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DNA Replication Fidelity and Cancer

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Abstract

Cancer is fueled by mutations and driven by adaptive selection. Normal cells avoid deleterious mutations by replicating their genomes with extraordinary accuracy. Here we review the pathways governing DNA replication fidelity and discuss evidence implicating replication errors (point mutation instability or PIN) in carcinogenesis.

Keywords

DNA polymerase; proofreading; mismatch repair; mutator; cancer

1. Genetic instability and cancer

Tumor development is a multistep process requiring the accumulation of mutations that activate oncogenes or inactivate tumor suppressors [1–3]. To maintain normal cell functions, genetic stability is strictly controlled [4]. Therefore it is argued that defects in pathways governing genetic stability will facilitate tumorigenesis by fueling the reiterative process of mutation, selection and clonal expansion that drives cancer progression (reviewed and debated in [5–13]).

There is considerable evidence that genetic instability plays a role in cancer (reviewed in [3,13–16]). Cancer cells exhibit high frequencies of chromosomal aberrations [17,18], and the rates of gene rearrangements and amplifications are increased in many tumor cell lines [19–21]. Moreover, inherited deficiencies in genome maintenance systems contribute to human cancer susceptibility syndromes [22–24]. Defects in genes required for nucleotide excision repair (NER; Xeroderma pigmentosum), double-strand break recognition and repair (Ataxia telangiectasia, Nijmegen breakage syndrome), genetic recombination (Bloom, Werner and Rothmund-Thomson syndromes and BRCA1/2) and mismatch repair (MMR; Lynch syndrome) all cause genetic instability and are associated with human cancer syndromes. Thus, it is well established that chromosome instability (CIN) and microsatellite instability (MIN or MSI) predispose to cancer. Recently, sporadic cancers were shown to have elevated frequencies of random nucleotide point mutations, thus implicating point mutation instability (PIN) in oncogenesis [25,26].

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Perhaps the strongest early evidence that increased spontaneous mutation (i.e., mutator phenotype) contributes to human cancer was the discovery that defective mismatch repair (MMR) causes hereditary colon cancer (reviewed in [27–31]). In the early 1990s, colorectal cancer samples from Lynch syndrome pedigrees (also called hereditary nonpolyposis colon cancer or HNPCC) were noted to have microsatellite instability, normal cytogenetics, and were associated with a unique clinical presentation. Two groups simultaneously reported that these families carried mutations in *MSH2*, the gene encoding one of the primary proteins required for MMR [32,33]. Shortly thereafter, *MLH1*, the gene encoding another essential MMR protein, was cloned and found to be mutated in additional Lynch syndrome families [34,35]. The majority of Lynch syndrome patients inherit a mutation in either *MSH2* or *MLH1*, with a smaller percentage inheriting mutations in *PMS2* or *MSH6* [36,37]. The wild-type allele is then lost in tumors through LOH or gene silencing. Patients with inherited MMR deficiency are primarily susceptible to early-onset colorectal cancer, but also have an increased risk for extra-intestinal neoplasms. Inherited MMR defects are only responsible for a small number (1–5%) of colorectal cancer cases; thus, most colorectal cancers with MSI (~15% of all colorectal cancer cases) result from acquired defects in MMR, almost exclusively due to *MLH1* promoter hypermethylation [38]. MMR defects and MSI are also detected in non-colonic sporadic tumors, most commonly in endometrial, lung and gastric cancer [38,39].

MMR-deficient human tumor cell lines display increased spontaneous mutation rates with a preference for frameshifts and base substitution mutations [40–43]. Accordingly, microsatellite instability is a hallmark of MMR loss [31]. Microsatellite instability may be particularly relevant for colorectal cancer as many genes involved in intestinal carcinogenesis (*TGFβR2*, *APC*, *KRAS*, *BRAF*, and others) have repetitive DNA in their coding regions [44]. *MSH6* defects are a less common cause of Lynch syndrome and result in predominantly extra-colonic tumors [45–49]. Interestingly, cells defective for *MSH6* have elevated rates of base-substitution mutations and lower levels of frameshifts, due to selective inactivation of MutSa [43,50,51]. Thus, intestinal carcinogenesis in Lynch syndrome may be directly related to MSI, whereas extra-colonic tumors may result from elevated base substitutions.

2. Determinants of DNA replication fidelity

Normal cells replicate their DNA with extraordinary fidelity ($\sim 10^{-10}$ mutations per base pair per cell division) [52]. This is achieved through the combined actions of polymerase base selectivity, 3'→5' exonucleolytic proofreading, mismatch correction and DNA damage repair (Fig. 1; reviewed in [53–71]).

Proofreading and MMR both contribute substantially to the overall fidelity of cellular DNA replication and mutation avoidance. Genetic experiments in *E. coli* [53,54] and *S. cerevisiae* [50,51,72–76] show that loss of either proofreading or MMR results in a 10- to 1000-fold increase in spontaneous mutation rate. Although studies in mammalian cells are more limited, cell-free fidelity assays [77,78] and experiments with MMR-deficient [40–43,79,80] and proofreading-deficient [81–84] cells also point to these repair pathways as major determinants of replication fidelity in higher eukaryotes. The prevailing model (Figs. 1 and 2) is that spontaneous errors by the replicative lagging- and leading-strand DNA polymerases (Pols δ and ε, respectively [85–87]) trigger proofreading by their intrinsic 3'-exonucleases. Occasional errors escape proofreading, and these are corrected by the MMR machinery. It is estimated that replicative eukaryotic DNA polymerases make errors approximately once every $10^4 - 10^5$ nucleotides polymerized [58,59]. Thus, each time a diploid mammalian cell replicates, at least 100,000 and up to 1,000,000 polymerase errors occur. The majority of these are base•base mispairs and ± 1 slippage events [58,59], which

must be corrected with almost 100% efficiency to achieve a spontaneous mutation rate of $\sim 10^{-10}$ per base pair per cell division [52].

