A cancer-associated DNA polymerase δ variant modeled in yeast causes a catastrophic increase in genomic instability

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Edited by Philip C. Hanawalt, Stanford University, Stanford, CA, and approved November 4, 2009 (received for review July 7, 2009)

Accurate DNA synthesis by the replicative DNA polymerases α, δ, and ε is critical for genome stability in eukaryotes. In humans, over 20 SNPs were reported that result in amino–acid changes in Polδ or Polε. In addition, Polδ variants were found in colon–cancer cell lines and in sporadic colorectal carcinomas. Using the yeast-model system, we examined the functional consequences of two cancer-associated Polδ mutations and four polymorphisms affecting well-conserved regions of Polδ or Polε. We show that the R696W substitution in Polδ (analog of the R689W change in the human cancer-cell line DLD-1) is lethal in haploid and homozygous diploid yeast. The cell death results from a catastrophic increase in spontaneous mutagenesis attributed to low-fidelity DNA synthesis by Polδ-R696W. Heterozygotes survive, and the mutation rate depends on the relative expression level of wild-type versus mutant alleles. Based on these observations, we propose that the mutation rate in heterozygous human cells could be regulated by transient changes in gene expression leading to a temporary excess of Polδ-R689W. The similarities between the mutational spectra of the yeast strains producing Polδ-R696W and DLD-1 cells suggest that the altered Polδ could be responsible for a significant proportion of spontaneous mutations in this cancer cell line. These results suggest that the highly error-prone Polδ-R689W could contribute to cancer initiation and/or progression in humans.

mutator | SNP | transient hypermutagenesis | DNA synthesis fidelity | colon cancer

Maintaining high-fidelity DNA replication is imperative for cells to avoid mutations that can lead to disease. In eukaryotes, chromosomal DNA is replicated by the concerted action of three DNA polymerases, Polα, Polδ, and Polε, although their exact roles at the replication fork are currently a subject of debate (1). Replication fidelity is maintained by accurate nucleotide selectivity of the three replicative enzymes and the exonucleolytic proofreading functions of Polδ and Polε. Fidelity is further increased *in trans* through the action of the DNA mismatch repair (MMR) pathway. Mutations affecting the fidelity of yeast Polα, Polδ, and Polε result in a spontaneous mutator phenotype (2–6). In the case of Polδ, when specific nucleotide selectivity and proofreading defects were engineered in mice, the resulting increased mutation rate was also accompanied by an accelerated tumorigenesis (7–9). This provided support for the mutator hypothesis for cancer, which states that the expression of a mutator phenotype is an early event in tumorigenesis and that it is required for the accumulation of multiple mutations typically observed in cancers (10).

The aforementioned studies examined DNA polymerase mutations expressly engineered to destroy specific polymerase functions. These are dramatic mutations, which, to our knowledge, have not been identified as naturally occurring in any human cells. In contrast, SNPs are naturally occurring DNA sequence variations that are found in most genes. Whereas some SNPs may have no functional significance, others may affect protein function and/or structure. In the frame of the National Institute of Environmental Health Sciences (NIEHS) Environmental Genome Project, 22 SNPs resulting in nonsynonymous amino acid substitutions in the catalytic subunits of Polδ and Polε have been found with frequencies ranging from 0.6% to 41% in the normal population (http://www.genome.utah.edu/genesnps/). Earlier studies also reported 11 mutations in colon–cancer cell lines and sporadic colorectal carcinomas that change the amino–acid sequence of the catalytic subunit of Polδ (11, 12). The functional consequences of these variants are not known. There is, however, evidence that variants of other DNA polymerases, such as Polγ, Polη, and Rev1, are associated with disease in humans (13). In addition, variants of Polβ found in human cancers were shown to contribute to cellular transformation in mouse cells (13, 14). These findings point to the importance of the functional analysis of naturally occurring DNA polymerase mutations.

In this study, we used the genetically tractable yeast model to examine the effects of Polδ and Polε variants found in human cells on viability, growth, mutagenesis, and DNA damage sensitivity. The results reveal that one Polδ variant (Polδ-R696W, which is analogous to the human Polδ-R689W variant) leads to a catastrophic increase in genomic instability that is incompatible with life in haploid and homozygous diploid cells. We present the genetic and biochemical data suggesting that the mutation catastrophe was caused by extremely low-fidelity DNA synthesis by the altered Polδ.

