PNAS RESEARCH ARTICLE | GENETICS OPEN ACCESS

Global genomic instability caused by reduced expression of DNA polymerase ε in yeast

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Contributed by Thomas D. Petes; received October 26, 2021; accepted February 14, 2022; reviewed by Catherine Freudenreich and Anna Malkova

DNA polymerase ε (Pol ε) is one of the three replicative eukaryotic DNA polymerases. Pol ε deficiency leads to genomic instability and multiple human diseases. Here, we explored global genomic alterations in yeast strains with reduced expression of *POL2*, the gene that encodes the catalytic subunit of Pol ε. Using whole-genome SNP microarray and sequencing, we found that low levels of Pol ε elevated the rates of mitotic recombination and chromosomal aneuploidy by two orders of magnitude. Strikingly, low levels of Pol ε resulted in a contraction of the number of repeats in the ribosomal DNA cluster and reduced the length of telomeres. These strains also had an elevated frequency of break-induced replication, resulting in terminal loss of heterozygosity. In addition, low levels of Pol ε increased the rate of single-base mutations by 13-fold by a Pol ζ-dependent pathway. Finally, the patterns of genomic alterations caused by low levels of Pol ε were different from those observed in strains with low levels of the other replicative DNA polymerases, Pol α and Pol δ, providing further insights into the different roles of the B-family DNA polymerases in maintaining genomic stability.

DNA replication stress | DNA polymerase | genome instability | mitotic recombination | loss of heterozygosity

Significance

Although most studies of the genetic regulation of genome stability involve an analysis of mutations within the coding sequences of genes required for DNA replication or DNA repair, recent studies in yeast show that reduced levels of wild-type enzymes can also produce a mutator phenotype. By whole-genome sequencing and other methods, we find that reduced levels of the wild-type DNA polymerase ε in yeast greatly increase the rates of mitotic recombination, aneuploidy, and single-base mutations. The observed pattern of genome instability is different from those observed in yeast strains with reduced levels of the other replicative DNA polymerases, Pol α and Pol δ. These observations are relevant to our understanding of cancer and other diseases associated with genetic instability.

Chromosome replication in eukaryotes requires three conserved B-family polymerases (Pols α, δ, and ε), with DNA polymerase ζ having an important role in the replication of damaged bases (1, 2). The roles of these polymerases in duplicating the genome are different. Polymerase (Pol) α initiates DNA replication by synthesizing short RNA/DNA primers, and Pol δ is required to extend these fragments to complete lagging-strand replication. Although it is widely accepted that the main role of Pol ε is to replicate the leading-strand template, more recent evidence suggests that in conditions of replication stress, Pol δ can also participate in leading-strand replication (3, 4). Although the catalytic domain of Pol ε is not required for viability in yeast (5), Pol ε is required for assembly of the replisome (6). Mutations of *POL2* (encoding the catalytic subunit of Pol ε) also result in defects in S-phase checkpoint activation (7), short telomeres (8), and modest defects in DNA repair (9). In addition, mutations of *DPB3* (encoding a nonessential subunit of Pol ε) lead to a reduction in the efficiency of silencing of markers inserted in the ribosomal DNA (rDNA) (10).

In humans, mutations in Pol ε result in several human immunodeficiency syndromes (11, 12). In addition, mutations in *POLE* (equivalent to the *POL2* gene in yeast) are associated with a variety of types of tumors with a mutator phenotype (13, 14). The cancer-associated mutations are found throughout the gene but concentrated within the exonuclease domain. Tumors with elevated mutation rates are often heterozygous for the *POLE* mutation (13), suggesting the possibility that a reduction in the amount of the wild-type enzyme might be relevant to the mutator phenotype. In this study, we examine the effects of reducing the amount of Pol ε on a variety of genomic alterations, including single-base substitutions, insertions/deletions (in/dels), changes in chromosome structure, and alterations in chromosome number.

Previously, we showed that reduced expression of *POL1* (encoding the catalytic subunit of Pol α) or *POL3* (encoding the catalytic subunit of Pol δ) in *Saccharomyces cerevisiae* greatly elevated aneuploidy, chromosome rearrangements, and loss of heterozygosity (LOH) (15–18). Our analysis suggested that most of these events reflected double-strand DNA breaks (DSBs) that occurred at stalled or broken replication forks. Although reduced levels of both Pol α and Pol δ greatly elevated the rates of LOH, and large deletions and duplications (often caused by homologous recombination between nonallelic Ty retrotransposons), there were a number of differences in the genomic alterations induced by these two polymerases (19). For example, the ratio of LOH events to chromosome losses is about fourfold higher in strains with low levels of Pol α than in strains with low Pol δ. The likely explanation of this ratio difference is that Pol δ has a more important role in the repair of DSBs by homologous

Author contributions: K.Z., T.D.P., and D.-Q.Z. designed research; K.Z. and Y.S. performed research; K.Z., Y.S., W.-L.L., G.C., X.-C.W., R.J.K., T.D.P., and D.-Q.Z. analyzed data; and K.Z., Y.S., T.D.P., and D.-Q.Z. wrote the paper.

Reviewers: C.F., Tufts University; and A.M., University of Iowa.

Competing interest statement: T.D.P. and the reviewers A.M. and C.F. were among 48 coauthors on a 2019 review article [H. L. Klein *et al.*, *Microb. Cell* **6**, 1–64 (2019)].

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This article contains supporting information online at http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2119588119/-/DCSupplemental.

Published March 15, 2022.

recombination than Pol α. Thus, many DSBs in strains with low Pol δ are not repaired, leading to chromosome loss (19).

In the present study, we explored the global genomic instability in yeast strains in which *POL2*, encoding the catalytic subunit of Pol ε, was down-regulated. Using a combination of single-nucleotide polymorphism (SNP) microarrays and whole-genome sequencing, we found that these strains have substantially elevated LOH events, chromosome aneuploidy, and single-base substitutions. We found that low Pol ε induced a different pattern of genomic instability than induced by low levels of Pol α or Pol δ, providing insights into how B-family DNA polymerases coordinately contribute to genomic stability in eukaryotic cells.

Results

Reduced Expression of *POL2* Leads to Aberrant Cell Cycle and Growth. A diploid *S. cerevisiae* strain DZP2 was constructed by mating two haploid strains (one isogenic with W303-1A and one isogenic with YJM789) in which the expression of *POL2* was regulated by the *GAL1* promoter (details provided in *SI Appendix*). This strain is isogenic with strains used previously to examine the effects of low levels of *POL1* (17) and *POL3* (18) on genome stability, and is heterozygous for about 50,000 SNPs that allow the mapping of mitotic recombination events. The isogenic wild-type diploid (JSC25) (20) was used as a control in our study. The genotypes of the strains and the primers used in strain construction are in *SI Appendix*, Tables S1 and S2, respectively.

By qRT-PCR, we showed that the mRNA level of *POL2* was down-regulated by 62% and 69%, respectively, when DZP2 was cultured in low-galactose (LG) medium (3% raffinose and 0.005% galactose) and YPD (2% glucose without galactose) compared to that in isogenic wild-type cells (Fig. 1*A*). The level of *POL2*-specific mRNA in DZP2 grown in high-galactose (HG) medium (3% raffinose, 0.05% galactose) was elevated about 30% relative to the wild-type levels. By Western analysis, we found that the level of the Pol2 in LG medium was reduced to <10% of the wild-type level (Fig. 1*B*). Lower expression of Pol2 resulted in very slow growth on LG or YPD solid media compared to growth on HG medium (Fig. 1*C*). The doubling times of the wild-type strain on HG and LG media were 1.7 h, and the growth rate of the *GAL-POL2* strain on HG medium was not significantly different (1.8 h). In contrast, the doubling time of the *GAL-POL2* strain on LG medium was about 2.7 h.

We also examined the two strains in the two types of media by FACS analysis of cells synchronized with α-pheromone. In our previous studies, we found the yeast cells with low levels of Pol α or Pol δ had an elongated S-phase (17, 18). In *SI Appendix*, Fig. S1, we show that the wild-type strain in both types of media, and the *GAL-POL2* strain in HG medium begin DNA replication synchronously 30 to 45 min after release from the α-pheromone block; replication was completed about 40 min after DNA synthesis was initiated. In contrast, the *GAL-POL2* strain grown in LG medium delayed the initiation of DNA synthesis until about 60 min after release from the α-block. The synchrony of this strain was poor, preventing an accurate measurement of the S-period. We also examined the fraction of the cells in exponentially dividing cultures that were doublets (mother and bud of approximately the same size). These proportions were: 7% (wild-type in HG), 5% (wild-type in LG), 4% (*GAL-POL2* in HG), and 32% (*GAL-POL2* in LG). These results, taken together, suggest that the *GAL-POL2* strain grown in LG medium has an extended cell cycle time, likely as a consequence of a transient arrest at a cell cycle checkpoint. This observation is consistent with the dual role of Pol ε in replisome assembly and DNA synthesis (6).

