R-Loop Mediated Transcription-Associated Recombination in trf4Δ Mutants Reveals New Links between RNA Surveillance and Genome Integrity

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Abstract

To get further insight into the factors involved in the maintenance of genome integrity we performed a screening of Saccharomyces cerevisiae deletion strains inducing hyperrecombination. We have identified trf4Δ, a gene encoding a non-canonical polyA-polymerase involved in RNA surveillance, as a factor that prevents recombination between DNA repeats. We show that trf4Δ confers a transcription-associated recombination phenotype that is mediated by the nascent mRNA. In addition, trf4Δ also leads to an increase in the mutation frequency. Both genetic instability phenotypes can be suppressed by overexpression of RNase H and are exacerbated by overexpression of the human cytidine deaminase AID. These results suggest that in the absence of Trf4 R-loops accumulate co-transcriptionally increasing the recombination and mutation frequencies. Altogether our data indicate that Trf4 is necessary for both mRNA surveillance and maintenance of genome integrity, serving as a link between RNA and DNA metabolism in S. cerevisiae.

Introduction

Maintenance of genome integrity is critical for cell homeostasis. Cells posses multiple mechanisms such as specific DNA repair pathways or cell cycle checkpoints to deal with DNA damage and the resulting genetic instability commonly associated with cancer and several genetic disorders [1]. Genomes are exposed to the action of physical and chemical agents, and metabolic processes that can cause lesions in the DNA. One such process is transcription, which has been established as an inducer of genome instability. Recombination and mutation frequencies are enhanced by transcription, leading to transcription-associated recombination (TAR) and transcription-associated mutation (TAM) [2,3]. Key to understanding how transcription increases genomic instability is the fact that single-stranded DNA (ssDNA) is chemically more unstable than double-stranded DNA (dsDNA). Transcription itself changes in topology and chromatin conformation associated with it may increase the probability of the occurrence of ssDNA. Consistently, DNA-damaging agents show a synergistic effect with transcription in the induction of recombination in yeast [4], and mutation rates correlate with the strength of transcription and superhelical stress [5]. In addition to a major ssDNA accessibility, transcription associated genomic instability could also be the result of the collision between the transcription and replication machineries [6,7]. A possible intermediate of transcription-associated genomic instability is an R-loop structure consisting of a RNA:DNA hybrid that displaces the non-template ssDNA strand. R-loops are transcription by-products rarely formed in the cell but they accumulate in a number of transcription and mRNP mutants with a genetic instability phenotype [8].

During transcription, the nascent pre-mRNA associates with mRNA-binding proteins and undergoes a series of processing steps resulting in an export competent mRNA ribonucleoprotein complexes (mRNP) [9,10]. Emerging evidence suggest that when mRNP biogenesis does not occur properly the RNA can hybridize with the DNA template, forming R-loops that would hinder transcription elongation and block replication. One of the best studied examples is the THO complex, which functions at the interface transcription-mRNA export. Mutations in THO lead to a transcription-associated hyperrecombination phenotype partially suppressed by overexpression of RNase H, an enzyme that degrades the RNA strand of DNA:RNA hybrids [11]. Moreover, in these mutants genome instability is exacerbated by the action of the human cytidine deaminase AID that acts on the displaced ssDNA of R-loops [12,13]. Similar R-loop-dependent transcriptional genome instability is observed in mammalian and chicken DT40 cells depleted of the ASF/SF2 splicing factor [14]. More recently, mutations in topoisomerase I, Sen1/SENASAIX and Sin3 have also been reported to cause genome instability via a common mechanism [15,16,17,18]. In addition, a number of RNA processing factors have been shown to be relevant for the maintenance of genome integrity by preventing R-loop accumulation by different genetic and cellular approaches in yeast and human cells [17,19,20].

