Harmful DNA:RNA hybrids are formed in cis and in a Rad51-independent manner

Juan Lafuente-Barquero, María Luisa García-Rubio, Marta San Martín-Alonso, Belén Gómez-González*, Andrés Aguilera*

Centro Andaluz de Biología Molecular y Medicina Regenerativa-CABIMER, Universidad de Sevilla-CSIC-Universidad Pablo de Olavide, Seville, Spain

Abstract DNA:RNA hybrids constitute a well-known source of recombinogenic DNA damage. The current literature is in agreement with DNA:RNA hybrids being produced co-transcriptionally by the invasion of the nascent RNA molecule produced in cis with its DNA template. However, it has also been suggested that recombinogenic DNA:RNA hybrids could be facilitated by the invasion of RNA molecules produced in trans in a Rad51-mediated reaction. Here, we tested the possibility that such DNA:RNA hybrids constitute a source of recombinogenic DNA damage taking advantage of Rad51-independent single-strand annealing (SSA) assays in the yeast Saccharomyces cerevisiae. For this, we used new constructs designed to induce expression of mRNA transcripts in trans with respect to the SSA system. We show that unscheduled and recombinogenic DNA:RNA hybrids that trigger the SSA event are formed in cis during transcription and in a Rad51-independent manner. We found no evidence that such hybrids form in trans and in a Rad51-dependent manner.

Introduction

R loops are structures formed by a DNA:RNA hybrid and the complementary displaced single stranded DNA (ssDNA). They were observed naturally as programmed events in specific genomic sites such as the S regions of Immunoglobulin genes in mammals or mitochondrial DNA (Chang et al., 1985; García-Muse and Aguilera, 2019; Yu et al., 2003), where they play specific functions by promoting class switch recombination or DNA replication, respectively; but also as unscheduled non-programmed structures upon dysfunction of RNA binding proteins involved in the assembly or processing and export of the protein-mRNA particle (mRNP) such as the THO complex or the SRSF1 splicing factor (Huertas and Aguilera, 2003; Li and Manley, 2005). Also, they have been inferred in the rDNA regions of the bacterial chromosome upon Topo I inactivation (Drolet et al., 1995). Accumulated evidence indicates that R loops are detected from yeast to humans in many transcribed regions of the eukaryotic genome in wild-type cells, in cells defective in several metabolic processes covering from RNA processing to DNA replication and repair and in cells deficient in specific chromatin factors (Bhatia et al., 2014; García-Muse and Aguilera, 2019; García-Rubio et al., 2015; Herrera-Moyano et al., 2014; Mischo et al., 2011; Paulsen et al., 2009; Schwab et al., 2015). The biological consequences of such R loop structures are diverse and include replication stress, DNA breaks and genome instability that can be detected as hyperrecombination, plasmid loss or gross chromosomal rearrangements (García-Muse and Aguilera, 2019). Indeed, DNA:RNA hybrids have been inferred by their potential to induce DNA damage and recombination, but they can also be directly detected via different methodologies. These include electrophoresis detection after nuclelease treatment, bisulfite mutagenesis or either in situ immunofluorescence or DNA:RNA Immuno-Precipitation (DRIP) using the S9.6 anti-DNA:RNA monoclonal antibody (García-Muse and Aguilera, 2019).
The increasing number of reports showing R loop accumulation in different organisms from bacteria to human cells, and the relevance of their functional consequences, whether on genome integrity, chromatin structure and gene expression suggest that most DNA:RNA hybrids are compatible with a co-transcriptional formation (García-Muse and Aguilera, 2019). This is consistent with the idea that it is the RNA produced in cis the one that invades the duplex DNA, a reaction that can be facilitated by DNA sequence and supercoiling (Stolz et al., 2019) as well as by nicking of the DNA template (Roy et al., 2010). The evidence of DNA:RNA hybrid formation at breaks has matured in the last years (Cohen et al., 2018; D’Alessandro et al., 2018; Li et al., 2016; Ohle et al., 2016; Teng et al., 2018; Yasuhara et al., 2018) although the source and role of such hybrids remains still controversial (Aguilera and Gómez-González, 2017; Puget et al., 2019). Of note, genome-wide mapping results have been interpreted in diverse manners by different labs. Whereas some claim that DNA:RNA hybrids detected around DNA breaks mostly accumulate at transcribing sites (Cohen et al., 2018), in agreement with their co-transcriptional formation, others suggest that there is no preference for DNA:RNA hybrids to form at transcribed loci in human cells (D’Alessandro et al., 2018), implying a scenario in which DNA:RNA hybrids at break sites would form either de novo or with RNAs produced at different loci (in trans). Moreover, it has been shown in yeast that short RNAs can be used as templates for the recombinational repair of DSBs in a reaction catalyzed by Rad52 (Keskin et al., 2014).

DNA:RNA hybrids can also form in vitro with the aid of the bacterial DNA strand exchange protein RecA (Kasahara et al., 2000; Zaitsev and Kowalczykowski, 2000). In vivo, DNA:RNA hybrids are formed with RNAs produced in trans as intermediates in the course of ribonucleoprotein-mediated reactions such as telomerase and CRISPR-Cas9 ribonucleoprotein involved in specific reactions (Collins, 2000; Jinek et al., 2012). They have also been reported to have regulatory roles in gene expression when formed by long non-coding RNAs (IncRNAs) at in trans loci such as the cases of the GAL IncRNA in yeast (Cloutier et al., 2016) or the APOLO IncRNA in plants (Ariel et al., 2020). In summary, despite the accumulating evidence that in vivo DNA:RNA hybrids formed in cis constitute a threat for genome stability, an open question is whether DNA:RNA hybrids also form in trans as a potential source of recombinogenic DNA damage. To our knowledge, this has only been addressed in the yeast Saccharomyces cerevisiae (Wahba et al., 2013). By S9.6 immunofluorescence (IF) and a yeast artificial chromosome-based genetic assay that measures gross chromosomal rearrangements, it was inferred that DNA:RNA hybrids could be formed with RNAs produced in trans by a reaction catalyzed by the eukaryotic DNA strand exchange protein Rad51 (Wahba et al., 2013). Nevertheless, the fact that the detected gross chromosomal rearrangements could depend on Rad51 and that the S9.6 antibody can also recognize dsRNAs (Hartono et al., 2018; König et al., 2017; Silva et al., 2018), prompted us to address this question using a different approach. Using Rad51-independent recombination assays in which the initiation region could be unambiguously delimited, we do not find evidence for recombinogenic DNA:RNA hybrids forming in trans. Instead, we provide genetic evidence that DNA:RNA hybrids compromising genome integrity are formed in cis and in a Rad51-independent manner.

Results

A new genetic assay to detect recombinogenic DNA:RNA hybrids with RNA produced in trans

We developed a new genetic assay to infer the formation of recombinogenic DNA:RNA hybrids with RNAs produced in trans. It is based on two plasmids, one containing the recombination system and the LacZ gene in cis (GL-LacZ recombination system), and another one providing the in trans LacZ transcripts (tet::LacZ) (Figure 1). The bacterial LacZ gene consists of a 3 Kb sequence with high G+C content previously reported to be hyper-recombinant and difficult to transcribe in DNA:RNA hybrid-accumulating strains, such as tho mutants (Chávez et al., 2001).

The GL-LacZ recombination system is a leu2 direct-repeat construct carrying the LacZ gene in between and under the GAL1 inducible promoter so that this construct is transcribed as a single RNA unit driven from the GAL1 promoter (Piruat and Aguilera, 1998). Single-Strand Annealing (SSA) events cause the deletion of the LacZ sequence and one of the leu2 repeats leading to Leu+ recombinants in a Rad51-independent manner (Figure 1A). To provide LacZ transcripts in trans, we
used a fusion construct containing the complete bacterial LacZ gene sequence under the doxycycline-inducible tet promoter (tet<sub>p</sub>:LacZ). As a control of no expression in trans, we used transformants with an empty plasmid to avoid any possible effect from leaky transcription from the tet<sub>p</sub>:LacZ construct.

