

Distinct effects of the recurrent *Mlh1*^{G67R} mutation on MMR functions, cancer, and meiosis

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Mutations in the human DNA mismatch repair (MMR) gene *MLH1* are associated with hereditary nonpolyposis colorectal cancer (Lynch syndrome, HNPCC) and a significant proportion of sporadic colorectal cancer. The inactivation of *MLH1* results in the accumulation of somatic mutations in the genome of tumor cells and resistance to the genotoxic effects of a variety of DNA damaging agents. To study the effect of *MLH1* missense mutations on cancer susceptibility, we generated a mouse line carrying the recurrent *Mlh1*^{G67R} mutation that is located in one of the ATP-binding domains of *Mlh1*. Although the *Mlh1*^{G67R} mutation resulted in DNA repair deficiency in homozygous mutant mice, it did not affect the MMR-mediated cellular response to DNA damage, including the apoptotic response of epithelial cells in the intestinal mucosa to cisplatin, which was defective in *Mlh1*^{-/-} mice but remained normal in *Mlh1*^{G67R/G67R} mice. Similar to *Mlh1*^{-/-} mice, *Mlh1*^{G67R/G67R} mutant mice displayed a strong cancer predisposition phenotype. However, in contrast to *Mlh1*^{-/-} mice, *Mlh1*^{G67R/G67R} mutant mice developed significantly fewer intestinal tumors, indicating that *Mlh1* missense mutations can affect MMR tumor suppressor functions in a tissue-specific manner. In addition, *Mlh1*^{G67R/G67R} mice were sterile because of the inability of the mutant *Mlh1*^{G67R} protein to interact with meiotic chromosomes at pachynema, demonstrating that the ATPase activity of *Mlh1* is essential for fertility in mammals.

DNA damage response | HNPCC | Meiosis | *Mlh1* | MMR

Mutations in the human DNA mismatch repair (MMR) genes *MSH2*, *MSH6* (MutS homolog), and *MLH1* (MutL homolog 1) are the cause of the majority of hereditary nonpolyposis colorectal cancer (Lynch syndrome, HNPCC) cases and also a significant number of sporadic cancers (1, 2). The DNA MMR system plays a critical role in the maintenance of genome integrity and functions in the postreplicative repair of base substitution mutations as well as small insertions/deletions (IDLs) caused by erroneous replication (3). In addition, studies in yeast and mice indicate that several of the MMR proteins have essential meiotic functions (4, 5). In eukaryotic cells, MMR is initiated by subsets of MutS homologs (MSH) that form heterodimeric complexes: MSH2–MSH6 (MutS α) and MSH2–MSH3 (MutS β). The MutS α complex recognizes base–base mismatches and single-base IDLs, whereas the MutS β complex detects single-base and larger IDLs to initiate the repair process. As a result of mismatch recognition, the MutS α and MutS β complexes undergo ATP-hydrolysis-dependent conformational transitions and recruit a heterodimeric complex of the MutL homologs MLH1 and PMS2 (MutL α). This interaction between MutS and the MutL complexes is essential for the activation of subsequent MMR steps, i.e., the excision of the mismatches and IDLs and the resynthesis of the excised DNA strand (6–8). Similar to MutS complexes, the mediation of repair excision and resynthesis by MutL α requires ATP binding and hydrolysis (9–11).

