DNA Replication Error-Induced Extinction of Diploid Yeast

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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 diploid *Saccharomyces 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 10 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.

EVOOLUTIONARY 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 hitchhike 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; L. N. Williams *et al.* 2013) or acquire antimutator 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; L. N. Williams *et al.* 2013). Error-induced extinction occurs within a few cell divisions in bacterial and haploid yeast cells lacking both DNA polymerase proofreading and mismatch repair (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; L. N. Williams *et al.* 2013). 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; L. N. Williams *et al.* 2013). 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.

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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; Cancer Genome Atlas Network 2012a; Palles *et al.* 2012; Church *et al.* 2013; Kandoth *et al.* 2013). Mice with engineered defects in MMR (Baker *et al.* 1995, 1996; De Wind *et al.* 1995; Edelmann *et al.* 1996, 1997; Reitmaier *et al.* 1996), polymerase proofreading (Goldsby *et al.* 2001, 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; Cancer Genome Atlas Network 2012a,b; Imielinski *et al.* 2012; Nik-Zainal *et al.* 2012; Palles *et al.* 2012; Pugh *et al.* 2012; Kandoth *et al.* 2013). Some hypermutated intestinal and endometrial tumors carry defects in both MMR and DNA polymerase proofreading, consistent with synergy between these two pathways in human disease (Cancer Genome Atlas Network 2012a; Palles *et al.* 2012; Church *et al.* 2013; Kandoth *et al.* 2013).

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 reveal 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 hypermutator 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; and 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 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 fivefold 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° 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 in Sherman (2002) from individual components purchased from Sigma-Aldrich or Fisher Scientific (Pittsburgh). To select for Ura[−] cells during plasmid shuffling or mutation rate assays, we used SC media containing 1 mg/ml 5-fluoroorotic acid (5-FOA) (Zymo Research) (Boeke *et al.* 1984). To select for Trp[−] cells during plasmid shuffling we used media containing 5-fluoroanthranilic acid (5-FAA) made as described in Toyn *et al.* (2000). Canavanine-resistant haploids for mutation rate assays were selected on SC plates lacking arginine that contained 60 μ g/ml canavanine (Sigma-Aldrich). For mutation rate assays in diploids, we used SC plates containing 60 μ g/ml canavanine and either 100 μ g/ml nourseothricin (NTC; Werner Bio-Agents) or 300 μ g/ml geneticin (G418; Sigma-Aldrich) with monosodium glutamate (MSG) (1 g/liter) as the nitrogen source rather than ammonium sulfate (Tong and Boone 2006).

Yeast plasmids and strains

Plasmids: pGL310 (Simon *et al.* 1991; Giot *et al.* 1995) is derived from YCp50 (*CEN4/ARS1/URA3*) (Rose *et al.* 1987) and carries *POL3* under control of the endogenous promoter. YCplac111*POL3* and YCplac111*pol3* derivatives (Herr *et al.* 2011) are derived from YCplac111 (*CEN6/ARS1/LEU2*) (Gietz and Sugino 1988) and carry the wild-type (WT) or mutant *pol3* coding and regulatory sequences, cloned between the *HindIII* and *EcoRI* restriction sites. pRS414*POL3* (Herr *et al.* 2011) is derived from pRS414 (*CEN6/ARS4/TRP1*) (Brachmann *et al.* 1998) and carries the *HindIII*–*EcoRI* *POL3* fragment from YCplac111*POL3*. Construction of YCplac111*pol3*-L612M, YCplac111*pol3*-L612G, YCplac111*pol3*-L612K, YCplac111*pol3*-01,L612M, YCplac111*pol3*-01,L612G, and YCplac111*pol3*-01,L612K was described previously (Venkatesan *et al.* 2006). 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 YCplac111-*pol3-01,L612M*, using the *EcoRI* and *BamHI* fragments from YCplac111-*pol3-01,D831G* and YCplac111-*pol3-01,K891T* (Herr *et al.* 2011). We isolated all other *pol3-01,L612M,exx* 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,exx* alleles, we subcloned DNA containing the *Q697R*, *A704V*, and *G818C* mutations into YCplac111-*pol3-01,L612G*, using *BamHI-EcoRI* fragments from the *pol3-01,L612M,exx* plasmids. The *S319F* allele was subcloned into YCplac111-*pol3-01,L612G*, using *NcoI-EagI* fragments. The entire *pol3* sequence was verified for all plasmids used in this study.

Strains: All strains are listed in [Supporting Information, Table S1](#). All PCR fragments used for strain construction are described in [Table S4](#) and were generated as described in [File S1](#).

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). 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 Figure 2 and Figure 3 were taken with backlighting, using an Olympus CX41 microscope, equipped with a DP12 digital camera at 40× or 100× magnification.

Plasmid shuffling

Plasmid shuffling (Boeke *et al.* 1984) with pGL310- or pRS414-*POL3*-containing strains has been described previously (Simon *et al.* 1991; Herr *et al.* 2011). Cells transformed with YCplac111-*pol3* plasmids, YCplac111-*POL3* (positive control), or YCplac111 (empty vector control) were plated on SC lacking uracil and leucine (pGL310 cells) or SC lacking tryptophan and leucine (pRS414-*POL3* cells). After 2–3 days incubation at 30°, individual colonies were picked and dispersed in sterile H₂O. Serial dilutions containing ~10⁵, 10⁴, 10³, and 10² cells were plated on SC or SC with 5-FOA (for pGL310 cells) (Boeke *et al.* 1984) or 5-FAA selection media (for pRS414-*POL3* cells) (Toyn *et al.* 2000) to select for cells that had spontaneously lost pGL310 or pRS414-*POL3*.

Mutation rates

Mutation rates were calculated by fluctuation analysis of 5-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 5-FAA shuffling plates and used to inoculate 100 µl overnight SC cultures in sterile PCR strip tubes and incubated without shaking at 30°. 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 2 days of growth at 30° the cells were suspended by vigorous vortexing for 2 min. The cells and liquid were recovered by a brief centrifugation. We added 200 µl of sterile water, resuspended the cells by pipetting, and plated 150 µl of nine replica cultures for each isolate on separate canavanine and 5-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 (*N_t*) 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^r mitotic recombinants. Individual transformant colonies were considered “replica” cultures for purposes of fluctuation analysis. Colonies were picked from the transformation plates and suspended with vigorous vortexing in 200 µl sterile H₂O, and 170 µl was plated on SC-MSG plates lacking arginine and containing canavanine and nourseothricin. For each genotype, 10 µl from each replica was pooled, serially diluted, and plated on SC media to determine the average number of colony-forming units per replica. The remaining cell suspension of each replica was treated with zymolyase and used for PCR genotyping as described in [File S1](#). 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 YCplac111-*pol3* 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-5-FOA and SC media to measure viability and shuffling efficiency (Figure 4). At the same time larger volumes of each dilution were plated on 5-FOA to obtain sufficient numbers of replica colonies for fluctuation analysis. After 2 days of growth, 8 well-isolated 5-FOA-resistant colonies from each of three independent shuffling experiments per genotype (24 total) were suspended in 100

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 5-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 5-FAA media (Toyn *et al.* 2000) to select for clones with *pol3-LEU2* as the sole source of Pol-δ. FAA^r clones were cultured in liquid SC media, divided in half, and plated on 5-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^r or Can^f mutants per cell division) are indicated on each column.

μ l of H₂O. For strains with low and moderate mutation rates, we plated 90 μ l on canavanine-G418 selection plates. The remaining 10 μ l was used for 10-fold serial dilutions for N_t 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^f 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, 2005, 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

Table 1 Mutation rates of *pol3* heterozygotes

Genotype	Mutation rate ($\times 10^{-7}$)	
<i>POL3/POL3</i>	5.4	(3.4, 8)
<i>POL3/pol3-01</i>	18	(12, 25)
<i>POL3/pol3-01,L612G</i>	8,800	(7,400, 10,000)
<i>POL3/pol3-01,L612M</i>	11,000	(9,100, 12,000)

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

using LRIntervalD or CILDplating in Salvador 2.3, both of which rely on likelihood ratios (Zheng 2002, 2005, 2008).

Can^f mutation rates were converted to per-base-pair mutation rates as described in Herr *et al.* (2011). The Can^f mutation rate was multiplied by a correction factor (4.53), which takes into account the number of scorable sites within *CAN1* (Herr *et al.* 2011), and divided by the size of *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

$$R_{cd} = (MR_{eg})^2(1000)(N_g), \quad (1)$$

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. One thousand 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^f 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):

$$MR_{eg} = \frac{1296}{1773} \times MR_{can1} = 0.73 MR_{can1}. \quad (2)$$

We substituted Equation 2 into Equation 1 to estimate the expected rate of cell death at different Can^f mutation rates:

$$R_{cd} = (0.73 MR_{can1})^2(1000)(N_g). \quad (3)$$

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

$$P_v = \frac{R_{cd}^k e^{-R_{cd}}}{k!} = e^{-R_{cd}}. \quad (4)$$

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 the 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 10⁸ 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); postindexed by PCR; and sequenced using 101-bp, paired-end reads on an Illumina 2500 platform.

