DNA replication error-induced extinction of diploid yeast

Alan J. Herr*, Scott R. Kennedy, Gary M. Knowels, Eric M. Schultz, and Bradley D. Preston

Department of Pathology, University of Washington, Seattle, Washington 98195
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*Correspondence: Alan J. Herr
Department of Pathology, Box 357705
University of Washington
1959 NE Pacific St.
Seattle, WA 98195-7705
alanherr@uw.edu
206-616-5063 (tel)
206-543-3967 (fax)
ABSTRACT

Genetic defects in DNA polymerase accuracy, proofreading, or mismatch repair (MMR) induce mutator phenotypes that accelerate adaptation of microbes and tumor cells. Certain combinations of mutator alleles synergistically increase mutation rates to levels that drive extinction of haploid cells. The maximum tolerated mutation rate of diploid cells is unknown. Here, we define the threshold for replication error-induced extinction (EEX) of diploidSaccharomyces cerevisiae. Double mutant pol3 alleles that carry mutations for defective DNA polymerase δ proofreading (pol3-01) and accuracy (pol3-L612M or pol3-L612G) induce strong mutator phenotypes in heterozygous diploids (POL3/pol3-01,L612M or POL3/pol3-01,L612G). Both pol3-01,L612M and pol3-01,L612G alleles are lethal in the homozygous state; cells with pol3-01,L612M divide up to 10 times before arresting at random stages in the cell cycle. Antimutator eex mutations in the pol3 alleles suppress this lethality (pol3-01,L612M,eex or pol3-01,L612G,eex). MMR defects synergize with pol3-01,L612M,eex and pol3-01,L612G,eex alleles, increasing mutation rates and impairing growth. Conversely, inactivation of the Dun1 S-phase checkpoint kinase suppresses strong pol3-01,L612M,eex and pol3-01,L612G,eex mutator phenotypes as well as the lethal pol3-01,L612M phenotype. Our results reveal that the lethal error threshold in diploids is ten times higher than in haploids and likely determined by homozygous inactivation of essential genes. Pronounced loss of fitness occurs at mutation rates well below the lethal threshold,
suggesting that mutator-driven cancers may be susceptible to drugs that exacerbate replication errors.

**INTRODUCTION**

Evolutionary selection for long-term fitness acts on the genes for DNA replication and repair, driving spontaneous mutation rates to a low level (Lynch 2010). Yet at times, selection favors cells with mutator phenotypes that increase the adaptive mutation rate (Chao and Cox 1983; Mao et al. 1997; Sniegowski et al. 1997; Giraud et al. 2001; Notley-McRobb et al. 2002; Nilsson et al. 2004; Loh et al. 2010). Many of the general principles governing mutators in replicating populations have been established using microbes. Mutators are common in microbial populations because they continually arise and hitch-hike on the fitness effects of rare adaptive mutations (Mao et al. 1997; Drake et al. 1998). The relative fitness of mutators is maximal when selection conditions require multiple adaptive mutations (Mao et al. 1997; de Visser 2002). But most mutations are deleterious (Sturtevant 1937; Funchain et al. 2000; Giraud et al. 2001), and for haploids, the short-term benefits of being a mutator rapidly erode as mutation burden increases.

To avoid extinction following adaptation, cells must either replace the mutator allele with a wild-type copy (Herr et al. 2011; Williams et al. 2013b) or acquire antimituator mutations that suppress the mutator phenotype (Tröbner and Piechocki 1984; Schaaper and Cornacchio 1992; Fijalkowska and Schaaper 1995; Giraud et al. 2001; Herr et al. 2011; Williams et al. 2013b). Error-induced extinction occurs within a few cell divisions in bacterial and haploid yeast cells.
lacking both DNA polymerase proofreading and MMR, because mutation rates exceed an error threshold of one inactivating mutation per essential gene per cell division (Morrison et al. 1993; Fijalkowska and Schaaper 1996; Herr et al. 2011; Williams et al. 2013b). When cells replicate near the haploid error threshold, antimutators readily emerge, indicating that strong mutator phenotypes may be inherently transient (Fijalkowska and Schaaper 1996; Herr et al. 2011; Williams et al. 2013b). Thus, while the need for genetic diversity selects for the emergence of mutators during adaptation, the accumulation of deleterious mutations limits the persistence of mutators.

Mammalian mutator phenotypes are theorized to play a role in the somatic evolution of tumor cells (Loeb et al. 1974; Loeb 2011). Ample support exists for this hypothesis. Families predisposed to colon and endometrial cancer encode defects in MMR components or polymerase proofreading that increase mutation rate ((Lynch et al. 2009; Palles et al. 2012; The Cancer Genome Atlas Network 2012a; Church et al. 2013)). Mice with engineered defects in MMR (Baker et al. 1995; de Wind et al. 1995; Baker et al. 1996; Edelmann et al. 1996; Reitmair et al. 1996; Edelmann et al. 1997), polymerase proofreading (Goldsby et al. 2001; Goldsby et al. 2002; Albertson et al. 2009) or polymerase accuracy (Venkatesan et al. 2007) also display elevated mutation rates and cancer predisposition. Finally, deep sequencing of cancer genomes from human patients reveals thousands of clonal mutations, the hallmark of a mutator phenotype (Loeb 2011; Imielski et al. 2012; Nik-Zainal et al. 2012; Palles et al. 2012; Pugh et al. 2012; The Cancer Genome Atlas Network 2012a; 2012b). Some hyper-mutated
intestinal and endometrial tumors carry defects in both MMR and DNA polymerase proofreading, consistent with synergy between these two pathways in human disease (Palles et al. 2012; The Cancer Genome Atlas Network 2012a).

Unlike haploid mutators, diploid mutators are buffered from the effects of recessive deleterious mutations (Morrison et al. 1993). Diploid yeast tolerate mutation loads that are lethal to their haploid offspring (Wloch et al. 2001). This tolerance for recessive mutations has the important benefit of allowing diploid mutators to remain competitive after the acquisition of adaptive mutations (Thompson et al. 2006). The mounting heterozygous mutation load, however, is unlikely to be neutral. Competition experiments with a comprehensive library of heterozygous-knockout diploid yeast strains reveals that 20% of heterozygotes are at a disadvantage in at least one growth condition (Delneri et al. 2008).

Several lines of evidence suggest that diploids, like haploids, may be subject to a lethal error threshold. First, diploid MMR-deficient yeast lineages propagated continually through bottlenecks occasionally go extinct (Zeyl et al. 2001). Second, a recessive allele encoding a hyper-mutator Pol δ variant drives diploid yeast to extinction when homozygous (Daee et al. 2010). Third, mice deficient in Pol δ proofreading and MMR die during embryogenesis by day E9.5; mice deficient in Pol ε proofreading and MMR die by day E14.5 (Albertson et al. 2009). Thus, all diploids may be limited in the number of random mutations they can tolerate. Defining the maximum mutation rate of diploids is important for understanding the mutational robustness of diploid cells, the long-term fitness of
Herr et al.

mutator-driven cancers, and the impact of a lifetime of mutation accumulation in somatic cells.

Previously, we utilized a series of mutator Pol δ variants expressed in MMR-defective strains to empirically define the lethal error threshold for haploid budding yeast (Herr et al. 2011). The isolation of error-induced extinction (eex) mutants encoding antimutator changes in Pol δ provided crucial support for the hypothesis of lethal mutagenesis. Several of these eex alleles lowered mutation rates only two to five-fold below the lethal error threshold and helped to refine our estimate of the threshold. Here, we take a similar approach to define the maximum mutation rate for diploid yeast. Diploids defective in both Pol δ proofreading and MMR are viable (Morrison et al. 1993). Thus, our strategy was to increase mutation rates by perturbing polymerase accuracy as well as proofreading and MMR. We find that combined defects in polymerase proofreading and accuracy are lethal to diploids, but suppressed by antimutator alleles. We then combine additional mutator and antimutator alleles to delineate the diploid error threshold.

