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Both R-loop removal and ribonucleotide excision repair activities of RNase H2 contribute substantially to chromosome stability

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Abstract

Cells carrying deletions of genes encoding H-class ribonucleases display elevated rates of chromosome instability. The role of these enzymes is to remove RNA-DNA associations including persistent mRNA-DNA hybrids (R-loops) formed during transcription, and ribonucleotides incorporated into DNA during replication. RNases H1 and H2 can degrade the RNA component of R-loops, but only RNase H2 can initiate accurate ribonucleotide excision repair (RER). In order to examine the specific contributions of these activities to chromosome stability, we measured rates of loss-of-heterozygosity (LOH) in diploid *Saccharomyces cerevisiae* yeast strains carrying the *rnh201-RED* separation-of-function allele, encoding a version of RNase H2 that is RER-defective, but partly retains its other activity. The LOH rate in *rnh201-RED* was intermediate between *RNH201* and *rnh201*. In strains carrying a mutant version of DNA polymerase ϵ (*pol2-M644G*) that incorporates more ribonucleotides than normal, the LOH rate in *rnh201-RED* was as high as the rate measured in *rnh201*. Topoisomerase 1 cleavage at sites of ribonucleotide incorporation has been recently shown to produce DNA double strand breaks. Accordingly, in both the *POL2* and *pol2-M644G* backgrounds, the LOH elevation in *rnh201-RED* was suppressed by *top1*. In contrast, in strains that incorporate fewer ribonucleotides (*pol2-M644L*) the LOH rate in *rnh201-RED* was low and independent of topoisomerase 1. These results suggest that both R-loop removal and RER contribute substantially to chromosome stability, and that their relative contributions may be variable across different regions of the genome. In this scenario, a prominent contribution of R-loop removal may be expected at highly transcribed regions, whereas RER may play a greater role at hotspots of ribonucleotide incorporation.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Keywords

ribonucleotides; R-loops; RNase H2; *RNH201*; Loss-of-Heterozygosity (LOH)

Introduction

RNA strands, as well as single and tandem ribonucleotides (rNMPs) can in some cases be transiently associated with chromosomal DNA [1, 2]. For example, during transcription the nascent mRNA chain may remain associated as a hybrid duplex with the DNA template strand (R-loops). During replication, a substantial number of single rNMPs are incorporated into newly synthesized DNA by the replicative polymerases, and delay or failure in the removal of tandem rNMPs used to prime lagging strand synthesis may cause these structures to persist. Any of these RNA-DNA associations may interfere with normal DNA transactions and thereby destabilize chromosomes. Two H-class ribonucleases are tasked with removing these RNA structures from DNA [3] (Fig. 1A). RNases H1 and H2 have a redundant role in degrading the RNA component of RNA-DNA hybrids and tandem rNMPs in DNA, with RNase H2 likely playing a more prevalent role [4]. In addition, RNase H2, but not RNase H1, has a second distinct activity, which is to initiate the accurate removal of single rNMPs incorporated into DNA (ribonucleotide excision repair - RER) [5].

Multiple studies have shown that in the absence of these enzymes (RNase H2 in particular) eukaryotic genomes can become destabilized in different ways. For example, 2–5 bp deletions accumulate at low complexity regions in RNase H2 mutants [6]. This phenotype stems from a defect in normal RER initiation, which then affords an opportunity for mutagenic processing by topoisomerase 1 at regions containing rNMPs [7–11]. In addition to nucleotide-level mutations, the absence of RNase H2 has also been shown to destabilize chromosome structure, leading to increased rates of gene conversion, gene duplication, chromosomal rearrangements, chromosome loss, and loss-of-heterozygosity (LOH) [12–18]. These structural mutations are presumably triggered by DNA lesions resulting from the accumulation and improper processing of RNase H2 substrates. For example, persistent R-loops stalling replication fork progression, leading to their eventual collapse [1]; and processing of single rNMPs by topoisomerase 1, resulting in DNA double strand breaks [9]. The relative contributions of these different sources to chromosome instability has not been fully ascertained, and contrasting views favoring one source over the other have been proposed [17, 18].

