

Emergence of DNA Polymerase ϵ Antimutators That Escape Error-Induced Extinction in Yeast

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ABSTRACT DNA polymerases (Pols) ϵ and δ perform the bulk of yeast leading- and lagging-strand DNA synthesis. Both Pols possess intrinsic proofreading exonucleases that edit errors during polymerization. Rare errors that elude proofreading are extended into duplex DNA and excised by the mismatch repair (MMR) system. Strains that lack Pol proofreading or MMR exhibit a 10- to 100-fold increase in spontaneous mutation rate (mutator phenotype), and inactivation of both Pol δ proofreading (*pol3-01*) and MMR is lethal due to replication error-induced extinction (EEX). It is unclear whether a similar synthetic lethal relationship exists between defects in Pol ϵ proofreading (*pol2-4*) and MMR. Using a plasmid-shuffling strategy in haploid *Saccharomyces cerevisiae*, we observed synthetic lethality of *pol2-4* with alleles that completely abrogate MMR (*msh2 Δ* , *mlh1 Δ* , *msh3 Δ msh6 Δ* , or *pms1 Δ mlh3 Δ*) but not with partial MMR loss (*msh3 Δ* , *msh6 Δ* , *pms1 Δ* , or *mlh3 Δ*), indicating that high levels of unrepaired Pol ϵ errors drive extinction. However, variants that escape this error-induced extinction (eex mutants) frequently emerged. Five percent of *pol2-4 msh2 Δ* eex mutants encoded second-site changes in Pol ϵ that reduced the *pol2-4* mutator phenotype between 3- and 23-fold. The remaining eex alleles were extragenic to *pol2-4*. The locations of antimutator amino-acid changes in Pol ϵ and their effects on mutation spectra suggest multiple mechanisms of mutator suppression. Our data indicate that unrepaired leading- and lagging-strand polymerase errors drive extinction within a few cell divisions and suggest that there are polymerase-specific pathways of mutator suppression. The prevalence of suppressors extragenic to the Pol ϵ gene suggests that factors in addition to proofreading and MMR influence leading-strand DNA replication fidelity.

ORGANISMS must accurately duplicate their genomes to avoid loss of long-term fitness. Consequently, cells employ high-fidelity DNA polymerases (Pols) equipped with proofreading exonucleases to replicate their DNA (reviewed in McCulloch and Kunkel 2008; Reha-Krantz 2010). Mismatch repair (MMR) further ensures the integrity of genetic information by targeting mismatches for excision from newly replicated DNA (reviewed in Kolodner and Marsischky 1999; Iyer *et al.* 2006; Hsieh and Yamane 2008). These polymerase error-correcting mechanisms, together with DNA damage repair (Friedberg *et al.* 2006), maintain the genome with less than one mutation per 10^9 nucleotides per cell division (Drake *et al.* 1998). Defects in proofreading or MMR result in mutator phenotypes characterized by increased rates of spontaneous

mutation (Kolodner and Marsischky 1999; Iyer *et al.* 2006; Hsieh and Yamane 2008; McCulloch and Kunkel 2008; Reha-Krantz 2010).

Mutator phenotypes can be an important source of genetic diversity, which facilitates adaptation to environmental change. In bacterial and yeast populations, unstable environments favor mutator strains that readily acquire adaptive mutations (Chao and Cox 1983; Mao *et al.* 1997; Sniegowski *et al.* 1997; Giraud *et al.* 2001a; Notley-McRobb *et al.* 2002; Nilsson *et al.* 2004; Thompson *et al.* 2006; Desai *et al.* 2007). In mammals, mutator phenotypes are proposed to accelerate the process of somatic cell evolution during tumorigenesis (Loeb *et al.* 1974, 2008). Deep sequencing of spontaneous tumors provides evidence for a mutator phenotype (Fox *et al.* 2009; Loeb 2011), and genetic defects in MMR or Pol proofreading elevate cancer susceptibility (Wei *et al.* 2002; Peltomäki 2005; Preston *et al.* 2010). However, mutator phenotypes do not persist indefinitely. Loss of fitness accompanies sustained expression of a mutator phenotype in a variety of organisms, including viruses (Smith *et al.* 2005), bacteria (Funchain *et al.* 2000; Giraud *et al.* 2001a), yeast (Wloch

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et al. 2001; Zeyl and De Visser 2001; Herr *et al.* 2011a), worms (Estes *et al.* 2004), and mammals (Albertson *et al.* 2009). Thus, following adaptation, selection pressure favors restoration of low mutation rates, which can occur through the elimination of mutator alleles or the acquisition of mutator suppressors (*i.e.*, antimutators). A limited number of antimutator variants in the DNA replication machinery have been described (reviewed in Herr *et al.* 2011b).

In the budding yeast *Saccharomyces cerevisiae*, DNA polymerases epsilon (Pol ϵ) and delta (Pol δ) are thought to perform the bulk of leading- and lagging-strand DNA synthesis, respectively (Pursell *et al.* 2007; Kunkel and Burgers 2008; Nick McElhinny *et al.* 2008; Larrea *et al.* 2010; Pavlov and Shcherbakova 2010). Both polymerases are accurate and possess intrinsic proofreading exonucleases that edit mispaired primer termini during polymerization (Morrison *et al.* 1991; Simon *et al.* 1991; Shimizu *et al.* 2002; Shcherbakova *et al.* 2003; Fortune *et al.* 2005). Defects in Pol ϵ or Pol δ proofreading increase the spontaneous mutation rate in a manner consistent with major roles for these polymerases in leading- and lagging-strand synthesis (Morrison *et al.* 1991; Simon *et al.* 1991; Morrison and Sugino 1994; Shcherbakova *et al.* 1996; Tran *et al.* 1999; Karthikeyan *et al.* 2000; Greene and Jinks-Robertson 2001). Interestingly, the Pol δ proofreading defect generates a mutator phenotype 5- to 30-fold greater than that observed in Pol ϵ proofreading-deficient strains (Morrison and Sugino 1994; Shcherbakova *et al.* 1996; Tran *et al.* 1999; Datta *et al.* 2000; Karthikeyan *et al.* 2000; Greene and Jinks-Robertson 2001; Pavlov *et al.* 2004), and the spectra of spontaneous mutations that arise in Pol ϵ and Pol δ proofreading-deficient strains differ, which may reflect distinct error specificities of the polymerases as well as strand-specific effects (Morrison and Sugino 1994; Karthikeyan *et al.* 2000; Pavlov *et al.* 2002, 2003; Shcherbakova *et al.* 2003; Fortune *et al.* 2005). Mouse cells with defects in Pol ϵ or Pol δ proofreading also exhibit increased mutation rates (Goldsby *et al.* 2002; Albertson *et al.* 2009), and, consistent with distinct roles in DNA replication, the types of tumors that develop in Pol ϵ and Pol δ proofreading-deficient mice differ markedly (Albertson *et al.* 2009). Thus, avoidance of errors during eukaryotic DNA replication depends on both Pol ϵ and Pol δ proofreading (McCulloch and Kunkel 2008; Preston *et al.* 2010).

The extent to which Pol ϵ and Pol δ proofreading contribute to DNA replication fidelity is obscured by MMR. The eukaryotic MMR machinery consists of homologs of bacterial MutS and MutL proteins (reviewed in Iyer *et al.* 2006; Hsieh and Yamane 2008). MutS homolog 2 (*Msh2*) associates with *Msh6* or *Msh3* to form two different heterodimers with partially overlapping activities. *Msh2-Msh6* recognizes and binds to base-base and small insertion/deletion mispairs, while *Msh2-Msh3* recognizes small and larger insertion/deletion mispairs and a subset of base-base mispairs (Harrington and Kolodner 2007). Once bound to mismatched DNA, the Msh proteins recruit heterodimers of MutL homologs (Mlh). *Mlh1* is the common subunit for two complexes. *Mlh1-Pms1* (Mlh1-Pms2 in mammals) functions with

both *Msh2-Msh6* and *Msh2-Msh3*, while *Mlh1-Mlh3* works primarily with *Msh2-Msh3*. *Pms1* and *Mlh3* contain latent endonucleases that cleave the nascent DNA strand, providing entry points for removal of mismatches and error-free DNA resynthesis (Kadyrov *et al.* 2006, 2007; Nishant *et al.* 2008).

Consistent with these biochemical properties, genetic studies in yeast reveal overlapping mutator phenotypes when individual MMR genes are deleted. Deletion of *MSH2* eliminates both *Msh6*- and *Msh3*-dependent repair, effectively abrogating MMR and conferring a strong base-substitution and frameshift mutator phenotype. Deletion of *MSH6* or *MSH3* alone only partially inactivates MMR. *msh6* Δ mutants are strong base-substitution but weak frameshift mutators, *msh3* Δ strains are weak frameshift and duplication/deletion mutators (with increases in some base substitutions), and *msh3* Δ *msh6* Δ double mutants recapitulate the strong mutator phenotype of *msh2* Δ (Reenan and Kolodner 1992; New *et al.* 1993; Johnson *et al.* 1996; Marsischky *et al.* 1996; Greene and Jinks-Robertson 1997; Sia *et al.* 1997; Flores-Rozas and Kolodner 1998; Tran *et al.* 1999; Harrington and Kolodner 2007). Similar to *msh2* Δ , deletion of *MLH1* inactivates MMR, resulting in a strong base-substitution and frameshift mutator phenotype. *pms1* Δ mutants are also strong base-substitution and frameshift mutators, while *mlh3* Δ strains are weak frameshift and duplication/deletion mutators (Williamson *et al.* 1985; Strand *et al.* 1993; Prolla *et al.* 1994; Greene and Jinks-Robertson 1997; Flores-Rozas and Kolodner 1998; Yang *et al.* 1999; Harfe *et al.* 2000; Harrington and Kolodner 2007). Yeast *pms1* Δ *mlh3* Δ double mutants have strong mutator phenotypes, similar to or stronger than *pms1* Δ and *mlh1* Δ single mutants (Flores-Rozas and Kolodner 1998). In mice, deletion of both *Mlh3* and *Pms2* (equivalent to yeast *PMS1*) is required to recapitulate the strong mutator and cancer phenotypes caused by deletion of *Mlh1* alone (Chen *et al.* 2005). Collectively, these studies indicate that *Msh2-Msh6* and *Mlh1-Pms1* (*Mlh1-Pms2* in mammals) are the primary complexes that function in eukaryotic MMR, while *Msh2-Msh3* and *Mlh1-Mlh3* play important secondary roles.

Elimination of MMR in Pol ϵ or Pol δ proofreading-deficient cells results in a multiplicative increase in mutation rate in diploid yeast, suggesting that proofreading and MMR act in series to correct polymerase errors (Morrison *et al.* 1993; Morrison and Sugino 1994). In haploids, combined inactivation of Pol δ proofreading (via the *pol3-01* allele) and any one of several MMR components (*msh6* Δ , *msh2* Δ , or *pms1* Δ) is lethal, presumably due to unrestrained mutagenesis during replication (Morrison *et al.* 1993; Tran *et al.* 1999; Greene and Jinks-Robertson 2001). We recently used a collection of *pol3* mutator alleles to define the maximal mutation rate compatible with haploid yeast viability (Herr *et al.* 2011a). Cell populations become inviable when mutation rates exceed $\sim 10^{-3}$ inactivating mutations/gene/cell division. This “error-induced extinction” (EEX) phenotype is readily suppressed by antimutator mutations encoding amino-acid substitutions in the catalytic subunit of Pol δ ,

as well as suppressor mutations in undefined genes. Thus, variants that escape error-induced extinction (*eex* mutants) provide a means to probe mechanisms of adaptation to mutator phenotypes (Herr *et al.* 2011a,b).

Whether Pol ϵ errors are also sufficient to trigger error-induced extinction remains unclear. Morrison and Sugino (1994) reported that the *pol2-4* allele, which inactivates Pol ϵ proofreading, was not synthetically lethal with *pms1* Δ in haploid yeast, but noted that the resulting colonies were heterogeneous in size and grew slowly. Interestingly, *pol2-4 pms1* Δ isolates exhibited varying mutation rates, suggesting that mutator suppressors may arise in these strong mutator strains. Tran *et al.* (1999) also described haploid strains with Pol ϵ proofreading and MMR defects: *pol2-4 msh2* Δ and *pol2-4 msh3* Δ *msh6* Δ . Mutation rate increases relative to *pol2-4*, *msh2* Δ , and *msh3* Δ *msh6* Δ strains were consistent with a multiplicative relationship between MMR and Pol ϵ proofreading (Tran *et al.* 1999). However, Greene and Jinks-Robertson (2001) later reported that they could not obtain a viable *pol2-4 msh2* Δ strain using either gene disruption or plasmid-shuffling methods. Thus, it remains unresolved whether the magnitude of Pol ϵ errors is sufficient for error-induced extinction.

Here, we show that defective Pol ϵ proofreading is lethal to haploid yeast in the absence of MMR, providing evidence that, when left unrepaired, leading-strand errors exceed a mutation threshold. Moreover, we show that spontaneous mutants escape this Pol ϵ error-induced extinction and that *eex* alleles function as antimutators. We discuss possible mechanisms of escape and mutator suppression. Our studies corroborate the unstable nature of mutators and provide a tractable system to investigate adaptive antimutator mutations that influence leading-strand DNA replication fidelity.