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In *Saccharomyces cerevisiae*, screenings based on marker stability provide a powerful approach for studying genes that preserve genome structure [21,22]. These screenings exploit the use of artificial chromosome (YAC) and endogenous loci to measure genome instability events such as gross chromosomal rearrangements (GCR) and chromosome loss. Artificially constructed DNA repeats have also been validated as models to study genomic instability involving homologous recombination [23,24]. To get further insight into the factors implicated in the maintenance of genome integrity we performed a screening of *S. cerevisiae* deletion strains for hyperrecombinant mutants. We analyzed a total of 610 viable deletion strains constructed by the EUROFAN consortium. All strains were transformed with pRS14 and pRS216 centromeric plasmids carrying three different recombination systems, L, LY and SU, as described previously [28]. These systems are based on direct (L and LY) or inverted (SU) repeats of a 0.6 kb internal fragment of the *LEU2* ORF generated with two truncated copies of the *LEU2* gene (leu2Δ3' and leu2Δ5') spaced by different DNA sequences. Deletions (L and LY systems) and inversions (SU) were scored as Leu+ events and quantified by fluctuation tests. Among the strains analyzed, we found seven deletion mutants that conferred a hyperrecombinant phenotype (Figure 1). Four out of these mutants correspond to genes involved in RNA related processes: **MED2**, a subunit of the RNA polymerase II mediator complex [34]; **RPL13A**, a component of the large (60S) ribosomal subunit [55]; **LSG1**, a GTPase involved in 60S ribosomal subunit biogenesis [36], and **TRF4**, a component of the TRAMP complex involved in RNA surveillance [25,26,27]. The other three mutants were in **TOS3**, a redundant kinase that activates the Sm1/AMPK pathway that controls nutrient and environmental stress response [37]; **ART1**, involved in regulating the endolysosome of plasma membrane proteins [38], and **APC9**, involved in the regulation of protein stability [39]. Next, we measured the frequency of direct-repeat recombination in the chromosomal *leu2-ΔADE2-URA3-lacZ·leu2-k* system. We constructed the different mutant strains carrying this chromosomal system and recombination leading to ura-deletions was scored. As shown in Figure 1, all mutants showed similar recombination frequencies to those of the wild-type strain, except **trf4Δ**. Thus we decided to focus our work on **trf4Δ** because it showed a hyperrecombination phenotype in all direct-repeat systems assayed, regardless of whether they were in plasmids or chromosomes.

** Materials and Methods **

**Strains and Plasmids**

Yeast strains used are listed in Table 1. Plasmids pRS314ΔL, pRS316ΔL, pRS314LY, pRS316LY, pRS316SU, pRS316SU and pRS316-ÝΔNS [29], pRS314ΔL-Δz:pRS314ΔL-Δz [29], pGL-ΔdΔG, pGL-ΔhΔG, pGAL-A:RH1 [11] p413GAL1, p416GAL1 [30] and p413GAL1AD [13] were used to determine recombination frequencies. Plasmid pCM184-LAUR was used for the analysis of mRNA expression levels as previously described [31]. Plasmids pNOPPATA1L, pNOPPATA1L-TRF4Δ-WT and pNOPPATA1L-TRF4Δ-DADA kindly provided by W. Keller, have been previously described [32].

**Recombination and Mutation Analysis**

Recombination frequencies were determined as described [33]. For each strain, the recombination frequencies are given as the average and standard deviation of the median recombination value obtained from fluctuation tests performed in 3–4 different transformants using 6 independent colonies per transformant. Recombinants were selected as Leu+ colonies for the plasmid containing *LEU2* truncated repeat systems. Recombination analyses for the chromosomal *ln2·ΔADE2-URA3·ln2-k* system (Lk-ŁU) were performed in wild-type and congenic mutants using 6 independent colonies grown in synthetic complete medium SC, and recombinants were selected in SC+F0A.

Mutation frequencies were determined in wild-type and mutant strains using the *Pet·lacZ·URA3* (pCM184-LAUR) fusion construct. Ura+ mutants were selected in SC+F0A. The human *AID* gene, present in p413GAL1AD, was used for overexpression in 2% galactose medium. Median mutation frequencies were obtained by fluctuation tests performed in 3–4 different transformants using 6 independent colonies per transformant.