Low Levels of Pol *ε* Stimulate Genomic Alterations in Subcultured Isolates. To determine the effects of low Pol ε on genomic integrity, we grew 37 DZP2-derived isolates on LG plates from a single cell to a colony (roughly 25 cell divisions) for two cycles. These 37 independent colonies were purified on HG plates, and then analyzed by whole-genome SNP microarray analysis (21 isolates) or whole-genome sequencing (18 isolates). Using whole-genome microarrays, we also examined six DZP2-derived isolates that were grown from a single cell to a colony on HG medium for two cycles; in these isolates, no genomic alterations were observed. Below, we summarize the rate of various types of genomic changes, including: recombination between homologs producing LOH events, changes in chromosome number, copy-number variations in tandem arrays of the ribosomal RNA (rRNA) and *CUP1* genes, and single-base substitutions.

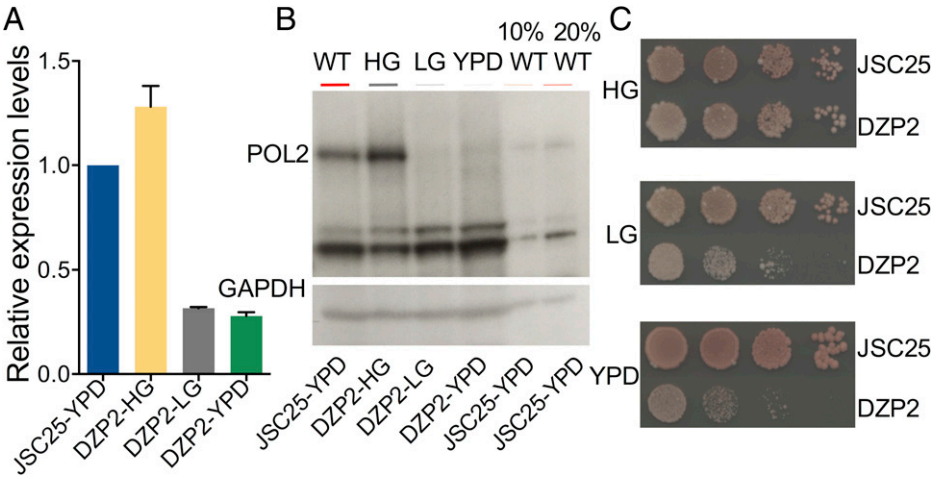

Fig. 1. Levels of Pol *ε* and growth rates of *GAL1-POL2* strains in media containing different concentrations of galactose. (*A*) The relative mRNA levels of *POL2* in strains JSC25 (wild-type) and DZP2 *(GAL1-POL2*). RNA was isolated from these strains grown in HG, LG, and no-galactose medium (YPD) (details in *Materials and Methods*). Levels of *POL2*-specific mRNA were normalized to the levels of *ACT1*-specific mRNA. (*B*) Western blot analysis of the levels of the Pol2p in JSC25 and DZP2 incubated in various types of media. The level of GAPDH was used as a control. (*C*) Comparative growth rates of JSC25 and DPZ2 on HG, LG, and YPD plates for strains JSC25 and DZP2. Ten-fold dilutions of each strain were spotted onto solid medium and grown at 30 °C.

Mitotic recombination (LOH) events. As reported in our previous studies (20, 21), the abundant heterozygous SNPs in diploids created by crossing W303-1A– and YJM789-derived sequences allow mapping of recombination events that cause LOH. The copy-number neutral LOH events were classified into interstitial LOH (I-LOH or gene conversion) and terminal LOH (T-LOH), shown in Fig. 2 *A* and *B*, respectively. In this figure, based on genome-wide sequence analysis, we show the relative number of "reads" for W303-1A– (red in Fig. 2*A*) and YJM789 (blue in Fig. 2*A*)-specific SNPs along the chromosome divided by the average of the two types of reads for all SNPs in the genome. Thus, a ratio of 0.5 indicates heterozygous SNPs, and LOH regions result in 0 reads for one type of SNP and a ratio of about 1 for the other type of SNP. In Fig. 2*A*, the repair of a DSB on the W303-1A–derived homolog near coordinate 270 kb results in a 10-kb I-LOH region; in the *Saccharomyces* Genome Database (SGD), coordinates are labeled from the left telomere to the right telomere. Fig. 2*B* shows a T-LOH event on chromosome XIII that extends from coordinate 750 kb to the end of the chromosome. T-LOH events could be a consequence of a reciprocal cross-over (RCO, resulting in reciprocal patterns of LOH in the two daughter cells) or the nonreciprocal break-induced replication (BIR) mechanism in which one end of the chromosome is lost. Although RCO and BIR cannot be distinguished without special procedures (to be described further below), in wild-type diploids engaged in allelic recombination, RCO occurs much more frequently than BIR (22–25).

Among the 37 isolates, we detected 27 gene conversions with a median tract size of 5.5 kb (4.2 kb to 16 kb, 95% confidence interval [CI]) and 89 T-LOH events (Dataset S1). In the strains examined by sequencing alone, there were 18 conversion tracts with a median size of 4.3 kb (1.9- to 16.4-kb CI). Based on the number of cell divisions during subcloning of the isolates, the rates of I-LOH and T-LOH were calculated as 1.5×10^{-2} and 4.8×10^{-2} events per cell division, respectively. The total LOH rate is 6.3×10^{-2} per division. Compared to the isogenic wild-type diploid yeast cells (21), the strains with low levels of Pol ε showed a 4.5-fold elevation in the frequency of I-LOH events, and a 36-fold elevation in T-LOH events. There is a striking difference in the proportion of I-LOH and T-LOH events between the low Pol ε strains and wild-type strains. In the strain with low levels of DNA polymerase ε, the proportion of I-LOH events (0.23) is about one-third of the proportion of T-LOH events (0.77). In the wild-type strain, these proportions are reversed, with the proportions of I-LOH events and T-LOH events being 0.71 and 0.29, respectively. Possible explanations for this difference will be considered in *Discussion*.

The positions (SGD coordinates) of the LOH breakpoints that likely reflect the positions of the DNA lesions that initiate those LOH events are listed in Dataset S1. All mitotic recombination events were assigned a class depending on whether the event was a T- or I-LOH event, and which chromatid was the recipient of information (depicted in Dataset S2). Using the statistical approaches described in our previous studies (17, 18), we determined whether certain chromosomal elements (for example, centromeres, transfer RNA genes, Ty transposons, replication termination sites, high-GC content regions, and low-GC content regions) were significantly overrepresented or underrepresented at the break points of the LOH events (*SI Appendix*, Table S3). Of the elements examined, only low-GC regions were significantly overrepresented at the recombination breakpoints after correction for multiple comparisons ($P < 0.001$).

Large (>1 kb) deletions and insertions. Excluding the rRNA and *CUP1* genes (to be described in *Copy-number variation within the rDNA and CUP1 clusters*), we identified 9 interstitial deletions/duplications (8 deletions and 1 duplication) and 22 terminal deletions/duplications (14 deletions and 8 duplications) (Dataset S3). These two classes of genomic alterations occurred at rates of 4.9×10^{-3} and 1.2×10^{-2} event per cell division, respectively; these rates are elevated 33- and 400-fold compared to the rates in the wild-type strain (21). The patterns of these deletions/duplications are shown in Dataset S4. Of the 31 deletions/duplications detected in the 37 DZP2-derived isolates, 29 involved Ty transposons or long terminal repeats at the breakpoints, and one was between *MAT* and *HMR* (Dataset S3). These data show that most of chromosomal rearrangements in low Pol ε strains are caused by homologous recombination between ectopic repeats. Interstitial deletions and duplications can reflect unequal cross-overs between nonallelic repeats on the same chromosome (17) or single-strand annealing, which preferentially produces deletions (19). Terminal deletions and duplications can be formed by recombination between repeats on nonhomologous chromosomes (*SI Appendix*, Fig. S2). Although

