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POLE Proofreading Defects: Contributions to Mutagenesis and Cancer

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Abstract

DNA polymerases are uniquely poised to contribute to the elevated mutation burdens seen in many human tumors. These mutations can arise through a number of different polymerase-dependent mechanisms, including intrinsic errors made using template DNA and precursor dNTPs free from chemical modifications, misinsertion events opposite chemically damaged template DNA or insertion events using modified nucleotides. While specific DNA repair polymerases have been known to contribute to tumorigenesis, the role of replication polymerases in mutagenesis in human disease has come into sharp focus over the last decade. This review describes how mutations in these replication DNA polymerases help to drive mutagenesis and tumor development, with particular attention to DNA polymerase epsilon. Recent studies using cancer genome sequencing, mutational signature analyses, yeast and mouse models, and the influence of mismatch repair on tumors with DNA polymerase mutations are discussed.

Keywords

DNA polymerase; mutagenesis; tumorigenesis; exonuclease proofreading

Introduction

DNA polymerases (Pols) are key enzymes involved in DNA replication and repair *in vivo*. Pols are phylogenetically classified into six families (A, B, C, D, X, and Y) based on amino acid sequence similarity. In humans, there are 15 identified DNA polymerases, which belong to the A-, B-, X-, and Y-families. DNA replication in eukaryotes requires the cooperative action of three DNA polymerase enzymes (Pols α , δ and ϵ), each of which is a member of the B family. Human Pol ϵ is a heterotetramer of p261 (POLE gene), p59 (POLE2), p17 (POLE3), and p12 (POLE4) subunits [1, 2]. The p261 subunit consists of an N-terminal domain containing the conserved polymerase and 3'→5' exonuclease subdomains. POLE

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also encodes a large C-terminal domain that is required for interaction with the three smaller subunits [3]. Human Pol δ is also a heterotetramer, comprised of p125 (POLD1), p66 (POLD3), p50 (POLD4) and p12 (POLD4) subunits. The p125 subunit is similar to the N-terminal half of Pol ϵ p261, in that it also contains polymerase and exonuclease domains. While the main purpose of this review is to discuss results specific to Pol ϵ , crucial differences from and similarities to Pol δ will be described. A number of excellent recent reviews can be consulted for more detailed descriptions of Pol δ [4–7].

DNA Replication

Pol α , along with its tightly associated primase subunits, is responsible for initiating DNA synthesis, including each Okazaki fragment on the lagging strand. The bulk of replication DNA synthesis is then carried out by Pols δ and ϵ . A large body of work over the last decade has demonstrated that normal replication fork synthesis duties are divided by strand: Pol δ is responsible for lagging strand synthesis with Pol ϵ responsible for leading strand synthesis [7–13]. The initial reports characterizing this division of labor at the replication fork made note that alternatives to this architecture were possible and not incompatible with these models. In fact, earlier reports showed the viability in yeast, albeit very sickly, of pol2–16 mutants, which completely lack the ability of Pol ϵ to synthesize DNA [3, 14]. This indicated that Pol δ leading strand synthesis was formally possible. Pol δ has been shown to replicate both strands under specific conditions, like fork restart via recombination [15]. Several recent reports have suggested that Pol δ is actually involved in initiating leading strand DNA synthesis [16–18]. Upon DNA helix unwinding at the origin, Pol α -primase initiates leading strand synthesis upstream of the origin. The evidence then points to Pol δ synthesis, either directly from the Pol α product or strand displacement synthesis on this product within 100–200 nt, handing off to Pol ϵ -CMG for bulk leading strand synthesis. These proposed mechanisms involve priming the leading strand in a fashion that essentially looks like Okazaki fragment synthesis, thus obviating the need for a dedicated Pol for initiating leading strand synthesis.

In addition to DNA polymerase activity, Pols δ and ϵ also possess 3'→5' exonuclease activity, which detects and removes primer 3'-terminal nucleotides misincorporated by the polymerase domain. This editing function enhances the fidelity of several replicative DNA polymerases by about 200-fold [19–21]. Use of *lacZ* forward mutation assays has shown that this 3'→5' exonuclease activity also enhances the *in vitro* replication fidelity of human Pol ϵ [22]. Interestingly, cancer-associated mutations affect the 3'→5' exonuclease activity of Pol ϵ , but to varying degrees [22, 23]. Two very recent studies are beginning to expose how these cancer mutations might be differentially affecting polymerase enzymology and cellular function [24, 25]. Parkash et al. showed that the yeast P301R mutant (equivalent to the human P286R mutant described below) is a more active polymerase, not only on standard DNA templates, but also for extending mispaired primer termini and bypassing hairpin structures [24]. The mechanism through which this might occur is shown through a companion structural study, where the mutant Arg residue bulges into the primer binding cleft in the exonuclease active site, thus preventing partitioning of the primer [25]. The authors speculate that this mutant maintains the enzyme in a hyperactive polymerization state, with particular mutagenic consequences for the nascent replication fork.

Human Pol ϵ also readily inserts and extends from incorporated ribonucleotides [26], while the exonuclease activity excises a matched primer 3'-terminal rNMP with a 3-fold higher rate than a corresponding dNMP [26]. This is notable as unrepaired ribonucleotides incorporated during replication can be mutagenic [27–32]. A series of elegant studies has now shown that both Pol ϵ and Pol δ , as well as Pol α , naturally incorporate rNMPs into genomic DNA during replication, along with the consequences that arise when genomic rNMP removal is compromised (reviewed in [33]). The extent to which this occurs in POLE and POLD1 tumors has not been explored.

