Genome-Wide Distribution of RNA-DNA Hybrids Identifies RNase H Targets in tRNA Genes, Retrotransposons and Mitochondria

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Introduction

During transcription, the RNA polymerase opens the DNA duplex, and in the process rotates the DNA double helix by approximately one turn per 10 bp. This generates positive torsional stress ahead, and negative torsional stress in the wake, of the transcribing polymerase [1]. Positive stress impedes further unwinding of the DNA duplex, potentially stalling the polymerase. In contrast, negative torsion can lead to DNA strand separation and opening of the duplex. The resulting template single-stranded DNA region can base-pair with the nascent RNA transcript, generating an RNA-DNA duplex and an unpaired non-template DNA strand, giving rise to the term “R-loop” for such structures (for reviews see [2,3,5,6,7,8]).

Other features besides negative topological stress strongly influence R-loop formation [3], e.g. the G.C content of the inherent sequence. In particular, R-loop formation can be favoured by a high guanine (G) density in the non-template DNA strand [property known as positive GC skew, see [9,10]], and this is specifically due to the higher thermodynamic stability of RNA-DNA hybrid sequences endowed with “G-rich purine RNA”/”C-rich pyrimidine DNA” duplexes [9,10,11,12,13,14]. Importantly, R-loops rich in G-clusters have been linked to immunoglobulin class switch recombination and Cpg methylation in mammals [9,10,14,15].

R-loops are generally regarded as highly deleterious, since the single stranded DNA is susceptible to damage. Moreover, it is believed that the structure can block both transcription and DNA replication, creating replicative stress and potentially causing further DNA damage [for reviews see [2,3,5,6,7]]. Highly transcribed genes in yeast exhibit greater mutation and recombination rates than genes transcribed at lower rates (reviewed in [7]), which might be related to R-loop formation.

R-loops can be resolved by RNase H1 and/or RNase H2 (Rnh201 is the catalytic subunit of a three subunit enzyme), either of which can cleave the RNA component in the RNA-DNA hybrid, albeit with different efficiencies (reviewed in [16]). However, loss of both RNase H1 and H2 activity is not lethal in yeast [17], strongly indicating that other cellular activities can resolve R-loops, such as the helicase Sgs1/Senataxin, THO/TREX RNA packaging complexes and the RNA exosome [2,5,8]. Moreover, RNase H2 plays dual roles in preserving genome integrity, processing both R-loops and ribonucleotides mis-incorporated in to DNA during replication, whereas RNase H1 is reported to resolve only R-loops (reviewed in [16,18]). In mammals both RNase H1 and H2 are required for cell viability and for embryonic development, and mutations in any of the three subunits of RNase H2 have been reported to cause the neuroinflammatory disease Aicardi-Goutières syndrome (AGS) [19,20,21,22].

In previous analyses of transcription by RNA polymerase I (Pol I) on the yeast ribosomal DNA (rDNA), we observed that R-loops are common at specific sites, in particular within the S′-region of the 18S rDNA [23]. These were readily detected in wild-type strains, although their abundance was increased in strains lacking the activity of DNA Topoisomerase I (Top1), which can resolve
RNA polymerase (mtRNAP). Notably, R-loops
accumulated unevenly across intron-containing genes with the
highest peak over exon 2. We show that R-loop accumulation was
strongly detected in the absence of both ribonucleases H1 and H2 (RNase H1 and H2), indicating that R-loops are inherently formed but rapidly cleared by RNase H. Importantly, stable R-loops lead to reduced synthesis of tRNA precursors in mutants lacking RNase H and DNA topoisomerase activities. RNA-DNA hybrids associated with TY1 cDNA retrotransposition intermediates were elevated in the absence of RNase H, and this was accompanied by increased retrotransposition, in particular to 5′-flanking regions of tRNAs. Our findings show that RNase H participates in silencing of TY1 life cycle. Interestingly, R-loops associated with mitochondrial transcription units were suppressed specifically by RNase H1. These findings have potentially important implications for understanding human diseases caused by mutations in RNase H.

Results

Genome-wide mapping of R-loop locations by ChIP-seq

We previously reported that R-loops can be identified robustly by ChIP-QPCR analyses using the S9.6 antibody [23], which is specific for the structure of RNA-DNA duplexes independently of their sequence [26,27]. To assess the genome-wide distribution of R-loops, formaldehyde-crosslinked and sonicated chromatin was incubated with antibody S9.6. The immunoprecipitated DNA and input chromatin were processed for high-throughput sequencing. Recovered DNA sequences were then mapped to the yeast genomic sequence (GEO Series accession number GSE53420).

ChIP-seq was applied to wild-type and to mutants double
mutants lacking both RNase H and double
Top1, or also Top2 activities. TY1 tRNA genes leads to reduced pre-tRNA synthesis specifically in
highest peak over exon 2. We show that R-loop accumulation was
strongly detected in the absence of both ribonucleases H1 and H2 (RNase H1 and H2), indicating that R-loops are inherently formed but rapidly cleared by RNase H. Importantly, stable R-loops lead to reduced synthesis of tRNA precursors in mutants lacking RNase H and DNA topoisomerase activities. RNA-DNA hybrids associated with TY1 cDNA retrotransposition intermediates were elevated in the absence of RNase H, and this was accompanied by increased retrotransposition, in particular to 5′-flanking regions of tRNAs. Our findings show that RNase H participates in silencing of TY1 life cycle. Surprisingly, R-loops associated with mitochondrial transcription units were suppressed specifically by RNase H1. These findings have potentially important implications for understanding human diseases caused by mutations in RNase H.

negative torsion behind the RNA polymerase (for a review on
DNA topoisomerases see [24]), and further increased in the absence of RNase H activity. R-loop formation by Pol I on the
highly transcribed rDNA array is favored by negative torsional stress [23,25], suggesting the possibility that R-loop formation in wild-type cells might also be associated with other RNA polymerases, particularly on actively transcribed genes.

Here we determined the genome-wide distribution of RNA-
DNA hybrids in budding yeast using chromatin immunoprecipitation (ChIP) with antibody S9.6 [26,27], followed by deep sequencing of immunopurified DNA fragments (ChIP-seq). The conclusions are related to, but not identical with, the results of recent microarray analyses [11]. R-loops were strongly associated with actively transcribed loci by all RNA polymerases including the mitochondrial RNA polymerase (mTRNAp). Notably, R-loops accumulated unevenly across intron-containing genes with the highest peak over exon 2. We show that R-loop accumulation at tRNA genes leads to reduced pre-rRNA synthesis specifically in mutants lacking both RNase H and Top1, or also Top2 activities. We also show that integration of TY1 retrotransposons in 5′-flanking regions of tDNAs is favored in cells depleted of both Top1 and cellular RNase H activities. We present evidence that both RNase H1 and RNase H2 are involved in cleaving RNA-DNA hybrids associated with cDNAs of TY1 retrotransposons. Unexpectedly, we also show that only RNase H1 is involved in processing of co-transcriptional R-loops at mtDNA transcription units.

Pol III genes are targets for RNase H activity

ChIP-QPCR for the 5S rRNA, the cytoplasmic RNA scR1, the small nuclear RNA U6 (SNR6) and three tRNA genes (Fig IA) revealed that R-loops are also associated with these loci. In the wild-type strain R-loops are detected at the 5S rDNA (compare dark blue +Ab and light blue −Ab bars), as also observed for the 35S rDNA. However, for other Pol III transcripts R-loops were strongly increased by the absence of RNase H activity (rnh1Δ rnh201Δ, red +Ab bars). In each case, R-loop accumulation was further increased by depletion of Top1, although this increase was relatively small in the case of tRNA genes (rnh1Δ rnh201Δ, light green +Ab bars). In vitro treatment of S9.6 ChIP samples with recombinant RNase H strongly reduced R-loops associated with Pol III genes (Fig 1B). This confirms that Pol III fragments, which are immunoprecipitated by antibody S9.6 indeed represent sites of RNA-DNA hybrid formation.

The conclusion that R-loop accumulation is highly increased over tRNA genes in strains lacking RNase H activities was supported by ChIP-Seq data (Figs 1C–D). The expression levels of tRNA genes are correlated with the usage of the corresponding codon in mRNAs, but this is offset by the greater numbers of genes present.
Figure 1. R-loops generated by RNA Polymerase III are substrates for cellular RNase H. A: Analysis of R-loops by ChIP-QPCR using antibody S9.6 in wild-type strain BY4741 (WT) and double mutant rnh1Δrnh201Δ, and in triple mutant PGAL-TOP1 rnh1Δrnh201Δ depleted of Top1 for 6h at 30°C. CEN16, the Pol II genes ADH1 and ACT1, the Pol I transcribed 35S rRNA gene, and the Pol III genes (5S rDNA, tRNA\textsubscript{tQ(UUG)l}, SCR1, SNR6, tRNA\textsubscript{tS(GCU)F} and tRNA\textsubscript{SUF2}) were analyzed by Q-PCR. Values for no-antibody (−Ab) and antibody S9.6 (+Ab) were calculated as described in...
encoding isoacceptors for the most common codons [28,29,30]. Separation of the tRNAs into 41 gene families, ranked by codon usage, indicated that R-loop occupancy is heterogeneous between isogenic tRNAs of each family (Figs. 1C–D; number 1 indicates the most common anticodon). Importantly also, some tRNA genes were enriched over their entire transcribed region, some showed higher levels of enrichment over their 5’ region, and some other showed enrichment over their 3’ region. This heterogeneity in R-loop occupancy between and within tRNA genes is likely to reflect heterogeneity in tRNA transcription rates among isoacceptors [28,29,30], as well as differences in the thermodynamic stability of the RNA-DNA hybrids [12,13]. It should be noted that the fold enrichment of R-loops at tRNA genes in strains lacking RNase H (or also Top1) relative to input chromatin (or to the WT) in ChIP-Seq data were generally lower than those in ChIP-QPCR data (compare Figs. 1C–D with 1A). This may reflect differences in ChIP-seq efficiencies within and between samples.

