During eukaryotic replication, DNA polymerases ε (Polε) and δ (Polδ) synthesize the leading and lagging strands, respectively. In a long-known contradiction to this model, defects in the fidelity of Polδ have a much weaker impact on mutagenesis than analogous Polε defects. It has been previously proposed that Polδ contributes more to mutation avoidance because it proofreads mismatches created by Polε in addition to its own errors. However, direct evidence for this model was missing. We show that, in yeast, the mutation rate increases synergistically when a Polδ nucleotide selectivity defect is combined with a Polε proofreading defect, demonstrating extrinsic proofreading of Polδ errors by Polε. In contrast, combining Polε nucleotide selectivity and Polδ proofreading defects produces no synergy, indicating that Polε cannot correct errors made by Polδ. We further show that Polε can remove errors made by exonuclease-deficient Polε in vitro. These findings illustrate the complexity of the one-strand-one-polymerase model where synthesis appears to be largely divided, but Polδ proofreading operates on both strands.

DNA replication | extrinsic proofreading | DNA polymerase δ | DNA polymerase ε

The most widely accepted model of eukaryotic DNA replication proposed in the 1990s suggests that Polα-primase synthesizes short RNA-DNA primers at the origins and at the beginning of the Okazaki fragments. Polε synthesizes the leading strand, and Polδ completes the lagging strand (1). During the three decades that passed since the landmark publication by Morrison et al., numerous reports have contributed evidence for the participation of Polε and Polδ in leading and lagging strand replication, respectively. Genetic studies detected strand-specific increases in mutagenesis in yeast and human cells carrying inaccurate Polδ or Polε variants (2–6). More sensitive assays monitoring ribonucleotide incorporation into DNA by Polδ or Polε variants with relaxed sugar selectivity confirmed ribonucleotide accumulation in the leading strand in Polδ mutants and in the lagging strand in Polδ mutants (7, 8). Polδ but not Polε was shown to proofread errors made by Polε (9) and participate in the maturation of Okazaki fragments on the lagging strand (10, 11). At the same time, Polε but not Polδ interacts with the Cdc45-MCM-GINS helicase on the leading strand (12). While the roles of Polδ in the synthesis of the leading strand near replication origins and termination zones have recently been detected (13–16), these stretches of Polδ synthesis appear to account for a relatively minor fraction of the leading strand (~18%, ref. 16). Overall, a bulk of evidence supports the originally proposed division of labor with Polε and Polδ predominately replicating opposite DNA strands.

In contradiction to this model, Polδ fidelity defects have long been known to have a greater impact on mutagenesis than analogous Polε defects. Both Polδ and Polε contribute to mutation avoidance via their intrinsic nucleotide selectivity conferred by the polymerase domain and the proofreading activity located in a separate exonuclease domain. The exonuclease activity of both polymerases can be abolished by alanine substitutions at the conserved carboxylate residues in the ExoI motif FDIET/C (17, 18). The resulting mutator phenotype of the Polδ-exo variant is an order of magnitude stronger than the phenotype of the analogous Polε-exo variant (2, 17–25). Furthermore, haploid yeast deficient in Polδ proofreading do not survive when DNA mismatch repair (MMR) is also inactivated with the death attributed to an excessive level of mutagenesis (26). In contrast, yeast lacking both proofreading by Polδ and MMR are viable, and while the mutation rate in these strains is high, it does not reach the lethal threshold (19, 21, 22, 25, 27). Similarly, when identical tyrosine to alanine substitutions were made in the conserved region III of the polymerase domains (Polδ-Y708A and Polε-Y831A), the Polδ variant produced a much stronger mutator effect than the analogous Polε variant (28).

To explain the controversy between the accepted fork model and the disparity of Polδ and Polε effects on mutagenesis, a hypothesis has been entertained that Polδ proofreads errors made by Polε in addition to its own errors, thus, contributing more significantly to mutation avoidance. This hypothesis, discussed in multiple publications (2, 29–31), stems from the original observation by Morrison and Sugino that the combination of Polδ and Polε proofreading defects results in a synergistic increase in mutation rate (19). The synergy implies that the exonucleases of Polδ and Polε act on the same pool of replication errors and could potentially mean Polε correcting errors made by Polδ, Polδ correcting errors made by Polε, or both polymerases proofreading for each other. In general, the possibility of extrinsic proofreading has been demonstrated in multiple in vivo and in vitro studies. Initial experiments showed that errors made by purified calf thymus Polδ could be corrected by the ε subunit of Escherichia coli DNA polymerase III or by Polδ (32, 33). Several mammalian autonomous exonucleases have also been shown to