POLE Mutations in Human Cancers

DNA polymerase dysfunction is linked to genome instability and cancer [34–40]. Since Pol δ and Pol ϵ are responsible for the vast majority of replicative DNA synthesis, any process that compromises their fidelity might be expected to have profound influence on mutagenesis and tumor development. While this has generally been shown to be the case, a number of critical questions remain that will be discussed in this review. These include determining precisely how a polymerase gene mutation leads to tumors with incredibly high mutation burdens. It is also not well understood how mutations in POLE affect mutagenesis and tumor development differently than do mutations in POLD1.

With the advent of low-cost, next-generation sequencing technology, it first became possible, and is now commonplace, to perform targeted, whole-exome (WES) or whole-genome (WGS) sequencing on sets of tumors. Since the first report in 2011 [41], several hundred case studies, retrospective reports, and international collaborative studies have identified more than 600 human tumors with mutations in the catalytic subunits of POLD1 and POLE [42–47]. POLE mutations are recurrent in endometrial (7–10%) and colorectal (~2%) cancers, but also occur at lower frequency in a wide range of cancers [48, 49]. A challenge now is to distinguish between POLE mutations that are responsible for driving a mutator phenotype from POLE mutations that are non-pathogenic, or passengers. The emerging consensus is that POLE mutation drivers can be defined by their occurrence in hypermutated tumors, those with >10 mutations per megabase (Mut/Mb). A query of cBioportal [50, 51] of all The Cancer Genome Atlas (TCGA) PanCancer studies, comprised of 10,967 samples as of Nov. 2018, showed 92 POLE exonuclease domain mutations in hypermutant tumors. While this is 0.84% of all tumor types, the distribution is highly uneven with enrichment in endometrial (9.7%), colorectal (2.2%), stomach, adrenocortical, pancreatic (1–2% each) and lesser but still prevalent occurrence in prostate, renal, bladder, cervical squamous cell carcinoma, melanoma and head and neck cancers (Fig. 1). Pan-cancer analysis also shows P286 and V411 are hotspots across tumor types (Fig. 2). POLD1 mutation prevalence follows the same trend as POLE with a slight enrichment in melanoma (Fig. 1). POLD1 hypermutant tumors occur in both the exo and pol domain with equal frequency (Fig. 2). The reasons for this frequency disparity are unknown, but several POLD1 pol domain mutations have been shown to cause increases in dNTP pools that contribute to replication infidelity [31, 52].

The first large-scale cancer genome sequencing study to identify POLE tumor mutations used whole-exome and whole-genome sequencing on DNA from 276 colorectal (CRC)

tumors. In this 2012 study, the TCGA Network identified a subset (16%) that was both hypermutated (i.e. mutation burden ≥ 10 Mut/Mb) and showed evidence of microsatellite instability (MSI) [47]. Another subset (3%) had tumor mutation burdens exceeding 100 Mut/Mb and contained POLE mutations. These POLE mutations were clustered in the exonuclease domain. Several subsequent studies found POLE mutations to be enriched in polyposis-unrelated early-onset CRC and either absent or greatly reduced in late-onset CRC, suggesting POLE mutations may contribute to cancer development in younger patients [53–56]. POLE-specific mutations have also been found in adenocarcinomas of the upper gastrointestinal tract with high mutational burden [57].

In a 2013 TCGA analysis of 373 endometrial carcinomas (EC), 7% of the tumors were found to have mutations in the POLE exonuclease domain and mutation burdens exceeding 100 Mut/Mb [44]. Recurrent hotspot mutations in POLE were identified for the first time at P286R and V411L (Table 1). Subsequent studies confirmed these hotspot mutations in South East Asian and Canadian women, among other studies [58, 59]. Analysis of British patients found POLE mutations in non-malignant precursors of EC and CRC, which suggest somatic POLE mutations are early/initiating events in EC and CRC [60]. POLE mutations confer very high progression-free or recurrence-free survival, especially when compared to MSI or chromosomal unstable tumors, though the reasons for this remain unclear [44, 58–61]. Two recent reports studying POLE endometrial tumors, one with a single patient sample [62] and one with a much larger combination of several sets [60], showed that POLE mutations are detectable in adjacent early neoplasia along with the main tumor. The larger study reanalyzed the TCGA data along with their own WGS to show that the POLE mutations are early, likely clonal/driver mutations, and that the POLE mutation signature is present in subsequent driver genes. These results support the idea that POLE mutations are early, driver events, at least in endometrial cancer. They further showed that the increased immune infiltrate seen in POLE tumors is also seen in the adjacent hyperplasias, specifically in areas that retain the POLE mutation.

Additional studies found POLE exonuclease domain mutations in uterine serous carcinoma and an inherited multiple adenoma defect with predisposition to CRC, but without evidence of MSI [43, 45, 63–65]. Also identified was D275A, a substitution in an invariant ExoI residue that coordinates Metal A in the exonuclease active site and is critical for the 3'→5' excision reaction [22, 66, 67]. In a Chinese study looking at adenocarcinomas in non-small cell lung cancer cohort, most of the POLE tumors had elevated mutational burden and higher levels of CD8-positive tumor infiltrating lymphocytes when compared to tumors that lacked the POLE mutation [68]. In another study, analysis of POLE-mutated lung cancers in TCGA tumors revealed that the POLE mutation in squamous cell carcinoma had better overall survival, but did not in appear to confer an advantage in adenocarcinoma patients [69].

