Stimulation of Chromosomal Rearrangements by Ribonucleotides

Hailey N. Conover,^{*,†} Scott A. Lujan,^{*} Mary J. Chapman,^{*} Deborah A. Cornelio,^{*} Rabab Sharif,^{*} Jessica S. Williams,^{*} Alan B. Clark,^{*} Francheska Camilo,^{*} Thomas A. Kunkel,^{*,1} and Juan Lucas Argueso^{*,†,1}

^{*}Department of Environmental and Radiological Health Sciences and Institute for Genome Architecture and Function, and [†]Cell and Molecular Biology Graduate Program, Colorado State University, Fort Collins, Colorado 80523, and ^{*}Genome Integrity and Structural Biology Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709

ORCID ID: 0000-0002-7157-1519 (J.L.A.)

ABSTRACT We show by whole genome sequence analysis that loss of RNase H2 activity increases loss of heterozygosity (LOH) in *Saccharomyces cerevisiae* diploid strains harboring the *pol2-M644G* allele encoding a mutant version of DNA polymerase ϵ that increases ribonucleotide incorporation. This led us to analyze the effects of loss of RNase H2 on LOH and on nonallelic homologous recombination (NAHR) in mutant diploid strains with deletions of genes encoding RNase H2 subunits (*rnh201 Δ* , *rnh202 Δ* , and *rnh203 Δ*), topoisomerase 1 (*TOP1 Δ*), and/or carrying mutant alleles of DNA polymerases ϵ , α , and δ . We observed an ~ 7 -fold elevation of the LOH rate in RNase H2 mutants encoding wild-type DNA polymerases. Strains carrying the *pol2-M644G* allele displayed a 7-fold elevation in the LOH rate, and synergistic 23-fold elevation in combination with *rnh201 Δ* . In comparison, strains carrying the *pol2-M644L* mutation that decreases ribonucleotide incorporation displayed lower LOH rates. The LOH rate was not elevated in strains carrying the *pol1-L868M* or *pol3-L612M* alleles that result in increased incorporation of ribonucleotides during DNA synthesis by polymerases α and δ , respectively. A similar trend was observed in an NAHR assay, albeit with smaller phenotypic differentials. The ribonucleotide-mediated increases in the LOH and NAHR rates were strongly dependent on *TOP1*. These data add to recent reports on the asymmetric mutagenicity of ribonucleotides caused by topoisomerase 1 processing of ribonucleotides incorporated during DNA replication.

KEYWORDS LOH; NAHR; genome stability; recombination; ribonucleotides

THE replicative DNA polymerases of *Saccharomyces cerevisiae*, DNA polymerases α (Pol α), δ (Pol δ), and ϵ (Pol ϵ), frequently incorporate ribonucleotides into DNA both *in vitro* and during nuclear DNA replication *in vivo* (Nick McElhinny *et al.* 2010a,b; Williams and Kunkel 2014; Williams *et al.* 2015). These ribonucleotides are efficiently removed when RNase H2 incises the DNA backbone containing a ribonucleotide to initiate ribonucleotide excision repair (RER) (Nick McElhinny *et al.* 2010a; Sparks *et al.* 2012). When the *RNH201*

gene that encodes the catalytic subunit of RNase H2 (Cerritelli and Crouch 2009) is deleted, RER is defective and many unrepaired ribonucleotides remain in the genome. A subset of these unrepaired ribonucleotides can be removed when topoisomerase 1 (*TOP1*) incises a DNA backbone containing a ribonucleotide (Williams *et al.* 2013). However, *TOP1* incision creates nicks with unligatable ends and elicits several RNA–DNA damage phenotypes, including slow growth, activation of the genome integrity checkpoint and altered progression through the cell cycle, sensitivity to the replication inhibitor hydroxyurea (HU), and strongly elevated rates for deletion of 2–5 bp from low-complexity DNA sequences (Nick McElhinny *et al.* 2010a; Clark *et al.* 2011; Kim *et al.* 2011). These effects are elicited primarily by ribonucleotides incorporated by Pol ϵ , but not by ribonucleotides incorporated by Pol α or Pol δ (Williams *et al.* 2015). Loss of RNase H2 is also associated with reduced efficiency of mismatch repair (MMR), thereby elevating the

Copyright © 2015 by the Genetics Society of America

doi: 10.1534/genetics.115.181149

Manuscript received August 5, 2015; accepted for publication September 12, 2015; published Early Online September 22, 2015.

Supporting information is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.181149/-/DC1.

¹Corresponding authors: 493 MRB, 1618 campus delivery, Colorado State University, Fort Collins, CO 80523. E-mail: lucas.argueso@colostate.edu; and Genome Integrity and Structural Biology Laboratory, National Institute of Environmental Health Sciences, NIH, P.O. Box 12233, Mail Drop E3-01, Research Triangle Park, NC 27709.

E-mail: kunkel@niehs.nih.gov

rate of single-base mutations (Ghodgaonkar *et al.* 2013; Lujan *et al.* 2013). This mutator phenotype is consistent with the hypothesis (Nick McElhinny *et al.* 2010a) that nicks resulting from RNase H2 incision at ribonucleotides can signal for strand discrimination during removal of DNA replication errors.

In addition to the point mutations mentioned above, larger types of genome instability have also been observed in RNase H2-defective cells. For example, in a study of gross chromosomal rearrangements (GCRs) in haploid yeast cells, RNase H2 defects alone had little effect, but GCR rates were elevated in double mutant strains lacking the noncatalytic Rnh203 subunit in combination with deletions of any of eight other genes affecting DNA metabolism (Allen-Soltero *et al.* 2014). An earlier GCR study reported that *rnh201Δ* single mutants displayed a fourfold increase in instability of a nonessential yeast artificial chromosome (YAC loss and terminal deletions) (Wahba *et al.* 2011). This instability may also be related to the fact that defects in the yeast Rnh202 subunit of RNase H2 increase the rate of gene conversion (also in haploids), an effect that is partially suppressed by deleting *TOP1* (Aguilera and Klein 1988; Li *et al.* 2011; Potenski *et al.* 2014). Similarly, mouse embryonic fibroblasts lacking the noncatalytic RNASEH2B subunit of RNase H2 have increased levels of micronuclei and chromosomal rearrangements (Reijns *et al.* 2012). The mechanisms responsible for these types of large-scale genome instability are not yet fully understood, but could involve DNA strand breaks arising during processing of unrepaired ribonucleotides incorporated during replication, processing of unresolved R-loops formed during transcription, or both.

The present study was designed to answer three questions. First, do ribonucleotides incorporated during nuclear DNA replication in RER-defective yeast strains elevate the rate of two types of large-scale genome instability in diploid cells: mitotic interhomolog allelic homologous recombination leading to LOH and nonallelic homologous recombination (NAHR) leading to chromosomal translocations and copy number alterations? Second, if so, do elevated LOH or NAHR rates depend on ribonucleotides incorporated by Pol ϵ , Pol α , or Pol δ ? Third, do elevated LOH or NAHR rates depend on *TOP1*? To answer these questions, we studied a series of homozygous diploid yeast strains that vary in RNase H2 and *TOP1* status and that also vary in the propensity to incorporate ribonucleotides by Pol ϵ (*pol2-M644G*, increased; *pol2-M644L*, decreased) or by Pols α and δ (increased in both *pol1-L868M* and *pol3-L612M*, respectively). These strains, and their parents encoding wild-type replicases, were modified to assay for LOH and NAHR. The results indicate that ribonucleotides incorporated by Pol ϵ cause *TOP1*-dependent chromosome instability, while the role of those incorporated by Pols α and δ is not as strong. The results support a model in which asymmetric processing of ribonucleotides in DNA by *TOP1* causes both local and large-scale genome destabilization.