**Miscellaneous**

β-galactosidase assays and Northern analyses were performed according to previously published procedures [31].

**Results**

**New proteins involved in genome instability**

To identify novel genes with a role in genome stability, we performed a screening of *S. cerevisiae* deletion strains for hyperrecombinant mutants. We analyzed a total of 610 viable deletion strains constructed by the EUROFAN consortium. All strains were transformed with pRS14 and pRS216 centromeric plasmids carrying three different recombination systems, L, LY and SU, as described previously [28]. These systems are based on direct (L and LY) or inverted (SU) repeats of a 0.6 kb internal fragment of the *LEU2* ORF generated with two truncated copies of the *LEU2* gene (leu2Δ3' and leu2Δ5') spaced by different DNA sequences. Deletions (L and LY systems) and inversions (SU) were scored as Leu+ events and quantified by fluctuation tests. Among the strains analyzed, we found seven deletion mutants that conferred a hyperrecombinant phenotype (Figure 1). Four out of these mutants correspond to genes involved in RNA related processes: **MED2**, a subunit of the RNA polymerase II mediator complex [34]; **RPL13A**, a component of the large (60S) ribosomal subunit [55]; **LSG1**, a GTPase involved in 60S ribosomal subunit biogenesis [36], and **TRF4**, a component of the TRAMP complex involved in RNA surveillance [25,26,27]. The other three mutants were in **TOS3**, a redundant kinase that activates the Sm1/AMPK pathway that controls nutrient and environmental stress response [37]; **ART1**, involved in regulating the endolysosome of plasma membrane proteins [38], and **APC9**, involved in the regulation of protein stability [39]. Next, we measured the frequency of direct-repeat recombination in the chromosomal *leu2-ΔADE2-URA3·lacZ·leu2-k* system. We constructed the different mutant strains carrying this chromosomal system and recombination leading to ura-deletions was scored. As shown in Figure 1, all mutants showed similar recombination frequencies to those of the wild-type strain, except **trf4Δ**. Thus we decided to focus our work on **trf4Δ** because it showed a hyperrecombination phenotype in all direct-repeat systems assayed, regardless of whether they were in plasmids or chromosomes.

**trf4Δ mutants confer transcription-dependent hyperrecombination**

We observed that the hyperrecombination phenotype of **trf4Δ** for the direct-repeat systems analyzed seems to be transcription-dependent (Figure 1). Recombination frequencies in **trf4Δ** strains were 2.6 and 8.7 times the WT levels for the L and LY systems, respectively. Both systems are based on the same direct repeats (an internal fragment of the *LEU2* gene) and differ in the length of the intervening sequence (51bp for L, and 5.57kb for LY) [28]. As in **trf4Δ** cells the recombination frequency is higher when there is a long DNA fragment transcribed between the two direct repeats, we wondered if deletion of **TRF4** indeed conferred a transcription-dependent genetic instability phenotype. To test this, we determined the effect of **trf4Δ** on recombination in the *L-Δz* and *GL-Δz* systems carrying 0.6-kb *ln2* direct repeats flanking the *Δz* ORF under conditions of low (GAL1 promoter in 2% glucose), medium (*LEU2* promoter) and high levels of transcription (GAL1 promoter in 2% galactose). As can be seen in Figure 2, the higher the strength of transcription the stronger the increase in recombination. Altogether, the data indicate a statistically significant increase in recombination levels in **trf4Δ** cells respect to the wild-type that is transcription-dependent.
The hyperrecombination phenotype of trf4Δ mutant is mediated by the nascent mRNA

The length and high GC content of lacZ gene makes transcription through this sequence poorly efficient in mutants impaired in transcription elongation [40,41]. As lacZ transcription impairment was linked in many cases to hyperrecombination phenotype in mutants of THO and other mRNP factors [42,43], we explored whether lacZ transcription was also affected in trf4Δ mutants. For this purpose, we analyzed gene expression in the LAUR expression system [31] that contains a 4.15-kb lacZ-URA3 translational fusion under the control of the Tet promoter. Defects in lacZ expression were determined as poor growth in the absence of uracil and as lack of ß-galactosidase activity. As shown in Figure 3A trf4Δ cells behave as wild-type cells, suggesting that this mutant does not have a negative effect on transcription of the lacZ-URA3 fusion.