Results

Effects of SNPs and the Cancer-Associated yR511H/hR506H Mutation of Polδ on Viability, Mutagenesis, and DNA Damage Sensitivity. The structural and functional conservation of the DNA replication machinery provides an opportunity to evaluate the consequences of human DNA polymerase variants by studying analogous mutations in yeast. A similar approach has been used previously for the identification of potentially pathogenic MMR gene variants (15). The application of this approach, by definition, is largely limited to mutations that affect evolutionarily conserved protein domains. Of the 22 Polδ and Polε SNPs identified in the NIEHS Environmental Genome Project (http://www.genome.utah.edu/genesnps/), five are located in conserved regions (Fig. 1*A*). Additionally, the two cancer-associated Polδ mutations (R689W and R506H substitutions), present in the colon cancer

Author contributions: P.V.S. designed research; D.L.D. and T.M.M. performed research; D.L.D., T.M.M., and P.V.S. analyzed data; and D.L.D., T.M.M., and P.V.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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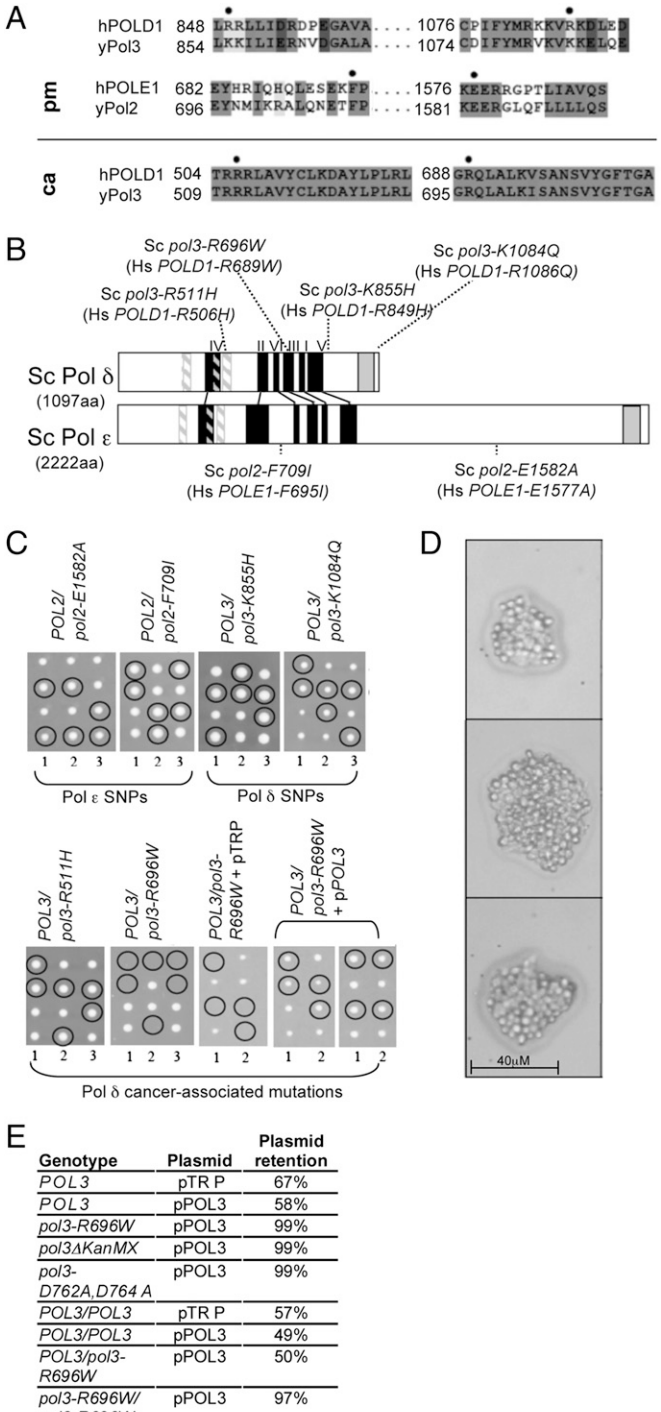
This article contains supporting information online at www.pnas.org/cgi/content/full/0907526106/DCSupplemental.



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Genotype	Plasmid	Plasmid retention
POL3	pTR P	67%
POL3	pPOL3	58%
pol3-R696W	pPOL3	99%
pol3ΔKanMX	pPOL3	99%
pol3-D762A,D764 A	pPOL3	99%
POL3/POL3	pTR P	57%
POL3/POL3	pPOL3	49%
POL3/pol3-R696W	pPOL3	50%
pol3-R696W/ pol3-R696W	pPOL3	97%

Fig. 1. Effects of DNA polymerase mutations on viability. (*A*) Alignment of hPOLD1 and hPOLE1 with yPol3 and yPol2. Dots show the sites of polymorphisms (pm) and cancer-associated mutations (ca). Basic (light gray), invariant (medium gray), and acidic (dark gray) residues are highlighted. pm, polymorphisms; ca, cancer-associated mutations. (*B*) The catalytic subunits of yeast Polδ and ε with DNA polymerase (black), exonuclease (hatched), and zinc finger (gray) motifs indicated. Mutation locations are approximated. (*C*) Representative tetrad dissections of heterozygous diploids. Colonies harboring the indicated polymerase mutation are circled in black. (*D*) Dead cell groups formed by *pol3-R696W* haploid cells after tetrad dissection. (*E*) Frequency of plasmid retention in haploid and diploid yeast.

cell line DLD-1 (11, 12), are in the conserved DNA polymerase III and exonuclease III motifs, respectively (Fig. 1 *A* and *B*). Remarkably, the degree of amino–acid sequence conservation was noticeably higher for the regions affected by the cancer-associated mutations than for the regions affected by SNPs (Fig. 1*A*). This is consistent with the expectation that the changes found in cancers may have a more severe effect on protein function than most SNPs found in the normal population.

