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Nucleotide selectivity defect and mutator phenotype conferred by a colon cancer-associated DNA polymerase δ mutation in human cells

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Abstract

Mutations in the *POLD1* and *POLE* genes encoding DNA polymerases δ (Polδ) and ε (Polε) cause hereditary colorectal cancer (CRC) and have been found in many sporadic colorectal and endometrial tumors. Much attention has been focused on *POLE* exonuclease domain mutations, which occur frequently in hypermutated DNA mismatch repair (MMR)-proficient tumors and appear to be responsible for the bulk of genomic instability in these tumors. In contrast, somatic *POLD1* mutations are seen less frequently and typically occur in MMR-deficient tumors. Their functional significance is often unclear. Here we demonstrate that expression of the cancer-associated *POLD1-R689W* allele is strongly mutagenic in human cells. The mutation rate increased synergistically when the *POLD1-R689W* expression was combined with a MMR defect, indicating that the mutator effect of *POLD1-R689W* results from a high rate of replication errors. Purified human Polδ-R689W has normal exonuclease activity, but the nucleotide selectivity of the enzyme is severely impaired, providing a mechanistic explanation for the increased mutation rate in the *POLD1-R689W*-expressing cells. The vast majority of mutations induced by the *POLD1-R689W* are GC→TA transversions and GC→AT transitions, with transversions showing a strong strand bias and a remarkable preference for polypurine/polypyrimidine sequences. The mutational specificity of the Polδ variant matches that of the hypermutated CRC cell line, HCT15, in which this variant was first identified. The results provide compelling evidence for the pathogenic role of the *POLD1-R689W* mutation in the development of the human tumor and emphasize the need to experimentally determine the significance of Polδ variants present in sporadic tumors.

Keywords

DNA polymerase δ; colorectal cancer; *POLD1*; mutator; nucleotide selectivity; mutational spectrum

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

INTRODUCTION

Mutations in genes that control cell division are the primary cause of cancer. Not surprisingly, malfunction of the cellular systems that maintain genome stability increases cancer risk. Among these systems, the pathway controlling accuracy of DNA replication is of utmost importance for preventing mutation and tumorigenesis.[1] It involves accurate selection of nucleotides by replicative DNA polymerases, proofreading of rare errors by the 3′→5′ exonuclease activity of these polymerases, and postreplicative error correction by the DNA mismatch repair (MMR). Inherited mutations in MMR genes cause a predisposition to colorectal cancer (CRC) in Lynch syndrome.[2,3] The MMR gene *MLH1* is also somatically inactivated in ~15% of sporadic colorectal, endometrial and gastric tumors.[4]

Germline mutations in the *POLD1* and *POLE* genes encoding the catalytic subunits of replicative DNA polymerases δ (Polδ) and ε (Polε) similarly cause hereditary CRC.[5,6] Furthermore, ultramutated sporadic colon and endometrial tumors (~3% and 8% of all CRC and endometrial cancer cases, respectively) were found to carry somatic changes in Polε.[7–10] The changes affect conserved amino acid residues in the exonuclease domains, suggesting that loss of proofreading must be responsible for the faulty DNA maintenance that leads to cancer. Many of these Polε variants, indeed, have impaired 3′→5′ exonuclease activity and reduced fidelity.[11] However, *in vivo* studies showed that the variant alleles cause very strong mutator effects far exceeding those expected from loss of proofreading.[12,13] The additional biochemical defect(s) that make the cancer-associated Polε variants so mutagenic remain to be identified. Because these *POLE* mutations primarily occur in tumors that do not have MMR defects, the encoded Polε variants are believed to be responsible for the high number of mutations present in these tumors.[14,15]

Somatic *POLD1* mutations have also been observed in up to 5% of sporadic colorectal and endometrial tumors,[8–11,16,17] as well as in other cancers (http://www.cbioportal.org/; http://cancer.sanger.ac.uk/cosmic). In contrast to the *POLE* variants that have garnered much interest, the majority of *POLD1* mutations are found in MMR-deficient tumors and do not show a noticeable concentration in the exonuclease domain. Approximately a quarter of these mutations affect conserved amino acid residues in the DNA polymerase motifs. Functional consequences of the vast majority of these mutations is unknown. We have previously shown that the yeast analog of one such variant allele, *POLD1-R689W*, encodes an error-prone DNA polymerase and causes a catastrophic increase in spontaneous mutagenesis.[18] This prompted us to investigate the effects of the R689W substitution on the function of human Polδ in the present study. The *POLD1-R689W* mutation was found in *MSH6*-deficient CRC cell lines DLD-1 and HCT15,[19,20] which were derived from the same tumor[21] and are among the most hypermutated cell lines known.[19,22] However, due to the presence of the MMR defect in these cells, the potential significance of the *POLD1-R689W* variant has been overlooked. We demonstrate that expression of *POLD1-R689W* is strongly mutagenic in human cells, and that the encoded enzyme, Polδ-R689W, has reduced base selectivity leading to frequent nucleotide misincorporation during DNA replication. The error signature of Polδ-R689W matches the mutational pattern of the HCT15 cell line, supporting the idea that the *POLD1-R689W* mutator played a primary role in the

development of the tumor. These findings argue that replicative DNA polymerase mutations that map outside of the exonuclease domain and/or occur in MMR-deficient tumors can be highly significant, and the need for functional analysis of such mutations is crucial.

RESULTS

Expression of *POLD1-R689W* elevates the mutation rate in MMR-proficient and MMR-deficient human cells

The *POLD1-R689W* mutation was originally identified in the CRC cell lines DLD-1 and HCT15.[19,20] While these cell lines show a mutator phenotype, the presence of MMR defect and multiple replicative DNA polymerase mutations[19,20] makes it difficult to determine whether the *POLD1-R689W* variant contributes to the genomic instability. We sought to compare the mutation rate in cells that differ only by the status of the *POLD1* allele (wild-type *versus POLD1-R689W*). We have chosen the CRC cell line HCT116, which has no mutations in the replicative DNA polymerase genes, as a model. These cells have been used extensively for mutagenesis studies[23–25] because of their near-diploid karyotype and the ability to form colonies readily when plated at low densities, which facilitates mutation rate measurements. A derivative of HCT116 is also available (HCT116+ch3), in which the MMR defect originally present in this cell line is corrected by microcell transfer of chromosome 3 containing the *MLH1* gene.[26] This allowed us to study the effects of *POLD1-R689W* in closely related MMR-proficient and MMR-deficient cells.

