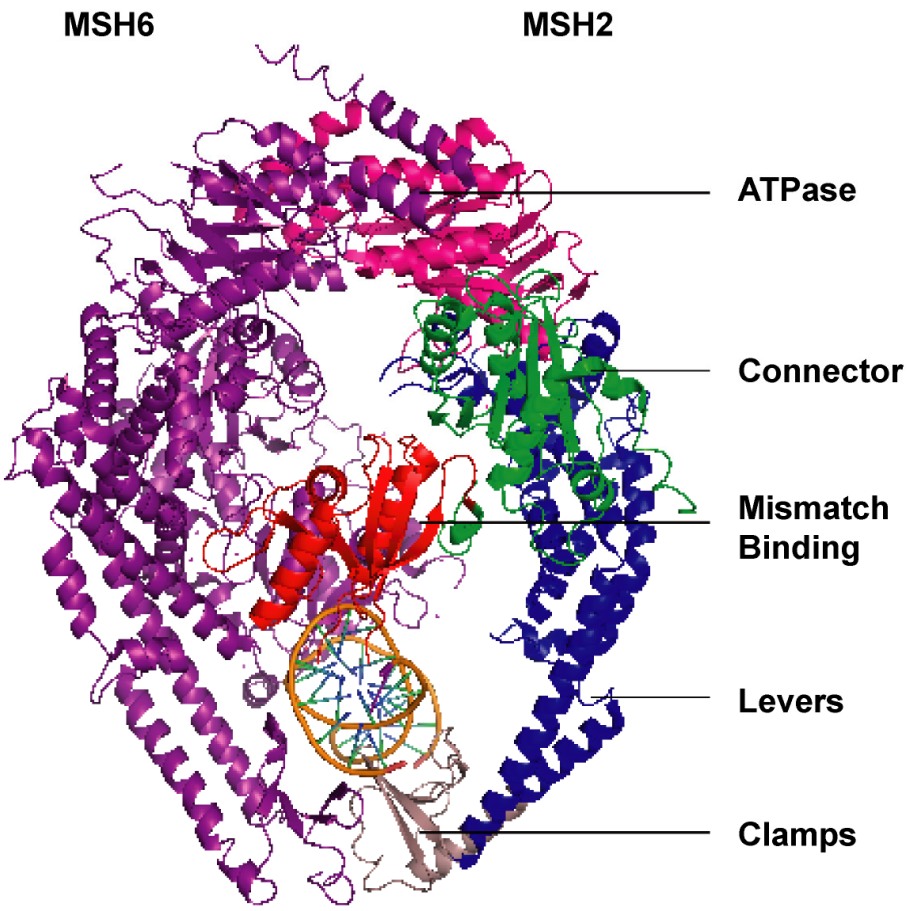

MSH6
MSH2
ATPase
Connector
Mismatch
Binding
Levers
Clamps

Figure 1-1. MSH2-MSH6 crystal structure. Complete crystal structure of the MSH2-MSH6 heterodimer bound to ADP and G/T mismatched DNA (protein data bank ID 2O8B).

binding reduces the affinity of MSH2 for ADP while increasing the MSH6 ATPase domain's affinity for ATP. Binding of ATP by the MSH6 subunit enhances the MSH2 subunit's affinity for ATP (Mazur et al., 2006, Heinen et al., 2011). When both subunits have bound ATP, the MSH2-MSH6 heterodimer has been converted into a stable, DNA-bound clamp that diffuses from the mismatch enabling it to be recognized by a new MSH2-MSH6 heterodimer and activate another round of clamp-loading.

D. MMR molecular mechanism hypotheses

How MMR proteins involved in mismatch recognition link mismatch binding with the excision of a mismatch-containing DNA strand remains unclear. There have been three models proposed based on both prokaryotic and eukaryotic MMR biochemistry: static transactivation, active translocation and molecular switch.

The static transactivation model proposes that MutS and MutL proteins complex directly at a mismatch and use intervening loops in DNA to bring distant d(GATC) sites to the mismatch, where MutS and MutL can activate MutH and incise the mismatch-containing DNA strand (Junop et al., 2001; Schofield et al., 2001). Further biochemistry has largely eliminated the static transactivation model of MMR. Additions of a protein "roadblock" and strand discontinuities, which should not affect a model in which the MMR proteins remain bound at the mismatch, severely inhibited activation of MutH and prevented excision (Pluciennik and Modrich, 2007; Wang and Hays, 2004). The remaining two mechanistic models of MMR both rely on loading of ATP-bound MutS or MSH2-MSH6 clamps followed by diffusion while maintaining residency on the DNA. However the active translocation model posits that ATP binding *then* hydrolysis is necessary for clamp movement from the mismatch (Allen et al., 1997) while the molecular switch model posits that only ATP binding is necessary for clamp formation and diffusion from

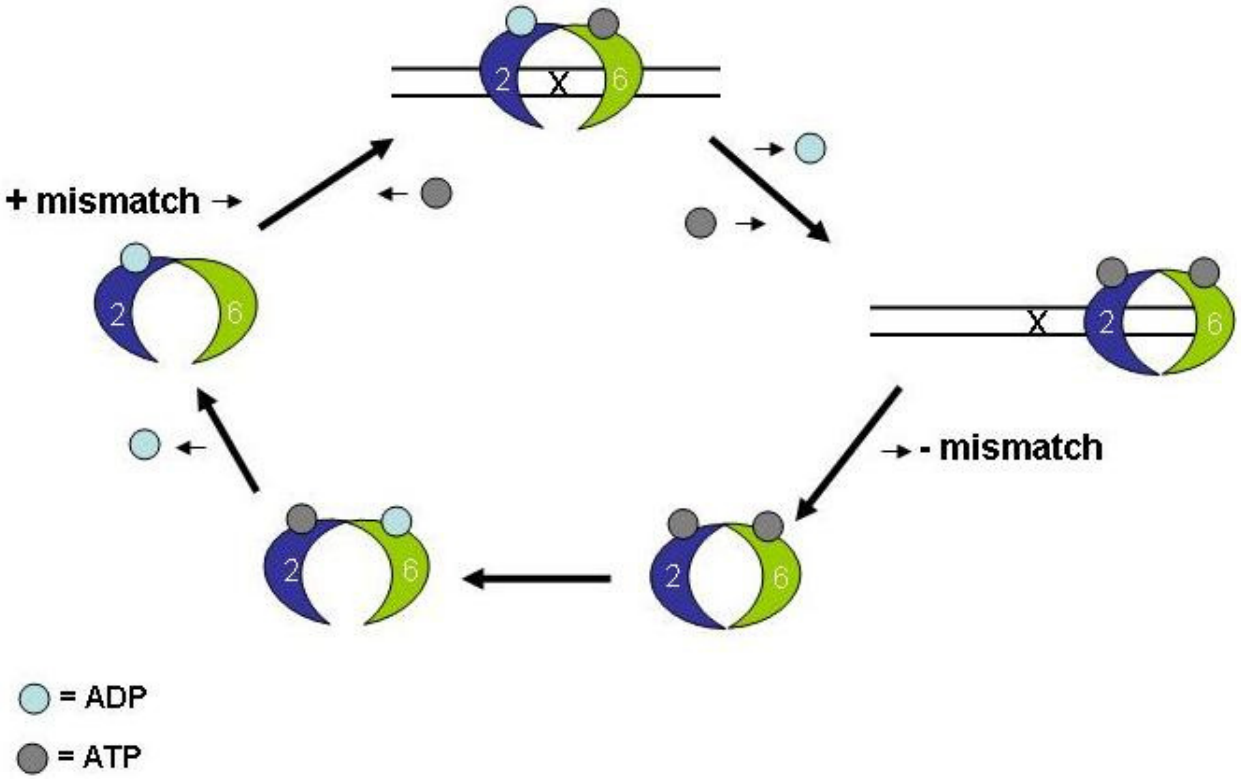

2
X
6
+ mismatch
- mismatch
= ADP
= ATP

Figure 1-2. Schematic illustration of the steps in the yeast molecular switch model of mismatch repair.

the mismatch (Figure 1-2; Gradia et al., 1999). Increasing biochemical evidence supports the molecular switch model of MMR *in vitro* for both prokaryotes and eukaryotes (Jeong et al., 2011; Cho et al., 2012), but little is known about the *in vivo* model of cellular MMR. The goal of this research project was to validate the molecular switch model in cellular MMR as there is currently a gap in knowledge regarding *in vivo* MMR.

E. MMR and the DNA damage response

Cells have evolved numerous, sometimes redundant, mechanisms to repair DNA damage and prevent the transmission of genetic mutations to daughter cells. Often these mechanisms involve detecting various DNA lesions during DNA replication and arresting the cell cycle while the lesions are repaired or committing to apoptosis (Lazzaro et al., 2009). That genetic mutations within the MMR pathway result in genomic instability can be understood given the biological function of the system, but the survival advantage and cancer predisposition resulting therein is somewhat paradoxical. If mutations are increasing in cells, it seems likely that those cells might die more rapidly than a normal cell in which mutations are not increasing, but that is not the case for cells that lack an intact MMR system. This is because the MMR pathway acts as a DNA damage sensor, in addition to its role in mismatch repair, and localizes to chromatin in response to alkylation damage to DNA (Adamson et al., 2005). The primary lesion that activates the MMR-dependent DNA damage response (DDR) is O^6-methylguanine (O^6-me-G) lesions caused by S_N1 methylating agents like *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; Christmann and Kaina, 2000). The cellular mechanism of MNNG is similar to other commonly used alkylating chemotherapeutics. There are two models to explain the role of the MMR system in O^6-me-G-induced cell cycle arrest and apoptosis: the futile repair cycle model and the direct signaling model.

a. Futile repair cycle

The name of this model is derived from the hypothesis that during DNA replication the replicative polymerase will persistently mispair a thymine opposite an O^6-me-G lesion in the template DNA strand. Iterative rounds of unsuccessful MMR to remove the O^6-me-G:T mispair will result in persistent single-stranded (ss) DNA stretches and replication fork collapses during another round of DNA replication (Kaina et al., 1997; Cejka et al., 2003). *In vitro* evidence for this model came from a study that utilized a plasmid with a O^6-me-G:T mismatch (York and Modrich, 2006). The O^6-me-G lesion could be repaired with eukaryotic, nick-directed MMR when the nick was on the same strand as the O^6-me-G lesion, but could not be repaired when the nick was on the opposite strand (York and Modrich, 2006). *In vivo* evidence arose from the observation that MNNG treatment caused persistent ssDNA gaps after the first cell cycle and cell cycle arrest during the second round (Mojas et al., 2007; Stojic et al., 2004). Additionally, persistent nuclear foci containing proteins associated with DNA repair and cell cycle arrest, including the kinase ataxia telangiectasia and Rad3-related (ATR), indicated that these were sites of DNA damage that were not being repaired (Stojic et al., 2004). Further work has supported the futile repair cycle model as evidenced by a second round cell cycle arrest, but also indicated that the concentration of MNNG used in experimentation may influence the cellular outcome. At low doses, there is a shift towards using sister chromatid exchanges and homologous recombination to overcome the O^6-me-G lesion without a cell cycle arrest (Roos et al., 2009; Quiros et al., 2010; Schroering and Williams, 2008). At high doses, cell death results from base excision repair processing N^3-me-A and N^7-me-G lesions (Stojic et al., 2005).

b. Direct signaling model

The second model to account for MMR-directed cell cycle arrest and apoptosis is the direct signaling model. This model requires no iterative rounds of unsuccessful MMR; instead cell cycle arrest effector proteins like ATR form complexes with MSH2-MSH6 and MLH1-PMS2 at O^6-me-G:T lesions that directly lead to activation of ATR-dependent Chk1 and cell cycle arrest (Yoshioka et al., 2006). Additionally, treatment of MMR-proficient cancer cells with cisplatin causes MSH2-MSH6-ATR binding and in MSH2-deficient cell lines this direct interaction is not observed (Pabla et al., 2011). It is unclear if this direct interaction is occurring at the cisplatin-induced DNA cross-links or nearby however.