MATERIALS AND METHODS

Media and Growth Conditions

Yeast were grown at 30°C using YPD, synthetic complete (SC) media or SC ‘drop-out’ media deficient in defined amino acids to select for prototrophic genetic markers (Sherman 2002). Premade nutrient supplements for SC and SC lacking uracil and leucine were purchased from Bufferad. Other drop-out nutrient supplements were made as described (Sherman 2002) from individual
components purchased from Sigma-Aldrich or Fisher Scientific. To select for
Ura\(^{-}\) cells during plasmid shuffling or mutation rate assays, we used SC media
containing 1 mg/ml 5-fluoorotic-acid (FOA; Zymo Research) (Boeke \textit{et al.} 1984).
To select for Trp\(^{-}\) cells during plasmid shuffling we used media containing 5-
fluoroanthranilic acid (FAA) made as described (Toyn \textit{et al.} 2000). Canavanine-
resistant haploids for mutation rate assays were selected on SC plates lacking
arginine that contained 60 \(\mu\)g/ml canavanine (Sigma-Aldrich). For mutation rate
assays in diploids, we used SC plates containing 60 \(\mu\)g/ml canavanine and either
50 \(\mu\)g/L nourseothricin (ClonNat, Werner BioAgents) or 300 \(\mu\)g/L geneticin
(G418, Sigma-Aldrich) with mono-sodium-glutamate (MSG) (1 g/L) as the
nitrogen source rather than ammonium sulfate (Tong and Boone 2006).

\textbf{Yeast Plasmids and Strains}

\textbf{Plasmids}: pGL310 (Simon \textit{et al.} 1991; Giot \textit{et al.} 1995) is derived from
YCP50 (CEN4/ARS1/URA3) (Rose \textit{et al.} 1987) and carries POL3 under control
of the endogenous promoter. YCplac111POL3 and YCplac111pol3 derivatives
(Herr \textit{et al.} 2011) are derived from YCplac111 (CEN6/ARS1/LEU2) (Gietz and
Sugino 1988), and carry the WT or mutant \textit{pol3} coding and regulatory
sequences, cloned between the \textit{HindIII} and \textit{EcoRI} restriction sites.
pRS414POL3 (Herr \textit{et al.} 2011) is derived from pRS414 (CEN6/ARS4/TRP1)
(Brachmann \textit{et al.} 1998) and carries the \textit{HindIII–EcoRI} \textit{POL3} fragment from
YCplac111POL3. Construction of YCplac111pol3-L612M, YCplac111pol3-
L612G, YCplac111pol3-L612K, YCplac111pol3-01,L612M, YCplac111pol3-
01,L612G, and YCplac111pol3-01,L612K were described previously
pRS416-POL3, pRS416-pol3-01, pRS416-pol3-01,L612M, and pRS416-pol3-01,L612G were generated by subcloning the HindIII–EcoRI fragments from the corresponding YCplac111 vectors into the HindIII and EcoRI sites of pRS416 (URA3) (Brachmann et al. 1998).

To generate pol3-01,L612M,D831G and pol3-01,L612M,K891T alleles, we subcloned DNA containing the D831G and K891T mutations into YCplac111pol3-01,L612M using the EcoRI and BamHI fragments from YCplac111pol3-01,D831G and YCplac111pol3-01,K891T (Herr et al. 2011). We isolated all other pol3-01,L612M,eex alleles from spontaneous mutants that escaped the pol3-01,L612M lethality in haploids during plasmid shuffling experiments. One pol3-01,L612M suppressor mutation (K559N) was also independently isolated as a pol3-01,L612G suppressor. To generate additional pol3-01,L612G,eex alleles, we subcloned DNA containing the Q697R, A704V, and G818C mutations into YCplac111pol3-01,L612G using BamHI–EcoRI fragments from the pol3-01,L612M,eex plasmids. The S319F allele was subcloned into YCplac111pol3-01,L612G using Ncol–Eagl fragments. The entire pol3 sequence was verified for all plasmids used in this study.

**Strains:** All strains are listed in Table S1. All PCR fragments used for strain construction are described in Table S4 and were generated as described in Supporting Information.

**Images**

Images of visible colonies on petri dishes were taken under ambient lighting using a Canon Powershot SD 630 Digital camera mounted to a copy
stand (Testrite Instruments Co). Images were imported into Photoshop, where the exposure and offset settings were adjusted (~1.5, 0.5 respectively) to maximize visualization of colonies. Images of microcolonies in Figures 2 and 3 were taken with backlighting using an Olympus CX41 microscope, equipped with a DP12 digital camera at 40X or 100X magnification.

**Plasmid Shuffling**

Plasmid shuffling (Boeke *et al.* 1984) with pGL310- or pRS414POL3-containing strains has been described previously (Simon *et al.* 1991; Herr *et al.* 2011). Cells transformed with YCplac111pol3 plasmids, YCplac111POL3 (positive control), or YCplac111 (empty vector control) were plated on SC lacking uracil and leucine (pGL310 cells) or SC lacking tryptophan and leucine (pRS414POL3 cells). After two to three days incubation at 30°C, individual colonies were picked and dispersed in sterile H2O. Serial dilutions containing approximately 10^5, 10^4, 10^3, and 10^2 cells were plated on SC or SC with FOA (for pGL310 cells) (Boeke *et al.* 1984) or FAA selection media (for pRS414POL3 cells) (Toyn *et al.* 2000) to select for cells that had spontaneously lost pGL310 or pRS414POL3.

**Mutation Rates**

Mutation rates were calculated by fluctuation analysis of FOA- and canavanine-resistant mutants in replica cultures (Foster 2006). For mutation rates of P3H3a-derived haploid strains (Figure 1), four independent freshly shuffled colonies per genotype were picked from FAA shuffling plates and used to inoculate 100 µl overnight SC cultures in sterile PCR strip tubes, and
incubated without shaking at 30°C. The following morning each culture was
diluted to 1000 cells/ml and divided into twelve 100 μl replica cultures, grown in a
sterile round-bottom 96-well polypropylene microtiter plate, sealed with PCR
plate adhesive sealers (ABgene, AB-0580) to minimize evaporation (Lang and
Murray 2008; Herr et al. 2011). After two days of growth at 30°C the cells were
suspended by vigorous vortexing for two minutes. The cells and liquid were
recovered by a brief centrifugation. We added 200 μl of sterile water, re-
suspended the cells by pipetting, and plated 150 μl of nine replica cultures for
each isolate on separate canavanine and FOA selection plates. The remaining
three replica cultures for each isolate were combined, diluted, and plated on SC
plates to determine the number of colony forming units, which we used to
estimate the total number of cells (Nt) for each mutation rate determination.

To measure forward mutation rates conferred by pol3 heterozygous alleles
(Table 1), we used a diploid strain with the nourseothricin-resistance gene
(natMX) inserted immediately downstream of a hemizygous CAN1 gene
(Goldstein and McCusker 1999). This allowed us to select against confounding
Can’ mitotic recombinants. Individual transformant colonies were considered
‘replica’ cultures for purposes of fluctuation analysis. Colonies were picked from
the transformation plates, suspended with vigorous vortexing in 200 μl sterile
H₂O, and 170 μl were plated on SC-MSG plates lacking arginine and containing
canavanine and nourseothricin. For each genotype, 10 μls from each replica
were pooled, serially diluted, and plated on SC media to determine the average
number of colony forming units per replica. The remaining cell suspension of
Herr et al.

each replica was treated with zymolyase and used for PCR genotyping as described in Supporting Information. For our calculations, we used 32 verified, independent transformants for POL3/POL3 cells, 33 for POL3/pol3-01, 25 for POL3/pol3-01,L612G, and 28 for POL3/pol3-01,L612M.

