Fall 12-18-2020

DNA Polymerase ε: Replication Error Prevention and Consequences of a Cancer-Associated Mutation

Chelsea R. Bulock
University of Nebraska Medical Center

DNA POLYMERASE EPSILON: REPLICATION ERROR PREVENTION AND CONSEQUENCES OF A CANCER-ASSOCIATED MUTATION

by

Chelsea Bulock

A DISSERTATION

Presented to the Faculty of
the University of Nebraska Graduate College
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

Genetics, Cell Biology & Anatomy
Graduate Program

Under the Supervision of Professor Polina V. Shcherbakova

University of Nebraska Medical Center
Omaha, Nebraska

August 2020

Supervisory committee:

Youri I. Pavlov, Ph.D.
Karen A. Gould, Ph.D.
James D. Eudy, Ph.D
Tadayoshi Bessho, Ph.D

Acknowledgments

Like many things in life, a Ph.D. is not something that can be done in isolation. The completion of my dissertation would not have happened without the guidance, encouragement, patience, and companionship of a great number of people.

"If I have seen further it is by standing on the shoulders of Giants." — **Isaac Newton**

Polina, though your physical stature may be small, you are the most prominent scientific "giant" whose shoulders I stand on. I cannot begin to express my appreciation for your mentorship over the last 6 years. From taking the time to demonstrate laboratory techniques to critically reading and providing thoughtful suggestions for everything I write; I thank you for taking the time to ensure I receive a proper graduate education. Thank you for celebrating my accomplishments, both professional and personal, and for supporting my career goals. I especially appreciate your encouragement when my results proved hypothesis after hypothesis to be wrong. I admire your leadership and aspire to your ability to communicate complicated ideas simply.

I also owe a debt a gratitude to my current and former Supervisory Committee members, Dr. Pavlov, Dr. Gould, Dr. Trujillo, Dr. Eudy, and Dr. Bessho. Thank you for valuable advice and practical suggestions regarding not only my research project, but also my interest in a teaching career. I would especially like to thank Dr. Pavlov for asking questions that made me think deeper about published literature, even if he often already knew the answers himself. I would also like to thank Dr. Gould and Dr. Solheim for always sending teaching opportunities my way.

I was fortunate to spend my graduate days with an especially wonderful group of people: Tony, Olga, Hollie, Yinbo, Dan, Xuan, Stephanie, Wyatt, Jian, Liz, Krista, and Annette. Krista and Liz, thank you for making countless liters of media, filling endless tip

boxes, and washing a never-ending pile of dishes. Olga, your vast knowledge of techniques, literature in the field, and exact placement of random reagents no one had used in years was invaluable to my graduate career. Xuan, thank you for taking the time to teach biochemistry to this geneticist. I could not have completed key experiments without your guidance and cheerful smile. Stephanie, you have been like a sister to me from the very beginning. Thank you for celebrating with me during exciting times, and for complaining with me when the PCR just wouldn't work. I will forever value our friendship.

I would not have made it into graduate school without the encouragement and inspiration of many educators. Mrs. Forster, thank you for believing in me and giving me the confidence in my academic abilities that allowed me to believe I could be a scientist. Mrs. Holm and Mrs. Ostlie, thank you for being role models to high school students of women in STEM, and for preparing me for scientific courses at an undergraduate level. I was inspired to want to educate undergraduates by the innovative teaching methods of Dr. Doan and Dr. Soneral. Dr. Neiwert and Dr. Maddox, thank you for encouraging me to be a scientist, even if I didn't change my major to chemistry. Dr. DeGolier, thank you for giving me a first taste of research experience and an appreciation for experimental work.

I was blessed to be raised by parents who provided me with exceptional opportunities and encouraged me to pursue my own goals and interests. Thank you for putting up with my independent attitude and never pressuring me into your own expectations. I love you both deeply. Emily, my sweet and sassy sister, thank you for always texting me back, for cheering me up and calming me down. Your work ethic, positive attitude, and spontaneous personality inspire me.

Finally, Logan, my loving husband. Thank you for embarking on this journey of life with me. We have had many ups and downs in our graduate school career, and I wouldn't want to do this life with anyone but you. Thank you for hours-long discussions

of scientific questions, and for inspiring me to think differently about my own work. Thank you for cheering me on in the midst of your own difficult research. And thank you for Leo, the greatest gift of my life. The two of you have shown me that I am capable of more than I could have ever dreamed, and I am forever grateful.

DNA Polymerase ε: Replication Error Prevention and Consequences of a Cancer-Associated Mutation

Chelsea R. Bulock, Ph.D.

University of Nebraska, 2020

Supervisor: Polina V. Shcherbakova, Ph.D.

Genome integrity is necessary to prevent mutations and disease. During eukaryotic DNA replication, DNA polymerases ε (Polε) and δ (Polδ) synthesize the leading and lagging strand, respectively. Polε and Polδ also have exonuclease activity that acts in series with post-replicative mismatch repair (MMR) to remove replication errors. Defects in proofreading and MMR lead to an increase in mutations and cause cancer in humans. This dissertation focuses on several unresolved issues involving the relationship between Polε and Polδ in replication error avoidance. First, despite an abundance of data supporting the one-strand-one-polymerase replication fork model, defects in the fidelity of Polε have a much weaker impact on mutagenesis than analogous Polδ defects. It has been proposed, but not directly tested, that Polδ contributes more to mutation avoidance because it proofreads mismatches created by Polε in addition to its own errors. In this work, we sought to explicitly test this idea. Second, the most common cancer-associated Polε variant, P286R, has recently been discovered to possess unusual and puzzling properties. Despite the location in the exonuclease domain, it produces a mutator effect far exceeding the effect of Polε exonuclease deficiency. The purified yeast analog, Polε-P301R, has increased DNA polymerase activity, which is thought to underlie its high mutagenicity, but the exact mechanism remains unclear. We aimed to investigate the impact of the P301R

substitution on the function of Polε as the leading strand polymerase, and the removal of Polε errors by error correction mechanisms *in vivo*.

To test the hypothesis that Polδ proofreads errors made by Polε, we measured mutation rates in yeast strains harboring a nucleotide selectivity defect in one polymerase and a proofreading defect in the other. We show that Polδ can proofread errors made by Polε, but Polε cannot proofread errors made by Polδ. To investigate the role of Polε-P301R at the replication fork, we measured the accumulation of strand-specific replication errors across a well-defined replicon in yeast. We found that, despite exceptional polymerase activity, Polε-P301R is a dedicated leading strand polymerase. We further show that both Polδ proofreading and MMR remove errors incorporated by Polε-P301R and are required for viability of Polε-P301R cells. In summary, by demonstrating Polδ-dependent extrinsic proofreading, we resolved the discrepancy between the one-strand-one-polymerase model and the stronger impact of Polδ defects on genome stability. Using the hyperactive Polε-P301R, we further demonstrate the unexpected ease of polymerase exchange *in vivo* and its critical role in preventing catastrophic accumulation of errors on the leading strand. Our results also explain the apparent incompatibility of Polε variants and MMR defects in cancers.

Table of contents

List of tables and figures

List of abbreviations

5-FOA	5-fluoroorotic acid
A	adenine
ARS	autonomous replicating sequence
C	cytosine
Can^r	canavanine-resistant
exo^-	exonuclease-deficient
G	guanine
His	histidine
Leu	leucine
MMR	DNA mismatch repair
PCNA	proliferating cell nuclear antigen
Polα	DNA polymerase α
Polδ	DNA polymerase δ
Polε	DNA polymerase ε
RFC	replication factor C
SC	synthetic complete
T	thymine
Ura	uracil
YPDAU	yeast extract peptone dextrose adenine uracil

Chapter 1: Introduction

DNA replication in eukaryotes

DNA replication is an essential process in every cell cycle, and it must be completed both accurately and efficiently to produce two identical daughter cells. The synthesis, or S phase of the cell cycle is tightly regulated to ensure that the cell completes DNA replication properly prior to mitotic cell division. Replication is initiated at DNA replication origins, which must be licensed prior to entrance into S phase, and activated only once S phase has begun. Although the general mechanisms of DNA replication are conserved in all eukaryotes, the discussion below will focus on DNA replication in the budding yeast *Saccharomyces cerevisiae*.

Series of events and required factors

In eukaryotic cells, replication origin licensing involves the binding of a complex of proteins termed the origin recognition complex to specific DNA regions during G1 phase of the cell cycle (1). In *Saccharomyces cerevisiae*, these include the proteins Orc1-6, Cdc6 and Cdt1 (2). This is followed by loading of a hexamer of MCM proteins, a required component of the helicase which will eventually unwind the duplex DNA, to establish the pre-replication complex (3,4). Origin licensing must be complete before the start of S phase, and prevented from occurring again during S phase, which would result in re-replication, replication stress, and potentially aneuploidy (5). Once origins have been licensed and S phase has begun, cell cycle kinases CDK and DDK phosphorylate the pre-replication complex, recruiting the remaining components of the helicase, including Cdc45, GINS, and DNA polymerase ε (Polε) to form the pre-initiation complex (6,7). At this point, replication origins are activated and the helicase begins to unwind the DNA, allowing DNA synthesis by DNA polymerases to proceed. Importantly, only a subset of licensed origins are activated in a given cell cycle (8). This appears to allow

the cell greater control over replication timing and responses to DNA damage and replication stress. Activation, or firing, of origins is strictly regulated spatially and temporally to produce exactly two identical copies of the genome (2,5,8-11). Replication timing and origin firing vary depending on the species, cell type and differentiation state of the cell (2,10). DNA synthesis then proceeds bidirectionally from origins, and replication forks merge forming replication termination zones. In *S. cerevisiae*, the location of termination zones depends on the timing of the firing of the two replication origins converging, but is generally at the midpoint between efficient replication origins (12).

Replicative polymerases and their functions

Eukaryotic DNA replication requires three DNA polymerases: Polα, Polδ, and Polε (13). The model was originally proposed by Morrison and co-authors (14) and remains the most widely accepted model at this time (**Figure 1.1**). It suggests that Polα-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.

Pols α, ε, and δ belong to the B family of DNA polymerases [(9), **Figure 1.2**]. *S. cerevisiae* Polα-primase complex consists of Pol1, Pol12, Pri1, and Pri2. The Pri1 subunit contains the primase activity, and the Pol1 subunit contains DNA polymerase activity (15). Polα synthesizes approximately 30 nucleotides at replication origins and the beginning of each Okazaki fragment (~10% of the genome), yet much of the Polα-synthesized DNA is removed by processing of Okazaki fragments (16). Recent analysis of ribonucleotide incorporation by a variant Polα suggested that only 1.5% of the mature genome consists of Polα-replicated DNA (17). Polε consists of the catalytic subunit Pol2,

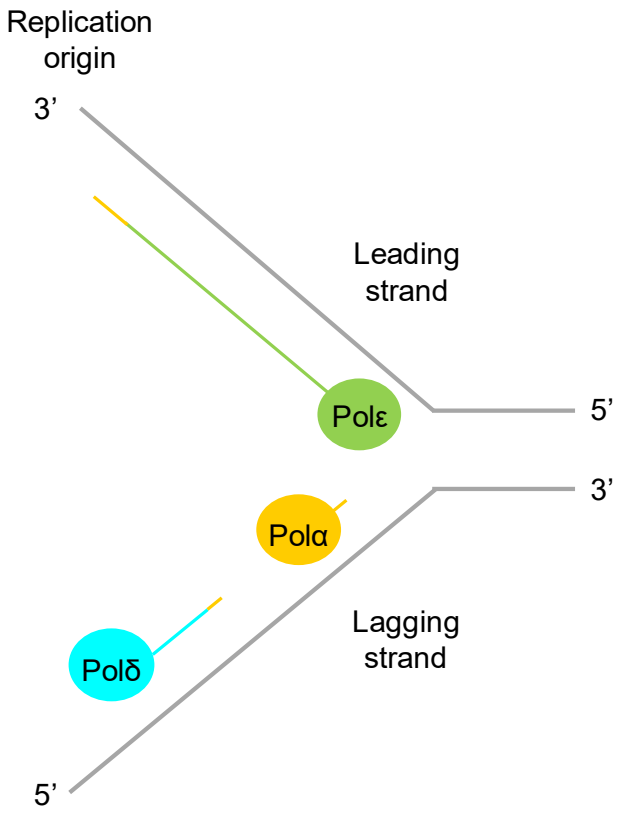


Figure 1.1. Replication fork model.

Polα-primase synthesizes primers at replication origins and the beginning of each Okazaki fragment on the lagging strand. Polε synthesizes the leading strand continuously, whereas Polδ synthesizes the remainder of Okazaki fragments on the lagging strand.

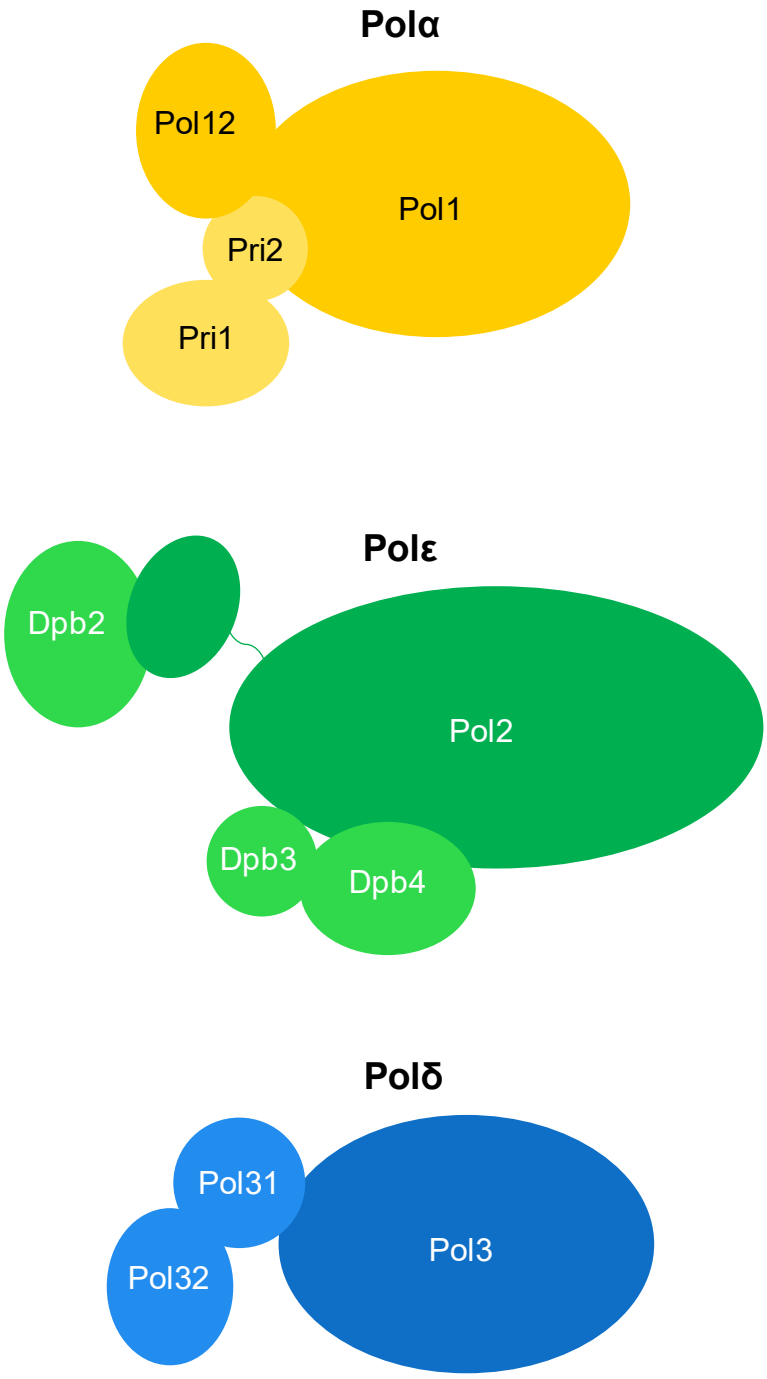


Figure 1.2. Catalytic and accessory subunits of three replicative DNA polymerases of *S. cerevisiae.*

Polα-primase contains primase activity in Pri1, and polymerase activity in Pol1, as well as two accessory subunits (Pol12 and Pri2). Polε contains catalytic subunit (Pol2) and three accessory subunits (Dpb2, Dpb3, and Dpb4). Polδ contains catalytic subunit (Pol3) and two accessory subunits (Pol31 and Pol32).

and three accessory subunits Dpb2, Dpb3, and Dpb4 (15). Pol2 contains an N-terminal portion and C-terminal portion, connected by a flexible linker region (18), though this region appears to be rigid in the presence of Dpb3 and Dpb4 (19). The DNA polymerase activity is located in the N-terminal portion, which also contains 3'-5' exonuclease activity for proofreading of replication errors. The C-terminal portion contains a second, inactivated exonuclease-polymerase module (20). While Pol2 is required for viability in yeast, the N-terminal portion is not, although strains lacking the N-terminus of Pol2 are very sick (21). The C-terminal portion of Pol2 is necessary for DNA replication, as it forms part of the helicase complex CMGE (Cdc45, Mcm2-7, GINS, and Pol ε) (7). *S. cerevisiae* Polδ consists of catalytic subunit Pol3 and accessory subunits Pol31 and Pol32 (15). Pol3 contains both DNA polymerase and 3'-5' exonuclease activities, and is required for viability (22). In addition to their vital roles in DNA replication, both Polε and Polδ have also been implicated in various forms of DNA repair (23).

Prevention of DNA replication errors

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 (24). Rare errors missed by the nucleotide selectivity and proofreading functions of replicative polymerases are further corrected by the DNA mismatch repair (MMR) system (25), 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 (26). Nucleotide selectivity, proofreading, and MMR act in series to ensure accurate DNA replication (**Figure 1.3**).

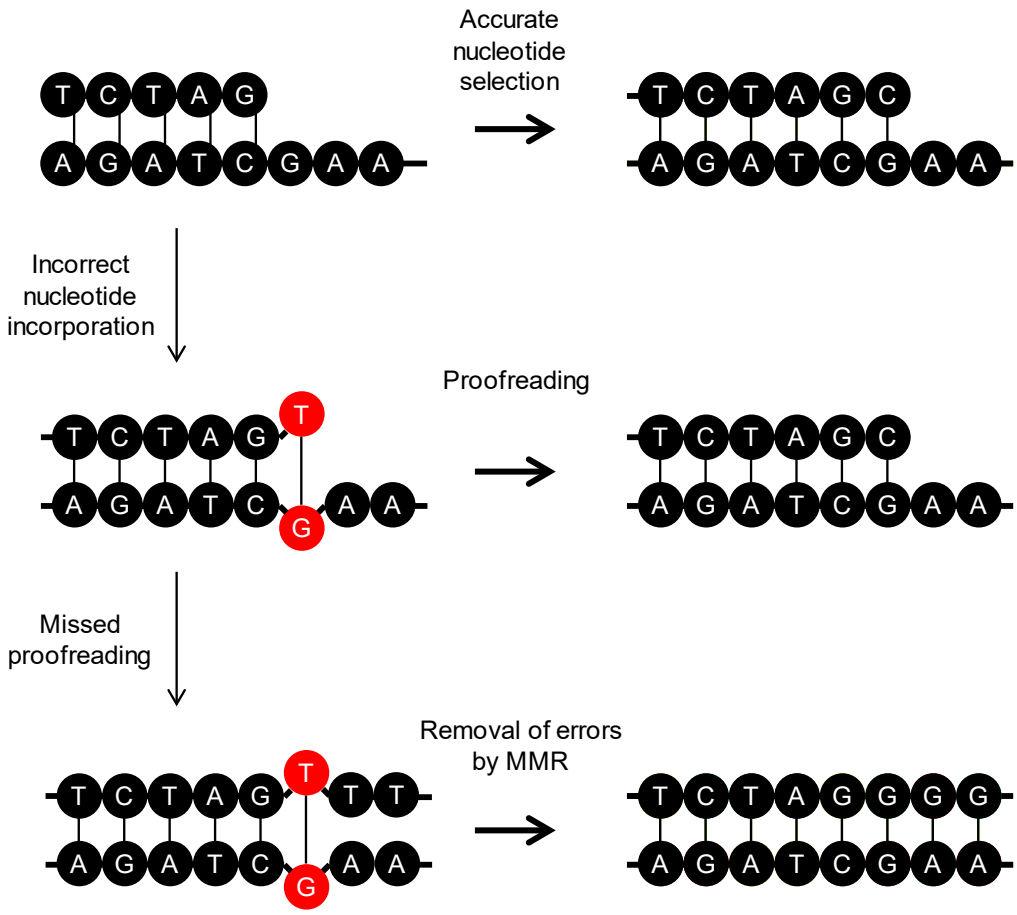


Figure 1.3. Replication error avoidance pathway.

Replicative polymerases accurately select correct nucleotides for incorporation (top). In the event a wrong nucleotide is incorporated, polymerases can remove the error by proofreading (middle). If an error is missed by proofreading, it can be corrected by post-replicative DNA mismatch repair (bottom).

Nucleotide selectivity

The highest contribution to DNA replication fidelity comes from the nucleotide selectivity of DNA polymerases. The error rates of exonuclease-deficient Polε and Polδ, measured *in vitro* are 1.6×10^{-4} and 1.3×10^{-4} mutations per nucleotide, respectively (27,28), although it has been suggested that the error rates could be lower *in vivo* (29). When the structure of DNA was first proposed, it was suggested that the specific hydrogen bonds formed between the base pairs could serve as a mechanism for making an exact copy of DNA (30,31). However, measurements of the free energy differences between correct and incorrect base pairs did not fully account for the extremely high selectivity of DNA polymerases (32). It was later demonstrated that hydrogen bonds and base stacking interactions both play a role in maintenance of the structure of DNA, but the geometric fit of the newly forming base pair to the active site in DNA polymerases provides the greatest nucleotide selection (32-34). To differentiate between hydrogen bonding and geometric contribution to nucleotide selectivity of DNA polymerases, base analogs that lack hydrogen bonding capability but maintain the same shape were used in *in vitro* polymerase assays. Difluorotoluene is a thymine analog that lacks hydrogen bonding capacity. It does not spontaneously pair with adenine in solution, yet Klenow fragment inserts it across from template adenine and not across from template thymine, cytosine, or guanine (35,36). Studies using other base analogs provided further support for the geometric fit hypothesis (36-41). In addition to selecting the correct base, DNA polymerases must discriminate between ribonucleotides and deoxyribonucleotides. Studies involving altered polymerases illuminated a specific region of the polymerase responsible for selection of the correct sugar (42).

Given the tight geometric fit necessary for accurate nucleotide selection, point mutations that alter the structure of the catalytic site of DNA polymerases would be

predicted to alter fidelity. Both mutator and antimutator polymerase variants have been isolated, containing substitutions in the polymerase domain predicted to affect the geometry of the catalytic site (43). The L868F substitution in Polα occurs at a structurally conserved position in the polymerase domain and affects the fidelity of the polymerase (44). Analogous mutations affecting Polδ (*pol3-L612M*) and Polε (*pol2-M644G*) also decrease the fidelity of the replicative polymerases, while leaving exonuclease activity intact (45,46). Both variants result in increased base-base mismatches *in vitro* and *in vivo*, as well as increased incorporation of ribonucleotides into DNA (47).

Proofreading

After nucleotide selectivity, the next highest contributor to replication fidelity is exonucleolytic proofreading. Because Polδ and Polε possess exonuclease activity, they are significantly more accurate than Polα (48-50). The proofreading capabilities of Polε and Polδ decrease the *in vivo* replication error rate in yeast by 160-fold and 1000-fold, respectively (29). The proofreading domains of the replicative polymerases contain three conserved motifs, Exo I, Exo II, and Exo III (51). Mutations that inactivate the metal ion-coordinating residues in the Exo I motif of Polε (*pol2-4*) and Polδ (*pol3-01*) result in proofreading-deficient polymerases (52,53). Similarly, an aspartate to valine substitution in the Exo III motif of Polδ (*pol3-D520V*) also results in a proofreading-deficient Polδ (54). Antimutator variants of polymerases have also been isolated that reduce replication errors by altering the balance between nucleotide incorporation and excision (43,55).

