

AN ABSTRACT OF THE DISSERTATION OF

Stephanie L. Smith-Roe for the degree of Doctor of Philosophy in Toxicology presented on May 2, 2006.

Title: DNA Mismatch Repair-Dependent Responses to the Food-Borne Carcinogen 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in the Mouse.

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The DNA mismatch repair (MMR) pathway maintains genomic stability and reduces cancer risk (colorectal and other internal cancers) by correcting polymerase errors and activating cell cycle checkpoints and apoptosis in response to DNA damage. Few studies have examined the influence of commonly encountered environmental mutagens/carcinogens on the etiology of MMR-deficient cancers. 2-Amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) is a cooked-meat mutagen implicated in human colorectal carcinogenesis. To determine whether PhIP represents a cancer risk to individuals with MMR-deficiency, I examined the effect of *Mlh1*-deficiency on PhIP-induced mutagenesis, tumorigenesis, and cell-turnover responses in mice following exposure to eight intraperitoneal (i.p.) injections of 50 mg/kg PhIP. *Mlh1*^{-/-} mice were hypermutable by PhIP in colon and small intestine (as measured using transgene shuttle

vectors), demonstrating specific increases in mutations not typically associated with PhIP. In contrast, G/C to T/A transversions, the "signature PhIP mutation" were similarly induced in *Mlh1*^{-/-} and wild-type mice. In cancer studies, *Mlh1*^{-/-} mice showed heightened susceptibility to induction of colonic aberrant crypt foci (a biomarker for colon carcinogenesis), whereas adenomas of the small intestine and colon were not induced. Cell-turnover responses in *Mlh1*^{+/+} and *Mlh1*^{-/-} mouse colon were evaluated following multiple i.p. injections or a single i.p. injection of 50 mg/kg PhIP. These responses were compared to a single i.p. injection of 80 mg/kg 1,2-dimethylhydrazine (DMH). Colonic apoptosis in response to PhIP increased, shifted to predominately the stem cell compartment of colon crypts, and was partially *Mlh1*-dependent. This response was also rapid, occurring at 8 h after treatment and diminishing by 16 h. Similar effects were observed in DMH-exposed animals. The apoptotic response to PhIP was greater after multiple exposures compared to a single exposure. PhIP-exposure did not notably alter cell proliferation, whereas proliferation was consistently reduced in DMH-treated mice. These results suggest that loss of *Mlh1* alters homeostatic functions of the colonic crypt that may contribute to suppression of the mutagenic effects of PhIP. These data are consistent with the hypothesis that PhIP exposure increases mutagenesis and carcinogenesis in *Mlh1*^{-/-} mice, and support further evaluation of the risk that consumption of heterocyclic amines may impart to MMR-deficient individuals.

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DNA Mismatch Repair-Dependent Responses to the Food-Borne Carcinogen
2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in the Mouse

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Stephanie L. Smith-Roe

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DNA Mismatch Repair-Dependent Responses to the Food Borne-Carcinogen 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in the Mouse

Chapter 1. Introduction

DNA Mismatch Repair

DNA mismatch repair (MMR) is an evolutionarily conserved excision repair pathway that contributes to the maintenance of genomic stability (Buermeier et al., 1999; Kunkel and Erie, 2005; Schofield and Hsieh, 2003). A primary function of MMR is to correct errors made in the nascent (newly synthesized) strand during DNA replication based on the sequence encoded by the template strand. These errors include mispaired bases that can generate base substitution mutations upon subsequent replication, and nucleotide loopouts that can generate insertion or deletion mutations (insertion if loops occur on the nascent strand, and deletion if they occur on the template strand). Nucleotide loopouts occur when the replicating polymerase extends from misaligned templates, which occur more frequently on repetitive sequences. The overall rate of mutation is increased 1,000 to 10,000-fold in MMR-deficient bacteria, and 10 to 1,000-fold in MMR-deficient eukaryotes. By directing excision to the newly synthesized strand (which will become the template in the next round of DNA replication) MMR specifically maintains the intended genomic sequence of an organism.

Prokaryotic MMR

The MutHLS methyl-directed MMR pathway of *Escherichia coli* (Modrich, 1991) is initiated when a MutS homodimer detects and binds a post-replication DNA mismatch. MutS recruits the MutL homodimer, an interaction that requires ATP (Grilley et al., 1993). MutS and MutL then activate a latent endonuclease activity of MutH, which makes a nick at non-methylated d(GATC) sites in nascent strand. *E. coli* DNA is normally methylated at adenine residues of d(GATC) sites, except for a transient under-methylation of the nascent strand that occurs during replication. Therefore, these hemi-methylated d(GATC) sites serve to direct excision to the nascent strand. MutH can incise at hemi-methylated d(GATC) sites either 5' or 3' to the location of the mismatch. Excision requires recruitment of DNA helicase II (UvrD) and exonuclease VII or RecJ exonuclease for 5' to 3' excision, or exonuclease I or X for 3' to 5' excision. Single-strand DNA binding protein (SSB) is required during excision, which stops at approximately 100 base pairs (bp) past the site of the mismatch for 3'-directed excision (Grilley et al., 1993), an observation made *in vitro*. The resulting excision tract is re-synthesized by DNA polymerase III holoenzyme, and the continuity of the nascent strand is restored by DNA ligase. This pathway has been reconstituted *in vitro* by examining the ability of purified proteins (as mentioned above) to repair a heteroduplex present in a covalently closed circular molecule derived from the bacteriophage ϕ 1 genome (Cooper et al., 1993; Lahue et al., 1989).

Eukaryotic MMR

Eukaryotes have multiple homologs of the prokaryotic MutS (MutS-homologs 2, 3, 4, 5, and 6) and MutL proteins (MutL-homologs 1 and 2, Post-Meiotic Segregation 1 and 2 - yeast PMS1 is mammalian PMS2). MSH2 dimerizes with MSH6 to form MutS α , which recognizes base-base mismatches and single nucleotide loops. MutS β is a dimer of MSH2 and MSH3, and recognizes nucleotide loops up to 4 bases in length. MSH4 and MSH5 do not have an error correction function but are required for successful meiotic recombination. In mammalian cells, MLH1 dimerizes with PMS2 to form MutL α or with MLH3 to form MutL γ (MutL α in yeast is a heterodimer of MLH1 and PMS1). MLH1 also dimerizes with PMS1 to form MutL β , but there does not appear to be a significant role for this heterodimer in excision repair. As in prokaryotes, MSH complexes interact with MLH complexes to link error identification with strand excision. There does not appear to be a eukaryotic homolog of the *E. coli* MutH endonuclease. Although the strand discrimination signal for eukaryotic MMR is unknown, it is hypothesized that the 3' end of the growing nascent strand or the 5' end of Okazaki fragments may serve as the point of excision (Kunkel and Erie, 2005). Exonuclease I (EXO1) participates in 5' to 3' excision, and may also contribute to 3' to 5' excision (Genschel et al., 2002). The 5' to 3' excision function of EXO1 can be compensated for by an as yet unidentified activity, as *Exo1*-deficient mice exhibit a very mild phenotype (median survival of 17 months) compared to *Mlh1*- or *Msh2*-deficient mice (median survival of 6 to 8 months) (Wei et al., 2003). Aphidicolon-sensitive polymerases (δ , ϵ)

and MRE11 also may participate in 3' to 5' excision (Vo et al., 2005; Wang and Hays, 2002).

5' and 3' eukaryotic MMR has been reconstituted *in vitro* using the following purified proteins: MutS α , MutL α , EXO1, replicating protein A (RPA), proliferating cell nuclear antigen (PCNA), recombinant factor C (RFC), and DNA polymerase δ (Constantin et al., 2005; Zhang et al., 2005). These reconstitution experiments describe a minimal complement of proteins that can perform a MMR-dependent excision reaction, with the caveat that the DNA substrate is an exogenous bacteriophage genome derivative with presumably a single nick to provide a point for strand-specific excision. This type of substrate is a logical model for prokaryotic MMR and a heuristically useful model for eukaryotic MMR, but does not necessarily recapitulate how eukaryotic MMR might interact with a replication fork in order to gain access to a nascent strand terminus or Okazaki fragment for strand discrimination and excision. Similar experiments using nuclear extracts from human HeLa cells (as opposed to purified components) suggest that other, auxiliary proteins also are important for eukaryotic MMR. For example, HMGB1, which binds to aberrant DNA structures and also tightly bends DNA, has been shown to interact with MutS α and participate in eukaryotic MMR (Yuan et al., 2004).

Models of MMR Excision Function

In both prokaryotic and eukaryotic MMR, the mismatch and the strand discrimination signal are separated in space, and MutS/MSH proteins interact with

MutL/MLH proteins in an ATP-dependent manner in order to link a mismatch-detection event with initiation of excision. Presently, there are three models in the literature that attempt to describe the as-yet elusive mechanism of MMR-initiated excision. The first of these models is termed, "active translocation" (Allen et al., 1997). In this model, MutS binds a mismatch, and subsequent binding of ATP reduces MutS binding of the mismatch and activates other DNA-binding sites used for translocation. Translocation is accomplished in an ATP hydrolysis-dependent manner, in which MutS draws flanking DNA towards itself bi-directionally, forming a loop with the once-bound mismatch in the center of the growing loop. MutL binds to MutS and facilitates growth of the loop. Partial release of the loop then places the MutS/MutL complex on a linear molecule at some distance from the mismatch. Hypothetically, depending on how MutS releases the loop, MutS could reside on either side of the mismatch in search of an excision signal (possibly accounting for bi-directional repair). A second model is called the "molecular-switch" model (Acharya et al., 2003). In this model, recognition of a mismatch by MutS provokes ADP to ATP exchange. ATP binding by MutS results in formation of a stable hydrolysis-independent sliding clamp that moves along DNA by diffusion in either direction from the mismatch. By moving away from the mismatch, additional MutS sliding clamps can continue to load onto the DNA. MutL is capable of binding to the diffusing MutS molecule. Eventually, a MutS/MutL complex will encounter MutH at a hemi-methylated GATC site. MutL will activate MutH to initiate excision. In these two models, MutS moves away from the mismatch in search of excision signals/machinery. These models require more than one MutS protein to interact with the mismatch in order

to assure translocation or diffusion in the correct direction of the excision signal/machinery.

A third model is the “static transactivation” model, or “passive DNA bending” model (Schofield et al., 2001; Wang and Hays, 2004). This model was proposed when it was observed that MutH could be activated by MutS and MutL in *trans*. Additional biochemical experiments showed that MutS can move bi-directionally on DNA in the presence of ATP, but when MutL is present, the movement of MutS appears to be restricted to the vicinity of the mismatch. Therefore, a MutS/MutL complex retained at or near a mismatch could activate MutH via DNA bending through space, and retention of MutS/MutL at the mismatch would serve as a signal to terminate excision. This third model is supported in part by the observation that DNA-hairpin or streptavidin-biotin structures engineered between a mismatch and nick to block hypothetical sliding MSH complexes do not disrupt MMR in experiments using HeLa cell nuclear extracts (Wang and Hays, 2003; Wang and Hays, 2004). Also, by varying the mismatch-to-nick distance and the molar ratio of DNA substrate to MutS α , it was shown that a single MutS α is sufficient for efficient MMR (Wang and Hays, in preparation). This finding lends weight to the passive DNA bending model, as the other models predict that multiple MutS/MutS α complexes are required to move from the mismatch to find the excision machinery.

MMR Responses to Spontaneous Polymerization Errors

Certain events that occur during polymerization cause spontaneous errors. DNA bases can undergo tautomeric shifts to form structural isomers, thereby allowing non-canonical base pairing to occur. For example, cytosine is normally in a keto form, but when cytosine is in a rare imino form, it can mispair with adenine and vice versa. Also, when thymine is in a rare enol form, it can mispair with guanine and vice versa. Another source of spontaneous error is primer-template misalignment of repetitive sequences, resulting in nucleotide loops of various sizes (Garcia-Diaz et al., 2006). Errors may also be introduced by nucleotide pool imbalance, in which a change in nucleotide concentration allows insertion of the wrong base (Kunz et al., 1994). In *E. coli*, spontaneous errors that escape the editing 3' to 5' exonuclease function of the replicating polymerase occur at a frequency of about one in every 10,000 to 100,000 nucleotides polymerized.

The process by which MutS or MutS α discriminates heteroduplex DNA from homoduplex DNA is under investigation. MutS from *E. coli* and *Thermus aquaticus* have been crystallized bound to a G:T mismatch or an unpaired thymine base, respectively (Lamers et al., 2000; Obmolova et al., 2000). These crystal structures show that MutS has five domains. Domains I and IV bind to DNA, and a composite ATPase domain is formed in the dimer by domain V of the monomer. Domain III links DNA binding by domains I and IV with the ATPase function of domain V. Domain I contains a conserved phenylalanine-X-glutamate (Phe-X-Glu) motif. The phenylalanine (Phe 36)

of this motif interchelates within DNA and forms stacking interactions with mismatched bases. The crystal structures of MutS with heteroduplexes show that the DNA is kinked by 60° to widen the minor groove and compress the major groove when one monomer of MutS interacts with the mismatch via Phe 36. The ability of MutS to bind homoduplex or heteroduplex DNA is greatly reduced if Phe 36 is mutated to alanine (Dufner et al., 2000; Malkov et al., 1997). In the eukaryotic heterodimer MutS α , Phe 36 is conserved in the MSH6 subunit, and only MSH6 interacts with DNA (Dufner et al., 2000). Further discrimination of homoduplex versus heteroduplex DNA may be provided by the conserved glutamate of the Phe-X-Glu motif (Glu 38), which forms a hydrogen bond with the mismatched base (Lebbink et al., 2006; Schofield et al., 2001). The binding process alone of a mismatch by MutS α does not account for the specificity of excision for heteroduplex DNA versus homoduplex DNA. MutS α binding preference for a mismatch ranges from 5- to 30-fold greater than homoduplex DNA, whereas excision is at least 300-fold more likely to occur in response to mismatched DNA (Hays et al., 2005; Schofield et al., 2001). It is hypothesized that asymmetry in ATP binding and rate of hydrolysis by the two ATPase sites of MutS/MutS α commit mismatch detection to progression of the MMR pathway (Junop et al., 2001; Lebbink et al., 2006).

Excision that follows MutS α detection of mispaired or unpaired normal bases will provide an unaltered template for the repair synthesis step that is performed by the replicating polymerase δ . The probability that the highly accurate polymerase δ will make an error upon resynthesis of a tract up to 1 kb in length is extremely low, and is likely to be a non-mutagenic process. Therefore, the excision function of MMR is

sufficient for suppression of mutation that arises from spontaneous errors that do not alter the coding properties of the template strand.

MMR Responses to DNA Damage

DNA is subjected to continuous chemical modification. Three of the four bases have exocyclic amino groups and undergo spontaneous deamination, and the bases themselves can be released from the DNA backbone by spontaneous hydrolysis (depurination and depyrimidination), generating abasic sites. DNA bases also are subject to oxidation by reactive oxygen species, and can be covalently modified by many different adducts. Damage to the genome is removed by several DNA repair pathways. Alkylation of DNA bases is directly removed by enzymes such as O⁶-methylguanine-DNA methyltransferase (MGMT). Base excision repair (BER) employs a variety of glycosylases and/or apyrimic/apyrimidinic (AP) endonuclease/lyase to remove alkylated, oxidized, and deaminated bases and abasic sites. Nucleotide excision repair (NER) detects small as well as large, more distorting modifications of DNA, such as bulky adducts and ultraviolet (UV) photoproducts. These excision repair pathways remove the damaged nucleotide and the opposite strand is used as the template for repair synthesis (Hoeijmakers, 2001; Sancar et al., 2004).

Chemical modifications present in the template strand during DNA synthesis can cause mispairing. Certain modifications, such as O⁶methylguanine (O⁶meG) and 8-oxo-guanine (8oxoG), tend to mispair with dTTP or dATP, respectively, and due to their

small size and Watson-Crick-like base pairing, are able to escape the editing function of the replicating polymerase δ (Khare and Eckert, 2002). Mismatching also occurs when polymerase δ is stalled by bulky adducts or base-base crosslinks in the template strand. In order to continue replication, these lesions are bypassed in an error-free or error-prone manner by specialized DNA polymerases called translesion polymerases (TLPs) (Lehmann, 2005; Prakash et al., 2005). Therefore, one result of these encounters between the replication machinery and template damage is the generation of compound damage – mispairs in which the template base is chemically altered.

Compound damage represents a particular challenge to cellular avoidance of mutagenesis. If the correct nucleotide is not restored to the newly synthesized strand, a mutation could be generated if the damage in the template strand is detected by BER or NER. Also, a mutation could be generated if the next round of DNA replication occurs before resolution of the mispair to the base intended by the template strand. There must be an efficient resolution to compound damage, however, as the mammalian genome of three billion base pairs is replicated with a level of fidelity of about one error per round of replication. To avoid mutagenesis, the correct base must be restored to the new strand despite the presence of damage in the template strand, thereby providing a non-mutagenic substrate for damage removal by BER or NER.

In addition to detecting and repairing mismatched normal DNA bases, MMR is also capable of responding to chemically modified DNA. MutS α has the greatest affinity for mispairs, but MutS α will also bind to various forms of damage, such as O⁶methylguanine (O⁶-meG) generated by alkylating agents, 8-oxoguanine (8-oxoG) generated by reactive

oxygen species, intrastrand crosslinks generated by cisplatin, UV photoproduct lesions, and bulky adducts such as *N*-acetyl-2-aminofluorene (AAF), benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (B[*a*]PDE), and benzo[*c*]phenanthrene-3,4-dihydro-1,2-epoxide (B[*c*]PDE) (Duckett et al., 1996; Larson et al., 2003; Li et al., 1996; Mu et al., 1997; Wang et al., 1999; Wu et al., 1999; Wu et al., 2003). In most cases in which it has been tested, MutS α prefers to bind to compound (“mismatched”) damage as opposed to damaged homoduplex DNA. MutS α -binding of compound damage also can be influenced by the sequence of the DNA surrounding the lesion (Hoffman et al., 2005).

Whereas MMR-proficient cell lines engage cell cycle checkpoints and apoptosis in response to DNA damage, these damage-surveillance responses are attenuated or absent in MMR-deficient cells. As such, MMR-deficient cells are tolerant to the cytotoxic effects of a variety of chemicals, such as alkylating agents, anti-cancer drugs, and environmentally encountered genotoxins (Aquilina and Bignami, 2001; Karran et al., 2003; Li, 1999; Meyers et al., 2004). A dramatic example is the MT1 (*MSH6*^{-/-}) lymphoblast cell line that is 500-fold more resistant to killing by the DNA-alkylating anti-cancer drug *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) compared to the parent TK6 cell line (Goldmacher et al., 1986; Kat et al., 1993). MT1 cells and HCT116 (*MLH1*^{-/-}) colorectal cancer cells are resistant to cytotoxic effects of bulky adduct-forming chemicals such as AAF and B[*a*]PDE, and these cell lines showed reduced apoptosis in response to these chemicals compared to MMR-proficient counterparts (Wu et al., 1999). Not only do MMR-deficient cells survive levels of pre-mutagenic damage that are toxic to MMR-proficient cells, they also exhibit hypermutability. MMR-

deficient cells are hypermutable in response to various chemicals, including MNNG, 6-thioguanine (6-TG), methyl methanesulfonate (MMS), *N*-ethyl-*N*-nitrosourea (ENU), and oxidative-damage inducing agents (Buermeier et al., 1999; Claij et al., 2003; Glaab et al., 1998; Sanada et al., 2004; Shin-Darlak et al., 2005). Therefore, this “damage surveillance” function of MMR may suppress tumorigenesis by preventing the clonal evolution of genetically unstable cells (Karran et al., 2003; Li, 1999).

The DNA damage-surveillance response of MMR remains to be fully characterized. Unlike BER and NER, MutS α binding of damaged DNA is not followed by removal of a damaged nucleotide. Instead, observations obtained from cell culture experiments suggest that mispair-generating damage in the template strand instigates multiple rounds of MMR excision and resynthesis. This hypothesis requires that the pre-mutagenic (compound) lesion be regenerated upon repair synthesis. These “futile cycles” lead to single-stranded regions that generate cytotoxic strand breaks in the next replication cycle (Stojic et al., 2004b; Stojic et al., 2004a; Yan et al., 2003). Alternative possible mechanisms are suggested by more recent reports. Foremost, MMR proteins may bind to a damaged mispair and signal directly to cell cycle checkpoint proteins. Thus far, MSH and MLH proteins are demonstrated to interact directly or indirectly with several checkpoint signaling proteins, including ATM, ATR, CHK2, p73, MRE11, and BRCA1 (Brown et al., 2003; Her et al., 2002; Shimodaira et al., 2003; Wang et al., 2001; Wang et al., 2000; Wang and Qin, 2003). Activation of p53 (although not always required) also can be a downstream event in response to MMR detection of DNA damage (Duc and Leong-Morgenthaler, 2004; Duckett et al., 1999; Wu et al., 1999).

Interestingly, cells from mice with point mutations in *Msh2* (Lin et al., 2004) or *Msh6* (Yang et al., 2004) that impede ATP hydrolysis by MutS α (required for excision of nascent DNA during correction of replication errors) have high levels of spontaneous mutation, yet are capable of engaging apoptotic pathways in response to various types of DNA damage. This observation would suggest that MMR-dependent activation of cell cycle signaling pathways and apoptosis is not necessarily dependent on an excision step. In the murine intestinal tract, MMR-dependent apoptosis in response to DNA-damaging agents occurs within 6 to 8 hours after exposure (Sansom et al., 2001; Toft et al., 1999; Wu et al., 2003). Such kinetics do not appear consistent with the two rounds of DNA replication necessary to generate double-strand breaks as proposed by the futile cycle hypothesis. Finally, one cell culture study suggests that apoptosis in response to DNA damage may be due to *Mlh1*-dependent inhibition of RNA synthesis (Yanamadala and Ljungman, 2003). These various observations suggest that MMR responses to DNA damage are complex and varied, and may depend on the properties of lesion and the context of the encounter (eg. post-replication v. repair synthesis v. transcription).

MMR and Cancer Risk

The discovery of MMR-deficiency as cancer risk factor was initiated in 1895 and again in 1962, when in each instance, a depressed individual lamented to a physician that he or she was sure to die from cancer of the bowel, as everyone in his or her family had died from such cancers (Lynch and Lynch, 2004). This strong familial predisposition to

early onset colorectal cancer (CRC) and other internal cancers was documented by physician Henry T. Lynch and others, and over time officially became known as Lynch syndrome, and it has also been known as hereditary non-polyposis colorectal cancer (HNPCC). A full list of the criteria for diagnosis of Lynch syndrome, including the Amsterdam I and Amsterdam II Criteria and Bethesda Guidelines is available in Lynch & Lynch (2004). Lynch syndrome is inherited in an autosomal dominant manner, with an ~85% lifetime risk of CRC, considerably greater than the 5% risk in the general population for CRC. Furthermore, Lynch syndrome cancers are diagnosed on average at 45 years of age, much earlier than the average age of 65 years for sporadic CRC. Cancers of the endometrium, ovary, stomach, small intestine, and urinary tract are also associated with Lynch syndrome.

In the search for a genetic component to this disease, linkage analysis studies of affected families showed that chromosomes 2 and 3 carried loci that segregated with risk for Lynch syndrome (Lindblom et al., 1993; Peltomaki et al., 1993). Tumors from Lynch syndrome patients exhibited a form of genetic instability called microsatellite instability (MSI), which was also found in 13% of sporadic cases of colorectal cancer (Aaltonen et al., 1993; Peltomaki et al., 1993; Thibodeau et al., 1993). Microsatellites are mono-, di-, tri-, and tetranucleotide repeats distributed throughout the genome, including the coding sequences of expressed genes. MSI results from an increased rate of change in number of repeats within microsatellite DNA. The cause for this instability in human tumors was unknown, but work with bacterial and yeast systems provided clues. For example, a dinucleotide repeat in a plasmid mutation reporter system exhibited 10-fold greater

instability when propagated in *E. coli* deficient for the *mutS* or *mutL* genes (Levinson and Gutman, 1987). More dramatically, mutations in yeast *MSH2* and *PMS1* resulted in 100- to 700-fold elevated rates of instability in a dinucleotide repeat of a plasmid reporter (Strand et al., 1993). Given that bacterial and yeast MMR genes suppressed insertion/deletion errors in dinucleotide repeats, Parsons et al (Parsons et al., 1993) asked whether cells derived from a Lynch syndrome-like human tumor were deficient for MMR activity. Similar to the yeast experiments, insertion/deletion errors were increased approximately 100-fold in a dinucleotide repeat of a plasmid reporter propagated in the tumor cells. Furthermore, tumor cell extracts were incapable of repairing mispaired DNA bases and di-, tri-, and tetranucleotide loopouts. These studies in bacteria, yeast, and human tumor cell lines suggested that the Lynch syndrome-linked loci at chromosome 2 and 3 were the locations of human homologs of MMR genes. Homology searches of the human genome using yeast gene sequences lead to cloning of *hMSH2* and *hMLH1* and mapping of these genes to chromosome 2 (Fishel et al., 1993; Leach et al., 1993), and chromosome 3 (Bronner et al., 1994; Papadopoulos et al., 1994), respectively. Analysis of cell lines from Lynch syndrome patients showed that *MSH2* and *MLH1* were mutated, and kindred analysis and analysis of normal cells from patients versus tumor cells showed that the mutations were heterozygous germline mutations (Bronner et al., 1994; Fishel et al., 1993; Leach et al., 1993; Papadopoulos et al., 1994).