In addition to repairing mismatched bases, MMR also contributes to genome stability by stimulating DNA damage-induced apoptosis as part of the cytotoxic response to DNA-damaging agents. Although MMR-proficient cells respond to exposure to DNA-damaging agents such as cisplatin by signaling cell cycle arrest at the G₂ checkpoint followed by apoptosis, MMR-deficient human tumor cells are defective in this response (8, 12, 13). As a result, they display increased resistance to the genotoxic effects of DNA damage-inducing agents. It has been proposed that both the DNA repair and DNA damage-response functions of MMR are important for the suppression of tumorigenesis. On the one hand, defects in MMR are associated with an increased mutator phenotype, resulting in the accumulation of mutations in tumor-suppressor genes and oncogenes and leading to the initiation of tumorigenesis (14, 15). Alternatively, defects in the MMR-dependent DNA damage response could lead to a failure in clearing DNA damage-bearing cells, which may confer a selective advantage in tumor cells (13, 16, 17). Consistent with this notion, *Msh2*^{G674A} and *Msh6*^{T1217D} mutant mice, which carry separation of function mutations that inactivate DNA repair but leave the DNA damage-response function intact, have delayed tumor onset compared with *Msh2*^{-/-} and *Msh6*^{-/-} mice, which are defective in both MMR functions (18, 19).

Although the majority of MMR mutations found in Lynch syndrome (HNPCC) patients are frameshift or nonsense mutations that result in an inactive protein, a significant number of MMR mutations are missense mutations [$>25\%$ of *MSH2* or *MLH1* mutations and $>45\%$ of *MSH6* mutations (20)]. The impact of these missense mutations on the individual MMR functions and their significance for cancer predisposition often remain uncertain. The generation and analysis of mouse lines with MMR point mutations that model Lynch syndrome (HNPCC) mutations provides the opportunity to determine their impact on DNA repair and DNA damage response on the organismal level and assess their impact on carcinogenesis. Here, we report on a knockin mouse line with the first missense mutation in a MutL homolog. This mouse line carries a germ-line

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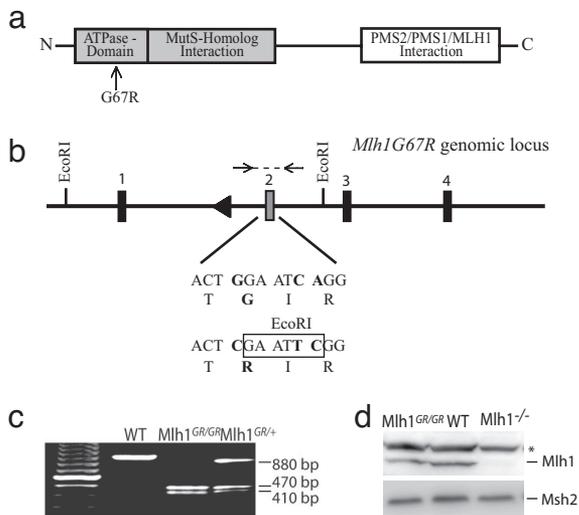


Fig. 1. The generation of *Mlh1*^{G67R} mutant mice. (a) Domain structure of Mlh1. The location of the G67R mutation is indicated. (b) Schematic representation of the modified *Mlh1*^{G67R} genomic locus and sequence characteristics of the G67R mutation. (c) PCR genotyping of tail DNA from *Mlh1*^{G67R} mutant mice. *Mlh1*^{G67R/+}, heterozygous mice; *Mlh1*^{G67R/G67R}, homozygous mutant mice. After *EcoRI* restriction digestion of the PCR product, the WT allele is indicated by an 880-bp fragment and the *Mlh1*^{G67R} mutant allele by 470- and 410-bp restriction fragments. (d) Western blot analyses of thymocyte cell extracts using anti-Mlh1 and anti-Msh2 antibodies. *, Unspecific protein recognized by the *Mlh1* antisera.

mutation that models the recurrent *MLH1*^{G67R} variant found in several Lynch syndrome (HNPCC) families from different countries (1). Patients carrying the *MLH1*^{G67R} mutation develop colorectal cancers that are characterized by microsatellite instability (MSI) (21). Consistent with the cancer phenotype in these Lynch syndrome (HNPCC) patients, *Mlh1*^{G67R/G67R} mice display a strong cancer phenotype. However, in contrast to *Mlh1*^{-/-} mice, the tumor incidence in *Mlh1*^{G67R/G67R} mice is significantly different, with a lower incidence of gastrointestinal tumors. This change appears to be caused by the different effects the *Mlh1*^{G67R} mutation exerts on the DNA repair and damage-response functions in the intestinal mucosa. In addition, the *Mlh1*^{G67R} mutation causes infertility in both male and female mice that is caused by the inability of the mutant Mlh1^{G67R} protein to localize to meiotic chromosomes. These studies demonstrate that the *Mlh1*^{G67R} mutation differentially affects the biological functions associated with Mlh1 with distinct phenotypic effects.