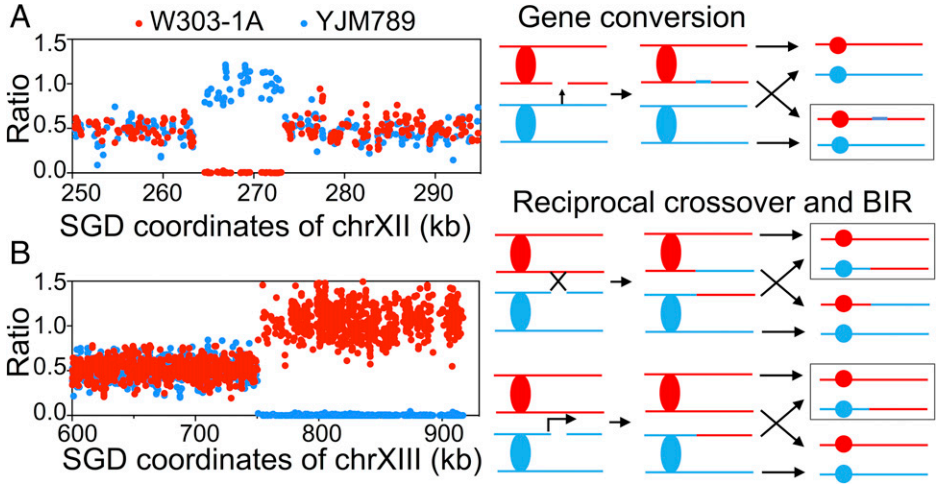

Fig. 2. Examples of I-LOH and T-LOH events. Red and blue dots reflect the ratio of coverage (RC) of W303-1A–specific and YJM789-specific SNPs, respectively. RC values of about 0, about 0.5, and about 1 indicate zero, one, or two copies of homolog-specific SNPs, respectively. (*A*) An I-LOH (gene conversion) event occurred near 270 kb on chromosome XII; the LOH region includes about 10 kb. Such an event could result from the repair of a double-strand break on the W303-1A–derived homolog. (*B*) A T-LOH event that might be caused by RCO or BIR near coordinate 750 kb on chromosome XIII.

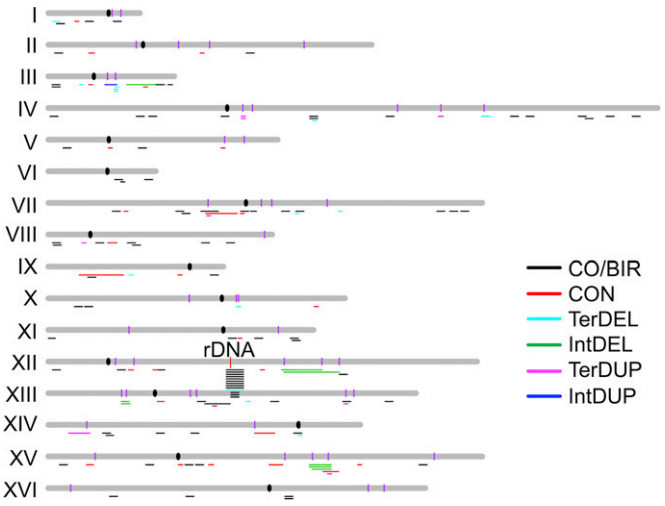

Fig. 3. The distribution of genomic alterations within the yeast genome. Centromeres are shown as black ovals, and Ty elements are shown violet vertical lines. CO/BIR, CON, TerDEL, TerDUP, IntDEL, and IntDUP are synonymous with T-LOH, gene conversion, terminal deletion, terminal duplication, interstitial deletion, and interstitial duplication. Note that the rDNA gene cluster occupies about 0.5 to 1 Mb in most laboratory strains.

terminal deletions can also reflect chromosome breakage followed by telomere addition to the broken end, coupled terminal deletions and duplications found in a single isolate are likely to reflect ectopic recombination (*SI Appendix*, Fig. S2).

Previously, we found that 30% of the interstitial deletions caused by low levels of Pol δ occurred within the *HXT7/6/3* cluster of chromosome IV (18). No deletions were observed within this cluster in the 37 DZP2 isolates, although with the small number of deletions observed in DZP2, this difference was not statistically significant ($P = 0.14$ by Fisher's exact test).

In summary, reduced expression of Pol2 greatly stimulates mitotic recombination in yeast. The distribution of all 147 mitotic recombination events (allelic and ectopic) on the 16 chromosomes is shown in Fig. 3. We observed that 12 of the 89 T-LOH events (0.13) were associated with the rDNA cluster on chromosome XII. In our recent study of the wild-type strain (21), we found 19 of the 356 T-LOH events (0.05) were located at the rDNA region. The higher ratio of T-LOH events in this region in the DZP2-derived isolates ($P = 0.02$, Fisher's exact test) suggests that the rDNA region is a preferred target for recombinogenic DNA lesions under conditions of low Pol ε, although this effect is not large.

Chromosomal aneuploidy. Among the 37 DZP2-derived isolates, we observed 94 aneuploidy events, including 56 monosomic chromosomes, 23 trisomic chromosomes, and 15 uniparental disomy (UPD) events (*SI Appendix*, Fig. S3 and Dataset S5). In strains with UPD, one homolog is lost and the other is duplicated. Each individual isolate of DZP2 had at least one change in chromosome numbers (*SI Appendix*, Fig. S3). The rate of aneuploidy was 0.05 events per cell division, which is about 800-fold higher than that observed in the isogenic wild-type diploid (21, 26).

In the wild-type isolates that were subcultured on rich medium for many generations, there were 5- to 10-fold more trisomic chromosomes than monosomic chromosomes (21, 26). In contrast, in DZP2-derived isolates, there were twofold more monosomes than trisomes (Dataset S5); possible reasons for this difference will be discussed below. Chromosomes III and XIV were the two chromosomes that were most readily lost, whereas chromosome IV tended to be duplicated (trisomy). In the wild-type diploid, chromosome III was lost in one of the three monosomes, and chromosome IV was duplicated in 3 of the 12 trisomes (21).

Copy-number variation within the rDNA and CUP1 clusters. In yeast, the rDNA cluster consists of ~75 to 150 tandem copies of a 9.1-kb sequence, accounting for about 10% of whole genome (27). By determining the sequencing coverage of SNPs specific for the W303-1A– and YJM789-derived rDNA genes and comparing that coverage with that of single-copy sequences, we calculated the numbers of each type of rDNA repeats in DZP2 and its sequenced subcultured isolates (*SI Appendix*, Fig. S4*A* and Dataset S6). In mutation-accumulation experiments done in wild-type diploids (21), the total number of repeats (average of 191 ± 6, 95% confidence limits [CL]) was not significantly different from the number in the starting strain (195). In the DZP2 strain, the total number of repeats before subculturing was 137, with many more repeats in the rDNA array derived from W303-1A (120 repeats) than in the array derived from YJM789 (17 repeats). The 18 sequenced isolates had an average of 78 (±14; 95% CL) repeats, roughly half the number of the starting strain. Strikingly, all sequenced isolates had reduced numbers of rDNA copies compared with DZP2 (*SI Appendix*, Fig. S4*A*). Similar reductions were also observed in strains with low levels of Pol α or Pol δ, and in strains with hypomorphic alleles of *POL1*, *POL2*, and *POL3* (18, 28, 29), as well as in other strains with mutations that likely generate replication stress (29, 30). It has been suggested that, under replication stress, competition for replication factors between rDNA replication origins and non-rDNA origins may select for strains that have reduced numbers of rDNA genes (31, 32). The number of repeats per rDNA array in a diploid can be altered by a number of different types of homologous recombination events, including unequal crossing over between sister chromatids (33, 34), crossing-over or gene conversion within a single chromatid (35), homologous recombination between homologs (18), and single-strand annealing (36).

We did not attempt to calculate a rate of alterations in the rDNA in the mutation-accumulation experiment under low Pol ε conditions, since the very frequent alterations in the number of repeats (Dataset S6) suggests that most isolates had more than one change during the subculturing. Even in the wild-type diploids, almost all subcultured isolates had altered numbers of repeats (21). For this reason, we did not include the rDNA in our estimates of the rates of internal deletions/duplications in Table 1.

DZP2-derived isolates also had variable numbers of the *CUP1* tandem repeats (total varying from 8 to 32 copies with an average of 17 ± 2 repeats). The starting strain had 19 repeats, 12 on the W303-1A–derived homolog and 7 on the YJM789-derived homolog. Although the total number of *CUP1* repeats is not substantially reduced in strains with low Pol ε, as we observed in strains with low levels of Pol α or Pol δ (17, 18), deletions (14 events) outnumber additions (4 events) significantly ($P = 0.03$ by χ^2 analysis) (*SI Appendix*, Fig. S4*B* and Dataset S6). Since we also found a slight, but significant decrease, in the number of *CUP1* repeats in the subcultured wild-type diploid (control strain, 18 repeats; subcultured isolates, 16 ± 0.6 repeats) (21), the reduction in the size of the *CUP1* array cannot be unambiguously attributed to low levels of the replicative DNA polymerases.