To assess the functional significance of Polδ and Polε variants that map to conserved regions, we constructed *Saccharomyces cerevisiae* strains with analogous changes in Polδ and Polε (Fig. 1*B*). The *POL3* and *POL2* genes encoding the catalytic subunits of these DNA polymerases are essential. Therefore, we began by determining the effect of the SNPs and cancer-associated mutations on cell growth and viability. Diploid strains heterozygous for the *pol3* or *pol2* mutations were generated, and after sporulation, tetrads were dissected to compare the colony growth of haploid cells expressing the mutant or wild-type polymerase alleles. The four SNPs examined and the cancer-associated mutation *pol3-R511H* had no apparent effect on viability or cell growth (Fig. 1*C*). We further examined these five DNA polymerase mutants with respect to spontaneous mutagenesis and DNA damage sensitivity. To reveal any subtle mutator phenotypes that may be masked by the action of MMR, we measured mutation rates in the absence of the MMR factor Msh6, which is involved in the recognition of base–base mismatches and small loops (16). The Polδ and Polε SNPs did not significantly increase the rate of mutation to canavanine resistance (Can^r) in the presence or absence of MMR (Table S1). The cancer-associated mutation *pol3-R511H* led to a small but significant (2.5-fold) increase in the rate of the Can^r mutation in the MMR-deficient strain Table S1). We observed no effects of any of the five mutations on the sensitivity of the cells to the DNA-damaging agent methyl methanesulfonate (MMS). Additionally, the *pol3-R511H* mutant was not appreciably sensitive to UV light, hydroxyurea, or hydrogen peroxide. Collectively, these results indicate that the *pol3-K855H*, *pol3-K1084Q*, *pol2-F709I*, and *pol2-E1582A* mutations do not significantly affect the function or fidelity of the respective polymerases. The subtle mutator effect of *pol3-R511H* suggests that this mutation slightly reduces the fidelity of DNA replication, which is compensated for in the wild-type cells by the action of MMR.

The *pol3-R696W* Mutation Is Lethal in Haploid and Homozygous Diploid Yeast. In contrast to the other mutants examined, spores carrying the cancer-associated allele *pol3-R696W* failed to produce visible colonies (Fig. 1*C*). The lethality is rescued by the ectopic expression of *POL3* (Fig. 1*C*), confirming that inviability is caused by the *pol3-R696W* mutation. The inviability of *pol3-R696W* could be caused by (i) severely impaired catalytic activity of Polδ or (ii) the accumulation of lethal mutations in other essential genes because of a dramatic decrease in the fidelity of Polδ. Such a "mutation catastrophe" has been previously described for yeast that are defective simultaneously in two replication error-avoidance mechanisms (exonucleolytic DNA polymerase proofreading and MMR) (17) and for *E. coli* carrying a defective exonuclease subunit of Pol III (18). If *pol3-R696W* encodes a nonfunctional polymerase, haploid cells expressing the mutant allele would not be able to replicate their DNA and divide, as observed previously (19) and in this study (Fig. S1), for cells completely lacking active Polδ. In the mutation–catastrophe scenario, lethal mutations would accumulate during replication, and *pol3-R696W* cells would be expected to proceed through a number of cell cycles before cell division ceases. This was previously observed for the proofreading- and MMR-deficient yeast cells (17). Microscopic examination after tetrad dissection showed that the *pol3-R696W* cells were able to divide and form microcolonies of varying size (estimated at ~100 cells on average) before cell division stopped (Fig. 1*D*). These dead cell groups contained a mixture of cells at various

stages of the cell cycle with no prevailing cell morphology. Counting cells from six microcolonies revealed on average 34% single, unbudded cells, 26% budded cells, 23% dumbbells, 11% aberrant, and 5% others (typically several cells that did not separate). The absence of a unique terminal morphology argues against a specific DNA replication defect or checkpoint activation as the cause of inviability. The capability of *pol3-R696W* cells to divide suggests that they can form a functional Polδ. Furthermore, the random nature of cell death is consistent with the accumulation of random mutations caused by the presence of a mutagenic DNA polymerase.

Because most mutations are recessive, diploid cells typically can tolerate higher levels of genome-wide mutagenesis than haploid cells. For example, the high spontaneous mutation rate resulting from a combination of defective Polδ proofreading and defective MMR is lethal in haploid but not diploid yeast cells (17). In surprising contrast, we found that, like haploids, the homozygous *pol3-R696W/pol3-R696W* diploids are inviable, which is indicated by their inability to lose the plasmid expressing an ectopic copy of *POL3* (pPOL3) (Fig. 1*E*).