To define the diploid error threshold, we engineered diploid plasmid-shuffling strains that were hemizygous for CAN1 (CAN1::kanMX/can1Δ::TRP1). The strains carried deletions of both copies of chromosomal POL3, complemented by the POL3-URA3 plasmid, pGL310. We transformed cells with YCplac111pol3 plasmid and selected for colonies on SC plates lacking uracil and leucine. Transformants were suspended in 100 μl of H2O, and 10-fold serial dilutions were plated onto SC-FOA and SC media to measure viability and shuffling efficiency (Figure 4). At the same time larger volumes of each dilution were plated on FOA to obtain sufficient numbers of replica colonies for fluctuation analysis. After 2 days of growth, 8 well-isolated FOA-resistant colonies from each of three independent shuffling experiments per genotype (24 total) were suspended in 100 μl of H2O. For strains with low and moderate mutation rates, we plated 90 μl on canavanine-G418 selection plates. The remaining 10 μl was used for 10-fold serial dilutions for Nt determinations. For strains with high mutation rates, 10-fold serial dilutions of each replica culture were plated on SC and canavanine-G418 selection plates to ensure accurate counting of the number of Can’ mutants in each replica.

Mutation rates were calculated from the number of mutant colonies in each replica by first estimating m by maximum likelihood (Rosche and Foster
2000) using newtonLD or newtonLDPlating in Salvador 2.3 (Zheng 2002; Zheng 2005; Zheng 2008) with Mathematica 8.0 (Wolfram Research) and then dividing by the number of cell divisions inferred from the number of colony forming units. Confidence intervals were calculated using LRIntervalLD or CILDplating in Salvador 2.3, which rely on likelihood ratios (Zheng 2002; Zheng 2005; Zheng 2008).

Can\(^{-}\) mutation rates were converted to per-base-pair mutation rates as described (Herr \textit{et al.} 2011). The Can\(^{-}\) mutation rate was multiplied by a correction factor (4.53), which takes into account the number of scorable sites within \textit{CAN1} (Herr \textit{et al.} 2011), and dividing by the size of \textit{CAN1} (1773 bp).

**Modeling the Diploid Error Threshold**

To model the influence of mutation rate on colony formation of diploids, we assumed that cell death results primarily from recessive lethal mutations in both copies of a random essential gene. We described the rate of cell death \((R_{cd})\) as follows:

\[
R_{cd} = (MR_{eg})^2(1000)(N_g)
\]

where \(MR_{eg}\) is the median mutation rate of the essential genes and is squared because both copies of an essential gene must be mutated for lethality. These mutations could occur in the same cell division or in two distinct cell divisions. 1000 is the number of essential genes in yeast and \(N_g\) is the number of cellular generations at the mutation rate, \(MR_{eg}\). \(MR_{eg}\) can be related to our measured
Can\(^r\) mutation rates \((MR_{can1})\) by applying a correction factor for size differences between \(CAN1\) (1773 bp) and the median essential gene (1296 bp) (Table S5).

\[
MR_{eg} = \frac{1296}{1773} \times MR_{can1} = 0.73 \times MR_{can1}
\]

We substituted equation (2) into equation (1) to estimate the expected rate of cell death at different Can\(^r\) mutation rates.

\[
R_{cd} = (0.73 \times MR_{can1})^2 (1000) (N_g)
\]

Using equation (3) and Microsoft Excel (Table S6) we calculated values for \(R_{cd}\) at different Can\(^r\) mutation rates for each of the first 20 cellular generations of a mutator cell line \((N_g = 1, 2, 3, \ldots, 20)\). We then used a Poisson distribution function (equation 4, where e is the base of the natural logarithm) to estimate the probability \((P_v)\) of cells at each generation remaining free of homozygous lethal mutations \((k = 0)\) (Table S6).

\[
P_v = \frac{R_{cd}^k e^{-R_{cd}}}{k!} = e^{-R_{cd}}
\]

Finally, we modeled colony growth at different mutation rates by multiplying the total cells produced at each division by the corresponding \(P_v\) value to estimate how many of these cells (rounded to nearest whole number) would be able to divide in the next generation (Table S6). The predicted numbers of dead and live cells at the end of 20 generations were summed to give an estimated colony size. The same procedure was used to model growth for 1000 generations (Table S7).
Whole Genome Sequencing

To obtain the POL3/POL3, POL3/pol3-01,L612G, and POL3/pol3-01,L612M strains used for whole genome sequencing (Table 2), we first subcloned the initial Ura+ transformants (Figure 2A) onto SC-URA media. Well-isolated colonies were then grown overnight in 10 ml YPD, and genomic DNA was purified from $10^8$ cells using a ZR Fungal/Bacterial Miniprep kit (Zymo Research). DNA was simultaneously fragmented and ligated to Illumina DNA adapters using the Nextera V2 Kit (Illumina), post-indexed by PCR, and sequenced using 101 bp, paired-end reads on an Illumina 2500 platform.

Reads were aligned to the *S. cerevisiae* S288C genome (Assembly R64-1-1) using the Burrows-Wheeler Aligner (Li and Durbin 2009). The aligned reads were then filtered to remove unmapped reads and reads that mapped to more than one location. PCR duplicates were evaluated using the MarkDuplicates option in the Picard suite of programs ((Li et al. 2009), URL: http://picard.sourceforge.net). In order to reduce false variant calls, The Genome Analysis Took Kit (GATK) suite of programs was used for local realignment (IndelRealigner and LeftAlignIndels) and base quality score recalibration (BQSR) (BaseRecalibrator and PrintReads) (DePristo et al. 2011). The BQSR step used a BY4743-specific SNP database for SNP masking (see next section). After processing, the final average sequencing depth ranged between 100 and 300-fold. Coding variants were identified using VarScan2 with the strand bias filter option invoked (Koboldt et al. 2012). Only regions of the genome with >15-fold coverage and a minimum average read quality of 15 were evaluated. The
BY4743 strain specific SNPs were filtered out of the final variants and were not scored as a mutation.

To build the BY4743 specific SNP database, 10 independent colonies of diploid BY4743 strain were sequenced, aligned, and processed as previously described with the exception that base quality score recalibration was not performed. Initial candidate SNPs were called using the GATK UnifiedGenotyper with default settings, as well as VarScan2 with a minimum coverage of 15-fold and a minimum average read quality of 15. Variants called in at least 40% the samples by both programs were considered candidate SNPs. BQSR was then performed on the processed sequencing data and a second round of candidate SNPs were called using both GATK’s UnifiedGenotyper and VarScan2, using the same criteria. BQSR was then repeated on the original processed sequencing data and candidate SNPs were called as previously described. This process was repeated until there were no further changes in the number of called SNPs. A total of 305 likely SNPs were recorded.

RESULTS

Synthetic Interactions Between Polymerase Accuracy, Proofreading, and MMR in Haploid Cells

In order to develop genetic tools to probe the diploid error threshold, we first used haploid strains (Table S1) to examine the synthetic interactions between mutations defective in Pol δ accuracy (pol3-L612G, pol3-L612M, and pol3-L612K) (Venkatesan et al. 2006), proofreading (pol3-01) (Morrison et al. 1993), and base-base MMR (msh6Δ) (Alani 1996; Iaccarino et al. 1996; Johnson
et al. 1996; Marsischky et al. 1996). Plasmid-shuffling experiments confirmed
that all three \( \textit{pol3-L612} \) alleles were tolerated in otherwise WT cells, although
\( \textit{pol3-L612G} \) markedly reduced colony sizes (Figure 1A). Each of the alleles
increased FOA- and canavanine-resistance (\( \text{Can}^+ \)) mutation rates, consistent with
a genome-wide elevation in mutation rates (Figure 1B). The \( \textit{pol3-L612M} \) and
\( \textit{pol3-L612K} \) alleles induced similar levels of mutagenesis (~10-fold above the WT
background), while mutation rates with \( \textit{pol3,L612G} \) were comparable to those
observed with the \( \textit{pol3-01} \) allele (~20 to 100-fold above background depending
on the locus; Figure 1B). Previous work indicated that \( \textit{pol3-L612M} \) and mutant
MMR alleles synergistically elevate mutation rate (Li et al. 2005; Nick McElhinny
et al. 2008). We found that \( \textit{pol3-L612G} \) was synthetically sick with \( \textit{msh6} \)\( \Delta \)
(Figure 1A), suggesting that \( \textit{pol3-L612G msh6} \)\( \Delta \) cells have mutation rates near
the haploid error threshold, as observed for \( \textit{pol3-01 msh6} \)\( \Delta \) cells (Figure 1A)
(Sokolsky and Alani 2000; Herr et al. 2011).