Materials and Methods

Media and growth conditions

Standard media and growth conditions were used in the propagation of yeast strains (Sherman 2002). Cells were grown nonselectively using YPD or synthetic complete (SC) media with 2% dextrose. Selective growth was on SC media containing 2% dextrose and lacking the appropriate amino acid(s). Preformulated SC amino acid supplement was purchased from Bufferad, and supplements lacking defined amino acids were made from individual components as described (Sherman 2002). *URA3*-deficient cells were selected with 5-fluoroorotic acid (FOA, 1 mg/ml; Zymo Research) media (Boeke *et al.* 1984). *can1* mutants were selected on SC lacking arginine and supplemented with 60 μ g/ml of canavanine. Unless otherwise specified, reagents were purchased from Sigma-Aldrich or Fisher Scientific.

Plasmids and strain constructions

***POL2* plasmids:** pRS416, a *CEN6/ARS4/URA3* plasmid (Brachmann *et al.* 1998), was engineered to carry the wild-type *POL2* gene under control of its native promoter. The

genomic sequence of *POL2* was amplified from BY4733 yeast using Expand Hi-Fidelity DNA polymerase (Roche) and the following primers and PCR conditions: *Pol2-XhoI* (5'-ACTCGGTACTCGAGGCGCTCTGCCCTAGTTGGAATG-3'; *XhoI* site underlined) and *PolED2* (5'-GATATTCCGAGCTCGCAACTTCCGGAGTGGTCAC-3'; *SacI* site underlined); 94°, 1 min; 29 \times (94°, 15 sec; 58°, 20 sec; 68°, 8 min); 68°, 16 min. The resulting 7.5-kb fragment and pRS416 were digested with *SacI* and *XhoI*, ligated together with T4 DNA ligase (Gibco BRL), and then introduced into *Escherichia coli*. Transformed clones were isolated, and a correct *POL2*-containing plasmid was confirmed by sequencing the entire insert. This vector (pRS416*POL2-5'YIF1*) did not fully complement the growth deficiency of our *pol2* Δ mutants. pRS416*POL2-5'YIF1* contains the entire *POL2* coding sequence as well as 592 bp of upstream sequence, including 371 bp of noncoding sequence containing the promoter and 221 bp of the 5' end of the *YIF1* gene, transcribed in the opposite direction. We hypothesized that transcription of the truncated *YIF1* gene may suppress *POL2* expression in our vector. Thus, we eliminated the *YIF1* sequences by replacing the *XhoI-SalI* 5' fragment of pRS416*POL2-5'YIF1* with DNA amplified from this plasmid using PCR primers *Pol2-7386bp-XhoIF* (5'-ATGACTCGAGGTATGGGCCCTTTGGTTTTTCGT-3') and *Pol2-8161bpR* (5'-GTTACACGCAATAAAGAAGTATGG-3'). The PCR product and pRS416*POL2-5'YIF1* were digested with *Bam*HI and *SalI* and ligated together, and *E. coli* were transformed with the ligation product. The entire *POL2* gene from a transformant was again sequenced to verify its integrity, and this new vector (pRS416*POL2*) fully complemented the growth deficiency of *pol2* Δ yeast. We subcloned the functional *POL2* fragment from pRS416*POL2* into the *XhoI* and *SalI* sites of the related plasmid pRS415 (*CEN6/ARS4/LEU2*) (Brachmann *et al.* 1998) to obtain pRS415*POL2*. pRS416*POL2* and pRS415*POL2* contain the full-length *POL2* coding sequence plus 368 bp upstream of the *POL2* start site (corresponding to nucleotides 147844 to 155125 of yeast chromosome XIV). The *pol2-4* mutation and all *eex* mutations were introduced into pRS415*POL2* using the primers listed in supporting information, Table S1 (see also References for Supporting Tables) the QuikChange protocol (Wang and Malcolm 1999); Phusion polymerase (New England Biolabs); and the following PCR conditions: 95°, 1 min; 16 \times (95°, 40 sec; 53°, 1 min; 68°, 7 min). The entire *pol2* gene was sequenced in each case to verify the presence of desired mutations and the absence of other mutations. All Pol δ -expressing plasmids are previously described (Herr *et al.* 2011a). pRS vectors (Brachmann *et al.* 1998) were used as templates for chromosomal gene disruptions (see below). pUG6 served as a template for *kanMX* (Guldener *et al.* 1996) and pFv199 as a template for *natMX* (Stulemeijer *et al.* 2011).

Strains: BY4733 and Y7092 haploid yeast strains were engineered to carry alleles of DNA polymerase and MMR genes (Table S2). BY4733 (*MATa leu2* Δ 0 *ura3* Δ 0 *met15* Δ 0 *trp1* Δ 63 *his3* Δ 200), a S288C descendant (Brachmann *et al.*

1998), was obtained by sporulating a BY4733 × BY4734 diploid (kindly provided by Tim Formosa, University of Utah). All engineered BY4733 strains (Table S2) originated from the same spore. Y7092 (*MAT α can1 Δ ::STE2pr_his5 lyp1 Δ ura3 Δ 0 leu2 Δ 0 his3 Δ 1 met15 Δ 0*), also a S288C descendant, is a BY4742 derivative (Brachmann *et al.* 1998) modified by Boone and colleagues to use in synthetic genetic array analyses (Tong and Boone 2007). Chromosomal gene disruptions were made using PCR products generated with Phusion polymerase (New England Biolabs) and the primers, templates, and PCR conditions indicated in Table S3. Yeast were transformed with the resulting PCR products using lithium acetate transformation (Gietz and Woods 2002). Cells from transformant colonies were treated with Zymolyase (ICN Biomedicals; 50 units/ml in 10 mM Tris-HCl/0.1 mM EDTA, pH 7.5, at 37° for 30 min and then at 95° for 10 min) and subjected to junction-specific PCR with primers in the transgenes and flanking endogenous loci to detect correct insertion/deletion mutants (primers and PCR conditions available upon request).

Plasmid shuffling

Plasmid shuffling strains contained either *pol2 Δ ::kanMX* or *pol3 Δ ::HIS3* chromosomal gene disruptions and the *URA3* plasmids pRS416*POL2* or pGL310 (*POL3*) (Simon *et al.* 1991) to provide the essential activity of Pol ϵ or Pol δ (Table S2). Following transformation with CEN/LEU2 plasmids encoding mutant polymerases (YCplac111*pol3-01*, pRS415*pol2-4*, pRS415*pol2-4,eex*, or pRS415*pol2-eex*), transformants were selected on SC media lacking leucine and uracil. YCplac111*POL3*, pRS415*POL2*, and the pRS415-unmodified plasmid were used as positive and negative controls. Colonies (1–2 mm) from the selection plates were picked and suspended in 200 μ l sterile water, and 25 μ l was plated onto FOA-containing media (Boeke *et al.* 1984) in serial dilutions to select for spontaneous loss of the *URA3* plasmid expressing the wild-type polymerase.

eex mutant screen

eex mutants were isolated using the shuffling protocol. *pol2 Δ msh2 Δ* strains with the pRS416*POL2-URA3* plasmid were transformed with pRS415*pol2-4-LEU2*. Forty-eight individual colonies (1–2 mm) were picked from the SC media lacking uracil and leucine transformation plates and separately suspended in 240 μ l sterile water, and 25- μ l aliquots from each independent transformant were spotted in separate patches on FOA-containing media in a 6 × 8 grid. pRS415*POL2-LEU* and unmodified pRS415-*LEU* plasmids were used as positive and negative controls, respectively. After incubation at 30° for 3 days, individual colonies were isolated and genotyped to identify clones carrying the *pol2-4* allele and no wild-type *POL2*. Sequences encoding the Pol ϵ exonuclease domain were amplified using primers Pol2-4U (5'-ATAACACTCTCAGGGGACAAGTATAT-3') and Pol2s5 (5'-AGAATATTCGGAAAGGTGCTG-3') and the following PCR conditions: 98°, 1 min; 30× (98°, 10 sec; 54°, 60 sec;

72°, 90 sec); 72°, 60 sec. PCR products were then digested with Alu1 at 37° for at least 6 hr. The wild-type *POL2* allele results in a prominent 824-bp product as well as a number of smaller DNA fragments (275, 114, 84, 79, 53, and 17 bp). The *pol2-4* mutation introduces an additional AluI site within the 824-bp fragment, resulting in 447- and 377-bp products. Using this assay, we eliminated strains with *pol2-4* to *POL2* gene conversions (colonies with only the *POL2* allele) or other mutations (*e.g.*, *ura3*) that allow cells to retain *POL2* when grown on FOA (colonies with both *pol2-4* and *POL2*). Strains containing only the *pol2-4* allele were considered *bona fide eex* mutants. To identify *eex* mutants that are intragenic to *pol2-4*, plasmids were recovered from each mutant, introduced into *E. coli*, purified, and reshuffled into a fresh *pol2 Δ msh2 Δ* yeast strain. Plasmids that retained the ability to induce *pol2-4 msh2 Δ* synthetic lethality were considered to be from strains with an *eex* mutation extragenic to the *pol2-4* plasmid (“chromosomal *eex*”). Plasmids that failed to recapitulate the *pol2-4 msh2 Δ* synthetic lethality were hypothesized to carry *eex* mutations within *pol2-4* (“intragenic *eex*”). These mutations were identified by sequencing the *pol2* gene. All intragenic *eex* alleles were re-engineered into fresh pRS415*POL2* and pRS415*pol2-4* plasmids and reshuffled for final confirmation of suppression. The re-engineered *eex* plasmids were used for all subsequent analyses.

Mutation and suppression rates

Mutation rates: Canavanine resistance (Can^r) mutation rates were measured as described (Herr *et al.* 2011a). Briefly, a freshly streaked *pol2 Δ msh6 Δ* strain containing pRS416*POL2* was transformed with pRS415*POL2*, pRS415*pol2-4*, pRS415*pol2-eex*, or pRS415*pol2-4,eex*, and multiple transformants were plated onto FOA media to obtain shuffled colonies. Twenty-four colonies for each allele were picked, suspended in water, and vigorously vortexed to disperse the cells. Diluted aliquots from each colony were then plated onto SC (to estimate the number of cell divisions during colony formation) and canavanine selection plates (to determine the number of Can^r mutants). Colonies were counted after 3–4 days at 30°. Mutation rates were calculated by the MMS maximum-likelihood method using the web-based program Fluctuation AnaLysis CalculatOR (FALCOR; <http://www.keshavsingh.org/protocols/FALCOR.html>) (Hall *et al.* 2009).

Rates of escape from error-induced extinction: Rates of escape were determined from the plates shown in Figure 3 by counting the number of colonies in grid positions with verified *eex* mutants (patches with colonies that had *pol2-4* → *POL2* gene conversions or other FOA-resistant mutations were not included in this calculation) and the number of colonies from wild-type controls. The shuffling efficiency was assessed by plating aliquots of cells from an individual colony onto FOA (to determine the number of viable cells that lose the *ura3* plasmid) and synthetic complete media

(to determine the total number of viable cells plated). The percentage of cells that lose the *ura3* plasmid (*i.e.*, the shuffling efficiency) was ~1% in our system. Therefore, colony counts were multiplied by 100, and FALCOR was used to calculate the rate of escape from error-induced extinction (Hall *et al.* 2009). Totals from the grid positions with verified *eex* mutants were entered into FALCOR as number of mutants (*r*), and totals from wild-type control positions were entered as number of viable cells (*Nt*). The FALCOR program uses these values to calculate a maximum-likelihood estimate of mutation rate expressed per cell division (see Hall *et al.* 2009 and references therein).

CAN1 mutation spectra

For each strain, ~55 Can^r colonies were isolated from 55 independent shuffling experiments. Cells were treated with Zymolyase, and the *can1* gene was PCR-amplified and sequenced as previously described (Herr *et al.* 2011a). Mutation spectra were compared statistically using iMARS (Morgan and Lewis 2006) and Fisher's exact test.

Results

Synthetic lethal interactions of Pol ε proofreading and MMR defects

To investigate error-induced extinction due to Pol ε replication errors, we introduced *pol2-4*, which encodes proofreading-deficient Pol ε, into newly constructed haploid strains containing deletions of individual MMR genes (*msh2Δ*, *msh3Δ*, *msh6Δ*, *mlh1Δ*, *mlh3Δ*, or *pms1Δ*). A plasmid-shuffling strategy was used, starting with MMR mutant strains that also carry a chromosomal deletion of the Pol ε gene (*pol2Δ*) covered by a *POL2-URA3* plasmid. Following transformation with a *pol2-4-LEU2* plasmid, cells were plated on 5-FOA-containing media to select for spontaneous loss of *POL2-URA3*, and the viabilities of the resultant *pol2-4 mmrΔ* double mutants were assessed following incubation at 30° for 2–3 days.

We observed that *pol2-4* was synthetically lethal with *msh2Δ* or *mlh1Δ*, but not with *msh3Δ*, *msh6Δ*, *pms1Δ*, or *mlh3Δ* (Figure 1). The *pol2-4 msh6Δ* and *pol2-4 pms1Δ* strains exhibited slow-growth phenotypes, as evidenced by the reduced size and number of colonies compared to *POL2 msh6Δ* and *POL2 pms1Δ* strains (Figure 1A). In contrast, when *pol3-01* (which encodes proofreading-deficient Pol δ) was introduced by plasmid shuffling into analogous MMR mutant strains (*pol3Δ mmrΔ*), synthetic lethality occurred with all MMR gene deletions except *msh3Δ* and *mlh3Δ* (Figure 1).