Frequency and patterns of point mutations. Among the 18 sequenced isolates, we detected 55 single-nucleotide variants (SNVs) and 4 in/dels (Dataset S7). We confirmed the existence of six randomly selected mutations by DNA sequence analysis

Table 1. Summary of rates of various genomic alteration in a wild-type diploid strain and in diploid strains with low levels of replicative DNA polymerases

Type of alterations	WT	Low Pol α	Low Pol δ	Low Pol ε
Point mutations	4.8 (4.5–5.1) E-3	7.6 (5.4–10) E-2*[16]	1.9 (1.5–2.4) E-1 [40]	6.1 (4.6–8.0) E-2 [13]
In/dels (<1 kb)	2.7 (2.1–3.4) E-4	1.1 (0.4–2.5) E-2*[42]	2.9 (1.5–5.2) E-2 [108]	4.4 (1.2–11) E-3 [16]
I-LOH	3.3 (3.0–3.5) E-3	1.4 (1.1–1.7) E-1 [42]	2.4 (1.5–3.7) E-2 [7]	1.5 (1.0–2.1) E-2 [4]
T-LOH	1.3 (1.2–1.5) E-3	1.8 (1.5–2.2) E-1 [136]	7.9 (6.1–10) E-2 [58]	4.8 (3.9–5.9) E-2 [36]
Total LOH	4.6 (4.3–4.9) E-3	3.2 (2.8–3.7) E-1 [70]	1.0 (0.8–1.3) E-1 [22]	6.3 (5.2–7.5) E-2 [14]
I-deletion/duplication[†]*	1.5 (1.1–2.0) E-4	3.7 (2.3–5.5) E-2 [249]	2.3 (1.4–3.5) E-2 [155]	4.9 (2.2–9.2) E-3 [33]
T-deletion/duplication	3.0 (1.3–6.0) E-5	5.1 (3.5–7.2) E-2 [1690]	1.8 (1.0–3.0) E-2 [603]	1.2 (0.8–1.8) E-2 [392]
Total deletion/duplication	1.8 (1.3–2.4) E-4	8.8 (6.6–11) E-2 [494]	4.1 (2.9–5.7) E-2 [231]	1.7 (1.1–2.4) E-2 [94]
Monosomy	1.1 (0.2–3.3) E-5	8.8 (6.6–11) E-2 [7744]	1.1 (0.9–1.4) E-1 [9755]	3.0 (2.3–3.9) E-2 [2664]
Trisomy	4.5 (2.3–7.9) E-5	1.9 (1.0–3.4) E-2 [422]	9.1 (3.9–18) E-3 [201]	1.2 (0.8–1.9) E-2 [274]
UPD	3.8 (0.1–21) E-6	1.9 (1.0–3.4) E-2 [5069]	1.3 (0.6–2.2) E-2 [3319]	8.1 (4.5–13) E-3 [2141]
Total aneuploidy	6.1 (3.5–9.8) E-5	1.3 (1.0–1.6) E-1 [2086]	1.3 (1.1–1.6) E-1 [2187]	5.1 (4.1–6.2) E-2 [838]
A3B-induced mutations[‡]	3.4 (3.3–3.5) E-0	9.6 (9.4–9.7) E+1 [28]	9.7 (9.5–9.8) E+1 [28]	3.0 (3.0–3.1) E+1 [9]
Rate of RCO on chrIV[§]	3.5 (2.5–4.7) E-5	1.3 (1.0–1.5) E-2 [364]	8.2 (6.2–11) E-3 [236]	3.0 (2.4–3.8) E-3 [87]
Rate of BIR on chrIV[§‡]	6.2 (3.2–11) E-6	1.1 (0.9–1.3) E-2 [1711]	5.1 (3.5–7.1) E-3 [818]	9.1 (8.0–10) E-3 [1474]

The rates (per genome per cell division) of various genomic alterations (except where indicated by superscripts) were measured by Sui et al. (WT) (21), Song et al. (low Pol α) (17), and Zheng et al. (low Pol δ) (18). The numbers in parentheses indicate the 95% CIs predicted from the Poisson distribution of the number of alterations. The numbers in brackets indicate the fold-increase relative to the wild-type rate.

*Rates of point mutations and in/dels (<1 kb) under low Pol α conditions for strain WS84 were measured by whole-genome sequencing.

[†]I-deletions/duplications >1 kb, excluding rDNA and the *CUP1* array.

[‡]APOBEC3B-induced mutations were analyzed by Sui et al. (46).

[§]Rates of cross-over and BIR events on chromosome IV under spontaneous (JSC25), low Pol α (MG47), low Pol δ (DZ12), and low Pol ε (DZP2) conditions were determined in this study. We used the approach of St. Charles et al. (20), as described in the main text.

of PCR fragments containing the relevant region. We calculated the frequencies of SNVs and in/dels as 2.7×10^{-9} and 1.9×10^{-10} per base per cell division, respectively. These rates are about 13-fold and 16-fold higher than that in the wild-type diploid strains for SNVs and in/dels (21). We also sequenced 21 isolates of a *GAL-POL1* diploid (WS84) grown on LG plates for one cycle (single cell to a colony) (Dataset S7). In this strain, the gene encoding the catalytic subunit of Pol α is fused to the *GAL1* promoter (17). The rates of SNVs and in/dels were elevated 16- and 41-fold, respectively, compared to the rates in wild-type cells.

The patterns of single-base mutations between the low-polymerase strains and wild-type strains are also significantly different (Fig. 4*A*) ($P < 0.001$ by Fisher's exact test). The proportion of G-to-C/C-to-G transversions is particularly high among the SNVs caused by low levels of all three replicative polymerases (Fig. 4*A*). Previously, Northam et al. (37) showed that one key feature of point mutations introduced by Pol ζ under replication stress is elevated G-to-C/C-to-G alterations.

To determine the effect of Pol ζ on point mutations in strains with low levels of Pol ε, we examined the mutation rate at the *CAN1* locus in four haploid strains: wild-type (MC42-2d), *rev3* (DZ27), *GAL-POL2* (DZ28), and *GAL1-POL2 rev3* (DZ29). Mutation rates in all four strains were measured on both HG and LG solid media. The rate of *can1* mutations in the wild-type strain was about 2×10^{-7} per division on both HG and LG plates (Fig. 4*B*). As expected, the rate of *can1* mutations in the *GAL1-POL2* strain grown on LG medium was greatly (33-fold) elevated with a much lower rate when grown on HG medium. The rate of mutations in the *GAL1-POL2 rev3* strain was reduced about fourfold with respect to the *GAL1-POL2* single mutant, indicating that about 80% of the mutations induced by low Pol ε are dependent on the error-prone DNA polymerase Pol ζ. Subtracting the contribution of Pol ζ to the mutation rate, we find that there is still a sixfold elevation in single-base substitutions. Although we do not have a simple explanation for this elevation, based on the observation that replication stress results in Pol δ recruitment to the leading strand of the replication fork (3, 4), it is possible that this unusual distribution of replicative DNA polymerases leads to more frequent misincorporation or less-efficient repair of misincorporated bases.

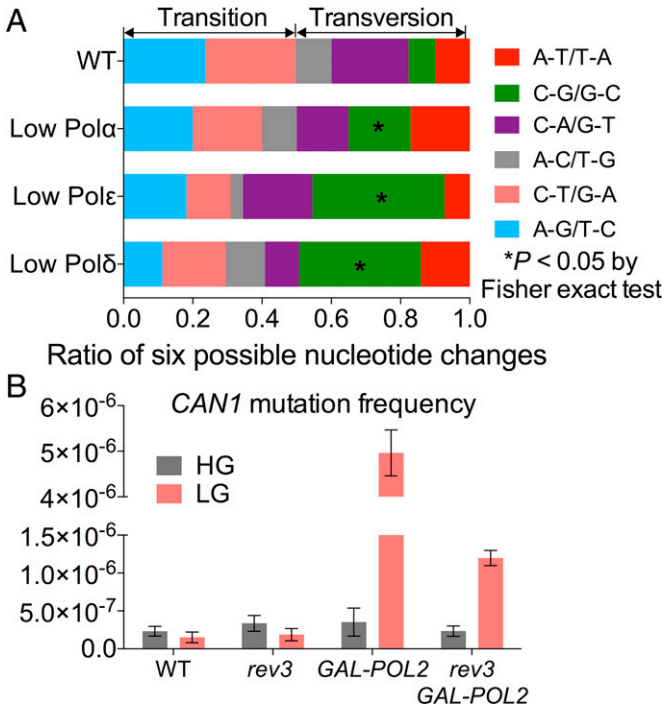

Fig. 4. Low levels of *POL2* stimulate single-base mutations. (*A*) Comparisons of patterns of single-base substitutions between wild-type cells and strains with low Pol α, ε, or δ. These data are based on whole-genome sequencing. Black asterisks indicate a significant difference ($P < 0.05$) from the wild-type based on Fisher's exact test. (*B*) *CAN1* mutation rates in the wild-type haploid strain MC42-2d and in mutant haploid strains DZ27 (*rev3*), DZ28 (*GAL1-POL2*), and DZ29 (*GAL1-POL2 rev3*) grown in HG and LG media.