Results

Generation and Cancer Susceptibility Phenotype of *Mlh1*^{G67R} Mutant Mice. The *Mlh1*^{G67R} mouse line was created by a knockin gene-targeting strategy [Fig. 1 a–c and supporting information (SI) Fig.

8 in SI Appendix]. Western blot analysis revealed slightly reduced levels of cellular Mlh1^{G67R} protein, indicating that the mutation affected protein stability (Fig. 1d). However, similar to WT Mlh1, the mutant Mlh1^{G67R} protein was still capable of interacting with Pms2 as assessed by coimmunoprecipitation of Flag-tagged Mlh1^{G67R} and HA-tagged Pms2 protein expressed in 293T cells (SI Fig. 9 in SI Appendix). Analysis of heterozygous or homozygous F2 animals revealed no developmental abnormalities.

Cohorts of *Mlh1*^{G67R/G67R}, *Mlh1*^{G67R/+}, *Mlh1*^{+/+}, and *Mlh1*^{-/-} mice were monitored for survival and cancer susceptibility. Both *Mlh1*^{G67R/G67R} and *Mlh1*^{-/-} mice showed severely reduced survival, with only 50% of animals surviving 30 weeks of age, and all animals had died by 64 weeks of age. In contrast, 100% of *Mlh1*^{+/+} and 96% of *Mlh1*^{G67R/+} mice survived this period (Fig. 2a and data not shown). There was no difference in survival between *Mlh1*^{G67R/G67R} and *Mlh1*^{-/-} mice. The reduced survival in the *Mlh1*^{G67R/G67R} and *Mlh1*^{-/-} mutant mice was caused by an increase in cancer predisposition. The histopathological analysis of moribund mice revealed that among 66 *Mlh1*^{-/-} mice, 35 had developed T and B cell lymphoma, 35 had developed small intestinal tumors (among these, 8 mice developed both lymphoma and intestinal tumors), and 4 mice developed squamous basal cell carcinoma of the skin. The analysis of 49 *Mlh1*^{G67R/G67R} mice revealed a similar tumor spectrum; however, we found a significant reduction in the number of mice with intestinal tumors (Fig. 2b). Although 34 mice had developed T and B cell lymphoma, only 16 mice developed small intestinal tumors (among these, 5 mice developed both types of tumors). Histopathological analysis revealed that, of 31 intestinal tumors in *Mlh1*^{-/-} mice, 19 were adenocarcinoma, and 12 were adenoma, whereas, of 18 intestinal tumors in *Mlh1*^{G67R/G67R} mice, 11 were adenocarcinomas, and 7 were adenomas. In addition, four *Mlh1*^{G67R/G67R} mice had also developed squamous basal cell carcinoma of the skin. Six *Mlh1*^{G67R/G67R} mice died prematurely, but their tumors were not available for analysis.

MSI in *Mlh1*^{G67R/G67R} Mice. To determine the MMR defect caused by the *Mlh1*^{G67R} mutation, we assessed the *in vivo* mutator phenotype in *Mlh1*^{G67R/G67R} mutant mice by analyzing MSI in tail genomic DNA (SI Fig. 10a in SI Appendix). At the dinucleotide marker *D17Mit123*, 25.4% of alleles tested were unstable in *Mlh1*^{-/-} mice, and 21.9% of alleles were unstable in *Mlh1*^{G67R/G67R} mice. In contrast, only 6.6% of alleles in *Mlh1*^{+/+} genomes were unstable, indicating highly significant increases in mutation frequency in the genomes of *Mlh1*^{-/-} and *Mlh1*^{G67R/G67R} mice at this marker ($P < 0.0001$ compared with *Mlh1*^{+/+}) (SI Fig. 10b in SI Appendix). Similarly, the same marker showed significantly increased instability in intestinal epithelial cells of *Mlh1*^{-/-} and *Mlh1*^{G67R/G67R} mice (24.0% and 23.4%, respectively) compared with WT mice (4.2%) ($P < 0.0001$) (SI Fig. 10c in SI Appendix). The analysis of five different microsatellite markers in the genomes of intestinal and lymphatic tumors showed that 80% of *Mlh1*^{G67R/G67R} tumors displayed a high MSI phenotype (22 of 28), with three or more