**Significance**

Polδ and Polε are the two major replicative polymerases in eukaryotes, but their precise roles at the replication fork remain a subject of debate. A bulk of data supports a model where Polδ and Polε synthesize leading and lagging DNA strands, respectively. However, this model has been difficult to reconcile with the fact that mutations in Polδ have much stronger consequences for genome stability than equivalent mutations in Polε. We provide direct evidence for a long-entertained idea that Polε can proofread errors made by Polδ in addition to its own errors, thus, making a more prominent contribution to mutation avoidance. This paper provides an essential advance in the understanding of the mechanism of eukaryotic DNA replication.
increase the fidelity of Polδ in vitro (34–36). Both E. coli and eukaryotic replicative polymerases can excise nucleotides incorporated by translesion synthesis polymerases at sites of DNA damage (37, 38). With respect to the extrinsic proofreading capabilities of Polδ and Polε in vivo, several studies have been illuminating. As already mentioned, Polδ but not Polε has been shown to proofread errors made by an error-prone Polα variant in yeast (9). Furthermore, Polδ exonuclease defects are almost completely recessive, indicating that wild-type Polδ can efficiently proofread errors created by Polδ-exo− (18, 26, 31). On the other hand, the mutant allele encoding Polε-exo− is semidominant, suggesting that wild-type Polδ does not correct errors in trans (31, 39). Flood et al. (31) further investigated extrinsic proofreading by Polδ and Polε: using transformation of yeast cells with oligonucleotides that, when annealed, create a 3′-terminal mismatch. These experiments showed that Polδ but not Polε can proofread in trans and that the exonuclease of Polδ can act on oligonucleotides annealed to both leading and lagging strands. However, it remained unknown whether the exonuclease of Polδ could proofread errors generated by Polε during normal chromosomal replication.

To answer this question, we used yeast strains harboring a nucleotide selectivity defect in one polymerase, Polδ or Polε, and a proofreading defect in the other. We compared mutation rates between the corresponding single and double mutants to determine whether the proofreading activity of one polymerase acts in series or in parallel with the nucleotide selectivity of the other. We also used an in vitro replication system to investigate whether Polδ can excise mismatched primer termini generated by exonuclease-deficient Polε. Our results show that Polδ can correct errors made by Polε, but Polδ cannot correct errors made by Polδ. This observation provides direct evidence that the remarkably mild in vivo consequences of severe Polδ fidelity defects are explained by the compensatory proofreading by Polδ. These findings support a replication fork model wherein synthesis on leading and lagging strands is primarily accomplished by separate polymerases, but proofreading is more dynamic and can be performed by the exonuclease of Polδ on both strands.

Results
Polδ Proofs Errors Made by Polε, but Polε Does Not Proofread Errors Made by Polδ In Vivo. The synergistic interaction between the two polymerases Polδ and Polε has been previously characterized using the pol2-4 and pol3-01 alleles, which result in the replacement of two catalytic carboxylates in the ExoI motif of the respective polymerase with alanines (FDIET/C → FAIAT/C; ref. 19). The pol3-01 mutation, however, may have consequences beyond simply destroying the exonuclease of Polδ as its extremely strong mutator phenotype has been reported to be partially dependent on the activation of the S-phase checkpoint (40), and a different allele, pol3-D520V, exists that also eliminates the exonuclease activity but is a weaker mutator (10). We started by verifying that the synergy between Polδ and Polδ could still be detected when the pol3-D520V allele is used instead of pol3-01 to produce exonuclease-deficient Polδ. While the pol2-4 pol3-01 double mutant haploids were inviable due to a catastrophically high mutation rate (19), the pol2-4 pol3-D520V haploids survived (SI Appendix, Fig. S1). The mutation rate in the pol2-4 pol3-D520V strains increased synergistically as compared with the single pol2-4 and pol3-D520V mutants (SI Appendix, Table S1), consistent with the idea that the exonucleases of Polδ and Polε act on the same pool of replication errors. We next ascertained that this synergistic interaction is not due to the pol3-D520V mutation disrupting MMR. If the exonuclease of Polδ is essential for functional MMR, combining pol3-D520V with a MMR defect would yield no further increase in mutation rate beyond the effect of pol3-D520V alone. On the other hand, if Polδ proofreading and MMR act in series, a synergistic increase in mutation rate would be expected in the double mutants. Haploid yeast deficient in MMR and harboring pol3-D520V are not viable (41); therefore, we assessed the epistatic relationship between pol3-D520V and MMR deficiency in diploid strains, which can tolerate a higher level of mutagenesis. We used the msh6 deletion to inactivate MMR as the Msh6-dependent pathway is primarily responsible for the repair of single-base mismatches (42), which is the predominant type of replication errors generated by exonuclease-deficient Polδ and Polε (43–45). Diploids homozygous for both pol3-D520V and msh6 mutations showed a strong synergistic increase in mutation rate as compared with the single pol3-D520V and msh6 mutants (SI Appendix, Table S2). A similar synergistic increase in mutagenesis in pol3-D520V/pol3-D520V msh6/msh6 diploids was observed by Flood et al. for base substitutions at a single nucleotide position in the TRP5 gene (31). We recapitulate and expand these earlier findings by using the forward mutagenesis reporter CAN1 that can detect a variety of base substitutions and indels in many DNA sequence contexts as well as the his7-2 frameshift reporter that is particularly sensitive to MMR defects. Together, these data demonstrate that pol3-D520V does not confer a MMR defect. Thus, the synergy between pol2-4 and pol3-D520V indicates proofreading of the same errors by Polδ and Polε. It also shows that the pol3-D520V allele provides an adequate model for the extrinsic proofreading studies described below.