This problem has been difficult to study directly because most prior studies have used full deletions of genes encoding essential subunits of RNase H2, in which R-loop, tandem and single rNMP removal activities are concomitantly eliminated. An approach that can illuminate this issue has been proposed [19] through the use of a separation-of-function allele of the *RNH201* gene that encodes the catalytic subunit of RNase H2. This mutant, *rnh201-RED* (Ribonucleotide Excision Defective) [10], is completely unable to remove single rNMPs, but retains partial enzymatic activity for the removal of tandem rNMPs, and presumably also R-loops (Fig. 1A). Two recent studies [4, 20] reported mildly elevated chromosome instability phenotypes in *rnh201-RED* compared to wild type, but the elevation

was not as high as that seen in *rnh201*. In each case the authors concluded that RER was not a substantial contributor to chromosome stability. In this report, we measured chromosome instability in the *rnh201-RED* mutant, and in a comprehensive panel of double and triple mutant combinations with *rnh1*, *top1* and two DNA Pol ϵ (*pol2*) alleles. We also observed elevated chromosome instability in *rnh201-RED*, and it was dependent on the presence of topoisomerase 1 and on the level of rNMP incorporation by Pol ϵ . We favor an interpretation of these results in which the RER activity of RNase H2 does play a meaningful role in promoting chromosome stability, particularly at regions of the genome that are prone to frequent rNMP incorporation. An alternative model in which RER plays a lesser role is also discussed.

2. Materials and Methods

2.1. Culture media, plasmids and yeast strains

Saccharomyces cerevisiae yeast cells were cultured in conventional YPD and dropout media at 30C [21]. The strains used in this study were isogenic with the CG379 background [22], with modifications described previously [23, 24]. The genotypes of all yeast strains used are detailed in Table S1. The *rnh201-RED* allele (*rnh201-P45D-Y219A*) [10] was integrated at the native *RNH201* locus using a two-step allele replacement strategy using the pRS306 vector [25] (construction details in Table S1 footnotes). The *RNH1* gene was deleted by PCR-mediated integration of the *KanMX4* cassette [26] into strain JAY1161.

2.2. Loss-of-heterozygosity (LOH) assay and statistical analyses

The LOH assays were conducted as previously described [17]. Part of the data presented in Figure 1C and Table S2 are composites of rates reported previously plus additional cultures generated from the same strains for the present study. Recombination rates and 95% confidence intervals (CI) were determined using the method of the median [27, 28]. Specific pairwise comparisons between LOH rates were assessed for statistical significance through a non-parametric Mann-Whitney test using GraphPad Prism software (Table S3).

3. Results and Discussion

3.1. Initial observations and experimental approach

We recently reported results from three different experimental approaches in diploid yeast cells (whole genome unselected LOH; right arm of chromosome 7 [Chr7] selected LOH; and Chr5/Chr14 translocations mediated by non-allelic homologous recombination) that suggested that a defect in RER resulting from absence of the catalytic subunit of RNase H2 (*rnh201*) may contribute significantly to chromosome instability [17]. Our conclusion was based on the observations that (1) the absence of topoisomerase 1 (*top1*) suppressed the high rates of recombination measured in *rnh201*; and (2) the rates of recombination in *rnh201* could be modulated up or down in mutant Pol ϵ backgrounds more or less prone to incorporate ribonucleotides into DNA (*pol2-M644G* or *pol2-M644L*, respectively). However, we were not able to estimate the specific extent of RER's contribution to these phenotypes, as all enzymatic activities of RNase H2 were eliminated in *rnh201* (Fig. 1A).

Of the three approaches listed above, the Chr7 LOH assay was both the most straightforward from a technical standpoint, and also showed the largest phenotypic differentials between the tested genotypes. In this assay (Fig. 1B), diploid cells have a hemizygous insertion of a double *URA3* counter-selectable cassette near the right end of Chr7 (*MAL13::CORE2*). Mutations leading to loss of the cassette can be selected for by resistance to 5-fluoroorotic acid (5-FOA). Our previous characterization of the spectrum of 5-FOA resistance mechanisms [17], showed that most clones examined (26/27) were consistent with allelic interhomolog recombination leading to LOH, with breakpoints in the right arm anywhere in the long 575 Kb interval between *CEN7* and *MAL13*. None of the 27 examined clones were due to double point mutations in the *URA3* genes or to chromosome loss. The high specificity of the 5-FOA selection for LOH is likely observed because the rate of double *URA3* inactivating point mutations is predicted to be below our detection level ($<10^{-12}$), and monosomy of large chromosomes (*e.g.* Chr7 or Chr4) might be deleterious and thus difficult to recover, whereas monosomy of small chromosomes (*e.g.* Chr1 or Chr3) is tolerated relatively well [23, 29, 30].