| Strain | Genotype | Source/Reference |
|--------|-----------|------------------|
| W303-1A | MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 | R. Rothstein |
| FY1679 | MATA ura3-52 his3Δ200 leu2Δ1 trp1Δ63 | Eurofan |
| FLRA006-01B(A) | MATA ura3-52 his3Δ200 leu2Δ1 lys2 trp1Δ63 lsg1Δ::KAN | Eurofan |
| FLP2022-088(AL) | MATA ura3-52 his3Δ200 leu2Δ1 lys2 trp1Δ63 rpl13Δ::KAN | Eurofan |
| FBS1008-02A(A) | MATA ura3-52 his3Δ200 leu2Δ1 lys2 trp1Δ63 apc9Δ::KAN | Eurofan |
| FPS023-03C(A) | MATA ura3-52 his3Δ200 leu2Δ1 lys2 trp1Δ63 art1Δ::KAN | Eurofan |
| WFB030 | MATA ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 trf4Δ::KAN | This study |
| WNS002 | MATA ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 med2Δ::KAN | This study |
| TRF4D-CS | MATA ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 trf4Δ::KAN | This study |
| MGY6-1A | MATA ade2 his3 trp1 ura3 leu2-k::ADE2-URA3::leu2-k | [64] |
| AFGL-7D | MATA ade2 his3 trp1 ura3 leu2-k::ADE2-URA3::leu2-k lsg1Δ::KAN | This study |
| WFDL-1D | MATA ade2 his3 trp1 ura3 leu2-k::ADE2-URA3::leu2-k rpl13Δ::KAN | This study |
| AFGL-2D | MATA ade2 his3 trp1 ura3 leu2-k::ADE2-URA3::leu2-k art1Δ::KAN | This study |
| WFLR-2B | MATA ade2 his3 trp1 ura3 leu2-k::ADE2-URA3::leu2-k apc9Δ::KAN | This study |
| AFOR-1A | MATA ade2 his3 trp1 ura3 leu2-k::ADE2-URA3::leu2-k rpl13Δ::KAN | This study |
| AWT4-1C | MATA ade2 his3 trp1 ura3 leu2-k::ADE2-URA3::leu2-k art1Δ::KAN | This study |
| MGY1-2D | MATA ade2 his3 trp1 ura3 leu2-k::ADE2-URA3::leu2-k med2Δ::KAN | This study |

The hyperrecombination phenotype of trf4Δ mutant is mediated by the nascent mRNA

The length and high GC content of lacZ gene makes transcription through this sequence poorly efficient in mutants impaired in transcription elongation [40,41]. As lacZ transcription impairment was linked in many cases to hyperrecombination phenotype in mutants of THO and other mRNP factors [42,43], we explored whether lacZ transcription was also affected in trf4Δ mutants. For this purpose, we analyzed gene expression in the LAUR expression system [31] that contains a 4.13-kb lacZ-URA3 translational fusion under the control of the Tet promoter. Defects in lacZ expression were determined as poor growth in the absence of uracil and as lack of ß-galactosidase activity. As shown in Figure 3A trf4Δ cells behave as wild-type cells, suggesting that this mutant does not have a negative effect on transcription of the lacZ-URA3 fusion.

