Yra1-bound RNA-DNA hybrids cause orientation-independent transcription-replication collisions and telomere instability

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ABSTRACT

R loops are an important source of genome instability largely due to its negative impact on replication progression. Yra1/ALY is an abundant RNA-binding factor conserved from yeast to humans and required for mRNA export, but its excess cause lethality and genome instability. Here, we show that, in addition to ssDNA and ssRNA, Yra1 binds RNA-DNA hybrids in vitro and when artificially overexpressed can be recruited to chromatin in an RNA-DNA hybrid-dependent manner stabilizing R loops and converting them into replication obstacles in vivo. Importantly, excess of Yra1 increases R loop-mediated genome instability caused by transcription-replication collisions regardless of whether they are co-directional or head-on. It also induces telomere shortening in telomerase-negative cells and accelerates senescence, consistent with a defect in telomere replication. Our results indicate that RNA-DNA hybrids form transiently in cells regardless of replication and, after stabilization by excess Yra1, they compromise genome integrity, in agreement with a two-step model of R loop-mediated genome instability. This work opens new perspectives to understand transcription-associated genome instability in repair-deficient cells, including tumoral cells.
INTRODUCTION

RNA-DNA hybrids are produced co-transcriptionally when the nascent transcript threads back hybridizing with the template DNA, leading together with the displaced non-template ssDNA to a structure termed R loop. Hybrids have a tendency to accumulate preferentially at highly transcribed protein-coding genes, peaking at promoters and terminators, rDNA, tRNA-coding genes, Ty elements, centromeres and telomeres (Ginno et al. 2012; Chan et al. 2014; El Hage et al. 2014; Wahba et al. 2016). RNA-DNA hybrids may benefit cell physiology as shown in some cases of transcription initiation and termination, mitochondrial DNA replication or immunoglobulin class switching (Aguilera and García-Muse 2012). However, RNA-DNA hybrids may also have a strong impact on genome instability as shown in cells defective in specific mRNP assembly factors such as the THO complex or the SRSF1 RNA binding protein, topoisomerase I, RNA-DNA helicases or RNase H, a ribonuclease that specifically degrades the RNA moiety of RNA-DNA hybrids (Santos-Pereira and Aguilera 2015; Sollier and Cimprich 2015). Accumulating evidence indicate that most of this genetic instability is due to the ability of R loops to stall the progression of the replication fork, leading to its collapse (García-Muse and Aguilera 2016). Thus, RNA-DNA helicases are required for the replication of highly transcribed regions (Boubakri et al. 2010) and R loop-accumulation impairs replication fork progression from bacteria to human cell lines (Wellinger et al. 2006; Tuduri et al. 2009; Gan et al. 2011; Castellano-Pozo et al. 2012; Hamperl et al. 2017). Alternatively, R loops may generate genomic instability if the displaced ssDNA is recognized and processed by flap endonucleases (Sollier et al. 2014). Despite reports showing that R loops alter replication causing genome instability, the mechanism is still unclear. However, recent evidence indicates that R loops are not deleterious per se, but require a second step, such as a local chromatin compaction, to compromise genome integrity (Castellano-Pozo et al. 2012; García-Pichardo et al. 2017).

RNA-DNA hybrids also have a physiological role at telomeres. Telomeres are transcribed into a long non-coding RNA (lncRNA) called TERRA that recruits telomeric proteins, contributes to heterochromatin formation (Maicher et al. 2014) and prevents activation of the DNA damage response (Flynn et al. 2011). A small proportion of TERRA RNA hybridizes with the DNA
forming telomeric RNA-DNA hybrids. This is restrained by the actions of RNase H and the THO complex (Balk et al. 2013; Pfeiffer et al. 2013; Arora et al. 2014; Yu et al. 2014) and favored by telomere shortening. TERRA RNA-DNA hybrids promote homologous recombination (Graf et al. 2017) between telomeric repeats enabling alternative lengthening of telomeres (ALT), a mechanism employed by telomerase-deficient tumor cells to prevent telomere shortening (Balk et al. 2013; Arora et al. 2014; Yu et al. 2014).

A number of transcription and RNA processing factors, such as THO or SRSF1, control co-transcriptional R loop formation (Santos-Pereira and Aguilera 2015). Yra1 is an RNA binding protein conserved in metazoans (ALY/REF) that acts as an adaptor for mRNA export factors (Kohler and Hurt 2007). It is cotranscriptionally loaded onto RNA by directly interacting with the carboxy-terminal domain (CTD) of RNA polymerase II (RNAP II) (MacKellar and Greenleaf 2011) or with other mRNP assembly factors (Johnson et al. 2011; Ma et al. 2013; Ren et al. 2017). Interestingly, Yra1 stoichiometry is tightly regulated in the cell via a mechanism relying on Yra1 inhibition of YRA1 pre-mRNA splicing (Rodriguez-Navarro et al. 2002; Preker and Guthrie 2006; Dong et al. 2007). Thus, removal of the YRA1 intron from the gene bypasses this autoregulatory circuit causing Yra1 overexpression with a strong negative impact on mRNA export and cell viability (Rodriguez-Navarro et al. 2002; Preker and Guthrie 2006). Interestingly, we have recently reported that high Yra1 intracellular levels alter genome dynamics by accumulating DNA damage and causing transcription-associated spontaneous recombination that is suppressed by RNase H overexpression (Gavaldá et al. 2016).

Aiming at understanding how Yra1 overexpression and transcription compromises genome integrity, here we demonstrate that excess Yra1 is recruited to chromatin in an RNA-DNA hybrid-dependent manner thereby stabilizing R loops and converting them into genome integrity threats. Importantly, excess of Yra1 increases R loop-mediated genome instability regardless of transcription-replication orientation. Consistent with the fact that Yra1 also binds to telomeres, where R loops accumulate, we show that Yra1 overexpression causes increase of RNA-DNA hybrids at telomeres, telomere alterations and accelerated senescence in telomerase-deficient cells. Our results demonstrate not only that excess of Yra1/ALY binds and stabilizes R
loops, but that R loops are transiently formed in cells regardless of replication, although they need to be stabilized to compromise genome integrity.