In a large pan-cancer study of 80,000 patients, Campbell *et al.* found that 15% of adult somatic tumors are hypermutated [42]. Across all adult tumor types, 0.6% had mutation burdens >100 Mut/Mb, many of which had POLE or POLD1 mutations. The number of patients in this study permitted unambiguous assignment of “driver” or “passenger” status to POLE and POLD1 mutations based on mutation burden in multiple tumor samples that each contain the same POLE mutation. For example, all tumors with P286R mutations in this

cohort had ~100 Mut/Mb. Tumors with A426V mutations, on the other hand, consistently had <10 Mut/Mb, thus making this POLE mutation highly unlikely to drive the mutator phenotype. Based on the nature of the amino acid change and the resulting mutation burden, this study then classified POLE mutations into several categories: invariable, insensitive and sensitive. Invariant mutants (e.g. V411L) are residues that only change to one other residue and result in mutation burdens near or above 100 Mut/Mb. Insensitive mutants (e.g. S461P, S461T) can change to multiple amino acids, each resulting in similarly high mutation burdens. Sensitive mutants can also change to different amino acids, but the mutation burden is dependent on the nature of the change. L424I and L424P tumors are classic ultramutated tumors, while L424V tumors have mutation burdens between 5–10 Mut/Mb. Another sensitive mutant of note is the D275 catalytic exonuclease residue. D275A and D275G tumors have mutation burdens well over 100 Mut/Mb, while D275V mutants are consistently 10-fold lower. Each change eliminates one of the catalytic exonuclease residues, yet the resulting mutagenesis varies over 10-fold. The causes of this mutational sensitivity are unknown, but perhaps reflect unknown underlying biochemical and/or structural differences.

POLE and POLD1 exonuclease mutations are also found as inherited germ-line alterations. WGS was performed on tumors from individuals diagnosed with 10 CRC adenomas prior to age 60 along with tumors from a paired first-order relative also diagnosed with CRC. These studies discovered POLE L424V and POLD1 S478N familial mutations responsible for the elevated tumor mutation burden [45]. These POLE mutations are associated with increased risk of developing polyposis, termed polymerase proofreading-associated polyposis (PPAP), and early-onset CRC [45, 70, 71]. A separate study of 529 kindred participants with either familial nonpolyposis CRC or polyposis, found that POLE/POLD1 mutation carriers are characterized by attenuated polyposis and CRC, while POLD1 carriers are at higher risk for endometrial and breast tumors [70]. A study of 155 Spanish patients with multiple polyps or early-onset CRC found a novel POLE variant, V474I, was predicted to be pathogenic and was functionally validated using *S. pombe* [72]. Estimated cumulative risk of developing CRC in POLE germline carriers is slightly higher in men than women, 40% and 32%, respectively. Likewise, the risk of CRC in POLD1 germline carriers is 63% in men and 52% in women [73]. In a familial case of CRC, WES of germline DNA revealed the novel POLE T278L mutation that is immediately adjacent to the catalytically active E277 residue. Affected members of this family presented with several dozen to over 100 colorectal adenomas, CRC including multiple diagnoses in specific individuals, as well as breast and ovarian cancer [74]. WES from four CRC tumors showed clear POLE mutation signature 10 (discussed below). The breast tumor lacked significant POLE mutation signature, but showed evidence of two mismatch repair (MMR)-associated signatures as well as loss of MLH1 and PMS2 by immunohistochemistry. This mutant was also shown to be a mutator in yeast. These results are notable for two reasons. First, they clearly show that sequential loss of POLE proofreading followed by MMR loss can drive hypermutated tumors. Delineating the order of inactivation can help to better understand the mechanisms driving tumor development. Second, based on their proximity to a catalytic exonuclease residue they provide supporting evidence that heterozygous loss of the exonuclease proofreading activity itself can possibly drive tumorigenesis. This is important because a growing body of

evidence, particularly from yeast and discussed further below, suggests that something more complicated than simple loss of exonuclease activity is responsible for the hypermutant phenotype.

The rich mutational datasets from tumor WGS and WES studies has permitted analyses of mutational signatures. Mutational signatures have long been known in certain cancers with known etiologies. For example, C→T transitions at Cs flanked by an adjacent pyrimidine are enriched in UV-exposed cancers like melanoma. Pan-cancer analyses have derived several dozen major mutational signatures as defined by their trinucleotide context [75–77]. The Catalog Of Somatic Mutations In Cancer (COSMIC) database identified and catalogued these signatures along with prevalent cancer types and etiologies for each signature, if known. For example, from over 12,000 WES/WGS samples, COSMIC signature 7 was identified as strong bias in C→T transitions with 5' and/or 3' flanking pyrimidines (e.g. CCT→CTC, TCG→TTG, etc.) enhanced in skin cancers as well as oral and head and neck squamous cell carcinomas. Thus, this type of analysis is a powerful tool to identify potential biochemical mechanisms for observed mutational outcomes. Tumors with exonuclease domain mutations in POLE have shown a strong enrichment in a unique mutational signature that distinguishes them from other cancers. TCT→TAT and TTT→TGT transversions along with TCG→TTG transitions are both significantly elevated in POLE tumors. This pattern was designated signature 10 in the COSMIC database and is found a number of different cancer types, with enrichment in colorectal and uterine cancers. While it has been shown biochemically that POLE P286R *in vitro* has a bias for TCT→TAT transversions over the complementary AGA→ACA transversions [13], the precise mechanism whereby POLE exonuclease domain mutations give rise to this specific mutational outcome remains unknown.