Since the early 1990s it has been established that Lynch syndrome accounts for 5 – 8% of all hereditary colorectal cancer, Lynch syndrome generally is associated with hereditary defects in MMR genes, and that MSI is a hallmark of MMR-deficiency (Lynch

and Lynch, 2004). Mutations in MMR genes have not been identified in all tumors that meet the diagnostic criteria of Lynch syndrome. This may be due to limitations of detection techniques or mutation of non-MutS/MutL homolog proteins that participate in the MMR pathway. In tumors for which a MMR gene defect has been detected, approximately 40% carry mutations in *MSH2* and 50% carry mutations in *MLH1*. The remainder carry mutations in MMR genes *MSH6* or *PMS2*. It is possible that identification of germline mutations in *PMS2* is underestimated due to interference from *PMS2* pseudogenes (Nakagawa et al., 2004). Germline mutations in MMR genes also are linked to Muir-torre syndrome, a rare disease of early onset skin cancer characterized by sebaceous tumors and basal-cell carcinomas in addition to risk of cancers associated with Lynch syndrome (Ponti and Ponz de Leon, 2005). Individuals with homozygous germline mutations in *MSH2* and *MLH1* develop neurofibromatosis and die very young from severe hematological cancers (Bandipalliam, 2005). In addition to inherited MMR-deficient cancer risk, approximately 15% of sporadic colorectal cancers exhibit MSI, primarily due to hypermethylation of the *MLH1* gene (Herman JG et al 1998 PNAS; Veigl ML 1998 PNAS). Taken together, MMR-deficiency is a common etiological factor in human carcinogenesis.

Mouse Models of MMR-Deficiency

Development of mouse lines with inactivating mutations for each of the genes specific to the MMR pathway has demonstrated unequivocally that MMR genes

contribute to suppression of mammalian tumorigenesis, and have allowed researchers to evaluate the importance of each MMR gene for tumor suppression (Chen et al., 2005; Edelman and Edelman, 2004; Wei et al., 2002). Mice deficient for *Msh2* or *Mlh1* are considered to be MMR-nulls. *Msh2*^{-/-} and *Mlh1*^{-/-} mice are prone to early onset gastrointestinal tumors and other tumors, and their average survival is shortened to 6 to 8 months of age due to aggressive hematopoietic cancers. The phenotypes of other MMR gene deficiencies in mice are not as severe. *Pms2*^{-/-} mice also are prone to aggressive hematopoietic cancers and have an average survival of 9 to 10 months, but lack intestinal tumorigenesis. *Msh6*^{-/-}, *Msh3*^{-/-}, and *Mlh3*^{-/-} mice are longer lived (12 to 18 months), and are susceptible to lymphomas and gastrointestinal tumors later in life.

Levels of genetic instability due to different MMR gene deficiencies appear to correlate with tumor susceptibility. Genetic instability measured in *Msh2*^{-/-} and *Mlh1*^{-/-} mice by analysis of microsatellite DNA, mutation frequency at endogenous loci, and mutation frequency at transgene reporters is higher than that of other MMR-deficient mice (Andrew et al., 1998; Andrew et al., 2000; Chen et al., 2005; Edelman et al., 2000; Mark et al., 2002; Xu et al., 2001; Yao et al., 1999). *Pms2*^{-/-} mice exhibit about one-third to half the mutation level of *Msh2*^{-/-} or *Mlh1*^{-/-} mice, and genetic instability in *Msh6*^{-/-}, *Msh3*^{-/-}, and *Mlh3*^{-/-} mice is somewhat lower than *Pms2*^{-/-} mice. These observations suggest that level of genetic instability can correlate with degree of cancer susceptibility, and that a critical level of mutation may need to be reached to promote intestinal tumorigenesis. Furthermore, both the error correction function and the damage surveillance response of MMR are important for suppression of spontaneous

tumorigenesis in mice, as illustrated by experiments using *Msh2*^{G674A/G674A} mice (Lin et al., 2004). This mutation disables the ATPase activity of MutS α , abolishing the excision function of MMR, yet the ability to engage MMR-dependent apoptosis in response to lesions generated by cisplatin and alkylating agents remains intact.

Comparison of survival and tumor onset in *Msh2*^{-/-} mice (median survival of 6 months) versus *Msh2*^{G674A/G674A} mice (median survival of 9 to 10 months) suggests that whereas accumulation of mutations (due to lack of the excision function) is sufficient for tumorigenesis, having an intact damage surveillance response slows this process.

The severity and spectrum of the cancer phenotypes of MMR-deficient mice correlates with the observation that the majority (~90%) of mutations detected in Lynch syndrome kindreds are present in either *MLH1* or *MSH2*, suggesting that mechanisms for MMR-dependent tumor suppression are similar across species. Indeed, the generation of *Msh6*^{-/-} mice, which have low levels of MSI and develop tumors at later age, predicted the finding of *MSH6* mutations in atypical Lynch syndrome cases, in which tumors with low levels of MSI were diagnosed in patients over 60 years of age (Edelmann and Edelmann, 2004). An obvious caveat for the comparison of mouse models with human MMR-deficient tumorigenesis is that mice must be homozygous deficient for MMR genes for a tumor-prone phenotype. This is likely due to the considerably shorter lifespan of mice compared to humans. It is interesting to note, however, that despite being completely MMR-deficient, the tumor spectrum of *Msh2*^{-/-} and *Mlh1*^{-/-} mice remains dominated by hematopoietic cancers and the gastrointestinal tract, which are both highly proliferative tissues. Lastly, mice are preferentially prone to spontaneous tumorigenesis

in the small intestine as opposed to colon, whereas the reverse is true in humans.

These differences may be due to mouse versus human physiology, as other mouse models for another human hereditary colorectal cancer syndrome called familial adenomatous polyposis (FAP) are also highly susceptible to small intestinal tumors as opposed to colon tumors (Oshima et al., 1995; Su et al., 1992; Yang et al., 1997).

The Mutator Hypothesis of Carcinogenesis and MMR-deficient Tumors

The mutation rate of normal cells is extremely low, about 10^{-9} (Bielas and Loeb, 2005), and the mutation rate of stem cells, the likely progenitors of cancer cells, may be as low as 10^{-11} (Cervantes et al., 2002). These numbers indicate that cells replicate their genomes with extraordinary accuracy. However, it is generally hypothesized that at least 6 mutations are needed to drive carcinogenesis, and cells obtained from cancers are observed to have hundreds of thousands of mutations in their genomes (Loeb et al., 2003). The mutator hypothesis (Loeb et al 1974; Loeb et al 1991) suggests that mutations in “care-taker” genes, those involved in the maintenance of genomic stability, will allow increased mutagenesis to occur in order to drive cancer and account for the extremely high numbers of mutations detected in cancers. The designation of “care-taker” can be applied to genes that contribute to the accuracy of DNA polymerization, to DNA repair, and to activation of cell cycle arrest and apoptotic pathways (Beckman and Loeb, 2005).

Given their roles in mutation suppression, MMR genes, particularly *MSH2* and *MLH1*, are undoubtedly care-taker genes. Loss of MMR increases random mutagenesis, and some of these mutations occur in other care-taker genes, as well as “gate-keeper” genes that govern cell growth or apoptosis (apoptosis as both an anti-mutagenic “care-taker” and anti-proliferation “gate-keeper” process). For example, simple repeats in genes that regulate cell growth (*TGF β RII*, *IGFR1I*), cell survival (*BAX*, *caspase-5*), and DNA repair (*MSH3*, *MSH6*, *MBD4*) undergo frameshift mutagenesis in MMR-deficient cancers in a selective manner (Miyaki et al., 2001; Mori et al., 2001; Yamamoto et al., 1998). Also, some MSI-positive cancers have truncating mutations in the adenomatous polyposis coli (*APC*) tumor suppressor gene (Huang et al., 1996), which is found to be mutated in the majority of sporadic colorectal cancers (Miyoshi et al., 1992; Powell et al., 1992).

The incidence of adenomas in Lynch syndrome patients is low, but once adenomas form, they progress rapidly (Calabrese et al., 2004; Leach et al., 1993). To illustrate, it may take 8 to 10 years for an adenoma to progress to carcinoma when sporadic in origin, whereas this process may take only 2 to 3 years in a Lynch syndrome patient (Lynch and Lynch, 2004). These observations suggest that loss of MMR increases cancer risk, in part, by accelerating mutagenesis. Furthermore, it is not precisely known why individuals with MMR deficiencies are susceptible to tumorigenesis of certain tissues. However, the requirement of cell replication to convert pre-mutagenic lesions to mutations may be why highly proliferative tissues such as the

colon, stomach, and endometrium are particularly at risk for carcinogenic transformation (Chao and Lipkin, 2006).

Suppression of DNA-Damage Induced Carcinogenesis in MMR-Deficient Mice

Given the role of MMR in suppression of both spontaneous and induced mutation, the question as to whether MMR-deficiency increases susceptibility to carcinogens has been investigated using mouse models. This line of research has focused on the effects of anti-cancer treatments, such as alkylating agents, cisplatin, and radiotherapy. MMR-deficient cell lines show varying degrees of resistance to the cytotoxic effects of such therapies, and theoretically, use of these treatments in patients could select for deleterious growth of MMR-deficient cells (Karran et al., 2003). The effects of these treatments on hypermutability, apoptosis, and cancer risk have been examined in MMR-deficient mice. Tissues of *Msh2*^{-/-} mice are hypermutable in response to the alkylating agent *N*-methyl-*N*-nitrosourea (MNU), and tissues of *Pms2*^{-/-} mice are hypermutable in response to ionizing treatment (Andrew et al 1998; Xu et al 2001). In the small intestine of *Msh2*^{-/-}, *Mlh1*^{-/-}, or *Pms2*^{-/-} mice, exposure to temozolomide (or a low dose of MNU) resulted in reduced apoptosis compared to wild-type levels and increased mutagenesis at the endogenous *Dlb-1b* locus (Sansom et al., 2003; Toft et al., 1999). With regard to cancer risk, *Msh2*^{-/-} mice exhibit accelerated lymphomagenesis when treated with ENU, MNU, or 1,2-dimethylhydrazine (DMH), and treatment with DMH also increased risk of colon tumors (Claij et al 2003; Jansen et al 2000; Colussi et al 2002). *Pms2*^{-/-} mice also are prone to

accelerated lymphomagenesis when treated with MNU (Qin et al 1998), whereas *Pms2*^{+/-} mice are susceptible to colon tumors induced by MNU (Qin et al., 2000). Additionally, the severe lymphoma induced in *Mgmt*^{-/-}*Mlh1*^{-/-} mice treated with MNU may be a result of reduced apoptosis and hypermutability (Kawate et al., 1998; Takagi et al., 2003). Taken together, the combination of selective advantage and increased mutation in response to DNA-damaging agents in MMR-deficient cells may have a considerable influence on carcinogenesis.

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), an Environmentally Relevant Cancer Risk for MMR-deficient Individuals?

Men who carry heterozygous germline mutations in MMR genes face an 85 to 90% lifetime risk of CRC, and women with such mutations face a ~50% lifetime risk of CRC, in addition to a ~60% lifetime risk of endometrial cancer and a ~13% lifetime risk for ovarian cancer (Aarnio et al., 1999; Watson and Lynch, 1993). The risk for gastric cancer is ~12% in men and women who carry MMR gene mutations. Presently, it is not known why the colon is the most prevalent site for MMR-deficient cancer risk. Given the well known association of diet with CRC risk, it may be possible that cancer risk in individuals with MMR-deficiency is influenced by dietary factors. Indeed, the question as to whether commonly encountered environmental risk factors contribute to carcinogenic processes in individuals with MMR-deficiencies is relatively unexplored. However, recent epidemiological studies suggest that consumption of red meat, alcohol intake, and cigarette smoking may increase risk of MSI-positive sporadic cancers

(Diergaarde et al., 2003; Luchtenborg et al., 2005; Satia et al., 2005; Wu et al., 2001), and one study suggests that MSI-positive sporadic colon cancer risk is specifically modulated by consumption of heterocyclic amines (HCAs) (Wu et al., 2001), which are present in heavily cooked meats, as well as tobacco smoke, beer, wine, and diesel fuel exhaust (Turesky, 2005).

HCAs are the condensation products of creatine and amino acids formed during the cooking of meat and fish, and the HCA content increases with higher cooking temperatures and longer cooking times (Gooderham et al., 2002; Sugimura et al., 2004; Turesky, 2005). Over twenty HCAs are present in cooked meat, and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is the most abundant by mass weight (Felton et al., 1986; Sinha et al., 1995). PhIP is initially *N*-hydroxylated in the liver by p450 enzymes, primarily CYP1A2 (Boobis et al., 1994). *N*-hydroxylation provides a site for esterification reactions by phase II enzymes, such as sulfotransferases and acetyltransferases. These ester derivatives are unstable and break down to form highly electrophilic products that adduct to DNA, primarily at the C8 and *N*² positions of guanine residues (Mauthe et al., 1998; Wohlin et al., 1996).

PhIP increases mutagenesis in wild-type cells (in cell culture or *in vivo*), and leaves a “mutational fingerprint” of G/C to T/A transversions and -1 G frameshifts as the result of its preference for adducting to guanine residues (Dashwood, 2003; Gooderham et al., 2002). PhIP induces colon, mammary, and prostate tumors in rats, as well as aberrant crypt foci (ACF, a pre-neoplastic lesion of the colon) (Ito et al., 1991; Ochiai et al., 1996). Mice exposed to PhIP are susceptible to lymphoma and ACF (Esumi et al.,

1989; Kristiansen et al., 1997; Ochiai et al., 2002; Sorensen et al., 1997; Sorensen et al., 1996). Neonatal exposure to PhIP increases intestinal tumorigenesis in *Min/+* mice, a model of Familial Adenomatous Polyposis (FAP), a hereditary colorectal cancer syndrome in which patients develop hundreds of colonic polyps at a young age (Fodde, 2002). Furthermore, co-exposure to PhIP and promoting agents enhances intestinal carcinogenesis in mice. For example, co-exposure with a high-fat diet increases small intestinal tumorigenesis (Ochiai et al., 2002), and co-exposure with sodium dextran sulfate (an inflammatory agent) targets PhIP-induced tumorigenesis to the colon (Nishikawa et al., 2005; Tanaka et al., 2005).

HCA's have been associated with increased colorectal cancer risk in the general population, and were recently described by the National Toxicology Program as "reasonably anticipated to be a human carcinogen" (NTP, 2004). Humans are exposed to approximately 1 µg HCA/kg weight/day. Compared to rodents, human liver microsomal samples are more proficient at bioactivation of PhIP, resulting in 10-times more PhIP-DNA adduct formation in the colons of humans (volunteers who were undergoing surgical removal of colon tumors) compared to rats (when using dietary-relevant, equivalent doses) (Turteltaub et al., 1999).

The hypothesis that MMR-deficiency may increase the mutagenic effects of PhIP was initially tested in cell culture. The colorectal cancer cell line HCT-116 (deficient for *hMLH1*) was shown to be resistant to PhIP-induced cytotoxicity and also to exhibit hypermutability with PhIP-treatment (Glaab and Skopek, 1999). Similar results were demonstrated in the MMR-deficient human lymphoblast cell line MT1 (deficient for

hMSH6) (Leong-Morgenthaler et al., 2001). Furthermore, the reduced effects of PhIP-cytotoxicity in MT1 cells could be explained in part by reduced activation of apoptosis (Duc and Leong-Morgenthaler, 2004). These studies suggested that MMR suppresses PhIP-induced mutation.

Therefore, as indicated by studies of alkylating agents in MMR-deficient mice, MMR-deficiency potentially could enhance the carcinogenic effects of PhIP *in vivo*. To test this possibility, I treated *Mlh1*-deficient mice with multiple injections of PhIP, and assessed mutagenic, tumorigenic, and cell-survival outcomes. Overall, my goal was to learn whether this component of the diet could be a specific cancer risk factor for individuals also at risk for MMR-deficiency. If so, avoiding PhIP in the diet could reduce cancer risk for such individuals.

Chapter 2. Materials and Methods

Animals and Genotyping

Heterozygous *Mlh1*-deficient mice (Baker et al., 1996) were crossed with the 3340 strain carrying the *supFG1* mutation reporter transgene (Narayanan et al., 1997) or Muta™ Mouse (Gossen et al., 1989). All mice were of the C57BL/6 background. Mice were bred in-house and litters were toe-clipped at 14 to 18 days of age for permanent identification and genotyping. Toes were digested overnight at 56 °C in 200 µl of PCR tissue lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2 mM MgCl₂, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween-20) and 5 µl of 20 mg/ml proteinase K (Fermentas). Mice were genotyped for *Mlh1*, *supFG1*, or *cII* by using PCR with primers obtained from Invitrogen (Carlsbad, CA). PCR mixes, thermocycler conditions, and primer sequences are listed in Tables 2.1, 2.2, and 2.3.

Table 2.1. PCR mixes used to amplify *Mlh1*, *cII*, or *supFG1* genes from mouse DNA (final volume of 25 µl). 10x buffer, 5x buffer, 25 mM MgCl₂, and taq polymerase are constituents of the Eppendorf Mastertaq kit (VWR). dNTPs were purchased from Fermentas.

Reaction constituents	Gene		
	<i>Mlh1</i>	<i>cII</i>	<i>supFG1</i>
10x Buffer	1x	1x	1x
5x Buffer	1x	1x	1x
25 mM MgCl ₂	1.2 µl	-	2.0 µl
Primer 1	0.25 mM <i>Mlh1</i> -A	0.2 mM <i>cII</i> p1	0.3 mM <i>T7</i> -12
Primer 2	0.50 mM <i>Mlh1</i> -U	0.2 mM <i>cII</i> p2	0.3 mM <i>Neo</i> -13
Primer 3	0.50 mM <i>Mlh1</i> -T5	-	-
10 mM dNTPs	0.5 µl	0.5 µl	0.5 µl
Taq polymerase	2 units	2 units	2 units
DNA	1.0 µl	2.0 µl	2.0 µl

Table 2.2. Thermocycler programs used to amplify *Mlh1*, *cII*, or *supFG1* genes from mouse DNA.

Gene	Number of Cycles	Temperature (°C)	Time (min)
<i>Mlh1</i>	1	94	4:00
	30	94	1:00
		60	0:30
		72	0:30
	1	72	2:00
<i>cII</i>	1	95	4:00
	35	95	1:00
		55	0:30
		72	0:30
	1	72	2:00
<i>supFG1</i>	1	94	4:00
	35	94	1:00
		55	0:40
		72	0:40
	1	72	2:00

Table 2.3. Sequences of primers used to amplify *Mlh1*, *cII*, and *supFG1* from mouse DNA

Gene	Primer name	Primer sequence
<i>Mlh1</i>	<i>Mlh1</i> -A	5'-AGGAGCTGATGCTGAGGC-3'
	<i>Mlh1</i> -U	5'-GATCTCGACGGTATCGATAAGC-3'
	<i>Mlh1</i> -T5	5'-TTTCATCTTGTCACCCGATG-3'
<i>cII</i>	<i>cII</i> p1	5'-ACCACACCTATGGTGTATGCA-3'
	<i>cII</i> p2	5'-GTCATAATGACTCCTGTTGA-3'
<i>supFG1</i>	<i>T7</i> -12	5'-CTCTAATACGACTCACTATAGGG-3'
	<i>Neo</i> -13	5'-GGACACCGCCAGCAAACGCGAGC-3'

A nested-primer technique was used to genotype for the *Mlh1* gene. A 258 base pair product from the wild-type allele was obtained with primers *Mlh1*-anchor (*Mlh1*-A) and *Mlh1*-untargeted (*Mlh1*-U), and a 198 base pair product from the targeted allele was obtained with primers *Mlh1*-A and *Mlh1*-targeted 5 (*Mlh1*-T5). These products were resolved by electrophoresis using 2% GenePure HiRes Agarose gels (ISC BioExpress). The *cIip1* and *cIip2* primers were used to amplify a 441 base pair product from the *cII* gene. The *T7-12* and *Neo-13* primers were used to amplify a 330 base pair product from the *supFG1* gene. It was assumed that mice were heterozygous for the transgene.

Mice were housed in a positive-ventilation caging unit with automatic water (Thoren Caging Systems, Inc., Hazelton, PA) and were fed a diet of laboratory chow (Picolab Rodent Diet 20, PMI Nutrition International, St. Louis, MO) ad libitum. Mice were cared for by the Oregon State University Laboratory Animal Resources Center according to institutional guidelines, and all procedures were approved by the Oregon State University IACUC. Mice generally were free of endotopic and exotopic parasites and pathogens (Basic Necropsy Profile) except for the *Helicobacter* strains *H. hepaticus*, *H. rodentium*, and *H. typhlonius* (Research Animal Diagnostic Laboratory, University of Missouri, Columbia, MO).

PhIP and Treatment of Mice

PhIP (Toronto Research Chemicals, Ontario, Canada) was dissolved in 0.05 M HCl, diluted with sterile 0.9% saline, and adjusted to pH 4.0 with 1 M NaOH. A final

concentration of 10 mg/kg PhIP was sterile filtered and stored in aliquots at -20°C.

PhIP was protected from exposure to light. Vehicle-treated mice were injected with 10 ml/kg 0.9% sterile saline, pH 4.0. *Mlh1*^{+/+} and *Mlh1*^{-/-} mice were weaned at 23-28 days of age and received their first interperitoneal (i.p.) injection of either 50 mg/kg PhIP or 0.01 ml/kg vehicle solution two to three days post-weaning. Eight injections were administered 48 hrs apart, alternating right- and left-side injections. PhIP exposure by this protocol was not overtly toxic in either *Mlh1*^{+/+} or *Mlh1*^{-/-} mice as assessed by average weight gain in the cohort during the course of treatment.

Materials and Methods specific to Chapter 3: *Mlh1*-dependent suppression of specific mutations induced *in vivo* by the food-borne carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)

Mlh1^{+/+} or *Mlh1*^{-/-} mice carrying either the *supFG1* reporter or the *cII* reporter were used for these studies. Treatment groups were comprised of nearly equal numbers of male and female mice.

Tissue Collection and Transgene Rescue

Mice treated with either PhIP or vehicle were killed two weeks after their last injection (approximately 8 weeks of age) by carbon-dioxide inhalation followed by cervical dislocation. Various tissues were harvested, including thymus, colon, and small intestine. Upon removal, tissues were rinsed with 4 °C sterile PBS. Intestinal tissue was cut longitudinally, and the colon was minced to obtain three representative aliquots of tissue. The small intestine was divided into proximal and distal portions. Tissues were snap-frozen in liquid nitrogen and stored at -80 °C.

High molecular weight genomic DNA was extracted from approximately 100 mg of intestinal tissue or approximately 50 mg of thymus tissue by using the components of a RecoverEase DNA Extraction Kit (Stratagene), summarized as follows. Frozen tissues were weighed and placed in 5 ml of cold lysis buffer (140 mM NaCl, 3 mM KCL, 35 mM sucrose, 1 mM EDTA, 1% Triton X-100, 10 mM Tris-HCl, pH 8.3) in a Wheaton Dounce tissue grinder and were homogenized with 8 – 12 strokes of a loose-fitting pestle followed by 8 strokes with a tight-fitting pestle in order to release cell nuclei. The

homogenate was strained into a 50 ml conical tube using a sterile cell strainer provided by the kit. The tissue grinder was rinsed with an additional 3 ml of cold lysis buffer and the rinse was also strained into the 50 ml conical tube. The homogenate was centrifuged at 1100 x g for 12 min at 4 °C. The supernatant was discarded and the tube was inverted on a paper towel for about 1 min to drain excess liquid. Residual drops were removed using sterile applicators provided by the kit. 70 µl of digestion buffer plus 1.4 µl of RNase-It™ were added to the pellet. The tube was placed in a 50 °C water bath and 70 µl of proteinase K solution (20 mg/ml) was added to the pellet. The pellet was incubated for 45 min with gentle swirling every 10 min. After incubation, the viscous genomic DNA was transferred from the tube to a dialysis cup floating in autoclaved Tris-EDTA (TE) buffer (10 mM Tris-HCl (pH 7.5) 1 mM EDTA), using a wide-bore pipet tip. A volume of 500 ml TE was used for dialysis of each DNA sample. The DNA was dialyzed for 46 to 48 h, with a change of buffer after 16 h. After dialysis, the DNA was transferred to a microfuge tube using a wide-bore pipet tip and stored at 4 °C.

To rescue shuttle vectors, high molecular weight mouse DNA was combined with Transpack phage packaging extracts (Stratagene) as follows. Five eppendorf tubes each containing ~10 µl of phage packaging extract were removed from the -80 °C freezer and placed in a bucket of crushed dry ice. One at a time, approximately 8 µl of genomic DNA was added to a tube of packaging extract that was first spun briefly at 14,000 rpm and finger-thawed. After adding DNA (using a wide-bore pipet tip), the tube was placed in a 30 °C water bath for 90 min. Near the end of the first incubation, a tube containing ~70 µl of phage packaging extract was removed from the -80 °C freezer, finger-thawed,

and placed on ice. For the second addition of packaging extract, 12 μ l of extract was added to each of the five samples, which were then incubated for another 90 min. The reactions were quenched by adding 970 μ l of sterile SM buffer (100 mM NaCl, 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 ml 1 M Tris-HCl pH 7.5, 5 ml of 2% gelatin, final volume of 1 L with ddH_2O) for a final volume of ~ 1000 μ l. The reactions were vortexed at maximum speed for 10 s, stored at 4 $^\circ\text{C}$, and plated generally within a week of the packaging reaction.