RESULTS

**Excess of Yra1 binds to chromatin in RNA-DNA hybrid-dependent manner.** Since *YRA1* overexpression increases genomic instability in an RNase H-sensitive manner (Gavalda et al. 2016), we wondered whether overexpressed Yra1 stabilized naturally-formed RNA-DNA hybrids. For this, we analyzed whether localization of overexpressed Yra1 to transcribed genes was dependent on RNA-DNA hybrids. We performed an Yra1 chromatin immunoprecipitation (ChIP) in wild-type cells overexpressing or not *YRA1* using an HA-tagged Yra1 protein expressed either from an intron-deficient (HA-YRA1\(\Delta\)i) or intron-containing (HA-YRA1) version of *YRA1* respectively (Gavaldá et al. 2016). To determine whether a putative Yra1 binding to chromatin was dependent on the presence of RNA-DNA hybrids, we overexpressed RNase H, an enzyme that specifically degrades the RNA moiety of RNA-DNA hybrids. Under *YRA1* overexpressing conditions, we found a clear increase of Yra1 recruitment to the endogenous *GCN4* and *PDR5* genes previously reported to accumulate RNA-DNA hybrids (García-Benitez et al. 2017) and to the *YRA1* intron region. Overexpressing RNase H in the cell, we restored Yra1 basal level in all the regions analyzed (Fig. 1A). These observations argue that Yra1 is recruited to RNA-DNA hybrids when artificially overexpressed in addition to their natural putative RNA

**Yra1 increases RNA-DNA hybrid accumulation *in vivo***

Next, we determined whether RNA-DNA hybrids were increased, presumably due to stabilization, when *YRA1* was overexpressed. We performed a DNA-RNA immunoprecipitation (DRIP) with the S9.6 antibody in wild-type cells overexpressing (YRA1\(\Delta\)i) or not (YRA1) *YRA1* to detect the hybrids. We focused on the genomic regions where we showed a hybrid-dependent Yra1 localization: *GCN4, PDR5* and *YRA1*-intron. We observed a S9.6 signal in the three regions analyzed that was significantly reduced by RNase H treatment.
The RNA-DNA hybrids detected in *GCN4*, *PDR5* and *YRA1*-intron were significantly increased when *YRA1* was overexpressed (Fig. 1B).

Next, we examined whether Yra1 recruitment was enriched at regions naturally forming RNA-DNA hybrids genome-wide when overexpressed. We performed S9.6 DRIP-seq analysis in wild-type cells and compared the data with our previously published ChIP-chip data on Yra1 recruitment (HA-Yra1 and HA-Yra1Δi) (Gavalda et al. 2016). Our DRIP-seq data are consistent with previously published S9.6 ChIP-seq data (el Hage et al., 2014), with a significant overlap in the mitochondrial DNA profile and nuclear peaks and genes (Figure 2A; Supplemental Figs. S1A, S1B). Comparison with the distribution of Yra1 at wild-type levels (HA-Yra1) or overexpressed (HA-Yra1Δi), revealed a significant correlation between hybrids and Yra1 signals. In our study, the majority of the DRIP signal mapped on ORFs, tRNA genes and mobile elements, consistent with other reports (El Hage et al., 2014), even though we detected less peaks (Supplemental Fig. S1C). Overexpressed Yra1 localized at 1923 genes (Fig 2B and Supplemental Fig S1D), with a significant overlap between Yra1-bound genes in cells overexpressing Yra1 and the RNA-DNA hybrid-accumulating genes in wild type cells (25 or 53% when compared with our new DRIP-seq or the previously published ChIP-seq data; Fig 2B and Supplemental Fig S1D). In summary, the results suggest that overexpressed Yra1 binds to R loop-accumulating regions genome-wide. Certainly, Yra1 still binds to regions without detectable DRIP signal as expected from an RNA-binding protein involved in mRNA export.

We finally investigated the effect of Yra1-mediated hybrid stabilization in cell fitness. For this, we overexpressed *YRA1* in mutants that accumulate R loops, such as *hpr1Δ*, *mft1Δ* or *tho2Δ* mutants lacking the THO complex involved in mRNP biogenesis or the double mutant *top1Δ* *top2-1* that accumulates negative supercoiling favoring R loops (Supplemental Fig. S2). As can be seen the results showing a decrease in cell viability suggest that the increase in persistent RNA-DNA hybrids in *YRA1*-overexpressing cells could contribute to cell death.

**An mRNA export defect is not sufficient to increase R loops**

Since overexpression of Yra1 impairs mRNA export (Rodríguez-Navarro et al. 2002), we wanted to confirm that the nuclear mRNA accumulation resulting
from an mRNA export defect was not sufficient to increase R loops. We analyzed three different nucleoporin mutants, nup42Δ, nup60Δ known to be affected in mRNA export and nup100Δ not affected (Bonnet and Palancade 2014) and the positive control mlp1Δ, a nuclear pore mutant known to accumulate R loops (García-Benítez et al, 2017). R loops were inferred using a genetic method based on the hyper-recombination ability of the human Activation-Induced Cytidine Deaminase (hAID), an enzyme that modifies cytidines in the ssDNA moiety of the R loop, as previously shown (García-Pichardo et al 2017; García-Benítez et al 2017). Recombination in nup42Δ and nup100Δ was similar to the wild type, whereas in nup60Δ was increased (x4.3) but this increase was not suppressed by RNase H overexpression, consistent with its known sensitivity to HU and MMS (Niño et al 2016), and in contrast to the positive control mlp1Δ (Supplemental Fig. S3). Therefore, we conclude that accumulation of mRNA in the nucleus due to an RNA export defect does not induce R loops per se.

Yra1 binds RNA-DNA hybrids in vitro
Since Yra1 localizes to RNA-DNA hybrid-enriched regions and is a well-characterized RNA binding protein, we reasoned that Yra1 might directly bind to RNA-DNA hybrids. To test this idea, we isolated a recombinant His₆-tagged version of Yra1 from bacteria confirming it by western blot (Supplemental Fig. S4). Next, we performed a gel mobility shift assay incubating increasing amounts of Yra1 with a 25-bp RNA-DNA hybrid formed annealing an RNA oligonucleotide to the complementary radioactively labeled DNA. In parallel, we assayed Yra1 binding to the same radioactive end-labeled RNA or DNA oligonucleotide that forms the hybrid and to a dsRNA constructed by annealing the same end-labeled RNA and a complementary RNA. We observed a shift in RNA migration, confirming the previously described Yra1 ability to bind RNA, but also in ssDNA, dsRNA and more importantly in RNA-DNA hybrid migration (Fig. 3A). Since the RNA-DNA hybrid and the ssDNA migrate differently (Fig. 3A, lanes 6 and 11) we concluded that all labeled ssDNA is present in the form of an RNA-DNA hybrid, as only one band is observed in the sample without Yra1 (Fig. 3A, lane 11). Therefore, Yra1 is able to bind in vitro not only ssRNA but also ssDNA, dsRNA and a RNA-DNA duplex. To confirm these interactions, we competed Yra1 binding with different cold nucleic acid species. First, we
incubated purified Yra1 with labeled dsRNA and increasing amounts of cold ssRNA or RNA-DNA hybrid. Both nucleic acids reverted Yra1 interaction with dsRNA at similar concentrations (5 µM) (Fig. 3B). Next, we challenged Yra1-hybrid interaction with increasing amounts of cold hybrid, ssRNA or ssDNA. In agreement with its binding to RNA-DNA hybrids, cold ssRNA, ssDNA or RNA-DNA hybrids competed for Yra1 binding, being RNA-DNA hybrids a slightly better competitor than ssRNA and ssDNA (Fig. 3C). Altogether, these results indicate that Yra1 is able to bind RNA-DNA hybrids supporting the conclusion that when overexpressed in vivo Yra1 can bind R loops in chromatin, contributing to their stabilization.