Reads were aligned to the *Saccharomyces 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) (<http://picard.sourceforge.net>). To reduce false variant calls, The Genome Analysis Toolkit (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 the 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% of the samples by both programs were considered candidate SNPs. BQSR was then performed on

Table 2 Whole-genome mutation spectra

Mutation type	Genotype			
	<i>POL3/pol3-01,L612G</i>		<i>POL3/pol3-01,L612M</i>	
Transitions				
C → T/G → A	351	(0.350)	546	(0.356)
A → G/T → C	165	(0.165)	285	(0.186)
Transversions				
C → G/G → C	2	(0.002)	3	(0.002)
C → A/G → T	270	(0.269)	368	(0.240)
A → T/T → A	103	(0.103)	110	(0.072)
A → C/T → G	80	(0.080)	151	(0.098)
Indels				
+1 insertion	8	(0.008)	29	(0.019)
–1 deletion	24	(0.024)	42	(0.027)
–2 deletion	0	—	1	(0.001)
	1003		1535	

For each genotype, a single colony of 10⁵ 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 for each genotype. The proportion is given in parentheses to the right.

the processed sequencing data and a second round of candidate SNPs was 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

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 *pol3-L612* alleles were tolerated in otherwise WT cells, although *pol3-L612G* markedly reduced colony sizes (Figure 1A). Each of the alleles increased 5-FOA- and canavanine-resistance (Can^r) mutation rates, consistent with a genome-wide elevation in mutation rates (Figure 1B). The *pol3-L612M* and *pol3-L612K* alleles induced similar levels of mutagenesis (~10-fold above the WT background), while mutation rates with *pol3-L612G* were comparable to those observed with the *pol3-01* allele (~20- to 100-fold above background depending on the locus; Figure 1B). Previous work indicated that *pol3-L612M* and mutant MMR alleles synergistically elevate mutation rate (Li *et al.* 2005; Nick McElhinny *et al.* 2008). We found that *pol3-L612G* was synthetically sick with *msh6Δ* (Figure 1A), suggesting that *pol3-L612G msh6Δ*

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 was 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°. Insets show a magnified view of the colonies. (B) The *pol3-01, L612M* and *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) *POL3/pol3-01, L612M* heterozygotes exhibit a strong mutator phenotype. The hemizygous *CAN1* mutation rate reporter is flanked by *natMX*, which confers resistance to nourseothricin (NTC). Eight independent *Ura⁺* colonies from WT *POL3* and *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 *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 hr.

cells have mutation rates near the haploid error threshold, as observed for *pol3-01 msh6Δ* 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 *pol3-01* and the *L612* mutations (Venkatesan *et al.* 2006). We found that cells could not survive with *pol3-01, L612M* or *pol3-01, L612G* as the sole source of Pol-δ, but in contrast to a previous report (Venkatesan *et al.* 2006), cells with the *pol3-01, L612K* allele readily formed colonies (Figure 1A). The *L612K* mutation lowered the *pol3-01* mutator phenotype between two- and threefold (Figure 1B) and suppressed the synthetic lethality between *pol3-01* and *msh6Δ* (Figure 1A). Consistent with this observation, the analogous L606K substitution in human Pol-δ suppresses the mutator phenotype of Pol-δ proofreading deficiency *in vitro* (Schmitt *et al.* 2010). Thus, although *pol3-01, L612K* is a moderate mutator, it clearly does not synergize with *pol3-01*. In contrast, the lethality conferred by *pol3-01, L612G* and *pol3-01, L612M* suggests that the *L612G* and *L612M* mutations may synergize with *pol3-01* to drive mutation rates above the lethal error threshold of haploid yeast.

Heterozygous effects of *pol3-01, L612M* and *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 *pol3-01, L612M* and *pol3-01, L612G* DNA linked to *URA3* (Figure S1A). Correct chromosomal integration occurred efficiently, resulting in numerous independent *POL3/pol3-01, L612M* and *POL3/pol3-01, L612G* transformants (Figure S1, B and C), which formed similar-

sized colonies to those of *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 *POL3/POL3* diploids lived, all spores from *POL3/pol3-01, L612M* and *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 *pol3-01, L612M* and *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 hemizygous for the *CAN1* gene (Figure 2C, top schematic), which allowed us to determine the frequency of Can^r mutant cells in independent transformant colonies (Figure 2C, Can + NTC) and then calculate mutation rates by fluctuation analysis (Rosche and Foster 2000). The *POL3/pol3-01, L612M* and *POL3/pol3-01, L612G* strains exhibited mutation rates >1000 times greater than those of the *POL3/POL3* diploid controls (Table 1). In absolute terms, the mutation rates of the heterozygous mutant diploids ($0.9\text{--}1.1 \times 10^{-3}$ Can^r mutants per cell division) were at the haploid error threshold ($\sim 10^{-3}$) (Herr *et al.* 2011), suggesting that they accumulated a recessive lethal mutation nearly every cell division (Herr *et al.* 2011).

To understand the nature of mutagenesis in these cells, we sequenced the genomes of individual *POL3/pol3-01, L612M*, *POL3/pol3-01, L612G*, and *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 *POL3/POL3* strain harbored a single T→G mutation in its genome. In contrast, the *POL3/pol3-01, L612M* subclone contained 1535 *de novo* point mutations and the *POL3/pol3-01, L612G* subclone had 1003 mutations (Table 2, Table 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 *POL3/pol3-01, L612M* strain contained three times the number of +1 frameshifts found in the *POL3/pol3-01, L612G* strain (Table 2, Table S3).

At the time of subcloning, the primary transformant colonies (Figure 2A) contained $\sim 10^5$ 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 (2.2×10^7 bp), divided by the number of generations (17) gives mutation rates of $\sim 3\text{--}4 \times 10^{-6}$ mutations per base pair per generation, consistent with the expected per-base-pair mutation rate of cells replicating with a mutation rate of 1×10^{-3} Can^r mutants per cell division (2.5×10^{-6} mutations per base pair per 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-lit images). Thus, heterozygous *pol3-01, L612M* and *pol3-01, L612G* alleles exert strong semidominant mutator phenotypes in diploid cells that severely compromise both replicative and reproductive fitness.

Error-induced extinction in diploids

To determine whether 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 5-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 ~ 1000 cells (Figure 3). No *pol3-01, L612G* microcolonies arose $> \sim 10$ 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; and 23 of 210 cells from the second microcolony divided a limited number of times, forming small microcolonies of 4–100 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 multibudded 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 (*dun1 Δ*) (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 *msh6 Δ* , 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 (*pol3-01, L612M, K559N* and *pol3-01, L612M, R674G*) to 1×10^{-3} Can^r mutants per cell division (*pol3-01, L612M, G447D*, *pol3-01, L612M, F467I*, and *pol3-01, L612M, R658G*). MMR deficiency (*msh2 Δ /msh2 Δ*) 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 < 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.

Figure 3 Lethality of *pol3-01,L612M* and *pol3-01,L612G* alleles in diploid yeast. A diploid plasmid-shuffling strain with chromosomal deletions of *POL3* (*pol3Δ*) complemented by a *POL3-URA3* plasmid (blue) was transformed with *pol3-LEU2* plasmids (red) on plates lacking leucine and uracil (–LEU–URA). Tenfold serial dilutions of transformants were plated on synthetic complete media (SC) or SC with 5-FOA to assess viability. Microcolonies of <1000 cells were observed for *pol3-01,L612M* but not *pol3-01,L612G*.