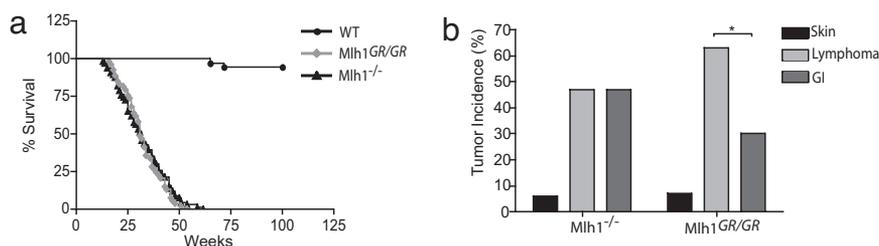


Fig. 2. Survival and tumor incidence in *Mlh1* mutant mice. (a) Survival of *Mlh1* mutant mice. Significantly reduced survival of *Mlh1*^{-/-} and *Mlh1*^{G67R/G67R} mice compared with WT mice ($P < 0.0001$; log-rank test) is shown. There was no difference in survival between *Mlh1*^{-/-} and *Mlh1*^{G67R/G67R} mice ($P = 0.915$; log-rank test). (b) Tumor incidence in *Mlh1*^{-/-} and *Mlh1*^{G67R/G67R} mutant mice. Reduced gastrointestinal (GI) tumor incidence in *Mlh1*^{G67R/G67R} mice ($P < 0.0296$) is shown.

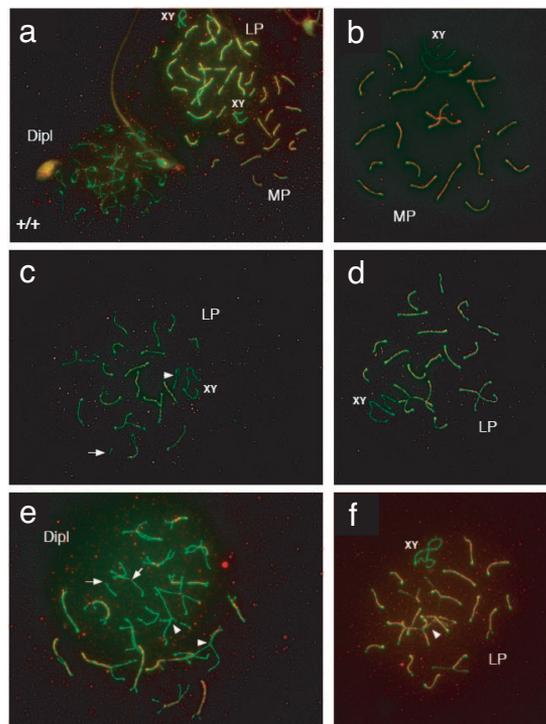


Fig. 6. Premature chromosome desynapsis in *Mlh1^{G67R/G67R}* mice. Sycp1 (red) and Sycp3 (green) staining shows pachytene to diplotene chromosome configurations in WT (a) and *Mlh1^{G67R/G67R}* (b–f) mouse spermatocytes. Mutant animals show premature separation of some chromosomes, particularly the XY in midpachynema (MP) and longer chromosomes in late pachynema (LP) to early diplotene (arrowheads in c) with abnormal intrahomolog associations being evident at late pachynema and diplotene (Dipl) (arrowheads in e and f). Broken synaptonemal complexes are also common (arrows in c and e). (See also larger images in SI Fig. 12 in SI Appendix).