Materials and Methods

Yeast strains

The *S. cerevisiae* strains used for the whole genome sequencing mutation accumulation experiment were diploids descended from $\Delta|(-2)|-7B-YUNI300$ (Pavlov *et al.* 2001). They were homozygous for *his7-2*, *leu2Δ::kanMX*, *ura3Δ*, *trp1-289*, *ade2-1*, *lys2ΔGG2899-2900*, and *agp1::URA3*. Sanger sequencing confirmed that the *pol2-M644G* mutation was homozygous, and *rnh201Δ::hphMX* was confirmed to be homozygous by PCR fragment-size analysis. The strains used in the main set of LOH assays and all NAHR assays (presented in Supporting Information, Table S1) were isogenic to the CG379 strain background (Morrison *et al.* 1991; Argueso *et al.* 2008). We also conducted a limited number of LOH assays using hybrid diploids resulting from the mating of *MATa* haploids isogenic to CG379 and *MATα* haploids isogenic with YJM789 (Wei *et al.* 2007). CG379 is a laboratory strain background closely related to the S288c reference yeast strain, while YJM789 is a diverged background derived from a clinical isolate. There are ~60,000 single nucleotide polymorphisms (SNPs) between two strains' genomes, some of which are associated with restriction fragment length polymorphisms (RFLPs) that can be used to monitor recombination between homologous chromosomes. For the isogenic and hybrid LOH assays, the CORE2 cassette containing the *Kluyveromyces lactis* *URA3* gene, the *S. cerevisiae* *URA3* gene, and the *kanMX* geneticin resistance gene was amplified from plasmid pJA40 (Zhang *et al.* 2013) and integrated at chromosome 7 (Chr7) downstream of the *MAL13* gene (distal side), ~20 kb from the right telomere. For the NAHR assay, we used a PCR-based approach to delete a 180-bp segment spanning the 3' end of the open reading frame (ORF) and the immediate downstream sequence of the *URA3* gene at its native position on Chr5. This *ura3Δ3'* allele corresponds to a clean (marker-less) deletion between Chr5 coordinates 116,823 and 117,003 from the reference *S. cerevisiae* S288c genome (Cherry *et al.* 2012). The specific coordinates given are from the release version 64-2-1 of the S288c reference genome. In addition, a cassette containing the *kanMX* marker and a segment of the *URA3* gene missing the promoter sequences and 34 bp from 5' end of the ORF was integrated on Chr14, downstream of the *PEX17* gene (proximal side). This nonallelic *PEX17::kanMX-ura3Δ5'* (-RA3) insertion shares 622 bp of perfect homology (RA region) with the *ura3Δ3'* allele described above. Both RA sequences are oriented 5' to 3' in the top strand of the reference genome (Watson strand), on the left arms of their respective chromosomes, and recombination between them can regenerate a fully functional *URA3* gene that can be selected for by cell growth on uracil drop-out medium.

The *RNH201*, *RNH202*, *RNH203*, and *TOP1* genes were deleted with the *hphMX* or *natMX* drug resistance cassettes using a PCR-based approach (Goldstein and McCusker 1999). The *pol2-M644G* and *pol2-M644L* alleles were integrated through a two-step allele replacement procedure

(*URA3* pop-in, pop-out) using plasmids described earlier (Kirchner *et al.* 2000). We built custom plasmids to integrate the *pol3-L612M* and *pol1-L868M* alleles, pHCI and pHCI2, respectively. We used overlapping PCR approaches to create mutant *pol3-L612M* and *pol1-L868M* fragments containing synonymous substitutions that created diagnostic *TaqI* restriction enzyme polymorphisms immediately adjacent to the codons encoding the Leu to Met mutations of interest in each gene. These PCR fragments were cloned into the pRS306 *URA3* integrative vector (Sikorski and Hieter 1989) and Sanger sequenced to validate the constructions. pHCI was linearized with *Bam*HI and pHCI2 was linearized with *Sal*I prior to integration into *POL3* and *POL1* homozygous diploid strains, respectively. Ura⁺ clones containing the respective hemizygous plasmid integrations were patched onto 5-FOA medium to select for plasmid pop-outs. PCR and *TaqI* restriction analysis were used to screen candidate 5-FOA-resistant derivatives and identify diploids heterozygous for *POL3/pol3-L612M* or *POL1/pol1-L868M*, from which haploids carrying the respective DNA polymerase δ and α mutations were obtained by sporulation and tetrad dissection. All combinations of LOH and NAHR experimental markers and mutant backgrounds were obtained by intercrossing isogenic haploid strains followed by sporulation, tetrad dissection, and genotyping of haploid spores. The DNA sequences of all plasmids and oligonucleotide primers used in the constructions above are available upon request.

Detection of LOH by whole genome sequencing

RNH201-dependent LOH events were monitored during mutation accumulation experiments performed as previously described (Lujan *et al.* 2014). LOH rates are lower bound estimates calculated from the number of homozygous mutations accumulated in a particular isolate, divided by both the total number of mutations and by the number of accumulated yeast generations for that isolate. *P*-values comparing LOH rates between strains were calculated with a one-tailed Welch's *t*-test (Welch 1947).

Quantitative recombination assays

Yeast cells were streaked to single colonies on solid YPD medium and incubated on plates at 30° for 3 days. For the LOH assays, fresh whole colonies were picked, resuspended in 1 ml of sterile distilled water, serially diluted, and plated on synthetic complete medium containing 1 g/liter of 5-FOA (selective) and YPD (permissive). For the NAHR assays, fresh single colonies were picked, inoculated in tubes containing 4 ml of liquid YPD, and incubated for 24 hr at 30° in a rotating drum. One-milliliter aliquots from these cultures were transferred to microcentrifuge tubes, pelleted, washed, and resuspended in 1 ml of sterile distilled water. Serial dilutions of cells were plated on uracil drop-out (selective) and YPD (permissive). For both assays, plates were incubated at 30°, and colonies were counted from the permissive plates after 2 days of growth and from the selective plates after 4 days. The colony counts were used to calculate recombination rates and

Table 1 LOH events detected by whole genome sequencing of mutation accumulation lines

Diploid homozygous genotype	<i>pol2-M644G</i> <i>RNH201</i>	<i>pol2-M644G</i> <i>rnh201Δ</i>
Number of isolates sequenced	8	7
Total generations elapsed	7200	6300
Total mutation count	436	912
LOH count	14	111
Mean LOH rate (per Mbp per generation)	38	140
Standard deviation (per Mbp per generation)	33	41
Fold difference in rate	3.7	
Welch's <i>t</i> -test <i>P</i> -value	0.00019	

All rates are lower bound estimates per mega base pairs (Mbp) per generation. Lower bound LOH rates are estimated for each isolate thus: (homozygous mutation count)/(mutation count)/(generations elapsed).

95% confidence intervals (Table S2) using the Lea and Coulson method of the median within the FALCOR web application (<http://www.keshavsingh.org/protocols/FALCOR.html>) (Lea and Coulson 1949; Hall *et al.* 2009). Statistical analyses of comparisons between recombination rates were performed using a two-sided nonparametric Mann–Whitney test in GraphPad Prism software. The calculated *P*-values for several of the most relevant pairwise recombination rate comparisons are shown in Table S3.

Molecular karyotype analysis

We analyzed the spectra of chromosomal rearrangements present in recombinant Ura⁺ clones derived from WT and *rnh201Δ* NAHR diploid strains. Independent Ura⁺ clones were picked from uracil drop-out plates, purified to single colonies in YPD plates, and then patched on to diagnostic plates containing uracil drop-out, 5-FOA, YPD geneticin, and YPD media. Full-length chromosomal DNA embedded in agarose was prepared and fractionated by pulse field gel electrophoresis (PFGE), and in some cases the DNA was used for comparative genome hybridization microarrays (array-CGH) using the methods and microarray design described previously (Zhang *et al.* 2013).

Data availability

Yeast strains and plasmids are available upon request. The whole genome sequencing data used to identify genome-wide LOH events (findings in Table 1) have been deposited at the Sequence Read Archive 10 (SRA) database under study no. SRP062900 and experiment nos. SRX1165947, SRX1165952, SRX1165955, and SRX1165953.

Results

Genome-wide ribonucleotide-dependent LOH in a *pol2-M644G rnh201Δ* strain

We constructed diploid yeast strains carrying the *pol2-M644G* allele and either *RNH201* or *rnh201Δ*. Multiple clonal isolates were passaged on solid, complete medium,

their genomes were sequenced, and mutations were identified by comparison to “zero passage” genomes for each strain (Lujan *et al.* 2014). In strains proficient in RNase H2, the majority of the mutations that accumulated were single base events whose allelic fractions were between 40 and 60%, as expected for a heterozygous state for new mutations. The identities, distributions in the genome, and rates of formation of these mutations in RNase H2-proficient strains have recently been described in detail (Lujan *et al.* 2014). In comparison, the *pol2-M644G rnh201Δ* strain accumulated single base mutations and 2- to 5-bp deletions at higher rates (to be described in detail elsewhere). However, what was not expected was that the allelic fraction of a substantial number of point mutations approached 100%. These LOH mutations suggested that a subset of ribonucleotide-induced mutations quickly became homozygous through interhomolog recombination. The number of these LOH events was larger in the *pol2-M644G rnh201Δ* strain as compared to the *pol2-M644G RNH201* strain (111 vs. 14; Table 1). Given the number of generations over which these events accumulated, we conservatively estimate that a defect in RNase H2 increased the rate of LOH in the *pol2-M644G* strain by 3.7-fold. This result motivated the more specific genetic analyses described next.