In contrast, mutation rates increased >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 $\geq 1 \times 10^{-3}$ Can^r mutants per 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 ($\geq 4 \times 10^{-3}$ Can^r mutants per 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 5.5×10^{-3} Can^r mutants per 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 1×10^{-2} Can^r mutants per cell division or as high as 3×10^{-1} Can^r mutants per 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 the nearest whole number) would divide in the next generation (Table S6). We then plotted expected col-

ony 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 1×10^{-3} Can^r mutants per cell division, which is the mutation rate at which we began to observe growth defects. Predicted and observed colony sizes declined dramatically between 1×10^{-3} and 1×10^{-2} Can^r mutants per cell division (Figure 4). At a mutation rate of 5×10^{-3} Can^r mutants per cell division colony size is predicted to be ~70,000 cells. The average colony size produced by *pol3-01,L612M dun1Δ/dun1Δ* cells (5.5×10^{-3} Can^r mutants per cell division) was 53,000 cells. At 1×10^{-2} Can^r mutants per 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 *pol3-01,L612M* (Figure 3). At 2×10^{-2} Can^r mutants per 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 $\sim 1 \times 10^{-2}$ Can^r mutants per 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 6×10^{-3} Can^r mutants per cell division (Table S7). Diploids with a mutation rate of 1×10^{-3} Can^r mutants per cell division are predicted to continue to divide through 1000 generations (assuming unlimited growth). Doubling the mutation rate to 2×10^{-3} Can^r mutants per cell division leads to extinction by 700 generations, whereas a mutation rate of 4×10^{-3} Can^r mutants per 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 proof-reading are lethal in both haploid (Figure 1) and diploid

Table 3 Mutation rates ($\times 10^{-7}$) defining the diploid error threshold

Allele ^a	WT ^b		<i>msh2Δ/msh2Δ</i> ^c		MMR effect ^d	<i>dun1Δ/dun1Δ</i> ^e		Dun1 effect ^f
<i>POL3</i>	1.6	(0.1, 7)	270	(180, 400)	174	2.4	(0.6, 6.3)	1
<i>pol3-01</i>	320	(210, 440)	13,000	(10,000, 17,000)	42	35	(21, 54)	9
<i>pol3-L612M</i>	24	(12, 40)	1,300	(936, 1,600)	54	7.1	(3.9, 12)	3
<i>pol3-L612G</i>	1,100	(870, 1,400)	40,000	(31,000, 49,000)	35	30	(12, 60)	38
<i>pol3-01,L612M</i>	—	—	—	—	—	55,000	(37,000, 75,000)	—
+ <i>S319F</i>	640	(450, 860)	18,000	(15,000, 21,000)	28	65	(35, 110)	10
+ <i>G447D</i>	8,000	(5,500, 11,000)	13,000	(10,000, 16,000)	2	340	(260, 420)	23
+ <i>F467I</i>	11,000	(8,200, 13,000)	21,000	(18,000, 25,000)	2	170	(120, 230)	63
<i>K559N</i>	2.1	(0.7, 4.9)	3,200	(2,600, 3,800)	1,509	—	—	—
+ <i>S611Y</i>	250	(200, 310)	6,400	(5,300, 7,600)	25	100	(63, 160)	2
+ <i>R658G</i>	8,700	(6,900, 11,000)	55,000	(41,000, 70,000)	6	520	(410, 640)	17
+ <i>R674G</i>	4.6	(2.0, 8.8)	3,900	(2,600, 5,400)	854	—	—	—
+ <i>Q697R</i>	17	(4.2, 44)	4,400	(3,500, 5,200)	257	—	—	—
+ <i>A704V</i>	38	(20, 65)	5,100	(4,000, 6,400)	134	22	(14, 31)	2
<i>G818C</i>	8.8	(1.5, 27)	4,600	(3,300, 5,900)	515	—	—	—
+ <i>D831G</i>	110	(68, 160)	3,300	(2,400, 4,300)	31	42	(27, 60)	3
+ <i>K891T</i>	260	(180, 360)	31,000	(27,000, 36,000)	119	—	—	—
<i>pol3-01,L612G</i>	—	—	—	—	—	—	—	—
+ <i>S319F</i>	—	—	—	—	—	14,000	(830, 21,000)	—
+ <i>K559N</i>	130	(68, 210)	21,000	(17,000, 25,000)	165	—	—	—
+ <i>Q697R</i>	52	(24, 95)	13,000	(10,000, 15,000)	243	—	—	—
+ <i>A704V</i>	2,900	(2,400, 3,500)	68,000	(45,000, 97,000)	23	66	(43, 94)	44

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.

^a All *pol3* alleles were carried on a YCplac111 plasmid.

^b The parental strain was BP8001, Table S1.

^c The parental strain was BP9101, Table S1.

^d The MMR effect was calculated by dividing the mutation rate in each viable *msh2Δ/msh2Δ* strain by the mutation rate in the corresponding *pol3* WT strain.

^e The parental strain was BP8301, Table S1.

^f The Dun1 effect was calculated by dividing the mutation rate in each viable *pol3* WT strain by the mutation rate in the *pol3 dun1Δ/dun1Δ* strain.

(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 1×10^{-2} Can^r mutants per 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 1×10^{-3} Can^r mutants per 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 per base pair to the overall mutation rate (Kunkel 2009). Pol-δ proofreading and MMR each reduce mutation rates by at least 1×10^{-2} mutations per base pair (Morrison *et al.*

1993; Herr *et al.* 2011) to give an overall DNA replication fidelity of $< 1 \times 10^{-9}$ mutations per base pair (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.* (2006) first reported that *pol3-01,L612M* and *pol3-01,L612G* were synthetically lethal in haploids. Nick McElhinny *et al.* (2006) also observed lethality when *pol3-L612M* was combined with the proofreading-deficient allele, *pol3-DV*. These observations suggested either that the mutant polymerases were 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

Figure 4 Defining the diploid error threshold. (A) Viability and mutation rates of *pol3* diploid strains. WT (BP8001), *msh2Δ/msh2Δ* (BP9101), and *dun1Δ/dun1Δ* (BP8301) diploid-shuffling strains with the *pol3-LEU2* plasmids indicated to the left were plated onto 5-FOA media to select for loss of the complementing *POL3-URA3* plasmid. The *CAN1* mutation rate determined by fluctuation analysis (10^{-7} Can^r mutants per 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 *msh2Δ/msh2Δ* 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 5-FOA are shown to illustrate wild-type (+++; BP8001/*POL3*), moderately defective (++; BP9101/*pol3-01*), severely defective (+; BP9101/*pol3-01,L612M,R658G*), and failed growth (-; BP8001/*pol3-01,L612M*). The vertical dashed lines indicate 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 (*msh2Δ/Δ*) or Dun1 deficient (*dun1Δ/Δ*). Solid symbols are rates measured by fluctuation analyses. Open symbols are rates estimated as described in the text.

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, 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 semidominant mutator phenotypes, capable of elevating mutation rates to the haploid error threshold [$\sim 10^{-3}$ Can^r mutants per 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 semidominance of *pol3-01,L612M* and *pol3-01,L612G* contrasts with the recessive nature of *pol3-01* (Table 1) (Morrison *et al.* 1993). Diminished nucleotide selectivity and saturation of

Figure 5 Predicted long-term viability of strong diploid mutators. Scatter plot depicts exponential growth of diploid yeast with different *Can^r* mutation 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.

MMR may contribute to the semidominance 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 Okazaki fragment maturation, nonhomologous 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 (J. S. Williams *et al.* 2013). 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 1×10^{-2} *Can^r* mutants per 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. Nonlethal 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 $>1 \times 10^{-3}$ *Can^r* mutants per 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 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, 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 to 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 affects overall fitness of the organism.

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Literature Cited

- Alani, E., 1996 The *Saccharomyces cerevisiae* Msh2 and Msh6 proteins form a complex that specifically binds to duplex oligonucleotides containing mismatched DNA base pairs. *Mol. Cell. Biol.* 16: 5604–5615.
- Albertson, T. M., M. Ogawa, J. M. Bugni, L. E. Hays, Y. Chen *et al.*, 2009 DNA polymerase ϵ and δ proofreading suppress discrete mutator and cancer phenotypes in mice. *Proc. Natl. Acad. Sci. USA* 106: 17101–17104.
- Baker, S. M., C. E. Bronner, L. Zhang, A. W. Plug, M. Robatzek *et al.*, 1995 Male mice defective in the DNA mismatch repair gene *PMS2* exhibit abnormal chromosome synapsis in meiosis. *Cell* 82: 309–319.
- Baker, S. M., A. W. Plug, T. A. Prolla, C. E. Bronner, A. C. Harris *et al.*, 1996 Involvement of mouse Mlh1 in DNA mismatch repair and meiotic crossing over. *Nat. Genet.* 13: 336–342.
- Beckman, R. A., and L. A. Loeb, 2005 Negative clonal selection in tumor evolution. *Genetics* 171: 2123–2131.
- Boeke, J. D., F. Lacroute, and G. R. Fink, 1984 A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol. Gen. Genet.* 197: 345–346.
- Brachmann, C. B., A. Davies, G. J. Cost, E. Caputo, J. Li *et al.*, 1998 Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* 14: 115–132.
- Cancer Genome Atlas Network, 2012a Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 487: 330–337.
- Cancer Genome Atlas Network, 2012b Comprehensive molecular portraits of human breast tumours. *Nature* 490: 61–70.
- Chao, L., and E. C. Cox, 1983 Competition between high and low mutating strains of *Escherichia coli*. *Evolution* 37: 125–134.
- Church, D. N., S. E. W. Briggs, C. Palles, E. Domingo, S. J. Kearsey *et al.*, 2013 DNA polymerase ϵ and δ exonuclease domain mutations in endometrial cancer. *Hum. Mol. Genet.* 22: 2820–2828.
- Clausen, A. R., S. Zhang, P. M. Burgers, M. Y. Lee, and T. A. Kunkel, 2013 Ribonucleotide incorporation, proofreading and bypass by human DNA polymerase delta. *DNA Repair* 12: 121–127.
- Cowen, L. E., and S. Lindquist, 2005 Hsp90 potentiates the rapid evolution of new traits: drug resistance in diverse fungi. *Science* 309: 2185–2189.
- Dae, D. L., T. M. Mertz, and P. V. Shcherbakova, 2010 A cancer-associated DNA polymerase δ variant modeled in yeast causes a catastrophic increase in genomic instability. *Proc. Natl. Acad. Sci. USA* 107: 157–162.
- Datta, A., J. L. Schmeits, N. S. Amin, P. J. Lau, K. Myung *et al.*, 2000 Checkpoint-dependent activation of mutagenic repair in *Saccharomyces cerevisiae* *pol3-01* mutants. *Mol. Cell* 6: 593–603.
- Delneri, D., D. C. Hoyle, K. Gkargkas, E. J. M. Cross, B. Rash *et al.*, 2008 Identification and characterization of high-flux-control genes of yeast through competition analyses in continuous cultures. *Nat. Genet.* 40: 113–117.