Polε can switch processively (without dissociating) from synthesis to proofreading, although it dissociates after approximately one out of every three misincorporations (56). Intramolecular switching between polymerase and exonuclease active sites has also been suggested for Polδ (57). The exonuclease activities of Polε

and Polδ have been shown to be important for removal of base analogs such as N^6-hydroxylaminopurine (HAP) (58). Polδ exonuclease activity can also compensate for lack of flap endonuclease Rad27 during Okazaki fragment processing (54). Another study found that combining of deletion of *EXO1*, a 5'-3' exonuclease involved in MMR, and Polδ proofreading deficiency resulted in a synergistic increase in the mutation rate of a long homonucleotide run, and suggested possible involvement of Polδ exonuclease in MMR (59). As originally hypothesized in the 1970s (60), mutations affecting the proofreading domains of the replicative DNA polymerases have recently been implicated in sporadic and hereditary cancers (61).

Mismatch repair

MMR is responsible for removal of DNA replication errors missed by the proofreading activities of the replicative polymerases. The proteins involved in MMR are conserved throughout prokaryotes and eukaryotes (62). In yeast, a mismatch is first recognized by one of two heterodimers, Msh2-Msh6 or Msh2-Msh3 (62). Msh2-Msh6 is responsible for recognizing base-base mismatches and single-nucleotide loops, whereas Msh2-Msh3 primarily recognizes single-nucleotide and larger loops, and, to a lesser extent, base-base mismatches. The binding of a second heterodimer consisting of Mlh1 and Pms1 results in an incision on the nascent strand. This is followed by excision of the mismatch, usually by Exo1. Polδ, or possibly Polε, re-synthesize the DNA before ligation.

The timing of MMR is coupled to DNA replication to allow for nascent strand discrimination and correction of errors prior to mitosis (63). Yet, MMR does not correct errors uniformly throughout the genome. Microsatellites are short repeat sequences of DNA found in various locations across the genome. MMR is very efficient in repairing

insertions and deletions at microsatellites. Accordingly, cells lacking MMR are microsatellite instable, meaning they have increases in mutations in these repeat sequences (64). Wild-type strains demonstrate variation in microsatellite stability across the genome, but inactivation of MMR by deletion of *MSH2* eliminated this variation (65). MMR of 8-oxoguanine-containing mismatches in *ogg1*Δ strains occurred with better efficiency on the lagging strand (66). Inactivation of MMR by deletion of *EXO1* (63,67) and *MSH2* (68) resulted in a larger increase in lagging strand mutations than leading strand mutations. MMR has a higher efficiency near origins on the leading strand and in early replicating regions of the genome (68). MMR also possesses a bias in the type of errors that are repaired, with the highest efficiency of repair for mispairs that lead to C→T mutations and lowest efficiency for mispairs that lead to A→T mutations (68).

Evidence for the DNA replication fork model

During the three decades that passed since the landmark publication by Morrison *et al.* (14) proposing the currently accepted replication fork model, numerous reports have contributed evidence for the participation of Polε and Polδ in leading and lagging strand replication, respectively. Several genetic studies detected strand-specific increases in mutagenesis in yeast and human cells carrying inaccurate Polε or Polδ variants. Experiments using proofreading-deficient polymerase variants demonstrated that the exonucleases of Polε and Polδ corrected errors induced by base analog N^6-hydroxylaminopurine (HAP) on opposite strands near a defined replication origin (58). Two studies using mutator variants of Pols ε and δ utilized the bias in the formation of reciprocal mispairs by the variant polymerases, and a reporter allele placed near a defined replication origin, to assign Polε to the leading strand and Polδ to the lagging strand (46,69). Deep sequencing of a yeast strain carrying a mutator Polδ assigned Polδ

to synthesis of the lagging strand, with strongest evidence near replication origins (70). Analysis of mutations in human tumors carrying Polε variants found that abrupt switches in strand specificity of mutagenesis coincided with replication origins (71). More sensitive assays monitoring ribonucleotide incorporation into DNA by Polε or Polδ variants with relaxed sugar selectivity confirmed ribonucleotide accumulation in the leading strand in Polε mutants and in the lagging strand in Polδ mutants (72,73). Polδ but not Polε was shown to proofread errors made by Polα (74) and participate in maturation of Okazaki fragments on the lagging strand (54,75). At the same time, Polε but not Polδ interacts with the CMG helicase on the leading strand (7). While the roles of Polδ in synthesis of the leading strand near replication origins and termination zones have recently been detected (76-79), these stretches of Polδ synthesis appear to account for a relatively minor fraction of the leading strand [~18%, (79)]. Overall, a bulk of evidence supports the originally proposed division of labor with Polε and Polδ predominantly replicating opposite DNA strands.

POLE mutations in cancer

Ultramutation in colorectal and endometrial cancers

Approximately 6-15% of endometrial tumors and .65-3% of colorectal tumors contain mutations in *POLE*, the gene encoding the catalytic subunit of Polε in humans (80-94). While mutations have been recorded across the entire gene, recurrent hotspot mutations cluster in the region encoding the exonuclease domain of the protein (61,95,96). These mutations are associated with extremely high tumor mutation burden, typically >100 mutations per megabase (termed ultramutated). While cases of germline mutations in *POLE* have been reported, most of these ultramutated cancers are sporadic. Patients presenting with ultramutated colon and endometrial tumors are

typically younger than patients without mutations in *POLE* (80,83,86,88,97-99). Notably, these patients also have better progression free survival than patients with wild-type *POLE* (80,83,85,87,97,98,100,101).

POLE mutations have been reported in cancer precursors (endometrial intraepithelial neoplasias, endometrial hyperplasias, and colorectal adenomas), suggesting these mutations are early events in sporadic tumors (102,103). Furthermore, *POLE* mutant tumors are associated with a specific mutation signature with characteristic C→A mutations in a TCT sequence context (71,82,104-106). Mutations in oncogenes in *POLE* mutant tumors have also been found to have C→A mutations in a TCT sequence context (93). While the tumors are ultramutated, they are typically microsatellite stable, distinguishing these from hypermutated MMR-deficient tumors which contain between 10 and 100 mutations per megabase (90,91).

POLE-mutant endometrial tumors are morphologically heterogeneous and highly immunogenic (87,107). The high levels of mutagenesis in *POLE*-mutant tumors leads to increased numbers of neo-epitopes (103,108). Associations have been shown with high levels of $CD8^+$ and $CD3^+$ tumor infiltrating lymphocytes (83,86,87,103,108-112). PD-1 expression in T-lymphocytes and PDL-1 expression in the tumors has also been shown, suggesting these tumors would respond well to immunotherapy (86,108,109,112-114). Indeed, anti-PD-1 drugs Nivolumab and Pembrolizumab have both been used to treat patients with *POLE*-mutant tumors with good outcomes (115,116).

Polε-P286R

The majority of suspected pathogenic *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 (61,95,96). *POLE-P286R* 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 (81,117). Studies in which the mutation was introduced in model organisms have illuminated some of the genetic, biochemical, and physical properties of this particular replicative polymerase variant.

Genetic modeling

After the mutation was found in a cohort of colorectal patients at the University of Nebraska Medical Center, Kane and Shcherbakova engineered the analogous mutation in *S. cerevisiae*. The *pol2-P301R* mutation caused a 150-fold increase in mutation rate over the wild-type strain (118). 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, $Pole^{wt/P286R}$ mice are dramatically more cancer-prone than mice deficient in Polε proofreading and, in fact, more cancer-prone than any existing monoallelic animal model (119,120). The attempts to introduce CRISPR-mediated P286R mutation into one copy of *POLE* in MMR-deficient colon cancer cell line HCT116 or its MMR-proficient derivative resulted in cells that were heterozygous at the DNA level but produced almost no *POLE-P286R* transcripts, suggesting that expression of the mutant allele could be deleterious for this cell line (121). However, colon cancer cell line HCC2998, which contains the P286R mutation and is one of the most hypermutated cell lines known, has elevated mutation frequency and increased C→A mutations (122,123). The mechanisms of these uniquely strong mutagenic and tumorigenic effects of the P286R variant remain to be determined.

Biochemical and structural properties

While initial hypotheses suggested that *POLE* mutations impacting the exonuclease domain inactivated proofreading, purified four-subunit yeast Polε-P301R retains residual exonuclease activity (28). The N-terminal part of the catalytic subunit of human Polε-P286R also retains residual exonuclease activity (71). It was recently reported that the purified yeast variant has an unusually high DNA polymerase activity in addition to a severe exonuclease defect (28). It extends matched and mismatched primer termini more efficiently than either wild-type or proofreading-deficient Polε, and particularly excels at synthesis through secondary structures that normally impede replicative polymerases (28). Crystallographic studies of Polε-P301R and molecular dynamics simulations suggested that the arginine side chain protrudes into the opening of the exonuclease active site, hindering access of the primer terminus to the catalytic residues (124). It was, 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 (28).

Dissertation overview

This dissertation addresses some unanswered questions regarding the interplay of Polδ and Polε in the prevention of replication errors, and the consequences of the most common cancer-associated Polε variant, Polε-P286R (Polε-P301R in *S. cerevisiae*). While a plethora of evidence supports the one-strand-one-polymerase model described above, several studies have indicated that Polε and Polδ do not operate completely independently. Defects in Polδ have stronger impacts on mutagenesis than analogous defects in Polε. The combination of proofreading defects in

Polδ and Polε results in a synergistic increase in mutation rate, which suggests the two polymerases compete to correct the same pool of replication errors. A hypothesis has been long-entertained that Polδ corrects errors made by Polε, but direct evidence for this idea was lacking. Previous studies also left uncertainty regarding leading strand replication by Polε near termination zones. Additionally, it remained unknown how the newly revealed biochemical properties of the Polε-P301R variant impact the role of Polε in replication and correction of its errors.

Chapter 3 describes genetic experiments designed to test the hypothesis that Polδ corrects errors made by Polε, in addition to its own errors. A synergistic increase in the mutation rate resulted from combining a Polε selectivity defect with a Polδ proofreading defect. However, only an additive increase in the mutation rate was observed from the combination of a Polδ selectivity defect and Polε proofreading defect. These results provide evidence for a model where Polδ can extrinsically proofread errors made by Polε, whereas Polε cannot proofread errors made by Polδ.

Chapter 4 focuses on how the Polε-P301R variant affects the role of Polε in DNA replication. By measuring the accumulation of strand-specific replication errors across a well-defined replicon, these experiments demonstrate that despite greatly increased polymerase activity, Polε-P301R remains a dedicated leading strand polymerase. Furthermore, our results reveal for the first time the strong contribution of Polε to leading strand replication in the vicinity of the termination zone.

Chapter 5 describes a series of genetic experiments designed to evaluate how the unprecedented mismatch extension capability of Polε-P301R impacts the correction of its errors by extrinsic correction mechanisms. The results establish that both extrinsic proofreading by Polδ and correction by MMR are necessary to maintain viability in cells expressing Polε-P301R. Moreover, extrinsic proofreading of Polε-P301R errors by Polδ

is extremely efficient, demonstrating the ease with which polymerase exchange occurs *in vivo*.

Chapter 2: Materials and methods

Plasmids

The plasmid used to construct *pol2-M644G* mutants was p173, a *URA3*-based yeast integrative vector containing a *Bam*HI-*Bsp*EI C-terminal fragment of *POL2* (125), in which the *pol2-M644G* mutation was created by site-directed mutagenesis (46). It was kindly provided by Youri Pavlov (University of Nebraska Medical Center). YIpJB1, a *URA3*-based yeast integrative vector, was used to construct the *pol2-4* mutant strains (52). To construct *pol3-D520V* and *pol3-L612M* mutants, we used p170, a *URA3*-based integrative plasmid containing an *Eco*RV-*Hind*III C-terminal fragment of *POL3* (126), in which the *pol3-D520V* and *pol3-L612M* were created by site-directed mutagenesis (54,127). These p170 derivatives were also provided by Youri Pavlov. To construct strains with the *ura3-29* or *ura3-24* reporter, we used derivatives of YIpGL1 to amplify the *ura3-29::LEU2* and *ura3-24::LEU2* cassettes (128,129). pBL304 is an episomal plasmid containing a *URA3* marker, and expressing *POL3* (130). YIpDK1-pol2-P301R or YIpCB2 was used to construct the *pol2-P301R* mutation. YIpDK1-pol2-P301R is a derivative of YIpJB1 in which the *pol2-4* mutation was changed to wild-type and the *pol2-P301R* mutation was introduced, both by site-directed mutagenesis (118). YIpCB2 was constructed by replacing the *URA3* marker in YIpDK1-pol2-P301R with the *LYS2* marker. *LYS2* was amplified from chromosomal DNA of a W303 derivative (*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) provided by Duncan Smith (New York University) using primers 5'-TTTTTTGCCAATTTGGCCTGGCTCACTTGAGGGCTAT-3' and 5'-TTTTTTTGGCCAAGCAGACTAACGCCAGCTGA-3'. The primers created *Bgl*I and *Msc*I cut sites at each end of the amplified DNA. The PCR fragment was digested with *Bgl*I and *Msc*I and ligated into YIpDK1-pol2-P301R digested with *Pfl*MI and *Msc*I to create YIpCB2.

Yeast strains

Construction of *ura3-29* and *ura3-24* reporter strains

The haploid *Saccharomyces cerevisiae* strains used to study mutagenesis across the *ARS306* replicon (**Appendix B**) were derived from CG379Δ, which contains a deletion of chromosomal *URA3* (*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3*Δ) (131). The CG379Δ n303::ura3-29inv or1 (and or2) as well as 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 using a derivative of YIpGL1 containing the *ura3-29* allele (128,129). The primers were designed such that the end of the amplified cassette contain homology to the target site on chromosomal DNA, so that it is inserted into a defined location upon transformation (**Figure 2.1**). Another derivative of YIpGL1 containing the *ura3-24* allele was used to amplify the *ura3-24::LEU2* cassette in the same manner. The *ura3-29* and *ura3-24* reporters were inserted in a total of six different locations (*HBN1*, *BIK1*, *HIS4*, *STE50*, *LSB5*, and *ATG22*). PCR primers for cassette amplification and insertion at each location are listed in **Table 2.1**.

Construction of DNA polymerase mutants

All DNA polymerase mutants were constructed by integration and excision of a yeast integrative plasmid containing the desired mutation (**Figure 2.2**).

Saccharomyces cerevisiae strains used to study extrinsic proofreading of Polε errors by Polδ (**Appendix A**) are derivatives of E134 (*MAT*α *ade5-1 lys2::InsE*$_{A14}$ *trp1-289 his7-2 leu2-3,112 ura3-52*) (132,133) and 1B-D770 (*MAT***a** *ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4*) (133). Strains used to study mutagenesis across the

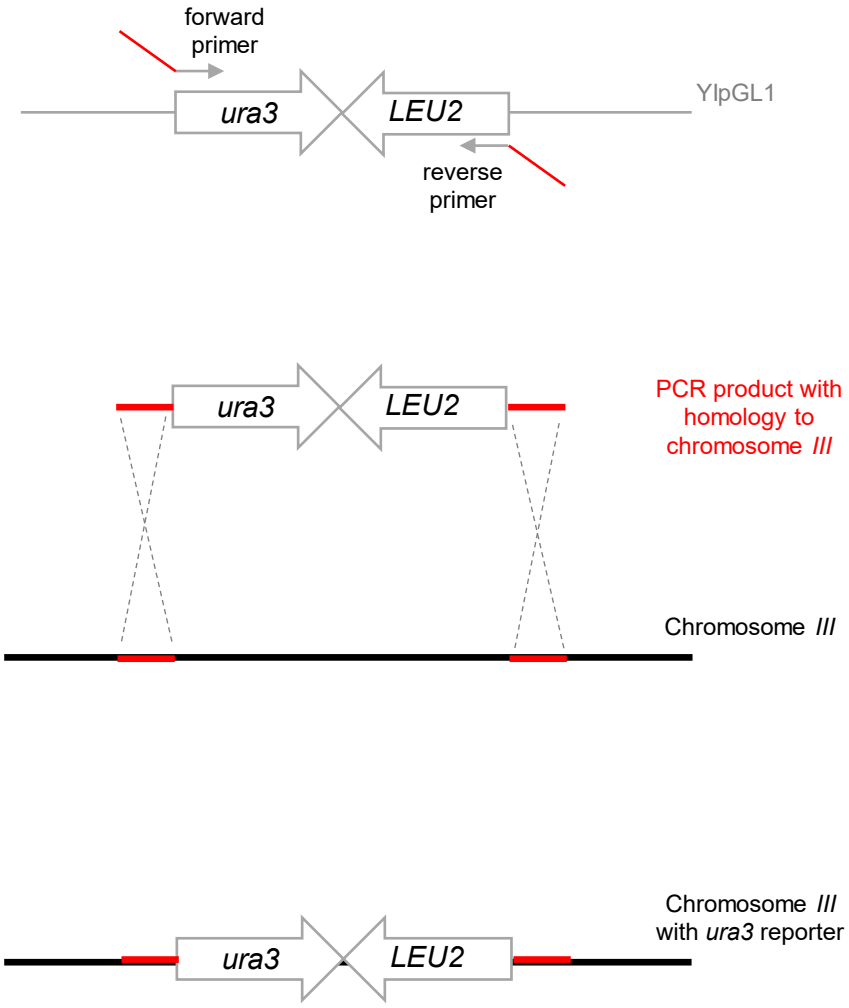


Figure 2.1. Integration of *ura3-29* and *ura3-24* reporters.

ura3-29::LEU2 and *ura3-24::LEU2* cassettes were amplified using primers with homology to replicon *ARS305-ARS306* in chromosome *III* at the 5' end and homology to plasmid YIpGL1 containing the reporter cassette at the 3' end (top). After PCR amplification of the cassette, yeast strains were transformed with the cassette, allowing the homologous regions to undergo recombination (middle), inserting the cassette into the defined location (bottom). Gray lines indicate plasmid DNA, black indicates chromosomal DNA, and red indicates homologous DNA sequence.

Table 2.1. Primers for amplification of *ura3-29* and *ura3-24* reporter cassettes

Integration Site[a]	Primer	Sequence[b]	Chromosomal position[c]
HBN1	n306-F	GCCGGTCAAAAGAGGCCTGCTTCAGCAAGGGAT GAGGCCaaacgacggccagtgccaag	73718
	n306-R	TACGCTGGGAAGTCAGCCTTTAGCTTTTCAGTTA CCTTGtgtgggaatactcaggtac	73650
BIK1	bik1-F	GCGCGGACAACTGAAATACGTGGGTCCAGTGGA CACGaaacgacggccagtgccaag	68968
	bik1-R	CTGTTGTCTTCCTGCCGTGGTATCGACTGGTGCA Ggttaactgtgggaatactcag	68679
HIS4	his4-F	GGCATCTTCATCGGCAATAACCAAAACTTCACTT GGaaacgacggccagtgccaag	66515
	his4-R	CCAGCACAAGTTGCCCAATGTAAGGAGATTGTGT TTGCgttaactgtgggaatactcag	66833
STE50	ste50-F	GGAGGACGGTAAACAGGCCATCAATGAGGGATC AAACGAaaacgacggccagtgccaag	63444
	ste50-R	CATCAATATTGTGCCATTCACGTCCAGATCCGGC GAAGgttaactgtgggaatactcag	63523
LSB5	lsb5-F	GGATCATCCGCATACAGCTATCACCGAGACGAT CTTTCGaaacgacggccagtgccaag	61673
	lsb5-R	CGTGGACGGCTGATAAGAAGACAAGCTCTCTTC CTCTGgttaactgtgggaatactcag	62688
ATG22	atg22-F	ATTGTTGAACAAACCAAGAACACACTTATCTGAaa acgacggccagtgccaag	56266
	atg22-R	GAGCTATGGAACTATAAATGATATGAATGAATCG GTAgttaactgtgggaatactcag	56525

[a]ORFs disrupted by insertion of the reporter cassette are indicated
[b]Primer homology to chromosome *III* is shown in uppercase, and homology to the plasmid containing the reporter cassette is shown in lowercase.
[c]The chromosomal position corresponding to the first nucleotide of the primer is shown with respect to the left telomere. Chromosomal sequences deleted within each ORF by insertion of the cassette are as follows: *HBN1* (73688-73717), *BIK1* (68714-69831), *HIS4* (66553-66797), *STE50* (63483-63485), *LSB5* (61712-62650), *ATG22* (56303-56308). The coordinates of replication origins *ARS306* and *ARS305* are 74458-74677 and 39508-39595, respectively.

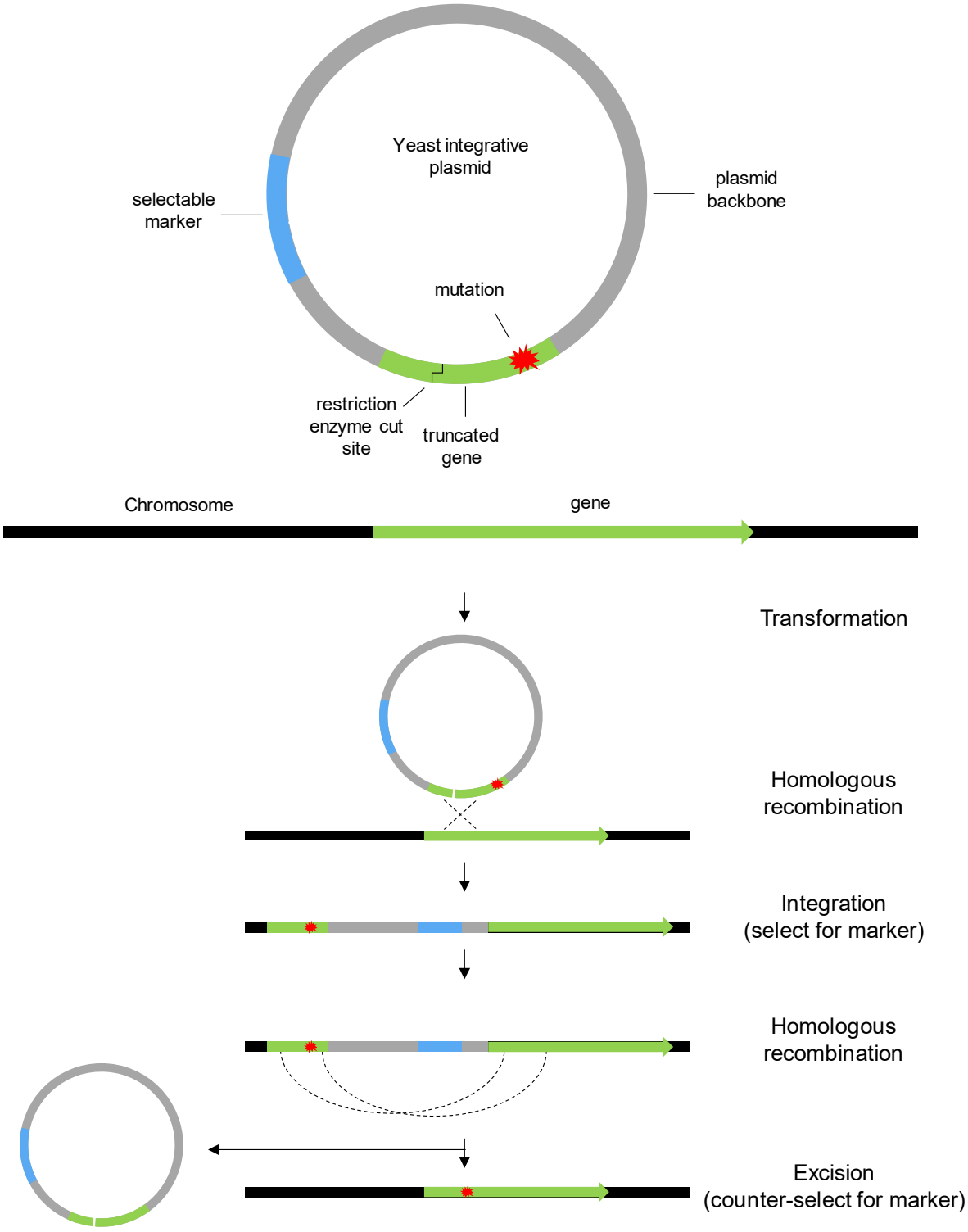

Yeast integrative plasmid
selectable marker
plasmid backbone
mutation
restriction enzyme cut site
truncated gene
Chromosome
gene
Transformation
Homologous recombination
Integration (select for marker)
Homologous recombination
Excision (counter-select for marker)

Figure 2.2. Integration-excision procedure.

