Cell, Vol. 85, 1125–1134, June 28, 1996, Copyright ©1996 by Cell Press

Meiotic Pachytene Arrest in MLH1-Deficient Mice

Winfried Edelmann,* Paula E. Cohen,† Michael Kane,‡ Kirkland Lau,* Bernice Morrow,* Samuel Bennett,§ Asad Umar,§ Thomas Kunkel,§ Giorgio Cattoretti,‖ Raju Chaganti,# Jeffrey W. Pollard,† Richard D. Kolodner,‡ and Raju Kucherlapati*
*Department of Molecular Genetics
†Department of Developmental and Molecular Biology
Albert Einstein College of Medicine
Bronx, New York
‡Dana Farber Cancer Institute
Boston, Massachusetts
§National Institute of Environmental Health Sciences
Research Triangle Park, North Carolina
‖Department of Pathology
College of Physicians and Surgeons
Columbia University
New York, New York
#Department of Genetics
Sloan Kettering Cancer Center
New York, New York

Summary

Germ line mutations in DNA mismatch repair genes including *MLH1* cause hereditary nonpolyposis colon cancer. To understand the role of *MLH1* in normal growth and development, we generated mice that have a null mutation of this gene. Mice homozygous for this mutation show a replication error phenotype, and extracts of these cells are deficient in mismatch repair activity. Homozygous mutant males show normal mating behavior but have no detectable mature sperm. Examination of meiosis in these males reveals that the cells enter meiotic prophase and arrest at pachytene. Homozygous mutant females have normal estrous cycles and reproductive and mating behavior but are infertile. The phenotypes of the *mlh1* mutant mice are distinct from those deficient in *msh2* and *pms2*. The different phenotypes of the three types of mutant mice suggest that these three genes may have independent functions in mammalian meiosis.

Introduction

Colorectal cancer (CRC) in humans constitutes an important cause of mortality and morbidity. There are two major inherited syndromes that exhibit predisposition to CRC. The first of these is the autosomal dominant familial adenomatous polyposis (FAP). Germ line mutations in the adenomatous polyposis coli (APC) gene cause FAP (Kinzler et al., 1991; Groden et al., 1991; Joslyn et al., 1991). The second syndrome with predisposition to CRC is hereditary nonpolyposis colon cancer (HNPCC) (Lynch syndrome). This syndrome is also inherited in an autosomal dominant fashion and is characterized by colonic and extra colonic tumors. The tumors in HNPCC patients have a characteristic replication error (RER^+) phenotype (Aaltonen et al., 1993, 1994; Parsons et al., 1993; Lothe et al., 1993; Lynch et al., 1993). Recent studies have shown that much of HNPCC is due to inheritance of germ line mutations in genes encoding proteins required for DNA mismatch repair. HNPCC-causing germ line mutations are most commonly found in the genes *MSH2*, located on human chromosome 2, and *MLH1*, located on chromosome 3, whereas germ line mutations are rare or not yet reported in the mismatch repair genes *PMS2* and *GTBP* (*MSH6*) (Drummond et al., 1995; Palombo et al., 1995) and the candidate mismatch repair gene *PMS1* (Peltomaki et al., 1993; Fishel et al., 1993; Leach et al., 1993; Liu et al., 1994, 1996; Lindblom et al., 1993; Bronner et al., 1994; Papadopoulos et al., 1994, 1995; Nicolaides et al., 1994; Drummond et al., 1995; Palombo et al., 1995; reviewed by Kolodner, 1996).

Much of our understanding of the mismatch repair proteins implicated in HNPCC is derived from studies of the Escherichia coli MutHLS mismatch repair system (reviewed by Modrich, 1991). The roles of many of the proteins that function in this reaction have been elucidated. The MutS protein binds to DNA at the site of a mispaired base and is responsible for mismatch recognition. No activity has been assigned to MutL, although it interacts with MutS bound to a mispaired base and is required for activation of MutH. MutH is an endonuclease that nicks hemimethylated DNA on the unmethylated strand when activated by MutS and MutL in the presence of a mismatch, and this directs repair to the newly synthesized DNA strand.

The mismatch repair system in eukaryotes is more complex and involves several MutS and MutL homologs. The yeast Saccharomyces cerevisiae has six homologs of mutS (Reenan and Kolodner, 1992a, 1992b; New et al., 1993; Ross-Macdonald and Roeder, 1994; Hollingsworth et al., 1995; Marsischky et al., 1996) and four homologs of mutL (Kramer et al., 1989; Prolla et al., 1994a; Prolla, 1994; H. Florez-Rozas and R. D. K., unpublished data) although not all of these proteins appear to be involved in mismatch repair. Genetic and biochemical analysis of mismatch repair in S. cerevisiae has indicated that mismatch repair requires a MSH2–MSH3 complex and a MSH2–MSH6 (GTBP in humans) complex that both recognize mispaired bases in DNA (Marsischky et al., 1996), and a MLH1–PMS1 (PMS2 in humans) complex that interacts with MSH2 (Prolla et al., 1994b).

There is considerable evidence that the products of mismatch repair genes are also important in meiosis. S. cerevisiae PMS1, MLH1 and MSH2, and MSH4 and MSH5 are required for normal meiosis (Petes et al., 1991; Reenan and Kolodner, 1992b; Prolla et al., 1994a; Ross-Macdonald and Roeder, 1994; Hollingsworth et al., 1995; Williamson et al., 1985). No definitive meiotic role has been detected yet for MSH3 and MSH6 (Marsischky et al., 1996). Because of the presence of numerous MutS and MutL homologs in mammals (reviewed by Kolodner, 1996), it is important to determine their relative roles in mismatch repair systems in mitosis and meiosis, as well as cancer susceptibility. A strategy to accomplish this goal is to generate mice, by the use of gene targeting,

that are mutant in each of the genes and examine the resulting phenotype. Two groups generated mice with mutations in *msh2* (de Wind et al., 1995; Reitmair et al., 1995). Mice that are homozygous for the mutation breed normally and do not show any major phenotypic abnormalities. They are mismatch repair–defective as evidenced by a RER^+ phenotype, and a fraction of them develop B cell and T cell lymphomas early in their lives. Mice that carry a mutation in the *pms2* gene (Baker et al., 1995) have been developed. A fraction of these mice developed lymphomas or sarcomas during their first year of life and also show microsatellite instability. PMS2-deficient males are sterile while the females have normal reproductive capacity. To understand the role of mammalian MLH1, we generated mice that carry a null mutation in the *mlh1* gene. Unlike *msh2* and *pms2* mutants, the male as well as female *mlh1* mutant mice are sterile. The males do not produce any spermatozoa or spermatids and show a meiosis I arrest. Females produce oocytes and mate normally with wild-type males, but the oocytes fail to develop beyond the single-cell stage.

Results

Isolation of a Mouse Genomic Clone for *MLH1*

The isolation of human *MLH1* cDNA and its organization has been described by several groups (Bronner et al., 1994; Papadopoulos et al., 1994; Kolodner et al., 1995). The human cDNA was used to screen a mouse genomic λ phage library derived from the strain 129/Ola. One of these λ phages contained the 5′-region of the gene as determined by the presence of sequences homologous to exons 1, 2 , 3, and 4. To ensure that the genomic clone corresponds to *MLH1*, a 12 kb SacI fragment from the phage was subcloned and the location of the first four exons was determined by hybridization with polymerase chain reaction (PCR) products specific for each of the exons. Sequencing of these four exons and the regions flanking them revealed that the organization of this region of the mouse gene is identical to that of the human *MLH1* gene. Analysis of the sequence data indicated that the region encoded by the four mouse *mlh1* exons showed 89.5% and 97.6% identity at the DNA and protein level, respectively, with human MLH1. Thus, we conclude that the 12 kb SacI fragment contains the 5′-part of mouse *mlh1* gene. The mouse *mlh1* gene was previously mapped to chromosome 9 (Kolodner et al., 1994) to a region that is homologous to human 3p21, the location of human *MLH1* (Bronner et al., 1994; Papadopoulos et al., 1994). Northern blot analysis revealed a single ~3 kb transcript that is expressed in all mouse tissues tested (heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis) (unpublished data) consistent with *mlh1* being unique in the mouse genome. These results provide further confirmatory evidence for the identity of the mouse *mlh1* gene used in our studies.