- Depristo, M. A., E. Banks, R. Poplin, K. V. Garimella, J. R. Maguire *et al.*, 2011 A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat. Genet.* 43: 491–498.
- Deutschbauer, A. M., D. F. Jaramillo, M. Proctor, J. Kumm, M. E. Hillenmeyer *et al.*, 2005 Mechanisms of haploinsufficiency revealed by genome-wide profiling in yeast. *Genetics* 169: 1915–1925.
- De Visser, J. A., 2002 The fate of microbial mutators. *Microbiol. Rev.* 66: 1247–1252.
- De Wind, N., M. Dekker, A. Berns, M. Radman, and H. Te Riele, 1995 Inactivation of the mouse *Msh2* gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. *Cell* 82: 321–330.
- Drake, J. W., B. Charlesworth, D. Charlesworth, and J. F. Crow, 1998 Rates of spontaneous mutation. *Genetics* 148: 1667–1686.
- Edelmann, W., P. E. Cohen, M. Kane, K. Lau, B. Morrow *et al.*, 1996 Meiotic pachytene arrest in *MLH1*-deficient mice. *Cell* 85: 1125–1134.
- Edelmann, W., K. Yang, A. Umar, J. Heyer, K. Lau *et al.*, 1997 Mutation in the mismatch repair gene *Msh6* causes cancer susceptibility. *Cell* 91: 467–477.
- Fijalkowska, I. J., and R. M. Schaaper, 1995 Effects of *Escherichia coli* *dnaE* antimutator alleles in a proofreading-deficient *mutD5* strain. *J. Bacteriol.* 177: 5979–5986.
- Fijalkowska, I. J., and R. M. Schaaper, 1996 Mutants in the Exo I motif of *Escherichia coli* *dnaQ*: defective proofreading and inviability due to error catastrophe. *Proc. Natl. Acad. Sci. USA* 93: 2856–2861.
- Fortune, J. M., Y. I. Pavlov, C. M. Welch, E. Johansson, P. M. Burgers *et al.*, 2005 *Saccharomyces cerevisiae* DNA polymerase δ : high fidelity for base substitutions but lower fidelity for single- and multi-base deletions. *J. Biol. Chem.* 280: 29980–29987.
- Foster, P. L., 2006 Methods for determining spontaneous mutation rates. *Methods Enzymol.* 409: 195–213.
- Fox, E. J., and L. A. Loeb, 2010 Lethal mutagenesis: targeting the mutator phenotype in cancer. *Semin. Cancer Biol.* 20: 353–359.
- Friedberg, E. C., G. C. Walker, W. Siede, R. D. Wood, R. A. Schultz *et al.*, 2006 *DNA Repair and Mutagenesis*. ASM Press, Washington, DC.
- Funchain, P., A. Yeung, J. L. Stewart, R. Lin, M. M. Slupska *et al.*, 2000 The consequences of growth of a mutator strain of *Escherichia coli* as measured by loss of function among multiple gene targets and loss of fitness. *Genetics* 154: 959–970.
- Gietz, R. D., and A. Sugino, 1988 New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* 74: 527–534.
- Giot, L., M. Simon, C. Dubois, and G. Faye, 1995 Suppressors of thermosensitive mutations in the DNA polymerase δ gene of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 246: 212–222.
- Giraud, A., I. Matic, O. Tenaillon, A. Clara, M. Radman *et al.*, 2001 Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut. *Science* 291: 2606–2608.
- Goldsby, R. E., N. A. Lawrence, L. E. Hays, E. A. Olmsted, X. Chen *et al.*, 2001 Defective DNA polymerase- δ proofreading causes cancer susceptibility in mice. *Nat. Med.* 7: 638–639.
- Goldsby, R. E., L. E. Hays, X. Chen, E. A. Olmsted, W. B. Slayton *et al.*, 2002 High incidence of epithelial cancers in mice deficient for DNA polymerase δ proofreading. *Proc. Natl. Acad. Sci. USA* 99: 15560–15565.
- Goldstein, A., and J. H. Mccusker, 1999 Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* 15: 1541–1553.
- Greene, C. N., and S. Jinks-Robertson, 2001 Spontaneous frameshift mutations in *Saccharomyces cerevisiae*: accumulation during DNA replication and removal by proofreading and mismatch repair activities. *Genetics* 159: 65–75.
- Harrison, R., B. Papp, C. Pal, S. G. Oliver, and D. Delneri, 2007 Plasticity of genetic interactions in metabolic networks of yeast. *Proc. Natl. Acad. Sci. USA* 104: 2307–2312.
- Herr, A. J., M. Ogawa, N. A. Lawrence, L. N. Williams, J. M. Egginton *et al.*, 2011 Mutator suppression and escape from replication error-induced extinction in yeast. *PLoS Genet.* 7: e1002282.
- Hillenmeyer, M. E., E. Fung, J. Wildenhain, S. E. Pierce, S. Hoon *et al.*, 2008 The chemical genomic portrait of yeast: uncovering a phenotype for all genes. *Science* 320: 362–365.
- Iaccarino, I., F. Palombo, J. Drummond, N. F. Totty, J. J. Hsuan *et al.*, 1996 MSH6, a *Saccharomyces cerevisiae* protein that binds to mismatches as a heterodimer with MSH2. *Curr. Biol.* 6: 484–486.
- Imielinski, M., A. H. Berger, P. S. Hammerman, B. Hernandez, T. J. Pugh *et al.*, 2012 Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing. *Cell* 150: 1107–1120.
- Johnson, R. E., G. K. Kovvali, L. Prakash, and S. Prakash, 1996 Requirement of the yeast *MSH3* and *MSH6* genes for *MSH2*-dependent genomic stability. *J. Biol. Chem.* 271: 7285–7288.
- Kennedy, S. R., L. A. Loeb, and A. J. Herr, 2011 Somatic mutations in aging, cancer and neurodegeneration. *Mech. Ageing Dev.* 133: 118–126.
- Kandoth, C., N. Schultz, A. D. Cherniack, R. Akbani, Y. Liu *et al.*, 2013 Integrated genomic characterization of endometrial carcinoma. *Nature* 497: 67–73.
- Koboldt, D. C., Q. Zhang, D. E. Larson, D. Shen, M. D. McEellan *et al.*, 2012 VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res.* 22: 568–576.
- Kuchta, R. D., P. Benkovic, and S. J. Benkovic, 1988 Kinetic mechanism whereby DNA polymerase I (Klenow) replicates DNA with high fidelity. *Biochemistry* 27: 6716–6725.
- Kunkel, T. A., 2009 Evolving views of DNA replication (in)fidelity. *Cold Spring Harb. Symp. Quant. Biol.* 74: 91–101.
- Lang, G. I., and A. W. Murray, 2008 Estimating the per-base-pair mutation rate in the yeast *Saccharomyces cerevisiae*. *Genetics* 178: 67–82.
- Li, H., and R. Durbin, 2009 Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25: 1754–1760.
- Li, H., B. Handsaker, A. Wysoker, T. Fennell, J. Ruan *et al.*, 2009 The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25: 2078–2079.
- Li, L., K. M. Murphy, U. Kanevets, and L. J. Reha-Krantz, 2005 Sensitivity to phosphonoacetic acid: a new phenotype to probe DNA polymerase δ in *Saccharomyces cerevisiae*. *Genetics* 170: 569–580.
- Loeb, L. A., 2011 Human cancers express mutator phenotypes: origin, consequences and targeting. *Nat. Rev. Cancer* 11: 450–457.
- Loeb, L. A., C. F. Springgate, and N. Battula, 1974 Errors in DNA replication as a basis of malignant changes. *Cancer Res.* 34: 2311–2321.
- Loh, E., J. J. Salk, and L. A. Loeb, 2010 Optimization of DNA polymerase mutation rates during bacterial evolution. *Proc. Natl. Acad. Sci. USA* 107: 1154–1159.
- Lujan, S. A., J. S. Williams, A. R. Clausen, A. B. Clark, and T. A. Kunkel, 2013 Ribonucleotides are signals for mismatch repair of leading-strand replication errors. *Mol. Cell* 50: 437–443.
- Lynch, H. T., P. M. Lynch, S. J. Lanspa, C. L. Snyder, J. F. Lynch *et al.*, 2009 Review of the Lynch syndrome: history, molecular genetics, screening, differential diagnosis, and medicolegal ramifications. *Clin. Genet.* 76: 1–18.
- Lynch, M., 2010 Evolution of the mutation rate. *Trends Genet.* 26: 345–352.