Repair of promutagenic DNA damage (both spontaneous and induced; [22,68–71]) and maintenance of normal dNTP pools [88–90] are also important determinants of replication fidelity. Similar to proofreading and mismatch repair, defects in individual enzymes affecting dNTP pool ratios confer spontaneous mutator phenotypes [88–90]. In contrast, most single-gene defects in DNA damage repair pathways exhibit near-normal spontaneous mutation rates and reveal themselves as “conditional mutators” when cells are challenged with DNA damaging agents [71]. One exception is the repair of 8-oxo-G lesions by the MutM/MutY/MutT “GO” system [54,91–95]. In *E. coli*, loss of either *MutM* or *MutT* confers a moderate-to-strong mutator phenotype in the absence of exogenous oxidative stress [54,92,96]. However, defects in homologous mouse genes have only modest effects on spontaneous mutation rates, presumably due to different interactions of redundant pathways that prevent or repair oxidative DNA damage in mammals [71,93–95].

Quantitative estimates of spontaneous DNA degradation in cells suggest that the daily dose of promutagenic damage is substantial [97–99] (Fig. 3A). These lesions result from the intrinsic chemical instability of DNA under physiological conditions and the exposure of DNA to active oxygen and other reactive metabolites and coenzymes that are generated by normal cells [97,99]. Altogether, it is estimated that $\sim 20,000$ potentially mutagenic lesions arise per diploid mammalian cell per day. Most of these lesions are repaired by the base excision repair (BER) pathway [68,69]. This repair must occur efficiently prior to DNA replication for cells to maintain a low spontaneous mutation rate. The toll of 20,000 spontaneous lesions per cell per day is high, and this is in addition to the 100,000 – 1,000,000 DNA polymerase errors that occur in replicating cells over the same approximate time frame (Fig. 3B).

Thus, there are a number of repair systems that ensure faithful DNA replication. Among these, polymerase proofreading and MMR play primary roles as evidenced by the strong mutator phenotypes conferred by loss of either pathway in the absence of exogenous DNA damage.

2.1. Proofreading polymerases

There are 3 eukaryotic DNA polymerases that have intrinsic 3'→5' exonucleolytic proofreading activity: Pol δ , Pol ϵ and Pol γ [60,100]. Only Pols δ and ϵ are nuclear, while Pol γ is mitochondrial. Similar to other proofreading DNA polymerases, Pols δ and ϵ are comprised of multiple subunits with the catalytic peptide of each enzyme harboring both polymerase (pol) and exonuclease (exo) activities. These proofreading polymerases, together with Pol α :primase, are the primary replicative enzymes functioning at DNA replication forks (reviewed in [87,101,102]). Pol α primes both leading- and lagging-strand synthesis and copies relatively short stretches of DNA, while Pols δ and ϵ 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 [85–87].

A large literature shows that Pol δ and Pol ϵ play essential roles in the replication and repair of chromosomal DNA [101–108]. These roles are most readily demonstrated using genetic

¹Calculated as follows: The mammalian diploid genome has 6×10^9 base pairs, which corresponds to 1.2×10^{10} nucleotides that must be polymerized each time a cell divides. DNA polymerases δ and ϵ replicate the bulk of genomic DNA and have error rates of 10^{-4} to 10^{-5} per nucleotide polymerized (prior to correction by proofreading or MMR). Thus, if the error rate is 10^{-5} , then 10^{10} nucleotides per genome $\times 10^{-5}$ errors per nucleotide = 100,000 polymerase errors per genome per cell division. If the error rate is 10^{-4} , then 10^{10} nucleotides per genome $\times 10^{-4}$ errors per nucleotide = 1,000,000 errors per genome per cell division.

approaches in yeast. Pol δ contributes to yeast lagging-strand DNA synthesis from replication origins [86,108] and the maturation of Okazaki fragments [102,109]. Pol ϵ participates in leading-strand elongation [85] (perhaps preferentially during late S phase [110]) in addition to its more specialized roles at DNA replication origins [111–114], in S phase checkpoint control [115], and sister chromatid cohesion [116]. Studies in mammalian cells also establish essential roles for Pol δ and Pol ϵ in chromosomal DNA replication. Both polymerases associate with nascent chromosomal DNA [110,117,118], and anti-Pol δ and -Pol ϵ antibodies inhibit chromosomal replication when introduced into human cells [118,119]. Interestingly, SV40 DNA replication requires only Pols α and δ and occurs by a Pol ϵ -independent mechanism [117,119]. Thus, SV40 replication is not a complete model of S phase-regulated cellular DNA replication [102]. Finally, Pols δ and ϵ catalyze DNA synthesis in several repair pathways including nucleotide excision repair, MMR, the long-patch pathway of base excision repair, and double-strand break repair [71,101,120]. Thus, Pols δ and ϵ participate in most cellular pathways that require nuclear DNA synthesis, and together they synthesize the bulk of chromosomal DNA during cell division.