Screening for Mutant *supFG1* Transgenes

Characterization of mutations using the *supFG1* reporter in a lambda shuttle vector has been described (Narayanan et al., 1997). Briefly, an estimate of the number of plaque forming units (PFU) in a packaged reaction was made (in duplicate) by combining a small volume of phage with *E. coli* Cla *lacZ125* (*am*) and plating the infected bacteria onto NZY agar plates (NZY agar consists of 100 mM NaCl, 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g yeast extract, 10 g NZ-amine, and 15 g agar per 1 L). The estimate was used to plate the remainder of the packaged reaction at a maximal density of 1,500 plaques per 150 mm plate using top agar containing 1 mM 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-gal) and 1 mM isopropyl β -D-thiogalactoside (IPTG). Phage with inactivating mutations in *supFG1* fail to suppress a nonsense mutation in the *lacZ* gene of the plating strain, thereby generating colorless or light blue plaques. Plates were placed on a light box for better identification of plaques and all plaques were counted by hand. Blue plaques were scored as wild-type for *supFG1*. The reported mutant frequency (MF) is the number of mutant plaques divided by the total number of plaques counted. The DNA obtained and

sequenced from every clear or light blue plaque carried a mutation in the *supFG1* gene. No adjustments were made for potential clonal expansion.

Selection for Mutant *cII* Transgenes

The *cII* mutant frequency was determined by using selection at 24 °C following infection of *E. coli* strain G1250, performed according to the “λ Select-*cII*TM Mutation Detection System for Big Blue® Rodents” protocol provided by Stratagene. Briefly, an estimate of the number of PFU in a packaged reaction was made (in triplicate) by combining a small volume of phage with *E. coli* G1250 and plating the infected bacteria onto TB-1 plates (100 mM NaCl, 10 g casein peptone, 3 μM thiamine hydrochloride, and 12 g agar per 1 L) at 37 °C for 24 hr. The remainder of the packaged reaction was plated and incubated at 24 °C for 46-48 hr to obtain mutant plaques. The density of mutant plaques per 90 mm plate ranged from zero to ~100 depending on the genotype and treatment of the animal from which the DNA was extracted.

For *Mlh1*^{-/-} mice, 1 to 3 packaging reactions were needed to obtain approximately 200,000 PFU per mouse, and for *Mlh1*^{+/+} mice, 1 to 6 packaging reactions were needed to obtain approximately 300,000 PFU per mouse. The raw MF for each mouse was determined by dividing the total number of mutant plaques by the total estimated PFU. By sequencing the mutant transgenes it was possible to discard redundant mutants and adjust the MF for “independent” mutants. For *Mlh1*^{+/+} mice, a total of 74 mutants were obtained from both treatment groups. For *Mlh1*^{-/-} mice, however, a total of 1720 mutants were obtained from both treatment groups. Therefore, it was not feasible to sequence all

mutants. Instead, almost all of the mutants recovered from *Mlh1*^{+/+} mice were sequenced, and enough mutants (172) were sequenced from the *Mlh1*^{-/-} mice to obtain ~10 independent mutants from each mouse. Furthermore, sequencing results showed that 2% of mutants that made a plaque at the selective temperature of 24 °C did not carry a mutation in the *cII* gene. Therefore, the overall MF was first adjusted for whether all phenotypic mutants carried mutations in the *cII* gene, by multiplying the raw MF by the fraction of genotypic *cII* mutants/phenotypic mutants sequenced. The resulting genotypic MF was then multiplied by the fraction of independent mutants/true *cII* mutants sequenced for each mouse. These adjustments were made per mouse, and the adjusted MF were averaged for the final MF per treatment and genotype.

Sequence Analysis of *supFGI* and *cII* Transgenes

Mutant plaques were cored into 100 µl SM buffer (100 mM NaCl, 10 mM g MgSO₄·7H₂O, 50 ml 1 M Tris-HCl, pH 7.5, 5 ml of 2% gelatin per 1 L) with 10 µl chloroform. Phage were eluted overnight at 24 °C and were stored at 4 °C. Phage were re-plated at low density on either TB-1 plates (*cII*) or NZYM plates with X-gal and IPTG (*supFGI*), picked and eluted overnight at 24 °C in 30 µl of PCR-grade water. Eluted phage were transferred to a fresh tube, boiled for 5 min, and placed on ice. Phage DNA was amplified by using PCR. PCR conditions are listed in Table 2.4. The thermocycler programs developed for amplification of *cII* and *supFGI* for genotyping also were used for amplifying phage DNA for sequencing. Primers and dNTPs were removed from PCR reactions by using QiaQuick PCR purification spin columns (Qiagen, La Jolla, CA).

Purified DNA was combined with sequencing primer (*T7-12* for *supFG1* or *cIIp1* for *cII*), ABI Prism Big Dye® terminator v. 3.1 (Applied Biosystems, Foster City, CA), and PCR-grade water for sequencing reactions run on an ABI 3100 capillary sequencer (Center for Gene Research and Biotechnology, Oregon State University). DMSO at 10% of the final volume was substituted for PCR water in *supFG1* reactions. For spectrum analysis all G/C base pair mutants were reported as G/C to A/T, G/C to C/G, or G/C to T/A mutations, based on the assumption that PhIP was adducted to guanine residues.

Table 2.4. PCR mixes used to amplify *cII* or *supFG1* from phage DNA for sequencing (final volume of 50 μ l). 10x buffer, 5x buffer, 25 mM MgCl₂, and taq polymerase are constituents of the Eppendorf Mastertaq kit (VWR). dNTPs were purchased from Fermentas.

Reaction constituents	Gene	
	<i>cII</i>	<i>supFG1</i>
10x Buffer	1x	1x
5x Buffer	1x	1x
25 mM MgCl ₂	4.0 μ l	-
Primer 1	0.2 mM <i>cIIp1</i>	0.3 mM (<i>T7-12</i>)
Primer 2	0.2 mM <i>cIIp2</i>	0.3 mM (<i>Neo-13</i>)
10 mM dNTPs	1.0 μ l	1.0 μ l
Taq polymerase	2 units	2 units
DNA	10.0 μ l	10.0 μ l

Statistical Analyses

Within each genotype, tissue, and reporter, MF for PhIP-treated mice were compared to those for vehicle treated with *t*-tests. A test for equality of variance preceded the *t*-tests (folded *F*-test) with $p > 0.12$ in all cases, but one. The exception was

MF data for *supFG1* in *Mlh1*^{+/+} colon ($p = 0.03$), which was analyzed on the log scale to achieve homogeneity of variance and reduce right skew. For the *cII* reporter, the estimated PhIP-induced MF in *Mlh1*^{-/-} mice was compared to that in *Mlh1*^{+/+} mice by testing for interaction (genotype-by-treatment, Satterthwaite approximate F -test) in a two-factor linear model allowing the variation between mice to be different (larger) in *Mlh1*^{-/-} mice versus *Mlh1*^{+/+} mice ($p = 0.0069$ likelihood ratio test for equality of variances). For *Mlh1*^{-/-} mice, differences in MF between colon and small intestine for PhIP-treated mice were compared to the differences in vehicle treated with a t -test. For tabulations of mutational spectra, mutations were pooled across mice within a group after first confirming that there was no evidence of differences between mice within a group (Fisher's exact test). All analyses were done with SAS for Windows, version 9.1 (2003, SAS Institute, Inc., Cary, NC) utilizing the Ttest, Mixed, and Freq procedures.

Materials and Methods specific to Chapter 4: Induction of aberrant crypt foci in DNA mismatch repair-deficient mice by the food-borne carcinogen 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP)

Mlh1^{+/+} or *Mlh1*^{-/-} mice carrying the *supFG1* reporter were entered into the study as available over a period of approximately 5 months. Treatment groups were comprised of nearly equal numbers of male and female mice.

Tissue Collection

Following treatment with PhIP or vehicle, *Mlh1*^{+/+} and *Mlh1*^{-/-} mice were allowed to live until they exhibited signs of morbidity, including weakness and/or labored breathing. Morbidity often was due to infiltration and enlargement of organs (liver, spleen) or formation of thoracic lymphatic masses by lymphoma. Mice were monitored every 2 to 3 days and, when moribund, were killed by carbon-dioxide inhalation followed by cervical dislocation. Mice found dead were examined for lymphoma, but necrosis prohibited intestinal tumor analysis. Upon necropsy, the colon was removed separately from the intestinal tract, flushed with 4 °C sterile PBS, cut lengthwise, pinned flat onto Whatman 3 filter paper, and fixed with 10% neutral buffered formalin. The intestinal tract (stomach to cecum) was filled with 10% neutral buffered formalin using a 20 gauge needle, then cut lengthwise. The tissue was placed in a 50 ml conical tube and rinsed in 70% ethanol, followed by tap water and a second rinse with 70% ethanol. The intestinal tract was further fixed overnight in 10% neutral buffered formalin and then stored in 70% ethanol. Liver, spleen, kidney, and any tissues that appeared abnormal (eg. thoracic

lymphoma, enlarged lymph nodes) were removed, rinsed in 4 °C sterile PBS and fixed in 10% neutral buffered formalin.

Pathogenic Analysis

Small Intestine

Adenomas identified in intestinal tissues using a Nikon SMZ-U Zoom 1:10 stereomicroscope were excised, embedded in paraffin, sectioned at 4 μm thickness, and stained for one minute with hematoxylin and eosin followed by a tap-water rinse.

Adenomas were defined histologically as a focal area of disorganized epithelial tissue exhibiting increased mitotic activity and cellular atypia. An epithelial neoplasm was considered an adenocarcinoma only if it invaded the muscularis mucosae. Of 93 total adenomas identified by visual inspection, 65 were available for analysis, and 59 were confirmed histologically.

Colon

To visualize ACF, formalin-fixed colons were rinsed in water and 70% ethanol, then stained with 0.2% methylene blue for 1 min. The mucosal surface was examined for ACF using the 6x and 10x objectives of a light microscope. ACF were identified by their thickened epithelia and enlarged luminal openings (two times larger than surrounding crypts), and by their elevation above adjacent normal crypts (Cheng and Lai, 2003). The experimenter was blind to treatment for scoring ACF.

Lymphoma-affected Tissues

Paraffin-embedded tissues were sectioned at 4 μm thickness and were first assessed for lymphoma by staining with hematoxylin. Lymphoma was diagnosed based on the presence of a monomorphic population of lymphocytes accompanied by effacement of normal tissue architecture and increased mitotic activity. Tissues infiltrated with lymphoma also were stained for the cell surface marker CD3, a T-cell-specific antigen, as a means to differentiate T-cell versus B-cell origin (tissues that lacked anti-CD3 staining were considered to be of B-cell origin). Sections were blocked with 1.5% goat serum in Automation buffer for 5 min, followed by a serum-free protein block (DakoCytomation) for 10 min. Polyclonal rabbit anti-CD3 (AbCam ab5690) was diluted 1:100 in Antibody diluent (DakoCytomation) and applied for 30 min at room temperature. After washing in Automation Buffer, Envision+ rabbit (DakoCytomation) was applied for 45 min at room temperature, followed by a wash with Automation Buffer. The chromagen Nova Red (Vector Laboratory) was applied for 5 min and quenched with a wash in ddH₂O followed by hematoxlin (Gill's diluted 1:10 (Shandon)) for 1 min. Slides were rinsed with tap water and blued in 1% lithium carbonate for 30 s.

Statistical Analyses

Kaplan-Meier (non-parametric) survival curves were compared within genotypes with the log rank test, using Prism for Macintosh, version 4.0 (GraphPad Software, San Diego, CA). ACF incidences were compared with the Fisher's exact test using Prism.

ACF counts were modeled without litter and gender as factors, after initial analysis within each treatment group revealed no evidence of those effects. ACF counts were compared between treatments and genotypes using a two-factor generalized linear model with identity link and with an over-dispersed Poisson distribution for the conditional response. The identity link was used to allow comparisons as differences on the same scale. For example, of primary interest is whether the effect of PhIP (as a difference on the original scale) differs between genotypes (which could be assessed as an interaction between treatment and genotype). The over-dispersed Poisson distribution captured the observed pattern of variation increasing with the mean, so that the model-based standard errors for each treatment reasonably matched the empirical standard errors from replicate animals. Analysis of ACF counts was done with SAS for Windows, version 9.1 (2003, SAS Institute, Inc., Cary, NC).

Materials and Methods specific to Chapter 5: *Mlh1*-dependent activation of apoptosis in the murine colon in response to the food-borne carcinogen 2-amino-1-methyl-6-phenylimidazo [4,5 *b*] pyridine (PhIP)

Mlh1^{+/+} or *Mlh1*^{-/-} mice carrying the *cII* reporter were used for these studies. Treatment groups were comprised of nearly equal numbers of male and female mice.

Treatment of Mice

Mice were exposed to multiple injections of 50 mg/kg PhIP or vehicle as described previously (Smith-Roe et al., 2006a) and were killed at approximately 8 weeks of age. Untreated mice and mice receiving a single injection of carcinogen (50 mg/kg PhIP or 80 mg/kg DMH) also were killed at approximately 8 weeks of age. Treatment groups were comprised of 4 to 7 mice, with nearly equal numbers of male and female mice for each treatment. DMH (Sigma-Aldrich CAS# 306-37-6) was dissolved in a solution of 0.9% saline and 1mM EDTA. 1 M NaOH was used to adjust the pH to approximately 7.0, and the solution was brought to a final volume for a final concentration of 8 mg/ml. DMH was used within 45 min of preparation.

Tissue Collection and Processing

Mice were injected i.p. with 20 mg/kg BrdU solution (BD Pharmingen) two hours prior to killing by CO₂ inhalation and cervical dislocation. The BrdU solution was prepared by thawing a vial of a 10 mg/ml solution of BrdU on ice and diluting it with sterile, cold PBS for a final concentration of 2 mg/ml. At necropsy, the colon was removed, flushed with cold, sterile PBS, cut lengthwise and laid flat on filter paper, rolled

into a “jelly-roll” using a transfer pipette, and placed into a 2 ml microfuge tube containing neutral-buffered formalin (VWR). Formalin-fixed colons were embedded in paraffin and were sliced 4 μm thick. Colons were sectioned in a manner to provide matched tissue samples to stain for either BrdU incorporation or the presence of cleaved caspase-3.

Immunohistochemistry

All sections were first de-paraffinized in a standard series of xylene and alcohol washes as follows: 4 washes in xylene, 2 washes in 100% ethanol, 1 wash in 80% ethanol, 1 wash in ddH₂O (30 s each). A micro-capillary staining system (Fisher Microprobe) was used for application of reagents to slides. Sections were stained for incorporated BrdU using the protocol and components of the BrdU In-Situ Detection Kit (BD Pharmingen), except that the biotinylated anti-BrdU antibody was diluted 1:40, and blocking with 3% H₂O₂ was performed after the antigen retrieval step. Apoptotic cells were identified using the polyclonal cleaved caspase-3 (Asp175) antibody (Cell Signaling Technology[®]). High temperature antigen retrieval in Dako antigen retrieval buffer (DAKO Cytomation) was followed by blocking with 3% H₂O₂ in Automation buffer (Biomedica Corp) for 10 min. Dako serum-free protein block was then applied for 10 min. Anti-CC-3 was diluted 1:200 and applied to slides for 30 min in a humidified chamber at room temperature. The Dako universal negative rabbit antibody was used for a negative control. Slides were washed with TBST and Dako anti-rabbit EnVision was applied for 30 min. Incorporated BrdU and cleaved caspase-3 antigens were visualized

using streptavidin-HRP with the Vector[®] NovaRED[™] Substrate Kit (Vector Laboratories). NovaRED was applied for 5 min and quenched with a wash in ddH₂O. Slides were counterstained with hematoxylin (Gill's diluted 1:10 (Shandon)) for 1 min. Slides were rinsed with tap water and blued in 1% lithium carbonate for 30 s.

Data Acquisition

Crypts that were open to the lumen and that extended to the musculature in the same plane were selected for counting. The entire length of the colon was surveyed for selectable crypts, although crypts from the proximal (ridged) portion of the colon were not selected as often due to difficulty in identifying individual cells within the crypt. Crypts were photographed at 20x using a Nikon digital camera mounted to a Leica microscope (Figure 5.1). Each crypt was divided longitudinally, and a total of 25 crypts (50 half-crypts), were counted per mouse per stain (BrdU or CC-3). Cells were numbered beginning at the bottom of the crypt and counted until the juncture of the crypt at the intercrypt table. The number of cells per half-crypt, the number of stained cells per half-crypt, and the location of each stained cell were recorded for both stains using the manual tag function of the Image-Pro Express software program for Windows (Media Cybernetics). The experimenter was blind to treatment when counting colon cells.

The proliferative index (%), a measure of the overall level of cell proliferation in colon crypts, was defined as the total number of BrdU-positive cells counted in 50 half-crypts divided by the total number of cells counted in 50 half-crypts, multiplied by 100. The index allows comparisons across mice, as the total number of cells in 50 half-crypts

is not exactly the same from animal to animal. The apoptotic index (%) was defined in the same manner for CC-3-positive cells. To compare groups, proliferative and apoptotic indices were averaged.

Chapter 3. *Mlh1*-dependent suppression of specific mutations induced in vivo by the food-borne carcinogen 2-amino-1-methyl-6-phenylimidazo[4-5-*b*]pyridine (PhIP)

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ABSTRACT

Disruption of the DNA mismatch repair (MMR) pathway results in elevated mutation rates, inappropriate survival of cells bearing DNA damage, and increased cancer risk. Relatively little is known about the potential impact of environmentally relevant carcinogens on cancer risk in individuals with MMR-deficiency. We determined the effect of MMR status (*Mlh1*^{+/+} versus *Mlh1*^{-/-}) on mutagenesis induced by the cooked-meat mutagen, 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) within *cII* and *supFGI* transgene reporters. Despite being a lymphomagen in mice, PhIP was not mutagenic in thymus. In colon, PhIP exposure induced 3-fold more mutations in *Mlh1*-deficient mice compared to their *Mlh1*^{+/+} littermates. Similar induction was seen in *Mlh1*^{-/-} small intestine. Analysis of mutational spectra revealed that G/C to T/A transversions, the “signature PhIP mutation”, were induced to similar levels regardless of *Mlh1* status. In contrast, *Mlh1*^{-/-} mice exhibited hypermutability to frameshifts, G/C to A/T transitions, and G/C to C/G transversions. Thus, both the level and types of mutation induced by PhIP are influenced by the activity of the MMR system. MMR may suppress PhIP-induced mutation through recognition and processing of specific mispairs (PhIP-G/T, PhIP-G/G, and PhIP-G/loop mispairs). In contrast, the PhIP-G/A mispair is unlikely to be a MMR substrate. In addition, the similar induction of both transversions and transitions in *Mlh1*^{-/-} mice suggests that mutagenic bypass of PhIP-G is similarly efficient with dATP, dTTP, and dGTP, in contrast to previously published conclusions. Our data suggests that MMR-deficiency would increase the likelihood of PhIP-induced

carcinogenic mutations. Further evaluation of the risk that consumption of heterocyclic amines may impart to MMR-deficient individuals therefore is warranted.

INTRODUCTION (abbreviated from original publication)

Few studies have examined the potential impact of environmentally relevant carcinogens on cancer risk in individuals with MMR-deficiency. 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) is a heterocyclic amine formed during the cooking of meat that is mutagenic in bacteria, and mutagenic and carcinogenic in rodents (Gooderham et al., 2002). PhIP forms adducts predominately to guanine residues in DNA, and mutagenesis in several systems is associated with G/C to T/A transversions and -1 bp deletions in G/C bp (Dashwood, 2003; Shibutani et al., 1999). Based on mutational spectrum in wild-type cells, mutagenic bypass of PhIP-G in DNA is proposed to occur most efficiently with dATP, although bypass has not been measured directly. Colon cancer risk is increased for persons who consume high levels of heterocyclic amines (Cross and Sinha, 2004; Layton et al., 1995), and PhIP has been classified by the National Toxicology Program as “reasonably anticipated to be a human carcinogen” (2004). Cell-culture studies suggest that MMR responds to PhIP-induced DNA damage. Cancer cell lines lacking MMR are resistant to cytotoxicity, and are hypermutable in response to PhIP at the *HPRT* gene (Duc and Leong-Morgenthaler, 2001; Glaab and Skopek, 1999; Leong-Morgenthaler et al., 2001). Consistent with *in vitro* studies, *Msh2* is involved in suppression of PhIP-induced mutation in mouse colon (Zhang et al., 2001), suggesting that exposure to PhIP may increase cancer risk for MMR-deficient individuals. However, *Msh2* has functions separate from canonical MMR that do not require MutL proteins (Sugawara et al., 1997), including the activation of apoptosis and

the suppression of mutation in murine small intestine in response to high doses of alkylating agents (Sansom et al., 2003). Therefore, to understand both the potential risk of PhIP exposure in individuals with different MMR deficiencies and the mechanism of cellular responses to PhIP-induced DNA damage, it is necessary to determine the involvement of both MSH and MLH proteins in response to damage *in vivo*.

We treated *Mlh1*^{-/-} mice with PhIP and measured induced mutant frequency in the colon, proximal small intestine, and thymus using the *cII* transgene reporter. Also, as PhIP is reported to induce frameshifts in runs of guanine residues (Dashwood, 2003), we exposed mice carrying the *supFGI* transgene to PhIP. *SupFGI* contains two mononucleotide repeats of G/C base pairs, similar to repeats in the coding regions of tumor suppressor genes that are hotspots for mutation in MMR-deficient tumors (Duval and Hamelin, 2002).

Although PhIP exposure was not mutagenic in thymus of MMR-proficient or deficient mice, PhIP exposure increased the *cII* mutant frequency (MF) approximately 3-fold more in the colon of *Mlh1*^{-/-} mice versus *Mlh1*^{+/+} littermates. A similar increase was detected in *Mlh1*^{-/-} small intestine. PhIP-induced hypermutability in *Mlh1*^{-/-} colon was characterized by the selective increase in G/C to A/T transitions, G/C to C/G transversions, and frameshifts within simple repeats, whereas G/C to T/A transversions, previously characterized as signature mutations, were similarly induced in both *Mlh1*^{-/-} and *Mlh1*^{+/+} mice. Thus, hypermutability to PhIP is a general response of MMR-deficiency resulting from an inability to suppress specific classes of PhIP-induced mutation. As such, the previously characterized signature mutation for PhIP exposure

may result from a lack of recognition and/or processing of a PhIP-G/A mispair by the MMR pathway. In addition, the similar induction of G/C to A/T, G/C to C/G, and G/C to T/A mutations in *Mlh1*^{-/-} mice suggests that mutagenic bypass of PhIP-G adducts *in vivo* is similarly efficient with dTTP, dGTP, and dATP.

RESULTS

PhIP-induced cII Mutant Frequency in Mlh1-proficient and -deficient mice

In colon tissue of *Mlh1*^{+/+} mice, PhIP exposure increased the average *cII* MF from 2.5×10^{-5} to 15.9×10^{-5} ($P < 0.005$), indicating a PhIP-induced MF of approximately 13×10^{-5} (Figure 3.1, Table 3.1). In contrast, the identical exposure protocol in *Mlh1*^{-/-} littermates resulted in an induced frequency of approximately 43×10^{-5} , increasing the average *cII* MF from 25.9×10^{-5} to 69.2×10^{-5} ($P < 0.005$). The 3-fold greater induction in MF in the colons of *Mlh1*^{-/-} mice relative to *Mlh1*^{+/+} mice was significant ($P < 0.01$). Thus, *Mlh1*-deficient mice are hypermutable by PhIP.

Although susceptible to colon tumors, the predominant cancer types that arise spontaneously in *Mlh1*^{-/-} mice are lymphomas and small intestinal adenomas. Also, PhIP is a lymphomagen in mice (Esumi et al., 1989; Ochiai et al., 2002; Sorensen et al., 1997). PhIP exposure increased the MF in proximal small intestine of *Mlh1*^{-/-} mice from 49×10^{-5} to 107×10^{-5} ($P < 0.01$) (Table 3.1), indicating an induced MF of approximately 58×10^{-5} . Thus, the PhIP-induced MF was similar in small intestine and colon of *Mlh1*^{-/-} mice ($P = 0.24$). Although we did not measure MF in *Mlh1*^{+/+} small intestine, previous studies

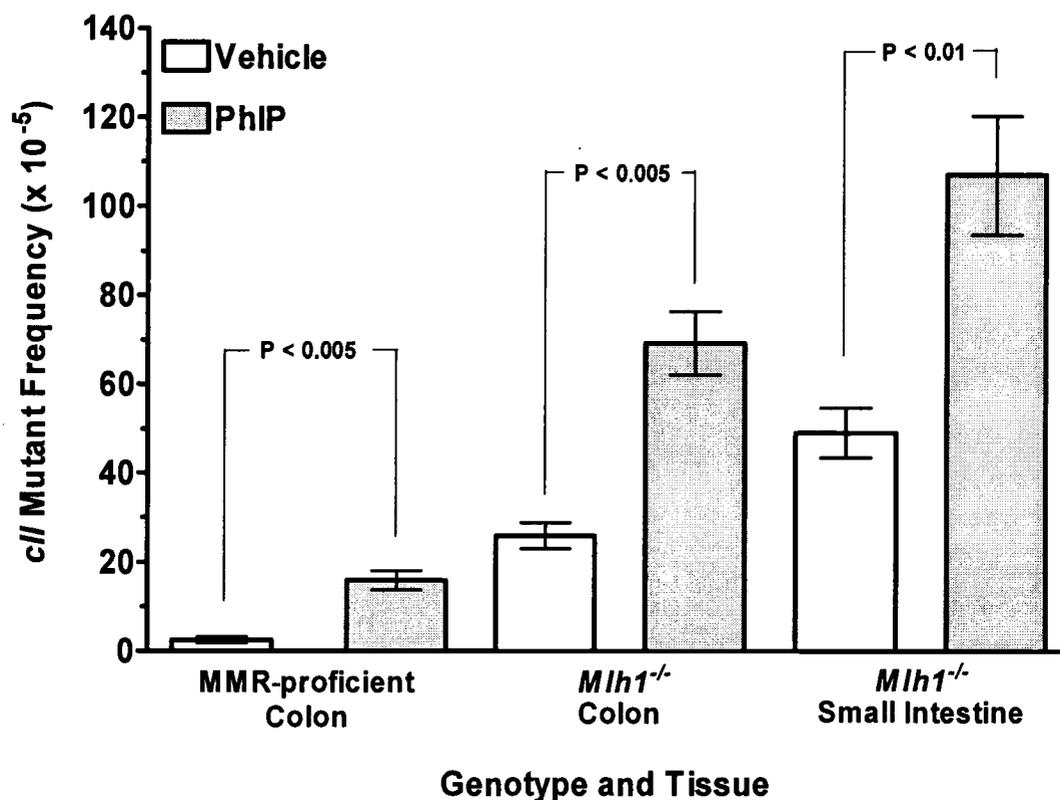


Figure 3.1. PhIP-induced *cII* mutagenesis in *Mlh1*-proficient and -deficient mice. *Mlh1*^{+/+} and *Mlh1*^{-/-} mice were exposed to eight doses of 50 mg/kg PhIP or vehicle via i.p. injection over a period of two weeks. Following two additional weeks, genomic DNA was isolated from the indicated tissues, and MF were determined in the *cII* gene. Plotted are the average MF (adjusted for potential clonal expansion) of three to five vehicle- (white bars) or PhIP-treated (gray bars) mice in each treatment group. Data = mean \pm standard error of the mean (S.E.M.).

