

## A Common Cancer-Associated DNA Polymerase $\epsilon$ Mutation Causes an Exceptionally Strong Mutator Phenotype, Indicating Fidelity Defects Distinct from Loss of Proofreading

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### Abstract

Exonucleolytic proofreading and DNA mismatch repair (MMR) act in series to maintain high-fidelity DNA replication and to avoid mutagenesis. MMR defects elevate the overall mutation rate and are associated with increased cancer incidence. Hypermutable colorectal and endometrial tumors with functional MMR were recently reported to carry amino acid substitutions in the exonuclease domain of DNA polymerase  $\epsilon$  (Pol $\epsilon$ ). This created a notion that loss of the proofreading activity of Pol $\epsilon$  is an initiating cause of some sporadic human cancers. In this study, we identified a somatic P286R substitution in the conserved ExoI motif of Pol $\epsilon$  in a collection of 52 sporadic colorectal tumor specimens. This change has been repeatedly observed in colorectal and endometrial tumors in previous studies despite many possible ways to inactivate Pol $\epsilon$  proofreading. To understand the reasons for the recurrent appearance of the P286R variant, we characterized its functional consequences using the yeast model system. An analogous substitution in the yeast Pol $\epsilon$  produced an unusually strong mutator phenotype exceeding that of proofreading-deficient mutants by two orders of magnitude. This argues that the P286R mutation acts at some level other than loss of exonuclease to elevate cancer risk. Heterozygosity for the variant allele caused a strong mutator effect comparable with that of complete MMR deficiency, providing an explanation for why loss of heterozygosity is not required for the development of Pol $\epsilon$ -mutant human tumors. *Cancer Res*; 74(7); 1895–901. ©2014 AACR.

### Introduction

Cancer cells contain thousands of mutations not present in normal cells. Changes in oncogenes and tumor suppressor genes directly promote carcinogenesis. In addition, the genomic instability leads to a genetic heterogeneity of tumors, contributing to their ability to adapt and develop resistance to therapy (1). For most cancers, the mechanisms underlying the high frequency of genetic changes are unknown. Maintenance of the low mutation rate relies heavily on three DNA replication fidelity mechanisms: (i) accurate nucleotide selection by the replicative DNA polymerases (Pol)  $\alpha$ ,  $\delta$ , and  $\epsilon$ , (ii) proofreading of errors by the 3'-exonuclease activity of Pol $\delta$  and Pol $\epsilon$ , and (iii) correction of mispairs by the DNA mismatch repair (MMR) system. These processes act in series keeping the mutation rate per cell division at a low level of less than  $10^{-10}$ . Defects in any of the three mechanisms accelerate tumorigenesis in mice (2, 3), and inherited mutations in the human MMR

genes cause predisposition to colorectal cancer in Lynch syndrome (4). The MMR gene *MLH1* is also somatically inactivated in approximately 12% of sporadic colorectal cancer.

A recent large-scale molecular characterization of sporadic colorectal cancer revealed that although MMR-deficient cancers are hypermutated, tumors with the highest mutational load had alterations in Pol $\epsilon$  rather than MMR defects (5). Similarly, hypermutated sporadic endometrial tumors frequently contained Pol $\epsilon$  alterations (6, 7). In both cases, the changes affected conserved amino acid residues in the exonuclease domain, suggesting that inactivation of exonuclease activity was responsible for the hypermutator phenotype. In addition, germline mutations affecting the exonuclease domains of Pol $\epsilon$  and Pol $\delta$  were found to cause a high-penetrance hereditary colorectal cancer predisposition (8). These discoveries strongly suggested that loss of proofreading activity of replicative DNA polymerases is the initiating cause of some hereditary and sporadic human cancers.