Point mutations that selectively inactivate exonucleolytic proofreading activities of Pol δ or Pol ϵ confer moderate-to-strong mutator phenotypes in yeast [75,76,121–128]. For reasons not fully understood, this mutator effect tends to be greater in Pol δ than Pol ϵ proofreading mutants. Consistent with their leading- and lagging-strand functions, there is good evidence that the proofreading exonucleases of Pol δ and Pol ϵ correct DNA replication errors on opposite strands [76,127,129]. However, it is not clear whether they always correct their own errors. Studies of double mutants show that the proofreading activities of Pol δ and Pol ϵ synergize to suppress spontaneous mutations and that this synergy reflects a competitive relationship [76,122,126]. This suggests that yeast Pol δ and Pol ϵ can act on the same pool of DNA replication errors and may proofread for each other. Recent studies indicate that Pol δ and Pol ϵ can also proofread for Pol α [130,131], suggesting that these and other 3'-exonucleases in the cell [60] could function as extrinsic proofreading enzymes [132,133]. Evidence for overlapping polymerase functions also comes from the observation that the polymerase domain of Pol ϵ is dispensable in yeast when it is deleted (albeit with severe growth consequences; [105,106,122,134]), while inactivating point mutations in the intact pol domain of Pol ϵ are lethal [105]. Thus, it appears that yeast Pols δ , ϵ , α and perhaps other polymerases interact and can serve partially redundant functions. The roles of Pol δ and Pol ϵ proofreading and their interactions in mammalian cells are largely unknown.

2.2. Mismatch repair

Eukaryotes have multiple MMR proteins that exhibit distinct substrate preferences (reviewed in [62–67]). In mammals, polymerase errors are recognized and bound by complexes containing the Msh2 protein. Both the Msh6 and Msh3 proteins heterodimerize with Msh2 and dictate substrate preferences. Biochemical studies show that base•base and small insertion/deletion mispairs are primarily targeted by the Msh2/Msh6 heterodimer (MutS α), while larger insertion/deletion loops are bound primarily by a Msh2/Msh3 complex (MutS β) [135–138]. Similar substrate binding preferences are observed for yeast and mammalian Msh proteins *in vitro* [138], and genetic studies in yeast confirm that MutS α preferentially repairs base•base mispairs and 1-bp loops while MutS β prefers larger loops [50,51,62]. However, MutS α and MutS β are redundant with respect to the repair of small insertion/deletion mispairs [50,51], and MutS β recognizes a limited set of base•base mispairs in certain sequence contexts [139]. There is also functional overlap between MutS α and MutS β in human cells [43]. To initiate repair, the MutS complexes interact with a heterodimer of MutL homologues (MutL α comprised of Mlh1/Pms1 in yeast, equivalent to Mlh1/Pms2 in mammals), which contains endonucleolytic activity that nicks the nascent strand both 5' and 3' of the mismatch [140,141]. These nicks provide entry points for

enzymes that remove the mismatched sequence, allowing re-synthesis of the DNA [140,142].

In addition to correcting mispairs, MMR proteins play an important role as sensors of DNA damage that signal cell death ([79,143]; reviewed in [67,144–148]). Although initially thought to confer sensitivity to a broad range of DNA damaging agents, it is now apparent that MMR-mediated cytotoxicity occurs primarily in response to S_n1 alkylating agents, 6-thioguanine and cisplatin [149]. There is also evidence that some DNA adducts (e.g., 8-oxo-G and O^6 -methyl-G) are repaired by MMR [150,151] and that misincorporated bases opposite some adducts are excised by MMR [152,153], potentially resulting in a futile repair cycle [154]. These MMR-dependent, damage-response functions are mediated by MutS α complexed with MutL α . Thus, cells with defective *Msh2*, *Msh6* or *Mlh1* are resistant to DNA damage-induced apoptosis, whereas *Msh3* mutants remain sensitive and indistinguishable from wild type [155–157]. Studies examining mechanisms of MMR-dependent apoptosis show that MMR proteins recognize and bind to DNA lesions and initiate a signal transduction cascade that activates G₂/M cell cycle checkpoints and engages the apoptotic machinery [67,147,158].

2.3. Cooperativity of proofreading and MMR

Genetic studies in yeast show that Pol proofreading and MMR serve overlapping functions in the repair of DNA replication errors and that they exhibit a synergistic relationship [75,76,121,126,159]. Proofreading defects in Pol δ or Pol ϵ are synthetically lethal in haploid cells when combined with loss of *pms1*, *mlh1* or *msh2* [75,126], and defective Pol δ proofreading is synthetically lethal with nullizygous *msh6* [160]. In diploids proofreading/MMR double mutants are viable, but grow slowly and exhibit extremely high spontaneous mutation rates [75,76,124–126]. This implies that deficiencies in either proofreading or MMR can be partially compensated by the function of the other and that loss of both pathways results in the accumulation of replication errors and compromised growth. These data also indicate that proofreading and MMR act in series along a common pathway (see discussion in [75,76]). This is supported by evidence that MMR proteins interact with components of the DNA replication apparatus [125,161–164]. Synergy occurs primarily with the Msh6 pathway of MMR [125], which is consistent with proofreading and Msh6-dependent MMR being the principal pathways that correct base•base mispairs [58–67]. Interestingly, the fidelity of lagging-strand DNA synthesis in yeast is greater than that of leading-strand [165], because MMR preferentially corrects lagging-strand replication errors [153].