Mismatch Repair and POLE Mutations

The role of mismatch repair in POLE tumor development remains a critical unanswered question. Loss of MMR alone can cause genome instabilities that can lead to cancer [78–81]. Since MMR is responsible for correcting replication errors, its loss in the context of polymerase mutants with reduced fidelity could greatly accelerate tumor development. Understanding how MMR function or dysfunction affects Pol ε-dependent mutagenesis is essential to our understanding of the mechanisms of tumor development.

Inactivating MMR in the context of POLE exonuclease mutants causes a synergistic increase in mutation rate in yeast [67, 82–84] and drives accelerated tumor development in mice [85]. The first reported human POLE tumor mutation, F367S, also contained a mutation in MutL homolog (MLH1) (L512I) and showed evidence of MSI [41]. The POLE mutant tumors identified by TCGA [44, 47] can be subdivided into the following categories based on MMR status: no mutation in any mismatch repair gene, mutation in a MMR gene and epigenetically silenced MLH1. Mutations in MutS homolog (MSH6) are particularly overrepresented in MMR genes in POLE tumors. These MSH6 mutations in POLE tumors cluster in the clamp and lever domains, which make non-specific DNA contacts adjacent to the mismatch and transduce signals from the mismatch to the ATPase domain, respectively [86]. Whole-exome sequencing of the NCI-60 panel of commonly used cancer cell lines [87]

identified the POLE P286R mutation in the HCC2998 cell line. This cell line also contains a mutation in the lever domain of MSH6 (K885N), similar to the TCGA findings and consistent with a role for MSH6 loss in accelerating Pol e tumor development. MMR-deficient human cell lines engineered to express exonuclease-inactivating active site alanine mutations are mutators [88]. However, restoration of MMR in these POLE exo-deficient cells is sufficient to suppress the mutator phenotype.

Several detailed studies have now shown that polymerase exonuclease deficiency combined with MMR loss in humans is compatible but tumorigenic [42, 46, 89, 90]. Patients with biallelic (or constitutional) mismatch repair disorder (bMMRD/cMMRD) are born with biallelic inactivating mutations in MMR genes. These are most commonly MSH6 or PMS2 mutations, unlike the MLH1 and MSH2 defects commonly seen in Lynch syndrome patients. A subset of bMMRD patients can develop aggressive pediatric brain tumors with a spontaneously acquired a POLE or POLD1 exonuclease domain mutation. WGS and WES from these tumors indicated an estimated mutation rate per cell doubling consistent with complete loss of MMR and polymerase proofreading *in vitro* [46]. The mutation signature identified in POLE-bMMRD tumors differed somewhat from the COSMIC signature 10 seen in sporadic POLE tumors and has been seen in subsequent studies. Haradhvala *et al.* [90] used a variant of NMF to identify novel mutation signatures from a dataset that contained a significant number of POLE/MSS, POLE/MSI-L and POLE/MSI-H endometrial tumors. Campbell *et al.* [42] used hierarchical clustering on their 80,000 tumor dataset tumors and found POLE/MSS and POLE/MSI clusters. Taken together these studies made several important observations. First is that POLE mutant tumors with no evidence of MMR loss generate mutations most closely resembling the COSMIC signature 10. POLE tumors with MSI generate a lower percentage of these signature 10 mutations along with a novel set of signatures that together somewhat resembles signature 14. Haradhvala *et al.* speculate that these mutations could represent errors that are corrected by either proofreading or MMR pathways and that their presence in the tumor would require inactivation of both pathways together. Based on the relative allelic fractions of these signature mutations, both studies were able to identify tumors in which POLE mutation preceded MMR loss. Further, Haradhvala *et al.* showed evidence that this MMR loss occurred either by epigenetic MLH1 inactivation or by MMR gene inactivation. Campbell *et al.* showed that MMR loss can precede POLE mutation in sporadic tumors as well as in bMMRD tumors. Another interesting observation was that mutations made in the POLE mutant tumors had a slight bias toward late-replicating regions that was not observed in POLD1 mutant tumors, consistent with a temporal division of labor that has been suggested by immunostaining studies [91].

In the endometrial tumor cohort from TCGA, P286R and V411L POLE mutations are underrepresented in tumors designated as MSI-H by a standard five locus PCR-based assay. While this seems to suggest that these particular mutations are incompatible with MMR defects, one study used an in-house genome-wide indel caller to show that V411L tumors, while enriched in MSI-L, actually contain indel burdens similar to MSI-H tumors [92]. Interestingly, this same genome-wide indel measurement showed that the MSI-L P286R tumors actually have low indel abundance. They speculate that perhaps the V411L POLE mutant tumors are in fact MSI-H, while the P286R mutant tumors are MSS, but, perhaps

because of the tumor high mutation rate, contain random mutations in the Bethesda microsatellite loci that resulted in misclassification.