Yeast strains carrying both GL-LacZ recombination system and the tet<sub>p</sub>:LacZ construct were used to assay SSA events in the four different possible conditions: i) no transcription, with GL-LacZ construct turned transcriptionally off (2% glucose) and an empty plasmid; ii) transcription in trans, with GL-LacZ construct turned transcriptionally off (2% glucose) and the tet<sub>p</sub>:LacZ construct; iii) transcription in cis, with GL-LacZ construct turned transcriptionally on (2% galactose) and an empty plasmid; and iv) transcription in cis and in trans, with GL-LacZ construct turned transcriptionally on (2% galactose) and the tet<sub>p</sub>:LacZ construct.

The online version of this article includes the following source data and figure supplement(s) for figure 1:

Figure supplement 1. LacZ expression levels in the GL-LacZ and tet<sub>p</sub>:LacZ constructs.

Figure supplement 1—source data 1. Relative RNA levels at the LacZ gene from GL-LacZ and tet<sub>p</sub>:LacZ constructs.
and iv) transcription in cis and in trans, with GL-LacZ construct turned transcriptionally on (2% galactose) and the tet<sub>p</sub>:LacZ construct (Figure 1B).

**RNAs produced in trans are not a spontaneous source of recombinogenic DNA damage**

The analysis of recombination in wild-type cells revealed that whereas the stimulation of transcription in cis elevated the frequency of recombination threefold, the stimulation of transcription in trans driven from the tet<sub>p</sub>:LacZ construct had no effect on recombination (Figure 2A). These results already suggest that homologous transcripts coming from a different locus do not represent a detectable source of genetic instability in wild-type conditions and thus argue against the hypothesis that spontaneous DNA:RNA hybrids could be formed with mRNAs generated in trans. However, it is known that mRNA coating protects DNA from co-transcriptional RNA hybridization. Thus, we wondered if transcripts produced in trans could induce recombination in mRNP-defective mutants such as those of the THO complex. Hence, we performed our experiments in mft1Δ and hpr1Δ mutant strains. mft1Δ and hpr1Δ enhanced recombination slightly when transcription in cis was switched off (Figure 2A), likely as a consequence of leaky transcription form the GAL1 promoter in glucose (Figure 1—figure supplement 1). More significantly and in agreement with previous reports (Chávez et al., 2000), recombination frequencies rocketed when transcription was stimulated in cis. However, transcription activation in trans did not enhance recombination, as it would be expected if additional DNA:RNA hybrids could form with RNA produced in trans.

Instead, under conditions of high transcription of the recombination system (transcription in cis), RNA driven from an ectopic locus (transcription in trans) led to a partial suppression of the hyper-recombination. The reason for such suppression might involve the potential ability of the remotely produced RNAs to interfere with transcription occurring at the GL-LacZ construct. Given that a DNA:RNA hybrid produced in the template DNA strand can impair transcription elongation (Tous and Aguilera, 2007), one possibility would be that this interference is mediated by DNA:RNA hybrids formed between the RNA produced in trans and the transcribed DNA strand of the GL-LacZ construct. To rule out this possibility, we used an alternative recombination system (GL-LacZi), in which the LacZ sequence was inverted so that the LacZ transcript produced in trans would not be able to anneal with the transcribed DNA strand of the GL-LacZi system (Figure 2B). We detected a strong hyper-recombination in hpr1Δ cells when the LacZ sequence was transcribed in agreement with previous reports and with the fact that it has been shown that it is the length (and the GC content) but not the orientation of the lacZ sequence what impairs transcription and triggers hyper-recombination (Chávez and Aguilera, 1997; Chávez et al., 2001). Surprisingly, the production of RNAs in trans from the tet::LacZ construct also led to a reduction of the hyper-recombination in this system. Furthermore, in this case, the suppression was stronger and was also observed in glucose, when transcription in cis was off. This could be explained because, in this scenario, the RNA produced in trans is complementary to the mRNA produced in cis. Consequently, they can hybridize together forming a dsRNA that would preclude the possibility to form DNA:RNA hybrids at the GL-LacZi construct.

Since transcription from the long LacZ gene is inefficient and leads to unstable RNA products, particularly in tho mutants (Chávez et al., 2001), we made a new construct with only the last 400 bp of LacZ (tet<sub>p</sub>:LacZ400) (Figure 2C). Strikingly, in this case, we observed no suppression of the tho-induced hyper-recombination by the production of RNA in trans. More importantly and again, recombination frequencies were not significantly enhanced by transcription in trans in any of the strains or conditions tested, further arguing against mRNA produced in trans as a possible source of recombinogenic DNA:RNA hybrids.

The THO complex is thought to prevent R-loops mainly by promoting a proper mRNA-protein assembly (Luna et al., 2019), whereas the two RNase H enzymes efficiently degrade the RNA moiety of DNA:RNA hybrids once formed (Cerritelli and Crouch, 2009). Thus, to favor DNA:RNA hybrid accumulation, we used cells lacking both RNases H1 and H2 and we determined the impact on SSA. Figure 2A and C show that rnh1Δ rnh201Δ cells elevated the recombination frequency when transcription was stimulated in cis, as expected. Importantly, the recombination frequencies were not altered by producing transcripts in trans, arguing again against the recombinogenic potential of putative DNA:RNA hybrids formed with RNAs produced in trans.

DOI: https://doi.org/10.7554/eLife.56674
Figure 2. Analysis of the effect on genetic recombination of RNA produced in cis or in trans. (A) Recombination analysis in WT (W303), mft1Δ (WMK.1A) and hpr1Δ (U678.4C) strains carrying GL-LacZ plasmid system (pRS314-GL-LacZ) plus either the pCM190 empty vector or the same vector carrying the LacZ gene (pCM179). (B) Recombination analysis in WT (W303), mft1Δ (WMK.1A) and hpr1Δ (U678.4C) strains carrying GL-LacZ plasmid plus either the pCM190 empty vector or the same vector carrying the sequence of the LacZ gene (pCM179). (C) Recombination analysis in WT (W303), mft1Δ mnh201Δ (HRN2.10C) and hpr1Δ (U678.4C) strains carrying GL-LacZ plasmid system (pRS314-GL-LacZ) plus either the pCM190 empty vector or the same vector carrying the last 400 bp from the 3′ end of the LacZ gene (pCM190: LacZ400). In all panels, average and SEM of at least three independent experiments consisting in the median value of six independent colonies each are shown. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ****, p ≤ 0.0001 (unpaired Student’s t-test).

The online version of this article includes the following source data for figure 2:

Source data 1. Analysis of the effect on genetic recombination of RNA produced in cis or in trans.
In order to confirm DNA:RNA hybrid formation in these different sequence contexts, we performed DRIP experiments at the LacZ sequence (Figure 3) and we observed that DNA:RNA hybrids accumulate in both, hpr1Δ and rnh1Δ rnh201Δ mutants, and in all GL-LacZ, tet:LacZ and GL::lacZ1 sequences as expected, despite the technical difficulty of detecting increases in hybrid accumulation in plasmids, since they cause plasmid loss.

Given that the levels of transcription from the GAL1 and tet promoters used for the constructs are very different (Figure 1—figure supplement 1), we decided to perform recombination tests with similar constructs in which the promoters were interchanged. Thus, we studied recombination in the TL-LacZ recombination system (Santos-Pereira et al., 2013) and used a GAL::LacZ construct to produce the LacZ transcripts from a remote locus. Figure 4 shows that, whereas transcription at the TL-LacZ recombination system enhanced recombination as previously published (Santos-Pereira et al., 2013), again no significant stimulation of recombination was detected when RNAs were produced in trans in either wild-type, hpr1Δ or rnh1Δ rnh201Δ cells even when the RNA was generated from the strong GAL1 promoter.