For tRNA heatmaps in Figs. 1C–D, only sequence reads mapping to unique locations on the genome were used, thus excluding the possibility that hits at one tRNA isoform could be erroneously attributed to other family members. Notably, the distribution of hits extends beyond the ends of the mature tRNA species into the non-conserved flanking regions (indicated by dotted lines in Figs. 1C–D), confirming that the recovered sequences are unique and arise from the genomic loci. The R-loops overlapping 5’ and 3’ flanking regions of mature tRNA species potentially play roles in initiation and termination of transcription by Pol III [31,32].

The substantial increase in R-loops on tRNA genes seen in strains lacking RNase H activity presumably reflects their high transcription rates, whereas low levels of R-loops in wild-type strains apparently shows that these are normally cleared rapidly by RNase H. However, the ChIP-QPCR data (Fig. 1A) revealed little further increase in strains also depleted of Top1. We speculate that due to their generally short lengths, Pol III genes are less dependent on topoisomerase activity than are the long Pol I transcripts [23,25,33].

Alterations in pre-tRNA metabolism in strains with increased R-loops at tRNA genes

The presence of R-loops is expected to impede transcription elongation, increasing the time required for pre-tRNA synthesis. In contrast, the accumulation of negative supercoiling behind the polymerase in strains with reduced topoisomerase activity can increase transcription initiation rates via promoter opening.

To assess the outcome of these potentially competing effects, pre-tRNA levels were assessed in strains genetically depleted of Top1 (single PGAL-TOP1), or of both Top1 and Top2 (double PGAL-TOP1 PGAL-TOP2, designated PGAL-TOP1/TOP2 in Fig. 2), or also lacking RNase H activity (triple PGAL-TOP1 rnh1Δ and quadruple PGAL-TOP1 PGAL-TOP2 rnh1Δ). Pre-tRNA synthesis was slightly affected under conditions of partial induction of PGAL-TOP1, specifically on 0 h depletion in medium containing galactose plus sucrose (which provides the cells with limited amounts of glucose), in both the triple and quadruple mutant strains, relative to the strains with functional RNase H (Figs. 2A–D panels I, compare lanes 7 and 13 with 1, 4 and 10; quantified in Figs. 2F–I).

Following a shift to repressive, glucose-containing media, Top1 depletion in the single mutant resulted in elevated pre-tRNA levels, and this was further increased when Top2 was also depleted in the double mutant (Figs. 2A–D, panels I, lanes 5–6 and 11–12; quantified in Figs. 2F–I). Elevated levels were seen for the unprocessed primary transcripts and the unspliced but end-matured pre-tRNAs (Figs. 2B–D, lanes 5–6 and 11–12, panels I). Similarly, elevated levels were also seen for the intronless pre-tRNA species (Fig. 2A, lanes 5–6 and 11–12, panel I). However, loss of RNase H activity reversed this accumulation in each case. Indeed, the triple and quadruple mutant strains had a reduced ratio of precursor to mature tRNA for several species tested at 0 h and 6–9 h post-shift to glucose-containing medium (Figs. 2A–D, lanes 7–9 and 13–15, compare panels I and II).

Changes in transcription elongation rates are reported to impact on pre-mRNA and pre-tRNA maturation pathways [reviewed in 34,35], and our data indicate that this may also be the case for pre-tRNAs.

Integration of TY1 retrotransposons at tDNAs is favored when Top1 and RNase H are lacking

TY1 LTR-retrotransposons are composed of 2 direct long terminal repeats (LTRs) flanking the TYA and TYB open reading frames (see Fig. 3B; and [36,37]). TYA encodes the Gag structural proteins of the virus-like particle (VLP), whereas TYB encodes the protease, the integrase and the reverse-transcriptase/RNase H (RT/RNase H). ChIP-QPCR analyses revealed only low levels of RNA-DNA hybrids over TY1 retrotransposons in the wild-type strain, but notable accumulation was seen in the double mutant rnh1Δ rhn201Δ, and even more in the triple mutant PGAL-TOP1 rnh1Δ rhn201Δ following depletion of Top1 for 6 h (Fig. 3A). S9.6 ChIP-seq profiles showed that RNA-DNA hybrids are unevenly enriched across the TY1 elements in the RNase H mutants (Figs. 3B and S2). In vitro treatment of S9.6 ChIP samples of double mutant rnh1Δ rhn201Δ with recombinant RNase H strongly reduced the signals over TY1 retrotransposons confirming thus that these elements are associated with RNA-DNA prone sites (Fig. 1B).

The life-cycle of TY1 involves transcription of a chromosomal element by Pol II, reverse transcription of TY1 genomic mRNA into cDNA by the RT/RNase H inside the VLPs and incorporation of the cDNA into the nuclear genome (reviewed in [36,37]). This raised the question of whether RNA-DNA hybrids mapped to TY1 elements in the absence of cellular RNase...
H enzymes are produced by Pol II co-transcriptionally on the chromosomal elements (R-loops) or by reverse transcription in the VLPs (Yt1cDNA:RNA hybrid molecules).

Transposition of endogenous Yt1 elements is most frequent during growth at 22°C and below, much less active at 30°C and undetectable at 37°C [38]. Yt1 cDNA levels were quantified by Southern analysis of PuVII digested, total DNA. This showed an ~3 fold increase in cDNA molecules in the double rnh1A rnh201A strain relative to the wild-type, in cultures grown at 22°C (Fig S3B). To directly assess the role of RT in generating RNA-DNA duplexes on Yt1, cultures of double rnh1A rnh201A strains were treated with the RT inhibitor phosphonoformic acid (PFA) (e.g. see [39]). This greatly reduced the accumulation of RNA-DNA hybrids by 3 fold in R-Loops (see Fig S4), but not at other sites. This indicates that RNA-DNA hybrids over rnh1A rnh201A strains are mostly associated with Yt1 cDNA molecules.

During growth at 30°C, Yt1 cDNA accumulated in the triple PGAL-TOP1 rnh1A rnh201A mutant following 6 h depletion of Top1 (Fig S3C; compare lane 12 with 4). Loss of the intron-lariat debranching enzyme Dbr1 was reported to silence Yt1 retrotransposition, possibly by suppressing Ty1 replication in the VLPs [40]. The basis of this effect is unclear, but may be a consequence of the accumulation of high levels of intron lariats. The triple PGAL-TOP1 rnh1A rnh201A and quadruple PGAL-TOP1 rnh1A rnh201A dbr1A strains were compared following 6 h depletion of Top1 at 30°C. Loss of Dbr1 reduced the accumulation of RNA-DNA hybrids at Yt1 elements in ChIP-QPCR analyses (Fig 3A), and Yt1 cDNA in Southern analysis (Fig S3C, compare lanes 12 and 16). Western blotting showed that the abundance of the Gag protein p45 was slightly increased in the PGAL-TOP1 rnh1A rnh201A strain depleted of Top1, relative to the wild-type, at 30°C (Fig S5). Together these data indicate that most RNA-DNA hybrids mapped to Yt1 elements in the absence of RNase H are associated with Yt1 mRNAs, undoubtedly arising during reverse transcription of these into cDNAs in the VLPs (see model in Fig 3E). However, we cannot exclude the possibility that some of these RNA-DNA hybrids are co-transcriptional R-loops generated by Pol II transcription of chromosomal Yt1 loci, as recently proposed [11].

To assess the effects of loss of RNase H and Top1 on endogenous Ty1 retromobility, we used a BY4741 strain carrying a his3A::LY5 (see model in Fig 3E). This greatly reduced the accumulation of RNA-DNA hybrids [42]. Ty1 mobility in this strain was ~30 fold higher than in the wild-type and ~5 fold greater than the rnh1A rnh201A strain (Fig 3C). These data show that RNase H and Top1 act together to suppress endogenous Ty1 retromobility (see model in Fig 3E).

Ty1 preferentially integrates in a ~1 kb window upstream of Pol III-transcribed genes, at the nucleosomal H2A/H2B interface, with an approximate 80-bp periodicity between integration hotspots [43,44]. To determine whether Top1 and/or RNase H play roles in the targeting of endogenous Ty1 to the 5′-flanking sequences of tRNA genes, we made use of a qualitative PCR assay (Fig 3D). This yields a PCR product whenever a TY1 element integrates upstream of any of the 16 different tRNA genes. Analysis of DNA samples from the cultures used in Fig 3C showed a large increase in Ty1 integration upstream of a Yt1 element (Fig 3D, lanes 21–25), relative to the wild-type (Fig 3D, lanes 1–5). However, there was only a small increase in Ty1 integration in the double rnh1A rnh201A mutant (Fig 3D, lanes 6–10; see also Fig S6, odd numbered lanes, rnh1A rnh201A mutants). Integration of Ty1 at tRNA genes was strongly suppressed in strains lacking only Dbr1 or both Dbr1 and RNase H, relative to the loss of only RNase H (Fig S6, even numbered lanes). We conclude that Top1 and RNase H act together to restrict Ty1 integration at sites 5′ to tRNA genes, presumably by suppressing R-loop formation, with integration also dependent on intact debranching activity (see model in Fig S7).

