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RNase H and multiple RNA biogenesis factors cooperate to prevent RNA-DNA hybrids from generating genome instability

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Abstract

Genome instability, a hallmark of cancer progression, is thought to arise through DNA double strand breaks (DSBs). Studies in yeast and mammalian cells have shown that DSBs and instability can occur through RNA-DNA hybrids generated by defects in RNA elongation and splicing. We report that in yeast hybrids naturally form at many loci in wild-type cells, likely due to transcriptional errors, but are removed by two evolutionarily conserved RNase H enzymes. Mutants defective in transcriptional repression, RNA export and RNA degradation show increased hybrid formation and associated genome instability. One mutant, *sin3Δ*, changes the genome profile of hybrids, enhancing formation at ribosomal DNA. Hybrids likely induce damage in G1, S and G2/M as assayed by Rad52 foci. In summary, RNA-DNA hybrids are a potent source for changing genome structure. By preventing their formation and accumulation, multiple RNA biogenesis factors and RNAse H act as guardians of the genome.

Keywords

RNA-DNA hybrids; transcription; genome instability; RNase H

Introduction

As cells grow and divide, preserving genome integrity is essential for survival and proper development. While changes in chromosome structure or number (henceforth referred to as gross chromosomal rearrangements or GCRs) can help generate genetic diversity and drive evolution, they also are associated with developmental disorders and increased cancer susceptibility (Motegi and Myung, 2007). GCRs are thought to occur through a double-strand break (DSB) intermediate. Studies of bacterial and yeast systems suggest that DSBs occur as a result of exogenous damaging agents, faulty repair processes and errors in DNA replication. Replication errors arise when a moving replisome encounters secondary

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structure or tightly bound proteins that cause the replication fork to stall and collapse, driving the generation of DSB-induced genome rearrangements (Myung et al., 2001).

Similarities between the types of rearrangements found in yeast replication and repair mutants and human cancer cells indicate they may be generated through analogous mechanisms. Thus far defects in repair and checkpoint genes but not replication genes have been found in human cancers (Aguilera and Gómez-González, 2008). Therefore, failures of the replication machinery may not be the main, or only source for instability in cancers.

A growing body of evidence points to transcription as another biological process that affects genome integrity. In addition to triggering mutations, an effect for transcription on mitotic recombination has been documented in yeast, prokaryotes and higher eukaryotes (Datta and Jinks-Robertson, 1995). One mechanism for transcription-induced genome instability is through the formation of RNA-DNA hybrids (Huertas and Aguilera, 2003; Tuduri et al., 2009). In budding yeast, deletions arising from recombination between direct repeats flanking bacterial sequences are elevated two to three orders of magnitude in mutants of the THO complex, a conserved four-protein complex involved in elongation (Prado et al., 1997; Piruat and Aguilera, 1998). THO mutants also exhibit increased levels of RNA-DNA hybrids between nascent transcripts of the bacterial sequences and their DNA template. The over-expression of RNase H, which specifically degrades the RNA portion of hybrids, suppresses both the formation of RNA-DNA hybrids and associated deletions. Similarly, depletion of specific splicing and spliceosome assembly factors in chicken and human cells significantly increases DSB formation and DNA fragmentation (Li and Manley, 2005; Paulsen et al., 2009). Damage in at least a subset of these splicing mutants appears to occur through RNA-DNA hybrids, as DSB formation is suppressed by RNase H. These results point to a role for the elongation and splicing machinery in safeguarding the genome from hybrid-mediated GCRs.

Despite the potential of RNA-DNA hybrids as a potent source of cancer-related rearrangements, it has not engendered many follow-up studies. A number of critical questions remain unanswered. For example, while the loss of splicing factors in mammalian cells increases DSB formation, whether these DSBs generate GCRs has not been demonstrated. In yeast, where a more direct link between elongation impairment and GCRs is established, it is not clear how universal the phenomenon is; how many genes and in how many different steps of RNA biogenesis when perturbed create RNA-DNA hybrids and elevated GCRs? This is especially critical given that yeast THO mutants increase GCRs of artificially constructed repeat substrates (Chavez et al., 2000), but not endogenous chromosomes (Aguilera and Klein, 1990; Gómez-González et al., 2009). Furthermore, are RNA-DNA hybrids more likely to form at unusual DNA structures or sequences? If not, how often and where do RNA-DNA hybrids occur in the genome? Do RNA-DNA hybrids generate DNA damage by a mechanism distinct from stalling of replisome progression in S phase?