Finally, since all experiments were performed in plasmid-born systems in the original W303 background bearing the rad5-G535R mutation (Fan et al., 1996), we integrated the GL-LacZ system in the chromosome of a RAD5 wild-type strain to ascertain that the rad5-G535R mutation did not affect the results as well as to confirm that the results were the same in a chromosome locus. As it can be seen in Figure 5, transcription of the chromosomal recombination system promoted a 30-fold increase in recombination levels in the ℎ mutant hpr1Δ with respect to the WT, in agreement with all previous data showing that co-transcriptional DNA:RNA hybrids are a potent source of recombination. By contrast, mRNA produced at a different locus had no effect on recombination, neither in wild-type cells nor in the ℎ mutant hpr1Δ. Hence, altogether, these results argue that, in contrast to mRNA produced in cis, RNA produced at a particular locus does not lead to recombinogenic DNA damage at regions located in trans.

**Rad51 is not required for DNA:RNA hybridization**

We next wondered about the possible role of the recombination protein Rad51 in DNA:RNA hybridization. To examine this, we analyzed in hpr1Δ cells the effect of transcribing the ectopic tet::LacZ construct on recombination in our direct-repeat systems when these were not transcribed (Figure 6). It is important to remark that the recombination events detected in our assays are deletions occurring by SSA between direct repeats, which do not require Rad51 (Pardo et al., 2009). Indeed, in agreement with SSA annealing being Rad51-independent, RAD51 deletion caused no significant changes in the recombination frequencies in our assay. Thus, any conclusion about Rad51-dependency or independency of the hybridization inferred from our assay is not contaminated by a possible direct role of Rad51 in the event we are studying. Importantly, we observed no differences when RAD51 was deleted in hpr1Δ cells even when the LacZ sequence was expressed from the plasmid containing the tet::LacZ construct. This result argues against Rad51 facilitating or impeding the formation of DNA:RNA hybrids with RNAs produced in trans.

We then wondered whether the formation of known recombinogenic DNA:RNA hybrids formed in cis, such as those reported in the hpr1Δ mutant, requires Rad51. For this purpose, we studied the effect in the strong hyper-recombination phenotype of hpr1Δ when transcription was induced in cis. As shown in Figure 6, the absence of Rad51 had no effect on the hyper-recombination observed, as hpr1Δ rad5Δ cells elevated the recombination frequency more than 70-fold with respect to rad5Δ, similarly to Rad51+ cells. This result clearly indicates that the in cis DNA:RNA hybrid-mediated hyper-recombination phenotype is actually independent on Rad51.

In parallel, we studied the formation of Rad52 foci, a marker of recombinogenic DNA breaks (Lisby et al., 2001), in which case we used AID overexpression to enhance the recombinogenic potential of R loops (Gómez-González and Aguilera, 2007) and RNase H overexpression to remove DNA:RNA hybrids (Figure 7A). In agreement with the role of the THO complex in R loop prevention, hpr1Δ caused an increase in Rad52 foci that was enhanced by AID overexpression and suppressed by RNase H overexpression, as previously reported (Alvaro et al., 2007; García-Pichardo et al., 2017; Wellinger et al., 2006). By contrast, the accumulation of Rad52 foci observed in rad5Δ cells was not affected by either AID or RNase H overexpression. This result argues that R loops are not the cause for the genetic instability observed in the absence of Rad51. The accumulation of Rad52 foci in rad5Δ cells is rather likely due to the accumulation of unrepaird recombination
Figure 3. Detection of co-transcriptional DNA:RNA hybrids in hpr1Δ and mh1Δ rhn201Δ mutants at the LacZ-containing constructs under the GAL1 or tet promoters. DNA:RNA Immuno-Precipitation (DRIP) with the S9.6 antibody in WT (W303), hpr1Δ (U678.4C) and mh1Δ rhn201Δ (HRN2.10C) strains in asynchronous cultures treated or not in vitro with RNase H in the GL-LacZ, tetp:LacZ and GL-LacZi constructs turned transcriptionally off (2%}

Figure 3 continued on next page
intermediates, as previously suggested (Alvaro et al., 2007). Importantly, hpr1Δ rad51Δ cells showed a similar result, further supporting that the accumulation of recombinogenic damage in hpr1Δ cells is independent on Rad51. Consequently, we next directly measured DNA:RNA hybrid accumulation by immunodetection with the S9.6 antibody on metaphase spreads. Figure 7B illustrates that the number of cells with S9.6 positive signal was similar in hpr1Δ and in hpr1Δ rad51Δ cells. Altogether, these results demonstrate that the Rad51 protein is not required for the DNA:RNA hybrid formation previously reported in THO mutants.

Figure 3 continued

Glucose or 5 μg/mL doxycycline or on (2% galactose and in the absence of doxycycline). Average and SEM of three independent experiments are shown *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001 (unpaired Student’s t-test).

The online version of this article includes the following source data for figure 3:

Source data 1. Detection of co-transcriptional DNA:RNA hybrids.

Figure 4. Analysis of the effect on genetic recombination of RNA produced in cis or in trans with different promoters. Recombination analysis in WT (W303), hpr1Δ (U678.4C) and mh1Δ mh201Δ (HRN2.10C) carrying TL-LacZ plasmid system (pCM184-TL-LacZ) plus either the pRS416 empty vector or the same vector carrying the LacZ gene (pRS416-GAL-LacZ). In this case, the four combinations studied were: i) no transcription, with TL-LacZ construct turned transcriptionally off (5 μg/mL doxycycline) and an empty plasmid; ii) transcription in trans, with TL-LacZ construct turned transcriptionally off (5 μg/mL doxycycline) and the GAL-LacZ construct switched on (2% galactose); iii) transcription in cis, with TL-LacZ construct turned transcriptionally on and an empty plasmid; and iv) transcription in cis and in trans, with TL-LacZ construct turned transcriptionally on and the GAL-LacZ construct switched on (2% galactose). Average and SEM of at least three independent experiments consisting in the median value of six independent colonies each are shown. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ****, p ≤ 0.0001 (unpaired Student’s t-test).

The online version of this article includes the following source data for figure 4:

Source data 1. Analysis of the effect on genetic recombination of RNA produced in cis or in trans with different promoters.

Lafuente-Barquero et al. eLife 2020;9:e56674. DOI: https://doi.org/10.7554/eLife.56674
Discussion