Next, we investigated whether Polδ proofreads errors made by Polε by combining a nucleotide selectivity defect in Polδ (pol2-M644G) with a proofreading defect in Polε (pol3-D520V). The pol2-M644G confers a change in the polymerase domain of Polδ, which causes promiscuity during nucleotide incorporation without compromising proofreading (3). The pol2-M644G strains, therefore, accumulate a high number of Pol-specific errors. We observed a synergistic increase in mutation rate in the double pol2-M644G pol3-D520V mutants (Table 1). This synergy indicates that the nucleotide selectivity of Polδ and the proofreading activity of Polε act consecutively to prevent replication errors and, thus, Polδ proofreads errors made by Polε in vivo. In a reciprocal experiment, we combined a Polδ nucleotide selectivity defect (pol3-L612M) with a Polδ proofreading defect (pol2-4) to determine whether Polδ can proofread errors made by Polδ. Similar to pol2-M644G, pol3-L612M increases the rate of nucleotide misincorporation by Polδ without impacting exonuclease activity (46). In contrast to the pol2-M644G pol3-D520V combination, the pol3-L612M pol2-4 combination resulted in only an additive increase in the mutation rate in the double mutant compared to the single pol3-L612M and pol2-4 mutants (Table 2). The additive interaction indicates that Polδ nucleotide selectivity and Polε exonuclease activity act in parallel nonoverlapping pathways, and, therefore, Polε does not proofread errors made by Polδ.

Polδ Removes Mismatched Primer Termini Created by Polε In Vitro. We next developed an in vitro assay to examine whether Polδ can excise mismatched primer termini generated by exonuclease-deficient Polε. In this assay, purified yeast Polδ-exo− is allowed to synthesize DNA on a 9-kb single-stranded circular template in the presence of accessory proteins proliferating cell nuclear antigen (PCNA), replication factor C (RFC), replication protein A (RPA), and highly imbalanced dNTP concentrations. dCTP and dGTP are provided at their physiological S-phase concentrations (47, 48). dATP is at ~1/5 of its S-phase concentration, and dTTP is in vast excess (~150-fold) over its S-phase concentration (Fig. L4). The dNTP imbalance results in a high rate of incorrect nucleotide incorporation, which inhibits synthesis because Polδ-exo− cannot proofread these errors. Although it is capable of extending mismatched primer termini (43), the number of mismatches under these conditions is overwhelming, and the continuous need to proofread these errors made by Polδ active. Therefore, accumulate a high number of Pol-specific errors. We observed a synergistic increase in mutation rate in the double pol2-M644G pol3-D520V mutants (Table 1). This synergy indicates that the nucleotide selectivity of Polδ and the proofreading activity of Polε act consecutively to prevent replication errors and, thus, Polδ proofreads errors made by Polδ in vivo. In a reciprocal experiment, we combined a Polδ nucleotide selectivity defect (pol3-L612M) with a Polδ proofreading defect (pol2-4) to determine whether Polδ can proofread errors made by Polδ. Similar to pol2-M644G, pol3-L612M increases the rate of nucleotide misincorporation by Polδ without impacting exonuclease activity (46). In contrast to the pol2-M644G pol3-D520V combination, the pol3-L612M pol2-4 combination resulted in only an additive increase in the mutation rate in the double mutant compared to the single pol3-L612M and pol2-4 mutants (Table 2). The additive interaction indicates that Polδ nucleotide selectivity and Polε exonuclease activity act in parallel nonoverlapping pathways, and, therefore, Polε does not proofread errors made by Polδ.