### Table 1. Table of Strains used in this work.

| Strain | Genotype | Source/Reference |
|--------|-----------|------------------|
| W303-1A | MATA ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1 | R. Rothstein |
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| FPS023-03C(A) | MATA ura3-52 his3Δ200 leu2Δ1 lys2 trp1Δ63 art1Δ::KAN | Eurofan |

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Figure 1. Recombination analyses of med2Δ, lsg1Δ, trf4Δ, rpl13Δ, tos3Δ, art1Δ and apc9Δ mutants. A diagram of each recombination system (not drawn to scale) is shown at the top. Repeats are shown as gray boxes. Arrows indicate relevant transcripts produced by the constructs. For the L, LY and SU systems, recombination frequencies were determined in wild-type (FY1679) and mutant strains transformed with plasmids pRS314-L and pRS314-LY carrying the leu2 direct-repeat systems, and pRS314-SU carrying an inverted repeats system. Recombinants were selected as Leu+. The average median value and SD of 3–4 fluctuation tests are shown. Recombination frequencies of med2Δ (MGY1-2D), lsg1Δ (AFGL-7D), trf4Δ (AWT4-1C), rpl13Δ (WFDL-1D), tos3Δ (AFGL-2D), art1Δ (AFOR-1A), apc9Δ (WFLR-2B) and wild-type (MGY6-1A) congenic strains carrying the chromosomal leu2-k::ADE2-URA3::leu2-k system are shown. For recombination analyses, independent colonies were obtained from SC and recombinants were selected in SC+FOA.

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Moreover, northern analyses show that lacZ mRNA levels are much higher in trf4Δ mutants than in wild-type cells (Figure 3B), consistent with the previously described role of this protein in mRNA degradation [27].

Our data indicate that although trf4Δ mutants show a transcription-dependent hyperrecombination phenotype (Figure 2), transcription seems not to be affected. Instead, higher amounts of mRNAs are accumulated, probably as a result of the defect in mRNA decay mediated by TRAMP. As the hyperrecombination phenotypes of several mRNP mutants depend on the nascent mRNA [11,44], we analyzed whether the hyperrecombination phenotypes of several mRNP mutants depend on the nascent mRNA. We measured recombination in isogenic strains W303-1A (WT) and TRF4D-C5 (trf4Δ) cells by the overexpression of RNase H. As Trf4 is a cofactor of the TRAMP complex involved in RNA surveillance, we determined whether hyperrecombination was dependent on its polyadenylation activity. We measured the recombination frequency in trf4Δ cells carrying the chromosomal leu2-k::ADE2-URA3::leu2-k system transformed with a plasmid expressing either the wild-type TRF4 allele or the polyadenylation-defective allele TRF4-DADA under the control of the NÖP1 promoter [32]. TRF4-DADA contains two aspartate to alanine mutations in the catalytic site of the polyA-polymerase that render the enzyme inactive [26]. Recombination levels were significantly reduced in trf4Δ cells by the overexpression of the TRF4-DADA allele, reaching values close to those of cells complemented with the wild-type TRF4 allele (Figure 5). This suggests that hyperrecombination in trf4Δ cells takes place through a mechanism that is independent of its polyadenylation activity.

Hyperrecombination in trf4Δ cells does not depend on the catalytic polyadenylation domain of Trf4

As Trf4 is a cofactor of the TRAMP complex involved in RNA surveillance, we determined whether hyperrecombination was dependent on its polyadenylation activity. We measured the recombination frequency in trf4Δ cells carrying the chromosomal leu2-k::ADE2-URA3::leu2-k system transformed with a plasmid expressing either the wild-type TRF4 allele or the polyadenylation-defective allele TRF4-DADA under the control of the NÖP1 promoter [32]. TRF4-DADA contains two aspartate to alanine mutations in the catalytic site of the polyA-polymerase that render the enzyme inactive [26]. Recombination levels were significantly reduced in trf4Δ cells by the overexpression of the TRF4-DADA allele, reaching values close to those of cells complemented with the wild-type TRF4 allele (Figure 5). This suggests that hyperrecombination in trf4Δ cells takes place through a mechanism that is independent of its polyadenylation activity.