Mutator Effect of the *pol3-R696W* Allele. To determine if both *pol3-R696W* haploids and *pol3-R696W/pol3-R696W* diploids die from a high level of mutagenesis, we examined the rate of spontaneous mutation in strains expressing the mutant allele. Because *pol3-R696W* is lethal when it is the sole source of Pol3, we measured the mutation rate under conditions where the expression of *pol3-R696W* from the natural chromosomal promoter is the primary source of Pol3 and the expression of the plasmid *POL3* from the *GAL1* promoter is repressed by the presence of glucose. Leaky *GAL1-POL3* expression is sufficient to rescue the lethality while it diminishes competition by wild-type Pol3, which may mask *pol3-R696W* effects. Under these conditions, the growth of *pol3-R696W*-expressing cells is similar to the wild type. The rate of spontaneous mutation was increased in both haploid and diploid *pol3-R696W* mutants for all reporter genes tested (Table 1 and Table S2). Most remarkably, the rate of Can^r mutation was increased at least 65- and 200-fold in two independently derived homozygous *pol3-R696W/pol3-R696W* diploids compared with wild-type diploids (Table 1). The slight difference in the mutation rate between the two diploids may reflect the differences in the genetic background of the strains used or the contribution of modifier mutations, which would likely accumulate in the mutator strains. Notably, *can1* mutations are recessive, so two *CAN1* alleles must be inactivated to generate a Can^r mutant. Sequencing of 10 Can^r mutants revealed that all resulted from simultaneous inactivation of both alleles by independent mutations rather than from the mutational inactivation of one allele followed by a loss of heterozygosity. The mutations are described in Table 2 and Table S3, and examples of sequencing electropherograms showing the heterozygosity at the mutation sites are shown in Fig. S2. The observed >65- and >200-fold increases in the rate of Can^r mutation in the *pol3-R696W/pol3-R696W* diploids, therefore, translate to at least a 6,000- and 11,000-fold increase in the rate of mutation at a single *CAN1* allele over the wild-type mutation rate. Even more striking, this supermutator effect is seen in the presence of wild-type Polδ that presumably reduces the contribution of mutagenic Polδ-R696W to replication. Calculations shown in the *SI Text* suggest that the high mutation rate in the haploid and diploid *pol3-R696W* mutants is expected to be incompatible with life. The spectrum of spontaneous mutations in the *pol3-R696W/pol3-R696W* diploid reveals a distinct mutational signature: 15 of 17 base substitutions resulted in the change to a T•A base pair (Table 2 and Table S3).

Previous studies illustrate that a low level of functional Polδ can promote mutagenesis (20). The R696W substitution could potentially reduce the stability or the catalytic activity of Polδ to the extent that the pPOL3-rescued *pol3-R696W* cells contain a low total level of functional Polδ under our experimental conditions. The normal growth rate and insensitivity of the cells to hydroxyurea argue that replication is not severely affected under these conditions. We, however, tested the possibility that the mutator effect of *pol3-R696W* was caused by a decreased level of Polδ rather than the low fidelity of the Polδ-R696W. We measured mutagenesis in pPOL3-rescued *pol3Δ* and *pol3-D762A, D764A* mutants that carry no chromosomally encoded Pol3 and the catalytically inactive Pol3, respectively. Similar to the experiments with the *pol3-R696W* mutants, the leaky expression of the plasmid *GAL1-POL3* on glucose-containing medium provided functional Polδ to rescue the viability of these strains. If the strong mutator phenotype of *pol3-R696W* cells was caused by a low level of active Polδ, then the same strong mutator effect should be seen when no functional Pol3 is encoded at the chromosomal locus. In contrast, we observed only a 3-fold increase in the rate of Can^r mutation in pPOL3-rescued *pol3-D762A,D764A* or *pol3Δ* mutants (Table 1). This nominal increase (compared with the 30-fold increase in *pol3-R696W*) suggests that the mutator effect in *pol3-R696W* cells is specifically attributed to the presence of the Polδ-R696W.

We have shown previously that many mutations in replicative DNA polymerases can lead to an increased spontaneous mutation rate indirectly through the recruitment of the error-prone Polζ (21). To determine if any of the spontaneous mutagenesis in

Table 1. Mutator effects of *pol3-R696W*, *pol3-D762A,D764A*, and *pol3ΔKanMX* alleles in haploid and diploid cells

Genotype	pPOL3	Can^r mutation (x 10^{-7})*
POL3	+	2.4 (2.2–3.2)
pol3-R696W	+	67 (60–73)
pol3-R696W rev3	+	58 (45–89)
pol3ΔKanMX	+	6.7 (5.4–17)
pol3-D762A,D764A	+	7.6 (6.9–9.1)
POL3/POL3	–	<0.3
POL3/pol3-R696W	–	<0.3
POL3/POL3	+	<0.3
POL3/pol3-R696W	+	<0.3
pol3-R696W/pol3-R696W (1)†	+	69 (56–90)
pol3-R696W/pol3-R696W (2)†	+	20 (13–28)
pol3-R696W/pol3-R696W rev3/rev3	+	41 (36–51)
pol3ΔKanMX/pol3-R696W	+	5.4 (4.2–6.0)

*The values are medians for at least nine cultures. The 95% confidence limits are given in parentheses.
†*pol3-R696W/pol3-R696W* (1) and (2) were constructed using PSD93 and E134×1B-D770 strains, respectively.