In this work, we assessed the status of the exonuclease domain of Pol $\epsilon$  in sporadic colon and rectum adenocarcinomas from 52 patients undergoing treatment at the University of Nebraska Medical Center (UNMC; Omaha, NE). We identified a P286R substitution in the conserved ExoI motif essential for the proofreading activity. This change was observed previously in two other colon tumors (9) and 14 endometrial tumors (6, 7), and is by far the most frequent somatic Pol $\epsilon$  mutation in both types of cancer. The same mutation was found in a colon carcinoma cell line HCC2998 (10). A P286H substitution was

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**Table 1.** Summary of sequence alterations in the *POLE* gene in colorectal adenocarcinoma samples from 52 patients

| Sample ID                   | Age at diagnosis | Tumor site   | Nucleotide change <sup>a</sup> | Amino acid change <sup>a</sup> |
|-----------------------------|------------------|--------------|--------------------------------|--------------------------------|
| T-07-285                    | 33               | Colon        |                                |                                |
| T-99-082                    | 36               | Colon        |                                |                                |
| T-00-028                    | 42               | Colon        |                                |                                |
| T-01-076                    | 47               | Colon        |                                |                                |
| T-08-756                    | 48               | Colon        |                                |                                |
| T-05-498                    | 53               | Colon        |                                |                                |
| <b>T-04-364<sup>b</sup></b> | <b>54</b>        | <b>Colon</b> | <b>C857C/G</b>                 | <b>P286P/R</b>                 |
| T-00-075                    | 54               | Colon        | C755C/T                        | A252A/V                        |
| T-02-465                    | 56               | Colon        |                                |                                |
| T-01-336                    | 58               | Rectum       |                                |                                |
| T-08-225                    | 60               | Colon        | C755C/T                        | A252A/V                        |
| T-98-285                    | 60               | Colon        |                                |                                |
| T-03-418                    | 61               | Colon        |                                |                                |
| T-00-067                    | 62               | Colon        | C755C/T                        | A252A/V                        |
| T-05-616                    | 63               | Colon        |                                |                                |
| T-99-114                    | 64               | Rectum       |                                |                                |
| T-09-130                    | 65               | Colon        |                                |                                |
| T-01-125                    | 66               | Colon        | C755C/T                        | A252A/V                        |
| T-99-040                    | 66               | Rectum       |                                |                                |
| T-04-019                    | 67               | Colon        |                                |                                |
| T-04-099                    | 67               | Colon        |                                |                                |
| T-09-113                    | 67               | Colon        | C1470C/T                       | synonymous                     |
| T-02-578                    | 67               | Colon        |                                |                                |
| T-03-492                    | 68               | Colon        |                                |                                |
| T-07-332                    | 68               | Colon        |                                |                                |
| T-99-068                    | 69               | Colon        | C755T                          | A252V                          |
| T-02-052                    | 69               | Colon        |                                |                                |
| T-10-274                    | 69               | Colon        |                                |                                |
| T-00-102                    | 69               | Colon        |                                |                                |
| T-09-366                    | 71               | Colon        |                                |                                |
| T-08-387                    | 72               | Colon        | C755C/T                        | A252A/V                        |
| T-08-483                    | 72               | Colon        |                                |                                |
| T-97-183                    | 72               | Colon        |                                |                                |
| T-07-595                    | 72               | Colon        |                                |                                |
| T-97-025                    | 73               | Colon        |                                |                                |
| T-04-683                    | 73               | Colon        | G776G/A                        | R259R/H                        |
| T-08-400                    | 75               | Colon        |                                |                                |
| T-09-675                    | 75               | Colon        | C755C/T                        | A252A/V                        |
| T-09-676                    | 75               | Colon        | C755C/T                        | A252A/V                        |
| T-10-257                    | 75               | Colon        |                                |                                |
| T-05-513                    | 76               | Rectum       |                                |                                |
| T-02-459                    | 77               | Colon        |                                |                                |
| T-01-235                    | 79               | Colon        |                                |                                |
| T-02-499                    | 80               | Colon        |                                |                                |
| T-04-727                    | 82               | Colon        |                                |                                |
| T-02-568                    | 86               | Colon        | C755C/T                        | A252A/V                        |
| T-08-129                    | 86               | Colon        |                                |                                |
| T-08-654                    | 87               | Colon        |                                |                                |
| T-97-010                    | 88               | Colon        |                                |                                |
| T-97-122                    | 88               | Colon        |                                |                                |

*(Continued on the following page)*

**Table 1.** Summary of sequence alterations in the *POLE* gene in colorectal adenocarcinoma samples from 52 patients (Cont'd)

| Sample ID | Age at diagnosis | Tumor site | Nucleotide change <sup>a</sup> | Amino acid change <sup>a</sup> |
|-----------|------------------|------------|--------------------------------|--------------------------------|
| T-08-754  | 88               | Colon      |                                |                                |
| T-01-149  | 96               | Colon      | C755C/T                        | A252A/V                        |

NOTE: Sample containing the P286R mutation is highlighted in bold.