**R loop-mediated genome instability is linked to head-on transcription-replication**

We previously showed that transcription causes hyper-recombination when occurring in a head-on orientation with respect to replication but not when occurring co-directionally (Prado and Aguilera 2005). Since RNA-DNA hybrids are an obstacle for replication fork progression (Wellinger et al. 2006; Gan et al. 2011), we examined whether they could explain the orientation-dependent transcription-replication conflicts responsible for genome instability. We used the previously reported plasmids pGAL-OUT and pGAL-IN (Prado and Aguilera 2005) that contain leu2 truncated repeats transcribed from an inducible GAL1 promoter either in a co-directional (OUT) or head-on (IN) orientation with respect to replication driven from the early replication origin ARSH4 (Fig. 4A), respectively. DNA damage driven by the transcription-replication collision would be repaired by recombination between the leu2 direct repeats generating a wild-type LEU2 gene. Consequently, we could measure genome instability as the frequency of Leu2+ recombinant colonies. We observed that the frequency of recombination in the absence of transcription was low and similar in both systems (Fig. 4B), consistent with previous results (Prado and Aguilera 2005). However, upon transcription induction recombination was highly increased (7.7 fold) in the IN system with head-on transcription-replication, whereas in the co-directional OUT system only a two-fold increase in recombination was observed (Fig. 4B). When similar experiments were performed in both systems after RNase H overexpression, the transcription-dependent hyper-recombination observed in the head-on IN system was suppressed, whereas the
recombination frequencies in the co-directional OUT system did not change (Fig. 4B). These results argue that RNA-DNA hybrids are an important source of genome instability in systems undergoing head-on transcription-replication conflicts, but not in those undergoing co-directional conflicts. This interpretation would be consistent with the recent observation in human cells using similarly designed plasmid-based constructs in which transcription is driven from a bacterial T7 promoter and replication from an Epstein-Barr virus replication origin (Hamperl et al. 2017) as well with R loop-dependent replication impairment observed in the *Bacillus subtilis* genome (Lang et al. 2017). However, none of these results provide any answer to whether R loops are formed only in head-on transcription-replication or after replication.

**Yra1-stabilized R loops induce instability regardless of transcription-replication orientation**

If Yra1 binds and stabilize transient RNA-DNA hybrids present in the genome, we might expect that Yra1 will increase transcription–replication conflicts mediated by these structures. To test this hypothesis, we measured recombination in the co-directional and head-on systems either overexpressing (YRA1<sup>Δi</sup>) or not (YRA1) YRA1. Yra1 overexpression enhanced recombination 7.2 times in the head-on IN system, an increase that was majorly suppressed by RNase H overexpression (Fig. 4C). Importantly, an 8.7-fold increase was also observed in the co-directional OUT system, which was also suppressed by RNase H (Fig. 4C). This result indicates that RNA-DNA hybrids also form under co-directional transcription-replication, but such hybrids are not stable enough as to drive high genome instability. However, under Yra1 overexpression, hybrids are stabilized resulting in a significant increase in recombination, presumably by constituting a stable block to replication fork progression, regardless of transcription-replication orientation.

Our hypothesis predicts therefore that RNA-DNA hybrids should be present in both plasmids. Consequently, we performed a DRIP analysis using the S9.6 antibody in samples with or without RNase H treatment. In the analysis, we included the previously described hybrid-accumulating *PDR5* gene as an internal positive control and *PRE1* as a negative control. The results confirmed the presence of RNA-DNA hybrids in the plasmid-born *LEU2* gene of the recombined head-on system IN, in agreement with the *in vivo* data (Fig. 5A).
Notably, we also detected hybrids in the recombined OUT-system where LEU2 transcription is co-directional to replication fork progression (Fig. 5A). Therefore, we conclude that RNA-DNA hybrids are formed during transcription in both co-directional and head-on constructs but they significantly induce genomic instability, measured as recombination, when replication and transcription are head-on and not when they are co-directional. However, when the hybrid is stabilized via binding to Yra1, replication would stall regardless of the transcription orientation, leading to a similar increase in recombination (7-8 fold above the wild-type levels). These results support clearly that R loops are formed transiently regardless of replication and not as a consequence of transcription-replication conflicts.

Although unlikely, it might be possible that circular plasmids impose a specific topological constrain different to chromosomes that could enhance R loop accumulation and Yra1 binding. To confirm that this was not the case and that R loops occurred in linear chromosomes, we constructed a transcription-replication collision system in chromosome III and analyzed RNA-DNA hybrids under the same conditions tested in the plasmid construct. To do so, we integrated the LEU2 gene under the inducible GAL1 promoter in a head-on orientation with respect to replication driven from the early-firing ARS315 origin (Fig. 5B), for being this orientation the one with the potential to cause DNA opening that could favor R loops. DRIP analyses of cells cultured in galactose-containing media (transcription ON) versus glucose-containing media (transcription OFF) revealed that RNA-DNA hybrids appeared in the LEU2 gene only when transcribed (Fig. 5B). In the PDR5 gene used as a positive control, hybrids were observed in both conditions consistent with the fact that PDR5 is constitutively transcribed in both media (Fig. 5B). Importantly, the RNA-DNA hybrids formed in the chromosomal transcription-replication collision system increased when YRA1 was overexpressed (Fig. 5B), in agreement with the increase in recombination observed in the plasmid (Fig. 4C).