Table 2. Spontaneous *can1* mutations in *pol3-R696W/pol3-R696W* diploids

can1 isolate	Nucleotide changes
1	C684A
2	G1018A, C1598A
3	T649C, G670A, C1240A, T1676C
4	G687A, G1261A
5	G1018A
6	G1018A
7	G671A, G718A
8	C648A
9	G671A, C732A
10	C1426T

All recovered mutations were in the heterozygous state (see Fig. S2). Only nucleotides 618–1773 of *CAN1* were analyzed, so the isolates could contain additional undetected mutations.

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pol3-R696W cells is caused by the activity of Polζ, we measured the mutator effect of *pol3-R696W* in a *rev3* strain, which lacks the catalytic subunit of Polζ. The Polζ⁻ derivative still showed the same high level of mutagenesis (Table 1). This is consistent with the idea that the mutator effect of *pol3-R696W* results from inaccurate DNA synthesis by Polδ-R696W itself rather than from the recruitment of a specialized polymerase.

Polδ-R696W Is an Error-Prone DNA Polymerase. We used a biochemical approach to determine whether or not the *pol3-R696W* allele does indeed encode for an error-prone DNA polymerase. We purified recombinant three-subunit yeast Polδ and its R696W variant, and then, we compared their catalytic activity and their ability to misincorporate nucleotides using oligonucleotide DNA substrates (Fig. 2). The R696W substitution did not affect the ability of the catalytic subunit (Pol3p) to associate with the accessory subunits Pol31p and Pol32p, as indicated by the similar subunit stoichiometry in the Polδ and Polδ-R696W preparations (Fig. 2*A*). In a primer extension assay using an oligonucleotide template, Polδ-R696W showed a reduced DNA polymerase activity in comparison with the wild-type Polδ. When equal concentrations of the enzymes were used, the Polδ-R696W reaction products contained significantly more unextended and less fully extended substrate (Fig. 2*B Left*). However, increasing the concentration of Polδ-R696W by 3-fold resulted in a comparable amount of primer extension (Fig. 2*B Right*). Multiple rounds of DNA synthesis initiation are not prohibited in these experiments. Thus, the decreased activity of Polδ-R696W could reflect less efficient nucleotide incorporation, reduced enzyme cycling, or both. Unlike the DNA polymerase activity, 3′-exonuclease activity of Polδ was not significantly affected by the R696W substitution (Fig. 2*C*).

To investigate whether or not Polδ-R696W has a reduced DNA synthesis fidelity, we compared the abilities of Polδ and Polδ-R696W to insert an incorrect nucleotide in a primer extension assay (Fig. 2*D*). The oligonucleotide substrate and the reaction conditions were identical to those used for the DNA polymerase activity assay in Fig. 2*B*, except that only a single nucleotide was present. In the reaction containing dGTP complementary to the template C, Polδ and Polδ-R696W generated a +1 product with similar efficiency. The Polδ-R696W, however, also had a profound ability to extend the primer using a noncomplementary dTTP and to a lesser extent, dATP. The amount of misinsertion is likely to be underestimated in these experiments, because some of the +1 product could be degraded by the exonuclease activity of the enzymes. Neither enzyme showed a high rate of dCMP misinsertion in this assay. The error specificity of Polδ-R696W in vitro, thus, is in excellent agreement with the spectrum of spontaneous mutations seen in the *pol3-R696W/pol3-R696W* mutant in vivo (CG to AT transversions and CG to TA transitions) (Table 2 and Table S3). The sequence of the DNA substrate used in the in vitro assays mimicked the *CAN1* gene sequence around position 732 where a C to A substitution was observed in the *pol3-R696W/pol3-R696W* diploid. The use of this particular sequence context could explain the fact that dTTP was more readily inserted at this site than dATP by Polδ-R696W (Fig. 2*D*).