- Lynch, M., W. Sung, K. Morris, N. Coffey, C. R. Landry *et al.*, 2008 A genome-wide view of the spectrum of spontaneous mutations in yeast. *Proc. Natl. Acad. Sci. USA* 105: 9272–9277.
- MacArthur, D. G., S. Balasubramanian, A. Frankish, N. Huang, J. Morris *et al.*, 2012 A systematic survey of loss-of-function variants in human protein-coding genes. *Science* 335: 823–828.
- Mao, E. F., L. Lane, J. Lee, and J. H. Miller, 1997 Proliferation of mutators in a cell population. *J. Bacteriol.* 179: 417–422.
- Marsischky, G. T., N. Filosi, M. F. Kane, and R. Kolodner, 1996 Redundancy of *Saccharomyces cerevisiae* *MSH3* and *MSH6* in *MSH2*-dependent mismatch repair. *Genes Dev.* 10: 407–420.
- Mendelman, L. V., J. Petruska, and M. F. Goodman, 1990 Base mispair extension kinetics. Comparison of DNA polymerase alpha and reverse transcriptase. *J. Biol. Chem.* 265: 2338–2346.
- Morrison, A., and A. Sugino, 1994 The 3'→5' exonucleases of both DNA polymerases δ and ϵ participate in correcting errors of DNA replication in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 242: 289–296.
- Morrison, A., A. L. Johnson, L. H. Johnston, and A. Sugino, 1993 Pathway correcting DNA replication errors in *Saccharomyces cerevisiae*. *EMBO J.* 12: 1467–1473.
- Nick McElhinny, S. A., C. M. Stith, P. M. J. Burgers, and T. A. Kunkel, 2006 Inefficient proofreading and biased error rates during inaccurate DNA synthesis by a mutant derivative of *Saccharomyces cerevisiae* DNA polymerase delta. *J. Biol. Chem.* 282: 2324–2332.
- Nick McElhinny, S. A., D. A. Gordenin, C. M. Stith, P. M. J. Burgers, and T. A. Kunkel, 2008 Division of labor at the eukaryotic replication fork. *Mol. Cell* 30: 137–144.
- Nick McElhinny, S. N., D. Kumar, A. B. Clark, D. L. Watt, B. E. Watts *et al.*, 2010 Genome instability due to ribonucleotide incorporation into DNA. *Nat. Chem. Biol.* 6: 774–781.
- Nik-Zainal, S., L. B. Alexandrov, D. C. Wedge, P. Van Loo, C. D. Greenman *et al.*, 2012 Mutational processes molding the genomes of 21 breast cancers. *Cell* 149: 979–993.
- Nilsson, A. I., E. Kugelberg, O. G. Berg, and D. I. Andersson, 2004 Experimental adaptation of *Salmonella typhimurium* to mice. *Genetics* 168: 1119–1130.
- Notley-McRobb, L., S. Seeto, and T. Ferenci, 2002 Enrichment and elimination of *mutY* mutators in *Escherichia coli* populations. *Genetics* 162: 1055–1062.
- Palles, C., J. B. Cazier, K. M. Howarth, E. Domingo, A. M. Jones *et al.*, 2012 Germline mutations affecting the proofreading domains of POLE and POLD1 predispose to colorectal adenomas and carcinomas. *Nat. Genet.* 45: 136–144.
- Perrino, F. W., and L. A. Loeb, 1989 Differential extension of 3' mispairs is a major contribution to the high fidelity of calf thymus DNA polymerase-alpha. *J. Biol. Chem.* 264: 2898–2905.
- Perrino, F. W., B. D. Preston, L. L. Sandell, and L. A. Loeb, 1989 Extension of mismatched 3' termini of DNA is a major determinant of the infidelity of human immunodeficiency virus type 1 reverse transcriptase. *Proc. Natl. Acad. Sci. USA* 86: 8343–8347.
- Petruska, J., M. F. Goodman, M. S. Boosalis, L. C. Sowers, C. Cheong *et al.*, 1988 Comparison between DNA melting thermodynamics and DNA polymerase fidelity. *Proc. Natl. Acad. Sci. USA* 85: 6252–6256.
- Pugh, T. J., S. D. Weeraratne, T. C. Archer, D. A. Pomeranz Krummel, D. Auclair *et al.*, 2012 Medulloblastoma exome sequencing uncovers subtype-specific somatic mutations. *Nature* 488: 106–110.
- Reitmair, A. H., J. C. Cai, M. Bjerknes, M. Redston, H. Cheng *et al.*, 1996 MSH2 deficiency contributes to accelerated APC-mediated intestinal tumorigenesis. *Cancer Res.* 56: 2922–2926.
- Rosche, W. A., and P. L. Foster, 2000 Determining mutation rates in bacterial populations. *Methods* 20: 4–17.
- Rose, M. D., P. Novick, J. H. Thomas, D. Botstein, and G. R. Fink, 1987 A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. *Gene* 60: 237–243.
- Schaaper, R. M., 1993 Base selection, proofreading, and mismatch repair during DNA replication in *Escherichia coli*. *J. Biol. Chem.* 268: 23762–23765.
- Schaaper, R. M., and R. Cornacchio, 1992 An *Escherichia coli* *dnaE* mutation with suppressor activity toward mutator *mutD5*. *J. Bacteriol.* 174: 1974–1982.
- Schmitt, M. W., Y. Matsumoto, and L. A. Loeb, 2009 High fidelity and lesion bypass capability of human DNA polymerase delta. *Biochimie* 91: 1163–1172.
- Schmitt, M. W., R. N. Venkatesan, M. J. Pillaire, J. S. Hoffmann, J. M. Sidorova *et al.*, 2010 Active site mutations in mammalian DNA polymerase delta alter accuracy and replication fork progression. *J. Biol. Chem.* 285: 32264–32272.
- Sherman, F., 2002 Getting started with yeast, pp. 3–41 in *Guide to Yeast Genetics and Molecular and Cell Biology, Part B*, edited by C. Guthrie, and G. R. Fink. Academic Press, San Diego.
- Simon, M., L. Giot, and G. Faye, 1991 The 3' to 5' exonuclease activity located in the DNA polymerase δ subunit of *Saccharomyces cerevisiae* is required for accurate replication. *EMBO J.* 10: 2165–2170.
- Sniegowski, P. D., P. J. Gerrish, and R. E. Lenski, 1997 Evolution of high mutation rates in experimental populations of *E. coli*. *Nature* 387: 703–705.
- Sokolsky, T., and E. Alani, 2000 *EXO1* and *MSH6* are high-copy suppressors of conditional mutations in the *MSH2* mismatch repair gene of *Saccharomyces cerevisiae*. *Genetics* 155: 589–599.
- Sturtevant, A. H., 1937 Essays on evolution. I. On the effects of selection on mutation rate. *Q. Rev. Biol.* 12: 464–467.
- Thompson, D. A., M. M. Desai, and A. W. Murray, 2006 Ploidy controls the success of mutators and nature of mutations during budding yeast evolution. *Curr. Biol.* 16: 1581–1590.
- Tong, A. H., and C. Boone, 2006 Synthetic genetic array analysis in *Saccharomyces cerevisiae*. *Methods Mol. Biol.* 313: 171–192.
- Toyn, J. H., P. L. Gunyuzlu, W. H. White, and L. A. Thompson, and G. F. Hollis, 2000 A counterselection for the tryptophan pathway in yeast: 5-fluoroanthranilic acid resistance. *Yeast* 16: 553–560.
- Tran, H. T., D. A. Gordenin, and M. A. Resnick, 1999 The 3'→5' exonucleases of DNA polymerases δ and ϵ and the 5'→3' exonuclease Exo1 have major roles in postreplication mutation avoidance in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 19: 2000–2007.
- Tröbner, W., and R. Piechocki, 1984 Selection against hypermutability in *Escherichia coli* during long term evolution. *Mol. Gen. Genet.* 198: 177–178.
- Venkatesan, R. N., J. J. Hsu, N. A. Lawrence, B. D. Preston, and L. A. Loeb, 2006 Mutator phenotypes caused by substitution at a conserved motif A residue in eukaryotic DNA polymerase δ . *J. Biol. Chem.* 281: 4486–4494.
- Venkatesan, R. N., P. M. Treuting, E. D. Fuller, R. E. Goldsby, T. H. Norwood *et al.*, 2007 Mutation at the polymerase active site of mouse DNA polymerase δ increases genomic instability and accelerates tumorigenesis. *Mol. Cell. Biol.* 27: 7669–7682.
- Wagner, A., 2000 Robustness against mutations in genetic networks of yeast. *Nat. Genet.* 24: 355–361.
- Williams, J. S., D. J. Smith, L. Marjavaara, S. A. Lujan, A. Chabes *et al.*, 2013 Topoisomerase 1-mediated removal of ribonucleotides from nascent leading-strand DNA. *Mol. Cell* 49: 1010–1015.
- Williams, L. N., A. J. Herr, and B. D. Preston, 2013 Emergence of DNA polymerase ϵ antimutators that escape error-induced extinction in yeast. *Genetics* 193: 751–770.
- Winzeler, E. A., D. D. Shoemaker, A. Astromoff, H. Liang, K. Anderson *et al.*, 1999 Functional characterization of the *S. cerevisiae*