Polδ removes mismatched primer termini created by Polδ in vitro. We next developed an in vitro assay to examine whether Polδ can excise mismatched primer termini generated by exonuclease-deficient Polε. In this assay, purified yeast Polδ-exo− is allowed to synthesize DNA on a 9-kb single-stranded circular template in the presence of accessory proteins proliferating cell nuclear antigen (PCNA), replication factor C (RFC), replication protein A (RPA), and highly imbalanced dNTP concentrations. dCTP and dTTP are provided at their physiological S-phase concentrations (47, 48). dATP is at ~1/5 of its S-phase concentration, and dGTP is in vast excess (~150-fold) over its S-phase concentration (Fig. L4). The dNTP imbalance results in a high rate of incorrect nucleotide incorporation, which inhibits synthesis because Polδ-exo− cannot proofread these errors. Although it is capable of extending mismatched primer termini (43), the number of mismatches under these conditions is overwhelming, and the continuous need to proofread these errors made by Polδ active. Therefore, accumulate a high number of Pol-specific errors. We observed a synergistic increase in mutation rate in the double pol2-M644G pol3-D520V mutants (Table 1). This synergy indicates that the nucleotide selectivity of Polδ and the proofreading activity of Polε act consecutively to prevent replication errors and, thus, Polδ proofreads errors made by Polδ in vivo. In a reciprocal experiment, we combined a Polδ nucleotide selectivity defect (pol3-L612M) with a Polδ proofreading defect (pol2-4) to determine whether Polδ can proofread errors made by Polδ. Similar to pol2-M644G, pol3-L612M increases the rate of nucleotide misincorporation by Polδ without impacting exonuclease activity (46). In contrast to the pol2-M644G pol3-D520V combination, the pol3-L612M pol2-4 combination resulted in only an additive increase in the mutation rate in the double mutant compared to the single pol3-L612M and pol2-4 mutants (Table 2). The additive interaction indicates that Polδ nucleotide selectivity and Polε exonuclease activity act in parallel nonoverlapping pathways, and, therefore, Polε does not proofread errors made by Polδ.
of proofreading (SI Appendix, Fig. S2). After several minutes of inefficient synthesis attempts by Polɛ-exo⁺, Polδ was added to the reactions, and its ability to assist with the removal of mismatched termini and rescue DNA synthesis was measured by the accumulation of long products (Fig. 1A). We found that the addition of Polδ rescued synthesis significantly (Fig. 1B and C), indicating that Polδ efficiently corrected misinsertions made by Polɛ-exo⁺. Importantly, the restoration of synthesis in this system could only be due to Polδ acting on Polɛ-exo⁺-generated primer termini as, in our reaction conditions, all originally available primers are extended by Polɛ-exo⁺ before Polδ is added (Fig. 1B and SI Appendix, Fig. S2). The polymerase exchange could conceivably involve physical interaction between Polδ and Polɛ; or simply reflect binding of Polδ to mismatched primer termini vacated by Polδ. While our in vitro assay cannot distinguish between these possibilities, it shows that the biochemical properties of Polδ are consistent with its ability to yield to Polδ in the context of ongoing DNA synthesis in the presence of accessory replication proteins.

**Discussion**

The accepted model for eukaryotic DNA replication is not easily reconciled with the stronger mutator effects of Polδ variants in comparison with analogous Polɛ variants. It has been proposed that Polδ can proofread errors made by Polɛ in addition to its own errors, which would explain its more prominent contribution to mutation avoidance. Currently available data suggest that, indeed, Polδ but not Polɛ can readily proofread errors in trans (9, 18, 26, 31, 39). However, evidence that Polδ can proofread DNA synthesized by Polɛ at the replication fork has been lacking. Using inaccurate variants of Polδ and Polɛ, here we demonstrate that incorrect nucleotides incorporated by Polɛ are efficiently removed by the exonuclease of Polδ, but Polδ cannot remove nucleotides misincorporated by Polδ (Fig. 2). This conclusion is supported by the following observations. i) Mutation rate increases synergistically when the Polɛ nucleotide selectivity defect is combined with Polδ proofreading defect. ii) Only an additive increase in mutagenesis is observed when the Polδ nucleotide selectivity defect is combined with the Polδ proofreading defect. iii) Mismatched primer termini generated by Polɛ-exo⁺ can be proofread by Polδ in an in vitro replication system.