We have devised a new genetic assay to infer whether the source of DNA:RNA hybrids compromising genome integrity could potentially come from RNAs produced in trans. To reach this conclusion, we used an SSA assay. It is well established that SSA events are Rad51-independent; they do not require DNA strand exchange, but just annealing between resected single-stranded DNA (ssDNA) for which the action of Rad52 is sufficient (Figure 1A; Pardo et al., 2009). Our constructs show that, in contrast to the RNA produced at the site where SSA occurs, an RNA produced in a remote locus does not induce an increase in homology-directed repair. Importantly, recombination is not induced by in trans RNA production even when the major DNA:RNA removal machinery is absent (rnh1Δ rnh201Δ mutant) or when the RNA coating functions preventing DNA:RNA hybrid formation are impaired (tho mutants), arguing against the idea that harmful DNA:RNA hybrids could spontaneously form in trans and constitute a menace for genome integrity. Co-transcriptional R-loops are responsible for the hyper-recombination of hpr1Δ as reported previously (Huertas and Aguilera, 2003). Putative DNA:RNA hybrids formed in trans would be expected to further increase recombination levels. Instead, the simultaneous induction of transcription in cis and in trans (Figure 2A) reduced the strong hyper-recombinogenic effect of tho mutants. The fact that this suppressor effect was augmented when one of the LacZ sequences was inverted (Figure 2B) and prevented by a shorter LacZ construct (Figure 2C), which was reported to be more stable in tho mutant backgrounds (Chávez et al., 2001), suggests that the free RNA itself, and not in the form of DNA:RNA hybrids formed at the template DNA strand, plays some role in preventing the hyper-recombination, likely because stable RNAs can interfere with transcription at a homologous locus. However, no suppressor effect was observed when the recombination system was placed in a chromosome (Figure 5) or when the ectopic RNA was transcribed from the GAL1 promoter (Figures 2 and 4).
DNA:RNA hybrids formed with an RNA produced in trans were previously suggested to threaten genome integrity (Wahba et al., 2013). This conclusion was based on experiments performed with a yeast artificial chromosome after the induction of transcription of a homologous region placed at chromosome III. Recombination involving multiple substrates was first reported in S. cerevisiae, in which an induced-DSB triggered recombination between two other homologous fragments at different chromosomes (Ray et al., 1989). Tri-parental recombination assays have been successfully used since then to define specific features of the HR reaction as well as for studies of Break-Induced Recombination (BIR) or translocations and chromosomal rearrangements occurring between ectopic regions (Pardo and Aguilera, 2012; Piazza et al., 2017; Ruiz et al., 2009). However, such events are not the most adequate to infer recombination initiation unless this has been artificially induced (as is the case of an HO-induced DSB). Hence, the assay used to infer the potential of DNA:RNA hybrids formed with RNAs produced in trans to induce genetic instability (Wahba et al., 2013) relied on an RNA fragment produced at a (first) DNA region that could form a DNA:RNA hybrid with a (second) ectopic homologous DNA region that would promote its deletion or loss, leading to a genetically detectable phenotype. Thus, this assay does not exclude the possibility that the RNA forms the hybrid in cis inducing subsequently a DSB that would stimulate the recombination events studied (Figure 6). Indeed, this event would demand the action of Rad51 for DNA strand invasion, consistent with the results obtained (Wahba et al., 2013). Therefore, the increased genetic instability observed could be explained by the invasion of the 3’ end of a DNA break induced by the DNA:RNA hybrid formed at the first site (Figure 8) rather than implying that Rad51 is required for the RNA to invade the second DNA sequence.

**Figure 6.** Analysis of the effect on genetic recombination of RNA produced in trans with or without Rad51. Recombination analysis in WT (W303), hpr1Δ (U678.1C), rad51Δ (WSR51.4A) and hpr1Δ rad51Δ (HPR51.15A) strains carrying GL-LacZ plasmid system (pRS314-GL-LacZ) plus either the pCM190 empty vector or the same vector carrying the LacZ gene (pCM179). Average and SEM of at least three independent experiments consisting in the median value of six independent colonies each are shown. *, p<0.05; **, p<0.01, ***, p<0.001 (unpaired Student’s t-test).

The online version of this article includes the following source data for figure 6:

**Source data 1.** Analysis of the effect on genetic recombination of RNA produced in trans with or without Rad51.
In our case, however, we show that the hyper-recombinogenic potential of DNA:RNA hybrids is Rad51-independent (Figure 4). Our assays involve two \textit{leu2} homologous repeats that recombine by Rad51-independent SSA. Indeed, as expected, \textit{RAD51} deletion caused no decrease in the observed recombination frequencies in our assay (Figure 5). Recombination between the \textit{leu2} repeats could be originated by either a DNA:RNA hybrid in cis or by a DSB occurring in between the repeats, or as suggested previously for \textit{tho} mutants, by a bypass mechanism involving template switching.
It is worth noting that although we are depicting the SSA reactions as being initiated by a DSB (Figures 1 and 8), we cannot discard that the initial lesion triggered by a hybrid is a nick or ssDNA gap, as previously proposed for the mutants (Gómez-González et al., 2009).

Similarly, a DSB occurring at the locus where the RNA in trans was generated could give rise to Leu+ recombinants in our assay. However, such recombination events would be Rad51-dependent, as they will require a Rad51-dependent invasion into the GL-LacZ construct (Figure 8). Hence, the Leu+ recombinants obtained in rad51Δ mutant cells (Figure 5) can only be explained by Rad51-independent events occurring in cis, at the GL-LacZ construct. Strikingly, the fact that we detected no significant increase in Leu+ recombinants by inducing transcription in trans, either in RAD51 or rad51Δ backgrounds rules out the possibility that recombinogenic DNA:RNA hybrids form in trans in our assay. It was previously shown that S9.6 signal detected by IF was reduced by rad51Δ in metaphase spreads (Wahba et al., 2013). By contrast, we detected S9.6 signal in metaphase spreads of the hpr1Δ mutant of the THO complex in both RAD51 and rad51Δ backgrounds (Figure 7B).

The uncertainty about the identity of the structures detected by IF using the S9.6 antibody, which also recognizes dsRNA (Hartono et al., 2018; König et al., 2017; Silva et al., 2018), and the possibility that chromosomal spreads could preferentially visualize the rDNA regions, in which high levels of dsRNA structures formed by the rRNAs, makes difficult to make conclusions on S9.6 IFs in this case.

Thus, we have found no evidence for a Rad51-facilitated strand invasion from RNAs produced in trans. Further arguing against any major role of this recombinase in R loop metabolism or function, none of the so far reported DNA:RNA hybrid interactomes has identified RAD51 (Cristini et al., 2018; Nadel et al., 2015; Wang et al., 2018). The fact that, in vitro, RecA can catalyze an inverse DNA strand exchange reaction with DNA or RNA thus promoting the assimilation of a transcript into duplex DNA (Kasahara et al., 2000; Zaitsev and Kowalczykowski, 2000) does not argue that this is the case for unscheduled recombinogenic R loops in vivo. More likely, the biological significance of this process relies on its use for replication initiation of prokaryotic cells as originally proposed (Zaitsev and Kowalczykowski, 2000), for replication-dependent recombination to restart stalled forks (Pomerantz and O’Donnell, 2008) or even for transcription-induced origin-independent replication (Stuckey et al., 2015). Hence, DNA:RNA hybridization could occur in trans under regulated conditions but not spontaneously as unscheduled and harmful structures that would put genome integrity into risk. Thus, the assimilation of a transcript into a duplex DNA in trans would be tightly regulated and limited to specific reactions such as the case of telomerase or CRISPR and possibly other proteins yet to be discovered. For other cases, such as that of the GADP45 factor that binds to promoters harboring hybrids formed by lncRNAs (Arab et al., 2019), it is unclear whether such hybrids are formed in trans and in a GADP45-dependent manner.

Altogether, our results suggest that RNAs do not form hybrids in trans, so that the previously reported induction of Rad51-dependent ectopic genetic instability would be explained by R loop-mediated DNA breaks in cis.
## Key resources table

| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|-------------|------------------------|
| **Genetic reagent** Saccharomyces cerevisiae | W303 background strains with different gene deletions | various |  | (See Materials and methods section) |
| **Recombinant DNA reagent** | Yeast expression plasmids and recombination systems | various |  | (See Materials and methods section) |
| **Sequence-based reagent** | Primers for DRIP and RT-PCR | Condalab |  | (See Materials and methods section) |
| **Antibody** | Cy3 conjugated anti-mouse (goat monoclonal) | Jackson laboratories | Cat# 115-165-003, RRID: AB_2338680 | IF (1:1000) |
| **Antibody** | S9.6 anti DNA-RNA hybrids (mouse monoclonal) | ATCC Hybridoma cell line | Cat# HB-8730, RRID: CVCL_G144 | DRIP (1 mg/ml) and IF (1:1000) |
| **Commercial assay, kit** | Macherey-Nagel DNA purification | Macherey- Nagel | Cat# 740588.250 |  |
| **Commercial assay, kit** | Qiagen’s RNeasy | Qiagen | Cat# 75162 |  |
| **Commercial assay, kit** | Reverse Transcription kit | Qiagen | Cat # 205311 |  |
| **Peptide, recombinant protein** | Zymolyase 20T | US Biological | Z1001 | (50 mg/ml) |
| **Chemical compound, drug** | Doxycyclin hyclate | Sigma-Aldrich | D9891 | (5 mg/ml) |
| **Peptide, recombinant protein** | Proteinase K (PCR grade) | Roche | Cat # 03508811103 |  |
| **Peptide, recombinant protein** | Rnase A | Roche | Cat # 10154105103 |  |
| **Software, algorithm** | GraphPad Prism V8.4.2 | GraphPad Software, La Jolla, CA, USA | RRID: SCR_002798 |  |
| **Other** | iTaq Universal SYBR Green | Bio-RAD | Cat # 1725120 |  |
| **Other** | DAPI stain | Invitrogen | D1306 | 1 µg/mL |  