To determine whether proofreading defects synergize with polymerase
accuracy defects, we analyzed double mutant alleles containing \( \textit{pol3-01} \) and the
\( L612 \) mutations (Venkatesan et al. 2006). We found that cells could not survive
with \( \textit{pol3-01,L612M} \) or \( \textit{pol3-01,L612G} \) as the sole source of Pol \( \delta \), but in contrast
to a previous report (Venkatesan et al. 2006), cells with the \( \textit{pol3-01,L612K} \) allele
readily formed colonies (Figure 1A). The \( L612K \) mutation lowered the \( \textit{pol3-01} \)
mutator phenotype between 2- and 3-fold (Figure 1B) and suppressed the
synthetic lethality between \( \textit{pol3-01} \) and \( \textit{msh6} \)\( \Delta \) (Figure 1A). Consistent with this
observation, the analogous L606K substitution in human Pol \( \delta \) suppresses the
mutator phenotype of Pol $\delta$ proofreading deficiency \textit{in vitro} (Schmitt \textit{et al.} 2010). Thus, although \textit{pol3-L612K} is a moderate mutator, it clearly does not synergize with \textit{pol3-01}. In contrast, the lethality conferred by \textit{pol3-01,L612G} and \textit{pol3-01,L612M} suggests that the \textit{L612G} and \textit{L612M} mutations may synergize with \textit{pol3-01} to drive mutation rates above the lethal error threshold of haploid yeast.

\textbf{Heterozygous effects of \textit{pol3-01,L612M} and \textit{pol3-01,L612G} Alleles in Diploid Cells}

To determine the effects of these haploid-lethal alleles in diploids, we generated heterozygous mutants by transforming diploid cells with \textit{pol3-01,L612M} and \textit{pol3-01,L612G} DNA linked to \textit{URA3} (Figure S1A). Correct chromosomal integration occurred efficiently, resulting in numerous independent \textit{POL3/pol3-01,L612M} and \textit{POL3/pol3-01,L612G} transformants (Figure S1B and S1C), which formed similar-sized colonies as \textit{POL3/POL3} control transformants (Figure 2A). We picked individual colonies of each genotype and plated them immediately onto solid sporulation media without subcloning or further propagation. All diploid strains formed tetrads with similar frequencies. However, while all haploid spores derived from \textit{POL3/POL3} diploids lived, all spores from \textit{POL3/pol3-01,L612M} and \textit{POL3/pol3-01,L612G} diploids were inviable whether or not they inherited the mutator allele (Figure 2B). The majority of these spores terminated as unbudded cells (Table S2). Thus, the \textit{pol3-01,L612M} and \textit{pol3-01,L612G} alleles exert dominant-lethal effects on spore viability.
The dominant spore lethality could be explained by the accumulation of recessive lethal mutations in the diploid parent cells prior to sporulation. Our diploid strains were hemizyous for the \textit{CAN1} gene (Figure 2C, top schematic), which allowed us to determine the frequency of Can\textsuperscript{r} mutant cells in independent transformant colonies, (Figure 2C, Can + NTC) and then calculate mutation rates by fluctuation analysis (Rosche and Foster 2000). The \textit{POL3/pol3-01,L612M} and \textit{POL3/pol3-01,L612G} strains exhibited mutation rates more than 1000-times greater than the \textit{POL3/POL3} diploid controls (Table 1). In absolute terms, the mutation rates of the heterozygous mutant diploids (0.9-1.1 x 10\textsuperscript{-3} Can\textsuperscript{r} mutants/cell division) were at the haploid error threshold (~10\textsuperscript{-3};(Herr \textit{et al.} 2011)), suggesting that they accumulated a recessive lethal mutation nearly every cell division (Herr \textit{et al.} 2011).

To understand the nature of mutagenesis in these cells, we sequenced the genomes of individual \textit{POL3/pol3-01,L612M}, \textit{POL3/pol3-01,L612G}, and \textit{POL3/POL3} colonies. Dispersed cells from a single transformant colony of each genotype (Figure 2A) were grown into subclones, and one subclone of each genotype was analyzed by whole genome sequencing. The \textit{POL3/POL3} strain harbored a single T→G mutation in its genome. In contrast, the \textit{POL3/pol3-01,L612M} subclone contained 1535 de novo point mutations and the \textit{POL3/pol3-01,L612G} subclone had 1003 mutations (Table 2, S3). The mutation spectra of the two mutator strains consisted of primarily base-substitutions with 3-5\% frameshifts. Similar percentages of transitions, transversions, and -1 frameshifts were observed; however the \textit{POL3/pol3-01,L612M} strain contained three times
Herr et al.

the number of +1 frameshifts found in the POL3/pol3-01, L612G strain (Tables 2, S3).

At the time of subcloning, the primary transformant colonies (Figure 2A) contained ~ $10^5$ cells, the result of 17 cellular generations ($10^5$ cells = $2^{17}$ cell divisions). The number of mutations (1003 or 1535), divided by the size of the diploid yeast genome ($2.2 \times 10^7$ bp), divided by the number of generations (17) gives mutation rates of ~3-4 $\times 10^{-6}$ mutations/bp/generation, consistent with the expected per-base-pair mutation rate of cells replicating with a mutation rate of 1 $\times 10^{-3}$ Can' mutants/cell division ($2.5 \times 10^{-6}$ mutations/bp/generation) (Herr et al. 2011). This is the cellular mutation rate. The actual error rates of the mutator polymerases depend on their contribution to genome replication and may be several-fold higher.

The accumulation of mutations in POL3/pol3-01, L612M and POL3/pol3-01, L612G diploids coincided with a loss in fitness. Although the initial transformants were indistinguishable from WT, subcloning revealed variably sized colonies, including numerous microscopic ones with limited proliferative capacity (Figure 2C, back-lite images). Thus, heterozygous pol3-01, L612M and pol3-01, L612G alleles exert strong semi-dominant mutator phenotypes in diploid cells that severely compromise both replicative and reproductive fitness.

**Error-Induced Extinction in Diploids**

To determine if diploid yeast are viable with pol3-01, L612M or pol3-01, L612G as the sole source of Pol δ, we constructed a diploid strain for plasmid shuffling, which contained deletions of both chromosomal copies of POL3,
complemented by a POL3–URA3 plasmid (Figure 3). Cells were readily transformed with LEU2–CEN plasmids carrying the pol3-01,L612M and pol3-01,L612G alleles. However, the pol3-01,L612M and pol3-01,L612G alleles could not serve as the sole source of Pol δ: the transformed cells failed to form visible colonies when plated on FOA media, which selects for spontaneous loss of the POL3–URA3 plasmid. Microscopic examination of the agar surface revealed that pol3-01,L612M cells formed microcolonies of around 1000 cells (Figure 3). No pol3-01,L612G microcolonies arose greater than ~10 cells. Thus, pol3-01,L612G rapidly kills diploids in the absence of POL3, while pol3-01,L612M may confer a mutation rate near the maximum threshold for diploid colony formation.