<sup>a</sup>Nucleotides and amino acids are numbered from the first codon of *POLE* and the first amino acid, respectively.

<sup>b</sup>Sample T-04-364 contained an additional heterozygous C→T mutation leading to Arg1826Trp substitution, although we could not determine whether it was present in *cis* or in *trans* with the codon 286 mutation. Arg1826 is a nonconserved residue away from the known functional domains, so the significance of this change is presently unclear.

reported in one additional colon tumor (5), and a germline mutation of the homologous Pro327 in Pol $\delta$  was present in a patient with a family history of colorectal cancer (8). The repetitive occurrence of changes at this proline was surprising, because studies in model organisms have demonstrated that proofreading can be inactivated by altering a variety of amino acid residues in the exonuclease domain (11–13). To gain insight into the unique properties of the P286R variant, we studied its genome-destabilizing potential in the yeast model system using several mutational assays. The corresponding P301R change in yeast Pol $\epsilon$  conferred an exceptionally strong mutator phenotype greatly exceeding that of any previously characterized Pol $\epsilon$  mutant, including proofreading-deficient mutants. Moreover, in contrast with mutations inactivating the exonuclease of Pol $\epsilon$ , which barely affects the mutation rate in the heterozygous state, heterozygosity for the P301R produced a strong mutator effect comparable with that of MMR deficiency. The results (i) suggest that the extraordinary mutagenic potential of this Pol $\epsilon$  variant results from functional defects distinct from loss of proofreading; (ii) explain earlier observations that loss of heterozygosity for the *POLE*-P286R mutation is not required for tumor development; and (iii) advocate an idea that the frequent occurrence of P286R in sporadic tumors is related to its stronger mutator effect in comparison with other exonuclease domain changes and the resulting greater chance of accumulating cancer-driving mutations.

## Materials and Methods

Snap-frozen colon and rectum adenocarcinoma tissue and matching normal mucosa were obtained from the UNMC Tissue Procurement Shared Resource. The study was approved by the UNMC Institutional Review Board. Exons 7 to 16 of *POLE* (or all coding exons for the tumor carrying P286R) were amplified by PCR using DNA isolated from the tissue (Supplementary Fig. S1 and Supplementary Table S1) and Sanger sequenced.

Yeast strains used in the study (Supplementary Table S2) are isogenic to E134 (14). Diploids contain the *Kluyveromyces lactis* *LEU2* gene 26 nucleotides downstream of *CAN1* in one copy of chromosome V and a deletion of *CAN1* in the homologous chromosome, allowing for selection of recessive *can1* mutants on medium containing canavanine and lacking leucine. To

replace the chromosomal *POL2* with the *pol2*-P301R allele, an integrative plasmid YIpDK1 was constructed by cloning a 2.1-kb *HpaI*-*EcoRI* *POL2* fragment into *NruI* and *EcoRI* sites of YIp5 (15). The *pol2*-P301R mutation was created in YIpDK1 by site-directed mutagenesis. The spontaneous mutation rate was measured by fluctuation analysis as described previously (14).

## Results and Discussion

Inspired by the association of Pol $\epsilon$  exonuclease domain mutations with intestinal cancer in mice (2) and humans (5, 8, 9), we began this study by analyzing this region of Pole in 52 patients with sporadic colorectal cancer treated at UNMC. The age distribution of the patients and the anatomical site of the tumor are listed in Table 1. Ten of the 52 tumors carried a common variant allele producing an A252V change in Pol $\epsilon$  (nine in heterozygous and one in homozygous state; Table 1). The variant allele frequency did not differ significantly ( $P = 0.11$ ;  $\chi^2$ ) from that reported for the general population (16). One tumor carried an R259H variant, also observed previously in the general population (allele frequency 0.5%–1%). Remarkably, one tumor (T-04-364) had a heterozygous mutation leading to a P286R substitution in the ExoI motif (Table 1; Fig. 1). This change was not present in the publicly available databases of common polymorphisms, but was reported previously as a somatic mutation in 16 other sporadic cancer cases (6, 7, 9) and as a potential cancer-specific variant in a colon carcinoma cell line HCC2998 (10). Other mutations affecting Pro286 in Pol $\epsilon$  or a homologous Pro327 in Pol $\delta$  were also observed in patients with colorectal cancer (5, 8). The P286R change found in tumor T-04-364 was not present in normal cells of the patient (Fig. 1A), indicating that it was a somatic mutation. The recurrent emergence of Pro286 alterations is in sharp contrast with the apparently random incidence in tumors of other changes in the exonuclease domain of Pol $\epsilon$ , most of which were only observed once (5–7, 9).