Loss of RNase H activity increases R-loop formation in mitochondrial DNA

Analysis of the S. cerevisiae mtDNA sequences enriched with RNA-DNA hybrids (Fig 4A). At all sites in the mtDNA, R-loop formation was stronger in strains lacking RNase H activity than in the wild-type strain, but this was not further increased by the additional loss of Top1 activity (Figs 4A and S8). Notably, the degree of R-loop accumulation varied within and between mtDNA transcription units (Figs 4 and S8, e.g. compare the different regions in the relatively long ~12.38 Kb COX1/Q0045 gene with other mt genes in Fig 4A), possibly reflecting variations in the transcription initiation and elongation rates of mRNAP [43], as well as differences in the thermodynamic stability of the RNA-DNA hybrids [12,13]. Reverse transcriptase activity has been reported in mitochondria of S. cerevisiae [46]. However, our data show that RNA-DNA hybrids accumulated on mtDNA transcription units are generated through transcription by mRNAP rather than reverse transcription (Fig S4).

In order to assess the contributions of RNase H1 and RNase H2 in resolving R-loops in mitochondria, we performed S9.6 ChIP-QPCR in single rnh1A, single rnh201A and double rnh1A

Figure 2. R-loops at tRNA genes affect pre-tRNA synthesis in strains lacking topoisomerase and RNase H activities. A–E: Wild-type strain BY4741 (WT) and isogenic mutant strains single PGAL-TOP1, triple PGAL-TOP1 rnh1A rnh201A, double PGAL-TOP1/ TOP2 rnh1A rnh201A, and quadruple PGAL-TOP1/ TOP2 rnh1A rnh201A, were grown at 30°C and harvested at 0 h (galactose- and sucrose-containing medium) and at 6–9 h post-shift to glucose-containing medium. Total RNAs were extracted and analyzed by northern hybridization. The membrane was hybridized separately with probes tRNA Met (A), tRNA Leu (B), tRNA Tyr (C) and tRNA Thr (D). Ethidium bromide staining of 5S rRNA is in (E). Precursor and mature tRNA species are in subpanels I and II, respectively. Exon and intron sequences are represented by filled boxes and horizontal lines, respectively, and probe locations are indicated as lines under the schematics of the pre-tRNA species. Short (UMT+1/UMT4) and long (UMT2+UMT3) forms of tRNA Met are indicated. F–I: Quantification of tRNA precursors (pre-tRNAs) from northern analysis data in panels A–D. Pre-tRNA ratios at each time point were generated by normalising the pre-tRNA species (subpanels I) to the loading control (5S rRNA) and by expressing all the values relative to the 0 h sample of the wild-type strain, which was set to 1.
Figure 3. Cellular RNase H suppresses the mobility of Ty1 LTR-retrotransposons. A: Ty1 elements were analyzed by ChIP-QPCR for distribution of RNA-DNA hybrids in WT (BY4741) and double mutant \( mh1\Delta \) \( mh201\Delta \), and in triple mutant \( P_{GAL1}\cdot TOP1\ mh1\Delta \ mh201\Delta \) and quadruple mutant \( P_{GAL1}\cdot TOP1\ mh1\Delta \ mh201\Delta \) \( dbr1\Delta \) depleted of Top1 for 6 h at 30°C. ChIP samples and normalization of Q-PCR values to CEN16 are as in Fig. 1A. The mean of three independent experiments is shown with standard error (two independent experiments for the quadruple mutant). Ab = antibody S9.6. B: S9.6 ChIP-seq profiles over the Ty1 retrotransposon YGRWTY1-1 in the WT (BY4741), double mutant \( mh1\Delta \ mh201\Delta \), and triple mutant...
Promoter region containing RNAi elements shown in green. Black arrow = positive regulation. Red thick arrow = negative regulation. Green arrow indicates that the mtDNA regions recovered with antibody S9.6 indicate the presence of R-loops. VLP = virus-like particle. (C) and (D): The same yeast cultures served for both analyses. (E): Model for the role of co-transcriptional R-loops in activation of Ty1 retrotransposition (see Discussion). VLP = virus-like particle. Red thick arrow = negative regulation. Green thick arrow = positive regulation. For a detailed review on the mechanisms of Ty1 retrotransposition see [36,37]. See also model in Fig. S7. doi:10.1371/journal.pgen.1004716.g003

R-loops are enriched at specific sites on intron-containing genes

In the wild-type strain, clear enrichment for R-loops in the S9.6 ChIP-seq data relative to the input chromatin was seen at highly expressed mRNA genes (Fig. 5A). Most mRNA genes showing clear enrichment for R-loops also have relatively high G+C contents (Fig. 5B). The ChIP-seq findings were confirmed by ChIP-QPCR for the highly expressed genes ADH1, ACT1 and RPL28, which showed a small but significant enrichment in R-loops (+Ab red bars) relative to no-antibody control (−Ab black bars) (Fig. 5C; see also gene PMAT in Fig. S9). The levels of R-loops detected by ChIP-QPCR at Pol II genes ACT1, ADH1, PMA1 and RPL28 were similar in the wild-type and the double mutant rnh1Δ rnh201Δ (Fig. S9). Since the numbers of mapped sequences in the S9.6 ChIP-seq samples are expressed relative to total reads, the high increase in signal over Pol III genes, retrotransposons and mitochondria in mutants double rnh1Δ rnh201Δ and triple Pgal-TOP1 rnh1Δ rnh201Δ is expected to overshadow the effects seen elsewhere, precluding genome-wide analysis of mRNA genes in these strains.

Treatment of S9.6 ChIPs with recombinant RNase H1 in vitro lead to slight decreases in R-loop signals over all mRNA genes tested (Fig. S9). Note, however, that this is a ChIP experiment in which the RNA, DNA and chromatin are all formaldehyde crosslinked, and these conditions, together with the bound antibody, potentially hinder cleavage of RNA-DNA hybrids by recombinant E.coli RNase H, which has different hybrid hydrolysis properties from eukaryotic RNase H enzymes (for a review on the mechanisms of action of RNase H enzymes see [16]). In the cases of Pol I, Pol III and mtDNA genes (Fig. 1B), RNA-DNA hybrids may be much more accessible to recombinant RNase H1 due to the relative lack of nucleosomes at these loci [29,47,48,49] and/or to the length/complexity of the RNA-DNA hybrids [50].

At intron-containing genes (designated here “i-genes”) the distribution of S9.6 ChIP-seq reads was distinctly different over exon and intron sequences (Figs. 5D–E). The density of R-loops was notably higher over the second exon (exon 2) than the first exon (exon 1), or intron, particularly for spliced ribosomal protein genes (RPG i-genes) (Figs. 5D–E). This was confirmed by ChIP-QPCR for the RPG i-gene RPL28 (Fig. 5C). The majority of yeast i-genes harbor a relatively short exon1 (≤150 bp; see Fig. S10B). The asymmetric distribution pattern of R-loops over i-genes, with low relative hit densities over exon 1, was most marked for this class and particularly for the highly expressed RPGs (Fig. S11). This pattern was also visible when individual, well-expressed i-genes were examined (i-genes colored in red in Fig. S12). The distribution of hits along i-genes was very different from that seen on intronless genes (designated here “e-genes”), clearly showing it to be splicing-specific (compare Figs. 5D–E and 5F). Moreover the signal on exon 2 of RPG i-genes was clearly higher than on the RPG e-genes (Fig. S13).

Remapping sequence reads across splice junctions using STAR, revealed no sequenced reads that map across annotated splice junctions in neither the S9.6 CHIP-seq nor the input chromatin. This confirms that R-loops were accumulated over genomic loci and not associated with spliced mRNAs. This is in contrast to RNA-DNA hybrids accumulated over Ty1 elements in the double mutant rnh1Δ rnh201Δ which could be associated with Ty1 cDNAs i.e. products of reverse transcription (see Figs. S3, S4).

Analysis of G+C content across i-genes revealed higher enrichment over exon 2 than exon 1 and intron regions, in particular the RPGs (Fig. S14). Prediction of the thermodynamic stability patterns of (pre-mRNA)/DNA and DNA/DNA duplexes across i-genes, indicated that (pre-mRNA)/DNA stability is likely to be particularly weak in the intron region adjacent to the 3′ splice site, relative to exon 2, in particular for the RPGs (Fig. S14, for a description of ΔGc calculations see Protocol S1 and [12])). This potentially contributes to the sharp rise in R-loops seen at the intron-exon2 boundary (Figs. 5D–E and S11). Altogether these data clearly show that there is a correspondence between G+C content, thermodynamic stability of (pre-mRNA)/DNA duplexes, R-loop accumulation and transcription activity over yeast i-genes.