F. Rationale

Development of MMR molecular mechanism models has largely been through the use of *in vitro* biochemistry, small DNA oligonucleotides and recombinant proteins. Comparatively little is known about human MMR *in vivo* where DNA exists as chromatin in association with numerous proteins and in tightly-wound conformations. Indeed, biochemical evidence suggests that nucleosomes reduce human MMR efficiency *in vitro* (Li et al., 2009). Development of MMR models through biochemistry means it is unclear whether the field can make statements about which MMR model is correct in human cells. The use of various MSH2 or MSH6 mutant proteins is a tool that can allow us to make hypotheses about MMR models then selectively inhibit the proposed mechanism at different points in order to generate data that might support a proposed mechanism in human cells. Hargreaves et al. details the *in vitro* biochemical study of eight Msh2 and Msh6 *Saccharomyces cerevisiae* mutants that all demonstrate differing levels of nucleotide and mismatch binding and Msh2-Msh6 sliding clamp formation (Hargreaves et al., 2010). The step-wise progression of Msh2-Msh6 binding mismatches, Msh2-Msh6 nucleotide

exchange, sliding clamp formation and iterative sliding clamp loading that defines the molecular switch model of MMR means that the Msh2 and Msh6 yeast mutants could be tools to validate the molecular switch model of MMR in human cells. This would be the first evidence to support a particular MMR model in human cells. We generated the human homologues of two Msh2 mutants from Hargreaves et al.: R711C and G827D. This study also utilized a previously-characterized MSH2 mutant, K675A, which contains a mutated conserved lysine residue in the Walker box A ATP binding motif that directly coordinates with the ATP molecule. MSH2 K675A, R711C and G827D are all ATPase domain mutants. Using purified wild-type and mutant MSH2-MSH6 proteins, we characterized the nucleotide binding, sliding clamp formation and ATPase activity statuses of each heterodimer. To investigate MMR *in vivo*, we used a lentivirus and an MSH2-null cancer cell line, Hec59. With the lentivirus, we restored expression of MSH2 in Hec59 cells using the MSH2 ATPase domain mutants as well as wild-type MSH2 and lentivirus vector-only Hec59 as controls. We found that all MSH2 ATPase domain mutants had decreased sliding clamp formation and ATPase activity relative to wild-type MSH2-MSH6. *In vivo*, we found that MNNG treatment of Hec59 cells failed to induce an increase in MSH2 ATPase domain mutants' chromatin localization despite being able to bind mismatches. MSH2 ATPase domain mutants were unable to repair a G/T mismatch-containing plasmid *in vivo* and did not exhibit a MMR-dependent G_2/M cell cycle arrest when mutant MSH2-expressing Hec59 cells were treated with MNNG. These data provide evidence that sliding clamp formation and iterative sliding clamp loading on DNA is a necessary step for functional downstream MMR in human cells. Support for sliding clamp formation and iterative sliding clamp loading in human cells validates the molecular switch model of MMR *in vivo*.

Chapter II:

Biochemical and cellular studies of MSH2 ATPase domain mutants support the molecular switch model of mismatch repair *in vivo*.

A. ABSTRACT

The DNA MMR pathway is crucial in preventing genomic instability. Much of what is known about the MMR pathway has been developed through *in vitro* biochemistry using bacteria and yeast. It is unclear if the current mechanistic models of MMR extend to human cells *in vivo* and if they have any functional relevance in the MMR-dependent DDR. We generated several MSH2 ATPase domain mutants and characterized them using *in vitro* biochemistry in order to investigate the molecular switch model of MMR in human cells. Using [γ-^{32}P]-ATP, we demonstrate that our MSH2 ATPase domain mutants do not bind ATP and this alters ATP binding in the MSH6 subunit. As well, the MSH2 ATPase domain mutations inhibit ATP hydrolysis by the MSH6 subunit. *In vitro* surface plasmon resonance (SPR) studies indicate that the mutant MSH2-MSH6 heterodimers bind mismatches with increased K_d and do not form sliding clamps efficiently. In an MSH2-null cell line, Hec59, we re-expressed the different MSH2 ATPase domain mutants using a lentivirus. Using an *in vivo* MMR assay, all the MSH2 mutants were unable to direct MMR *in vivo*. Treatment of the lentiviral Hec59 cell lines with a DNA alkylating agent failed to induce increased chromatin localization of the MSH2 ATPase domain mutants or generate a MMR-dependent G_2/M cell cycle arrest. All the MSH2 ATPase domain mutants displayed significantly increased tolerance of DNA alkylation damage relative to wild-type MSH2 as assessed using a clonogenic survival assay. This survival advantage was eliminated using different DNA damaging agents. Taken together, the data suggest that the molecular switch model of MMR is accurate for human cells *in vivo* and the cycle of nucleotide

binding and sliding clamp formation is important for the MMR-dependent DDR to lesions caused by alkylation damage, but not other DNA lesions.

B. INTRODUCTION

The MMR pathway is a system for recognizing and repairing single nucleotide mispairs that arise through DNA replication or following DNA damage. The MMR pathway is integral in the maintenance of cell genomic stability. Mutations in the proteins that comprise the MMR system cause LS, a hereditary cancer syndrome (Martín-López and Fishel, 2013). Broadly, DNA mismatches are bound by a heterodimer of MSH2-MSH6, which forms a complex with a second MMR protein heterodimer of MLH1-PMS2. This protein complex recruits and regulates the activity of an exonuclease, EXO1, which removes the mismatch-containing strand. Resynthesis of the degraded DNA strand and sealing is done by DNA polymerase δ and DNA ligase (Zhang et al., 2005; Constantin et al., 2005; Dzantiev et al., 2004).

The exact molecular mechanism of coordinating mismatch binding with excision remains unclear and multiple models have been developed. One model hypothesizes that MSH2 and MSH6, the mismatch binding proteins, function as molecular switches activated by a mismatch and ATP binding. Accordingly, it is called the molecular switch model of MMR (Gradia et al., 1997). Multiple observations contributed to the development of this model. MSH2 and MSH6 both contain a highly-conserved ABC-ATPase domain capable of binding ADP and ATP and hydrolyzing ATP (Hollenstein et al., 2007; Warren et al., 2007). Absent DNA binding, *in vitro* biochemistry has demonstrated that MSH2 binds ADP while the MSH6 site is empty (Mazur et al., 2006; Heinen et al., 2011). There is a conformational change in the MSH2-MSH6 heterodimer following mismatch binding that increases affinity in both MSH2 and MSH6 ATPase domain subunits for binding ATP (Gradia et al., 1999). Pre-binding ATP to MSH2-MSH6 blocks mismatch binding (Gradia et al., 1997). Because of these observations, the molecular switch model states that a DNA mismatch acts like a nucleotide exchange factor

similar to those found in G-protein signaling (Fishel, 1998). Off of DNA, the MSH2 ATPase subunit contains ADP while the MSH6 subunit is empty (Mazur et al., 2006; Heinen et al., 2011). Upon binding to a mismatch, there is a conformational change that increases the affinity of the MSH6 site for binding ATP. Once MSH6 has bound ATP, there is another small conformational change that drives an ADP-ATP exchange at the MSH2 ATPase site. Off of DNA MSH2-MSH6 are "off" and there is no MMR. Following mismatch and ATP binding, MSH2-MSH6 are "on" and downstream MMR can proceed (Figure 1-2).

Other observations contributing to the formation of the molecular switch model of MMR were the significantly increased MSH2-MSH6 residence time on DNA following ATP binding as well as the loading of multiple MSH2-MSH6 heterodimers on the same mismatch-containing DNA strand *in vitro* (Gradia et al., 1999). This observation led to the hypothesis that the MSH2-MSH6 heterodimers were diffusing from the mismatch bidirectionally, but remaining on the DNA. This allowed the mismatch to be recognized by a new MSH2-MSH6 heterodimer and would lead to the iterative loading of hundreds to thousands of MSH2-MSH6 heterodimers. These ultra-stable MSH2-MSH6 heterodimers are called sliding clamps in the molecular switch model of MMR.

In addition to repairing single nucleotide mismatches, the MMR system is involved in a DDR that can lead to cell cycle arrest and apoptosis. The MMR pathway recognizes numerous DNA lesions, but the best-characterized and most cytotoxic are O^6-me-G lesions caused by alkylating agents like *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; Christmann and Kaina, 2000). Cells with a functional MMR pathway are approximately 50-fold more sensitive to MNNG than cells without functioning MMR (Karran, 2001). If cells replicate their DNA with O^6-me-G lesions, it can lead to O^6-me-G:T mismatches that are recognized by the MMR

pathway which causes a G_2/M cell cycle arrest and can lead to apoptosis (Duckett et al., 1996; Adamson et al., 2005).

Much of the mechanistic work on the MMR pathway has been developed through *in vitro* biochemistry in bacteria and yeast. It is still unclear if the molecular switch model of MMR is accurate in human cells or what this model's contribution to the MMR-dependent DDR might be, if any. Previous work identified yeast Msh2 and Msh6 ATPase domain mutants that were deficient in mismatch repair (Hargreaves et al., 2010). One yeast Msh2 mutant, G855D, was unable to bind ATP in the Msh2 subunit and had deficient sliding clamp formation, but was still able to form complexes with Mlh1-Pms1. A second yeast Msh2 mutant, R730C, was able to bind ATP in both the Msh2 and Msh6 subunits and bind Mlh1-Pms1, but was unable to form sliding clamps. Given the fundamental importance of ATP binding and sliding clamp formation to the molecular switch model of MMR, we hypothesized that generating the human homologs of the yeast Msh2 ATPase domain mutants would selectively inhibit steps of the proposed mechanism of the molecular switch model. By re-expressing the human MSH2 ATPase domain mutants, G827D and R711C, and a previously described MSH2 Walker box A ATP binding motif mutant, K675A (Heinen et al., 2011), in an MSH2-null Hec59 cell background we would be able to make functional observations about the molecular switch model of MMR in human cells. These observations could allow us to validate the molecular switch model of MMR in human cells for the first time as well as investigate the requirement for the molecular switch model in the MMR-dependent DDR.