Analysis of Selected RCO and BIR Events on Chromosome IV. As mentioned above, a terminal LOH event could reflect either an RCO or BIR (Fig. 2*B*). To determine the relative contributions of these two pathways to the LOH events in DZP2, we used a colony-sectoring assay that can distinguish between these two types of events (20). DZP2 is homozygous for the *ade2-1* ochre mutation, and has one copy of *SUP4-o* (an ochre suppressor) inserted at the end of the right arm of the YJM789-derived copy of chromosome IV. Diploids homozygous for an *ade2-1* mutation, in the absence of *SUP4-o*, form red colonies, and strains with one or two copies of *SUP4-o* form pink or white colonies, respectively (20). A BIR event that occurs at the time that the diploid DZP2 cells are plated produces a white/pink- or red/pink-sectored colony (*SI Appendix*, Fig. S5 *A* and *B*), whereas RCO events give rise to white/red-sectored colonies (*SI Appendix*, Fig. S5 *C* and *D*).

To determine the effect of low Pol ε on the rates of RCO and BIR, we incubated DZP2 cells in the absence of galactose (YPD medium, 2% glucose, 2% peptone, and 1% yeast extract) for 12 h, and then plated the cells on HG plates to allow colony formation. We found that the rate of red/white-sectored colonies was 3.1×10^{-3} per division (2.2 to 3.9×10^{-3}, 95% CL), about 100-fold higher than that in the isogenic wild-type strain (20). In addition, we found the rate of white/pink- and red/pink-sectored colonies was 9.1×10^{-3} (8 to 10×10^{-3}, 95% CLs), indicating that BIR was the preferred pathway for T-LOH in conditions of low Pol ε. In contrast, in the wild-type diploid examined using a similar assay, RCO is about four times more frequent than BIR (25). An elevated ratio of BIR to RCO (relative to wild-type) was also observed in the strains with low levels of Pol α and Pol δ (Table 1).

Previously, we showed that most mitotic recombination events between homologs under conditions of low Pol α or Pol δ were induced by DSBs generated in S/G_2 (17, 18), while most spontaneous recombination events were induced by DSBs in G_1 (20, 38). The phase of the cell cycle in which DSBs occurred can be distinguished by analysis of the patterns of gene-conversion tracts associated with RCOs (20). As shown in *SI Appendix*, Fig. S5*C*, a DSB occurred in the S/G_2 phase will usually lead to a 3:1 conversion tract (three chromatids were derived from one homolog and one chromatid was derived from the other). In contrast, a 4:0 or 4:0/3:1 hybrid conversion tract indicates a cross-over was initiated by a G_1-phase DSB in which the broken chromosome was replicated to generate two sister chromatids with breaks at the same position (*SI Appendix*, Fig. S5*D*).

To determine the nature of the conversion event associated with the cross-over, we isolated DNA from both sides of red/white sectors and used SNP-specific chromosome IV-specific microarrays to map LOH events in both sides of the sectored colony (20). In these microarrays, we monitored about 2,300 SNPs located between *CEN4* and the *SUP4-o* insertion. Among 57 cross-overs examined, 11 had no detectable gene conversion tract (class A1 in Datasets S8 and S9). Thirty-eight events showed simple 3:1 gene conversions associated with cross-overs (classes B1 to B6 in Datasets S8 and S9), indicating that most (83%) cross-over events associated with conversions induced by low Pol ε were a consequence of recombinogenic lesions in the S/G_2 phase. The remainder of the events (17%) had 4:1/3:1 hybrid tracts or more complicated conversion events (classes C1 to C7 in Datasets S8 and S9) that likely reflect the repair of G_1-initiated DSBs.

We found that most T-LOH events on IV were biased toward the right end of this chromosome for both cross-overs and BIR events (Fig. 5 *A* and *B*). Twenty-nine of the 57 cross-overs occurred in the region between 1,245,000 and the insertion site of *SUP4-o* (1510386). Although this region represents only one-quarter of the distance between *CEN4* and *SUP4-o*, 51% of the cross-over breakpoints are in this region, a significant overrepresentation ($P < 0.001$ by χ^2 analysis). This bias of cross-overs in the terminal quarter of the *CEN4–SUP4-o* interval in the low ε strain is not seen in the cross-over distributions in the wild-type (20), low Pol α (17), or low Pol δ strains (18) (Fig. 5 *C–E*); these differences are statistically significant by the Fisher's exact test with $P < 0.01$ for comparisons of the low ε with wild-type, low Pol α, and Pol δ, respectively. Thus, it is likely that low levels of Pol ε often result in recombinogenic DNA lesions that are displaced toward the telomere. This displacement could reflect either degradation of the chromosome initiated by telomere defects or elevated levels of DSBs in the late-replicating telomere regions.

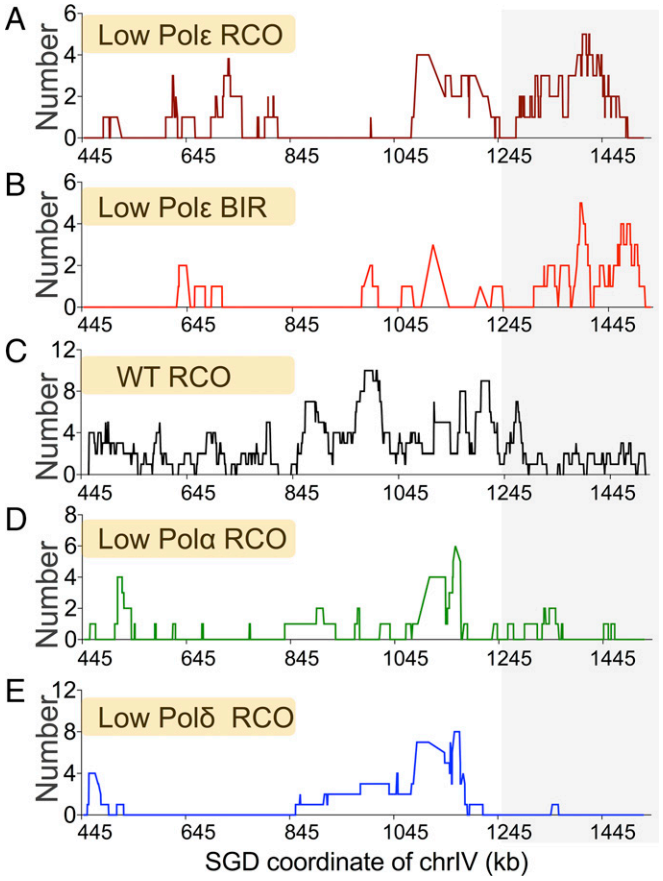

Fig. 5. Low levels of Pol ε lead to a distribution of T-LOH events that is skewed toward the telomere. Sectoring and microarray analyses (described in the text) were used to map RCO (*A*) and BIR (*B*) events on the right arm of chromosome IV in a diploid with low Pol ε. From previous studies, we summarize the location of RCOs in a wild-type diploid (*C*) (20), low Pol α (*D*) (17) or low Pol δ (*E*) (18). The *x* axis shows SGD coordinates on the right arm of chromosome IV; the centromere is located at coordinate 449 kb. Gray shading indicates the terminal 25% of the *CEN4* to *SUP4-o* interval.