Cumulative evidence with concomitant studies on RNA-DNA hybrids, γH2AX and 53BP1 foci, recombination and comet and DNA combing assays suggests that R loop-dependent γH2AX are the result of DNA damage (García-Rubio et al, 2015; Schwab et al, 2015; Salas-Armentero et al, 2017). To assess whether the RNA-DNA hybrids detected in the LEU2 gene cause DNA damage we measured H2A-P (the equivalent in yeast to γH2AX) levels by ChIP under
conditions of both active and inactive transcription of the \textit{GAL1::LEU2} fusion, using telomeric repeats as a positive control (Kitada et al. 2011). An increase in H2A-P was observed when transcription of \textit{LEU2} was active, but not when was inactive, as well as in the telomeric controls (Fig. 5C). Therefore, head-on transcription of the chromosomal \textit{LEU2} gene also generates DNA breaks. Notably, such breaks were suppressed by RNase H overexpression (Fig. 5C), confirming that they were R loop-dependent. Most important, such H2A-P foci were significantly increased when Yra1 was overexpressed, and this increase suppressed by RNH1 overexpression. The results, apart of validating our studies in plasmid-borne constructs, indicate that the genomic instability derived from transcription-replication collisions is mediated by RNA-DNA hybrids and not by any specific topological constrains that could potentially accumulate in circular plasmids and that the negative impact of those collisions is enhanced by Yra1 overexpression.

**Excess of Yra1 produces telomere shortening and premature senescence in telomerase-deficient cells.**

We recently showed that excess of Yra1 causes a cell senescence-like phenotype and a slight telomere shortening in telomerase positive cells and is enriched at Y' telomeric regions (Gavalda et al. 2016). Since Yra1 is a highly efficient RNA-binding protein (Strasser and Hurt 2000; Fig. 3) and telomerase activity in wild-type cells relies on an RNA molecule that is used as template for telomere synthesis, it was possible that an excess of Yra1 sequestered the telomerase RNA molecule TLC1 leading to a telomerase-deficient phenotype instead of acting directly on the telomere. To test this possibility, we determined the effects of Yra1 overexpression in telomerase-minus cells (\textit{est2Δ}). Heterozygous \textit{EST2/est2Δ} diploids harboring the \textit{pGAL::Yra1Δi} plasmid were sporulated in glucose medium. Next, haploid \textit{est2Δ pGAL::Yra1Δi} spore clones were selected in glucose medium and further propagated in galactose liquid medium via serial dilutions (Hardy et al. 2014). The senescence profiles, kinetics of telomere shortening, and the type of survivors formed in multiple \textit{est2Δ} \textit{and est2Δ pGAL::Yra1Δi} clones grown in galactose were analyzed (Fig. 6). We showed that \textit{est2Δ pGAL::Yra1Δi} clones (overexpressing Yra1) exhibited a rapid and deep premature senescence compared to \textit{est2Δ} clones (Fig. 6A). Strikingly, telomere length analysis at the first time point of the senescence
assay indicated that telomeres were shorter in the \textit{est2\textDelta\ pGAL::Yra1\textDelta\i} clones compared to \textit{est2\textDelta} clones (Fig. 6B,C,D). This result suggests an abrupt telomere shortening upon overexpression of Yra1 different from the progressive telomere erosion normally observed in the absence of telomerase. Consistently, survivors appeared earlier in \textit{est2\textDelta} cells overexpressing \textit{YRA1} (Fig. 6C). As predicted, RNA-DNA hybrids were increased in \textit{est2\textDelta} cells (Fig. 6E), as shown in the single \textit{TelVI-R} telomere and consistent with previous results indicating that shortening of telomeres in \textit{est2\textDelta} cells triggers TERRA transcription and RNA-DNA hybrid accumulation (Graf et al, 2017). Importantly, such hybrids were significantly increased under Yra1 overexpression, consistent with a putative role of Yra1 overexpression in RNA-DNA hybrids stabilization (Fig. 6E).

As expected, in \textit{est2\textDelta} cells after the onset of senescence, the liquid culture was mainly dominated by type II survivors because of their growth advantage over type I survivors. In contrast, \textit{est2\textDelta\ pGAL::Yra1\textDelta\i} cultures appeared to produce few type II survivors and a higher proportion of type I survivors. Quantification of the occurrence of type II recombination events at a single telomere, \textit{TELVI-R}, further confirmed that overexpression of \textit{YRA1} impairs type II recombination (Fig. 7). These results unequivocally indicate that it is the high accumulation of overexpressed Yra1 at telomeres (Gavaldá et al. 2016) rather than an inhibition of telomerase activity due to the sequestering of the RNA template, which causes a quick and profound shortening of telomeres and premature senescence. We also noticed an increase of \textit{Y'} amplification upon Yra1 overexpression although this increase did not reach a statistical significance (Supplemental Fig. S5).

Finally, we tried to determine the impact of RNH1 overexpression on senescence and telomere dynamics in \textit{est2\textDelta} cells overexpressing Yra1. However, RNase H overexpression leads to a very sick phenotype of telomerase-deficient \textit{est2\textDelta} cells overexpressing Yra1 (Supplemental Fig. S6). These cells entered quickly in crisis with a very low viability compared to \textit{est2\textDelta} cells overexpressing only Yra1. Unfortunately, it is not possible to know whether this behavior is the result of two independent types of stresses (Rnh1 and Yra1 overexpression) or whether the two processes are interdependent and any interpretation about the type of survivors was hampered by the very low cell density at crisis in cells co-overexpressing Rnh1 and Yra1. Indeed, since survivors result from clonal events, the large heterogeneity might simply reflect
differences among their very low cell densities. Therefore, we cannot conclude whether RNase H overexpression counteracts Yra1 overexpression or potentiate its effect on telomere dynamics, both possibilities being consistent in any case with the increased presence of RNA-DNA hybrids at telomeres (Fig. 6E).

**DISCUSSION**

After demonstrating that the RNA-binding factor Yra1 can bind RNA-DNA hybrids \textit{in vitro} and \textit{in vivo}, we show that dynamic DNA-RNA hybrids are bound by overexpressed Yra1 and threaten genome integrity by promoting transcription-replication collisions. This is a general effect occurring all over the genome. Hyper-recombination caused by transcription-replication collisions is R loop-dependent and specific of head-on orientation, not being observed for co-directional transcription. Importantly, overexpression of Yra1 causes a similar and significant 7 to 8-fold increase in R loop-dependent recombination in systems undergoing either head-on or co-directional transcription-replication collisions. This suggests that RNA-DNA hybrids are co-transcriptionally formed independent of replication, but only when the replication fork approaches an R loop from downstream the RNA polymerase it does compromise genome integrity. Thus, the replication fork progressing in the same direction as transcription may easily resolve an R loop, unless Yra1 is bound to it. The ability of Yra1 to bind R loops and block replication dynamics is supported by the fact that Yra1 overexpression shortens telomeres by a telomerase-independent mechanism presumably linked to R loops generated by TERRA RNA, yet to be understood. These data indicate that R loops form transiently in the cell regardless of replication and generate genome instability mainly in head-on orientation or when they are stabilized by proteins like Yra1, by impeding the progression of replication.