Chromosome instability as measured by LOH

We investigated the role that ribonucleotides play in stimulating structural genomic alterations using assays specifically designed to measure allelic and nonallelic homologous recombination in proliferating yeast diploid cells. The first assay was designed to characterize the LOH effect observed in the mutation accumulation experiment described above. We used isogenic diploids (Figure 1; Table S1) that were homozygous at all positions on Chr7, except that they contained a single hemizygous copy of the CORE2 counter-selectable cassette with two diverged orthologous copies of the *URA3* gene and a marker for geneticin resistance (Zhang *et al.* 2013) inserted near the right end of Chr7. An allelic interhomolog recombination event occurring anywhere within the 575-kb region between *CEN7* and the CORE2 insertion can result in LOH that may cause a diploid to become homozygous for the distal regions of the right arm of Chr7. This strain setup allows selection for homozygosity of the homolog lacking the CORE2 insertion, therefore the recombination rates measured by this approach correspond to only half of the LOH events that occur in the region.

We also conducted a limited number of experiments using hybrid diploids that resulted from mating between two diverged haploids (Materials and Methods; Figure S1). In this case, the genotype for two heterozygous positions along Chr7 were monitored in clones selected for the loss of the hemizygous CORE2 insertion. All examined 5-FOA^R clones derived from wild-type and RNase H2-deficient hybrid diploids remained heterozygous at a position on the left arm of Chr7, and most became homozygous for a marker located on the right arm ~11 kb proximal to the CORE2 insertion. This

Figure 1 Loss-of-heterozygosity (LOH) assay system in isogenic diploids. Schematic representation of the karyotype of the isogenic diploid strains used in the LOH assays in Figure 2A and Table S2. A hemizygous counter-selectable CORE2 cassette was inserted near the right end of one of the Chr7 homologs. Allelic homologous recombination between the two homologs may result in homozygosity for the region lacking the CORE2 insertion. Derived clones carrying this LOH event were resistant to 5-FOA and sensitive to geneticin. Terminal boxes labeled L and R correspond to the left and right telomeres, respectively. The circle corresponds to the centromere.

result indicated that the majority of 5-FOA^R clones selected through this approach formed by interhomolog recombination leading to LOH, and that chromosome loss was not a frequent occurrence. In both the isogenic (Figure 1) and hybrid (Figure S1) diploid backgrounds, the LOH rates measured from diploids carrying the hemizygous CORE2 insertion were in the 10^{-5} to 10^{-4} 5-FOA^R/cell/division range, and all 5-FOA^R clones examined became concomitantly sensitive to geneticin. When similar measurements were made from haploid strains, the rates were in the 10^{-8} range (data not shown). Thus, this assay provides a specific measurement for allelic interhomolog mitotic recombination that happens frequently in diploid genomes, without interference from chromosome loss, or from rare and more complex mutational mechanisms such as gross chromosomal rearrangements or clustered point mutations that simultaneously inactivate the two *URA3* genes.

Ribonucleotide-dependent LOH

We analyzed isogenic diploid strains (Figure 1 and Figure 2A) lacking each of the three genes encoding subunits of the RNase H2 enzyme: *rnh201Δ*, *rnh202Δ*, and *rnh203Δ*. All three single mutants showed a similarly significant six- to eightfold elevation of the LOH rate relative to wild type (Table S2 and Table S3; *RNH201* vs. *rnh201Δ*; $P < 0.0001$). A similar phenotypic differential was observed between the LOH rate measurements made using the hybrid diploid strain background (rate in CG379 × YJM789 *RNH201* 1.8×10^{-5} , and 8.5×10^{-5} in CG379 × YJM789 *rnh201Δ*). These direct measurements were consistent with the genome-wide result presented in Table 1, confirming that a defect in RER can cause chromosomal instability in the form of LOH.

Figure 2 Quantitative analyses of mitotic recombination. The graphs show the recombination rates determined from the LOH (A) and NAHR (B) assays, described in Figure 1 and Figure 3, respectively. The bars correspond to the median recombination rates and the error bars represent the 95% confidence intervals. The results are grouped according to DNA polymerase genotype, and the bars are color coded according to RNAse H2 and topoisomerase 1 genotypes. The same numerical values presented graphically in A and B are reproduced in Table S2 for reader reference, also including the number of cultures assayed for each genotype. Statistical significance analyses of specific pairwise comparisons between genotypes are shown in Table S3.

The mutagenic effects of unrepaired ribonucleotides have previously been determined to be dependent on inappropriate cleavage by *TOP1*, including induction of 2- to 5-bp deletions, gene conversions, and chromosomal rearrangements. We found this to also be true for LOH stimulation, as the *rnh201*Δ *TOP1*Δ double mutant displayed a rate that was significantly lower than that of the *rnh201*Δ single ($P < 0.0001$). The LOH rate in the *TOP1*Δ single mutant was slightly lower than that of wild type, but not significantly so ($P < 0.0853$).

A recent study used haploid strains carrying mutant alleles of three different DNA polymerase genes that differentially alter the rate of incorporation of ribonucleotides into DNA (Williams *et al.* 2015). We pursued a similar approach to investigate whether the asymmetric mutagenic effect of ribonucleotides also applies to LOH stimulation. Diploids homozygous for the *pol2-M644G* allele that encodes a mutant version of Pol ε that increases ribonucleotide incorporation showed an 8-fold elevation in LOH ($P < 0.0001$). This rate was further elevated to 23-fold above wild type ($P < 0.0001$)

when the ability to remove these extra ribonucleotides was eliminated in a *pol2-M644G rnh201*Δ double mutant. We also examined strains carrying the *pol2-M644L* allele that encodes a mutant version of Pol ε that incorporates fewer ribonucleotides. By itself this mutation did not significantly alter the LOH rate, but in combination with *rnh201*Δ, the LOH rate was lower than that measured for the wild-type version of Pol ε (*POL2 rnh201*Δ vs. *pol2-M644L rnh201*Δ; $P = 0.0349$). For both alleles of *POL2*, deletion of *TOP1* caused significant decreases in the LOH rate. Together, these results support the existence of a strong direct relationship between the frequency of ribonucleotide incorporation by Pol ε and the rate of *TOP1*-dependent LOH.

Diploids homozygous for the *pol1-L868M* or the *pol3-L612M* alleles that respectively encode mutant versions of Pol α and Pol δ, which increase ribonucleotide incorporation, did not display altered rates of LOH relative to diploids with wild-type polymerases. Neither mutation increased the rate of LOH when combined with loss of RNase H2 activity. The

rate of LOH in *pol1-L868M rnh201Δ* was similar to the rate in *POL1 rnh201Δ* ($P = 0.1821$), and surprisingly, the rate of LOH in *pol3-L612M rnh201Δ* was ~50% lower than that in *POL3 rnh201Δ* strains ($P = 0.0026$). Finally, deletion of *TOP1* from strains containing the *pol1-L868M* or the *pol3-L612M* alleles in combination with *rnh201Δ* significantly decreased the LOH rate. We conclude from these results that ribonucleotides incorporated by Pol α or Pol δ do not induce *TOP1*-dependent LOH as potently as those incorporated by Pol ϵ .