A yeast integrative plasmid containing a selectable marker and a truncated gene harboring a mutation is cut with a restriction enzyme and introduced into cells via transformation. The cut vector integrates at the corresponding genomic site via homologous recombination (dashed lines), leaving the entire plasmid backbone in the chromosome. Further selection against the marker in the plasmid selects for cells which have undergone homologous recombination (dashed lines) again to excise the backbone of the plasmid.

replicon are derivatives of the *ura3-29* and *ura3-24* reporter strains described above (**Appendix B**).

The *pol2-M644G* mutation was introduced by transformation with p173 containing *pol2-M644G* mutation linearized with *Bsr*GI, followed by selection for the loss of the plasmid backbone on medium containing 5-fluoroorotic acid (5-FOA). *POL3* mutations were introduced by integration-excision of *Bse*RI-linearized p170 with the D520V mutation and *Hpa*I-linearized p170 with the L612M mutation. The *pol2-4* mutation was introduced into strains by integration-excision of *Bam*HI-linearized YIpJB1 (52). The *pol2-P301R* was introduced by integration-excision of *Bam*HI-linearized YIpDK1-pol2-P301R.

Deletion of *MSH6*

The *MSH6* gene was deleted by transformation with a PCR-generated DNA fragment carrying the *kanMX* marker. The *kanMX* marker flanked by approximately 300 base pairs of sequence homology to each side of *MSH6*, was amplified from chromosomal DNA of yeast strain TM45 (*MATα ade5-1 lys2-InsE$_{A14}$ trp1-289 his7-2 leu2-3,112 ura3-52 can1Δ::loxP msh6Δ::kanMX*) (134). PCR primers used to amplify the cassette were 5'-AGTCTCCATTTCCAACTAATG-3' and 5'-CACTCAAGAAATGGAAAATAC-3'.

Construction of double mutant strains

Haploid double mutants

Single-mutant *pol2-M644G*, *pol2-4*, *pol3-D520V*, and *pol3-L612M* haploids were crossed to make the desired 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.

We created double mutant *pol2-4 msh6*Δ strains by first transforming with *Bgl*II-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.

Diploid double mutants

Diploid strains used to study the synergistic interaction of various mutations with (**Appendix C**) were derived from TM30 (same as 1B-D770 but *CAN1::Kl.LEU2*) and TM44 (same as E134 but *can1*Δ*::loxP*) (134). Crosses of TM30 and TM44 derivatives produce diploids with a single copy of *CAN1* linked to a selectable marker, *K. 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::Kl.LEU2* locus by mitotic recombination, and nearly all Leu^+ Can^r colonies result from intragenic mutations in *CAN1* (134). *msh6*Δ*::kanMX* (*msh6*Δ), *pol2-4*, *pol2-P301R*, and *pol3-D520V* mutations were introduced into TM30 and TM44 as described above.

To make diploid strains heterozygous for *pol2-P301R* and homozygous for *msh6*Δ (**Figure 2.3**), we first transformed TM30 and TM44 with *Bgl*II-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

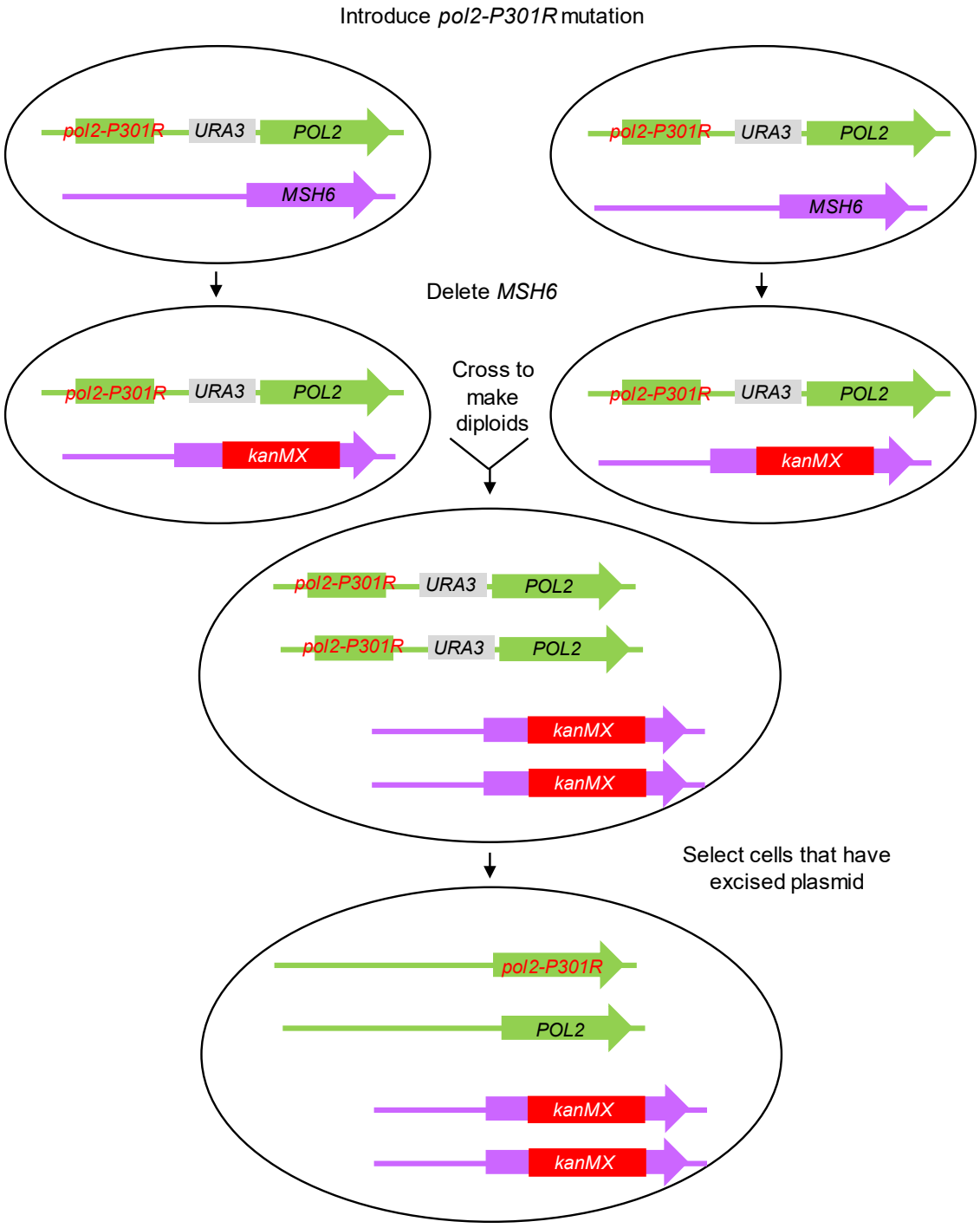

Introduce pol2-P301R mutation
pol2-P301R
URA3
POL2
MSH6
Delete MSH6
kanMX
Cross to make diploids
Select cells that have excised plasmid

Figure 2.3. Creation *of POL2/pol2-P301R msh6Δ/msh6Δ* strains.

The *pol2-P301R* mutation was first introduced into one *MAT***a** and one *MATα* strain from a *URA3*-based integrative plasmid so that the mutant allele was in the truncated copy. *MSH6* was then deleted by disruption with the *kanMX* cassette. Haploids were crossed to make diploids, and cells that lost the *URA3*-based plasmid from both *POL2* loci and retained the *pol2-P301R* mutation in one copy were selected.

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* were made by crossing TM30 containing the *pol2-P301R* mutation and TM44 containing the *pol3-D520V* mutation. To create double homozygous *pol2-P301R/pol3-P301R pol3-D520V/pol3-D520V* diploid strains containing a plasmid expressing wild-type *POL3* (**Figure 2.4**), we transformed *pol2-P301R/POL2 pol3-D520V/POL3* diploids with pBL304 (*POL3*). 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 (**Figure 2.5**). TM30 was first transformed with *Bse*RI-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 *Sal*I-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.

To construct the *pol3-D520V*/*pol3-D520V msh6*Δ/*msh6*Δ diploids (**Figure 2.6**), we first transformed both TM30 and TM44 with a *Bse*RI-linearized p170 plasmid containing the *pol3-D520V* mutation, such that the *pol3-D520V* mutation was in the

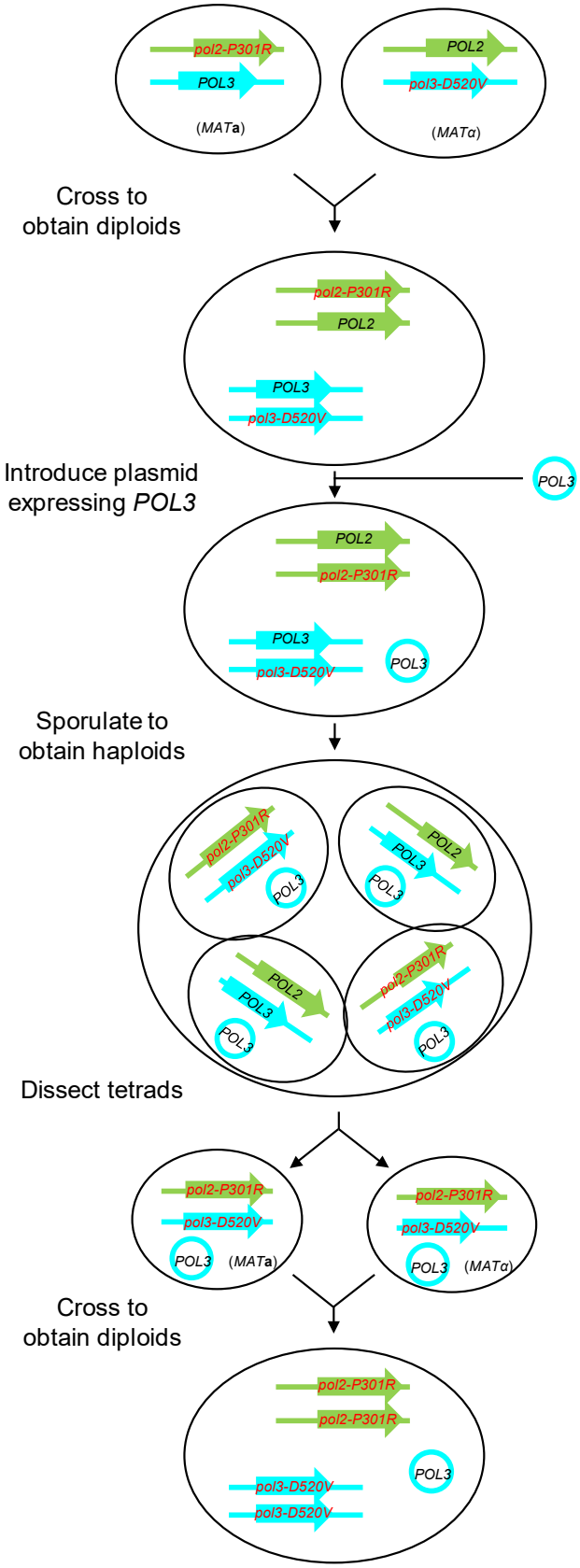

pol2-P301R
POL3
(MATa)
POL2
pol3-D520V
(MATα)
Cross to
obtain diploids
pol2-P301R
POL2
POL3
pol3-D520V
Introduce plasmid
expressing POL3
POL3
POL2
pol2-P301R
POL3
pol3-D520V
POL3
Sporulate to
obtain haploids
pol2-P301R
pol3-D520V
POL3
POL2
POL3
POL3
POL2
POL3
POL3
pol2-P301R
pol3-D520V
POL3
Dissect tetrads
pol2-P301R
pol3-D520V
POL3
(MATa)
pol2-P301R
pol3-D520V
POL3
(MATα)
Cross to
obtain diploids
pol2-P301R
pol2-P301R
POL3
pol3-D520V
pol3-D520V

Figure 2.4. Creation of strains for plasmid loss assay.

Individual haploid mutants were crossed to make double-heterozygous diploids. A plasmid expressing wild-type *POL3* was introduced, and the cells were forced to undergo meiosis (only one possible variant of segregation is shown). Individual double mutant haploids of opposite mating type containing the wild-type plasmid were then crossed to create double homozygous diploids, which were used to evaluate loss of the plasmid expressing wild-type *POL3*.

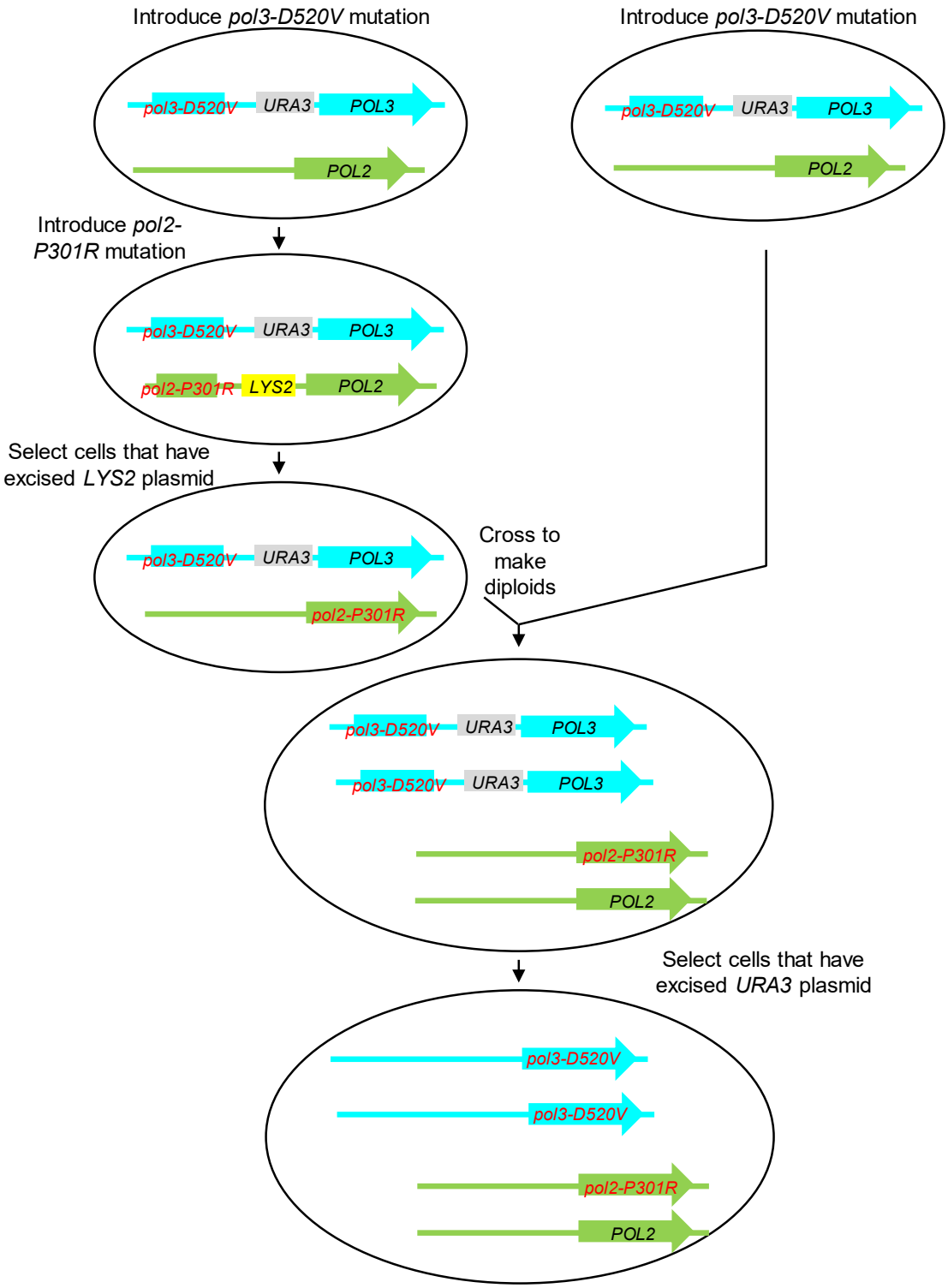

Introduce pol3-D520V mutation
pol3-D520V
URA3
POL3
POL2
Introduce pol3-D520V mutation
pol3-D520V
URA3
POL3
POL2
Introduce pol2-P301R mutation
pol3-D520V
URA3
POL3
pol2-P301R
LYS2
POL2
Select cells that have excised LYS2 plasmid
pol3-D520V
URA3
POL3
pol2-P301R
Cross to make diploids
pol3-D520V
URA3
POL3
pol3-D520V
URA3
POL3
pol2-P301R
POL2
Select cells that have excised URA3 plasmid
pol3-D520V
pol3-D520V
pol2-P301R
POL2

Figure 2.5. Creation of *POL2/pol2-P301R pol3-D520V/pol3-D520V* strains.

The *pol3-D520V* mutation was first introduced (from a *URA3*-based integrative plasmid) into strains of opposite mating type such that the mutation was in the truncated copy of *POL3*. In one strain, the *pol2-P301R* mutant allele was then introduced (from a *LYS2*-based integrative plasmid) such that the mutant allele was in the truncated copy of *POL2*. Cells that lost the *LYS2*-based plasmid and retained the *pol2-P301R* mutation in the genome were selected and crossed to cells of opposite mating type containing the *pol3-D520V* mutation in the truncated copy. Cells that lost the *URA3*-based plasmid and retained the *pol3-D520V* mutation in both chromosomes were selected.

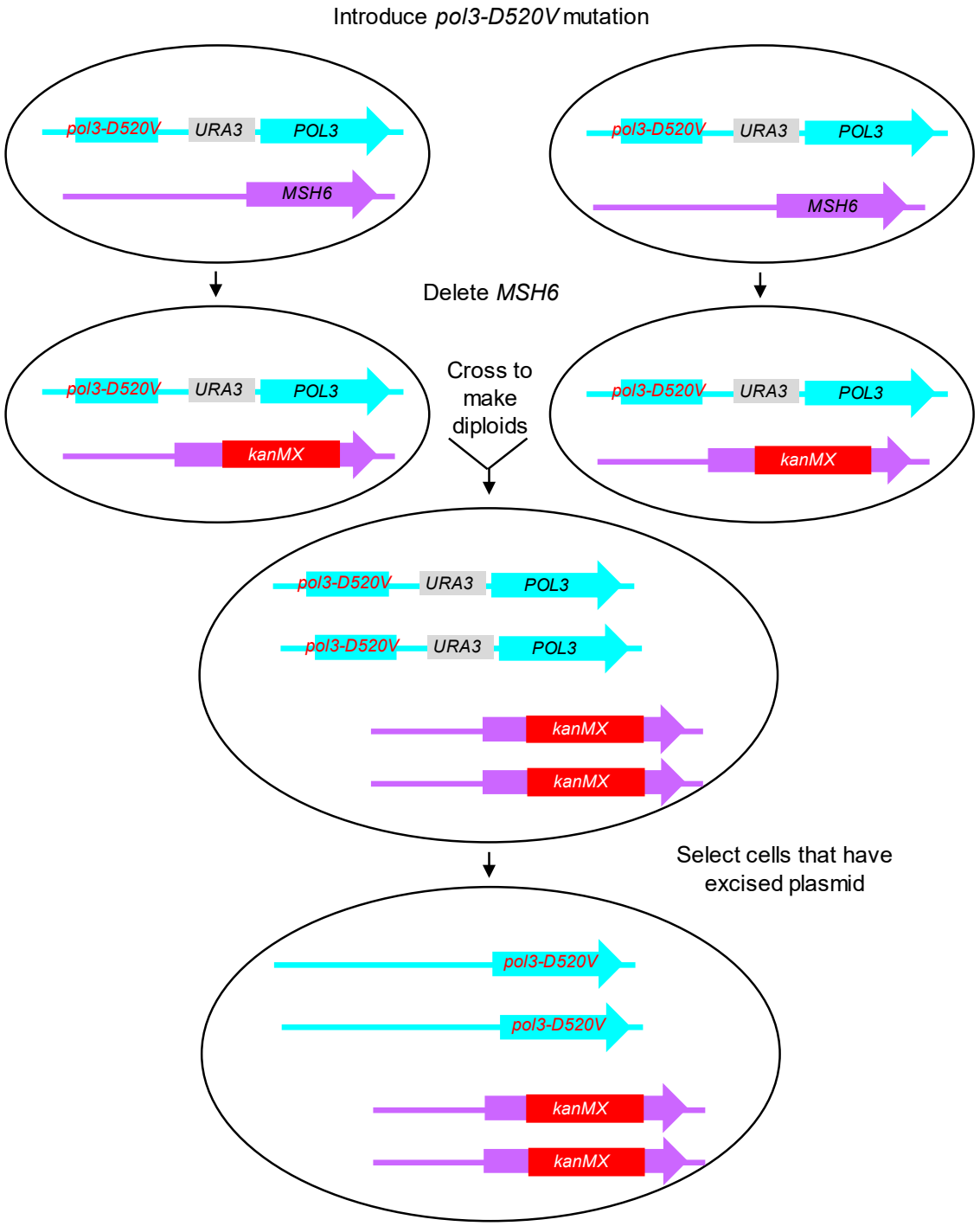


Figure 2.6. Creation of *pol3-D520V/pol3-D520V msh6Δ/msh6Δ* strains.

The *pol3-D520V* mutant allele was first introduced into haploid strains using a *URA3*-based integrative plasmid so that the mutation was in the truncated copy of *POL3*. Then, *MSH6* was disrupted with a *kanMX* cassette. The strains were crossed to obtain diploids, and cells that lost the *URA3*-based plasmid and retained the *pol3-D520V* mutation in both chromosomes were selected.

truncated, non-expressed portion of *POL3*. Then we deleted chromosomal *MSH6* in these strains as described above, and crossed to obtain diploids. Finally, we selected for cells which had lost the p170 plasmid from both chromosomes simultaneously on medium containing 5-FOA, and used Sanger sequencing to find clones homozygous for the *pol3-D520V* mutation, now present in the full-length, expressed alleles. Isogenic single-mutant diploids (*pol3-D520V*/*pol3-D520V* or *msh6*Δ/*msh6*Δ) and wild-type controls were constructed similarly, omitting the *MSH6* disruption step, the p170-*pol3-D520V* transformation step, or both.

Mutation rate measurements

The rate of *ura3-29* reversion, *ura3-24* reversion, *CAN1* forward mutation, and *his7-2* reversion in haploids was measured by fluctuation analysis as described previously (135). *ura3-29* and *ura3-24* reversion score single point mutations in a TCT sequence context, *CAN1* scores a variety of base substitutions, insertions, and deletions in many sequence contexts, and *his7-2* scores +1 frameshift mutations in an A7 run (58,128,133,136). For each strain, nine single colonies were inoculated separately into rich yeast extract peptone dextrose liquid medium supplemented with uracil and adenine (YPDAU) (135), and the cultures were grown to saturation overnight. The cultures were appropriately diluted and plated on synthetic complete (SC) medium or selective medium. SC medium lacking uracil or histidine was used as selective medium for Ura^+ and His^+ reversion measurements. For Ura^+ reversion, the cells were washed with sterile H_2O before dilution. SC medium containing 0.006% L-canavanine and lacking arginine was used for *CAN1* mutation measurements in haploids, whereas SC medium containing 0.006% L-canavanine and lacking both arginine and leucine was used for *CAN1* mutation measurements in diploids generated from crossing derivatives of TM30

and TM44. 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 the Drake equation (137). Medians and 95% confidence intervals (138) are reported, and comparison between mutation rates of different strains was done using the Wilcoxon-Mann-Whitney test.

In vitro error specificity assay

DNA substrates and proteins

Substrates for primer extension assays were prepared by annealing primer P1 (5'-Cy5-ATTTGACTGTATTACCAATGTCAGCAAATTTTCTGTCTTCGAAGAGTAAA) to template BT1 (5'-Bio-AAGGCATTATCCGCCAAGTACAATTCTTTACTCTTCGAAGACAGAAAATTTGCTGACATTGGTAATACAGTCAAATTGCAGTACTCTGCGGGTGTATACAG-Bio) and primer P2 (5'-Cy5-CATGGAGGGCACAGTTAAGCCGCTAAAGGCATTATCCGCCAAGTACAATT) to template BT2 (5'-Bio-AAATTTTCTGTCTTCGAAGAGTAAAGAATTGTACTTGGCGGATAATGCCTTTAGCGGCTTAACTGTGCCCTCCATGGAAAAATCAGTCAAGATATCCACAT-Bio). Primer and template were combined in a ratio of 1:1.5 in the presence of 150 mM NaAc and 20mM Hepes (pH 7.8), and annealed by incubating the mixture at 95˚C for 3 min and then cooling to room temperature slowly over approximately 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 and allow stable loading of the clamp Proliferating Cell Nuclear Antigen (PCNA) by the clamp loader Replication Factor C (RFC).