## Yeast strains and Plasmids

Strains used were the wild-type W303-1A (MATa ade2-1 can1- 100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-G535R) and its isogenic hpr1Δ::HIS3 mutant U678-1C (MATa) and U678-4C (MATa), mft1Δ::KANMX mutant (WMK.1A) (Chávez and Aguilera, 1997), rnh1Δ::KANMX rnh201Δ::KANMX (RNH-R), rad51Δ::KANMX (WSR51.4A) (González-Barrera et al., 2002), and hpr1Δ::HIS3MX rad51Δ::KANMX (HPR51.15A) from this study. rnh1Δ::KAN rnh201Δ::KAN (HRN2.8A) and the wild-type HRN2.8A were from Huertas and Aguilera, 2003. Wild-type (WGLZN) and hpr1ΔHIS3 mutant were made in this study by insertion of the GL-LacZ::NATMX at the LEU2 locus in Chromosome III of a W303-1A strain corrected for RADS (Moriel-Carretero and Aguilera, 2010).

Yeast plasmids pCM179, pCM184, pCM189 and pCM190 were previously published (Garí et al., 1997). pRS314-GL-LacZ (Piruat and Aguilera, 1998) and pRS314-GL-LacZi plasmids with recombination systems were built as follows. The BarnHI fragment containing the LacZ sequence from pPZ (Straka and Hörz, 1991), was inserted in both sense and antisense orientations with respect to the
promoter, respectively, into the BglII site of pRS314GLB (Piruat and Aguilera, 1998). pCM184TL-lacZ (Santos-Pereira et al., 2013) pRS416 and pRS416-GALlacZ were described previously (Prado et al., 1997). pCM184:AID was built by inserting the AID ORF from pCM189:AID (Santos-Pereira et al., 2013) into the NotI site of pCM190. pCM190-tet::LacZ400 was built by cloning the KpnI-BamHI 400 bp fragment of the 3′ from the LacZ gene into KpnI-BamHI digested pCM190. Plasmids pCM189:AID, pCM184:RNH1 (Santos-Pereira et al., 2013), and pWJ1344 (Lisby et al., 2001) were also previously published.

Yeast transformation
Yeast transformation was performed using the lithium acetate method as previously described (Gietz et al., 1995).

Recombination assays
Cells transformed were grown in selective media containing 2% glucose and 5 μg/mL of doxycycline (kept in the dark) to repress transcription from the GAL1 and tet promoter, respectively. Recombination frequencies were calculated as previously described as means of at least three median frequencies obtained each from six independent colonies isolated in the appropriate medium for the selection of the required plasmids (Gómez-González et al., 2011). Briefly, transformants were cultured for at 3–4 days (until acquiring similar colony size) in the appropriate selective media containing either 2% glucose or 2% galactose and recombinants were obtained by plating appropriate dilutions in selective medium. To calculate total number of cells, plates with the same requirements as for the original transformation were used. All plates were grown for 3–4 days at 30°C. The average and SEM of at least three independent transformants was plotted for each figure but the numerical data can be seen in Figure 2—source data 1, Figure 4—source data 1, Figure 5—source data 1 and Figure 6—source data 1.

Transcription analysis
Mid-log cultures were grown with either glucose or galactose and with or without 5 μg/ml doxycycline (kept in the dark). Total RNA was obtained using Qiagen’s RNeasy kit and used for cDNA synthesis with the QuantiTect Reverse Transcription kit with random primers (Qiagen) according to instruction. Real-time quantitative PCR was performed using iTaq universal SYBR Green (Biorad) with a 7500 Real-Time PCR machine (Applied Biosystems). Primers sequences used for this analysis were LacZT1-Fw (GCGCCGTGGCCTGAT), LacZT1-Rv (GTGCAGCGCGATCGTAATC), Intergenic-Fw (TGTTTCTTTAAGAGGTGATGGTGAT) and Intergenic-Rv (GTGCGCAGTACTTGTGAAAACC). The exact values obtained are shown in Figure 1—figure supplement 1—source data 1.

DRIP assays
DNA:RNA hybrids were measured in cultures with either glucose or galactose and either with or without 5 μg/ml doxycycline (kept in the dark). Cultures were collected, washed with chilled water, resuspended in 1.4 mL spheroplasting buffer (1 M sorbitol, 10 mM EDTA pH 8, 0.1% β-mercaptoethanol, 2 mg/ml Zymoliase 20T) and incubated at 30°C for 30 min. The spheroplasts were pelleted (5 min at 7000 rpm) rinsed with water and homogeneously resuspended in 1.65 mL of buffer G2 (800 mM Guanidine HCl, 30 mM Tris-Cl pH 8, 30 mM EDTA pH 8, 5% Tween-20, 0.5% Triton X-100). Samples were treated with 40 μl 10 mg/ml RNase A for 30 min at 37°C and 75 μl of 20 mg/ml protease K (Roche) for 1 hr at 50°C. DRIP was performed mainly as described (Ginno et al., 2012) with few differences. DNA was extracted gently with chloroform:isoamyl alcohol 24:1. Precipitated DNA, washed twice with 70% EtOH, resuspended gently in TE and digested overnight with 50 U of HindIII, EcoRI, BsrGI, XbaI and SspI, 2 mM spermidine and 2.5 μl BSA 10 mg/ml. Half of the DNA was treated with 8 μl RNase H (New England BioLabs) overnight 37°C as RNase H control. RNA-DNA hybrids were immunoprecipitated using 59.6 monoclonal antibody (hybridoma cell line HB-8730) coupled to Dynabeads Protein A (Invitrogen) for 2 hr at 4°C and washed 3 times with 1x binding buffer. DNA was eluted in 100 μL elution buffer (50 mM Tris pH 8.0, 10 mM EDTA, 0.5% SDS) treated 45 min with 7 μL proteinase K 20 mg/ml at 55°C and purified with Macherey-Nagel DNA purification kit. Primers sequences used for this analysis were LacZT1-Fw (GCGCCGTGGCCTGAT)
and LacZT1-Rv (GTGCAGCGCGATCGTAATC). The average and SEM of at least three independent transformants was plotted but the numerical data can be seen in Figure 3—source data 1.

**Detection of Rad52-YFP foci**

Spontaneous Rad52-YFP foci from mid-log growing cells carrying plasmid pWJ1344 were visualized and counted by fluorescence microscopy in a Leica DC 350F microscope, as previously described (Lisby et al., 2001). More than 200 S/G2 cells were inspected for each experimental replica. The average and SEM of at least three independent transformants was plotted but the numerical data can be seen in Figure 7—source data 1.

**S9.6 immunofluorescence of yeast chromosome spreads**

The procedure performed is similar to Chan et al., 2014 with some modifications. Briefly, mid-log cultures (OD600 = 0.5–0.8) were grown at 30°C; 10 ml of them were collected, washed in cold spheroplasting buffer (1.2 M sorbitol, 0.1 M potassium phosphate and 0.5 MgCl$_2$ at pH 7) and then digested by adding 10 mM DTT and 150 mg/ml of Zymolyase 20T to the same buffer. The digestion was performed for 10 min (37°C) and stopped by mixing the samples with the solution 2 (0.1 M MES, 1M sorbitol, 1 mM EDTA, 0.5 mM MgCl$_2$, pH 6.4). Later, spheroplasts were centrifuged carefully 8 min at 800 rpm, lysed with 1% vol/vol LipsoI and fixed on slides using Fixative solution (4% paraformaldehyde/3.4% sucrose). The spreading was carried out using a glass rod and the slides were dried from 2 hr to overnight in the extraction hood.

