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DNA Rereplication Is Susceptible to Nucleotide-Level Mutagenesis

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ABSTRACT The sources of genome instability, a hallmark of cancer, remain incompletely understood. One potential source is DNA rereplication, which arises when the mechanisms that prevent the reinitiation of replication origins within a single cell cycle are compromised. Using the budding yeast Saccharomyces cerevisiae, we previously showed that DNA rereplication is extremely potent at inducing gross chromosomal alterations and that this arises in part because of the susceptibility of rereplication forks to break. Here, we examine the ability of DNA rereplication to induce nucleotide-level mutations. During normal replication these mutations are restricted by three overlapping error-avoidance mechanisms: the nucleotide selectivity of replicative polymerases, their proofreading activity, and mismatch repair. Using lys2InsEA14, a frameshift reporter that is poorly proofread, we show that rereplication induces up to a 30× higher rate of frameshift mutations and that this mutagenesis is due to passage of the rereplication fork, not secondary to rereplication fork breakage. Rereplication can also induce comparable rates of frameshift and base-substitution mutations in a more general mutagenesis reporter CAN1, when the proofreading activity of DNA polymerase ε is inactivated. Finally, we show that the rereplication-induced mutagenesis of both lys2InsEA14 and CAN1 disappears in the absence of mismatch repair. These results suggest that mismatch repair is attenuated during rereplication, although at most sequences DNA polymerase proofreading provides enough error correction to mitigate the mutagenic consequences. Thus, rereplication can facilitate nucleotide-level mutagenesis in addition to inducing gross chromosomal alterations, broadening its potential role in genome instability.

KEYWORDS DNA replication fidelity; rereplication; mismatch repair; genome instability; DNA polymerase proofreading

To ensure that every chromosomal segment is replicated once and only once, eukaryotic cells initiate DNA replication at thousands of replication origins scattered throughout their genome and employ multiple overlapping mechanisms to prevent reinitiation at these origins (Nguyen et al. 2001; Truong and Wu 2011; Abbas et al. 2013; Siddiqui et al. 2013). These mechanisms are regulated during the cell cycle and inhibit proteins required for the initiation of DNA replication after they have executed key initiation functions. In G1 phase, the Origin Recognition Complex (ORC), Cdc6, Cdt1, and the Mcm2-7 core replicative helicase license origins for initiation in S phase by loading the ring-shaped Mcm2-7 helicase around origin DNA. Subsequently, during the exit from G1 phase, the licensing activities of these proteins are downregulated in a multitude of ways to prevent relicensing and reinitiation for the remainder of the cell cycle.

These inhibitory mechanisms cooperate in a multiplicative fashion to minimize the probability that any origin reinitiates within the genome. When only a single mechanism is disrupted, the increase in reinitiation frequency is apparently too low to result in enough rereplication to be detected by current assays. However, as more and more mechanisms are disrupted, reinitiation and rereplication become detectable, and progressively larger in amount (Nguyen et al. 2001; Green et al. 2010). Understanding the consequences of disrupting reinitiation controls may be of relevance to cancer, as the deregulation of replication initiation proteins has both been observed in cancers (Karakaidos et al. 2004; Tatsumi et al. 2006; Liontos et al. 2007) and shown to potentiate oncogenesis in animal models (Arentson et al. 2002; Seo et al. 2005; Liontos et al. 2007).
We have previously established in the budding yeast *Saccharomyces cerevisiae* that rereplication arising from disruptions in reinitiation control can be an extremely potent source of gross chromosomal alterations, specifically intrachromosomal gene amplification (Green et al. 2010) and whole-chromosome aneuploidy (Hanlon and Li 2015). These gross chromosomal alterations are stimulated in part because rereplication forks are highly susceptible to breakage (Finn and Li 2013; Alexander and Orr-Weaver 2016), which in turn triggers recombinational repair pathways that can generate rearrangements (Finn and Li 2013). Although the basis for the rereplication fork breakage that we see is not clear, the compromised integrity of these forks suggests they might differ in fundamental ways from S-phase replication forks. This prompted us to ask whether the fidelity of DNA replication might also be compromised during rereplication.

Three mechanisms ensure the fidelity of normal S-phase replication [reviewed in Kunkel (2009) and Ganai and Johansson (2016)]: (1) the nucleotide selectivity of the polymerase activity of the replicative DNA polymerases, polymerase ε (Pol ε) and polymerase δ (Pol δ); (2) the proofreading excision of misincorporated and/or mismatched primer nucleotides by the 3′ to 5′ exonuclease activity of Pol ε and Pol δ; and (3) a postreplicative mismatch repair (MMR) system that cooperates with DNA replication to detect, excise, and replace any remaining mismatched nucleotides on newly replicated daughter strands. These mechanisms work in series and cooperate in a multiplicative manner (Morrison et al. 1993; Tran et al. 1999; Greene and Jinks-Robertson 2001; Lujan et al. 2012; Buckland et al. 2014; St Charles et al. 2015; Schmidt et al. 2017) to reduce the rate of nucleotide-level errors (e.g., base substitutions and small insertions/deletions) to ~10^{-10} per replicated nucleotide. They also appear to operate with some redundancy, because the loss of fidelity due to partial disruption of an error-avoidance mechanism is often not fully manifested until another mechanism is disrupted (Tran et al. 1999; Deem et al. 2011; St Charles et al. 2015; Schmidt et al. 2017).

When replication forks are stalled or stressed by local impediments, such as DNA damage or short-hairpin structures, these mechanisms may be circumvented by the transient substitution of error-prone polymerases for replicative polymerases as the cell tries to bypass the problem (Northam et al. 2014; Makarova and Burgers 2015; Vaisman and Woodgate 2017). Not only do these polymerases have lower nucleotide selectivity and lack proofreading function, the errors introduced by DNA polymerase ζ (Pol ζ), the error-prone polymerase required for much of the mutagenesis in budding yeast, do not seem to be edited by MMR (Huang et al. 2002; Lehner and Jinks-Robertson 2009; Aksenova et al. 2010; Kohenanova et al. 2015).