Genetic instability in trf4Δ is mediated by R-loops

Next we determined whether the mRNA dependency of the hyperrecombination phenotype of trf4Δ cells was linked to the co-transcriptional formation of R-loops. To address this possibility we assayed the effect of RNase H overexpression in the trf4Δ mutant carrying the direct-repeat recombination system LYΔNS [28]. As can be seen in Figure 6 the hyperrecombination phenotype of trf4Δ was suppressed by the overexpression of RNase H.
trf4Δ Transcription-Associated Genome Instability

Figure 4. Nascent mRNA-dependency of the hyperrecombination phenotype of trf4Δ mutants. (A) Direct-repeat recombination systems GL-ribm and GL-ribm containing the PHOS-Rib or PHOS-ribm sequences flanked by two truncated copies of LEU2 in direct orientation under the GAL1 promoter. These systems contain respectively an active or inactive 52-bp ribozyme (Rib). The ribm system (inactive ribozyme) yields a long transcript, whereas in the Rib+ system (active ribozyme) self-cleavage of the PHOS-Rib transcript leads to a shorter mRNA (represented by arrows). (B) Recombination frequencies in W303-1A (WT) and TRF4Δ cells containing the recombination systems GL-Rib+ and GL-ribm. Experiments were performed in 2% galactose to allow expression of the direct repeats. The average median value and SD of 3–4 fluctuation tests are shown. Asterisk indicates statistically significant differences, according to Student’s t-tests (*, P<0.05).

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As we have previously shown that as a consequence of R-loop formation expression of AID is able to strongly induce both mutation and recombination in yeast THO mutants [13], next we analyzed whether this was also the case for trf4Δ cells. As can be seen in Figure 6 AID expression increased recombination in trf4Δ mutants 8.2 times above the WT levels, which was suppressed by RNase H overexpression, consistent with the conclusion that R-loops are formed in trf4Δ mutants. To confirm this, we assayed the effect of AID expression on the mutation frequency in trf4Δ. We analyzed the frequency of Ura- mutations in the LAUR expression system. We observed that AID expression increased the frequency of Ura- colonies 3-fold in wild-type cells, consistent with previously reported data [13]. However this increase was of 13-fold in trf4Δ cells (Figure 7). As expected if this specific enhancement of trf4Δ was linked to R-loop formation, overexpression of RNase H reduced the frequency of Ura- mutations to values close to those of the wild-type (Figure 7). Altogether the data indicate that R-loops accumulate in the absence of TRF4 and mediate the genomic instability of trf4Δ cells.

Discussion

Here, we show the results of a screening for mutations that increased homologous recombination between repeated DNA fragments in yeast using genetic assays based on artificially constructed DNA repeats. We identified different mRNP biogenesis and transcription related proteins, as well as other factors, whose deletions lead to an increase in recombination in plasmid borne assays. We focused our studies in TRF4, a factor involved in RNA surveillance, because its absence causes a transcription-associated hyperrecombination phenotype both in chromosome and plasmid-borne systems. Using different genetic tools we show that this phenotype is dependent on the presence of the nascent mRNA and is mediated by R-loops. Our results therefore provide a new link between RNA quality control and genetic instability involving R-loops.

Our screening has permitted to identify a number of transcription and RNA related factors as suppressors of genome instability (Figure 1). These include MED22, one subunit of the transcription Mediator complex; RPL13A, a ribosomal subunit; LSG1, a GTPase involved in ribosomal biogenesis; and TRF4, a poly(A) polymerase of the TRAMP complex. Our results provide new evidence for the link between mRNA biogenesis and genome instability. Mutants affecting various steps of transcription, from initiation to termination, and RNA processing have been shown to lead to an increase of γH2A foci, YAC instability or hyperrecombination in yeast and human cells [14,17,19,20,31,43,44]. In addition, our screening identified other non-RNA related factors whose deletion could have an indirect impact on genome instability: APC9, encoding a subunit of the Anaphase-Promoting Complex/Cyclosome (APC/C), and TOS3, considered as the functional orthologous of LKB1, a mammalian kinase associated with Peutz-Jeghers cancer-susceptibility syndrome (Figure 1).