Table 3.1. Spontaneous and PhIP-induced *cII* mutant frequency in colon and small intestine of *Mlh1*^{+/+} and *Mlh1*^{-/-} mice

Tissue & Genotype	No. of mouse	Vehicle-treated			PhIP-treated			PhIP-induced MF (x10 ⁻⁵) ^d
		pfu ^a	No. of mutants ^b	MF (x10 ⁻⁵) ^c	pfu	No. of mutants	MF (x10 ⁻⁵)	
Colon <i>Mlh1</i> ^{+/+}	1	254500	4	1.7	359400	56	15.6	
	2	210400	8	3.9	294700	36	12.2	
	3	217500	4	1.8	237100	47	19.8	
Mean ± s.e.m. ^e				2.5 ± 0.7			15.9 ± 2.2	13.4 ± 2.3
Colon <i>Mlh1</i> ^{-/-}	1	236000	77	32.6	397600	362	91.1	
	2	159500	33	20.6	298500	217	72.8	
	3	79900	23	28.6	124400	59	47.2	
	4	268400	58	21.6	126400	90	71.0	
	5				268300	171	63.7	
Mean ± s.e.m.				25.9 ± 2.9			69.2 ± 7.1	43.3 ± 7.7
Small Intestine <i>Mlh1</i> ^{-/-}	1	207200	111	53.7	223500	338	151.2	
	2	280100	173	61.6	226600	268	118.0	
	3	273100	96	35.2	243600	220	90.3	
	4	106000	48	45.6	188600	195	103.4	
	5				172000	124	72.1	
Mean ± s.e.m.				49.0 ± 5.6			107.0 ± 13.4	58.0 ± 14.5

^aPfu: plaque forming units ^bNumber of mutants has been corrected for clonal expansion and rounded to the nearest whole number

^cMutant frequency (MF) is the number of mutants divided by pfu ^dInduced MF is the within-genotype difference in MF due to

PhIP treatment ^eS.e.m: standard error of the mean.

with the reporter transgenes *cII* (Itoh et al., 2003), *lacI* (Zhang et al., 1996), and *lacZ* (Lynch et al., 1996) indicate that PhIP-induced mutation is similar in small intestine and colon of wild-type mice. Thus, our data are consistent with a MMR-dependent suppression of PhIP-mutagenesis in both small intestine and colon. In contrast to results using intestinal tissue, no significant increase in MF was detected in thymus of *Mlh1*^{+/+} or *Mlh1*^{-/-} mice with PhIP treatment ($2.5 \pm 1.0 \times 10^{-5}$ versus $2.9 \pm 0.5 \times 10^{-5}$ and $41.1 \pm 7.8 \times 10^{-5}$ versus $51.2 \pm 5.5 \times 10^{-5}$, respectively). Therefore, despite a previously described lymphomagenic activity, PhIP is not a strong mutagen in either *Mlh1*^{+/+} or *Mlh1*^{-/-} mice with the current protocol.

***cII* Mutational Spectra in Colon and Small Intestine**

To determine whether MMR status influenced the types of mutations resulting from PhIP exposure, we compared the induced MF of different classes of mutations (Figure 3.2, Tables 3.2 and 3.3). The mutational spectra in vehicle-treated *Mlh1*-proficient and -deficient mice were similar to previous reports of spontaneous spectra (Andrew et al., 1998; Andrew et al., 1997; Andrew et al., 2000; Baross-Francis et al., 2001; Mark et al., 2002; Xu et al., 2001; Zhang et al., 2002). In *Mlh1*^{+/+} mouse colon, 69% (9×10^{-5}) of the PhIP-induced MF resulted from G/C to T/A transversions, consistent with their reported predominance among PhIP-induced mutations. In addition, the PhIP-induced MF for single-bp frameshifts within the G/C repeat and for G/C to A/T transitions was approximately 1×10^{-5} . In contrast to *Mlh1*^{+/+} mice, G/C to T/A transversions in *Mlh1*^{-/-} colon accounted for only 23% of the total PhIP-induced MF.

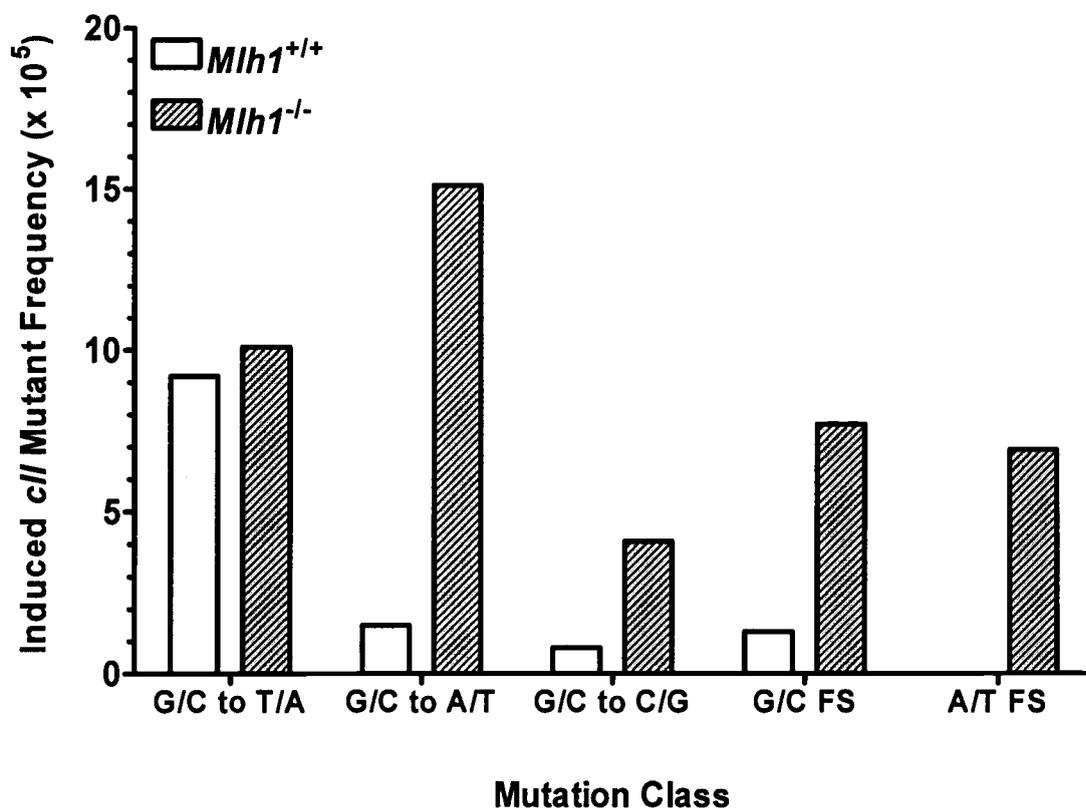


Figure 3.2. PhIP-induced *cII* mutations in colon of *Mlh1*-proficient and -deficient mice. The spectrum of *cII* mutations in the colon induced by PhIP exposure of *Mlh1*^{+/+} and *Mlh1*^{-/-} mice was determined by sequencing of independent mutant phage. Plotted are the induced MF (calculated as the difference in average frequency in PhIP-treated versus vehicle-treated mice) for several classes of mutation in *Mlh1*^{+/+} (white bars) and *Mlh1*^{-/-} (stripped bars) mice.

The increased frequency of these mutations was essentially the same in both *Mlh1*-deficient (10×10^{-5}) and -proficient colon (9×10^{-5}). Thus, induction of G/C to T/A transversions, a signature mutation of PhIP exposure, was unaffected by *Mlh1* genotype. Other mutations were induced to a greater extent in *Mlh1*^{-/-} relative to *Mlh1*^{+/+} mice. The PhIP-induced MF for frameshifts within the G/C bp repeat in *Mlh1*^{-/-} colon was approximately 8×10^{-5} , compared with 1×10^{-5} in *Mlh1*^{+/+} mice. In addition, an increase in the MF of one bp deletions within an A/T repeat of about 7×10^{-5} was detected in *Mlh1*^{-/-} colon. Similar A/T frameshifts were not detected in *Mlh1*^{+/+} colon. With regard to base substitutions other than G/C to T/A transversions, PhIP exposure increased the MF of G/C to A/T transitions by 15×10^{-5} and of G/C to C/G transversions by 4×10^{-5} . The induction of frameshifts, G/C to A/T and G/C to C/G base substitutions in *Mlh1*^{-/-} colon was 15-, 8-, and 4-fold greater (respectively) than in *Mlh1*^{+/+} colon. PhIP-induced mutations in *Mlh1*^{-/-} small intestine also were comprised of several classes other than G/C to T/A transversions (Table 3.4) in a pattern similar to that of colon. Therefore, hypermutability to PhIP in *Mlh1*^{-/-} intestine is associated with an increased frequency of a broad spectrum of mutations.

Table 3.2. Spectrum of PhIP-induced *cII* mutations in colon of *Mlh1*^{+/+} mice

Mutation Type	Vehicle-treated			PhIP-treated			PhIP-induced MF ($\times 10^{-5}$) ^c
	No. of mutants ^a	% Recovered	MF \pm s.e.m ($\times 10^{-5}$) ^b	No. of mutants	% Recovered	MF \pm s.e.m ($\times 10^{-5}$)	
Total	18 ^d		2.5 \pm 0.7	35		15.9 \pm 2.2	13.4 \pm 2.3
Transitions							
G/C to A/T	12	66.7	1.7	7	20.0	3.2	1.5
A/T to G/C	2	11.1	0.3	1	2.9	0.5	0.2
Transversions							
G/C to T/A	2	11.1	0.3	21	60.0	9.5	9.2
G/C to C/G	1	5.6	0.1	2	5.7	0.9	0.8
A/T to T/A	N.D. ^e			1	2.9	0.5	(< 0.5)
A/T to C/G	N.D.			N.D.			N.D.
Frameshifts							
-1 G/C	N.D.			2	5.7	0.9	(< 0.9)
+1 G/C	1	5.6	0.1	1	2.9	0.5	0.4
-1 A/T	N.D.			N.D.			N.D.
+1 A/T	N.D.			N.D.			N.D.
Other	N.D.			N.D.			

^aNumber of mutants has been corrected for clonal expansion ^bMutant frequency for each class of mutations is the percent recovered multiplied by the total MF ^cInduced MF is the within genotype difference in MF due to PhIP treatment ^dTwo mutants identified in packaging reactions for which titers were not available are included in the total count, but were excluded from the calculations of total mutant frequency in Table 1 ^eN.D.: none detected.

Table 3.3. Spectrum of PhIP-induced *cII* mutations in colon of *Mlh1*^{-/-} mice

Mutation Type	Vehicle-treated			PhIP-treated			PhIP-induced MF (x10 ⁻⁵) ^c
	No. of mutants ^a	% Recovered	MF ± s.e.m (x10 ⁻⁵) ^b	No. of mutants	% Recovered	MF ± s.e.m (x10 ⁻⁵)	
Total	43		25.9 ± 2.9	52		69.2 ± 7.1	43.3 ± 7.7
Transitions							
G/C to A/T	17	39.5	10.2	19	36.5	25.3	15.1
A/T to G/C	8	18.6	4.8	2	3.8	2.6	N.D. ^d
Transversions							
G/C to T/A	1	2.3	0.6	8	15.4	10.7	10.1
G/C to C/G	2	4.7	1.2	4	7.7	5.3	4.1
A/T to T/A	1	2.3	0.6	N.D.			N.D.
A/T to C/G	N.D.			N.D.			N.D.
Frameshifts							
-1 G/C	4	9.3	2.4	5	9.6	6.6	4.2
+1 G/C	3	7.0	1.8	4	7.7	5.3	3.5
-1 A/T	4	9.3	2.4	7	13.5	9.3	6.9
+1 A/T	2	4.7	1.2	N.D.			N.D.
Other	1 ^e	2.3	0.6	3 ^f	5.8	4.0	3.4

^aNumber of mutants has been corrected for clonal expansion ^bMutant frequency for each class of mutations is the percent recovered multiplied by the total MF ^cInduced MF is the within genotype difference in MF due to PhIP treatment ^dN.D.: none detected ^eDeletion ^fTwo deletions, one double mutant.

Table 3.4. Spectrum of PhIP-induced *cII* mutations in small intestine of *Mlh1*^{-/-} mice

Mutation Type	Vehicle-treated			PhIP-treated			PhIP-induced MF (x10 ⁻⁵) ^c
	No. of mutants ^a	% Recovered	MF ± s.e.m (x10 ⁻⁵) ^b	No. of mutants	% Recovered	MF ± s.e.m (x10 ⁻⁵)	
Total	31		49.0 ± 5.6	49		107.0 ± 13.4	58.0 ± 14.5
Transitions							
G/C to A/T	12	38.7	19.0	17	34.7	37.1	18.1
A/T to G/C	2	6.5	3.2	1	2.0	2.1	N.D. ^d
Transversions							
G/C to T/A	3	9.7	4.8	10	20.4	21.8	17.0
G/C to C/G	1	3.2	1.6	4	8.2	8.8	7.2
A/T to T/A	1	3.2	1.6	N.D.			N.D.
A/T to C/G	N.D.			N.D.			N.D.
Frameshifts							
-1 G/C	4	12.9	6.3	4	8.2	8.8	2.5
+1 G/C	3	9.7	4.8	2	4.1	4.4	N.D.
-1 A/T	4	12.9	6.3	5	10.2	10.9	4.6
+1 A/T	N.D.			N.D.			
Other	1 ^e	3.2	1.6	6 ^f	12.2	13.1	11.5

^aNumber of mutants has been corrected for clonal expansion ^bMutant frequency for each class of mutations is the percent recovered multiplied by the total MF ^cInduced MF is the within genotype difference in MF due to PhIP treatment ^dN.D.: none detected ^eDeletion ^fOne deletion, five double mutants.

supFG1 Mutant Frequency and Mutational Spectra in Colon

To characterize further the interactions between PhIP-induced frameshift mutagenesis and MMR-deficiency, we exposed mice carrying the *supFG1* gene to PhIP. *supFG1* contains two mononucleotide repeats of G/C bp, one of seven bp and one of eight bp (compared to a repeat of six G/C bp in *cII*) that are highly sensitive to insertion and deletion in MMR-deficient mice (Andrew et al., 2000; Narayanan et al., 1997; Yao et al., 1999). Based on the adduction of metabolized PhIP to guanine in DNA (Gooderham et al., 2002), we expected *supFG1* to be highly sensitive to PhIP-induced insertions and deletions.

In colon tissue of *Mlh1*^{+/+} mice, PhIP exposure increased the average *supFG1* MF from 7.4×10^{-5} to 32.0×10^{-5} ($P < 0.0001$), indicating an induced MF of approximately 24.6×10^{-5} (Table 3.5). Surprisingly, sequencing of mutant *supFG1* genes demonstrated that the majority of the increased MF (53%, or 13×10^{-5}) was due to G/C to T/A transversions, not insertions and deletions. Insertions and deletions of one bp within G/C bp repeats also were induced, representing 22% (5×10^{-5}) of the total (all mutants recovered from the PhIP-treated mice were independent). Additional mutations (G/C to C/G and A/T to T/A transversions) were detected only in the PhIP-treated group, although the relatively small number recovered limited a conclusive analysis. As reported previously (Yao et al., 1999), the spontaneous *supFG1* MF in colon tissue of *Mlh1*^{-/-} mice was greatly elevated (484×10^{-5} versus 7.4×10^{-5} in *Mlh1*^{+/+} colon) due predominately to insertions or deletions of one bp within G/C repeats. When treated with

PhIP, the average MF was increased (689×10^{-5} versus 484×10^{-5} vehicle). However, the high spontaneous background and variability precluded detection of a significant increase due to PhIP ($p = 0.24$). Accounting for clonogenicity did not significantly alter the results, nor was there a significant shift in mutational spectrum in colon of PhIP-treated *Mlh1*^{-/-} mice (data not shown).

Table 3.5. Spontaneous and PhIP-treated *supFGI* mutant frequency in colon tissue of *Mlh1*^{+/+} and *Mlh1*^{-/-} mice

Genotype	No. of mouse	Vehicle-treated			PhIP-treated			PhIP-induced MF (x10 ⁻⁵) ^c
		pfu ^a	No. of mutants	MF (x10 ⁻⁵) ^b	pfu	No. of mutants	MF (x10 ⁻⁵)	
<i>Mlh1</i> ^{+/+}	1	55780	2	3.6	15825	11	69.5	
	2	104505	7	6.7	19373	5	25.8	
	3	53280	2	3.8	17435	3	17.2	
	4	19623	3	15.3	19622	3	15.3	
Mean ± s.e.m. ^d				7.4 ± 2.7			32.0 ± 12.7	24.6 ± 13.0
<i>Mlh1</i> ^{-/-}	1	24722	139	562	13429	124	923	
	2	14018	65	464	22514	126	560	
	3	15013	64	426	14690	86	585	
Mean ± s.e.m.				484 ± 41			689 ± 117	205 ± 124

^aPfu: plaque forming units ^bMutant frequency is the number of mutants divided by pfu ^cInduced MF is the within-genotype difference in MF due to PhIP treatment ^dS.e.m: standard error of the mean.

DISCUSSION

Defects in MMR are associated with increased susceptibility to colorectal and other cancers. Although spontaneous mutagenesis is greatly elevated in the absence of functional MMR, MMR also is important for suppression of mutation induced by exogenous DNA-damaging agents (Buermeyer et al., 1999). Few studies, however, have examined the potential contribution of commonly encountered environmental mutagens to MMR-deficient cancer risk. In the present study, the PhIP-induced *cII* MF in colon tissue of *Mlh1*^{-/-} mice was 3-fold greater than that in *Mlh1*^{+/+} mice. Sequencing of mutants revealed that hypermutability of *Mlh1*^{-/-} mice was not due to a simple increase in previously described PhIP signature mutations. Specifically, whereas G/C to T/A transversions were induced to the same extent in both *Mlh1*-proficient and -deficient mice, the PhIP-induced MF of G/C to A/T transitions, G/C to C/G transversions, and frameshifts was 8-, 4-, and 15-fold greater (respectively) in *Mlh1*^{-/-} mice. Similar results were observed in proximal small intestine. Therefore, *Mlh1* has a significant role in the intestine in suppressing specific classes of mutations induced by PhIP.

In contrast to results obtained using colon and small intestine, no significant increase in mutant frequency was detected in thymus of *Mlh1*-proficient or -deficient mice two weeks after exposure to PhIP. This lack of mutation induction was surprising, considering that PhIP is a lymphomagen in mice (Esumi et al., 1989; Ochiai et al., 2002; Sorensen et al., 1997; Sorensen et al., 1996). However, PhIP-DNA adduct levels in thymus are reported to be one-tenth of the level induced in the intestine (Turteltaub et al., 1992). Possibly significant mutations would be detected in thymus with higher doses, at

different time points, or with a different treatment protocol. As a comparison, lymphoma was detected following long-term exposure in the diet (300 – 400 ppm PhIP, 7 months to life-time exposure). Alternatively, the recently recognized estrogenic potential of PhIP (Lauber et al., 2004) may contribute to lymphomagenesis in mice.

The PhIP-induced hypermutability in *Mlh1*^{-/-} colon described in the current study is similar to a reported 3-fold greater induction of mutation by PhIP in the *lacI* gene in *Msh2*^{-/-} mouse colon (Zhang et al., 2001). Therefore, a generalized MMR response involving both MSH and MLH proteins is engaged *in vivo* to reduce mutations due to PhIP exposure. Similar hypermutability first was reported *in vitro* with *MLH1*-*MSH2*- and *MSH6*-deficient cancer cell lines (Glaab and Skopek, 1999; Leong-Morgenthaler et al., 2001). Hypermutability to PhIP in *Msh2*^{-/-} colon was associated with increased G/C to A/T transitions and G/C base pair frameshifts relative to *Msh2*^{+/+} colon, whereas the frequency of PhIP-induced G/C to T/A transversions was nearly the same in both *Msh2*^{+/+} and *Msh2*^{-/-} mice. Unlike the current study, induction of G/C to C/G transversions and A/T base pair frameshifts was not detected. The broader spectrum of induced mutations detected in *Mlh1*^{-/-} mice likely is due the use of different transgene reporters (*cII* versus *lacI*), although differences in loss of *Mlh1* versus *Msh2* also may contribute. In addition, our mice received twice the dose of PhIP as those in the previous study, perhaps allowing for greater sensitivity for detection of PhIP-specific versus spontaneous mutations. Taken together, our results and those obtained in *Msh2*^{-/-} colon demonstrate a hypermutable response to PhIP in MMR-deficient mice that is due to the accumulation of mutations not typically associated with PhIP-mutagenesis.

Hypermutable to PhIP in MMR-deficient mice may result from inappropriate survival of cells bearing persistent damage, thereby heightening the probability for mutagenesis. MMR-deficient cell lines that exhibit PhIP-induced hypermutability also are resistant to the cytotoxic effects of PhIP, in part due to reduced apoptosis (Duc and Leong-Morgenthaler, 2001; Glaab and Skopek, 1999). In addition, a loss of MMR-dependent apoptosis is correlated with hypermutability induced by alkylating agents in small intestine of MMR-deficient mice (Sansom et al., 2003; Takagi et al., 2003; Toft et al., 1999). Although a similar loss of MMR-dependent apoptosis may contribute to increased levels of mutation induced by PhIP, it is not obvious how enhanced survival of damaged cells would alter the spectrum of induced mutations. Therefore, loss of MMR-dependent apoptosis cannot entirely explain the mutagenic response of MMR-deficient mice to PhIP.

An alternative explanation for suppression of PhIP-induced mutation by MMR involves excision targeted to the nascent DNA strand following bypass of a damage-containing template. As metabolically activated PhIP adducts almost exclusively to guanine (Frandsen et al., 1992; Lin et al., 1992), the increased PhIP-induced G/C to A/T and G/C to C/G base substitutions in MMR-deficient mice are consistent with MMR recognition and processing of PhIP-G/T and PhIP-G/G mispairs. Excision of T- or G-containing nascent DNA would allow a second chance for replication bypass to insert dCTP correctly opposite PhIP-G. In contrast, the similar levels of G/C to T/A transversions in both MMR-proficient and -deficient cells suggests that PhIP-G/A mispairs are not recognized and/or processed by MMR. Similar results have been

reported for 7,8-dihydro-8-oxoguanine, which can be processed by MMR *in vitro* when mispaired with dTTP or dGTP, but not dATP (Larson et al., 2003). Our results and that of Zhang et al. (2001) also are consistent with MMR processing of loop intermediates (generated by slip mispairing) to reduce the mutagenicity of PhIP. Additional biochemical studies are necessary to confirm whether MMR recognition and processing of different PhIP-G mispairs varies in the manner suggested.

Previous studies in wild-type cells identifying the predominance of G/C to T/A transversions among mutations induced by PhIP have concluded that PhIP-G adducts are bypassed most efficiently with dATP (Carothers et al., 1994; Shibutani et al., 1999). However, the similar induction of G/C to A/T, G/C to C/G, and G/C to T/A mutations in *Mlh1*-deficient mice suggests that bypass of PhIP-G with dTTP or dGTP may be as efficient as with dATP. As with PhIP, the structurally related environmental mutagen *N*-acetyl-2-aminofluorene (AAF) adducts to guanine and is associated with G/C to T/A transversions (Shibutani et al., 1998). However, bypass of AAF-G (using polymerase ϵ) with different dNTPs does not correlate with the mutational spectrum. Instead, bypass efficiencies with dATP, dTTP, or dGTP are nearly equivalent, at approximately 2-10% of the efficiency of non-mutagenic bypass with dCTP (Masutani et al., 2000). Similar bypass efficiencies with PhIP could generate a mutational spectrum like that seen in our *Mlh1*^{-/-} mice. Direct measurement of bypass of PhIP-G in the absence of MMR would help clarify the relationship between bypass efficiencies and mutational spectra.