Chromosome instability as measured by NAHR

In the second assay, we investigated the formation of chromosomal translocations that result from NAHR in diploid cells. We used a classic heteroallele recombination approach in which two incomplete overlapping sequences can recombine to regenerate a functional selectable marker. In this case, a 3' truncation of the *URA3* gene designated *URA⁻* was present on Chr5 while a 5' truncation (*-RA3*) was located on Chr14 (Figure 3A). Homologous recombination involving the shared central regions (*RA*; 622 bp, 100% sequence identity) can create a functional copy of the *URA3* gene at the breakpoint of a Chr14/Chr5 translocation. This event may occur through a reciprocal mitotic crossover, or nonreciprocal mechanisms such as break-induced replication (BIR) or half crossover (HC) (Malkova and Ira 2013; Symington *et al.* 2014; Vasan *et al.* 2014). In the simple recombination scenarios presented in Figure 3, if a reciprocal crossover occurs between the *RA* substrates, depending on how the recombinant chromatids segregate in the following cell division, the karyotype of the resulting *Ura⁺* cells may contain either two balanced reciprocal translocations (class 1; Figure 3B) or only the nonreciprocal Chr14/Chr5 translocation associated with a terminal deletion on the left arm of Chr14 and a terminal amplification on the left arm of Chr5 (class 2; Figure 3C). If BIR or HC were the mechanisms of recombination between the *RA* sequences, the same class 2 outcome would be observed. Additionally, since the *kanMX* marker is distal to the *-RA3* sequence on Chr14, the expectation is that *Ura⁺* clones carrying a terminal deletion of Chr14 should lose *kanMX*, thus becoming sensitive to geneticin. The class 1 and class 2 karyotype configurations predicted from this model were validated using PFGE and array CGH (Figure 3, B and C).

To characterize this NAHR assay system, we analyzed the karyotypes and the presence of the *kanMX* marker in 67 independent *Ura⁺* clones derived from *RNH201* and *rnh201Δ* diploids (Table 2). We analyzed the PFGE profiles of these clones to determine the number of copies of the parental-sized Chr5 and Chr14, the presence of the 650-kb Chr14/Chr5 translocation with *URA3* at the breakpoint, and the reciprocal 700-kb Chr5/Chr14 translocation with *kanMX-RA* at the breakpoint. In addition, we inspected the PFGE karyotypes for the presence of any other chromosomal rearrangements not predicted by the model in Figure 3. The results showed that the reciprocal crossover outcome (class 1) was infrequent, having only one example detected from each

genotype. This was expected, since the reciprocal crossover mechanism requires the spontaneous initiating double-strand break (DSB) lesion to occur within the relatively small *RA* region of the recipient chromosome. In contrast, nonreciprocal outcomes can be initiated by DSBs within *RA* or anywhere in the 245-kb distal region of Chr14. This higher probability for the formation of spontaneous initiating lesions was reflected in the higher abundance of nonreciprocal recombination outcomes (classes 2–7). Interestingly, while class 2 clones were the most abundant category recovered, other configurations were also detected at substantial frequency, particularly classes 3 and 4 (Figure S2). Class 3 clones had karyotypes and copy number profiles that were indistinguishable from those of class 2, with the exception that they were resistant to geneticin. Because class 3 clones contained only one parental-sized copy of Chr14 and retained the *kanMX* marker, they must somehow have lost the copy of Chr14 that did not contain the *RA* recombination substrate. Class 4 was also resistant to geneticin, but contained two parental-sized copies of Chr14. Finally, the relatively rare nonreciprocal classes 5, 6, and 7 involved loss of one of the parental copies of Chr5. Although these alternative events were quite interesting, the detailed characterization of the recombination mechanisms leading to their formation was beyond the scope of this project. Regardless of the final karyotype configurations of the *Ura⁺* clones, all of them resulted from homologous recombination between nonallelic *RA* repeats leading to gross chromosomal rearrangements.

The overall distribution of *Ura⁺* clones in the various karyotype classes was very similar between *RNH201* and *rnh201Δ*. However, we detected six cases in which *rnh201Δ*-derived clones each carried one additional chromosomal rearrangement band with size different from those predicted for the *URA⁻/-RA3* translocations between Chr14 and Chr5 (Table 2). No such other chromosomes were detected among the wild-type-derived *Ura⁺* clones. While we did not characterize the structures of these other rearrangements, and the numbers of clones analyzed were relatively small, their presence exclusively in the *rnh201Δ* background suggested a higher occurrence of complex genome rearrangements in diploids defective for RNase H2.

Ribonucleotide-dependent NAHR

The experiments described above showed that the recombinogenic effect of RNA–DNA damage, first demonstrated for intramolecular gene conversion (Aguilera and Klein 1988; Ii *et al.* 2011; Potenski *et al.* 2014), also extend to allelic inter-homolog recombination leading to LOH. In the absence of RNase H2, these recombination events are presumably initiated by DNA breaks that accumulate following processing of ribonucleotides or R-loops by pathways other than RER. Therefore, it is to be expected that the same lesions may also increase the formation of more complex outcomes such as gross chromosomal rearrangements. This hypothesis was tested previously using the original version of the haploid *URA3-CAN1* GCR assay (Allen-Soltero *et al.* 2014), and a

Figure 3 Nonallelic homologous recombination (NAHR) assay and predicted recombination outcomes. (A) Schematic representation of the karyotype of the diploid strain used in the NAHR assay. Chr5 (blue) and Chr14 (red) are drawn to approximate scale. Terminal boxes labeled L and R correspond to the left and right telomeres, respectively, and the numbered circles correspond to the centromeres. Only one of the Chr14 homologs contains a *Kan-RA3* insertion, and only one of the Chr5 homologs contains a *URA3* sequence. Both recombination substrates are present in Watson orientation and no other *URA3* sequences are present in the genome. Recombination between the *RA* sequences can regenerate a full-length functional copy of *URA3*, selectable on uracil drop-out medium. (B) The class 1 reciprocal crossover outcome is shown to the left, with the respective sizes and structure of the associated chromosomal rearrangements. The array-CGH plots for Chr5 and Chr14 and PFGE from a representative class 1 clone are shown. The array-CGH plots y-axis corresponds to copy number [Log₂ (Cy5-labeled Ura⁺ clone DNA/Cy3-labeled parental DNA)]. The x-axis corresponds to the probe coordinates along the respective chromosomes. The white circles indicate the positions of *CEN5* and *CEN14*. The gray dots indicate the Log₂ Cy5/Cy3 values and chromosome position of each array probe. The copy number profile of class 1 clones was fully balanced, with no gains or losses relative to the parental diploid. The PFGE was cropped for emphasis, showing only the region from Chr8 (540 kb) to Chr2 (815 kb), with lane quantification traces flanking the image. Chr5 and Chr14 trace peaks are shaded in blue and red, respectively. (C) The class 2 nonreciprocal karyotype outcome is shown to the right. The array-CGH plots for a representative class 2 clone show a deletion (one copy; pink shaded) on the left arm of Chr14 from *TEL14L* to *PEX17*, and an amplification (three copies; purple shaded) on the left arm of Chr5 from *TEL05L* to *URA3*. The class 2 PFGE profile and its quantitative analysis are also shown. Note that the WT diploid strain used as reference in the PFGEs in B and C, and in Figure S2, has a slightly longer Chr5 band because it is homozygous for the *ura3-52* allele (Ty1 insertion) rather than the *ura3Δ3'* and *ura3Δ10* alleles present in the NAHR parent diploid strain. The overall difference in Chr5 sizes caused by the Ty1 insertion and *ura3* deletions is ~7 kb.

Table 2 Classes of Ura⁺ NAHR clones

NAHR class	Phenotype		PFGE karyotype ^a				Clones in each Ura ⁺ class ^b	
	Ura	Gen	Chr14/Chr5, 650 kb	Chr5/Chr14, 700 kb	Chr5, 570 kb	Chr14, 780 kb	<i>RNH201</i>	<i>rnh201Δ</i>
Parent diploids	—	R	NA	NA	2	2	NA	NA
1	+	R	1	1	1	1	1	1
2	+	S	1	0	2	1	14	17 ⁽¹⁾
3	+	R	1	0	2	1	9	15 ⁽⁴⁾
4	+	R	1	0	2	2	5	1
5	+	R	1	0	1	1	1	0
6	+	R	0	0	1	1	1	1 ⁽¹⁾
7	+	S	1	0	1	1	1	0
Total							32	35 ⁽⁶⁾

NA, not applicable.

^a The numbers (0, 1, or 2) indicate the number of copies of the indicated chromosomes (parental size Chr5 and Chr14, and the two translocations) determined by measuring the ethidium bromide staining intensity of the corresponding band in PFGE relative to the parental bands in the parent strains (representative examples shown in Figure 3 and Figure S2).

^b The number of Ura⁺ clones in each class is shown, and when present, the superscript number in parentheses indicates the number of clones that contained additional chromosomal rearrangements of size other than 650 kb or 700 kb. Six Ura⁺ clones derived from *rnh201Δ* diploids contained such uncharacterized rearrangements as observed by PFGE; no rearrangements of unexpected sizes were observed in the wild-type control. Note that all six rearrangements in question had sizes <1200 kb and, therefore, were unlikely to correspond to contractions of the ribosomal DNA tandem repeat cluster on Chr12.