To determine if *POLD1-R689W* expression elevates the mutation rate, we created stable clonal cell lines by transducing HCT116 and HCT116+ch3 with retroviral vectors expressing the wild-type *POLD1* or *POLD1-R689W.* At least six clones of each transgenic cell line were created. The majority of clonal cell lines had morphology and growth rate similar to the respective parental cell line. In all of these clones, POLD1 was significantly overproduced (Figure 1), indicating that the exogenously introduced alleles served as the primary source of the polymerase. Next, we determined the effects of *POLD1-R689W* on the rate of mutation at the *HPRT1* locus, which confers 6-thioguanine resistance (6-TG^r).[27] The 6-TG^r mutation rate in the original HCT116+ch3 and HCT116 cell lines was 1.2×10^{-6} and 8.1×10^{-6}, respectively (Table 1), consistent with earlier data.[24] Overexpression of wild-type *POLD1* had no effect on mutagenesis in either cell line (Table 1). In contrast, mutagenesis was increased 2.0-, 3.6-, and 3.9-fold in three independent derivatives of HCT116+ch3 overexpressing *POLD1-R689W*. Clones with higher levels of overexpression had a slightly higher mutation rate, similar to our previous findings with the yeast analog of *POLD1-R689W*.[18] MMR deficiency enhanced the mutator effect of *POLD1-R689W*: the mutation rate was increased 2.9-, 11-, and 12-fold in three HCT116 derivatives expressing *POLD1-R689W* in comparison to the original HCT116 cells (Table 1). The smaller increase in one of the clones did not appear to result from a lower expression of *POLD1-R689W* (Figure 1) and is likely a consequence of a suppressor mutation that strong mutators frequently accumulate.[28] Overall, the data show that the *POLD1-R689W* allele imparts a mutator phenotype in the presence and absence of a MMR defect.

Because MMR primarily corrects errors that occur during DNA replication, the effect of MMR status on the mutagenic potential of *POLD1-R689W* can be used to determine if this

mutator promotes replication errors. If the majority of mispairs created by Polδ-R689W are corrected by MMR, a combination of *POLD1-R689W* with a MMR defect should result in a synergistic increase in the mutation rate. We have used such synergy analysis previously to demonstrate that mutations induced by the yeast analog of *POLD1-R689W* result from replication errors.[29] We analyzed the mutation rates in HCT116- and HCT116+ch3-derived cell lines (Table 1) to determine if this is also true for the human *POLD1-R689W.* MMR deficiency increases the mutation rate 6.8-fold (compare the values for HCT116 and HCT116+ch3 cell lines). The expression of *POLD1-R689W* in HCT116+ch3 cells increases the mutation rate 3.2-fold, on average. In HCT116 cells expressing *POLD1-R689W,* the combination of the MMR defect and *POLD1-R689W* results, on average, in a 58-fold increase in the mutation rate (compared to HCT116+ch3). This strong synergistic interaction indicates that errors resulting from *POLD1-R689W* expression are normally corrected by MMR and, therefore, are made during replicative DNA synthesis.

The mutational signature of *POLD1-R689W* in human cells

We next determined the mutational specificity of human *POLD1-R689W.* We sequenced the *HPRT1* gene in 96 independent 6-TG^r mutant clones that arose in the *POLD1-R689W*-expressing HCT116 cells (clone 1). The mutation rate in this clone exceeds the mutation rate in the original HCT116 cells 12-fold (Table 1), so at least 92% of the 6-TG^r mutants must have resulted from Polδ-R689W errors. Four of the mutants had no mutation present in the *HPRT1* coding sequence and could possibly have a mutation(s) in the 5′ or 3′ untranslated region. An additional 11 mutants likely had large deletions within the *HPRT1* locus, as suggested by the failure to amplify some stretches of adjacent exons by PCR or the entire *HPRT1* cDNA by rtPCR. Among the remaining 81 mutants, 70 contained base substitutions leading to nonsynonymous amino acid changes, premature stop codons, or ablation of correct exon splicing, and 11 contained −1 or +1 frameshift mutations within the *HPRT1* coding sequence (Table 2 and Supplementary Figure S1). A total of 83% of base substitutions were GC→TA transversions or GC→AT transitions (Table 2).

Notably, the expression of *POLD1-R689W* dramatically changed the specificity of spontaneous mutagenesis in HCT116 cells making it resemble the mutational specificity of HCT15 cells (Figure 2). The spectrum of *HPRT1* mutations in the parental HCT116 cells, in which we introduced *POLD1-R689W,* showed a high frequency of small insertions/deletions (indels) (Table 2), similar to earlier data for this cell line.[25] In contrast, spontaneous mutations in HCT15 cells are almost exclusively base substitutions.[25] The expression of *POLD1-R689W* in HCT116 cells decreased the proportion of indels and increased the proportion of base substitutions, particularly GC→TA transversions, making the spectrum very similar to that of HCT15 (Figure 2). The minor differences between HCT15 and HCT116 POLD1-R689W spectra (e.g. still a slightly higher proportion of indels in the latter) are likely due to the fact that up to 8% of mutations in the *POLD1-R689W*-expressing cells are contributed by the HCT116 background. Indeed, the majority of indels in HCT116 POLD1-R689W cells (nine out of 11) occurred in a run of six consecutive G-C base pairs (Supplementary Figure S1) that is a hot spot for indels in HCT116 ([25]; Supplementary Figure S2). Taken together, these observations strongly argue that the *POLD1-R689W* variant plays a major role in shaping the mutational specificity of HCT15

cells. The results also suggest that the additional mutations present in the *POLD1* gene in these cells, including the R506H variant in the exonuclease domain, contribute very little, if at all, to the mutational spectrum.