In addition to base substitutions, *Mlh1*^{-/-} mice also were hypermutable to PhIP-induced frameshifts at both G/C and A/T bp repeats. PhIP-induced frameshift

mutagenesis at G/C bp repeats was expected and has been reported on extensively in the literature (Dashwood, 2003); however, the induction of A/T bp frameshifts in *Mlh1*^{-/-} mice is surprising, and is the first report of non-G/C bp repeat mutagenesis by PhIP. DNA-damage induced frameshifts result from increased slippage/misalignment of the template and nascent strands of DNA during replication, especially when the misaligned intermediate is stabilized by local complementary bases (eg. within repetitive sequences). Products of such bypass are detected in biochemical experiments with AAF-G adducts and purified polymerases (Shibutani et al., 2004; Thomas et al., 1995; Yasui et al., 2004). Slip-strand bypass of PhIP-G adducts within G/C bp repeats could account for G/C bp frameshifts in both MMR-proficient and -deficient cells, whereas deletions within the A/T bp repeat in *Mlh1*-deficient mice might be caused by misalignment during bypass of a PhIP-G adduct in the flanking G/C bp.

We have demonstrated in mice that MMR-deficient intestinal tissues are subjected to a wider range and a higher level of PhIP-induced mutations compared to repair-proficient tissues. Therefore, MMR-deficiency may increase likelihood of PhIP-induced carcinogenic mutations. As such, exposure to PhIP may be a significant concern for individuals at risk for MMR-deficiency. Such risk is not limited to those with an inherited deficiency, as MMR activity can be down-regulated by cellular environments such as chronic oxidative stress (Chang et al., 2002) or hypoxia (Mihaylova et al., 2003). Reduced exposure to PhIP is a modifiable factor that may reduce intestinal carcinogenesis in particularly susceptible populations. Additional studies of the risk that

may be imparted to MMR-deficient individuals when they consume heterocyclic amines, such as tumorigenesis studies in MMR-deficient mice, are warranted.

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Chapter 4. Induction of aberrant crypt foci in DNA mismatch repair-deficient mice by the food-borne carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)

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ABSTRACT

Disruption of the DNA mismatch repair (MMR) pathway results in elevated mutation rates, inappropriate survival of cells bearing DNA damage, and increased cancer risk. Relatively little is known about the impact of environmentally relevant carcinogens on cancer risk in individuals with MMR-deficiency. We evaluated the effect of MMR status (*Mlh1*^{+/+} versus *Mlh1*^{-/-}) on the carcinogenic potential of the cooked-meat mutagen, 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) in mice. PhIP exposure did not obviously increase lymphoma or small intestinal tumorigenesis in either *Mlh1*-deficient or -proficient mice. In contrast, the frequency of aberrant crypt foci (ACF), a preneoplastic biomarker for colon tumorigenesis, was increased by PhIP, and the increase due to PhIP was significantly greater in *Mlh1*^{-/-} versus wild-type littermates. This apparent heightened susceptibility to induction of ACF parallels the previously reported hypermutability of *Mlh1*-deficient mice to PhIP and is consistent with the hypothesis that MMR-deficiency would increase the likelihood of PhIP-induced carcinogenic mutations. Further evaluation of the risk that consumption of heterocyclic amines may impart to MMR-deficient individuals therefore is warranted.

INTRODUCTION (abbreviated from original publication)

To determine whether deficiency for the *Mlh1* gene would affect the carcinogenic response to PhIP, we treated weanling *Mlh1*^{+/+} and *Mlh1*^{-/-} littermates with eight injections of 50 mg/kg PhIP or vehicle and subsequently monitored for the development of cancer. Although there was no obvious effect on hematological cancer or small intestinal tumorigenesis, PhIP-exposure did induce ACF in both *Mlh1*-deficient and wild-type littermates. The induced frequency of ACF in *Mlh1*^{-/-} mice was 4-fold greater than in *Mlh1*^{+/+} mice, similar to the increased frequency of induced mutation in *Mlh1*-deficient mouse colon reported previously (Smith-Roe et al., 2005), suggesting loss of MMR increases the likelihood PhIP-induced ACF. Although premature death due to lymphoma limited the analysis of colon tumors, a heightened susceptibility to induction of ACF in *Mlh1*^{-/-} mice is consistent with the hypothesis that MMR-deficiency increases risk of carcinogenesis in colon due to PhIP.

RESULTS

Survival and hematopoietic tumorigenesis

Following exposure to PhIP, mice were monitored for signs of cancer over a period of 17 months. *Mlh1*^{-/-} mice were sacrificed when moribund, whereas wild-type mice generally were killed between the ages of 12 and 17 months, bracketing the age of the longest surviving *Mlh1*^{-/-} mouse (Figure 4.1). Survival, hematopoietic cancer

development, and intestinal tumorigenesis (see below) in vehicle-treated *Mlh1*^{-/-} mice generally were similar to previous reports of spontaneous tumorigenesis in *Mlh1*-deficient mice (Edelmann et al., 1999; Prolla et al., 1998). Morbidity in most *Mlh1*-deficient mice was associated with metastatic lymphoma (both T-cell and non-T-cell, data not shown) or leukemia affecting multiple organs (Table 4.1). There was, however,

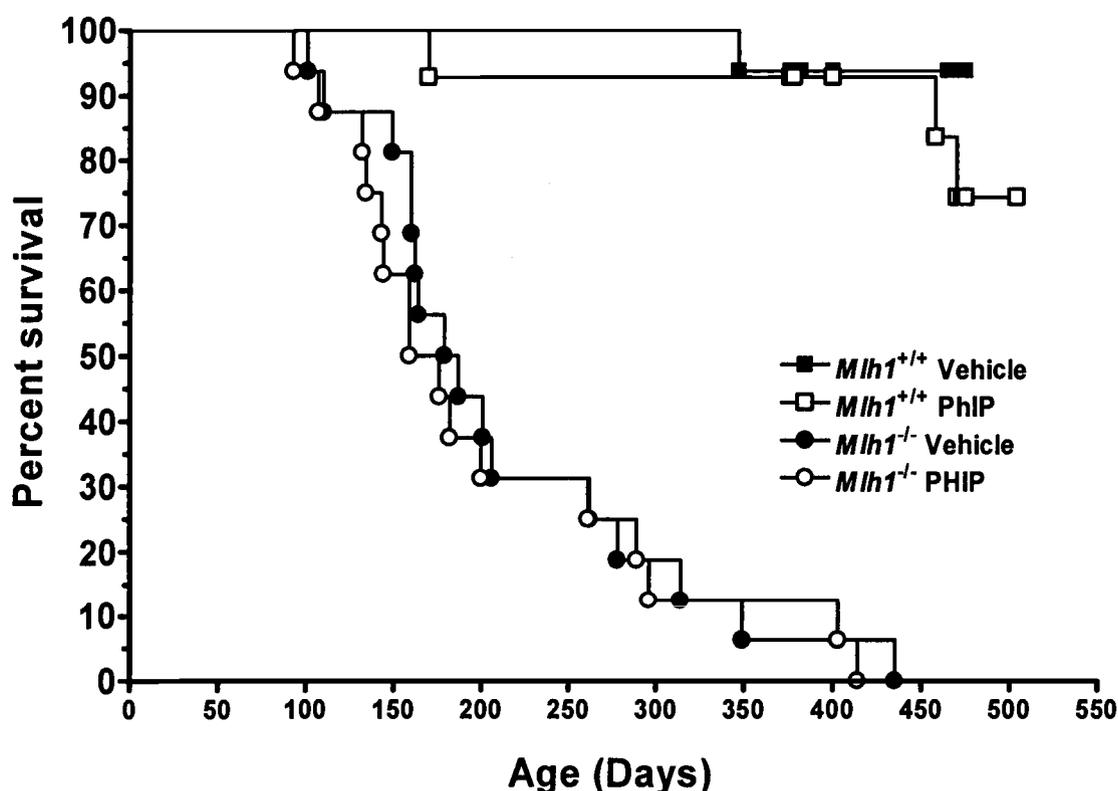


Figure 4.1. Survival of *Mlh1*^{+/+} (squares) and *Mlh1*^{-/-} (circles) mice. Mice were treated with 8 injections of 50 mg/kg PhIP (open symbols) or vehicle (filled symbols) and monitored for cancer development. Mice were euthanized when moribund or at the end of the study. The median survival times for *Mlh1*^{-/-} mice treated with vehicle or PhIP were 183 days and 167 days, respectively.

no significant difference in the median survival of *Mlh1*^{-/-} mice treated with PhIP (167 days) or vehicle (183 days) ($P = 0.6$). Most wild-type mice in both treatment groups survived until sacrifice without obvious signs of disease (Figure 4.1). There was no significant difference in survival (up to approximately 500 days, $p=0.3$), and the incidence of hematological cancer in PhIP- and vehicle-treated wild-type mice was similar (Table 4.1). Thus, PhIP exposure by the current protocol did not appear to increase risk of hematological cancer in wild-type or *Mlh1*-deficient mice.

Table 4.1. Incidence of hematopoietic cancers in PhIP-treated *Mlh1*^{-/-} and *Mlh1*^{+/+} mice

Genotype	Treatment	Total hematopoietic cancers (%)	Thoracic lymphoma (%)
<i>Mlh1</i> ^{-/-}	Vehicle	16/16 (100) ^a	7/16 (44)
	PhIP	14/15 (93) ^b	11/15 (73)
<i>Mlh1</i> ^{+/+}	Vehicle	4/15 (27) ^{c,d}	0/15 (0)
	PhIP	4/15 (27) ^{c,d}	1/15 (7)

^aIncludes two mice with leukemia ^bThe one lymphoma-free mouse became moribund at 13.5 months due to histiocytic sarcoma present in liver, spleen, kidney, and uterus
^cOne mouse not available for histology ^dIncludes one mouse with leukemia.

Colonic tumorigenesis and ACF development

Several *Mlh1*^{-/-} mice older than 250 days developed colon adenomas (Table 4.2). Of five *Mlh1*^{-/-} mice in each treatment group older than 250 days at time of sacrifice, one tumor was found in a single mouse treated with vehicle, whereas three tumors were found

in two mice exposed to PhIP (Table 4.2). Although the small number of surviving *Mlh1*^{-/-} mice of this age precluded a definitive analysis, these findings supported the need to evaluate other potential markers of colonic tumorigenesis.

We therefore determined the effect of *Mlh1*-deficiency on PhIP-induction of aberrant crypt foci (ACF), a monoclonal, preneoplastic lesion thought to represent an early step in colorectal carcinogenesis (Bird and Good, 2000; Mori et al., 2005). In *Mlh1*^{-/-} mice, PhIP exposure increased the incidence of ACF from 31% (5/16) to 87% (13/15) ($p=0.003$) and the average frequency of ACF from 0.4 ± 0.2 to 2.7 ± 0.6 ($p<0.0001$), indicating a PhIP-induced frequency of approximately 2.3 ACF per mouse (PhIP-treated frequency minus vehicle-treated frequency) (Figure 4.2). In contrast, the PhIP-induced frequency of ACF in wild-type littermates was 0.6 per mouse (0.1 ± 0.07 to 0.7 ± 0.3 ; $p=0.04$), associated with a corresponding increase in incidence from 7% (1/15) to 45% (5/11) ($p=0.05$). The approximately 4-fold greater PhIP-induced frequency of ACF in *Mlh1*^{-/-} mice versus *Mlh1*^{+/+} mice was significant (2.3 versus 0.6; $p<0.01$), suggesting that MMR may suppress the induction of ACF by PhIP. Although not quite significant statistically, vehicle-treated *Mlh1*^{-/-} mice also had an observed higher incidence (31% versus 7%; $p = 0.17$) and average frequency of ACF (0.4 ± 0.2 versus 0.1 ± 0.07 ; $p < 0.08$) than vehicle-treated wild-type mice, suggesting a trend towards higher spontaneous risk of ACF similar to mice lacking *Msh2* (Reitmair et al., 1996).

Small intestinal tumorigenesis

Adenomas or adenocarcinomas of the small intestine were detected in 75% (12/16) of vehicle-treated *Mlh1*^{-/-} mice with an average of 2.7 ± 0.7 tumors per mouse (Table 4.2). PhIP-exposure did not increase the incidence (47%; 7/15) or average number of tumors (2.8 ± 1.4 per mouse). There also was no apparent difference in the incidence or number of carcinomas in PhIP- versus vehicle-treated *Mlh1*^{-/-} mice, suggesting that PhIP exposure did not increase the likelihood of progression of adenomas to carcinomas. Wild-type mice were free of small intestinal tumors except for two PhIP-treated wild-type mice that each developed a single adenoma.

Table 4.2. Intestinal tumors in PhIP-treated *Mlh1*^{-/-} and *Mlh1*^{+/+} mice

Genotype	Treatment	Incidence of small intestinal adenomas (%)	Incidence of small intestinal carcinomas (%)	Number of tumors (adenomas plus carcinomas) per mouse (Mean ± SEM)	Incidence of colon adenomas (%)
<i>Mlh1</i> ^{-/-}	Vehicle	12/14 (86)	4/14 (29) ^a	3.1 ± 0.2	1/14 (7) ^b
	PhIP	7/15 (47)	4/15 (27)	3.0 ± 0.6	2/15 (13) ^c
<i>Mlh1</i> ^{+/+}	Vehicle	0/15 (0)	0/15 (0)	None detected	0/15 (0)
	PhIP	2/11 (18)	0/11 (0)	0.1 ± 0.3	0/15 (0)

^aA single tumor from this group was adenoma mixed with carcinoma and is designated as "carcinoma" ^bThis mouse was 8 months old when moribund and had one colon tumor ^cThese mice were both 15 months old when moribund; one mouse had one tumor, the other had two tumors.

DISCUSSION

We report the first comparison of the carcinogenic response of MMR-deficient and -proficient mice to PhIP. Although there was no obvious effect on survival (generally reflective of the development of lethal lymphoma) or on small intestinal tumorigenesis, the induced frequency of ACF was significantly greater in *Mlh1*^{-/-} versus *Mlh1*^{+/+} mice, suggesting that loss of MMR increases the likelihood of PhIP-induced ACF. The increased induced frequency of ACF in *Mlh1*^{-/-} mice parallels the 3-fold greater induction of mutation by PhIP in the colons of mice deficient for *Mlh1* or *Msh2* versus MMR-proficient mice (Smith-Roe et al., 2005; Zhang et al., 2001), suggesting that MMR-dependent suppression of PhIP-induced mutation helps prevent the development of ACF. One potential confounding factor is that wild-type mice were significantly older at the time of analysis than the *Mlh1*-deficient mice (Figure 4.1). As carcinogen-induced ACF may regress after cessation of exposure (Mori et al., 2005), additional ACF induced by PhIP-treatment might have been missed. However, the incidence and frequency of ACF detected in the PhIP-exposed wild-type mice are similar to or greater than those previously reported in normal mice after four weeks of dietary exposure, or 10 weeks after i.p. exposure of PhIP (Kristiansen, 1996; Steffensen et al., 1997), suggesting that earlier assessment would not have altered the current conclusions. Due to a limited number of tumors detected in colon, effects on PhIP-induced colon tumorigenesis could not be assessed directly. Increasing the number of mice in the study or chronically exposing *Mlh1*^{-/-} mice to dietary PhIP may have increased the probability of detecting

PhIP-induced colon tumors. However, the induction of ACF in *Mlh1*^{-/-} mice is consistent with the hypothesis that PhIP-exposure would increase risk of colon carcinogenesis in individuals with MMR-deficiency.

ACF exhibiting microsatellite instability have been identified in human patients with Lynch Syndrome, suggesting that ACF may be a premalignant lesion of MMR-deficient cancer (Pedroni et al., 2001). However, the significance of ACF as biomarker for PhIP-induced cancer in mice has been questioned (Kristiansen, 1996) as the mouse colon is not a strong target tissue for PhIP carcinogenesis (Kristiansen, 1996). In rats, a subset of PhIP-induced ACF do progress to adenoma (Mori et al., 2005); these ACF appear dysplastic histologically, and are associated with cellular accumulation of β -catenin and specific mutations activating *β -catenin* (*Cntb1*) or inactivating the tumor suppressor *adenomatous polyposis coli* (*Apc*) (Ochiai et al., 2003; Tsukamoto et al., 2000). The types of mutations and the genes targeted in PhIP-induced ACF in mice are likely to be similar to those in rats, as *β -catenin* and *Apc* mutant mice show increased spontaneous and PhIP-induced ACF (Kristiansen, 1996; Orner et al., 2002; Paulsen et al., 2000). The rapid induction of adenocarcinoma in mice following a single exposure of PhIP (200 mg/kg) and post-treatment with dextran sodium sulfate (Tanaka et al., 2005) indicates that PhIP-initiated colonic tumors can develop in mice given an appropriate tumor-promoting environment. The robust induction of ACF in *Mlh1*^{-/-} mice, possibly in combination with appropriate tumor promoters, represents a potentially useful model system for identification of the molecular events associated with the development and progression of PhIP-induced ACF and colon cancers.

An increased risk of carcinogenesis due to PhIP was not apparent in the lymph system (lymphoma) or in the small intestine, in contrast to the significant sensitivity of MMR-deficient mice to lymphoma induced by alkylating agents (Colussi et al., 2001; Kawate et al., 2000; Qin et al., 1999). However, PhIP-exposure also did not significantly induce mutation in the thymus of *Mlh1*-deficient (or wild-type) mice (Smith-Roe et al., 2005), and lymphoma in wild-type or in nucleotide-excision repair-deficient mice apparently requires long-term exposure (Kristiansen et al., 1998; Ochiai et al., 2002). Thus, PhIP is a weak lymphomagen even in the absence of important DNA repair pathways. The lack of significant increase in small intestinal tumorigenesis was not predicted by a similar induction of mutation by PhIP in both small intestine and colon in *Mlh1*^{-/-} mice (Smith-Roe et al., 2005). However, most small intestinal tumors in mice are initiated very early in life (Shoemaker et al., 1995), and significant induction of such tumors by PhIP in genetically susceptible *Apc* mutant mice requires neonatal exposure (Steffensen et al., 2001). Similarly, induction of small intestinal tumorigenesis in *Mlh1*-deficient mice might require exposure prior to weaning.

We have demonstrated an induction of ACF by PhIP in *Mlh1*^{-/-} mice that correlates with a previously described hypermutability in *Mlh1*-deficient mouse colon. These findings are consistent with the hypothesis that loss of MMR increases the likelihood of PhIP-induced carcinogenic mutations. As such, exposure to PhIP may be a significant concern for individuals at risk for MMR-deficiency. Such risk is not limited to those with an inherited deficiency, as MMR activity can be down-regulated by cellular environments such as chronic oxidative stress (Chang et al., 2002) or hypoxia (Mihaylova

et al., 2003). Reduced exposure to PhIP is a modifiable lifestyle factor that may reduce intestinal carcinogenesis, particularly in susceptible populations. Additional studies of the risk that may be imparted to MMR-deficient individuals when they consume heterocyclic amines are warranted.

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Chapter 5: *Mhl1*-dependent apoptotic responses to the food-borne carcinogen 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) and 1,2-dimethylhydrazine (DMH) in murine colon

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ABSTRACT

The DNA mismatch repair (MMR) pathway maintains genomic stability and reduces cancer risk (colorectal and other internal cancers) by correcting polymerase errors and activating cell cycle checkpoints and apoptosis in response to DNA damage. The influence of commonly encountered environmental mutagens/carcinogens on the etiology of MMR-deficient cancers is not well understood. 2-Amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) is a cooked-meat mutagen implicated in human colorectal carcinogenesis. Previously, we demonstrated that *Mlh1*^{-/-} mouse colon was hypermutable in response to PhIP, and that hypermutability correlated with increased susceptibility for development of colonic aberrant crypt foci (a biomarker for colon carcinogenesis), suggesting that PhIP may increase cancer risk for individuals with MMR-deficiencies. MMR-deficient colorectal cancer cells are resistant to PhIP-induced cytotoxicity, suggesting that activation of cell cycle checkpoints and apoptosis by MMR may contribute to suppression of PhIP-induced hypermutability. Therefore, we evaluated the effect of *Mlh1*-deficiency on apoptosis (detected by staining for cleaved caspase-3) and cell proliferation (detected by staining for bromodeoxyuridine) in *Mlh1*^{+/+} and *Mlh1*^{-/-} mouse colon following either multiple interperitoneal (i.p.) injections or a single i.p. injection of 50 mg/kg PhIP. These responses were compared to a single i.p. injection of 80 mg/kg 1,2-dimethylhydrazine (DMH). Colonic apoptosis in response to PhIP increased, shifted to predominately the stem cell compartment, and was partially *Mlh1*-dependent. Similar effects were observed in DMH-exposed animals, although the level of induced apoptosis was 3 times higher versus PhIP-treated mice. The apoptotic

response to PhIP was greater after multiple exposures compared to a single exposure.

PhIP-exposure did not notably alter cell proliferation, whereas proliferation was

consistently reduced in DMH-treated mice. These results suggest that loss of *Mlh1* alters

homeostatic functions of the colonic crypt that may contribute to suppression of the

mutagenic effects of PhIP, and therefore, cancer risk.

INTRODUCTION

Both the error correction function and the DNA-damage surveillance function of MMR appear important for suppression of tumorigenesis. Tissues of MMR-deficient mice are hypermutable in response to treatment with alkylating agents, and demonstrate heightened susceptibility to lymphoma and colon tumors when treated with alkylating agents (Andrew et al., 1998; Colussi et al., 2001; Kawate et al., 2000; Qin et al., 1999). Escape from MMR-initiated cell cycle checkpoints and apoptosis may be important for these tumorigenic effects. For example, the apoptotic response to the alkylating agent 1,2-dimethylhydrazine (DMH) is reduced in the colons of *Msh2*^{-/-} mice, possibly contributing to the increased susceptibility of *Msh2*^{-/-} mice to DMH-induced colon tumors (Colussi et al., 2001). In humans, resistance to cytotoxic chemotherapy may drive selection of MMR-deficient secondary cancers of hematopoietic origin (Karran et al., 2003). However, the extent to which exposure to commonly encountered environmental genotoxins modulates cancer risk in individuals at risk of MMR-deficiency (due to either genetic or epigenetic mechanisms) is not known. Recent epidemiological studies suggest long duration exposure to tobacco smoke (containing polycyclic aromatic hydrocarbons among other mutagens) or high consumption of heavily grilled meat (containing heterocyclic amines (HCAs)) may increase risk for MSI-positive CRC (Luchtenborg et al., 2005; Slattery et al., 2000; Slattery et al., 2002; Wu et al., 2001). Avoidance of these factors may reduce cancer risk in individuals with MMR deficiencies.

In cultured cells, exposure to PhIP affects cell cycle progression and apoptosis, and can alter expression of cell-cycle related genes (Creton et al., 2005; Duc and Leong-

Morgenthaler, 2004; Zhu et al., 2000; Zhu et al., 2005). *In vivo*, exposure to PhIP increased apoptosis in the colons of rats 24 h after treatment, but did not notably alter cell proliferation (Hirose et al., 1998; Ochiai et al., 1996). Apoptosis was modestly increased in the small intestine of very young wild-type and *Min/+* (multiple intestinal neoplasia) mice 24 h after exposure to PhIP (Steffensen et al., 2005). These studies suggest that PhIP represents a challenge to cellular repair mechanisms, culminating in apoptosis if PhIP-induced DNA damage is not properly repaired. Presently, it is not known whether the MMR damage-surveillance response participates in activation of cell cycle checkpoints and apoptosis in response to PhIP *in vivo*. Loss of this function of MMR could contribute to the hypermutability and increased risk for neoplasia in *Mlh1^{-/-}* mouse colon resulting from PhIP exposure.

Similar to our previous studies, we treated *Mlh1^{+/+}* and *Mlh1^{-/-}* littermate weanlings with 8 interperitoneal (i.p.) injections of 50 mg/kg PhIP administered 48 hours apart (Smith-Roe, 2006b; Smith-Roe et al., 2006a). We assessed apoptotic and proliferative responses to PhIP at 8, 16, 24, and 48 h after the last injection of PhIP and compared these results to treatment with a single injection of 80 mg/kg DMH. DMH is a metabolically activated S_N1 alkylating agent that produces O⁶methylguanine (O⁶meG) lesions (Swenberg et al., 1979), which are a likely substrate for the MMR damage-surveillance response (Duckett et al., 1996; Hickman and Samson, 2004). We found that an *Mlh1*-dependent apoptotic response occurs in the mouse colon 8 h after treatment with PhIP or DMH. PhIP- and DMH-induced apoptosis was apparent in the stem cell compartment of colonic crypts, while apoptosis was reduced at the lumen of the crypts,

and crypt height was maintained. Induction of apoptosis was greater following multiple injections of PhIP versus a single exposure. Increased apoptosis in the stem cell compartment due to DMH-exposure also was accompanied by reduced cellular proliferation in the crypt. Taken together, MMR is one of several pathways that contribute to the extreme sensitivity of colonic-crypt stem cells to DNA damage.

RESULTS

To assess the role of *Mlh1* on cell turnover in the mouse colon in response to PhIP, the number and location of apoptotic (CC-3-positive) cells and replicating (BrdU-positive) cells were recorded from 50 half-crypts per mouse at 8, 16, 24, and 48 h time points after mice were given their last injection of PhIP. The number of cells lining the half-crypt (crypt height) also was recorded. Vehicle-treated *Mlh1*^{+/+} and *Mlh1*^{-/-} controls were included for the 8, 24, and 48 h time points. Cell turnover also was assessed in untreated *Mlh1*^{+/+} and *Mlh1*^{-/-} mice, and in mice exposed to a single i.p. injection of DMH.