Generation of Mice with a Disrupted *mlh1* Gene

The gene targeting scheme that we used to generate mouse embryonic stem (ES) cells containing the *mlh1* gene modification is shown in Figure 1. The gene targeting vector, designated pMLH1dex2, did not contain exon 2 of the *mlh1* gene and, if a transcript is made from the mutated gene and spliced from exon 1 to exon 3, it would contain an in-frame stop codon at the novel 5′-junction. If no splicing between exons 1 and 3 occurs, the chimeric transcript would contain multiple in-frame chain termination codons. Regardless of what type of mature transcript the mutated gene directs, a correct gene targeting event would yield a nonfunctional *mlh1* gene. pMLH1dex2 was linearized and used to transfect E14-1 ES cells. 544 G418 and gancyclovir resistant ES cell clones were isolated and screened for the desired recombination events by a PCR-based assay. We found that 3 of the 544 cell lines had the desired modification. The targeting event was verified by Southern blot analysis.

The three cell lines, MLH1-2011, MLH1-2106, and MLH1-3210, were injected into blastocysts derived from C57BL/6 females. All three cell lines yielded chimeric animals, and several of them derived from each of the three cell lines transmitted the ES cell genome through their germ line.

Homozygous *mlh1* Mutant Animals Are Viable

To ascertain the viability of *mlh1* mutant homozygotes, F1 animals that are heterozygous for the mutation were interbred and the offspring from several such matings were genotyped by Southern blot analysis. Representative results from this experiment are shown in Figure 1B. DNA was digested with EcoRI and hybridized with a probe that will detect both the wild-type and mutant alleles (Figure 1A). Hybridization with this probe revealed three classes of animals, some containing a 4.8 kb band corresponding to the wild type, some containing a 3.5 kb fragment corresponding to the mutant allele, and others containing both bands. Of 77 animals from 7 litters produced from founders derived from all 3 original disrupted cell lines, we obtained 24 +/+, 34 +/−, and 19 −/− animals, consistent with normal Mendelian segregation. These results clearly indicate that animals that are homozygous for deletion of exon 2 of the *mlh1* gene are viable.

mlh1 Mutant Mice Do Not Have MLH1 Protein

Based on the structure of the gene targeting construct, we expected that mice that are homozygous for the mutation would be unable to produce a functional MLH1 protein. To confirm this notion, protein extracts from 12-day-old mouse embryo–derived fibroblasts (MEF) were tested by Western blot analysis with an antibody against the human MLH1 protein (Figure 2). The antibody reacted with the expected 90 kDa protein species present in extracts of normal human lymphoblastoid cells, wild type, and *mlh1* +/− MEF. However, no such protein species was detectable in extracts prepared from *mlh1* −/− cells.

mlh1 −/− Cells Show a High Degree of Microsatellite Instability

The RER^+ phenotype, as manifested by microsatellite instability, is a hallmark of tumors in HNPCC patients,

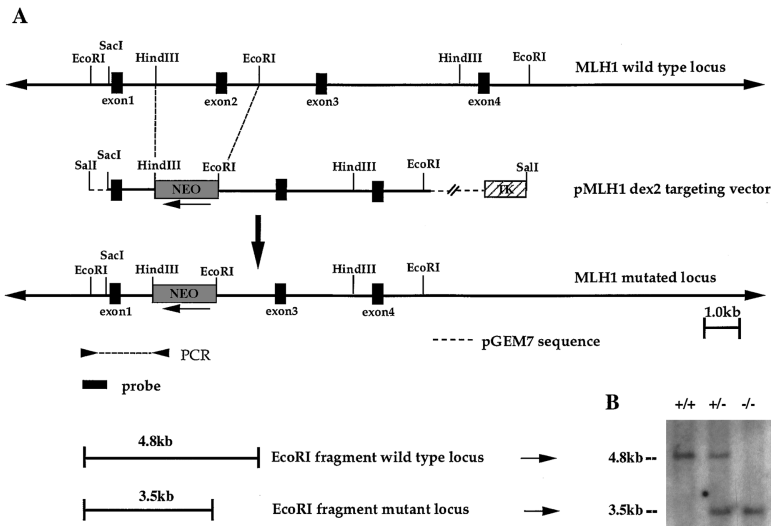

Figure 1. Targeted Disruption of the *mlh1* Gene

(A) The organization of the wild-type gene, the targeting construct, and the structure of the locus following gene targeting are shown. Exons are shown as filled boxes. The location of PCR primers for detecting gene targeting events, as well as the expected EcoRI digestion products that will be recognized by the probe, are also shown.

(B) Southern blot hybridization of DNA from mice from F_2 generation. DNA was digested with EcoRI and hybridized with probe shown in (A). The 4.8 kb band corresponds to wild type, and the 3.5 kb band corresponds to the mutant locus.

and this phenotype can result from the inactivation of MLH1-, PMS2- and MSH2-dependent mismatch repair. To ascertain if cells from *mlh1* −/− mice exhibit this phenotype, cellular DNA was prepared from 1.2 × 10^6 MEF that had undergone eight to ten cell divisions in culture. The DNA was diluted to a level where each aliquot contained one to three genome equivalents of DNA and used for PCR reactions with four different sets of PCR primers: D1Mit36, D7Mit91, D10Mit2, and D14Mit15 (Dietrich et al., 1994). The allele sizes of these markers are distinct in C57Bl/6 and 129/Ola strains of mice. Results from use of one of the four sets of primers are shown in Figure 3. With D1Mit36, we observed that 5 of 21 (24%) samples tested contained novel size alleles. Similar results were obtained with the other three markers. Taken together, 10%–25% of the samples had novel alleles at each of the four loci tested. These results indicate that mismatch repair, as determined by microsatellite instability, is severely impaired in the somatic cells of *mlh1* −/− mice.

Extracts from *mlh1* −/− Cells Show Impaired Mismatch Repair Activity

To assess if the RER^+ phenotype is the result of impaired mismatch repair activity, we prepared cell-free extracts from MEF and assayed them for mismatch repair activity in vitro. The results are presented in Figure 4. G–G mismatches were repaired efficiently by HeLa cell extracts, wild-type (+/+) embryo fibroblast extracts (34%), and, to a lesser extent, in an extract of +/− cells (15%). In contrast, *mlh1* −/− embryo fibroblast extracts were not significantly different from those observed with the unincubated control, i.e., this extract was repair-deficient. Similar results were obtained using a G–T heteroduplex (data not shown). These data clearly show that lack of MLH1 protein results in impaired mismatch repair activity.

mlh1 −/− Mice Have Impaired Meiosis in the Male Germ Line

Li and Modrich (1995) have shown that human MLH1 and PMS2 (PMS1 in S. cerevisiae) form a functional heterodimer that appears to be the equivalent of MutL in bacteria. Baker et al. (1995) generated mice deficient in PMS2 and showed that *pms2* −/− males are sterile. This observation prompted us to examine the *mlh1* −/− males. Homozygous mutant males exhibited normal sexual behavior towards female mice, and their mating frequency, as assessed by the presence of vaginal plugs, was identical to that of wild-type males. However, the matings with mutant males did not result in any pregnancies, while all females possessing vaginal plugs after mating with wild-type males were found to be pregnant on day 5 (as assessed by the uterine extravascular accumulation of pontamine blue dye). Furthermore, epididymal extrusion revealed a complete absence of spermatozoa in the *mlh1* −/− males, while wild-type males

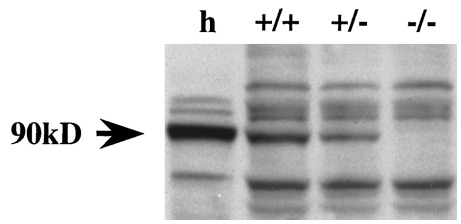

Figure 2. Detection of MLH1 Protein by Western Blot Analysis

h, extract from a normal human lymphoblastoid cell line; +/+, wild type; +/−, heterozygote; and −/−, homozygote.