In this study, we show that the fidelity of DNA rereplication is reduced > 30-fold relative to replication as assayed by reversion of *lys2-InsEA14*, a sensitized frameshift reporter that is poorly proofread (Tran et al. 1997). We provide evidence that the mutagenesis is caused by rereplication per se and not by break repair-induced mutagenesis arising from nearby rereplication fork breakage. At a more general mutagenesis reporter, *CAN1*, the compromised fidelity of rereplication is partially masked by the redundancy of fidelity mechanisms but can be uncovered by inactivating the exonuclease proofreading activity of Pol ε. This rereplication-induced *lys2-InsEA14* reversion and *CAN1* mutagenesis is not dependent on Pol ζ, suggesting that rereplication is not increasing error rates by promoting more frequent bypass of the canonical error-avoidance mechanisms. Instead, we find that the higher mutagenesis of rereplication vs. replication is lost in MMR null strains, suggesting that rereplication compromises nucleotide fidelity by attenuating MMR. Thus, in addition to inducing extremely high levels of gross chromosomal rearrangements, rereplication can facilitate nucleotide-level mutagenesis.

### Materials and Methods

### Nucleotide and chromosomal positions

Nucleotide positions in the chromosomes are reported based on the S288c reference sequence in the *Saccharomyces* Genome Database (R64.2.1, 2014-11-18) (Engel et al. 2014). Nucleotide positions of deleted gene segments are reported relative to the A of the start codon, with positive numbers 3′ of the A (+1) and negative numbers 5′. Chromosomal positions of mutagenesis reporter genes are reported to the nearest kilobase (kb) of their insertion site. *URA3* fragments were inserted at the boundaries of the amplified segment in place of clusters of repetitive sequences elements (Ty elements and adjacent tRNA genes, and long terminal repeats) (Finn and Li 2013); the insertion sites of these fragments are reported as the positions at the edges of the amplified segment.

### Oligonucleotides

Oligonucleotides used as PCR or sequencing primers in plasmid constructions, strain constructions, and mutational analyses are listed in Supplemental Material, Table S1.

### Plasmids

All plasmids used in this study are listed in Table S2. pDB101 contains a *LYS2-CAN1* cassette and was used as the PCR template for generating cassette fragments that were integrated at various positions in chromosome IV. The cassette was generated by two rounds of PCR. In the first round, the *LYS2* (−450 to +4524) and *CAN1* (−829 to +2515) genes were separately amplified by PCR from S288c genomic DNA with primer tails that generate 40 bp of overlap between the two fragments. In the second round, these fragments were then used as templates for fusion PCR to generate a SacI-*LYS2-CAN1*-XmaI fragment containing both genes in the same orientation with *LYS2* upstream of *CAN1*. This fragment was cloned into the SacI and XmaI sites of pRS306 (Sikorski and Hieter 1989) to form pDB101.

pDB107 is derived from a clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 targeting vector (pRS425-CAS9-2XSapI) developed in Bruce Futcher’s
laboratory (Zhao et al. 2016), pRS425-CAS9-2XSapl constitutively expresses Cas9 from the TEF1 promoter and a CRISPR single-guide RNA (sgRNA) from the SNR52 promoter. Two closely spaced SapI sites in a nonspecific 20-bp targeting sequence at the beginning of the sgRNA allow replacement of the nonspecific sequence with a desired 20-bp targeting sequence. pRS425-CAS9-2XSapl also contains a LEU2 marker, and a 2 micron replication origin and stability locus. In pDB107 two changes were made: (1) complementary oligonucleotides OJL4249 and OJL4250 were cloned between the two SapI restriction sites to target POL2 for CRISPR-Cas9 cleavage and mutation to pol2-4, and (2) the LEU2 marker was replaced by hphMX from pRS40H (Chee and Haase 2012). pDB114 was constructed from pDB107 by replacing the 2 micron elements with the CEN6-ARS209 cassette from pRS414 (Sikorski and Hieter 1989).

Strains

Yeast strains used in this study were descendants of YJL8363 and YJL9149 (Green et al. 2010; Finn and Li 2013), and are listed in Table S3. The YJL8363 genotype deregulates three replication initiation proteins that are normally inhibited by Cln cyclin-dependent kinase (CDK) to prevent the reinitiation of DNA replication: (1) MCM7-2NLS confers constitutive nuclear localization on the Mcm2-7 core replicative helicase (Nguyen et al. 2000); (2) ORC6-cdk1A(S116A) disrupts one of four consensus CDK phosphorylation sites on Orc6 (Nguyen et al. 2001); and (3) pGAL-ΔntCDC6-cdk2A provides galactose-inducible overexpression of stabilized ΔntCDC6-cdk2A on top of the normal cell cycle-regulated expression of Cdc6 from the wild-type CDC6 gene (Nguyen et al. 2001; Perkins et al. 2001). Exposing cells to galactose induces detectable reinitiation most prominently from one origin, ARS317 (Green et al. 2006; Richardson and Li 2014). In YJL8363, this origin has been removed from its endogenous location on chromosome III and inserted into chromosome IV (Figure 1A). YJL8363 also contains a split URA3 reporter for rereplication-induced gene amplification (RRIGA) of a 124-kb segment of chromosome IV spanning the integrated ARS317. The 3′ (RA3) and 5′ (UR) portions of this split reporter replace the Ty retrotransposons that originally formed the boundaries of this amplified segment in the predecessor strain for YJL8363 (Green et al. 2010; Finn and Li 2013). Intrachromosomal amplification via nonallelic homologous recombination between the 390 bp of overlapping sequence identity in RA3 and UR reconstitutes an intact URA3 reporter at the junction between adjacent tandem amplified segments (Finn and Li 2013). YJL9149, the nonrereplicating control strain, is congenic with YJL8363, but has pGAL in place of pGAL-ΔntCDC6-cdk2A.

YJL11037 and YJL11035 were derived from YJL8363 and YJL9149, respectively, by introducing precise deletions of both LYS2 (lys2Δ) and CAN1 (can1Δ) open reading frames (ORFs) into the latter two strains. The lys2Δ and can1Δ deletion fragments were both generated by two successive rounds of PCR: the first round synthesized a primer dimer with 40 bp homology to the sequences just outside both ends of the deleted ORF; the second round extended the homology to 70 bp using the primer dimer as template. lys2Δ isolates were obtained by transforming cells with the lys2Δ deletion fragment and selecting for survivors on α-aminoadipic acid plates (Chattoo et al. 1979). can1Δ isolates were obtained by transforming cells with the can1Δ deletion fragment and selecting for survivors on canavanine plates. Chromosomal deletions were confirmed by PCR of the expected size fragments spanning the deletions.