- genome by gene deletion and parallel analysis. *Science* 285: 901–906.
- Wloch, D. M., K. Szafraniec, R. H. Borts, and R. Korona, 2001 Direct estimate of the mutation rate and the distribution of fitness effects in the yeast *Saccharomyces cerevisiae*. *Genetics* 159: 441–452.
- Yoshida, S., 2008 Spermatogenic stem cell system in the mouse testis. *Cold Spring Harb. Symp. Quant. Biol.* 73: 25–32.
- Yu, L., L. Pena Castillo, S. Mnaimneh, T. R. Hughes, and G. W. Brown, 2006 A survey of essential gene function in the yeast cell division cycle. *Mol. Biol. Cell* 17: 4736–4747.
- Zeyl, C., M. Mizesko, and J. G. M. De Visser, 2001 Mutational meltdown in laboratory yeast populations. *Evolution* 55: 909–917.
- Zheng, Q., 2002 Statistical and algorithmic methods for fluctuation analysis with SALVADOR as an implementation. *Math. Biosci.* 176: 237–252.
- Zheng, Q., 2005 New algorithms for Luria-Delbruck fluctuation analysis. *Math. Biosci.* 196: 198–214.
- Zheng, Q., 2008 A note on plating efficiency in fluctuation experiments. *Math. Biosci.* 216: 150–153.

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Supporting Information

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DNA Replication Error-Induced Extinction of Diploid Yeast

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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.

File S1

Supplemental Methods

All PCR products for strain construction were amplified with Phusion polymerase using primers and templates found in Table S4 and the following conditions: [98°C 1 min., 30 x (98°C, 10 sec.; 54°C, 30 sec.; 72°C, 1.5 min.), 72°C 1 min.] unless specified otherwise. For PCR screening of transformants, cells were lysed by a 30 min. treatment with 2.5 mg/ml Zymolyase (MP Biomedicals) in TE⁻¹ (10 mM Tris•HCl, pH 7.5; 0.1 mM EDTA) followed by a 10 min. incubation at 90°C. Zymolyase was freshly diluted from a stock solution of 25 mg/ml Zymolyase stored at -20°C in 0.01M Na₂HPO₄ pH 7.5, 50% glycerol.

To make a diploid mutation rate reporter strain suitable for introduction of chromosomal *pol3-01*, *L612M* and *pol3-01*, *L612G* mutations, we first transformed BY4743 with a *can1Δ::HIS3* PCR fragment to obtain AH0301. Positive clones were identified by PCR of the *CAN1* locus with CAN1-321F and CAN1R1 [98°C 1 min., 30 x (98°C, 10 sec.; 50°C, 30 sec.; 72°C, 1 min.), 72°C 1 min.], which generates DNA fragments of 2.4 and 1.9 kb for *CAN1* and *can1::HIS3* alleles, respectively. We integrated the nourseothricin-resistance gene, *natMX*, immediately downstream of the intact *CAN1* gene to create AH0401 (Goldstein and Mccusker 1999) (Figure 2C). Nourseothricin-resistant colonies were PCR screened for loss of a 623 bp fragment generated by amplification of WT *CAN1* with can1f4 and can1R2 [98°C 1 min., 30 x (98°C, 10 sec.; 52°C, 30 sec.; 72°C, 45 sec.), 72°C 1 min.]. All clones lacking the 623 bp fragment were screened for loss of the high rates of canavanine-resistant colonies due to mitotic recombination by plating on media containing canavanine and nourseothricin. Simultaneous selection for resistance to canavanine and nourseothricin dramatically lowers the background rate of Can^r mutants. To generate chromosomal insertions of *pol3* alleles, AH0401 was transformed with *URA3::POL3*, *URA3::pol3-01*, *URA3::pol3-01,L612M*, or *URA3::pol3-01,L612G*, amplified with the following conditions [98°C 1 min., 30 x (98°C, 10 sec.; 54°C, 30 sec.; 72°C, 2 min.), 72°C 1 min.]. Correct *URA3* insertions at the *POL3* locus were identified by screening Ura⁺ colonies with an insertion-specific PCR assay using primers lac111pol3-1 and URA3intR to produce a 1.1 kb fragment [98°C 1 min., 30 x (98°C, 10 sec.; 54°C, 30 sec.; 72°C, 40 sec.), 72°C 1 min.]. All Ura⁺ colonies assayed contained the correctly integrated *URA3* transgene. These clones were subjected to two genotyping PCR assays. In the first assay, a fragment of *POL3* spanning the *pol3-01* and *L612* mutation sites was amplified with primers POL3GTF and pldr6 and then digested with EcoRV, which unambiguously distinguishes *pol3-01* and *pol3-L612G* from WT *POL3* sequence (Figure S1) (98°C, 10 sec.; 54°C, 30 sec.; 72°C, 40 sec.). In the second assay, *L612M*-positive clones were identified by PCR amplification with

primers pldr6 and L612M-specific, which preferentially amplifies the *pol3-L612M* allele (Figure S3) [98°C 1 min., 28 x (98°C, 10 sec.; 69°C, 30 sec.; 72°C, 30 min.), 72°C 1 min.].

To construct a diploid shuffling strain suitable for canavanine-mutation rate assays, we constructed P3H3 α by transforming BY4734 with pGL310 and then with the *pol3 Δ ::HIS3* PCR fragment. Correct insertions were confirmed by PCR of the *POL3* locus using pldf629 and pldr4818 [98°C 1 min., 30 x (98°C, 10 sec.; 54°C, 30 sec.; 72°C, 2 min.), 72°C 1 min.], which are not contained in the *POL3* sequence found in pGL310. Amplification of WT *POL3* sequence with these primers produces a 4.2 kb fragment, while amplification of *pol3 Δ ::HIS3* produces a 2.2 kb fragment. We constructed BP7801 by transforming P3H3 α with *can1 Δ ::TRP1*. Correct insertions were identified by screening for canavanine resistance. We constructed BP7901 by transforming P3H3a (Herr *et al.* 2011) with *CAN1::kanMX*. Positive clones were identified by insertion-specific PCRs using can1F4 and pUG-KanMX-1073R [98°C 1 min., 30 x (98°C, 10 sec.; 51°C, 30 sec.; 72°C, 1 min.), 72°C 1 min.], which amplifies a 1.3 kb fragment, and pUG-KanMX-701fp and can1R2, which amplifies a 0.9 kb fragment. BP7801 and BP7901 were mated to produce the diploid BP8001, which was selected for the ability to grow on SC-MSG plates lacking tryptophan and containing G418.

To construct an Msh2-deficient diploid, BP7801 and BP7901 were transformed with a *msh2 Δ ::MET15* PCR fragment to produce BP8402 and BP9014, respectively. We have found that *MET15* PCR fragments generated with just 50 nt of targeting homology often produce a high background of incorrect insertions, which can be minimized by constructing DNA fragments with extended upstream and downstream homology. To generate *msh2 Δ ::MET15* with extended homology, we first amplified a 430 bp upstream fragment with primers msh2-560f and msh2-965r, a 404 bp downstream fragment with primers msh2-2674f and MSH2-downstream [98°C 1 min., 30 x (98°C, 10 sec.; 60°C, 30 sec.; 72°C, 1 min.), 72°C 1 min.], and a 1.8 kb *MET15* fragment with MSH2U and MSH2D [98°C 1 min., 30 x (98°C, 10 sec.; 54°C, 30 sec.; 72°C, 1.5 min.), 72°C 1 min.]. We gel-purified the above DNA fragments, combined them at an equal molar ratio, and amplified the chimeric DNA fragment with msh2-560f and MSH2-downstream. We screened transformants for correct insertions by PCR amplification across the *MSH2* locus with outside primers msh2-537f and msh2-3109r [98°C 1 min., 30 x (98°C, 10 sec.; 53°C, 30 sec.; 72°C, 2 min.), 72°C 1 min.], which generates a 3.7 kb fragment for *MSH2* and a 2.7 kb fragment for *msh2 Δ ::MET15*. BP8402 and BP9014 were mated and plated on SC-MSG plates lacking tryptophan and containing G418 to select for BP9101.

