DNA polymerase ε and δ proofreading suppress discrete mutator and cancer phenotypes in mice

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Organisms require faithful DNA replication to avoid deleterious mutations. In yeast, replicative leading- and lagging-strand DNA polymerases (Pols ε and δ, respectively) have intrinsic proofreading exonucleases that cooperate with each other and mismatch repair to limit spontaneous mutation to less than 1 per genome per cell division. The relationship of these pathways in mammals and their functions in vivo are unknown. Here we show that mouse Pol ε and δ proofreading suppress discrete mutator and cancer phenotypes. We found that inactivation of Pol ε proofreading elevates base-substitution mutations and accelerates a unique spectrum of spontaneous cancers; the types of tumors are entirely different from those triggered by loss of Pol δ proofreading. Intercrosses of Pol ε-, Pol δ-, and mismatch repair-mutant mice show that Pol ε and δ proofreading act in parallel pathways to prevent spontaneous mutation and cancer. These findings distinguish Pol ε and δ functions in vivo and reveal tissue-specific requirements for DNA replication fidelity.

DNA replication | genetic instability | DNA polymerase fidelity | mismatch repair

Eukaryotes replicate their DNA with remarkable accuracy (1). This accuracy is achieved through a network of conserved pathways that repair DNA damage and correct DNA polymerase errors (2–6). Studies in yeast show that polymerase proofreading and postreplication mismatch repair (MMR) are the primary guardians of DNA replication fidelity (7–10). Loss of either alone significantly increases spontaneous mutation, and combined defects reveal strong synergistic interactions among proofreading and MMR pathways (9–11).

The prevailing model is that polymerase fidelity, proofreading, and MMR act in series (3, 6, 9, 10). Replicative DNA polymerases err approximately once every 10^4-10^5 nucleotides polymerized (3). Most misincorporated nucleotides are removed by 3′-exonucleases intrinsic to proofreading polymerases (4, 5). Occasional errors escape proofreading, and these errors are corrected by the MMR machinery (6). The majority of polymerase errors are base−base mispairs and ±1 slippage events (3) that must be corrected at nearly 100% efficiency to achieve a spontaneous mutation rate of $\approx 10^{-10}$ per base pair per cell division (1).

Eukaryotes have 2 nuclear DNA polymerases with intrinsic proofreading activity: Pol ε and Pol δ (3, 7, 8). These polymerases, together with Pol α, are the primary replicative enzymes functioning at DNA replication forks (12–14). Pol α primes both leading- and lagging-strand synthesis and copies relatively short stretches of DNA, whereas Pols ε and δ are responsible for the bulk of chromosomal DNA synthesis during cell division. In yeast, Pols ε and δ are principal leading- and lagging-strand DNA polymerases, respectively (15, 16).

Alleles that selectively inactivate the proofreading exonucleases of Pol ε or δ significantly increase spontaneous mutation rates in yeast (7, 8). This mutator effect is particularly strong in cells defective for Pol δ proofreading (9–11, 17). Consistent with their leading- and lagging-strand functions, Pols ε and δ proofread opposite DNA strands (10, 17, 18). However, it is not clear whether these polymerases always correct their own errors. Studies of double mutants show that Pol ε and δ proofreading synergize to suppress spontaneous mutations and potentially compete for nascent mispairs (10).

To determine the nature and significance of these pathways in mammals, we engineered mice with "knockin" point mutations that selectively inactivate the proofreading exonuclease of Pol ε. We show that loss of Pol ε proofreading confers a strong mutator phenotype and high incidence of spontaneous neoplasms. Surprisingly, Pol ε and Pol δ proofreading-deficient mice exhibited very different survival rates and tissue-specific cancer susceptibilities. Our findings establish Pol ε proofreading as a unique tumor suppressor and show that the proofreading exonucleases of Pols ε and δ act in discrete tissue-specific pathways to prevent spontaneous cancer.

Results and Discussion

Using an allelic-replacement strategy, we created mice with alanine substitutions at residues D272 and E274 in the highly conserved 3′-exonuclease active site of Pol ε (4, 5) [supporting information (SI) Fig. S1]. The resultant exonuclease-mutant allele, *Pole*^{e}, is equivalent to yeast *pol2–4*, which selectively inactivates the proofreading exonuclease of Pol ε while preserving normal polymerase activity (8). Litters from *Pole*$^{+/e}$ × *Pole*$^{+/e}$ matings exhibited a normal Mendelian distribution of *Pole* alleles. Both male and female *Pole*$^{e/e}$ mice were fertile. Thus, Pol ε proofreading is not essential for embryogenesis or development to sexual maturity.