The LYS2-CAN1 cassette from pDB101 was integrated into the rereplicating strain YJL11037 at one of four locations on chromosome IV to form YJL11071 (ChrIV_570 kb), YJL11073 (ChrIV_637 kb), YJL11075 (ChrIV_795 kb), and YJL11077 (ChrIV_1081 kb). The cassette was also integrated into the nonrereplicating strain YJL11035 at the same positions to form YJL11049 (ChrIV_570 kb), YJL11066 (ChrIV_637 kb), YJL11067 (ChrIV_795 kb), and YJL11069 (ChrIV_1081 kb). Integrating fragments containing 60 bp of homology flanking the integration sites were generated by two successive rounds of PCR from pDB101. After selecting for Lys+ yeast transformants, integration at the correct sites was confirmed by PCR across the integration junctions on both sides of the cassette. The sequence of the integrated CAN1 was confirmed by PCR amplification and sequencing.

The lys2InsE_A14 and lys2InsE_A10 reporters were substituted for LYS2 in strains containing the LYS2-CAN1 cassettes. Fragments containing almost the entire lys2InsE_A14 and lys2InsE_A10 reporters were excised (with Ntul and HindIII), respectively, from plasmids p233 and p10A-2-Int1, which were obtained from the Gordenin/Resnick laboratories (Tran et al. 1997). Strains transformed by these fragments were plated on α-aminoadipic acid plates (Chattoo et al. 1979) to select for integration of the reporters, and successful replacement of the LYS2 sequence was confirmed by PCR with OJL4084 and OJL4218 to check for the presence of the InsE insert.

The pol2-4 mutation (Morrison et al. 1991) was introduced into lys2InsE_A14-CAN1 reporter strains by CRISPR-Cas9 editing. The edited strains were the rereplicating strains YJL11108 (ChrIV_570 kb reporters) and YJL11112 (ChrIV_637 kb reporters), and the nonrereplicating strains YJL11130 (ChrIV_570 kb reporters) and YJL11090 (ChrIV_637 kb reporters). These strains were cotransformed with a CRISPR-Cas9 plasmid targeting POL2 (either pDB107 or pDB114) and a pol2-4 donor template generated by two rounds of PCR. In the first round of PCR, genomic DNA from S288c was used as template to PCR two overlapping segments from POL2 with the pol2-4 mutation (D290A and E292A) and a silent mutation (V286V) in the overlap region. The silent mutation disrupts the sequence targeted by the CRISPR-Cas9 plasmid, so once Cas9-cleaved POL2 is repaired with the pol2-4 donor template, the resulting pol2-4 allele is protected from further cleavage. In the second round, the
two overlapping segments were used as templates for fusion PCR. Surviving yeast isolates from the CRISPR-Cas9 transformation were screened for the presence of the pol2-4 mutation by PCR amplification and sequencing, using primers OJL4255 and OJL4258.

YJL11234-YJL11236 were generated from YJL11108 (lys2InsE_{A14}-CAN1 reporter inserted in ChrIV_{570} kb) by introducing a deletion of the MSH2 ORF marked by TRP1. A msh2\Delta::TRP1 deletion fragment was generated by two successive rounds of PCR: in the first round, pRS414 (Sikorski and Hieter 1989) was used as a template to PCR a TRP1 fragment flanked by 30 bp of homology to sequences just outside the MSH2 ORF; in the second round, this fragment was used as a template to extend the flanking homology to 60 bp. After selecting for Trp\(^+\) transformants, we screened for the msh2\Delta::TRP1 deletion by PCR across the junctions on both sides of the deletion.

**Strain growth and media**

For standard growth, yeast were grown on rich yeast extract, peptone media (YPD) or synthetic complete media (SDC) containing 2% w/v dextrose (D16; Fisher Scientific, Pittsburgh, PA) as previously described (Green et al. 2010). Before the induction of rereplication with 2.7% w/v galactose (G0750; Sigma [Sigma Chemical], St. Louis, MO), cells were grown overnight in YPRd, rich yeast extract peptone media containing 3% w/v raffinose (R1030; US Biological) and 0.1% w/v dextrose, or in a few experiments for 3 hr in YPR, rich media containing 3% w/v raffinose. These media allow gradual release from dextrose-mediated repression of the pGAL promoter, so that the promoter can be rapidly induced upon the addition of galactose (Johnston 1987). To quantify Lys\(^+\) revertants, cells were plated on SDC-LYS. To quantify canavanine-resistant mutants, cells were plated on SDC-ARG plates containing 60 \(\mu\)g/ml canavanine (C9758; Sigma). To quantify cells that had undergone segmental amplification and reconstituted the URA3 amplification reporter at the amplification junction, cells were plated on SDC-URA. To determine the total number of colony forming units in experimental cultures, cells were plated on SDC.

**Measuring basal and rereplication-induced mutation rates**

Basal mutation rates were determined by measuring the rate of mutant accumulation during the expansion of yeast cultures from single cells (Drake 1991). Rereplication-induced mutation rates were determined by measuring the increase in mutants after 3 hr of rereplication in nocodazole-arrested cells.

Specifically, yeast strains were struck for single colonies from frozen yeast stocks onto YPD plates and grown at 30\(^\circ\)C. For most strains, an entire colony grown from a single cell for 30 hr was transferred into 50–100 ml of liquid YPRd, and grown for an additional 13–16 hr at 30\(^\circ\)C until the culture reached an OD\(_{600}\) between 0.2 and 0.5 (~0.4–1.0 \(\times\) 10\(^7\) cells/ml). At that point, 10 mg/ml nocodazole (N3000; US Biological) in DMSO (ICN19481950; Fisher Scientific) was added to the culture to a final concentration of 15 \(\mu\)g/ml. After 2.5 hr incubation, mitotic arrest was confirmed microscopically (> 90% large-budded cells), and 40% galactose was added to a final concentration of 2.7% w/v to induce rereplication. At T = 0 and T = 3 hr of galactose induction, cells were plated on SDC plus one or more of the following plates: SDC-LYS, SDC-URA, and canavanine. Cells were concentrated or diluted before plating such that most plates yielded 50–250 colonies. These were counted after 2–3 days of incubation at 30\(^\circ\)C.