Proliferation and Crypt Height in Murine Colon

BrdU-positive cells (Figure 5.1) in untreated and vehicle-treated mice accounted for approximately 8 - 11% of all counted colon crypt cells (Table 5.1), with no apparent differences in proliferative index between *Mlh1*^{+/+} and *Mlh1*^{-/-} mice. Approximately 90% of all BrdU-positive cells were identified in positions 1 – 10 in the crypt (Figure 5.2).

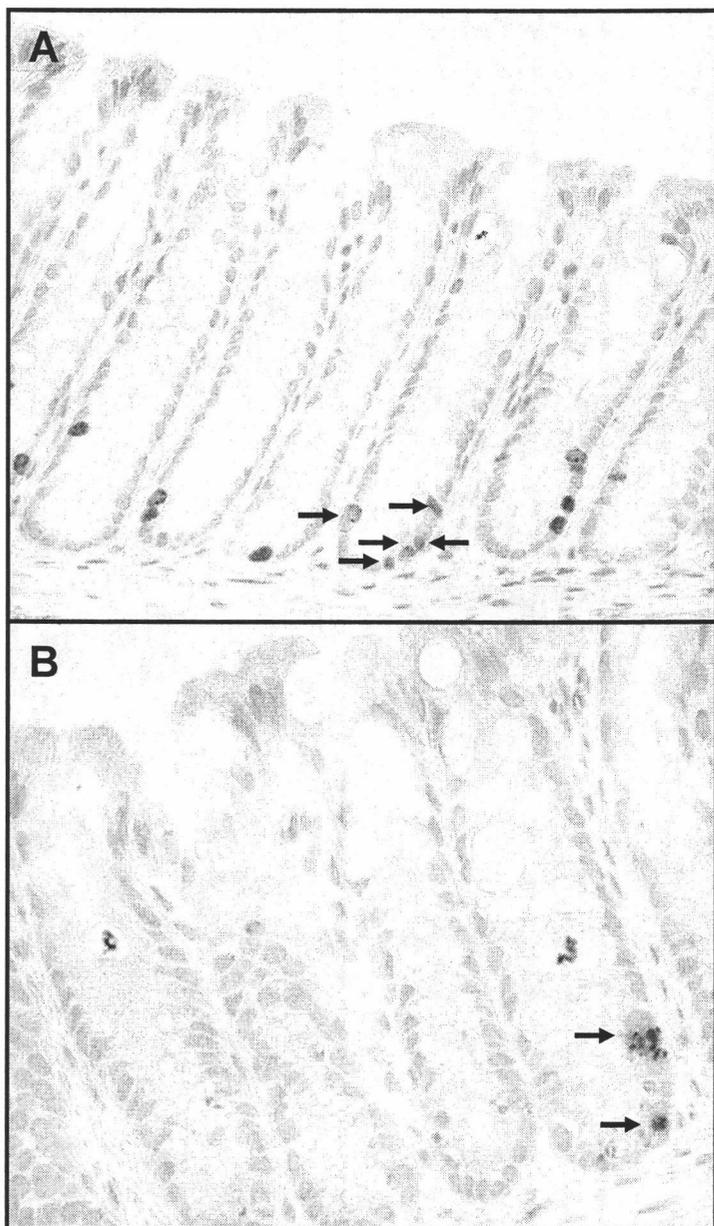


Figure 5.1A and B. Cross-section (20x) of the mouse colon stained for BrdU (A) or CC-3 (B) and counterstained with hematoxylin and lithium carbonate. Arrows (within countable crypts) point to cells that are positive for BrdU incorporation or presence of CC-3.

Table 5.1. Mean proliferative index (%) and crypt height in wild-type and *Mlh1*-deficient mouse colon in response to PhIP or DMH

Treatment	Time Point ^a	<i>Mlh1</i> ^{+/+}		<i>Mlh1</i> ^{-/-}	
		Proliferative Index (%) ^b	Crypt Height ^c	Proliferative Index (%) ^b	Crypt Height ^c
Untreated		11.2 ± 1.6	24.7 ± 0.03	9.9 ± 0.9	23.7 ± 1.2
8 injections of 50 mg/kg PhIP	8 h	8.3 ± 0.6	24.5 ± 0.5	9.8 ± 2.1	24.5 ± 0.5
	16 h	8.2 ± 1.1	25.7 ± 0.4	9.0 ± 1.2	25.6 ± 0.7
	24 h	7.9 ± 0.4	23.4 ± 1.0	7.1 ± 0.6	23.7 ± 0.5
	48 h	8.1 ± 2.4	25.0 ± 0.8	8.8 ± 1.2	23.9 ± 0.6
8 injections of vehicle	8 h	6.1 ± 0.4	23.3 ± 0.2	N.D. ^d	N.D.
	24 h	7.9 ± 1.2	22.8 ± 0.3	9.1 ± 1.3	22.5 ± 0.5
	48 h	9.6 ± 0.6	24.5 ± 0.4	10.0 ± 1.0	22.9 ± 0.5
1 injection of 50 mg/kg PhIP	8 h	8.5 ± 1.1	26.2 ± 0.4	7.1 ± 0.9	24.9 ± 0.3
	16 h	4.3 ± 0.6	24.8 ± 0.5	7.1 ± 0.5	25.2 ± 0.4
1 injection of 80 mg/kg DMH	8 h	6.4 ± 1.2	23.9 ± 1.0	6.5 ± 0.8	24.5 ± 0.9
	16 h	4.8 ± 0.9	23.2 ± 0.6	5.4 ± 0.6	24.8 ± 1.1

^aTime after treatment. ^bPresented is the average proliferative index ± standard error of the mean (S.E.M.) scored in 3 – 6 mice.

^cNumber of cell positions counted from the base of the crypt to the luminal surface. Presented is the mean ± S.E.M. in 50 half crypts. counted per mouse. ^dNot determined.

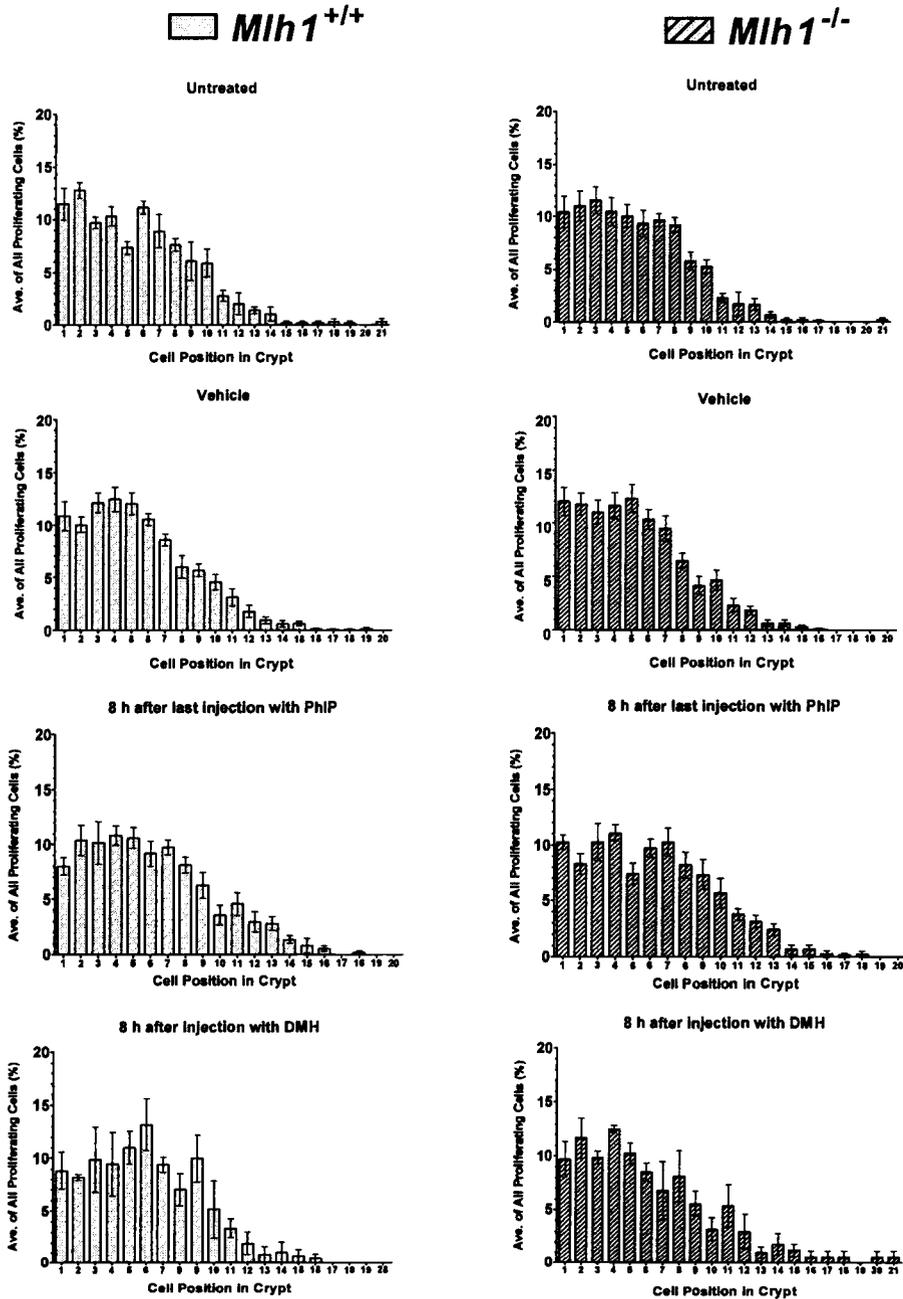


Figure 5.2. Representative histograms of cellular proliferation in $Mlh1^{+/+}$ and $Mlh1^{-/-}$ mouse colon by cell position. Each bar of the histogram represents the % of BrdU-positive cells detected at each position averaged among mice in the experimental group. ($n = 3$ to 6 mice per group, except for $Mlh1^{+/+}$ vehicle $n = 12$ and $Mlh1^{-/-}$ vehicle $n = 9$). Error bars are the standard error of the mean (S.E.M.).

Therefore, positions 1 – 10 were designated as the proliferative zone of the crypt, the 1 to 2 cells at the juncture of the crypt and the intercrypt table were designated as the luminal zone of the crypt, and the intervening cells were considered to comprise the transit zone of the crypt. The level (8 – 9%) and locations of BrdU-positive cells in *Mlh1*^{+/+} and *Mlh1*^{-/-} mice exposed to multiple injections of PhIP were similar to vehicle-treated mice. Likewise, the average crypt height of *Mlh1*^{+/+} and *Mlh1*^{-/-} mice exposed to multiple injections of PhIP was not significantly different from that of untreated and vehicle-treated mice (Table 5.1). In contrast, the proliferative index was reduced to approximately 5 – 6% at the 8 and 16 h time points in both *Mlh1*^{+/+} and *Mlh1*^{-/-} mice exposed to DMH. Despite the reduction in overall proliferation observed in DMH-treated mice at the 8 and 16 h time points, the average crypt height was similar to all other experimental groups.

Mlh1-dependent Apoptosis in Murine Colon

Compared to vehicle-treated and untreated background levels, the apoptotic index in the colons of *Mlh1*^{+/+} mice treated with 8 injections of PhIP was significantly increased (from 0.16 ± 0.02 to 0.83 ± 0.3) at 8 h after treatment (Figure 5.3), and decreased to background levels ($\sim 0.16\%$) in a time-dependent manner. Exposure to PhIP also increased apoptosis in *Mlh1*^{-/-} mouse colon. However, the apoptotic index at 8 h was lower in *Mlh1*^{-/-} mice (0.41 ± 0.09) compared to *Mlh1*^{+/+} mice, suggesting that approximately half of the apoptotic response to PhIP in mouse colon is *Mlh1*-dependent. In response to a single injection of 80 mg/kg DMH, the apoptotic index was increased (up

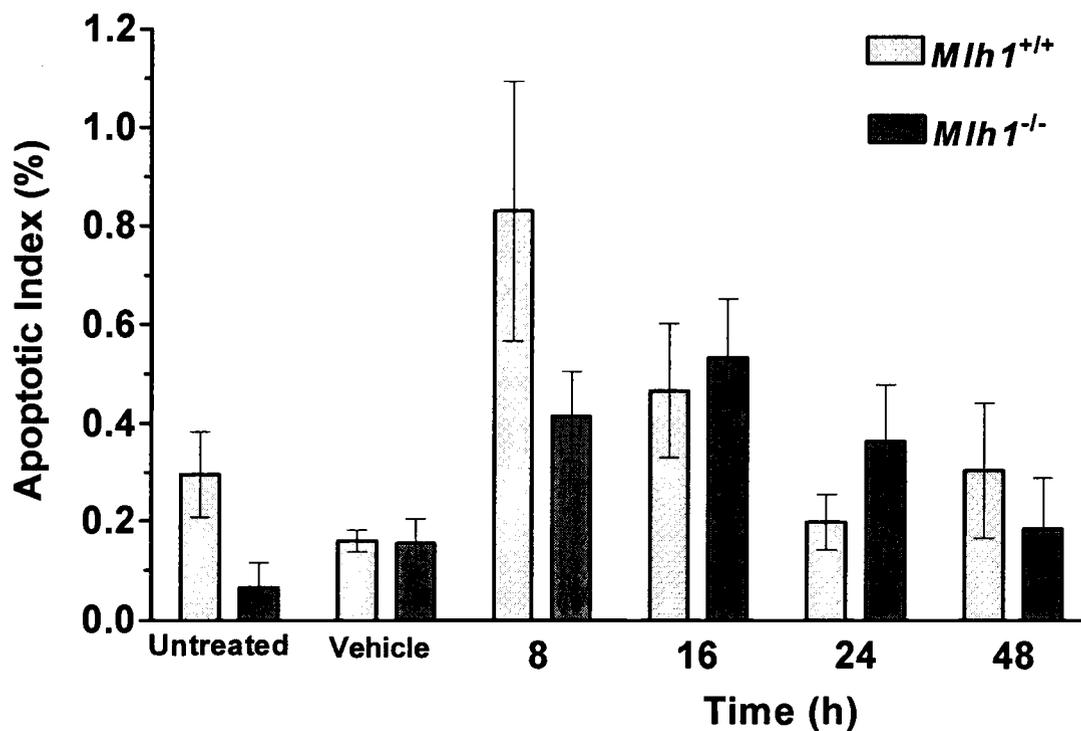


Figure 5.3. Apoptotic index (%) in untreated mice or mice given multiple injections of PhIP or vehicle, evaluated at several time points after treatment (4 to 6 mice per group). The apoptotic index is the number of CC-3 positive cells counted in 50 half-crypts divided by the total number of cells counted in 50 half-crypts, multiplied by 100. "Vehicle" includes pooled results from 8, 24, and 48 h after last injection. Plotted is average \pm standard error of the mean.

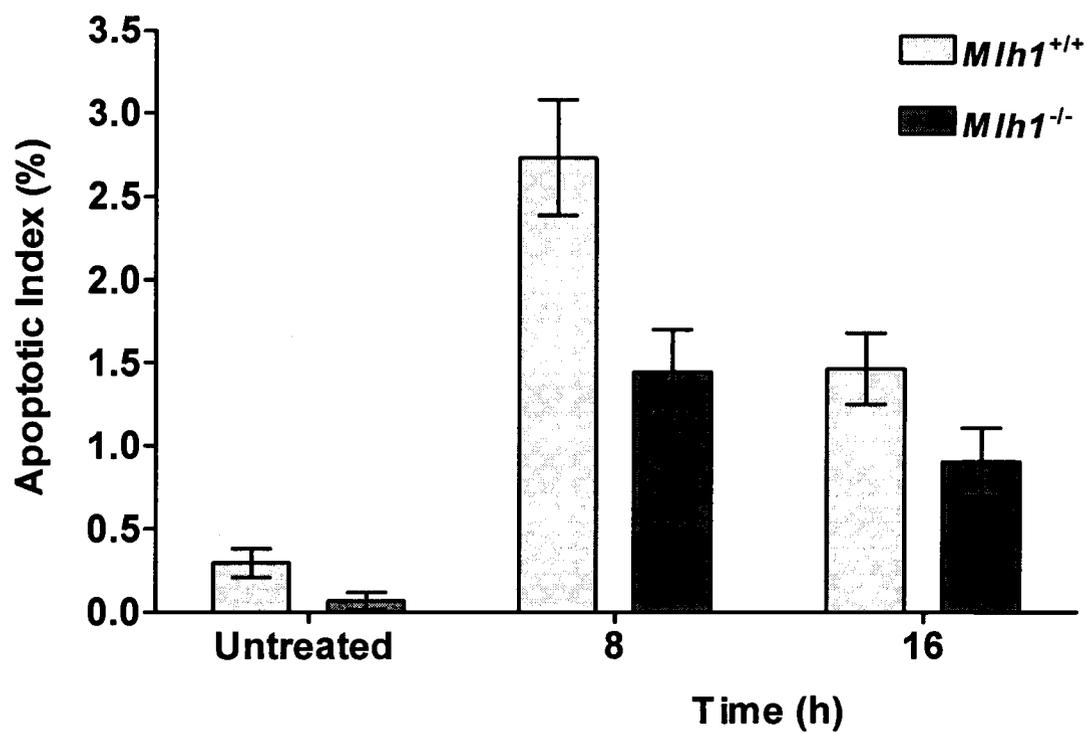


Figure 5.4. Apoptotic index (%) of mice exposed to a single injection of DMH, evaluated at 8 and 16 h after injection (4 to 7 mice per group) as described in Figure 5.3.

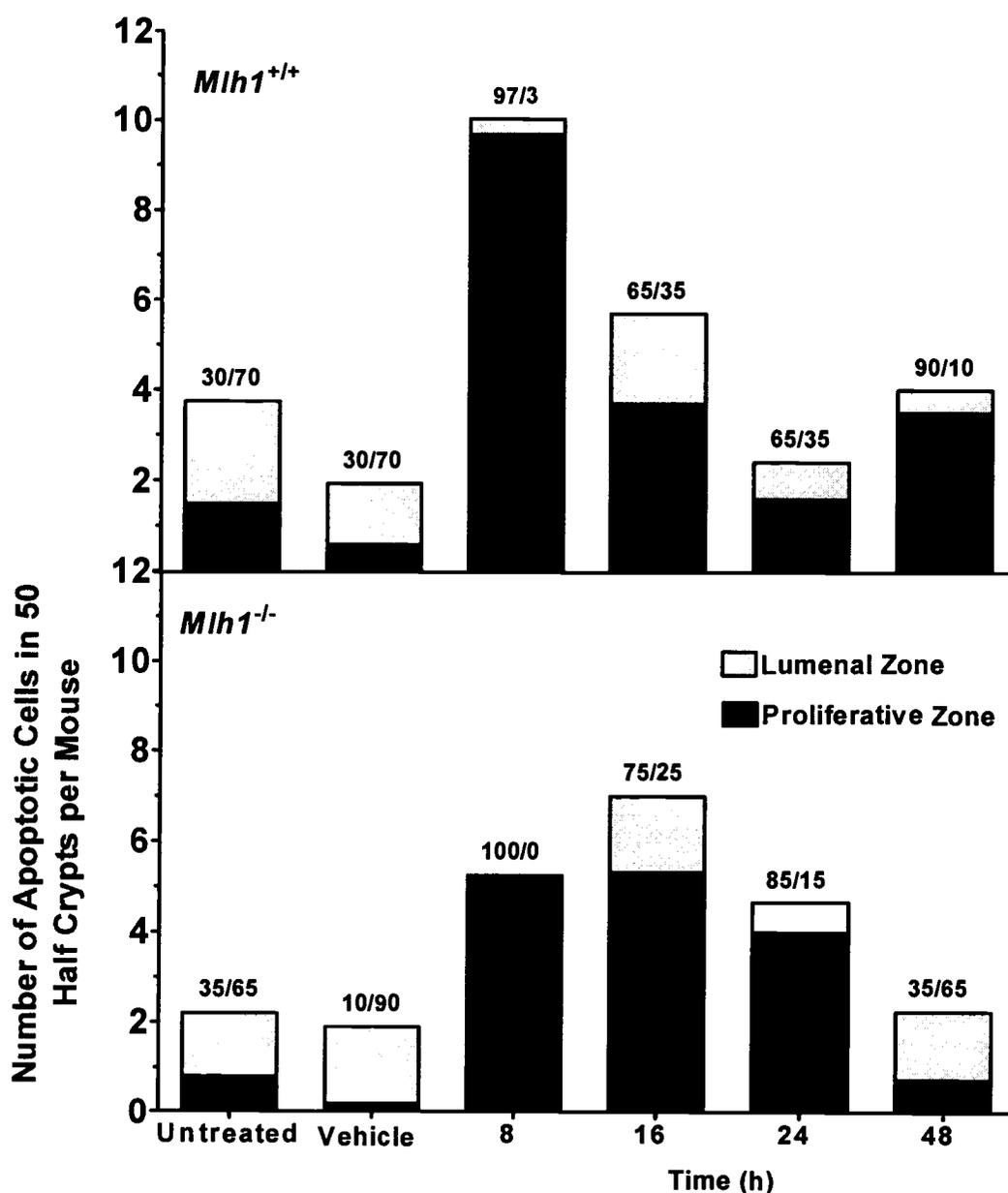


Figure 5.5. Location of apoptotic (CC-3-positive) cells in *Mlh1*^{+/+} or *Mlh1*^{-/-} mice that were untreated, vehicle-treated, or that received multiple injections of PhIP. PhIP-treated mice were evaluated at several time points following treatment. Cells attributed to the proliferative zone were those detected in positions 1 – 10, cells attributed to the luminal zone were those detected at the juncture of the crypt with the intercrypt table. Plotted are the total number of CC-3-positive cells in each zone divided by the number of mice in each group. Numbers above the bars indicate the percentage of the total number of apoptotic cells observed in the proliferative zone versus the luminal zone.

to 2.7%) in *Mlh1*^{+/+} mice 8 h after treatment, and this increase also was partially *Mlh1*-dependent (Figure 5.4). A similarly reduced apoptotic response to DMH was reported previously in *Msh2*^{-/-} mice (Colussi et al., 2001).

Apoptotic cells (CC-3 positive) were detected in the proliferative zone or the luminal zone of the crypt, and were never observed in the transit zone. In untreated or vehicle-treated mice, apoptotic cells were identified more often in the luminal zone: ~70% of total in *Mlh1*^{+/+} mice, and 65 - 95% of total in *Mlh1*^{-/-} mice (Figure 5.5). Following exposure to multiple injections of PhIP, the balance of apoptosis was shifted dramatically. Apoptosis in the proliferative zone in *Mlh1*^{+/+} mice increased from 0.8 – 1.5 per 50 half crypts in vehicle and untreated mice to ~10 per 50 half crypts (~97% of all apoptosis) in PhIP-exposed animals (8 h time point). Similarly, apoptosis in the proliferative zone in *Mlh1*^{-/-} mice increased with PhIP exposure from < 0.8 to 5.5 (~100%). In contrast, luminal zone apoptosis decreased with PhIP exposure from ~2 per 50 half crypts to < 0.3 in *Mlh1*^{+/+} mice, and from ~1 to undetectable in 50 half crypts in *Mlh1*^{-/-} mice. At later time points, apoptosis was again detected at the lumen in both *Mlh1*^{+/+} and *Mlh1*^{-/-} mice. Similar to PhIP-treated mice, nearly 100% of apoptosis in both *Mlh1*^{+/+} and *Mlh1*^{-/-} mice 8 h following exposure to DMH occurred in the proliferative zone of the crypts, with a loss of apoptosis at the luminal zone (Figure 5.6). In both PhIP- and DMH-exposed mice, most (60% to 70%) apoptosis occurred within positions 1 – 4 within the proliferative zone (see Figures 5.8 and 5.9 and additional discussion below), reported to be the location of stem cells in the crypts (Potten, 1998). Taken

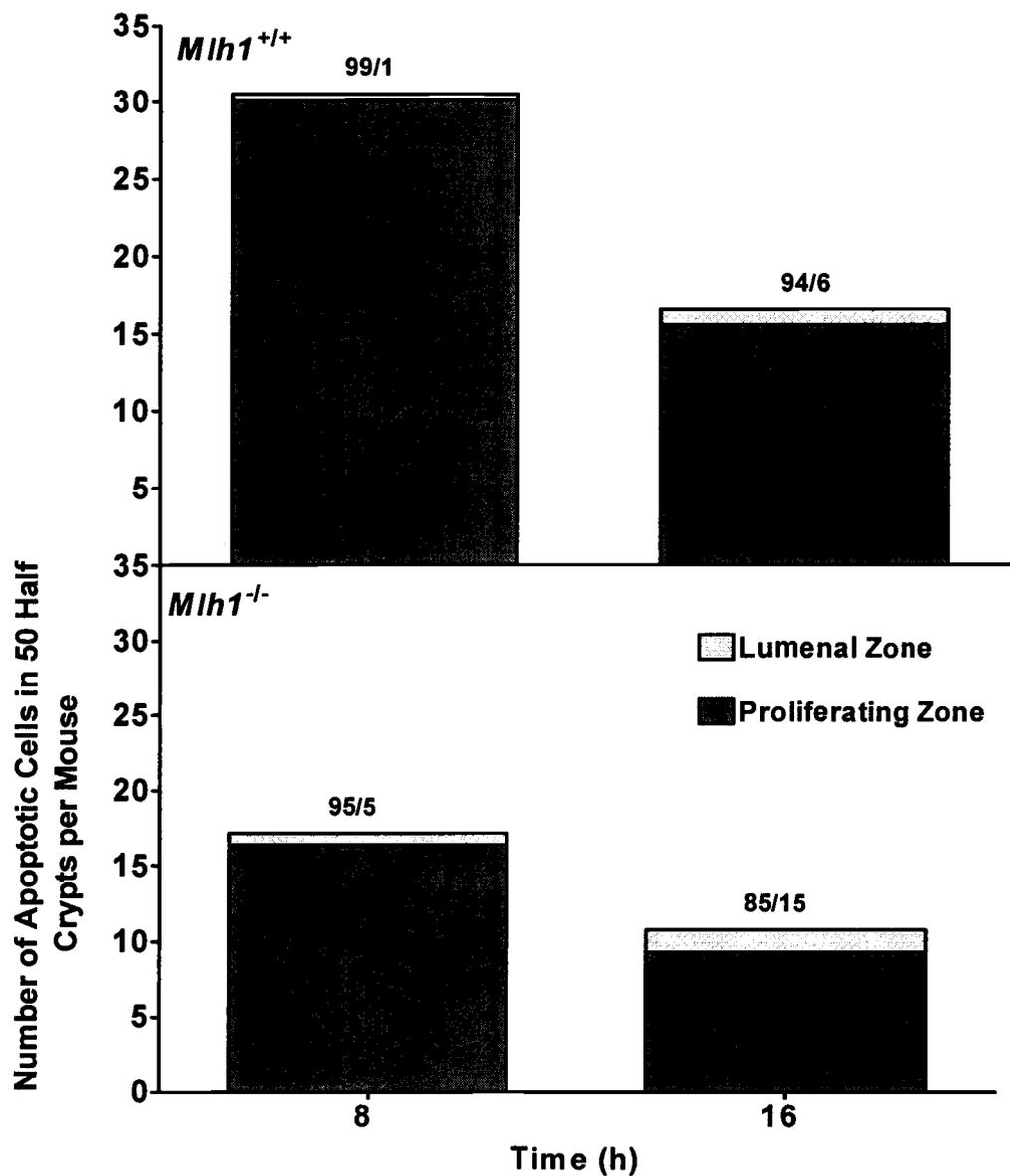


Figure 5.6. Location of apoptotic (CC-3-positive) cells in *Mlh1*^{+/+} or *Mlh1*^{-/-} mice that received a single injection of DMH, evaluated at 8 and 16 h after treatment, analyzed as described in Figure 5.5. Vehicle and untreated data are reproduced from Figure 5.5.

together, the data indicate two changes in apoptosis in response to DNA damaging agents – increased “stem cell apoptosis” and decreased luminal surface apoptosis.

