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Mismatch repair and DNA polymerase δ proofreading prevent catastrophic accumulation of leading strand errors in cells expressing a cancer-associated DNA polymerase ε variant

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ABSTRACT

Substitutions in the exonuclease domain of DNA polymerase ε cause ultramutated human tumors. Yeast and mouse mimics of the most common variant, P286R, produce mutator effects far exceeding the effect of Polε exonuclease deficiency. Yeast Polε-P301R has increased DNA polymerase activity, which could underlie its high mutagenicity. We aimed to understand the impact of this increased activity on the strand-specific role of Polε in DNA replication and the action of extrinsic correction systems that remove Polε errors. Using mutagenesis reporters spanning a well-defined replicon, we show that both exonuclease-deficient Polε (Polε-exo$^-$) and Polε-P301R generate mutations in a strictly strand-specific manner, yet Polε-P301R is at least ten times more mutagenic than Polε-exo$^-$ at each location analyzed. Thus, the cancer variant remains a dedicated leading-strand polymerase with markedly low accuracy. We further show that P301R substitution is lethal in strains lacking Polδ proofreading or mismatch repair (MMR). Heterozygosity for *pol2-P301R* is compatible with either defect but causes strong synergistic increases in the mutation rate, indicating that Polε-P301R errors are corrected by Polδ proofreading and MMR. These data reveal the unexpected ease with which polymerase exchange occurs *in vivo*, allowing Polδ exonuclease to prevent catastrophic accumulation of Polε-P301R-generated errors on the leading strand.

INTRODUCTION

Accurate DNA replication is the primary defense against mutation accumulation in cells. Elevated mutation rates contribute to genome instability and oncogenesis. Replicative DNA polymerases are responsible for the selection of correct nucleotides during DNA synthesis and exonucleolytic proofreading of errors, thus being a major safeguard against genome instability (1). Rare errors missed by the nucleotide selectivity and proofreading functions of replicative polymerases are further corrected by the DNA mismatch repair (MMR) system (2), ultimately resulting in a low mutation rate of 2.6×10^{-10} and 3.3×10^{-10} per base pair in prokaryotic and eukaryotic genomes, respectively (3). Eukaryotic DNA replication requires three DNA polymerases: Polα, Polδ and Polε (4). Polδ and Polε possess a proofreading exonuclease activity and are significantly more accurate than Polα (5–7). The current model of eukaryotic DNA replication was originally proposed by Morrison *et al.* (8) and remains the most widely accepted model at this time. It suggests that Polα associated with the primase creates short RNA–DNA primers at replication origins and at the beginning of each Okazaki fragment on the lagging strand, Polδ synthesizes the remaining portion of Okazaki fragments, and Polε synthesizes the bulk of the leading strand. Accordingly, the nucleotide selectivity and proofreading activities of Polδ and Polε are mainly responsible for the fidelity of synthesis on opposite DNA strands (9–13), and MMR corrects errors on both strands, albeit with unequal efficiency (14,15). Furthermore, we recently showed that Polδ is capable of proofreading Polε-generated errors, further increasing the fidelity of DNA replication (16).

Ultramutated colorectal and endometrial tumors almost invariably contain mutations in the *POLE* gene which encodes the catalytic subunit of Polε in humans (17,18). The mutation load in these tumors is over 100 mutations per megabase genome-wide, an order of magnitude higher than

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in MMR-deficient tumors with microsatellite instability (19,20). The majority of *POLE* mutations result in amino acid changes in the exonuclease domain of the polymerase, yet the impact of these mutations goes far beyond a simple loss of proofreading. This is best illustrated by the properties of *POLE-P286R*, which is the most common *POLE* variant in sporadic tumors. It has been reported in over 200 tumors to date, predominantly endometrial and colorectal but also across other tissue types including ovary, urinary tract, pancreas, breast, prostate, and brain (21,22). When modeled in haploid budding yeast, the P286R variant caused a 150-fold increase in mutation rate over the wild-type strain (23). This is 50-fold higher than the mutator effect of Polε proofreading deficiency and also overwhelmingly exceeds the effect of any previously studied Polε mutation. Furthermore, $Pole^{P286R}$ mice are dramatically more cancer-prone than mice deficient in Polε proofreading and, in fact, more cancer-prone than any existing monoallelic animal model (24,25).

The mechanisms of these uniquely strong mutagenic and tumorigenic effects of P286R variant remain to be determined. We recently reported that the purified yeast variant, Polε-P301R, has an unusually high DNA polymerase activity in addition to a severe exonuclease defect (26). It extends matched and mismatched primer termini more efficiently than either wild-type Polε or Polε-exo$^-$ and particularly excels at synthesis through secondary structures that normally impede replicative polymerases (26). Crystallographic studies of Polε-P301R and molecular dynamics simulations suggested that the arginine protrudes into the opening of the exonuclease active site, hindering access of the primer terminus to the catalytic residues (27). We, therefore, proposed that the robust increase in polymerase activity is caused by the inability to accommodate the 3′ end in the exonuclease site, which prompts Polε-P301R to stay in the polymerization mode (26). How these unusual biochemical properties of Polε-P301R affect DNA replication *in vivo* remained unclear.

In the present work, we aimed to understand the consequences the increased polymerase activity of Polε-P301R has for the role of Polε in replication and the ability of extrinsic mechanisms to correct its errors. By analyzing strand-specific mutation accumulation across a well-defined replicon in yeast, we demonstrate that Polε-P301R is strictly a leading strand replicase. We further show that mismatch repair (MMR) and extrinsic proofreading by Polδ are both required to maintain viability of cells that carry Polε-P301R as the sole source of Polε. We conclude that MMR and Polδ proofreading prevent catastrophic accumulation of leading strand errors in yeast harboring Polε-P301R. These data provide an explanation for the apparent incompatibility of Polε-P286R and MMR defects in human cancers. They also illustrate the robustness of the extrinsic proofreading mechanism that can effectively fight a leading strand error burden much higher than what eukaryotic cells typically encounter.

