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’ 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 pdlr6 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 P3H3α by transforming BY4734 with pGL310 and then with the pol3Δ::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 pol3Δ::HIS3 produces a 2.2 kb fragment. We constructed BP7801 by transforming P3H3α with can1Δ::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 msh2Δ::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 msh2Δ::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.], 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 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 msh2Δ::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 dun1Δ::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, 1.5 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.