The largest class of *HPRT1* mutations induced by *POLD1-R689W* expression, GC→TA transversions, showed a strong DNA sequence context preference. Nearly all occurred in polypurine/polypyrimidine tracts, with all but one having the polypurine sequence in the non-transcribed strand (Figure 3, *left*). To further support the premise that the *POLD1-R689W* variant was primarily responsible for the hypermutability of the original tumor, we analyzed the DNA sequence context of GC→TA transversions present in a set of 26 cancer-related genes in the HCT15 cell line.[19] This set of genes was used as a sample of genomic sequence potentially enriched for mutations that played a role in the development of the tumor. All GC→TA transversions in these genes were analyzed without consideration of their possible functional significance. Twelve GC→TA transversions were found in the 26 genes. Strikingly, all occurred in polypurine/polypyrimidine tracts, with many of the sequences matching precisely the context of *POLD1-R689W*-induced mutations (Figure 3, *right*). In contrast, two GC→TA transversions found in the 26 cancer genes in HCT116 cell line[19] did not share this sequence context. Thus, the context analysis suggests that not only does the *POLD1-R689W* allele determine the specificity of new mutations arising in the HCT15 cell line, but it likely shaped the genome of the original hypermutated tumor as well.

Purified human Polδ-R689W has reduced base selectivity

To further understand the mechanisms responsible for the mutator effect of *POLD1-R689W*, we purified four-subunit human Polδ and Polδ-R689W using an insect cell/baculovirus-based protein production system (Figure 4a) and compared their biochemical properties. On an oligonucleotide substrate, the 3′→5′ exonuclease and DNA polymerase activities of Polδ-R689W and the wild-type Polδ were nearly identical (Figure 4b,c). The intact exonuclease activity indicates that Polδ-R689W must be fully capable of proofreading its errors, consistent with the location of Arg689 outside of the exonuclease domain. Next, we examined whether *POLD1-R689W* had a base selectivity defect by monitoring the insertion of individual nucleotides, correct or incorrect, across from a template C. The correct dGTP was inserted by the wild-type and mutant Polδ equally well. However, unlike the wild-type enzyme, Polδ-R689W also showed a profound ability to misincorporate dTTP at this position (Figure 4d). The C-dTTP is one of the two mispairs that could lead to GC→TA transversions, the most frequent type of mutation induced by *POLD1-R689W* expression. Thus, the results of oligonucleotide-based assays provide strong support for the idea that the increased mutation rate in cell lines expressing *POLD1-R689W* is due to a reduced base selectivity of the encoded polymerase.

DISCUSSION

Hypermutated tumors with replicative DNA polymerase defects carry up to one million clonal mutations in their genomes.[8,10] At such a density of mutations, nearly every gene is affected, and multiple alterations in the DNA maintenance pathways are often present. Identification of mutations responsible for the high level of genome instability requires

experimental assessment of their significance. A number of replicative DNA polymerase variants, particularly Polε exonuclease domain variants, have been suggested to be functionally important by bioinformatics analysis and/or by functional studies in yeast or *in vitro*.[5,11,13] However, the ability to increase the mutation rate in human cells has not been demonstrated for any of the cancer-associated polymerase variants. Prompted by the extraordinary mutator effect of the yeast *POLD1-R689W* analog,[18] here we analyzed the functional consequences of the *POLD1-R689W* mutation in human cells. We show that expression of the *POLD1-R689W* allele is mutagenic in both MMR-deficient and MMR-proficient cells. Synergistic interaction with the MMR defect indicated that *POLD1-R689W*-induced mutations result from DNA replication errors. Biochemical studies of purified human Polδ-R689W indicated that decreased base selectivity of this polymerase is likely responsible for the mutator effect. Finally, we show that the mutational signature of *POLD1-R689W* is consistent with the pivotal role of the encoded polymerase in the development of the human tumor, in which this variant was found.

This study indicates that *POLD1* mutations seen in sporadic human tumors can be highly significant. Furthermore, DNA polymerase mutations in MMR-deficient tumors can be highly significant, as appears to be the case for *POLD1-R689W.* While MMR deficiency amplifies the mutator effect, the initial replication error load and mutational specificity are determined by the DNA polymerase variant in such tumors. It is worth noting that the HCT15 and DLD-1 cell lines are deficient only in the *MSH6*-dependent MMR subpathway. It remains to be determined whether strong DNA polymerase mutators are compatible with full MMR deficiency, such as that caused by inactivation of *MSH2* or *MLH1.* Curiously, the *POLD1-R689W* variant has been reported in two other sporadic tumors, a hepatocellular carcinoma and another colon tumor (http://www.cbioportal.org/), neither of which was hypermutated. While relative levels of the wild-type and mutant allele expression and/or tissue- and tumor-specific factors could have affected the expression of the mutator phenotype, it is also possible that a combination with a MMR defect is required for *POLD1-R689W* to cause significant hypermutation. Finally, this study demonstrates that functionally important DNA polymerase mutations can occur outside the exonuclease domain and affect base selectivity rather than the much discussed proofreading. Interestingly, a L606M substitution in the DNA polymerase domain of Polδ, which is analogous to a well-known yeast mutator variant Polδ-L612M,[30–32] has been reported as a somatic mutation in brain tumors of children with hereditary biallelic MMR gene defects,[33] providing another example of a likely significant somatic *POLD1* mutation. Future studies of other *POLD1* changes reported in sporadic tumors will help identify additional pathogenic variants.