The pattern of *pol2-4* (in)viability with each of the single MMR gene deletions suggested that complete loss of MMR is required for synthetic lethality. To further investigate this relationship, we shuffled *pol2-4* into strains lacking both Msh2 partners (*msh3Δ* and *msh6Δ*) or both Mlh1 partners (*pms1Δ* and *mlh3Δ*). *pol2-4 msh3Δ msh6Δ* and *pol2-4 pms1Δ mlh3Δ* triple mutants were inviable (Figure 1B). Considered together, these data suggest that Msh2-Msh3, Msh2-Msh6,

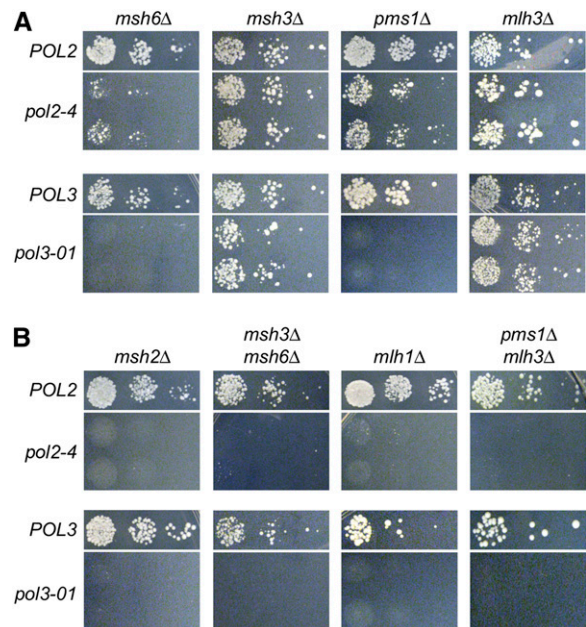


Figure 1 Genetic interactions between Pol ε and Pol δ proofreading and MMR. Using a plasmid-shuffling strategy, proofreading-deficient variants of Pol ε (encoded by *pol2-4*) or Pol δ (encoded by *pol3-01*) or wild-type controls (*POL2* or *POL3*) were introduced via *LEU2* plasmids into BY4733 *pol2Δ* or *pol3Δ* strains harboring deletion mutations that partially (A) or completely (B) abrogate MMR. Serial dilutions were plated on FOA-containing media to select for loss of complementing *POL2-URA3* or *POL3-URA3* plasmids and reveal the synthetic phenotype. *POL* and *MMR* alleles are indicated at left and above the corresponding panels, respectively.

Mlh1-Pms1, and *Mlh1-Mlh3* complexes all play important roles in suppressing lethal Pol ε errors and that the *Msh2-Msh6* and *Mlh1-Pms1* complexes predominate.

The reduced growth of strains lacking Pol ε proofreading and either *Msh2-Msh6* (*msh6Δ*) or *Mlh1-Pms1* (*pms1Δ*) suggests that these strains accumulate deleterious mutations that compromise replicative fitness. *pol2-4 msh6Δ* cells were most compromised (Figure 1A, top left), resembling strong mutator variants previously shown to exist near the maximum tolerated mutation rate of haploid yeast (Herr *et al.* 2011a). *msh2Δ* and *mlh1Δ* are stronger mutator alleles than *msh6Δ* (Greene and Jinks-Robertson 1997; Flores-Rozas and Kolodner 1998; Tran *et al.* 1999; Li *et al.* 2005), suggesting that *pol2-4 msh2Δ* and *pol2-4 mlh1Δ* cells are inviable because they exceed the maximum tolerated rate. Although *pol2-4 msh2Δ* cells did not form visible colonies (Figure 1B, top left), when viewed under the microscope we found abundant microcolonies of ~100 cells. Thus, *pol2-4 msh2Δ* cells initially divide but fail to continue after 6–7 mitotic cycles. This pattern of abortive growth typifies cells undergoing replication error-induced extinction (Morrison *et al.* 1993; Herr *et al.* 2011a).

Pols ζ and η do not mediate pol2-4 msh2Δ synthetic lethality

The mutator phenotypes of many DNA replication mutants are dependent on the specialized DNA polymerase Pol ζ (Shcherbakova *et al.* 1996; Pavlov *et al.* 2001; Kai and Wang

2003; Northam *et al.* 2006; Aksenova *et al.* 2010; Northam *et al.* 2010). This dependency may reflect a role for Pol ζ in rescuing stalled DNA replication forks (Northam *et al.* 2010) and may relate to Pol ζ 's ability to efficiently extend primers with 3'-terminal mismatches (Johnson *et al.* 2000; Guo *et al.* 2001; Haracska *et al.* 2001; Simhadri *et al.* 2002). To determine whether Pol ζ is required for *pol2-4 msh2 Δ* lethal mutagenesis, we used our plasmid-shuffling strategy to introduce *pol2-4* into *msh2 Δ* cells that also lack *REV3* (which encodes the catalytic subunit of Pol ζ). *rev3 Δ* did not rescue *pol2-4 msh2 Δ* synthetic lethality (Figure 2). Similarly, deletion of *RAD30*, which encodes the translesion DNA polymerase η (Waters *et al.* 2009), also failed to rescue *pol2-4 msh2 Δ* synthetic lethality (Figure 2). These data show that Pols ζ and η do not mediate *pol2-4 msh2 Δ* synthetic lethality.

Mutants escape *pol2-4 msh2 Δ* synthetic lethality

We occasionally observed macroscopic colonies that survived when *pol2-4* was shuffled into the *msh2 Δ* , *mlh1 Δ* , *msh3 Δ* , *msh6 Δ* , or *pms1 Δ mlh3 Δ* strains. In light of our recent discovery of Pol δ antimutators (Herr *et al.* 2011a), we hypothesized that these surviving colonies may have acquired suppressor mutations that improve Pol ϵ fidelity. Thus, we systematically screened for *error-induced extinction* (*eex*) mutants that survived *pol2-4 msh2 Δ* synthetic lethality. Using the plasmid-shuffling strategy, multiple independent *pol2-4 msh2 Δ* transformants were separately plated on FOA-containing medium so that rare survivors could be detected and counted (Figure 3, A and B). There was wide fluctuation in the number and size of surviving colonies, suggesting that escape variants arise randomly prior to selection on FOA.

After eliminating clones with *pol2-4* \rightarrow *POL2* gene conversions or other mutations conferring FOA resistance (*e.g.*, *ura3*), 81 independent *eex* mutants were isolated (Table 1). To distinguish between *eex* mutations in the plasmid-borne *pol2-4* gene (intragenic *eex*) and *eex* mutations located elsewhere in the yeast genome (chromosomal *eex*), *pol2-4* plasmids were rescued from the *eex* mutants and used to transform a fresh *msh2 Δ* strain. Among the 81 *eex* mutants characterized, 76 (94%) carried plasmids that were lethal when reshuffled into *msh2 Δ* cells. Thus, the majority of *eex* mutants contained suppressor mutations extragenic to *pol2-4*. Five mutants carried *pol2-4* plasmids that did not induce *pol2-4 msh2 Δ* synthetic lethality. DNA sequencing revealed that, in addition to the *pol2-4* allele, each of these plasmids encoded a single amino-acid substitution in Pol ϵ : G435C, V522A, T850M, K966Q, or A1153D. The amino-acid changes were located in regions that are moderately or highly conserved in DNA polymerases ϵ and δ (Figure 4). When the corresponding mutations were re-engineered into fresh *pol2-4* plasmid backbones and shuffled into *msh2 Δ* cells, they conferred an *eex* phenotype (Figure 5A). *pol2-4,eex msh2 Δ* strains were viable, forming small clearly visible colonies (Figure 5A, right). Moreover, the *eex* alleles completely rescued the slow-growth phenotype of *pol2-4 msh6 Δ* cells (Figure 5A, center). These data show that secondary amino-acid substitutions

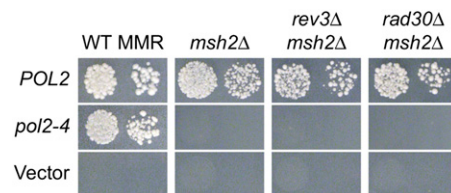


Figure 2 Effects of Pols ζ and η on *pol2-4 msh2 Δ* synthetic lethality. *pol2 Δ msh2 Δ POL2-URA3* cells defective for Pol ζ (*rev3 Δ*) or Pol η (*rad30 Δ*) were transformed with *POL2-* or *pol2-4-LEU2* plasmids, and 10-fold serial dilutions of isolated transformants were plated onto FOA-containing media as in Figure 1. For comparison, the *POL2-* and *pol2-4-LEU2* plasmids were similarly shuffled into strains that were wild type (WT) for Pols ζ and η (*REV3 RAD30*) and either *msh2 Δ* or *MSH2* (WT MMR). The *LEU2* plasmid with no *POL2* or *pol2-4* gene served as the vector-only control. Colony formation was assessed after incubation at 30° for 3 days. Neither *rev3 Δ* nor *rad30 Δ* rescued *pol2-4 msh2 Δ* synthetic lethality.

within proofreading-deficient Pol ϵ can rescue *pol2-4 msh2 Δ* synthetic lethality and restore normal growth to *pol2-4 msh6 Δ* cells.

Strain-dependent differences in *pol2-4 msh2 Δ* suppression

In our screen for *eex* mutants, we used two related yeast strains derived from S288C: BY4733 (Brachmann *et al.* 1998) and Y7092 (Tong and Boone 2007). *eex* alleles intragenic and extragenic to *pol2-4* were recovered from both strains (Table 1). However, BY4733 displayed a reproducibly lower frequency of *pol2-4 msh2 Δ* suppression than Y7092 (compare Figures 3, A and B). Independent *pol2-4 msh2 Δ* clones from either genetic background yielded variable numbers of escape mutants (compare different grid positions on the same plate), further indicating that suppressors originate during clonal expansion prior to plating on FOA. Accordingly, intragenic escape mutants isolated from a common parent clone (*i.e.*, from the same grid position) harbored identical *eex* mutations. Based on these observations, we used fluctuation analyses to calculate the rates of escape from error-induced extinction in each strain. Transformation of *POL2-URA3 msh2 Δ* cells with the wild-type *POL2-LEU2* plasmid served as a control to assess shuffling efficiencies and estimate the number of cell divisions during colony outgrowth (see *Materials and Methods*). Escape mutants arose at a rate of 2×10^{-4} per cell division in the BY4733 strain and at a 20-fold higher rate in the Y7092 strain (4×10^{-3} escape mutants per cell division). These results show that genetic background influences the propensity of strains to escape the synthetic lethal interaction of *pol2-4* and *msh2 Δ* .

To compare rates of escape from Pol ϵ and Pol δ proofreading deficiency, we quantified *eex* mutants in *pol3-01 msh2 Δ* Y7092 cells using an analogous plasmid shuffling strategy (Herr *et al.* 2011a) (Figure 3C). Suppressors arose at a rate 450 times lower in the *pol3-01 msh2 Δ* strain (9×10^{-6} escape mutants per cell division) compared to *pol2-4 msh2 Δ* (4×10^{-3}) in the same genetic background. Thus, escape from error-induced extinction occurs more readily in Pol ϵ than Pol δ proofreading-deficient *msh2 Δ* cells.

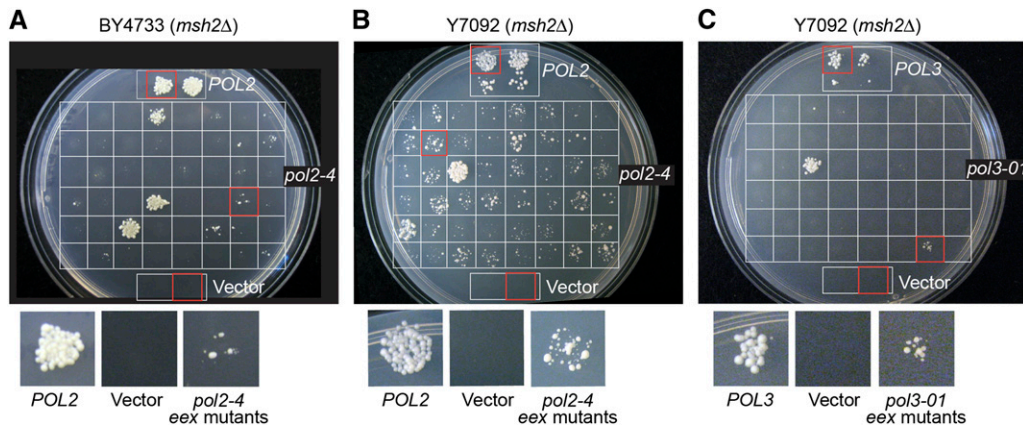


Figure 3 Escape from error-induced extinction. (A and B) Plasmid shuffling was used to screen for *eex* mutants that suppress *pol2-4 msh2Δ* synthetic lethality. *pol2Δ msh2Δ POL2-URA3* strains derived from (A) BY4733 and (B) Y7092 were transformed with *pol2-4*, *POL2*, or vector-only *LEU2* plasmids. Approximately 10^4 – 10^5 cells from 48 independent *pol2-4* transformants of each strain were spotted separately in a 6 × 8 grid on FOA-containing media to select for loss of the *POL2-URA3* plasmid and to isolate suppressor mutants. *Bona fide* suppressors containing

pol2-4 as the sole source of Pol ε arose at a rate of 1.9×10^{-4} *eex* mutants/cell division in the BY4733 strain [95% confidence interval (C.I.) = 2.6×10^{-4} – 1.3×10^{-4}] and 4.0×10^{-3} in the Y7092 background (95% CI = 4.4×10^{-3} – 3.6×10^{-3}). (C) A similar plasmid-shuffling strategy was used to estimate the rate of escape from *pol3-01 msh2Δ* synthetic lethality in the Y7092 background (8.8×10^{-6} *eex* mutants/cell division; 95% CI = 2.0×10^{-5} – 1.6×10^{-6}). In C, 10-fold fewer viable cells were plated in each grid position compared to A and B. The *POL2* control patches in A are two independent transformants. The *POL2* and *POL3* control patches in B and C are also from replicate transformants and include 10-fold dilutions of each. Rates (*eex* mutants/cell division) were calculated as described in *Materials and Methods*. Red boxes indicate grid positions magnified below each plate.