Evidence for cooperativity in mammals comes from recent studies of mice with defects in both proofreading and MMR [83,84]. Loss of either Pol δ or Pol ϵ proofreading combined with defective MMR results in embryonic lethality [83]. Interestingly, Pol δ and Pol ϵ proofreading defects confer different phenotypes in embryos. In the absence of MMR, early mouse development requires proofreading by Pol δ but not Pol ϵ , and this dependency is reversed later in development (see discussion in [83]). These results suggest that Pols δ and ϵ have unique roles at different stages of mammalian development. Moreover, they indicate that embryogenesis requires faithful DNA replication and that dual loss of proofreading and MMR exceeds an error threshold.

3. Mutator mice

Although mechanisms of DNA replication fidelity have been studied extensively in simple microbes, less is known about the corresponding pathways and their functions in higher eukaryotes. Mammals require faithful DNA replication to avoid deleterious mutations in critical subpopulations of cells (gametes, stem cells, developing embryos) and to sustain

essential somatic functions throughout the adult reproductive life span. At the same time, increased mutation (hypermutation) is required for normal functions in some somatic tissues [166]. Thus, the complexities of mammalian biology impose unique constraints on DNA replication fidelity that vary among tissues, cell lineages and genetic loci.

To study the effects of repair deficiencies on tumor predisposition, mice containing knockout and knockin alleles of a number of DNA repair genes have been constructed [167]. These mouse lines are powerful experimental tools that reveal the biological functions of mammalian mutation-avoidance pathways and the genetic interactions that occur among repair genes, oncogenes and tumor suppressors. Furthermore, cells obtained from these animals are used to probe mechanisms of mammalian DNA repair at the cellular level.

Interestingly, deficiencies in individual components of the major DNA damage repair pathways (BER and NER) do not generally manifest spontaneous cancer phenotypes. This contrasts with deficiencies in MMR (reviewed in [168,169]) or proofreading [81–84] which, by themselves, confer strong spontaneous cancer phenotypes in the absence of DNA damaging agents or other cancer-susceptibility genes. Thus, MMR and Pol proofreading are different from most DNA repair pathways studied in mice in that the simple loss of either pathway alone is sufficient to cause spontaneous cancer with almost 100% penetrance.

3.1. DNA damage repair

A number of laboratories have produced mice with mutations in genes that affect repair of endogenous DNA damage (summarized in [167]). Knockouts of uracil-DNA glycosylase (*Ung/Udg*), O⁶-methylguanine-DNA methyltransferase (*Mgmt*), 3-methyladenine-DNA glycosylase (*Aag/Apg*), 8-oxoguanine-DNA glycosylase (*Ogg1*), or NER genes do not result in a significant spontaneous cancer predisposition, although indolent lung tumors are observed in *Xpc* mutant mice late in life [170]. Mice defective for *Mth1* (an 8-oxo-dGTPase) or *Myh* (an adenine DNA glycosylase that repairs A:8-oxo-G mispairs) have relatively weak spontaneous cancer phenotypes [171–174]. However, when *Myh*-null mice are exposed to oxidative stress, the incidence and numbers of intestinal tumors are dramatically increased [174]. *Mgmt*-deficient mice as well as mice lacking components of the NER pathway also exhibit conditional cancer-susceptibility phenotypes when exposed to exogenous DNA damaging agents [22,167,175–177].

Some repair gene knockouts reveal phenotypes when combined with defects in other cancer-susceptibility genes [167,178–181] or combined with defects in redundant repair pathways. A good example of redundancy is in the repair of 8-oxo-G lesions. Mice with deficient *Ogg1* or *Myh* alone develop few tumors and live normal lifespans [172–174,182,183]. In contrast, the combined loss of *Ogg1* and *Myh* results in a high spontaneous tumor incidence and cancer mortality [173]. Thus, different pathways of repair often have overlapping functions that can influence the biological impact of a single class of DNA lesions [71].

3.2. Mismatch repair

Soon after the discovery of MMR defects in Lynch syndrome, several groups generated and characterized mouse strains with knockout and knockin alleles of the MMR genes (reviewed in [168,169]). Many aspects of the Lynch syndrome phenotype are recapitulated in mice deficient for *Msh2*, *Mlh1*, or *Msh6*. Homozygous mutant mice display increased tumor susceptibility, consisting primarily of gastrointestinal tract tumors and lymphomas. Mouse embryonic fibroblasts obtained from *Msh2* or *Mlh1* knockouts have increased frameshift and base-substitution mutation rates and exhibit resistance to DNA alkylating agents much like human MMR-deficient tumor cells [80,184,185]. Tumors as well as somatic tissue in these

mice show microsatellite instability [186] and increased mutant frequencies as revealed in transgenic reporter systems [187–190].

It is tempting to conclude that cancer results from the mutator phenotype associated with loss of MMR. However, as discussed above, MMR proteins also play an important role as sensors of DNA damage that signal apoptosis [67,144–148]. This has important implications for tumorigenesis [191]. In addition to acquiring a mutator phenotype, inactivation of MMR reduces the apoptotic response to endogenous and exogenous sources of DNA damage. The combination of a mutator phenotype and defective apoptosis is expected to accelerate the reiterative process of mutation and clonal expansion that drives tumor progression [5]. While a mutator phenotype will affect any rapidly dividing cell, defective apoptosis will have the greatest impact in tissues under genotoxic stress (*e.g.*, gut, skin, lungs and inflammation). Thus, MMR-defective tumors may result from loss of mismatch repair, loss of apoptosis or both, and the relative contribution of these factors likely varies in different tissues. Studies of mice carrying mutations that selectively inactivate the mismatch repair activities of Msh2 or Msh6 but preserve their damage-response functions indicate that the increased mutation rate in MMR-deficient cells is sufficient to drive tumorigenesis [192,193] and that defective apoptosis can accelerate this tumorigenesis [193].