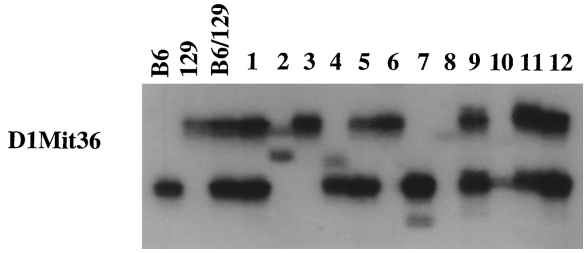

Figure 3. Microsatellite Instability in *mlh1* −/− -Derived MEF

B6, C57Bl/6; 129, 129/Ola; B6/129, F1 animal; 1–12, individual samples of DNA. Note abnormal size bands in lanes 2, 4, and 7.

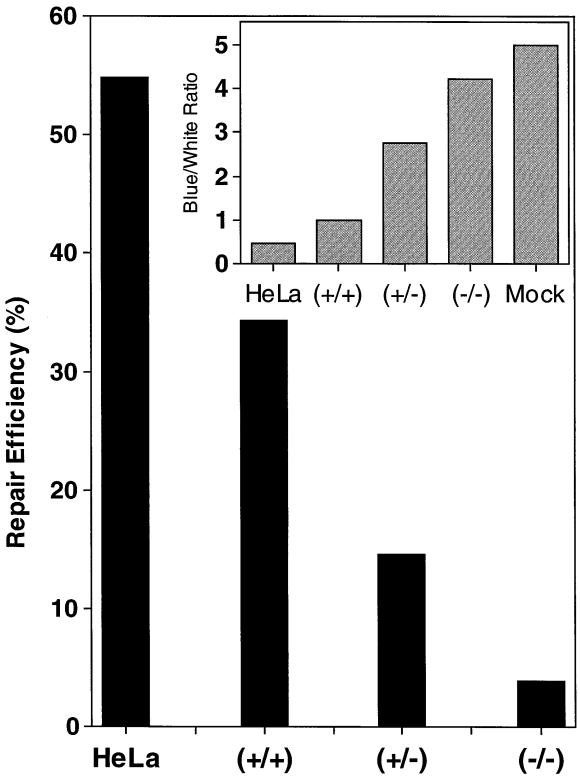

Figure 4. Mismatch Repair Activity in +/+, +/*mlh1*$^-$, and *mlh1*$^-$/*mlh1*$^-$ Embryo Fibroblasts

The analysis was performed as described in Experimental Procedures using a M13mp2 DNA substrate containing a nick in the minus strand at position 276 (where position 1 is the first transcribed nucleotide of the *lacZ* gene) and a G–G mismatch at position 88. Results are expressed as percent repair determined from counting several hundred plaques per variable. Similar results (data not shown) were obtained when the analysis was repeated. Inset: ratio of blue to white plaques.

contained $2.48 \times 10^7 \pm 5.65 \times 10^5$ sperm per pair of epididymides.

Further detailed analysis of the testes of *mlh1* −/− males revealed that they are approximately half the size of those of normal males. Transverse and sagittal sections of the testes showed profoundly abnormal spermatogenesis (Figure 5). The testicular parenchyma showed a slight reduction in the size of the seminiferous tubules. All tubules showed a basal single layer of spermatogonia. Most of the tubules contained Sertoli cells along with two to five layers of primary spermatocytes at the leptotene to pachytene stage, but no spermatocytes beyond this stage were observed. The remaining tubules showed a germinal epithelium of reduced thickness composed of primary spermatocytes undergoing nuclear condensation, coarse chromatin clumping, karyorexis, and pycnosis. Eosinophilic staining of the spermatocyte cytoplasm indicated a progressive degeneration and apparent apoptosis (Figure 5). Abnormal meiotic figures were often observed, as were luminal giant cells and syncytial rounded elements. No mature spermatozoa were present in the lumen of the seminiferous tubules. The testicular interstitium was of increased thickness and contained increased numbers of Leydig cells, fibroblasts, and collagen. The vas deferens was normal. These results suggested that MLH1-deficient males have abnormal meiosis.

To understand further the stage at which the meiosis is blocked in *mlh1* −/− mice, meiotic chromosome preparations were made from isolated spermatocytes. The preparations from wild-type littermates exhibited all stages of normal meiosis with the normal preponderance of leptotene–pachytene stage spermatocytes. The mutant mice had an over abundance of leptotene–pachytene stage cells with a conspicuous absence of post-pachytene stages. Even the cells that contained early stage meiotic figures were abnormal, characterized by chromatin despiralization. To confirm these observations, primary spermatocytes were isolated, gently lysed, and chromosomes stained with silver nitrate and examined by electron microscopy (EM). No stages beyond pachytene were observed in preparations from *mlh1* −/− animals. The chromosomes in pachytene showed normal pairing (Figures 5E–5F). The overall picture was that of meiotic arrest post-synapsis but prior to chiasma formation. This is unlike the *pms2* mutant mice, where abnormal pairing and subsequent development of abnormal sperm was seen. Taken together, the histology and the cytogenetic and EM examination revealed that *mlh1* −/− mice failed to produce spermatids and spermatozoa and the spermatocytes fail to progress normally beyond the pachytene stage of meiosis. To understand the mechanism of the progressive degeneration of spermatocytes, we used the TdT-mediated dUTP–biotin nick end labeling (TUNEL) assay to determine if the cells become apoptotic. Results from these experiments (Figure 6) show a preponderance of luminal spermatocytes undergoing apoptotic cell death. Although the *mlh1* −/− mice share their male sterility phenotype with *pms2* −/− mice (Baker et al., 1995), the cause of sterility in the two types of mice seems to be very different.

mlh1 −/− Females Are Also Sterile

We ascertained the reproductive behavior of females that are MLH1-deficient. *mlh1* −/− females undergo normal estrous cycles and are receptive to mating at estrus. However, these mice fail to become pregnant, and no implantation sites were visible on day 5 of pregnancy. In contrast, 75% of plugged wild-type females became pregnant, and possessed viable implantation sites on day 5 of pregnancy. Ovaries from −/− animals were indistinguishable from those of wild-type animals. Sections of ovaries (Figure 7) contained follicles at all stages of development as well as corpora lutea, indicating that ovaries from *mlh1* −/− females are capable of follicular development and ovulation.

To ascertain further the basis for female sterility, we superovulated *mlh1* −/− and wild-type females and examined them at different times post-insemination. The results are presented in Table 1. The mutant mice superovulated with the same efficiency as wild-type females, with 77 day one oocytes being recovered from two wild-type females and 75 from two *mlh1* −/− females. At 22 hr post-insemination, 71.9% of the eggs from wild-type animals progressed to the two-cell stage while only 13.6% of the eggs from mutant animals showed a similar progression. At 32 hr post-insemination, only 20% of

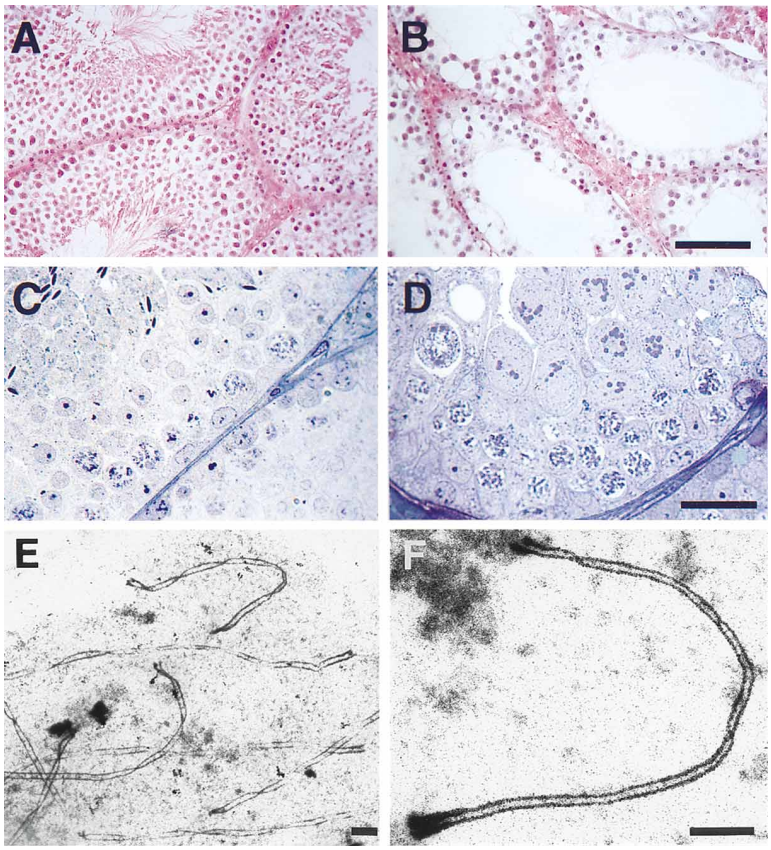

Figure 5. Histological Examination of *mlh1* −/− Male Testes

(A)–(D) Cross sections of testes from *mlh1* +/+ (A and C) and *mlh1* −/− (B and D) males. (A) and (B) Sections stained with H and E, showing the gross morphological appearance of seminiferous tubules. Note the absence of round and elongating spermatids and mature spermatozoa in the tubules of *mlh1* −/− males, as well as the relative increase in interstitial tissue around the seminiferous tubules (scale bar, 500 μm).