however, spermatocytes from *Mlh1^{G67R/G67R}* males displayed abnormal spindle structures with chromosomes misaligned across the spindles (Fig. 5d, arrows). Further analysis of metaphase chromosomes in *Mlh1^{G67R/G67R}* animals revealed mainly univalent chromosomes with only a few bivalents (Fig. 5f, arrow), indicating that the chromosomes undergo premature separation before aligning at the metaphase plate. These results suggest that the *Mlh1^{G67R}* mutation causes the loss of chiasmata at or before metaphase I, and, as a result, most of the *Mlh1^{G67R/G67R}* spermatocytes failed to progress beyond metaphase and became apoptotic. In contrast, in *Mlh1^{+/+}* males the seminiferous epithelium displayed a normal range of spermatogenic cells and contained mature spermatozoa within their lumen, indicating normal progression through metaphase and full completion of spermatogenesis (Fig. 5a, b, and e).

We monitored meiotic progression in adult WT and *Mlh1^{G67R/G67R}* males by analyzing the assembly of synaptonemal complex proteins, Sycp1 and Sycp3, during prophase I. Although the meiotic chromosomes in *Mlh1^{G67R/G67R}* mice display the typical pachytene-to-diplotene configurations similar to WT (Fig. 6a and b), some spermatocytes from these mutant animals display premature separation of some of the chromosomes (Fig. 6c and d) as well as abnormal intrahomolog associations at late pachytene and diplotene (Fig. 6e and f).

We also examined the localization of Mlh1 to meiotic foci in WT and mutant animals. Although Mlh1 containing foci, which represent sites of crossing-over, were found at the predicted frequency and localization in WT mice (Fig. 7a), the analysis of *Mlh1^{G67R/G67R}* males revealed either the complete loss or a decrease in the frequency and intensity of Mlh1^{G67R} staining

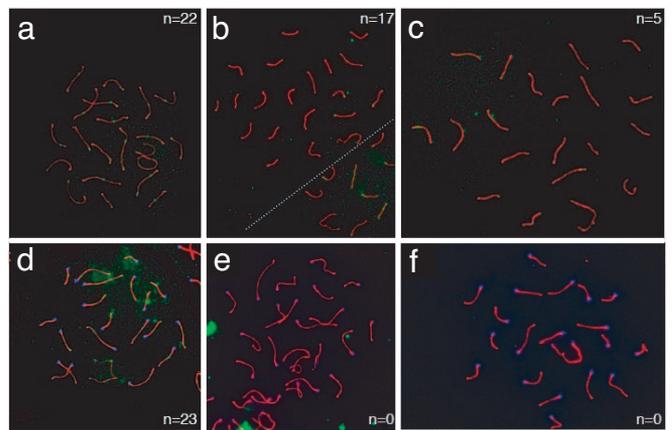


Fig. 7. Localization of Mlh1 (a–c) and Mlh3 (d–f) on meiotic chromosomes. (a–c) Localization of Mlh1 (green foci) on pachytene chromosome spreads from WT (a) and *Mlh1^{G67R/G67R}* (b and c) males reveals a dramatic decrease in frequency and intensity of Mlh1 staining in the *Mlh1^{G67R/G67R}* mutant animals. Many cells show a complete absence of Mlh1^{G67R} staining (data not shown), but others show distinct Mlh1^{G67R} reduction as exemplified in b and c. (d–f) Mlh3 staining (green foci) of WT (d) and *Mlh1^{G67R/G67R}* (e and f) males reveals a complete absence of Mlh3 staining in pachytene spreads from *Mlh1^{G67R/G67R}* mutant animals. (See also larger images in SI Fig. 13 in SI Appendix). Synaptonemal complexes are stained with anti Sycp3 antiserum (red), and centromeres are detected by CREST autoimmune serum (blue).