To determine the effects of Pol ε proofreading on longevity and disease risk, *Pole*$^{+/e}$ heterozygotes (> 98% C57BL/6 genetic background) were bred to generate *Pole*$^{+/+}$, *Pole*$^{+/e}$, and *Pole*$^{e/e}$ offspring that were monitored for health throughout their natural life spans (Fig. 1*A*). All mice developed into adulthood with no evident disease. However, between 9 and 24 months of age, *Pole*$^{e/e}$ animals progressively succumbed to cancer (median survival = 16 months), whereas *Pole*$^{+/e}$ heterozygotes were indistinguishable from wild-type mice (median survival = 25 months; $P > 0.05$). Similar results were obtained with the *Pole*$^{e/e}$ founder strain (75:25 genetic mix of C57BL/6:129/Sv) housed in a separate facility during previous calendar years (Fig. 1*A*). These data show that homozygous loss of Pol ε proofreading significantly and reproducibly increases cancer mortality in mice.

The most frequent tumors were intestinal adenomas and adenocarcinomas, which arose spontaneously in almost half of

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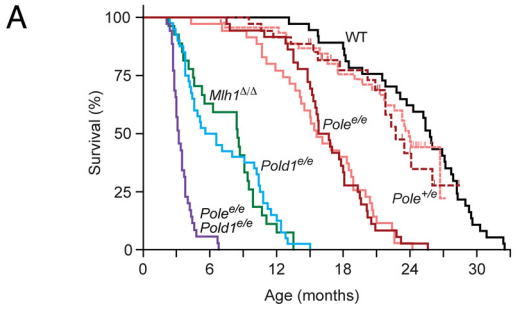
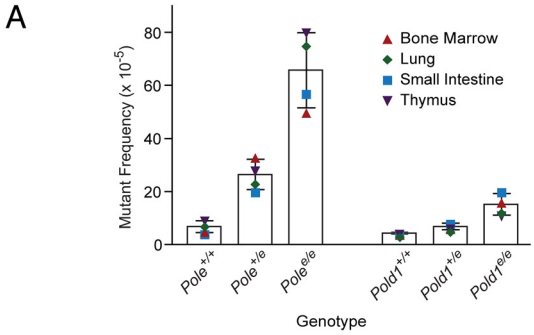


B

Tumor†	Incidence (%)* WT	*Pole*$^{e/e}$	*Pold1*$^{e/e}$	*Mlh1*$^{\Delta/\Delta}$	*Pole*$^{e/e}$ *Pold1*$^{e/e}$
Lymphoma					
Thymic	0	0	42	38	82
Nodal	6	24	14	4	12
Follicular	38	15	8	31	3
Squamous Papilloma/Carcinoma					
Tail Skin	0	0	25	0	6
Adenoma/Adenocarcinoma					
Intestine	6	45	14	42	3
Lung	9	12	28	4	3
Histiocytic Sarcoma	22	36	3	4	0

Fig. 1. Survival and cancer phenotypes of Pol ε proofreading-deficient mice. (*A*) Kaplan-Meier survival estimates. Mice were followed for long-term survival and observed daily until moribund or unexpected natural death. Dark red indicates *Pole*$^{e/e}$ (n = 36) and *Pole*$^{+/e}$ (n = 35) in C57BL/6 genetic background after removal of the neomycin selection cassette (*Neo*$^{-}$); light red indicates *Pole*$^{e/e}$ (n = 35) and *Pole*$^{+/e}$ (n = 45) in a mixed C57BL/6:129/Sv genetic background with the neomycin selection cassette still present (*Neo*$^{+}$); blue indicates *Pold1*$^{e/e}$ (n = 40) in C57BL/6 (*Neo*$^{-}$); purple indicates *Pole*$^{e/e}$*Pold1*$^{e/e}$ (n = 35) in C57BL/6 (*Neo*$^{-}$); black indicates wild-type (WT; n = 37) C57BL/6; green indicates *Mlh1*$^{\Delta/\Delta}$ (n = 27) in C57BL/6. One month = 30.4 days. (*B*) Spontaneous tumor incidences. Moribund mice were euthanized and necropsied, and tumors were diagnosed by histology. *, Incidences among 32 wild-type (WT), 33 *Pole*$^{e/e}$, 36 *Pold1*$^{e/e}$, 26 *Mlh1*$^{\Delta/\Delta}$, and 34 *Pole*$^{e/e}$*Pold1*$^{e/e}$ mice. †, Tumors with 15% or greater incidence in 1 or more groups. See Table S1 for details and rare tumors.