(C) and (D) Sections stained with toluidene blue, showing more detailed nuclear structures (scale bar, 125 μm). In (D), note the large numbers of pachytene spermatocytes close to the basement membrane (bottom) in the seminiferous tubules and increasing nuclear fragmentation approaching the lumen (top). Also note the absence of mature sperm heads in *mlh1* −/− testes, which are clearly visible in wild-type controls (C).

(E) and (F) Pachytene chromosome spreads from *mlh1* −/− mice (scale bars, 1 μm for [E] and [F]).

the eggs from the wild-type animals were at the one-cell stage or were abnormal while 75% of the eggs from the mutant animals were at the one-cell stage or were abnormal. The eggs from the mutant animals had one polar body, and we never observed the formation of a second polar body during the 32 hr over which meiotic progression was studied, suggesting that the eggs never completed meiosis II. These results show that the absence of the MLH1 protein causes both male and female sterility as a result of the absence of viable gametes in both sexes. While it is more difficult to pinpoint the stage at which oogenesis is disrupted in the mutant females, it is possible that female ovarian meiosis is impaired at the same stage as in the testis. The phenotype of *mlh1* −/− females is also distinctly different from that of *pms2* −/− females, which exhibit normal fertility.

Discussion

To examine the function of the mismatch repair gene *mlh1* in normal growth and development, we generated

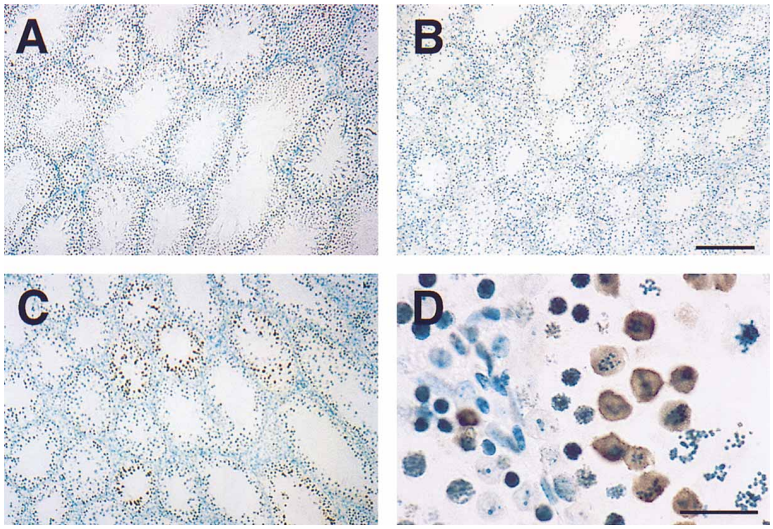

Figure 6. Detection of Apoptosis

In situ labeling of apoptotic cells in the seminiferous tubules of *mlh1* +/+ (A) and *mlh1* −/− (B–D) males. Note the luminal distribution of apoptotic (dark brown) cells in the seminiferous tubules of *mlh1* −/− males (C) as opposed to the lack of such cells in *mlh1* +/+ controls (A) and in the negative control (B). (D) shows a higher magnification of a seminiferous tubule from a homozygous mutant male showing numerous apoptotic cells and cellular fragments in the layers closest to the lumen (scale bars, 1000 μm for [A]–[C] and 125 μm for [D]).

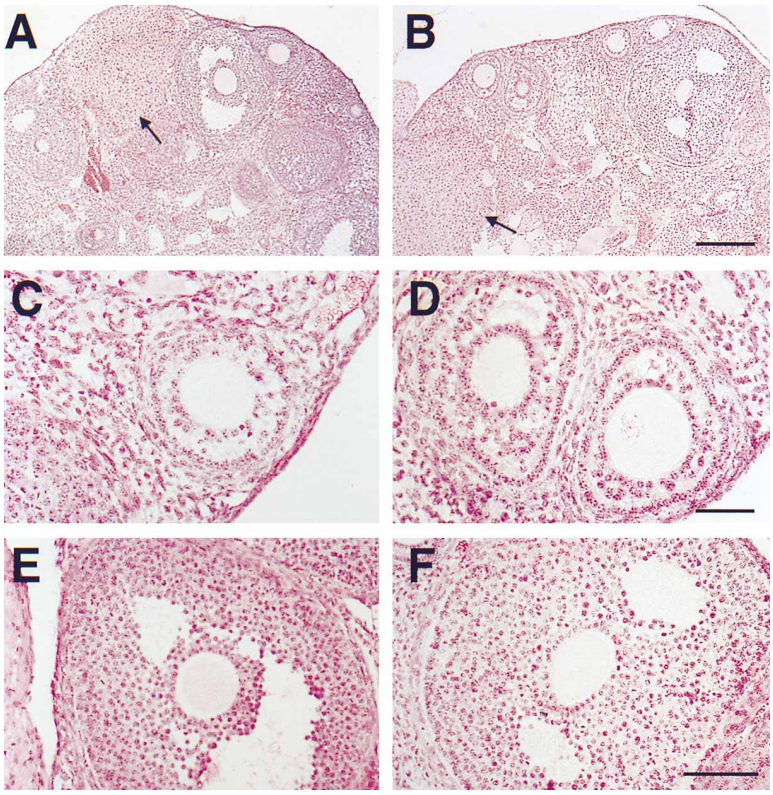

Figure 7. Histological Examination of Ovaries

H and E staining of ovaries from *mlh1* +/+ (A, C, and E) and *mlh1* −/− (B, D, and F) females.

(A) and (B) Overview of ovarian morphology showing the presence of oocytes from all developmental stages, along with corpora lutea (arrows) in both genotypes (scale bar, 1000 μm).

(C) and (D) Higher magnification of developing pre-antral follicles containing immature oocytes (scale bar, 250 μm).

(E) and (F) Higher magnification of antral follicles containing mature oocytes, well developed granulosa cell layers, and large antral cavities (scale bar, 500 μm).

mice that carry a null mutation in the *mlh1* gene. Evidence in support of the view that we targeted the appropriate gene was obtained from nucleotide sequence, map location, and Northern blot analysis. Western blot analysis of proteins obtained from embryo-derived fibroblasts revealed that the wild-type and heterozygous mice contained the MLH1 protein, but no MLH1 protein was detectable in extracts from the homozygous mutant animals. These results indicate that *mlh1* is unique in the mouse genome and that the *mlh1* gene has been inactivated in the ES cell lines and mutant mice constructed here.

mlh1 −/− Mice Are Mismatch Repair–Defective

We have shown that somatic cells from mutant mice are mismatch repair–defective by the microsatellite instability assay as well as by a direct test of mismatch repair. Tumors from HNPCC patients, or cell lines derived from them, exhibit microsatellite instability when tested at a single locus. As many as 4.4%–11% of the cells were estimated to show abnormalities (Parsons et al., 1993). In our assay, using four different microsatellite markers, we observed as much as 25% instability. We also utilized a biochemical assay to measure the mismatch repair proficiency of extracts prepared from early passage MEFs. The level of mismatch repair activity in extracts from *mlh1* +/− cells was 44.1% and from *mlh1* −/− cells was 11.8% of that observed with extracts of wild-type cells. These results clearly show that MLH1 is required for normal mismatch repair and the RER^+ phenotype we observed is the result of the lack of mismatch repair proficiency.