A number of questions also arise from the ability of RNAse H over-expression to suppress the DSBs and genome instability caused by defects in yeast elongation and mammalian splicing factors (Huertas and Aguilera, 2003; Li and Manley, 2005). This observation would predict that endogenous RNase H might act to suppress hybrid-mediated genome instability. However, inactivation of yeast RNAse H activity does not increase recombination between direct repeats. In fact, while RNase H1 and RNase H2 are highly conserved from prokaryotes to eukaryotes (Cerritelli and Crouch, 2009), their *in vivo* functions remain an enigma. The uncertainties regarding RNase H function question whether RNA-DNA hybrids occur spontaneously in normal cells and if RNAse H is important for their removal and for suppressing GCRs.

To address these important questions we use *Saccharomyces cerevisiae* as a model system to further explore the impact of perturbing transcription, RNA-DNA hybrids, and RNase H function on GCRs and genome instability. We exploit genetic and cytological tools to address many of the questions posed above. We provide insights into the number and type of genes that guard the genome against RNA-DNA hybrids, the frequency and genome distribution of hybrid formation with and without perturbing transcription, and the cell cycle dependence of DNA damage induced by hybrid formation.

Results

Monitoring genomic instability with a yeast artificial chromosome (YAC)

Because GCRs can dramatically change genetic content, essential cellular processes are often disrupted, leading to cell inviability. As a result, detecting GCRs in rare surviving cells requires very sensitive assays applied to screening large cell populations (Chen and Kolodner, 1999). To overcome these limitations, our lab has developed an assay using YACs as substrates to monitor GCR formation in haploid budding yeast cells (Huang and Koshland, 2003; this study) (Figure 1). The dispensability of YACs for viability allows us to detect GCRs resulting from lesions along the entire length of the chromosome, even when a mutagenic event generates large deletions. The two classes of GCRs recovered in this system are terminal deletions and chromosome loss (Figure 1). In WT cells, the rate of GCRs on the YAC is on the order of $6*10^{-4}$, and results from a comparable number of terminal deletion and chromosome loss events. Notably, rates for terminal deletion and loss events on the YAC are comparable to rates of mitotic recombination and chromosome loss observed for native chromosomes in diploids (this study; Hartwell and Smith, 1985). In summary the YAC provides a rapid haploid-based assay to monitor GCRs that would only be detected on native chromosomes in diploids.

Perturbing RNA biogenesis increases YAC instability

Using the YAC to monitor the occurrence of GCR events, we explored the impact of perturbing transcription and RNA processing on genome stability. Our work was motivated by two main observations. First previous work has established strong links between transcription and recombination rates (Saxe et al., 2000; Aguilera and Klein, 1990). Second, results from our screen of the non-essential knockout collection for mutants with elevated YAC GCR showed a 6-fold enrichment for RNA biogenesis genes (Vuica-Ross and Koshland, unpublished). Deletions of genes acting at various stages, from transcription initiation to RNA degradation and export, increase the rate of instability 4- to 16- fold over wild type (WT) (Figure 2a, Figure S1a). These rates are similar to that of well-established chromosome integrity determinants, including DNA damage checkpoint and repair mutants, which increase YAC GCRs 4- to 19-fold over WT (Figure 2b; Figure S2). Thus errors in RNA biogenesis pose a major threat to genome integrity.

Previously, impairment of transcription elongation has been linked to genome instability through mutations in the THO complex. THO mutants stimulate direct-repeat recombination, but not terminal deletions on Chromosome V (Gómez-González et al., 2009). Consistent with these previous observations, deletion of the Hpr1p subunit of the THO complex does not induce GCRs of the YAC (data not shown). Therefore,, the THO complex appears to have a specialized role for the complex in guarding repetitive elements from transcription-induced instability. In contrast, the formation of YAC GCRs are significantly elevated in other transcription elongation mutants including the PAF complex (*cdc73Δ*, *leo1Δ*) and *spt2Δ* (Figure 2a). PAF complex mutants have no effect on direct-repeat recombination (Rondón et al., 2004). Substrate-dependent variation in genome

stability indicates there are two distinct classes for elongation factors: those that prevent GCRs in non-repetitive DNA and others specific to repetitive DNA.

Elevated rates of YAC GCRs are not restricted to mutants affecting transcription elongation. GCRs also occur more frequently in mutants defective in master repressors of transcription, namely *sin3Δ, sds3Δ, rpd3Δ, not5Δ, stb3Δ, med13Δ, cdk8Δ, med12Δ, cycCΔ*. One likely scenario for why these mutants elevate YAC GCRs is as a result of elevated transcript levels. To test this hypothesis we needed to inhibit the elevated transcription in these mutations. Elevated transcription in mutants of the repressive module of the mediator complex (*med13Δ, cdk8Δ, med12Δ*, and *cycCΔ*) is dependent upon the Mediators' activation module, which includes the Med2p subunit (Van de Peppel et al., 2005). Indeed, we find that deletion of *med2* in a *cdk8Δ* mutant background completely abrogates its genome instability phenotype (Figure S1c). Therefore, inappropriate transcription in either amount and/or position leads to genome instability.