C. MATERIALS AND METHODS

Mismatch Repair Protein Preparation

R711C and G827D MSH2 ATPase domain mutations were generated using QuikChange®-based PCR mutagenesis (Agilent Technologies). Wild-type, R711C and G827D MSH2 heterodimers were expressed and purified as previously described (Heinen, 2002). Generation and purification of the K675A MSH2 mutant is described elsewhere (Heinen, 2011).

Adenosine Nucleotide Binding

200 nM wild-type or MSH2 mutant-MSH6 protein was incubated with 250 nM [γ-^{32}P]-ATP for 20 minutes at room temperature in 25 mM Hepes (pH 8.1), 110 mM NaCl, 1 mM DTT, 5% glycerol, (+/-) 5 mM $MgCl_2$ and (+/-) 1 mM EDTA. Samples were cross-linked for 10 minutes using a UV Stratalinker 1800 (Stratagene) and resolved using 6% SDS-PAGE. Gels were dried and bands visualized using a Cyclon Storage Phosphor System (PerkinElmer). Bands were quantified using ImageJ.

ATPase Assay

15-100 nM wild-type or MSH2 mutant-MSH6 protein was incubated with 16.5 nM [γ-^{32}P]-ATP, 20-240 µM unlabeled ATP and a G/T mismatch-containing 41-bp oligonucleotide for 30 minutes at 37^{o}C in 25 mM Hepes (pH 8.1), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 15% glycerol (20 µl reaction volume). Reactions were quenched with 400 µl of 10% charcoal and 1mM EDTA. Hydrolyzed [γ-^{32}P]-ATP was detected using liquid scintillation following charcoal pelleting.

Surface Plasmon Resonance Analysis

SPR analysis was carried out using the ProteOn™ XPR36 protein interaction array system (Bio-Rad). A 60-base pair oligonucleotide with a central G/T mismatch and 5' biotinylation was

bound to a neutravidin-containing NLC ProteOn™ sensor chip (Bio-Rad). Mismatch binding strength analysis was carried out by injecting 0-120 nM protein over the chip at a rate of 30 µl/min for 3 min in a buffer containing 25 mM Hepes (pH 8.1), 110 mM NaCl, 1 mM DTT, 2 mM $MgCl_2$ and 2% glycerol. Dissociation with buffer alone was observed for 3 min. Samples were kept on ice prior to injection and experiments were carried out at 25°C. The sensor chip was regenerated after each run by injecting 2.5 M NaCl at 100 µl/min for 1 min. Data were analyzed using the ProteOn Manager™ software with a Langmuir fit. Non-specific binding was subtracted using an interspot reference.

ATP-dependent dissociation off-rate analysis was completed by injecting 50 nM protein over the chip for 3 min as above. ATP-dependent dissociation was observed by injecting an identical buffer containing 50 nM ATP over the chip at 30 µl/min for 3 min. Data were exported and analyzed using Prism 5 software (GraphPad) and a least-squares fit.

Generation of lentiviral cell lines

Wild-type and MSH2 mutant cDNAs were PCR-amplified and sub-cloned into the lentiviral vector pCDH-EF1-MCS-T2A-Puro (System Biosciences). Lentivirus was generated according to manufacturer's protocol. Following transduction, Hec59 cells were maintained in DMEM:F12 with 10% fetal bovine serum (Gibco) and 0.5 µg/mL puromycin (Gibco). Cells were incubated at 37°C and 5% CO_2.

Chemicals, reagents and antibodies

N-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (CAS: 70-25-7). O^6-Benzylguanine (O^6-BG) and hydroxyurea (HU) were from Sigma. Antibodies were: MSH2 (Western blot; BD Biosciences 556349; 1:1000), MSH2 (Immunoprecipitation; Calbiochem

NA27), MSH6 (Bethyl A300-023A; 1:850), MLH1 (BD Biosciences 550838; 1:1000), TATA-binding protein (TBP; Abcam ab63766; 1:2000), Histone 3 (H3; Abcam ab24834; 1:2000) and Actin (Sigma A5060; 1:5000).

Chromatin Localization Assay

Hec59 lentiviral cell lines were synchronized in S-phase with 2 mM HU for 18 h in complete medium. At 16 h, cells were incubated with 25 μM O^6-BG or an equivalent volume of DMSO for 2 h. Cells were washed twice in phosphate-buffered saline (PBS) and treated with 25 μM O^6-BG and 5 μM MNNG or an equivalent volume of DMSO for 4 h in complete medium before harvesting. Cells were harvested with 0.25% trypsin (Gibco) then washed in PBS and 10 mM NaCl, 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$. To remove cytosolic and nuclear soluble proteins, cells were incubated on ice for 10 minutes in 10 mM NaCl, 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 0.5 mM DTT, 0.5% NP-40 and protease and phosphatase inhibitors. Cells were homogenized with a dounce homogenizer then nuclei were collected via centrifugation at 3400 rpm for 15 minutes. Nuclei were rotated at 4^{o}C for approximately 45 minutes in 420 mM NaCl, 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM DTT, 5% glycerol, 0.75% Triton-X 100, 1 U DNase I and protease and phosphatase inhibitors to create a protein fraction from chromatin-associated proteins. Protein extracts were separated by 10% SDS-PAGE and examined by Western blot. Western blot images were developed using a ChemiDoc™ XRS+ imaging system (Bio-Rad) and bands quantified with ImageJ. MSH2 band intensity was normalized to wild-type MSH2 DMSO-treated. At least six separate experiments were quantified and the average fold induction of MSH2 chromatin localization was determined using Prism 5 (GraphPad). Statistical significance was determined with analysis of variance and Tukey's post-test.

In vivo MMR Assay

The heteroduplex repair plasmid (Zhou et al., 2009) was produced by transforming XL-1 blue cells with p111 plasmid. Infection with the M13KO7 phage (New England Biolabs) produced viral particles that could be collected using polyethylene glycol precipitation. Single-stranded circular plasmid DNA was collected using CsCl centrifugation and phenol-chloroform precipitated. Linearized p189 was denatured and annealed to the single-stranded p111 plasmid to create a nick-directed heteroduplex repair plasmid. To assess *in vivo* MMR, cells were transfected with 1.5 µg repair plasmid and 1.0 µg of an RFP-expressing plasmid as a transfection efficiency control. Approximately 36 hours after transfection, GFP and RFP expression was detected using a LSRII-B flow cytometer (BD Biosciences). GFP fluorescence was normalized to RFP fluorescence to account for transfection efficiency differences. Analysis of variance was performed to detect statistical differences between groups.

Cell Cycle Analysis

Hec59 lentiviral cell lines were synchronized with HU as above. At 16 h cells were treated with 25 µM O^6-BG for 2 h then with 1 µM MNNG plus 25 µM O^6-BG for 48 h. At 48 h cells were either released into normal growth medium to recover for 24 h (72 h time point) or harvested and fixed in 70% ethanol at -20^{o}C. Cells were stained in 20 µg/mL propidium iodide and 200 µg/mL RNase A for 1 h at 37 ^{o}C. Cell cycle analyis was on a FACS Calibur flow cytometer (BD Biosciences) using Modfit software (Verity Software House). All time points were completed in duplicate.

Cell Survival Assays

Approximately 500 cells were plated and allowed to attach overnight. Cells were treated with 25 µM O^6-BG for 2 h then with the indicated [MNNG] and 25 µM O^6-BG for 48 h. At 48 h,

fresh growth medium was added and cells were allowed to recover for approximately 10 days. To quantify, cells were harvested with 0.25% trypsin, washed with PBS, fixed in 100% methanol and stained with crystal violet. 6-Thioguanine (6-TG; Meyers et al., 2005), 5-fluorouracil (5-FU; Chen et al., 2010) and cisplatin (Aebi et al., 1996) survival assays were done as previously described. Cells were harvested, fixed and stained as above. Student's t-test was performed to detect statistical differences between groups.

D. RESULTS

MSH2 ATPase domain mutants display differential nucleotide binding affinities in both MSH2 and MSH6

The goal of this study was to validate the molecular switch model of MMR *in vivo* and assess the contribution of ATP binding and sliding clamp formation to downstream MMR and the MMR-dependent DDR in cells. To accomplish this, we generated MSH2 ATPase domain mutations that were predicted to be deficient in one or more aspects of the molecular switch model of human MMR based on previous biochemical work (Hargreaves et al., 2010; Heinen et al., 2011; Figure 2-1).

Following recognition of a mismatch by the MSH2-MSH6 heterodimer, MSH6 and MSH2 sequentially bind ATP and complete a conformational change that converts the MSH2-MSH6 heterodimer into a MSH2-MSH6 sliding clamp that diffuses away from the mismatch while remaining on the DNA according to the molecular switch model (Gradia et al., 1999). Since sliding clamp formation is a fundamental step in the molecular switch model, we first wanted to test the ability of the R711C and G827D mutants to bind ATP in both MSH2 and MSH6 subunits. R711C and G827D mutants have a reduced ability to bind to ATP in MSH6 in the absence of magnesium, which prevents hydrolysis of the gamma phosphate of ATP (Figure 2-2). The R711C mutant has an over 90% reduction in ATP binding by MSH2 and the G827D mutant has no measurable ATP binding by MSH2 (Figure 2-2). However both mutants have significantly increased binding by MSH6 in the presence of magnesium relative to the wild-type control (Figure 2-2). The G827D mutation could occlude ATP from the MSH2 binding site while