We analyzed five studies comprised of 8,000 tumors of different types and found 1,144 tumors that had microsatellite status and WGS/WES reported for each sample, 64% of which were MSS and 36% were MSI (Fig. 3) [44, 47, 51, 93, 94]. Of these tumors, approximately 3% of MSS and 10% of MSI tumors also had a POLE mutation while 6% of MSI tumors had POLD1 mutations. When we compared the incidence of polymerase gene mutation to the overall tumor mutation burden in these samples, 21 MSS and 27 MSI POLE exo mutant tumors had hypermutant tumor mutation burden. The overall tumor mutation burden in POLE exo domain tumors is similar in both MSS and MSI tumors (Fig. 4). There are also 1 MSS and 11 MSI tumors with mutations in the POLE pol domain and no exo domain mutation. These POLE pol domain mutant MSI tumors actually have a significantly lower mutation burden than the POLE exo domain MSI tumors. In fact, this mutation burden is similar to MSI tumors lacking any POLE mutation, suggesting these pol domain mutations contribute little if any to mutagenesis in these tumors. Unlike POLE tumors, the 24 POLD1 MSI tumors have a similar mutation burden regardless of whether the mutation is in the exo or pol domain. What is striking, however, is the complete absence of POLD1 mutations in MSS tumors. This was noted previously for the smaller TCGA colorectal cancer set [95], but the current analysis expands to a larger number of POLD1 mutations across multiple tumor types and still none are MSS. One possibility is that POLD1 MSS tumors do arise, but the mutation rate is so high as to be lethal. Another possibility is that as cells acquire POLD1 mutations, they do not acquire a hypermutant phenotype since MMR is more efficient on the lagging strand [96–98]. The order of POLD1 mutation and MMR loss is not known. The absence of POLD1/MSS tumors suggests POLD1 mutation occurs after MMR loss. A close measurement of allelic frequency and mutation signature would be very helpful in resolving this.

Immune System: Surveillance and Checkpoint Blockade Therapy

A number of recent studies suggest that better overall survival and prognosis observed in patients with POLE-mutant and MSI tumors is due to an upregulated immune response. This upregulation is likely due to the increased mutagenesis creating large numbers of potential neoepitopes [99], which then recruit tumor-infiltrating lymphocytes (TILs) [99, 100]. POLE-mutated and MSI tumors in endometrial, colorectal, lung and brain cancers have shown increases in TILs over POLE-wild type and MSS tumors [55, 60, 63, 101–104]. An in-depth analysis of CRC patients determined that measuring lymphocyte type and abundance in a tumor is a better predictor of prognosis than MSI status in MSI cases [105]. These links to immune response led in part to investigating POLE-mutant and MSI tumor responses to immune checkpoint blockade therapies, like α -PD-1, α -PD-L1 and α -CTLA-4. Better response to immunotherapy is now understood to correlate with a number of factors, including high mutation burden, high numbers of neoantigens, high immune cell infiltrate and low clonality [106]. These relationships are extensively explored in a number of excellent reviews [106, 107]. Case reports of MSI and POLE-mutated EC, CRC, and pediatric glioma and glioblastoma multiforme reported encouraging responses to immune checkpoint inhibitors [102, 104, 108, 109]. It is now clear that high mutation burden alone is

insufficient to predict POLE-mutant response to immune checkpoint blockade therapy as POLE patients have been observed in both responding and non-responding groups. MSI and POLE-mutant responding tumors had significantly higher densities of CD8+ TILs than the non-responding tumors [104]. Levels of Th1-type CD4+ lymphocytes were also higher in responders, suggesting a more complicated effect. While much more needs to be understood, the opportunity for targeted immunotherapy in tumors from patients with POLE mutations or loss of mismatch repair is quite high as evidenced by a number of proposed and ongoing clinical trials.

Models of POLE Mutagenesis and Tumorigenesis

The use of model systems is critical to understanding human tumorigenesis and developing diagnostic and therapeutic techniques. Yeast and mouse models using mutant alleles of DNA polymerase genes have been particularly important in deciphering the contributions of polymerase dysfunction to disease [110–113]. Proofreading impairment in either Pol ϵ or δ has long been known to cause increased mutagenesis using reporter genes in yeast [83, 84, 114–116]. The original exonuclease-deficient POLE mutant in yeast, pol2–4, was a targeted inactivation of two acidic metal-binding residues critical for driving 3'→5' exonuclease catalysis. This impaired proofreading is synthetic lethal with complete, but not partial, inactivation of mismatch repair [82, 117]. The multiplicative increase in mutation rate when pol2–4 is combined with partial MMR loss supports the idea that POLE replication errors are subsequently corrected by MMR in series. In a series of elegant experiments, Preston and Herr showed that there is a ceiling on the mutation rate that is compatible with normal life [116, 118]. Single or combinations of mutations exceeding this ceiling either trigger cell death or require suppressor mutations to lower the mutation rate to tolerable levels. Indeed, suppressor mutations arise both in cis (i.e. within the polymerase gene itself) and trans (i.e. within different genes) [30, 116, 117, 119].