YAC stability assay (Wahba *et al.* 2011). Importantly, the majority of the GCR events detected with these two specific strain setups were formed through mechanisms other than homologous recombination, as there were no significant proximal homologous sequence substrates in the regions assayed (Chen and Kolodner 1999). Allen-Soltero *et al.* (2014). showed that single mutants lacking RNase H2 did not alter the GCR rate, but synergistic stimulation was observed in double mutant combinations with specific suppressors of chromosomal instability. Interestingly, deletion of *RAD51* partly rescued this phenotype, suggesting that a substantial fraction of the GCR events that are increased in RNase H2 mutants form through the homologous recombination pathway, possibly involving nonallelic repeats. We specifically investigated this possibility using the diploid NAHR assay described above, which was designed to detect chromosomal translocations formed by recombination between homologous substrates present on Chr5 and Chr14 (Figure 3A).

Our quantitative analyses of chromosomal instability (Figure 2, Table S2, and Table S3) showed that Chr14/Chr5 NAHR events were quite rare compared to Chr7 LOH events (the baseline rate of NAHR was two to three orders of magnitude lower than LOH). Only minor, not significant, stimulation of the NAHR rate was detected in single mutants lacking the RNase H2 catalytic subunit (1.3-fold up in *rnh201Δ* vs. *RNH201*; $P = 0.2817$). A similarly insignificant increase was observed in *rnh202Δ*, and no alteration at all in *rnh203Δ*. These small (or no) rate differentials in the single mutants, within a context of rare mutational events, indicated that the NAHR rate measurements were more susceptible than LOH to interference from other confounding pleiotropic effects of the various DNA polymerase genotypes tested, such as differences in cell growth kinetics, mutator phenotypes, roles in replicative repair, or others. Specifically, single mutants carrying either the *pol1-L868M* or *pol2-M644G*,

which result in higher incorporation of ribonucleotides by *Pol α* and *Pol ε*, respectively, showed mild, yet significant decreases in the NAHR rate (each ~40% reduction relative to wild type, each with $P < 0.0001$). Despite this complication, the results obtained when we combined RNase H2 deficiency with mutant replicases and *TOP1Δ* showed a trend analogous to that observed in the LOH experiments, and suggested that ribonucleotides incorporated into DNA also contribute to NAHR.

Within each of the four mutant replicase genetic backgrounds tested, the largest NAHR rate increase between *RNH201* and *rnh201Δ* was observed in strains carrying the *pol2-M644G* allele encoding a mutant version of *Pol ε* that increases the incorporation of ribonucleotides (5.4-fold NAHR rate elevation in *pol2-M644G rnh201Δ* vs. *pol2-M644G RNH201*; $P < 0.0001$). *pol2-M644G* strains also displayed the largest *TOP1*-dependent NAHR rate reduction (9.4-fold decrease in *pol2-M644G rnh201Δ TOP1Δ* vs. *pol2-M644G rnh201Δ TOP1*; $P < 0.0001$). *pol2-M644L* strains that have lower *Pol ε* ribonucleotide incorporation did not significantly increase the NAHR rate in combination with *rnh201Δ* (only 1.6-fold elevation in *pol2-M644L rnh201Δ* vs. *pol2-M644L RNH201*; $P = 0.0871$). These results were consistent with our observations for LOH and showed that the frequency of ribonucleotide incorporation by *Pol ε* is an important contributor to the formation of chromosomal rearrangements by NAHR.

The NAHR results obtained within the other two mutant replicase backgrounds that increase ribonucleotide incorporation were not as straightforward as in the LOH experiments. Deficiency of RNase H2 in combination with either *pol1-L868M* or *pol3-L612M* resulted in ~2.3-fold increases in the NAHR rate. These increases were not as robust as the 5.4-fold effect observed for *pol2-M644G*, but they were both significant (*pol1-L868M rnh201Δ* vs. *pol1-L868M RNH201* and *pol3-L612M rnh201Δ* vs. *pol3-L612M RNH201*; each had

$P < 0.0001$). In both cases, the increases in the NAHR rate were dependent on *TOP1*. A notable result was the significant ~60% increase in the NAHR rate in *pol3-L612M rnh201Δ* compared to *POL3 rnh201Δ* ($P < 0.0001$), a recombination rate change of approximately the same magnitude but opposite direction as observed in the LOH assay (~50% decrease; Figure 2A). One possible reason for these contrasting results might be related to the fact that Pol δ is known to participate in the BIR mechanism (Symington *et al.* 2014), which we showed accounts for the majority of the NAHR events detected in our assay (Table 2). In this scenario, the *pol3-L612M* mutation might somehow make Pol δ more efficient at initiating or sustaining BIR. This would promote the non-reciprocal recombination mechanism associated with the NAHR assay, and conversely, might disfavor the reciprocal interhomolog mitotic crossover pathway most often associated with LOH.

The results obtained for NAHR with the mutant alleles of *POL1* and *POL3* can be interpreted as a sign that ribonucleotides incorporated by these polymerases have a larger contribution to structural chromosomal rearrangements than they do to allelic interhomolog recombination (*i.e.*, LOH). However, even in this scenario, the ribonucleotides incorporated by Pol ϵ remain as the ones with the most destabilizing effect in both recombination assays used in this study. Alternatively, the inconsistencies in *pol1-L868M* and *pol3-L612M* behavior between our two assays might be primarily the result of interference in NAHR rate measurements caused by confounding phenotypes associated with these mutations, and not by their higher inherent rate of ribonucleotide incorporation.

Discussion

A number of recent studies have shown that ribonucleotides are incorporated throughout the genome during DNA replication (reviewed in Jinks-Robertson and Klein 2015). Incorrect removal of such abundant ribonucleotides can lead to genomic instability in the form of point mutations, short deletions within low complexity regions, and chromosomal rearrangements. Interestingly, the mutagenic effect of ribonucleotides appears to be asymmetric, according to which DNA polymerase was responsible for their incorporation. Specifically, *TOP1*-dependent 2- to 5-bp deletions in an RNase H2 mutant background were shown to be the product of ribonucleotides incorporated by Pol ϵ , but not by Pol α and Pol δ (Williams *et al.* 2015).

Earlier reports described increased chromosome-scale instability in RNase H2 mutant haploid yeast cells, including *TOP1*-dependent gene conversion (Potenski *et al.* 2014) and gross chromosomal rearrangements (Allen-Soltero *et al.* 2014). Another study measured the rate of LOH at Chr3 in RNase H homozygous mutant diploids (Wahba *et al.* 2011). Although Wahba *et al.* (2011) did not observe significant change in LOH in *rnh1Δ* or *rnh201Δ* single mutants, they did report an eightfold elevation in *rnh1Δ rnh201Δ* double mutants lacking both RNase H1 and H2 activities. Finally, a

study complementary to our own has been reported recently using hybrid yeast diploids to quantify LOH stimulation genome-wide and characterize the associated recombination tracts in RNase H defective cells. These experiments showed elevated LOH in *rnh201Δ* and *rnh1Δ rnh201Δ*, but not in *rnh1Δ* single mutants (O'Connell *et al.* 2015). Neither of the diploid studies described above investigated the *TOP1* dependency of the reported increases in LOH.

In this study, we show that genome-wide LOH occurs more frequently in diploids lacking RNase H2 activity, and that the rate of LOH on Chr7 is elevated in mutants carrying deletion of RNase H2 subunits. The increased LOH phenotype in *rnh201Δ* was almost entirely suppressed by deletion of the *TOP1* gene. Further, we showed that the rate of LOH is modulated by increasing or decreasing the number of ribonucleotides incorporated by mutant alleles of Pol ϵ , and this effect was also dependent on *TOP1*. In contrast, alleles of Pol α or Pol δ that increase ribonucleotide incorporation did not alter the rate of allelic interhomolog recombination.

We also observed a role for ribonucleotides incorporated by Pol ϵ in stimulating chromosomal translocations formed by NAHR. Although the phenotypic differential provided by this assay system is small, the highest rate of NAHR was measured in the *rnh201Δ pol2-M644G* strain, and chromosomal size polymorphisms other than the selected translocations were only detected in the *rnh201Δ* background. Together, these observations were consistent with earlier reports for a role of ribonucleotides in the generation of gross chromosomal rearrangements in yeast (Wahba *et al.* 2011; Allen-Soltero *et al.* 2014) and increased cytogenetic abnormalities in mammalian cells (Reijns *et al.* 2012).