Preparations of four-subunit Polε (exo$^-$ and P301R) and PCNA used in this work have been described (28,134). Purified yeast RFC was kindly provided by Peter Burgers (Washington University School of Medicine).

Primer extension assay

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 those measured for wild-type yeast strains to mimic intracellular conditions (30 µM dCTP, 80 µM dTTP, 38 µM dATP, and 26 µM dGTP) (134). PCNA was first loaded onto templates by RFC for 5 min at 30˚C before 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 2x loading buffer containing 95% formamide, 100 mM EDTA and 0.025% Orange G. Samples were boiled for 5 minutes, 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 1x TBE. Quantification of fluorescent products was carried out on a Typhoon imaging system (GE Healthcare). Averages of three separate runs were compared using an unpaired t-test.

Determination of *ura3-29* reversion specificity

Single colonies of *ura3-29* strains containing either the *pol2-4* or *pol2-P301R* mutation were inoculated into YPDAU and the cultures were grown to stationary phase overnight. The cultures were washed in sterile H_2O, diluted and plated on SC medium lacking uracil. The plates were incubated for 5 days at 30˚C and a single Ura$^+$ revertant colony from each culture was randomly picked for DNA isolation. A fragment including

122 nucleotides upstream of *URA3* and nucleotides 1-721 of the *URA3* open reading frame was amplified using primers 5’-GGAAGGAGCACAGACTTAGATT-3’ and 5’-CCTTTGCAAATAGTCCTCTTCC-3'. The products were purified and Sanger sequencing was done with primer 5’-GTTAGTTGAAGCATTAGGTCC-3’.

Plasmid loss assay

To determine whether yeast strains could survive without a plasmid expressing wild-type *POL3*, diploid strains harboring pBL304 were grown in YPDAU to saturation and then serially diluted in a sterile 96-well plate. A 48-pronged replicator was used to transfer diluted cultures to SC medium, or medium containing 5-FOA to select against the pBL304 plasmid. The ability to survive without wild-type *POL3* was determined by comparing growth on SC *versus* growth on 5-FOA medium.

Chapter 3: Extrinsic proofreading of Polε errors by Polδ

Introduction and rationale

As described in Chapter 1, the most widely-accepted model for eukaryotic DNA replication suggests that Polα-primase synthesizes short RNA-DNA primers at replication origins and the beginning of Okazaki fragments on the lagging strand, Polε synthesizes the leading strand and Polδ synthesizes the lagging strand. An abundance of evidence from genetic and biochemical studies supports this model. In contradiction to this model, however, Polδ fidelity defects have long been known to have a greater impact on mutagenesis than analogous Polε defects. The mutator phenotype resulting from *pol3-01*, which encodes proofreading-deficient Polδ (Polδ-exo$^-$), is an order of magnitude stronger than the phenotype of the analogous *pol2-4* mutation, which encodes proofreading-deficient Polε (Polε-exo$^-$) (52,53,58,59,130-132,139-141). Furthermore, haploid yeast deficient in Polδ proofreading do not survive when MMR is also inactivated, with the death attributed to an excessive level of mutagenesis (142). In contrast, yeast lacking both proofreading by Polε and MMR are viable, and while the mutation rate in these strains is high, it does not reach the lethal threshold (29,59,130,132,141). 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 (143). 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 (58,144-146), 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 (130). The synergy implies that the exonucleases of Polε and

Polδ 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 Polα could be corrected by the ε subunit of *E. coli* DNA polymerase III or by Polδ (147,148). Several mammalian autonomous exonucleases have also been shown to increase the fidelity of Polα *in vitro* (149-151). Both *E. coli* and eukaryotic replicative polymerases can excise nucleotides incorporated by translesion synthesis polymerases at sites of DNA damage (152,153). In respect to the extrinsic proofreading capabilities of Polδ and Polε *in vivo*, several studies have been illuminating. Polδ but not Polε has been shown to proofread errors made by an error-prone Polα variant in yeast (74). Further, Polδ exonuclease defects are almost completely recessive indicating that wild-type Polδ can efficiently proofread errors created by Polδ-exo$^-$ (53,142,146). On the other hand, the mutant allele encoding Polε-exo$^-$ is semidominant, suggesting that wild-type Polε does not correct errors *in trans* (118,146). Experiments employing transformation of yeast cells with oligonucleotides that, when annealed, create a 3'-terminal mismatch also showed that Polδ but not Polε can proofread *in trans* (146). These experiments further showed that the exonuclease of Polδ can act on oligonucleotides annealed to both leading and lagging strands (146). 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. The results show that Polδ can correct errors made by Polε, but Polε cannot correct errors made by Polδ. This observation provides direct evidence that the remarkably mild *in vivo* consequences of severe Polε 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.

The *pol3-D520V* mutation as a tool to study Polδ proofreading

The synergistic interaction between the exonucleases of Polε and Polδ has been previously demonstrated using the *pol2-4* and *pol3-01* alleles, which result in the replacement of two catalytic carboxylates in the Exo I motif of the respective polymerase with alanines (FDIET/C→FAIAT/C; (130)). 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 S-phase checkpoint (154), and a different allele, *pol3-D520V*, exists that also eliminates the exonuclease activity but is a weaker mutator (54). We started by verifying that the synergy between Polε 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 (130), the *pol2-4 pol3-D520V* haploids survived (**Figure 3.1**). The mutation rate in the *pol2-4 pol3-D520V* strains increased synergistically as compared with the single *pol2-4* and *pol3-D520V* mutants (**Table 3.1**), consistent with the idea that the exonucleases of Polδ and Polε act on same pool of replication errors. We next

pol2-4 x *pol3-D520V*

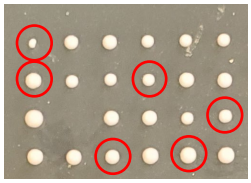


Figure 3.1. *pol2-4 pol3-D520V* haploid yeast are viable.

Tetrad analysis of yeast strains heterozygous for the *pol3-D520V* and *pol2-4* alleles, encoding exonuclease-deficient Polδ and Polε, respectively. Red circles indicate double mutant haploid spores.

Table 3.1. Synergistic interaction of *pol2-4* and *pol3-D520V*

Genotype	*CAN1* mutation			*his7-2* reversion		
	Mutation rate ($x10^{-7}$)		Fold increase	Mutation rate ($x10^{-8}$)		Fold increase
POL2 POL3	2.5	(2.1-2.9)	1	0.83	(0.70-0.97)	1
pol2-4 POL3	7.6	(6.8-8.7)	3.0	6.3	(5.6-6.9)	7.6
POL2 pol3-D520V	19	(16-21)	7.6	8.0	(7.0-9.6)	9.6
pol2-4 pol3-D520V	200	(130-260)	80	100	(61-290)	120

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

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 (57); 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 (155), which is the predominant type of replication errors generated by exonuclease-deficient Polδ and Polε (28,49,50). Diploids homozygous for both *pol3-D520V* and *msh6*Δ mutations showed a strong synergistic increase in mutation rate as compared with the single *pol3-D520V* and *msh6*Δ mutants (**Table 3.2**). Similar synergistic increase in mutagenesis in *pol3-D520V*/*pol3-D520V* *msh6*Δ/*msh6*Δ diploids was observed in an earlier study that scored base substitutions at a single nucleotide position in the *TRP5* gene (146). 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 Polε and Polδ. It also shows that *pol3-D520V* allele provides an adequate model for the extrinsic proofreading studies described below.

Table 3.2. Synergistic interaction of *pol3-D520V* and MMR deficiency

Genotype	*CAN1* mutation		
	Mutation rate ($x10^{-7}$)		Fold increase
POL3/POL3 MSH6/MSH6	3.4	(3.0-4.0)	1
POL3/POL3 msh6Δ/msh6Δ	31	(28-36)	9.1
pol3-D520V/pol3-D520V MSH6/MSH6	46	(35-69)	14
pol3-D520V/pol3-D520V msh6Δ/msh6Δ	4400	(3100-6000)	1300

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

Interplay of Polε and Polδ in replication error avoidance

Polδ proofreads errors made by Polε

Next, we investigated whether Polδ proofreads errors made by Polε by combining a nucleotide selectivity defect in Polε (*pol2-M644G*) with a proofreading defect in Polδ (*pol3-D520V*). The *pol2-M644G* confers a change in the polymerase domain of Polε, which causes promiscuity during nucleotide incorporation without compromising proofreading (46). The *pol2-M644G* strains, therefore, accumulate a high number of Polε-specific errors. We observed a synergistic increase in mutation rate in the double *pol2-M644G pol3-D520V* mutants **(Table 3.3)**. This synergy indicates that the nucleotide selectivity of Polε and the proofreading activity of Polδ act consecutively to prevent replication errors and, thus, Polδ proofreads errors made by Polε *in vivo*.

Polε does not proofread errors made by Polδ

In a reciprocal experiment, we combined a Polδ nucleotide selectivity defect (*pol3-L612M*) with a Polε proofreading defect (*pol2-4*) to determine whether Polε can proofread errors made by Polδ. Similar to *pol2-M644G*, *pol3-L612M* increases the rate of nucleotide misincorporation by Polδ without impacting exonuclease activity (45). 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 3.4**). The additive interaction indicates that Polδ nucleotide selectivity and Polε exonuclease activity act in parallel, non-overlapping pathways, and, therefore, Polε does not proofread errors made by Polδ.

Table 3.3. Synergistic interaction of Polε nucleotide selectivity and Polδ proofreading defects

Genotype	*CAN1* mutation			*his7-2* reversion		
	Mutation rate ($x10^{-7}$)		Fold increase	Mutation rate ($x10^{-8}$)		Fold increase
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. 95% confidence intervals are shown in parentheses.

Table 3.4. Additive interaction of Polδ nucleotide selectivity and Polε proofreading defects

Genotype	*CAN1* mutation			*his7-2* reversion		
	Mutation rate ($x10^{-7}$)		Fold increase	Mutation rate ($x10^{-8}$)		Fold increase
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. 95% confidence intervals are shown in parentheses.

Discussion

The accepted model for eukaryotic DNA replication is not easily reconciled with the stronger mutator effects of Polδ variants in comparison with analogous Polε variants. It has been proposed that Polδ can proofread errors made by Polε 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 Polε can readily proofread errors in trans (53,74,118,142,146). However, evidence that Polδ can specifically proofread DNA synthesized by Polε at the replication fork has been lacking. Using inaccurate variants of Polδ and Polε, here we demonstrate that incorrect nucleotides incorporated by Polε are efficiently removed by the exonuclease of Polδ but Polε cannot remove nucleotides misincorporated by Polδ (**Figure 3.2**). This conclusion is supported by two observations. (i) The mutation rate increases synergistically when the Polε nucleotide selectivity defect is combined with Polδ proofreading defect. (ii) Only an additive increase in mutagenesis is observed when Polδ nucleotide selectivity defect is combined with Polε proofreading defect.

Multiple studies suggested that Polδ is more efficient at extrinsic proofreading than Polε. Polδ can remove mismatches generated by Polα both *in vitro* and *in vivo* (74,148). Since Okazaki fragments are all initiated by exonuclease-deficient Polα, there is a clear need for extrinsic proofreading by the lagging strand polymerase, whereas there is less of a need for Polε to carry this out on the leading strand. Indeed, Polε does not appear to correct errors made by Polα *in vivo* (74). It is particularly interesting to note the recent evidence that initial leading strand synthesis is performed by Polδ (76-78), which further diminishes the need for extrinsic proofreading of Polα-generated errors by Polε on the leading strand. Additionally, the semidominance of the *pol2-4* mutation and almost complete dominance of *POL3* over the *pol3-01* and *pol3-D520V* mutations

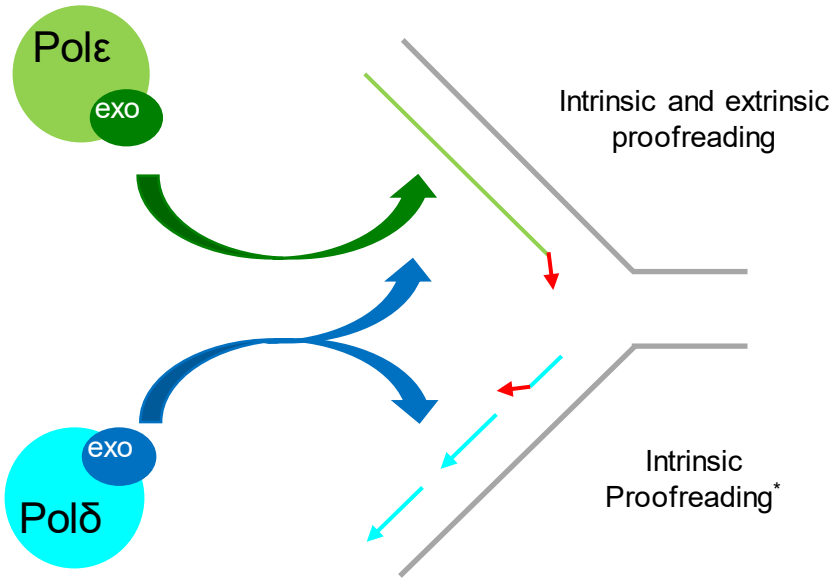


Figure 3.2. Interplay of Polε and Polδ proofreading and synthesis activities at the replication fork.

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.
*The intrinsic and extrinsic proofreading shown in the model relates specifically to the interplay between Polε and Polδ. There also exists evidence to suggest Polδ can carry out extrinsic proofreading of Polα and Polδ errors on the lagging strand as well.

demonstrates that only Polδ can remove errors inserted by a different polymerase molecule (53,118,142,146). The removal of 3' terminal mismatches during oligonucleotide-mediated transformation by Polδ but not Polε (146) also suggests that Polδ is much better suited to extrinsic proofreading than Polε. Finally, this study provides evidence that Polδ proofreads errors made by Polε *in vivo*, while Polε cannot proofread for Polδ.

Thus, the competition of Polδ and Polε exonucleases for correcting the same pool of replication errors originally demonstrated by Morrison and Sugino in the 1990s (130) is apparently one-sided. Perhaps the different properties and regulatory mechanisms of the two polymerases leave them appropriately suited to their own specialized roles. Polε is a component of the replication initiation complex, where it associates with origins during the G1/S phase transition (156,157). Polε remains bound to the moving helicase via the C-terminus of its catalytic subunit, Pol2, as the N-terminus copies the leading strand (7,46). A flexible region between the two halves of Pol2 could allow the polymerase to dissociate from the DNA while remaining bound to the replication machinery upon dissociation of accessory subunits (18,19). This association with the helicase indicates that Polε 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 re-association of Polδ with the primer terminus occurs routinely during lagging strand synthesis, and Polδ is loaded much faster than Polε onto PCNA-primer-template junction (158). Thus, the high efficiency of Polδ at correcting errors made by Polε may result from a combination of two factors: the high proclivity of Polε to yield to another polymerase, and the greater flexibility and robustness of Polδ when associating with new primer termini.

Chapter 4: Strand specificity of Polε-P301R errors

Introduction and rationale

As described in Chapter 1, the mutation conferring P286R substitution in human *POLE* is the most common *POLE* mutation observed in cancers (61,95,96). Initial genetic analysis in yeast demonstrated a strong increase in mutation rate for cells with the *pol2-P301R* mutation (118). Recent structural and biochemical analysis of the purified yeast variant Polε-P301R indicated that the arginine substitution blocks the primer terminus from accessing the exonuclease active site, increasing polymerase activity (28,124). The model proposed by these studies suggested that the increased polymerase activity causes a strong propensity to extend from mismatches, leading to the high mutation rate in these strains. The experiments described in this chapter were designed to test if the increased polymerase activity affected whether Polε-P301R replicated the leading strand. A genetic system was designed to measure strand-specific mutation accumulation across a well-defined replicon in yeast, and the results presented in this chapter demonstrate that Polε-P301R is strictly a leading strand replicase.

A genetic system to study strand-specific replication errors

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 *Saccharomyces cerevisiae* (12). 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 (12). 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 4.1a**). 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 4.1b**, left) (58,143). 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 4.1b**, 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 (28,159), in line with the mutational specificity of *POLE* mutant tumors (71,82,106). 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 4.2** and **Table 4.1**). 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 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 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 (45,160). 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 transcribed or non-transcribed strand of the

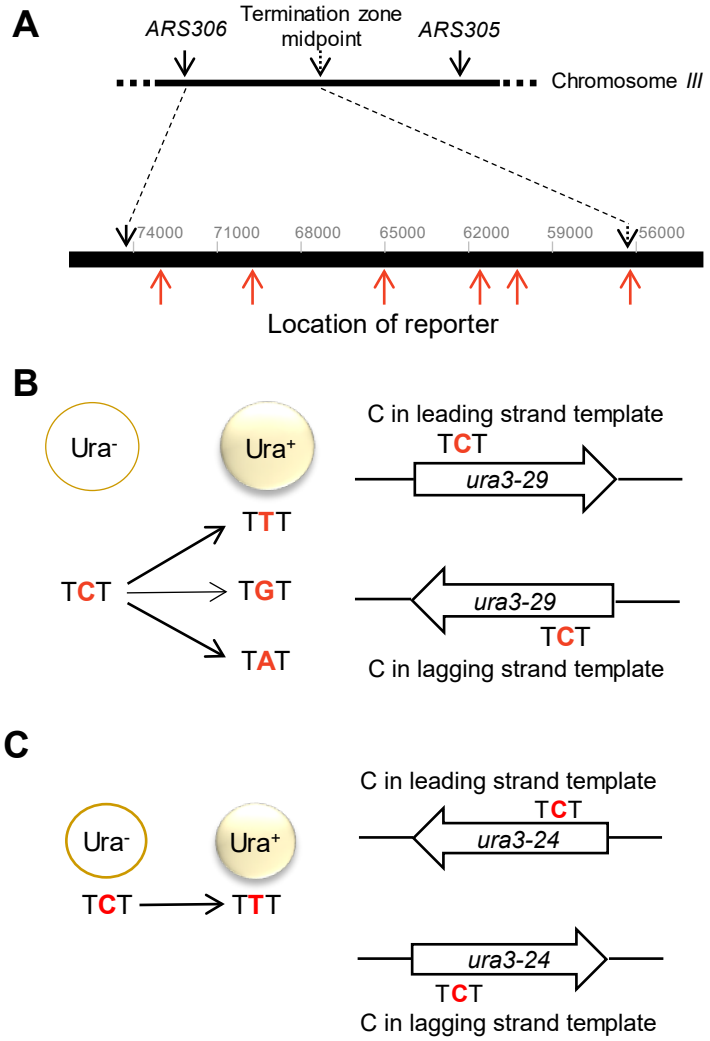


Figure 4.1. A genetic system for analysis of mutagenesis across a replicon.

A. A reversion reporter was placed at six locations between *ARS306* and the nearest replication termination zone. Grey 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 (58,143)**.** 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 template.

C. *ura3-24* strains cannot grow on medium lacking uracil and revert to Ura$^+$ phenotype via C→T transitions in a TCT sequence context. This is the same sequence context as in *ura3-29* but positioned in the opposite strand in respect to the direction of transcription (58). The *ura3-24* reporter was also placed in two orientations at each location shown in (**A**).

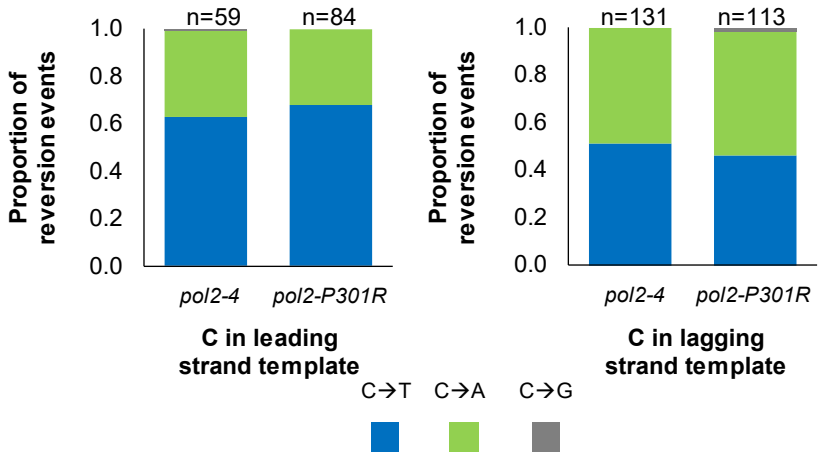


Figure 4.2. Reversion specificity of *ura3-29* in *pol2*-mutant strains.

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 to 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 **Table 4.1**.

Table 4.1. *ura3-29* reversion specificity.

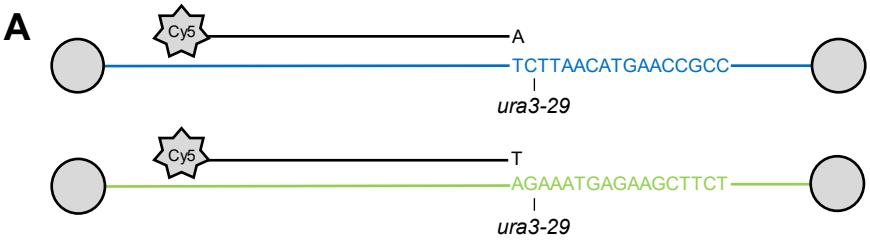
Reporter position	C in leading or lagging strand	*pol2-4*			*pol2-P301R*		
		C→T	C→A	C→G	C→T	C→A	C→G
hbn1	leading	8	4	1	5	14	0
hbn1	lagging	8	8	0	10	16	1
bik1	leading	10	6	0	5	4	0
bik1	lagging	0	3	0	0	11	0
his4	leading	0	5	0	9	9	0
his4	lagging	12	0	0	6	2	1
ste50	leading	7	2	0	18	16	0
ste50	lagging	2	5	0	1	4	0
lsb5	leading	0	0	0	10	17	0
lsb5	lagging	10	6	0	10	17	0
atg22	leading	12	4	0	20	4	0
atg22	lagging	25	5	0	10	9	0

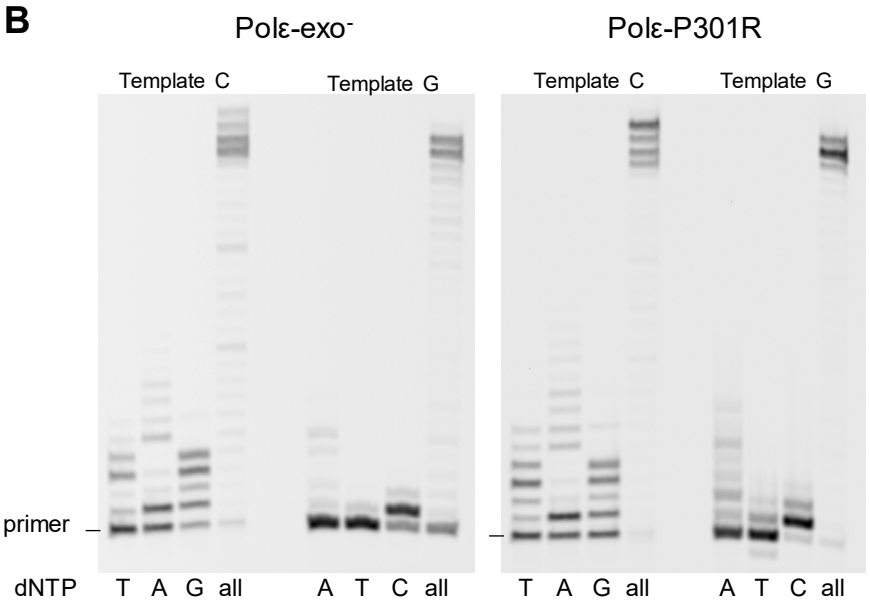
Number of reversions of each type observed at each position of the *ura3-29* reporter.

ura3-29 as a template (template G or template C, respectively; **Figure 4.3a**). The templates contained streptavidin bumpers on each end to allow stable loading of PCNA by RFC. Primers were positioned such that the first nucleotide incorporated would be at the site of the *ura3-29* mutation. Both Polε variants generated transition- and transversion-type mispairs significantly more efficiently when C was the templating base (**Figure 4.3b,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.