This study also validates the use of the *Saccharomyces cerevisiae* model system for functional analysis of cancer-associated DNA polymerase mutations. We have previously shown that the yeast *pol3-R696W* allele mimicking the human *POLD1-R689W* confers a strong mutator phenotype, interacts synergistically with the MMR defect, and encodes a Polδ variant with normal exonuclease activity but severely reduced base selectivity.[18,29] All of these properties were recapitulated in the present study of the human *POLD1-R689W* variant. Even the propensity of human Polδ-R689W for misincorporation of dTTP across from a template C on an oligonucleotide template *in vitro* (Figure 4d) is almost identical to that of yeast Polδ-R696W.[18] Like the human *POLD1-R689W*, the yeast *pol3-R696W*

mutator produced almost exclusively GC→AT transitions and GC→TA transversions *in vivo*.[18,29] The only difference was that transitions predominated in the mutational spectrum of *msh6 pol3-R696W* yeast strains,[29] and transversions were slightly more frequent in HCT116 POLD1-R689W cells (Figure 2 and Table 2). This could potentially be due to different reporter genes used or slight differences in the properties of the enzymes. Overall, the remarkable similarity of the effects of human *POLD1-R689W* and its yeast mimic illustrates the value of the yeast system for the identification of functionally significant polymerase variants. This is particularly important, because the number of *POLD1* and *POLE* mutations that await functional analysis will likely increase substantially as additional cancer genomes are sequenced.

Despite the advantages of the yeast system, mutations that affect poorly conserved amino acid residues might require analysis directly in human cells. The present work describes a simple and rational strategy for such an analysis. An approach has been recently proposed for the characterization of *POLD1* mutations, wherein the endogenous protein is inducibly replaced with the mutant variant.[34] While elegant and undoubtedly valuable for genes where the effects of mutations need to be analyzed in the absence of the wild-type allele expression, such inducible replacement may not be necessary for *POLD1* or *POLE* variants. As a rule, replicative DNA polymerase variants are present in cancer patients in the heterozygous state. Loss of heterozygosity is not required for the patients with germline *POLD1* or *POLE* mutations to develop tumors.[5] Studies of sporadic tumors with *POLD1* or *POLE* variants always report the presence of both wild-type and mutant alleles.[7,8,13,35] While we cannot exclude the possibility that non-tumor cells in the sequenced samples were responsible for the wild-type signal in some cases, cell lines established from hypermutated tumors are invariably heterozygous for the DNA polymerase mutations.[19,20,35] This is consistent with the view that the cancer-associated variant alleles encode active error-prone polymerases that function in the heterozygous cells along with the wild-type enzyme. Indeed, when tested in the model systems, the cancer-associated alleles appear to be semi-dominant.[13,29] Thus, a reasonable criterion for the functional significance of *POLD1* and *POLE* variants would be their ability to confer the mutator phenotype in the presence rather than in the absence of the wild-type allele. Development of cell-based assays where the wild-type and mutant alleles are expressed at comparable levels could allow for a more accurate prediction of the *in vivo* effects of DNA polymerase variants in the future.

The analysis of sequence specificity of *POLD1-R689W*-induced GC→TA transversions in the *HPRT1* gene revealed a strong preference for polypurine/polypyrimidine tracts and a highly asymmetric occurrence of mutations in respect to the two strands (Figure 3). The preference for GC→TA transversions in polypurine/polypyrimidine sequences is also evident in the genomic landscape of the HCT15 cell line (Figure 3), confirming the biological relevance of this signature. However, tracts with purines in the transcribed and non-transcribed strands were nearly equally affected in the HCT15 cells (not shown in Figure 3, but the original data can be found in [19]), suggesting that the bias we observed in the *HPRT1* gene is related to the strand-specific function of Polδ in DNA replication rather than the transcriptional asymmetry. A preferential occurrence of mutations in polypurine/polypyrimidine tracts has been observed previously in several other situations where increased mutagenesis results from DNA replication errors. Some examples include

mutations induced by the *pol3-R696W* allele or by treatment with a base analog 6-*N*-hydroxylaminopurine, a potent inducer of replication errors, in yeast, with a strong bias for G in the non-transcribed strand in both cases.[29,36] Curiously, the same context specificity could be seen for GC→TA transversions present in the genome of a hypermutated CRC cell line HCC2998, which carries a strongly mutagenic *POLE-P286R* variant (Supplementary Figure S3). The mechanism of preferential mutability of polypurine/polypirimidine tracts remains to be determined. One possibility we have previously discussed[36] is that stacking interactions between purine bases contribute to this phenomenon. Regardless of the mechanism, this signature could be useful for tracking the activity of mutator Polδ and Polε variants in human cancers.

MATERIALS AND METHODS

Cell lines

HCT116 and HCT116+ch3 were obtained from Robert Lewis and Michael Brattain (University of Nebraska Medical Center) and were last authenticated by STR profiling and tested for mycoplasma contamination in November 2016. All cell lines were maintained in Dulbecco's Modified Eagle's medium (DMEM; Hyclone) containing 10% fetal bovine serum (FBS; Atlanta Biologicals). HCT116+ch3 and its derivatives were grown in media containing 200 μg/ml G418 to prevent loss of chromosome 3.

Clonal cell lines expressing *POLD1* or *POLD1-R689W* were constructed by retroviral transduction. Retroviral particles were created by transfection of HEK293-GP cells (Clontech) with plasmids pVSV-G and MXIVpuro-POLD1 or MXIVpuro-POLD1-R689W using FuGENE6 (Promega) or TurboFect (Thermo Scientific) reagents and recommended manufacturer protocols. Media containing viral particles was collected 24 and 48 h after transfection and passed through a 0.45-μM syringe filter to remove unattached cells. Transductions of HCT116 and HCT116+ch3 were performed when cells were ~60% confluent by replacing the growth media with the retroviral media mixed with an equal volume of fresh DMEM+10%FBS and 10 μg/ml Hexadimethrine bromide (Polybrene). Cells with integrated retroviral vectors were selected for 24 h after transduction with 0.35 μg/ml puromycin (Life Technologies). Individual clones were isolated from separate transduction dishes by serial dilution in 96-well culture plates such that ~10% of the wells were positive for growth. Wells were examined after nine days, and only wells with a single colony were expanded for future use.