(Fig. 7b and c). The frequency of Mlh1 foci in *Mlh1^{G67R/G67R}* mice ranged from 20% to 77% of that observed in WT mice (data not shown). This analysis also showed a complete absence of Mlh3 staining in pachytene spreads of *Mlh1^{G67R/G67R}* animals, whereas WT animals displayed normal staining (Fig. 7d–f).

Discussion

We generated a mouse line carrying the *Mlh1^{G67R}* missense mutation that corresponds to a recurrent mutation in Lynch syndrome (HNPCC) patients and studied the consequences on individual MMR functions, cancer susceptibility, and fertility. The *Mlh1^{G67R}* mutation had distinct effects on the DNA repair and damage-response functions of Mlh1. Similar to the *Msh2^{G674A}* and *Msh6^{T1217D}* missense mutations that we previously analyzed in mice, these functions are clearly distinguishable by the *Mlh1^{G67R}* mutation. In homozygous mutant *Msh2^{G674A}* and *Msh6^{T1217D}* animals, the tumor onset was delayed compared with *Msh2^{-/-}* and *Msh6^{-/-}* mice (18, 19), consistent with the idea that the MMR-dependent DNA damage-induced apoptotic response could play an important tumor-suppressing role in the initial stages of tumorigenesis (13). Similar to *Mlh1^{-/-}* mice, *Mlh1^{G67R/G67R}* mice were characterized by a strong cancer predisposition phenotype. However, the number of intestinal tumors in *Mlh1^{G67R/G67R}* mice was significantly reduced compared with *Mlh1^{-/-}* mice. We observed the retention of a robust apoptotic response to cisplatin exposure in the epithelial mucosa of *Mlh1^{G67R/G67R}* animals similar to WT mice, whereas this response was severely affected in *Mlh1^{-/-}* animals. Similarly, WT and *Mlh1^{G67R/G67R}* T lymphocytes displayed a robust cisplatin response, whereas T lymphocytes in *Mlh1^{-/-}* mice showed increased resistance to cisplatin (Fig. 3 and SI Fig. 10 in SI Appendix). However, in contrast to intestinal epithelial cells, in T lymphocytes, the difference in the DNA damage response was not associated with a reduction in the incidence of lymphomas, although the time of death due to lymphoma was slightly delayed in *Mlh1^{G67R/G67R}* mice (data not shown). These results demonstrate that the effect of missense mutations on MMR functions can affect cancer susceptibility in a tissue-specific manner in mice. They also suggest that the MMR-dependent DNA damage

response in patients carrying the MLH1^{G67R} might differ compared with patients carrying complete MLH1 loss-of-function mutations, and it is possible that some Lynch syndrome (HNPCC)-associated cancers with missense mutations respond more favorably to chemotherapeutic treatment.

The G67R mutation affected the stability of the mutant protein and led to slightly reduced cellular levels of Mlh1^{G67R} protein. This finding is consistent with the observation that ectopic expression of human MLH1^{G67R} in *N*-methyl-*N*-nitrosourea (MNU)-resistant A2780MNU ovarian carcinoma cells did only yield reduced levels of protein compared with WT MLH1. In addition, ectopic expression of MLH1^{G67R} in A2780MNU cells did not restore sensitivity to MNU (27). We found that the cells from different tissues in *Mlh1*^{G67R/G67R} mutant mice remained sensitive to cisplatin exposure. It is possible that primary cells and transformed cancer cells derived from different tissue behave differently in the MMR-dependent DNA damage response.