B

Genotype	Mutation Rate (x 10^{-7})* Oua^{R}	6-TG^{R}	Mutation Spectrum BS:FS†	MIN‡
WT	<0.07 (0.01-0.4)	0.07 (0.02-0.1)	ND§	0/23
Pole$^{+/e}$	ND	0.3 (0.02-1)	ND	ND
Pole$^{e/e}$	5 (3-7)	12 (10-14)	41:0	0/11
Pold1$^{+/e}$	1 (0.4-2)	0.08 (0.04-0.4)	ND	ND
Pold1$^{e/e}$	5 (4-7)	4 (3-5)	54:4	1/26
Mlh1$^{\Delta/\Delta}$	9 (7-11)	ND	36:17‖	18/18
Pold1$^{e/e}$ *Pole*$^{e/e}$	7 (5-10)	6 (4-8)	10:0	0/12

Fig. 2. Mutator phenotypes conferred by defective Pol ε and Pol δ proofreading. (*A*) Mutant frequencies in vivo. Wild-type, *Pole*^{e}, and *Pold1*^{e} mice harboring the *cII* transgene were euthanized at 6–8 weeks of age, and *cII* mutant frequencies were determined in DNA isolated from whole thymus (*purple inverted triangles*), whole lung (*green diamonds*), unfractionated bone marrow (*red triangles*), and small intestine epithelium (*blue squares*). Each datum point is the average of 5–7 mice. Bar graphs are averages of 4 tissues with standard deviations. (*B*) Mutation rates and spectra in cultured fibroblasts. Cell lines were derived from 14- to 16-day embryos, and spontaneous mutant rates were determined by fluctuation analyses using maximum likelihood estimates. Data are from multiple experiments with 2–6 independently derived cell lines of each genotype (except for *Pole*$^{+/e}$, in which 1 cell line was analyzed in a single experiment). Parentheses indicate 95% confidence intervals. *, Ouabain-resistant (Oua^{R}) or 6-thioguanine–resistant (6-TG^{R}) mutants per cell division. †, Number of 6-TG^{R} clones with *Hprt* base substitutions (BS) or ±1 frameshifts (FS). ‡, Microsatellite instability (MIN) expressed as number of clones with variant microsatellites/total number of clones screened. §, ND, not determined. ‖, Base-substitution and frameshift values from 6-TG^{R} *Msh2*$^{\Delta/\Delta}$ clones. See Fig. S3 and Table S2 for details of mutation spectra.

the *Pole*$^{e/e}$ mice but rarely in wild-type littermates (Fig. 1*B*, Table S1, and Fig. S2*A*). The majority of tumors (in 13 of 15 *Pole*$^{e/e}$ mice with intestinal tumors) were adenocarcinomas of the small intestine with no evident metastases. Six animals had multiple gastrointestinal tumors, including 1 mouse with both a colonic adenocarcinoma and a gastric adenoma. *Pole*$^{e/e}$ mice also died with histiocytic sarcomas and non-thymic lymphomas, tumor types that occur frequently in wild-type mice later in life (Fig. 1*B*, Table S1, and Figs. S2 *B* and *C*). These results suggest that defective Pol ε proofreading accelerates neoplasms that naturally arise in the C57BL/6 strain. The effect on intestinal cancers was particularly striking.

This constellation of tumors is distinct from that previously observed in mice defective for Pol δ proofreading (19, 20). To compare Pol ε and Pol δ proofreading mutants directly in a uniform genetic background, C57BL/6 mice deficient for Pol δ proofreading (*Pold1*$^{e/e}$) were generated and housed side-by-side with the C57BL/6 *Pole*$^{e/e}$ cohort. Similar to the original *Pold1*$^{e/e}$ founder strain (50:50 mix of C57BL/6:129/Sv) (19, 20), mice expressing *Pold1*$^{e/e}$ in a C57BL/6 background died during the first year of life, primarily with thymic lymphomas, tail-skin squamous cell neoplasms, and lung epithelial tumors (Fig. 1 and Table S1). Thus, the 3′ exonucleases of Pol ε and δ suppress discrete tissue-specific cancers. The tumor types differed in the *Pole*$^{e/e}$ and *Pold1*$^{e/e}$ cohorts but overlapped with those observed in a contemporary cohort of C57BL/6 mice defective for MMR (*Mlh1*$^{\Delta/\Delta}$; Fig. 1 and Table S1) (21).