**Polδ Is a Versatile Extrinsic Proofreading Enzyme.** Multiple studies suggested that Polδ is more efficient at extrinsic proofreading than Polɛ. Polδ can remove mismatches generated by Polɛ both in vitro and in vivo (9, 33). Since Okazaki fragments are all initiated by exonuclease-deficient Polɛ, there is a clear need for extrinsic proofreading by the lagging strand polymerase, whereas there is less of a need for Polδ to carry this out on the leading strand. Indeed, Polδ does not appear to correct errors made by Polδ in vivo (9). It is particularly interesting to note the recent evidence that initial leading strand synthesis is performed by Polδ (13–15), which further diminishes the need for extrinsic proofreading of Polδ-generated errors by Polɛ on the leading strand. Additionally, the semidominance of the polδ-4 mutation and almost complete dominance of POL3 over the polɛ3-01 and polδ3-D520V mutations demonstrates that only Polδ can remove errors inserted by a different polymerase molecule (18, 26, 31, 39). The removal of 3′-terminal mismatches during oligonucleotide-mediated transformation by Polδ but not Polɛ (31) also suggests that Polδ is much better suited to extrinsic proofreading than Polɛ. Finally, this study provides evidence that Polδ proofreads errors made by Polɛ in vivo, while Polɛ cannot proofread for Polδ.

Thus, the competition of Polδ and Polɛ exonucleases for correcting the same pool of replication errors originally demonstrated by Morrison and Sugino in the 1990s (19) is apparently one sided. Perhaps the different properties and regulatory mechanisms of the two polymerases leave them appropriately suited to their own specialized roles. Polδ is a component of the replication initiation complex where it associates with origins during the G1/S phase transition (49, 50). Polɛ remains bound to the moving helicase via the C terminus of its catalytic subunit Pol2 as the N terminus copies the leading strand (3, 12). A flexible region between the two halves of Pol2 allows the polymerase to dissociate from the DNA while remaining bound to the replication machinery (51). This association with the helicase indicates that Polɛ may not be free to carry out extrinsic proofreading, but the flexibility of the N terminus could allow a different polymerase access to the 3′ end of the leading strand. On the other hand, dissociation and reassociation of Polδ with the primer terminus occurs routinely during lagging strand synthesis, and Polδ is loaded much faster than Polɛ onto the PCNA-primer-template junction (52). Thus, the high efficiency of Polδ at correcting errors made by Polɛ may result from a

**Table 1. Synergistic interaction of Polɛ nucleotide selectivity and Polδ proofreading defects**

| Genotype       | Mutation rate (×10⁻⁷) | Fold increase over wild type | Mutation rate (×10⁻⁸) | Fold increase over wild type |
|----------------|-----------------------|------------------------------|-----------------------|------------------------------|
| POL2 POL3      | 2.5 (2.1–2.9)         | 1.0                          | 0.83 (0.70–0.97)       | 1.0                          |
| pol2-M644G POL3| 9.7 (8.2–12)          | 3.9                          | 1.4 (1.0–1.6)          | 1.7                          |
| POL2 pol3-D520V| 19 (16–21)            | 7.6                          | 8.0 (7.0–9.6)          | 9.6                          |
| pol2-M644G pol3-D520V | 92 (77–110) | 37                            | 13 (11–15)           | 16                            |

Mutation rates are medians for at least 18 cultures from two to three independently constructed strains of the same genotype. The 95% confidence intervals are shown in parentheses.

**Table 2. Additive interaction of Polδ nucleotide selectivity and Polɛ proofreading defects**

| Genotype       | Mutation rate (×10⁻⁷) | Fold increase over wild type | Mutation rate (×10⁻⁸) | Fold increase over wild type |
|----------------|-----------------------|------------------------------|-----------------------|------------------------------|
| POL2 POL3      | 2.5 (2.1–2.9)         | 1.0                          | 0.83 (0.70–0.97)       | 1.0                          |
| pol2-4 POL3    | 7.6 (6.8–8.7)         | 3.0                          | 6.3 (5.6–6.9)          | 7.6                          |
| POL2 pol3-L612M| 11 (9.7–13)           | 4.4                          | 5.0 (4.1–5.9)          | 6.0                          |
| pol2-4 pol3-L612M | 17 (16–18) | 6.8                            | 8.9 (7.6–11)           | 11                            |

Mutation rates are medians for at least 18 cultures from two to three independently constructed strains of the same genotype. The 95% confidence intervals are shown in parentheses.
Errors made by Pol-exo- are removed by Polδ in vitro. (A) Schematic of polymerase rescue assay. A Cy5-labeled primer (wavy black line) annealed to single-stranded plasmid template M13/CAN1(1-1560-F) was extended by purified Pol-exo- (green line) in the presence of highly imbalanced dNTPs. dNTP concentrations below or above the normal S-phase concentrations are indicated in bold font. Synthesis is inefficient under these conditions due to frequent nucleotide misincorporation (shown in red). Polδ was then added to the reactions, and its ability to assist Pol-exo- with the removal of misincorporated nucleotides was monitored by the restoration of DNA synthesis (blue line). For experimental details, see the Materials and Methods section. (B) Analysis of M13/CAN1(1-1560-F) replication products by electrophoresis in a 1% alkaline agarose gel. The primer was elongated by Pol-exo- for 7 min, followed by synthesis with 0, 10, or 50 fmol of Polδ for an additional 3 min. (C) Quantification of long products (above 2.5 kb) from A. Synthesis is restored due to frequent misinsertion.