YAC GCRs are also observed at elevated rates in mutants defective for transcription initiation (*bur2Δ*), RNA degradation (*kem1Δ, air1Δ, rrp6Δ, trf4Δ*) and mRNA export (*npl3Δ*). Thus defects in many different transcription factors encompassing diverse aspects of mRNA biogenesis lead to genome instability. It is noteworthy that not all perturbations of transcription necessarily enhance genomic instability. YAC GCRs occur at WT levels in deletion mutants with impaired transcriptional activation because of deletions in chromatin remodeling (*snf2Δ*), histone acetylation (*hpa2Δ, elp3Δ, sas2Δ*), and mediator activator factors (*med2Δ, med3Δ, med31Δ*) (Figure S1b). Hence, reducing transcript levels appears to have no adverse effect on genome integrity.

Perturbing RNA biogenesis increases instability at chromosomal loci

The results described thus far demonstrate a role for RNA biogenesis factors in guarding genomes against instability. However, because instability was monitored on a YAC, we considered the possibility that the GCR rates in mutants is exaggerated due to the foreign nature of most of the YAC sequence. The impact of foreign DNA has been observed with the repeat recombination assay, where rates were highest when transcription proceeded through bacterial *lacZ* sequence (Chávez and Aguilera, 1997). Therefore, we assayed whether perturbing RNA biogenesis affects genomic integrity of natural chromosomes. For this purpose, we monitored loss of heterozygosity (LOH) of marker alleles on chromosome III and XII in diploids, due to terminal events (loss of markers from one arm) and/or chromosome loss (loss of markers from both arms (Figure S3a; Figure S4a). If RNA biogenesis mutants affect native chromosomes as much as the YAC, we expect total rates of genome instability to be similar. However the type of instability is likely to differ. A damaged YAC has no homologous sequence elsewhere in the genome to act as a repair template, hence YAC LOH occurs by truncations. In contrast, a damaged native chromosome in a diploid is likely to cause LOH by homologous recombination with the homolog.

In WT cells, the rate of instability on chromosome III and XII is $4*10^{-4}$ and $2*10^{-3}$ respectively, comparable to the rate observed on the YAC (Figures S3a, S3d). Altering transcription by deleting *bur2*, *med13* or *sin3* causes respectively a 4, 8 and 14-fold increase in instability on chromosome III (Figure 3a; Figure S3b). These mutants affect both terminal and chromosome loss events, to a degree similar to that on YAC terminal deletions and loss (Figure S3c). On chromosome XII, deleting *sin3* causes a 5-fold increase in instability (Figure 3b). The size of chromosome III and XII in cells suffering terminal LOH events were characterized by pulsed-field gel and Southern analysis. As expected, few if any of the terminal events result from truncations (data not shown). Phenotypic analysis of terminal events on chromosome XII in *sin3Δ* cells revealed that the majority occur by non-reciprocal

LOH, specifically break-induced replication (Figure S4a and S4b). These results are consistent with the YAC and native chromosomes suffering similar elevated levels of damage in RNA biogenesis mutants, but the repair of that damage leads to different outcomes.

Instability in most RNA biogenesis mutants is suppressed by RNase H over-expression

Previous work from Aguilera and colleagues suggested that genome instability observed in transcription elongation and splicing mutants results from the formation of RNA-DNA hybrids (Huertas and Aguilera, 2003; Li and Manley, 2005). To test whether RNA-DNA hybrids are responsible for GCRs in our RNA biogenesis mutants, we over-expressed RNAse H1. Over-expressing RNase H suppressed the YAC instability observed in 18 of the 21 mutants to near WT levels (Figure 2a; Figure S1a). RNase H1 over-expression also suppressed GCRs in diploids of representative RNA biogenesis mutants (Figure S3b). Importantly, we find that over-expressing RNase H1 does not suppress instability in DNA checkpoint and repair mutants (Figure 2b; Figure S2). Furthermore, over-expressing an RNase H1 with a mutation in its catalytic domain also does not suppress instability (Figure S7c). Taken together these results suggest that defects in many different aspects of RNA biogenesis lead to a common mechanism of genome instability that involves a RNA-DNA hybrid intermediate.