Pro286 is located at the end of the ExoI motif several amino acids downstream of the carboxylate residues Asp275 and Glu277 that are essential for catalysis of the exonuclease reaction (Fig. 1B). Mutations affecting these carboxylates abolish the exonuclease activity of Pol $\epsilon$  (15), and so do analogous mutations in other proofreading exonucleases (13). In addition, multiple other amino acid residues in the exonuclease domain,

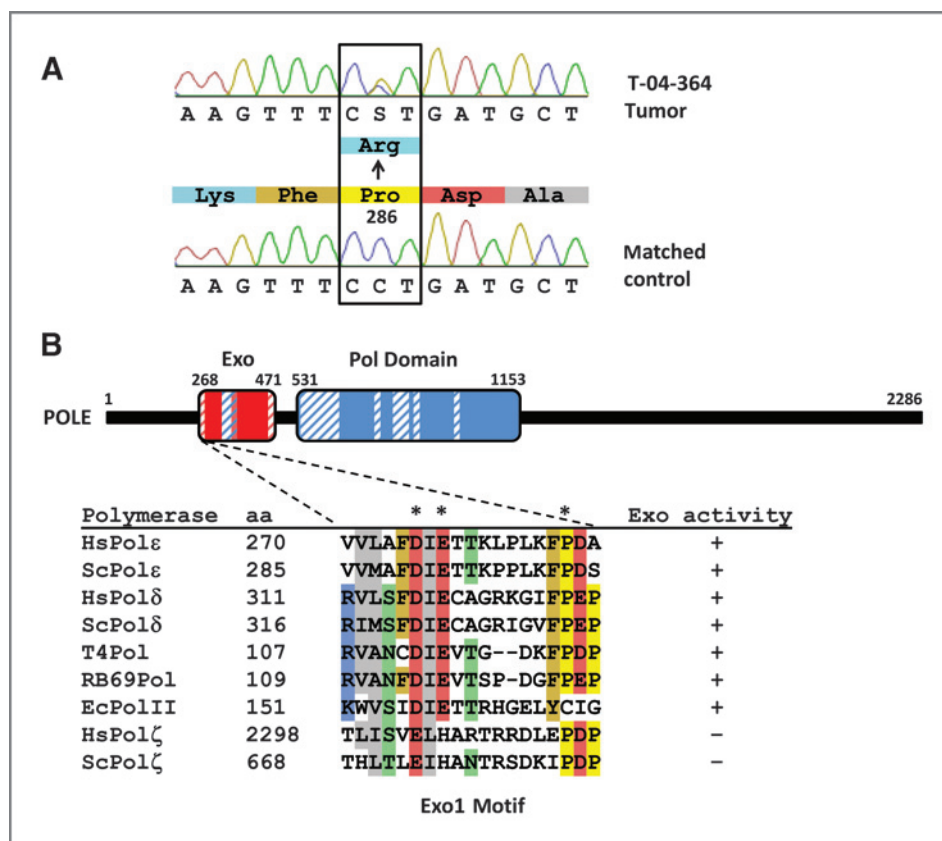


Figure 1. Mutation at codon 286 of *POLE* in a sporadic colon tumor. A, DNA sequencing analysis of sample T-04-364 and matching normal mucosa. B, conservation of Pro286 in B family DNA polymerases. Hatched boxes on the *POLE* schematic show conserved exonuclease and polymerase motifs. Hs, human; Sc, *S. cerevisiae*; Ec, *E. coli*. Asterisks mark Pro286 and the catalytic carboxylates affected by the *pol2-4* mutation.