Genome Stability Requires Redundancy of Replication Fidelity Mechanisms.

The overlap in replication and repair mechanisms is essential to prevent lethal and pathogenic mutations and ensure the stability of DNA. For example, several DNA glycosylases function in base excision repair such that, when one is compromised, the others can compensate (53). Multiple translesion synthesis polymerases provide redundant mechanisms of lesion bypass (54, 55). Cancer cells in which one DNA repair pathway has been compromised become resistant to DNA-damaging therapeutic drugs, in part, due to the redundancy that exists to repair the damage and prevent mutations. Targeting a redundant repair pathway in combination with a DNA damaging agent is a promising approach to overcome resistance (56). A recent example is the inclusion of nucleoside analog 5-NiDRI, an inhibitor of translesion synthesis with temozolomide in the treatment of homologous-recombination-impaired tumors to promote cancer cell death (57, 58).

The redundancy that serves to protect the genome is also found in the DNA replication process. It is well established that three different mechanisms, nucleotide selectivity, exonucleolytic proofreading, and MMR, act to prevent and correct replication errors. A combination of nucleotide selectivity and proofreading defects in Polδ results in a catastrophically high mutation rate incompatible with life in haploid yeast (59), indicating that proofreading normally compensates for reduced nucleotide selectivity. Haploid yeast deficient in Polδ proofreading require functional MMR for survival (26). Recent work has demonstrated that polymerase fidelity and MMR can compensate for defects in cellular metabolism that lead to dNTP pool imbalances and help maintain a normal low mutation rate despite the abnormal dNTP levels (60, 61). Extrinsic proofreading of Polε errors by Polδ shown here as well as proofreading of Polε errors by Polδ shown previously (9) is yet another mechanism of redundancy to prevent accumulation of DNA replication errors.

The redundancy in replication fidelity mechanisms has implications for human cancer biology. Mutations in the POLE gene, which encodes the catalytic subunit of Polε in humans, are found in 5–8% of sporadic colorectal and endometrial cancers and define a unique subset of these cancers with a so-called ultramutated phenotype (62). The POLE mutations predominantly affect the exonuclease domain of Polε and cause strong mutator and cancer susceptibility...
Intrinsic and extrinsic proofreading

Intrinsic proofreading

Intrinsic and extrinsic proofreading

polδ

polε

exo

Fig. 2. Interplay of Polδ and Polε proofreading and synthesis activities at the replication fork. Polε replicates the leading strand and proofreads its own errors. Polδ replicates the lagging strand but can remove errors made by Polε in addition to its own errors.

phenotypes in model systems (39, 63, 64). Although MMR defects are also common in colorectal and endometrial tumors, strong Polε mutators are never seen in combination with MMR deficiency, suggesting that MMR is critical to keep the mutation rate at a level compatible with cell survival. Curiously, mutations affecting the exonuclease domain of Polδ are seen much less frequently in sporadic tumors. While never explicitly tested, it is possible that these result in much stronger mutator phenotypes that hamper cell proliferation, and Polε-mutant cancers survive because extrinsic proofreading by Polδ helps reduce the number of errors to a tolerable level. Studies in mouse models suggested that the relative contributions of Polδ and Polε proofreading activities to replication fidelity and cancer prevention could vary depending on the cell and tissue types as well as the developmental stage. In a MMR-deficient background, both Polδ and Polε proofreading defects are lethal, but embryos lacking Polδ proofreading die earlier than those lacking Polε proofreading (65). In a MMR-proficient background, a Polδ proofreading defect leads to a significantly earlier onset of cancer than the analogous defect in Polε (Fig. 2). In this way, the plasmid into the chromosomal POL3 locus places the polδ-D520V mutation in a truncated, nonexpressed portion of POL3. Then, we deleted MSH6 in these strains by transformation with a PCR-generated DNA fragment containing the kanMX cassette flanked by homology to MSH6 (72). We crossed derivatives of TM30 and TM44 harboring the deletion of MSH6 and the integrated nonexpressed polδ-D520V mutation to obtain dips. Finally, we selected for cells that had lost the p170 plasmid from both chromosomes simultaneously on medium containing 5-fluoroorotic acid and used Sanger sequencing to find clones homozygous for the polδ-D520V mutation, now present in the full-length expressed alleles. Isogenic single-mutant diploids (polδ-D520V/polδ-D520V or msh6-kanMX/msh6-kanMX) and wild-type controls were constructed similarly, omitting the MSH6 disruption step, the p170-polδ-D520V transformation step, or both.