The simple model that developed to explain the high mutation burden in POLE and POLD1 tumors was that loss of proofreading increased the abundance of replication errors, perhaps by saturating MMR. Recent experiments using yeast have helped show that this simple model does not tell the full story. P286R is by far the most recurrent POLE mutant in human tumors (Fig. 2). This mutant was shown to compromise steady state exonuclease activity *in vitro*, but to roughly the same extent as the pol2–4 mutation [13]. Haploid yeast containing the P286R mutation, however, saw mutation rates increase by 150-fold over wild type using a forward mutation reporter [120]. This is 50-fold over the exo active site pol2–4 mutant. More importantly, this same study showed the P286R allele is a strong mutator (27-fold) even when present as a heterozygous allele in diploid yeast, the state most closely resembling the presumed tumorigenic state. This is unlike the pol2–4 allele, which is insufficient to drive strong mutagenesis as a heterozygous allele. More recent work modeling a panel of human POLE mutants in yeast has very nicely shown a gradient of POLE mutant allele-dependent mutagenesis [121]. The common thread being that only mutant alleles exposed to the DNA binding cleft induce mutagenesis. All cleft mutants increase the mutation rate equal to or higher than that measured in the pol2–4, suggesting that a function more complex than simple loss of exonuclease activity is likely driving mutagenesis. Recent structural and functional studies using the P286R equivalent mutant in

yeast lend support to this idea. Mutant alleles that protrude into this cleft cause hyperactive DNA synthesis on normal primer-template substrates as well as mispaired termini and those that undergo strand displacement synthesis [24, 25].

One curious finding from these studies concerns the V411L mutant. This allele is not located in the exonuclease single strand DNA binding cleft and does not drive a mutator effect in yeast, yet it is the second most recurrent POLE mutant found in tumors. The authors mention several intriguing possibilities, including that V411L may actually occur with high frequency based on the presence of a mutagenic hairpin sequence in the genomic sequence itself. Taking these yeast results together with the POLE/MSI studies discussed above, perhaps the V411L mutant requires MMR deficiency to drive mutagenesis in yeast. Further genetic and biochemical characterization of this intriguing mutant will be critical to our full understanding of how POLE mutants drive tumorigenesis.

POLE proofreading was first shown to play a direct role in suppressing tumorigenesis using the pol2-4 allele engineered into a mouse model [85, 110]. Mice with the homozygous $Pole^{(D275A/E277A)/(D275A/E277A)}$ mutation had two engineered exonuclease-compromised alleles and saw an accelerated cancer mortality, with 50% mortality at around 15 months. The equivalent POLD1 homozygous mutant mice, however, had a much more aggressive cancer phenotype, with 50% survival around 6 months, similar to that of complete loss of MMR [110, 111, 122]. The situation is different in humans, though, as the all POLE mutations to date have been found to be heterozygous with no evidence of LOH. Interestingly, heterozygous $Pole^{WT/(D275A/E277A)}$ mice did not develop tumors at a rate different than controls [44, 47, 110]. This absence of accelerated tumorigenesis is consistent with our own finding that diploid heterozygous $Pole^{WT/(D275A/E277A)}$ cells are weak mutators in the presence of MMR, both in yeast and in human cells [88].

This past year marks the first report of a human POLE cancer allele engineered into a mouse model. Li *et al.* engineered a conditional P286R knock-in mutation in mice [123]. $POLE^{+/P286R}$ in mice drives rapid tumorigenesis in multiple target tissues, including T cell lymphomas, angiosarcomas, small cell lung cancer and several adenocarcinomas in the endometrium and colon. All mice died in less than 10 months with an average of 2 tumors per mouse. This is considerably more aggressive than even the homozygous $POLE^{(D275A/E277A)/(D275A/E277A)}$ mutation. The construct design permitted testing viability and tumor incidence in mice expressing only the mutant P286R POLE via a hemizygous allele ($POLE^{P286R/null}$). $POLE^{P286R/null}$ mice were essentially embryonic lethal, with less than 5% of the expected number of offspring were born (n=2 vs. expected 41). The two viable $POLE^{P286R/null}$ mice were both minute and died from thymic lymphomas within two months. This situation could arise via suppressor mutations elsewhere in the genome, much like has been seen in yeast with mutator POLE mutations [117]. WGS sequencing from 11 primary tumors, from both $POLE^{+/P286R}$ and $POLE^{P286R/null}$ mice, showed tumor mutation burdens in the same >10 Mut/Mb seen in human tumors with POLE mutants. The WGS data are particularly interesting in that the POLE signature 10 is not recapitulated in this mouse model. Signature 10 is comprised largely by three specific trinucleotide-specific mutations: $TCT \rightarrow TAT$, $TCG \rightarrow TTG$ and $TTT \rightarrow TGT$. Enrichment is seen for C $\rightarrow$ A transversions at TCT (4–9% of the total base pair substitutions), however this enrichment is also seen in

MEFs with POLE^{+/+} genotype (4–7% of total base pair substitutions). The T→G transversion enrichment in the TTT context is observed in 0 of 6 POLE^{+/P286R} MEF single cell clones and 3 of 4 POLE^{P286R/null} tumors. The mutation signatures of heterozygous POLE^{+/P286R} tumors were not reported, and no evidence of C→T transitions at TCG was observed in any of the samples. These differences could be explained by several possibilities that will be of great interest to solve. One possibility is that, while general replication infidelity may be increased solely by presence of the POLE mutant, additional factors are needed to help drive the very specific signature (several possible sources are described below). Another possibility that cannot currently be excluded is that the unique strain construction in the current model is influencing mutagenesis and tumorigenesis to an unknown degree. These POLE^{+/P286R} mice were generated by crossing POLE^{+/LSL-P286R} hemizygous mice made in the 129SvEvTac background with the DDX4-Cre mice made in the FVB background. Our lab has generated a P286R knock-in mouse in a different mouse strain that has a similar cancer mortality phenotype, but has clear evidence of the POLE-dependent mutation signature, including TCG→TTG (unpublished results). While no phenotypes were reported for the POLE^{+/LSL-P286R} hemizygous mice [123], humans are sensitive to Pol ε gene dosage, with measureable reductions in immune function and stature [124, 125].