Strand specificity of 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 4.4a**). 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 (**Figure 4.1c**). 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 4.1b** and **c**). 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 (**Figure 4.5a**). 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 (**Figure 4.5b**). Neither *ura3-29* nor *ura3-24* reversion showed a bias in strains with wild-type Polε (**Figure 4.6**). These results are consistent with the replication


A
Cy5
A
TCTTAACATGAACCGCC
ura3-29
Cy5
T
AGAAATGAGAAGCTTCT
ura3-29


B
Polε-exo-
Polε-P301R
Template C
Template G
Template C
Template G
primer
dNTP T A G all A T C all T A G all A T C all

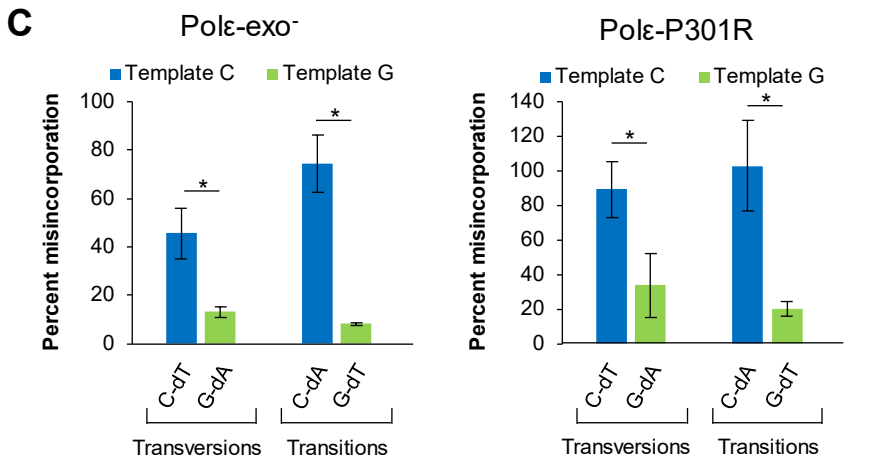

C
Polε-exo-
Polε-P301R
Template C
Template G
Percent misincorporation
100
80
60
40
20
0
140
120
C-dT
G-dA
C-dA
G-dT
Transversions
Transitions
*

Figure 4.3. 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. 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. Asterisks indicate $p<0.05$ by t-test.

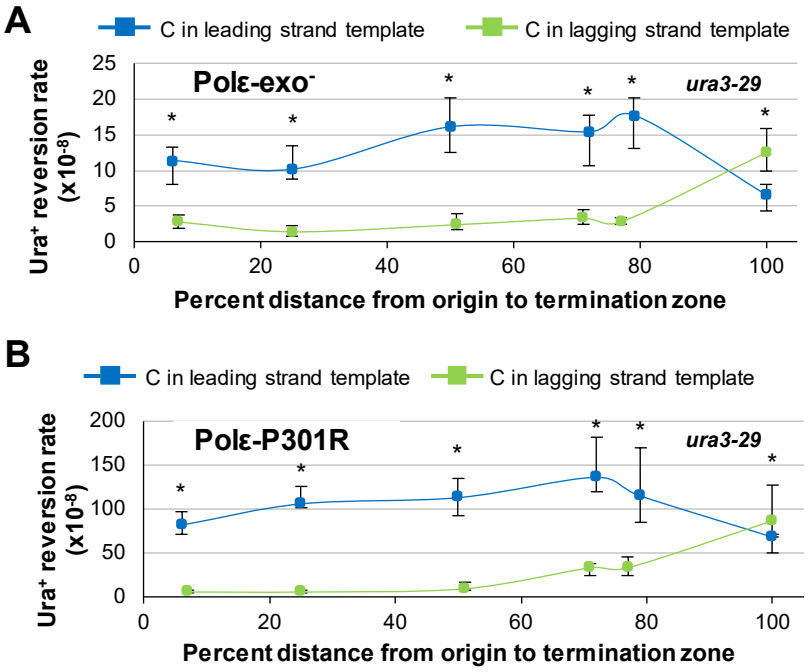


Figure 4.4. Polε-P301R, like Polε-exo-, is a dedicated leading strand polymerase.

The rate of Ura+ reversion in *pol2-4* (**A**) and *pol2-P301R* (**B**) strains with the different locations of *ura3-29* reporter shows the bias toward errors at leading strand cytosines. Data are medians for at least 18 cultures from two to six independent clones. Error bars represent 95% confidence intervals and asterisks indicate $p<0.05$ by Wilcoxon-Mann-Whitney test.

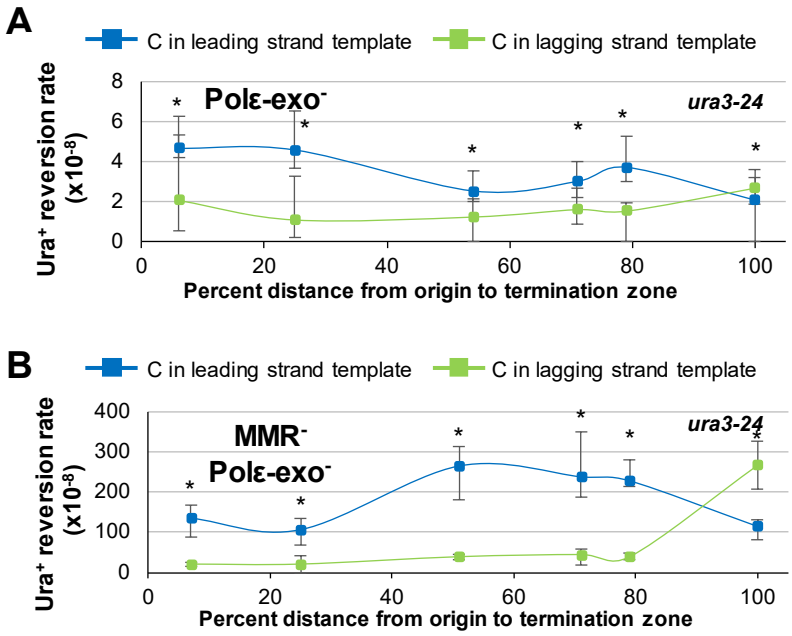


Figure 4.5. The higher mutability of leading strand cytosines in *pol2-4* strains is observed regardless of their position in the transcribed *vs.* non-transcribed strand, and regardless of MMR activity.

The rate of Ura$^+$ reversion in *pol2-4* (**A**) and *pol2-4 msh6*Δ*::kanMX* (**B**) strains with the different locations of *ura3-24* reporter shows the bias toward errors at leading strand cytosines. Data are medians for at least 18 cultures from two to six independent clones. Error bars represent 95% confidence intervals and asterisks indicate $p<0.05$ by Wilcoxon-Mann-Whitney test.

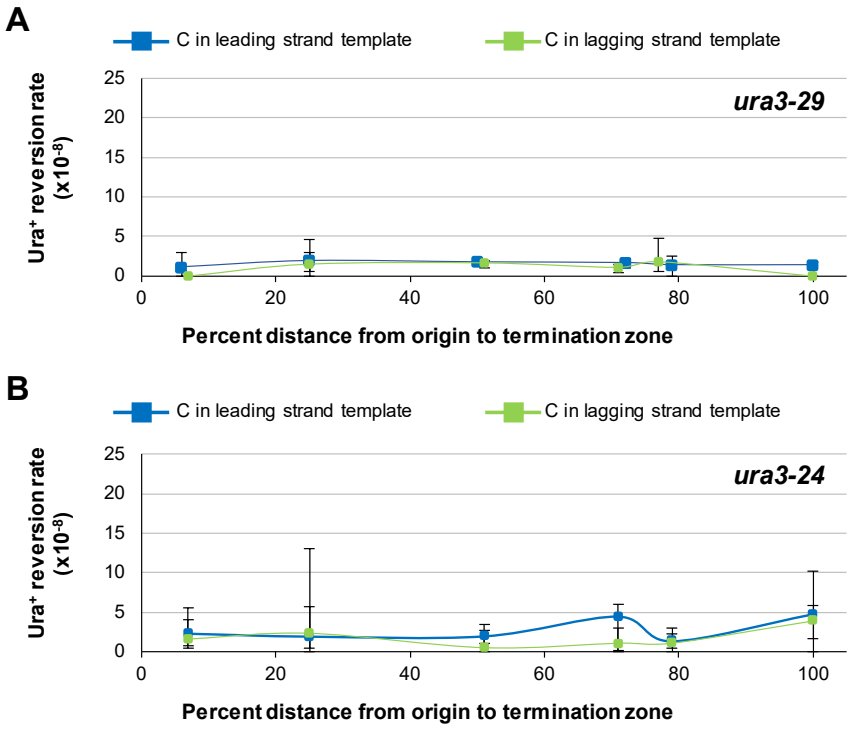


Figure 2.6. The rate of Ura$^+$ reversion in wild-type (*POL2*$^+$) strains is similar for the two orientations of *ura3-29* or *ura3-24* alleles.

Data are medians for at least 18 cultures from two to six independent clones. Error bars represent 95% confidence intervals and asterisks indicate $p<0.05$ by Wilcoxon-Mann-Whitney test.

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 4.4b**). 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 (28), Polε-P301R remains a strict leading strand polymerase.

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 (28,118,120). Here we used the yeast model to assess the impact of this variant on the strand-specificity of Polε in DNA replication. The results presented in this chapter demonstrate that, despite the dramatic change in biochemical properties, Polε-P301R remains a dedicated leading strand replicase.

The assay for the detection of leading and lagging strand errors developed in this chapter provided new information on the mechanism of DNA replication in *S. cerevisiae*. The currently accepted fork model, originally proposed by the Sugino group (14), 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 yeast strains with reduced fidelity of Polε or Polδ (46,58,69-73). 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 [(46,58,69,130); discussed further in (144)]. Subsequent genome-wide studies of mutation and ribonucleotide incorporation in Polε and Polδ mutants extended the division-of-labor model to multiple replicons (70,72,73). 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 (70,72,73). 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 4.4**), demonstrating that the majority of leading strand synthesis is completed by Polε from origin to termination zone. Recently published data mapping ribonucleotide incorporation by mutator Polε and Polδ variants revealed less synthesis by Polε and more synthesis by Polδ at termination zones (≤10 kb from the termination zone midpoint) than expected from the one-strand-one-polymerase model (79). 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 (less than 1 kb from the midpoint). A slight decrease in Polε synthesis in the 10-kb segment, however, may not be detected in our experiments.

Chapter 5: Extrinsic correction of Polε-P301R errors

Introduction and rationale

As described in Chapter 1, nucleotide selectivity, proofreading, and MMR act in series. The results presented in Chapter 3 demonstrated that extrinsic proofreading of Polε-generated errors by Polδ is an additional proofreading mechanism by which replication errors are minimized. Because of the hyperactivity and extraordinary mismatch extension ability of the purified Polε-P301R (28), and the extreme levels of *in vivo* mutagenesis (118), we designed experiments to probe the extent of error correction by MMR and extrinsic proofreading by Polδ in *pol2-P301R S. cerevisiae* strains. The results show that MMR and extrinsic proofreading by Polδ are both required to maintain viability of cells that carry Polε-P301R as the sole source of Polε. Additionally, lack of Polδ proofreading or MMR in diploid strains heterozygous for the *pol2-P301R* mutation leads to near-lethal levels of mutagenesis. We conclude that MMR and Polδ proofreading efficiently correct Polε-P301R errors to prevent catastrophic accumulation of leading strand errors in *pol2-P301R* strains.

Proofreading of Polε-P301R errors by Polδ

Prior studies have shown that Polδ can proofread errors made by Polα and Polε [(74,161) and Chapter 3]. 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. As discussed in Chapter 3, the *pol3-D520V* mutation results in D520V substitution in the conserved Exo III motif and a severe reduction in the exonuclease activity of Polδ (54). A combination of *pol3-D520V*

and *pol2-4* mutations results in a strong synergistic increase in mutation rate in both haploid and diploids, as expected from previous studies and consistent with Polδ proofreading errors made by Polε [(161);**Table 3.1; Table 5.1**]. Experiments described in Chapter 3 and reference (161) demonstrated 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. To study the genetic interaction of the *pol3-D520V* mutation with *pol2-P301R*, we first attempted to combine the mutations by crossing single *pol3-D520V* and *pol2-P301R* mutants and sporulating heterozygous diploids. This procedure yielded no viable double mutant spores (**Figure 5.1a**). The inviable spores formed microcolonies before cell division stopped (**Figure 5.1b**). This phenotype is characteristic of a replication error catastrophe (142). To test this hypothesis, we sought approaches to determine whether the combination of *pol2-P301R* and *pol3-D520V* 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 (57,59,130,142). 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 5.1c**). 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. Indeed, the levels of mutagenesis in diploids homozygous for the *pol2-P301R* alone already approach the viability threshold for diploid cells (118,162), and further increase due to the loss of proofreading by Polδ may be fatal.

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*. Heterozygosity for *pol2-P301R*

Table 5.1. Synergistic interaction of heterozygosity for *pol2-4* with Polδ proofreading deficiency

Genotype	*CAN1* mutation		*his7-2* reversion	
	Mutation rate ($x10^{-7}$)	Fold increase	Mutation rate ($x10^{-8}$)	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/pol2-4 POL3/POL3	5.5 (4.6-6.6)	1.6	4.5 (3.9-6.1)	4.1
POL2/pol2-4 pol3-D520V/pol3-D520V	130 (100-160)	39	70 (58-93)	64

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

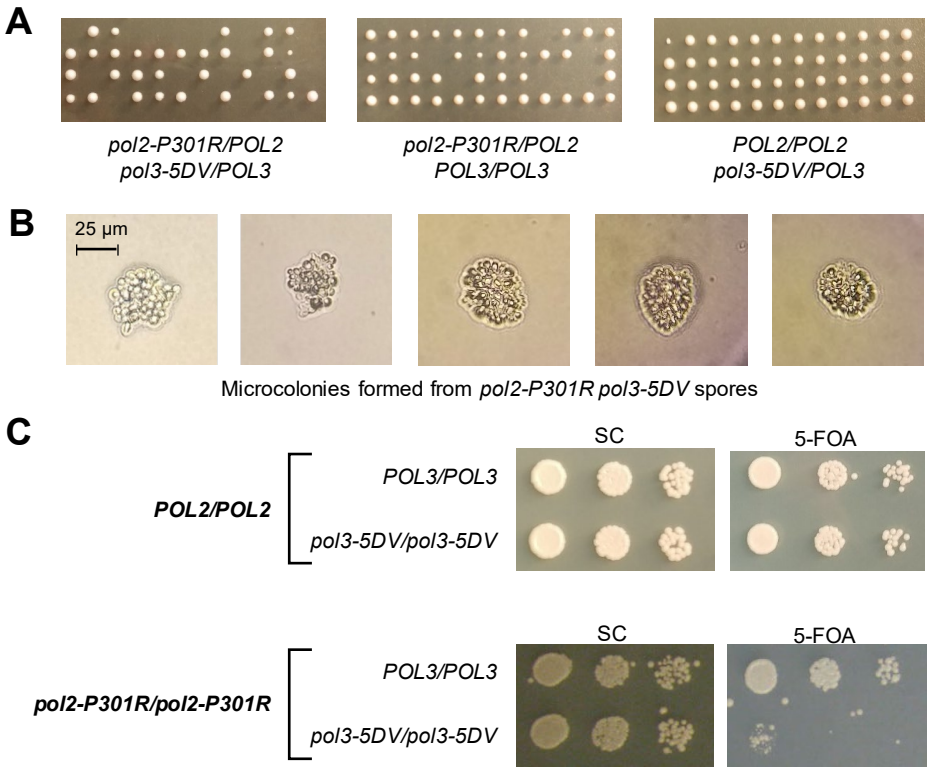


Figure 5.1. *pol2-P301R* mutants require functional Polδ proofreading for viability.

A. Tetrad analysis of yeast strains heterozygous for the *pol3-D520V* 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.

produces a rather strong mutator phenotype (118). Thus, we used these strains to assess the effect of the combination of *pol3-D520V* and *pol2-P301R* on mutagenesis. We measured the mutation rate at two reporter loci, *CAN1* and *his7-2*. All diploid strains used in this chapter contain the *CAN1::Kl.LEU2/can1*Δ configuration described in Chapter 2. We observed a strong synergistic increase in both *CAN1* mutation and *his7-2* reversion in the double mutant strains (**Table 5.2**), indicating that Polδ proofreading removes a majority of Polε-P301R errors *in vivo*.

Correction of Polε-P301R errors by DNA mismatch repair

Haploid *pol2-P301R msh6*Δ strains are inviable, but the double mutant cells can divide and form microcolonies before the growth stops (28). Like the inviability of *pol2-P301R pol3-D520V* haploids, this phenotype is characteristic of death from excessive levels of mutagenesis. It suggests that the number of mismatches generated by Polε-P301R is overwhelming, even after extrinsic proofreading by Polδ, and Msh6-dependent MMR is required to keep the mutation rate below the lethal threshold. We attempted to construct diploid strains homozygous for both *pol2-P301R* and *msh6*Δ mutations, but were unsuccessful, which suggested that the mutation rate was too high even for diploid cells. 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 the haploid state (28,59,145) as *pol2-4* is a much weaker mutator.

Diploids heterozygous for the *pol2-P301R* mutation and homozygous for *msh6*Δ, however, were viable. The combination of heterozygosity for *pol2-P301R* with homozygosity for *msh6*Δ resulted in a synergistic increase in mutation rate for both the

Table 5.2. Synergistic interaction of *pol2-P301R* and Polδ proofreading deficiency

Genotype	*CAN1* mutation		*his7-2* reversion	
	Mutation rate ($x10^{-7}$)	Fold increase	Mutation rate ($x10^{-8}$)	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.

CAN1 and *his7-2* reporters (**Table 5.3**). This demonstrates that MMR removes a majority of Polε-P301R errors missed by proofreading and 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 (**Table 5.4**), in line with the synergy between *pol2-4* and *msh6*Δ in haploids (28,59,145). However, the absolute mutation rate in *pol2-P301R/POL2 msh6*Δ/*msh6*Δ diploids is an order of magnitude higher than *pol2-4/pol2-4 msh6*Δ/*msh6*Δ diploids, once again illustrating the unprecedented level of replication errors generated by Polε-P301R *in vivo.*

Discussion

The results presented in this chapter derive from experiments using the yeast model to assess the impact of the P301R substitution in Polε on extrinsic error correction systems. 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.

Studies of the Polε-P301R variant described here uncover the remarkable efficiency at which extrinsic proofreading by Polδ operates to correct Polε errors. The data presented in Chapter 3 demonstrated that the exonuclease of Polδ readily proofreads errors made by Polε-exo$^-$ and another inaccurate Polε variant, Polε-M644G. This extrinsic correction must involve dissociation of Polε from the primer terminus to

Table 5.3. Synergistic interaction of *pol2-P301R* and MMR deficiency

Genotype	*CAN1* mutation		*his7-2* reversion	
	Mutation rate ($x10^{-7}$)	Fold increase	Mutation rate ($x10^{-8}$)	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.

Table 5.4. Synergistic interaction of heterozygosity for *pol2-4* with MMR deficiency

Genotype	*CAN1* mutation		*his7-2* reversion	
	Mutation rate ($x10^{-7}$)	Fold increase	Mutation rate ($x10^{-8}$)	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/*pol2-4 MSH6/MSH6*	5.5 (4.6-6.6)	1.6	4.5 (3.9-6.1)	4.1
POL2/pol2-4 msh6Δ/msh6Δ	450 (390-530)	130	36 (29-42)	33

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

allow Polδ access to the mismatch. The dissociation is presumably facilitated 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 (28). Structural studies showed that the arginine side chain protrudes into the space normally occupied by the 3'-terminal nucleotide in the exonuclease active site (124). The inability of Polε-P301R to accommodate the primer terminus in the exonuclease site was proposed to not only dramatically reduce exonuclease activity, but also to prompt Polε-P301R to stay in the polymerization mode, resulting in increased polymerase activity, mismatch extension, and ultimately an unprecedented mutator effect (28). The discovery that a majority of errors generated by Polε-P301R are proofread by the exonuclease of Polδ was, therefore, surprising. The strong synergistic interaction of *pol2-P301R* and *pol3-D520V* mutations (**Table 5.2**) 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. 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. 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 (76-78). On the other hand, intramolecular switching from the exonuclease to the polymerase active site has been suggested for Polδ (57). Intramolecular switching between active sites has also

been demonstrated for bacteriophage RB69 and T4 DNA polymerases, as well as for the eukaryotic Polε (56,163,164). The results presented in Chapter 4 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.

Chapter 6: Discussion, conclusions, and future directions

Discussion

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 (165). Multiple translesion synthesis polymerases provide redundant mechanisms of lesion bypass (166,167). 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 (168). A recent example is the inclusion of nucleoside analog 5-NIdR, an inhibitor of translesion synthesis, with temozolomide in treatment of homologous-recombination-impaired tumors to promote cancer cell death (169,170).

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 (162), indicating that proofreading normally compensates for reduced nucleotide selectivity. Haploid yeast deficient in Polδ proofreading require functional MMR for survival (142). 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 (171,172). Extrinsic proofreading of Polε errors by Polδ shown in Chapter 3, as well as proofreading of Polα

errors by Polδ shown previously (74) is yet another mechanism of redundancy to prevent accumulation of DNA replication errors.

Implications for the etiology of *POLE*-mutant tumors

The studies presented in this dissertation have implications for human cancer biology. Mutations in the *POLE* gene, which encodes the catalytic subunit of Polε in humans, are found in sporadic colorectal and endometrial tumors (61). *POLE*-mutant tumors have the highest mutation load across different cancer types [>100 mutations per Mb; (90,91,105)]. The *POLE* mutations predominantly affect the exonuclease domain of Polε and cause strong mutator and cancer susceptibility phenotype in model systems (118,120,173). Although MMR defects are also common in colorectal and endometrial tumors, strong *POLE* 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. 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 (61), these *POLE* alleles confer only a weak mutator effect in functional assays (173). Brain tumors in children with biallelic MMR deficiency often contain *POLE* mutations (101,174), 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* 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* (81). 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 (90,91). The second explanation suggested by our finding in yeast (Chapter 5) 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 (162), 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 (61,95) but are still not seen together with MMR defects, a combination that is viable in yeast (Chapter 5). 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. Our results strongly suggest that the corresponding defects in human cells are mutually exclusive because of a catastrophically high mutation rate.

Curiously, mutations affecting the exonuclease domain of Polδ are seen much less frequently than mutations affecting the exonuclease domain of Polε in sporadic

tumors. While never explicitly tested, it is possible that these result in much stronger mutator phenotypes that hamper cell proliferation, and *POLE*-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 type, as well as 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 (119). In MMR-proficient background, a Polδ proofreading defect leads to a significantly earlier onset of cancer than the analogous defect in Polε (119,175,176). These observations are reminiscent of the stronger effects of Polδ mutations in yeast, although dramatic differences in the spectrum of tumors in Polδ *versus* Polε mutant mice preclude accurate comparison of cancer susceptibility. A combination of Polδ and Polε proofreading defects, however, greatly accelerates the development of tumors characteristic of Polδ proofreading deficiency (119), consistent with the idea that tumors in Polδ proofreading-deficient mice result, in part, from Polε errors. Curiously, neither the stronger effects of Polδ exonuclease nor synergy between Polδ and Polε was detected when the mutation rate was measured in fibroblast cell lines derived from the mutant embryos (119). These studies illuminate the complexity of the mammalian developmental and tissue biology, and highlight the importance of investigating possible cooperation of Polδ and Polε exonucleases in cancer-relevant cells and tissues.

Conclusions

The results presented in Chapter 3 of this dissertation established a one-sided extrinsic proofreading mechanism for maintaining DNA replication fidelity. The synergy in mutation rate between a Polε nucleotide selectivity defect and a Polδ proofreading defect indicate that Polδ proofreading acts in series with Polε nucleotide selectivity, establishing that Polδ can and does proofread errors generated by Polε. Furthermore, the additivity in mutation rate observed between a Polδ nucleotide selectivity defect and a Polε proofreading defect indicate that Polδ nucleotide selectivity and Polε proofreading act in independent pathways, establishing that Polε cannot proofread errors generated by Polδ.