We showed here that the Yra1 RNA-binding factor can bind and presumably stabilize RNA-DNA hybrids \textit{in vitro} and \textit{in vivo} when overexpressed, although this would not be its role when present at wild-type levels in the cell. Interestingly, \textit{Arabidopsis} AtNDX, a homeodomain-containing protein, stabilizes R loops by binding to the displaced ssDNA (Sun et al. 2013).
In contrast to AtNDX, Yra1 is an RNA binding protein that presumably affects R loop dynamics by binding to the RNA-DNA hybrids, likely impeding the action of helicases and RNase H and leading to the accumulation of stable or more persistent R loops. Indeed, Yra1 inhibits loading of Dbp2, an RNA helicase with \textit{in vitro} RNA-DNA unwinding activity (Ma et al. 2013; Ma et al. 2016). However, the \textit{in vitro} ssDNA binding ability observed for Yra1 (Fig. 3) suggests that Yra1 would also bind the displaced ssDNA of the R loops, potentially strengthening the stabilization and lengthening the persistency of the three stranded R loop structure \textit{in vivo}. Although Yra1 overexpression impairs mRNA export (Rodriguez-Navarro et al. 2002), we showed that a defect in mRNA export is not sufficient to produce either R loop accumulation or R loop-dependent hyper-recombination (Supplemental Fig. S3) and that Yra1 binding to transient RNA-DNA hybrids and other RNA structures may also contribute to its deleterious effect (Supplemental Fig. S2). Indeed, RNaseH expression not only reduces recombination and DNA damage, but also reduces recruitment of Yra1 to chromatin at transcribed genes (Fig. 1A). Thus, the ability of Yra1, in cells overexpressing it, to bind and fix R loops and compromise genome integrity might explain, at least in part, its specific regulation to avoid its over-expression.

How transcription induces genome instability is non-fully understood yet, but research conducted in the last two decades points to conflicts with replication as a major source of transcription-associated instability (García-Rubio et al. 2003; Prado and Aguilera 2005; Mirkin and Mirkin 2007; Boubakri et al. 2010). The transcription-dependent recombination results obtained in the co-directional and head-on systems when Yra1 is overexpressed reveal that R loops are a key player in transcription-replication conflicts (Fig. 4C). This is in agreement with previous work showing that R loop-accumulating mutants hinder replication in yeast, \textit{C. elegans} or human cell lines (Wellinger et al. 2006; Gan et al. 2011; Castellano-Pozo et al. 2012) and that in bacteria the RNA-DNA helicase \textit{DinG} is essential for replication of highly transcribed regions (Boubakri et al. 2010). Interestingly, we have shown that R loops by themselves may not be strong inducers of genome instability; instead they rely on a subsequent step to compromise genome integrity (García-Pichardo et al. 2017). One such step is linked to the modification of chromatin via histone H3 serine 10 phosphorylation (H3S10-P), a mark of chromatin condensation, which reveals that a local chromatin compaction triggered by an R loop may be behind its negative impact
on replication progression and genome integrity (Castellano-Pozo et al. 2012; García-Pichardo et al. 2017). Thus, naturally formed R loops are presumably transient and with no consequence on genome integrity. However, here we demonstrate that by artificially stabilizing transient R loops by binding to overexpressed Yra1, they accumulate at high levels and compromise genome integrity.

We had previously shown that transcription induces hyper-recombination only when it occurs concomitantly with replication and in head-on orientation (Prado and Aguilera 2005). Now we show that the hyper-recombination induced in the head-on orientation is largely dependent on R loops (Fig. 4), but not co-directional collisions. These results are consistent with two recent reports in bacteria and human cells showing that co-directional transcription-replication conflicts have little impact on the stability of the construct-containing plasmid or in bacteria viability, while head-on orientation is unstable and causes lethality in RNaseH-deficient bacteria (Hamperl et al. 2017; Lang et al. 2017). Nevertheless, the S9.6 DRIP analysis reveals that RNA-DNA hybrids are formed in LEU2 not only in the head-on but also in the co-directional system although they do not largely compromise the genome integrity. We detect R loops regardless of transcription-replication orientation, but when Yra1 is overexpressed, an R loop-dependent increase in recombination occurred in both systems, whether head-on or co-directional, demonstrating that they are present in both situations. Therefore, our data indicate that R loops are formed co-transcriptionally regardless of replication, and may be present before the replication fork arrives, that is, it is not the transcription-replication collisions the cause of the R loops (Bermejo et al. 2011; Hamperl et al. 2017; Lang et al. 2017). Consistently, R loops are also observed in the co-directional orientation in a bacterial chromosome too (Lang et al. 2017) and are detected in G1 cells in yeast and human cells (Castellano-Pozo et al. 2012; Bhatia et al. 2014). Therefore, we conclude that R loops are the cause and not the consequence of transcription-replication collisions, consistent with the genome-wide accumulation of RNA-DNA hybrids detected in our study (Fig. 2 and Supplemental Fig. S1) or others previously published (El Hage et al, 2014; Chan et al 2014; Wahba et al. 2016).

We propose that the different outcome of a replisome encountering an R-loop co-directionally or head-on may depend on the structure encountered. In
co-directional orientation the replisome would meet the RNA-DNA hybrid directly in the leading strand template, presumably being able to remove it easily with the help of some helicase function (Fig. 8A). However, in the head-on orientation the fork would encounter first the RNA polymerase that would be thus trapped ahead of the nascent RNA forming the RNA-DNA hybrid in the lagging strand template. In this orientation, the RNA polymerase would be a major contributor to the stalling of the replisome, presumably incapable of backtracking due to the hybrid behind (Fig. 8A). Indeed, it has been shown in bacteria that backtracked RNA polymerase unable to resume transcription would cause DNA breaks when encountered by a replication fork (Nudler 2012).