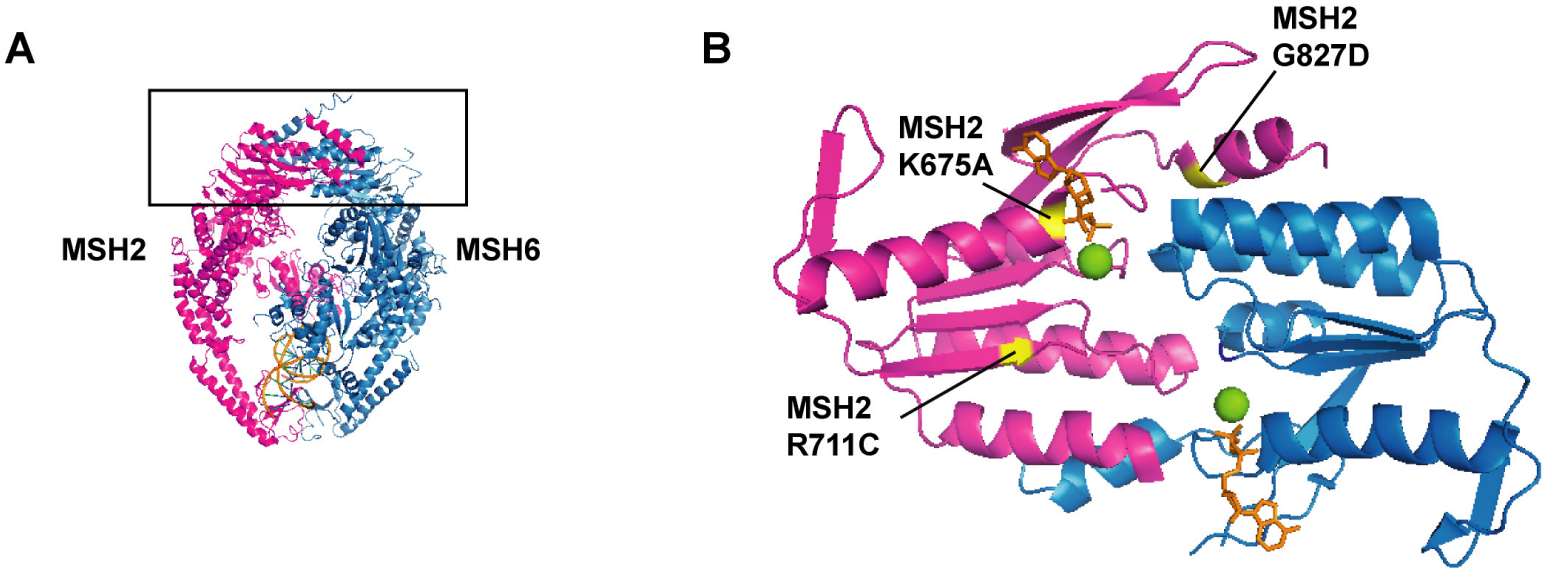
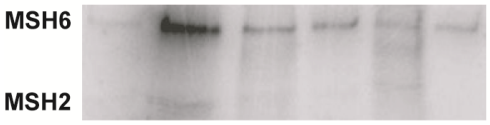
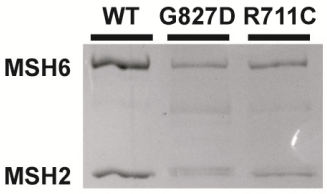

A
MSH2
MSH6
B
MSH2
G827D
MSH2
K675A
MSH2
R711C

Figure 2-1. MSH2 ATPase domain mutations modeled in the MSH2-MSH6 heterodimer crystal. A. Complete crystal structure of the MSH2-MSH6 heterodimer bound to ADP and G/T mismatched DNA (protein data bank ID 2O8B) with the ATPase domains highlighted. **B.** Top view of the ATPase domains of MSH2 and MSH6 with the locations of the MSH2 mutations indicated in yellow, ADP in orange and magnesium in green.





	WT		R711C		G827D	
Mg^{2+}	+	-	+	-	+	-
MSH6	0.13 ± 0.05	1.00 ± 0.00	0.32 ± 0.06	0.37 ± 0.07	0.13 ± 0.07	0.14 ± 0.04
MSH2	ND[a]	1.00 ± 0.00	0.07 ± 0.01	0.01 ± 0.01	0.06 ± 0.06	ND[a]

[a] Not Determinable

Figure 2-2. MSH2 ATPase domain mutations affect ATP binding. Representative autoradiograph showing [γ-^{32}P]-ATP binding by recombinant wild-type and MSH2 mutant heterodimers with coomassie-stained total protein loading control below. 200 nM wild-type and MSH2 mutant protein was incubated at room temperature with 250 nM [γ-^{32}P]-ATP, cross-linked and separated using SDS-PAGE. Dried gels were exposed overnight and developed using a phosphoimager. Quantification was carried out using ImageJ and is the result of three separate experiments. Quantification is displayed with standard error of the mean.

the R711C mutation is in a location that contributes to the integrity of the MSH2 ATP binding site. The increased ATP binding seen in the presence of magnesium could indicate an inability of these mutants to hydrolyze ATP. The K675A mutant is able to bind ATP at wild-type levels by MSH6, but is completely unable to bind ATP by MSH2 (Heinen et al., 2011). This is not surprising given that MSH2 lysine 675 is a highly conserved residue that is part of a Walker Box A motif involved in ATP binding (Iaccarino et al., 1998). In the presence of magnesium, there is no measurable detection of ATP binding by both MSH2 and MSH6 in the K675A mutant (Heinen et al., 2011).

MSH2 ATPase domain mutants do not hydrolyze ATP

According to the molecular switch model of MMR, ATP-bound MSH2-MSH6 sliding clamps must hydrolyze ATP following repair of a mismatch in order to return to a state that is capable of recognizing mismatches once again (Gradia et al., 1997). W next asked if the mutants could hydrolyze ATP. Both R711C and G827D had no ATPase activity above background as measured by the release of the terminal ATP phosphate, γ-^{32}P (Figure 2-3). The K675A mutant had very modest ATPase activity (Heinen et al., 2011). This finding supports our hypothesis that the increased ATP binding seen in the MSH6 subunit by the R711C and G827D mutants in the presence of magnesium is due to a lack of ATPase activity (Figure 2-2). Taken together, the R711C and G827D ATP binding and ATPase data indicate that both MSH2 and MSH6 make important contributions to each other's ATPase domains that are greater than the sum of the parts. The K675A mutation is within a highly conserved ATP binding motif and only affects ATP binding in the MSH2 subunit of the heterodimer, however the overall ATPase activity of the MSH2-MSH6 heterodimer is significantly affected.

G/T DNA stimulated-ATPase activity

Protein	k_{cat}	$K_m \times 10^{-6}$
	min^{-1}	M
WT	22.0 ± 5.8	72.2 ± 47.6
R711C	ND[a]	ND[a]
G827D	ND[a]	ND[a]

[a] Not Determinable

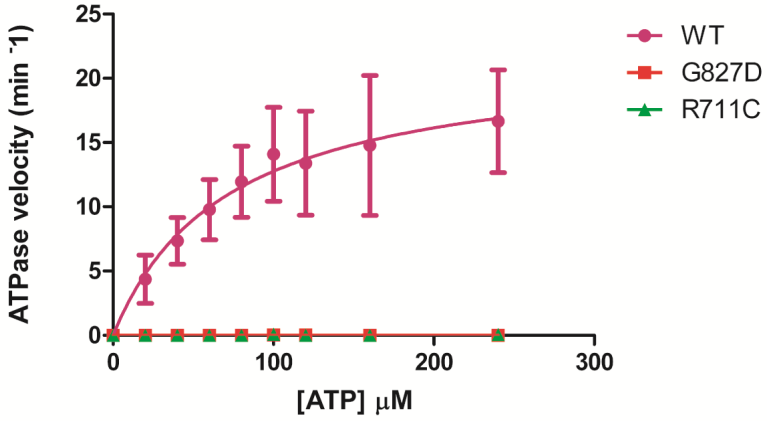


Figure 2-3. MSH2 ATPase domain mutations affect ATP hydrolysis. 15-100 nM wild-type and MSH2 mutant protein was incubated with a G/T mismatch-containing 41-base pair oligonucleotide and [γ-^{32}P]-ATP for 30 min. at 37^{o}C. ATPase velocity was determined by measuring the release of γ-^{32}P using charcoal binding (Gradia et al., 1997). The curve was fitted using a Michaelis-Menten equation. Error bars represent the standard error of the mean of at least four separate experiments.

MSH2 ATPase domain mutants bind G/T mismatches, but less efficiently than wild-type

Previous biochemical work (Mazur et al., 2006; Heinen et al., 2011) has supported the molecular switch model of MMR in which the MSH2 subunit of the mismatch recognition heterodimer binds ADP off of DNA while the ATP binding domain of MSH6 remains empty. Binding to a mismatch increases ATP binding by MSH6, which drives an ADP-ATP exchange in the MSH2 subunit. The dual ATP-bound form of MSH2-MSH6 is now free to diffuse from the mismatch, but stably remain on the DNA. Since this is a fundamental step in the molecular switch model of MMR, we first wanted to ask if R711C and G827D would bind to mismatches. We used SPR and increasing concentrations of wild-type and MSH2 mutant MSH2-MSH6 to measure the rate of association with a G/T mismatch (k_{on}) and the strength of mismatch binding (K_d). Both the R711C and G827D mutants were able to bind mismatches, but displayed slower k_{on} values relative to wild-type MSH2-MSH6 as well as increased K_d values (Table 2-1). The increased K_d values of mismatch binding indicate that the MSH2 mutants may have structural abnormalities that prevent mismatch binding as tightly as wild-type MSH2-MSH6.

MSH2 mutants undergo delayed ATP-dependent dissociation from mismatches

Since our MSH2 mutants were able to bind a G/T mismatch, but had significantly reduced ATP binding by MSH2, we then asked whether there were any differences in their ATP-dependent dissociation from mismatches. According to the sliding clamp model of MMR, ATP-dependent dissociation of MSH2-MSH6 heterodimers results in the iterative loading of MSH2-MSH6 heterodimers. Using SPR analysis as previously described (Cyr and Heinen, 2008), we pre-bound wild-type and MSH2 mutant MSH2-MSH6 heterodimers to G/T mismatches then dissociated using 50µM ATP (Figure 2-4). Experiments at 500µM and 250µM ATP showed no difference in dissociation (data not shown) so we utilized a decreased [ATP] based on the

Table 1

DNA mismatch binding affinity

Protein	k_{on}	k_{off}	K_D
	$10^6\ M^{-1}\ s^{-1}$	s^{-1}	nM
WT	0.86	0.0011	1.3
R711C	0.63	0.0033	5.3
G827D	0.0086	0.00031	36.3

ATP-dependent mismatch dissociation

Protein	k_{off}
	s^{-1}
WT	0.004
R711C	0.0005
G827D	0.00008

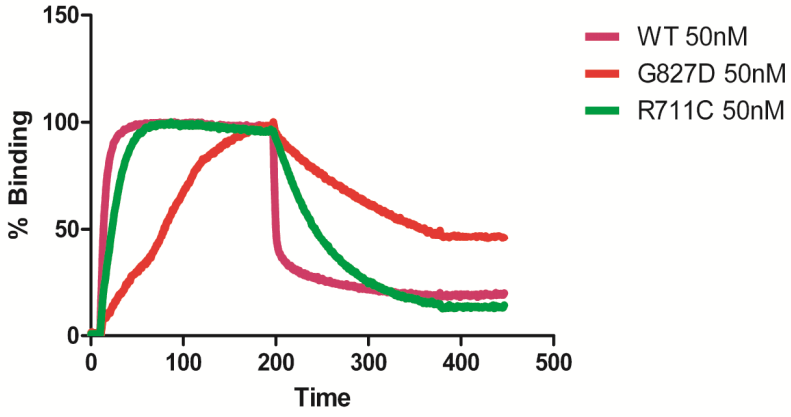


Figure 2-4. MSH2 ATPase domain mutants bind G/T mismatches, but do not undergo efficient ATP-dependent mismatch dissociation. SPR analysis of 50 nM wild-type or MSH2 mutant protein pre-bound to an oligonucleotide containing a central G/T mismatch and dissociated with 50 nM ATP.

hypothesis that the higher [ATP] were forcing MSH2 and MSH6 to bind ATP and form sliding clamps. Both the R711C and G827D mutants showed slower ATP-dependent dissociation at 50μM indicating decreased sliding clamp formation. The K675A mutant did not form sliding clamps as efficiently as wild-type MSH2-MSH6 in the presence of 250μM ATP (Heinen et al., 2011). These data are consistent with the molecular switch model and indicate that the inability of MSH2 ATPase domain mutants to properly bind ATP may result in these mutants remaining associated with mismatches for longer periods of time. This would prevent the mismatches from being recognized by additional MSH2-MSH6 heterodimers and accumulating multiple sliding clamps on damaged DNA.