From the T = 0-hr plating on SDC we obtained N, the total number of cells that grew out from a single cell. From the T = 0-hr plating on SDC-LYS, SDC-URA, and canavanine (and the value of N) we obtained f\(_0\), the fraction of mutant cells that had accumulated in the culture with Lys\(^+\), Ura\(^+\), and Can\(^R\) phenotypes, respectively. N and f\(_0\) were then used to solve u, the basal mutation rate, in Drake's formula \(\mu = f/\ln(N)\) (Drake 1991). Similarly, from the T = 3-hr plating we obtained f\(_3\), the fraction of mutant cells present after the induction of rereplication for each of the mutant phenotypes that were plated (Lys\(^+\), Ura\(^+\), or Can\(^R\)). For each phenotype, the difference f\(_3\) – f\(_0\) yielded the rereplication-induced mutation rate. For each genotype, experiments were performed with 3–25 biological replicas using at least two congenic sister isolates. For each pair of basal and induced rates, we reported the mean rate and SEM, and perform statistical comparison between the two rates using the Mann–Whitney U-test (Mann and Whitney 1947). For the results described in Table S11, we used the Mann–Whitney U-test to compare the ratios of (induced rate of Lys\(^+\) Ura\(^+\)) / (induced rate of Ura\(^+\)) for two different genotypes. The Mann–Whitney Test Calculator from Social Science Statistics was used to calculate the P-value for significance (https://www.socscistatistics.com/tests/mannwhitney/default2.aspx). Throughout the manuscript we have used a significance level of 0.01.

Several experiments involving the msh2\(\Delta\) strains YJL11234–YJL11236 used a modified outgrowth and induction protocol. Because of their high basal mutation rate, in some experiments these strains were grown for fewer generations during the mutation accumulation period. Colonies grown from single cells for 35–42 hr on YEPD plates were transferred to 50 ml of liquid YPR and incubated for only 3 hr till they reached an OD\(_{600}\) of only 0.01–0.10 before the addition of nocodazole. After arrested cells were plated for the T = 0-hr time point, the remaining culture was split into two. Galactose was added to one culture (final concentration 2.7% w/v) to induce rereplication and dextrose was added to the other (final concentration 2.7% w/v) as a negative rereplication control.

Finally, in some experiments, we wished to measure the rereplication-induced reversion rate for the subpopulation of lys2InsE_{A14} reporters that had experienced a rereplication-induced gene amplification. In principle, one could first select for the latter population by plating on SDC-URA, then replica...
DNA Rereplication Is Error Prone

Results

DNA rereplication induces an elevated frameshift mutation rate

To determine whether rereplication induces increased rates of nucleotide-level mutations, we used our previously published \textit{S. cerevisiae} system for conditionally inducing transient and localized rereplication (Green et al. 2010; Finn and Li 2013). In this system, we conditionally deregulate a subset of replication initiation proteins in metaphase-arrested cells by the addition of galactose to the media. Using aCGH to monitor the increase in copy number across the genome, we have previously shown that this deregulation induces a stereotypical rereplication profile with overt reinitiation occurring most prominently from one origin, \textit{ARS317}, at position 567 kb on chromosome IV (Figure 1A). By 3 hr of induction, \textasciitilde50% of the \textit{ARS317} has reinitiated with rereplication forks proceeding bidirectionally away, but progressively dropping out with increasing distance from the origin. After induction, cells are released from the arrest in the presence of dextrose, which represses further rereplications. This protocol allows us to assess the genomic consequences of a transient, limited, and localized induction of rereplication within one cell cycle.

One of these consequences, amplification of a large 124-kb chromosomal segment spanning \textit{ARS317}, can be detected with a split \textit{URA3} reporter (Finn and Li 2013). In this system, a 3’ \textit{RA3} segment and a 5’ \textit{UR} segment, sharing 390 bp of overlapping sequence identity, are positioned at the boundaries of the amplified segment at 521 and 645 kb (Figure 1A). Rereplication induced gene amplification (RRIGA) of this segment reconstitutes \textit{URA3} at the interamplicon junction, allowing for selection of these recombination events. Seeing a strong persistent induction of RRIGA in the experiments described below provides some assurance that the degree of rereplication, the extent of rereplication fork progression and breakage, and the recombinational repair of these breaks is not significantly affected in most of these experiments.

To measure the rate of mutagenesis during rereplication, we inserted a cassette containing two reporter genes at one of four positions in the path of rereplication forks originating from \textit{ARS317}. As expected, these insertions—at 3, 68, 228, and 514 kb to the right of \textit{ARS317} (Figure 1A)—did not alter the rereplication profiles of these strains (Figure S1). One of these reporters, \textit{lys2InsEA14}, has previously been used as a sensitized detector of frameshift mutagenesis (Tran et al. 1997). It contains a small insertion with an A14 homopolymeric tract that inactivates the \textit{LYS2} gene by introducing a frameshift (Figure 1B). DNA polymerases replicating through the A14 tract are susceptible to replication slippage, which can introduce compensatory frameshift mutations that generate selectable \textit{Lys}+ revertants. The other reporter, \textit{CAN1}, detects more generic frameshifts or base substitutions (Huang et al. 2002; Lang and Murray 2008), which inactivate the gene and confer resistance to the toxic arginine analog canavanine (Figure 1C).
Figure 2A shows how we measured the baseline and rereplication-induced mutation rates of these reporters. Cell cultures derived from single cells were arrested in metaphase with nocodazole, then galactose was added for 3 hr to transiently induce rereplication. Just before ($T = 0$ hr) and after ($T = 3$ hr) this induction, cells were plated to quantify the frequency of Lys$^+$ and CanR mutants. To determine the baseline mutation rate of these reporter genes during the expansion of the culture from a single cell, we applied Drake's formula for mutation accumulation (Drake 1991) to the mutant frequency at $T = 0$ hr. To determine the rereplication-induced mutation rate we took the difference in mutant frequency at $T = 3$ hr and $T = 0$ hr.