3.2. Chromosome stabilization role of RNase H2 in the presence of RNase H1

We revisited the Chr7 LOH assay ([17]; graphic data representation in Fig. 1C; numerical data in Table S2; statistical analyses in Table S3), now including data for the *rnh201-RED* separation-of-function allele. We initially measured Chr7 LOH in the *RNH1 POL2* background, thus cells retain some tandem rNMP and R-loop removal ability, and have basal levels of rNMP incorporation (first set of columns from the left in Fig. 1C). We found that the rate of LOH in *rnh201-RED* was significantly higher than in *RNH201* ($p<0.0001$), and slightly more than half of the phenotype stimulation measured in *rnh201* (3.1-fold and 5.8-fold higher than wild type, respectively). In addition, the LOH stimulation seen in the *rnh201-RED* single mutant was significantly lowered ($p<0.0001$) in the *rnh201-RED top1* double mutant, thus the chromosome instability phenotype was dependent on the action of topoisomerase 1, possibly through double strand break formation at sites of single rNMP incorporation [9]. Since cells with the *RNH1 rnh201-RED* genotype are equipped to remove tandem rNMPs and R-loops, using the full RNase H1 and partial RNase H2 activities, we suggest that perturbation of proper RER initiation plays a meaningful role in destabilizing yeast chromosomes. According to this interpretation, our results would indicate that, in the absence of other genetic defects, the RER and tandem rNMP/R-loop removal activities of RNase H2 make approximately equal contributions to preventing LOH on the right arm of Chr7.

A plausible alternative interpretation would be that RER does not play a role in preventing LOH, and that the mild phenotype observed in *rnh201-RED* is caused by the partial defect in cleavage of tandem rNMPs [10]. This model was favored by Epshtein *et al.* in their recent analysis of gene duplication at two independent reporter loci [20]. These authors reported intermediate recombination rates in *rnh201-RED*, reaching approximately one third of the stimulation seen in *rnh201*, and concluded that the RER defect was not a prominent contributor to locus-specific instability, since changing the frequency of rNMP incorporation with mutant Pol ϵ alleles did not significantly influence the duplication rates. These Pol ϵ

results were in contrast to our earlier and present measurements of regional chromosomal instability (*i.e.* 575 Kb Chr7 LOH; see item 3.4 below).

3.3. Chromosome stabilization role of RNase H2 in the absence of RNase H1

Next we investigated how the absence of tandem rNMP and R-loop removal carried out by RNase H1 can influence the phenotypes of RNase H2 mutants (second set of columns from the left in Fig. 1C). While in principle RNases H1 and H2 both have redundant tandem rNMP and R-loop removal activities, RNase H2's activity is thought to be more prevalent, having a global genomic role, whereas RNase H1's role might be confined to a subset of R-loop forming regions [4]. Thus, loss of RNase H1 by itself should have a limited impact on chromosome instability. Consistently, the rate of Chr7 LOH in the *rnh1* single mutant did not significantly change relative to wild type ($p=0.8133$), but increased by twelve fold in the *rnh1 rnh201* double ($p<0.0001$), in agreement with previous reports [4, 18]. This pronounced elevation in LOH when both RNase H's are absent underscores how important tandem rNMP/R-loop removal is for chromosome stabilization as a whole. However, the rate of LOH was elevated 3-fold in the *rnh1 rnh201-RED* double mutant compared to *rnh1 RNH201* ($p<0.0001$), and was similar to the rate in *RNH1 rnh201-RED* ($p=0.4111$). Likewise, the degree of suppression of the LOH rate was also similar between *rnh1 rnh201-RED top1* and *RNH1 rnh201-RED top1* ($p=0.6190$). Therefore, if we assume that the residual tandem rNMP removal activity present in *rnh201-RED* is sufficient to adequately process these substrates and R-loops, then an approximately 3-fold elevation in Chr7 LOH can be assigned to a specific defect in RER. Following this rationale, about 25% of the elevation in Chr7 LOH in the *rnh1 rnh201* double mutant can be attributed to a defect in RER, while the remaining 75% was due to a defect in tandem rNMP/R-loop removal.

These results align well with those reported recently for a Chr3 instability assay that detects LOH within a relatively narrow region (98 Kb) plus Chr3 monosomy, each class accounting for about half of the overall instability rate [4]. However, that study did not report chromosome instability rates for the *RNH1 rnh201-RED* single mutant, and therefore was unable fully contextualize the difference between the chromosome stabilization contributed by the RER and tandem rNMP/R-loop removal activities of RNase H2, versus the contributions of RER and the combined tandem rNMP/R-loop removal activities from RNase H1 and H2.

3.4. The abundance of rNMPs in DNA modulates the contribution of RER to chromosome stability

We interpret the results above to mean that RER does play a role in promoting chromosome stability. If this were indeed the case, then its contribution should be modulated up or down depending on the frequency of rNMP incorporation during DNA replication. We generated *rnh201-RED* mutant combinations with *pol2-M644G* and *pol2-M644L*, which respectively, encode versions of Pol ϵ that incorporate ~11-fold more or ~3-fold fewer rNMPs compared to the wild type enzyme (third and fourth set of columns from the left in Fig. 1C). As we reported previously, the *pol2-M644G* mutation causes an overall elevation in Chr7 LOH [17]. In this study, we found that the LOH rate in the *rnh201-RED pol2-M644G* was very

high and was indistinguishable from *rnh201 pol2-M644G* ($p=0.9330$). In both *rnh201-RED pol2-M644G* and *rnh201 pol2-M644G* backgrounds, similarly robust reductions in LOH resulted when combined with *top1*. These data suggested that under conditions in which DNA is overloaded with rNMPs, the contribution of RNase H2 to chromosome stability is strongly dependent on its RER role, and the contribution from tandem rNMP/R-loop removal becomes relatively minor.