Restoring MSH2 expression in an MSH2-null cancer cell line, Hec59, leads to MSH6 protein stability

Following the biochemical characterization of the MSH2 ATPase domain mutants, we used a lentivirus to transduce MSH2-null Hec59 endometrial cancer cells with wild-type, ATPase domain mutant MSH2 or empty lentiviral vector under puromycin selection. The presence of a T2A peptide, which adds water during protein translation (Donnelly et al., 2001), in the puromycin resistance gene in the lentiviral vector ensures that anything cloned in-frame into the multiple cloning site will be co-translated with puromycin resistance. Since Hec59 cells are MSH2-null, using a lentivirus to restore MSH2 expression creates cell lines that stably express wild-type or mutant MSH2 with which to study *in vivo* MMR. Restoring the expression of any form of MSH2 in Hec59 cells stabilizes MSH6 (Figure 2-5). The G827D MSH2 Hec59 line appeared to have decreased MSH2 expression indicating that the G827D MSH2 protein may not be as stable as the others. The lentiviral cell lines grew normally relative to non-transduced Hec59 cells. MLH1 expression was unaffected by the re-expression of MSH2 (Figure 2-5).

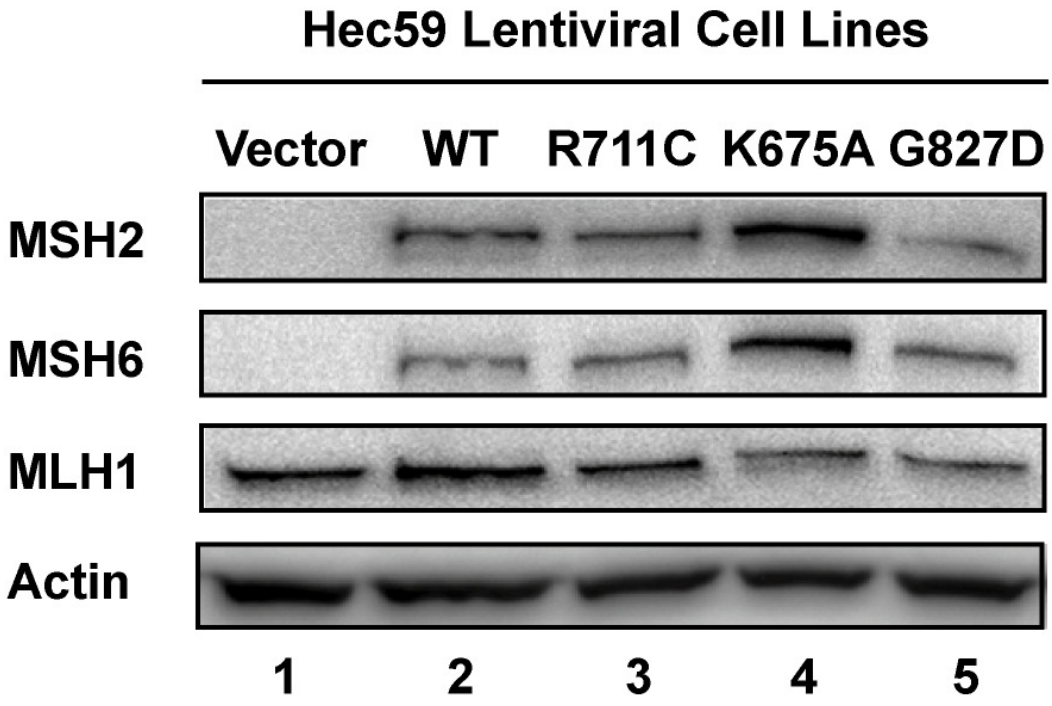

Hec59 Lentiviral Cell Lines
Vector
WT
R711C
K675A
G827D
MSH2
MSH6
MLH1
Actin
1
2
3
4
5

Figure 2-5. Expression of wild-type or mutant MSH2 stabilizes MSH6 protein in Hec59 cells. Wild-type or mutant MSH2 expression was restored in Hec59 cells using lentivirus transduction. Approximately 50 µg of total protein was separated using 10% SDS-PAGE and analyzed by Western blot with antibodies against MSH2, MSH6, MLH1. Actin was used as a loading control.

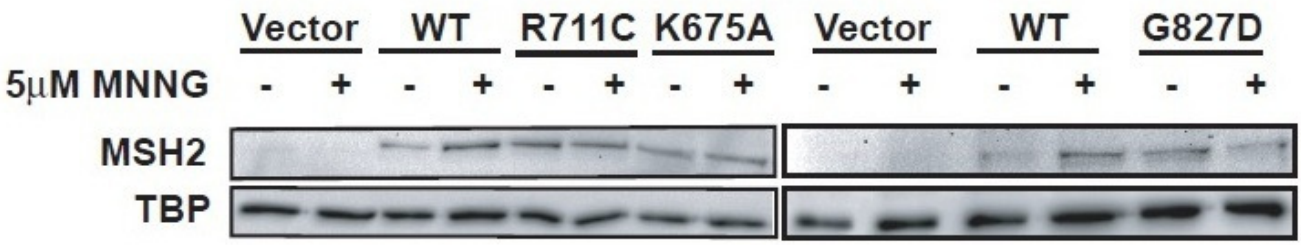
These data indicate it is possible to stably restore MSH2 expression in a human MSH2-null cell background in order to ask questions about human cellular MMR.

MSH2 mutants do not localize to chromatin in response to DNA alkylation damage

With the new MSH2 Hec59 lentiviral cell lines, we first wanted to know if DNA alkylation damage would induce the chromatin localization of our MSH2 mutants (Mastrocola and Heinen, 2010a). Recruitment of MSH2-MSH6 heterodimers to mismatches and the formation of multiple sliding clamps is necessary for downstream MMR and damage response functions based on the molecular switch model of MMR. We synchronized the MSH2 Hec59 lentiviral cell lines with HU, treated them with 5μM MNNG for 4 hours then harvested the cells and isolated soluble protein- and chromatin protein-enriched fractions. After treatment with MNNG, the chromatin enriched levels of MSH2 increased almost 4-fold in the wild-type MSH2 Hec59 cells when compared to the mock (DMSO)-treated wild-type MSH2 cells (Figure 2-6). None of the MSH2 mutants displayed an MSH2 chromatin localization induction in response to alkylation damage (Figure 2-6). Combined with the SPR biochemical data that shows MSH2 mutants binding mismatches, but do not dissociate efficiently and form ATP-dependent sliding clamps well, these data suggest that the MNNG-induced chromatin localization observed in wild-type cells is due to sliding clamp formation, consistent with the molecular switch model.

MSH2 mutants can not repair mismatches *in vivo*

Since alkylation damage by MNNG did not induce increased chromatin localization of the MSH2 mutants, the next question we wanted to ask was whether the MSH2 mutants were able to repair mismatches in cells using a mismatch-containing GFP reporter plasmid (Zhou et al., 2009). Repair of the mismatch results in GFP expression that can be detected by flow cytometry. The lentiviral vector-only Hec59 cell line was unable to repair mismatches and


Vector
WT
R711C
K675A
Vector
WT
G827D
5μM MNNG
- + - + - + - + - + - + - +
MSH2
TBP

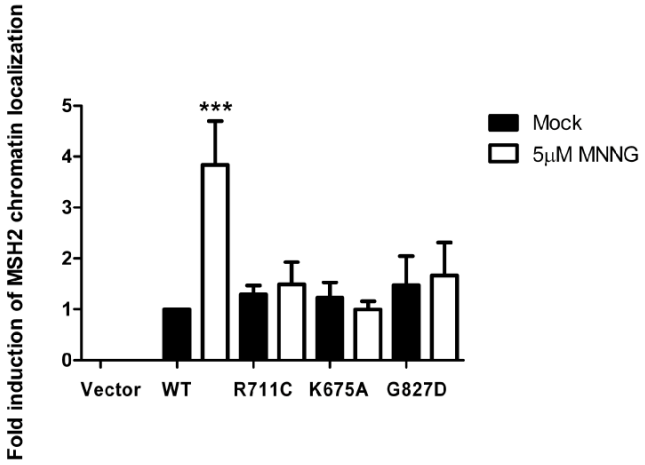

Fold induction of MSH2 chromatin localization
5
4
3
2
1
0
***
Vector
WT
R711C
K675A
G827D
Mock
5μM MNNG

Figure 2-6. MSH2 mutants do not localize to chromatin in response to MNNG treatment. Representative Western blot image showing MSH2 chromatin recruitment in lentiviral Hec59 cell lines in response to MNNG. Lentiviral Hec59 cells were synchronized in S-phase using HU and treated with O^6-BG and 5 µM MNNG or DMSO for 4 hours. Chromatin-enriched extracts were prepared 4 hours following HU release. Approximately 28 µg of total protein was separated using 10% SDS-PAGE and analyzed by Western blot with antibodies against MSH2 and TBP as a loading control. Quantification is the result of six separate experiments and represents the average MSH2 fold change relative to DMSO-treated WT MSH2 Hec59 cell levels.