Several studies have explored the role of LKB1 as a major actor of the AMPK/mTOR pathway connecting cellular metabolism, cell growth and tumorigenesis [43]. We have focused our interest in deciphering the genetic basis of genome instability in trf4Δ mutants. TRF4 is a non-canonical poly(A) polymerase that acts as a cofactor of the exosome complex
Figure 6. Genetic evidence for R-loop formation in trf4Δ mutants. Effect of RNaseH1 and AID over-expression on the mutation frequency in trf4 mutants. Upper panel shows the analysis of recombination frequencies in W303-1A (WT) and TRF4Δ-C5 (trf4Δ) cells containing the recombination system LYANS, without RNaseH1 overexpression (-RNH1) or with over-expression of RNaseH1 (+RNH1). The latter was achieved with the multicopy plasmid pGAL-RNH1 carrying RNH1 under the GAL1 promoter. Lower panel shows recombination frequencies as in the upper one, but over-expressing AID from plasmid p413GALAID. The average median value and SD of 3–4 fluctuation tests are shown. Other details as in Figure 2. Asterisks indicate statistically significant differences, according to Student’s t tests (**, P < 0.005).

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Figure 7. Spontaneous and AID-induced mutation frequencies in wild-type and trf4Δ strains. Mutation frequency of W303-1A (WT), TRF4Δ-C5 (trf4Δ) strains, using the LAUR fusion construct. Ura+ mutants are selected in SC-FOA. The human AID gene was overexpressed in 2% galactose medium using plasmid p413GALAID. The median values of mutation frequencies and SD of 3–4 different fluctuation tests are shown. Asterisks indicate statistically significant differences, according to Student’s t tests (*, P < 0.05; **, P < 0.005).
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for the quality control of different types of RNAs [46]. Interestingly, it was originally isolated in a synthetic growth screen with top1 [topoisomerase one-requiring function] [47]. This is a notable observation because THO mutations also show a synthetic growth defect with top1 [48] and indeed hpr1Δ was also recovered in that screen. In addition, trf4 interacts genetically with mutations in different components of transcription, histone modification and histone remodeling complexes, and proteins involved in cohesion and DNA repair (reviewed in [49]). Beside its function in RNA surveillance, Trf4 has been shown to control chromid cohesion, mitotic chromosome condensation and mitotic segregation [50,51,52], rDNA copy number [53], and telomere length [32]. Therefore, it seems that Trf4 plays a role in DNA metabolism of unknown nature.

We show that TRF4 prevents genetic instability, as trf4Δ was identified as in our screening with recombination systems containing truncated repeats of the LEU2 gene (Figure 1). Modulating the transcription levels of the repeats through constitutive and regulatable promoters, we have demonstrated that the hyperrecombination phenotype of trf4Δ is transcription-dependent (Figure 2). In addition, the hyperrecombination phenotype of trf4Δ cells can be suppressed by the action of a ribozyme inserted at the nascent mRNA as well as by RNase H overexpression (Figure 4 and Figure 6). The data indicate that RNA:DNA hybrids accumulate in the absence of this mRNA surveillance factor. Interestingly, we have previously reported that mutation in RRP6, the exonuclease subunit of the nuclear exosome, has an effect on transcription elongation and genome integrity [43]. Recently, it has been shown that deletion of TRF4, and of other mRNA surveillance factors, such as KEM1 (an exonuclease involved in cytoplasmic mRNA decay), ARI1 (a RNA-binding protein of the TRAMP complex) and RRP6 lead to elevated GCRs in the form of terminal deletions and mini-chromosome losses using YACs. These events were partially suppressed by RNase H overexpression [17], although its dependency on transcription was not established. Our results indicate that Trf4 is a factor that prevents different forms of genome instability, including that associated with transcription (Figure 1 and Figure 2). Importantly, we provide evidence that both recombination and mutation were enhanced in trf4Δ mutants (Figure 7). Altogether, our data suggest that cotranscriptional R-loop are responsible for both phenotypes, consistent with the exacerbated recombination and mutation phenotypes of trf4Δ cells upon AID cytidine deaminase overexpression (Figure 6 and Figure 7).