The recombinogenic effects associated with RNase H2 mutants may result from misprocessing of scattered ribonucleotides incorporated into DNA, misprocessing of R-loops, or a combination of these two defects. The observation that increased and decreased ribonucleotide incorporation by Pol ϵ correlates with the LOH rate leads us to propose that much of the LOH observed here in RNase H2 mutants is triggered by ribonucleotides incorporated by Pol ϵ during leading strand replication. This idea is consistent with our earlier proposal that ribonucleotides incorporated by Pol ϵ , but not (or less so) by Pals α and δ , result in 2- to 5-bp deletions that result from incisions by *TOP1* (Williams *et al.* 2015). Thus, it appears that after Pol ϵ incorporates a ribonucleotide, topoisomerase 1 cleavage of the DNA backbone at a ribonucleotide provides opportunities for multiple types of genome instability, including loss of a short repeat, LOH and NAHR. Possible mechanistic reasons for this observed asymmetry have been proposed (figure 5 in Williams *et al.* 2015) and are currently being tested. The first centers on the fact that the RER-defective *pol2-M644G* strain has a higher density of ribonucleotides in the nascent leading strand than is observed in the nascent lagging strand for the *pol1-L868M* and *pol3-L612M* mutator strains lacking *RNH201*, suggesting that ribonucleotide density must exceed a certain threshold to observe *TOP1*-dependent recombination events. Alternatively,

there may be additional pathways involved in removal of nascent lagging strand ribonucleotides that are either specific for this DNA strand or less available for ribonucleotide removal from the nascent leading strand. Finally, we propose that negative supercoils may accumulate in the continuous nascent leading strand in the wake of the replisome and *TOP1*, through its interaction with the CMG helicase (Gambus *et al.* 2006), would be in an ideal physical position to relieve this torsional stress. Such helical tension may not build up in the discontinuous nascent lagging strand because of the presence of DNA ends that could allow rotation. As with more localized forms of RNA–DNA damage, the asymmetry of *TOP1*-dependent recombination events support the concept that failure to remove ribonucleotides incorporated into DNA by Pol ϵ during leading strand synthesis puts genome stability at risk.

Acknowledgments

We thank Clinton Orebaugh and Marta Garbacz for helpful comments on the manuscript; Adam Burkholder and the National Institute of Environmental Health Sciences (NIEHS) Integrative Bioinformatics Support Group for help with whole genome mutation calling; Matheus A. Pichorim for offering technical support during the recombination rate assays; and Karen O'Connell, Sue Jinks-Robertson, and Tom Petes for helpful discussions and for sharing unpublished data. We are also thankful for the valuable feedback provided by the associate editor and anonymous reviewers. This work was supported by project Z01 ES065070 to T.A.K. from the Division of Intramural Research of the National Institutes of Health, NIEHS, and by a Webb-Waring biomedical research award from the Boettcher Foundation (to J.L.A.). D.A.C. received a postdoctoral fellowship from CNPq/Brazil. F.C. was supported by the National Science Foundation Research Experience for Undergraduates program at Colorado State University. The authors have no conflict of interest.

Note added in proof: See O'Connell *et al.* 2015 (pp. 963–975) in this issue for a related work.

Literature Cited

- Aguilera, A., and H. L. Klein, 1988 Genetic control of intrachromosomal recombination in *Saccharomyces cerevisiae*. I. Isolation and genetic characterization of hyper-recombination mutations. *Genetics* 119: 779–790.
- Allen-Soltero, S., S. L. Martinez, C. D. Putnam, and R. D. Kolodner, 2014 A *Saccharomyces cerevisiae* RNase H2 interaction network functions to suppress genome instability. *Mol. Cell. Biol.* 34: 1521–1534.
- Argueso, J. L., J. Westmoreland, P. A. Mieczkowski, M. Gawel, T. D. Petes *et al.*, 2008 Double-strand breaks associated with repetitive DNA can reshape the genome. *Proc. Natl. Acad. Sci. USA* 105: 11845–11850.
- Cerritelli, S. M., and R. J. Crouch, 2009 Ribonuclease H: the enzymes in eukaryotes. *FEBS J.* 276: 1494–1505.
- Chen, C., and R. D. Kolodner, 1999 Gross chromosomal rearrangements in *Saccharomyces cerevisiae* replication and recombination defective mutants. *Nat. Genet.* 23: 81–85.
- Cherry, J. M., E. L. Hong, C. Amundsen, R. Balakrishnan, G. Binkley *et al.*, 2012 *Saccharomyces* Genome Database: the genomics resource of budding yeast. *Nucleic Acids Res.* 40: D700–D705.
- Clark, A. B., S. A. Lujan, G. E. Kissling, and T. A. Kunkel, 2011 Mismatch repair-independent tandem repeat sequence instability resulting from ribonucleotide incorporation by DNA polymerase epsilon. *DNA Repair (Amst.)* 10: 476–482.
- Gambus, A., R. C. Jones, A. Sanchez-Diaz, M. Kanemaki, F. van Deursen *et al.*, 2006 GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. *Nat. Cell Biol.* 8: 358–366.
- Ghodgaonkar, M. M., F. Lazzaro, M. Olivera-Pimentel, M. Artola-Boran, P. Cejka *et al.*, 2013 Ribonucleotides misincorporated into DNA act as strand-discrimination signals in eukaryotic mismatch repair. *Mol. Cell* 50: 323–332.
- Goldstein, A. L., and J. H. McCusker, 1999 Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* 15: 1541–1553.
- Hall, B. M., C. X. Ma, P. Liang, and K. K. Singh, 2009 Fluctuation analysis CalculatOR: a web tool for the determination of mutation rate using Luria-Delbruck fluctuation analysis. *Bioinformatics* 25: 1564–1565.
- Ii, M., T. Ii, L. I. Mironova, and S. J. Brill, 2011 Epistasis analysis between homologous recombination genes in *Saccharomyces cerevisiae* identifies multiple repair pathways for Sgs1, Mus81-Mms4 and RNase H2. *Mutat. Res.* 714: 33–43.
- Jinks-Robertson, S., and H. L. Klein, 2015 Ribonucleotides in DNA: hidden in plain sight. *Nat. Struct. Mol. Biol.* 22: 176–178.
- Kim, N., S. N. Huang, J. S. Williams, Y. C. Li, A. B. Clark *et al.*, 2011 Mutagenic processing of ribonucleotides in DNA by yeast topoisomerase I. *Science* 332: 1561–1564.
- Kirchner, J. M., H. Tran, and M. A. Resnick, 2000 A DNA polymerase epsilon mutant that specifically causes +1 frameshift mutations within homonucleotide runs in yeast. *Genetics* 155: 1623–1632.
- Lea, D. E., and C. A. Coulson, 1949 The distribution of the numbers of mutants in bacterial populations. *J. Genet.* 28: 491–511.
- Lujan, S. A., J. S. Williams, A. R. Clausen, A. B. Clark, and T. A. Kunkel, 2013 Ribonucleotides are signals for mismatch repair of leading-strand replication errors. *Mol. Cell* 50: 437–443.
- Lujan, S. A., A. R. Clausen, A. B. Clark, H. K. MacAlpine, D. M. MacAlpine *et al.*, 2014 Heterogeneous polymerase fidelity and mismatch repair bias genome variation and composition. *Genome Res.* 24: 1751–1764.
- Malkova, A., and G. Ira, 2013 Break-induced replication: functions and molecular mechanism. *Curr. Opin. Genet. Dev.* 23: 271–279.
- Morrison, A., J. B. Bell, T. A. Kunkel, and A. Sugino, 1991 Eukaryotic DNA polymerase amino acid sequence required for 3'–5' exonuclease activity. *Proc. Natl. Acad. Sci. USA* 88: 9473–9477.
- Nick McElhinny, S. A., D. Kumar, A. B. Clark, D. L. Watt, B. E. Watts *et al.*, 2010a Genome instability due to ribonucleotide incorporation into DNA. *Nat. Chem. Biol.* 6: 774–781.
- Nick McElhinny, S. A., B. E. Watts, D. Kumar, D. L. Watt, E. B. Lundstrom *et al.*, 2010b Abundant ribonucleotide incorporation into DNA by yeast replicative polymerases. *Proc. Natl. Acad. Sci. USA* 107: 4949–4954.
- O'Connell, K., S. Jinks-Robertson, and T. D. Petes, 2015 Elevated genome-wide instability in yeast mutants lacking RNase H activity. *Genetics* 201: 959–971.
- Pavlov, Y. I., D. Nguyen, and T. A. Kunkel, 2001 Mutator effects of overproducing DNA polymerase eta (Rad30) and its catalytically inactive variant in yeast. *Mutat. Res.* 478: 129–139.