In Chapter 4, the genetic system we developed allows for discrimination between leading and lagging strand errors because both Polε-exo$^-$ and Polε-P301R are biased in the formation of reciprocal mispairs. Both polymerase variants are more prone to misinsertion across from template C than from template G, so when the reporter allele has C in the template strand, then the majority of errors made by either Polε-exo$^-$ or Polε-P301R occur during replication of that strand. Our results show that both Polε-exo$^-$ and Polε-P301R synthesize the leading DNA strand from the origin of DNA replication to the termination zone.

The experiments in Chapter 5 analyzed removal of Polε-P301R-generated errors by extrinsic proofreading and MMR. Combining either a Polδ proofreading defect or a MMR defect with *pol2-P301R* in haploid yeast is lethal. Furthermore, diploid yeast homozygous for *pol2-P301R/pol2-P301R* and lacking either MMR or Polδ proofreading also did not survive. When we combined a Polδ proofreading defect with heterozygosity for *pol2-P301R*, we observed a large synergistic increase in mutation rate for two reporters, which demonstrated that Polδ proofreads major portion of Polε-P301R-

generated errors. Similarly, there was a synergistic increase in the mutation rate when a MMR defect was added to strains heterozygous for *pol2-P301R*, indicating that MMR corrects the majority of Polε-P301R-generated errors that remain after extrinsic proofreading by Polδ.

Together, the results presented in this dissertation establish that Polε copies the leading strand from origin to the termination zone during DNA replication, and any errors missed by intrinsic proofreading are subject to correction by the exonuclease of Polδ. Importantly, the experiments presented here demonstrate that two fundamental properties of Polε (strand specificity and correction of its errors by Polδ and MMR) are not lost with the P301R substitution, even though Polε-P301R is a hyperactive enzyme with unrivaled mismatch extension and generates errors at a rate an order of magnitude higher than proofreading-deficient Polε.

Future directions

Does Polε participate in leading strand replication in late replicating regions?

The replicon used in Chapter 4 to measure mutation accumulation between the origin and termination zones occurs between two very efficient and early firing replication origins. Earlier genome-wide studies of ribonucleotide incorporation averaged data from all replicons and did not see a strong bias for leading strand replication by Polε near termination zones. If there is a difference in the organization of the replication fork near termination zones in early and late replicating regions, this could reduce the apparent bias of Polε for the leading strand near termination zones when averaging all replicons. To answer whether Polε participation in leading strand synthesis is maintained during

late replication, the mutation accumulation studies could be repeated in a late-replicating region of the genome. *ARS727* and *ARS728* are two efficient replication origins that fire later in S-phase (12), and thus the termination zone is defined. The *ura3-29* reporter could be inserted at several locations between coordinates 659806 and 715187 on chromosome *VII*, and mutation rates measured in *pol2-4* strains to determine the participation and strand bias of Polε in this late-replicating region.

How does extrinsic proofreading of Polε errors by Polδ vary across the replicon?

The results in Chapter 3 demonstrated that Polδ proofreads Polε-generated errors, and results in Chapter 4 showed a strict leading strand bias of Polε-generated errors. Given that MMR balances differences in fidelity on leading and lagging strands, as well as in early *versus* late replicating regions, the extent of extrinsic proofreading of Polε errors by Polδ may also vary across the replicon. To probe the extent of extrinsic proofreading across the replicon, the mutation accumulation experiments described in Chapter 4 could be adapted to measure the extent of synergy between *pol2-4* and *pol3-D520V* mutations. Measuring *ura3-29* reversion at each of the six locations in strains with *pol2-4* mutation, *pol3-D520V* mutation, or both mutations would give a picture of the extent of extrinsic proofreading at various locations between the origin of replication and termination zone.

Why are so many *pol2-P301R*-generated mutations observed *in vivo*?

Previous studies established that Polε-P301R is not more mutagenic than Polε-exo$^-$ *in vitro*, yet the *in vivo* mutation rate is an order of magnitude higher (28,118). The

increased polymerase activity and mismatch extension capabilities of Polε-P301R lead to the hypothesis that Polε-P301R extends and incorporate mismatches into the genome better than Polε-exo$^-$ (28). The results presented in this dissertation show that Polε-P301R errors are corrected by both Polδ proofreading and MMR, yet the mutation rate in *pol2-P301R* strains lacking MMR or Polδ proofreading is still much higher than isogenic *pol2-4* strains. So, what is the mechanism by which more errors generated by Polε-P301R are incorporated into the genome? One hypothesis is that there is an unknown factor capable of extrinsically proofreading Polε-exo$^-$ errors that is blocked by Polε-P301R. To test this possibility, one could screen for genes that, when deleted or mutated, are synergistic with *pol2-4* but not *pol2-P301R*. Alternatively, one could identify mutations that suppress the mutator phenotype in *pol2-P301R* strains. Uncovering the differences between *pol2-4* and *pol2-P301R* strains would help illuminate the molecular mechanism by which Polε-P301R errors become inserted in the genome at such high rates.

Does MMR saturation occur in diploid strains harboring error-prone Polε variants and lacking Polδ proofreading?

The mutation rate in *pol2-4/POL2 pol3-D520V/pol3-D520V* is approximately two times greater than expected from a multiplicative interaction between *pol2-4* and *pol3-D520V* (**Table 5.1**). Likewise, the mutation rate in *pol2-P301R/POL2 pol3-D520V/pol3-D520V* strains is approximately three times greater than expected from a multiplicative interaction between *pol2-P301R* and *pol3-D520V* (**Table 5.2**). This could be expected if there is a partial MMR defect in these strains due to saturation of MMR. To test whether there is an MMR defect, a triple mutant strain harboring a Polε variant (either exo$^-$ or P301R) and lacking Polδ proofreading and MMR would be necessary. Since the

expected multiplicative mutation rate in a *pol2-P301R/POL2 pol3-D520V/pol3-D520V msh6Δ/msh6Δ* strain would exceed the viability threshold for diploids, higher ploidy would be required. However, a *pol2-4/POL2 pol3-D520V/pol3-D520V msh6Δ/msh6Δ* could potentially survive, though careful construction would be necessary to avoid potential accumulation of suppressor mutations. Measuring mutation rate in single, double, and triple mutant cells would reveal whether the relationship is fully multiplicative or not, and thus whether there is a MMR defect in strains lacking Polδ proofreading and harboring a Polε variant. If we observe a multiplicative increase in mutation rate when we delete *MSH6* in *pol2-4/POL2 pol3-D520V/pol3-D520V* strains, then it will be clear that there is no MMR defect in the *pol2-4/POL2 pol3-D520V/pol3-D520V* strains.

To what extent do intrinsic proofreading, extrinsic proofreading, and MMR contribute to the fidelity of the leading strand replication?

If an incorrect nucleotide is incorporated by Polε during replication of the leading strand, there are at least three ways the error can be removed: intrinsic proofreading by Polε, extrinsic proofreading by Polδ, and post-replicative MMR. It is not known how much each error correction system does to maintain the low rate of mutations during DNA replication. To address the extent of intrinsic proofreading, the *pol2-M644G* mutation could be combined with the *pol2-4* mutation in the absence of Polδ proofreading, MMR, or both. Measuring mutation rates in these yeast strains would establish the extent of removal for each error correction system. Since previous studies and experiments presented in this dissertation have established that all three mechanisms act in series with nucleotide selectivity of Polε, diploid strains, or potentially strains with higher ploidy, would likely be necessary.

How much does extrinsic proofreading by Polδ contribute to replication fidelity on the lagging strand?

It has been established that Polδ proofreads errors made by Polα and Polε [(74,161) and Chapter 3]. Additionally, both *pol3-D520V* and *pol3-01* alleles appear to be nearly recessive (53,142,146), suggesting that Polδ molecules can proofread for other Polδ molecules on the lagging strand. The extent to which Polδ intrinsically and extrinsically proofreads lagging strand errors has not been studied. To investigate intrinsic proofreading by Polδ, combining the *pol3-L612M* and *pol3-D520V* mutations into a single allele (in diploid strains as haploids are likely to be inviable), could establish the extent of intrinsic proofreading. Combining this double mutant allele with a *pol3-D520V* mutation in the other allele of a diploid, and measuring mutation rates, could establish the extent of extrinsic proofreading of Polδ errors. It may also be important to conduct these mutation rate measurements in the absence of MMR, though the mutation rates may exceed the viability threshold, preventing inactivation of MMR.

References

1. Bell, S.P. and Stillman, B. (1992) ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. *Nature*, 357, 128-134.

2. Symeonidou, I.E., Taraviras, S. and Lygerou, Z. (2012) Control over DNA replication in time and space. *FEBS Lett.*, 586, 2803-2812.

3. Evrin, C., Clarke, P., Zech, J., Lurz, R., Sun, J., Uhle, S., Li, H., Stillman, B. and Speck, C. (2009) A double-hexameric MCM2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication. *Proc. Natl. Acad. Sci. U.S.A.*, 106, 20240-20245.

4. Remus, D., Beuron, F., Tolun, G., Griffith, J.D., Morris, E.P. and Diffley, J.F. (2009) Concerted loading of Mcm2-7 double hexamers around DNA during DNA replication origin licensing. *Cell*, 139, 719-730.

5. Hanlon, S.L. and Li, J.J. (2015) Re-replication of a centromere induces chromosomal instability and aneuploidy. *PLoS Genet.*, 11, e1005039.

6. Yeeles, J.T., Deegan, T.D., Janska, A., Early, A. and Diffley, J.F. (2015) Regulated eukaryotic DNA replication origin firing with purified proteins. *Nature*, 519, 431-435.

7. Langston, L.D., Zhang, D., Yurieva, O., Georgescu, R.E., Finkelstein, J., Yao, N.Y., Indiani, C. and O'Donnell, M.E. (2014) CMG helicase and DNA polymerase ε form a functional 15-subunit holoenzyme for eukaryotic leading-strand DNA replication. *Proc. Natl. Acad. Sci. U.S.A.*, 111, 15390-15395.

8. Boos, D. and Ferreira, P. (2019) Origin Firing Regulations to Control Genome Replication Timing. *Genes (Basel)*, 10.

9. Doublie, S. and Zahn, K.E. (2014) Structural insights into eukaryotic DNA replication. *Front. Microbiol.*, 5, 444.

10. Marks, A.B., Fu, H. and Aladjem, M.I. (2017) Regulation of Replication Origins. *Adv. Exp. Med. Biol.*, 1042, 43-59.

11. Parker, M.W., Botchan, M.R. and Berger, J.M. (2017) Mechanisms and regulation of DNA replication initiation in eukaryotes. *Crit. Rev. Biochem. Mol. Biol.*, 52, 107-144.

12. McGuffee, S.R., Smith, D.J. and Whitehouse, I. (2013) Quantitative, genome-wide analysis of eukaryotic replication initiation and termination. *Mol. Cell*, 50, 123-135.

13. Garg, P. and Burgers, P.M. (2005) DNA polymerases that propagate the eukaryotic DNA replication fork. *Crit. Rev. Biochem. Mol. Biol.*, 40, 115-128.

14. Morrison, A., Araki, H., Clark, A.B., Hamatake, R.K. and Sugino, A. (1990) A third essential DNA polymerase in *S. cerevisiae*. *Cell*, 62, 1143-1151.

15. Baranovskiy, A.G., Siebler, H.M., Pavlov, Y.I. and Tahirov, T.H. (2018) Iron-Sulfur Clusters in DNA Polymerases and Primases of Eukaryotes. *Methods Enzymol.*, 599, 1-20.

16. Muzi-Falconi, M., Giannattasio, M., Foiani, M. and Plevani, P. (2003) The DNA polymerase alpha-primase complex: multiple functions and interactions. *ScientificWorldJournal*, 3, 21-33.

17. Reijns, M.A.M., Kemp, H., Ding, J., de Proce, S.M., Jackson, A.P. and Taylor, M.S. (2015) Lagging-strand replication shapes the mutational landscape of the genome. *Nature*, 518, 502-506.

18. Sun, J., Yuan, Z., Georgescu, R., Li, H. and O'Donnell, M. (2016) The eukaryotic CMG helicase pumpjack and integration into the replisome. *Nucleus*, 7, 146-154.

19. Yuan, Z., Georgescu, R., Schauer, G.D., O'Donnell, M.E. and Li, H. (2020) Structure of the polymerase epsilon holoenzyme and atomic model of the leading strand replisome. *Nat Commun*, 11, 3156.

20. Tahirov, T.H., Makarova, K.S., Rogozin, I.B., Pavlov, Y.I. and Koonin, E.V. (2009) Evolution of DNA polymerases: an inactivated polymerase-exonuclease module in Pol ε and a chimeric origin of eukaryotic polymerases from two classes of archaeal ancestors. *Biol. Direct*, 4, 11.

21. Kesti, T., Flick, K., Keranen, S., Syvaoja, J.E. and Wittenberg, C. (1999) DNA polymerase ε catalytic domains are dispensable for DNA replication, DNA repair, and cell viability. *Mol. Cell*, 3, 679-685.

22. Boulet, A., Simon, M., Faye, G., Bauer, G.A. and Burgers, P.M. (1989) Structure and function of the *Saccharomyces cerevisiae CDC2* gene encoding the large subunit of DNA polymerase III. *EMBO J.*, 8, 1849-1854.

23. Garcia-Diaz, M. and Bebenek, K. (2007) Multiple functions of DNA polymerases. *CRC Crit Rev Plant Sci*, 26, 105-122.

24. Bebenek, A. and Ziuzia-Graczyk, I. (2018) Fidelity of DNA replication-a matter of proofreading. *Curr. Genet.*, 64, 985-996.

25. Kunkel, T.A. and Erie, D.A. (2015) Eukaryotic mismatch repair in relation to DNA replication. *Annu. Rev. Genet.*, 49, 291-313.

26. Lynch, M. (2010) Evolution of the mutation rate. *Trends Genet.*, 26, 345-352.

27. Fortune, J.M., Pavlov, Y.I., Welch, C.M., Johansson, E., Burgers, P.M. and Kunkel, T.A. (2005) Saccharomyces cerevisiae DNA polymerase delta: high fidelity for base substitutions but lower fidelity for single- and multi-base deletions. *J. Biol. Chem.*, 280, 29980-29987.

28. Xing, X., Kane, D.P., Bulock, C.R., Moore, E.A., Sharma, S., Chabes, A. and Shcherbakova, P.V. (2019) A recurrent cancer-associated substitution in DNA polymerase ε produces a hyperactive enzyme. *Nat. Commun.*, 10, 374.

29. St Charles, J.A., Liberti, S.E., Williams, J.S., Lujan, S.A. and Kunkel, T.A. (2015) Quantifying the contributions of base selectivity, proofreading and mismatch repair to nuclear DNA replication in *Saccharomyces cerevisiae*. *DNA Repair*, 31, 41-51.

30. Watson, J.D. and Crick, F.H. (1953) Genetical implications of the structure of deoxyribonucleic acid. *Nature*, 171, 964-967.

31. Watson, J.D. and Crick, F.H. (1953) Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature*, 171, 737-738.

32. Echols, H. and Goodman, M.F. (1991) Fidelity mechanisms in DNA replication. *Annu. Rev. Biochem.*, 60, 477-511.

33. Goodman, M.F. (1997) Hydrogen bonding revisited: geometric selection as a principal determinant of DNA replication fidelity. *Proc. Natl. Acad. Sci. U.S.A.*, 94, 10493-10495.

34. Kunkel, T.A. (2004) DNA replication fidelity. *J. Biol. Chem.*, 279, 16895-16898.

35. Moran, S., Ren, R.X. and Kool, E.T. (1997) A thymidine triphosphate shape analog lacking Watson-Crick pairing ability is replicated with high sequence selectivity. *Proc. Natl. Acad. Sci. U.S.A.*, 94, 10506-10511.

36. Moran, S., Ren, R.X., Rumney, S. and Kool, E.T. (1997) Difluorotoluene, a Nonpolar Isostere for Thymine, Codes Specifically and Efficiently for Adenine in DNA Replication. *J. Am. Chem. Soc.*, 119, 2056-2057.

37. Lessor, R.A., Gibson, K.J. and Leonard, N.J. (1984) Synthesis and biochemical evaluation of 2'-deoxy-lin-benzoadenosine phosphates. *Biochemistry*, 23, 3868-3873.

38. Morales, J.C. and Kool, E.T. (1998) Efficient replication between non-hydrogen-bonded nucleoside shape analogs. *Nat. Struct. Biol.*, 5, 950-954.

39. Matray, T.J. and Kool, E.T. (1999) A specific partner for abasic damage in DNA. *Nature*, 399, 704-708.

40. Kool, E.T., Morales, J.C. and Guckian, K.M. (2000) Mimicking the Structure and Function of DNA: Insights into DNA Stability and Replication. *Angew. Chem. Int. Ed. Engl.*, 39, 990-1009.

41. O'Neill, B.M., Ratto, J.E., Good, K.L., Tahmassebi, D.C., Helquist, S.A., Morales, J.C. and Kool, E.T. (2002) A highly effective nonpolar isostere of deoxyguanosine: synthesis, structure, stacking, and base pairing. *J. Org. Chem.*, 67, 5869-5875.

42. Joyce, C.M. (1997) Choosing the right sugar: how polymerases select a nucleotide substrate. *Proc. Natl. Acad. Sci. U.S.A.*, 94, 1619-1622.

43. Herr, A.J., Williams, L.N. and Preston, B.D. (2011) Antimutator variants of DNA polymerases. *Crit. Rev. Biochem. Mol. Biol.*, 46, 548-570.

44. Niimi, A., Limsirichaikul, S., Yoshida, S., Iwai, S., Masutani, C., Hanaoka, F., Kool, E.T., Nishiyama, Y. and Suzuki, M. (2004) Palm mutants in DNA polymerases α and η alter DNA replication fidelity and translesion activity. *Mol. Cell. Biol.*, 24, 2734-2746.

45. Nick McElhinny, S.A., Stith, C.M., Burgers, P.M. and Kunkel, T.A. (2007) Inefficient proofreading and biased error rates during inaccurate DNA synthesis by a mutant derivative of *Saccharomyces cerevisiae* DNA polymerase δ. *J. Biol. Chem.*, 282, 2324-2332.

46. Pursell, Z.F., Isoz, I., Lundstrom, E.B., Johansson, E. and Kunkel, T.A. (2007) Yeast DNA polymerase ε participates in leading-strand DNA replication. *Science*, 317, 127-130.

47. Nick McElhinny, S.A., Watts, B.E., Kumar, D., Watt, D.L., Lundstrom, E.B., Burgers, P.M., Johansson, E., Chabes, A. and Kunkel, T.A. (2010) Abundant ribonucleotide incorporation into DNA by yeast replicative polymerases. *Proc. Natl. Acad. Sci. U.S.A.*, 107, 4949-4954.

48. Kunkel, T.A., Hamatake, R.K., Motto-Fox, J., Fitzgerald, M.P. and Sugino, A. (1989) Fidelity of DNA polymerase I and the DNA polymerase I-DNA primase complex from Saccharomyces cerevisiae. *Mol. Cell. Biol.*, 9, 4447-4458.

49. Shcherbakova, P.V., Pavlov, Y.I., Chilkova, O., Rogozin, I.B., Johansson, E. and Kunkel, T.A. (2003) Unique error signature of the four-subunit yeast DNA polymerase ε. *J. Biol. Chem.*, 278, 43770-43780.

50. Fortune, J.M., Stith, C.M., Kissling, G.E., Burgers, P.M. and Kunkel, T.A. (2006) RPA and PCNA suppress formation of large deletion errors by yeast DNA polymerase delta. *Nucleic Acids Res.*, 34, 4335-4341.

51. Blanco, L., Bernad, A. and Salas, M. (1992) Evidence favouring the hypothesis of a conserved 3'-5' exonuclease active site in DNA-dependent DNA polymerases. *Gene*, 112, 139-144.

52. Morrison, A., Bell, J.B., Kunkel, T.A. and Sugino, A. (1991) Eukaryotic DNA polymerase amino acid sequence required for 3'----5' exonuclease activity. *Proc. Natl. Acad. Sci. U.S.A.*, 88, 9473-9477.

53. Simon, M., Giot, L. and Faye, G. (1991) The 3' to 5' exonuclease activity located in the DNA polymerase δ subunit of *Saccharomyces cerevisiae* is required for accurate replication. *EMBO J.*, 10, 2165-2170.

54. Jin, Y.H., Obert, R., Burgers, P.M., Kunkel, T.A., Resnick, M.A. and Gordenin, D.A. (2001) The 3'-->5' exonuclease of DNA polymerase δ can substitute for the

5' flap endonuclease Rad27/Fen1 in processing Okazaki fragments and preventing genome instability. *Proc. Natl. Acad. Sci. U.S.A.*, 98, 5122-5127.

55. Reha-Krantz, L.J. (1998) Regulation of DNA polymerase exonucleolytic proofreading activity: studies of bacteriophage T4 "antimutator" DNA polymerases. *Genetics*, 148, 1551-1557.

56. Ganai, R.A., Bylund, G.O. and Johansson, E. (2015) Switching between polymerase and exonuclease sites in DNA polymerase ε. *Nucleic Acids Res.*, 43, 932-942.

57. Jin, Y.H., Garg, P., Stith, C.M., Al-Refai, H., Sterling, J.F., Murray, L.J., Kunkel, T.A., Resnick, M.A., Burgers, P.M. and Gordenin, D.A. (2005) The multiple biological roles of the 3'-->5' exonuclease of *Saccharomyces cerevisiae* DNA polymerase δ require switching between the polymerase and exonuclease domains. *Mol. Cell. Biol.*, 25, 461-471.

58. Shcherbakova, P.V. and Pavlov, Y.I. (1996) 3'-->5' exonucleases of DNA polymerases ε and δ correct base analog induced DNA replication errors on opposite DNA strands in *Saccharomyces cerevisiae*. *Genetics*, 142, 717-726.

59. Tran, H.T., Gordenin, D.A. and Resnick, M.A. (1999) The 3'-->5' exonucleases of DNA polymerases δ and ε and the 5'-->3' exonuclease Exo1 have major roles in postreplication mutation avoidance in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, 19, 2000-2007.

60. Loeb, L.A., Springgate, C.F. and Battula, N. (1974) Errors in DNA replication as a basis of malignant changes. *Cancer Res.*, 34, 2311-2321.

61. Barbari, S.R. and Shcherbakova, P.V. (2017) Replicative DNA polymerase defects in human cancers: Consequences, mechanisms, and implications for therapy. *DNA Repair (Amst)*, 56, 16-25.

62. Modrich, P. and Lahue, R. (1996) Mismatch repair in replication fidelity, genetic recombination, and cancer biology. *Annu. Rev. Biochem.*, 65, 101-133.

63. Hombauer, H., Srivatsan, A., Putnam, C.D. and Kolodner, R.D. (2011) Mismatch repair, but not heteroduplex rejection, is temporally coupled to DNA replication. *Science*, 334, 1713-1716.

64. Thomas, D.C., Umar, A. and Kunkel, T.A. (1996) Microsatellite instability and mismatch repair defects in cancer. *Mutat. Res.*, 350, 201-205.

65. Hawk, J.D., Stefanovic, L., Boyer, J.C., Petes, T.D. and Farber, R.A. (2005) Variation in efficiency of DNA mismatch repair at different sites in the yeast genome. *Proc. Natl. Acad. Sci. U.S.A.*, 102, 8639-8643.

66. Pavlov, Y.I., Mian, I.M. and Kunkel, T.A. (2003) Evidence for preferential mismatch repair of lagging strand DNA replication errors in yeast. *Curr. Biol.*, 13, 744-748.

67. Lujan, S.A., Williams, J.S., Pursell, Z.F., Abdulovic-Cui, A.A., Clark, A.B., Nick McElhinny, S.A. and Kunkel, T.A. (2012) Mismatch repair balances leading and lagging strand DNA replication fidelity. *PLoS Genet.*, 8, e1003016.

68. Lujan, S.A., Clausen, A.R., Clark, A.B., MacAlpine, H.K., MacAlpine, D.M., Malc, E.P., Mieczkowski, P.A., Burkholder, A.B., Fargo, D.C., Gordenin, D.A. *et al.* (2014) Heterogeneous polymerase fidelity and mismatch repair bias genome variation and composition. *Genome Res.*, 24, 1751-1764.

69. Nick McElhinny, S.A., Gordenin, D.A., Stith, C.M., Burgers, P.M. and Kunkel, T.A. (2008) Division of labor at the eukaryotic replication fork. *Mol. Cell*, 30, 137-144.