While the mouse MMR gene knockouts have a phenotype similar to that found in Lynch syndrome patients, there are also notable differences. The *Msh2* and *Mlh1* knockout mice primarily develop lymphomas (which are not generally found in Lynch syndrome) as well as intestinal adenomas and adenocarcinomas. However, a major difference is that only homozygous null mice (*i.e.*, *Msh2*^{-/-} and *Mlh1*^{-/-}) develop tumors, while the heterozygotes do not. Lynch syndrome patients carry heterozygous mutations of the MMR genes, and with rare exceptions [194], normal somatic tissues from these individuals do not show evidence of microsatellite instability or increased mutation rate. There are a number of factors that could account for these discrepancies: life span, diet, environmental exposures, differences in human and mouse somatic cell biology or DNA repair pathways.

3.3. Polymerase proofreading

The studies of MMR-deficient mice clearly demonstrate that MMR genes function as tumor suppressors and suggest that unrepaired polymerase errors may contribute to tumorigenesis. To more directly examine the role of polymerase errors in cancer, we created mice with inactivating point mutations in the proofreading exonuclease domains of DNA Pols δ and ϵ and examined the effects of defective proofreading on spontaneous mutation and tumorigenesis [81–84].

Our initial studies focused on Pol δ [81,82]. We used a knockin strategy to introduce a single mutation in *Pold1*, the gene encoding the catalytic subunit of Pol δ . This mutation (designated *Pold1*^e) eliminates a catalytic carboxylate residue (D400A) in the exonuclease domain of Pol δ that is required for proofreading activity. The analogous substitution in yeast Pol δ (D407A) confers a strong spontaneous mutator phenotype [72] characterized by elevated base-substitutions and ± 1 frameshifts. We observed that *Pold1*^e inactivates the 3'→5' exonuclease of mouse Pol δ and causes a recessive mutator and cancer phenotype. Greater than 95% of homozygous *Pold1*^{e/e} mice spontaneously develop cancer and die by 15 months of age (Fig. 4A). Most of the cancers are epithelial in origin, and one-third of the animals develop tumors in more than one tissue. Interestingly, the prevalent cancers in *Pold1*^{e/e} mice (thymus, skin & lung tumors, but not intestine) differ from those observed in MMR knockout mice (thymus and intestinal tumors, but not skin; [168,169]; Fig. 4B). Thus, MMR is rate-limiting for tumorigenesis in intestinal epithelium and both thymic and splenic lymphocytes, while Pol δ proofreading is rate-limiting in lung epithelium, skin epidermis and only thymic lymphocytes. This study shows that mammalian Pol δ proofreading

suppresses spontaneous mutation and tumor development and strongly suggests that unrepaired polymerase errors contribute to carcinogenesis.

Using a similar allelic replacement strategy, we recently engineered mice with defective Pol ϵ proofreading [83]. In this case, two amino acid substitutions (D272A and E274A) were introduced into the highly conserved Exo I motif of the Pol ϵ exonuclease domain. The encoding allele, *Pole^e*, is equivalent to yeast *pol2-4*, which selectively inactivates proofreading while preserving normal polymerase activity [73]. To directly compare the effects of defective Pol ϵ proofreading, Pol δ proofreading and MMR, mutant congenic mouse strains were generated in the C57BL/6 genetic background. We found that homozygous loss of Pol ϵ proofreading confers a strong, base-substitution mutator phenotype and high incidence of spontaneous neoplasms. Surprisingly, *Pold1^{e/e}* and *Pole^{e/e}* mice exhibit very different survivals and cancer susceptibilities (Fig. 4). Consistent with our initial findings, *Pold1^{e/e}* mice died with thymic lymphomas, lung adenocarcinomas and skin squamous cell carcinomas during the first year of life. In contrast, *Pole^{e/e}* mice developed spontaneous intestinal adenocarcinomas, histiocytic sarcomas and nonthymic lymphomas and survived up to 24 months of age. The combined loss of both Pol δ and Pol ϵ proofreading (*Pold1^{e/e} Pole^{e/e}*) further accelerates cancer mortality, while proofreading/MMR double mutant mice die *in utero* [83]. Strong cooperativity between proofreading and MMR was also demonstrated in MMR nullizygotes expressing heterozygous proofreading alleles; these mice rapidly succumb to aggressive metastatic lymphomas [84]. Taken together, these studies distinguish Pol ϵ and δ functions *in vivo* and reveal tissue-specific requirements for DNA replication fidelity. Moreover, they demonstrate strong synthetic interactions between proofreading and MMR in mammals.