The few RNA biogenesis mutants that fail to be suppressed by RNAse H are informative. Mutants in the Mediator middle/tail submodule, Med5p and Med16p are only partially suppressed by the over-expression of RNase H. Even more strikingly, the instability resulting from loss of the histone deacetylase Rpd3p is completely RNase H-resistant. This contrasts with the RNAse H-sensitive nature of GCRs of two of its binding partners, Sin3p and Sds3p. The contrasting RNase-H sensitivity among subunits of a single complex indicate specialized roles for the various proteins, and suggests such roles may be distinct from previously characterized functions within their respective complexes.

Cytological detection of RNA-DNA hybrids

Results from our work and that of others suggests that perturbing RNA biogenesis may allow RNA-DNA hybrids, the intermediate responsible for instability, to form readily. In fact, there is molecular evidence that RNA-DNA hybrids form at a specific locus in the *hpr1Δ* elongation mutant (Mischo et al., 2011). To corroborate the induction of RNA-DNA hybrids in the RNA biogenesis mutants, and to assess how often and where hybrids occur, we developed a cytological assay. We made chromosome spreads and detected the presence of hybrids by indirect immunofluorescence using an antibody against RNA-DNA hybrids. We used this cytological assay to monitor hybrids in WT and two representative mutants, *sin3Δ* and *med13Δ*.

In WT cells, 95% of the nuclei exhibit very little staining with the RNA-DNA hybrid antibody (Figure 4a-b). Thus most wild-type cells are able to prevent hybrids from initially forming or from persisting once they form. Our failure to detect hybrids in WT cells is consistent with the fact that RNase H over-expression does not change the rate of GCRs in WT cells. In addition, wild-type like staining of RNA-DNA hybrids is observed in *rad52Δ*, and *rpd3Δ*, which cause elevated rates of GCR by hybrid-independent mechanisms (Figure S5b-c).

In contrast, high levels of RNA-DNA hybrids are detected in the transcription mutants *sin3Δ* and *med13Δ* (Figure 4a, Figure S5a). 86% of nuclei in *sin3Δ* and *med13Δ* exhibit substantial levels of RNA-DNA hybrids, with a median intensity in mutants roughly 7-fold over background staining in WT. Staining with the RNA-DNA antibody is reduced to that seen in

WT spreads by the over-expression of RNAse H in cells, or post treatment of chromosome spreads with RNAse H (Figure 5c, Figure S5e). Our findings provide direct evidence for the elevated formation of RNA-DNA hybrids in mRNA biogenesis mutants previously inferred by the genetic suppression of GCRs upon RNase H over-expression.

These spreads lead to two additional conclusions. The diffuse staining pattern over the entire chromosome spread demonstrates that Sin3 and Med13 prevent the formation of hybrids at multiple loci genome-wide. Furthermore the high percentage of nuclei with staining indicates that hybrids are forming in most mutant cells. It is noteworthy that this only translates into an instability event in less than 1% of cells. This difference between the frequency of hybrid and GCR formation signifies that cells proficiently resolve either RNA-DNA hybrids or any resulting DNA damage into non-deleterious outcomes.

The apparent diffuse hybrid-staining pattern in RNA biogenesis mutants, suggesting that multiple loci are susceptible to hybrid-mediated instability. This conclusion is validated by the elevated instability of the YAC, chromosome III and chromosome XII. However, analysis of the staining pattern in WT and *sin3Δ* nuclei indicates the level of RNA-DNA hybrids are not always uniform throughout the genome. In the *sin3Δ* mutant, 64% of nuclei analyzed are enriched for RNA-DNA hybrids at the nucleolus when compared to staining intensity over the total chromatin mass (Figure 4a). The nucleolus is the site of ribosomal DNA (rDNA), and in yeast it is sequestered from the bulk chromatin mass, appearing as either a diffuse ball or a distinct loop, depending on cell cycle stage (Guacci et al., 1994). In contrast, in *med13Δ*, only 15% of cells exhibit a bias for RNA-DNA hybrids at the rDNA locus. We reasoned that these results may reflect the importance of *SIN3* for transcriptional repression at the rDNA such that a *sin3Δ* would disproportionately affect transcription levels from rDNA loci. *In vivo* studies in mammalian cell lines as well as yeast support a specialized role for *SIN3* in rDNA silencing (Silverstein and Ekwall, 2005).