The baseline mutation rates at the four insertion sites on chromosome IV for the $\text{lys}2\text{InsEA}_{14}$ reporter were similar ($3 \pm 3 \times 10^{-7}$) (Figure 2B and Table S4) and within the range observed in previous reports (Tran et al. 1997; Deem et al. 2011; Bui et al. 2015). After transient induction of rereplication, the $\text{lys}2\text{InsEA}_{14}$ reporter at site 1 (within 3 kb from $\text{ARS317}$) experienced a 15-fold increase in reversion rate above its basal rate (Figure 2B and Table S4). At this position, 50% of chromosome IV is rereplicated (Figure 1A), making the normalized reversion rate for a fully rereplicated $\text{lys}2\text{InsEA}_{14}$ reporter 30-fold above basal rates. At site 2 (68-kb away from $\text{ARS317}$), where ~25% of chromosome IV is rereplicated (Figure 1A), the induced reversion rate was...
lower (12-fold). Further away, at sites 3 and 4, where there is little or no detectable rereplication, the \( \text{lys2InsEA14} \) reporter showed no significant increase (\( P \)-value > 0.01) in reversion rate above basal rates (Figure 2B and Table S4). Control strains that do not rereplicate in the presence of galactose also displayed no significant increase (\( P \)-value > 0.01) over basal reversion rates at all four reporter sites (Figure 2B and Table S4). Thus, there was a correspondence between the amount of rereplication experienced by the \( \text{lys2InsEA14} \) reporter and the amount of Lys\(^+\) reversion induced. In contrast, the level of rereplication-induced gene amplification was similar regardless of the position of the \( \text{lys2InsEA14} \) reporter, confirming that the overall effect of rereplication on the chromosome was the same for all reporters (Figure S2 and Table S5). Finally, \( \text{lys2InsEA10} \), a frameshift reporter containing a smaller A10 homopolymeric tract, also exhibited a significant degree of rereplication-induced Lys\(^+\) reversion (18-fold; \( P \)-value, 0.01) when inserted at site 1 (Figure S3 and Table S6). Together, these results suggest that the passage of a rereplication fork can induce frameshift mutations.

**Rereplication-induced mutagenesis can be exposed by compromising exonuclease proofreading**

The \( \text{CAN1} \) reporter exhibited a small rereplication-induced increase in reversion rate at site 1 (Figure 2C and Table S7). This fourfold increase had a \( P \)-value < 0.01 based on the Mann–Whitney \( U \)-test and was not seen at site 1 for its congenic nonrereplicating control strain. However, the significance of this induced mutation rate was somewhat clouded by the roughly equivalent increases (albeit with \( P \)-value ≥ 0.01) seen in the nonrereplicating strains at sites 2 and 4. What is clear is that the \( \text{CAN1} \) reporter did not show the same degree of rereplication-induced mutagenesis as the \( \text{lys2InsEA14} \) reporter.

The difference in results for the two reporters could be due to the fact that frameshift errors in long homopolymeric runs, like A14 in the \( \text{lys2InsEA14} \) reporter, are poor substrates for...
proofreading by replicative DNA polymerases (Tran et al. 1997; Deem et al. 2011). Unpaired nucleotides resulting from such frameshifts can be displaced away from the 3’ end of the primer-template junction, where proofreading polymerases sense and correct mismatches. Consequently, the \textit{lys2InsE}_{A14} reporter is a highly sensitized frameshift reporter for detecting defects in nonproofreading fidelity mechanisms. In contrast, the general mutations detected by the \textit{CAN1} reporter are readily proofread by replicative DNA polymerases. Hence, the discrepancy between the rereplication-induced mutagenesis of the \textit{lys2InsE}_{A14} and \textit{CAN1} reporters suggests that the proofreading of nucleotide misincorporations in \textit{CAN1} may be masking the impaired rereplication fidelity detected by the \textit{lys2InsE}_{A14} reporter.

To test this hypothesis, we reexamined the mutagenesis of \textit{CAN1} when the proofreading function of the replisome was compromised. Pol ε and Pol δ are responsible, respectively, for synthesizing the leading and lagging daughter strands at the replication fork [reviewed in Burgers and Kunkel (2017)]. Budding yeast can tolerate inactivation of one but not both of their proofreading exonuclease activities (Morrison and Sugino 1994). Because Pol δ has a prominent role in break-induced repair through homologous recombination [reviewed in McVey et al. (2016)] and has been implicated in vivo in extrinsic exonuclease proofreading for other polymerases (Pavlov et al. 2006; Flood et al. 2015), its proofreading function may be less confined to the replisome. Hence, we repeated our \textit{CAN1} mutagenesis analysis in the presence of a \textit{pol2-4} mutation, which specifically disrupts the exonuclease activity of Pol ε (Morrison et al. 1991).

Consistent with the redundancy of replication fidelity mechanisms and as reported by others (Tran et al. 1997, 1999), the basal mutation rate of the \textit{lys2InsE}_{A14} and \textit{CAN1} reporters increased by only two- and fivefold, respectively, in the \textit{pol2-4} background (Figure 3, and Tables S8 and S9). This higher basal mutation rate is reflected in the change in scale of the y-axis of Figure 3 compared to that in Figure 2. Given that frameshifts in the \textit{lys2InsE}_{A14} A14 tract are already poorly proofread, it was not surprising that the rereplication-induced increase in Lys\textsuperscript{+} reversion rate was not greatly affected by the addition of the \textit{pol2-4} allele. The induced reversion rate changed by only a factor of two at site 1 (from 15-fold to 31-fold) and did not change significantly at site 2 (from 12-fold to 11-fold) (Figure 3B and Table S8). As expected, the frequency of rereplication-induced gene amplification also did not change much (Figure S4 and Table S10). However, for the \textit{CAN1} reporter there was an obvious rereplication-induced increase in the mutation rate relative to the basal rate for both sites 1 and 2 (13-fold and eightfold, respectively) (Figure 3B and Table S9). Normalizing for 50\% rereplication at site 1 yields a rereplication-induced increase in \textit{CAN1} mutagenesis of 26-fold. Such an increase lends credence to the small and statistically significant increase in \textit{CAN1} mutagenesis observed at site 1 in the wild-type \textit{POL2} background (Figure 2C). Thus, the fidelity of DNA synthesis is compromised during rereplication, but much of this effect...
can be masked by redundancy in error correction provided by DNA polymerase proofreading.

Rereplication-induced mutagenesis is not an indirect consequence of rereplication-induced breaks

We have previously reported that the rereplication forks that we induce are highly susceptible to breakage (Finn and Li 2013). In that report, we quantified the amount of rereplication-induced double-stranded breaks starting ~50-kb away from ARS317 (near site 2) and extending further out. At these distances from the reinitiating origin, this break profile parallels the reinitiation profile. Hence, for sites 2–4, the level of rereplication-induced mutagenesis not only corresponds to the level of rereplication, but also to the level of rereplication-induced breaks. Several studies have shown that DNA break repair through various homologous recombination mechanisms (gene conversion, gene conversion with gap repair, and break-induced replication) are error-prone, increasing reporter mutagenesis by as much as hundreds to thousands of fold (Strathern et al. 1995; Hicks et al. 2010; Deem et al. 2011). Thus, we had to consider the possibility that the rereplication-induced mutagenesis we observed might be secondary to rereplication fork breakage.