- Potenski, C. J., H. Niu, P. Sung, and H. L. Klein, 2014 Avoidance of ribonucleotide-induced mutations by RNase H2 and Srs2-Exo1 mechanisms. *Nature* 511: 251–254.
- Reijns, M. A., B. Rabe, R. E. Rigby, P. Mill, K. R. Astell *et al.*, 2012 Enzymatic removal of ribonucleotides from DNA is essential for mammalian genome integrity and development. *Cell* 149: 1008–1022.
- Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122: 19–27.
- Sparks, J. L., H. Chon, S. M. Cerritelli, T. A. Kunkel, E. Johansson *et al.*, 2012 RNase H2-initiated ribonucleotide excision repair. *Mol. Cell* 47: 980–986.
- Symington, L. S., R. Rothstein, and M. Lisby, 2014 Mechanisms and regulation of mitotic recombination in *Saccharomyces cerevisiae*. *Genetics* 198: 795–835.
- Vasan, S., A. Deem, S. Ramakrishnan, J. L. Argueso, and A. Malkova, 2014 Cascades of genetic instability resulting from compromised break-induced replication. *PLoS Genet.* 10: e1004119.
- Wahba, L., J. D. Amon, D. Koshland, and M. Vuica-Ross, 2011 RNase H and multiple RNA biogenesis factors cooperate to prevent RNA:DNA hybrids from generating genome instability. *Mol. Cell* 44: 978–988.
- Wei, W., J. H. McCusker, R. W. Hyman, T. Jones, Y. Ning *et al.*, 2007 Genome sequencing and comparative analysis of *Saccharomyces cerevisiae* strain YJM789. *Proc. Natl. Acad. Sci. USA* 104: 12825–12830.
- Welch, B. L., 1947 The generalisation of student's problems when several different population variances are involved. *Biometrika* 34: 28–35.
- Williams, J. S., and T. A. Kunkel, 2014 Ribonucleotides in DNA: origins, repair and consequences. *DNA Repair (Amst.)* 19: 27–37.
- Williams, J. S., D. J. Smith, L. Marjavaara, S. A. Lujan, A. Chabes *et al.*, 2013 Topoisomerase 1-mediated removal of ribonucleotides from nascent leading-strand DNA. *Mol. Cell* 49: 1010–1015.
- Williams, J. S., A. R. Clausen, S. A. Lujan, L. Marjavaara, A. B. Clark *et al.*, 2015 Evidence that processing of ribonucleotides in DNA by topoisomerase 1 is leading-strand specific. *Nat. Struct. Mol. Biol.* 22: 291–297.
- Zhang, H., A. F. Zeidler, W. Song, C. M. Puccia, E. Malc *et al.*, 2013 Gene copy-number variation in haploid and diploid strains of the yeast *Saccharomyces cerevisiae*. *Genetics* 193: 785–801.

Communicating editor: N. Hollingsworth

GENETICS

Supporting Information

www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.181149/-/DC1

Stimulation of Chromosomal Rearrangements by Ribonucleotides

**Hailey N. Conover, Scott A. Lujan, Mary J. Chapman, Deborah A. Cornelio, Rabab Sharif,
Jessica S. Williams, Alan B. Clark, Francheska Camilo, Thomas A. Kunkel, and Juan Lucas Argueso**

Table S1. Yeast strains used in quantitative recombination assays.

		DIPLOIDS NAHR assay	HAPLOIDS PARENTS			DIPLOIDS LOH assay
			NAHR	Shared	LOH	
EXPERIMENTAL GENOTYPES	MAT	a/α	a	α	a	a/α
	MAL13	+/+	+	+	::CORE2	+/:::CORE2
	ura3	Δ3'/Δ0	Δ3'	Δ0	Δ0	Δ0/Δ0
	PEX17	+/:kanMX-ura3Δ5'	::kanMX-ura3Δ5'	+	+	+/+
		JAY#	JAY#	JAY#	JAY#	JAY#
MUTANT BACKGROUNDS		↓	↓	↓	↓	↓
Wild Type		1296	1087	1167	1168	1201
rnh201Δ::hphMX		1297,1563	1301	1161	1165	1487,1488
top1Δ::natMX		1401	1393	1163	1166	1402
rnh201Δ::hphMX top1Δ::natMX		1299	1303	1164	1162	1200
rnh202Δ::natMX		1403,1404	1359, 1360	1361, 1362	1353, 1354	1405,1406
rnh203Δ::natMX		1424,1562	1314,1315	1308	1309	1310
pol1-L868M		1489,1490	1415	1482	1497, 1498	1516,1517
pol1-L868M rnh201Δ::hphMX		1474,1475	1416	1389	1421	1476,1477
pol1-L868M rnh201Δ::hphMX top1Δ::natMX		1499,1505	1392	1400	1407,1408	1472,1473
pol1-L868M top1Δ::natMX		NA	NA	1586,1588	1585,1587	1589,1590
pol3-L612M		1495,1496	1419	1411	1417	1446,1447
pol3-L612M rnh201Δ::hphMX		1442,1443, 1597	1376	1387	1418	1440,1441
pol3-L612M rnh201Δ::hphMX top1Δ::natMX		1444,1445	1414	1412	1450,1451	1483,1484
pol3-L612M top1Δ::natMX		NA	NA	1582,1584	1581,1583	1591,1592
pol2-M644G		1449,1758, 1761,1564	1334	1313	1318	1432
pol2-M644G rnh201Δ::hphMX		1470,1471	1336	1320	1321	1428,1429
pol2-M644G rnh201Δ::hphMX top1Δ::natMX		1438,1439	1343	1324	1325	1430,1431
pol2-M644G top1Δ::natMX		NA	NA	1574,1576	1573,1575	1593,1594
pol2-M644L		1468,1469	1335	1312	1319	1466,1467
pol2-M644L rnh201Δ::hphMX		1462,1463	1425,1426	1322	1323	1464,1465
pol2-M644L rnh201Δ::hphMX top1Δ::natMX		1458,1459	1427	1342	1422,1423	1515,1461
pol2-M644L top1Δ::natMX		NA	NA	1578,1580	1577,1579	1595,1596

Strain table notes: The numbers in the table represent the JAY strain collection numbers for all haploid parents and the resulting diploids used in the LOH and NAHR assays. Two or more independently built strains were used where more than one number appears. The diploids were made by mating a *MATα* assay-specific haploid to a shared *MATα* haploid parent. For example, the JAY1296 NAHR assay wild type diploid was constructed by mating JAY1087 to JAY1167, while the JAY1201 LOH assay wild type diploid was constructed by mating JAY1168 to JAY1167.

Table S2. Quantitative analyses of recombination.

Genotype (stID)		LOH assay			NAHR assay		
		Cultures (n)	rate $\times 2 \times 10^{-5}$ /cell/division		Cultures (n)	rate $\times 10^{-7}$ /cell/division	
			Median	95% CI		Median	95% CI
POL+	WT (A)	40	3.71	3.06 - 4.84	28	2.04	1.92 - 2.59
	<i>rnh201</i> (B)	45	23.65	17.85 - 34.66	50	2.73	1.92 - 3.24
	<i>rnh202</i> (C)	20	30.45	24.29 - 36.39	35	2.73	1.91 - 3.12
	<i>rnh203</i> (D)	29	24.90	20.24 - 36.87	35	2.02	1.23 - 3.1
	<i>rnh201 top1</i> (E)	34	7.70	6.75 - 9.62	20	1.93	1.69 - 2.36
	<i>top1</i> (F)	20	3.13	2.3 - 3.67	20	1.62	1.12 - 2.02
pol2-M644G	WT (G)	19	28.42	25.71 - 36.77	21	1.17	0.7 - 1.6
	<i>rnh201</i> (H)	32	84.58	60.33 - 95.46	35	6.30	4.38 - 7.73
	<i>rnh201 top1</i> (I)	21	14.24	8.18 - 27.02	28	0.67	0.55 - 0.9
	<i>top1</i> (J)	21	5.38	3.81 - 8.25			
pol2-M644L	WT (K)	21	5.64	4.77 - 12.62	30	0.60	0.5 - 0.89
	<i>rnh201</i> (L)	20	15.42	12.34 - 21.87	30	0.96	0.6 - 1.5
	<i>rnh201 top1</i> (M)	21	8.81	5.81 - 15.52	30	0.34	0.21 - 0.39
	<i>top1</i> (N)	21	3.54	2.56 - 6.62			
pol1-L868M	WT (O)	21	4.30	3.51 - 7.77	30	1.31	0.95 - 1.76
	<i>rnh201</i> (P)	21	33.16	19.05 - 45.58	30	2.97	2.27 - 3.59
	<i>rnh201 top1</i> (Q)	21	8.24	5.41 - 24.4	30	0.63	0.57 - 0.74
	<i>top1</i> (R)	21	3.21	1.68 - 5.23			
pol3-L612M	WT (S)	21	4.04	2.61 - 6.94	30	1.94	1.24 - 2.75
	<i>rnh201</i> (T)	21	12.20	7.81 - 17.52	45	4.39	3.67 - 5.87
	<i>rnh201 top1</i> (U)	21	2.85	1.62 - 4.42	30	1.04	0.63 - 1.17
	<i>top1</i> (V)	21	2.07	1.38 - 4.85			