MATERIALS AND METHODS

Plasmid construction

YEp181MSH6 is a *LEU2*-based expression vector containing the *Saccharomyces cerevisiae MSH6* gene cloned into BamHI and HindIII sites of YEp181spGAL (28), which places the gene under control of the *GAL1* promoter. YIpCB2 was constructed by replacing the *URA3* marker in YIpDK1-pol2-P301R (23) with the *LYS2* marker as follows. The *LYS2* gene with 1053 nucleotides of upstream and 172 nucleotides of downstream region was amplified from chromosomal DNA of DS2 strain, a derivative of W303 (kindly provided by Duncan Smith, New York University), using primers 5′-TTTTTTGCCAATTTGGCCTGGCTC ACTTGAGGGCTAT-3′ and 5′-TTTTTTTGGCCAAG CAGACTAACGCCAGCTGA-3′ (Eurofins), which created BglI and MscI restriction sites, respectively, at the ends of the PCR fragment. Both the PCR fragment and YIpDK1-pol2-P301R were digested with BglI and MscI and ligated to create YIpCB2.

Yeast strains

The haploid *Saccharomyces cerevisiae* strains used to study mutagenesis across the *ARS306* replicon (Supplementary Table S1) were derived from CG379Δ, which contains a deletion of chromosomal *URA3* (29). The CG379Δ n303::ura3-29inv or1 (and or2) and CG379Δ atg22::ura3-29 or1 (and or2) strains were created by Olga Kochenova in the Shcherbakova laboratory by amplification of a *ura3-29::LEU2* cassette from a *URA3-LEU2* integrative vector containing the *ura3-29* mutation (30,31), and integration of the cassette into the corresponding chromosomal position by transformation. Reporter strains with other locations of the *ura3-29* allele and *ura3-24* reporter strains were constructed similarly. Primers used for amplification of the cassettes were obtained from Eurofins and are listed in Supplementary Table S2. *pol2-4* and *pol2-P301R* derivatives of *ura3-29* and *ura3-24* strains were created by an integration-excision procedure using BamHI-linearized YIpJB1 and YIpDK1-pol2-P301R plasmids, respectively, as described previously (23,32). The *MSH6* gene was deleted in *ura3-24* reporter strains by transformation with a PCR-generated DNA fragment carrying the *kanMX* cassette flanked by short sequence homology to *MSH6* (33). To minimize accumulation of mutations during strain construction, we created double-mutant *pol2-4 msh6*Δ derivatives of *ura3-24* reporter strains by first transforming them with BglII-linearized YIpJB1 such that the *pol2-4* mutation was in the truncated, non-expressed copy. We then deleted *MSH6* as described above, and finally used 5-FOA-containing medium to select for cells that had lost the YIpJB1 plasmid sequence through recombination and retained the *pol2-4* allele to obtain the double-mutant strains.

The strains used for the synergistic interaction studies were derived from TM30 and TM44 (34). *msh6*Δ, *pol2-4*, and *pol2-P301R* mutations were introduced into TM30 and TM44 as described above. The *pol3-D520V* mutation was introduced by integration-excision using BseRI-linearized p170 harboring the *pol3-D520V* (p170-pol3-D520V) (35). To make diploid strains heterozygous for *pol2-P301R* and homozygous for *msh6*Δ, we first transformed TM30 and TM44 with BglII-linearized YIpDK1-pol2-P301R to create haploid strains with the *pol2-P301R* mutation in the truncated, non-expressed copy of *POL2*. We then deleted chromosomal *MSH6* in both the TM30 YIpDK1-pol2-

P301R and TM44 YIpDK1-pol2-P301R strains as described above, and crossed the haploids. To obtain the heterozygous *pol2-P301R* mutation in these strains, we used 5-FOA medium to select for strains that had lost the YIpDK1-pol2-P301R plasmid from both chromosomes, and used Sanger sequencing to identify clones that maintained the *pol2-P301R* mutation in one chromosome. Diploid strains heterozygous for *pol2-P301R* and *pol3-D520V* (or *pol2-4* and *pol3-D520V*) were made by crossing TM30 containing the *pol2-P301R* (or *pol2-4*) mutation and TM44 containing the *pol3-D520V* mutation. To create double homozygous *pol2-P301R/pol2-P301R pol3-D520V/pol3-D520V* diploid strains containing a plasmid expressing wild-type *POL3*, we transformed *pol2-P301R/POL2 pol3-D520V/POL3* diploids with pBL304, an episomal plasmid expressing *POL3* (36). The transformants were subjected to sporulation and tetrad dissection, and haploid *pol2-P301R pol3-D520V* pBL304 segregants were identified by Sanger sequencing. The double-mutant segregants of opposite mating type were then crossed to obtain double-homozygous diploids for analysis of plasmid loss. Diploid strains heterozygous for *pol2-P301R* and homozygous for *pol3-D520V* were created as follows. TM30 was first transformed with BseRI-linearized p170-pol3-D520V, which placed the mutation in the truncated, non-expressed copy of *POL3*. TM30 containing the *pol3-D520V* mutation (in the non-expressed copy) was then transformed with SalI-linearized YIpCB2, which placed the *pol2-P301R* mutation in the truncated, non-expressed copy of *POL2*. We then used medium containing α-aminoadipic acid to select for cells which had lost YIpCB2 to obtain the *pol2-P301R* mutant. To obtain diploids, we crossed this strain to a TM44 derivative which contained the p170-pol3-D520V plasmid integrated such that the mutation was also in the truncated, non-expressed copy of *POL3*. We used 5-FOA medium to select for cells which had lost the p170-pol3-D520V plasmid from both chromosomes simultaneously, and the genotype was confirmed by Sanger sequencing.

ura3-29 revertant sequencing

Single colonies of *ura3-29* strains containing either the *pol2-4* or *pol2-P301R* mutation (strains #13-36 in Supplementary Table S1) were inoculated into rich yeast extract peptone dextrose liquid medium supplemented with uracil and adenine (YPDAU) (37) and the cultures were grown to stationary phase overnight. The cultures were diluted and plated on synthetic complete medium lacking uracil, and a single colony from each culture was randomly picked for DNA isolation. A fragment corresponding to 122 nucleotides upstream of *URA3* and nucleotides 1–721 of the *URA3* gene was amplified using primers 5′-GGAAGGAG CACAGACTTAGATT-3′ and 5′-CCTTTGCAAATAGT CCTCTTCC-3′ (Eurofins). The PCR products were purified and Sanger-sequenced with primer 5′-GTTAGTTGAA GCATTAGGTCC-3′ (Eurofins).