We also asked whether the absence of RNase H1 could influence the LOH rates in the *RNH201 pol2-M644G* and *rnh201-RED pol2-M644G* backgrounds. The LOH rate in *rnh1 RNH201 pol2-M644G* was marginally lower (-25%) than the rate in *RNH1 RNH201 pol2-M644G* ($p=0.0288$, but with overlapping CIs). In the case of *rnh1 rnh201-RED pol2-M644G*, the LOH rate was slightly higher ($+50\%$, $p=0.0068$, also with overlapping 95% CIs) than the rate in *RNH1 rnh201-RED pol2-M644G*. This mild elevation might suggest the possibility that a small number of tandem rNMPs are occasionally incorporated by the mutant Pol ϵ . These rNMP cluster regions could lead to transcription stalling and thereby to stable R-loops.

In contrast to the *pol2-M644G* background, the LOH rate in the *rnh201-RED pol2-M644L* was very low, and actually somewhat lower than in *RNH201 pol2-M644L* ($p=0.0007$, with overlapping CIs), but no further LOH decrease was observed between *rnh201-RED pol2-M644L TOP1* and *rnh201-RED pol2-M644L top1* ($p=0.3795$). This indicated that when rNMPs are not incorporated into DNA at an appreciable frequency, the RER activity of RNase H2 no longer plays a meaningful role in chromosome stability, shifting the balance completely toward its role in tandem rNMP/R-loop removal.

4. Conclusions

Taken together, our results support the conclusion that both enzymatic activities of RNase H2 contribute substantially to the preservation of chromosomal integrity. We showed that the relative contribution of the RER activity is higher when more rNMPs are incorporated into the DNA, and is negligible when the ribonucleotide load is light. A similar modulation of the RER role might also be observed if the ribo- to deoxyribo-ratio in the cell's nucleotide pool is altered, for example by inhibition of ribonucleotide reductase. Likewise, the relative contribution of tandem rNMP/R-loop removal activities to chromosomal stability can be modulated by the frequency of R-loop formation, as had been demonstrated previously and further elucidated recently [1, 4]. This flexibility suggests that both activities might, in fact, be critically and simultaneously important. Long and functionally diverse regions of chromosomes, such as the right arm of Chr7 examined here, include loci that are both prone to stable R-loop formation [31] and hotspots for ribonucleotide incorporation [32]. Therefore, in order to globally maintain whole chromosomes as stable structures, the two RNase H's must coordinate their enzymatic activities to efficiently remove each type of RNA structure from DNA according to the local context in which they form.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Both enzymatic activities of RNase H2 contribute to chromosome stability
- Each activity makes a roughly equal contribution across a long chromosomal region
- The role of RER is dependent on Top1 and ribonucleotide incorporation by Pol ϵ

Figure 1. Substrates of H-Class RNases, experimental system, and LOH rate analysis

(A) Schematic representation of the substrate specificity of H-Class RNases. The black arrow lines link the various enzymes to their RNA-DNA substrates. The gray dashed arrow line indicates that evidence of topoisomerase 1 cleavage at ribonucleotides has only been observed in the absence of functional RER. (B) Depiction of the hemizygous chromosomal configuration used in the LOH assay. One of the homologs of Chr7 (dark green) has an insertion of the counter-selectable CORE2 cassette (K_U URA3- S_C URA3-KanMX4) [24] downstream of the *MAL13* gene (distal side), ~20 kb from the right telomere (7R); the second homolog (light green) does not. A DNA lesion on the right arm of the dark green homolog may initiate an allelic mitotic recombination event leading to LOH, making the distal portion of the chromosome homozygous for the light green DNA sequence, and thus rendering that cell resistant to 5-FOA. (C) Quantitative analysis of LOH. The columns represent the median Chr7 right arm LOH rate for each genotype, and the error bars represent 95% confidence intervals (CI). The same data are presented in numeric form in Table S2, and statistical significance of pairwise comparisons are available in Table S3. All genotypes indicated in the X-axis are homozygous in the experimental diploids. The *greater than* (>) symbol indicates that the Y-axis was cropped to save space and to facilitate visualization of differences between the lower rates. The upper 95% CI limit of the *pol2-M644G rnh1 rnh201-RED TOP1* LOH rate was 182.48×10^{-5} /cell/division. Intentional gaps were left in the data columns for the *POL2 rnh1 rnh201 top1* and the *pol2-M644G*

rnh1 rnh201 TOP1 genotypes to emphasize the fact that these triple mutant combinations are synthetic lethal as reported previously [33–35].