POLE cancer alleles have also been shown to be mutators in mouse cell lines. Using fluctuation assays in independent MEF lines, Albertson *et al.* showed that the homozygous POLE^{(D275A/E277A)/(D275A/E277A)} mutation caused a more than 70-fold increase in the mutation rate almost exclusively due to an increase in base pair substitutions [110]. The mutation frequency was also elevated at a reporter transgene in several different tissues from adult POLE^{(D275A/E277A)/(D275A/E277A)} mice. A more recent study used CRISPR/Cas9 gene editing to generate mouse embryonic stem cell lines containing several different POLE mutants, including D275A/E277A, P286R, S297F and V411L, as homozygous mutations [126]. Similar to what was seen before, the homozygous POLE^{(D275A/E277A)/(D275A/E277A)} mutant conferred a modest mutator phenotype to the cells. The POLE^{P286R/P286R} clones were 2–3-fold stronger mutators than the active site inactivating mutant. These results are similar to what was seen in yeast, strengthening the argument that some function beyond exonuclease-deficiency is playing the major driving role in mutagenesis and likely tumorigenesis. One observation from these studies is that the mutant frequency of individual clones made of the same mutant can vary by over 100-fold. An implication, consistent with yeast POLE mutant data, is that different genotypic/phenotypic backgrounds can suppress or activate POLE-dependent mutagenesis. This would have implications for POLE tumor mutational heterogeneity. Another interesting observation is POLE mutants have different sensitivities to nucleoside analogs cytarabine and fludarabine. The POLE^{(D275A/E277A)/(D275A/E277A)} cells are no different than POLE wild type cells, while POLE^{P286R/P286R} and POLE^{V411L/V411L} cells are sensitive. These results have obvious potential clinical relevance, while also further supporting the idea of something more complicated than strict exonuclease impairment underpinning the mutagenesis.

POLE Accessory Subunits and Genome Stability

In addition to mutations affecting catalytic activity, mice with alterations affecting the Pol ϵ holoenzyme display altered phenotypes including increased tumorigenesis. When the POLE4 accessory subunit is disrupted, POLE4^{-/-} mice exhibit skeletal dysmorphias and immune defects [127] reminiscent of human patients with facial dysmorphism, immunodeficiency, livedo, and short stature (FILS syndrome) [124]. FILS syndrome patients contain mutations in POLE and POLE2 that reduce the gene dosage of the active Pol ϵ holoenzyme complex. Cells from POLE4^{-/-} mice also contain reduced active Pol ϵ complex protein levels. In addition, these mice show origin activation defects that contribute to HU sensitivity and increased chromosome breaks and rearrangements when challenged with low doses of aphidicolin. One observation from this study also strengthens the argument that strain differences should be carefully considered when measuring phenotypic outcomes in mice. The POLE4^{-/-} allele was essentially embryonic lethal when constructed in a C57BL/6J background. The lethality was rescued in one of two ways: inactivation of p53 or outcrossing to FVB/S129 strain. This rescue is not without cost, as even POLE4^{-/-} p53^{+/-} subsequently developed accelerated lymphomas.

Previous studies have implicated Pol ϵ in playing a role in chromatin and epigenetic inheritance [128–130]. Several recent reports show evidence that the small non-catalytic subunits physically interact with histone H3-H4 and facilitate deposition on the leading strand in both yeast and mice [131, 132]. Elimination of these subunits impairs leading strand-specific deposition of parental H3-H4, helicase unwinding and DNA supercoiling. Since deletion of these subunits in yeast causes a moderate mutator phenotype that is independent of MMR [133], it will be interesting to see if epigenetic defects and mutagenesis are related and whether histone deposition defects play any role in POLE tumorigenesis.

Possible Influence of Modified DNA

Tomkova *et al.* evaluated mutational signatures for replication timing and strand asymmetries using over 3,000 tumors from 19 different types of cancer [134]. Of the 29 mutation signatures evaluated, 21 showed some degree of strand asymmetry, meaning that a given mutational signature showed strand specificity in the regions surrounding origin sequences. The strongest strand asymmetry was seen for signature 10 in tumors with mutant POLE, consistent with POLE replicating the leading strand. However, a weaker abundance of signature 10 was seen in POLE wild type tumors, but with the bias now flipped toward the lagging strand. The authors offer a hypothesis to explain this strand switch: these mutations might actually be from replication errors made at DNA lesions. In tumors with wild type POLE, the mutations occur more frequently in the lagging strand due to less accurate synthesis at these lesions by Pols δ and/or α . In POLE mutant cells, the proofreading deficient Pol ϵ is responsible for the great abundance of mutagenic synthesis at these putative lesions on the leading strand. They also found a smaller degree of strand asymmetry for signature 1 that is consistent with their previous study suggesting that CpG>TpG mutations seen in POLE mutant tumors are due to mutant POLE synthesis across methylated cytosines situated on the leading strand [135]. While TCG>TTG transitions

predominate in POLE mutant tumors, and there is a leading strand bias for these mutations in heavily modified 5methylC regions, the leading strand bias remains even in regions of low modification. It will be interesting to determine if this persisting bias represents regions that are actually highly modified specifically in POLE mutant tumors, if these regions contain some uncharacterized C modification or if some other factor is at work. This remains an important open question considering these transitions in TCG context dominate the POLE mutant tumor mutational landscape.