Mutation rate measurements

The rate of *ura3-29* reversion, *ura3-24* reversion, *CAN1* forward mutation, and *his7-2* reversion was measured by fluctuation analysis as described previously (37). For each strain, nine independent cultures were started from single colonies in YPDAU broth and grown to saturation overnight. The cultures were appropriately diluted and plated on synthetic complete (SC) medium for viable cell count or selective medium. SC medium lacking uracil or histidine was used to select for Ura^+ and His^+ revertants. For Ura^+ reversion, the cells were washed with sterile water before dilution. SC medium containing 60 mg/l L-canavanine and lacking arginine and leucine was used to select for Can^r mutants. Mutation frequency was calculated by dividing the number of mutant cells in a culture by the total number of cells in that culture. The mutation rate was derived from the calculated mutation frequency using Drake equation (38).

Plasmid loss assays

Diploid strains harboring pBL304 (*POL3*) were grown in YPDAU broth to saturation and then serially diluted in a sterile 96-well plate. A 48-pronged replicator was used to transfer diluted cultures to plates containing either SC medium or 5-FOA medium selective for cells that have lost the pBL304 plasmid with the *URA3* marker. The ability to survive without wild-type *POL3* was determined by comparing growth on SC versus growth on 5-FOA medium.

Proteins

Preparations of four-subunit yeast Polε variants (Polε-exo$^-$ and Polε-P301R) and proliferating cell nuclear antigen (PCNA) used in this work have been described previously (26,34). Purified yeast replication factor C (RFC) was kindly provided by Peter Burgers (Washington University School of Medicine).

Primer extension assays

Substrates for primer extension assays were prepared by annealing primer P1 (5′-Cy5-ATTTGACTGTATTACCAA TGTCAGCAAATTTTCTGTCTTCGAAGAGTAAA) to template BT1 (5′-Bio-AAGGCATTATCCGCCAAG TACAATTCTTTACTCTTCGAAGACAGAAAATT TGCTGACATTGGTAATACAGTCAAATTGCAGT ACTCTGCGGGTGTATACAG-Bio) and primer P2 (5′-Cy5-CATGGAGGGCACAGTTAAGCCGCTAAAG GCATTATCCGCCAAGTACAATT) to template BT2 (5′-Bio-AAATTTTCTGTCTTCGAAGAGTAAAGAA TTGTACTTGGCGGATAATGCCTTTAGCGGCTT AACTGTGCCCTCCATGGAAAAATCAGTCAAGA TATCCACAT-Bio). All oligonucleotides were obtained from IDT. Primer and template were combined in a ratio of 1:1.5 in the presence of 150 mM NaAc and 20 mM HEPES (pH 7.8), and annealed by incubating the mixture at 95°C for 3 min and then cooling to room temperature slowly over ∼2 h. Streptavidin (NEB #N7021S) was added in 2-fold molar excess for 10 min at room temperature to block the ends of the substrate to allow stable loading of PCNA by RFC. The 10-μl primer extension reaction contained 40 mM Tris–HCl pH 7.8, 1 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, 8 mM $MgAc_2$, 125 mM NaAc, 25 nM DNA substrate, 1 mM ATP, 20 nM RFC,

60 nM PCNA, 6.25 nM Polε and the indicated dNTP. We used dNTP concentrations equivalent to intracellular concentrations estimated for wild-type yeast strains to mimic *in vivo* conditions [30 μM dCTP, 80 μM dTTP, 38 μM dATP and 26 μM dGTP; (34,39)]. RFC and PCNA were added first followed by 5-min incubation at 30°C to allow PCNA loading, and DNA synthesis reactions were then initiated by the addition of Polε. The synthesis reactions were carried out for 5 min at 30°C and stopped by the addition of an equal volume of 2× loading buffer containing 95% formamide, 100 mM EDTA and 0.025% Orange G. Samples were boiled for 5 min, cooled on ice for 5 min, and 6 μl of each sample was separated by electrophoresis in a 10% denaturing polyacrylamide gel containing 8 M urea in 1× TBE. Quantification of fluorescent products was carried out on a Typhoon imaging system (GE Healthcare).

RESULTS

Polε-P301R is a dedicated leading strand polymerase

The contribution of error-prone Polε variants to DNA replication can be monitored by measuring their mutator effects at various locations within replicons. Replication origins and termination zones are well-defined in *S. cerevisiae* (40). Autonomous replicating sequence 306 (*ARS306*) and *ARS305* are two adjacent early-firing replication origins, and termination of replication consistently occurs at the midpoint between these two origins (40). We developed a genetic system to study the effects of the *pol2-P301R* allele encoding Polε-P301R and *pol2-4* allele encoding Polε-exo$^-$ on mutagenesis at different positions within this replicon. This system comprises a series of strains with a reversion reporter allele, *ura3-29*, at six locations between *ARS306* and the termination zone (Figure 1A). The *ura3-29* strains can revert to a Ura$^+$ phenotype via C→T, C→A or C→G substitutions in a TCT sequence context (Figure 1B, left) (9,41). We placed the reporter allele in two orientations at each location within the replicon, such that the TCT sequence was either in the leading strand or the lagging strand (Figure 1B, right), producing a total of 12 reporter strains. The *ura3-29* reporter is particularly well suited to characterize Polε-P301R- and Polε-exo$^-$-induced mutagenesis as both Polε variants predominantly generate C→T transitions and C→A transversions (26,42), in line with the mutational specificity of *POLE* mutant tumors (43–45). Sequencing of Ura$^+$ revertants arising in the *pol2-P301R* and *pol2-4* derivatives of our reporter strains confirmed that reversion occurs via C→T transitions and C→A transversions, and C→G transversions are extremely rare (Figure 1C). Both C→T and C→A were observed at comparable frequencies regardless of the orientation of the reporter allele.