On spliced genes, R-loops are less abundant over exon1 and intron sequences than on the second exon, particularly on highly expressed intron-genes. We speculate that R-loops are suppressed at intron regions to ensure proper recognition of 5′ and 3′ splice sites by the splicing machinery, whereas they are favored over exon 2 to decelerate elongation of Pol II and by doing so to promote co-transcriptional splicing [51,52,53].
Discussion

New RNA-DNA substrates for RNase H

Here we report the application of ChIP-seq to systematically identify sites of RNA-DNA duplex formation throughout the nuclear and mitochondrial genomes in budding yeast. Numerous, prominent sites of R-loop enrichment were identified in actively transcribed genes by all RNA polymerases, Pol I, II, III and mtRNAP. Over the Pol I transcribed rDNA, the distribution of R-loops agreed well with the pattern previously observed in conventional ChIP analyses [23]. In addition, loss of cellular RNase H activity resulted in strong accumulation of RNA-DNA hybrids at Pol III transcribed genes, Ty1 retrotransposons, the second exon of spliced genes and over transcription units of the mtDNA. While this work was in preparation, an analysis of R-loop prone-sites in budding yeast arrived at similar, but not identical, conclusions [11].

Stable R-loops affect pre-tRNA synthesis

R-loops were strongly detected over the Pol I transcribed 35S rDNA and the 5S rRNA genes in the wild-type. Various other Pol III transcripts including tRNAs, scr1, U6 snRNA and the snoRNA snRNA52 were also enriched in R-loops particularly in the absence of RNase H activities.

Over the Pol I promoter and the rDNA 5’-ETS, R-loop formation was increased in mutants lacking both Top1 and RNase H (data herein and [23]), presumably also reflecting the effects of DNA strand separation over these regions (see Fig. 4 in [25]). The unusual high rates of transcription initiation by Pol I and Pol III (reviewed in [31,32,35,54]) may be facilitated by negative DNA supercoiling and strand separation over the promoter regions [33,53,56]. However, this can also favor formation of R-loops that interfere with transcription elongation [23,25]. The accumulation of negative supercoils behind transcription bubbles is expected to be enhanced by loss of Top1 or also Top2 [23,25,33,57]. Consistent with this, increased pre-tRNA accumulation was observed in strains lacking Top1 or also Top2. However, the increase in pre-tRNAs was reversed when RNase H was also absent. We therefore propose that increased transcription initiation of tRNA genes due to promoter opening, particularly when Top1 or also Top2 is absent, can be offset by impaired elongation due to stable R-loop accumulation in strains also lacking RNase H. This is reminiscent with the reduced rates of pre-tRNA synthesis in strains lacking both Top1 and RNase H due to stable R-loop accumulation at the rDNA repeats (data herein and [23,25]).

Our data agree with the recent report [11] that R-loops at tRNA genes are processed by RNase H. It is possible that nascent tRNAs engaged in R-loops in wild-type yeast are rapidly cleaved by RNase H and/or resolved by helicase Sen1/Senataxin [11] and targeted to degradation by the TRAMP/exosome 3’-5’ surveillance machinery [13,23,58,59,60].

Roles of Top1 and cellular RNase H in Ty1 retrotransposition

Analyses of the Ty1 class of endogenous LTR-retrotransposons in strains lacking cellular RNase H and Top1 activities revealed marked increases in the abundance of RNA-DNA hybrids and in the frequency of retrotransposition.

Most of the RNA-DNA hybrids mapped to Ty1 elements in strains lacking cellular RNase H or also Top1 activities are associated with Ty1 cDNAs rather than chromosomal genes. These hybrids are products of reverse transcription that may have escaped cleavage by the RT/RNase H protein (for a review on Ty1 replication see [61]). Ty1 RNA:DNA hybrid molecules could be produced during synthesis of either the first (minus) DNA or the second (plus) DNA strand in the VLPs. Minus strand synthesis requires reverse transcription of the highly structured Ty1 genomic RNA, which could be hampered by potential RT pausing/stalling events [62]. Plus strand synthesis requires priming at specific polypurine tracts, which are resistant to cleavage by the RNase H domain of the RT [61].

Ty1 cDNAs and/or retromobility are increased in a variety of different genome-maintenance mutants (reviewed in [36]). It is possible that DNA damage inflicted on the genome by co-transcriptional R-loops, e.g. in mutants lacking cellular RNase H or also Top1, leads to the alleviation of Ty1 dormancy (see model in Fig. 3E). Additionally, RNase H1 and H2 may directly cleave RNA-DNA hybrids generated by reverse transcription of Ty1 genomic RNAs. Cleavage of Ty1 RNA:DNA hybrid molecules by cellular RNase H could occur inside and/or outside the VLPs (see model in Fig. 3E and [36,63]). Notably, RNA-DNA hybrids associated with cDNAs of endogenous retroelements may play roles in the pathogenesis of autoimmune diseases in humans, e.g. in RNase H2-AGS [64,65].

The LTR-retrotransposons Ty3 and Ty5 in S. cerevisiae and Tt1 in S. pombe are selectively targeted to nuclear genomic regions through interactions between the retrotransposon and specific transcription factors and/or chromatin [reviewed in [36,37,66]]. Ty1 incorporation most commonly occurs by integrase-mediated integration at the nucleosomal H2A/H2B interface upstream of Pol III-transcribed genes [43,44,67], with a periodicity of ~80 bp that is mediated by interactions between the ATP-dependent chromatin remodeling factor Isw2 and the TFIIB transcription complex [68,69]. While pre-tRNA synthesis was reduced in strains lacking both Top1 and cellular RNase H activities, the integration of Ty1 at target sites upstream of tRNA^4L^3A was on the contrary strongly increased in these mutants (see model in Fig. 3E). tRNA genes act as nucleosome phasing signals in both directions, possibly due to specific properties of the TFIIBB-TFIHC transcription complex [49], and R-loops might affect the stability/flexibility of this complex leading to altered nucleosome dynamics/phasing, thus creating an environment that is conducive to Ty1 integration (see model in Fig. S7). It is possible that collisions between the DNA...
Figure 5. R-loops are enriched over the second exon of intron-containing genes. A–B: Box plots of mean sequence read distribution per gene across all yeast protein-coding genes (n = 5864, see Fig. S10A) in samples “input chromatin” and “S9.6 ChiP-seq” from the wild-type strain (BY4741) grown at 30°C in YEPD medium (glucose 2%). The y-axis represents the relative enrichment of sequencing reads where values >1 are above the background level of sequencing (i.e. general intergenic mean, see Materials and Methods). (A) Genes were clustered arbitrarily in 6 main categories based on the strength of their mRNA expression (X-axis): C1-0 (very low), C1 (low), C2 (medium-low), C3 (medium-high), C4 (high) and C4-max (very high) (see Fig. S10A and [101]). We used a Kolmogorov-Smirnov test to determine whether levels of mean sequence distribution differ significantly between the input chromatin and ChIP-seq data within each mRNA expression group: C1-0 (D = 0.5, p-value = 1.85E-008), C1 (D = 0.8109685, p-value = 0), C2 (D = 0.8624161, p-value = 0), C3 (D = 0.8607383, p-value = 0), C4 (D = 0.8439024, p-value = 0) and C4-max (D = 0.7888889, p-value = 4.440892E-16). (B) Genes were clustered based on their GC composition across the entire length of the gene (X-axis). Box-plots show median values (black line) +/− 25% quartiles in the box and minimum/maximum distribution of the values (excluding outliers) in the
whiskers. The width of the boxes reflects the number of genes in each group. C: ChiP-QPCR analysis of R-loops over control CEN16 and mRNA genes ADH1, ACT1 and intron-gene RPL28 (CHY2) in strain wild-type (BY4741), grown at 30°C in YEPD medium (glucose 2%). Q-PCR values were calculated and normalized to CEN16 as in Fig. 1A. The mean of three independent experiments is shown with standard error. Ab = antibody S9.6. Exon1 and intron regions of RPL28 are represented with a filled box and a horizontal line, respectively. D-F: Box plots of S9.6 ChiP-seq profiles of R-loops over mRNA genes in the wild-type (BY4741) (same samples as in panel A). (D) Intron-containing, non-ribosomal protein genes (NRPG i-genes). (E) Intron-containing, ribosomal protein genes (RPG i-genes). (F) Top 387 highly expressed intronless genes (e-genes). Each box plot represents the log, fold-change of S9.6 ChiP-seq relative to input chromatin, so the regions above zero value on the Y-axis are enriched with R-loops (see also Fig. S13). For ease of comparison between panels the horizontal dotted green line points to the position of the top R-loop signal on exon 2 in panel E. For i-genes in panels D-E, R-loop profiles are plotted across the Exon1-Intron-Exon2 region. The 5′ end of Exon 1 is defined either as the AUG start codon, or 100 bp upstream of the 5′ splice site for genes with Exon 1 <100 pb (see also Protocol S1). For e-genes in panel F (top 387 highly expressed, see Fig. S10A), R-loop profiles are plotted across the entire length of the gene.

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R-loops associate with yeast mitochondrial DNA

We observed the accumulation of R-loops over the mitochondrial DNA (mtDNA) transcription units, specifically in strains lacking RNase H1. The ~80 Kb S. cerevisiae mt chromosome comprises relatively long transcription units such as the genes COX1/Q0045 and COB/Q0105, which expression and polycistronic structure (with multiple exons and introns) are extremely complex [71]. Mitochondrial transcription-translation coupling is expected to suppress R-loop formation, as is believed to be the case in bacteria [reviewed in [72,73]]. However, gene structure and expression, poor packaging of nascent transcripts, G.C composition of the sequence (see Fig. 4A), transcription-mediated topological stress and other factors may favor R-loop formation on mtDNA [4].