Plasmids

Plasmid pVSV-G was from Clontech. Retroviral vectors MXIV-puro-POLD1 and MXIV-puro-POLD1-R689W expressing the *POLD1* alleles from the *CMV* promoter were constructed as follows. The R689W mutation was first introduced into the *POLD1* cDNA cloned in the bacterial expression plasmid pET-POLD4/1[37] by site-directed mutagenesis. Plasmids pET-POLD4/1 and pET-POLD4/1-R689W were digested with EcoRI, and fragments containing the *POLD1* and *POLD1-R696W* cDNA were cloned into the EcoRI site of pBABE-puro-based retroviral expression vector MXIV-puro.[38] For overproduction in insect cells, cDNAs for all full-length human Polδ subunits were obtained from Open

Biosystems, cloned into pFastBac-1 transfer vector (Life Technologies), and the R689W mutation was introduced by site-directed mutagenesis.

HPRT1 mutation rate and mutation spectra analysis

The *HPRT1* mutation rate was determined by measuring the accumulation of mutants during serial passaging as described.[24] To characterize the spectrum of *HPRT1* mutations, cells were cleansed of pre-existing mutants by passaging ten times in DMEM+10%FBS containing 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine. Independent cultures were then started in DMEM+10%FBS from <10 cells and expanded to ~4×10^7 cells. Approximately 1×10^5 cells from each culture were plated in 10-cm dishes containing DMEM+10%FBS with 30 μM 6-TG and incubated for 14 days. The media was changed every three days. One randomly selected colony was isolated by the use of cloning cylinders and expanded to 1×10^7 cells. Genomic DNA and total RNA were extracted using the Puregene DNA cell kit (Qiagen) and total RNA isolation kits (Omega Bio-Tek and IBI). The cDNA was produced using the SMARTScribe reverse transcriptase kit and oligo$(dT)_{18}$ primers. The *HPRT1* cDNA was amplified with primers flanking the entire coding region and sequenced. If sequencing of rtPCR products revealed skipped exons, corresponding genomic DNA samples were used as templates to amplify exons and flanking intronic sequence.

Immunoblots

Following dissociation with trypsin, ~5×10^6 cells were collected by centrifugation at 500 RCF, washed with 1 ml of Phosphate Buffered Saline, and flash-frozen in liquid nitrogen. Cells were re-suspended in 350 μl of NP-40 buffer (50 mM Tris-HCl, pH 7.6, 1% NP-40, 150 mM NaCl, 1 mM $MgCl_2$) containing 1x complete protease inhibitor cocktail (Roche) and incubated at 4 °C for 30 min with gentle agitation. The lysate was then centrifuged at 10,000 RCF at 4 °C for 10 min. The supernatant was either used immediately for immunoblotting or stored at −80 °C. For immunoblots, 30 μg of protein was separated in a 4–12% bis-tris gel and transferred to a nitrocellulose membrane. This membrane was subsequently cut horizontally at a position corresponding to a protein molecular mass of ~70 kDa. The top and bottom halves were probed with mouse monoclonal anti-POLD1 (Abcam, ab10362) and rabbit polyclonal anti-α-tubulin (Abcam, ab4074) antibodies, respectively. The secondary antibodies were HRP-conjugated goat anti-mouse (Genscript, A00160) and goat anti-rabbit (Genscript, A00160). Proteins were detected by using chemiluminescent SuperSignal West Pico kit (Thermo Scientific) and autoradiography.

Purification of human Polδ and Polδ-R689W

Polδ and Polδ-R689W with 6xHis-tagged p125 subunits were produced in insect cells co-infected with four baculoviruses, each encoding one of the four Polδ subunits. Preparation of high-titer baculoviruses and protein production were done using the Bac-to-Bac baculovirus expression system (Life Technologies) and the manufacturer instructions. Buffer N (20 mM Tris-HCl, pH 7.9, 5% glycerol, 10 mM KH_2PO_4/K_2HPO_4, pH 7.9, 0.005% Nonidet P-40, 3 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, 2 μg/ml leupeptin) was used during all purification steps. The concentration of NaCl (in mM) is denoted by subscripts. Insect cells (~7 g) were lysed in buffer N_{100} containing 1 mM

imidazole. After centrifugation at 40,000 RCF for 30 min, the cleared lysate was incubated with 5 ml of Ni-IDA resin (Bio-Rad) for 60 min with gentle agitation. The mix was loaded onto a Biorad econocolumn, washed with N_{100} containing 1 mM imidazole, and Polδ or Polδ-R689W was eluted with a 1 mM to 150 mM imidazole gradient in Buffer N_{100}. Fractions containing Polδ or Polδ-R689W were combined with an equal volume of N_0, dialyzed to N_{25}, filtered, and loaded to a Mono S column (GE Life Sciences). Polδ was eluted with an N_{25} to N_{800} gradient, the NaCl concentration was adjusted to ~250 mM with N_{100}, and the fractions were loaded to a 1-ml HiTrap Heparin HP column (GE Life Sciences). Polδ was eluted with an N_{250} to N_{800} gradient. Fractions containing Polδ were dialyzed in N_{100} and loaded to a 1-ml Mono Q column (GE Life Sciences). Polδ was eluted with an N_{100} to N_{800} gradient. Peak fractions were concentrated and stored in 20 mM Tris-HCl, pH 7.9, 10% glycerol, 10 mM KH_2PO_4/K_2HPO_4, pH 7.9, 0.005% NP-40, 2 mM dithiothreitol, 150 mM NaAc, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin and 2 μg/ml leupeptin.