To determine whether the dissimilar cancer susceptibilities of *Pole*$^{e/e}$ and *Pold1*$^{e/e}$ mice reflect differences in mutator phenotypes, we examined mutagenesis in vivo using a *cII* reporter transgene (22). Spontaneous mutant frequencies were higher in *Pole*$^{e/e}$ mice than in *Pold1*$^{e/e}$ mice in all tissues assayed and were similar among different tissues of the same genotype (Fig. 2*A*). MMR-deficient mice exhibit *cII* mutant frequencies (35×10^{-5}) (23), intermediate between those of *Pole*$^{e/e}$ mice (65×10^{-5}) and *Pold1*$^{e/e}$ mice (15×10^{-5}). Despite their low cancer susceptibility, heterozygous *Pole*$^{+/e}$ mice also had elevated mutant frequencies, comparable to those in homozygous *Pold1*$^{e/e}$ animals (Fig. 2*A*). Therefore, similar to findings in previous studies of MMR (24) and chemical carcinogens (25, 26), tissue-specific cancer susceptibilities in Pol ε and Pol δ proofreading-deficient mice do not correlate with transgene mutant frequencies in vivo.

A limitation of the *cII* assay is that it measures mutant frequency (not rate) at a single genetic locus in all cell types of a tissue. To determine rates of mutation per cell division in a standardized cell type, fluctuation analyses were performed on fibroblast cell lines derived from *Pole*$^{e/e}$ and *Pold1*$^{e/e}$ embryos. Homozygous loss of Pol ε proofreading increased spontaneous mutation rates more than 70 times above wild-type levels at 2 genetic loci (Fig. 2*B*). Cells deficient for Pol δ proofreading or

MMR exhibited similar increases in spontaneous mutation rate (Fig. 2*B*). In contrast, yeast defective for Pol ε proofreading are relatively weak mutators compared with Pol δ proofreading mutants (9–11, 17). This disparity suggests that Pol ε and δ proofreading influence different mutation pathways in mouse and yeast cells and/or that proofreading by each polymerase varies at different genetic loci (*URA3, CAN1*, and *SUP4-o* in yeast; *Atp1a1* and *Hprt* in mouse). $Pole^{e}$ and $Pold1^{e}$ were partially dominant in mouse cells as evidenced by a measurable mutator effect in $Pole^{+/e}$ and $Pold1^{+/e}$ heterozygotes (Fig. 2); equivalent yeast alleles also exhibit partial dominance (7, 9, 10). We estimate that 1–10 mutations are introduced for every 10^9 nucleotides polymerized in fibroblasts homozygous-deficient for Pol ε or Pol δ proofreading (Fig. 2*B*) (27, 28).

Although proofreading- and MMR-deficient cells exhibited similar overall mutation rates, their mutation spectra were different (Fig. 2*B*, Fig. S3, and Table S2). 6-Thioguanine–resistant (6-TG^R) $Pole^{e/e}$ clones harbored primarily base-substitution mutations (> 90%) in the *Hprt* gene. This prevalence of base substitutions agrees with the elevated rate of ouabain resistance, which results from base substitutions in the *Atp1a1* gene (27). Pol δ proofreading-deficient cells also exhibited a strong bias toward base substitutions. However, the distribution of mutation types (Table S2) and their positions in the *Hprt* gene (Fig. S3) were significantly different in $Pole^{e/e}$ and $Pold1^{e/e}$ cells. These mutation spectra reflect polymerase errors that escape MMR (3, 6) and do not reveal whether mouse Pols ε and δ function on opposite DNA strands (15, 16). Frameshifts and microsatellite instability, which are hallmarks of defective MMR (Fig. 2*B*) (6, 21), were not significantly increased in $Pole^{e/e}$ or $Pold1^{e/e}$ cells. Thus, loss of mouse polymerase proofreading confers a base-substitution mutator phenotype resembling that observed in human cancers (29).