Intragenic *eex* mutants suppress mutation rates

If *pol2-4 msh2Δ* synthetic lethality is due to error-induced extinction, then Pol ε *eex* substitutions may promote escape by increasing the fidelity of Pol ε. To determine whether the *eex* substitutions suppressed the *pol2-4* mutator phenotype, we compared mutation rates conferred by *pol2-4* and *pol2-4, eex* alleles in an *msh6Δ* strain derived from BY4733. All of the *eex* mutations suppressed the *pol2-4 msh6Δ* mutator phenotype between 3- and 23-fold (Figure 5B, shaded box). Thus, the *eex* mutations likely promote escape from *pol2-4 msh2Δ* synthetic lethality by lowering mutation rates below an error threshold. We also determined whether the *eex* alleles influence mutation rates in the presence of Pol ε proofreading by introducing each *eex* mutation into the otherwise wild-type *POL2* gene. The resultant *pol2-eex* alleles had modest effects on background *msh6Δ* mutation rates (Figure 5B, right). *pol2-G435C msh6Δ* and *pol2-A1153D msh6Δ* were indistinguishable from *POL2 msh6Δ*. *pol2-K966Q* and *pol2-T850M* increased the *msh6Δ* mutation rate two- to threefold, while *pol2-V522A* lowered the rate threefold.

Distinct mutation spectra of *eex* mutants

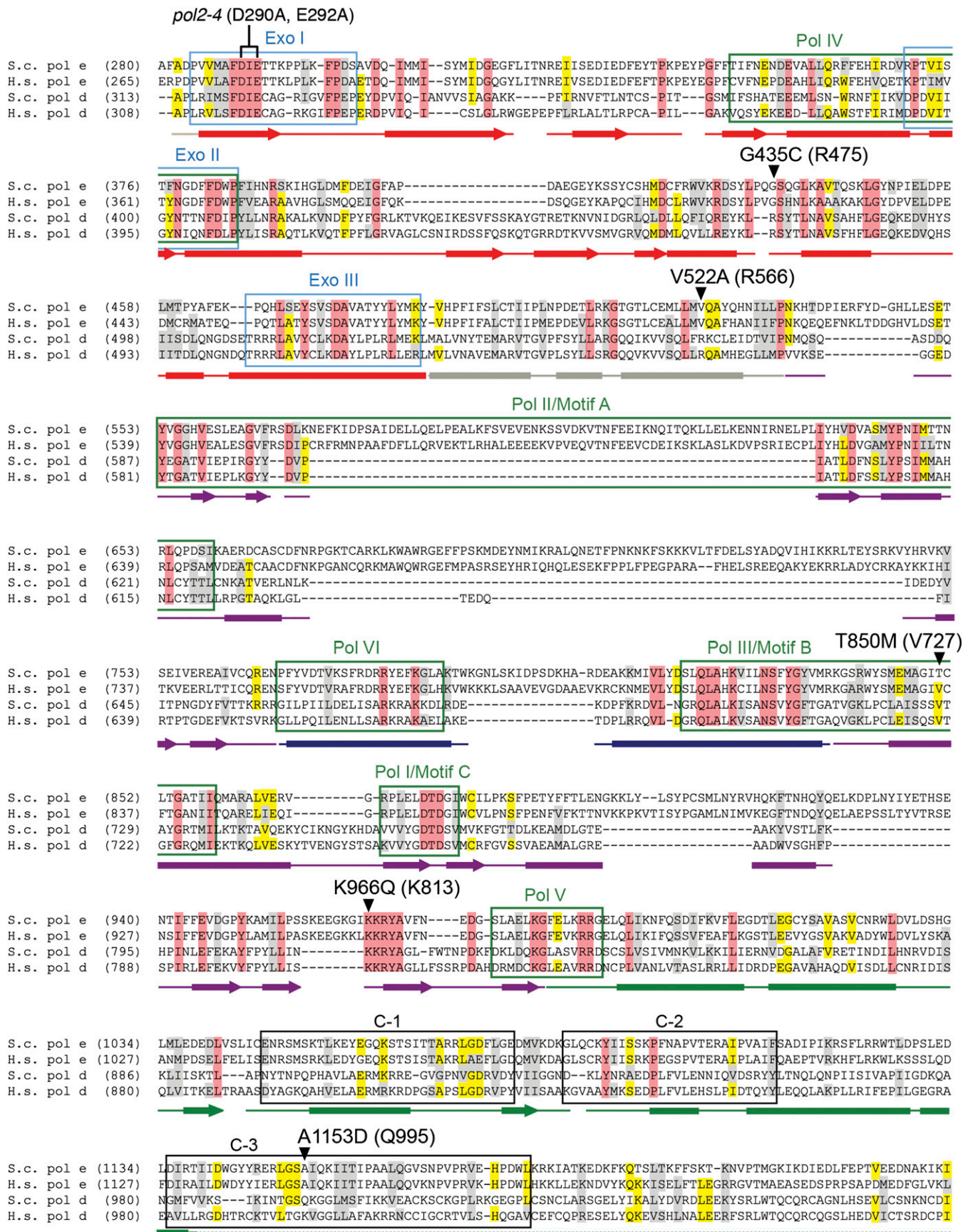
To gain insight into how the *eex* substitutions suppress mutation rates, we determined the types of mutations that spontaneously arise in *pol2-4 msh6Δ* and *pol2-4, eex msh6Δ* strains (Figure 6 and Table 2). Multiple independent *Can^r* colonies were isolated (~50 per strain), and the *CAN1* genes were sequenced to determine the spectrum of spontaneous mutations in each strain. Mutations were distributed throughout the *CAN1* sequence (Figure 6), with recurrent mutations observed at several nucleotide positions (*i.e.*, “hotspots”). In all strains, the majority of mutations were base substitutions (Table 2), consistent with the expected synergy of a proofreading-deficient polymerase with *msh6Δ* (Tran *et al.* 1999).

We analyzed the mutation spectra using iMARS (Morgan and Lewis 2006) to compare each *pol2-4, eex msh6Δ* spectrum to that produced by *pol2-4 msh6Δ* in a pair-wise fashion. This statistical analysis considers both the type of mutation and its position in the *CAN1* sequence (Figure 6). All but one *eex* mutant, *pol2-4, A1153D msh6Δ* ($P = 0.10$), displayed significantly different mutation spectra from the *pol2-4 msh6Δ* strain ($P \leq 0.05$; Monte Carlo hypergeometric test). When only base substitutions are considered with no regard to sequence position (Table 2), the distributions of mutation types in *pol2-4, T850M msh6Δ* and *pol2-4, K966Q msh6Δ* were significantly different from the distribution in *pol2-4 msh6Δ*. Specifically, *K966Q* increased the percentage of T→C substitutions in the *CAN1*-coding sequence, and *T850M* increased the C→A percentage while decreasing

Table 1 Genotypes of candidate *eex* mutants

Class of FOA ^r mutant	Mutants isolated from:		Total no. of mutants
	BY4733	Y7092	
<i>eex</i>			
Intragenic to <i>pol2-4</i>	4	1	5
Extragenic to <i>pol2-4</i>	50	26	76
Total	54	27	81
<i>pol2-4</i> → <i>POL2</i> conversion	26	3	29
Other (<i>e.g.</i> , <i>ura3</i> mutations)	23	0	23
Total	103	30	133

FOA-resistant (FOA^r) colonies from 133 different *pol2-4-LEU2 POL2-URA3 msh2Δ* parent clones were isolated, and *POL2* was genotyped to distinguish genuine *eex* mutants from *pol2-4* → *POL2* gene conversions and other mutations that confer FOA resistance (*e.g.*, *ura3*). Clones harboring only *pol2-4* were considered *eex* mutants. Plasmids from *eex* mutants were recovered and retested in a fresh *msh2Δ* strain to distinguish second-site suppressors within *pol2-4* (“intragenic *eex*”) from *eex* mutations located extragenic to *pol2-4* (“chromosomal *eex*”). *eex* mutants were isolated from two different strains: BY4733 and Y7092 (Figure 3, A and B).



the proportion of G→T substitutions. *T850M* also increased the percentage of -1 frameshifts, and *G435C* and *K966Q* decreased the proportion of +1 frameshifts.

To quantify the effects of *eex* alleles on individual mutation types, we converted mutation frequencies (Table 2) to mutation rates (Table 3). Only the most frequent mutation types were included in this analysis. When type-specific rates in *pol2-4 msh6Δ* and *pol2-4,eex msh6Δ* strains were compared (Table 3), several patterns of mutator suppression were evident. The two strongest *eex* alleles (*T850M* and *A1153D*) suppressed all mutation types to a similar degree (94–99% suppression relative to *pol2-4 msh6Δ*). *K966Q* also exhibited a nearly uniform pattern of suppression (70–94%) with some preference for +1 frameshifts. In contrast, *G435C* only weakly suppressed base substitutions (50–60%) while strongly suppressing +1 frameshifts (>95%), and *V522A* preferentially suppressed both G→A substitutions and +1 frameshifts (94–96%) with a somewhat weaker effect on C→T and G→T substitutions (80%). Collectively, the data in Tables 2 and 3 indicate that there are multiple mechanisms of mutator suppression.

Discussion

Adaptation necessitates genetic diversity. Large cell populations with low DNA replication error rates may contain sufficient variation to surmount a single selective barrier. However, rapid environmental changes impose additional selection pressures that may exceed the ability of wild-type cells to adapt. Natural populations of bacteria and yeast often harbor low levels of mutator cells that emerge under such conditions (Giraud *et al.* 2001b; Elena and Lenski 2003). However, sustained expression of mutator phenotypes compromises long-term fitness through the accumulation of deleterious mutations. Thus, following adaptation to external pressures, selection would favor cells that have either evolved mutator suppressors or eliminated the mutator allele. In this manner, mutator phenotypes rise and fall as cells shift between periods of relative stability and environmental change (Taddei *et al.* 1997; Giraud *et al.* 2001b).

Here we investigated pathways of mutator suppression that restore fidelity to an error-prone variant of Pol ε, the primary leading-strand DNA polymerase in yeast (Pursell *et al.* 2007; Nick McElhinny *et al.* 2008). We show that cells defective for both Pol ε proofreading and MMR are inviable (Figure 1). However, spontaneous mutants readily escape this synthetic lethality (Figure 3). Five percent of these es-

cape mutants encode secondary amino-acid substitutions in Pol ε (Figure 4) that suppress the Pol ε proofreading-deficient phenotype (Figure 5). This study complements our recent investigation of Pol δ (Herr *et al.* 2011a), where we defined the maximal mutation rate sustainable by haploid yeast cells and described variants that suppress the mutator phenotype of cells deficient for Pol δ proofreading. Our findings highlight the unstable nature of mutator phenotypes and suggest multiple mechanisms for their suppression.

Pol ε errors and lethal mutagenesis

It is well established that combined defects in Pol δ proofreading and MMR are synthetically lethal in haploid yeast (Morrison *et al.* 1993; Tran *et al.* 1999; Greene and Jinks-Robertson 2001). Loss of *Msh6* or *Pms1* (*msh6Δ* or *pms1Δ*) is sufficient to extinguish Pol δ proofreading-deficient (*pol3-01*) cells (Figure 1), the apparent consequence of mutation accumulation during DNA replication (Morrison *et al.* 1993; Herr *et al.* 2011a). Consistent with a mechanism of error-induced lethality, yeast cells with Pol δ proofreading and MMR defects form microcolonies of ~100 cells arrested at various stages of the cell cycle (Morrison *et al.* 1993) and with diverse cell morphologies resembling inactivation of different essential genes (Yu *et al.* 2006). The maximum tolerated mutation rate in haploid yeast (~10⁻³ inactivating mutations/gene/cell division ≈ 3 × 10⁻⁶ mutations/base pair/cell division) is consistent with random inactivation of essential genes as the cause of extinction (Herr *et al.* 2011a).

In contrast to Pol δ, the combined loss of Pol ε proofreading (*pol2-4*) and *Msh6* (*msh6Δ*) is not synthetically lethal, although it does compromise growth (Figure 1). The different fates of *msh6Δ* cells with defects in Pol δ proofreading (inviable) or Pol ε proofreading (slow-growing, strong mutators) are likely due to quantitative differences in cellular mutation burden. While *pol3-01 msh6Δ* cells exceed the maximum tolerated mutation rate (Herr *et al.* 2011a), *pol2-4 msh6Δ* cells mutate at a rate 10 times lower than the maximum (10⁻⁴ Can^r mutants/cell division ≈ 10⁻⁴ inactivating mutations/gene/cell division) (Figure 5B). We observe that *pol2-4* cells become inviable when both *Msh6*- and *Msh3*-dependent MMR are disrupted (Figure 1B). Thus, the additional burden of unrepaired *Msh2-Msh3* substrates is sufficient to push *pol2-4 msh6Δ* cells over the lethal threshold. Similarly, *pol2-4 pms1Δ* cells become inviable when *MLH3* is deleted. Our finding that unrepaired Pol ε errors are lethal provides additional evidence that Pol ε

Figure 4 Amino acid changes in Pol ε *eex* mutants. Aligned amino-acid sequences of the catalytic subunits of Pol ε and δ (S.c. pol e, *S. cerevisiae* Pol ε; H.s. pol e, *Homo sapiens* Pol ε; S.c. pol d, *S. cerevisiae* Pol δ; H.s. pol d, *H. sapiens* Pol δ). Secondary structural elements of yeast Pol δ (Swan *et al.* 2009) are indicated below the alignment and color-coded to depict their domain location (as in Figure 7): rectangles, α-helices; arrows, β-strands; solid lines, loops; dotted line, structure unknown. Amino-acid substitutions encoded by Pol ε *eex* mutations are shown in black type above the sequence, with the corresponding yeast Pol δ residues in parentheses. Conserved polymerase and exonuclease motifs are framed in green and blue, respectively (Bernad *et al.* 1989; Wang *et al.* 1989). Three additional regions of homology (C-1, C-2, and C-3) are framed in black (Huang *et al.* 1999). Amino-acid conservation is indicated using the following color scheme: red, residues conserved in all four sequences; yellow, residues conserved in three sequences; gray, similar amino acids in at least three sequences.