In vitro DNA polymerase and exonuclease assays

The reactions were performed at 37°C with 25 nM Cy5-labeled oligonucleotide substrate (5′-Cy5-CAGCACCACAAACCATACAAAAACA-3′/5′-GCCATTATCGGGTTTCTAATATACTGTTTTTGTATGGTTTGTGGTGCTG-3′),[18] 40 mM Tris-HCl, pH 7.8, 150 mM NaAc, 10 mM MgAc, 0.2 mg/mL bovine serum albumin, 4% polyethylene glycol 8000, 1 mM dithiothreitol, 25 nM Polδ or Polδ-R689W, and 100 μM dNTPs or no dNTPs. Reactions were terminated by placing the tubes on ice and adding 15 μl of formamide loading dye. The products were separated by electrophoresis in a 16% denaturing polyacrylamide gel, and detected and quantified as described.[18]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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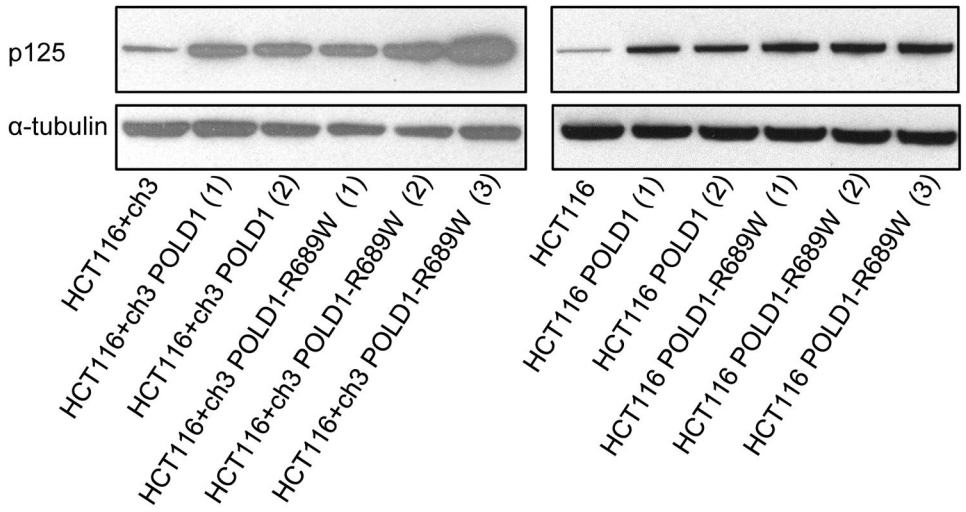

Figure 1.

Immunoblots showing p125 (POLD1) levels in clonal cell lines overexpressing *POLD1* or *POLD1-R689W*. The numbers in parenthesis indicate independently derived cell lines. The left and right panels are from separate SDS-PAGE gels.

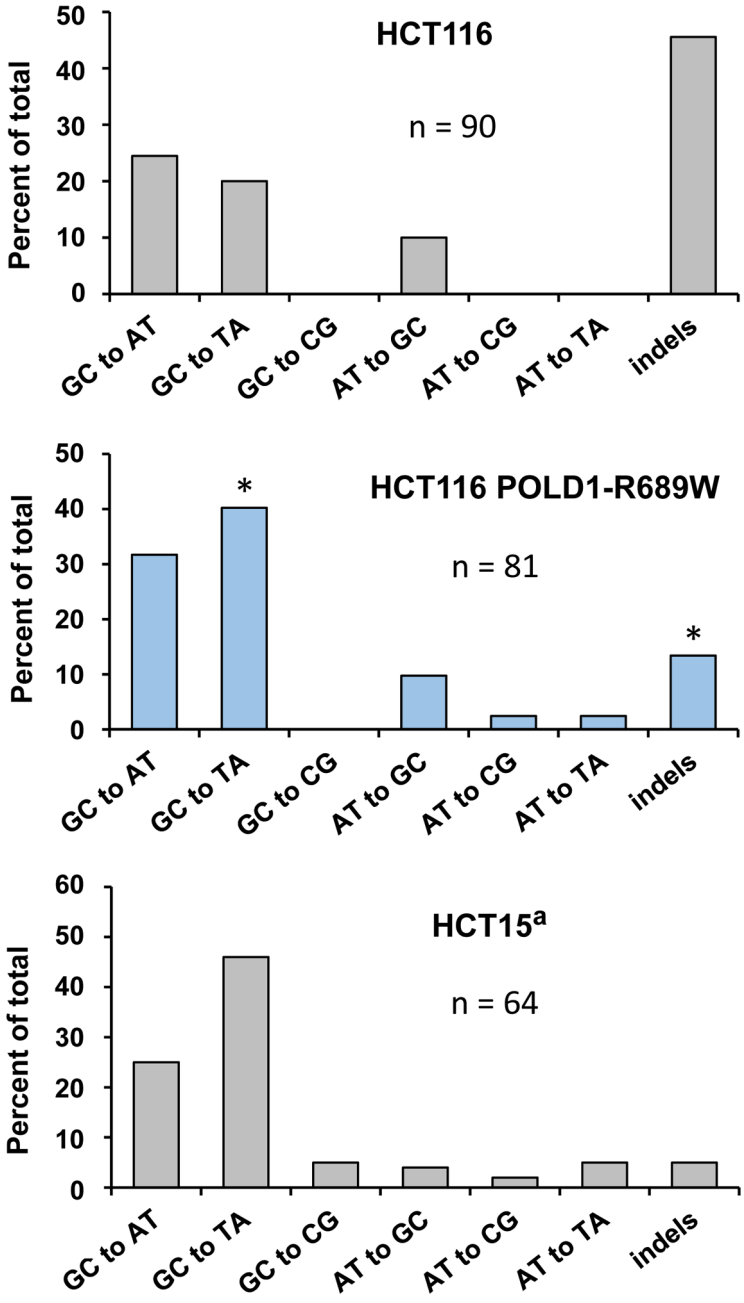

Figure 2.

Expression of *POLD1-R689W* alters the specificity of spontaneous mutagenesis in HCT116 cells making it resemble the mutational specificity of HCT15. The diagrams show the *HPRT1* mutation spectra of HCT116, HCT116 POLD1-R689W and HCT15 cell lines. The number of independent *HPRT1* mutants analyzed (n) is indicated for each spectrum. Data for HCT116 POLD1-R689W and HCT116 are from Table 2, and data for HCT15 is from reference 25. Asterisks indicate statistically significant differences in the proportion of indels and GC→TA transversions between HCT116 and HCT116 POLD1-R689W spectra (p=0.000005 for indels and p=0.0043 for GC→TA transversions, Fisher's exact text). No

significant differences were observed between HCT116 POLD1-R689W and HCT15 spectra.