We examined whether cells in the pol3-01,L612M microcolonies were still able to divide. Using micromanipulation, we dissected 100 cells away from one pol3-01,L612M microcolony and 210 cells from another and monitored growth. After 2 days, all 100 cells from the first microcolony failed to divide; 23 out of 210 cells from the second microcolony divided a limited number of times, forming small microcolonies of 4 to 100 cells. Cells that failed to divide arrested in a variety of terminal stages: 114 were unbudded, 48 were budded, and 45 were arrested as a dumbbell or multi-budded cell. Thus, as with haploids undergoing error-induced extinction (Morrison et al. 1993), pol3-01,L612M diploids arrest throughout the cell cycle, consistent with death by random mutations.
Defining the Diploid Error Threshold

To experimentally define the maximum mutation rate of diploids, we created a series of strains exhibiting a wide range of spontaneous mutation rates. This allowed us to titrate mutation rates up to the lethal limit of diploids. Key to this approach was the use of pol3 alleles that confer escape from error-induced extinction and function as antimutators (eex; Table 3). We also exploited the antimutator effect of Dun1 deficiency (dun1Δ) (Datta et al. 2000) and the synergistic relationship between Pol δ mutators and defective MMR (Morrison et al. 1993; Herr et al. 2011).

We previously isolated eex mutations in pol3-01 that lowered the mutator phenotype between 3- and 100-fold. These pol3-01,eex alleles, in combination with msh6Δ, allowed us to refine the estimate of the maximum mutation rate of haploids (Herr et al. 2011). To obtain a similar collection of alleles to define the diploid error threshold we took two strategies. First, we engineered two known pol3-01 antimutator alleles into pol3-01,L612M (K891T and D831G (Herr et al. 2011)). Second, we identified antimutator mutations in spontaneous mutants that emerged during pol3-01,L612M haploid plasmid shuffling experiments. We subsequently engineered several of these spontaneous eex mutations into pol3-01,L612G. All of the pol3-01,L612M,eex and pol3-01,L612G,eex alleles retain the pol3-01 and L612M or L612G mutations in addition to the eex mutation.

We found that engineered and spontaneous eex mutations suppressed the lethal pol3-01,L612M and pol3-01,L612G phenotypes in MMR-proficient diploids. Mutation rates conferred by the alleles varied from near WT levels.
Herr et al.  

(pol3-01,L612M,K559N, pol3-01,L612M,R674G) to 1 x 10^{-3} Can^{r} mutants/cell division (pol3-01,L612M,G447D, pol3-01,L612M,F467I, and pol3-01,L612M,R658G). MMR deficiency (msh2Δ/msh2Δ) elevated mutation rates an average of 222-fold, consistent with previous estimates of MMR efficiency in haploids (Nick McElhinny et al. 2008; Herr et al. 2011). However, the magnitude of the MMR effect depended on the pol3 allele. Mutation rates increased less than 7-fold with the strongest mutator alleles (pol3-01,L612M,G447D, pol3-01,L612M,F467I, and pol3-01,L612M,R658G), which suggests that MMR may be saturated in these strains. In contrast, mutation rates increased more than 500-fold with the weakest mutator alleles (pol3-01,L612M,G818C, pol3-01,L612M,R674G, and pol3-01,L612M,K559N), indicating that Pol δ errors in these strains may be recognized more efficiently by MMR. Half of the pol3 MMR-deficient strains had mutation rates greater than or equal to 1 x 10^{-3} Can^{r} mutants/cell division and exhibited slow growth phenotypes. MMR-deficient strains expressing pol3-L612G, pol3-01,L612G,A704V, or pol3-01,L612M,R658G (Figure 4A) had the highest mutation rates (≥4 x 10^{-3} Can^{r} mutants/cell division) and formed small colonies, suggesting that they exist on the verge of error-induced extinction.  

Dun1-deficiency was previously reported to suppress the pol3-01 mutator phenotype (Datta et al. 2000). We found that Dun1 deficiency suppressed the lethality of pol3-01,L612M, but not pol3-01,L612G (Figure 4A). The pol3-01,L612M dun1Δ/dun1Δ cells had mutation rates of 5.5 x 10^{-3} Can^{r} mutants/cell division. We hoped to use this mutation rate and the average Dun1 effect on
other pol3 mutator alleles to estimate the lethal error rate of pol3-01,L612M Dun1-proficient cells. Dun1-deficient cells expressing a subset of pol3 alleles exhibited synthetic growth defects and were not analyzed further (Figure 4A). With the remaining pol3 alleles, Dun1 deficiency lowered mutation rates an average of 19-fold (Table 3). However, we observed a wide variance in the effect of Dun1 deficiency (2 to 63-fold), suggesting that the rate of pol3-01,L612M Dun1-proficient diploids could be as low as 1 x 10^{-2} Can^{r} mutants/cell division or as high as 3 x 10^{-1} Can^{r} mutants/cell division. In view of this uncertainty, we mathematically modeled the influence of mutation rate on colony growth.

We assumed that cell death most often results from recessive lethal mutations in both copies of a random essential gene. We calculated the expected rate of cell death at different Can^{r} mutation rates for each of the first 20 cellular generations of a mutator cell line (Table S6). We then used a Poisson distribution function to estimate the probability of cells remaining free of homozygous lethal mutations at each generation (Table S6). Finally, to model colony growth we multiplied the number of cells at each generation by the corresponding probability to estimate how many cells (rounded to nearest whole number) would divide in the next generation (Table S6). We then plotted expected colony sizes relative to Can^{r} mutation rate and compared this curve to the experimental observations (Figure 4B).

We observed striking similarities between our experimental observations and the sizes of colonies predicted by the model. Our calculations predict unimpaired growth until mutation rates reach 1 x 10^{-3} Can^{r} mutants/cell division,
which is the mutation rate at which we began to observe growth defects. Predicted and observed colony sizes declined dramatically between $1 \times 10^{-3}$ and $1 \times 10^{-2}$ Can\(^r\) mutants/cell division (Figure 4). At a mutation rate of $5 \times 10^{-3}$ Can\(^r\) mutants/cell division colony size is predicted to be around 70,000 cells. The average colony size produced by \textit{pol3-01,L612M dun1\(\Delta\)/dun1\(\Delta\)} cells ($5.5 \times 10^{-3}$ Can\(^r\) mutants/cell division) was 53,000 cells. At $1 \times 10^{-2}$ Can\(^r\) mutants/cell division colony size is predicted to be just over 700 cells, which is similar to the number of cells estimated in WT diploids shuffled with \textit{pol3-01,L612M} (Figure 3). At $2 \times 10^{-2}$ Can\(^r\) mutants/cell division the model predicts colony sizes of only 16 cells. Thus, we conclude that the maximum mutation rate for colony formation of diploid yeast is around $1 \times 10^{-2}$ Can\(^r\) mutants/cell division.

Strains with mutation rates within an order of magnitude of the diploid error threshold exhibit growth defects, suggesting that they may eventually undergo extinction. We modeled long-term growth of cells with mutation rates between $1$ and $6 \times 10^{-3}$ Can\(^r\) mutants/cell division (Table S7). Diploids with a mutation rate of $1 \times 10^{-3}$ Can\(^r\) mutants/cell division are predicted to continue to divide through 1000 generations (assuming unlimited growth). Doubling the mutation rate to $2 \times 10^{-3}$ Can\(^r\) mutants/cell division leads to extinction by 700 generations, whereas a mutation rate of $4 \times 10^{-3}$ Can\(^r\) mutants/cell division drives extinction by 200 generations. Thus, error-induced extinction of diploids may be inevitable if mutation rates are maintained within an order of magnitude of the maximum mutation rate.
DISCUSSION

Our studies define for the first time the maximum tolerated mutation rate for a diploid organism. We demonstrate that alleles encoding combined defects in Pol δ fidelity and proofreading are lethal in both haploid (Figure 1) and diploid (Figure 3) yeast and confer strong mutator phenotypes in heterozygous diploids (Table 1, Figure 2). Mutations that exert an antimutator effect on the mutator alleles rescue diploids from lethality (Figure 4). Viability and mutation rate measurements of cells with combinations of mutator and antimutator alleles define a mutation rate threshold of $1 \times 10^{-2}$ Can[$r$] mutants/cell division at which colony forming capacity is lost (Figure 4). Finally, mathematical modeling recapitulates the experimentally defined error threshold (Figure 4) and suggests that extinction becomes inexorable once mutation rates rise above $1 \times 10^{-3}$ Can[$r$] mutants/cell division (Figure 5). In what follows, we discuss the lethal synergy between defects in polymerase fidelity and proofreading. We then explore determinants of the diploid error threshold and the implications for mutational robustness in evolution, cancer, and aging.