The Mutator Effect of the *pol3-R696W* Allele Depends on the Relative Levels of Polδ-R696W and the Wild-Type Polδ. Remarkably, in contrast to the high mutagenesis in *pol3-R696W* strains rescued with the low expression of wild-type *POL3*, the mutation rate in the heterozygous *pol3-R696W/POL3* diploid strains was at least two orders of magnitude lower (Table 1 and Table S2). This suggests that (*i*) Polδ-R696W is not able to compete efficiently with the wild-type Polδ when the latter is produced at normal endogenous levels in heterozygous diploids, and (*ii*) the mutagenicity of Polδ-R696W becomes a factor when the ratio of mutant to wild-type Pol3 is shifted toward the mutagenic polymerase as in the pPOL3-rescued haploids. If this hypothesis is correct, we should be able to suppress the mutator phenotype of the haploid *pol3-R696W* strain by increasing wild-type *POL3* expression. To test this, we measured spontaneous mutagenesis in *pol3-R696W* cells

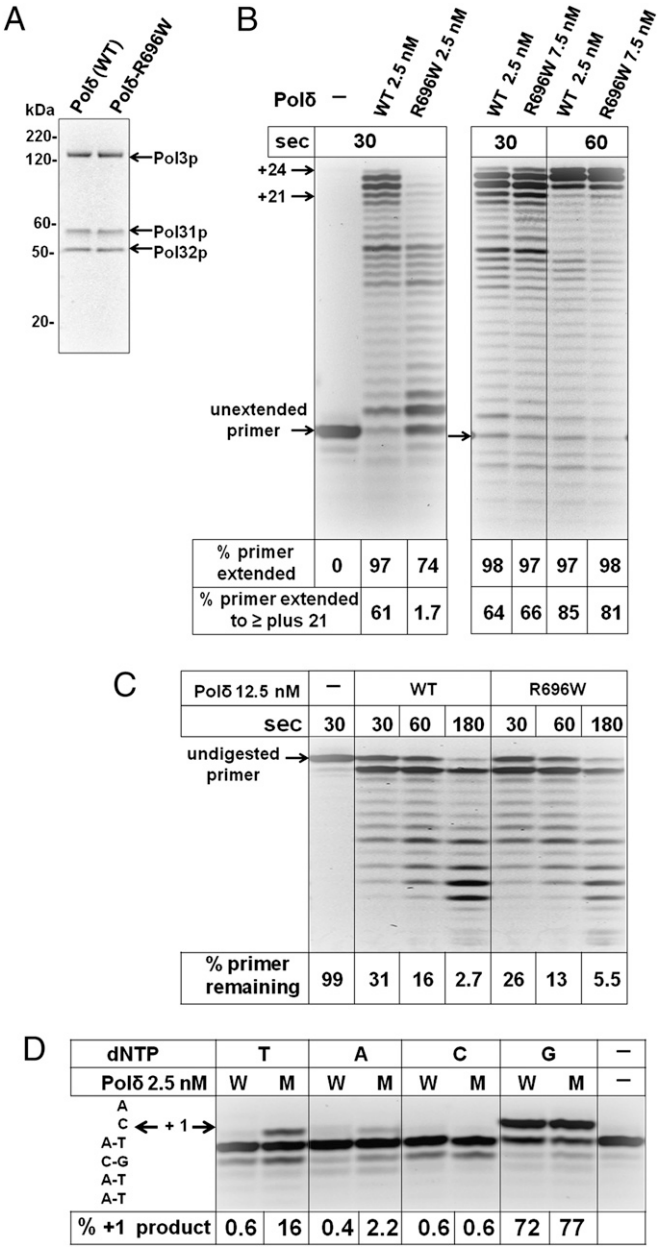

Fig. 2. Polδ-R696W is a functional but highly error-prone DNA polymerase. (*A*) SDS/PAGE analysis of purified recombinant Polδ and Polδ-R696W. The proteins were visualized by Coomassie staining. WT, wild-type Polδ. (*B*) DNA polymerase activity of Polδ and Polδ-R696W was assayed on an oligonucleotide substrate as described in *Materials and Methods*. Incubation time and enzyme concentration is indicated. The percentage of primer extended (+1 products and longer) and the percentage of primer extended nearly to the end of the template (+21 and longer) are shown below each lane. (*C*) Analysis of exonuclease activity of Polδ and Polδ-R696W. The percentage of substrate remaining is shown below each lane. (*D*) Specificity of nucleotide incorporation by Polδ and Polδ-R696W. Nucleotide sequence of the DNA substrate in the vicinity of the primer–template junction is shown to the left of the gel image. Wild-type Polδ or Polδ-R696W were incubated with this substrate in the presence of a single dNTP (T, A, C, G) as indicated for 30 s. The percentage of primer extension (+1 product) is shown below each lane. W, wild-type Polδ; M, Polδ-R696W.

containing pPOL3 and grown at various galactose concentrations, which would modulate the level of the wild-type *POL3* expression. As expected, increasing the expression of wild-type *POL3* (Fig. 3 *Inset*) leads to a concurrent decrease in the rate of spontaneous mutagenesis in the *pol3-R696W* strain (Fig. 3). By contrast, in wild-type cells containing a galactose-inducible *pol3-R696W* allele, mutagenesis was stimulated on galactose-containing medium (Fig. S3). Moreover, diploids with two chromosomal *pol3-R696W* alleles and the plasmid wild-type *POL3* had a 4- to 12-fold higher mutation rate than diploids with one chromosomal *pol3-R696W*, a deletion of the *POL3* locus on the homologous chromosome, and the plasmid wild-type *POL3* (Table 1). This suggests that even small changes in the ratio of Polδ-R696W to wild-type Polδ can significantly affect mutagenesis.