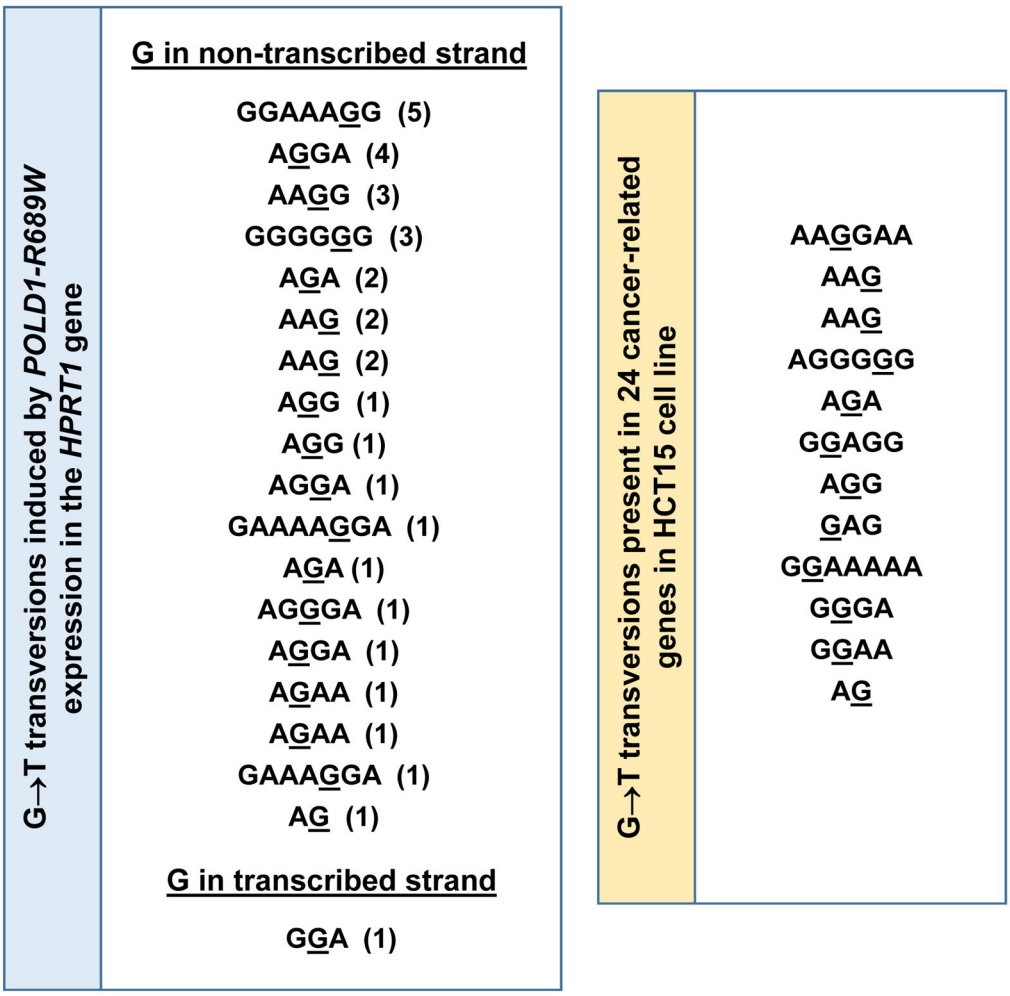

Figure 3.

DNA sequence context of GC→TA transversions in cancer-related genes in the HCT15 cell line matches the mutational specificity of the *POLD1-R689W* mutator. (A) Expression of *POLD1-R689W* results in GC→TA transversions at polypurine/polypyrimidine tracts. Genomic sequence context is shown for all GC→TA transversions observed at the *HPRT1* gene of the HCT116 POLD1-R689W cells. Each mutated site is shown as a separate entry with the number of mutations at this site in parentheses. The mutated base is underlined. (B) Sequence context of GC→TA transversions found in a set of 26 cancer-related genes in the HCT15 cell line. The set of genes was the same as the one used previously to characterize the mutational specificity of the NCI-60 panel of cell lines[19] and included *APC*, *ARID1A*, *BRAF*, *DNMT1*, *DNMT3A*, *DNMT3B*, *EGFR*, *EPHA3*, *EPHA5*, *EPHA7*, *FBXW7*, *GRIN2A*, *KRAS*, *LRP1B*, *NF2*, *NRAS*, *PBRM1*, *PIK3CA*, *POLE*, *PTEN*, *SETD2*, *SPTA1*, *STAG2*, *SYNE1*, *TP53*, and *TRRAP*. Sequence of the G-containing strand is shown. The mutated base is underlined.