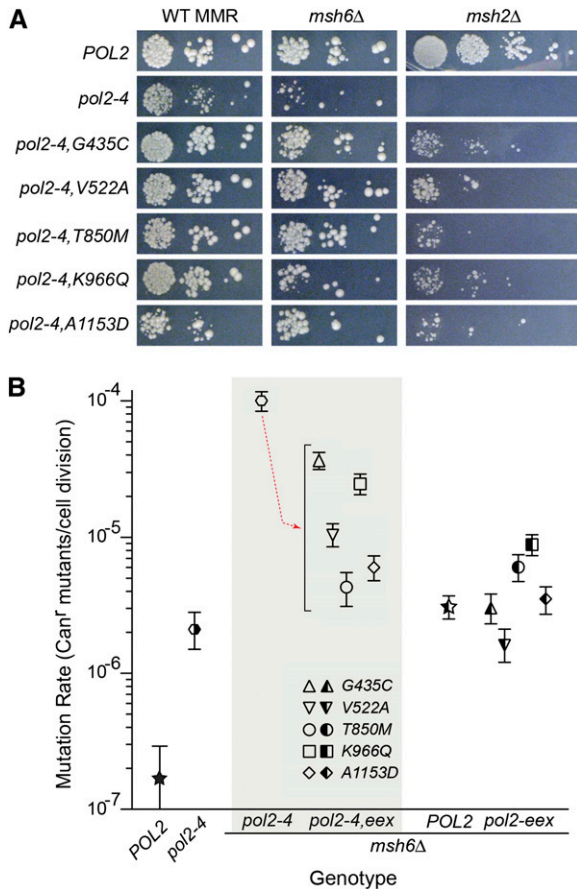


Figure 5 Growth and antimutator phenotypes conferred by *pol2-4* intragenic eex. (A) eex mutations reverse synthetic growth defects associated with *pol2-4*. The *pol2-4, eex* mutations were re-engineered into fresh *pol2-4-LEU2* plasmids and introduced into wild-type (WT) MMR, *msh6Δ*, or *msh2Δ* strains for plasmid shuffling. Strains harboring *POL2*- or *pol2-4-LEU2* plasmids served as controls. Transformants were serially diluted and spotted onto FOA-containing media to assess colony-forming capacity after incubation at 30° for 3 days (WT MMR and *msh6Δ*) or 4 days (*msh2Δ*). (B) eex mutations confer antimutator phenotypes. Rates of spontaneous mutation, expressed as Can⁺ mutants/cell division, were determined from multiple independent fluctuation analyses of each strain. Confidence intervals (95%) for each mutation rate are shown as error bars. The downward red arrow in the gray box indicates the antimutator effect of eex alleles on the *pol2-4* mutator phenotype. Symbol patterns indicate *POL2* and *MSH6* allele status: black left half, *POL2*; black right half, *MSH6*; solid black, *POL2 MSH6*; unfilled left half, *pol2-4*; unfilled right half, *msh6Δ*; completely unfilled, *pol2-4 msh6Δ*. Symbol shapes indicate eex allele status (see key insert): star and hexagon, no eex; triangle, G435C; inverted triangle, V522A; circle, T850M; square, K966Q; diamond, A1153D.

plays a substantial role in genome replication (Pursell and Kunkel 2008). Our data also indicate that loss of genetic information on either DNA strand [Pol ϵ errors on leading-strands and Pol δ errors on lagging-strands (Pursell *et al.* 2007; Nick McElhinny *et al.* 2008)] is sufficient to drive extinction of a population of cells.

MMR pathways suppressing lethal Pol ϵ errors

The MMR pathways that suppress deleterious Pol ϵ errors are sharply delineated in proofreading-MMR double mutants

(Figure 1). Pol ϵ 's bias for generating more base substitutions than frameshifts (Shcherbakova *et al.* 2003) is consistent with our observation that *pol2-4* cells defective for base-base mismatch repair (*msh6Δ* or *pms1Δ*) exhibit slow-growth phenotypes, while *pol2-4* cells defective primarily for frameshift repair (*msh3Δ* or *mlh3Δ*) grow normally (Figure 1). Morrison and Sugino (1994) also observed compromised growth of *pol2-4 pms1Δ* cells. Surprisingly, we found that deletion of *MSH6* impacted cell growth more severely than deletion of *PMS1* (Figure 1A), even though Msh2-Msh6 mediates repair through its interaction with Mlh1-Pms1 (Habraken *et al.* 1998; Iyer *et al.* 2006; Hsieh and Yamane 2008). This indicates that some Msh6-dependent repair can occur in the absence of Mlh1-Pms1, perhaps by using Mlh1-Mlh3 in place of Mlh1-Pms1 (Cannavo *et al.* 2005). Our observation that *pol2-4 pms1Δ* cells become inviable when *MLH3* is deleted (Figure 1) further implicates Mlh1-Mlh3 in the repair of deleterious Pol ϵ errors. Thus, both Pms1 and Mlh3 play important protective roles. Synthetic lethality with *pol2-4* occurs only when both Mlh1-Pms1 and Mlh1-Mlh3 are eliminated by deleting either *MLH1* alone or *PMS1* and *MLH3* together (Figure 1). Mouse *Mlh1*, *Mlh3*, and *Pms2* (equivalent to yeast *PMS1*) have a similar relationship in preventing spontaneous mutations and cancer (Chen *et al.* 2005). Considered together, our data show that multiple MMR components protect cells from deleterious Pol ϵ errors, which include base substitutions, frameshifts, and possibly other mutation types (Harrington and Kolodner 2007).

Contribution of Pol ϵ errors to cellular mutation burden

What accounts for the differences in synthetic phenotypes of *pol2-4* and *pol3-01* with MMR defects? Biochemical studies indicate that exonuclease-deficient Pols ϵ and δ have similar overall fidelities *in vitro* (Shimizu *et al.* 2002; Shcherbakova *et al.* 2003; Fortune *et al.* 2005). They generate distinct mutational spectra, but both primarily produce base-base mispairs and frameshift errors, the preferred substrates for MMR (Kolodner and Marsischky 1999; Iyer *et al.* 2006; Hsieh and Yamane 2008). Nevertheless, in MMR-proficient cells, the *pol3-01* mutation rate is 5–30 times higher than that of *pol2-4* (Morrison and Sugino 1994; Shcherbakova *et al.* 1996; Tran *et al.* 1999; Datta *et al.* 2000; Karthikeyan *et al.* 2000; Greene and Jinks-Robertson 2001; Pavlov *et al.* 2004). MMR preferentially repairs lagging-strand errors (Pavlov *et al.* 2003; Hombauer *et al.* 2011). Consistent with this bias, we observe that deletion of *MSH6* increases the mutation rate of *pol3-01* variants ~160-fold (Herr *et al.* 2011a) and the *pol2-4* mutation rate ~50-fold (Figure 5B). Shcherbakova *et al.* (2003) see a similar impact of *msh6Δ* on *pol2-4* mutation rates (40- to 50-fold). However, this preference of MMR for Pol δ errors contradicts the observation that loss of Pol δ proofreading results in higher mutation rates than loss of Pol ϵ proofreading.

There are several possible explanations for this apparent discrepancy. As previously suggested by others (Morrison and Sugino 1994; Pavlov and Shcherbakova 2010), Pol ϵ

1 ATGACAAATT CAAAAGAAGA CGCCGACATA GAGGAGAAGC ATATGTACAA TGAGCCGGTC ACAACCCCTCT TTCACGACGT TGAAGCTCA CAAACACACC
M T N S K E D A D I E E K H M Y N E P V T T L F H D V E A S Q T H H

101 ACAGACGTGG GTCAATACCA TTGAAAGATG AGAAAAGTAA AGAATTGTAT CCATTGCGCT CTTTCCCAGC GAGAGTAAAT GGCAGGATA CGTTCTCTAT
R R G S I P L K D E K S K E L Y P L R S F P T R V N G E D T F S M

201 GGAGGATGGC ATAGGTGATG AAGATGAAGG AGAAGTACAG AACGCTGAAG TGAAGAGAGA GCTTAAGCAA AGACATATTG GTATGATTGC CCTTGGTGGT
E D G I G D E D E G E V Q N A E V K R E L K Q R H I G M I A L G G

301 ACTATTGGTA CAGGTCTTTT CATTGGTTTA TCCACACCTC TGACCAACGC CGGCCAGGTG GGCCTCTTTA TATCATATT ATTTATGGGT TCTTTGGCAT
T I G T G L F I G L S T P L T N A G P V G A L I S Y L F M G S L A Y

401 ATTCTGTACG GCAGTCTCTG GGTGAAATGG CTACATTCAT CCCTGTACA TCCTCTTCA CAGTTTCTC ACAAGATTC CTTTCTCAG CATTGGGTC
S V T Q S L G E M A T F I P V T S S F T V F S Q R F L S P A F G A

501 GGCAATGGT TACATGATT GGTTCCTTG GCAATCACT TTTGCCCTGG AACTTAGTGT AGTTGGCCAA GTCATTCAAT TTTGGAGTA CAAAGTCCA
A N G Y M Y W F S W A I T F A L E L S V V G Q V I Q F W T Y K V P

601 CTGGCGCAT GGATTAGTAT TTTTGGGTA ATTATCACA TAATGAACCT GTTCCTCTG AAATATTACG GTGAATTCGA GTTCTGGTC GCTTCCATCA
L A A W I S I F W V I I T I M N L F P V K Y Y G E F E F W V A S I K

701 AAGTTTACG CATTATCGG TTCTAATAT ACTGTTTGG TATGTTTGT GGTCCTGGG TTACCGGCC AGTTGGATTC CGTTATTGGA GAAACCCAGG
V L A I I G F L I Y C F C M V C G A G V T G P V G F R Y W R N P G

801 TGCTGGGGT CCAGGTATAA TATCTAAGGA TAAAAACGAA GGGAGTCTCT TAGTGGGGT TTCCTCTTGG ATTAACGCTG CCTTCAATC TCAAGTACT
A W G P G I I S K D K N E G R F L G W V S S L I N A A F T F Q G T

901 GAAGTAGTGG GTATCACTGC TGGTGAAGCT GCAAAACCCCA GAAAATCCGT TCCAAGAGCC ATCAAAAAAG TTGTTTCCG TATCTAAC TTTACATTTG
E L V G I T A G E A A N P R K S V P R A I K K V V F R I L T F Y I G

1001 GCCTCTATT ATTCATTGA CTTTGTATTC CATAACAATG CCCTAAACTA ACACAATCTA CTTCTACTGT TTTACTTCT CCCTTTATTA TTGCTATTGA
S L L F I G L L V P Y N D P K L T Q S T S Y V S T S P F I I A I E

1101 GAAGTCTGGT ACAAGGTTT TGCCACATAT CTTCAACGCT GTTATCTTAA CAACCATAT TTCGCGCA AATTCAAATA TTTACGTTGG TTCCCGTATT
N S G T K V L P H I F N A V I L T T I I S A A N S N I Y V G S R I

1201 TTATTGGTC TATCAAGAA CAAGTTGGCT CCTAAATCC TGTCAAGGAC CACCAAAGT GGTGTTCCAT ACATTGCAGT TTTCTGTTACT GCTGCATTGG
L F G L S K N K L A P K F L S R T T K G G V P Y I A V F V T A A F G

1301 GCGCTTGGC TTACATGGAG ACATCTACTG GTGGTGACAA AGTTTTCGAA TGGCTATTAA ATATCACTGG TGTTCAGGC TTTTTCGAT GGTATTATT
A L A Y M E T S T G G D K V F E W L L N I T G V A G F F A W L F I

1401 CTCAATCTCG CACATCAGAT TTATGCAAGC TTTGAAATAC CGTGGCATCT CTCGTGACGA GTTACCATT AAAGTAAAT TAATGCCGG CTTGGCTTAT
S I S H I R F M Q A L K Y R G I S R D E L P F K A K L M P G L A Y

1501 TATGCGGCA CATTATGAC GATCATTATC ATTATTCAAG GTTTCACGGC TTTTGACCA AAATTCAATG GTGTTAGCTT TGCTGCCGC TATATCTCTA
Y A A T F M T I I I I I Q G F T A F A P K F N G V S F A A A Y I S I

1601 TTTTCTGTT CTTAGCTGTT TGGATCTTAT TTCAATGCAT ATTCAGATGC AGATTTATTT GGAAGATTGG AGATGTCGAC ATCGATTCCG ATAGAAGAGA
F L F L A V W I L F Q C I F R C R F I W K I G D V D I D S D R R D

1701 CATGAGGCA ATTGTATGGG AAGATCATGA ACCAAAGACT TTTTGGGACA AATTTTGGAA TGTTGTAGCA TAG
I E A I V W E D H E P K T F W D K F W N V V A

not pictured: 1 deletion of *CAN1* locus

MMR+ *msh6Δ*
POL2 *POL2 pol2-4,G435C pol2-4,T850M pol2-4,A1153D*
pol2-4 pol2-4,V522A pol2-4,K966Q

may replicate less of the genome than Pol δ . Replacement of Pol ϵ with Pol δ near the end of a replicon may be necessary to enable ligation of the leading strand to the downstream Okazaki fragment from an adjacent replicon (Garg *et al.* 2004). If replacement occurs randomly or in response to Pol ϵ errors or pausing at DNA lesions (Pavlov and Shcherbakova 2010), then Pol δ would synthesize variable amounts of each leading-strand fragment and overall more of the genome than Pol ϵ . Gap-filling synthesis is also catalyzed predominantly by Pol δ during DNA repair, recombination, and telomere replication (Pavlov *et al.* 2006b).