Apoptosis and Proliferation in $Mlh1^{+/+}$ and $Mlh1^{-/-}$ Mice in Response to a Single Injection of PhIP

At the 8 h time point, the apoptotic index in $Mlh1^{+/+}$ mice treated with DMH was approximately 3 times greater than that of $Mlh1^{+/+}$ mice treated with multiple injections of PhIP. To determine if the lower apoptotic response to PhIP may have been due to tolerance generated after multiple injections, $Mlh1^{+/+}$ and $Mlh1^{-/-}$ mice were given a single injection of 50 mg/kg PhIP and cell turnover endpoints were evaluated at 8 and 16 h after injection. As with multiple injections, overall levels of proliferation and average crypt height were not considerably altered (Table 5.1). Compared to vehicle, the apoptotic index was significantly increased in $Mlh1^{+/+}$ colon at 8 and 16 h after treatment (0.16 ± 0.02 with vehicle versus 0.38 ± 0.1 and 0.51 ± 0.13 , respectively, with PhIP). However, these induced levels of apoptosis after single injections were lower than that observed for multiple injections (0.83 ± 0.3 , 8 h time point). As with multiple injections, PhIP-induced apoptosis after a single injection was detected in the proliferative zone, and appeared partially dependent on *Mlh1* (Figure 5.7).

Comparison of Apoptosis and Proliferation in $Mlh1^{+/+}$ and $Mlh1^{-/-}$ Mice

To determine if the reduced apoptotic response detected in $Mlh1^{-/-}$ mice was due to loss of apoptosis within a specific subset of cells in colonic crypts, and to compare more precisely the distributions of apoptosis versus proliferation, we normalized the

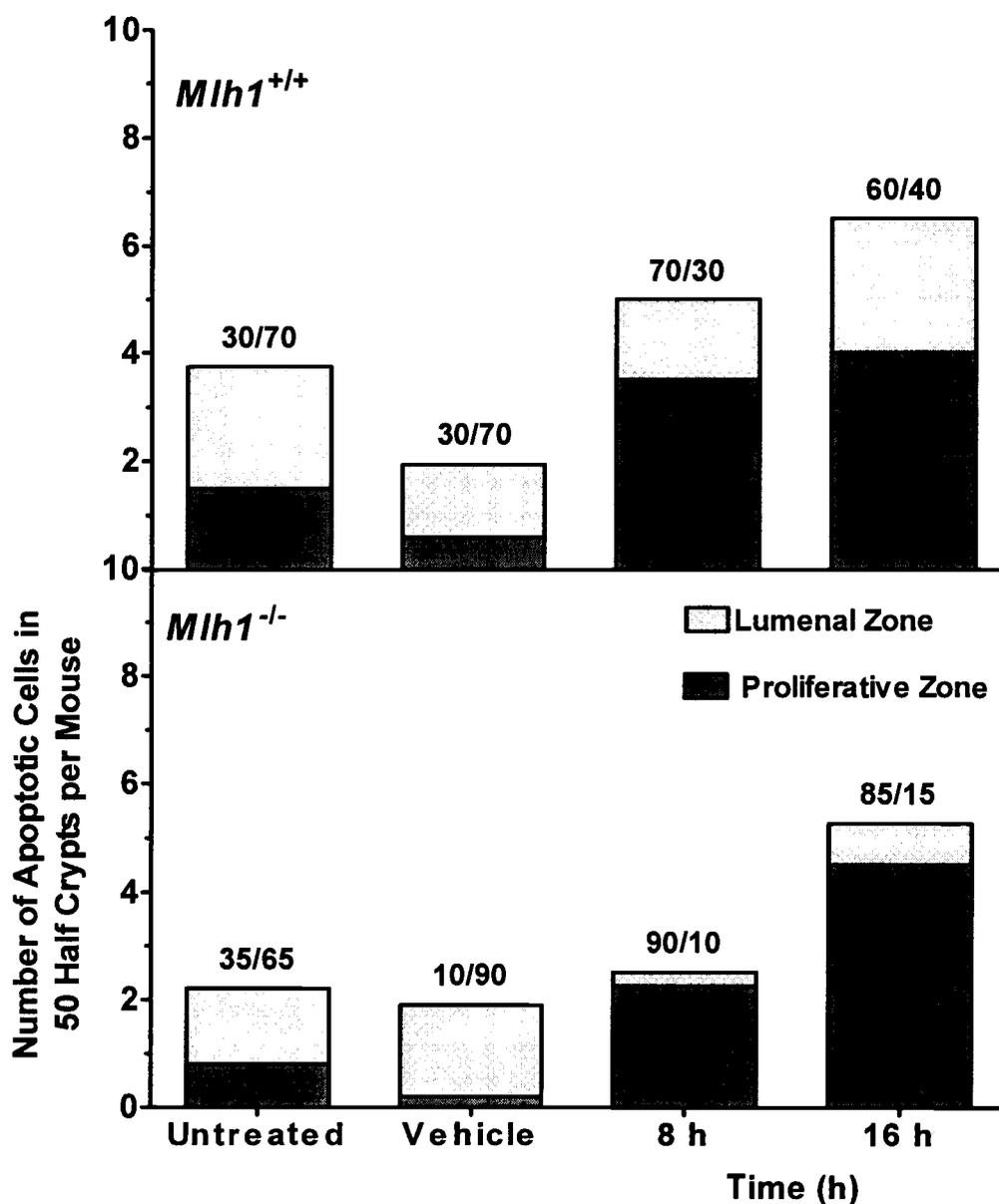


Figure 5.7. Location of apoptotic (CC-3-positive) cells in *Mlh1*^{+/+} or *Mlh1*^{-/-} mice that received a single injection of PhIP, evaluated at 8 and 16 h after treatment (4 mice per group) analyzed as described in Figure 5.5. Vehicle and untreated data are reproduced from Figure 5.5.

different absolute levels of apoptosis or proliferation by calculating the percent of total labeled cells (CC-3-positive or BrdU-positive, respectively) detected at each cell position in the crypt. Calculated percent values were plotted for each cell position, and the resulting distributions were compared (Figures 5.8 and 5.9 for PhIP- and DMH-treatments, respectively). For cell proliferation, data from all vehicle- and untreated *Mlh1*^{+/+} and *Mlh1*^{-/-} mice (28 total) were pooled as no significant differences were detected among the groups (Table 5.1 and Figure 5.2). Distributions of PhIP- and DMH-induced apoptosis (8 h time point) were similar in both *Mlh1*^{+/+} and *Mlh1*^{-/-} animals with approximately 60 – 70% of the total CC-3-positive cells detected in positions 1 – 4, suggesting that similar cells within the stem cell compartment are undergoing PhIP- and DMH-induced apoptosis in wild-type and *Mlh1*-deficient mice. Reductions in the apoptotic response in *Mlh1*^{-/-} mice therefore reflect a general reduced susceptibility of stem cells to DNA damage-induced apoptosis.

Compared to the profiles of PhIP- and DMH-induced apoptosis, the profile of proliferation (BrdU-positive cells) appeared different. BrdU-positive cells were detected with approximately equal frequency between positions 1 – 6, with only 45% of the total proliferation detected in positions 1 – 4 (versus 60 – 70% of apoptotic cells). Significant numbers of proliferating cells also were detected in positions 7 – 10 (26% of total) versus less than 14% of the apoptotic cells in PhIP- and DMH-treated mice. This comparison of the distribution of apoptotic cells and replicating cells suggests that not all replicating cells in the murine colonic crypt are equally sensitive to the presence of DNA damage.

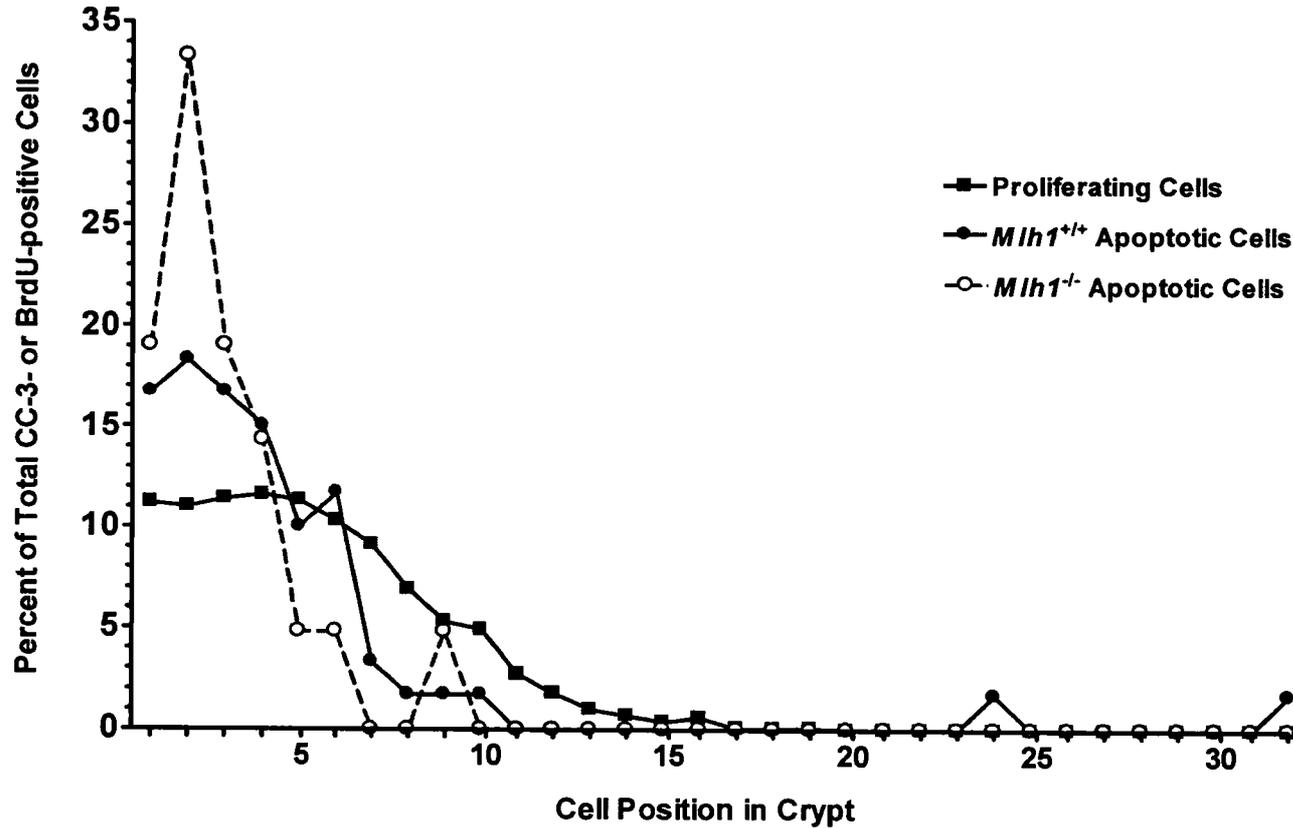


Figure 5.8. Positions of apoptotic (CC-3-positive) cells in *Mlh1*^{+/+} (n = 6) and *Mlh1*^{-/-} mice (n = 4) treated with multiple injections of PhIP (8 h time point) compared to the positions of replicating (BrdU-positive) cells detected in untreated and vehicle-treated *Mlh1*^{+/+} and *Mlh1*^{-/-} mice (n = 28, pooled data from 8, 16, and 24 h time points). Data are depicted as the average of % calculated for each mouse. For both *Mlh1*^{+/+} and *Mlh1*^{-/-} mice, 70% of apoptosis in the proliferative zone occurred within the stem cell compartment.

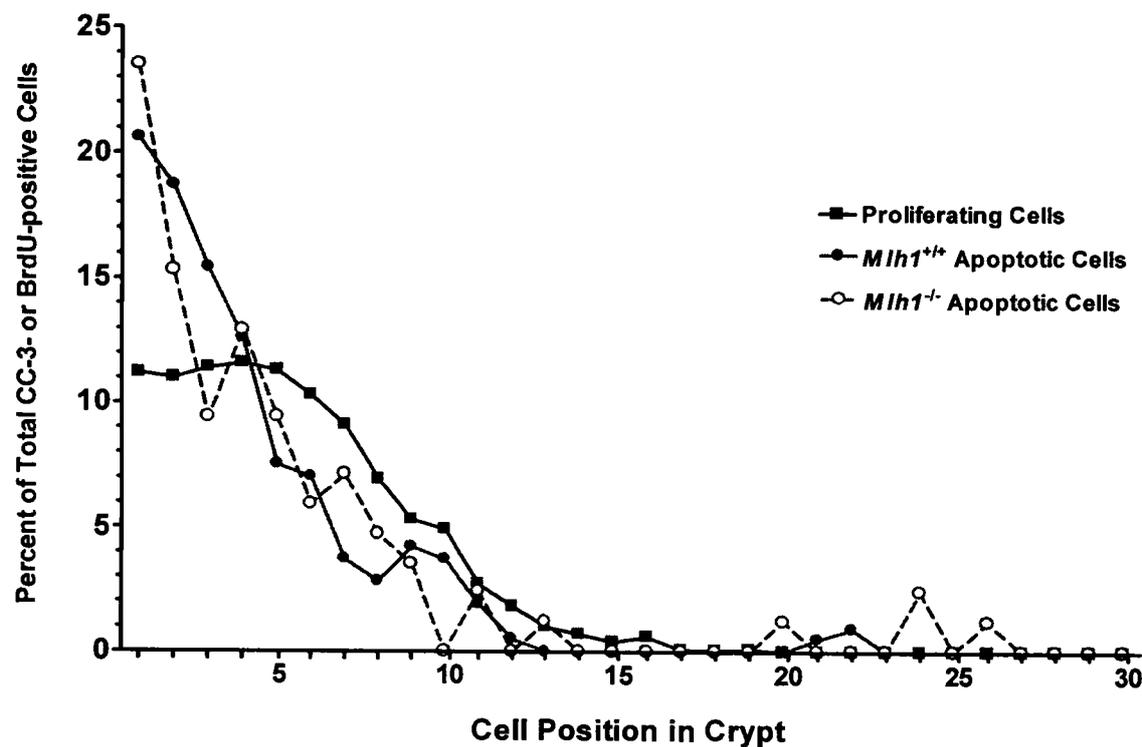


Figure 5.9. Positions of apoptotic (CC-3-positive) cells in *Mlh1*^{+/+} (n = 6) and *Mlh1*^{-/-} mice (n = 4) treated with a single injection of DMH (8 h time point) compared to the positions of replicating (BrdU-positive) cells detected in untreated and vehicle-treated *Mlh1*^{+/+} and *Mlh1*^{-/-} mice (n = 28, pooled data from 8, 16, and 24 h time points). Data are depicted as the average of % calculated for each mouse. For both *Mlh1*^{+/+} and *Mlh1*^{-/-} mice, 70% of apoptosis in the proliferative zone occurred within the stem cell compartment.

DISCUSSION

We previously reported that *Mlh1*-deficient mouse colon tissue is hypermutable in response to PhIP, and that *Mlh1*-deficient mice are more susceptible to ACF caused by PhIP compared to wild-type littermates. Here, we evaluated apoptotic and cell proliferation outcomes at several time points (8, 16, 24, and 48 h) in *Mlh1*^{+/+} and *Mlh1*^{-/-} mice exposed to the same PhIP-treatment protocol used in our previous studies. Our goal was to determine *in vivo* whether *Mlh1* might provide protection against the mutagenic and carcinogenic effects of PhIP in part by participating in the activation of apoptotic pathways.

We found that apoptosis in response to multiple injections of PhIP was significantly induced in *Mlh1*^{+/+} mouse colon (Figure 5.3). The highest level of apoptosis was detected at 8 h and had declined by 16 h, indicating that apoptosis was induced with rapid kinetics. A similar level of apoptosis was reported previously within the proliferative zone of rat colon crypts 24 h after a single exposure to PhIP, the onset of which could be reduced to 8 h by co-exposure to β -naphthoflavone, an inducer of the PhIP-metabolizing enzyme CYP1A2 (Hirose et al., 1998). In addition, while this study was in progress, a modest apoptotic response in the small intestine and colon of *Min*⁺ and wild-type mice was reported to occur at 24 h after a single subcutaneous injection of PhIP (Steffensen et al., 2005). The lack of significantly increased apoptosis at 24 h in the current study may reflect differences in staining methods (Marshman et al., 2001) or exposure protocol. In Steffensen et al (2005), PhIP-DNA adducts were detected in

throughout the mouse intestinal tract as early as 8 h after exposure to PhIP, declining to near baseline levels by 24 h after treatment. We also have detected significant PhIP-DNA adducts 8 h after exposure to PhIP, the levels of which are similar in *Mlh1*^{+/+} and *Mlh1*^{-/-} mice (Herman A. J. Schut, personal communication). Rapid induction of apoptosis with decreased levels 24 h after exposure appears to correlate well with the kinetics of PhIP-DNA adduct formation and repair.

Whereas apoptosis in untreated or vehicle-treated mice was detected primarily in the lumen of the crypt, treatment with PhIP caused the majority of apoptosis (95%) to occur in the proliferative zone of the crypt, predominately within positions 1 – 4. Within crypts in the colon, positions 1 – 4 are proposed to be the location of stem cells (Marshman et al., 2002). These results suggest that stem cells undergoing replication are the major target of PhIP-induced cytotoxicity. Previous studies examining the apoptotic response of cells in the murine colon crypt to gamma irradiation have concluded that stem cells are extremely sensitive to DNA damage, engaging apoptotic pathways instead of repair (Clarke et al., 1994; Potten, 1998). This sensitivity diminishes as daughters of stem cells divide and lose their clonogenic properties after two or three divisions. It is hypothesized that apoptotic killing of damaged stem cells reduces the chance of propagating mutated genomes that are prone to carcinogenic transformation, thereby suppressing tumor formation (Potten, 1998; Zabkiewicz and Clarke, 2004). Furthermore, MSH proteins are expressed in the bottom two-thirds of the crypt in mouse and human colon (Colussi et al., 2001; Leach et al., 1996), which highly correlates with our detection of BrdU-positive cells in positions 1 – 16 (Figure 5.8). As these cells are likely to

express MMR proteins, yet the majority of apoptosis takes place in positions 1 – 4, MMR may have different roles in DNA damage surveillance in stem cells versus other proliferating cells in the colon crypt.

PhIP-induced apoptosis detected within mouse colon was partially *Mlh1*-dependent, with MMR responsible for approximately half of the apoptotic response to PhIP. This finding is consistent with a previous study demonstrating a partial dependence on *MSH6* for PhIP-induced apoptosis in lymphoblast cells grown in cell culture (Duc and Leong-Morgenthaler, 2004). We demonstrated a similar *Mlh1*-dependence for DMH-induced apoptosis, although absolute levels of apoptosis in *Mlh1*^{+/+} and *Mlh1*^{-/-} mice were higher with DMH versus PhIP. As the relative levels of O⁶meG adducts versus PhIP adducts generated by our exposure protocols are not known, it is unknown whether O⁶meG adducts are inherently more capable of initiating apoptosis versus PhIP adducts. However, both PhIP- and DMH-induced apoptosis exhibit similar timing, and similarly targeted the stem cell compartment, suggesting that the mechanism of signaling apoptosis may be the same. Others have demonstrated rapid *Mlh1*- and/or *Msh2*-dependent apoptosis in response to benzo[c]phenanthrene diol epoxide (B[c]PhDE) in mouse small intestine and DMH in mouse colon (Colussi et al., 2001; Wu et al., 2003). Thus, MMR is an important component of apoptotic signaling pathways *in vivo* in response to several different types of DNA damage. Such a role is likely to contribute to MMR-dependent suppression of mutation and carcinogenesis in response to DNA damage.

Cell culture studies of the MMR response to O⁶meG lesions suggest that repeated processing of O⁶meG/T mismatches by MMR (futile cycling) generates single stranded DNA breaks that are detected after a second round of S-phase, culminating in a G₂M arrest that is dependent on the ATR-CHK1 pathway, accompanied by apoptosis (Hickman and Samson, 2004; Stojic et al., 2004a). Alternatively, MMR proteins may be able to activate apoptosis directly, possibly in the absence of an excision reaction. Numerous studies have shown that MMR proteins interact directly with cell cycle checkpoint and apoptosis-related proteins (Brown et al., 2003; Her et al., 2002; Mac Partlin et al., 2003; Shimodaira et al., 2003; Wang et al., 2001; Wang et al., 2000; Wang and Qin, 2003), and that MMR-recognition of some compound lesions fails to provoke MMR-dependent excision in an *in vitro* assay (Wang et al., 2006). Furthermore, apoptotic responses to lesions generated by cisplatin and alkylating agents are maintained in *Msh2*^{G674A/G674A} mice (Lin et al., 2004). This mutation abrogates the excision function of MMR by disabling the ATPase activity of MutS α . Currently, it is not known whether PhIP-DNA adducts can promote MMR-dependent excision, although we previously proposed such a mechanism to explain the altered spectrum of PhIP-induced mutation in the intestinal tissue of *Mlh1*^{-/-} mice (Smith-Roe et al., 2006a). Regardless of initial MMR activities involved in initiating apoptotic signaling, one key difference between cell culture and *in vivo* data appears to be the timing of apoptosis. In the current study, the highest level of apoptosis in response to both PhIP and DMH was detected at 8 h, similar to previous studies with DMH and other genotoxins, in both small intestine and colon tissue of mice (Colussi et al., 2001; Li et al., 1992; Potten et al., 1992; Sansom et al.,

2003; Sansom et al., 2001; Toft et al., 1999). In contrast, MMR-dependent apoptosis is significantly delayed in cell culture models (Stojic et al., 2004b). Although exact cell replication kinetics are not known *in vivo*, rapid induction of apoptosis in small intestine and colon appears inconsistent with mechanisms requiring more than one round of DNA replication. If MMR-dependent excision opposite template damage during replication contributes to signaling apoptosis, the signaling pathway initiated by such excision would need to result in apoptosis rapidly, within one replication cycle. A critical issue that remains unresolved is the level of DNA damage induced *in vivo* versus cell culture models. MMR-dependent checkpoint signaling may vary in cells exposed to low v. high doses (Jiricny). Clearly, additional studies are required to identify signaling factors, and to clarify potential mechanistic differences between apoptosis triggered in cell culture versus *in vivo*.

In addition to evaluating apoptotic responses to PhIP and DMH, we also investigated DNA damage-induced changes in cell proliferation, as both PhIP and alkylating agents are associated with various forms of cell cycle arrest in cell-culture studies (Creton et al., 2005; Duc and Leong-Morgenthaler, 2004; Stojic et al., 2004b). In the current study, PhIP-exposure did not affect the level or location of BrdU-positive cells. However, BrdU staining is not strictly quantitative (cells are scored as positive or negative), and moderately reduced incorporation of nucleotide due to transient cell cycle delay may not result in significant reductions in proliferation index. Therefore, occurrence and significance of PhIP-induced cell-cycle delay *in vivo* remains unresolved. In contrast, a consistent reduction in the overall percentage of BrdU-positive cells was

detected in mice treated with DMH. This general effect indicates that no particular subpopulation of proliferating cells was targeted to halt replication. The overall reduction in proliferation also was not *Mlh1*-dependent, and therefore appears not to be equivalent to a MMR-dependent G₂M arrest described in cell culture. Slowed proliferation may be due to other lesions generated by DMH that are not substrates for MMR, such as 3-methyladenine, which effectively induces S-phase arrest through multiple mechanisms (disruption of DNA chain elongation and inhibition of replication firing) (Engelward et al., 1998). A very similar reduction in proliferation in response to a single injection of azoxymethane (AOM) was reported in rat colon (Hirose et al., 1996). In this study, proliferative levels were reduced by approximately half at the 8 h time point, which was also the peak of detected apoptosis. Examination of additional time points revealed a dramatic increase (2- to 3-fold) in BrdU-staining 3 days after treatment, suggesting a delayed compensatory effect in proliferation in response to early, increased apoptosis (Hirose et al., 1996). The later increase in proliferation detected by Hirose et al (1996) may result in enhanced replication of damaged genomes that have escaped apoptosis, contributing to the ability of repeated DMH exposures to be complete carcinogens. We would predict that such a compensatory increase in proliferation might occur in response to DMH, but not PhIP, with the current exposure protocol.