Table 1. Post-Insemination Oocyte Progression Following Fertilization In Vitro

Post Insemination (hours)	Nature of Oocyte	Genotype +/+	Genotype −/−
0	Abnormal eggs	4.6% (3/65)	29.5% (13/44)
7.5	1 male pronucleus	83.9% (47/56)	35.3% (12/34)
	>1 male pronucleus	0% (0/56)	32.3% (11/34)
	Unfertilized	16.1% (9/56)	32.4% (11/34)
22	2-cell stage	71.9% (23/32)	13.6% (3/22)
	1-cell stage	18.8% (6/32)	50% (11/22)
	Abnormal	9.4% (3/32)	36.4% (8/22)
32	4–8-cell stage	65% (13/20)	0% (0/12)
	2-cell stage	15% (3/20)	25% (3/12)
	1-cell stage	15% (3/20)	33.3% (4/12)
	Abnormal	5% (1/20)	41.7% (5/12)

Meiotic Abnormalities in the Mutant Mice

The most dramatic phenotypes of the MLH1-deficient mice are that the mutant males and females are sterile. The males appear normal and have normal reproductive behavior in that they mate regularly with females and vaginal plugs were detectable post-mating. The testes are smaller than those of wild-type mice, and we did not observe any mature spermatozoa in the caudal epididymis or in the seminiferous tubules. The spermatocytes failed to proceed beyond the pachytene stage. The presence of Sertoli cells in the seminiferous tubules suggests that the intratubular environment is normal. Chromosomal pairing was normal, as determined by light and electron microscopy. The failure of the spermatocytes to go beyond the pachytene stage, eventually leading to apoptosis, suggests that normal MLH1 function is critical for normal meiotic progression. In this regard, it is of interest to note that mice that are deficient in PMS2 (Baker et al., 1995) also exhibit male sterility and abnormal chromosome pairing, but there are distinct differences in meiotic progression in these two types of mice. In *pms2* −/− males, the spermatocytes complete meiosis and mature spermatozoa with heads and tails are observed, although the heads were reported to be abnormal. No spermatozoa or spermatids were observed in the seminiferous tubules of MLH1-deficient males. Since the *mlh1* mice and the *pms2* mice have null mutations in the respective genes, these results suggest that MLH1 and PMS2 have distinct functions in male meiosis. The fact that male meiosis is arrested in the prophase of meiosis I in MLH1-deficient mice suggests that the MLH1 protein is critical at an earlier stage of meiosis than PMS2. This also might explain the differences in the fertility of *mlh1* and *pms2* mutant females (see below).

The observation that the arrest of the spermatocytes at pachytene is coupled to apoptosis suggests that in mouse male meiosis there is a checkpoint arrest similar to that known in mitosis and that cells that are arrested become slated for apoptosis. A role for mismatch repair proteins in mitotic checkpoint arrest was postulated in humans (Hawn et al., 1995) and suggested that mismatch repair proteins could be a sensor of DNA damage during the mitotic cell cycle. In our mice, a mismatch repair defect leads to meiotic arrest. These results suggest that the meiotic and mitotic checkpoints are different and that in *mlh1* mutant animals some type of aberrant product of meiotic recombination, most likely the failure to repair heteroduplexes, is likely to trigger arrest. This type of meiotic checkpoint has been suggested from the analysis of S. cerevisiae mutants that affect meiotic recombination (Bishop et al., 1992).

Female Sterility in MLH1-Deficient Mice

Unlike the PMS2-deficient mice that show normal levels of fertility, the *mlh1* −/− females are completely sterile. The fact that the mice enter estrous indicate that there are no hormonal imbalances in these animals and the females mate normally. Examination of the ovaries show normal structure with all stages of oogenesis and corpora lutea being present. The mice can be superovulated to levels comparable with those seen in wild-type females, again suggesting normal oocyte development. Because the oocytes normally are arrested at the pachytene stage of meiosis, the failure of the fertilized embryos to proceed beyond the single-cell stage is consistent with an arrest in meiosis I, as seen in the mutant male mice. This is corroborated further by the fact that we have not observed any eggs with two polar bodies, even 32 hr post-insemination of mutant animals.

Roles of MSH2, MLH1, and PMS2

In bacteria, both *mutS* and *mutL* mutations cause hypermutability and mismatch repair deficiency. With the exception of MSH3 and MSH6, which are partially redundant, all of the eukaryotic mismatch repair proteins appear to be of similar importance in mitotic mismatch repair, and MLH1, PMS1, and MSH2 appear to be of similar importance in meiotic mismatch repair in S. cerevisiae. (Williamson et al., 1985; Petes et al., 1991; Reenan and Kolodner, 1992b; Prolla et al., 1994a; Li and Modrich, 1995; Drummond et al., 1995; Boyer et al., 1995; Risinger et al., 1995; Kolodner, 1996). However, there are several observations that suggest that MSH2, MLH1, and PMS1 (PMS2 in humans) may not be entirely equivalent during mitotic growth: PMS1 and MSH2 do not appear to be of equal importance in suppressing mitotic recombination between divergent DNA sequences in S. cerevisiae (Datta et al., 1996); and mutations in MSH2 and MLH1 appear to be equally prevalent in HNPCC families, whereas mutations in PMS2 (PMS1 in S. cerevisiae) appear to be much more rare (Liu et al., 1996).

A different picture is emerging for the role of these proteins in mammalian meiosis. Mice with mutations in *msh2*, *pms2*, and *mlh1* have very similar somatic phenotypes. They have a RER^+ phenotype and a fraction of the *msh2* and *pms2* homozygous mice develop lymphomas. The MLH1-deficient mice also show a RER^+ phenotype, and our mice are not yet old enough for us to determine their tumor predisposition. The meiotic phenotypes of the three classes of mice are distinctly different. The *msh2* −/− mice have normal fertility and *pms2* mice have male infertility with normal female fertility, while MLH1-deficient males and females are sterile. These results suggest that MSH2 may not play a critical role in meiotic mismatch repair. If complexes involving MSH2, GTBP, MLH1, PMS2, and possibly MSH3 are necessary for mitotic mismatch repair, the observations from the gene-targeted mice suggest that MLH1 and PMS2 may function in a meiotic process, such as chromosome pairing or mismatch repair, that does not require MSH2. Studies in S. cerevisiae have indicated that PMS1 and MSH2 do not appear to be of equal importance in suppressing meiotic recombination between divergent DNA sequences (Hunter et al., 1996). Given the known interactions between the MutL-related and the MutS-related proteins, it is conceivable that a meiosis-specific function for MLH1 observed here could involve the action of one or more MutS homologs other than MSH2. Indeed, other homologs of MutS have been identified in eukaryotes (e.g., MSH3, GTBP [MSH6 in S. cerevisiae], MSH4, and MSH5); however, additional investigations are necessary to assess if any of these MSH proteins or other yet to be discovered proteins are critical for mammalian

meiosis. Possibly the most logical candidates for such MSH proteins are the homologs of S. cerevisiae, MSH4 and MSH5, since these proteins are required for meiotic crossing over and proper segregation of chromosomes during meiosis I. Expression studies of these different *MSH* and *MLH* genes and analysis of mice harboring mutations in each of these genes would help clarify their roles in meiosis. We presently are constructing mice containing mutations in other *msh* genes to test this idea.

Experimental Procedures

Construction of the MLH1dex2 Targeting Vector

A genomic *MLH1* fragment containing the first four exons was obtained by screening a mouse genomic Charron 35, 129/Ola phage library with a full-length human *MLH1* cDNA probe (Bronner et al., 1994). A 12 kb SacI fragment was subcloned into pcDNAII (Invitrogen) and the intron–exon structure of part of the gene was determined using sequencing and restriction mapping (Figure 1). A 1.3 kb SacI–HindIII fragment containing exon 1 was cloned into the SacI–HindIII sites of PGK*neo*. A 6 kb EcoRI fragment containing exons 3 and 4 was cloned into the EcoRI site of the resulting subclone. Finally, a 1.7 kb XhoI–BamHI fragment from pMC1tk (Mansour et al., 1988) was cloned into the single SacI restriction site, treated with T4 DNA polymerase. The resulting gene targeting clone was designated pMLH1dex2.