Another possibility is that Pol ϵ creates fewer mutations *in vivo* than predicted by *in vitro* measurements of fidelity. The overall error rate of Pol ϵ *in vivo* should approximately correspond to the mutation rate of cells that lack both Pol ϵ proofreading and MMR. *pol2-4 msh6 Δ* cells generate 10^{-4} Can^r mutants/cell division or $\sim 3 \times 10^{-7}$ mutations/base pair/cell division (calculated as described in Herr *et al.* 2011a). However, this likely underestimates the Pol ϵ error rate. Other enzymes may excise Pol ϵ errors [*e.g.*, Pol δ may proofread for Pol ϵ (Pavlov and Shcherbakova 2010)], and Msh2-Msh3-mediated MMR, which is still active in these cells, also repairs some Pol ϵ errors. Tran *et al.* (1999) show that loss of Msh2-Msh3 (*msh3 Δ*) further increases the mutation rate of *pol2-4 msh6 Δ* cells about threefold, while we find that *msh3 Δ* is lethal in *pol2-4 msh6 Δ* cells and therefore must increase mutations ≥ 10 -fold. A 10-fold increase corresponds to $\sim 3 \times 10^{-6}$ mutations/base pair, which is substantially lower than the error rate of proofreading-deficient Pol ϵ *in vitro* [$\sim 3 \times 10^{-4}$ errors/base pair (Shcherbakova *et al.* 2003)]. Furthermore, the spectra of mutations observed *in vivo* and *in vitro* are quite different (Figure 6 and Table 2) (Shcherbakova *et al.* 2003). These differences are not likely due to differential extension of mispairs *in vitro* because Pol ϵ is present in great excess to maximize mispair extension in the M13 fidelity assay (Bebenek and Kunkel 1995; Shcherbakova *et al.* 2003). Thus, *in vitro* fidelity measurements do not recapitulate the mutational events caused by Pol ϵ *in vivo*. As previously suggested (Shcherbakova *et al.* 2003), Pols ϵ and δ may replicate similar amounts of the yeast genome, but may do so with different accuracies, perhaps by utilizing strand- or polymerase-specific accessory factors or repair pathways that decrease mutations from Pol ϵ or by triggering the mutagenic Dun1 pathway that increases mutations from Pol δ (Datta *et al.* 2000; Reha-Krantz *et al.* 2011). Interestingly, the error-prone DNA polymerases ζ and η do not significantly impact mutagenesis by proofreading-deficient Pol ϵ or Pol δ (Figure 2) (Shcherbakova *et al.* 1996; Datta *et al.* 2000). Additional studies are required to examine in greater detail the roles

of Pol ϵ and Pol δ *in vivo* and the pathways that mediate mutagenesis when these polymerases err.

Escape from Pol ϵ error-induced extinction

In our plasmid-shuffling experiments, colonies frequently emerged that escape *pol2-4 msh2 Δ* lethality (Figure 3). This escape from Pol ϵ error-induced extinction is similar to that recently described for Pol δ (Herr *et al.* 2011a). Morrison and Sugino (1994) also observed mutator suppression in a *pol2-4 pms1* clone, but this clone was not further characterized. In our studies, escape results from genetic suppressors that are either second-site mutations within *pol2-4* (intragenic suppressors) or alleles affecting unknown genes elsewhere in the genome and extragenic to *pol2-4* (chromosomal suppressors) (Table 1). Five intragenic suppressors were identified (Figure 4). Each of these individually conferred escape from *pol2-4 msh2 Δ* lethality (Figure 5A) and suppressed the mutator phenotype of Pol ϵ proofreading deficiency 3- to 23-fold (Figure 5B). These data strongly suggest that *pol2-4 msh2 Δ* inviability results from lethal mutagenesis and that intragenic suppressors lower the spontaneous mutation rate below the maximum tolerated threshold. The existence of a relatively narrow lethal threshold was previously shown using a collection of *pol3* mutator and antimutator alleles (Herr *et al.* 2011a). Our new data with *pol2* alleles validate this threshold and are consistent with a maximal mutation rate in haploid yeast of $\sim 10^{-3}$ inactivating mutations/gene/cell division (Herr *et al.* 2011a).

Both *pol3-01 msh6 Δ* and *pol2-4 msh2 Δ* strains exceed the lethal error threshold, and antimutator variants that lower mutation rates below the threshold were readily obtained in both strains (data herein and Herr *et al.* 2011a). Yet chromosomal suppressors of *pol2-4 msh2 Δ* lethality represented a greater proportion of *eex* mutants (94%) (Table 1) than those that suppressed *pol3-01 msh6 Δ* lethality (65%) (Herr *et al.* 2011a). We also observed that *eex* mutants arise more readily in Pol ϵ than in Pol δ proofreading-deficient *msh2 Δ* cells (Figure 3, B and C). This may reflect differences in how close the mutation rates of the strains are to the lethal threshold. Both weak and strong antimutators would rescue *pol2-4 msh2 Δ* and *pol3-01 msh6 Δ* cells, which reside relatively close to the threshold (see above and Herr *et al.* 2011a). In contrast, the combination of *pol3-01* and *msh2 Δ* imparts a much higher mutation rate, which would be overcome by only the strongest antimutators. It is also possible that a wider array of genes influences Pol ϵ replication fidelity than Pol δ fidelity, and thus the probability of isolating chromosomal suppressor alleles is greater for Pol ϵ . *pol3-01 msh6 Δ* cells still have Msh2-Msh3 MMR activity that protects against frameshift mutations (Marsischky *et al.* 1996;

Figure 6 Spontaneous *CAN1* mutations from *pol2-4* and *pol2-4,eex msh6 Δ* cells. The *can1* genes from ~ 50 independent Can^r mutants of each strain were PCR-amplified and sequenced. Mutations identified in different strains are color-coded according to the key at the bottom. Each base letter above the wild-type *CAN1* sequence indicates an independent base substitution or frameshift (+ or -) mutation. Horizontal lines indicate *CAN1* sequences involved in complex mutations, duplications, and deletions. The wild-type *POL2* and *POL2 msh6 Δ* spectra are from Herr *et al.* (2011a).

Table 2 Types of spontaneous *CAN1* mutations in *pol2-4,eex msh6Δ* strains

	WT ^a	<i>msh6Δ</i> ^a	<i>pol2-4 msh6Δ</i>	<i>pol2-4,eex msh6Δ</i>				
				<i>G435C</i>	<i>V522A</i>	<i>T850M</i> ^b	<i>K966Q</i> ^b	<i>A1153D</i>
Transitions								
G → A	8 (24)	16 (34)	20 (36)	19 (41)	10 (19)	21 (41)	26 (48)	21 (41)
C → T	3 (9)	2 (4)	5 (9)	4 (9)	8 (15)	4 (8)	6 (11)	4 (8)
A → G	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)	0 (0)
T → C	0 (0)	3 (6)	0 (0)	0 (0)	0 (0)	1 (2)	4 (7) ^c	1 (2)
Total	11 (33)	21 (45)	25 (45)	23 (50)	18 (35)	27 (51)	36 (67) ^c	26 (51)
Transversions								
G → T	2 (6)	5 (11)	21 (38)	23 (50)	29 (56)	6 (12) ^c	12 (22)	19 (37)
C → A	3 (9)	10 (21)	1 (2)	0 (0)	0 (0)	8 (16) ^c	4 (7)	1 (2)
G → C	1 (3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2)
C → G	2 (6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
A → C	1 (3)	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
T → G	1 (3)	2 (4)	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)	0 (0)
A → T	1 (3)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)	0 (0)
T → A	1 (3)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)	0 (0)
Total	12 (36)	18 (38)	22 (39)	23 (50)	29 (56)	17 (33)	16 (30)	21 (41)
Frameshifts								
+1	2 (6)	3 (6)	9 (16)	0 (0) ^c	3 (6)	3 (6)	2 (4) ^c	3 (6)
-1	2 (6)	1 (2)	0 (0)	0 (0)	2 (4)	5 (10) ^c	0 (0)	1 (2)
Other	4 (12)	4 (9)	9 (16)	0 (0) ^c	5 (10)	8 (16)	2 (4) ^c	4 (8)
Other	6 (18)	4 (9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Multiple	0 (0)	0 (0)	1 (2)	1 (2)	1 (2)	0 (0)	1 (2)	0 (0)
Total	33	47	56	46	52	51	54	51

The *CAN1* genes were sequenced from independent *Can^r* mutants of each strain. The numbers of mutations of each subtype are shown with percentages in parentheses. Transitions and transversions indicate base changes observed in the coding strand of *CAN1* (as in Figure 6). "Other" includes duplications, deletions, and complex mutations. Some mutants had two mutations separated by >10 bp (61–1150 bp); these are reported under "Multiple"; each mutation in this category was scored as an independent event and added to the relevant subclass tally, although they may be mechanistically linked. See Figure 6 for locations of mutations in the *CAN1* gene.

^a WT and *msh6Δ* data from Herr *et al.* (2011a).

^b Distribution of base substitutions significantly different from *pol2-4 msh6Δ* ($P \leq 0.05$, Monte Carlo hypergeometric test).

^c Significantly different from *pol2-4 msh6Δ* ($P \leq 0.05$, Fisher's exact test).

Palombo *et al.* 1996), while Pol ϵ error-induced extinction depends on abrogation of the *Msh2-Msh3* pathway (Figure 1). Thus, the higher proportion of chromosomal suppressors in *pol2-4 msh2Δ* cells may also be due to mutations in genes encoding replication components that affect frameshift mutagenesis.

An unexpected finding from our study is that different strains escape from error-induced extinction at different rates (Figure 3). The facile emergence of *pol2-4 msh2Δ eex* mutants in Y7092 compared to BY4733 cells suggests that the former strain acquired a weak antimutator prior to our screening experiments. A pre-existing weak antimutator allele may be insufficient to trigger escape but could cooperate with a second nascent antimutator to shift mutation rates below the lethal threshold and into the viable range. Alternatively, a pre-existing weak mutator allele in Y7092 cells may generate suppressors at a higher rate. However, the infrequency of FOA resistance due to *ura3* and other mutations is inconsistent with a higher overall mutation rate in Y7092 (Table 1). The Y7092 strain has undergone a number of genetic manipulations (Tong and Boone 2007), thus providing opportunity for variants to arise. These data suggest that strain differences or the presence of unknown suppressor alleles may explain inconsistencies in the literature

regarding Pol ϵ proofreading and MMR synthetic lethal interactions (Morrison and Sugino 1994; Tran *et al.* 1999; Greene and Jinks-Robertson 2001).

Possible mechanisms of mutator suppression by Pol ϵ variants

Our screen for suppressors uncovered five novel Pol ϵ antimutator variants: G435C, V522A, T850M, K966Q, and A1153D (Figure 4). The Pol ϵ structure is unknown, but the positions of antimutator residues in Pol ϵ can be deduced from structures of related B-family DNA polymerases (Wang *et al.* 1997; Swan *et al.* 2009). Mapping the Pol ϵ *eex* substitutions onto the *S. cerevisiae* Pol δ structure indicates that the Pol ϵ substitutions are scattered throughout the domains of the protein (Figure 7A), similar to the wide distribution of *eex* substitutions found in Pol δ (Figure 7B) (Herr *et al.* 2011a). No *eex* mapped to the C-terminal half of Pol ϵ , which performs an essential, noncatalytic role in yeast cells (Dua *et al.* 1999; Kesti *et al.* 1999; Feng and D'Urso 2001) and is not present in Pol δ or other B-family DNA polymerases.

The locations of the *eex*-encoded amino-acid substitutions (Figure 7) and the mutation spectra resulting from each (Figure 6 and Tables 2 and 3) suggest several possible

Table 3 Rates of spontaneous *CAN1* mutations in *pol2-4, eex msh6Δ* strains

	<i>pol2-4</i> <i>msh6Δ</i>	<i>pol2-4, eex msh6Δ</i>				
		<i>G435C</i>	<i>V522A</i>	<i>T850M</i>	<i>K966Q</i>	<i>A1153D</i>
Transitions ^a						
G → A (C•dATP)	360 (1.0)	150 (0.4)	20 (0.06)	18 (0.05)	120 (0.3)	25 (0.07)
C → T (G•dTTP)	90 (1.0)	~32 (~0.4)	16 (0.2)	~3 (~0.04)	27 (0.3)	~5 (~0.05)
Transversions ^a						
G → T (C•dTTP)	380 (1.0)	180 (0.5)	58 (0.2)	5 (0.01)	55 (0.1)	22 (0.06)
C → A (G•dATP)	~18 (1.0)	<8 (ND)	<2 (ND)	7 (ND)	~18 (ND)	~1 (ND)
Frameshifts						
+1	160 (1.0)	<8 (<0.05)	~6 (~0.04)	~3 (~0.02)	~9 (~0.06)	~4 (~0.02)
-1	<18 (1.0)	<8 (ND)	~4 (ND)	4 (ND)	<5 (ND)	~1 (ND)
Overall	1001 (1.0)	365 (0.36)	104 (0.10)	43 (0.04)	247 (0.25)	60 (0.06)

Expressed as the number of Can^r mutants per cell division ($\times 10^{-7}$). Rates for individual mutation types were calculated by multiplying the overall Can^r mutation rate of a strain (last row of table) by the percentage of the corresponding mutation in the *CAN1* mutation spectrum from that strain (Table 2). Rates relative to those in *pol2-4 msh6Δ* are in parentheses. Values were calculated to three decimal places and then rounded to one or two significant figures. < or ~: incidence values (Table 2) were zero or not significantly different from zero, respectively ($P > 0.05$, Fisher's exact test). ND: relative rates could not be reliably determined because mutations of this type were not detected at significant levels in the *pol2-4 msh6Δ* strain (Table 2).