The yeast mt DNA encodes proteins and RNAs with key roles in mitochondrial function [74,75,76], but most proteins that are needed in mitochondria are encoded by the nuclear genome and imported from the cell cytoplasm. A mitochondrial function for RNase H1 has previously been reported in mammals [19,77], but this was not known to be the case for yeast. However, dramatic accumulation of R-loops was seen over transcription units in the mtDNA in the absence of RNase H1, but not RNase H2, strongly indicating that RNase H1 does function in this organelle. Notably, the absence of Top1 failed to exacerbate the accumulation of R-loops at mtDNA in yeast strains lacking also RNase H activity.

In yeast the mtDNA is not essential for viability on glucose-containing medium and many mutations cause instability of the mtDNA, leading to a complete loss (rho(-) petites) or truncations of rho(-) petite formation 2–3 fold, to 45% (Fig. S15). Processing of mRNA 3′ cleavage and polyadenylation (mCP) machinery [60,81,82,83,84], have all been associated with R-loop formation, with deleterious effects on genome stability. In mammalian cells, R-loops can result in silencing of protein coding genes, with potentially pathogenic outcomes [8,85,86].

Most intron-containing genes in yeast, particularly ribosomal protein genes, have a short exon 1, and this correlates with higher levels of expression relative to genes with longer exon 1 (see Fig. S10). The close proximity of the 5′ splice site to the promoter region may stimulate transcription via coupling of the splicing and Pol II initiation machineries [87,88]. In the S9.6 ChiP-seq data, R-loops were reduced over short exon 1 regions and the accompanying intron, relative to the second exon, on most spliced genes (see Figs. 5D–E and S11). It was recently proposed that R-loop formation downstream from CpG-rich regions of strong promoters of highly expressed spliced genes in mammalian cells may be more favored over longer than shorter first exon regions [9]. R-loops can slow down elongation of the RNA polymerase (reviewed in [2,3]), and co-transcriptional splicing is kinetically coupled to transcription elongation by Pol II [reviewed in [89]]. We speculate that co-transcriptional R-loops have been counter selected over short exon 1 and the associated introns. This may promote high expression of these genes, together with proper recognition of their 5′ and 3′ splice sites (S5). Notably, depletion of the splicing factor ASF/SF2 (alternative splicing factor/splicing factor 2) in mammalian cells can lead to increased R-loop formation and genome instability [53,90]. Whereas R-loops were reduced over exon 1 and intron regions of spliced genes, they were increased over exon 2 (see Figs. 5D–E and S11). We speculate that

Exon 2 is a favored site for R-loop formation in spliced, protein-coding genes

As for RNA Pol I, Pol III and mtRNAP genes, the actively transcribed mRNA genes were also associated with R-loops, albeit at much lower rates. Thus there appear to be a general link between transcriptional activity and R-loop formation in budding yeast, as recently reported in [11].

Competition between RNA packaging and R-loop formation is a normal feature of mRNA synthesis. R-loop formation at mRNA genes could be dictated by factors including increased residence in proximity to the DNA template of poorly packaged transcripts and the G.C content of the sequence (reviewed in [2,3,5]). Indeed the thermodynamic helical stability of (pre-mRNA/DNA) duplexes at highly expressed mRNA genes in yeast is higher than DNA/DNA duplexes mostly due to the relatively higher content in G.C of these genes in comparison to the less-well expressed mRNA genes (Fig. S16). More generally, there was a correspondence between transcription activity, R-loop formation and G.C. content at most yeast mRNA genes (see Figs. 5A–B and [11]). Yeast mutants of RNA biogenesis factors, including the helicase Sen1/SENA-TAXIN, the THO/TREX RNA packaging complexes, the RNA exosome, the RNA-binding protein Npl3 and components of RNA 3′ cleavage and polyadenylation (mCP) machinery [60,81,82,83,84], have all been associated with R-loop formation, with deleterious effects on genome stability. In mammalian cells, R-loops can result in silencing of protein coding genes, with potentially pathogenic outcomes [8,85,86].
R-loops over exon 2 could decelerate elongation of Pol II [2,3], and/or create a chromatin environment favorable for Pol II pausing [59], thus promoting co-transcriptional splicing [51,52]. Supporting these models, a computational, thermodynamic study covering many genomes including *H.sapiens*, predicted that R-loops will generally be less stable around the 5′ and 3′ SS, due to differences in the helical stability of (pre-mRNA)/DNA and DNA/ DNA duplexes [12]. The application of this approach to yeast indicated that (pre-mRNA)/DNA duplexes are indeed intrinsically less favored on introns compared to exons, in particular around the 3′ SS regions of ribosomal protein genes (see Fig S14), and this is potentially related to the high splicing efficiency of these pre-mRNAs [91].

**Materials and Methods**

**Strains, plasmids and growth conditions**

Yeast strains and plasmids used in this study are listed in Table S1. Growth and handling of *S.cerevisiae* were by standard techniques. For Top1 and Top2 depletion, cells (PGAL-TOP1 and PGAL-TOP1/2 strains) were grown at 30°C to OD600 ~0.3–0.4 in complete Kaiser synthetic SGS minimal medium (2% glucose). Growth was continued for several hours and maintained in exponential phase by dilution with pre-warmed SD medium.

**Chromatin immunoprecipitation (ChIP) analyses of RNA-DNA hybrids**

Immunoglobulin IgG2a of monoclonal antibody S9.6 [26,27] were purified from mouse hybridoma cell line supernatants by Eurogentec. ChIP of RNA-DNA hybrids using the antibody S9.6 was performed mainly as described in [23]. Crosslinking of exponentially growing cells (OD600 ~0.6, 50 OD600/ChIP sample) with formaldehyde (1%) was for 25 min at room temperature. Pellets were resuspended with 400 μL of FA-1 1x buffer [50 mM HEPES-KOH at pH 7.5, 140 mM NaCl, 1 mM EDTA at pH 8, 1% Triton X-100, 0.1% w/v sodium deoxycholate, plus CPI-EDTA 1x (Protease inhibitor cocktail, Roche 11697498001)], mixed with 500 μL of glass beads (Sigma, G8772), and vortexed (Vortex Genie 2T, Scientific Industries) for 45 min at full speed at 4°C. Glass beads were removed and cross-linked chromatin was recovered by centrifugation at full speed for 10 min at 4°C (supernatant discarded). Eight-hundred microliters of FA-1 buffer were added on the top of the pellet. Sonication of chromatin was performed for 2 min (10 sec ON, 15 sec OFF, 20% amplitude; Branson Digital Sonifier) to yield an average DNA fragment size of ~500 bp. Sonicated chromatin were spun for 15 min at full speed at 4°C and glycerol 5% was added to supernatants. Sonicated chromatin were mixed with sepharose CL-4B beads (Sigma CL4B200) and cleared for 1 h at 4°C. Twenty microliters were kept for control Input chromatin. Immunoprecipitations were performed by mixing ‘cleared-sonicated chromatin’ with 35–40 μg of IgG2a of antibody S9.6 together with 100 μL bed of Protein A sepharose CL-4B beads (GE Healthcare 17-0780-01) on a rotating wheel overnight at 4°C. To assess contribution of background, a ‘beads-only’ internal control was prepared in parallel to immunoprecipitated samples but without addition of any antibody. Beads were recovered (see also paragraph “Treatment of S9.6 ChIP with recombinant RNase H”) and washed successively with FA-1 buffer (plus CPI-EDTA 1x), FA-2 buffer (as FA-1 buffer but with 500 mM NaCl, plus CPI-EDTA 1x), FA-3 buffer (10 mM Tris-HCl at pH 8, 0.25 M LiCl, 0.5% NP-40, 0.5% w/v sodium deoxycholate, 1 mM EDTA at pH 8, plus CPI-EDTA 1x), and TE 1× (10 mM Tris-Cl at pH 8, 10 mM EDTA at pH 8) at 4°C. Cross-link reversal of sonicated chromatin from samples ‘input chromatin’, ‘beads-only’ and ‘S9.6 immunoprecipitate’ were performed by incubating the washed beads overnight at 65°C in 250 μL of TE buffer containing 1% SDS and 1 mg/mL proteinase K. DNA was purified using Qiagen PCR purification kit and eluted with 55 μL of buffer EB containing RNase A (0.5 μg/μL). 10–20 ng/μL of DNA were recovered from ‘S9.6 immunoprecipitates’ from wild-type cells as measured by Qubit dsDNA HS Assay Kit (Invitrogen, Q32851). Quantitative PCRs (qPCRs) were performed in triplicate in a MX3000P real-time PCR machine (Agilent Technologies) in 10 μL reaction containing: 5 μL of 2× TaKara SYBR premix Ex Taq II Ti Rnase H Plus (Clontech RR020L), 1 μL DNA (but 10% of input chromatin), 0.4 μL of 10 μM primers (see Table S3), 0.04 μL of Rox II and 3.56 μL of water. Values for ChIPs were calculated using the formulas ΔΔCt “no antibody” = 2−ΔΔCt ‘beads only’ - Ct ‘input chromatin’) and ΔΔCt ‘S9.6 immunoprecipitate’ = 2−ΔΔCt ‘S9.6 immunoprecipitate’ - Ct ‘input chromatin’.