Discussion

High-fidelity DNA replication is essential for mitigating cancer risk by reducing the DNA mutation load. Because single amino acid changes in Polα, Polδ, and Polε can dramatically affect DNA polymerase fidelity (2, 5, 6, 22–24), understanding the functional consequences of naturally occurring variants of these polymerases provides invaluable knowledge of potential cancer risk for individuals with these variants. To this end, we examined reported SNPs and cancer-associated mutations affecting Polδ and Polε. We showed that yeast analogs of the following human variants do not significantly affect the function (Fig. 1*C*) of the respective DNA polymerases or mutagenesis (Table S1): Polδ-K849H, Polδ-K1086Q, Polε-F695I, and Polε-E1577A. The relatively mild effect of the human cancer-associated mutation Polδ-R506H (yeast Polδ-R511H) is somewhat surprising, because the corresponding arginine residue is part of the highly conserved ExoIII motif essential for the proofreading activity of Polδ. Any proofreading defect is expected to result in increased spontaneous mutagenesis, particularly in combination with the MMR defect (17, 25). In the recently reported crystal structure of the yeast Pol3, however, Arg511 is found away from the exonuclease active site (26). This may explain why the function of the exonuclease domain is not severely compromised in the R511H variant. The nearly wild-type phenotype of the other Polδ and Polε mutants is consistent with the presence of the corresponding mutations in normal human population and in less conserved regions of the proteins.

One Polδ mutant, R696W (analogous to human R689W), forms a functional but extremely inaccurate DNA polymerase. We present several pieces of evidence that support this conclusion. First, yeast cells containing the mutant allele as the only source of Pol3 die after multiple cell divisions (Fig. 1 *C–E*). Second, the *pol3-R696W* mutants rescued by a low-level expression of wild-type *POL3* show a strong mutator phenotype (Table 1). Third, purified Polδ-R696W has only a moderately reduced DNA polymerase activity but a dramatically increased nucleotide misinsertion rate (Fig. 2). Taken together, these observations provide strong support for the idea that the death of the *pol3-R696W* mutants occurs because of the accumulation of random mutations during DNA replication.

These results indicate that Arg696 is a crucial residue for proper Polδ function. Defects in several DNA polymerase functions, including nucleotide selectivity, exonuclease, processivity, and protein–protein interactions, could contribute to the high mutagenicity of this polymerase. The initial biochemical characterization of Polδ-R696W reported here suggested that this Polδ mutant is severely compromised at the stage of nucleotide selectivity. Arg696 is located in the fingers domain but does not constitute a part of the nascent base pair–binding pocket that plays a primary role in the high fidelity of Polδ (26). We speculate that the replacement of the basic Arg with a neutral aromatic Trp residue alters the structure of the active site in a way that allows misinsertion. It has also been suggested that the R696W change could affect the partitioning of the primer terminus between the polymerase and exonuclease active sites (26). Future, more extensive biochemical and structural studies will help define the molecular basis for the low fidelity of Polδ-R696W as well as the possible effects of the accessory proteins, such as PCNA, on the activity and fidelity of this polymerase. Arg696 residue is conserved in some of the other B family DNA polymerases, including Polα and Polζ (Table S4), suggesting that its function in DNA synthesis fidelity likely extends to other DNA polymerases as well.

The human Polδ-R689W substitution was originally identified in the colon cancer cell line DLD-1 (12). The DLD-1 cell line is a MMR-deficient cell line that displays microsatellite instability and increased spontaneous mutagenesis (27). In addition to the mutation of the MMR gene *MSH6*, this heterogeneous cell line has four documented, heterozygous Polδ mutations, including the R506H and R689W mutations examined here (12, 28). Experiments with the derivatives of DLD-1 expressing exogenous *MSH6* and *POLD1* showed that, although the *MSH6* defect is responsible for a significant fraction of the mutator phenotype of this cell line, the contribution of the *POLD1* mutations could not be excluded (28–30). About half of the spontaneous *hprt* gene mutations in the DLD-1 cells are GC→TA transversions and GC→AT transitions (29, 31, 32), which constitute the characteristic mutational signature of the yeast *pol3-R696W* cells (Table 2 and Table S3). The proportion of these mutations becomes even higher when the MMR defect of DLD-1 cells is suppressed by exogenous expression of *MSH6* (29). The DLD-1 cells are also known to carry homozygous, nonsilent mutations in the *APC* gene, as well as mutations affecting RAS, p53, and αE-catenin (12, 33–35). All of them, with the exception of the *APC* mutations, are GC→AT transitions or GC→TA transversions. These observations are consistent with the idea that the putative Polδ-R689W mutator could contribute to the generation of these mutations and tumorigenesis.