For the immuno-staining, the slides were first washed in PBS 1X in coplin jars and then blocked in blocking buffer (5% BSA, 0.2% milk in PBS 1X) over 10 min in humid chambers. Afterwards, slides were incubated with the primary monoclonal antibody S9.6 (1 mg/ml) in a humid chamber 1 hr at 23°C. After washing the slides with PBS 1X for 10 min, the slides were incubated 1 hr at 23°C in the dark with the secondary antibody Cy3 conjugated goat anti-mouse (Jackson laboratories, #115-165-003) diluted 1:1000 in blocking buffer. Finally, the slides were mounted with 50 µl of Vectashield (Vector laboratories, CA) with 1X DAPI and sealed with nail polish. For each experiment, more than 100 nuclei were visualized and counted to obtain the fraction of nuclei with DNA:RNA hybrids. The average and SEM of at least three independent transformants was plotted but the numerical data can be seen in Figure 7—source data 1.

**Acknowledgements**

Research was supported by the Spanish Ministry of Economy and Competitiveness (BFU2016-75058-P) and the European Union (FEDER). BG-G was funded by a grant from the Spanish Association Against Cancer (AECC).

**Additional information**

**Competing interests**

Andrés Aguilera: Reviewing editor, eLife. The other authors declare that no competing interests exist.

**Funding**

| Funder                                      | Grant reference number | Author                  |
|---------------------------------------------|------------------------|-------------------------|
| Ministerio de Economía y Competitividad     | BFU2016-75058-P        | Andrés Aguilera         |
| European Regional Development Fund          |                        | Andrés Aguilera         |
| Spanish Association Against Cancer          |                        | Belén Gómez-González    |

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.
Author contributions
Juan Lafuente-Barquero, Conceptualization, Data curation, Formal analysis, Validation, Writing - review and editing; María Luisa García-Rubio, Formal analysis, Investigation, Methodology; Marta San Martín-Alonso, Formal analysis; Belén Gómez-González, Conceptualization, Formal analysis, Supervision, Funding acquisition, Writing - original draft, Writing - review and editing; Andrés Aguilera, Conceptualization, Supervision, Funding acquisition, Writing - original draft, Writing - review and editing

Author ORCIDs
Belén Gómez-González https://orcid.org/0000-0003-1655-8407
Andrés Aguilera https://orcid.org/0000-0003-4782-1714

Decision letter and Author response
Decision letter https://doi.org/10.7554/eLife.56674.sa1
Author response https://doi.org/10.7554/eLife.56674.sa2

Additional files
Supplementary files
- Transparent reporting form

Data availability
All data generated or analysed during this study are included in the manuscript and supporting files. Source data files have been provided for all graphs.

References
Aguilera A, Gómez-González B. 2017. DNA-RNA hybrids: the risks of DNA breakage during transcription. Nature Structural & Molecular Biology 24:439–443. DOI: https://doi.org/10.1038/nsmb.3395, PMID: 28471430

Alvaro D, Lisby M, Rothstein R. 2007. Genome-wide analysis of Rad52 foci reveals diverse mechanisms impacting recombination. PLOS Genetics 3:e228. DOI: https://doi.org/10.1371/journal.pgen.0030228, PMID: 18085829

Arab K, Karaulanov E, Musheev M, Trnka P, Schafer A, Grummt I, Niehrs C. 2019. GADD45A binds R-loops and recruits TET1 to CpG island promoters. Nature Genetics 51:217–223. DOI: https://doi.org/10.1038/s41588-018-0306-6, PMID: 30617255

Ariel F, Lucero L, Christ A, Mammarella MF, Jegu T, Veluchamy A, Mariappan K, Latrasse D, Blein T, Liu C, Benhamed M, Crespi M. 2020. R-Loop mediated trans action of the APOLO long noncoding RNA. Molecular Cell 77:1055–1065. DOI: https://doi.org/10.1016/j.molcel.2019.12.015, PMID: 31952990

Bhatia V, Barroso SI, García-Rubio ML, Tumini E, Herrera-Moyano E, Aguilera A. 2014. BRCA2 prevents R-loop accumulation and associates with TREX-2 mRNA export factor PCID2. Nature 511:362–365. DOI: https://doi.org/10.1038/nature13374, PMID: 24896180

Cerritelli SM, Crouch RJ. 2009. Ribonuclease H: the enzymes in eukaryotes. FEBS Journal 276:1494–1505. DOI: https://doi.org/10.1111/j.1742-4658.2009.06908.x, PMID: 19228196

Chan YA, Aristizabal MJ, Lu PY, Luo Z, Hamza A, Kobor MS, Stirling PC, Hieter P. 2014. Genome-wide profiling of yeast DNA:RNA hybrid prone sites with DRIP-chip. PLOS Genetics 10:e1004288. DOI: https://doi.org/10.1371/journal.pgen.1004288, PMID: 24743342

Chang DD, Hauswirth WW, Clayton DA. 1985. Replication priming and transcription initiate from precisely the same site in mouse mitochondrial DNA. The EMBO Journal 4:1559–1567. DOI: https://doi.org/10.11002/j.1460-2075.1985.tb03817.x, PMID: 24114313

Chávez S, Beilharz T, Rondón AG, Erdjument-Bromage H, Tempst P, Svejstrup JQ, Lithgow T, Aguilera A. 2000. A protein complex containing Tho2, Hpr1, Mrt1 and a novel protein, Thp2, connects transcription elongation with mitotic recombination in Saccharomyces cerevisiae. The EMBO Journal 19:5824–5834. DOI: https://doi.org/10.1093/emboj/19.21.5824, PMID: 11060033

Chávez S, García-Rubio M, Prado F, Aguilera A. 2001. Hpr1 is preferentially required for transcription of either long or G+C-Rich DNA sequences in Saccharomyces cerevisiae. Molecular and Cellular Biology 21:7054–7064. DOI: https://doi.org/10.1128/MCB.21.20.7054-7064.2001, PMID: 11564888

Chávez S, Aguilera A. 1997. The yeast HPR1 gene has a functional role in transcriptional elongation that uncovers a novel source of genome instability. Genes & Development 11:3459–3470. DOI: https://doi.org/10.1101/gad.11.24.3459, PMID: 9407037
Cloutier SC, Wang S, Ma WK, Al Husini N, Dhoondia Z, Ansari A, Pascuzzi PE, Tran EJ. 2016. Regulated formation of IncRNA-DNA hybrids enables faster transcriptional induction and environmental adaptation. Molecular Cell 61:393–404. DOI: https://doi.org/10.1016/j.molcel.2015.12.024, PMID: 26833086

Cohen S, Puget N, Lin YL, Clouaire T, Aguirrebengoa M, Rocher V, Pasero P, Canitrö Y, Legube G. 2018. Senataxin resolves RNA:dna hybrids forming at DNA double-strand breaks to prevent translocations. Nature Communications 9:533. DOI: https://doi.org/10.1038/s41467-018-02894-w, PMID: 29416669

Collins K. 2000. Mammalian telomeres and telomerase. Current Opinion in Cell Biology 12:378–383. DOI: https://doi.org/10.1016/1044-7497(00)00103-4, PMID: 10801465

Cristini A, Groh M, Kristiansen MS, Gromak N. 2018. RNA:DNA hybrid interactome identifies DXH8 as a molecular player in transcriptional termination and R-Loop-Associated DNA damage. Cell Reports 23:1891–1905. DOI: https://doi.org/10.1016/j.celrep.2018.04.025, PMID: 29742442