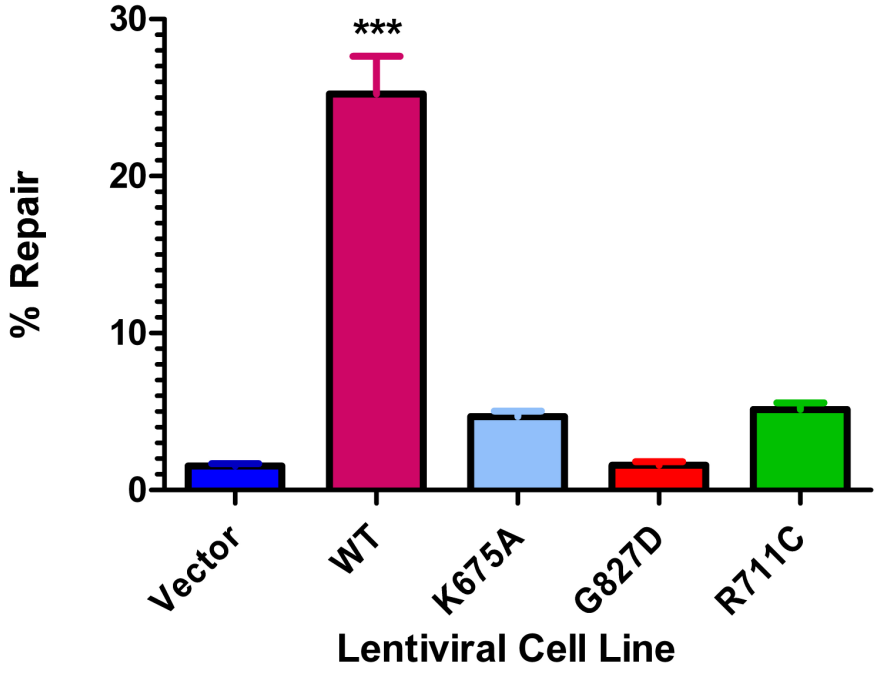

30
20
10
0
% Repair
***
Vector
WT
K675A
G827D
R711C
Lentiviral Cell Line

Figure 2-7. MSH2 ATPase domain mutations eliminate *in vivo* MMR activity. A GFP-encoding heteroduplex plasmid containing a premature stop codon was transfected into the lentiviral Hec59 cell lines. A plasmid encoding RFP was co-transfected as a transfection efficiency control. After 48 hours, fluorescence was detected using flow cytometry. Error bars indicate the standard error of the mean of the averages of three separate experiments. Asterisks indicate $p < 0.001$.

restore GFP expression whereas re-expression of wild type MSH2 resulted in a significant increase in GFP-positive cells (Figure 2-7). The G827D MSH2 Hec59 cell line was similarly unable to repair mismatches *in vivo*. The R711C and K675A MSH2 Hec59 cell lines were unable to significantly increase mismatch repair levels beyond the background vector-only level even though they did display a slightly increased repair percent. Together, the MMR assay suggests that all the MSH2 mutants are unable to repair mismatches *in vivo*.

MSH2 ATPase domain mutations increase alkylation resistance, but have no effect on other DNA lesions

Cells deficient in MMR demonstrate increased resistance to alkylation damage-induced cell cycle arrest and apoptosis (Adamson et al., 2005). To determine if the MSH2 mutants were resistant to alkylation damage, we used a colony-forming assay with the MSH2 Hec59 cells (Figure 2-8). Cells were plated out at low density and treated with low-doses of MNNG for 48 hours then allowed to recover for approximately 10 days. Following recovery, cells were fixed, stained with crystal violet and colonies counted. At all doses of MNNG, the MSH2 mutant Hec59 cells were significantly more resistant to the MNNG than wild-type MSH2 Hec59 cells (Figure 2-8). However at 1μM MNNG, the R711C and K675A MSH2 Hec59 cell lines were more sensitive to the alkylation damage than the G827D and LV vector-only Hec59 cell lines (Figure 2-8). This indicates that the MMR-dependent DDR in the R711C and K675A MSH2 Hec59 cells could be partially intact though blunted. The MMR pathway is known to be involved in detecting additional DNA lesions in addition to the cytotoxic O^6-me-G caused by MNNG. Because of this, we investigated the response of our Hec59 lentiviral cell lines to 6-TG, 5-FU and cisplatin (Figure 2-9). A previously published report demonstrated a small difference between Hec59 and Hec59 2-4 cells, in which MSH2 is re-expressed off chromosome 2, to 6-

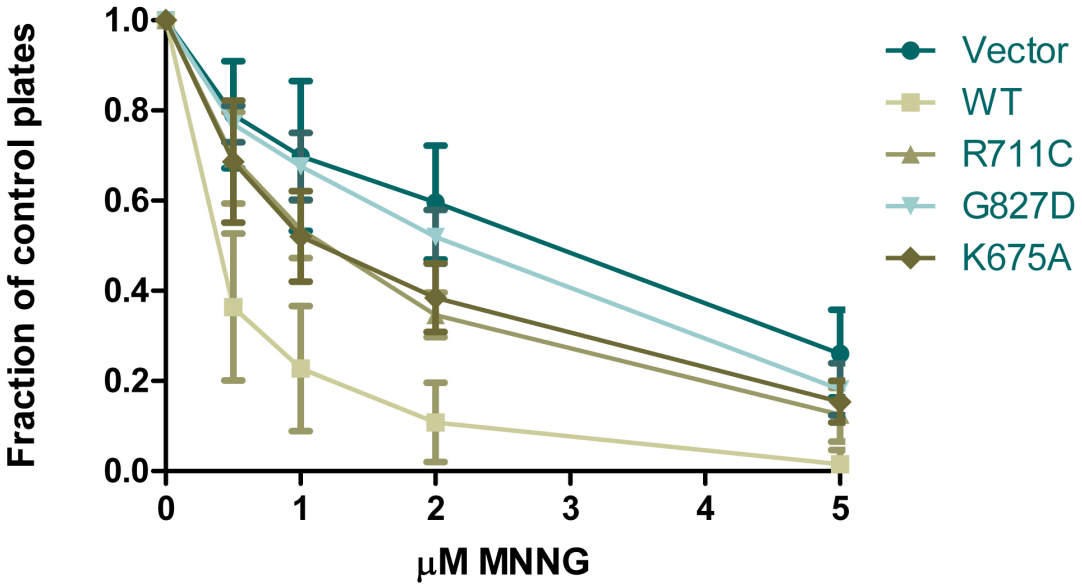
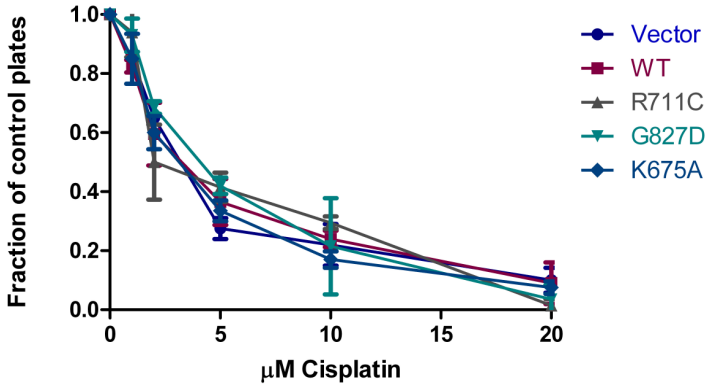
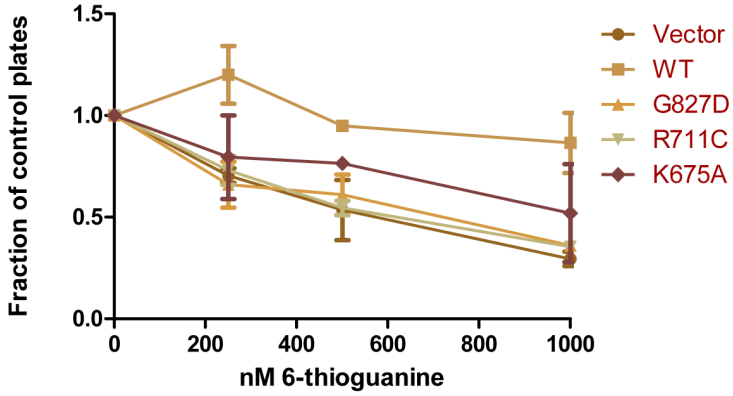

Fraction of control plates
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
μM MNNG
Vector
WT
R711C
G827D
K675A

Figure 2-8. MSH2 ATPase domain mutations affect Hec59 cell survival in response to alkylation damage. Lentiviral Hec59 cell lines were plated at low density and treated with O^6-BG and MNNG for 48 hours then allowed to recover for approximately 10 days in normal growth media. Cells were washed, fixed, stained and counted to determine colony number. Error bars indicate the standard error of the mean of the averages of three separate experiments done in duplicate. Asterisk indicates $p < 0.05$.


Fraction of control plates
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
μM Cisplatin
Vector
WT
R711C
G827D
K675A


Fraction of control plates
1.5
1.0
0.5
0.0
0
200
400
600
800
1000
nM 6-thioguanine
Vector
WT
G827D
R711C
K675A

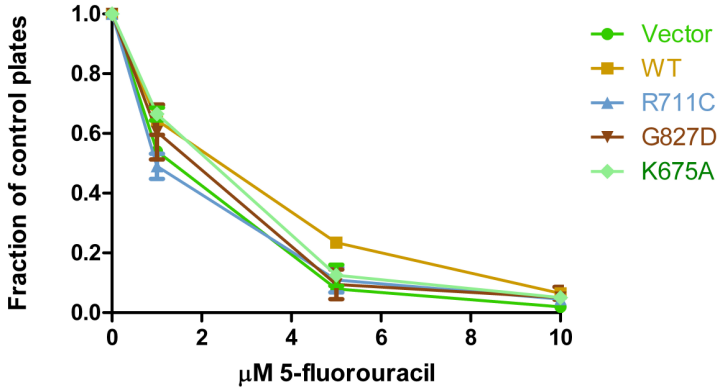

Fraction of control plates
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
μM 5-fluorouracil
Vector
WT
R711C
G827D
K675A

Figure 2-9. MSH2 ATPase domain mutations do not affect Hec59 cell survival in response to cisplatin, 6-TG and 5-FU. Lentiviral MSH2 mutant Hec59 cell lines were plated out at low density and treated as previously described (Aebi et al., 1996; Chen et al., 2010; Meyers et al., 2005).

TG, but it was not clear if this difference was significant or not (Meyers et al., 2005). We did not detect any significant differences between our Hec59 lentiviral cell lines. Another previously published report did not find any significant difference between Hec59 and Hec59 2-4 cells in response to 5-FU treatment (Chen et al., 2010). There was no difference between our Hec59 lentiviral cell lines in response to 5-FU treatment. It has previously been reported that there is an approximately 2-fold increase in cisplatin tolerance in Hec59 cells relative to Hec59 2-4 cells (Aebi et al., 1996). However, we detected no significant difference between our Hec59 lentiviral cell lines when treated with various concentrations of cisplatin. All together, this indicates that MMR is primarily involved in the detection of alkylation damage and the O^6-me-G lesion caused by MNNG in particular and plays a smaller role in the detection and potential repair of these other lesions.