The tissue-specific cancer phenotypes of *Pole^{e/e}* and *Pold1^{e/e}* mice suggest that Pol ϵ and Pol δ proofreading participate in separate pathways of tumor suppression that are contingent on cell type. Several molecular and cellular mechanisms may contribute. Pols ϵ and δ are involved in DNA damage repair and lesion bypass [71,101]. Thus, Pol ϵ may preferentially protect intestinal epithelium from natural carcinogens in the gut, while Pol δ may be required for faithful repair or bypass of DNA damage unique to skin. Cell lineage-specific differences in the expression levels of each polymerase, of MMR or other repair pathways that interact with Pol ϵ or Pol δ would similarly shape the cancer phenotype. There is also evidence that Pols ϵ and δ can function independently in the cell [110,118], perhaps replicating different regions of the genome that encode tissue-specific oncogenes or tumor suppressors. And these cancer genes may have “hotspots” that are preferentially mutated by one polymerase or the other. Finally, tissue specificity may not be a direct consequence of increased spontaneous mutation [8]. Secondary functions of proofreading exonucleases [60,61,195–197] and actions of Pol ϵ in cell cycle control [115] and gene silencing [198] could modify cell turnover and oncogenesis in select tissues. Other models have been proposed to explain the unique cancer spectrum caused by defective MMR [199]. Additional studies are needed to identify mechanisms that govern tissue-specific DNA replication fidelity and somatic disease.

4. Pol δ and ϵ mutations in human cancer

The studies of Pol δ and ϵ proofreading-deficient mice underscore the importance of DNA polymerase fidelity in the suppression of spontaneous tumorigenesis. It is reasonable to speculate that polymerase mutator alleles also increase the risk of human cancers [200,201]. The mouse studies suggest roles for Pol ϵ mutators in the genesis of intestinal tumors and Pol δ mutators in skin and lung carcinogenesis. Pol ϵ and δ mutators are also implicated in hematologic malignancies, and aggressive lymphomas or other pediatric neoplasms may result from the combined loss of proofreading and MMR. In addition to proofreading

defects, mutations affecting base selectivity by Pols δ and ϵ also confer mutator phenotypes in yeast [85,86,202] and mice [203]. Thus, similar mutations or polymorphisms could be oncogenic in humans.

4.1. Single-nucleotide polymorphisms (SNPs) in Pol δ and Pol ϵ genes

The catalytic subunits of human Pols δ and ϵ are encoded by *POLD1* and *POLE*. Review of the NCBI dbSNP (www.ncbi.nlm.nih.gov/SNP) and NIEHS geneSNP (www.genome.utah.edu/genesnps) databases currently identifies 18 SNPs in *POLD1* and 33 SNPs in *POLE* that result in amino acid changes (Fig. 5). Several SNPs (*POLD1* G321S and E673K; *POLE* N336S) are located in Pol or Exo motifs, which are conserved between diverse species and contain important residues for polymerase and exonucleolytic proofreading functions [60,204,205]. Only four SNPs (*POLD1* R849H and R1086Q; *POLE* F695I and E1577A) have been modeled in yeast, and none were found to be mutators in the presence or absence of MMR [206].

There are relatively few studies examining the cancer risk associated with non-synonymous SNPs in *POLD1* or *POLE*. *POLE* E2140K has been associated with an increased risk of breast cancer [207], but not cervical cancer [208]; A252V is associated with decreased overall survival in lung cancer patients [209]; and N1396S is not associated with meningioma risk [210]. The *POLD1* R119H SNP has been reported in multiple genome-wide SNP studies, and no association was detected with meningioma, bladder or breast cancer risk [210–213]. Surprisingly, most studies looking for associations with increased cancer risk have studied *POLD1* or *POLE* intronic or synonymous coding SNPs that are unlikely to affect polymerase expression or fidelity.

4.2. Pol δ variants in cancer

Several *POLD1* mutations have been found in colorectal cancer cell lines and primary tumors [214,215], and one mutation was identified in a rat hepatoma cell line [216] (Fig. 5). The MMR-deficient human cancer cell lines HCT15 and DLD-1 carry four heterozygous mutations that result in non-synonymous amino acid changes in Pol δ : G10V, R506H, R689W, and S746I [214,215]. Shcherbakova *et al.* [206] recently used a yeast system to study phenotypes conferred by R506H and R689W, which are positioned in Pol δ 's Exo III and Pol III motifs, respectively. They found that R506H was a mild mutator (2.5-fold over wild-type rates) in an MMR-defective background, while R689W was lethal in haploid cells. Heterozygous R689W did not influence the spontaneous mutation rate in diploid yeast proficient for MMR; MMR-deficient diploids were not examined [206]. Interestingly, haploid or diploid R689W yeast expressing low levels of wild-type Pol δ exhibited extreme rates of spontaneous mutation, and the purified Pol δ R689W enzyme was highly error-prone [206]. The R648Q substitution present in rat hepatoma cells [216] is also located in a conserved polymerase region (equivalent to human R652Q next to Pol VI), and this substitution affects the biochemical properties and fidelity of partially purified Pol δ [216–219].

Two additional *POLD1* mutations, D502N and R990L, were identified in primary colorectal cancer samples [214,215]. Both alleles were also present in surrounding normal colon tissue. Thus, these variants could be low-incidence SNPs, pre-neoplastic mutations in the colonic epithelium, or novel inherited mutations. D502N was introduced into homologous positions of Pol δ in yeast (D506N and E508N) and found not to be a mutator in the presence or absence of MMR (Singh and Preston, unpublished data).