Next, we examined whether our system could distinguish between leading and lagging strand errors. A C→T transition can occur via mispairing between an incoming dATP with template C, or dTTP with template G during copying of the opposite strand. Similarly, a C→A transversion can result from a dTTP mispairing with template C, or dATP with template G in the opposite strand. C→T and C→A

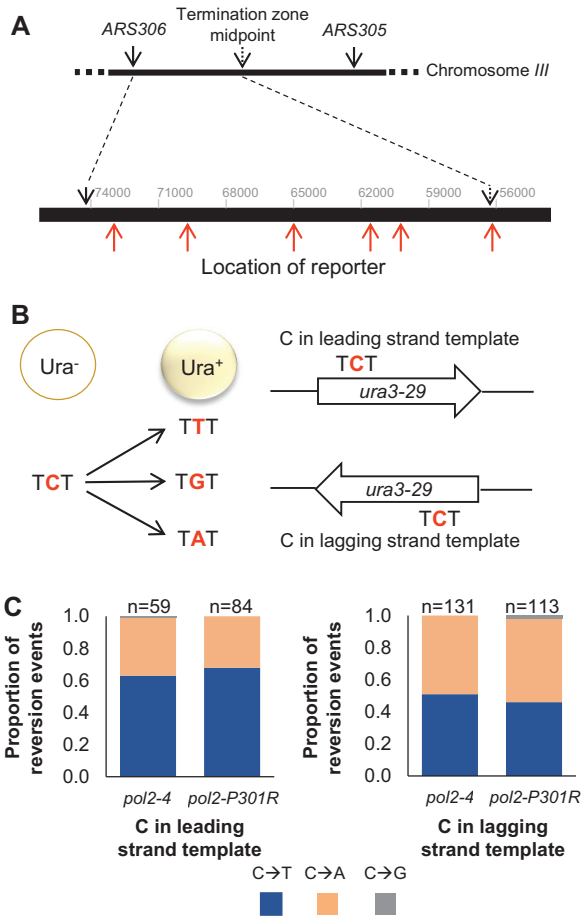

Figure 1. A *ura3-29* reporter system for analysis of mutagenesis across a replicon. (**A**) A reversion reporter was placed at six locations between *ARS306* and the nearest replication termination zone. Gray numbers show nucleotide position with respect to the left telomere on chromosome *III*. (**B**) *ura3-29* strains cannot grow on medium lacking uracil and revert to a Ura$^+$ phenotype via C→T, C→A or C→G mutations in a TCT context (9,41). The *ura3-29* reporter was inserted in two orientations at each location shown in (A), placing the TCT sequence in either the leading or the lagging strand. (**C**) *ura3-29* reverts primarily via C→T transitions and C→A transversions in *pol2-4* and *pol2-P301R* strains. The results shown are based on sequencing 3–34 independent revertants for each location and orientation of the *ura3-29* allele; data for the six locations are combined. Data for individual strains are shown in Supplementary Table S5.

mutations observed *in vivo* could be ascribed to either leading or lagging strand errors if there is a bias in the formation of reciprocal mispairs, as described previously (46,47). To compare the frequency at which Polε-exo$^-$ and Polε-P301R generate reciprocal mispairs at the *ura3-29* mutation site, we studied the incorporation of correct and incorrect nucleotides by purified polymerases *in vitro* on templates mimicking the *ura3-29* sequence. We used two oligonucleotide substrates containing either the transcribed or non-transcribed strand of the *ura3-29* as a template (template G or template C, respectively; Figure 2A). Primers were positioned such that the first nucleotide incorporated would be at the site of the *ura3-29* mutation. The reactions were carried out in the presence of accessory proteins PCNA and RFC, and the templates contained streptavidin bumpers on each end to allow stable loading of PCNA (Figure 2A).

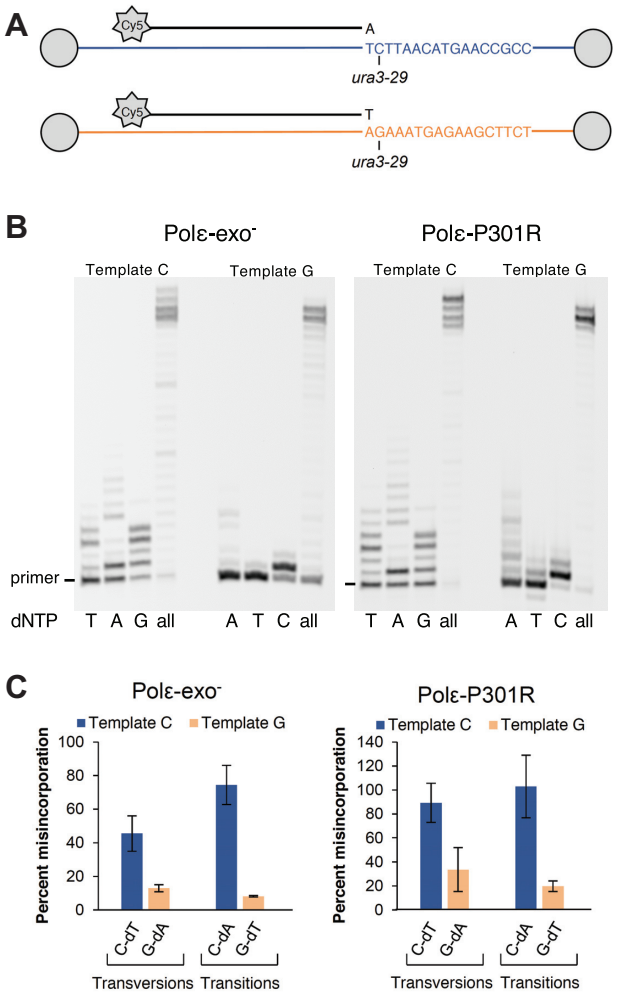

Figure 2. A bias in the formation of reciprocal mispairs at the *ura3-29* mutation site. (**A**) Oligonucleotide substrates for primer extension assays. The DNA sequence of the substrates corresponds to the sequence context of the *ura3-29* mutation. Sequences of the non-transcribed and transcribed strands serve as templates in the top and bottom substrates, respectively. The mutation site is indicated. For complete primer and template sequences, see Materials and Methods. Streptavidin bumpers are shown as grey circles. (**B**) Primer extension by Polε-exo$^-$ and Polε-P301R on substrates described in (A). Reactions were carried out for 5 min using a 4:1 ratio of substrate to polymerase, and the products were separated by denaturing polyacrylamide gel electrophoresis. The dNTPs present in each reaction are indicated below the gel image. (**C**) The efficiency of nucleotide misincorporation by Polε-exo$^-$ and Polε-P301R at the *ura3-29* mutation site. Percent misincorporation was calculated by dividing the fraction of primer extended with an incorrect nucleotide by the fraction of primer extended with the correct nucleotide. Data are averages of three experiments. Error bars represent standard deviation.