70. Larrea, A.A., Lujan, S.A., Nick McElhinny, S.A., Mieczkowski, P.A., Resnick, M.A., Gordenin, D.A. and Kunkel, T.A. (2010) Genome-wide model for the normal eukaryotic DNA replication fork. *Proc. Natl. Acad. Sci. U.S.A.*, 107, 17674-17679.

71. Shinbrot, E., Henninger, E.E., Weinhold, N., Covington, K.R., Goksenin, A.Y., Schultz, N., Chao, H., Doddapaneni, H., Muzny, D.M., Gibbs, R.A. *et al.* (2014) Exonuclease mutations in DNA polymerase epsilon reveal replication strand specific mutation patterns and human origins of replication. *Genome Res.*, 24, 1740-1750.

72. Clausen, A.R., Lujan, S.A., Burkholder, A.B., Orebaugh, C.D., Williams, J.S., Clausen, M.F., Malc, E.P., Mieczkowski, P.A., Fargo, D.C., Smith, D.J. *et al.* (2015) Tracking replication enzymology in vivo by genome-wide mapping of ribonucleotide incorporation. *Nat. Struct. Mol. Biol.*, 22, 185-191.

73. Daigaku, Y., Keszthelyi, A., Muller, C.A., Miyabe, I., Brooks, T., Retkute, R., Hubank, M., Nieduszynski, C.A. and Carr, A.M. (2015) A global profile of replicative polymerase usage. *Nat. Struct. Mol. Biol.*, 22, 192-198.

74. Pavlov, Y.I., Frahm, C., Nick McElhinny, S.A., Niimi, A., Suzuki, M. and Kunkel, T.A. (2006) Evidence that errors made by DNA polymerase α are corrected by DNA polymerase δ *Curr. Biol.*, 16, 202-207.

75. Garg, P., Stith, C.M., Sabouri, N., Johansson, E. and Burgers, P.M. (2004) Idling by DNA polymerase δ maintains a ligatable nick during lagging-strand DNA replication. *Genes Dev.*, 18, 2764-2773.

76. Yeeles, J.T.P., Janska, A., Early, A. and Diffley, J.F.X. (2017) How the eukaryotic replisome achieves rapid and efficient DNA replication. *Mol. Cell*, 65, 105-116.

77. Aria, V. and Yeeles, J.T.P. (2018) Mechanism of bidirectional leading-strand synthesis establishment at eukaryotic DNA replication origins. *Mol. Cell*, 73, 199-211.

78. Garbacz, M.A., Lujan, S.A., Burkholder, A.B., Cox, P.B., Wu, Q., Zhou, Z.X., Haber, J.E. and Kunkel, T.A. (2018) Evidence that DNA polymerase δ contributes

to initiating leading strand DNA replication in *Saccharomyces cerevisiae*. *Nat. Commun.*, 9, 858.

79. Zhou, Z.X., Lujan, S.A., Burkholder, A.B., Garbacz, M.A. and Kunkel, T.A. (2019) Roles for DNA polymerase δ in initiating and terminating leading strand DNA replication. *Nat. Commun.*, 10, 3992.

80. Billingsley, C.C., Cohn, D.E., Mutch, D.G., Stephens, J.A., Suarez, A.A. and Goodfellow, P.J. (2015) Polymerase ε (*POLE*) mutations in endometrial cancer: clinical outcomes and implications for Lynch syndrome testing. *Cancer*, 121, 386-394.

81. Cerami, E., Gao, J., Dogrusoz, U., Gross, B.E., Sumer, S.O., Aksoy, B.A., Jacobsen, A., Byrne, C.J., Heuer, M.L., Larsson, E. *et al.* (2012) The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov.*, 2, 401-404.

82. Church, D.N., Briggs, S.E., Palles, C., Domingo, E., Kearsey, S.J., Grimes, J.M., Gorman, M., Martin, L., Howarth, K.M., Hodgson, S.V. *et al.* (2013) DNA polymerase ε and δ exonuclease domain mutations in endometrial cancer. *Hum. Mol. Genet.*, 22, 2820-2828.

83. Domingo, E., Freeman-Mills, L., Rayner, E., Glaire, M., Briggs, S., Vermeulen, L., Fessler, E., Medema, J.P., Boot, A., Morreau, H. *et al.* (2016) Somatic *POLE* proofreading domain mutation, immune response, and prognosis in colorectal cancer: a retrospective, pooled biomarker study. *Lancet Gastroenterol. Hepatol.*, 1, 207-216.

84. Guerra, J., Pinto, C., Pinto, D., Pinheiro, M., Silva, R., Peixoto, A., Rocha, P., Veiga, I., Santos, C., Santos, R. *et al.* (2017) POLE somatic mutations in advanced colorectal cancer. *Cancer Med.*, 6, 2966-2971.

85. Haruma, T., Nagasaka, T., Nakamura, K., Haraga, J., Nyuya, A., Nishida, T., Goel, A., Masuyama, H. and Hiramatsu, Y. (2018) Clinical impact of endometrial cancer stratified by genetic mutational profiles, POLE mutation, and microsatellite instability. *PLoS One*, 13, e0195655.

86. Hino, H., Shiomi, A., Kusuhara, M., Kagawa, H., Yamakawa, Y., Hatakeyama, K., Kawabata, T., Oishi, T., Urakami, K., Nagashima, T. *et al.* (2019) Clinicopathological and mutational analyses of colorectal cancer with mutations in the POLE gene. *Cancer Med.*, 8, 4587-4597.

87. Hussein, Y.R., Weigelt, B., Levine, D.A., Schoolmeester, J.K., Dao, L.N., Balzer, B.L., Liles, G., Karlan, B., Kobel, M., Lee, C.H. *et al.* (2015) Clinicopathological analysis of endometrial carcinomas harboring somatic POLE exonuclease domain mutations. *Mod. Pathol.*, 28, 505-514.

88. Imboden, S., Nastic, D., Ghaderi, M., Rydberg, F., Rau, T.T., Mueller, M.D., Epstein, E. and Carlson, J.W. (2019) Phenotype of POLE-mutated endometrial cancer. *PLoS One*, 14, e0214318.

89. Levine, D.A., The Cancer Genome Atlas Research, N., Getz, G., Gabriel, S.B., Cibulskis, K., Lander, E., Sivachenko, A., Sougnez, C., Lawrence, M., Kandoth, C. *et al.* (2013) Integrated genomic characterization of endometrial carcinoma. *Nature*, 497, 67.

90. Cancer Genome Research Network. (2012) Comprehensive molecular characterization of human colon and rectal cancer. *Nature*, 487, 330-337.

91. Cancer Genome Research Network, Kandoth, C., Schultz, N., Cherniack, A.D., Akbani, R., Liu, Y., Shen, H., Robertson, A.G., Pashtan, I., Shen, R. *et al.* (2013) Integrated genomic characterization of endometrial carcinoma. *Nature*, 497, 67-73.

92. Stenzinger, A., Pfarr, N., Endris, V., Penzel, R., Jansen, L., Wolf, T., Herpel, E., Warth, A., Klauschen, F., Kloor, M. *et al.* (2014) Mutations in POLE and survival of colorectal cancer patients--link to disease stage and treatment. *Cancer Med.*, 3, 1527-1538.

93. Yao, J., Gong, Y., Zhao, W., Han, Z., Guo, S., Liu, H., Peng, X., Xiao, W., Li, Y., Dang, S. *et al.* (2019) Comprehensive analysis of POLE and POLD1 Gene Variations identifies cancer patients potentially benefit from immunotherapy in Chinese population. *Sci. Rep.*, 9, 15767.

94. Yu, S., Shao, H., Ban, X., Zhang, H., You, Y., Zhou, N., Mao, X., Zhao, H., Chen, J. and Lu, Z. (2019) Detection of POLE Subtypes in High-Grade Endometrioid Carcinoma by BaseScope-ISH Assay. *Front. Oncol.*, 9, 831.

95. Rayner, E., van Gool, I.C., Palles, C., Kearsey, S.E., Bosse, T., Tomlinson, I. and Church, D.N. (2016) A panoply of errors: polymerase proofreading domain mutations in cancer. *Nat. Rev. Cancer*, 16, 71-81.

96. Park, V.S. and Pursell, Z.F. (2019) POLE proofreading defects: Contributions to mutagenesis and cancer. *DNA Repair (Amst)*, 76, 50-59.

97. Church, D.N., Stelloo, E., Nout, R.A., Valtcheva, N., Depreeuw, J., ter Haar, N., Noske, A., Amant, F., Tomlinson, I.P., Wild, P.J. *et al.* (2015) Prognostic significance of POLE proofreading mutations in endometrial cancer. *J. Natl. Cancer Inst.*, 107, 402.

98. Ahn, S.M., Ansari, A.A., Kim, J., Kim, D., Chun, S.M., Kim, J., Kim, T.W., Park, I., Yu, C.S. and Jang, S.J. (2016) The somatic POLE P286R mutation defines a unique subclass of colorectal cancer featuring hypermutation, representing a potential genomic biomarker for immunotherapy. *Oncotarget*, 7, 68638-68649.

99. Kothari, N., Teer, J.K., Abbott, A.M., Srikumar, T., Zhang, Y., Yoder, S.J., Brohl, A.S., Kim, R.D., Reed, D.R. and Shibata, D. (2016) Increased incidence of FBXW7 and POLE proofreading domain mutations in young adult colorectal cancers. *Cancer*, 122, 2828-2835.

100. Meng, B., Hoang, L.N., McIntyre, J.B., Duggan, M.A., Nelson, G.S., Lee, C.H. and Kobel, M. (2014) POLE exonuclease domain mutation predicts long

progression-free survival in grade 3 endometrioid carcinoma of the endometrium. *Gynecol. Oncol.*, 134, 15-19.

101. Erson-Omay, E.Z., Caglayan, A.O., Schultz, N., Weinhold, N., Omay, S.B., Ozduman, K., Koksal, Y., Li, J., Serin Harmanci, A., Clark, V. *et al.* (2015) Somatic POLE mutations cause an ultramutated giant cell high-grade glioma subtype with better prognosis. *Neuro Oncol.*, 17, 1356-1364.

102. Miyamoto, T., Ando, H., Asaka, R., Yamada, Y. and Shiozawa, T. (2018) Mutation analysis by whole exome sequencing of endometrial hyperplasia and carcinoma in one patient: Abnormalities of polymerase epsilon and the phosphatidylinositol-3 kinase pathway. *J. Obstet. Gynaecol. Res.*, 44, 179-183.

103. Temko, D., Van Gool, I.C., Rayner, E., Glaire, M., Makino, S., Brown, M., Chegwidden, L., Palles, C., Depreeuw, J., Beggs, A. *et al.* (2018) Somatic POLE exonuclease domain mutations are early events in sporadic endometrial and colorectal carcinogenesis, determining driver mutational landscape, clonal neoantigen burden and immune response. *J. Pathol.*, 245, 283-296.

104. Haradhvala, N.J., Kim, J., Maruvka, Y.E., Polak, P., Rosebrock, D., Livitz, D., Hess, J.M., Leshchiner, I., Kamburov, A., Mouw, K.W. *et al.* (2018) Distinct mutational signatures characterize concurrent loss of polymerase proofreading and mismatch repair. *Nat. Commun.*, 9, 1746.

105. Campbell, B.B., Light, N., Fabrizio, D., Zatzman, M., Fuligni, F., de Borja, R., Davidson, S., Edwards, M., Elvin, J.A., Hodel, K.P. *et al.* (2017) Comprehensive analysis of hypermutation in human cancer. *Cell*, 171, 1042-1056 e1010.

106. Alexandrov, L.B., Nik-Zainal, S., Wedge, D.C., Aparicio, S.A., Behjati, S., Biankin, A.V., Bignell, G.R., Bolli, N., Borg, A., Borresen-Dale, A.L. *et al.* (2013) Signatures of mutational processes in human cancer. *Nature*, 500, 415-421.

107. Bellone, S., Centritto, F., Black, J., Schwab, C., English, D., Cocco, E., Lopez, S., Bonazzoli, E., Predolini, F., Ferrari, F. *et al.* (2015) Polymerase epsilon (POLE) ultra-mutated tumors induce robust tumor-specific CD4+ T cell responses in endometrial cancer patients. *Gynecol. Oncol.*, 138, 11-17.

108. Eggink, F.A., Van Gool, I.C., Leary, A., Pollock, P.M., Crosbie, E.J., Mileshkin, L., Jordanova, E.S., Adam, J., Freeman-Mills, L., Church, D.N. *et al.* (2017) Immunological profiling of molecularly classified high-risk endometrial cancers identifies POLE-mutant and microsatellite unstable carcinomas as candidates for checkpoint inhibition. *Oncoimmunology*, 6, e1264565.

109. Howitt, B.E., Shukla, S.A., Sholl, L.M., Ritterhouse, L.L., Watkins, J.C., Rodig, S., Stover, E., Strickland, K.C., D'Andrea, A.D., Wu, C.J. *et al.* (2015) Association of Polymerase e-Mutated and Microsatellite-Instable Endometrial Cancers With Neoantigen Load, Number of Tumor-Infiltrating Lymphocytes, and Expression of PD-1 and PD-L1. *JAMA Oncol.*, 1, 1319-1323.

110. van Gool, I.C., Eggink, F.A., Freeman-Mills, L., Stelloo, E., Marchi, E., de Bruyn, M., Palles, C., Nout, R.A., de Kroon, C.D., Osse, E.M. *et al.* (2015) POLE

Proofreading Mutations Elicit an Antitumor Immune Response in Endometrial Cancer. *Clin. Cancer. Res.*, 21, 3347-3355.

111. Bakhsh, S., Kinloch, M., Hoang, L.N., Soslow, R.A., Kobel, M., Lee, C.H., McAlpine, J.N., McConechy, M.K. and Gilks, C.B. (2016) Histopathological features of endometrial carcinomas associated with POLE mutations: implications for decisions about adjuvant therapy. *Histopathology*, 68, 916-924.

112. Bellone, S., Bignotti, E., Lonardi, S., Ferrari, F., Centritto, F., Masserdotti, A., Pettinella, F., Black, J., Menderes, G., Altwerger, G. *et al.* (2017) Polymerase epsilon (POLE) ultra-mutation in uterine tumors correlates with T lymphocyte infiltration and increased resistance to platinum-based chemotherapy in vitro. *Gynecol. Oncol.*, 144, 146-152.

113. Liu, L., Ruiz, J., O'Neill, S.S., Grant, S.C., Petty, W.J., Yang, M., Chen, K., Topaloglu, U., Pasche, B. and Zhang, W. (2018) Favorable outcome of patients with lung adenocarcinoma harboring POLE mutations and expressing high PD-L1. *Mol. Cancer*, 17, 81.

114. Wang, C., Gong, J., Tu, T.Y., Lee, P.P. and Fakih, M. (2018) Immune profiling of microsatellite instability-high and polymerase epsilon (POLE)-mutated metastatic colorectal tumors identifies predictors of response to anti-PD-1 therapy. *J. Gastrointest. Oncol.*, 9, 404-415.

115. Mehnert, J.M., Panda, A., Zhong, H., Hirshfield, K., Damare, S., Lane, K., Sokol, L., Stein, M.N., Rodriguez-Rodriquez, L., Kaufman, H.L. *et al.* (2016) Immune activation and response to pembrolizumab in POLE-mutant endometrial cancer. *J. Clin. Invest.*, 126, 2334-2340.

116. Santin, A.D., Bellone, S., Buza, N., Choi, J., Schwartz, P.E., Schlessinger, J. and Lifton, R.P. (2016) Regression of Chemotherapy-Resistant Polymerase epsilon (POLE) Ultra-Mutated and MSH6 Hyper-Mutated Endometrial Tumors with Nivolumab. *Clin. Cancer. Res.*, 22, 5682-5687.

117. Forbes, S.A., Beare, D., Gunasekaran, P., Leung, K., Bindal, N., Boutselakis, H., Ding, M., Bamford, S., Cole, C., Ward, S. *et al.* (2015) COSMIC: exploring the world's knowledge of somatic mutations in human cancer. *Nucleic Acids Res.*, 43, D805-811.

118. Kane, D.P. and Shcherbakova, P.V. (2014) A common cancer-associated DNA polymerase ε mutation causes an exceptionally strong mutator phenotype, indicating fidelity defects distinct from loss of proofreading. *Cancer Res.*, 74, 1895-1901.

119. Albertson, T.M., Ogawa, M., Bugni, J.M., Hays, L.E., Chen, Y., Wang, Y., Treuting, P.M., Heddle, J.A., Goldsby, R.E. and Preston, B.D. (2009) DNA polymerase ε and δ proofreading suppress discrete mutator and cancer phenotypes in mice. *Proc. Natl. Acad. Sci. U.S.A.*, 106, 17101-17104.

120. Li, H.D., Cuevas, I., Zhang, M., Lu, C., Alam, M.M., Fu, Y.X., You, M.J., Akbay, E.A., Zhang, H. and Castrillon, D.H. (2018) Polymerase-mediated

ultramutagenesis in mice produces diverse cancers with high mutational load. *J. Clin. Invest.*, 128, 4179-4191.

121. Hodel, K.P., Sun, M.J.S., Ungerleider, N., Park, V.S., Williams, L.G., Bauer, D.L., Immethun, V.E., Wang, J., Suo, Z., Lu, H. *et al.* (2020) POLE Mutation Spectra Are Shaped by the Mutant Allele Identity, Its Abundance, and Mismatch Repair Status. *Mol. Cell*.

122. Abaan, O.D., Polley, E.C., Davis, S.R., Zhu, Y.J., Bilke, S., Walker, R.L., Pineda, M., Gindin, Y., Jiang, Y., Reinhold, W.C. *et al.* (2013) The exomes of the NCI-60 panel: a genomic resource for cancer biology and systems pharmacology. *Cancer Res.*, 73, 4372-4382.

123. Hodel, K.P., de Borja, R., Henninger, E.E., Campbell, B.B., Ungerleider, N., Light, N., Wu, T., LeCompte, K.G., Goksenin, A.Y., Bunnell, B.A. *et al.* (2018) Explosive mutation accumulation triggered by heterozygous human Pol ε proofreading-deficiency is driven by suppression of mismatch repair. *Elife*, 7.

124. Parkash, V., Kulkarni, Y., ter Beek, J., Shcherbakova, P.V., Kamerlin, S.C.L. and Johansson, E. (2019) Structural consequence of the most frequently recurring cancer-associated substitution in DNA polymerase ε. *Nat. Commun.*, 10, 373.

125. Kirchner, J.M., Tran, H. and Resnick, M.A. (2000) A DNA polymerase ε mutant that specifically causes +1 frameshift mutations within homonucleotide runs in yeast. *Genetics*, 155, 1623-1632.

126. Kokoska, R.J., Stefanovic, L., Tran, H.T., Resnick, M.A., Gordenin, D.A. and Petes, T.D. (1998) Destabilization of yeast micro- and minisatellite DNA sequences by mutations affecting a nuclease involved in Okazaki fragment processing (*rad27*) and DNA polymerase δ (*pol3-t*). *Mol. Cell. Biol.*, 18, 2779-2788.

127. Li, L., Murphy, K.M., Kanevets, U. and Reha-Krantz, L.J. (2005) Sensitivity to phosphonoacetic acid: a new phenotype to probe DNA polymerase δ in *Saccharomyces cerevisiae*. *Genetics*, 170, 569-580.

128. Shcherbakova, P.V. and Pavlov, Y.I. (1993) Mutagenic specificity of the base analog 6-N-hydroxylaminopurine in the *URA3* gene of the yeast *Saccharomyces cerevisiae*. *Mutagenesis*, 8, 417-421.

129. Pavlov, Y.I., Newlon, C.S. and Kunkel, T.A. (2002) Yeast origins establish a strand bias for replicational mutagenesis. *Mol. Cell*, 10, 207-213.

130. Morrison, A. and Sugino, A. (1994) The 3'-->5' exonucleases of both DNA polymerases δ and ε participate in correcting errors of DNA replication in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, 242, 289-296.

131. Shcherbakova, P.V., Noskov, V.N., Pshenichnov, M.R. and Pavlov, Y.I. (1996) Base analog 6-N-hydroxylaminopurine mutagenesis in the yeast *Saccharomyces cerevisiae* is controlled by replicative DNA polymerases. *Mutat. Res.*, 369, 33-44.

132. Tran, H.T., Keen, J.D., Kricker, M., Resnick, M.A. and Gordenin, D.A. (1997) Hypermutability of homonucleotide runs in mismatch repair and DNA polymerase proofreading yeast mutants. *Mol. Cell. Biol.*, 17, 2859-2865.

133. Shcherbakova, P.V. and Kunkel, T.A. (1999) Mutator phenotypes conferred by *MLH1* overexpression and by heterozygosity for *mlh1* mutations. *Mol. Cell. Biol.*, 19, 3177-3183.

134. Mertz, T.M., Sharma, S., Chabes, A. and Shcherbakova, P.V. (2015) Colon cancer-associated mutator DNA polymerase δ variant causes expansion of dNTP pools increasing its own infidelity. *Proc. Natl. Acad. Sci. U.S.A.*, 112, E2467-2476.

135. Northam, M.R., Robinson, H.A., Kochenova, O.V. and Shcherbakova, P.V. (2010) Participation of DNA polymerase ζ in replication of undamaged DNA in *Saccharomyces cerevisiae*. *Genetics*, 184, 27-42.

136. Whelan, W.L., Gocke, E. and Manney, T.R. (1979) The CAN1 locus of Saccharomyces cerevisiae: fine-structure analysis and forward mutation rates. *Genetics*, 91, 35-51.

137. Drake, J.W. (1991) A constant rate of spontaneous mutation in DNA-based microbes. *Proc. Natl. Acad. Sci. U.S.A.*, 88, 7160-7164.

138. Dixon, W.J. (1969) *Introduction to statistical analysis*. Macgraw Hillkogakusha, Tokyo.

139. Ohya, T., Kawasaki, Y., Hiraga, S., Kanbara, S., Nakajo, K., Nakashima, N., Suzuki, A. and Sugino, A. (2002) The DNA polymerase domain of pol ε is required for rapid, efficient, and highly accurate chromosomal DNA replication, telomere length maintenance, and normal cell senescence in *Saccharomyces cerevisiae*. *J. Biol. Chem.*, 277, 28099-28108.

140. Pavlov, Y.I., Maki, S., Maki, H. and Kunkel, T.A. (2004) Evidence for interplay among yeast replicative DNA polymerases α, δ and ε from studies of exonuclease and polymerase active site mutations. *BMC Biol.*, 2, 11.

141. Lee, M.B., Dowsett, I.T., Carr, D.T., Wasko, B.M., Stanton, S.G., Chung, M.S., Ghodsian, N., Bode, A., Kiflezghi, M.G., Uppal, P.A. *et al.* (2019) Defining the impact of mutation accumulation on replicative lifespan in yeast using cancer-associated mutator phenotypes. *Proc. Natl. Acad. Sci. U.S.A.*, 116, 3062-3071.

142. Morrison, A., Johnson, A.L., Johnston, L.H. and Sugino, A. (1993) Pathway correcting DNA replication errors in *Saccharomyces cerevisiae*. *EMBO J.*, 12, 1467-1473.

143. Pavlov, Y.I., Shcherbakova, P.V. and Kunkel, T.A. (2001) *In vivo* consequences of putative active site mutations in yeast DNA polymerases α, ε, δ, and ζ. *Genetics*, 159, 47-64.

144. Pavlov, Y.I. and Shcherbakova, P.V. (2010) DNA polymerases at the eukaryotic fork-20 years later. *Mutat. Res.*, 685, 45-53.

145. Williams, L.N., Herr, A.J. and Preston, B.D. (2013) Emergence of DNA polymerase ε antimutators that escape error-induced extinction in yeast. *Genetics*, 193, 751-770.

146. Flood, C.L., Rodriguez, G.P., Bao, G., Shockley, A.H., Kow, Y.W. and Crouse, G.F. (2015) Replicative DNA polymerase δ but not ε proofreads errors in *cis* and in *trans*. *PLoS Genet.*, 11, e1005049.

147. Perrino, F.W. and Loeb, L.A. (1989) Proofreading by the ε subunit of *Escherichia coli* DNA polymerase III increases the fidelity of calf thymus DNA polymerase α. *Proc. Natl. Acad. Sci. U.S.A.*, 86, 3085-3088.