The ‘S9.6 immunoprecipitate’ and ‘input chromatin’ were further processed for ChIP-seq as described below.

**Preparation of Sollexa/Illumina libraries for S9.6 ChIP-seq**

200 ng of DNA (‘S9.6 immunoprecipitate’ and ‘input chromatin’) were linker-ligated as described mainly in the Illumina’s hand book with some modifications. Step1 “Repair DNA ends”: 100 μl reactions contained, DNA, 1× T4 DNA ligase buffer (NEB, B0202S), 0.4 mM dNTP mix, 15 units T4 DNA polymerase (NEB, M0203L), 50 units T4 Polynucleotide Kinase (NEB, M0201L) and 5 units DNA Polymerase I- Large (Klenow) Fragment (NEB, M0210L). Reactions were incubated at 20°C for 30 min. DNA was purified using QiAquick PCR Purification Kit (Qiagen, 28106). DNA columns were centrifuged several times up to 10 min to ensure that no residual traces of ethanol are left in the column. DNA was eluted with 32 μL EB buffer (10 mM Tris-HCl, pH 8.5) which was pre-heated to 55°C. Step2 “Add A”: 50 μl reactions contained DNA from step 1, 1 x NEB buffer 2 (NEB, B7002S), 0.2 mM dATP and 15 units Klenow Fragment (NEB, M7200S), 0.2 mM dCTP and 1600 units Quick DNA ligase (NEB, M2200S), 3.33 nM PE adapter mix and 1600 units Quick T4 DNA ligase (NEB, M2200S) (for primer sequences see Table S3). Reactions were incubated at room temperature (18–22°C) for 30 min. DNA was purified and eluted with 36.5 μL EB buffer as described in step 1. Step 4 ‘PCR amplification’: 50 μl reactions contained: DNA from step three, 1 unit Phusion high fidelity (HF) DNA polymerase (NEB, B0518S), 1× HF buffer (NEB, B0518S), 0.2 μM primer 1.2, 0.2 μM primer 2.2 and 0.2 mM dNTP mix (for primer sequences see Table S3). Cycling conditions were 98°C for 30 sec; followed by 18 cycles (98°C for 10 sec, 65°C for 30 sec and 72°C for 1 min); followed by 72°C for 5 min; followed by cooling to 4°C. PCR DNA was purified using MinElute spin column (Qiagen, 28006) and eluted with 10 μl EB buffer as described in step 1. Step 5 “Size selection”: DNA from step 4 was well resolved on 2% agarose gel [mixture 3:1 of standard agarose: Metaphor agarose (Lonza, 50180)] in 1× TBE, alongside with a DNA ladder, stained with SYBR safe (Invitrogen) and visualised with Fuji FLA-5100 PhosphorImager. 300 ± 50 bp DNA were excised from the gel and purified using QIAquick Gel Extraction Kit (Qiagen, 28706). Note that in order to improve the
representation of A+T rich-DNA sequences agarose gel slices were melted at room temperature (18–22°C). DNA was eluted using MiniElute spin column (Qiagen, 20006) with 10 μl EB buffer as described in step 1 and sent for high-throughput sequencing.

**Treatment of S9.6 ChIP with recombinant RNase H**

One-hundred microliters of bed of Protein A beads incubated with sonicated-crosslinked-chromatin and antibody S9.6 (see paragraph 'Chromatin immunoprecipitation analyses of RNA-DNA hybrids') were washed successively with FA-1 buffer (plus CPE-EDTA 1×), TE 1× buffer (plus CPE-EDTA 1×) and 10 mM Tris-HCl pH 8 (plus CPE-EDTA-free 1×, Roche 1187580001). Washed beads were re-suspended in 300 μl of reaction buffer containing RNAse H buffer 1× (NEB, M0297L), 4% glycerol and 20 μg/ml BSA. Beads were incubated for 2.5 h at 37°C in absence or presence of 15 μl of recombinant E. coli RNase HI (75 units, NEB, M0297L), with shaking at 1000 rpm (Eppendorf Thermomixer). RNase H reactions were stopped by adding 10 mM EDTA. Beads were washed successively with buffers FA-2, FA-3, and TE 1×, sonicated-chromatin was reverse-crosslinked, and DNA was recovered and analysed by q-PCR as described for the standard ChIP protocol.

**Northern analyses**

Equal amounts of total RNA (10 μg) were resolved on standard 8% polyacrylamide-8.3M urea gels for low molecular weight RNAs. Northern hybridizations for tRNAs were performed overnight at 37°C in ULTRAHyb-oligo buffer (Ambion, Invitrogen, AM8063) and washed done at 37°C in SSC 6×. Northern signals were generated by a Fuji FLA-5100 PhosphorImager and quantified with AIDA software (Raytest). For primer sequences see Table S2.

**Ty1 retrotransposition assay**

In strain JCY3212 (BY4741, TY1his3Ai-[A1]-3114; HIS3) gene was inserted in the TYB sequence of a Ty1 element in the antisense orientation (see Table S1 and [41]). HIS3 RNA synthesis from TY1his3Ai element was interrupted by an artificial Ai intron which is only spliced during transcription of TY1 RNA. TY1his3Ai retrotransposability occurs only when the TY1his3Ai RNA is spliced, reverse transcribed and the resulting Ty1HIS3 cDNA is incorporated into the genome by integration or recombination. For the activation of Ty1 retrotransposition in conditions non-permissive for Top1 expression (Figs. 3C–D), cells were: 1) grown overnight at 30°C in Kaiser synthetic SGS Drop-Out minimal medium, 2) diluted in SD Drop-Out medium to OD600 ~0.05 and grown for 3 doublings at 30°C, 3) re-diluted to OD600 ~0.01 in the same medium and aliquoted in 5 cultures of 10 ml each, and, 4) grown at 18°C until saturation (4–6 d). Cells were harvested, washed and re-suspended in 3 ml sterile water. For total number of colonies, aliquots of each culture (dilution 1:105) were plated on SD Drop-Out -Leu minimal medium and incubated at 30°C. For HIS+ colonies, aliquots of each culture (dilution 1:2) were plated on SD Drop-Out -Leu-His plates and incubated at 30°C. The rate of TY1his3Ai transposition is the number of HIS+ colonies divided by the total number of colonies (as described in [92]).

**Integration assay for Ty1 elements at tRNAGLY sites**

Spontaneous Ty1 insertions upstream of 16 tRNAGLY genes were detected as described previously [93,94] with some modifications. To confirm that the genomic DNA samples were in the linear range for PCR, DNA concentrations were measured with a Qubit dsDNA BR Assay Kit (Invitrogen, Q28250) and equal amounts of 6 or 30 ng DNA were assayed by PCR. Reactions of 50 μl contained 1× Phusion HF buffer (NEB, B05185), 0.2 μM primer ‘TYB OUT’, 0.2 μM primer ‘SUF16’ (for primer sequences see Table S3), 0.2 mM dNTP mix, 1 unit Phusion HF (NEB, B05185), and genomic DNA. Cycling conditions were 98°C for 30 sec; followed by 30 cycles (98°C for 10 sec, 57°C for 30 sec and 72°C for 1 min); followed by 72°C for 10 min; followed by cooling to 4°C. PCR DNA fragments were resolved on a standard 1.5% agarose gel (stained with SYBR safe, Invitrogen) in 1× TBE and visualized with a Fuji FLA-5100 PhosphorImager. For primer sequences see Table S3.

**Southern blot analysis of Ty1 cDNA**

Total genomic DNA were extracted by standard glass-bead/phenol lysis (e.g. see [95]). DNA concentrations were measured with a Qubit dsDNA BR Assay Kit (Invitrogen, Q28250). 2 μg DNA were incubated overnight at 37°C in presence of 200 units of restriction endonuclease PvuII-HF (NEB, R3151). DNA samples were resolved on a standard 1% agarose gel (stained with ethidium bromide) in 1× TBE. Washes and blotting of the gel were performed mainly as described in [95], but the depurination step was omitted. DNA random priming probes were prepared using DECAPrime II Random Primed DNA Labelling Kit (Ambion, Invitrogen, AM1455; for primer sequences see Table S2), hybridized overnight at 42°C in Hybridization Buffer (50% formamide, 5× SSC, 5× Denhardt’s solution, 0.5% SDS and 100 μg/ml sonicated salmon sperm DNA), and washed at 55°C with 2× SSC, 0.1% SDS and 0.1× SSC, 0.1% SDS. Southern signals were generated by a Fuji FLA-5100 PhosphorImager and quantified with AIDA software (Raytest).

**Western blotting**

Total protein extracts and Western blot analysis were performed using standard procedures. Mouse anti-Ty1 Gag antibodies, raised against the Ghu-Val-His-Thr-Asn-Gln-Asp-Pro-Leu-Asp peptide (Diagenode, anti-Ty1-tag, MAB-054-050; and see [96]), and rabbit anti-beta-actin antibodies (Abcam, ab 34731) were used as primary antibodies. Horseradish peroxidase-conjugated antibodies (GE Healthcare) were used as secondary antibodies.