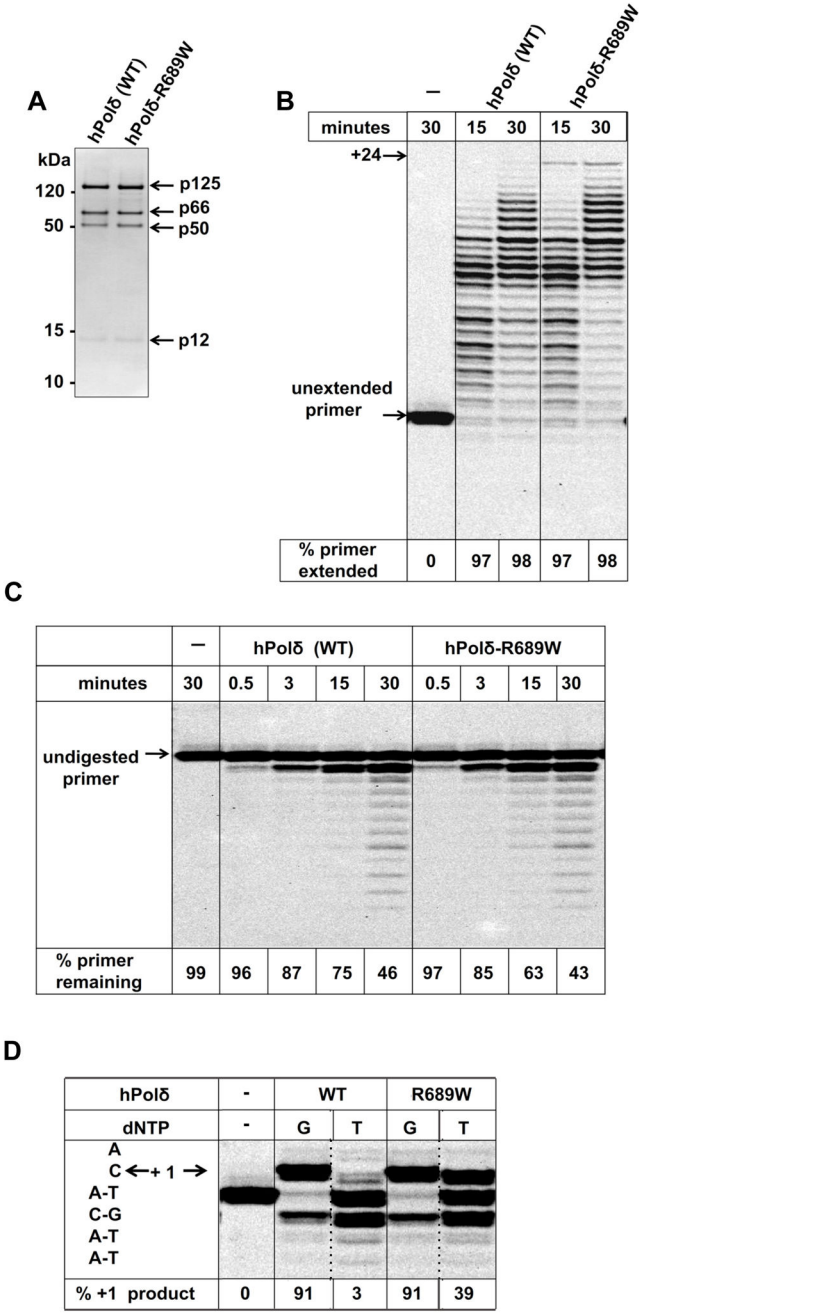

Figure 4.

Polδ-R689W is an active and highly error-prone DNA polymerase. (A) Purified Polδ and Polδ-R689W were separated by SDS-PAGE in a 10% Bis-Tris gel and visualized with Coomassie staining. (B) DNA synthesis by Polδ and Polδ-R689W was analyzed by incubating the purified enzymes with all four dNTPs and an oligonucleotide template (see Materials and Methods) for the times indicated. (C) Exonuclease activity was assayed by incubating the purified enzymes with the oligonucleotide substrate and no dNTPs. (D) The efficiency of correct and incorrect nucleotide insertion across from a template C was analyzed by incubating the enzymes and the oligonucleotide substrate for 15 min in the

presence of dGTP or dTTP as indicated. Dashed lines indicate that the lanes were not adjacent to each other in the original gel.

Table 1

HPRT1 mutation rate in HCT116-derived cell lines overexpressing *POLD1* or *POLD1-R689W*

Cell line[1]	MMR status	*HPRT1* mutation rate ($\times 10^{-6}$)[2]	Standard error ($\times 10^{-6}$)[2]	Fold Increase[3]
HCT116+ch3	+	1.2	0.08	1.0
HCT116+ch3 POLD1 (1)	+	0.94	0.17	0.78
HCT116+ch3 POLD1 (2)	+	1.4	0.41	1.2
HCT116+ch3 POLD1-R689W (1)	+	2.4	0.64	2.0
HCT116+ch3 POLD1-R689W (2)	+	4.3*	0.49	3.6
HCT116+ch3 POLD1-R689W (3)	+	4.7*	0.48	3.9
HCT116	−	8.1	1.0	6.8
HCT116 POLD1 (1)	−	8.9	1.6	7.4
HCT116 POLD1 (2)	−	8.8	1.2	7.3
HCT116 POLD1-R689W (1)	−	96*	11	80
HCT116 POLD1-R689W (2)	−	24*	2.6	20
HCT116 POLD1-R689W (3)	−	91*	20	76

[1] The numbers in parentheses designate independently derived cell lines.

[2] The mutation rate was calculated by plotting the mutation frequency as a function of population doublings, and determining the slope of the resulting line and standard error by linear regression using the LINEST function (Microsoft Excel). Asterisks indicate significant difference ($p<0.01$) from the respective parental cell line, HCT116+ch3 or HCT116. The p-values were calculated with Minitab statistical software using the "Fit Regression Model" function.

[3] Fold increase in the mutation rate is relative to HCT116+ch3.

Table 2

The spectrum of spontaneous *HPRT1* mutations in HCT116 POLD1-R689W cell line and the parental HCT116 cells

Mutation	HCT116 POLD1-R689W		HCT116	
	Number	Percent of total	Number	Percent of total
Base substitutions	70	86	49	54
GC to AT	26	32	22	24
GC to TA	33	41	18	20
GC to CG	0	<1.2	0	<1.1
AT to GC	8	9.9	9	10
AT to CG	1	1.2	0	<1.1
AT to TA	2	2.5	0	<1.1
Indels	11	14	41	46
Minus 1	6	7.4	18	20
Minus 3	0	<1.2	2	2.2
Plus 1	5	6.2	21	23

The data is based on DNA sequence analysis of 81 and 90 independent *HPRT1* mutants of the HCT116 POLD1-R689W (1) cell line and the parental HCT116 cell line, respectively. An additional silent mutation (not included in the table), AT to CG, was observed in one *HPRT1* mutant of HCT116 POLD1-R689W (1) that also contained a detectable change. The location of individual mutations in the *HPRT1* sequence is shown in Supplementary Figures S1 and S2.