Previously, we demonstrated that *Mlh1*^{-/-} mice are hypermutable to PhIP and have increased susceptibility to PhIP-induced ACF. Loss of apoptosis is suggested to be important for the development of cancer, by allowing propagation of mutagenized cells. Thus, loss of MMR-dependent signaling for apoptosis could contribute to increased

development of ACF and ultimately carcinogenesis. PhIP-induced apoptosis in the current study was more dramatic following multiple exposures. Presently, it is unknown whether this increased apoptotic response reflects increased numbers of DNA adducts, which could accumulate as a result of increased metabolism, altered repair, or altered signaling mechanisms. Regardless, this finding suggests that the consequences of genotoxic damage may increase following repeated exposure. This observation is particularly relevant in human populations, where exposure to PhIP and other heterocyclic amines is chronic. Although PhIP generally is not carcinogenic in mouse colon, a dramatic and synergistic induction of colon adenocarcinomas in mice occurs with co-administration of an inflammation-inducing agent (Nishikawa et al., 2005; Tanaka et al., 2005). The Western diet is associated with high levels of heterocyclic amines, high fat content, low vitamin content, and low fiber (Yang et al., 2005), and Northern, industrialized countries are more prone to inflammatory bowel diseases (Danese et al., 2004). As a Western diet and industrialization are being adopted on a global basis, it is important to model how exposure to PhIP and dietary promoters could influence cancer risk in individuals at risk for MMR-deficiency.

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Chapter 6. General Discussion and Future Directions

Since the mid 1990s, cellular loss of functional MMR through genetic or epigenetic mechanisms has been definitively linked to greatly increased cancer risk, particularly for CRC. Subsequent research in this field with regard to understanding cancer risk has focused primarily on refinement of diagnostic criteria for Lynch syndrome, determination of the pathogenicity of mutated MMR genes present in microsatellite-unstable cancers, and identification of oncogenic and tumor suppressor genes that may be targets of MMR-induced mutagenesis (Lynch and Lynch, 2004; Mitchell et al., 2002). Additionally, the resistance of MMR-deficient cells to certain anti-cancer drugs has launched investigation as to whether such drugs may influence prognosis with various chemotherapeutics or cause increased risk for MMR-deficient secondary cancers (Karran et al., 2003). The contribution of environmental factors to MMR-deficient cancer risk, however, is an emerging field of research. Identification and avoidance of such factors may delay the onset, or reduce the risk of cancer in individuals with MMR-deficiencies. Thus far, recent epidemiological studies suggest that heavily-cooked meat, alcohol, and cigarette smoke influence sporadic MSI-positive cancer risk (Diergaarde et al., 2003; Luchtenborg et al., 2005; Satia et al., 2005; Wu et al., 2001). Heterocyclic amines such as PhIP are present in heavily-cooked meat and cigarette smoke, and exposure to these chemicals is associated with increased colorectal cancer risk in the general population (NTP, 2004; Sinha and Norat, 2002). As cell culture studies showed an interaction between the MMR pathway and the food-borne carcinogen

PhIP (Glaab and Skopek, 1999; Leong-Morgenthaler et al., 2001), I used mice deficient for the *Mlh1* gene to investigate whether exposure to PhIP might represent a cancer risk factor for individuals with MMR-deficiencies. I found that *Mlh1*-deficient mice were susceptible to 3-fold greater levels of PhIP-induced mutation in the intestinal tract and were 4-fold more susceptible to ACF compared to *Mlh1*-proficient mice. Furthermore, *Mlh1*-deficient mouse intestinal tissue failed to fully engage PhIP-induced apoptotic responses, thereby potentially allowing clonal expansion of PhIP-damaged genomes. Taken together, these findings indicate that PhIP represents a cancer risk to individuals with MMR-deficiencies, and further studies are needed to fully characterize this risk.

In Chapter 3, I reported that *Mlh1*-deficient mouse colon and small intestinal tissue, but not thymus tissue, were hypermutable in response to PhIP-exposure, as measured at the *cII* locus in a transgenic shuttle vector. While this study was in progress, another group reported a similar finding in *Msh2*^{-/-} mouse colon in response to PhIP (Zhang et al., 2001), illustrating that both *Msh2* and *Mlh1* are necessary for suppression of PhIP-induced mutagenesis. Comparison of the PhIP-induced mutational spectra of *Mlh1*^{+/+} versus *Mlh1*^{-/-} mice lead to the unique observation that MMR appears to suppress mutations that arise from PhIP-G/T and PhIP-G/G mispairs, but not PhIP-G/A mispairs. This observation weakens a generally held hypothesis that the predominance of G/C to T/A transversions observed in wild-type mutation spectra are the result of preferential bypass of the PhIP-G adduct with dATP during DNA replication (Carothers et al., 1994; Shibutani et al., 1999). Also, this observation strengthens the possibility that MMR may

have a “blind spot” for certain types of compound DNA damage. For example, MutS binds to 8-oxoG with the following preference: 8-oxoG/T > 8-oxoG/G > 8-oxoG/A > 8-oxoG/C (Larson et al., 2003; Mazurek et al., 2002), but excision only is provoked in response to 8-oxoG/T and 8-oxoG/G (Larson et al., 2003). In crystal structures of bacterial MutS, the mismatched base is contacted via the Phe 36 and Glu 38 residues of one dimer. Otherwise, only loose associations are made with the DNA backbone of the other strand (Lamers et al., 2000; Obmolova et al., 2000). The ability of MutS to wedge Phe 36 against a mismatched base is dependent on the flexibility of the DNA due to the presence of the mismatch (Phe 36 stacking with a mismatched base is linked to bending of the DNA in a 60° kink, with widening of the minor groove). The structure of the PhIP-G adduct in homoduplex DNA as determined by nuclear magnetic resonance shows that PhIP-G adducts can reside within DNA in two forms (Brown et al., 2001). In the major form, the PhIP adduct prefers to displace guanine and intercalate within the helix, compressing the major groove and widening the minor groove. In the minor form, the PhIP adduct resides outside the helix, resulting in minimal distortion of the helix. These observations suggest that most of the time, PhIP-adducted DNA may already be in a conformation that is amenable to MutS binding, although it is not known how the PhIP-G adduct might alter the helix if it is mispaired with A, T, or G. Therefore, it is intriguing that MMR fails to suppress lesions that arise from PhIP-G/A mispairs, and even more so when MMR apparently responds to a wide variety of chemical modifications of DNA (Duckett et al., 1996; Larson et al., 2003; Li et al., 1996; Mu et al., 1997; Wang et al., 1999; Wu et al., 1999; Wu et al., 2003). Crystalization of MutS with a PhIP-G/T versus a

PhIP-G/A mismatch may offer insight as to why MMR responds differently to what would appear to be highly similar lesions.

Crystallization, however, will reveal only the most stable conformation of MutS with PhIP-adducted DNA, and other experiments could be performed to investigate biochemical responses of MMR to PhIP-G mispairs. First, binding studies (electrophoretic-mobility-shift analysis) can be performed with PhIP-G/C and the various mispairs with purified MutS α (or MutS, which is easier to purify) to determine whether MutS α recognizes PhIP-G/A. For the design of these studies, it is important to consider the effect of sequence context on MutS binding affinity (Hays et al., 2005). For example, MutS binding of a mismatched cyclobutane pyrimidine dimer (T \diamond T/AG) was similar to homoduplex DNA-binding in 3 out of 4 different sequence contexts (Hoffman et al., 2005). In the fourth sequence context, MutS binding of T \diamond T/AG was improved above homoduplex levels, but did not reach the same affinity as for G/T mispairs. Determining the appropriate sequence context to use for binding studies would seem to be a formidable task. However, analysis of PhIP-induced *cII* mutations *in vivo* has revealed several G/C to A/T transition mutations (resulting from a PhIP-G/T mispair) specifically induced in *Mlh1*^{-/-} mouse colon. Assuming that these mutations would normally have been suppressed by MMR, sequence contexts of these mutations are likely candidates for constructing DNA substrates for binding studies.

Binding does not necessarily correlate with initiation of excision, however, as exemplified by studies of compound UV damage. In these studies, T \diamond T/AG and a mismatched pyrimidine-(6,4)-pyrimidone photoproduct (T[6,4]T/AG) apparently do not

provoke excision in an *in vitro* MMR assay even when embedded in sequence contexts that promote binding by MutS (Wang et al., 2006). Therefore, the next step would be to test the ability of the various PhIP-G mispairs to provoke excision in an appropriate sequence context. Given the *in vivo* mutational data in response to PhIP as well as MMR responses to mismatched UV photoproducts and 8-oxoG/A mispairs, I would hypothesize that MutS would bind all PhIP-G mispairs, but only PhIP-G/T and PhIP-G/G would provoke excision.

In Chapter 3, we hypothesized that MMR suppressed PhIP-induced mutagenesis by an excision response, in which the repair synthesis step could allow a second opportunity for the correct nucleotide to be inserted across from the PhIP-G adduct. This hypothesis suggests that accurate translesion synthesis could occur during repair-initiated synthesis. Furthermore, this hypothesis implies that MMR proteins could recruit translesion polymerases to repair synthesis tracts. *In vitro* translesion synthesis experiments with purified translesion polymerases and primer/template substrates with a single PhIP-G adduct could allow identification of which translesion polymerases can generate specific PhIP-G mispairs. Polymerase η is a likely candidate for bypass of PhIP-G, as this polymerase can bypass other bulky adducts, such as UV photoproducts and *N*-2-acetylaminofluorene (AAF)-G (Masutani et al., 1999; Masutani et al., 2000). Notably, polymerase η inserted C across from AAF-G (an adduct similar in structure to PhIP) most of the time, with occasional insertions of dTTP, dGTP, and dATP (Masutani et al., 2000). This pattern of nucleotide insertion across from AAF-G is similar to the

mutational spectrum of PhIP-treated *Mlh1*^{-/-} mouse colon tissue, in which dTTP, dGTP, and dATP appeared to be inserted across from PhIP-G with similar frequencies.

If PhIP-adducts are bypassed by certain translesion polymerases *in vitro*, these studies would provide a basis for testing translesion synthesis in response to PhIP-adducts in cell lines in the presence or absence of MMR. In order to evaluate the effect of MMR on PhIP-G bypass events, experiments could be performed using mouse embryonic fibroblast (MEF) cell lines derived from littermate mouse embryos that are *Mlh1*-proficient or -deficient and that also carry the *cII* shuttle vector. The use of small interfering RNA (siRNA) could knock down expression of specific translesion polymerases in order to determine their roles in PhIP-G bypass in these cells. At this time, interactions between MMR and translesion synthesis are largely unexplored. These experiments would lend insight into the mechanism by which MMR suppresses PhIP-induced mutagenesis.

If PhIP-G/A mispairs escape a MMR-directed excision response, as observed for 8-oxoG (Larson et al., 2003), this effect might be generalized to other adducted guanine residues that mispair with dATP. For example, an extremely potent mutagen and suspected human carcinogen found in diesel exhaust, 3-nitrobenzanthrone (3-NBA), forms adducts at the C8 and N² position of guanine (Arlt et al., 2006), and increases the *cII* mutation frequency of DNA extracted from Muta Mouse liver and colon tissue (Arlt et al., 2004). Similar to PhIP, the mutation spectrum of 3-NBA-treated wild-type mouse liver is dominated by G/C to T/A transversions, with low levels of G/C to A/T and G/C to C/G mutations. It would be of interest to determine whether MMR modulates the

mutation spectrum of this nitropolycyclic aromatic hydrocarbon in a manner similar to PhIP. Testing for MMR responses to various forms of DNA damage by comparing mutational spectra of wild-type and MMR-deficient cells could be expedited by generating MEF cell lines from *Mlh1*^{+/+} and *Mlh1*^{-/-} mouse embryos that also carry the *cII* transgene. These cell lines would not only be useful for quicker and less expensive screening of carcinogen-DNA damage likely to be detected by MMR (as well as identification of mutational “hot spots”), but would also facilitate assays in which mutant *Mlh1* DNAs are transfected into MEFs for detection of aberrant MMR activity (Mohd et al., 2006).

Mice exposed to chronic, dietary PhIP are susceptible to lymphoma, but do not develop small intestinal or colon tumors (Esumi et al., 1989). However, genetic susceptibility to intestinal tumorigenesis (*Min*^{+/+} mice) (Paulsen et al., 1999) or co-exposure of PhIP with a promoting agent (dextran sodium sulfate or high fat diet) (Nishikawa et al., 2005; Ochiai et al., 2002; Tanaka et al., 2005) can target PhIP-induced tumorigenesis to the intestinal tract of mice. As *Mlh1*^{-/-} mice are susceptible to intestinal tumorigenesis, and as *Mlh1*-deficiency resulted in 3-fold greater PhIP-induced mutation in the intestinal tracts of mice, it was predicted that *Mlh1*-deficiency would increase the tumor-initiation potential of PhIP in mouse intestine. I tested this hypothesis by exposing *Mlh1*^{+/+} and *Mlh1*^{-/-} mice to PhIP and assessing tumorigenic outcomes (Chapter 4). *Mlh1*-deficiency increased sensitivity to development of PhIP-induced ACF by 4-fold, which correlated well with the 3-fold induction of mutation due to PhIP in *Mlh1*^{-/-} colon

(Chapter 3). However, *Mlh1*-deficiency did not affect the ability of PhIP to cause tumors in the intestinal tracts of mice.

There are several possibilities as to why *Mlh1*-deficiency did not result in generation of PhIP-induced intestinal tumors. The first may have to do with the shortened lifespans of *Mlh1*^{-/-} mice. In previous studies of spontaneous tumorigenesis in *Mlh1*^{-/-} mice, colon tumors appeared in mice that were 8 months of age or older, with an incidence of ~1.5 tumors per mouse (Prolla et al., 1998; Tsao et al., 2002). In my tumor study, only 5 vehicle-treated and 5 PhIP-treated *Mlh1*^{-/-} mice lived to be 8 months or older (approximately one-third of the mice in each treatment group). Within these groups, 3 colon tumors were detected in PhIP-treated mice versus 1 in vehicle-treated mice. Therefore, the possibility exists that if the size of the *Mlh1*^{-/-} treatment groups were larger an effect of colon tumor induction due to PhIP might be detected in older mice using the present treatment protocol. Obtaining a sufficient number of long-lived mice is difficult, however, as *Mlh1*^{-/-} mice have a median survival of about 6 – 8 months due to lymphoma, and can die as early as 3 months. A way to circumvent this problem would be to develop mice that are conditional knockouts for *Mlh1* in the intestinal tract using the *cre-lox* system.

It would be of considerable interest to perform a larger study or a study with conditional *Mlh1*^{-/-} mice not only to determine whether *Mlh1*-deficiency can enhance PhIP-induced colon tumorigenesis, and therefore provide stronger evidence of cancer risk due to PhIP, but also to further evaluate the possibility that ACF in *Mlh1*^{-/-} mice progress to colon tumors. Both *Mlh1*^{-/-} (Chapter 4) and *Msh2*^{-/-} mice appear to be more susceptible

to ACF compared to wild-type mice, and ACF exhibiting microsatellite instability have been detected in human colon tissue, suggesting that ACF may be precursors of microsatellite-unstable (MMR-deficient) tumors (Pedroni et al., 2001; Reitmair et al., 1996). Although the data in Chapter 4 were not depicted in this manner, the number of ACF per mouse in the PhIP-treated *Mlh1*^{-/-} group peaked at about 8 – 10 months of age and declined thereafter. Additionally, ACF were not detected in older *Mlh1*^{-/-} mice with colon tumors. These observations suggest that ACF regress with age, and/or that ACF may have progressed to colon tumors. If additional studies suggest that ACF are precursors of colon tumors in MMR-deficient mice, analysis of early genetic changes in ACF and in tumors (if apparent) obtained from *Mlh1*-deficient mice could provide candidate oncogenes and tumor suppressor genes to examine for carcinogen-induced mutations. As oncogenes are activated by base substitution mutations, a prediction would be that G/C to T/A transversions would be the activating mutation in PhIP-treated wild-type mice, whereas activating mutations in PhIP-treated *Mlh1*^{-/-} mice would be primarily G/C to A/T transitions and G/C to T/A transversions, with a few G/C to C/G transversions.

A second possibility for the lack of observation of PhIP-induced intestinal tumors in *Mlh1*^{-/-} mice may be related to the exposure protocol. In the tumor study, mice were exposed to PhIP over a two-week period (an acute exposure), as opposed to lifetime (chronic) exposure. In Chapter 5, I observed that a greater apoptotic response to PhIP was generated in *Mlh1*^{+/+} mouse colon after multiple exposures to PhIP versus a single exposure. It may be possible that continual exposure to PhIP is necessary to maintain a

selective advantage for the clonal outgrowth of potentially mutagenized stem-cell genomes (please see paragraph after next). Therefore, ACF may reflect the initiating effects of PhIP in the absence of MMR (increased mutagenesis), but not the promoting effects of PhIP in the absence of MMR (loss of activation of apoptotic pathways).

Taken together, the data obtained in Chapter 4 provide support for conducting additional studies with certain modifications (neonatal exposure or chronic exposure to PhIP, possible use of conditional *Mlh1* knockouts) to determine whether *Mlh1*^{-/-} mice are susceptible to PhIP-induced tumors in the colon, and to further establish the strength of ACF as predictors of colon tumor risk in *Mlh1*-deficient mice. These studies may provide a framework for testing of other carcinogens that may be of particular concern for individuals with MMR-deficiencies.

Stem cells that permanently reside at or near the bottom of colon crypts divide and produce epithelial cells that undergo differentiation as they migrate up the wall of the crypt. These epithelial cells emerge from the crypt and become part of the luminal surface of the colon. They perform their function at the luminal surface for a few days and then deteriorate. As a result, the surface epithelium of the colon is regenerated every 2 to 3 days (Potten, 1998). Due to the rapid, ongoing regeneration of the colonic epithelium, the mutation frequencies obtained from colon tissue in Chapter 3 reflect genetic changes that have occurred within resident colonic stem cells. Stem cells are extremely sensitive to DNA damage, and this sensitivity rapidly declines as stem-cell daughters divide and differentiate (Marshman et al., 2002). The sensitivity of stem cells to DNA damage-induced apoptosis is thought to prevent continued proliferation of stem

cells that otherwise may have been mutagenized by unrepaired or mis-repaired DNA damage, and inappropriate survival of such cells could lead to neoplastic transformation (Booth and Potten, 2000; Karran et al., 2003; Zabkiewicz and Clarke, 2004). In Chapter 5, I determined that apoptosis is increased in *Mlh1*^{+/+} mouse colon in response to PhIP-treatment, and was first to demonstrate that the apoptotic response to PhIP *in vivo* is partially *Mlh1*-dependent. By carrying out a cell-by-cell analysis, I showed that the majority of PhIP-induced apoptosis occurred within the hypothetical stem cell compartment of colon crypts (cell positions 1 – 4). Similar results were observed in the colons of *Mlh1*^{+/+} and *Mlh1*^{-/-} mice treated with DMH. Therefore, *Mlh1* contributes to the extreme sensitivity of stem cells to DNA damage.

The highest detected apoptotic response to PhIP and DMH occurred rapidly, at 8 h after treatment, and *Mlh1* clearly contributed to activation of this apoptotic response, similar to other reports of *in vivo*, MMR-dependent apoptosis in response to DNA damaging-agents (Sansom et al., 2003; Sansom et al., 2001; Toft et al., 1999). This rapid response suggests that cells undergoing S-phase at the time of carcinogen exposure are the targets of damage-induced apoptosis (although Figures 5.8 and 5.9 suggest that some replicating cells are not quite as sensitive to damage-induced apoptosis). Alternatively, direct signaling by MMR-binding of lesions could result in rapid activation of apoptosis. However, the timing of MMR-dependent checkpoints and apoptosis in response to DNA damaging-agents is much later in cell culture, with alkylating agents apparently requiring two S-phase cycles before arresting in G₂M or engaging apoptosis (Stojic et al., 2004b). The cell culture study in which two S-phases were required for MMR-dependent G₂M

arrest invokes a “futile-cycling” response of MMR that generates low levels of secondary DNA-lesions during the first S-phase that are not detected until the second round of replication (Stojic et al., 2004a). A MMR-dependent excision response to DNA damage is essential to the futile-cycling hypothesis. Discrepancies between *in vivo* and *in vitro* observations may be reconciled by acknowledging that MMR-dependent responses to DNA damage may be different in the damage-sensitive stem cells of colon crypts compared to immortalized cells in culture. Presently, mechanisms of stem cell DNA metabolism, particularly with regard to repair of DNA damage, remain largely unexplored.

Considering the results of Chapter 3 and Chapter 5, it is possible that both the error-correction function of MMR and the damage response of MMR may contribute to suppression of PhIP-induced mutagenesis. The biochemical experiments proposed earlier in this discussion would help to clarify the role of the error-correction function of MMR in response to PhIP-DNA damage. To better understand the role of MMR-dependent apoptosis in suppression of PhIP-induced mutagenesis and carcinogenesis, PhIP-induced hypermutability could be tested in *Msh2*^{-/-} mice and *Msh2*^{G674A/G674A} mice that have both been crossed to Muta Mouse. As described in the introduction (page 17), the G674A mutation in *Msh2* abrogates the excision function of MMR, but MMR DNA-damage responses to chemicals such as cisplatin and 6-TG are retained. If MMR-dependent apoptosis is important for suppressing PhIP-induced mutation, the mutation frequency of *Msh2*^{G674A/G674A} mouse colon exposed to PhIP would be lower than that observed in similarly exposed *Msh2*^{-/-} mice.

If both mechanisms contribute to suppression of PhIP-induced mutagenesis, I would propose the following model in the attempt to incorporate the error-correction function of MMR into the MMR-damage response. MMR detects PhIP-G mispaired with T or G. Excision is initiated, and if repair-initiated synthesis allows insertion of the correct nucleotide, then the potential mutation is suppressed, and the PhIP-G adduct becomes a non-mutagenic substrate for NER. If the correct nucleotide is not inserted upon repair synthesis (perhaps due to error-prone translesion bypass), then MMR may facilitate activation of checkpoints or apoptosis in order to suppress these mutations. The mutational spectra of MMR-deficient cells would then reflect bypass events in the absence of MMR, which would persist in the absence of MMR-initiated apoptosis. This model is similar to the futile cycle model, except that in this case, MMR "knows" that it has attempted repair once already, and upon the second detection, would signal directly to checkpoints and apoptotic pathways. Presently, it is not known for certain whether detection of a mispair by MMR results in stalling of the replication fork in eukaryotes. If MMR uses the nascent strand (or Okazaki fragment) of DNA in eukaryotes as a strand-discrimination signal and as a point for initiation of exonuclease activity for nascent-strand excision, does this process halt replication, or can replication proceed? If replication can proceed after MMR detects a mispair (the replicating polymerase releases the nascent strand, but is able to continue replication if RNA primase primes the template strand again), MMR proteins will possess the nascent strand from where replication left off for initiation of excision and resynthesis. If the lesion is generated a second time upon resynthesis, if resynthesis is completed (and DNA is ligated) before "re-detection"

of the lesion by MMR, and if the replication fork has moved on, MMR may lose access to nascent strands and may engage a different function in response to compound damage, such as direct signaling to checkpoint proteins. It may be possible that a “critical mass” of MMR-dependent signaling in response to damage is needed to fully activate checkpoints and apoptosis.

Overall, the work presented in this thesis indicates that *Mlh1*-deficiency heightens the mutagenic and carcinogenic effects of PhIP in mice. When extrapolating these findings to predict cancer risk in humans, there are of course several caveats, as is true for the majority of toxicological studies in rodents. Mice were exposed to a high dose of PhIP over a short time in a controlled environment, the murine colon tends to be resistant to tumor induction in the absence of a promoter, and mice were of the same genetic background except for *Mlh1*-proficiency or –deficiency. In contrast, humans are chronically exposed to low levels of a combination of heterocyclic amines in complex environments, the colon is the major target of human intestinal tumorigenesis, and humans are genetically diverse. All of these factors impinge on the degree to which PhIP will be metabolically activated, how well PhIP will be excreted, and whether PhIP-DNA damage will be successfully repaired. Nevertheless, the underlying molecular mechanisms among mammals for cancer risk are similar, and MMR-deficiency *in vivo* clearly compromises suppression of PhIP-induced mutation and ACF. Furthermore, despite the diversity of human genetics and environments, epidemiological studies still demonstrate that HCA exposure via meat consumption and smoking are capable of increasing cancer risk in the general population, as well as in individuals with sporadic

microsatellite-unstable cancers. Therefore, PhIP represents a cancer risk to individuals with MMR-deficiencies, and further investigation of PhIP-related cancer risk, as well as other environmental carcinogens that provoke MMR responses, should be pursued.

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