The impact of mutations in the RNA surveillance machinery on genome integrity reveals the global relevance of RNA metabolism in genome dynamics. Interestingly, in mammalian cells the core nuclear exosome subunit Rrp40 has been shown to be recruited to S regions of Ig genes and to be required for optimal class switching recombination [54]. It has been proposed that the exosome could provide the ribonucleolytic activity for degradation of the RNA strand of RNA-DNA hybrids exposing the template DNA strand to AID activity [54], but this is in principle unrelated with the...
AID-independent phenomenon described here. As AID induces mutation and recombination in different yeasts [55,13], including trf4 cells (this study) we believe that the loss of Trf4 may cause an accumulation of transcripts at the site of transcription with the potential to form R-loops, which are highly susceptible of AID, as previously shown for THO mutants [13].

R-loops structures accumulate in different mutants of mRNP biogenesis. mRNA processing and transcription factors could prevent R-loop formation by facilitating assembly of the nascent mRNA into a ribonucleoprotein particle, therefore limiting its ability to rehybridize with the template DNA strand (reviewed in [8]). Accumulative data indicate that R-loops in THO mutants hinder transcription elongation and generate recombinitogenic structures that could represent an obstacle for the replication machinery [11,56,57,58]. However, these properties are not shared by every mutant impairing mRNA biogenesis [43]. Indeed trf4 mutants do not show a reduction in the expression level the GC-rich lacZ gene from E. coli (Figure 3), in contrast to mutants of THO/TREX and other transcription elongation factors [31,44]. On the other hand, mRNA processing and assembly into an export-competent mRNP is a tightly regulated process and a defect in mRNA processing can affect downstream steps [10,59,60]. TRAMP together with the nuclear exosome have been proposed to mediate a quality-control checkpoint activated upon mRNA export blockage [61]. Therefore, it is possible that aberrant mRNA transcripts that escape degradation in trf4 cells hybridize with the DNA contributing to R-loop formation and genome instability.

TRAMP plays a role in polyadenylation and stimulates RNA degradation mediated by the nuclear exosome [25,26,27]. However, polyadenylation is not essential for active degradation in vitro [27] and a polyadenylation-defective Trf4 protein is fully active, suggesting that mRNA degradation triggered by Trf4 is independent of its polyadenylation activity [61]. Indeed, genome-wide expression analysis shows that the overexpression of TRF4-DADA restores the levels of most RNAs with an altered expression in trf4 cells, except a small fraction corresponding to highly expressed and structured RNAs [32]. The fact that hyper-recombination phenotype of trf4A cells is suppressed by the overexpression of the TRF4-DADA mutant allele (Figure 5) indicates that occurs via a polyadenylation-independent mechanism. Interestingly, other DNA-related phenotypes, such as the maintenance of telomere, have also been shown to be polyadenylation independent [32]. In addition, although Trf4 has been defined as a non-canonical poly-A-polymerase playing a role in RNA surveillance, its function role is not restricted to RNA degradation, but rather contributes to the processing of different RNAs such as rRNAs, snoRNA, snRNAs and RNA precursors [32,62,63]. Indeed, Trf4 has been shown recently to be associated with introns in vivo, as shown by crosslinking-RNA-immunoprecipitation, and to regulate degradation of spliced-out introns [32]. Therefore Trf4 could link RNA processing with the maintenance of genome integrity.

A number of reports suggest that R-loops are formed at a higher frequency in the genome than previously anticipated with an impact in both gene expression and genome integrity [8]. Given the role of Trf4 in the processing and degradation of different types of RNAs [46], it is also possible that the loss of rDNA repeats observed previously in trf4A cells, could be associated with the formation of R-loops [53,13]. In summary our work suggests that trf4A leads to a general transcription-associated genome instability phenotype that is mediated by the cotranscriptional formation of R-loops, providing a further connection between genome dynamics and RNA metabolism.

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Author Contributions

Conceived and designed the experiments: SG MG RL AA. Performed the experiments: SG MG RL. Analyzed the data: SG MG RL AA. Wrote the paper: RL AA.

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