The Synthetic Relationship between Polymerase Fidelity and Proofreading

The remarkable DNA replication fidelity found in all cells results from multiple error avoidance mechanisms acting in series to restrict the inheritance of polymerase errors. Together, Watson-Crick base-pairing interactions and Pol δ nucleotide selectivity contribute $\sim 1 \times 10^5$ mutations/bp to the overall mutation rate (Kunkel 2009). Pol δ proofreading and MMR each reduce mutation rates by at least $1 \times 10^{-2}$ mutations/bp (Morrison et al. 1993; Herr et al. 2011) to give an
Herr et al.

overall DNA replication fidelity of less than 1 x 10^{-9} mutations/bp (Drake et al. 1998; Lynch et al. 2008). Because of this cooperativity, tandem defects in polymerase accuracy and proofreading, polymerase accuracy and MMR, or polymerase proofreading and MMR are expected to produce multiplicative increases in overall mutation rate (Morrison et al. 1993).

Numerous studies have observed synergistic increases in mutation rate in double mutants deficient in polymerase proofreading and MMR (Morrison et al. 1993; Schaaper 1993; Morrison and Sugino 1994; Tran et al. 1999; Sokolsky and Alani 2000; Greene and Jinks-Robertson 2001; Albertson et al. 2009; Herr et al. 2011) or polymerase accuracy and MMR (Li et al. 2005; Nick McElhinny et al. 2008). Few studies have characterized the consequences of combining defects in polymerase accuracy and proofreading. Venkatesan et al. first reported that pol3-01,L612M and pol3-01,L612G were synthetically lethal in haploids (Venkatesan et al. 2006). Nick McElhinny et al. also observed lethality when pol3-L612M was combined with the proofreading-deficient allele, pol3-DV (Nick McElhinny et al. 2006). These observations suggested that the mutant polymerases were either nonfunctional or that they caused mutation rates to exceed the haploid error threshold.

Polymerases normally extend mispaired primer termini poorly, thereby amplifying proofreading efficiency (Kuchta et al. 1988; Petruska et al. 1988; Perrino and Loeb 1989; Perrino et al. 1989; Mendelman et al. 1990). In the absence of proofreading, Pol δ eventually extends mismatches into duplex DNA (Fortune et al. 2005). In contrast, Pol δ-L612M readily extends mispairs in the
presence of proofreading, resulting in base-substitutions and -1 frameshifts (Nick McElhinny et al. 2006). The equivalent human Pol δ L606M and L606G enzymes exhibit similar mutator phenotypes in vitro and proofreading-deficient Pol δ L606G is highly processive and inaccurate (Schmitt et al. 2009; Schmitt et al. 2010). Thus, the existing biochemical evidence suggests that the polymerases derived from pol3-01,L612G and pol3-01,L612M are likely active and error-prone.

We show that heterozygous pol3-01,L612M or pol3-01,L612G alleles confer strong semi-dominant mutator phenotypes, capable of elevating mutation rates to the haploid error threshold (~10^{-3} Can′ mutants/cell division; (Herr et al. 2011)) (Figure 2C, Table 1). The mutations accumulate throughout the genome in a random fashion and are overwhelmingly base-substitution errors, with equal numbers of transitions and transversions (Table 2). The semi-dominance of pol3-01,L612M and pol3-01,L612G contrasts the recessive nature of pol3-01 (Table 1) (Morrison et al. 1993). Diminished nucleotide selectivity and saturation of MMR may contribute to the semi-dominance of pol3-01,L612M and pol3-01,L612G. In addition, Pol δ-01 may dissociate from mispaired primer termini, allowing editing by WT Pol δ, whereas efficient mispair extension by Pol δ-01,L612M and Pol δ-01,L612G may minimize such proofreading in trans.

The pol3-01,L612M and pol3-01,L612G alleles are lethal in the homozygous state, which we interpret as evidence for lethal mutagenesis. However, other mechanisms of cell death merit consideration. Pol δ participates in a wide range of DNA transactions. These include Okasaki fragment maturation, non-homologous end-joining, break-induced recombination,
nucleotide excision repair, long patch base excision repair, and mismatch repair (Friedberg et al. 2006). Pol δ also incorporates ribonucleotides into DNA (McElhinny et al. 2010), which are removed by RNaseH (McElhinny et al. 2010) and topoisomerase I (Williams et al. 2013a). Although the contribution of Pol δ proofreading to the removal of ribonucleotides is thought to be negligible, (Clausen et al. 2013), the Pol δ-L612M polymerase increases ribonucleotide incorporation by 10-fold (Lujan et al. 2013).

Perturbation of these pathways may contribute to lethality by increasing the point mutation burden or by compromising DNA strand integrity. Unrepaired single or double stranded breaks would arrest cells in S-phase or G2. In pol3-01,L612M microcolonies (Figure 3), G1-arrested cells outnumbered the combined total of S-phase- and G2-arrested cells. This distribution of arrested phenotypes is consistent with random inactivation of essential genes (Herr et al. 2011): a similar distribution was observed when 700 of the 1000 essential genes of yeast were individually turned off by tetracycline repression (Yu et al. 2006). The hypothesis of lethal mutagenesis gains additional support from the ability of antimutator alleles to suppress pol3-01,L612M or pol3-01,L612G lethality and in the reduction of colony growth as mutation rates approach the error threshold.

The Diploid Error Threshold

Experimental and theoretical lines of evidence support a maximum mutation rate for diploid yeast of $1 \times 10^{-2}$ Can' mutants/cell division, which corresponds to 280 base substitutions per cell division (Herr et al. 2011). Our modeling suggests that the main determinant of the error threshold is the
probability of acquiring homozygous recessive mutations in random essential genes. We cannot exclude a role for dominant lethal mutations and synthetic lethal interactions between heterozygous mutations, as these occur with an unknown frequency. Non-lethal interactions between heterozygous mutations may also contribute to reduced growth. Under rich conditions ~3% of heterozygous single gene deletions undermine fitness (Deutschbauer et al. 2005; Delneri et al. 2008). Up to 20% reduce fitness under limiting conditions (Delneri et al. 2008; Hillenmeyer et al. 2008). At a minimum, the accumulation of multiple haplo-insufficiencies may erode fitness in an additive fashion. But given the integration of yeast metabolic networks, ample opportunity exists for synthetic interactions, and these increase exponentially with mutation burden.

The diploid error threshold describes the maximum mutation rate compatible with growth of a mutator clone during the first 20 cellular generations. Our modeling suggests that “viable” strains with mutation rates higher than $1 \times 10^{-3}$ Can$^r$ mutants/cell division go extinct within 1000 generations (Figure 5). Such lineages may be rescued by mutator suppression. For strains with high mutation rates, we frequently observe antimutator mutants arising even within the first 20 cellular generations.

**Error Thresholds in Evolution, Cancer, and Aging**

The ability of cells to withstand mutation accumulation depends on several factors, including the tolerance of protein structures to amino acid substitutions (Wagner 2000), the capacity of chaperones to assist folding and function of mutant proteins (Cowen and Lindquist 2005), and the flexibility of metabolic
networks to compensate for genetic deficiencies (Harrison et al. 2007). Diploidy provides additional protection by buffering cells from recessive mutations (Wloch et al. 2001). The diploid error threshold described here applies to mitotically dividing cells grown in complete media, where only one out of six genes is essential (Winzeler et al. 1999). The threshold may decrease considerably under nutrient-limiting growth conditions.