D'Alessandro G, Whelan DR, Howard SM, Vitelli V, Renaudin X, Adamowicz M, Ianneli F, Jones-Weinert CW, Lee M, Matti V, Lee WTC, Morton MJ, Venkitaraman AR, Cejka P, Rothenberg E, d’Adda di Fagagna F. 2018. BRCA2 controls DNA:rna hybrid level at DSBs by mediating RNase H2 recruitment. Nature Communications 9:5376. DOI: https://doi.org/10.1038/s41467-018-07799-2, PMID: 30560994

Drolet M, Phoenix P, Menzel R, Massé E, Liu LF, Crouch RJ. 1995. Overexpression of RNase H partially complements the growth defect of an Escherichia coli Delta topA mutant: r-loop formation is a major problem in the absence of DNA topoisomerase I. PNAS 92:3526–3530. DOI: https://doi.org/10.1073/pnas.92.8.3526, PMID: 7536935

Fan HY, Cheng KK, Klein HL. 1996. Mutations in the RNA polymerase II transcriptional machinery suppress the hyperrecombination mutant hpr1 Delta of Saccharomyces cerevisiae. Genetics 142:749–759. PMID: 8849885

García-Muse T, Aguilera A. 2019. R loops: from physiological to pathological roles. Cell 179:604–618. DOI: https://doi.org/10.1016/j.cell.2019.08.055, PMID: 31607512

García-Pichardo D, Cañas JC, García-Rubio ML, Gómez-González B, Rondón AG, Aguilera A. 2017. Histone mutants separate R loop formation from genome instability induction. Molecular Cell 66:597–609. DOI: https://doi.org/10.1016/j.molcel.2017.05.014, PMID: 28575656

García-Rubio ML, Pérez-Calero C, Barroso SI, Tumini E, Herrera-Moyano E, Rosado IV, Aguilera A. 2015. The fanconi Anemia pathway protects genome integrity from R-loops. PLOS Genetics 11:e1005674. DOI: https://doi.org/10.1371/journal.pgen.1005674, PMID: 26584049

Gari E, Piedrafita L, Aldea M, Herrero E. 1997. A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in Saccharomyces cerevisiae. Yeast 13:837–848. DOI: https://doi.org/10.1002/1097-0061(19970713)13:9<837::AID-YEA145>3.0.CO;2-T, PMID: 9243672

Gietz RD, Schiestl RH, Willems AR, Woods RA. 1995. Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11:355–360. DOI: https://doi.org/10.1002/yea.320110408, PMID: 7785336

Ginno PA, Lott PL, Christensen HC, Korf I, Chédin F. 2012. R-loop formation is a distinctive characteristic of unmethylated human CpG island promoters. Molecular Cell 45:814–825. DOI: https://doi.org/10.1016/j.molcel.2012.01.017, PMID: 22870027

Gómez-González B, Felipe-Abrio I, Aguilera A. 2009. The S-Phase checkpoint is required to respond to R-Loops accumulated in THO mutants. Molecular and Cellular Biology 29:5203–5213. DOI: https://doi.org/10.1128/MCB.00402-09, PMID: 19651896

Gómez-González B, Ruiz JF, Aguilera A. 2011. Genetic and molecular analysis of mitotic recombination in Saccharomyces cerevisiae. Methods in Molecular Biology 745:151–172. DOI: https://doi.org/10.1007/978-1-61779-129-1_10, PMID: 21660594

Gómez-González B, Aguilera A. 2007. Activation-induced cytidine deaminase action is strongly stimulated by mutations of the THO complex. PNAS 104:8409–8414. DOI: https://doi.org/10.1073/pnas.0702836104, PMID: 17488823

González-Barrera S, García-Rubio M, Aguilera A. 2002. Transcription and double-strand breaks induce similar mitotic recombination events in Saccharomyces cerevisiae. Genetics 162:603–614. PMID: 12399375

Hartono SR, Malapert A, Legros P, Bernard P, Chédin F, Vanooosthuyse V. 2018. The affinity of the S9.6 Antibody for Double-Stranded RNAs Impacts the Accurate Mapping of R-Loops in Fission Yeast. Journal of Molecular Biology 430:272–284. DOI: https://doi.org/10.1016/j.jmb.2017.12.016, PMID: 29289576

Herrera-Moyano E, Mergui X, García-Rubio ML, Barroso S, Aguilera A. 2014. The yeast and human FACT chromatin-reorganizing complexes solve R-loop-mediated transcription-replication conflicts. Genetics & Development 28:735–748. DOI: https://doi.org/10.1101/gad.234070.113, PMID: 24636987

Huertas P, Aguilera A. 2003. Cotranscriptionally formed DNA:rna hybrids mediate transcription elongation impairment and transcription-associated recombination. Molecular Cell 12:711–721. DOI: https://doi.org/10.1016/j.molcel.2003.08.010, PMID: 14527416

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Nature 498:338–343. DOI: https://doi.org/10.1038/nature12252, PMID: 22745249

Kasahara M, Clikeman JA, Bates DB, Kogoma T. 2000. RecA protein-dependent R-loop formation in vitro. Genes & Development 14:360–365. PMID: 10673507

Keskin H, Shen Y, Huang F, Patel M, Yang T, Ashley K, Mazin AV, Storici F. 2014. Transcript-RNA-templated DNA recombination and repair. Nature 518:436–439. DOI: https://doi.org/10.1038/nature13682, PMID: 25186730
König F, Schubert T, Längst G. 2017. The monoclonal S9.6 antibody exhibits highly variable binding affinities towards different R-loop sequences. PLOS ONE 12:e0178875. DOI: https://doi.org/10.1371/journal.pone.0178875, PMID: 28594954

Li L, Germain DR, Poon HY, Hildebrandt MR, Monckton EA, McDonald D, Hendzel MJ, Godbout R. 2016. DEAD box 1 facilitates removal of RNA and homologous recombination at DNA Double-Strand breaks. Molecular and Cellular Biology 36:2794–2810. DOI: https://doi.org/10.1128/MCB.00415-16, PMID: 27550810

Li X, Manley JL. 2005. Inactivation of the SR protein splicing factor ASF/SF2 results in genomic instability. Cell 122:365–378. DOI: https://doi.org/10.1016/j.cell.2005.06.008, PMID: 16096057

Lisby M, Rothstein R, Mortensen UH. 2001. Rad52 forms DNA repair and recombination centers during S phase. PNAS 98:8276–8282. DOI: https://doi.org/10.1073/pnas.121006298, PMID: 11459964

Luna R, Rondón AG, Pérez-Calero C, Salas-Armenteros I, Aguilera A. 2019. The THO complex as a paradigm for the prevention of cotranscriptional R-Loops. Cold Spring Harbor Symposia on Quantitative Biology 84:105–114. DOI: https://doi.org/10.1101/sqb.2019.04.039594, PMID: 32493765

Mischo HE, Gómez-González B, Grzechnik P, Rondón AG, Wei W, Steinmetz L, Aguilera A, Proudfoot NJ. 2011. Yeast Sen1 helicase protects the genome from transcription-associated instability. Molecular Cell 41:21–32. DOI: https://doi.org/10.1016/j.molcel.2010.12.007, PMID: 21211720

Moriel-Carretero M, Aguilera A. 2010. A postincision-deficient TFIIH causes replication fork breakage and unveils alternative Rad51- or Pol32-mediated restart mechanisms. Molecular Cell 37:690–701. DOI: https://doi.org/10.1016/j.molcel.2010.02.005, PMID: 20227372

Nadel J, Athanasiadou R, Lemetre C, Wijetunga NA, Ó Broin P, Sato H, Zhang Z, Jeddeloh J, Montagna C, Golden A, Scoighe C, Grealli JM. 2015. RNA:DNA hybrids in the human genome have distinctive nucleotide characteristics, chromatin composition, and transcriptional relationships. Epigenetics & Chromatin 8:46. DOI: https://doi.org/10.1186/s13072-015-0040-6, PMID: 26579211