148. Perrino, F.W. and Loeb, L.A. (1990) Hydrolysis of 3'-terminal mispairs in vitro by the 3'----5' exonuclease of DNA polymerase δ permits subsequent extension by DNA polymerase α. *Biochemistry*, 29, 5226-5231.

149. Belyakova, N.V., Kleiner, N.E., Kravetskaya, T.P., Legina, O.K., Naryzhny, S.N., Perrino, F.W., Shevelev, I.V. and Krutyakov, V.M. (1993) Proof-reading 3'-->5' exonucleases isolated from rat liver nuclei. *Eur. J. Biochem.*, 217, 493-500.

150. Huang, P. (1998) Excision of mismatched nucleotides from DNA: a potential mechanism for enhancing DNA replication fidelity by the wild-type p53 protein. *Oncogene*, 17, 261-270.

151. Brown, K.R., Weatherdon, K.L., Galligan, C.L. and Skalski, V. (2002) A nuclear 3'-5' exonuclease proofreads for the exonuclease-deficient DNA polymerase α. *DNA Repair (Amst)*, 1, 795-810.

152. McCulloch, S.D., Kokoska, R.J., Chilkova, O., Welch, C.M., Johansson, E., Burgers, P.M. and Kunkel, T.A. (2004) Enzymatic switching for efficient and accurate translesion DNA replication. *Nucleic Acids Res.*, 32, 4665-4675.

153. Fuchs, R.P. and Fujii, S. (2007) Translesion synthesis in *Escherichia coli*: lessons from the NarI mutation hot spot. *DNA Repair (Amst)*, 6, 1032-1041.

154. Datta, A., Schmeits, J.L., Amin, N.S., Lau, P.J., Myung, K. and Kolodner, R.D. (2000) Checkpoint-dependent activation of mutagenic repair in *Saccharomyces cerevisiae pol3-01* mutants. *Mol. Cell*, 6, 593-603.

155. Marsischky, G.T., Filosi, N., Kane, M.F. and Kolodner, R. (1996) Redundancy of Saccharomyces cerevisiae MSH3 and MSH6 in MSH2-dependent mismatch repair. *Genes Dev.*, 10, 407-420.

156. Aparicio, O.M., Weinstein, D.M. and Bell, S.P. (1997) Components and dynamics of DNA replication complexes in *S. cerevisiae*: redistribution of MCM proteins and Cdc45p during S phase. *Cell*, 91, 59-69.

157. Muramatsu, S., Hirai, K., Tak, Y.S., Kamimura, Y. and Araki, H. (2010) CDK-dependent complex formation between replication proteins Dpb11, Sld2, Pol ε, and GINS in budding yeast. *Genes Dev.*, 24, 602-612.

158. Chilkova, O., Stenlund, P., Isoz, I., Stith, C.M., Grabowski, P., Lundstrom, E.B., Burgers, P.M. and Johansson, E. (2007) The eukaryotic leading and lagging strand DNA polymerases are loaded onto primer-ends via separate mechanisms but have comparable processivity in the presence of PCNA. *Nucleic Acids Res.*, 35, 6588-6597.

159. Grabowska, E., Wronska, U., Denkiewicz, M., Jaszczur, M., Respondek, A., Alabrudzinska, M., Suski, C., Makiela-Dzbenska, K., Jonczyk, P. and Fijalkowska, I.J. (2014) Proper functioning of the GINS complex is important for the fidelity of DNA replication in yeast. *Mol. Microbiol.*, 92, 659-680.

160. Fijalkowska, I.J., Jonczyk, P., Tkaczyk, M.M., Bialoskorska, M. and Schaaper, R.M. (1998) Unequal fidelity of leading strand and lagging strand DNA replication on the *Escherichia coli* chromosome. *Proc. Natl. Acad. Sci. U.S.A.*, 95, 10020-10025.

161. Bulock, C.R., Xing, X. and Shcherbakova, P.V. (2020) DNA polymerase δ proofreads errors made by DNA polymerase ε. *Proc. Natl. Acad. Sci. U.S.A.*, 117, 6035-6041.

162. Herr, A.J., Kennedy, S.R., Knowels, G.M., Schultz, E.M. and Preston, B.D. (2014) DNA replication error-induced extinction of diploid yeast. *Genetics*, 196, 677-691.

163. Reddy, M.K., Weitzel, S.E. and von Hippel, P.H. (1992) Processive proofreading is intrinsic to T4 DNA polymerase. *J. Biol. Chem.*, 267, 14157-14166.

164. Fidalgo da Silva, E. and Reha-Krantz, L.J. (2007) DNA polymerase proofreading: active site switching catalyzed by the bacteriophage T4 DNA polymerase. *Nucleic Acids Res.*, 35, 5452-5463.

165. Boiteux, S. and Jinks-Robertson, S. (2013) DNA repair mechanisms and the bypass of DNA damage in *Saccharomyces cerevisiae*. *Genetics*, 193, 1025-1064.

166. Sharma, S., Helchowski, C.M. and Canman, C.E. (2013) The roles of DNA polymerase ζ and the Y family DNA polymerases in promoting or preventing genome instability. *Mutat. Res.*, 743-744, 97-110.

167. Jansen, J.G., Temviriyanukul, P., Wit, N., Delbos, F., Reynaud, C.A., Jacobs, H. and de Wind, N. (2014) Redundancy of mammalian Y family DNA polymerases in cellular responses to genomic DNA lesions induced by ultraviolet light. *Nucleic Acids Res.*, 42, 11071-11082.

168. Hosoya, N. and Miyagawa, K. (2014) Targeting DNA damage response in cancer therapy. *Cancer Sci.*, 105, 370-388.

169. Choi, J.S., Kim, S., Motea, E. and Berdis, A. (2017) Inhibiting translesion DNA synthesis as an approach to combat drug resistance to DNA damaging agents. *Oncotarget*, 8, 40804-40816.

170. Choi, J.S. and Berdis, A. (2018) Combating resistance to DNA damaging agents. *Oncoscience*, 5, 134-136.

171. Manhart, C.M. and Alani, E. (2017) DNA replication and mismatch repair safeguard against metabolic imbalances. *Proc. Natl. Acad. Sci. U.S.A.*, 114, 5561-5563.

172. Schmidt, T.T., Reyes, G., Gries, K., Ceylan, C.U., Sharma, S., Meurer, M., Knop, M., Chabes, A. and Hombauer, H. (2017) Alterations in cellular metabolism triggered by URA7 or GLN3 inactivation cause imbalanced dNTP pools and increased mutagenesis. *Proc. Natl. Acad. Sci. U.S.A.*, 114, E4442-E4451.

173. Barbari, S.R., Kane, D.P., Moore, E.A. and Shcherbakova, P.V. (2018) Functional analysis of cancer-associated DNA polymerase ε variants in *Saccharomyces cerevisiae*. *G3 (Bethesda)*, 8, 1019-1029.

174. Shlien, A., Campbell, B.B., de Borja, R., Alexandrov, L.B., Merico, D., Wedge, D., Van Loo, P., Tarpey, P.S., Coupland, P., Behjati, S. *et al.* (2015) Combined hereditary and somatic mutations of replication error repair genes result in rapid onset of ultra-hypermutated cancers. *Nat. Genet.*, 47, 257-262.

175. Goldsby, R.E., Lawrence, N.A., Hays, L.E., Olmsted, E.A., Chen, X., Singh, M. and Preston, B.D. (2001) Defective DNA polymerase-δ proofreading causes cancer susceptibility in mice. *Nat. Med.*, 7, 638-639.

176. Goldsby, R.E., Hays, L.E., Chen, X., Olmsted, E.A., Slayton, W.B., Spangrude, G.J. and Preston, B.D. (2002) High incidence of epithelial cancers in mice deficient for DNA polymerase δ proofreading. *Proc. Natl. Acad. Sci. U.S.A.*, 99, 15560-15565.

Appendix A: Yeast strains used to study extrinsic proofreading

	Strain name	Genotype	Source
Single-mutant strains crossed to make diploids	CB404	*MATα ade5-1 lys2::InsE$_{A14}$ trp1-289 his7-2 leu2-3,112 ura3-52 pol2-4*	This work
	CB405	*MATα ade5-1 lys2::InsE$_{A14}$ trp1-289 his7-2 leu2-3,112 ura3-52 pol2-M644G*	This work
	CB414	*MAT**a** ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 pol3-D520V*	This work
	CB415	*MAT**a** ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 pol3-L612M*	This work
Relevant genotypes of segregants used to measure mutation rates	CB420	(*POL2 POL3*)	This work
	CB421	*pol2-4*	This work
	CB422	*pol2-M644G*	This work
	CB423	*pol3-D520V*	This work
	CB424	*pol3-L612M*	This work
	CB425	*pol2-4 pol3-L612M*	This work
	CB426	*pol2-M644G pol3-D520V*	This work

Appendix B: Yeast strains used for replicon studies

	Strain name	Genotype	Source
ura3-29 reporter strains *POL2*	CG379Δ n306::ura3-29inv or1	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ hbn1Δ::ura3-29::LEU2or1*	Kochenova and Shcherbakova, unpublished
	CG379Δ n306::ura3-29inv or2	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ hbn1Δ::ura3-29::LEU2or2*	Kochenova and Shcherbakova, unpublished
	CG379-3-29RL	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ bik1Δ::ura3-29RL*	(58,143)
	CG29LR	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ bik1Δ::ura3-29LR*	(58)
	CB105	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ his4Δ::ura3-29::LEU2or1*	This work
	CB106	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ his4Δ::ura3-29::LEU2or2*	This work
	CB107	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ ste50Δ::ura3-29::LEU2or1*	This work
	CB108	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ ste50Δ::ura3-29::LEU2or2*	This work
	CB109	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ lsb5Δ::ura3-29::LEU2or1*	This work
	CB110	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ lsb5Δ::ura3-29::LEU2or2*	This work
	CG379Δ atg22::ura3-29 or1	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ atg22Δ::ura3-29::LEU2or1*	Kochenova and Shcherbakova, unpublished
	CG379Δ atg22::ura3-29 or2	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ atg22Δ::ura3-29::LEU2or2*	Kochenova and Shcherbakova, unpublished

	Strain name	Genotype	Source
ura3-29 reporter strains *pol2-4*	CG379Δ n306::ura3-29inv or1 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ hbn1Δ::ura3-29::LEU2or1 pol2-4*	This work
	CG379Δ n306::ura3-29inv or2 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ hbn1Δ::ura3-29::LEU2or2 pol2-4*	This work
	CG379-3-29RL pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ bik1Δ::ura3-29RL pol2-4*	This work
	CG29LR pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ bik1Δ::ura3-29LR pol2-4*	This work
	CB105 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ his4Δ::ura3-29::LEU2or1 pol2-4*	This work
	CB106 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ his4Δ::ura3-29::LEU2or2 pol2-4*	This work
	CB107 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ ste50Δ::ura3-29::LEU2or1 pol2-4*	This work
	CB108 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ ste50Δ::ura3-29::LEU2or2 pol2-4*	This work
	CB109 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ lsb5Δ::ura3-29::LEU2or1 pol2-4*	This work
	CB110 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ lsb5Δ::ura3-29::LEU2or2 pol2-4*	This work
	CG379Δ atg22::ura3-29 or1 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ atg22Δ::ura3-29::LEU2or1 pol2-4*	This work
	CG379Δ atg22::ura3-29 or2 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ atg22Δ::ura3-29::LEU2or2 pol2-4*	This work

	Strain name	Genotype	Source
ura3-29 reporter strains *pol2-P301R*	CG379Δ n306::ura3-29inv or1 pol2-P301R	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ hbn1Δ::ura3-29::LEU2or1 pol2-P301R*	This work
	CG379Δ n306::ura3-29inv or2 pol2-P301R	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ hbn1Δ::ura3-29::LEU2or2 pol2-P301R*	This work
	CG379-3-29RL pol2-P301R	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ bik1Δ::ura3-29RL pol2-P301R*	This work
	CG29LR pol2-P301R	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ bik1Δ::ura3-29LR pol2-P301R*	This work
	CB105 pol2-P301R	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ his4Δ::ura3-29::LEU2or1 pol2-P301R*	This work
	CB106 pol2-P301R	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ his4Δ::ura3-29::LEU2or2 pol2-P301R*	This work
	CB107 pol2-P301R	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ ste50Δ::ura3-29::LEU2or1 pol2-P301R*	This work
	CB108 pol2-P301R	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ ste50Δ::ura3-29::LEU2or2 pol2-P301R*	This work
	CB109 pol2-P301R	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ lsb5Δ::ura3-29::LEU2or1 pol2-P301R*	This work
	CB110 pol2-P301R	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ lsb5Δ::ura3-29::LEU2or2 pol2-P301R*	This work
	CG379Δ atg22::ura3-29 or1 pol2-P301R	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ atg22Δ::ura3-29::LEU2or1 pol2-P301R*	This work
	CG379Δ atg22::ura3-29 or2 pol2-P301R	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ atg22Δ::ura3-29::LEU2or2 pol2-P301R*	This work

	Strain name	Genotype	Source
ura3-24 reporter strains *POL2*	CB201	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ hbn1Δ::ura3-24::LEU2or1*	This work
	CB202	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ hbn1Δ::ura3-24::LEU2or2*	This work
	CB203	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ bik1Δ::ura3-24::LEU2or1*	This work
	CB204	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ bik1Δ::ura3-24::LEU2or2*	This work
	CB205	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ his4Δ::ura3-24::LEU2or1*	This work
	CB206	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ his4Δ::ura3-24::LEU2or2*	This work
	CB207	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ ste50Δ::ura3-24::LEU2or1*	This work
	CB208	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ ste50Δ::ura3-24::LEU2or2*	This work
	CB209	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ lsb5Δ::ura3-24::LEU2or1*	This work
	CB210	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ lsb5Δ::ura3-24::LEU2or2*	This work
	CB211	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ atg22Δ::ura3-24::LEU2or1*	This work
	CB212	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ atg22Δ::ura3-24::LEU2or2*	This work

	Strain name	Genotype	Source
ura3-24 reporter strains *pol2-4*	CB201 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ hbn1Δ::ura3-24::LEU2or1 pol2-4*	This work
	CB202 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ hbn1Δ::ura3-24::LEU2or2 pol2-4*	This work
	CB203 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ bik1Δ::ura3-24::LEU2or1 pol2-4*	This work
	CB204 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ bik1Δ::ura3-24::LEU2or2 pol2-4*	This work
	CB205 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ his4Δ::ura3-24::LEU2or1 pol2-4*	This work
	CB206 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ his4Δ::ura3-24::LEU2or2 pol2-4*	This work
	CB207 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ ste50Δ::ura3-24::LEU2or1 pol2-4*	This work
	CB208 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ ste50Δ::ura3-24::LEU2or2 pol2-4*	This work
	CB209 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ lsb5Δ::ura3-24::LEU2or1 pol2-4*	This work
	CB210 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ lsb5Δ::ura3-24::LEU2or2 pol2-4*	This work
	CB211 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ atg22Δ::ura3-24::LEU2or1 pol2-4*	This work
	CB212 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ atg22Δ::ura3-24::LEU2or2 pol2-4*	This work

	Strain name	Genotype	Source
ura3-24 reporter strains *msh6Δ POL2*	CB301	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ hbn1Δ::ura3-24::LEU2or1 msh6Δ::kanMX*	This work
	CB302	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ hbn1Δ::ura3-24::LEU2or2 msh6Δ::kanMX*	This work
	CB303	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ bik1Δ::ura3-24::LEU2or1 msh6Δ::kanMX*	This work
	CB304	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ bik1Δ::ura3-24::LEU2or2 msh6Δ::kanMX*	This work
	CB305	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ his4Δ::ura3-24::LEU2or1 msh6Δ::kanMX*	This work
	CB306	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ his4Δ::ura3-24::LEU2or2 msh6Δ::kanMX*	This work
	CB307	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ ste50Δ::ura3-24::LEU2or1 msh6Δ::kanMX*	This work
	CB308	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ ste50Δ::ura3-24::LEU2or2 msh6Δ::kanMX*	This work
	CB309	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ lsb5Δ::ura3-24::LEU2or1 msh6Δ::kanMX*	This work
	CB310	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ lsb5Δ::ura3-24::LEU2or2 msh6Δ::kanMX*	This work
	CB311	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ atg22Δ::ura3-24::LEU2or1 msh6Δ::kanMX*	This work
	CB312	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ atg22Δ::ura3-24::LEU2or2 msh6Δ::kanMX*	This work

	Strain name	Genotype	Source
ura3-24 reporter strains *msh6Δ pol2-4*	CB301 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ hbn1Δ::ura3-24::LEU2or1 msh6Δ::kanMX pol2-4*	This work
	CB302 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ hbn1Δ::ura3-24::LEU2or2 msh6Δ::kanMX pol2-4*	This work
	CB303 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ bik1Δ::ura3-24::LEU2or1 msh6Δ::kanMX pol2-4*	This work
	CB304 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ bik1Δ::ura3-24::LEU2or2 msh6Δ::kanMX pol2-4*	This work
	CB305 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ his4Δ::ura3-24::LEU2or1 msh6Δ::kanMX pol2-4*	This work
	CB306 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ his4Δ::ura3-24::LEU2or2 msh6Δ::kanMX pol2-4*	This work
	CB307 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ ste50Δ::ura3-24::LEU2or1 msh6Δ::kanMX pol2-4*	This work
	CB308 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ ste50Δ::ura3-24::LEU2or2 msh6Δ::kanMX pol2-4*	This work
	CB309 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ lsb5Δ::ura3-24::LEU2or1 msh6Δ::kanMX pol2-4*	This work
	CB310 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ lsb5Δ::ura3-24::LEU2or2 msh6Δ::kanMX pol2-4*	This work
	CB311 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ atg22Δ::ura3-24::LEU2or1 msh6Δ::kanMX pol2-4*	This work
	CB312 pol2-4	*MATα ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3Δ atg22Δ::ura3-24::LEU2or2 msh6Δ::kanMX pol2-4*	This work

Appendix C: Yeast strains used to study correction of Polε-P301R errors

	Strain name	Genotype	Source
Haploid strains used to make diploids	TM30	*MAT***a** *ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::Kl.LEU2*	(134)
	CB401	*MAT***a** *ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::Kl.LEU2 pol2-4*	This work
	CB402	*MAT***a** *ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::Kl.LEU2 pol2-P301R*	This work
	CB403	*MAT***a** *ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::Kl.LEU2 pol3-D520V*	This work
	TM44	*MATα ade5-1 lys2::InsE$_{A14}$ trp1-289 his7-2 leu2-3,112 ura3-52 can1Δ::loxP*	(134)
	CB323	*MATα ade5-1 lys2::InsE$_{A14}$ trp1-289 his7-2 leu2-3,112 ura3-52 can1Δ::loxP msh6Δ::kanMX*	This work
	CB411	*MATα ade5-1 lys2::InsE$_{A14}$ trp1-289 his7-2 leu2-3,112 ura3-52 can1Δ::loxP pol2-4*	This work
	CB412	*MATα ade5-1 lys2::InsE$_{A14}$ trp1-289 his7-2 leu2-3,112 ura3-52 can1Δ::loxP pol2-P301R*	This work
	CB413	*MATα ade5-1 lys2::InsE$_{A14}$ trp1-289 his7-2 leu2-3,112 ura3-52 can1Δ::loxP pol3-D520V*	This work

	Strain name	Genotype	Source
pBL304 plasmid loss strains	CB615	*MAT**a**/MATα ade5-1/ade5-1 lys2-InsE$_{A14}$/lys2-Tn5-13 trp1-289/trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-4 can1Δ::loxP/CAN1::Kl.LEU2 pol2-P301R/pol2-P301R pol3-D520V/pol3-D520V [pBL304]*	This work
	CB616	*MAT**a**/MATα ade5-1/ade5-1 lys2-InsE$_{A14}$/lys2-Tn5-13 trp1-289/trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-4 can1Δ::loxP/CAN1::Kl.LEU2 pol3-D520V/pol3-D520V [pBL304]*	This work
	CB617	*MAT**a**/MATα ade5-1/ade5-1 lys2-InsE$_{A14}$/lys2-Tn5-13 trp1-289/trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-4 can1Δ::loxP/CAN1::Kl.LEU2 pol2-P301R/pol2-P301R [pBL304]*	This work
	CB618	*MAT**a**/MATα ade5-1/ade5-1 lys2-InsE$_{A14}$/lys2-Tn5-13 trp1-289/trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-4 can1Δ::loxP/CAN1::Kl.LEU2 [pBL304]*	This work

	Strain name	Genotype	Source
Interaction of Polδ proofreading deficiency and Polε variants	CB511	*MAT**a**/MATα ade5-1/ade5-1 lys2-InsE$_{A14}$/lys2-Tn5-13 trp1-289/trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-4 can1Δ::loxP/CAN1::KI.LEU2 pol2-4/POL2 pol3-D520V/POL3*	This work
	CB512	*MAT**a**/MATα ade5-1/ade5-1 lys2-InsE$_{A14}$/lys2-Tn5-13 trp1-289/trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-4 can1Δ::loxP/CAN1::KI.LEU2 pol2-P301R/POL2 pol3-D520V/POL3*	This work
	CB513	*MAT**a**/MATα ade5-1/ade5-1 lys2-InsE$_{A14}$/lys2-Tn5-13 trp1-289/trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-4 can1Δ::loxP/CAN1::KI.LEU2 pol2-4/POL2*	This work
	CB514	*MAT**a**/MATα ade5-1/ade5-1 lys2-InsE$_{A14}$/lys2-Tn5-13 trp1-289/trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-4 can1Δ::loxP/CAN1::KI.LEU2 pol2-P301R/POL2*	This work
	CB515	*MAT**a**/MATα ade5-1/ade5-1 lys2-InsE$_{A14}$/lys2-Tn5-13 trp1-289/trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-4 can1Δ::loxP/CAN1::KI.LEU2 pol3-D520V/POL3*	This work
	CB613	*MAT**a**/MATα ade5-1/ade5-1 lys2-InsE$_{A14}$/lys2-Tn5-13 trp1-289/trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-4 can1Δ::loxP/CAN1::KI.LEU2 pol3-D520V/pol3-D520V*	This work
	CB711	*MAT**a**/MATα ade5-1/ade5-1 lys2-InsE$_{A14}$/lys2-Tn5-13 trp1-289/trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-4 can1Δ::loxP/CAN1::KI.LEU2 pol2-4/POL2 pol3-D520V/pol3-D520V*	This work
	CB712	*MAT**a**/MATα ade5-1/ade5-1 lys2-InsE$_{A14}$/lys2-Tn5-13 trp1-289/trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-4 can1Δ::loxP/CAN1::KI.LEU2 pol2-P301R/POL2 pol3-D520V/pol3-D520V*	This work

	Strain name	Genotype	Source
Interaction of MMR deficiency and polymerase variants	TM63	*MAT**a**/MATα ade5-1/ade5-1 lys2-InsE$_{A14}$/lys2-Tn5-13 trp1-289/trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-4 can1Δ::loxP/CAN1::Kl.LEU2*	(134)
	CB326	*MAT**a**/MATα ade5-1/ade5-1 lys2-InsE$_{A14}$/lys2-Tn5-13 trp1-289/trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-4 can1Δ::loxP/CAN1::Kl.LEU2 msh6Δ::kanMX/msh6Δ::kanMX*	This work
	CB331	*MAT**a**/MATα ade5-1/ade5-1 lys2-InsE$_{A14}$/lys2-Tn5-13 trp1-289/trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-4 can1Δ::loxP/CAN1::Kl.LEU2 msh6Δ::kanMX/msh6Δ::kanMX POL2/pol2-P301R*	This work
	CB341	*MAT**a**/MATα ade5-1/ade5-1 lys2-InsE$_{A14}$/lys2-Tn5-13 trp1-289/trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-4 can1Δ::loxP/CAN1::Kl.LEU2 msh6Δ::kanMX/msh6Δ::kanMX POL2/pol2-4*	This work
	CB352	*MAT**a**/MATα ade5-1/ade5-1 lys2-InsE$_{A14}$/lys2-Tn5-13 trp1-289/trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-4 can1Δ::loxP/CAN1::Kl.LEU2 msh6Δ::kanMX/msh6Δ::kanMX pol3-D520V/pol3-D520V*	This work