Both Polε variants generated transition- and transversion-type mispairs significantly more efficiently when C was the templating base in this sequence context (Figure 2B, C). This strong bias allowed us to use the *ura3-29* reporter to determine the rate of strand-specific errors in cells harboring Polε-exo$^-$ and Polε-P301R.

In haploid *pol2-4* strains containing Polε-exo$^-$, the rate of Ura$^+$ reversion was consistently higher for the orientation of *ura3-29* that scores leading strand errors (Figure 3, top). The bias persisted across the entire replicon and disappeared abruptly at the termination zone. To confirm that the bias was not due to the differences in the direction of transcription relative to DNA replication between the two orientations of *ura3-29*, we used a second set of strains containing a different reporter allele, *ura3-24*, placed in the same six chromosomal locations (Supplementary Figure S1A, B). The *ura3-24* strains revert to a Ura$^+$ phenotype via C→T substitutions in the same TCT sequence context but the TCT sequence is in the transcribed DNA strand in the *ura3-24* while it is in the non-transcribed strand in *ura3-29* (compare Figure 1B to Supplementary Figure S1B). The rates of *ura3-24* reversion in *pol2-4* strains were still higher when C was in the leading strand, confirming that the bias was due to replication and not transcription asymmetry (Supplementary Figure S1C). We also verified that the bias was not due to the differential MMR activity on the two strands as it was also observed, even to a greater extent, in *pol2-4 msh6* strains lacking Msh6-dependent MMR (Supplementary Figure S1D). Neither *ura3-29* nor *ura3-24* reversion showed a bias in strains with wild-type Polε (Supplementary Figure S2). These results are consistent with the replication fork model wherein Polε synthesizes the leading strand. We observed a similar pattern of mutagenesis in *pol2-P301R* strains harboring the cancer-associated variant Polε-P301R (Figure 3, bottom). The reversion rates were up to 17 times higher when C was in the leading strand, and the bias disappeared at the termination zone. The only major difference between *pol2-4* and *pol2-P301R* strains was in the absolute rate of leading strand errors, which was an order-of-magnitude higher for *pol2-P301R* across the entire replicon. We conclude that, despite the dramatic change in the biochemical properties (26), Polε-P301R remains a strict leading strand polymerase.

Survival of *pol2-P301R* strains requires correction of Polε-P301R errors by MMR

Haploid *pol2-P301R msh6*Δ strains are inviable, but the double mutant cells can divide and form microcolonies before the growth stops (26). This phenotype is characteristic of a replication error catastrophe (48). It suggests that the number of mismatches generated by Polε-P301R is overwhelming, and Msh6-dependent MMR is required to keep the mutation rate below the lethal threshold. To test this hypothesis, we sought approaches to determine whether the combination of *pol2-P301R* with a MMR defect results in a synergistic increase in the mutation rate. Diploids can tolerate higher levels of mutagenesis, and mutator effects of many allele combinations lethal in haploids could be studied in diploids (36,48–50). We attempted to construct diploid strains homozygous for both *pol2-P301R* and *msh6*Δ mutations but were unsuccessful, which suggested that the mutation rate in the double mutants was too high even for diploid cells. Indeed, the levels of mutagenesis in MMR-proficient diploids homozygous for the *pol2-P301R* alone already approach the viability threshold for diploid cells (23,51), and further increase due to the loss of MMR may be fatal. Thus, MMR appears to be required for survival of strains containing Polε-P301R as the sole source of Polε. This is in striking contrast to the *pol2-4* strains containing Polε-exo$^-$ that can tolerate a loss of MMR even in

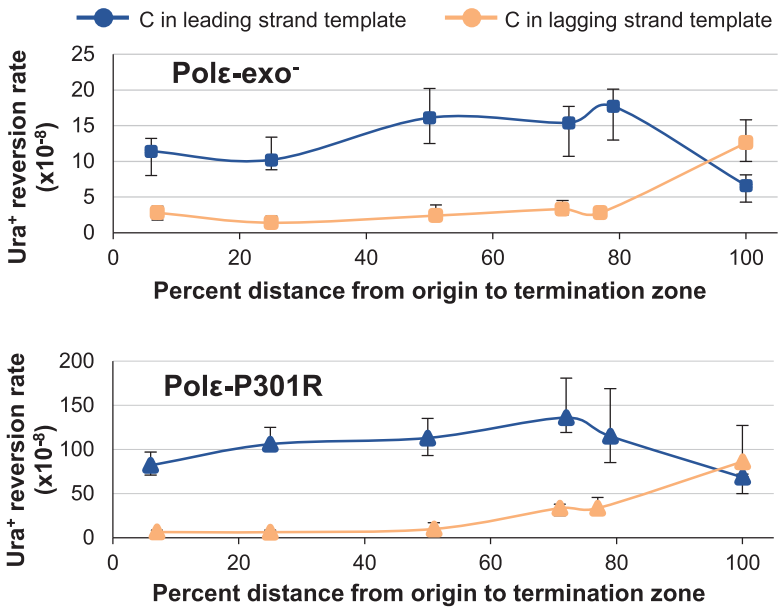

Figure 3. Polε-P301R, like Polε-exo$^-$, is a dedicated leading strand polymerase. The reversion rate of the *ura3-29* allele in two orientations at each location is shown for *pol2-4* (top) and *pol2-P301R* (bottom) strains. Data are medians for at least 18 cultures from two to six independent clones. Error bars represent 95% confidence intervals.

the haploid state (26,49,52) as *pol2-4* is a much weaker mutator.