Reduced Telomere Length in Strains with Low Pol ε. The 3′ end of yeast chromosomes terminate in a tract of $TG_{1–3}$ repeats that vary in length from about 350 to 600 bp in various wild-type strains (39, 40). In yeast strains with mutations affecting the telomerase pathway of telomere elongation, telomeres can be elongated by homologous recombination (40). Since this process requires some of the same proteins required for BIR (41), it is assumed that this pathway of telomere elongation is a

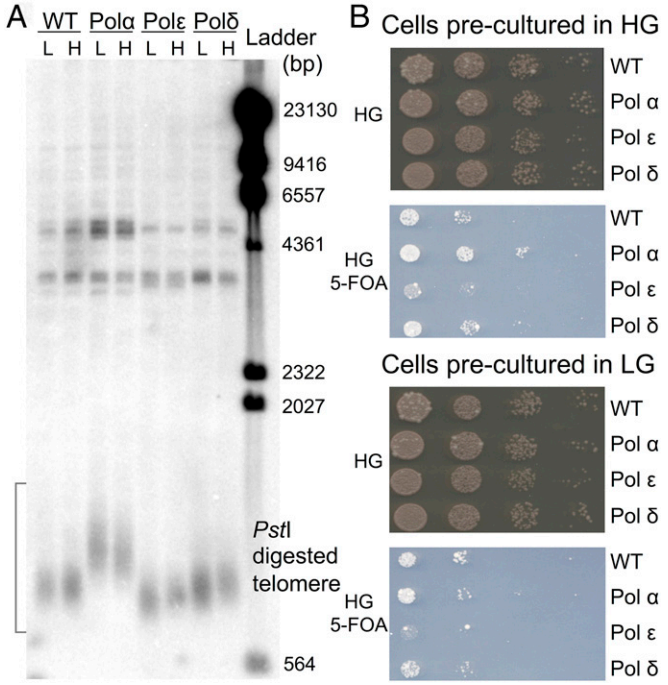

Fig. 6. Low levels of Pol ε reduce telomere length and telomere silencing. (*A*) Different effects of low levels of Pol α, ε and δ on telomere length. We examined telomere length in diploids of the following genotypes: wild-type (JSC25), *GAL-POL1* (MG47), *GAL-POL2* (DZP2), and *GAL-POL3* (DZ12). Each strain was grown in liquid medium (HG or LG) for two passages; DNA was then isolated, and telomere length examined by Southern analysis (details in *Materials and Methods*. (*B*) Low Pol ε reduces TPE. The wild-type haploid RCY308-3a has a *URA3* gene inserted near the telomere of chromosome XV (45). In this location, expression of *URA3* is often silenced, resulting in a high frequency of 5-FOA–resistant derivatives. We constructed isogenic derivatives of RCY308-3a with the *GAL1-POL1* (DZ30), *GAL1-POL2* (DZ31), and *GAL1-POL3* (DZ32). Each of these strains was grown in liquid medium containing either HG or LG, and then serial dilutions were plated onto solid media containing either HG or HG plus 5-FOA. The number of 5-FOA–resistant derivatives is reduced in strains with low Pol ε, but not in strains with low levels of Pol α or δ.

specialized type of BIR. Although most genomic rearrangements initiated by telomere degradation are likely restricted to the telomeric or subtelomeric regions, Hackett et al. (42) showed that telomere dysfunction could elevate LOH events for genes located more than 50 kb from the telomere.

To determine the effects of low levels of DNA polymerases on telomere integrity, we measured telomere lengths of the isogenic diploid strains that were wild-type (JSC25), *GAL-POL1* (MG47), *GAL-POL2* (DZP2), and *GAL-POL3* (DZ12) and were cultured either in HG or LG medium. In LG medium, low levels of Pol α elongated telomeres by about 200 bp relative to wild-type (Fig. 6*A*); previous studies found a similar elongation in *pol1-17* strains (8, 43). In contrast, telomere length in the *GAL-POL2* strain grown in LG was reduced by about 50 bp; a similar reduction was observed in strains with the *pol2-16* mutation (8). Low levels of Pol δ did not have a substantial effect on telomere length (Fig. 6*A*).

We also examined the effects of limited DNA polymerases on telomere-position effect (TPE), the transcriptional silencing of genes located near the telomeres. TPE can be monitored by inserting the *URA3* gene near the telomere; silencing is detected by the high-frequency of 5-FOA–resistant derivatives (44). We used the wild-type haploid strain RCY308-3a in which the *URA3* gene was inserted near the telomere of chromosome XV (45). We constructed isogenic derivatives of this strain containing *GAL1-POL1* (DZ30), *GAL2-POL2* (DZ31), and *GAL1-POL3* (DZ32). As shown in Fig. 6*B*, we found that the low Pol ε strain, when precultured in LG medium and then plated on HG plates containing 5-FOA, had fewer 5-FOA–resistant papillations than any of the other strains, suggesting that low Pol ε leads to reduced TPE.

Discussion

This study shows that low levels of Pol ε elevate numerous types of genomic alterations that range in size from single-base changes to large-scale chromosomal rearrangements and aneuploidy. Low levels of Pol ε: 1) greatly elevate the rates of genetic rearrangements that are likely induced by DSBs; 2) alter the distribution of cross-overs from that observed in wild-type strains or in strains with low levels of Pol α and Pol δ; 3) reduce telomere length and TPE; 4) elevate single-base mutations by a Pol ζ-dependent pathway; and 5) reduce the number of repeats in the rRNA gene cluster. We will also discuss the similarities and differences in the patterns of genomic instability resulting from the reduced expression of three replicative polymerases (α, δ, and ε).

Recombinogenic Lesions Induced by Low Levels of Replicative Polymerases. We summarize these data in two ways. Fig. 7*A* and Table 1 have the rates of each class of event with comparisons to the wild-type rates, whereas Fig. 7*B* shows the various types of chromosome alterations as ratios (number of specific classes of events/total number of events) for each strain. As observed for strains with low levels of Pol α and Pol δ (17, 18), low levels of Pol ε substantially increased the rates of a number of genomic alterations that likely reflect the repair of DSBs, including I- and T-LOH events, and large (>5 kb) deletions and duplications. In general, these effects were smaller for the low Pol ε strains than for the low Pol α and Pol δ strains (Table 1). For all strains with low levels of replicative DNA polymerases, most of the recombinogenic lesions are formed during or after DNA replication (17, 18) (Datasets S8 and S9). In contrast, in wild-type strains, about two-thirds of the lesions that initiate recombination between homologs are induced prior to DNA replication (20, 38). This difference may be relevant to the observation that the conversion tract lengths in the low Pol ε and Pol δ strains are similar (median lengths of 4.3 and 4.5 kb, respectively) but longer than observed in the wild-type strain (median length of 2.8 kb) (21). The difference between the conversion tract lengths in the wild-type strain and the low Pol ε strain is significant ($P = 0.05$ by Mann–Whitney *U* test).

As monitored by the level of mutations induced by expression of the single-strand–specific APOBEC protein, yeast strains with low levels of the replicative DNA polymerases have greatly elevated levels of single-stranded DNA (Table 1), about 30-fold elevated for low Pol α or Pol δ and 10-fold elevated for low Pol ε (46). These single-stranded regions may be more susceptible to cleavage, resulting in DSBs. Alternatively, single-stranded DNA may be more prone to formation of secondary DNA structures that can be cleaved by structure-specific endonucleases. In addition, as described below, in the low Pol ε strains, some of the T-LOH events could reflect repair of a terminally degraded chromosome by BIR.

Distribution of Cross-Overs and BIR Events in Strains with Low Levels of Pol ε. In T-LOH events selected on the right arm of chromosome IV, in the low Pol ε strain, BIR events were about threefold more common than RCOs, whereas in the wild-type strain, cross-overs are about four times more common than BIR events (24, 25). The BIR events are located primarily in the 250-kb interval that is closest to the telomere