Passage through a mitotic haploid state imposes an important constraint on the maximum mutation load of diploids. As illustrated by the dominant spore lethality observed with POL3/pol3-01,L612M diploids (Figure 2), haploid spore viability rapidly declines as the number of recessive lethal mutations increases. Interestingly, haploid spermatocytes and oocytes from animals do not divide mitotically, which may lessen selection against deleterious alleles. Moreover, spermatogonial stem cells, by developing and differentiating synchronously into spermatids as syncytia (Yoshida 2008), may be further buffered from phenotypic expression of deleterious alleles by the diffusion of gene products between cells.

The existence of a diploid error threshold has important implications for the role of mutator phenotypes in cancer. Our previous work with mutator mice indicates that proofreading defects increased mutation rates 100-fold and greatly accelerated tumorigenesis (Goldsby et al. 2001; Goldsby et al. 2002; Albertson et al. 2009). Mathematical modeling indicates that a 100-fold increase over background is insufficient to limit tumor growth (Beckman and Loeb 2005). However, simultaneous MMR and proofreading defects may produce even more rapidly evolving tumors and these may be susceptible to treatments that enhance
mutation rates (Fox and Loeb 2010). We previously observed embryonic lethality of mice defective in proofreading and MMR, indicating that mouse development is subject to an error threshold (Albertson et al. 2009). Mutation rates in these mice are predicted to be ~10,000-fold above background, consistent with the increases in mutation rate associated with reduced fitness in diploid yeast. Because tumor cells need only divide mitotically and not differentiate into specialized tissues, they may tolerate a higher mutational load than the organism from which they develop. The extent to which antimutators will thwart a strategy of lethal mutagenesis also remains to be determined.

Humans inherit an estimated 100 recessive loss-of-function alleles (MacArthur et al. 2012). Additional mutations accumulate in somatic cells during development and over a lifetime (reviewed in (Kennedy et al. 2011)). A long-standing question is whether this cumulative mutation burden contributes to aging. We have demonstrated that diploid cells can tolerate substantial genetic loads before succumbing to extinction. Nevertheless, a high heterozygous mutation burden may compromise fitness. It remains to be determined whether the heterozygous mutation burden of somatic cells during aging ever reaches a threshold that impacts overall fitness of the organism.

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Herr et al.


Herr et al.


Herr et al.


Herr et al.


FIGURE LEGENDS

Figure 1. Synthetic relationships between polymerase accuracy, proofreading and MMR.

(A) Plasmid shuffling assays to reveal synthetic interactions between mutant alleles affecting MMR, proofreading, and polymerase accuracy. Strains P3H3a (MSH6) or BP1506 (msh6Δ), transformed with the pol3–LEU2 plasmids indicated to the left, were plated onto FOA media to select for loss of the complementing POL3–URA3 plasmid. Failed growth indicates synthetic lethality. (B) Mutation rates conferred by pol3 mutator alleles. Strain BP4001 was transformed with LEU2 plasmids carrying the alleles indicated below the x axis. BP4001 carries a pol3 deletion mutation complemented by a POL3-TRP1 plasmid. Leu⁺ Trp⁺ transformants were plated on FAA media (Toyn et al. 2000) to select for clones with pol3–LEU2 as the sole source of Pol δ. FAA⁺ clones were cultured in liquid SC media, divided in half, and plated on FOA and canavanine-containing plates to detect mutations at the endogenous CAN1 locus or URA3 integrated at AGP1 (agp1::URA3). Mutation rates were calculated by maximum likelihood using Salvador 2.3 (Zheng 2005) and plotted on a logarithmic scale; error bars represent 95% confidence intervals; mutation rate values (10⁻⁸ FOA⁺ or Can⁺ mutants/cell division) are indicated on each column.

Figure 2. Heterozygous mutator phenotypes of pol3-01,L612M and pol3-01,L612G alleles

(A) Initial growth characteristics of POL3/POL3, POL3/pol3-01,L612M, and POL3/pol3-01,L612G diploid strains. Each of the pol3 alleles were introduced into the endogenous POL3 locus by transformation with URA3::pol3 PCR
fragments (see Figure S1). Transformation plates (SC-URA) are shown after 3
days of growth at 30°C. Insets show a magnified view of the colonies. (B) The
\textit{pol3-01,L612M} and \textit{pol3-01,L612G} alleles exert a dominant spore-lethal
phenotype. Diploid transformant colonies were spread onto sporulation agar and
the resulting tetrads were dissected by micromanipulation. (See Table S2 for
morphologies of inviable spores). (C) \textit{POL3/pol3,01-L612M} heterozygotes
exhibit a strong mutator phenotype. The hemizygous \textit{CAN1} mutation rate
reporter is flanked by \textit{natMX}, which confers resistance to nourseothricin (NTC).
Eight independent \textit{Ura}^+ colonies from WT \textit{POL3} and \textit{pol3-01,L612M}
transformation plates were suspended in water and plated on Can + NTC plates
(See Table 1 for mutation rates of these and other \textit{POL3/pol3} strains). The
backlit images to the right show colony forming capacity of cells from each clone
on synthetic complete (SC) media (10^{-3} dilution) after 48 hours.

\textbf{Figure 3. Lethality of \textit{pol3-01,L612M} and \textit{pol3-01,L612G} alleles in diploid yeast.}

A diploid plasmid shuffling strain with chromosomal deletions of \textit{POL3} (pol3Δ)
complemented by a \textit{POL3–URA3} plasmid (blue) was transformed with \textit{pol3-LEU2}
plasmids (red) on plates lacking leucine and uracil (-LEU -URA). 10-fold
serial dilutions of transformants were plated on synthetic complete media (SC) or
SC with FOA to assess viability. Microcolonies of less than 1000 cells were
observed for \textit{pol3-01,L612M} but not \textit{pol3-01,L612G}.

\textbf{Figure 4. Defining the diploid error threshold.}

(A) Viability and mutation rates of \textit{pol3} diploid strains. WT (BP8001),
\textit{msh2Δ/msh2Δ} (BP9101), and \textit{dun1Δ/dun1Δ} (BP8301) diploid shuffling strains
Herr et al.

with the pol3–LEU2 plasmids indicated to the left were plated onto FOA media to select for loss of the complementing POL3–URA3 plasmid. The CAN1 mutation rate determined by fluctuation analysis ($10^{-7}$ Can mutant/cell division, rounded to the nearest whole number) is indicated to the left of each serial dilution. A dash indicates that inviability precluded mutation rate measurements. Mutants are arranged based on the severity of the mutator phenotype in $msh2\Delta/msh2\Delta$ cells. (A complete list of mutation rates and 95% confidence intervals can be found in Table 3). (B) Relationship between growth capacity and CAN1 mutation rate in 42 diploid strains. Colonies of plasmid-shuffling strains grown on FOA are shown to illustrate wild-type (+++; BP8001POL3), moderately defective (++; BP9101pol3-01), severely defective (+; BP9101pol3-01,L612M,R658G), and failed growth (-; BP8001pol3-01,L612M). The vertical dashed lines indicates the estimated maximum mutation rate compatible with haploid growth (blue, Haploid Error Threshold) (Herr et al. 2011) and diploid growth (red, Diploid Error Threshold). The solid light blue curve depicts the theoretical error threshold defined by homozygous inactivation of essential genes (see Table S6). The labels indicate the pol3 allele carried on the shuffled plasmid and whether the strain is MMR deficient ($msh2\Delta\Delta$) or Dun1 deficient ($dun1\Delta\Delta$). Filled symbols are rates measured by fluctuation analyses. Open symbols are rates estimated as described in the text.