Diploids heterozygous for the *pol2-P301R* mutation and homozygous for *msh6*Δ, however, were viable. Heterozygosity for *pol2-P301R* produces a rather strong mutator phenotype (23). Thus, we used these strains to assess the effect of the combination of *msh6*Δ and *pol2-P301R* on mutagenesis. We measured the mutation rate at two reporter loci, *CAN1* and *his7-2*. The *CAN1* forward mutation reporter scores a wide variety of base-substitution and indel mutations inactivating the gene and resulting in resistance to the toxic arginine analog canavanine. These mutations are recessive, but we have previously developed an assay to study *CAN1* mutagenesis in diploid strains with a single copy of the gene (34). In this assay, the diploids carry a selectable marker, *Kluyveromyces lactis LEU2*, next to the *CAN1* open reading frame in one chromosome, and a deletion of the entire *CAN1* locus in the homologous chromosome. While loss of the entire *CAN1* locus in these diploids occurs frequently due to mitotic recombination, the presence of the *K. lactis LEU2* allows us to select against the recombination events and score intragenic mutations in *CAN1*. Accordingly, all diploid strains used for mutation rate measurements in our work contain the *CAN1::K.l.LEU2/can1*Δ configuration. The second reporter, *his7-2*, scores +1 frameshift mutations in an A_7 run in the *HIS7* gene (53). The combination of heterozygosity for *pol2-P301R* with homozygosity for *msh6*Δ resulted in a synergistic increase in mutation rate for both the *CAN1* and *his7-2* reporters (Table 1). This synergy demonstrates that MMR removes most of Polε-P301R errors present as mismatches in double-stranded DNA upon completion of DNA replication. It further supports the premise that diploids homozygous for both *pol2-P301R* and *msh6*Δ die due to high levels of mutagenesis. A synergistic increase in mutation rate was also observed when heterozygosity for *pol2-4* was combined with homozygosity for *msh6*Δ (Supplementary Table S3), in line with the synergy between *pol2-4* and *msh6*Δ in haploids (26,49,52). However, the absolute mutation rate in *pol2-P301R/POL2 msh6*Δ*/msh6*Δ diploids is an order of magnitude higher than in *pol2-4/POL2 msh6*Δ*/msh6*Δ diploids, once again illustrating the unprecedented level of replication errors generated by Polε-P301R *in vivo*.

Survival of *pol2-P301R* strains requires correction of Polε-P301R errors by the exonuclease activity of Polδ

Prior studies have shown that Polδ can proofread errors made by inaccurate variants of Polα and Polε (11,16). We aimed to determine if the *pol2-P301R* mutation, which greatly increases DNA polymerase activity and mismatch extension ability of Polε, affects the efficiency of extrinsic proofreading by Polδ. To generate strains deficient in Polδ proofreading, the chromosomal wild-type *POL3* gene encoding the catalytic subunit of Polδ was replaced with the *pol3-D520V* allele. The *pol3-D520V* mutation results in a D520V substitution in the conserved ExoIII motif and a severe reduction in the exonuclease activity of Polδ (35). A combination of *pol3-D520V* and *pol2-4* mutations results in a strong synergistic increase in mutation rate in both haploids and diploids, as expected from previous studies and consistent with Polδ proofreading errors made by Polε [(16,36); Supplementary Table S4]. We have shown previously that this synergistic interaction reflects proofreading of errors made by Polε-exo$^-$ by the exonuclease of Polδ, and not the involvement of the exonuclease of Polδ in MMR as suggested earlier (16). To study the genetic interaction of *pol3-D520V* mutation with *pol2-P301R*, we first attempted to combine the mutations by crossing single *pol3-D520V* and *pol2-P301R* mutants and sporulating heterozy-

Table 1. Synergistic interaction of *pol2-P301R* and MMR deficiency

	CAN1 mutation		*his7-2* reversion	
Genotype	Mutation rate ($\times 10^{-7}$)	Fold increase	Mutation rate ($\times 10^{-8}$)	Fold increase
POL2/POL2 MSH6/MSH6	3.4 (3.0–4.0)	1	1.1 (0.85–1.3)	1
POL2/POL2 msh6Δ/msh6Δ	31 (28–36)	9.1	4.6 (4.1–5.3)	4.2
POL2/pol2-P301R MSH6/MSH6	75 (70–93)	22	29 (25–33)	26
POL2/pol2-P301R msh6Δ/msh6Δ	4300 (3300–6000)	1300	105 (73–230)	95

Mutation rates are medians for at least 18 cultures from two to three independent clones. 95% confidence intervals are shown in parentheses.

gous diploids. This procedure yielded no viable double mutant spores (Figure 4A). The inviable spores formed microcolonies before cell division stopped (Figure 4B), suggesting death from a high level of mutagenesis. Diploid yeast homozygous for both *pol3-D520V* and *pol2-P301R* also did not survive, as indicated by their inability to lose an episomal plasmid expressing wild-type *POL3* (Figure 4C). These observations were consistent with the idea that Polδ exonuclease is required to keep the level of replication errors in *pol2-P301R* strains below the lethal threshold.

To further determine whether Polδ exonuclease activity proofreads Polε-P301R errors, we created diploid yeast homozygous for *pol3-D520V* and, thus, lacking Polδ proofreading, and heterozygous for *pol2-P301R*. We observed a strong synergistic increase in both *CAN1* mutation and *his7-2* reversion in the double mutant strains (Table 2), indicating that Polδ proofreading removes a majority of Polε-P301R errors.

DISCUSSION

The most common cancer-associated Polε variant, Polε-P286R, has elevated DNA polymerase activity and causes an exceptionally strong mutator effect and tumor susceptibility when modeled in yeast or mice (23,24,26). Here, we used the yeast model to assess the impact of this variant on the role of Polε in DNA replication and the ability of extrinsic correction mechanisms to act on Polε errors. We determined that, despite the dramatic change in biochemical properties, Polε-P301R remains a dedicated leading strand replicase. Due to a catastrophically high rate of leading strand errors, both MMR and extrinsic proofreading by the exonuclease of Polδ are required for viability when Polε-P301R is the sole Polε variant present in a cell. Synergistic increases in mutagenesis in diploids heterozygous for the *pol2-P301R* allele and lacking either MMR or Polδ exonuclease further demonstrate that Polε-P301R errors are efficiently corrected by Polδ proofreading and MMR.