It is worth noting that a physical contact between the replisome and the RNA polymerase might not be necessary as the positive topological constraint generated in between the two advancing polymerases may be sufficient to block both processes. However, if the R loop is blocked artificially by binding with Yra1, when it is in excess in the cell, then it becomes an obstacle to replication no matter from which direction is encountered (Fig. 8B).

The importance of R loop stabilization to become a general obstacle to replication is supported by the analysis of the impact of Yra1 on telomere dynamics and homeostasis, where R loops have been shown to play a role. We have shown that Yra1 overexpression causes a telomere shortening phenotype that is not explained by a potential ability of overexpressed Yra1 to sequester the telomerase TLC RNA. Telomeres are over-shortened in the absence of telomerase when Yra1 is overexpressed (Fig. 6), favoring the idea that Yra1 excess would be shortening the telomere through R loop stabilization. Telomere repeats and subtelomeric regions are transcribed in long non-coding RNAs known as TERRA, which form unstable R loops (Maicher et al. 2014; Azzalin and Lingner 2015) that need to be removed before chromosomal ends are replicated (Graf et al. 2017). Therefore, it is likely that by stabilizing TERRA R loops Yra1 overexpression impairs telomere replication progression. Consistent with this idea, overexpressed Yra1 is recruited to telomeres and Rrm3, the replicative helicase that resolves replication fork stalls, accumulates at telomeres in this situation (Gavalda et al. 2016). Importantly, the presence of RNA-DNA hybrids at telomeres is higher in est2Δ cells, as expected, and significantly enhanced under Yra1 overexpression (Fig. 6E). Fork collapse mediated by stable R loops might be responsible of the telomere shortening,
thus causing premature senescence (Simon et al. 2016). Indeed, deletion of RNase H or the THO complex in cells unable to undergo recombination (rad52Δ) increases TERRA R loops and leads to rapid telomere loss and senescence (Balk et al. 2013). We observed a reduced appearance of type II telomeres and slightly higher Y’ subtelomeric amplification in the survivors after senescence. This suggests that Yra1 overexpression may favor type I recombination between subtelomeric Y’ elements at the expense of type II recombination. This is consistent with the observation that RNA-DNA hybrids formed by TERRA hybridization at telomeres promote the recruitment of Rad51 (Graf et al. 2017). Collectively, these data support our model that Yra1 binding to R loops causes replication impairment and hyper-recombination all over the genome. The fact that simultaneous overexpression of RNase H and Yra1 makes telomerase deficient cells extremely sick, triggering a quick entry into crisis, suggests an involvement of RNA-DNA hybrids in this behavior, but we cannot predict whether RNase H overexpression should either counteract or strengthen the impact of Yra1 overexpression on telomere dynamics and senescence without further mechanistic understanding (Fig. S6).

In conclusion, we have uncovered the role of RNA-DNA hybrids in transcription–replication conflicts regardless of the orientation, indicating that R loop formation is independent of replication. Artificially stabilized RNA-DNA hybrids cause genome instability when encountered by the replication fork both in head-on and co-directional orientation, otherwise only RNA-DNA hybrids involved in head-on transcription-replication collisions have a major effect on genome instability. These findings may help understand the role in preventing R loop accumulation and R loop-mediated genome instability of specific functions such as histone deacetylase Sin3A, the THO complex or the Fanconi Anemia pathway, which have been shown to impair replication fork progression through R loops (García-Rubio et al. 2015; Schwab et al. 2015; Madireddy et al. 2016; Salas-Armenteros et al. 2017). Similarly, these results rise the question of whether overabundance of the Yra1 ortholog ALY protein in a significant proportion of tumoral cells may be related with high levels of stabilized RNA-DNA hybrids that contributes to genome instability (Domínguez-Sanchez et al. 2011). This work should, thus, open new perspectives to understand the mechanisms of R loop-mediated genome instability and its implications in cancer development.
MATERIALS AND METHODS

Strains, primers and plasmids
Yeast strains, primers and plasmids used are listed in Tables S1, S2 and S3 respectively. The BGL2 strain was generated by integrating in BY4741 the GAL1::LEU2 (URA3) construct at position 225807 of Chr. III in a head-on orientation to replication starting at ARS315. This strain was crossed with Ybp250 to generate BYGL2-10B.

The recombined plasmid pARSGLBIN-Leu2 was used as a template to amplify the GAL1::LEU2 (URA3) construct adding the BglII restriction site and 40-bp of homology to region upstream or downstream to position 225807 of ChrIII at both sides. The linear PCR fragment was purified and transformed in BY4741 strain. Ura+ transformants were checked for GAL1::LEU2 (URA3) integration by Southern blot.

Genome-wide experiments
DRIP was performed as described (García-Pichardo et al. 2017). For seq IP and INP samples were used as template with a GenomePlex® Complete Whole Genome Amplification Kit (Merck) to amplify the DNA. Library was prepared to obtain a fragment size of 200bp according to manufacturer’s protocol (Ion Torrent PGM). The quality of the samples was certified with Bionalyzer and High Sensitivity DNA analysis Kit and runs performed in Ion 316™ Chip v2. Data generated are available under GEO accession number GSE113580. Bioinformatic analysis was performed by as described (see Supplemental Material). DRIP-seq signal values were multiplied by 3 for visualization.

Yra1 expression and purification.
His6-tagged Yra1 was expressed from pET-Yra1 (MacKellar and Greenleaf 2011) in BL21 Rosetta E. coli (DE3) cells (Novagen). Bacteria were grown in 1L of LB medium with ampicillin and chloramphenicol, and protein expression was induced with 0.2 mM IPTG o/n at 16°C. Cells were lysed and His6-Yra1 purified through Ni-Sepharose Fast Flow resin (GE Healthcare) as described
(MacKellar and Greenleaf 2011) followed by SP-sepharose (GE Healthcare) (Ma et al. 2013).

**Electrophoretic mobility shift assay**
RNA and DNA oligonucleotides purchased from Integrated DNA Technologies (IDT) were end-labeled with $^{32}$P-ATP using T4 polynucleotide kinase according to manufacturer’s instructions. The same nucleic acids were used as cold competitors, added at the concentration indicated. The binding reactions were performed with the indicated nucleic acids at 30-50 nM final concentration and increasing amounts of recombinant Yra1 (170 nM to 1.4 mM) in binding buffer (10 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1mM DTT and 10% glycerol) supplemented with 0.05 mg/ml BSA, 1X Cocktail protease inhibitors and RNaseOUT, at 30°C 10 min. Samples were resolved in 6% PAGE 0.5X TBE run 1h at 15 mA. Gels were dried and images were taken with Fujifilm Life Science FLA-5100 imaging system.