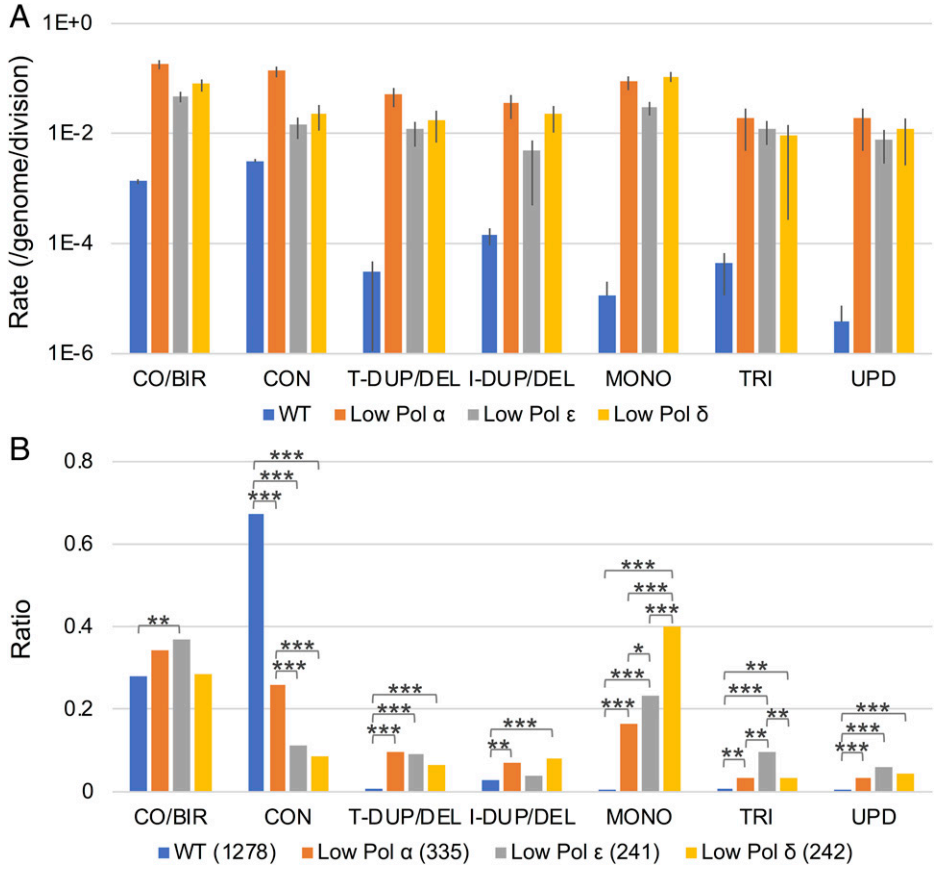

Fig. 7. Comparison of patterns of chromosomal alterations caused by lowered expression of *POL1*, *POL2* and *POL3*. (*A*) The rates of T-LOH (CO/BIR), I-LOH (gene conversions, CON), large (> 5 kb) terminal duplications and deletions (T-DUP/DEL), large (> 5 kb) interstitial duplications and deletions (I-DUP/DEL), monosomy (MONO), trisomy (TRI), and UPD observed in strains with low levels of Pol α, Pol ε, and Pol δ. Error bars represent 95% CIs predicted from the Poisson distribution shown in Table 1. (*B*) Proportions of various chromosomal alterations. The numbers in parentheses indicate the total number of events in different strains. The difference in ratio between any two conditions in each group is assessed by Fisher's exact tests followed by correction for multiple comparisons (69). *$P < 0.05$, **$P < 0.01$; ***$P < 0.001$.

(Fig. 5*B*). This observation suggests the possibility that these events are initiated by terminal degradation of the chromosome, followed by BIR. However, it should be noted that RCOs in the low Pol ε strain show a similar distribution to the BIR events (Fig. 5*A*). Since RCOs are not a product of BIR, this observation is puzzling. It is possible that the broken chromosome end that is normally lost during BIR is repaired to generate a double-stranded DNA molecule (*SI Appendix*, Fig. S6). Subsequent processing of this repair end could result in formation of a double Holliday junction that is repaired to generate a cross-over.

Alternatively, the elevated levels of DSBs in the telomeric regions might be produced by a mechanism unrelated to terminal degradation. One characteristic of telomeric and subtelomeric regions (within 50 kb of the telomere) in *S. cerevisiae* is that these regions are late-replicating (47). It is possible that such regions are particularly sensitive to a reduction in the level of Pol ε and, therefore, have elevated levels of recombinogenic DSBs.

Finally, we note that in all strains with low levels of replicative DNA polymerases, T-LOH events exceeded I-LOH events (Table 1). In wild-type strains, however, I-LOH events were about two-fold more common than T-LOH events (21). One interpretation of this result is that, in all of the strains under replication stress, one-ended DNA breaks are formed, resulting in BIR. This hypothesis is supported by the elevated ratio of BIR event to RCOs in the low-polymerase strains (Table 1). Alternatively, recombination events initiated in S-phase (as are most of the events observed in the low-polymerase strains) may result in broken ends that are processed differently than those initiated in G_1, leading to a difference in subsequent outcomes.

Low Levels of Pol *ε* Lead to High Levels of Aneuploidy. As described previously (Table 1), low levels of Pol ε lead to very high levels of monosomy (about 3,000-fold elevated compared to wild-type) and high levels of trisomy (about 300-fold elevated). High levels of monosomy could be a consequence of high levels of chromosome nondisjunction, failure to replicate a chromosome, or failure to repair a chromosome with a DSB. We attribute the high level of monosomy to a replication defect for a number of reasons. First, elevated chromosome nondisjunction would be expected to induce comparably high levels of monosomy and trisomy, and monosomy in the low Pol ε is about threefold more frequent that trisomy. This difference cannot be explained by a selective growth advantage of the monosomic strains relative to the trisomic strains, since monosomic strains have a growth disadvantage (48); in addition, in subcultured wild-type diploids, trisomy is about four times more common than monosomy (Table 1) (21).

It is more likely that the high rate of monosomy is a consequence of an elevated frequency of DSBs in the low Pol ε coupled with less-efficient DSB repair. Holmes and Haber (49) showed that Pol ε was required for the efficient repair of HO-induced DSBs. Loss of homologous recombination leads to very elevated rates of monosomy (50). Another possible factor in generating aneuploidy may be the failure to complete

DNA replication before mitosis, leading to the segregation of partly replicated chromosomes. By this mechanism, one would expect to produce both monosomic and trisomic strains. Since monosomes substantially outnumber trisomes, it is likely that failure to repair DSBs is the more important factor.

Elevated levels of aneuploidy are observed in strains with reduced levels of any of the replicative DNA polymerases (Table 1). In Dataset S10, we summarize the 289 aneuploidy events detected in strains with low levels of Pol α (17), Pol δ (18), and Pol ε. By χ^2 analysis, we found chromosomes III, V, XIII, and XIV are prone to be aneuploidy (accounting for about half of all aneuploidy events), while chromosomes II, X, XI, and XVI are more stable.

An interesting observation in Dataset S10 is that in most (25 of 31) of the strains that were monosomic for chromosome III, the missing chromosome was from the W303-1A–derived homolog (significant difference at $P = 0.001$). This skewed distribution likely reflects the existence of the recombinogenic inverted pair of Ty elements that is located on the W303-1A of III, but not the YJM789-derived copy (15).

DNA Replication Stress Leads to Reduced Copy Numbers of rDNA Genes. In our recent study (21), we found that the average number of rDNA repeats among 93 subcultured isolates of a wild-type strain (191) was very similar to the number of repeats in the strain before subculturing (195). In contrast, in our previous studies (17, 18) and this study (*SI Appendix*, Fig. S4), we found that lower levels of all three replicating polymerases led to decreased numbers of rDNA repeats. As stated previously, under conditions of replication stress, this reduction may alleviate the competition of rDNA and non-rDNA replication origins for replication factors (31, 32). Similar reductions have been observed in cancer cells (29, 51–53). It is possible that rDNA repeats act as a ubiquitous "buffer" to tackle various stressors encountered by eukaryotic cells.

Summary of Similarities and Differences in Genomic Alterations Induced by Low Levels of Pol *α*, *δ*, and *ε* in yeast. In Table 1, we summarize the elevated levels of genomic alterations (relative to wild-type) in strains with low levels of Pol α, δ, and ε. Low levels of any of the replicative DNA polymerases greatly stimulate both T-LOH and I-LOH. These LOH events likely have several sources. Since low levels of all three polymerases lead to elevated rates of single-stranded DNA at the replication fork (46, 54), the replication forks in all three strains are likely to be susceptible to DSBs (19). In the low Pol ε strain, the relatively high frequency of BIR events and the telomere-biased distribution of these events suggest that terminal degradation of chromosome ends may also contribute to T-LOH events. In addition, the bias in favor of BIR over reciprocal events could be explained if Pol ε has an important role in the process by which one-ended breaks are converted to two-ended breaks.

Low levels of all of the replicative DNA polymerases lead to elevated rates of single-base mutations (Table 1). Most of the mutations in the low Pol ε strain were dependent on the error-prone polymerase Pol ζ. In a previous study, Northam et al. (37) observed that strains with *pol2* or *pol3* mutations had substantial mutator phenotypes that were dependent on Pol ζ. They suggested that certain polymerase mutations resulted in a defective replisome, and subsequent recruitment of Pol ζ. Our results suggest that low levels of replicative polymerases may also produce a defective replisome. It is also possible that low levels of DNA polymerases may have a less-direct effect on mutation rates and spectra. Mertz et al. (55) showed that the *pol3-R696W* mutation resulted in the activation of the S-phase checkpoint, and an elevation of cellular dNTP pools. This elevation produced a substantial increase in the mutation rate that could be alleviated by reducing the concentration of dNTPs. Low levels of the replicative DNA polymerases may exert a similar effect.