Implications for the mechanism of chromosomal DNA replication

The assay for the detection of leading and lagging strand errors developed in this work provided new information on the mechanism of DNA replication in *S. cerevisiae*. The currently accepted fork model, originally proposed by the Sugino group (8), posits that Polε and Polδ synthesize the bulk of leading and lagging DNA strands, respectively. The most compelling evidence for this model comes from genetic studies that monitor strand-specificity of mutation or ribonucleotide incorporation in cells with reduced fidelity of Polε or Polδ (9,12,13,45,54–56). Earlier studies used reporter alleles placed in different orientations near a replication origin, and, thus, could deduce the roles of Polε and Polδ only in the vicinity of the origin [(9,12,13,36); discussed further in (57)]. Subsequent genome-wide studies of mutation and ribonucleotide incorporation in Polε and Polδ mutants extended the division-of-labor model to multiple replicons (54–56). However, because the genome-wide analysis relied on averaging data for many replicons where the location of the termination zone can vary, this analysis, too, was most efficient at assigning the polymerase roles in the vicinity of the origins. The bias for Polε errors on the leading strand and Polδ errors on the lagging strand was significantly reduced toward the termination zone (54–56). It remained unclear whether the reduced bias was due to the limitations of the genome-wide analysis or if the forks rearranged as they moved further away from the origins. The reversion assay used in our study is more sensitive and allowed us to detect a strong bias in the proximity of the termination zone (Figure 3), demonstrating that the majority of leading strand synthesis is completed by Polε from origin to termination zone. Recent genome-wide analysis of ribonucleotide incorporation by mutator Polε and Polδ variants revealed less synthesis by Polε and more synthesis by Polδ at termination zones ($\leq$10 kb from the average termination zone midpoint) than expected from the one-strand-one-polymerase model (58). Our data shows a strong bias for Polε participation in leading strand synthesis at 10, 8 and 6 kb from the calculated inter-origin midpoint and a loss of bias only at the very last reporter location ($<$1 kb from the midpoint). However, a slight decrease in Polε synthesis in the 10-kb segment may not be detected in our experiments. The sharp switching at the termination zone observed in the *ARS306* replicon likely also applies to other genomic regions with efficient, early-firing origins. Further studies with highly sensitive reversion reporters could help determine whether similar abrupt polymerase switching occurs in late-replicating DNA segments.

Cooperation of Polε and Polδ in error avoidance

Studies of the Polε-P301R variant described here uncover the remarkable efficiency at which extrinsic proofreading by Polδ operates to correct Polε errors. We showed previously that the exonuclease of Polδ readily proofreads errors made by Polε-exo$^-$ and another inaccurate Polε variant, Polε-M644G (16). This extrinsic correction must involve dissociation of Polε from the primer terminus to allow Polδ access to the mismatch. The dissociation is presumably fa-

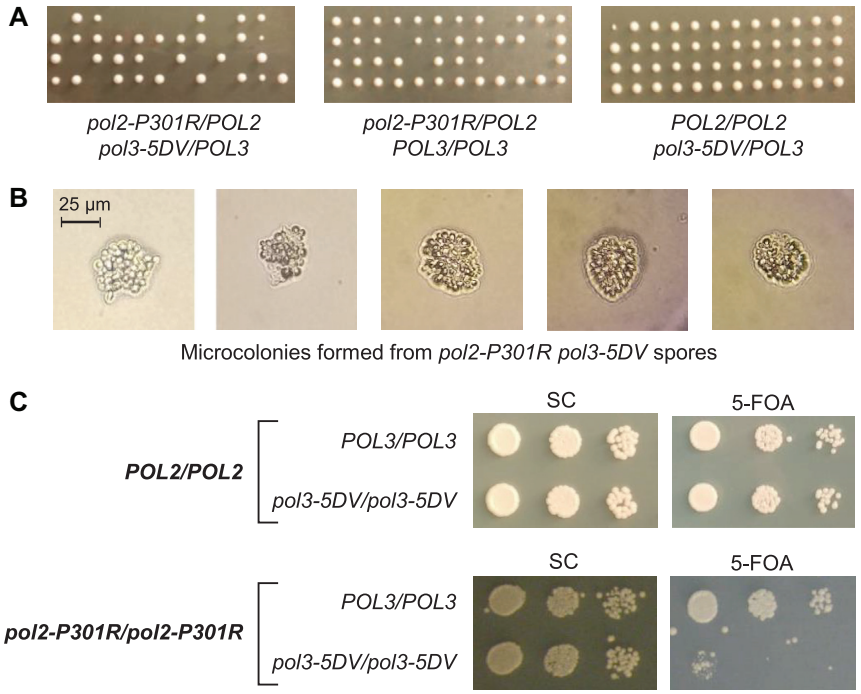

Figure 4. *pol2-P301R* mutants require functional Polδ proofreading for viability. (**A**) Tetrad analysis of yeast strains heterozygous for the *pol3-D520V* (*pol3-5DV*) allele encoding exonuclease-deficient Polδ, *pol2-P301R*, or both *pol3-D520V* and *pol2-P301R*. No viable *pol3-D520V pol2-P301R* spores were obtained from the *pol3-D520V/POL3 POL2/pol2-P301R* diploid. (**B**) Microcolonies formed by haploid *pol3-D520V pol2-P301R* spores**.** Photographs were taken at 200x magnification three days after placement of spores. (**C**) Diploids homozygous for both *pol3-D520V* and *pol2-P301R* are inviable. Cultures of diploid strains carrying the indicated chromosomal alleles and pBL304 were serially diluted and plated onto synthetic complete medium (SC, *left*) or medium containing 5-FOA to select for cells that have lost pBL304 (*right*). The inability of *pol3-D520V/pol3-D520V pol2-P301R/pol2-P301R* diploids to grow without pBL304 indicates synthetic lethality.