**DNA-RNA immunoprecipitation (DRIP) assays**
DRIP in est2Δ strains were performed as described (García-Rubio et al. 2018) in haploid spore products of diploid PAY316 obtained by crossing PAY76 with PAY321 that are heterozygous for EST2 (EST2/est2Δ) and carry the pRS415GAL or pRS413GAL::YRA1Δi plasmid. After 3 days of growth at 30°C, the entire spore colonies were transferred to 10 ml of liquid SRaf.

**ChIP assays**
Yeast mid-log cultures growing at 30°C were processed as described (Hecht and Grunstein 1999) with minor modifications. Cells were broken in a multi-beads Shocker at 4°C in lysis buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxicholate). Chromatin was sonicated to an average fragment size of 400–500bp and immunoprecipitated with 5 µl of anti-histone H2A (phospho 129) or anti-HA antibody coated to Protein A Dynabeads (Invitrogen).

**Genome instability analysis in S. cerevisiae**
Spontaneous recombination frequencies were obtained as the average value of median frequencies obtained by 6–10 fluctuation tests performed with 2–3
independent transformants. For each fluctuation test, six independent colonies were analysed as previously described (Prado and Aguilera 2005).

**Senescence assays and Telomere analysis**

Senescence assays in liquid media were performed as previously described (Churikov et al. 2014) from haploid spore products of diploids PAY247 or PAY249 obtained by crossing PAY267 or PAY269 with PAY76 that are heterozygous for EST2 (EST2/est2Δ) and carry the pEST2-URA3 plasmid and the pRS413GAL or pRS413GAL::YRA1Δi plasmid. To ensure homogeneous telomere length before sporulation, the diploids were propagated for at least 50 generations on YPD plates. After 3 days of growth at 30°C, the entire spore colonies were transferred to 2 ml of liquid SGal-his to estimate the number of population doublings (PDs), and the suspensions were immediately diluted to 10^5 cells/ml. Cells were serially passaged in 15 ml liquid SGal-his at 10^5 cells/ml at 48h intervals. Replicative senescence was calculated as the average of 2-10 independent spores with identical genotype. Telomere analysis of the samples was performed as described (Churikov et al. 2014; Hardy et al. 2014).

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**Author contributions:** MGR, PA and AGR performed all experiments, with the exception of the DRIP-seq and its bioinformatic analysis, performed by JLB and the construction of the head-on T-R system, performed by JFR. The study was designed by MGR, PA, AGR, MNS, VG, and AA who also analyzed the data. AGR and AA wrote the first draft of the manuscript. All authors contributed to the final version.
REFERENCES


Preker PJ, Guthrie C. 2006. Autoregulation of the mRNA export factor Yra1p requires inefficient splicing of its pre-mRNA. RNA 12: 994-1006.


FIGURE LEGENDS

Figure 1. *YRA1* overexpression causes RNA-DNA hybrid accumulation.

(A) ChIP analysis of Yra1 using anti-HA antibody in a wild-type strain overexpressing (green, HA-YRAΔi) or not (blue, HA-YRA1) *YRA1* and RNaseH (RNH+ or -) at the *GCN4* and *PDR5* genes or the *YRA1* intron (n >3).

(B) DRIP with the S9.6 antibody in wild-type asynchronous cultures overexpressing (green, HA-YRAΔi) or not (blue, HA-YRA1) *YRA1* at the *GCN4* and *PDR5* genes or the *YRA1* intron (n >3). Samples were treated (+) or not (-) *in vitro* with RNase H (RNH) prior to the immunoprecipitation. Means and SEM are plotted in all panels. *p < 0.05; (two-tailed Student’s t test).

Figure 2. Genome-wide correlation between *YRA1* overexpression and RNA-DNA hybrids.

(A) Genomic view of Yra1 recruitment in a wild-type strain overexpressing (HA-YRAΔi) or not (HA-YRA1) *YRA1* and RNA-DNA hybrid distribution. Fragments of chrI (top) and chrIV (bottom) are plotted with the signal log2 ratio values of the ChIP-chip from Gavaldá et al. (2016), the logFE of IP over input for the WT (W303) DRIP-seq and the relative enrichment of reads over the background level of sequencing in a WT strain (El Hage et al. 2014). Blue (HA-YRA1 IP), red (HA-YRA1Δi) and green (DRIP-seq or ChIP-seq) histograms represent the significant clusters. SGD features are represented below as blue bars. Profiles were represented using the UCSC Genome Browser.

(B) Venn diagrams showing the overlap between gene sets with significant Yra1 binding in the different ChIP-chip experiments from Gavaldá et al. (2016) (HA-YRA1 IP, blue; HA-YRA1Δi, red) and significant RNA-DNA hybrids accumulation (DRIP-seq, from this study or ChIP-seq from El Hage et al. (2014), green). P(x) was calculated with the Hypergeometric distribution formula.

Figure 3. *YRA1* binds to RNA-DNA hybrids *in vitro*.

(A) Electrophoretic mobility shift assay (EMSA) of His6-Yra1 and either ssRNA, ssDNA, RNA-DNA hybrids formed by annealing the RNA and DNA probes used in the single experiments or dsRNA.
(B) EMSA of His6-Yra1 and dsRNA competed with increasing amounts of cold RNA or RNA-DNA hybrids (0.6 µM to 40 µM).
(C) EMSA of His6-Yra1 and RNA-DNA hybrids competed with increasing amounts of cold RNA-DNA hybrids (0.25 µM to 8 µM), or RNA or DNA (0.25 µM to 16 µM).

**Figure 4.** R loop-mediated transcription-replication conflicts are orientation-independent in YRA1-overexpressing cells.

(A) Schemes of the centromeric plasmids harboring the recombination systems in head-on (IN) and co-directional (OUT) orientation. The arrows indicate the RNAPII-driven transcription orientation of the leu2 repeats from the GAL1 promoter and the direction of replication forks initiated at ARSH4.

(B) Effect of RNaseH1 overexpression on the recombination of the head-on (IN) and co-directional (OUT) systems in wild-type cells. Cells grown either in glucose (TRX -) or galactose (TRX +) and overexpressing (RNH +) or not (RNH -) RNase H. The fold-increase over no transcription and no RNase H expression is indicated on the right.