Electroporation of Embryonic Stem Cells

The targeting vector pMLH1dex2 (30 μg) was linearized at the single SalI site and electroporated into 2.5×10^7 E14-1 ES cells and selected with G418 and gancyclovir as described previously (Sirotkin et al., 1995). Colonies were picked after 10 days and their DNA was screened by PCR using forward primer 5′-*GGCATTCATGCTGCCCA ATC*-3′ and reverse primer 5′-*TGTCAATAGGCTGCCCTAGG*-3′. The reaction was performed in a 50 μl reaction mixture containing 100 ng of DNA, 5 ng/ml each primer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, and 0.5 U Taq polymerase. Cycling conditions were as follows: 5 min at 94°C (1 cycle), 1 min at 94°C, 1 min at 56°C, 1 min at 72°C (40 cycles), 5 min at 72°C (1 cycle). Positive ES cell colonies were identified by a 1.6 kb PCR fragment specific for the targeting event.

Generation of MLH1-Deficient Mice

Chimeric mice were generated by injecting C57Bl/6 blastocysts with 8–12 ES cells derived from the MLH1-2011, MLH1-3210, and MLH1-2106 colonies. All three cell lines gave rise to male chimeric animals that were mated with C57Bl/6 females. Chimeras obtained from all three cell lines transmitted the MLH1dex2 mutation through the germ line. F1 heterozygotes were interbred to obtain homozygous MLH1dex2 mutant animals.

Isolation of Mouse Embryonic Fibroblasts

Day 12 embryos were minced and incubated in 2 ml of 0.25% trypsin at 4°C overnight. After an additional incubation at 37°C for 20 min, a 5× excess of Dulbecco's modified Eagle's medium was added and the suspensions were triturated. The cells were gently pelleted, resuspended in medium, and a portion was used for genotyping with the rest plated for growth.

Western Blot Analysis

For Western blot analysis, equal amounts of protein from cultured cell extracts were separated on a 10% SDS–polyacrylamide gel and transferred onto an Immobilon-P (Millipore) membrane. The membrane was blocked in Tris-buffered saline (TBS), 0.1% Tween-20, 5% nonfat dry milk, 10% donkey serum (Sigma), and incubated with 3 mg/ml primary antibody in TBS, MLH1(Ab-2) (Oncogene Science). Bound protein was detected by chemiluminescence, using a donkey anti-rabbit IgG horseradish peroxidase conjugate (Amersham).

Microsatellite Instability in Mouse Embryonic Fibroblasts

DNA was extracted from a total of 1.2×10^6 MEF (eight to ten cell divisions), diluted to 1–2.5 cellular equivalents, and subjected to PCR (Parsons et al., 1995). Four end-labeled primer pairs were used: D1Mit36, D7Mit91, D10Mit2, and D14Mit15 (Dietrich et al., 1994). Amplified PCR products were separated on a denaturing polyacrylamide gel and autoradiographed for analysis.

Cell-Free Extracts and Mismatch Repair Assay

Cell-free extracts were prepared from logarithmically growing cultures as described (Thomas et al., 1995). Procedures for measuring mismatch repair have been described (Thomas et al., 1991, 1995). The reaction products were processed and introduced into E. coli NR9162 (mutS) and plaque colors were determined. Repair efficiency is expressed in percent as 100 × (1 minus the ratio of the percentages of mixed bursts obtained from extract-treated and untreated samples).

Examination of Reproductive Performance

Females

Eight- and ten-week-old female +/+ and −/− mice were used for the study. The ability of these females to undergo normal estrous cycles was assessed by taking vaginal smear samples daily for up to four complete cycles. Mating with wild-type males was assessed by the presence of a vaginal plug (day 1). Pregnancy was confirmed on day 5 by the uterine extravascular accumulation of pontamine blue dye at the implantation sites (Psychoyos, 1961).

For in vitro fertilization studies, females were superovulated with 5 IU PMS (i.p.) followed, 48 hr later, by 3 IU hCG (i.p.). Eggs were removed the following morning and fertilized with sperm from wild-type males. The fertilized oocytes were incubated in KSOM media at 37°C for up to 32 hr post-insemination and examined for developmental progression.

Males

Males were used between 8 and 10 weeks of age. The mating behavior of these males was analyzed as described (Cohen et al., 1996), by placing each male with a receptive female (C57Bl/6, 8 weeks of age) each night for 4 consecutive nights. For epididymal sperm assessment, epididymides were removed and their contents extruded into 1 ml of modified Tyrode's medium containing bovine serum albumin (4 mg/ml) overlaid with sterile mineral oil (Fraser, 1983). The sperm were allowed to disperse into the medium for 15 min at 34°C, and sperm concentrations were assessed with the aid of a hemocytometer.

Examination of Spermatogenesis

Male testes were dissected and fixed in buffered 10% formalin for 18 hr, progressively dehydrated with ethanol and embedded in paraffin. Four-μm thick deparaffinized sections were stained with hematoxylin-eosin (H and E). Meiotic chromosome preparations for light microscopy were obtained following a slight modification of previously described methods (Chaganti et al., 1980).

Histology and Electron Microscopy

Ovaries and testes were prepared for histological analysis by conventional methods. For electron microscopy, chromosome spreads were prepared and silver-stained by previously described methods (Counce and Meyer, 1973; Goodpasture and Bloom, 1975). In situ labeling of apoptotic cells was performed on 5-μm tissue sections using the ApoTag Plus kit (Oncor, Incorporated, Gaithersburg, MD), according to the instructions of the manufacturer.

Acknowledgments

This work is supported by grants from the National Institutes of Health (CA67944 to R. K., GM50006 and CA67151 to R. D. K., HD/AI30280 to J. W. P.), American Cancer Society grant CN132 to R. K., and a Center grant, CA13330, to the Albert Einstein College of Medicine. J. W. P. is a Monique Weill-Caulier Scholar. We acknowledge the assistance of Harry Hou, Jr. for blastocyst injections, Frank Macaluso, Leslie Gunther, and William Schubert in the preparation

of EM sections, Liyin Zhu for technical assistance, and Vivian Gradus for manuscript preparation.

Received May 6, 1996; revised June 4, 1996.

References

Aaltonen, L.A., Peltomaki, P., Leach, F., Sistonen, P., Pylkkanen, S.M., Mecklin, J.-P., Jarvinen, H., Powell, S., Jen, J., Hamilton, S.R., Petersen, G.M., Kinzler, K.W., Vogelstein, B., and de la Chapelle, A. (1993). Clues to the pathogenesis of familial colorectal cancer. Science *260*, 812–816.

Aaltonen, L.A., Peltomaki, P., Mecklin, J.P., Jarvinen, H., Jass, J.R., Green, J.S., Lynch, H.T., Watson, P., Tallqvist, G., Juhola, M., Sistonen, P., Hamilton, S.R., Kinzler, K.W., Vogelstein, B., and de la Chapelle, A. (1994). Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients. Cancer Res. *54*, 1645–1648.

Baker, S.M., Bronner, C.E., Zhang, I., Plug, A., Robatzek, M., Warren, G., Elliott, E.A., Yu, J., Ashley, T., Arnheim, N., Flavell, R.A., and Liskay, R.M. (1995). Male mice defective in the DNA mismatch repair gene *PMS2* exhibit abnormal chromosome synapsis in meiosis. Cell *82*, 309–320.

Bishop, D.K., Park, D., Xu, L., and Kleckner, N. (1992). *DMC1*: a meiosis-specific yeast homolog of E. coli *recA* required for recombination, synaptonemal complex formation, and cell cycle progression. Cell *69*, 439–456.

Boyer, J.C., Umar, A., Risinger, J., Kane, M.F., Lipford, J., Barrett, J.C., Kolodner, R.D., and Kunkel, T.A. (1995). Microsatellite instability, mismatch repair deficiency and genetic defects in human cancer cell lines. Cancer Res. *55,* 6062–6070.