Interestingly, the heterozygous *POL3*/*pol3-R696W* diploid yeast strain did not show a strong mutator phenotype (Table 1 and Table S2). This result is not completely unexpected, because recessive or nearly recessive DNA polymerase mutations have

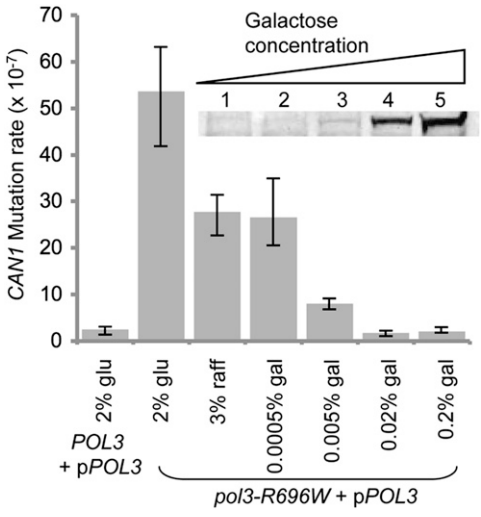

Fig. 3. Increasing the level of wild-type Polδ mitigates the mutagenic potential of Polδ-R696W. The rate of *can1* mutation at different galactose concentrations was determined for haploid cells expressing the chromosomal *pol3-R696W* allele from the natural promoter and wild-type plasmid-borne *POL3* from the galactose-inducible *GAL1* promoter. The rates are medians for at least nine cultures. Error bars show the 95% confidence intervals. The inset Western blot shows increasing Pol3 production with increasing galactose concentration (lane 1, 3% raffinose; lane 2, 0.0005% galactose; lane 3, 0.005% galactose; lane 4, 0.02% galactose; lane 5, 0.2% galactose). A total of 30 μg of protein extract was analyzed for each condition.

been reported previously (17, 36). Titration of wild-type *POL3* expression in *pol3-R696W* cells (Fig. 3) and the analysis of DNA polymerase activity of the purified enzymes (Fig. 2) revealed that Polδ-R696W is likely unable to compete efficiently with wild-type Polδ when the latter is produced at normal endogenous levels. The dependence of mutagenesis on the relative levels of wild-type Polδ and Polδ-R696W (human Polδ-R689W) has important implications for human cancer cells that are frequently aneuploid and/or could have other allele dosage alterations. Additionally, the dosage dependence of Polδ-R689W is relevant to the concept of transient hypermutability in cancers. This concept suggests that tumors that do not display high mutator phenotypes may have accumulated transforming mutations during a transient burst of hypermutability (37). Although largely speculative at this point, this transient hypermutability may arise because of the transient up-regulation of a stress response (38, 39), random translational or transcriptional errors that generate aberrant proteins, or deregulation of various processes (37). Along those lines, deregulation of transcription that results in a transcriptional bias for the expression of *POLD1-R689W* versus the wild-type *POLD1* allele in heterozygous humans could tip the balance to a hypermutable state, thereby generating cancer-initiating or metastasis-promoting mutations. Thus, given the critical roles that mutations and mutator phenotypes are postulated to play in cancer development (40), our study points to the importance of future investigations of the causative relationship between the Polδ-R689W variant and cancer in humans.

Materials and Methods

All *S. cerevisiae* strains used in this study are isogenic to the haploid strains E134 (*MATα ade5-1 lys2::InsE$_{A14}$ trp-289 his7-2 leu2-3,112 ura3-52*) or 1B-D770 (*MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4*) (41). Heterozygous diploid *pol3* or *pol2* mutants were constructed by replacing one chromosomal copy of the corresponding gene with the mutant allele (confirmation primers listed in Table S5), Table S5 and haploids were obtained by tetrad dissection. The rate of forward mutation to Can^r was measured by fluctuation analysis as described previously (41). Immunoblot analysis of whole-cell extracts was performed as described (21) with rabbit polyclonal antiserum to yeast Polδ (42) provided by Peter Burgers (St. Louis, MO). Polδ and Polδ-R696W were overproduced and purified as previously described (43) with minor modification (see *SI Text*). DNA substrates for DNA polymerase and exonuclease assays were created by annealing a Cy5-labeled primer to an oligonucleotide template, and reactions were performed at 30°C with 40 mM Tris-HCl (pH 7.8), 125 mM NaAc, 8 mM MgAc, 1 mM DTT, 0.2 mg/mL BSA, 4% polyethylene glycol 8000, 25 nM DNA substrate, and 100 μM each dNTPs. For exonuclease assays, dNTPs and polyethylene glycol were omitted. A more thorough description of methods can be found in the *SI Text*.

ACKNOWLEDGMENTS. We thank Peter Burgers for *POL3*, *POL31*, and *POL32* overexpression plasmids and Polδ antiserum, Victoria Liston and Corinn Grabow for technical assistance, and Tadayoshi Bessho and Youri Pavlov for critical manuscript reading. This work was supported by the Eppley Institute for Research in Cancer and Allied Diseases and by the National Institutes of Health Grant ES015869 (to P.V.S.).

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