Lastly, a *POLD1* frameshift mutation, which results in an early stop codon, was identified in the colon cancer cell lines HCT116 and SW48 [215]. The resultant Pol δ lacks two

conserved DNA binding domains in the carboxy terminus and is expressed at decreased levels in mutant cells [215]. Depletion of Pol δ or Pol α is known to increase insertion/deletion events and chromosomal instability in yeast [220–224]. Alleles impacting mouse Pol δ activity also increase spontaneous mutation rates and chromosomal instability and accelerate tumorigenesis [203]. Taken together, these findings suggest that decreased polymerase fidelity or activity may play a role in tumorigenesis in humans. A weakness of many studies examining tumor cell lines, however, is the lack of control DNA from uncultured tumor samples and normal tissues from the patient. Thus, it is unclear whether the observed *POLD1* mutations were present *in vivo* or acquired during establishment of the cell lines.

Interestingly, most heterozygous *POLD1* mutations are found in MMR-deficient tumors and cell lines. This may simply be a bias due to the limited number and types of human tumors analyzed for *POLD1* mutations. Alternatively, heterozygous mutations in polymerase genes may be more oncogenic when MMR is also defective. In yeast and mammalian cells, heterozygous mutations that affect the fidelity of Pols δ or ϵ result in mild mutator phenotypes [75,83,130,225]. However, when MMR is inactivated in yeast that are heterozygous for these mutator polymerases, the mutation rate is synergistically elevated [75,159,225]. Similarly, mice heterozygous for polymerase proofreading and nullizygous for MMR exhibit extreme mutator phenotypes and rapidly develop aggressive disseminated lymphomas ([84] and unpublished results). Thus, heterozygous mutations in human *POLD1* or *POLE* may have an enhanced tumorigenic effect in a MMR-deficient cell. Moreover, SNPs that mildly decrease polymerase fidelity could have strong mutator effects in cells that have epigenetically silenced MMR.

4.3. Pol ϵ variants in cancer

Very few studies have examined human tumors for mutations in the Pol ϵ genes. Zhou *et al.* surveyed cancer-associated *POLE* or *POLE2* mutations in tumors from breast, colon and brain [226–229]. Analyses of ~160 breast tumor samples revealed no mutations in the highly conserved regions of *POLE* [226]. However, when the survey was expanded to include the coding region and flanking intronic sequences of *POLE2*, the gene for Pol ϵ 's 55-kD subunit, they found an intronic 4-bp deletion in breast and colorectal tumors that was not present in control tissues from the same patients [227,228]. In contrast, no *POLE2* mutations were found in a series of brain tumors [229]. The authors propose that this *POLE2* site may be a mutational hotspot in some cancers, although the distance of this intronic deletion from the nearest exon suggests it is not pathogenic. Additional work is required to determine if this small *POLE* intronic deletion affects splicing or expression of *POLE2*, supporting its role in tumorigenesis.

5. Summary and perspectives

At the beginning of the 20th century, cytogenetic techniques revealed the first chromosomal abnormalities in malignant cells and led to the hypothesis that genetic instability fueled cancer [230,231]. Since then it has become clear that multiple changes in the genome, including point mutations, insertion/deletion events, large chromosomal rearrangements and epigenetic modifications of gene expression dysregulate cellular pathways on the road to malignant transformation [1–3,13,14]. In cultured human cells, the introduction of only three oncogenes is sufficient for malignant transformation, and rodent cells can be transformed with as few as two oncogenes [232]. Kinetic analyses of age-related tumor incidences in humans suggests 4 to 6 events are required *in vivo* [233]. Recent genome-wide sequence analyses show that, although a small number of mutations may be required for tumor formation, most cancer cells contain significantly more: as many as 50,000 clonal mutations

[234–237] and a large, undetermined number of non-clonal mutations that occur randomly throughout the genome of each cancer cell [238,239].

Loeb *et al.* proposed the “mutator hypothesis” in 1974 to reconcile the number of mutations needed for carcinogenesis and the low spontaneous mutation rate of normal cells [6]. He argued that a cell must acquire a mutator phenotype, through defects in DNA replication or repair, early in the evolution of a tumor. As described in this review, multiple pathways function to maintain genetic integrity in normal cells. Accordingly, tumors exhibit different types of genetic instability. These can be categorized into three general classes: 1) chromosome instability (CIN) involving gross chromosomal aberrations (translocations, large deletions, gene amplifications, chromosome losses or gains), 2) microsatellite instability (MSI or MIN) involving widespread expansions and contractions of short tandem repeat sequences (“microsatellites”), and 3) point mutation instability (PIN) involving subtle nucleotide sequence changes (base substitutions, small deletions/insertions). Chromosome instabilities in cancer are readily revealed through cytogenetic and molecular analyses and are abundantly documented in the literature. Microsatellite instability is also relatively easy to detect and is observed in cancers associated with loss of mismatch repair as typified by Lynch syndrome. In contrast, nucleotide sequence instabilities are experimentally difficult to detect, and this difficulty has limited the search for PIN mutator phenotypes in tumors [25,26].

PIN is a potentially important pathway to cancer. Nucleotide sequence changes activate oncogenes and inactivate tumor suppressors, and at least one human cancer syndrome (Lynch syndrome with defective Msh6) is associated with PIN, minimal MSI and no CIN. The recent demonstration of increased spontaneous cancer in proofreading-deficient mice [81–84] provides additional evidence that PIN causes cancer and suggests that other PIN-associated cancers will be discovered in humans as improved methods of detection are developed. Additional studies are needed to screen tumors for DNA polymerase variants that may be error-prone. Because Pols δ and ϵ are required for cell viability, error-prone variants will likely result from missense mutations and not large insertions, deletions, translocations or LOH that would disrupt vital polymerase functions. Thus, it will be necessary to sequence the coding regions of *POLD1*, *POLE* and other essential “fidelity genes” to detect mutator variants. Mutations affecting conserved regions of Pol δ and Pol ϵ are of interest, particularly loss-of-function alleles that disrupt proofreading, which is not essential for cell viability.