**Figure 5. Predicted long-term viability of strong diploid mutators**

Scatter plot depicts exponential growth of diploid yeast with different Can mutant rates (see Table S7) using a mathematical model in which the only limit
on growth is the accumulation of homozygous mutations in random essential genes. Growth curves are color coded according to the key on the right.

**Figure S1. Construction of chromosomal *pol3* alleles.**

(A) Construction of heterozygous *POL3* diploid yeast strains. A DNA fragment containing *URA3* (blue), *POL3* regulatory sequences (green) and mutant *pol3* sequences (white with red lines) was PCR amplified from plasmid templates and integrated (sites of recombination depicted as dashed ‘X’s) at one of two *POL3* loci in the diploid strain, AH0401 (Table S1). (B) Genotyping assays. Schematic illustrates two assays used for genotyping *pol3* alleles (mutations highlighted in red): the L612M-specific PCR utilizes mismatched primer termini to selectively amplify *pol3*-L612M; the restriction-fragment-length-polymorphic PCR (RFLP-PCR) assay monitors *EcoRV* restriction sites lost in *pol3*-01 and gained in *pol3*-L612G. Green and blue hashed lines correspond to PCR amplicons. Staggered lines indicate expected fragment sizes for each allele following digestion. (C) Integration efficiency of *URA3-pol3* constructs. Agarose gels show results from 10 random transformants of each genotype from the plates pictured in Figure 2A.
Table 1: Mutation rates of *pol3* heterozygotes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mutation rate (x10⁻⁷)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POL3 / POL3</td>
<td>5.4</td>
</tr>
<tr>
<td>POL3 / pol3-01</td>
<td>18</td>
</tr>
<tr>
<td>POL3 / pol3-01,L612G</td>
<td>8800</td>
</tr>
<tr>
<td>POL3 / pol3-01,L612M</td>
<td>11000</td>
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</table>

Mutation rates (canavanine-resistant mutants per cell division) were calculated using Salvador 2.3 in Mathematica 8.0 from the fluctuation of Can¹ cells arising in colonies of new *URA3::pol3* transformants (see Figure 2). Confidence intervals (95%) are in parentheses.
### Table 2: Whole genome mutation spectra

<table>
<thead>
<tr>
<th>Genotype</th>
<th>transitions</th>
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<tr>
<td></td>
<td>C→T / G→A</td>
<td>351</td>
<td>546</td>
</tr>
<tr>
<td></td>
<td>A→G / T→C</td>
<td>165</td>
<td>285</td>
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<tr>
<td>transversions</td>
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<td>3</td>
</tr>
<tr>
<td></td>
<td>C→A / G→T</td>
<td>270</td>
<td>368</td>
</tr>
<tr>
<td></td>
<td>A→T / T→A</td>
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<td>151</td>
</tr>
<tr>
<td>indels</td>
<td>+1 insertion</td>
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<td>29</td>
</tr>
<tr>
<td></td>
<td>-1 insertion</td>
<td>24</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>-2 insertion</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1003</td>
<td>1535</td>
</tr>
</tbody>
</table>

For each genotype, a single colony of $10^5$ cells was suspended in water, diluted, and subcloned by plating for single colonies. DNA from a single subclone of each genotype was then analyzed by Illumina high-throughput sequencing. The total of each class of mutation is indicated to the left in each column. The proportion is given in parentheses.
Table 3. Mutation Rates ($x \times 10^{-7}$) Defining the Diploid Error Threshold\(^a\)

<table>
<thead>
<tr>
<th>Allele(^b)</th>
<th>WT(^c)</th>
<th>$msh2\Delta msh2\Delta$</th>
<th>MMR effect(^d)</th>
<th>$dun1\Delta dun1\Delta$</th>
<th>Dun1 effect(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POL3</td>
<td>1.6 (0.1, 7)</td>
<td>270 (180, 400)</td>
<td>174</td>
<td>2.4 (0.6, 6.3)</td>
<td>1</td>
</tr>
<tr>
<td>pol3-01</td>
<td>320 (210, 440)</td>
<td>13000 (10000, 17000)</td>
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<td>35 (21, 54)</td>
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<td>1300 (936, 1600)</td>
<td>54</td>
<td>7.1 (3.9, 12)</td>
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<tr>
<td>pol3-L612G</td>
<td>1100 (870,1400)</td>
<td>40000 (31000, 49000)</td>
<td>35</td>
<td>30 (12, 60)</td>
<td>38</td>
</tr>
<tr>
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<td>—</td>
<td>—</td>
<td>55000 (37000, 75000)</td>
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<td>640 (450, 860)</td>
<td>18000 (15000, 21000)</td>
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<td>13000 (10000, 16000)</td>
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<td>21000 (18000, 25000)</td>
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<td>3200 (2600, 3800)</td>
<td>1509</td>
<td>—</td>
<td>—</td>
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<tr>
<td>+ S611Y</td>
<td>250 (200, 310)</td>
<td>6400 (5300, 7600)</td>
<td>25</td>
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<tr>
<td>+ R658G</td>
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<td>55000 (41000, 70000)</td>
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<td>3900 (2600, 5400)</td>
<td>854</td>
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<tr>
<td>+ Q697R</td>
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<td>4400 (3500, 5200)</td>
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<td>—</td>
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<tr>
<td>+ A704V</td>
<td>38 (20, 65)</td>
<td>5100 (4000, 6400)</td>
<td>134</td>
<td>22 (14, 31)</td>
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<tr>
<td>+ G818C</td>
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<td>+ D831G</td>
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<tr>
<td>+ K991T</td>
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<td>14000 (830, 21000)</td>
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<tr>
<td>+ S319F</td>
<td>130 (68, 210)</td>
<td>21000 (17000, 25000)</td>
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<tr>
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<tr>
<td>+ A704V</td>
<td>2900 (2400, 3500)</td>
<td>68000 (45000, 97000)</td>
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<td>66 (43, 94)</td>
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</table>

\(^a\)Mutation rates (canavanine-resistant mutants per cell division) were calculated using Salvador 2.3 in Mathematica 8.0 from the fluctuation of mutants arising in three strains per allele, isolated by independent plasmid shuffling experiments. A dash (—) indicates the strain was inviable.
\(^b\)All pol3 alleles were carried on a YCplac111 plasmid.
\(^c\)The parental strain was BP8001, Table S1.
\(^d\)The parental strain was BP9101, Table S1.
\(^e\)The MMR effect was calculated by dividing the mutation rate in each viable $msh2\Delta msh2\Delta$ strain by the mutation rate in the corresponding pol3 WT strain.
\(^f\)The parental strain was BP8301, Table S1.
\(^g\)The Dun1 effect was calculated by dividing the mutation rate in each viable pol3 WT strain by the mutation rate in the pol3 $dun1\Delta dun1\Delta$ strain.
Figure 1
Herr et al.

A

<table>
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<tr>
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<td><img src="msh6%CE%94.png" alt="Image" /></td>
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B

<table>
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<tr>
<th>Mutation Rate (mutants/cell division)</th>
<th>Canfr</th>
<th>FOAr (agp1::URA3)</th>
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<td>210</td>
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<td>270</td>
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<td>pol3-01,L612G</td>
<td>270</td>
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Figure 3
Herr et al.
### Table:

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<th>Locus</th>
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<td>pol3-01,L612M,D631G</td>
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<td>3900</td>
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<td>pol3-01,L612M,Q697R</td>
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<td>4400</td>
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### Figure B:

- **Growth Index**: The graph shows the growth index as a function of mutation rate. The growth index is higher for WT compared to mutants such as pol3-L612M, pol3-01, and pol3-01,L612G,A704V.
- **Mutation Rate (Can' mutants/cell division)**: The x-axis represents the mutation rate ranging from $10^{-7}$ to $10^{-1}$. The y-axis shows the growth index.
- **Error Thresholds**: The graph indicates the error thresholds for both haploid and diploid conditions.
Figure 5
Herr et al.