Pol ε proofreading, Pol δ proofreading, and MMR coordinately prevent spontaneous mutation in yeast (9–11). To explore the functional relationship of these fidelity pathways in mice, we established $Pole^{+/e}Pold1^{+/e} \times Pole^{+/e}Pold1^{+/e}$ breeding pairs and generated animals deficient for both proofreading activities. Using similar breeding strategies, we also attempted to generate mice with combined defects in proofreading and MMR ($Mlh1^{\Delta/\Delta}$ or $Msh2^{\Delta/\Delta}$). $Pole^{e/e}Pold1^{e/e}$ mice were born in normal Mendelian ratios (Table S3) with no apparent developmental abnormalities. Therefore, mice deficient for Pol ε proofreading do not require Pol δ proofreading for viability. The viability of $Pole^{e/e}Pold1^{e/e}$ mice contrasts with the embryonic lethality caused by loss of either Pol ε or Pol δ proofreading combined with homozygous-defective MMR (Table S3). Timed matings of $Pold1^{e/e}Mlh1^{+/\Delta}$ parents yielded normal Mendelian ratios of implanted embryos, but $Pold1^{e/e}Mlh1^{\Delta/\Delta}$ embryos developed slowly and failed to survive beyond embryonic day E9.5. In contrast, $Pole^{e/e}Mlh1^{\Delta/\Delta}$ embryos were present in normal ratios up to E14.5. Thus, in the absence of MMR, early mouse development requires proofreading by Pol δ but not Pol ε. This dependency is reversed later in development, as evidenced by perinatal lethality in $Pole^{+/e}MMR^{\Delta/\Delta}$ but not $Pold1^{+/e}MMR^{\Delta/\Delta}$ animals (Table S3). These data show that genetic interactions of $Pole^{e/e}$ with $Pold1^{e/e}$ are weaker than those of $Pole^{e/e}$ or $Pold1^{e/e}$ with defective MMR. Moreover, they suggest that embryogenesis requires faithful DNA replication and that dual loss of proofreading and MMR exceeds an error threshold.

To assess functional interactions of Pol ε and δ proofreading at the cellular level, we measured spontaneous mutation rates in fibroblast cell lines derived from $Pole^{e/e}Pold1^{e/e}$ embryos. In contrast to the synergistic relationship observed in yeast (10), the combined loss of mouse Pol ε and δ proofreading did not increase spontaneous mutation rates above the levels of single $Pole^{e/e}$ or $Pold1^{e/e}$ mutants (Fig. 2*B*). Similar results were observed in fluctuation assays of ouabain and 6-thioguanine resistance and in multiple experiments with $Pole^{e/e}Pold1^{e/e}$ cell lines independently derived from separate embryos. These results suggest 2 possibilities: mouse Pol ε and δ proofreading may be required for the same step in a single pathway, or they may act in parallel, noncompeting pathways (9, 10). $Pole^{e/e}$ and $Pold1^{e/e}$ clearly do not have a multiplicative relationship (Fig. 2*B*) and thus do not act in series (9). The dissimilarities in $Pole^{e/e}$ and $Pold1^{e/e}$ mutation spectra (Fig. S3 and Table S2) argue against a requirement for both enzymes at a single molecular step and indicate that Pols ε and δ function in parallel, noncompeting pathways. Statistical variations in our mutation rate data (Fig. 2*B*) would mask the additive relationship predicted by this model. Therefore, action in parallel is consistent with our mutation data (Fig. 2*B* and Fig. S3) and with the divergent cancer susceptibilities observed in $Pole^{e/e}$ and $Pold1^{e/e}$ mice (Fig. 1*B*).

$Pole^{e/e}Pold1^{e/e}$ mutant animals died significantly younger than mice with either $Pole^{e/e}$ or $Pold1^{e/e}$ alone (Fig. 1*A*). Most $Pole^{e/e}Pold1^{e/e}$ animals succumbed to thymic lymphoma by 6 months of age (Fig. 1*B*); skin papillomas also were observed occasionally on their tails. Thus, defective Pol ε proofreading accelerates tumors that characterize the $Pold1^{e/e}$ cancer phenotype (thymic lymphoma and skin squamous cell neoplasms). We cannot assess whether Pol δ proofreading also modifies the $Pole^{e/e}$ cancer phenotype, because $Pole^{e/e}Pold1^{e/e}$ mice did not live long enough to develop tumors characteristic of older $Pole^{e/e}$ mice.

These data show that the combined loss of Pol ε and δ proofreading confers an intermediate in vivo phenotype that is stronger than loss of either proofreading activity alone but weaker than dual loss of proofreading and MMR. Assuming parallel modes of action (see earlier discussion), we infer that accelerated tumorigenesis in $Pole^{e/e}Pold1^{e/e}$ mice reflects additive cooperativity. The embryonic lethal phenotype observed in $Pole^{e/e}MMR^{\Delta/\Delta}$ and $Pold1^{e/e}MMR^{\Delta/\Delta}$ mice (Table S3) is consistent with action in series, a multiplicative relationship previously demonstrated for proofreading and MMR in yeast (9–11). Taken together, these studies of double mutants suggest that mammalian Pol ε and δ proofreading function in parallel pathways and that each has a sequential relationship with MMR.