Ohle C, Tesorero R, Schermann G, Dobrev N, Sinning I, Fischer T. 2016. Transient RNA-DNA hybrids are required for efficient Double-Strand break repair. Cell 167:1001–1013. DOI: https://doi.org/10.1016/j.cell.2016.10.001, PMID: 27881299

Pardo B, Gómez-González B, Aguilera A. 2009. DNA repair in mammalian cells: DNA double-strand break repair: how to fix a broken relationship. Cellular and Molecular Life Sciences : CMLS 66:1039–1056. DOI: https://doi.org/10.1007/s00018-009-0840-3, PMID: 19153654

Pardo B, Aguilera A. 2012. Complex chromosomal rearrangements mediated by break-induced replication involve structure-selective endonucleases. PLOS Genetics 8:e1002979. DOI: https://doi.org/10.1371/journal.pgen.1002979, PMID: 23071463

Paulsen RD, Soni DV, Wollman R, Hahn AT, Yee MC, Guan A, Hayes JA, Miller SC, Cromwell EF, Solow-Cordero DE, Meyer T, Cimprich KA. 2009. A genome-wide siRNA screen reveals diverse cellular processes and pathways that mediate genome stability. Molecular Cell 35:229–239. DOI: https://doi.org/10.1016/j.molcel.2009.06.021, PMID: 19647519

Piazza A, Wright WD, Heyer WD. 2017. Multi-invasions are recombination byproducts that induce chromosomal rearrangements. Cell 170:760–773. DOI: https://doi.org/10.1016/j.cell.2017.06.052, PMID: 28781165

Piruat JI, Aguilera A. 1998. A novel yeast gene, THO2, is involved in RNA pol II transcription and provides new evidence for transcriptional elongation-associated recombination. The EMBO Journal 17:4859–4872. DOI: https://doi.org/10.1093/emboj/17.16.4859, PMID: 9707445

Pomerantz RT, O’Donnell M. 2008. The replisome uses mRNA as a primer after colliding with RNA polymerase. Nature 456:762–766. DOI: https://doi.org/10.1038/nature07527, PMID: 19020502

Prado F, Piruat JI, Aguilera A. 1997. Recombination between DNA repeats in yeast hpr1 delta cells is linked to transcription elongation. The EMBO Journal 16:2826–2835. DOI: https://doi.org/10.1093/emboj/16.10.2826, PMID: 9184227

Pugnet N, Miller KM, Legube G. 2019. Non-canonical DNA/RNA structures during Transcription-Coupled Double-Strand break repair: roadblocks or bona fide repair intermediates? DNA Repair 81:102661. DOI: https://doi.org/10.1016/j.dnarep.2019.102661, PMID: 31331819

Ray A, Machin N, Stahl FW. 1989. A DNA double chain break stimulates triparental recombination in Saccharomyces cerevisiae. PNAS 86:6225–6229. DOI: https://doi.org/10.1073/pnas.86.16.6225, PMID: 2668958

Roy D, Zhang Z, Lu Z, Hsieh CL, Lieber MR. 2010. Competition between the RNA transcript and the nontemplate DNA strand during R-loop formation in vitro: a nick can serve as a strong R-loop initiation site. Molecular and Cellular Biology 30:146–159. DOI: https://doi.org/10.1128/MCB.00897-09, PMID: 19841062

Ruiz JF, Gómez-González B, Aguilera A. 2009. Chromosomal translocations caused by pol32-dependent or pol32-independent triparental break-induced replication. Molecular and Cellular Biology 29:5441–5454. DOI: https://doi.org/10.1128/MCB.00256-09, PMID: 19651902

Santos-Pereira JM, Herrero AB, García-Rubio ML, Marin A, Moreno S, Aguilera A. 2013. The Npl3 hnRNP prevents R-loop-mediated transcription-replication conflicts and genome instability. Genes & Development 27:2445–2458. DOI: https://doi.org/10.1101/gad.229880.113, PMID: 24240235

Schwab RA, Niemiuszczy J, Shah F, Langton J, Lopez Martinez D, Liang CC, Cohn MA, Gibbons RJ, Deans AJ, Niedzwiedz W. 2015. The fanconi Anemia pathway maintains genome stability by coordinating replication and transcription. Molecular Cell 60:351–361. DOI: https://doi.org/10.1016/j.molcel.2015.09.012, PMID: 26593718

Silva S, Camino LP, Aguilera A. 2018. Human mitochondrial degradosome prevents harmful mitochondrial R loops and mitochondrial genome instability. PNAS 115:11024–11029. DOI: https://doi.org/10.1073/pnas.1807258115, PMID: 30301808

Lafuente-Barquero et al. eLife 2020;9:e56674. DOI: https://doi.org/10.7554/eLife.56674
Stolz R, Sulthana S, Hartono SR, Malig M, Benham CJ, Chedin F. 2019. Interplay between DNA sequence and negative superhelicity drives R-loop structures. PNAS 116:6260–6269. DOI: https://doi.org/10.1073/pnas.1819476116, PMID: 30850542

Straka C, Hörz W. 1991. A functional role for nucleosomes in the repression of a yeast promoter. The EMBO Journal 10:361–368. DOI: https://doi.org/10.1002/j.1460-2075.1991.tb07957.x, PMID: 1899374

Stuckey R, García-Rodríguez N, Aguilera A, Wellinger RE. 2015. Role for RNA:DNA hybrids in origin-independent replication priming in a eukaryotic system. PNAS 112:5779–5784. DOI: https://doi.org/10.1073/pnas.1501769112, PMID: 25902524

Teng Y, Yadav T, Duan M, Tan J, Xiang Y, Gao B, Xu J, Liang Z, Liu Y, Nakajima S, Shi Y, Levine AS, Zou L, Lan L. 2018. ROS-induced R loops trigger a transcription-coupled but BRCA1/2-independent homologous recombination pathway through CSB. Nature Communications 9:4115. DOI: https://doi.org/10.1038/s41467-018-06586-3, PMID: 30297739

Tous C, Aguilera A. 2007. Impairment of transcription elongation by R-loops in vitro. Biochemical and Biophysical Research Communications 360:428–432. DOI: https://doi.org/10.1016/j.bbrc.2007.06.098

Wahba L, Gore SK, Koshland D. 2013. The homologous recombination machinery modulates the formation of RNA–DNA hybrids and associated chromosome instability. eLife 2:e00505. DOI: https://doi.org/10.7554/eLife.00505

Wang IX, Grunseich C, Fox J, Burdick J, Zhu Z, Ravaian N, Hafner M, Cheung VG. 2018. Human proteins that interact with RNA/DNA hybrids. Genome Research 28:1405–1414. DOI: https://doi.org/10.1101/gr.237362.118, PMID: 30108179

Wellinger RE, Prado F, Aguilera A. 2006. Replication fork progression is impaired by transcription in hyperrecombinant yeast cells lacking a functional THO complex. Molecular and Cellular Biology 26:3327–3334. DOI: https://doi.org/10.1128/MCB.26.8.3327-3334.2006, PMID: 16581804

Yasuhara T, Kato R, Hagiwara Y, Shiotani B, Yamauchi M, Nakada S, Shibata A, Miyagawa K. 2018. Human Rad52 promotes XPG-Mediated R-loop processing to initiate Transcription-Associated homologous recombination repair. Cell 175:558–570. DOI: https://doi.org/10.1016/j.cell.2018.08.056, PMID: 30245011

Yu K, Chedin F, Hsieh CL, Wilson TE, Lieber MR. 2003. R-loops at immunoglobulin class switch regions in the chromosomes of stimulated B cells. Nature Immunology 4:442–451. DOI: https://doi.org/10.1038/ni919, PMID: 12679812

Zaitsev EN, Kowalczykowski SC. 2000. A novel pairing process promoted by Escherichia coli RecA protein: inverse DNA and RNA strand exchange. Genes & Development 14:740–749. PMID: 10733533