To separate the effect of rereplication per se from rereplication fork breakage, we examined the reversion rate of the lys2InsE_{A14} reporter in the subpopulation of sister chromatids that had undergone RRIGA. As discussed above, we can select for sister chromatids that have amplified a 124-kb segment encompassing the reinitiation origin ARS317 and the two reporter sites closest to this origin. At the boundaries of this amplified segment, inserted at positions 521 and 645 kb of chromosome IV, are two fragments of the selectable URA3 genes with 390 bp of overlapping sequence identity. As we previously showed (Finn and Li 2013), RRIGA occurs when bidirectional rereplication forks emanating from ARS317 progress beyond both URA3 fragments before breaking (Figure 4A). Nonallelic homologous recombination mediated by single-strand annealing between the rereplicated URA3 fragments closest to the breakpoints then results in tandem duplication of the segment and reconstitution of a complete URA3 gene at the boundary between segments.

Based on this mechanism of RRIGA, we inferred that the rereplication profile of sister chromatids that experienced RRIGA would show full and equal rereplication throughout the entire amplified segment before declining beyond its boundaries (Figure 4A). Importantly, rereplication fork breakage in this subpopulation of sister chromatids is excluded from within the amplified segment and clustered in two zones flanking the segment (Figure 4A). The lys2InsE_{A14} reporter inserted at site 1 is positioned toward the middle of this 124-kb-segment far away from these breaks, while the reporter inserted at site 2 is positioned ~10-kb away from the right boundary, closer to the zone of fork breakage. Hence, if passage of the rereplication fork were responsible for the rereplication-induced mutagenesis, we would expect the increase in lys2InsE_{A14} reversion rates for sister chromatids that experience RRIGA to be (1) equivalent at the two sites and (2) comparable to that for the bulk population of sister chromatids. On the other hand, if rereplication fork breakage were primarily responsible for the rereplication-induced mutagenesis, we would expect (1) the increase in lys2InsE_{A14} reversion rate at site 1 to be much lower in sister chromatids experiencing RRIGA than in the bulk population, and (2) the increase in lys2InsE_{A14} reversion rate at site 2 to be equivalent or higher than site 1 in cells experiencing RRIGA.

To quantify the sister chromatids that were induced by rereplication to undergo both RRIGA and reversion of the lys2InsE_{A14} reporter, we selected for cells that were both Ura+ and Lys+. For better statistical reliability, we performed these experiments in a pol2-4 background to increase the number of rare Ura+ Lys+ cells present before rereplication induction. For the bulk population of pol2-4 cells, rereplication induces a 31- and 11-fold higher reversion rate of lys2InsE_{A14} at site 1 and site 2, respectively (Figure 3A). After normalizing for 50% rereplication of site 1 and 25% rereplication of site 2 (Figure 1A), this induction comes out to 62-fold for site 1 and 44-fold for site 2. Figure 4B and Table S11 show the rereplication-induced lys2InsE_{A14} reversion rate for the subpopulation of sister chromatids that experienced RRIGA. For this subpopulation, rereplication increases the lys2InsE_{A14} reversion rate by 81-fold for site 1 and 71-fold for site 2. These inductions are similar to each other and, despite the dearth of nearby rereplication fork breakage, comparable to the inductions for the bulk population. We conclude that the lys2InsE_{A14} reversion arises from passage of the rereplication fork and not from rereplication-induced breaks.

Rereplication induces both simple frameshift mutations and base substitutions

To examine the nature of the mutations induced during rereplication, we sequenced the lys2InsE_{A14} reporter for a subset of the Lys+ revertants from YJL11215, a rereplicating pol2-4 strain with the reporter inserted at site 1. All 11 of the spontaneous Lys+ revertants and 16 out of 17 of the rereplication-induced Lys+ revertants had a -1A frameshift mutation in the A14 homopolymeric tract. The one exception had a -1C frameshift mutation immediately after the homopolymeric tract. This result confirmed that both the spontaneous and rereplication-induced Lys+ revertants in our strains occur through a simple frameshift, consistent with previous observations (Tran et al. 1997) and most readily explained by uncorrected replication slippage.

Notably, none of these frameshift mutations were accompanied by nearby base substitutions, a strong signature of frameshifts generated by DNA polymerase ζ (Pol ζ) in a similar LYS2 frameshift assay (Harfe and Jinks-Robertson 2000). This error-prone polymerase is responsible for much of the spontaneous and DNA damage-induced mutagenesis in budding yeast, and has been implicated in mutagenesis associated with replication stress and break repair (Lawrence 2004; Northam et al. 2014; Makarova and Burgers 2015; McVey et al. 2016). However, our sequence data suggest that it does not play a
major role in rereplication-induced mutagenesis, and we have confirmed this directly by deletion analysis (see below).

We also sequenced the can1R alleles from reporters at site 1 (YJL11215) and site 2 (YJL11213 and YJL11214), both before and after the induction of rereplication in the pol2-4 background (Table S12). With the exception of one double-base pair substitution 12-nt apart, these mutations consisted of single-base pair substitutions or single-base pair frameshifts scattered throughout the CAN1 coding sequence (Figure 5). Most of the frameshift mutations were observed in small homonucleotide runs, again suggestive of uncorrected replication slippage events. Together, the sequence analyses of the two mutagenesis reporters are consistent with error correction being partially compromised during rereplication.

**Rereplication induces mutagenesis by compromising MMR**

Like replication, rereplication is subject to three error-avoidance mechanisms working in series and cooperating in a multiplicative manner to reduce the rate of nucleotide misincorporation: nucleotide selectivity during the initial incorporation, proofreading, and MMR. One model for how the fidelity of rereplication could be less than the fidelity of replication is that one (or more) of these mechanisms is compromised during rereplication. If we disrupt that mechanism, the fidelity of both replication and rereplication would be equally compromised, precluding the induction of a higher mutation rate by rereplication. On the other hand, if the disrupted mechanism is not the one compromised by rereplication, then we should still observe rereplication-induced mutagenesis, albeit starting from a higher basal rate.