To construct a Dun1-deficient diploid, BP7801 and BP7901 were transformed with *dun1 Δ ::MET15* to produce BP8202 and BP8301, respectively. The *DUN1* locus is adjacent to *POL3*, and we generated a chimeric PCR

product with extended upstream and downstream homology. We first amplified a 602 bp upstream fragment with dun1met15-439f and dun1met15-1013r and a 436 bp downstream fragment with dun1met15-2905f and pol3KI-361R [98°C 1 min., 30 x (98°C,10 sec.; 60°C, 30 sec.; 72°C, 1 min.), 72°C 1 min.], and a 1.8 kb *MET15* fragment with dun1FTKO and dun1RTKO [98°C 1 min., 30 x (98°C,10 sec.; 54°C, 30 sec.; 72°C, 1.5 min.), 72°C 1 min.]. The three fragments were combined, and the chimeric PCR fragment was amplified with dun1met15-439f and pol3KI-361R [98°C 1 min., 30 x (98°C,10 sec.; 60°C, 30 sec.; 72°C, 2.5 min.), 72°C 1 min.]. Positive clones were identified by two insertion-specific PCR assays. The first assay utilized primers Dun1probeR and Met15d2rp to amplify a 1.9 kb fragment [98°C 1 min., 30 x (98°C,10 sec.; 58°C, 30 sec.; 72°C, 1.5 min.), 72°C 1 min.]. The second assay utilized primers pol3KI-668R and Met15d1fp to amplify a 2.3 kb fragment [98°C 1 min., 30 x (98°C,10 sec.; 58°C, 30 sec.; 72°C, 1.5 min.), 72°C 1 min.]. BP8101 and BP8202 were mated and plated on SC-MSG plates lacking tryptophan and containing G418 to select for BP8301.

Table S1 Yeast Strains

Strain	Relevant Genotype	Reference
BY4733 ^a	<i>MATα leu2Δ0 ura3Δ0 met15Δ0 trp1Δ63 his3Δ200</i>	(Brachmann <i>et al.</i> 1998)
BY4734 ^a	<i>MATα leu2Δ0 ura3Δ0 met15Δ0 trp1Δ63 his3Δ200</i>	(Brachmann <i>et al.</i> 1998)
P3H3a	<i>MATα leu2Δ0 ura3Δ0 met15Δ0 trp1Δ63 his3Δ200 pol3Δ::HIS3 + pGL310 [URA3/POL3]</i>	(Herr <i>et al.</i> , 2011)
BP1506	<i>MATα leu2Δ0 ura3Δ0 met15Δ0 trp1Δ63 his3Δ200 pol3Δ::HIS3 msh6::kanMX + pGL310 [URA3/POL3]</i>	(Herr <i>et al.</i> , 2011)
BP4001	<i>MATα leu2Δ0 ura3Δ0 met15Δ0 trp1Δ63 his3Δ200 pol3Δ::HIS3 agp1::URA3 + pRS414POL3 [TRP1/POL3]</i>	(Herr <i>et al.</i> , 2011)
P3H3 α	<i>MATα leu2Δ0 ura3Δ0 met15Δ0 trp1Δ63 his3Δ200 pol3Δ::HIS3 + pGL310 [URA3/POL3]</i>	This Study
BP7801	<i>MATα leu2Δ0 ura3Δ0 met15Δ0 trp1Δ63 his3Δ200 can1Δ::TRP1 pol3::HIS3 + pGL310 [URA3/POL3]</i>	This Study
BP7901	<i>MATα leu2Δ0 ura3Δ0 met15Δ0 trp1Δ63 his3Δ200 CAN1::kanMX pol3::HIS3 + pGL310 [URA3/POL3]</i>	This Study
BP8001	<i>MATα/α leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/met15Δ0 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 CAN1::kanMX/can1Δ::TRP1 pol3::HIS3/pol3::HIS3 + pGL310 [URA3/POL3]</i>	This Study
BP8101	<i>MATα leu2Δ0 ura3Δ0 met15Δ0 trp1Δ63 his3Δ200 can1Δ::TRP1 dun1Δ::MET15 pol3::HIS3 + pGL310 [URA3/POL3]</i>	This Study
BP8202	<i>MATα leu2Δ0 ura3Δ0 met15Δ0 trp1Δ63 his3Δ200 CAN1::kanMX dun1Δ::MET15 pol3::HIS3 + pGL310 [URA3/POL3]</i>	This Study
BP8301	<i>MATα/α leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/met15Δ0 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 CAN1::kanMX/can1Δ::TRP1 dun1Δ::MET15/dun1Δ::MET15 pol3::HIS3/pol3::HIS3 + pGL310 [URA3/POL3]</i>	This Study
BP8402	<i>MATα leu2Δ0 ura3Δ0 met15Δ0 trp1Δ63 his3Δ200 CAN1::TRP1 msh2Δ::MET15 pol3::HIS3 + pGL310 [URA3/POL3]</i>	This Study
BP9014	<i>MATα leu2Δ0 ura3Δ0 met15Δ0 trp1Δ63 his3Δ200 CAN1::kanMX msh2Δ::MET15 pol3::HIS3 + pGL310 [URA3/POL3]</i>	This Study
BP9101	<i>MATα/α leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/met15Δ0 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 CAN1::kanMX/can1Δ::TRP1 msh2Δ::MET15/msh2Δ::MET15 pol3::HIS3/pol3::HIS3 + pGL310 [URA3/POL3]</i>	This Study
BY4743 ^b	<i>MATα/α leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 his3Δ200/his3Δ200 LYS2/lys2Δ0 MET15/met15Δ0</i>	(Brachmann <i>et al.</i> 1998)
AH0301	<i>MATα/α leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 his3Δ200/his3Δ200 LYS2/lys2Δ0 MET15/met15Δ0 CAN1/can1Δ::HIS3</i>	This Study
AH0401	<i>MATα/α leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 his3Δ200/his3Δ200 LYS2/lys2Δ0 MET15/met15Δ0 CAN1::natMX/can1Δ::HIS3</i>	This Study

^aP3H3a, P3H3 α , and the BP series were constructed from BY4733 and BY4734. BY4733 and BY4734 are S288C descendants that we re-derived via sporulation of a BY4733 X BY4734 diploid (kindly provided by Tim Formosa, University of Utah). P3H3a and P3H3 α were generated by replacing *POL3* with *HIS3* in BY4733 or BY4734 carrying pGL310. pGL310 is the *CEN4/ARS1/URA3* plasmid YCp50 (Rose *et al.* 1987) modified to carry *SUP11* and *POL3* (Simon *et al.* 1991; Giot *et al.* 1995). pRS414POL3 is the *CEN6/ARSH4/TRP1* plasmid pRS414 (Brachmann *et al.* 1998) carrying wild-type *POL3* with its natural promoter. BP4001 was derived from P3H3a by integration of *URA3* at the *agg1* locus. BP7801 was derived from P3H3 α by replacing the *CAN1* coding sequence with *TRP1*. BP7901 was generated by integrating kanMX immediately downstream of *CAN1*, leaving canavanine sensitivity intact. BP8101 and BP8402 were generated by replacing *DUN1* or *MSH2* with MET15 in BP7801. BP8202 and BP9014 were generated in a similar manner from BP7901. BP7801 was crossed with BP7901 to generate BP8001; BP8101 and BP8202 were crossed to produce BP8301; BP8402 was crossed with BP9014 to generate BP9101.

^bAH0301 was generated from BY4743, by replacing the *CAN1* coding sequence with *HIS3*. AH0401 was generated from AH0301 by integration of the natMX cassette downstream of *CAN1*, which leaves canavanine sensitivity intact.

Table S2 Terminal morphologies of inviable spores^a

Strain	Genotype of sporulated diploid	growth-arrested state			
		U	B	D	M
1	<i>POL3/pol3-01,L612M</i>	23	2	8	7 ^b
2	<i>POL3/pol3-01,L612M</i>	33	1	7	–
3	<i>POL3/pol3-01,L612G</i>	33	–	4	–
4	<i>POL3/pol3-01,L612G</i>	32	–	3	–

^a Morphologies were scored after 48 hours of continuous growth at 30°C. U, unbudded; B, small bud; D, large symmetrical bud; M, microcolony (4-10 cells).

^b Three microcolonies were from the same ascus and had 7 cells (1 U, 6 D), 10 cells (5 U, 5 B), and 5 cells (5 U). Two were from another ascus, and both formed 4 large unbudded cells. The remaining two microcolonies were from unrelated spores and were composed of 4 cells (B) and 2 multi-budded cells, respectively.