The Mlh1^{G67R} mutation caused a strong DNA repair defect as indicated by the high MSI in the tissues and tumors of *Mlh1*^{G67R/G67R} mice, which is consistent with studies in bacteria, yeast, and human cells and further demonstrates that normal ATPase activity is essential for the activation of the repair processes that facilitate the removal of mismatched bases (9, 11, 28–32). However, because the *Mlh1*^{G67R} mutation did not significantly affect the MMR-dependent cytotoxic response, normal Mlh1-mediated ATP processing is apparently not essential for the DNA damage-signaling function of Mlh1 in mammalian tissues. Different models have been proposed to explain the role of MMR proteins in DNA damage response: The futile cycle model proposes that MMR engages in futile repair cycles after treatment with alkylating agents that ultimately lead to the formation of double-strand breaks that signal cell-cycle arrest and apoptosis (33). A second model suggests that MMR proteins function as damage sensors and directly link cell cycle and apoptotic mediators to the site of DNA damage (13, 34, 35). The molecular analysis of the *Mlh1*^{G67R} mouse line showed that the *Mlh1*^{G67R} mutation resulted in reduced levels of Mlh1^{G67R} protein but had no effect on the stability of other proteins such as Pms2 and p73 (data not shown) with which it interacts during MMR and apoptosis signaling (36, 37). The presence of Mlh1^{G67R} protein that has lost its ability to mediate excision repair but is still capable of DNA damage signaling is consistent with the idea that the MutS α and MutL α complexes can act as molecular scaffolds to recruit downstream effectors to sites of DNA damage. In this model, MutS α and MutL α bind to either mismatched or damaged bases. Depending on the DNA ligand, the MutS α and MutL α complexes then recruit either DNA repair factors to mediate either excision repair or downstream effectors such as ATR (ataxia-, telangiectasia-, and Rad3-related) and ATRIP (ATR-interacting protein) or p73 to mediate cell-cycle arrest and apoptosis (37, 38). This scenario would also predict that, although the Mlh1^{G67R} mutation prevents MutL α from functional interaction with downstream repair factors to initiate mismatch excision similar to other previously reported ATPase mutations in Mlh1 or Pms2 (9, 11), it should still allow the interaction of MutL α with ATM/ATRIP, p73, or other factors to signal cell-cycle arrest and apoptosis. Recently it was discovered that MutL α contains a latent endonuclease activity that depends on the DQHA(X)₂E(X)₄E motif in Pms2 (39, 40), and it was shown that this activity is important in the cellular response to 6-thioguanine exposure (41). It has also been reported that mutations in the ATPase centers of both MLH1 and PMS2 impair MutL α endonuclease activity (39). Our results suggest that either the Mlh1^{G67R} mutation does not completely impair the ATPase activity of MutL α or the endonuclease

activity may not be required for the cisplatin response. These predictions can be tested in future studies.

In mice, Mlh1 also has distinct functions in meiotic recombination, mismatch repair, and, possibly, DNA damage response. The *Mlh1*^{G67R} mutation caused a strong meiotic defect that resulted in male and female sterility. The histopathological analysis of meiotic progression in *Mlh1*^{G67R/G67R} male mice revealed that, similar to *Mlh1*^{-/-} mice, spermatogenesis proceeded normally through pachynema but failed to progress through metaphase I (42, 43). Consistent with this observation, cytogenetic analysis of prophase I chromosomes in *Mlh1*^{G67R/G67R} males showed normal synapsis and typical pachytene and diplotene configurations of the chromosomes, however, a significant number of nuclei also showed premature desynapsis of chromosomes. In addition, metaphase chromosomes also showed premature separation of chromosomes, suggesting that the Mlh1^{G67R} protein interfered with meiotic recombination. Consistent with this idea, mutations affecting the ATPase domain of yeast MLH1 resulted in reduced meiotic crossing-over (44). Localization studies showed that this meiotic defect in *Mlh1*^{G67R/G67R} mice was caused by the inability of the mutant protein to efficiently localize to meiotic chromosomes. Therefore, at the cytogenetic level the Mlh1^{G67R} mutation resulted in an “Mlh1 knockout” phenotype. Interestingly, the meiotic defect is different in *Mlh1*^{-/-} mice, in that Mlh3 localization to chromosomes is absent in *Mlh1*^{G67R/G67R} mice, whereas Mlh3 localization occurs in *Mlh1*^{-/-} mice (45). Mlh1 and Mlh3 are known to form a heterodimeric complex (termed MutL γ) (5) whose function is essential for meiotic progression, and our studies demonstrate that the Mlh1^{G67R} mutation interferes with the function of this complex and its chromosomal localization. It is possible that the mutant Mlh1^{G67R} also interferes with MutL γ function in heterozygous mice, and it will be interesting to evaluate the possibility of more subtle meiotic defects in heterozygous animals, which may provide a model for human aneuploidy.