**Bioinformatic analysis**

**Read alignment and normalization.** 50 bp reads were sequenced by an Illumina MiSeq Benchtop Sequencer, quality trimmed and filtered with Trimomatic and aligned to the *S.cerevisiae* sacCer3 genome assembly using Novaalign V2.07. Reads mapping to more than 1 location were assigned a random alignment (or uniquely mapped for tRNA heatmaps in Figs. 1C–D). Reads aligning to rDNA (except for Fig. S1) were further removed from the analysis. Alignments (BAM files) were transformed into coverage files (bigWig) using the deepTools package, which extends reads to the estimated fragment length (200 bp) and applies a windowing function to calculate the average read depth in 50 bp windows. A smoothing function was also applied so each 50 bp window contained the average value of the surrounding 100 bp. In order to compensate for differences in ChIP-seq efficiencies a background level of sequencing (see e.g. Fig. S12) was attained by calculating the mean read depth of all intergenic regions covered by at least 1 read (regions not annotated by SacCer3 genome http://www.yeastgenome.org). A measure of enrichment for each window was given as the ratio of the windows mean depth vs the intergenic mean (i.e. 50 bp window score = “average read depth in surrounding 100 bp”/“intergenic
mean depth”). This method of normalization was mainly adapted from [97].

**Accession numbers**

The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus [98] and are accessible through GEO Series accession number GSE53420.

**Supporting Information**

**Figure S1** R-loops over the rDNA repeats are detected by S9.6 ChiP-seq. A: Analysis of R-loops by ChiP-seq using antibody S9.6 in the wild-type strain BY4741 (WT) and double mutant rnh1Δ rnh201Δ, and in triple mutant P_{GAL-TOP1} rnh1Δ rnh201Δ depleted of Top1 for 6 h at 30°C. Also shown is the input chromatin profile of the wild-type strain. Raw read counts were not corrected by the number of rDNA repeats. G+C content of the DNA sequence was calculated for 100 bp windows and is depicted as a blue intensity. Shown below the profiles is a diagram of one rDNA repeat which comprises the 35S rDNA gene, transcribed by Pol I in to the 35S pre-rRNA which is processed to 18S, 5.8S, and 25S rRNAs. 35S genes are flanked by the intergenic spacers IGS1 and IGS2 and the 58 rDNA gene, transcribed by Pol III. The direction of transcription is indicated by a tailed arrow. The diagram is not drawn to scale.

(TIF)

**Figure S2** Average profiles of RNA-DNA hybrids over all Ty1 elements. Median S9.6 ChiP-seq profiles of RNA-DNA hybrids over the 31 Ty1 elements in strains WT (BY4741) and double mutant rnh1Δ rnh201Δ, and in triple mutant P_{GAL-TOP1} rnh1Δ rnh201Δ depleted of Top1 for 6 h at 30°C. Median profiles of control input chromatin from WT are also shown. The y-axis represents the relative enrichment of reads where values >1 are above the background level of sequencing (i.e. general intergenic mean, see Materials and Methods).

(TIF)

**Figure S3** TY1 cDNAs accumulate in mutants lacking cellular RNAse H or also depleted of Top1. A: Diagrams of unintegrated TY1 cDNA and a genomic Ty1 element, indicating the location of the TY1B hybridization probe (filled black rectangle) and relevant PvuII cleavage sites. Probe TY1B detects an ~2 Kb PvuII DNA fragment of unintegrated TY1 cDNA and variably sized PvuII DNA fragments >2 Kb containing the junction of Ty1 elements with chromosomal DNA at different locations in the genome. B: Southern analyses of TY1 cDNA from strain WT (BY4741) and double mutant rnh1Δ rnh201Δ, grown at 3°C in YEHP medium (glucose 2%) until saturation, in the absence or presence of 600 mg/ml of phosphonoformic acid (PFA), which is an inhibitor of TY1 RT [39]. The ratio of the ~2 Kb TY1 cDNA was determined by normalising the intensity of the TY1 cDNA band relative to the average intensities of 3 genomic Ty1 junction bands (filled black circles). Values were expressed relative to the wild-type (-PFA, lane 1) which was set to 1. TY1 cDNA band is indicated by a tailed arrow. Indicated to the left of the gel the migration position of a 2 Kb band from a DNA size ladder. C: Southern analyses of TY1 cDNAs from strain WT (BY4741) and mutant strains double rnh1Δ rnh201Δ, single P_{GAL-TOP1}, triple P_{GAL-TOP1} rnh1Δ rnh201Δ, single dbr1Δ, triple rnh1Δ rnh201Δ dbr1Δ, double P_{GAL-TOP1} dbr1Δ, and quadruple P_{GAL-TOP1} rnh1Δ rnh201Δ dbr1Δ. Cultures were grown at 30°C in medium containing galactose and sucrose (permissive for P_{GAL-TOP1} expression, lanes 1–9) and shifted for 6 h to medium containing glucose (non-permissive for P_{GAL-TOP1} expression, lanes 9–16). TY1 cDNA band is indicated by a tailed arrow. Indicated to the left of the gel the migration position of a 2 Kb band from a DNA size ladder.

(TIF)

**Figure S4** Reduced accumulation of RNA/DNA hybrids at Ty1 in the absence of RT activity. Double mutant rnh1Δ rnh201Δ was grown in YEHP medium (glucose 2%) at 22°C in the absence or presence of 600 mg/ml of phosphonoformic acid (PFA), which is an inhibitor of TY1 reverse transcriptase (RT) [39]. ChiPs were performed with no-antibody (−Ab) or antibody S9.6 (+Ab), The Pol I transcribed gene (18S rDNA), Ty1 retrotransposons, mtDNA transcription units (COX1 and 21S rDNA), Pol III gene tRNA SUF2, mRNA gene RPL28 and CEN16 were analysed by Q-PCR as described in Fig. 1A. The mean of three independent experiments is shown with standard error.

(TIF)

**Figure S5** Gag proteins are slightly increased in mutants lacking both Top1 and cellular RNAse H. Immunoblots of cellular homogenates from strain WT (BY4741) and mutant strains double rnh1Δ rnh201Δ, triple P_{GAL-TOP1} rnh1Δ rnh201Δ and quadruple P_{GAL-TOP1} rnh1Δ rnh201Δ dbr1Δ. Yeast cultures grown at 30°C were shifted from galactose- and sucrose-containing medium to glucose medium and harvested at 6 h depletion of Top1. Panel I. Blot probed with antibody anti-Ty1 Gag. The Gag-p49 and processed-Gag-p45 bands each appear as doublets. Panel II. Blot probed with antibody anti-beta-actin.

(TIF)

**Figure S6** PCR analyses of integration of Ty1 at tRNA{Gly} in mutants lacking RNAse H and/or Dbr1. Four independent isolates for each strain, WT (BY4741) and mutant strains single dbr1Δ, double rnh1Δ rnh201Δ, triple rnh1Δ rnh201Δ dbr1Δ, triple P_{GAL-TOP1} rnh1Δ rnh201Δ, and quadruple P_{GAL-TOP1} rnh1Δ rnh201Δ dbr1Δ, were grown until saturation at 18°C in medium containing both galactose and sucrose (permissive for P_{GAL-TOP1} expression). Panel I. See legend in Fig. 3D. Panel II. Representative examples of SYBR-stained gels are shown, revealing integration of Ty1 cDNA upstream of the 16 tRNA{Gly} gene loci. Shown to the right of the gels DNA ladders with lengths in base-pairs (bp).

(TIF)

**Figure S7** Model: Pol III-associated R-loops facilitate targeting of Ty1 at 5' flanking regions of tRNA genes. Ty1 integration upstream of tRNA genes is specifically targeted to the H2A/H2B interface of nucleosomal DNA in a ~1 kb window [43,44,67]. The nascent transcript behind elongating Pol III can invade the DNA duplex and hybridize with the DNA template strand, generating a three-stranded R-loop structure, composed of an RNA-DNA duplex and an unpaired non-template DNA strand. We postulate that alterations in chromatin structure due to R-loop formation [102,103] at Pol III genes, favor recruitment of the Ty1 pre-integration complex formed by the integrase (IN) and the DNA (green thick arrow = positive regulation). Black thick arrow = transcription direction. The diagram is not drawn to scale.

(TIF)

**Figure S8** ChiP-QPCR of R-loops in mutants lacking both Top1 and cellular RNAse H. ChiP samples using antibody S9.6 (same as in Fig. 3A) are from strains WT (BY4741) grown in YEPD medium to glucose medium and harvested at 6 h depletion of Top1.

(TIF)
double mutant \( \text{rnh1}\Delta \text{rnh201}\Delta \) and from mutants triple \( P_{CAY}^{\text{TOP1}} \text{rnh1}\Delta \text{rnh201}\Delta \) and quadruple \( P_{CAY}^{\text{TOP1}} \text{rnh1}\Delta \text{rnh201}\Delta \text{dbr1}\Delta \) depleted of Top1 for 6 h at 30°C. \( \text{CEN16} \) and four different regions of \( \text{COX1} \) genes were analysed by Q-PCR as described in Fig. 1A. \( \text{Ab} \) = antibody S9.6.