The tissue-specific phenotypes conferred by $Pole^{e}$ and $Pold1^{e}$ can be taken as qualitative indicators of the sites of action of Pols ε and δ in vivo, with the assumption that each polymerase preferentially proofreads its own errors and that the phenotypes result from unrepaired errors. Thus, Pol ε errors affect tumorigenesis in intestinal epithelial cells, histiocytes, and non-thymic lymphocytes (or their progenitors), whereas Pol δ fidelity is rate limiting for malignant conversion of thymic lymphocyte and skin squamous cell lineages. These differences suggest that Pol ε and Pol δ participate in separate pathways of tumor suppression that are contingent on cell type. Similarly, Pol ε and δ fidelities are rate limiting at different stages of embryogenesis in MMR nullizygotes, thus suggesting unique roles for Pols δ and ε in early and late development, respectively.

The disparate phenotypes of $Pole^{e/e}$ and $Pold1^{e/e}$ mice probably reflect different actions of Pol ε and Pol δ during chromosomal replication. Both polymerases act at DNA replication forks and are required for normal DNA replication (12–14). Accordingly, we observed that $Pole^{e}$ and $Pold1^{e}$ primarily affect dividing cell populations, i.e., embryos and tumors derived from tissues with high cell turnover. Although both polymerases also participate in DNA repair (30, 31), their role in the repair of spontaneous DNA damage (≈10^4 lesions per cell per day) (32) is relatively minor compared with the demands of normal genomic replication (≈10^{10} nucleotides polymerized per cell division). Studies in yeast show that Pols ε and δ synthesize and proofread opposite DNA strands at defined replication origins (10, 14–18). However, Pols ε and δ also may act independently (33, 34), and some replication forks may involve only 1 proofreading polymerase

(14). Considered together, our results suggest a model in which Pol ε and Pol δ replicate and proofread different regions of the mouse genome encoding tissue-specific genes that suppress cancer or are required for embryogenesis. Mutation hotspots (Fig. S3), tissue-specific damage/repair, and secondary functions of Pols ε and δ in DNA metabolism, cell cycle control, chromatin maintenance, and gene silencing (30, 31) also could shape the in vivo phenotype.

In summary, our findings show that proofreading by Pols ε and δ serve distinct and separate functions in vivo and act in parallel pathways to prevent spontaneous mutation and cancer and to ensure normal embryo development. These data underscore the importance of DNA replication fidelity in mammals and suggest that defects in proofreading increase the risk of human cancers.

Materials and Methods

Mutant Mice and Survival Studies. Molecular clones of the mouse Pol ε gene (*Pole*) were isolated, and codons D272 and E274 in the exonuclease active site were mutated to encode alanines. The resultant *Pole*^{e} allele was introduced into 129/Sv embryonic stem cells by targeted homologous recombination. Chimeric mice were generated, and germline transmission of the *Pole*^{e} allele was achieved by breeding with C57BL/6J mice. *Pold1*^{e} mutant mice were created using a similar strategy (20). The loxP-flanked neomycin selection cassettes were removed by matings with cre-transgenic mice, and the resultant *Pole*$^{+/e}$ and *Pold1*$^{+/e}$ mice were separately backcrossed into the C57BL/6J genetic background (> 98%) and maintained as heterozygotes. *Mlh1*$^{+/\Delta}$ and *Msh2*$^{+/\Delta}$ mice were obtained from the National Cancer Institute (NCI) Mouse Models of Human Cancers Consortium. Cohorts for study were established by appropriate matings and were housed concurrently in a shared room. Mice were necropsied at the time of natural death or were euthanized and necropsied when moribund. Formalin-fixed tissues were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined histologically. Additional details are provided in *SI Materials and Methods*.

Mutation Frequencies, Rates, and Spectra. *Pole*^{e} and *Pold1*^{e} homo- and heterozygous mutant mice were crossed with C57BL/6 Big Blue mice (Stratagene), and *cII* transgene mutant frequencies were determined in tissues isolated from 6- to 8-week-old offspring. Fluctuation analyses were performed with immortalized embryonic fibroblasts, and mutation rates were determined by maximum likelihood estimates. Microsatellite and *Hprt* mutations were identified by PCR and DNA sequencing. Additional details are provided in *SI Materials and Methods*.

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