The fact that rereplication induces significant mutagenesis in the proofreading-defective pol2-4 strain background directed us toward nucleotide selectivity and/or MMR as the possible error-avoidance mechanism(s) impaired by rereplication. To test whether MMR is impaired, we quantified the basal and rereplication-induced reversion rate of lys2InsEA14 at site 1 in an msh2Δ background, which ablates MMR. Similar to previous reports, the basal lys2InsEA14 reversion rates in the msh2Δ strains increased by several thousand-fold (Tran et al. 1997). However, the induction of a 15-fold higher reversion rate by rereplication was lost, resembling the lack of induction observed when rereplication is prevented (Figure 6 and Table S13). A similar result was observed if MMR was crippled using an mlh1Δ deletion instead of msh2Δ (Figure SSA and Table S14). Both deletion backgrounds still induced robust increases in Ura+ rates (Figure S6A, and Tables S15 and S16), suggesting that these deletions do not perturb the induction of rereplication nor its expected stimulation of amplification. Together, these results raise the possibility that rereplication-induced mutagenesis operates primarily through the impairment of MMR. However, one can imagine three alternative explanations that require consideration.

One is that we failed to see a rereplication-induced increase in the lys2InsEA14 reversion rate because the overall error rate caused by the loss of MMR function is near the limit of what
cells can tolerate due to error-induced extinction. Indeed, an upper limit for the reversion rate of the *lys2InsEA14* reporter in budding yeast can be observed as an increasing number of fidelity mechanisms are genetically ablated, eventually resulting in lethality (Tran et al. 1999). However, this limit is at least five times higher than the reversion rate in an *msh2Δ* strain (Tran et al. 1999). Moreover, even this fivefold limit may not apply to our experiments, as our transient and limited pulse of rereplication does not cause any increased inviability in the MMR mutant strains (data not shown). In other words, the rereplication in our experiments is not pushing the MMR mutant strains over the threshold for error-induced extinction.

A second alternative possibility is that there is some intrinsic saturation of the *lys2InsEA14* reversion assay, and we are approaching the saturation limit in the MMR mutants. To address this possibility, we examined rereplication-induced *CAN1* mutagenesis in the MMR mutants. In contrast to *lys2InsEA14* reversion, the basal rate of *Can^R* generation increases only 20–70-fold in MMR mutants (Figure S5B, and Tables S17 and S18) (Johnson et al. 1996; Marsischky et al. 1996; Shcherbakova and Kunkel 1999; Tran et al. 1999) and has room to increase ~50-fold more before being limited by error-induced extinction (Tran et al. 1999). As discussed previously, in MMR-proficient cells rereplication induces a small (fourfold) but reproducible increase in the *CAN1* mutation rate at site 1 (Figure 2C). Despite the large dynamic range still available to the *CAN1* reporter, this induction was lost in both an *msh2Δ* and an *mlh1Δ* background (Figure S5B, and Tables S17 and S18). These results argue against assay saturation being the underlying cause for the loss of the rereplication-induced mutagenesis in the MMR mutant background.

The third alternative possibility to explain the disappearance of rereplication-induced mutagenesis in MMR mutants is that rereplication induces mutagenesis through a mechanism that does not operate in series with MMR. In such a situation, the increase in mutagenesis from rereplication and MMR disruption would cooperate in an additive rather than multiplicative manner, and the small increase from rereplication could be eclipsed by the larger increase due to MMR disruption. The predominant replication-associated mechanism that is known to act in such an additive fashion is mutagenesis by the error-prone polymerase Pol ζ. Pol ζ is enlisted in response to stalled or stressed replication repair (Lawrence 2004; Northam et al. 2014; Makarova and Burgers 2015; McVey et al. 2016), but its errors do not appear to be subject to MMR correction (Huang et al. 2002; Lehner and Jinks-Robertson 2009; Aksenova et al. 2010; Kochenova et al. 2015). As discussed earlier, our sequencing of rereplication-induced *can1^R* mutations suggested that Pol ζ might not be responsible for generating these mutations. More direct analysis by deletion of *REV3*, which encodes the catalytic subunit of Pol ζ, indicates that the rereplication-induced mutagenesis of both *lys2InsEA14* and *CAN1* does not require Pol ζ (Figure 7, and Tables S19 and S20). As with previous deletion mutants, control experiments confirm that RRIGA is induced normally in the *rev3Δ* strains (Figure S7 and Table S21).

Taken together, these results reinforce the notion that rereplication is likely inducing mutagenesis by compromising one or more of the three sequential error-avoidance mechanisms that ensure replication fidelity. Thus, the simplest explanation for why this induction is lost in the MMR mutants is because this induction arises from partial impairment of MMR.

**Discussion**

**A wider effect of rereplication on genome stability**

We have previously shown that rereplication is an extremely potent source of gross chromosomal rearrangements such as gene amplification (Green et al. 2010; Finn and Li 2013) and whole-chromosome aneuploidy (Hanlon and Li 2015). In this study, we establish that rereplication also makes cells more prone to nucleotide-level mutagenesis, broadening the consequences of rereplication on genome stability. Thus, mechanisms important for the control of replication initiation also contribute to the fidelity of DNA synthesis.

The effect of rereplication on nucleotide-level mutagenesis is less dramatic than its effect on gross chromosomal rearrangements and can be masked by the partial redundancy of replication fidelity mechanisms. However, when we alleviated this redundancy by using the proofreading-resistant *lys2InsEA14* reporter or by inactivating the proofreading exonuclease of Pol ζ for the *CAN1* reporter, we observed a 26–30-fold increase in the rate of frameshift and/or base substitutions. As discussed below, such a moderate effect on the fidelity of DNA synthesis may be relevant to the accumulation of mutations in cancers.

**How does rereplication compromise nucleotide fidelity?**

Given that rereplication forks show increased susceptibility to breakage and that recombinational repair of double-strand breaks has been associated with mutagenesis [reviewed in Malkova and Haber (2012)], one trivial reason for
recombination-induced mutagenesis is increased break repair. However, when we required a large 124-kb segment to be reeplicated intact by selecting for reeplication events that result in amplification of the segment, we saw no decrease in mutation rate for reporters within that segment. Thus, the extent of mutagenesis correlates with the level of reeplication and not with the level of fork breakage, arguing that the mutagenesis occurred during DNA synthesis by the reeplication fork.