Table S3 is available for download as an Excel file at
<http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.160960/-/DC1>

Table S3A De novo mutations in a single *POL3/pol3-01,L612G* isolate

Table S3B De novo mutations in a single *POL3/pol3-01,L612M* isolate

Table S4 Construction of Chromosomal Gene Disruptions^a

Allele	PCR Primer	Primer Sequence	Template
<i>can1Δ::HIS3</i> or <i>can1Δ::TRP1</i>	CAN1KOfp	5'-TGTCGTCAATCGAAAGTTTATTTTCAGAGTTCTTCAGACT TCTTAACCTCTAGATTGTA <u>CTGAGAGTGCAC</u> -3'	pRS413 or pRS414 (Brachmann <i>et al.</i> 1998)
	CAN1KOrp	5'-TGAGAATGCGAAATGGCGTGGAATGTGATCAAAGGT AATAAACGTCATATCTACTGTGCGGTATTTCA <u>CACCG</u> -3'	
	CAN1-321F	5'-GAACTCTTGTCCTTATTAGCCTTGATAGTGC-3'	
	CAN1-R1	5'-TATGAGGGTGAGAATGCGAAATGGCG-3'	
<i>CAN1::natMX</i> or <i>CAN1::kanMX</i>	CAN1::KanMXfp	5'-ACCAAAGACTTTTTGGGACAAATTTTGAATGTTGTAGC ATAGATATGACACATGGAGGCCAGAA <u>TACCCT</u> -3'	natMX (pFvL099, Stulemeijer <i>et al.</i> 2011); kanMX (pUG6; Guldener <i>et al.</i> 1996)
	CAN1::KanMXrp	5'-ATGAGGGTGAGAATGCGAAATGGCGTGGAATGTGAT CAAAGGTAATAAAAC <u>CAGTATAGCGACCAGCATT</u> CAC-3'	
	can1f4	5'-GAGACATCTACTGGTGGTGAC-3'	
	pUG-KanMX- 1073R	5'-AATCACCATGAGTGACGACTGAATCC-3'	
	pUG-KanMX- 701fp	5'-CTGACGGAATTTATGCCTCTTCCG-3'	
	can1R2	5'-GGAGTTTCAAATGCTTCTACTCCGTCTGC-3'	
<i>URA3::POL3</i> , <i>URA3::pol3-01</i> , <i>URA3::pol3-01,L612M</i> , or <i>URA3::pol3-01,L612G</i>	Pol3U	5'-ATATTGAGCACTTGCTATTAAGCATTAACTTTATACATA TACGCACAGCA <u>AGATTGTA</u> CTGAGAGTGCAC-3'	pRS406- <i>POL3</i> ; pRS406-pol3-01; pRS406- <i>pol3-01,L612M</i> ; pRS406- <i>pol3-01,L612G</i> (this study)
	pldr6	5'-AAACCACAACGGCATCGTGC-3'	
	lac111pol3-1F	5'-AAGCTTTCCCAATTGTGGAAGTTGACTTT-3'	
	URA3intR	5'-CAATGTCAACAGTACCCTTAGTATATTCTCCAG-3'	
	POL3GTF	5'-AGAGTTTCTCTTGTCAGTTGGAAGTTTCAATTA-3'	

	L612M-specific	5'-CGATTGCAACTTTGGATTTCATTCTATG-3'	
<i>pol3Δ::HIS3</i>	Pol3U	5'-ATATTGAGCACTTGCTATTAAGCATTAACTTTATACATA TACGCACAGCAAGATTGTACTGAGAGTGCAC-3'	pRS413 (Brachmann <i>et al.</i> 1998)
	Pol3D	5'-TTGTTAGCCTTTCTTAATCCTAATATGATGTGCCACCCTA TCGTTTTTACTGTGCGGTATTTACACCG-3'	
	pldf629	5'-GGTCTTTGCGAGACAAGCTTTCCC-3'	
	pldr4818	5'-CAAACACACTTTGTGGTACGCCTTCTTA-3'	
<i>msh2Δ::MET15</i>	msh2-560f	5'-TCCAGCGGAGTACTCGAAAAGATTAGAA-3'	Genomic DNA
	msh2-965R	5'-GAGGATTTTGATGTTAGGTCAGCAG-3'	
	msh2-2674f	5'-GGAAAACGATAATTACCTGAAATATATAAAAGCCTTG-3'	Genomic DNA
	MSH2- downstream	5'-GATGACTCATCTTTGTACCCAATTCG-3'	
	MSH2U	5'-AAAAATCTCTTTATCTGCTGACCTAACATCAAAATCCTCA GATTAAGAGTAGATTGTACTGAGAGTGCAC-3'	pRS411 (Brachmann <i>et al.</i> 1998)
	MSH2D	5'-TTATAACAACAAGGCTTTTATATATTTAGGTAATTATCG TTTTCTTTTCTGTGCGGTATTTACACCG-3'	
	msh2-536f	5'-CCATGTGTCTTCTCTGACGAGCCTC-3'	
	msh2-3109r	5'-TATCGCCATCTTCAAATAGGAAA-3'	
	dun1met15-439f	5'-TTCAGGAATTGATTCCTACGATATGGACG-3'	Genomic DNA
	dun1met15- 1013r	5'-TTACATCACCAGAGTGCTCTCTTTTCGT-3'	
<i>dun1Δ::MET15</i>	dun1met15- 2905f	5'-GCATTATTTCAAAAACATTGTTTGAATCTGGATTTTCCA AGTATC-3'	Genomic DNA
	pol3KI-361R	5'-GCCCAATGGTACCAACGATGTTCC-3'	
	dun1FTKO	5'-ATGAGTTTGTCCACGAAAAGAGAGCACTCTGGTGATGT AACTGACTCTTCCAGAGCAGATTGTACTGAGAGTGCACCA- 3'	pRS411 (Brachmann <i>et al.</i> 1998)
	dun1RTKO	5'-GGAAAAATCCAGATTCAAACAATGTTTTGAAATAATGC TTCTCATGTTTAGGAACAACACTCAACCTATCTCGGTCTA- 3'	

Dun1probeR	5'-CGACTTTTGTTCGCTTTGTATCAGTGAATTCTT-3'
Met15d2rp	5'-CGAAAGATAAGACACCACCGAAACCGTT-3'
Met15d1fp	5'-CTCATTTGATACTGTTCAACTACACGCCG-3'
pol3KI-668R	5'-GGTCAAGCTTTTAAAGAGGCCCTACTGG-3'

^aMutations were introduced into yeast using PCR products generated with the primers and template DNAs found at the top of each section. Additional primers used in the identification of correct insertions are found at the bottom of each section. Underlined sequences are complementary to the PCR template plasmids, 5' and 3' of each marker gene (*kanMX*, *natMX*, *HIS3*, *TRP1*, *URA3*, or *MET15*). Non-underlined sequences correspond to portions of each yeast gene targeted for homologous disruption (*CAN1*, *POL3*, *MSH2*, or *DUN1*). To improve targeting specificity, three separate PCR fragments were combined by chimeric PCR to generate *msh2Δ::MET15* and *dun1Δ::MET15* PCR fragments with extended homology 5' and 3' of the targeted gene. Each primer pair and corresponding template is delineated with a dashed line. All PCR amplification conditions are described in Supporting Information.

Tables S5-S7 are available for download as Excel files at
<http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.160960/-/DC1>

Table S5 Median Size of the Essential Genes of Yeast

Table S6 Growth of diploid yeast for 20 generations modeled as a function of *can1* mutation rate

Table S7 Growth of diploid yeast for 1000 generations modeled as a function of *can1* mutation rate

References

- Brachmann, C. B., A. Davies, G. J. Cost, E. Caputo, J. Li *et al.*, 1998 Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14**: 115-132.
- Giot, L., M. Simon, C. Dubois and G. Faye, 1995 Suppressors of thermosensitive mutations in the DNA polymerase δ gene of *Saccharomyces cerevisiae*. *Mol Gen Genet* **246**: 212-222.
- Goldstein, A., and J. H. Mccusker, 1999 Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* **15**: 1541-1553.
- Guldener, U., S. Heck, T. Fielder, J. Beinhauer and J. H. Hegemann, 1996 A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res* **24**: 2519-2524.
- Herr, A. J., M. Ogawa, N. A. Lawrence, L. N. Williams, J. M. Eggington *et al.*, 2011 Mutator Suppression and Escape from Replication Error–Induced Extinction in Yeast. *PLoS Genet* **7**: e1002282.
- Rose, M. D., P. Novick, J. H. Thomas, D. Botstein and G. R. Fink, 1987 A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. *Gene* **60**: 237-243.
- Simon, M., L. Giot and G. Faye, 1991 The 3' to 5' exonuclease activity located in the DNA polymerase δ subunit of *Saccharomyces cerevisiae* is required for accurate replication. *EMBO J* **10**: 2165-2170.
- Stulemeijer, I. J., B. L. Pike, A. W. Faber, K. F. Verzijlbergen, T. Van Welsem *et al.*, 2011 Dot1 binding induces chromatin rearrangements by histone methylation-dependent and -independent mechanisms. *Epigenetics Chromatin* **4**: 2.