including those in the conserved motifs ExoII and ExoIII, are known to be essential for proofreading (11–13). To understand why the P286R variant is uniquely observed at a high frequency in tumors, we examined its effects on mutagenesis using the yeast *S. cerevisiae* model system. Haploid and diploid strains with the corresponding *pol2-P301R* allele were constructed,

and mutagenesis was measured at three different reporter loci (Table 2). The Can<sup>r</sup> forward mutation assay scores a variety of base substitutions, frameshifts and larger rearrangements that inactivate the *CAN1* gene. The *lys2-InsE<sub>A14</sub>* and *his7-2* reporters are frameshift alleles that revert to wild-type predominantly via -1 and +1 events, respectively. In haploid *pol2-P301R*

**Table 2.** Rate of spontaneous mutation in haploid and diploid *pol2-P301R* mutants

| Ploidy    | Genotype                     | Can <sup>r</sup> mutation                       |               | Lys <sup>+</sup> reversion                      |                  | His <sup>+</sup> reversion                      |               |
|-----------|------------------------------|---|---------------|---|------------------|---|---------------|
|           |                              | Mutation rate ( $\times 10^{-8}$ ) <sup>a</sup> | Fold increase | Mutation rate ( $\times 10^{-8}$ ) <sup>a</sup> | Fold increase    | Mutation rate ( $\times 10^{-8}$ ) <sup>a</sup> | Fold increase |
| <i>n</i>  | +                            | 15 (10–20)                                      | 1             | 14 (11–16)                                      | 1                | 0.57 (0.42–0.75)                                | 1             |
|           | <i>pol2-P301R</i>            | 2,300 (1,800–2,800)                             | 150           | 610 (450–710)                                   | 44               | 210 (160–250)                                   | 370           |
|           | <i>pol2-4</i>                | 44 (39–51)                                      | 2.9           | 14 (11–15)                                      | 1                | 3.3 (2.6–4.2)                                   | 5.8           |
| <i>2n</i> | +/+                          | 19 (16–24)                                      | 1             | 16 (14–18)                                      | 1                | 0.87 (0.62–0.92)                                | 1             |
|           | <i>pol2-P301R</i> /+         | 510 (430–680)                                   | 27            | 29 (23–53)                                      | 1.8              | 25 (20–31)                                      | 29            |
|           | <i>pol2-4</i> /+             | 41 (37–46)                                      | 2.2           | 24 <sup>b</sup> (19–29)                         | 0.8 <sup>b</sup> | 3.3 (2.5–4.1)                                   | 3.8           |
|           | <i>pol2-P301R/pol2-P301R</i> | 14,000 (6,900–27,000)                           | 740           | 4,100 (2,000–7,500)                             | 280              | 1,400 (590–3,100)                               | 1,600         |
|           | <i>pol2-4/pol2-4</i>         | 97 (73–110)                                     | 5.1           | 22 (18–27)                                      | 1.4              | 7.6 (5.7–8.6)                                   | 8.7           |

<sup>a</sup>Mutation rates are given as medians for at least nine cultures, with 95% CIs in parentheses.