Mutation Rate Measurements. The rate of Can1+ forward mutation and his2-7 two-reversion was measured by fluctuation analysis as described previously (73). Briefly, multiple independent cultures of each strain were grown from single colonies in liquid medium overnight. Appropriate dilutions were plated on complete and selective medium, and colonies counted to obtain the mutation frequency (the total number of mutant in the culture divided by the total number of viable cells in the culture). The mutation rate was calculated from the mutant frequency using the Drake equation (74). The mutation rate reported for each strain is the median mutation rate for at least 18 cultures from two or more independently constructed clones of the same genotype. The Wilcoxon–Mann–Whitney test was used to determine whether differences between the mutation rates are statistically significant.

Proteins. Preparations of four-subunit S. cerevisiae Pol, Polε-exo-, two-subunit Polε, PCNA, and RPA used in this work have been described (45, 47, 73). Purified yeast RFC was kindly provided by Peter Burgers (Washington University School of Medicine).

In Vitro Replication Assay. Singly primed circular DNA substrates for in vitro replication assays were prepared by annealing the Cy5-labeled oligonucleotide P50-1M3 (Cy5-5-AAGGAACTTTCGAGAAACCTGGAAAGGATCGGATAGT-3′) to the M13 CAN1(1-1560-F) single-stranded DNA (76) by incubating the primer and template at a ratio of 1:1 in the presence of 150 mM NaAc at 92 °C for 2 min and then cooling slowly to room temperature (∼2 h). The 10-μL replication reactions contained 40 mM Tris HCl pH 7.8, 8 mM MgAc₂, 125 mM NaAc, 1 mM DTT, 0.2 mg/mL bovine serum albumin, 1 mM ATP, dNTPs at S-phase concentrations (30 μM dCTP, 80 μM dTTP, 38 μM dATP, and 26 μM dGTP) (47, 48) or imbalanced concentrations (30 μM dCTP, 80 μM dTTP, 7.9 μM dATP, and 4 μM dGTP), 2 nM singly primed M13/CAN1(1-1560-F), 790 nM RPA, 2 nM RFC, 21 nM PCNA, 50 nM Polδ, or Polε-exo-, and, when indicated, 1 or 5 mM wild-type Polλ. RPA was the first protein added to the reaction, followed by a 1-min incubation at 30 °C, then RFC and PCNA were added, followed by another 1-min incubation at

Materials and Methods

Yeast Strains and Plasmids. All Saccharomyces cerevisiae strains used in this study are derivatives of E134 (MATα ade5-1 lys2-α2 leu2-3,112 ura3-52 [21, 68] and B770 [MATα ade5-1 lys2-α2 trp1-189 hisα-2 leu2-3,112 ura3-4-3]) (68). The plasmid used to construct pol2-M644G mutants was p170, a URA3-based integrative plasmid containing an EcoRV-HindIII C-terminal fragment of POL3 (70) in which the pol2-D520V and pol3-L612M were created by site-directed mutagenesis (10, 71). These p170 derivatives were also provided by Youri Pavlov. The mutations were introduced into 1B-D770 by integration–excision of BseRI-linearized p170 with the D520V mutation and HpaI-linearized p170 with the L612M mutation. The pol2-D4 mutation was introduced into E134 by integration–excision of BamH1-linearized Yip81 (17). Single-mutant pol2 and pol3 haploids were crossed to make double-heterozygous diploids, which were then sporulated, and tetrads were dissected to obtain double-mutant pol2 pol3 haploids. The presence of pol2 and pol3 mutations was confirmed by Sanger sequencing prior to mutation rate measurements.