(C) Effect of RNH1 overexpression on the recombination of transcribed IN or OUT plasmids in wild-type cells overexpressing (YRA1Δi) or not (YRA1) YRA1 and RNaseH (RNH + or -). The fold-increase over no overexpression of YRA1 and RnaseH is indicated on the right.

Means and SEM are plotted in all panels. Asterisks indicate statistically significant differences between the strains indicated, according to Student’s t-tests (*, P< 0.05; **, P< 0.005; ***, P< 0.0005).

**Figure 5.** Effect of Yra1 overexpression on transcription-replication collision-mediated genome instability.

(A) DRIP with the S9.6 antibody in a wild-type strain carrying the recombined head-on (IN') and co-directional (OUT') plasmid. Samples from asynchronous cultures grown in galactose were treated (+) or not (-) in vitro with RNase H (RNH). The regions assayed were the LEU2 gene of the plasmid and the chromosomal PDR5 and PRE1 (negative control) genes (n >3).

(B) DRIP assay in wild-type strain with the GAL1::LEU2 (URA3) system integrated in chromosome III close to ARS315 in head-on orientation. Nucleic acids from cells grown either in glucose (Trx -) or galactose (Trx +) were treated
with RNase H (RNH +) prior to the immunoprecipitation with S9.6 antibody. Samples from cells grown in galactose (Trx +) overexpressing (GAL::YRA1Δi) YRA1 are shown in green. The regions assayed were the LEU2 gene at the head-on OUT′ integrated system and the chromosomal PDR5 gene (n >3).

(C) ChIP analysis of H2A-P in the GAL1::LEU2 (URA3) construct integrated in chromosome III in a head-on orientation to replication. Cells transformed with GAL1::RNH1 (RNH +) or with the empty vector (RNH-), were grown in glucose (Trx -) or in galactose (Trx +) (n >3). Cells overexpressing (GAL::YRA1Δi) YRA1 are shown in green. The regions assayed were the LEU2 gene at the head-on IN′ integrated system and the telomere (TELVI-R) (n >3).

Means and SEM are plotted in all panels. Asterisks indicate statistically significant differences between the strains indicated, according to Student’s t-tests (*, P< 0.05; **, P< 0.005, ***, P< 0.0005).

Figure 6. Premature senescence and accelerated shortening of telomeres promoted by Yra1 overexpression.
(A) Growth curves over time of the WT (n=2), or G-Yra1Δi (n=2), est2Δ (n=10) and est2Δ G-Yra1Δi (n=10). Each clone corresponds to a spore isolated from heterozygous diploid strain, propagated in liquid culture through daily serial dilutions every 2 days. OD_{600} was measured every 2 days to estimate the cell density. Population doublings (PDs) were estimated from the initial spores.
(B) Top, Schematic representation of wild type, type I and type II telomeres. All telomeres contain one X element in the subtelomeric region and from zero to four long (L) or short (S) Y′ subtelomeric sequences. Type I survivors show an amplification of Y′ sequence and interstitial TG_{1-3} repeats. Type II survivors display an elongation of TG_{1-3} terminal repeats. Positions of the Xhol sites are shown.
(C) TG_{1-3} -probed Southern-blot analysis of telomere length of representative clones of est2Δ and est2Δ G-Yra1Δi during senescence.
(D) Southern-blot analysis and mean telomere length at the first time-point (n=10).
(E) DRIP with the S9.6 antibody in WT and est2Δ strains. Nucleic acids were treated with RNase H (RNH +) prior to the immunoprecipitation with S9.6 antibody. Samples from cells grown in galactose (Trx +) overexpressing
(GAL::YRA1Δi) YRA1 are shown in green. The region assayed was the telomere (TELVI-R) (n >3). Means and SEM are plotted in all panels. Asterisks indicate statistically significant differences between the samples indicated, according to Student's t-tests (*, P< 0.05; **, P< 0.005, ***, P< 0.0005).

**Figure 7.** Analysis of type II recombination events at TELVI-R.

(A) The clones used in Figure 6 were analyzed by single-telomere Southern blot at the first time-point of senescence and after appearance of survivors. Each band corresponds to a single type II recombination event in the cell population independently of its intensity.

(B) Frequency of type II recombination events at TELVI-R. Details as in Fig. 6D

**Figure 8.** Model to explain Yra1 mediated transcription–replication collisions.

(A) In the co-directional orientation, the replication fork (RF) encounters the R loop removing it. In the head-on orientation either the positive supercoiling accumulated ahead or the RNA polymerase (RNAP) itself would block the replication fork.

(B) Overexpressed Yra1 (+Yra1 OE) binds to the R-loop stabilizing it and blocking replication whether it proceeds co-directionally or in head-on orientation.
A

chrI: 57,652-114,393

HA-YRA1 (Gavaldá et al. 2016)

HA-YRA1 ∆i (Gavaldá et al. 2016)

DRIP-seq

ChIP-seq (El Hage et al. 2014)

chrIV: 1,179,971 - 1,248,315

HA-YRA1 (Gavaldá et al. 2016)

HA-YRA1 ∆i (Gavaldá et al. 2016)

DRIP-seq

ChIP-seq (El Hage et al. 2014)

B

HA-YRA1 genes (1748) 734 1014 909 HA-YRA1 ∆i genes (1923) 1428 495 781 DRIP-seq genes (1276) 897 1026 1223 ChIP-seq genes (2249)

p = 3.42e-197 p = 1.93e-16 p = 1.57e-95
A

WT est2Δ est2Δ G-YraΔi G-YraΔi

Cell Density (OD_{600})

Population Doublings

B

Wild type telomeres

Type I telomeres

Type II telomeres

C

Mw (kb)

est2Δ  est2Δ G-Yra1Δi

est2Δ  est2Δ G-Yra1Δi

All telomeres, TG_{1-3} probe

Y' amplification

telomeres

D

Mw (kb)

est2Δ  est2Δ G-Yra1Δi

est2Δ  est2Δ G-Yra1Δi

Terminal fragments (Y telomeres) [pb]

E

S9.6 Signal (A.U.)

TelVI-R

pRS413 GAL::YRA1Δi

*  **  ***
A

Telomere VI-R

Mw (Kb) 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

WT  G-YRA1A Δi

Δ est2Δ G-Yra1Δi

Type II Y’ translocation

B

Type II recombinant events

WT  G-YRA1A Δi

Δ est2Δ G-Yra1Δi

***
A
Head-on (IN)  -Yra1 OE

Genome instability

Co-directional (OUT)

Genome Stability

B
Head-on (IN)  +Yra1 OE

Genome instability

Co-directional (OUT)

Genome instability