MSH2 ATPase domain mutations prevent MMR-dependent G_2/M cell cycle arrest

Because of the decreased survival at 1μM MNNG by the R711C and K675A MSH2 Hec59 cells, we wanted to investigate whether this decreased colony-forming response was due to apoptosis or an activated cell cycle checkpoint. Cells with intact MMR, which are treated with an alkylating agent, cell cycle arrest in the second G_2/M and undergo apoptosis (Mastrocola and Heinen, 2010b). We treated the MSH2 Hec59 lentiviral cell lines with 1μM MNNG for 48 hours then harvested or allowed 24 hours recovery in normal growth media before harvesting and staining with propidium iodide for flow cytometry analysis. All mock-treated and untreated cells cycled normally. After 48 hours in 1μM MNNG, wild-type MSH2 Hec59 cells displayed a strong G_2/M arrest characteristic of cells with an intact MMR response while lentiviral vector-only Hec59 cells showed no G_2/M arrest (Figure 2-10). None of the MSH2 mutant Hec59 cell lines showed any G_2/M arrest in response to MNNG alkylation damage. After 24 hours recovery,

wild-type MSH2 Hec59 cells remain arrested in G_2/M, but now have a substantial sub-G_1 peak indicating apoptosis. Even after 24 hours recovery, none of the MSH2 mutant Hec59 cells arrest in G_2/M or undergo apoptosis. These data suggest that the DDR is impaired by the mutations in the MSH2 ATPase domain.

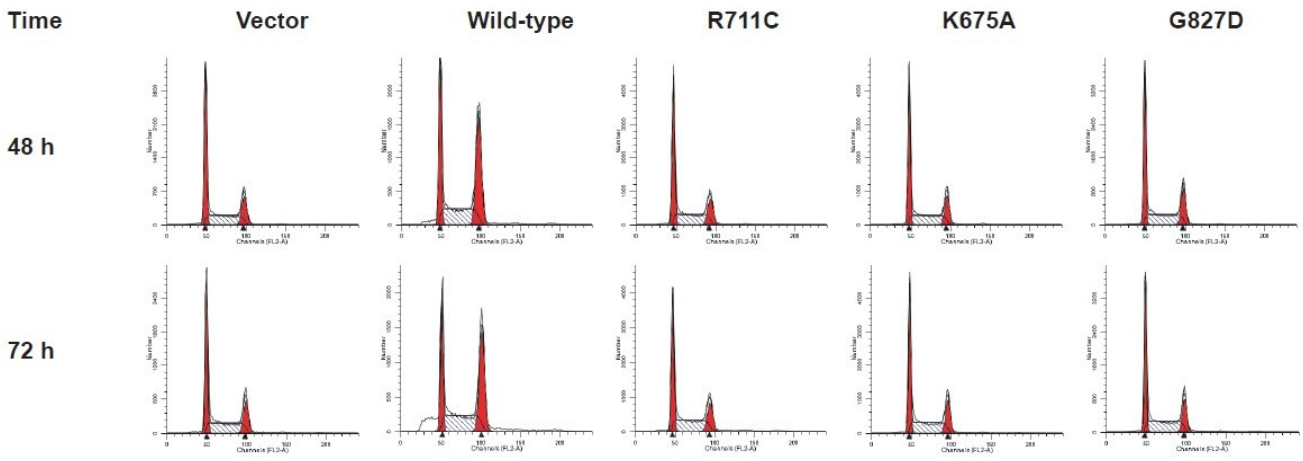

Time
Vector
Wild-type
R711C
K675A
G827D
48 h
72 h

Figure 2-10. MSH2 ATPase domain mutations prevent MMR-dependent G_2/M cell cycle arrest in Hec59 cells. Representative flow cytometry plots showing cell cycle progression in lentiviral Hec59 cell lines after MNNG treatment. Lentiviral Hec59 cell lines were treated with O^6-BG and 1 μM MNNG for 48 hours then harvested or allowed to recover for 24 hours. Cells were washed, fixed, stained with propidium iodide then analyzed for DNA content using flow cytometry.

E. DISCUSSION

The MMR pathway is an important system to preserve genomic integrity as evidenced by the hereditary cancer syndrome, LS, which is caused by inherited mutations in the MMR pathway. Most of the work done on the MMR pathway has been *in vitro* biochemistry in yeast and bacteria. There is far less data about MMR in humans. The eukaryotic biochemical data has suggested three possible mechanisms whereby mismatch recognition, strand discrimination and excision are linked: static transactivation, active translocation and molecular switch. The static transactivation model posits that MSH2-MSH6 remain bound at a mismatch and bend the DNA until a distant initiation site for strand excision is encountered (Junop et al., 2001; Schofield et al., 2001). This model has fallen into disfavor since the observation that protein "roadblocks" prevent strand excision, an outcome not predicted by the model (Pluciennik and Modrich, 2007; Wang and Hays, 2004). The active translocation model hypothesizes that MSH2-MSH6 bind ATP while bound to a mismatch, but hydrolysis of the ATP provides energy for movement along the DNA helix until a site for initiation of strand excision is found (Allen et al., 1997). The third model, molecular switch, is similar to the active translocation model except movement from the mismatch is ATP hydrolysis-independent and only requires ATP binding (Gradia et al., 1997). In this manner, the mismatch is continually available to be bound by MSH2-MSH6 heterodimers until excision removes it. Thus numerous MSH2-MSH6 sliding clamps accumulate along the DNA bidirectionally (Gradia et al., 1999).

Since these models were all developed through *in vitro* biochemistry it is unclear which one, if any, reflect human cellular MMR. The biochemistry used to generate mechanistic MMR models utilizes small linear and circular heteroduplexes of naked DNA, which is unlike the chromatin MMR would be repairing in cellular nuclei. Indeed, there is biochemical evidence that

nucleosomes inhibit MMR efficiency *in vitro* (Li et al., 2009). The goal of this study was to validate the molecular switch model of MMR in human cells using MSH2 ATPase domain mutants, unable to complete key steps predicted by the molecular switch model like ATP binding and sliding clamp formation, then re-expressed in an MSH2-null background, Hec59, to create stable cell lines to work with and study *in vivo* human MMR.

Previously published work detailed numerous yeast Msh2 ATPase domain mutants that were deficient in nucleotide binding, sliding clamp formation and Mlh1-Pms1 binding (Hargreaves et al., 2010). The mismatch-provoked ADP to ATP exchange by MSH2-MSH6 heterodimers and subsequent sliding clamp formation are key steps in the molecular switch model of MMR. In order to investigate the molecular switch model *in vivo*, we had to biochemically characterize MSH2 homologues of the yeast Msh2 mutants. Both MSH2 and MSH6 have composite ATPase sites with each protein subunit contributing to the other's ATPase site. The MSH6 ATPase domain is more disordered and flexible than the MSH2 ATPase domain (Warren et al., 2007). The two MSH2 mutants we generated based on the work of Hargreaves et al., R711C and G827D, plus the MSH2 Walker A motif mutant, K675A, are all deficient at binding ATP in the MSH2 subunit, but all three retain some ATP binding capacity in the MSH6 subunit with the MSH2 K675A mutant having wild-type MSH2-MSH6 levels (Heinen et al., 2011). The inability of the R711C and G827D mutants to bind ATP in the MSH2 site could be explained by these mutations disrupting the more structured MSH2 ATPase domain thereby making it more disordered like the MSH6 site. Although the R711C and G827D MSH2 mutations are not near the MSH6 ATPase site (Figure 2-1), the greatly reduced ATP binding observed in the MSH6 subunit for these mutations suggests that these regions are structurally involved both in the conformational change that accompanies mismatch binding and the general

structure of the composite MSH6 ATPase site. The K675A MSH2 mutation is highly-conserved in directly coordinating with the ATP molecule that it is not surprising that this mutation has no effect on ATP binding by the MSH6 subunit. It is not involved structurally in the composite ATPase domain sites of MSH2 and MSH6. SPR studies indicated that all three MSH2 ATPase domain mutants were able to bind mismatches albeit G827D doing so much less efficiently than the K675A and R711C mutants. All three MSH2 mutants formed ATP-dependent sliding clamps less efficiently than wild-type MSH2-MSH6 with the G827D MSH2 mutant being the least efficient. None of the MSH2 mutants had any significant ATPase activity. Our results suggest that the MSH2 ATPase domain mutants can not dissociate readily from mismatches because of the ATP binding deficiencies that prevent them from finalizing the conformational change necessary to form sliding clamps. This observation and the lack of ATPase activity indicates that the MSH2 ATPase domain mutants may act as dominant negatives in the cell both by preventing other MSH2-MSH6 heterodimers from recognizing mismatches and being unable to hydrolyze bound ATP, a necessary step towards recycling sliding clamps into a MSH2-MSH6 heterodimer that is capable of binding mismatches once more.

Since our goal was to validate the molecular switch model of MMR *in vivo* and we had MSH2 mutants that were deficient at key steps of the model, we re-expressed these MSH2 mutants in an MSH2-null cell line, Hec59, to prevent any interference from wild-type MSH2. Using these MSH2 mutants we were able to stabilize MSH6. Using an established *in vivo* MMR assay (Zhou et al., 2005) we observed that the MSH2 mutants were unable to direct MMR in cells. This is consistent with our hypothesis that ATP binding and sliding clamp formation are key steps to *in vivo* human MMR. Treating MMR-proficient cells with MNNG induces chromatin localization of MSH2-MSH6 followed by MLH1-PMS2 (Mastrocola and Heinen,

2010b). We observed no increase in MSH2 mutant chromatin localization following MNNG treatment of the Hec59 cell lines, but a significant increase in wild-type MSH2 chromatin localization. Given the ability of the MSH2 mutants to bind mismatches, especially the K675A and R711C mutants, it seems likely that the nearly four-fold increase in wild-type MSH2 on chromatin in response to MNNG we observed is due to repetitive loading of MSH2-MSH6 sliding clamps at single mismatches.