Materials and Methods

PCR Genotyping of *Mlh1*^{G67R} Mutant Mice. Genomic tail DNA was PCR amplified by using forward primer 5'-TGC-TGA-AAG GAC-TGC-CCT AC-3' and reverse primer 5'-GCC-CTC-CTG-AAT-GAC-CAA-TA-3', and the 880-bp PCR was subsequently digested with EcoRI.

Western Blot Analysis. Thymocyte cell extracts (50 μ g of protein) were separated on two 10% SDS/PAGE gels. Protein was transferred onto Protran membranes, and the membranes were incubated either with rabbit polyclonal antibodies directed against Mlh1 (AB 9144; Abcam) or Msh2 (PharMingen).

Histopathological Analysis of Tumors. Gastrointestinal tumors were dissected, fixed in 10% neutral buffered formalin, and processed for paraffin embedding and H&E staining. Lymphomas were analyzed for surface markers by three-color flow cytometry.

MSI Analysis. Genomic DNA from tail and intestinal mucosa was analyzed at dinucleotide markers *D17Mit123* and *D7Mit91* as described (22). For tumor tissue, three mononucleotide markers (*U12235*, *Aa006036*, and *L24372*) and two dinucleotide markers (*D7Mit91* and *D17Mit123*) were analyzed. PCR was performed by using Cy5-end-labeled primers (30 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 2 min). PCR products were separated on denaturing acrylamide gels and autoradiographed.

Cytotoxicity Assays. MEFs were exposed to cisplatin and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays were performed according to standard procedures. MEFs were seeded at 2×10^4 cells per well in a 24-well plate. The cells were exposed to various concentrations of cisplatin and analyzed after 24, 48, and 72 h of treatment. The percentage of cell survival was calculated as (treated cells per untreated cells \times 100). The apoptotic response to drug exposure was measured by TUNEL (Promega). The experiments were performed for three different MEF strains for each *Mlh1* genotype and repeated at least three times for each strain.

In Vivo DNA Damage Response. Three animals of each *Mlh1*^{+/+}, *Mlh1*^{-/-}, and *Mlh1*^{G67R/G67R} genotype were injected intraperitoneally with cisplatin (10

mg/kg of body weight) or saline solution. All animals were killed 24 h after injection, and their intestines were fixed in 10% formalin. Paraffin-embedded histological sections from small and large intestines were stained with TUNEL (Promega). The number of TUNEL-positive cells per 50 crypts in the small and large intestines were counted, and the means of three mice in each group were calculated.

Cell Cycle Analysis. MEF cells (1.0×10^6) were either treated with 40 μ M cisplatin for 36 h or left untreated. The cells were subsequently fixed in 70% ethanol, stained with propidium iodide (PI), and analyzed by flow cytometry.

Analysis of Meiotic Prophase I. The testes from male mice were fixed in Bouin's or 10% formalin, embedded in paraffin, and sectioned. Chromosome spreads

were prepared as described (23) and subjected to indirect immunofluorescence using antibodies against synaptonemal complex proteins (Sycp1 and Sycp3), MutL homologs (Mlh1 and Mlh3), and centromeres (CREST). Secondary antibodies were conjugated to Cy3, Cy5, or fluorescein (Jackson Immunochemicals). Images were visualized with the aid of a Zeiss Axioimager Z1 equipped with a cooled CCD AxioCam MRM camera, and processed by using Axiovision 4.0 software (Zeiss).

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