^a Base changes in the *CAN1* coding sequence are shown with presumed causal mispairs in parentheses (template base•incoming dNTP), if Pol ϵ catalyzes leading-strand DNA synthesis (Pursell *et al.* 2007; Kunkel and Burgers 2008; Pavlov and Shcherbakova 2010) initiated from the *ARS507* origin of replication (Raghuraman *et al.* 2001; Yabuki *et al.* 2002; Kumar *et al.* 2011; Siow *et al.* 2012).

antimutator mechanisms for the Pol ϵ variants. Two amino-acid substitutions, T850M and K966Q, are within the predicted palm domain of Pol ϵ (Figure 7A). T850 is in conserved motif B (Figure 4), proximal to the polymerase active site (Figure 7, A and C) and adjacent to Y831, a highly conserved residue, which, when mutated, also suppresses the *pol2-4* mutator phenotype (Pavlov *et al.* 2004). This suggests that T850M increases Pol ϵ fidelity through its interaction with Y831, thereby changing the active-site geometry and reducing the formation or extension of mispairs and strand slippage intermediates (Table 3). The K966Q *eex* substitution, also located in the palm domain, maps to the first lysine in an absolutely conserved KKRYA sequence (Figure 4) that interacts with the minor groove of the primer•template (Figure 7, A and D). K966Q significantly decreased the rate of +1 frameshift mutations (Table 3), suggesting that K966Q decreases Pol ϵ pausing through homonucleotide runs, thereby reducing the opportunity for slippage (Viguera *et al.* 2001; Johnson *et al.* 2003).

Interestingly, the T850M and K966Q antimutator variants confer a mild mutator phenotype (two- to threefold) in *msh6Δ* cells when the catalytic residues of Pol ϵ 's exonuclease are restored to wild type (Figure 5B). Studies of T4 antimutators show that enhanced discrimination against some errors is commonly accompanied by weakened discrimination against others (Drake 1993). Polymerase variants with increased fidelity often have reduced overall activity (Loh *et al.* 2007), which can improve accuracy (Clayton *et al.* 1979; Kunkel *et al.* 1994; Joyce and Benkovic 2004; Johnson 2010; Herr *et al.* 2011b), but will also increase error rates for some mutation types (Kunkel *et al.* 1994) and may trigger mutagenic replication processes in the cell (Northam *et al.* 2006; Aksenova *et al.*

2010; Northam *et al.* 2010). Additionally, T850M and K966Q would increase mutagenesis if they hamper partitioning of nascent errors to the exonuclease active site (Donlin *et al.* 1991; Reha-Krantz 2010).

The remaining Pol ϵ antimutator substitutions affect other domains in the polymerase structure (Figures 4 and 7). V522A maps to the amino domain in an α -helix previously implicated in *pol3-01* mutator suppression (Figure 7, A, B, and E) (Herr *et al.* 2011a). The same types of spontaneous mutations arise in *pol2-4 msh6Δ* cells with or without the V522A allele (Table 2). Thus, V522A suppresses the most prevalent mutations generated by proofreading-deficient Pol ϵ to a similar degree. This pattern of suppression is consistent with a mutant polymerase that exhibits either increased overall nucleotide selectivity or increased facility to undergo extrinsic proofreading (Albertson and Preston 2006; Nick McElhinny *et al.* 2006; Pavlov *et al.* 2006a). The G435C antimutator maps to Pol δ R475 in a loop of the exonuclease domain that extends into and interacts with the thumb domain (Figure 7, A and F). We previously identified R475 substitutions in Pol δ that suppress *pol3-01 msh6Δ* synthetic lethality (Herr *et al.* 2011a). The recurrence of *eex* mutants with substitutions at this position is striking and suggests that Pols δ and ϵ share structural features that govern fidelity. Finally, the Pol ϵ A1153D antimutator likely resides in the thumb domain of the polymerase (Figure 4), although the structure of this region in Pol δ is unknown (Swan *et al.* 2009). A1153D suppressed the overall mutation rate 17-fold (Figure 5B), but did not significantly alter the mutation spectrum (Tables 2 and 3). Thus, A1153D may uniformly increase dNTP selectivity by Pol ϵ or may promote uniform extrinsic proofreading of Pol ϵ errors.

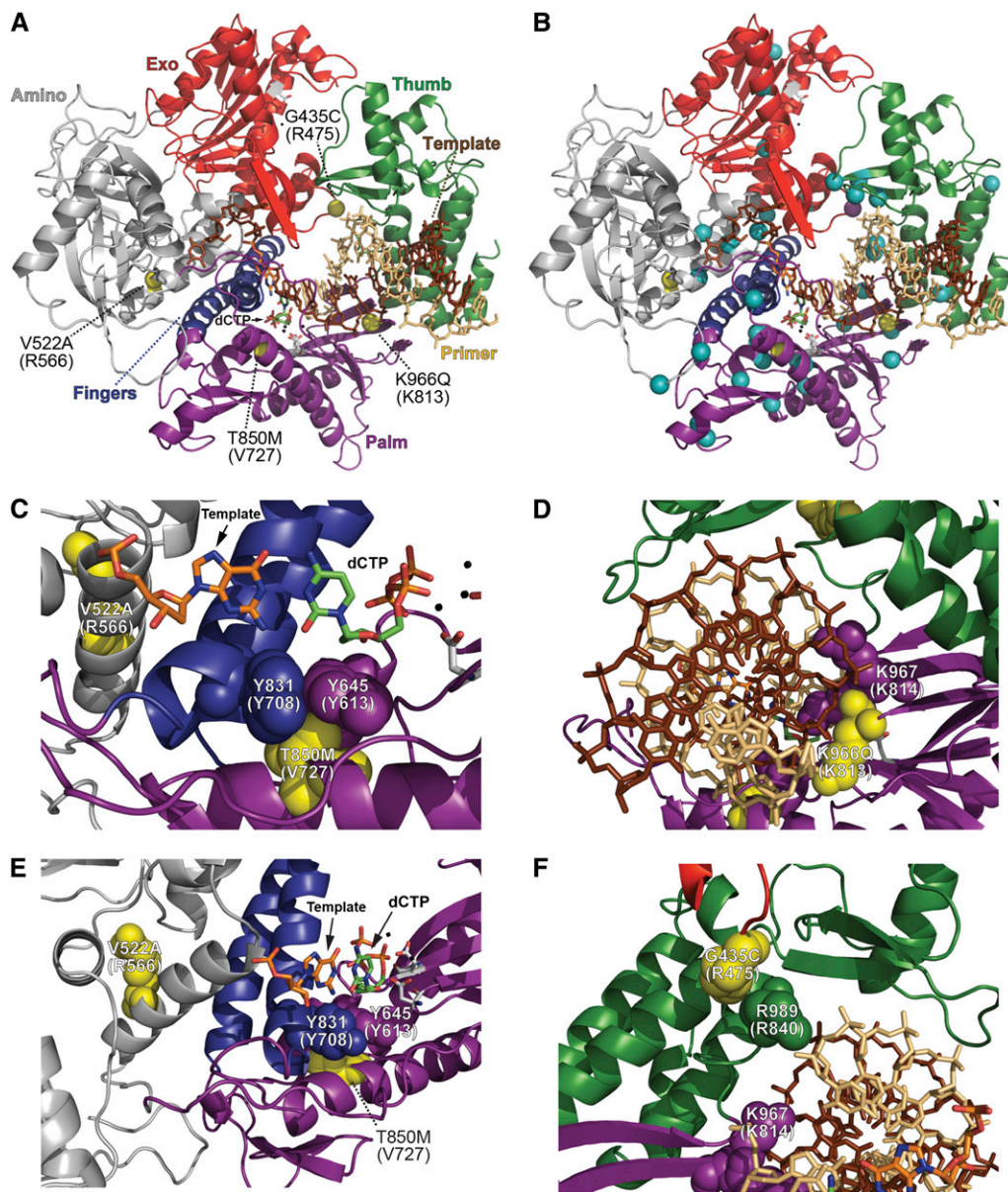


Figure 7 Locations of Pol ϵ eex amino-acid substitutions mapped onto the Pol δ structure. (A) Overall distribution of Pol ϵ eex amino-acid substitutions. The catalytic subunit of yeast Pol δ is depicted as a ribbon diagram with the following color-coded elements: exonuclease domain, red; thumb domain, green; fingers domain, blue; palm domain, purple; amino domain, gray; DNA template strand, brown sticks; primer strand, tan sticks; catalytic carboxylate residues in the polymerase and exonuclease active sites, gray CPK sticks; metal ions, small black spheres; incoming dCTP, green CPK sticks; template G nucleotide, orange CPK sticks. Locations of eex-encoded changes are shown as yellow spheres and labeled by the Pol ϵ amino-acid substitution with the corresponding Pol δ residue in parentheses. The A1153D substitution is not pictured because it falls in a region where the Pol δ structure is unknown. (B) Locations of Pol ϵ eex substitutions (yellow spheres) relative to Pol δ eex substitutions (aqua spheres; see (Herr *et al.* 2011a)). The purple sphere at the Exo-Thumb interface is Pol ϵ G435C, which aligns with Pol δ R475I/G. (C, D, E, and F) Close-up depictions of Pol ϵ eex substitutions. Important residues are highlighted as space-filling spheres, with yellow indicating positions corresponding to Pol ϵ eex substitutions. Structure of *S. cerevisiae* Pol δ is from Swan *et al.* (2009) (Protein Data Bank accession code 3IAY).

Perspectives and conclusions

Previous studies identified antimutator variants of bacteriophage T4 and *E. coli* DNA polymerases (reviewed in Reha-Krantz 1995; Schaaper 1998; Herr *et al.* 2011b). Similar to the yeast variants that we describe (data herein and Herr *et al.* 2011a), *E. coli* antimutators were isolated in the absence of native proofreading and are due to individual amino-acid substitutions throughout the polymerase structures (Schaaper 1998; Loh *et al.* 2007; Herr *et al.* 2011b). Accordingly, Schaaper and colleagues proposed two general mechanisms for polymerase antimutators: (1) increased dNTP discrimination and (2) increased dissociation from DNA to allow editing by alternate pathways (Fijalkowska and Schaaper 1995). Our data are consistent with these mechanisms. Biochemical analyses will be required to assess the fidelities and

processivities of our Pol ϵ variants and the potential contributions of these parameters to antimutagenesis.

The idea of dissociation and alternative editing raises the question: What other enzymes might remove Pol ϵ errors in the antimutator strains? One candidate is proofreading by Pol δ . Yeast defective for both Pol δ and Pol ϵ proofreading exhibit a synergistic increase in mutation rate, suggesting that one or both polymerases may proofread for the other (Morrison and Sugino 1994). Pavlov and Shcherbakova (2010) present evidence that mistakes made by Pol ϵ are corrected by the proofreading activity of Pol δ , but not vice versa. Other candidates for extrinsic proofreading include the 3' \rightarrow 5' exonuclease activities of *Mre11* (Trujillo and Sung 2001) and *Apn2* (Unk *et al.* 2001) or endonucleases such as *Rad1/Rad10* or *Mus81/Mms4* that cleave 3' flap structures during replication

fork restart (Bardwell *et al.* 1994; Boddy *et al.* 2001; Chen *et al.* 2001; Kaliraman *et al.* 2001; Bastin-Shanower *et al.* 2003).

The majority of *eex* mutants isolated in our screen were extragenic to *pol2-4* (Table 1). Which genes mediate antimutagenesis in these *eex* mutants? What are the functions of the proteins affected by these *eex* alleles? And what are the roles of the proteins in eukaryotic DNA replication? Antimutators could result from upregulation of DNA repair or recombination proteins that, when overexpressed, are able to remove nascent 3' mispairs or mismatches in duplex DNA. MMR proteins, other 3' nucleases (see above), and recombination proteins and resolvases are potential candidates for this mechanism. Antimutators could also result from changes in proteins that function at the DNA replication fork. Proteins directly affecting stalled replication forks are candidates as are checkpoint sensors and indirect effectors of these processes. The error-prone Pols ζ and η are unlikely *eex* candidates because neither polymerase is required for *pol2-4 msh2 Δ* synthetic lethality (Figure 2). Previous studies show that the mutator phenotype caused by defective Pol δ proofreading (*pol3-01*) is partially dependent on *Dun1* (Datta *et al.* 2000), a checkpoint kinase that up-regulates dNTP synthesis (Zhou and Elledge 1993; Zhao and Rothstein 2002; Tsaponina *et al.* 2011). However, the *pol2-4* mutator phenotype does not appear dependent on *Dun1* (Datta *et al.* 2000). Thus, mutations in *DUN1* are unlikely *eex* candidates. There are a number of other possible antimutagenesis mechanisms involving multiple pathways and candidate genes (Herr *et al.* 2011b). Whole-genome sequencing should greatly facilitate identification of the chromosomal *eex* alleles (Birkeland *et al.* 2010; Wenger *et al.* 2010; Dunham 2012). These *eex* alleles may reveal novel pathways of mutation suppression and help clarify the division of labor between Pols ϵ and δ at the replication fork.