Summary

It is now clear that a mutation of a single allele in the exonuclease domain of POLE is sufficient to drive tumor development. That simple statement belies a complex relationship between the mutant polymerase enzyme, the particular mutant allele, the genetic background, the tissue involved, the possible nature and abundance of DNA lesions and the resulting mutation burden in that tumor. There are many important mechanistic insights to be gained into what distinguishes POLE cancer mutants from POLE engineered exonuclease inactivating mutations as well as what distinguishes POLE mutants from POLD1 mutants. Other important and outstanding issues remain. To what extent do the following factors play roles in POLE tumor development: rNMPs, histone dysregulation, epigenetics, modified DNA, other protein factors, immune system status and other DNA pols. Addressing these issues is likely to help treatment of polymerase mutant and other hypermutant cancers.

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Abbreviations:

Pol	polymerase
CMG	Cdc45-Mcm-GINS complex
WES	whole exome sequencing
WGS	whole genome sequencing
TCGA	The Cancer Genome Atlas
CRC	colorectal cancer
EC	endometrial cancer
MSI	microsatellite instability
MSS	microsatellite stability
MMR	mismatch repair
PPAP	polymerase proofreading-associated polyposis

bMMRD/cMMRD	biallelic/constitutional mismatch repair deficiency
CRISPR	clustered regularly interspaced short palindromic repeats
dNTP	deoxyribonucleotide triphosphate
dNMP	deoxyribonucleotide monophosphate
rNMP	ribonucleotide monophosphate
Mb	megabase
Mut/Mb	mutations per megabase
MSH6	MutS homolog
MLH	MutL homolog
PMS2	postmeiotic segregation
MEF	mouse embryonic fibroblasts
PD-1	programmed cell death 1
PD-L1	programmed cell death 1 ligand 1
CTLA-4	cytotoxic T-lymphocyte associated protein 4
TIL	tumor infiltrating lymphocytes
Th1	T helper cell

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Figure 1.

POLE and POLD1 exonuclease domain mutation frequencies in select cancer types.

Frequency data was collected from TCGA PanCancer Atlas studies and included 10,953 patients (10,967 samples) as of November 2018 48,49. POLE and POLD1 mutations were scored positive for those with hypermutant tumors (reported tumor mutation burden 10 mutations/Mb). Hypermutant frequency was determined as a percentage of all TCGA tumors of that particular cancer.

Figure 2.

Location and occurrence of POLE and POLD1 exonuclease and polymerase mutations. Data was collected from TCGA PanCancer Atlas studies 48,49. Only variants identified to have POLE and POLD1 exonuclease or polymerase domain missense mutations and were associated with hypermutant status (>10 mutations/Mb) were included, regardless of MSI status. The height of the lollipop represents the number of times the mutation was reported cBioPortal/TCGA PanCancer Atlas.

Figure 3.

Proportion of MSS and MSI tumors containing POLE or POLD1 mutations. Five studies that reported MSI status, tumor mutation burden and POLE/ POLD1 mutation status were used in this analysis 42,45,48,49,102,103. We compiled only those POLD1 and POLE variants that were found to contain each of the following: 1) a POLE or POLD1 missense mutation in the exonuclease or polymerase domain; 2) hypermutated (≥ 10 mutations/Mb); and 3) reported microsatellite status (MSS or MSI) determined either through locus PCR, IHC, genome sequencing, or some combination thereof.

Figure 4.

Mutation burden of POLE and POLD1 tumors subdivided by mutation location and by MSI status. Tumors described above (Figure 3) were categorized by the gene mutated (POLE or POLD1), the domain containing the mutation (exo or pol) and microsatellite status (MSS or MSI). Mutation burden per megabase was plotted for each sample. For each category, mean (+), median (–) and 25–75 percentile are shown. For comparisons of the means between two groups, unpaired two-tailed t-test and Welch’s correction for nonequal SD was used.

Significance: (**) denotes p value = 0.0019, and (ns) denotes p value = 0.1510 between the means. Analysis was conducted using GraphPad Prism 7.

Table 1.
POLE mutation occurrence in human tumors.

POLE mutations discussed in the text are shown along with their domain location, cancer types they are observed in and references cited. CRC, colorectal; EC, endometrial; GBM, glioblastoma multiforme; AA, anaplastic astrocytoma; PN, primitive neuroectodermal tumor

Mutation	Domain	Cancer Type	Reference
D275A	Exol	CRC	42
T278L	Exol	CRC, EC	50, 51, 74
P286R	Exol	CRC, EC, Breast, Pancreatic	42, 44, 50, 51, 92
S297F	between Exol-II	EC, GBM, Cervical	44, 50, 51
F367S	Exoll	CRC	41, 50, 51
V411L	between Exoll-III	CRC, EC, GBM, Cervical	42, 44, 45, 50, 51, 92
L424V	between Exoll-III	CRC, EC	42, 45, 50, 51
S459F	ExoIII	CRC, EC, AA	46, 50, 51
S461P	ExoIII	EC, PN	42, 46, 50, 51