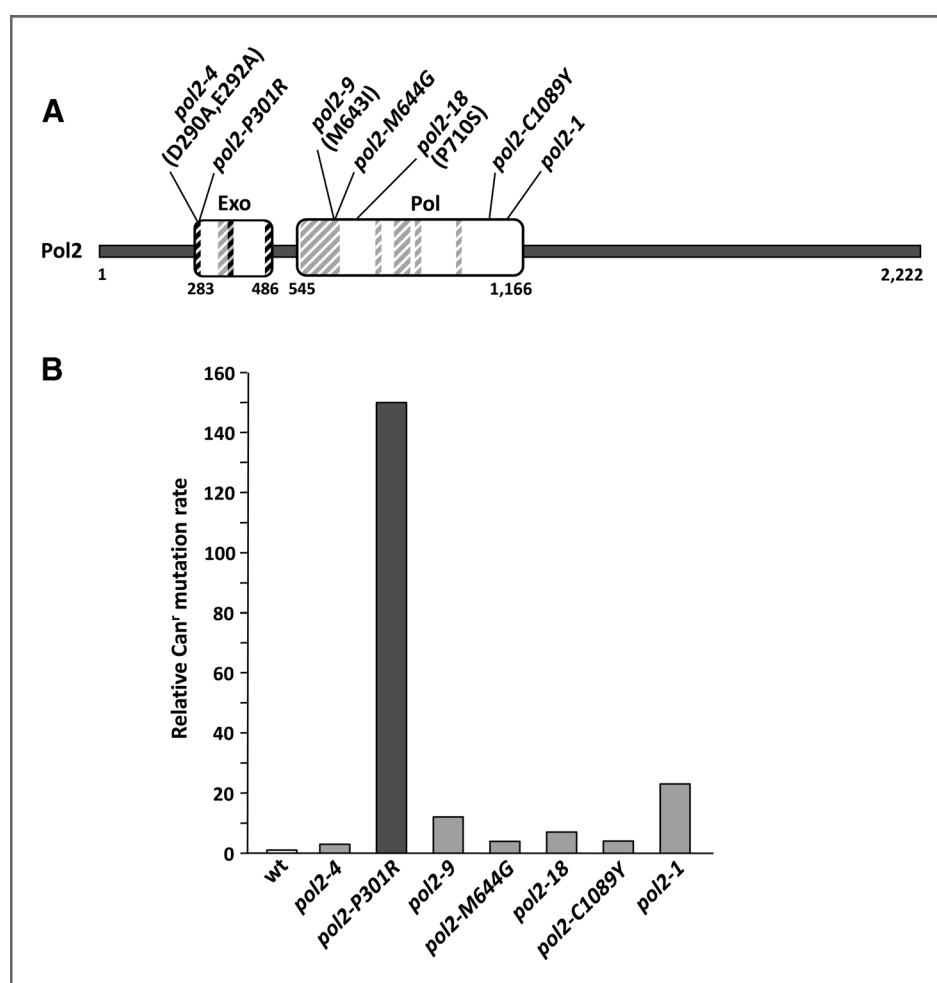
<sup>b</sup>The *pol2-4/POL2* heterozygote contains two copies of the *lys2-InsE<sub>A14</sub>* allele. All other diploids have one copy of *lys2-InsE<sub>A14</sub>* and a nonrevertible mutation at the same position of the *LYS2* gene in the homologous chromosome (Supplementary Table S2). The fold increase shown is the fold difference in the Lys<sup>+</sup> reversion rate between the wild-type and *pol2-4/POL2* diploids divided by two.

mutants, the rate of  $\text{Can}^r$ ,  $\text{Lys}^+$ , and  $\text{His}^+$  events was increased 150-, 44-, and 370-fold, respectively, in comparison with the wild-type strain. Strikingly, for all three markers, the mutator effect of *pol2-P301R* was approximately 50-fold higher than that of the *pol2-4* mutation inactivating Pol $\epsilon$  proofreading due to the replacement of both catalytic carboxylates at the ExoI motif with alanines (15). Thus, despite the location of Pro286/301 in the ExoI motif, its alteration results in fidelity defects substantially more severe than disruption of proofreading. Moreover, the mutator effect of *pol2-P301R* greatly exceeded that of any previously characterized Pol $\epsilon$  mutation, including mutations strongly reducing nucleotide selectivity (Fig. 2).

The mutant *POLE* alleles were always found in the heterozygous state in sporadic human tumors (Fig. 1A; refs. 5, 6, 9). Diploid yeast strains heterozygous for the *pol2-P301R* showed a remarkable increase in the rate of  $\text{Can}^r$  mutation and *his7-2* reversion (27- and 29-fold, respectively; Table 2). This is drastically different from strains heterozygous for the *pol2-4* mutation inactivating proofreading that showed only a 2.2-fold elevated  $\text{Can}^r$  mutation and 3.8-fold elevated *his7-2* reversion. The  $\text{Can}^r$  mutation rate in the *POL2/pol2-P301R* heterozygotes was comparable with that in strains with completely inactivated MMR, which is a well-known cancer-driving replication fidelity