**Figure S9** Formaldehyde-crosslinked R-loops associated with mRNA genes are seldom cleaved in vivo by recombinant RNase H1. ChIP samples are from strains WT (BY4741) and double mutant \( \text{rnh1}\Delta \text{rnh201}\Delta \) (same as in Fig. 1B) grown at 30°C in YEpd (glucose 2%). ChIPs were performed with no-antibody (−Ab) or antibody S9.6 (+Ab), or with antibody S9.6 but beads were further incubated for 2.5 h at 37°C in absence [+Ab]/+ RNase H1) or presence [+Ab]/+RNase H1) of recombinant RNase H1 (see Material and Methods). \( \text{CEN16} \), the highly Pol II transcribed mRNA genes \( \text{ADHI} \), \( \text{ACTI} \), \( \text{PMAI} \) and \( \text{RPL28} \) (exon 2), and the telomeric region \( \text{Tel01L} \), were analyzed by Q-PCR as described in Fig. 1A.

**Figure S10** Expression of spliced genes with a “short-exon1” is generally higher than those with a “long-exon1.” A: Plot of RNA expression of yeast mRNA genes. Panel I. Raw transcriptome sequencing (RNA-seq) reads of exponentially growing wild-type strain BY4741 [101] were processed as described in Protocol S1. Normalised reads per base of exon of the 5864 protein-coding genes were plotted on the Y-axis. The top value of the Y-axis was arbitrarily set to 15. We clustered the mRNA genes into four groups of RNA expression, and indicated their boundaries by red vertical lines on the plot: C1 (low, \( n = 1788 \)), C2 (medium-low, \( n = 1788 \)), C3 (medium-high, \( n = 1788 \)) and C4 (high, \( n = 500 \)). The seventy four very-lowly expressed mRNA genes (subgroup “C1-0” in Fig. 5A) were included in group C1. The top ninety very-highly expressed mRNA genes (subgroup “C4-max” in Fig. 5A) were included in group C4. Ribosomal protein genes (RPG) and non-ribosomal protein genes (NRPG) are represented by a green or black vertical line, respectively. The plot region featuring the 500 highly expressed mRNA genes of group C4 including all the RPGs is magnified in panel II. \( n \) = number of genes. B: Plot of yeast mRNA intron-genes (i-genes) grouped according to the length of their first exon (exon 1) [panel I]. The region of the plot featuring the i-genes with exon 1 <300 bp is magnified in panel II: 87 RPGs (out of 89 i-genes) and 144 NRPGs (out of 101 i-genes) have an Exon 1 <300 bp. C: Boxplots of RNA-seq and Net-seq data from wild-type strain BY4741 [101] covering the 181 i-genes of NRPG (the 3 dubious open reading frames \( \text{YDR535C} \), \( \text{YLR202C} \) and \( \text{YOR318C} \) were excluded from our analysis in Figs. 5, S10, S11, S13, S14), which were divided into to two groups based on the length of Exon1 (<100 and >100 bp). Normalised average read coverage of RNA-seq and NET-seq data (the number of reads per base of exon; see Protocol S1 and [101]) were calculated for each i-gene and the two Exon1-groups were represented as boxplots. Box-plot representation shows median values (black line) +/− 25% quartiles in the box and minimum/maximum distribution of the values (excluding outliers) in the whiskers. We used a Kolmogorov-Smirnov test to show that levels of expression differ significantly between the two groups of Exon 1 (<100 and >100): RNA-seq (\( D = 0.4426 \), p-value = 1.392e-06) and NET-seq (\( D = 0.3463 \), p-value = 0.0002292). \( n \) = number of genes.

**Figure S11** R-loop distribution over mRNA spliced genes according to the length of their first exon. Average profiles of S9.6 ChIP-seq and input chromatin of mRNA intron-genes (i-genes) in the wild-type strain (BY4741), grown at 30°C in YEpd medium (glucose 2%). Averaged reads were plotted on sequences encompassing Exon1-intron-Exon2 regions as described in Protocol S1. The 5′ end of Exon 1 is defined either as the AUG start codon, or 100 bp upstream of the 5′ splice site for genes with Exon 1 <100 pb (see also Protocol S1). The i-genes were split in to the ribosomal protein genes (RPG) (panel A) and the non-ribosomal-protein genes (NRPG) (panel B), and further segregated in to four sub-categories according to the length of their first exon (Exon 1): (0–50 bp), (50–100 bp), (100–150 bp) and (>300 bp). The y-axis represents the relative enrichment of reads where values >1 are above the background level of sequencing (i.e. general intergenic mean, see Materials and Methods).

**Figure S12** Examples of S9.6 ChIP-seq profiles of mRNA genes. Profiles of input chromatin and S9.6 ChIP-seq are for the wild-type strain (BY4741) grown at 30°C in YEpd medium (glucose 2%). Shown for spliced genes \( \text{RPL28} \) (A), \( \text{ACT1} \) (B) and \( \text{EFR1} \) (E) and intronless genes \( \text{SEHI} \) (A), \( \text{FET5} \) and \( \text{YPT1} \) (B), \( \text{DUF1} \), \( \text{MHFI} \) and \( \text{ADHI} \) (C), \( \text{LEU1} \) and \( \text{PMA1} \) (D), \( \text{SSAI} \) and \( \text{VPS58} \) (E), and \( \text{IBA57} \), \( \text{RPS5} \) and \( \text{ENT7} \) (F). The y-axis represents the relative enrichment of reads where values >1 are above the background level of sequencing (i.e. general intergenic mean, see Materials and Methods). G+C content of the DNA sequence was calculated for 100 bp windows and is depicted as a blue intensity. Shown below the profiles is a graphical representation of genomic features, with exon and intron sequences depicted as filled boxes and horizontal lines, respectively. The direction of transcription is indicated by a tailless arrow. Highly expressed genes are colored in red. Chr = chromosome. Profiles were generated using Integrative Genomics Viewer [100].

**Figure S13** Comparison of R-loop distribution across Exon 2 of spliced-genes and across non-spliced ribosomal protein genes. Box plots of mean sequence read distribution of R-loops per gene across the second exon of the i-genes NRPG (I) and RPG (II), and the entire length of the e-genes RPG (III). Each box plot represents the log2 fold change of mean S9.6 ChIP-seq relative to input chromatin in the wild-type (BY4741), grown at 30°C in YEpd medium (glucose 2%). Box-plot representation shows median values (black line) +/− 25% quartiles in the box and maximum/maximum distribution of the values (excluding outliers) in the whiskers (the regions above zero value on the Y-axis are enriched with R-loops; see also Fig. 3). \( n \) = number of genes. RPG = ribosomal protein genes. NRPG = non-ribosomal protein genes. i-genes = intron-containing genes. E-genes = intronless genes. We used a Kolmogorov-Smirnov test to determine whether levels of log2 fold change of mean sequence distribution differ significantly between box plots I and II (\( D = 0.449314044047675 \), p-value = 6.90119072999096e-11), and II and III (\( D = 0.3959925093633 \), p-value = 6.5907560322966e-05).
start codon, or 100 bp upstream of the 5’ splice site for genes with Exon 1 <100 pb (see also Protocol S1). n = number of genes. 5’SS = 5’ splice site. 3’SS = 3’ splice site.

**Figure S15** Petit frequency is higher in yeast rnh1A mutants than in isogenic wild-type W303a. Strains wild-type W303a and mutant rnh1A were grown at 30°C in YEP medium containing 3% glycerol. After four days cells were washed with sterile water, diluted appropriately, plated onto YEP medium containing 2% glucose and incubated at 30°C. One feature of ‘petite’ ade2 cells is to have a small size and the inability to convert an intermediate to impaired respiratory functions (e.g. see [104]). Conversely, in the adenine biosynthesis pathway (AIR) into a red pigment due to functional mitochondria. For each strain a total of 8000 colonies were counted and petite/white and grande/ red colonies were scored. Values are means of ten independent isolates with standard errors.

**Figure S16** Thermodynamic helical stability of DNA/DNA and (pre-mRNA)/DNA duplexes of yeast mRNA intronless genes. The mRNA intronless genes (e-genes) were split in to four groups according to their mRNA expression; panel A (group C1, low expression, n = 1674), panel B (group C2, medium-low expression, n = 1754), panel C (group C3, medium-high expression, n = 1708) and panel D (group C4, high expression, n = 387). For ΔG0 values of DNA/DNA and (pre-mRNA)/DNA duplexes and [G +C] nucleotides see Fig. S14 and Protocol S1. Averaged values of ΔG0 and [G +C] for the e-genes in each group were plotted on sequences encompassing the entire length of the gene (0–100%). n = number of genes.

### References


**Table S1** List of strains and plasmids. A: Strains. B: Plasmids.

**Table S2** Oligonucleotides used in Northern and Southern analysis. A: Oligonucleotides for Northern analysis of rRNAs and rRNAs. B: Oligonucleotides for Southern analysis of Yt1 cDNAs.

**Table S3** Oligonucleotides used in ChIP-QPCR, PCR and ChIP-seq analysis. A: ChIP-QPCR primers. B: Standard PCR primers. C: ChIP-seq primers.

**Protocol S1** Bioinformatic analysis.

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Conceived and designed the experiments: AEH DT. Performed the experiments: AEH SW AK. Analyzed the data: AEH SW AK DT. Contributed reagents/materials/analysis tools: AEH SW AK. Wrote the paper: AEH SW AK DT.
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