Our inquiry of how reeplication forks might generate an elevated error rate was directed by our understanding of how errors are generated and handled during DNA replication. Three error-avoidance mechanisms that cooperate in a multiplicative manner to reduce the error rate of DNA replication—polymerase nucleotide selectivity, polymerase proofreading, and MMR—are also available to reduce the error rate during reeplication. Compromising one or more of these mechanisms could provide one way in which reeplication could increase mutations rates. In principle, mutations can also be generated during DNA reeplication by recruitment of the error-prone DNA polymerase, Pol ζ, which is responsible for much of the mutagenesis that arise spontaneously, or in response to DNA damage or replication stress in budding yeast (Lawrence 2004; Northam et al. 2014; Makarova and Burgers 2015; McVey et al. 2016). However, our finding that REV3, the gene encoding the catalytic subunit of Pol ζ, is not required for reeplication-induced mutagenesis argues against this possibility. Given this result, the loss of reeplication-induced mutagenesis in MMR mutant backgrounds suggests that this mutagenesis arises primarily by compromising MMR.

Future studies will be needed to determine how MMR might be compromised during reeplication. We offer one suggestion based on the prevailing notion that eukaryotic DNA replication and MMR are somehow coupled [reviewed in Kunkel and Erie (2015)]. This coupling is conceptually appealing because MMR requires replacement of mismatched sequences specifically on the daughter strand, which is most readily distinguished from parental strands at newly replicated DNA. Two types of observations have encouraged this notion: (1) the temporal and spatial association of MMR with DNA replication in eukaryotic cells (Kleckzkowska et al. 2001; Hombauer et al. 2011a,b), and (2) the interaction of MMR proteins with PCNA (Clark et al. 2000; Flores-Rozas et al. 2000; Dzantiev et al. 2004; Lee and Alani 2006), the sliding clamp for Pol ε and Pol δ. PCNA, we note, is loaded around replicating DNA with an orientation specified by which strand is the daughter strand [reviewed in Modrich (2016)], so, in principle, PCNA can remember daughter strand identity and transmit this information to interacting MMR proteins. In budding yeast, the two MMR complexes that first bind and recognize mismatched DNA, Msh2-Msh6 and Msh2-Msh3, also bind PCNA (Clark et al. 2000; Flores-Rozas et al. 2000). Later, daughter-specific strand excision of the mismatched segment is facilitated by Mlh1-Pms1, whose endonuclease-nicking activity is directed to daughter strands through its interaction with PCNA (Pluciennik et al. 2010). These molecular connections hint at coordination between DNA replication and MMR designed to enhance the efficiency of the latter. We speculate that this coordination may somehow be suboptimal during DNA reeplication, causing indirect attenuation of MMR.

Possible role of weak MMR defects in cancer

More than 40 years ago, Loeb proposed that an increase in mutation rate, which he termed a mutator phenotype, could promote tumor initiation and/or progression (Loeb 2016). Strong MMR defects arising from genetic or epigenetic inactivation of key MMR genes (e.g., hMSH2 and hMLH1) provided the first validation of this hypothesis (Fishel and
Kolodner 1995; Kinzler and Vogelstein 1996; Modrich 2016). These defects generate a mutator phenotype (detected as a high level of microsatellite repeat instability or MSI-H) that is responsible for a small subset of colorectal, endometrial, and stomach cancers. Many of these cancers appear as part of Lynch syndrome, an inherited disorder where germline inactivation of MMR genes causes a strong familial susceptibility to early cancer development [reviewed in Peltomäki (2016)].

Recent findings suggest that weaker MMR defects with subtler mutator phenotypes may be associated with a broader range of cancers. Diagnostic sequencing of suspected Lynch syndrome patients, whose cancers exhibit low penetrance and/or late occurrence, has identified a growing number of missense mutations that have uncertain functional consequences in MMR genes. Assays attempting to assess the pathogenicity of these “variants of uncertain clinical significance” often show weak MMR defects (Heinen and Juel Rasmussen 2012; Wielders et al. 2014; Peltomäki 2016). This has led to speculation that weak MMR defects, possibly in synergism with other weak repair defects, generate enough of a mutator phenotype to promote sporadic cancers (Liccardo et al. 2017). Importantly, even if a cancer is initiated by other means, its progression or eventual therapeutic resistance might still be facilitated by a weak MMR defect.

Whole-exome and whole-genome sequencing of thousands of cancer samples suggests that such weak mutator phenotypes may be more prevalent than previously realized (Hause et al. 2016; Cortes-Ciriano et al. 2017). These studies reveal a broad and continuous range in the number of microsatellite alterations present in cancers, with many cancer samples displaying moderate to low amounts. Interestingly, some of these samples have no detectable genetic or epigenetic defects in MMR genes (Cortes-Ciriano et al. 2017), raising the possibility that MMR in these cancers is somehow attenuated by indirect means.

Another potential role of rereplication in tumor biology

Our observation that MMR is compromised during rereplication offers one possible means for the indirect attenuation of MMR in cancer. This possibility is encouraged by growing circumstantial evidence that rereplication occurs in some cancers. First, moderately elevated levels of the replication initiation proteins Cdc6 and Cdt1, which in high amounts induce overt rereplication in cell culture and model organisms, has been observed in several types of primary human tumors (Karakaidos et al. 2004; Liontos et al. 2007). Such widespread overexpression may be due in part to the fact that the Rb-E2F pathway, which controls CDC6 and CDT1 transcription (Truong and Wu 2011), is often deregulated in cancer cells (Vogelstein and Kinzler 2004). Second, moderate overexpression of Cdc6 and Cdt1 can potentiate oncogenesis in mouse models (Arentson et al. 2002; Seo et al. 2005; Liontos et al. 2007). Overt rereplication was not detected in these experiments, as expected given that currently detectable levels of rereplication are associated with extensive DNA damage and cell lethality (Vaziri et al. 2003; Melietzian et al. 2004; Zhu et al. 2004; Archambault et al. 2005; Green and Li 2005; Jin et al. 2006; Green et al. 2010; Hanlon and Li 2015). However, the possibility remains open that rereplication does occur in these and other cancer cells, but at low levels that are both currently undetectable and compatible with viability.

Our previous finding that rereplication efficiently induces gross chromosomal alterations has provided a potential source for these alterations in cancers. For example, rereplication has been speculated to contribute to the large number of tandem segmental duplications (tandem duplicator phenotype) observed in a subset of breast, ovarian, and endometrial carcinomas (Menghi et al. 2016). The results described here suggest that rereplication may also be relevant to some of the extensive nucleotide-level mutagenesis observed in cancers (Hollstein et al. 2017).
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