defect. For example, the haploid *mlh1* strain is a 26-fold mutator (14). This suggests that heterozygosity for the *POLE-P286R* seen in tumors can have a potent genome-destabilizing effect and explain the hypermutated phenotype of these tumors. Diploid yeast homozygous for the *pol2-P301R* allele showed extreme hypermutability (a 740-, 280-, and 1,600-fold increase in the rate of  $\text{Can}^r$ ,  $\text{Lys}^+$ , and  $\text{His}^+$  events; Table 2). The dramatic difference in the apparent mutation rate between diploids and haploids is commonly observed when the mutation load approaches a lethal threshold. It is explained by death in haploids of a fraction of cells with the highest level of mutagenesis that survives and produces the majority of mutants in diploids (17–19, and references therein). In contrast, the *pol2-4/pol2-4* diploids are only slightly more mutable than *pol2-4* haploids (Table 2; ref. 19), consistent with a modest level of replication errors and fewer cells accumulating lethal mutations. This further emphasizes the differences between the mutator effects of *pol2-P301R* and the proofreading deficiency.

Because of the location of cancer-associated Pol $\epsilon$  changes at conserved residues in the exonuclease domain, a concept has formed in the past year that loss of Pol $\epsilon$  proofreading is responsible for the development of hypermutated human tumors. The functional consequences of the Pol $\epsilon$  variants



**Figure 2.** Yeast mimic of *POLE-P286R* is the strongest Pol $\epsilon$  mutator known. **A**, location of the known mutator mutations on the yeast Pol2 schematic. Hatched boxes, conserved exonuclease and polymerase motifs. **B**, rate of  $\text{Can}^r$  mutation (relative to wild-type, wt) in haploid *pol2* mutants. Data are from Table 2 and Supplementary Table S3.

found in cancers, however, have not been tested previously. The studies described here indicate that the P286R variant confers fidelity defects well beyond those expected from proofreading deficiency. Pro286 is conserved in a B-family Pol $\zeta$  lacking proofreading activity (Fig. 1B), consistent with its possible other functions. Thus, the higher frequency of P286R in cancers in comparison with other exonuclease domain changes could be explained by its exceptionally strong mutator effect and the resulting increased chances of accumulating cancer-driving mutations. Interestingly, we found the P286R variant in a tumor from a 54-year-old individual, indicating a relatively early cancer onset (the median age at diagnosis in our randomly selected group of patients was 69 with 95% confidence interval (CI) of 66–71; Table 1). Further studies could determine whether this mutation is more frequent in younger patients.

The uniquely strong mutator effect of the yeast P286R analog resembles our earlier observations with an R696W variant of Pol $\delta$ , the only extensively characterized colorectal cancer-associated Pol $\delta$  variant (17). Yeast strains expressing Pol $\delta$ -R696W had a mutation rate at least two orders of magnitude higher than any known Pol $\delta$  mutator and were generally the strongest eukaryotic mutators ever seen. Together with the present studies of Pol $\epsilon$ -P286R, this draws an interesting and unexpected picture wherein the cancer endpoint acts as an efficient selective system for the strongest mutators. Characterization of other replicative polymerase variants present in tumors in future studies will help expand and circumstantiate this view. Long-term expression of a strong mutator phenotype is believed to be disadvantageous, because it leads to an accumulation of deleterious mutations that could impede tumor growth (20). The mutagenic potential of the mutator polymerase variants, however, is buffered by the presence of the wild-type polymerases in the heterozygous tumors. Importantly,

heterozygosity for the mutator alleles is still quite mutagenic (Table 2), because both wild-type and mutator enzymes contribute to replication. Thus, DNA polymerase genes constitute an uncommon class of tumor suppressors, for which loss of heterozygosity is not required to promote tumorigenesis. Although limiting the genome-damaging activity of supermutator enzymes, the heterozygosity also creates opportunities for driving the genome instability by transient spikes of hypermutation due to imbalanced expression of the wild-type and mutant alleles as we discussed previously (17).

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Authors' Contributions

**Conception and design:** D.P. Kane, P.V. Shcherbakova

**Development of methodology:** D.P. Kane

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** D.P. Kane, P.V. Shcherbakova

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** D.P. Kane, P.V. Shcherbakova

**Writing, review, and/or revision of the manuscript:** D.P. Kane, P.V. Shcherbakova

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** D.P. Kane

**Study supervision:** P.V. Shcherbakova

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