Bronner, C.E., Baker, S.M., Morrison, P.T., Warren, G., Smith, L.G., Lescoe, M.K., Kane, M., Earabino, C., Lipford, J., Lindblom, A., Tannergard, P., Bollag, R.J., Godwin, A.R., Ward, D.C., Nordenskjold, M., Fishel, R., Kolodner, R., and Liskay, R.M. (1994). Mutation in the DNA mismatch repair gene homologue *hMLH1* is associated with hereditary nonpolyposis colon cancer. Nature *368*, 258–261.

Chaganti, R.S., Jhanwar, S.C., Ehrenbard, L.T., Kourides, I.A., and Williams, J.J. (1980). Genetically determined asynapsis, spermatogenic degeneration, and infertility in men. Am. J. Hum. Genet. *32*, 833–848.

Cohen, P.E., Chisholm, O., Arceci, R.J., Stanley, E.R., and Pollard, J.W. (1996). Absence of colony stimulating factor-1 in osteopetrotic (*csfm^{op}/csfm^{op})* mice results in male fertility defects. Biol. Reprod. *55*, in press.

Counce, S.J., and Meyer, G.F. (1973). Differentiation of the synaptonemal complex and the kinetochore in *Locusta* spermatocytes studied by whole mount electron microscopy. Chromosome *44*, 231–253.

Datta, A., Adjiri, A., New, L., Crouse, G.F., and Jinks-Robertson, S. (1996). Crossovers between diverged sequences are regulated by mismatch repair proteins in yeast. Mol. Cell. Biol. *16*, 1085–1093.

de Wind, N., Dekker, M., Berns, A., Radman, M., and te Riele, H. (1995). Inactivation of the mouse *Msh2* gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. Cell *82*, 321–330.

Dietrich, W.F., Miller, J.C., Steen, R.G., Merchant, M., Damron, D., Nahf, R., Gross, A., Joyce, D.C., Wessel, M., Dredge, R.D., Marquis, A., Stein, L.D., Goodman, N., Page, D.C., and Lander, E.C. (1994). A genetic map of the mouse with 4,006 simple sequence length polymorphisms. Nature Genet. *7*, 220–245.

Drummond, J.T., Li, G.M., Longley, M.J., and Modrich, P. (1995). Isolation of an hMSH2-p160 heterodimer that restores DNA mismatch repair to tumor cells. Science *268*, 1909–1912.

Fishel, R.A., Lescoe, M.K., Rao, M.R.S., Copland, N., Jenkins, N., Garber, J., Kane, M., and Kolodner, R. (1993). The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. Cell *75*, 1027–1038.

Fraser, L.R. (1983). Mouse sperm capacitation assessed by kinetics and morphology of fertilization in vitro. J. Reprod. Fertil. *69*, 419–428.

Goodpasture, C., and Bloom, S.E. (1975). Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. Chromosoma *53*, 37–50.

Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M., Sargeant, L., Krapcho, K., Wolff, E., Burt, R., Hughes, J.P., Warrington, J., McPherson, P., Wasmuth, J., LePaslier, D., Abderrahim, H., Cohen, D., Leppert, M., and White, R. (1991). Identification and characterization of the familial adenomatous polyposis coli gene. Cell *66*, 589–600.

Hawn, M.T., Umar, A., Carethers, J.M., Marra, G., Kunkel, T.A., Boland, C.R., and Koi, M. (1995). Evidence for a connection between the mismatch repair system and the G2 cell cycle checkpoint. Cancer Research *55*, 3721–3725.

Hollingsworth, N.M., Ponte, L., and Halsey, C. (1995). *MSH5*, a novel MutS homolog, facilitates meiotic reciprocal recombination between homologs in *Saccharomyces cerevisiae* but not mismatch repair. Genes Dev. *9*, 1728–1739.

Hunter, N., Chambers, S.R., Lewis, E.J., and Borts, R.H. 1996. The mismatch repair system contributes to meiotic sterility in an interspecific yeast hybrid. EMBO J. *15*, 1726–1733.

Joslyn, G., Carlson, M., Thliveris, A., Albertsen, H., Gelbert, L., Samowitz, W., Groden, J., Stevens, J., Spirio, L., Robertson, M., Sargeant, L., Krapcho, K., Wolff, E., Burt, R., Hughes, J.P., Warrington, J., McPherson, P., Wasmuth, J., LePaslier, D., Abderrahim, H., Cohen, D., Leppert, M. and White R. (1991). Identification of deletion mutations and three new genes at the familial polyposis locus. Cell *66*, 601–613.

Kinzler, K.W., Nilbert, M.C., Su, L.K., Vogelstein, B., Bryan, T.M., Levy, D.B., Smith, K.J., Preisinger, A.C., Hedge, P., McKechnie, D., Finniear, R., Markham, A., Groffen, J., Boguski, M.S., Altschul, S.F., Horii, A., Ando, H., Miyoshi, Y., Miki, Y., Nishisho, I., and Nakamura Y. (1991). Identification of FAP locus genes from chromosome 5q21. Science *253*, 661–665.

Kolodner, R. (1996). Biochemistry and genetics of Eukaryotic mismatch repair. Genes Dev. *10*, 1433–1442.

Kolodner, R.D., Hall, N.R., Lipford, J.R., Kane, M.F., Rao, M.R.S., Morrison, P., Wirth, L., Finan, P.J., Burn, J., Chapman, P., Earabino, C., Merchant, E., Bishop, D.T., Garber, J., Bronner, C.E., Baker, S.M., Warren, G., Smith, L.G., Lindblom, A., Tannergard, P., Bollag, R.J., Godwin, A.R., Ward, D.C., Nordenskjold, M., Liskay, R.M., Copeland, N., Jenkins, N., Lescoe, M.K., Ewel, A., Lee, S., Griffith, J., and Fishel, R. (1994). The human mismatch repair genes and their association with hereditary non-polyposis colon cancer. Cold Spring Harbor Symp. Quant. Biol. *59*, 331–338.

Kolodner, R.D., Hall, N.R., Lipford, J.R., Kane, M.F., Morrison, P., Finan, P.J., Burn, J., Chapman, P., Earabino, C., Merchant, E., and Bishop, D.T. (1995). Structure of the human MLH1 locus and analysis of HNPCC kindreds for mlh1 mutations. Cancer Res. *55*, 242–248.

Kramer, W., Kramer, B., Williamson, M.S., and Fogel, S. (1989). Cloning and nucleotide sequence of DNA mismatch repair gene *PMS1* from *Saccharomyces cerevisiae*: homology of PMS1 to procaryotic MutL and HexB. J. Bacteriol. *171*, 5339–5346.

Leach, F.S., Nicolaides, N.C., Papadopoulos, N., Liu, B., Jen, J., Parsons, R., Peltomaki, P., Sistonen, P., Aaltonen, L.A., Nystrom-Lahti, M., Guan, X.-Y., Zhang, J., Meltzer, P.S., Yu, J.-W., Kao, F.-T., Chen, D.J., Cerosaletti, K.M., Fournier, R.E. K., Todd, S., Lewis, T., Leach, R.J., Naylor, S.L., Weissenbach, J., Mecklin, J.-P., Jarvinen, H., Petersen, G.M., Hamilton, S.R., Green, J., Jass, J., Watson, P., Lynch, H.T., Trent, J.M., de la Chapelle, A., Kinsler, K.W., and Vogelstein, B. (1993). Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. Cell *75*, 1215–1225.

Li, G.-M., and Modrich, P. (1995). Restoration of mismatch repair to nuclear extracts of H6 colorectal tumor cells by a heterodimer of human MutL homologs. Proc. Natl. Acad. Sci. USA *92*, 1950–1954.

Lindblom, A., Tannergard, P., Werelius, B., and Nordenskjold, M. (1993). Genetic mapping of a second locus predisposing to hereditary non-polyposis colon cancer. Nature Genet. *5*, 279–282.

Liu, B., Parsons, R.E., Hamilton, S.R., Petersen, G.M., Lynch, H.T., Watson, P., Markowitz, S., Willson, J.K., Green, J., de la Chapelle, A., Hamilton, S.R., Kinzler, K.W., and Vogelstein, B. (1994). hMSH2

mutations in hereditary nonpolyposis colorectal cancer kindreds. Cancer Res. *54*, 4590–4594.