The same numerical values presented in this table are shown graphically in Fig. 2 in the main manuscript. The genotype statistics identity (stID) for each genotype was used for reference in the pairwise comparisons shown in Table S3.

Table S3. Statistical significances of pertinent pairwise recombination rates comparisons.

Genotype 1	stID 1	Genotype 2	stID 2	LOH assay <i>p</i> -value	NAHR assay <i>p</i> -value
<i>WT</i>	A	<i>rnh201</i>	B	< 0.0001*	0.2817
<i>rnh201</i>	B	<i>rnh201 top1</i>	E	< 0.0001*	0.068
<i>WT</i>	A	<i>top1</i>	F	0.0853	0.0201*
<i>pol2-M644G</i>	G	<i>pol2-M644G rnh201</i>	H	< 0.0001*	< 0.0001*
<i>pol2-M644G rnh201</i>	H	<i>pol2-M644G rnh201 top1</i>	I	< 0.0001*	< 0.0001*
<i>pol2-M644G</i>	G	<i>pol2-M644G top1</i>	J	< 0.0001*	NA
<i>pol2-M644L</i>	K	<i>pol2-M644L rnh201</i>	L	0.0004*	0.0871
<i>pol2-M644L rnh201</i>	L	<i>pol2-M644L rnh201 top1</i>	M	0.0071*	< 0.0001*
<i>pol2-M644L</i>	K	<i>pol2-M644L top1</i>	N	0.0058*	NA
<i>pol1-L868M</i>	O	<i>pol1-L868M rnh201</i>	P	< 0.0001*	< 0.0001*
<i>pol1-L868M rnh201</i>	P	<i>pol1-L868M rnh201 top1</i>	Q	0.0001*	< 0.0001*
<i>pol1-L868M</i>	O	<i>pol1-L868M top1</i>	R	0.0181*	NA
<i>pol3-L612M</i>	S	<i>pol3-L612M rnh201</i>	T	< 0.0001*	< 0.0001*
<i>pol3-L612M rnh201</i>	T	<i>pol3-L612M rnh201 top1</i>	U	< 0.0001*	< 0.0001*
<i>pol3-L612M</i>	S	<i>pol3-L612M top1</i>	V	0.077	NA
<i>WT</i>	A	<i>pol2-M644G</i>	G	< 0.0001*	< 0.0001*
<i>WT</i>	A	<i>pol2-M644L</i>	K	0.011*	< 0.0001*
<i>WT</i>	A	<i>pol1-L868M</i>	O	0.3027	< 0.0001*
<i>WT</i>	A	<i>pol3-L612M</i>	S	0.7835	0.3928
<i>pol2-M644G rnh201</i>	H	<i>rnh201</i>	B	< 0.0001*	< 0.0001*
<i>pol2-M644L rnh201</i>	L	<i>rnh201</i>	B	0.0349*	< 0.0001*
<i>pol1-L868M rnh201</i>	P	<i>rnh201</i>	B	0.1821	0.4479
<i>pol3-L612M rnh201</i>	T	<i>rnh201</i>	B	0.0026*	< 0.0001*
<i>pol3-L612M</i>	S	<i>pol2-M644L</i>	K	0.0253*	< 0.0001*
<i>pol3-L612M rnh201</i>	T	<i>pol2-M644L rnh201</i>	L	0.1799	< 0.0001*
<i>pol2-M644G rnh201 top1</i>	I	<i>rnh201 top1</i>	E	0.0016*	< 0.0001*
<i>pol2-M644L rnh201 top1</i>	M	<i>rnh201 top1</i>	E	0.5972	< 0.0001*
<i>pol1-L868M rnh201 top1</i>	Q	<i>rnh201 top1</i>	E	0.4415	< 0.0001*
<i>pol3-L612M rnh201 top1</i>	U	<i>rnh201 top1</i>	E	< 0.0001*	< 0.0001*

* Asterisks indicate cases in which the pairwise comparison between the recombination rates obtained for genotype 1 were significantly different (*p*-value <0.05) from the recombination rates obtained from genotype 2. stID 1 and stID 2 correspond to the statistics identity for each genotype from Table S2. Statistical analyses of comparisons between recombination rates were performed using a two-sided nonparametric Mann Whitney test in GraphPad Prism.

LOH assay: Hybrid diploid strain setup

Figure S1

Figure S1. Loss-of-heterozygosity (LOH) assay system in hybrid diploids.

(A) Schematic representation of the karyotype of the hybrid diploid strains used in LOH assays. A hemizygous counter-selectable CORE2 cassette was inserted near the right end of the Chr7 homolog from the CG379 parent haploid. The Chr7 homolog from the YJM789 parent haploid did not contain this insertion. Two heterozygous sites (position 94 Kb and 1061 Kb) that coincide with the recognition sequence for the *HhaI* restriction endonuclease in one of the two Chr7 homologs are shown as hatched circles corresponding to the CG379 alleles and open circles corresponding to the YJM789 alleles. Allelic homologous recombination between the two Chr7 homologs may result in homozygosity for the YJM789 region lacking the CORE2 insertion (position 1072 Kb), and often also for the nearby 1061 Kb site within the same LOH tract. (B) and (C) Representation of the *HhaI* restriction maps of PCR products obtained using primers flanking the 94 Kb and 1061 Kb sites, respectively. The numbers indicate the expected sizes in bp of the *HhaI* restriction fragments from each allele. (D) and (E) *HhaI* digested PCR products from the 94 Kb and 1061 Kb loci, respectively, resolved by agarose gel electrophoresis showing the banding patterns for the CG379 and YJM789 alleles, and the pattern found in the parent heterozygous hybrid diploids. The numbers indicate the band sizes of the ladder (left) and restriction fragments (right). (F) Genotypes at the 94 Kb and 1061 Kb markers from 27 independently selected 5-FOA^R clones derived from hybrid diploids. All 5-FOA^R clones remained heterozygous for the 94 Kb marker, and all but one clone became homozygous for the YJM789 allele.

A. Class 3: Ura⁺, Gen^R
Complex non-reciprocal translocation

Class 3 array-CGH and PFGE:
Chr14 deletion and Chr5 amplification

B. Class 4: Ura⁺, Gen^R
Complex non-reciprocal translocation

Class 4 array-CGH and PFGE:
Chr14 amplification and Chr5 amplification

Figure S2

Figure S2. Examples of unexpected outcomes obtained from the NAHR assay.

Labeling is as described for Figure 3 in the main text. **(A)** The class 3 non-reciprocal outcome is shown to the left, with the respective sizes and structures of the associated chromosomes. The array-CGH plots and PFGE for a representative Class 3 clone shows a deletion (1 copy; pink-shaded) on the left arm of Chr14 from *TEL14L* to *PEX17*, and an amplification (3 copies; purple-shaded) on the left arm of Chr5 from *TEL05L* to *URA3*. **(B)** The class 4 non-reciprocal outcome is shown to the right, and the array-CGH plots and PFGE of a representative clone show an amplification (3 copies; purple-shaded) of part of the left arm of Chr14 from *PEX17* to *CEN14* and the entire right arm from *CEN14* to *TEL14R*. Class 4 clones have two complete copies of Chr14 and one copy of a Chr14/Chr5 translocation. The Chr5 array-CGH plot shows an amplification (3 copies; purple-shaded) on the left arm of Chr5 from *TEL05L* to *URA3*.