Table 2. Synergistic interaction of *pol2-P301R* and Polδ proofreading deficiency

	CAN1 mutation		*his7-2* reversion	
Genotype	Mutation rate ($\times 10^{-7}$)	Fold increase	Mutation rate ($\times 10^{-8}$)	Fold increase
POL2/POL2 POL3/POL3	3.4 (3.0–4.0)	1	1.1 (0.85–1.3)	1
POL2/POL2 pol3-D520V/pol3-D520V	46 (35–69)	14	17 (15–23)	15
POL2/pol2-P301R POL3/POL3	75 (70–93)	22	29 (25–33)	26
POL2/pol2-P301R pol3-D520V/pol3-D520V	3100 (2100–4500)	910	2800 (2200–3600)	2500

Mutation rates are medians for at least 18 cultures from two to three independent clones. 95% confidence intervals are shown in parentheses.

cilitated by a pause in DNA synthesis, as replicative DNA polymerases are rather inefficient at extending mismatched primer termini. Polε-P301R, however, is a hyperactive polymerase far superior to other Polε variants in the ability to utilize a variety of DNA substrates, including those with incorrectly paired primer ends (26). Structural studies showed that the arginine side chain protrudes into the space normally occupied by the 3′-terminal nucleotide in the exonuclease active site (27). We proposed that the inability of Polε-P301R to accommodate the primer terminus in the exonuclease site not only dramatically reduces exonuclease activity, but also prompts Polε-P301R to stay in the polymerization mode, resulting in increased polymerase activity, mismatch extension, and ultimately an unprecedented mutator effect (26). The discovery that a majority of errors generated by Polε-P301R are proofread by the exonuclease of Polδ was, therefore, surprising. The >40-fold difference in the *CAN1* mutation rate between *POL2/pol2-P301R* and *POL2/pol2-P301R pol3-D520V/pol3-D520V* strains (Table 2) suggests that, despite superior mismatch extension capability, Polε-P301R dissociates from the primer terminus upon misinserting a nucleotide in >97% of cases and allows Polδ to correct the error. These numbers could overestimate the efficiency of extrinsic proofreading if some of the mutator effect in *POL2/pol2-P301R pol3-D520V/pol3-D520V* diploids results from a saturation of MMR by the high number of replication errors. Although we cannot rule out this possibility, it is of note that neither homozygosity for *pol3-D520V* nor heterozygosity for *pol2-P301R* alone saturate MMR [(16) and Table 1]. The strong synergistic interaction of *pol3-D520V* and *pol2-P301R* alleles, whether or not it involves saturation of MMR, indicates efficient extrinsic proofreading of Polε-P301R-generated errors by Polδ. This finding illustrates the robustness of the extrinsic proofreading mechanism and suggests that the switch from Polε to Polδ on the leading strand is easier than one could expect,

as it is much preferred to even a very efficient mismatch extension by Polε-P301R.

Completion of leading strand synthesis after removal of the mismatch could conceivably occur by Polδ or, alternatively, involve switching back to Polε-P301R. Recent findings that DNA replication begins with Polδ extending Polα-synthesized primers on both the leading and lagging strands suggests that there is, indeed, a mechanism for Polδ to hand off the leading strand to Polε as synthesis catches up with the moving helicase (59–61). On the other hand, intramolecular switching from the exonuclease to the polymerase active site has been suggested for Polδ (50). Intramolecular switching between active sites has also been demonstrated for bacteriophage RB69 and T4 DNA polymerases, as well as for the eukaryotic Polε (62–64). Our data (Figure 3) indicate that in the vast majority of cases, the leading strand is synthesized by Polε until the termination zone, but a small proportion synthesized by Polδ, such as that expected from extrinsic proofreading and subsequent Polδ-driven extension, would not be detected.

Implications for the etiology of *POLE*-mutant tumors

POLE-mutant tumors have the highest mutation load across different cancer types [>100 mutations per Mb; (19,20,65)]. Although MMR defects are also common in cancers, tumors harboring a *POLE* mutation are typically microsatellite stable, indicating functional MMR. Thus, *POLE* and MMR defects appear to be mutually exclusive. While a small number of tumors with a combination of a *POLE* mutation and a MMR defect have been reported (17), these *POLE* alleles confer only a weak mutator effect in functional assays (66). Brain tumors in children with biallelic MMR deficiency often contain *POLE* mutations (67,68), but, again, these tumors usually harbor only partial MMR defects and weaker *POLE* mutators. No tumors with microsatellite instability and the *POLE-P286R* mutation have been found to date. There could be two possible explanations for the apparent incompatibility of strong *POLE* mutators with MMR deficiency. First, since either defect is sufficient to cause a tumor, the combination of a strong *POLE* mutator with a loss of MMR would only be detected if it occurred by chance, and the probability of acquiring both defects simultaneously is relatively low. This explanation seems unlikely given the large number of *POLE-P286R* tumors reported (>200) and no documented cases of MMR deficiency among those. One pancreatic tumor in TCGA database carried *POLE-P286R* along with two nonsense mutations in *MSH6* (22). However, there is no evidence that the *MSH6* mutations impacted different alleles or that the tumor had microsatellite instability. For comparison, approximately 10% of colorectal and 28% of endometrial cancers without *POLE* mutations are MMR deficient (19,20). The second explanation suggested by our finding in yeast (Table 1) is that the combination of strong *POLE* mutators with MMR deficiency is incompatible with cell viability because the mutation rate in such cells exceeds the maximum tolerated threshold. Although diploid cells can withstand relatively high levels of mutagenesis, they do have a viability threshold (51), and, indeed, we observed that yeast diploids homozygous for both *pol2-P301R* and *msh6* mutations do not survive.

It is noteworthy that the *POLE* mutations are usually present in heterozygous state in tumors (17,18) but are still not seen together with MMR defects, a combination that is viable in yeast (Table 1). It is possible that human cells, due to their more complex biology, have a lower viability threshold. It is also possible that while formally compatible with cell viability, the high mutation rate resulting from a combination of heterozygous *POLE* variants with a MMR defect is not compatible with the level of fitness required for the sustained proliferation of cancer cells within the human organism. Finally, it is possible that a full MMR defect such as that resulting from an *mlh1* or *msh2* mutation would be incompatible with the heterozygosity for *pol2-P301R* in yeast either, as the *msh6* mutation we employed leaves the Msh3-dependent MMR functional. These possibilities could be further investigated in the future. The data on the synergistic interaction of *pol2-P301R* allele with the MMR deficiency presented here, however, strongly suggest that the corresponding defects in human cells are mutually exclusive because of a catastrophically high mutation rate.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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