Liu, B., Parsons, R., Papadopoulos, N., Nicolaides, N.C., Lynch, H.T., Watson, P., Jass, J.R., Dunlop, M., Wyllie, A., Peltomaki, P., de la Chapelle, A., Hamilton, S.R., Vogelstein, B., and Kinzler, K.W. (1996). Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. Nature Med. *2*, 169–174.

Lothe, R.A., Peltomaki, P., Meling, G.I., Aaltonen, L.A., Nystrom-Lahti, M., Pylkkanen, L., Heimdal, K., Andersen, T.I., Moller, P., Rognum, T.O., Fossa, S.D., Haldorsen, T., Langmark, F., Brogger, A., de la Chapelle, A., and Borresen, A.-L. (1993). Genomic instability in colorectal cancer: relationship to clinicopathological variables and family history. Cancer Res. *53*, 5849–5852.

Lynch, H.T., Smyrk, T.C., Watson, P., Lanspa, S.J., Lynch, J.F., Lynch, P.M., Cavalieri, R.J., and Boland, C.R. (1993). Genetics, natural history, tumor spectrum, and pathology of hereditary nonpolyposis colorectal cancer: an updated review. Gastroenterology *104*, 1535–1549.

Mansour, S.L., Thomas, K.R., and Capecchi, M.R. (1988). Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature *336*, 348–352.

Marsischky, G.T., Filosi, N., Kane, M.F., and Kolodner, R. (1996). Redundancy of *Saccharomyces cerevisiae* MSH3 and MSH6 in MSH2-dependent mismatch repair. Genes Dev. *10*, 407–420.

Modrich, P. (1991). Mechanisms and biological effects of mismatch repair. Annu. Rev. Genet. *25*, 229–253.

New, L., Liu, K., and Crouse, G.F. (1993). The yeast gene *MSH3* defines a new class of eukaryotic MutS homologues. Mol. Gen. Genet. *239*, 97–108.

Nicolaides, N.C., Papadopoulos, N., Liu, B., Wei, Y., Carter, K.C., Ruben, S.M., Rosen, C.A., Haseltine, W.A., Fleischmann, R.D., Fraser, C.M., Adams, M.D., Venter, J.C., Dunlop, M.G., Hamilton, S.R., Petersen, G.M., de la Chapelle, A., Vogelstein, B., and Kinzler, K. (1994). Mutations of two PMS homologues in hereditary nonpolyposis colon cancer. Nature *371*, 75–80.

Palombo, F., Gallinari, P., Iaccarino, I., Lettieri, T., Hughes, M., D'Arrigo, A., Truong, O., Hsuan, J.J., and Jiricny, J. (1995). GTBP, a 160-kilodalton protein essential for mismatch-binding activity in human cells. Science *268*, 1912–1914.

Papadopoulos, N., Nicolaides, N.C., Wei, Y.-F., Ruben, S.M., Carter, K.C., Rosen, C.A., Haseltine, W.A., Fleischmann, R.D., Fraser, C.M., Adams, M.D., Venter, J.C., Hamilton, S.R., Petersen, G.M., Watson, P., Lynch, H.T., Peltomaki, P., Mecklin, J.-P., de la Chapelle, A., Kinzler, K.W., and Vogelstein, B. (1994). Mutation of a *mutL* homolog in hereditary colon cancer. Science *263*, 1625–1629.

Papadopoulos, N., Nicolaides, N.C., Liu, B., Parsons, R., Lengauer, C., Palombo, F., D'Arrigo, A., Markowitz, S., Willson, J.K., Kinzler, K.W., Jiricny, J., and Vogelstein, B. (1995). Mutations of GTBP in genetically unstable cells. Science *268*, 1915–1917.

Parsons, R., Li, G.-M., Longley, M.J., Fang, W.-H., Papadopoulos, N., Jen, J., de la Chapelle, A., Kinzler, K.W., Vogelstein, B., and Modrich, P. (1993). Hypermutability and mismatch repair deficiency in RER$^+$ tumor cells. Cell *75*, 1227–1236.

Parsons, R., Li, G.-L., Longley, M., Modrich, P., Liu, B., Berk, T., Hamilton, S.R., Kinzler, K.W., and Vogelstein, B. (l995). Mismatch repair deficiency in phenotypically normal human cells. Science *268*, 738–740.

Peltomaki, P., Aaltonen, L.A., Sistonen, P., Pylkkanen, L., Mecklin, J.-P., Jarvinen, H., Green, J.S., Jass, J.R., Weber, J.L., Leach, F.S., Petersen, G.M., Hamilton, S.R., de la Chapelle, A., and Vogelstein, B. (1993). Genetic mapping of a locus predisposing to human colorectal cancer. Science *260*, 810–812.

Petes, T.D., Malone, R.E., and Symington, L.S. (1991). Recombination in yeast. In The Molecular and Cellular Biology of the Yeast *Saccharomyces*, Volume 1, J. Broach, E. Jones, and J. Pringle, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 407–521.

Prolla, T.A. (1994). Dual requirement in yeast DNA mismatch repair for MLH1 and PMS1, two homologs of bacterial MutL. PhD thesis, Yale Univeristy, New Haven, Connecticut.

Prolla, T.A., Christie, D.-M., and Liskay, R.M. (1994a). A requirement in yeast DNA mismatch repair for *MLH1* and *PMS1*, two homologs of the bacterial *mutL* gene. Mol. Cell. Biol. *14*, 407–415.

Prolla, T.A., Pang, Q., Alani, E., Kolodner, R.D., and Liskay, R.M. (1994b). Interactions between the MSH2, MLH1 and PMS1 proteins during the initiation of DNA mismatch repair. Science *265*, 1091–1093.

Psychoyos, A. (1961). Permeabilite cappilaire et decidualisation uterine. CR Acad. Sci. (Paris) *252*, 1515–1517.

Reenan, R.A.G., and Kolodner, R.D. (1992a). Characterization of insertion mutations in the *Saccharomyces cerevisiae MSH1* and *MSH2* genes: evidence for separate mitochondrial and nuclear functions. Genetics *132*, 975–985.

Reenan, R.A.G., and Kolodner, R.D. (1992b). Isolation and characterization of two *Saccharomyces cerevisiae* genes encoding homologs of the bacterial HexA and MutS mismatch repair proteins. Genetics *132*, 963–973.

Reitmair, A.H., Schmits, R., Ewel, A., Bapat, B., Redston, M., Mitri, A., Waterhouse, P., Mittrucker, H.W., Wakeham, A., Liu, B., Thomason, A., Mitri, A., Redston, M., Gallinger, S., Bristol, R., Hills, R., Meuth, M., Ballhausen, W., Fishel, R., and Mak, T. (1995). MSH2 deficient mice are viable and susceptible to lymphoid tumours. Nature Genet. *11*, 64–70.

Risinger, J.I., Umar, A., Barrett, J.C., and Kunkel, T.A. (1995). A *hPMS2* mutant cell line is defective in strand-specific mismatch repair. J. Biol. Chem. *270*, 18183–18186.

Ross-Macdonald, P., and Roeder, G.S. (1994). Mutation of a meiosis-specific MutS homolog decreases crossing over but not mismatch correction. Cell *79*, 1069–1080.

Sirotkin, A.M., Edelmann, W., Cheng, G., Klein-Szanto, A., Kucherlapati, R., and Skoultchi, A.I. (1995). Mice develop normally without the H1(0) linker histone. Proc. Natl. Acad. Sci. USA *92*, 6434–6438.

Thomas, D.C., Roberts, J.D., and Kunkel, T.A. (1991). Heteroduplex repair in extracts of human HeLa cells. J. Biol. Chem. *266*, 3744–3751.

Thomas, D.C., Umar, A., and Kunkel, T.A. (1995). Measurement of heteroduplex repair in human cell extracts. Methods: A Companion to Methods in Enzymology *7*, 187–197.

Williamson, M.S., Game, J.C., and Fogel, S. (1985). Meiotic gene conversion mutants in *Saccharomyces cerevisiae*. I. Isolation and characterization of *pms1–1* and *pms1–2*. Genetics *110*, 609–646.

GenBank Accession Numbers

The accession numbers for the mouse *mlh1* sequences reported in this paper are U59881 to U59884 and U60872.