Cells that lack an intact MMR system have an almost 50-fold higher tolerance for alkylation damage caused by MNNG than do cells with intact MMR (Karran, 2001). It is still unclear if key steps in the molecular switch model, namely sliding clamp formation, are necessary to mediate the MMR-dependent DDR. There are two models to account for MMR involvement in detecting and communicating DNA damage, the futile repair cycle model and direct signaling model. The futile repair cycle model posits that MMR does not actually remove the O^6-me-G, but instead removes the mismatched thymine opposite it, which will lead to repetitive and unsuccessful repair attempts. This led to the hypothesis that replication fork collapses, double-strand breaks and stretches of ssDNA during the second cell cycle were responsible for a DNA damage-induced cell cycle arrest (Kaina et al., 1997; Mojas et al., 2007; Stojic et al., 2004). This model is supported by the observation of persistent ssDNA gaps and a MMR-dependent G_2/M arrest in the second cell cycle. The direct signaling model posits that MSH2-MSH6 and MLH1-PMS2 directly signal to ATR and ATR-interacting protein (ATRIP) while bound to a mismatch (Yoshioka et al., 2006; Adamson et al., 2005; Liu et al., 2010). This model is supported by a co-immunoprecipitation study that found direct interactions between a complex of MMR proteins and ATR-ATRIP when O^6-me-G:T lesions were present (Yoshioka et al., 2006). The differences between the two models, namely one requires actual MMR and the

other merely requires the presence of MMR proteins at mismatches, allows us to use our MSH2 mutants to observe whether the mutant MSH2 Hec59 lines would activate a MMR-dependent G_2/M arrest. We observed that all three MSH2 mutants failed to activate a G_2/M cell cycle arrest in response to alkylation damage induced by MNNG. As well, all three MSH2 mutants displayed significantly increased alkylation tolerance relative to wild-type MSH2 Hec59 cells using a clonogenic survival assay. The inability of the MSH2 mutants to carry out *in vivo* MMR, localize to chromatin in response to MNNG and lack of G_2/M arrest seems to support the futile repair cycle model and demonstrate a requirement for the molecular switch model of MMR *in vivo*. However it is unclear if the MSH2 mutants can bind to MLH1-PMS2 *in vivo* so the direct signaling model can not be dismissed. Taken together, the data suggest that ATP binding by both MSH2 and MSH6 and sliding clamp formation and accumulation on DNA are essential steps for both *in vivo* human MMR and the MMR-dependent DDR. This suggests that the molecular switch model of MMR is correct in human cells *in vivo*.

Chapter III: Conclusions and Future Directions

The aim of this project was to validate the molecular switch model of MMR in human cells and investigate a contribution for this model towards the MMR-dependent DDR. We generated MSH2 ATPase domain mutants predicted to be deficient at key steps of the molecular switch model and validated them using *in vitro* biochemistry. We re-expressed these MSH2 mutants in MSH2-null Hec59 cells and treated them with MNNG in order to study the role of the molecular switch model of MMR *in vivo* and its role in the MMR-dependent DDR.

A. MSH2 ATPase domain mutations impair MSH2-MSH6 sliding clamp formation

MSH2 ATPase domain mutants were unable to bind ATP in the MSH2 subunit. MSH6 ATP binding was unaffected for K675A, but significantly reduced for R711C and G827D. ATP hydrolysis was deficient for all MSH2 mutants and efficient sliding clamp formation was impaired relative to wild-type MSH2-MSH6 sliding clamps.

Previous work from our lab observed a negative correlation between mismatch binding and ATPase activity in MSH2 mutants (Heinen et al., 2002), however also uncovering some MSH6 mutations that uncoupled ATPase activity from mismatch binding (Cyr and Heinen, 2008). The R711C MSH2 mutant appears to uncouple mismatch binding from the MSH2-MSH6 heterodimer's ATPase activity while the G827D mutant has reduced mismatch-binding capabilities, although it is still able, as well as a complete lack of ATPase activity. Both the R711C and G827D MSH2 mutants have difficulty with ATP-dependent dissociation from mismatches. Binding to a mismatch by MSH2-MSH6 is known to be asymmetric with MSH6 being the actual mismatch-binding subunit. While both MSH2 and MSH6 have composite ATPase domains comprised of amino acid contributions from the other half, the MSH6 site is less structured than the MSH2 site (Warren et al., 2007). The data here suggest that some regions

of the more-structured MSH2 ATPase domain can occlude the MSH6 ATP binding site. Given the nature of the MSH2 ATPase mutations it is possible that this occlusion is either based on steric hindrance, in the case of the G827D mutation, or due to a charge attraction/repulsion. This would inhibit the mismatch binding-provoked ATP binding by the MSH6 ATPase domain. In turn, this would disrupt the conformational change that the MSH6 ATPase domain is predicted to communicate to the MSH2 ATPase domain that increases its affinity for ATP and allows MSH2-MSH6 sliding clamp formation. Previous work has demonstrated that mutations of the lysine in the Walker A ATP binding motif of MSH2, which eliminates ATP binding, does not affect the ATPase activity of the MSH2-MSH6 heterodimer as strongly as mutating the Walker A lysine in the MSH6 ATPase domain (Iaccarino et al., 1998). Additionally, MSH6 has a stronger affinity for ATP than does MSH2 (Mazur et al., 2006; Heinen et al., 2011). The data here support the previous findings that ATP binding by both MSH2 and MSH6 are crucial for overall ATPase activity by the MSH2-MSH6 heterodimer. Based on our observations of the MSH2 mutants, the ATPase activity of the MSH2-MSH6 heterodimer does not appear necessary for some ATP-dependent dissociation from mismatches. Overall, the data support a model where our MSH2 ATPase domain mutants bind and remain at mismatches longer than wild-type MSH2-MSH6 heterodimers and do not undergo efficient ATP-dependent mismatch dissociation. The study of single amino acid substitutions in MSH2 and MSH6 can be valuable in MMR research whether they are obtained naturally from LS patients or generated through hypothesis-driven research. The predicted MSH6 ATPase domain occlusion and MSH2 ATPase domain conformational change inhibition by the R711C and G827D MSH2 mutants could be tested with crystallography or NMR to illuminate the details of how the two MSH2 and MSH6 composite ATPase sites communicate with one another. Trypsin proteolysis could be used prior to determine if there is

an actual difference in conformational change or not. Additional amino acid substitutions in so-called "intersite" areas (Hargreaves et al., 2010) between the two ATPase domains, such as MSH2 R711C, provide the best opportunity to study the composite nature of the two ATPase domains. The Walker A motif that coordinates nucleotide binding in the MSH2 and MSH6 ATPase domains is complemented by the Walker B ATPase motif that coordinates ATP hydrolysis. The Walker B hydrolyzes ATP using a highly-conserved hhhhDE where 'h' is any hydrophobic amino acid and the glutamate is the essential amino acid residue for ATP hydrolysis (Hanson and Whiteheart, 2005). As there is still a mechanistic model of MMR that requires ATP hydrolysis to initiate downstream MMR, the active translocation model, an investigation into a Walker B mutant with the essential catalytic glutamate mutated could eliminate the active translocation model provided ATP binding by both MSH2 and MSH6 was unaffected and the ATPase activity of the MSH2-MSH6 heterodimer was eliminated. SPR experiments with longer, mismatch-containing oligonucleotides and a blocked-end could determine if the MSH2 mutant proteins are dissociating from mismatches and remaining on DNA or simply falling off when challenged with ATP. Knowing this could clarify results obtained from *in vivo* experiments.

B. MSH2 ATPase domain mutants are alkylation damage tolerant and can not repair mismatches

All MSH2 ATPase domain mutants were unable to repair mismatches *in vivo*. Treating our lentiviral MSH2 Hec59 cell lines with MNNG failed to induce increased chromatin localization to O^6-me-G:T mismatches and did not cause a MMR-dependent G_2/M cell cycle arrest. Using a clonogenic survival assay, the MSH2 mutant Hec59 cell lines were significantly more alkylation tolerant than wild-type MSH2 when treated with MNNG, but not in response to 6-TG, 5-FU or cisplatin.

Based on the results of the MSH2 ATPase domain mutant *in vitro* biochemistry, we hypothesized that the K675A MSH2 mutant could be capable of *in vivo* MMR, but the R711C and G827D mutants would not be. All three MSH2 mutants were incapable of coordinating nick-directed human MMR *in vivo*. This supports previous findings from our lab that MSH2 ATPase domain mutants tend to have more severe phenotypes than mutations found elsewhere (Mastrocola and Heinen., 2010a). Our hypotheses based on the molecular switch model of MMR were that ATP binding by both MSH2 and MSH6 and sliding clamp formation were crucial steps for *in vivo* MMR. The *in vivo* MMR data support our hypothesis that the molecular switch model of MMR is accurate in human cells. In response to MNNG treatment and generation of O^6-me-G:T mismatches, the Hec59 cells with mutant MSH2 failed to undergo an increase in chromatin localization as predicted by our previous work (Mastrocola and Heinen, 2010b) and also failed to activate a MMR-dependent G_2/M cell cycle arrest. Since the MSH2 mutants do bind mismatches, this indicates that the lack of chromatin localization observed is not from an inability to bind mismatches. It appears to be an inability to repetitively load MSH2-MSH6 heterodimers at mismatches, which supports the molecular switch model of MMR. We observed no MMR-dependent G_2/M cell cycle arrest following MNNG treatment in any of the MSH2 mutants. This could indicate that repetitive formation of MSH2-MSH6 sliding clamps is a necessary step in the activation of MMR-dependent cell cycle arrest, which would place the molecular switch model of MMR upstream of any MMR-dependent DDR. Additionally, this observation could indicate that *in vivo* repair is required to activate the MMR-dependent DDR, which would support the futile repair cycle model. An important future direction for this work is to determine if the ATPase domain mutant MSH2-MSH6 heterodimers can interact with the second MMR heterodimer, MLH1-PMS2. Binding ATRIP and ATR at O^6-me-G:T mismatches in a complex

with MLH1-PMS2 is a key feature of the direct signaling model (Yoshioka et al., 2006) and both MSH2-MSH6 and MLH1-PMS2 form complexes with ATR and additional DNA damage signaling proteins after MNNG-induced alkylation damage (Liu et al., 2010). If the MSH2 mutants can interact with MLH1-PMS2, it could be strong evidence to support the futile repair cycle model of MMR-dependent DDR. We observed that the Hec59 cells re-expressing ATPase domain mutant MSH2 were significantly more alkylation tolerant than Hec59 cells re-expressing wild-type MSH2 using a clonogenic survival assay, however the K675A and R711C MSH2 Hec59 cells were slightly more sensitive than the lentiviral vector and G827D Hec59 cells. Our work has demonstrated that the MSH2 mutant Hec59 cells can not repair mismatches *in vivo* and do not undergo a typical MMR-dependent G_2/M arrest or apoptosis. A possible explanation is that the K675A and R711C MSH2 mutant Hec59 cells have restored expression of MGMT, an enzyme that non-reversibly catalyzes the removal of the methyl group from the O^6 of guanine (Kaina et al., 2007), which is inhibited during our assays. This might slow down cell growth giving the appearance of slightly increased sensitivity to MNNG when none actually exists. Given the subjectivity of the clonogenic survival assay, repeating a few more times may clarify whether there is a true separation for the K675A and R711C mutants or whether it is an experimental artifact. Previous reports have all documented differences between MMR-deficient Hec59 cells and MMR-proficient Hec59 2-4, which have had human chromosome 2 containing *MSH2* restored, following treatment with 6-TG (Meyers et al., 2005) , 5-FU (Chen et al., 2010) and cisplatin (Aebi et al., 1996). The failure of our clonogenic survival assay to detect any differences between the various MSH2-expressing Hec59 cells could be due to the subjective nature of the assay as well as the slight differences in tolerance between the MMR-proficient

Hec59 2-4 and MMR-deficient Hec59 cells relative to the much more pronounced tolerance of alkylation damage demonstrated by MMR-deficient Hec59 cells.

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