Finally, cooperation of polymerase mutators with reduced MMR may significantly impact cancer risk and tumor progression. As tumor cells evolve and transit through new microenvironments, changing selective pressures will favor high mutation rates, which increase the likelihood of genetic adaptation. However, after adaptation to a new niche or tissue subcompartment in the body, these mutator cells will progressively lose fitness as they accumulate deleterious mutations in essential genes. Thus, mutators may rise and fall in tumor populations during the course of cancer progression [240]. Transient mutator phenotypes are possible via epigenetic silencing of repair genes and the emergence of mutator suppressors.

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Fig. 1.

Determinants of DNA replication fidelity. Schematic of a DNA replication fork with Pol ϵ and Pol δ on the leading- and lagging-strands, respectively. Major determinants of faithful DNA synthesis are highlighted in yellow. The polymerase domains (POL) of Pols ϵ and δ discriminate between correct and incorrect dNTPs prior to phosphodiester bond formation. If an error occurs, these are corrected primarily by the intrinsic proofreading exonuclease (EXO) present in each polymerase. Errors that escape proofreading are rectified by mismatch repair (MMR), which acts on both lagging (shown here) and leading (not shown) strands. DNA damage repair and dNTP pool ratios also influence replication fidelity.

Fig. 2.

Pathways correcting DNA polymerase errors. During DNA synthesis, rare polymerase errors [base•base mispairs (left) or primer•template slippage (right)] impede primer extension and thus trigger transfer of the growing DNA strand from the polymerase active site (POL) to the 3'→5' exonuclease active site (EXO) where the errant bases are excised by proofreading. Errors that escape 3'→5' exonucleolytic proofreading are corrected, at least in part, by two partially redundant pathways of the MMR system. Studies in yeast indicate that Pol proofreading and MMR act in series along a common pathway [75,76]. MutS α and MutS β have overlapping substrate specificities, and MutS α also recognizes DNA damage and signals apoptosis. [Note: mammalian Pms2 (shown here) is equivalent to yeast Pms1.]

Fig. 3. DNA “lesions” arising spontaneously in mammalian cells. (A) Spontaneous chemical decay of deoxyribonucleic acid. Rates are expressed as the number of decay events estimated to occur per mammalian cell per day under physiological conditions ([97,98]; T. Lindahl, personal communication). (B) Distribution of DNA lesions formed per cell per day in dividing mammalian cells. Values for hydrolysis, oxidation and methylation products are from panel A. Polymerase errors (100,000 or more per cell division) are based on measurements of Pol δ and Pol ϵ error rates *in vitro* in the absence of proofreading as described in the text and footnote 1. This assumes that actively dividing cells replicate approximately once every 24 hours. Shorter or longer cell cycles will occur *in vivo* depending on cell type, tissue, stage of embryonic development, and responses to environmental conditions. Panel B shows the distribution when there are 100,000 polymerase errors per day, but this could be as high as 1,000,000 errors per day (see footnote 1).

Fig. 4.

Survival and cancer phenotypes of Pol δ and 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, *Pole^{e/e}* (n=36) and *Pole^{+/-}* (n=35) in C57BL/6 genetic background after removal of the neomycin selection cassette (*Neo*⁻); light red, *Pole^{e/e}* (n=35) and *Pole^{+/-}* (n=45) in a mixed C57BL/6:129/Sv genetic background with the neomycin selection cassette still present (*Neo*⁺); blue, *Pold1^{e/e}* (n=40) in C57BL/6 (*Neo*⁻); purple, *Pole^{e/e}Pold1^{e/e}* (n=35) in C57BL/6 (*Neo*⁻); black, wild-type (WT; n=37) C57BL/6; green, *Mlh1^{Δ/Δ}* (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^{Δ/Δ}* and 34 *Pole^{e/e}Pold1^{e/e}* mice. [†]Tumors with $\geq 15\%$ incidence in one or more groups. Figure from Albertson *et al.* [83].

Fig. 5.

Pol δ and Pol ε amino acid changes encoded by human SNPs and found in tumor cells. Alignment of human Pol δ and Pol ε (H.s. Pol d and H.s. Pol e, respectively). To generate this alignment, yeast and human Pol δ and ε sequences were first aligned as described [205], and conserved amino acid residues were highlighted as follows: red, absolutely conserved among the four sequences; yellow, identical in the majority of sequences; gray, similar in majority of sequences. Yeast sequences were then removed from the figure for clarity. Conserved polymerase and exonuclease motifs are indicated by colored frames: blue, exonuclease motifs; green, polymerase motifs [204]. The zinc finger in Pol ε (residues 2146–2246) is underlined with component cysteines highlighted in green. Amino acid changes encoded by SNPs are placed above (Pol δ, black) or below (Pol ε, cyan) the alignment at the relevant positions. Data are from the NCBI dbSNP (www.ncbi.nlm.nih.gov/SNP) and NIEHS geneSNP (www.genome.utah.edu/genesnps) databases. Pol δ changes found in human colorectal cancer cell lines or primary tumors [214,215] are highlighted in gray above the Pol δ sequence. Asterisk (*) marks the amino acid substitution found in rat Novikoff hepatoma cells (rat R648Q equivalent to human R652Q [216]).