Low levels of Pol α, δ, or ε also elevate the rate of small (<1 kb) in/dels relative to the wild-type strain (Table 1). The types of in/dels, however, in the three types of strains are not identical. In the low Pol α and ε strains, almost all (9 of 10) of the in/dels are one or two base insertions or deletions. In contrast, in the low Pol δ strain, most (8 of 11) of the short in/dels were between 59 and 656 bp and were flanked by short (3 to 9 bp) direct repeats (18). This significant ($P < 0.01$, by Fisher's exact test) difference suggests an elevated rate of DNA polymerase slippage in the low Pol δ, but not the low Pol α or ε strains.

One of the clearest distinctions of the effects of the different low-polymerase strains is the distributions of the breakpoints of LOH events. Certain chromosomal motifs, such as *HXT6/7/3* cluster, high GC-content regions, and replication termination sites were significantly enriched near the breakpoints of LOH events in the low Pol α and Pol δ strains, but not in the low Pol ε strain. This difference suggests that these hard-to-replicate motifs in the low Pol α and Pol δ strains can be replicated with fork breakage under low Pol ε conditions, possibly because Pol δ can aid in the replication of the leading strand in conditions of perturbed leading strand synthesis (4). In contrast, Pol α and Pol δ may be unable to complement the role of Pol ε in maintaining telomere stability.

Implication of Yeast Models of DNA Replication Stress for Cancer. Mutations in human genes encoding the catalytic subunits of Pol ε (POLDE) and Pol δ (POLD1), often resulting in a mutator phenotype, have been detected in certain classes of tumors (13). These mutations are nonrandomly associated with the proofreading exonuclease domain for POLDE (56), but are distributed throughout the coding sequence of POLD1 (13). A straightforward interpretation of the connection between proofreading defects and mutator phenotype is complicated by the observation that the mutator phenotype of one of the exonuclease-defective alleles of Pol ε has a stronger mutator phenotype than can be explained by lack of the proofreading activity (57). Based on our results, another possibility is that some of the mutations of POLDE and POLD1 result in an unstable protein, mimicking the instability observed in our yeast studies.

Finally, we should mention that possibility that an epigenetic reduction (perhaps transient) in the levels of one of the replicative DNA polymerases may contribute to the genomic instability observed in some classes of tumors. Hypermethylation of the *hMLH1* promoter is common in colorectal cancers (58). An important extension of our study would be to monitor the level of the replicative DNA polymerases in cell lines derived from tumors that have patterns of genomic instability similar to those observed in yeast. In particular, since we found that low levels of Pol ε resulted in shorter telomeres and an elevated rate of T-LOH events located near the telomere, it would be of interest to determine whether tumor cell lines that have elevated levels of end-to-end chromosome associations have mutations in POLDE and a reduction in Pol ε.

Although some genome-destabilizing phenotypes are likely to be conserved in yeast and humans (for example, the reduction in the number of ribosomal RNA genes in conditions of replication stress), it is unlikely that identical phenotypes will

be exhibited by tumor cells and yeast even if the basic genome-destabilizing DNA lesions are similar. For example, in mammalian cells, the nonhomologous end-joining repair of DSBs is utilized more efficiently than in *S. cerevisiae* (59); consequently, deletions are likely to be elevated relative to mitotic cross-overs or gene conversion events. Despite this reservation, a detailed study of multiple types of genomic alterations in tumor cells, coupled with analysis of the amount and function of DNA replication proteins, should illuminate the connection between defects in DNA replication and tumorigenesis.

Materials and Methods

Strains and Medium. All haploid strains used in this study were isogenic to two previously described strains, W303-1A (60) and YJM789 (61), except for changes introduced by transformation. The details of strain construction are described in *SI Appendix*. YPD medium was used for cultures of yeast strains with the wild-type *POL2* promoter. HG and LG medium contained 0.05% and 0.005% galactose, respectively, in addition to 1% yeast extract, 2% peptone, and 3% raffinose (YPR). The medium used for selection of canavanine-resistant colonies contained 0.05% galactose, 3% raffinose, 0.17% YNB, 0.5% ammonium sulfate, 0.14% complete amino acid mixture, 0.12% canavanine, and 2% agar.

qRT-PCR. Total RNA was extracted from yeast cells that were cultured for 16 h in HG, LG, or YPD medium with an initial cell density of OD_{600} of 0.1. The RNeasy Mini Kit (Qiagen) was used for the isolation. RNA samples were reverse-transcribed into cDNA using the SuperScript First-Strand Synthesis System for RT-PCR with gDNA eraser (Invitrogen). The PerfeCTa SYBR Green SuperMix ROX (Quanta Biosciences) was used to prepare the PCR solution (20 μL), and samples were tested in triplicate in a 96-well plate using an ABI StepOnePlus instrument (Applied Biosystems). The relative expression of *POL2* was quantified using the comparative $2^{-\Delta\Delta CT}$ method with *ACT1* as the reference gene (62).

Western Blot. Cells of the strains JSC25 (wild-type) and DZP2 (*GAL1-POL2*) were grown in YPD, YPR with 0.05% or 0.005% galactose, and YPG (1% yeast extract, 2% peptone, and 2% galactose) liquid media for 16 h. About 10^6 cells were collected for protein extractions using the TCA method modified from Foiani et al. (63).

SNP Microarray Analysis. Custom-designed whole-genome SNP microarrays (Amadid # 027438) and chromosome IV-specific SNP microarrays (Amadid #047217) were ordered from Agilent. The protocols of DNA sample preparation, hybridization of these samples to the microarrays, and data analyses are described in our previous studies (20, 64).

Whole-Genome Sequencing. Whole-genome sequencing of *S. cerevisiae* strains was performed on an Illumina HiSEq. 2500 sequencer using a paired-end indexing protocol. Mapping of reads and detection of genomic variations were described in Zheng et al. (18). Briefly, the Burroughs–Wheeler Alignment software was used to map reads onto the reference genome (65). VarScan 2.3.9 software was then used to detect de novo base substitutions and in/dels (66). Only those genomic alterations that were supported by more than 25 reads were counted.

***CAN1* Mutation Rate.** Yeast cells were spread onto HG plates and incubated for 2 d at 30 °C. For each strain, patches (1 cm × 2 cm) were plated from 15 colonies onto HG or LG plates. These patches were incubated at 30 °C for 3 d. Cells from each patch were collected in 250 μL of sterile water, and 200 μL were plated on canavanine-containing plates. The cells were also diluted and plated on HG plates to count the number of cells per isolate. We counted the number of colonies on the canavanine-containing plates after 5 d of growth. The frequencies of canavanine-resistant mutations were converted into rates using the method of the median (67).

Southern Analysis of Telomere Length. Strains were grown from single cells to colonies on solid HG medium. The resulting colonies were resuspended and inoculated into liquid HG or LG media, and grown into stationary phase (first passage). For the second passage, the stationary-phase cultures from the first passage were diluted 1:1,000 and grown to stationary phase again. Yeast DNA was isolated, treated with PstI, and analyzed by gel electrophoresis. For Southern analysis, the DNA fragments were transferred to nitrocellulose filters, and hybridized to a telomere-specific probe as described in Ritchie et al. (68); PstI has a restriction site in the conserved Y′ subtelomeric repeat.

Statistical Analysis. The χ^2 tests were performed using VassarStat (http://vassarstats.net/). Corrections of *P* values for multiple comparisons were performed as described by Hochberg and Benjamini (69). Fisher exact tests with two-tailed *P* values were analyzed using the software GraphPad Prism 6.

Data Availability. The Illumina sequencing data are available in the Sequence Read Archive database, https://www.ncbi.nlm.nih.gov/sra (accession no. PRJNA314677) (70). The raw data of all microarray results are available in Gene Expression Omnibus database, https://www.ncbi.nlm.nih.gov/geo/ (accession no. GSE185366) (71).

ACKNOWLEDGMENTS. We thank P. Shcherbakova, S. Jinks-Robertson, J. Sheltzer, and all members of the T.D.P. and D.-Q.Z. laboratories for useful discussions; and G. Bylund and E. Johansson for the anti-Pol ε antibody. This study was supported by National Key Research and Development Program of China Grant 2021YFA0909500 (to D.-Q.Z.); National Natural Science Foundation of China Grants 32022004 and 32170078 (to D.-Q.Z.); Fundamental Research Funds for the Central Universities (2021XZZX018) (to D.-Q.Z.); NIH Grant R35GM118020 (to T.D.P.); and US Army Grant W911NF1920082 (to T.D.P.).

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