Our studies illustrate the inherent instability of eukaryotic mutators. Antimutators readily emerge from yeast harboring defects in either Pol ϵ or Pol δ proofreading (Figure 3) (Herr *et al.* 2011a). Suppressors of diverse mutator phenotypes (proofreading, MMR, and DNA damage repair) also frequently arise in *E. coli* (Tröbner and Piechocki 1984; Schaaper and Cornacchio 1992; Fijalkowska *et al.* 1993; Fijalkowska and Schaaper 1995; Schaaper 1996; Giraud *et al.* 2001b; Notley-McRobb *et al.* 2002). Thus, it appears that mutator phenotypes in general are prone to suppression. We speculate that antimutators moderate high mutation rates and minimize deleterious mutations during microbial adaptation and mammalian oncogenesis.

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Supporting Information

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Emergence of DNA Polymerase ϵ Antimutators That Escape Error-Induced Extinction in Yeast

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Table S1 Oligonucleotides used for site-directed mutagenesis

Allele	PCR Primer Name	PCR Primer Sequence
<i>pol2-4</i>	pol2-4F1	5'-CTGTGGTAATGGCATTGCTATAGCTACCACGAAGCCGCC-3'
	pol2-4R1	5'-GGCGGCTTCGTGGTAGCTATAGCAAATGCCATTACCACAG-3'
<i>pol2-G435C</i>	eex51qcF	5'-ATTCTTATTTACCACAATGTTCCAGGGTTTAAAA-3'
	eex51qcR	5'-TTTTAAACCCTGGGAACATTGTGGTAAATAAGAAT-3'
<i>pol2-V522A</i>	eex20qcR	5'-GAAATGTTGTTGATGGCTCAAGCTTATCAACAT-3'
	eex20qcR	5'-ATGTTGATAAGCTTGAGCCATCAACAACATTC-3'
<i>pol2-T850M</i>	eex26qcF	5'-GAAATGGCGGGGATTATGTGTTAACAGGTGCC-3'
	eex26qcR	5'-GGCACCTGTAAACACATAATCCCCGCCATTC-3'
<i>pol2-K966Q</i>	eex55qcF	5'-GAAGGAAAAGGTATACAGAAAAGATATGCTGTC-3'
	eex55qcR	5'-GACAGCATATCTTTCTGTATACCTTTTCCTTC-3'
<i>pol2-A1153D</i>	eex12qcF	5'-GAAAGACTTGGATCTGATATACAAAAGATAATT-3'
	eex12qcR	5'-AATTATCTTTGTATATCAGATCCAAGTCTTC-3'

Mutations were introduced into pRS415POL2 or pRS415pol2-4 using the QuickChange protocol (Wang and Malcolm 1999), Phusion Polymerase (New England Biolabs), the indicated primers, and PCR conditions of 95°C, 1 min; 16x (95°C, 40 sec.; 53°C, 60 sec., 68°C, 7 min.).

Table S2 Yeast strains

Strain	Relevant Genotype	Reference
LW01 ^a	<i>pol2::kanMX msh2::HIS3</i> + pRS416POL2	This study
LW02 ^a	<i>pol2::kanMX msh3::MET15</i> + pRS416POL2	This study
LW03 ^a	<i>pol2::kanMX msh6::HIS3</i> + pRS416POL2	This study
LW04 ^a	<i>pol2::kanMX mlh1::HIS3</i> + pRS416POL2	This study
LW05 ^a	<i>pol2::kanMX pms1::HIS3</i> + pRS416POL2	This study
LW06 ^a	<i>pol2::kanMX mlh3::MET15</i> + pRS416POL2	This study
LW07 ^a	<i>pol2::kanMX msh6::HIS3 msh3::LEU2</i> + pRS416POL2	This study
LW08 ^a	<i>pol2::kanMX pms1::HIS3 mlh3::TRP1</i> + pRS416POL2	This study
LW09 ^a	<i>pol2::kanMX msh2::HIS3 rev3::TRP1</i> + pRS416POL2	This study
LW10 ^a	<i>pol2::kanMX msh2::HIS3 rad30::TRP1</i> + pRS416POL2	This study
BP0109 ^b	<i>pol3::HIS3 msh2::TRP1</i> + pGL310 [URA3/POL3]	(Herr <i>et al.</i> 2011)
BP0210 ^b	<i>pol3::HIS3 msh3::TRP1</i> + pGL310 [URA3/POL3]	This study
BP0301 ^b	<i>pol3::HIS3 msh6::TRP1</i> + pGL310 [URA3/POL3]	This study
BP0412 ^b	<i>pol3::HIS3 mlh1::TRP1</i> + pGL310 [URA3/POL3]	This study
BP0502 ^b	<i>pol3::HIS3 pms1::TRP1</i> + pGL310 [URA3/POL3]	This study
LW09 ^b	<i>pol3::HIS3 mlh3::TRP1</i> + pGL310 [URA3/POL3]	This study
LW10 ^b	<i>pol3::HIS3 msh6::TRP1 msh3::MET15</i> + pGL310 [URA3/POL3]	This study
LW11 ^b	<i>pol3::HIS3 pms1::TRP1 mlh3::MET15</i> + pGL310 [URA3/POL3]	This study
LW12 ^c	<i>pol2::NAT1 msh2::MET15</i> + pRS416POL2	This study
LW13 ^c	<i>pol3::NAT1 msh2::MET15</i> + pGL310 [URA3/POL3]	This study

^a Strains engineered from the BY4733 strain (*MATa leu2Δ0 ura3Δ0 met15Δ0 trp1Δ63 his3Δ200*), an S288C descendent (Brachmann *et al.* 1998) that we re-derived via sporulation of a BY4733 X BY4734 diploid (kindly provided by Tim Formosa, University of Utah). The *pol2::kanMX* strains were constructed from this re-derived BY4733 strain by first introducing pRS416POL2 (to provide a wild-type plasmid copy of *POL2*) and then replacing the entire chromosomal *POL2* gene with a *kanMX* cassette. pRS416POL2 is the *CEN6/ARSH4/URA3* plasmid pRS416 (Brachmann *et al.* 1998) carrying wild-type *POL2* with its natural promoter.

^b These strains were engineered from P3H3a described in (Herr *et al.* 2011).

^c These strains were engineered from the Y7092 strain (*MATα can1Δ::STE2pr-his5 lyp1Δ ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*), an S288C descendent modified by the Boone lab to be used as a query strain in Synthetic Genetic Analysis (Tong and Boone 2007).

Table S3 Construction of chromosomal gene disruptions

Allele	PCR Primer Name	PCR Primer Sequence	PCR Template ^a
<i>pol2Δ::kanMX</i>	Pol2-kanMXkoF	5'-ATGATGTTTGGCAAGAAAAAACAACGGAGGATCTTCCACTGCAAGATATTCAGCTGGCGAAGTTATTAGGTCTAGAGATCTG-3'	pUG6 (Guldener <i>et al.</i> 1996)
	Pol2-kanMXkoR	5'-TCATATGGTCAAATCAGCAATACAACCTCAATAATATATCAAACCGTAATACTTGGCTACTACGAAGTTATATTAAGGGTTCTCG-3'	
<i>pol2Δ::natMX</i>	Pol2::nat1-for2	5'-AGAGCATATGATGATGAAAGAGCACATTCTATCAAGATAAACACTCTCAGGGGACAAGTATACATGGAGGCCAGAATACCCT-3'	pFvL99 (Stulemeijer <i>et al.</i> 2011)
	Pol2::nat1-rev2	5'-TTTTTTTTTTTTTTTTTTCATGGTAAAGAGGCCATTGAACCTCGCGTTATATAC TGCTTACCAGTATAGCGACCAGCATTAC-3'	
<i>pol3Δ::natMX</i>	Pol3MXF	5'-ATAGATATTGAGCACTTGCTATTAAGCATTAACTTTATACATATACGCACAGCAACATGGAGGCCAGAATACCCT-3'	pFvL99 (Stulemeijer <i>et al.</i> 2011)
	Pol3MXR	5'-GCAAAAAGTTGTTAGCCTTTCTTAATCCTAATATGATGTGCCACCCTATCGTTTTCAGTATAGCGACCAGCATTAC-3'	
<i>msh2Δ::HIS3</i> or <i>msh2Δ::TRP1</i>	Msh2U	5'-AAAAATCTCTTTATCTGCTGACCTAACATCAAATCCTCAGATTA AAAAGTAGA TTGTACTGAGAGTGCAC-3'	pRS413 or pRS414 (Brachmann <i>et al.</i> 1998)
	Msh2D	5'-TTATAACAACAAGGCTTTTATATATTTTCAGGTAATTATCGTTTTCTTTCTGTGCGGTATTTACACCG-3'	
<i>msh2Δ::MET15</i>	Msh2::Met15F	5'-AAAAATCTCTTTATCTGCTGACCTAACATCAAATCCTGCTGGCTTAACTATGCGGCATC-3'	pRS411 (Brachmann <i>et al.</i> 1998)
	Msh2::Met15R	5'-TTATAACAACAAGGCTTTTATATATTTTCAGGTAATTATGTTTACAATTTCTGTATGCGGT-3'	
<i>msh6Δ::HIS3</i> or <i>msh6Δ::TRP1</i>	Msh6U	5'-TTAATTGGAGCAACTAGTTAATTTTGACAAAGCCAATTTGAACTCCAAGAAGTTATTAGGTCTAGAGATCTG-3'	pRS413 or pRS414 (Brachmann <i>et al.</i> 1998)
	Msh6D	5'-ACTTTAAAAAAAATAAGTAAAAATCTTACATACATCGTAAATGAAAATACACGAAGTTATATTAAGGGTTCTCG-3'	
<i>mlh1 Δ::HIS3</i> or <i>mlh1Δ::TRP1</i>	Mlh1U	5'-ATAGTGATAGTAAATGGAAGGTA AAAATAACATAGACCTATCAATAAGCAA TGCTCTCAGAATAAAAGCAGATTGTA CTGAGAGTGCAC-3'	pRS413 or pRS414 (Brachmann <i>et al.</i> 1998)
	Mlh1D	5'-CTCAGGAAATAAACAAAAA ACTTTGGTATTACAGCCAAAACGTTTTAAAGTT AACACCTCTCAAAA ACTTTACTGTGCGGT ATTTACACCG-3'	
<i>pms1Δ::HIS3</i> or <i>pms1Δ::TRP1</i>	Pms1U	5'-GAACGCGAAAAGAAAAGACGCTCTCTTAATAATCATTATGCGATAAAATGTTTCACCACATCGAAAAAGATTGTA CTGAGAGTGCAC-3'	pRS413 or pRS414 (Brachmann <i>et al.</i> 1998)
	Pms1D	5'-TGTATATAATGATTTGTTAATTATATAATGAATGAATATCAAAGCTAGATCA TATTCGTAATCCTTCGACTGTGCGGTATTTACACCG-3'	

<i>mlh3::MET15</i> or <i>mlh3Δ::TRP1</i>	Mlh3KO- upstream	5'-ACATAAACCCAGCGAGGCTTTCAAGGAAGAATGAACGTGAACTCGTCAACTC AAAAAGAAAAGATTGTACTGAGAGTGCAC-3'	pRS411 or pRS414 (Brachmann <i>et al.</i> 1998)
	Mlh3KO- downstream	5'-TGCATATCCGCGCAATTTAAAATGCAGGCGACAAACCTTGTTCCAGGATTAA GGTTCTCTGTGCGGTATTTACACCG-3'	
<i>msh3::MET15</i> or <i>msh3Δ::LEU2</i>	Msh3KO- upstream	5'-GTACTTTTGAGAGCCAAAAGCAGTGCAAATAGATTTATTTTGTGAATCTATT AACAATAAGATTGTACTGAGAGTGCAC-3'	pRS411 or pRS415 (Brachmann <i>et al.</i> 1998)
	Msh3KO- downstream	5'-TCAGTGGATATCCAATGATAGTAATTTGCGGAGTTTATCCGTTGCTGTTATAT TATCTGTGCGGTATTTACACCG-3'	
<i>rev3Δ::TRP1</i>	Rev3U	5'-ATTTGAGTCAATACAAAACACTACAAGTTGTGGCGAAATAAAATGTTTGAAAG ATTGTAAGTACTGAGAGTGCAC-3'	pRS414 (Brachmann <i>et al.</i> 1998)
	Rev3D	5'-TTACCAATCATTTAGAGATATTAATGCTTCTTCCCTTGAACAGATTGATCTGT GCGGTATTTACACCG-3'	
<i>rad30Δ::TRP1</i>	Rad30U	5'-TAGCGCAGGCCTGCTCATTTTTGAACGGCTTTGATAAAACAAGACAAAGCAG ATTGTAAGTACTGAGAGTGCAC-3'	pRS414 (Brachmann <i>et al.</i> 1998)
	Rad30D	5'-TCATTTTTTTCTTGAAAAAATGATAAGATGTTTTGGAAGATGTAACCTTCTG TGCGGTATTTACACCG-3'	

^a pRS411 was used as template for gene replacement with *MET15*; pRS413 with *HIS3*; pRS414 with *TRP1*; pRS415 with *LEU2*.

Mutations were introduced into yeast using PCR products generated with the indicated primers and template DNAs. The PCR conditions for all primers used here were: 98°C, 1 min.; 30x (98°C, 10 sec.; 55°C, 30 sec.; 72°C, 90 sec.); 72°C, 60 sec.

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