The haploid strains TM30 (the same as 1B-D770 but CAN1::KLEU2) and TM44 (the same as E134 but can1::loxP [47] were used to construct diploid strains homozygous for pol3-D520V, msh6-kanMX or both mutations as well as the isogenic wild-type diploids. Crosses of TM30 and TM44 derivatives produce diploids with a single copy of CAN1 linked to a selectable marker, Klyuyveromyces lactis LEU2. In this system, recessive can1 mutations can be scored on medium lacking leucine and containing canavanine. The selection for leucine prototrophy discriminates against cells that acquire resistance to canavanine due to a loss of the entire CAN1::KLEU2 locus by mitotic recombination, and nearly all Leu+ Can+ colonies result from intragenic mutations in CAN1 (47). To construct the pol2-D520V/pol3-D520V msh6-kanMX diploids, we first transformed both TM30 and TM44 with a BseRI-linearized p170 plasmid containing the pol2-D520V mutation. In this way, incorporation of the plasmid into the chromosomal POL3 locus places the polδ-D520V mutation in a truncated, nonexpressed portion of POL3. Then, we deleted MSH6 in these strains by transformation with a PCR-generated DNA fragment containing the kanMX cassette flanked by homology to MSH6 (72). We crossed derivatives of TM30 and TM44 harboring the deletion of MSH6 and the integrated nonexpressed polδ-D520V mutation to obtain dips. Finally, we selected for cells that had lost the p170 plasmid from both chromosomes simultaneously on medium containing 5-fluoroorotic acid and used Sanger sequencing to find clones homozygous for the polδ-D520V mutation, now present in the full-length expressed alleles. Isogenic single-mutant diploids (polδ-D520V/polδ-D520V or msh6-kanMX/msh6-kanMX) and wild-type controls were constructed similarly, omitting the MSH6 disruption step, the p170-polδ-D520V transformation step, or both.

Mutation Rate Measurements. The rate of Can1+ forward mutation and his2-7 two-reversion was measured by fluctuation analysis as described previously (73). Briefly, multiple independent cultures of each strain were grown from single colonies in liquid medium overnight. Appropriate dilutions were plated on complete and selective medium, and colonies counted to obtain the mutation frequency (the total number of mutants in the culture divided by the total number of viable cells in the culture). The mutation rate was calculated from the mutant frequency using the Drake equation (74). The mutation rate reported for each strain is the median mutation rate for at least 18 cultures from two or more independently constructed clones of the same genotype. The Wilcoxon–Mann–Whitney test was used to determine whether differences between the mutation rates are statistically significant.

Proteins. Preparations of four-subunit S. cerevisiae Pol, Polε-exo-, two-subunit Polε, PCNA, and RPA used in this work have been described (45, 47, 73). Purified yeast RFC was kindly provided by Peter Burgers (Washington University School of Medicine).

In Vitro Replication Assay. Singly primed circular DNA substrates for in vitro replication assays were prepared by annealing the Cy5-labeled oligonucleotide P50-M13 (Cy5-5-AAGGAACTTTCGAGAAACCTGGAAAGGATCGGATAGT-3′) to the M13 CAN1(1-1560-F) single-stranded DNA (76) by incubating the primer and template at a ratio of 1:1 in the presence of 150 mM NaAc at 92 °C for 2 min and then cooling slowly to room temperature (∼2 h). The 10-μL replication reactions contained 40 mM Tris HCl pH 7.8, 8 mM MgAc₂, 125 mM NaAc, 1 mM DTT, 0.2 mg/mL bovine serum albumin, 1 mM ATP, dNTPs at S-phase concentrations (30 μM dCTP, 80 μM dTTP, 38 μM dATP, and 26 μM dGTP) (47, 48) or imbalanced concentrations (30 μM dCTP, 80 μM dTTP, 7.9 μM dATP, and 4 μM dGTP), 2 nM singly primed M13/CAN1(1-1560-F), 790 nM RPA, 2 nM RFC, 21 nM PCNA, 50 nM Polδ or Polε-exo-, and, when indicated, 1 or 5 mM wild-type Polλ. RPA was the first protein added to the reaction, followed by a 1-min incubation at 30 °C, then RFC and PCNA were added, followed by another 1-min incubation at
30 °C. Reaction was initiated by the addition of Polc. For the extrinsic proofreading assay, replication by Polc-exo− was allowed to proceed for 7 min. After which, Polc was added, and the reaction was incubated for an additional 3 min. Reactions were stopped by the addition of 1 μL of 500 mM ethylenediaminetetraacetic acid (EDTA) and 1 μL of 10% sodium dodecyl sulfate, incubated with 2 μL of 20 mg/mL Proteinase K (ThermoFisher Scientific) at 55 °C for 1 h and purified by phenol/chloroform extraction and ethanol precipitation. DNA pellets were dissolved in 20 μL ddH2O and mixed with 4 μL of 5X alkaline loading buffer containing 300 mM NaOH, 6 mM EDTA, 1.8% (v/v) Ficol, 0.15% (v/v) bromocresol green, and 0.25% (v/v) xylene cyanol. The reaction products were separated in a 1% alkaline with 46040 7m i na ft e r w h i c hP o l 22. H. T. Tran, D. A. Gordenin, M. A. Resnick, The 3
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