To determine whether the enrichment of RNA-DNA hybrids at the rDNA locus correlated with higher levels of instability in the region, we examined the rate of excision of a URA3 marker inserted into the *RDN* locus. In the *sin3Δ* mutant, the GCR rate at rDNA was 285-fold over WT, as compared to the 12 to 14-fold effect observed on the YAC and chromosome III (Figure 5b, Figure S5d). The instability is mediated through RNA-DNA hybrids, as over-expression of RNase H suppresses both the instability of the rDNA locus and its staining with the hybrid antibody (Figure 5b-c, S5e). The *med13Δ*, which does not show elevated rDNA staining, increases the rate of loss of URA3 13-fold over WT, similar to the 8 to 9-fold effect observed on the YAC and chromosome III. Thus Sin3p has an additional specialized role in guarding against hybrid-mediated instability at the rDNA locus.

RNA-DNA hybrids cause Rad52 foci outside of S phase

Because DNA repair proteins accumulate at sites of damage, the occurrence of damage can be monitored by the accumulation of repair foci using green fluorescent protein (GFP)-tagged proteins (Lisby et al., 2004). To determine whether RNA-DNA hybrids lead to an accumulation of damage, we monitored the formation of Rad52-GFP foci in *sin3Δ* cells (Figure S6a). In a *sin3Δ* mutant, 14% of cells accumulate Rad52-GFP foci, as compared to 3% in WT (Figure 6a). The induction of Rad52-GFP foci by RNA-DNA hybrids is consistent with previous studies of THO elongation complex, and RNA helicase Sen1 mutants (Gómez-González et al., 2009 and Mischo et al., 2011). The increase in *sin3Δ* is completely dependent on RNA-DNA hybrids, since the over-expression of RNase H1 suppresses the percent of cells with Rad52-GFP foci to 3% (Figure 6a). This observation suggests that RNA-DNA hybrids increase DNA damage, likely DSBs.

Evidence that DNA damage in THO mutants activates the S-phase checkpoint suggests that RNA-DNA hybrids may be causing damage during replication (Gómez-González et al., 2009). To determine whether the damage in *sin3Δ* cells is dependent on progression through S phase, we measured % Rad52-GFP foci as a function of the cell cycle (Figure S6b-c). As previously reported, in WT cells <1% have foci when arrested in G1. Upon release from G1, the number of foci peak during S phase at 8 (Alabert et al., 2009). In *sin3Δ* cells arrested in G1, the number of foci increases to 7-8% and increases to 37% when allowed to progress through S phase. These increases in the number of foci are suppressed by overexpression of RNAse H. These observations suggest that while some hybrid-mediated damage is dependent on S phase, it can also occur independent of S phase progression. However, we could not eliminate the possibility that the Rad52 foci observed in G1 cells are in fact unrepaired breaks carried over from a previous S phase.

To eliminate this uncertainty, we established a system that would allow us to control the timing of Sin3p depletion, and hence of hybrid formation. Degradation of Sin3p was dependent on the auxin-inducible degron (AID) system developed in the Kanemaki lab (Nishimura et al., 2009). Briefly, the AID was fused to the C terminus of Sin3p and expressed along with the required ubiquitin ligase TIR1; the presence of auxin should promote the interaction of TIR1 with the AID-tagged Sin3p, leading to its degradation. This system allowed us to limit the formation of hybrids to specific points in the cell cycle, and address what affects this has on Rad52 foci formation.

The auxin-mediated depletion of Sin3p caused RNA-DNA hybrids and Rad52 foci to accumulate at levels comparable to that of *sin3Δ* cells (Figure 6c). In an asynchronous culture, growth in auxin-containing media caused greater than 95% of cells to exhibit significant levels of RNA-DNA hybrids. Approximately 16% of cycling cells accumulated Rad52-GFP foci similar to that seen in cycling *sin3Δ* cells. Therefore we conclude that depleting Sin3p by the AID system is comparable to complete removal of the gene.

With this system in hand, we could now address whether hybrids can induce Rad52 foci outside of S phase. We arrested *sin3-AID* cells in either G1 or G2/M of the cell cycle, and then induced Sin3p degradation by the addition of auxin. In both cases we find that Rad52-GFP foci accumulate in 7-8% of cells, as compared to 1-2% in control untreated cells (Figure 6d). When compared to the total number of foci observed in asynchronous cultures, we estimate that at least a third of Rad52 foci are induced outside of S phase in the absence of Sin3p.

Endogenous RNase H interacts with transcriptional machinery to suppress genomic instability

To determine whether endogenous RNase H activity helps remove RNA-DNA hybrids, we monitored the rate of YAC and chromosome instability in *rnh1Δ*, *rnh201Δ* and *rnh1Δ rnh201Δ* mutants. We find that partial removal of RNase H activity from cells in either single deletion mutants causes a 3- to 4- fold increase in instability on the YAC (Figure 7a; Figure S7a). This phenotype is exacerbated when both RNase H complexes are inactivated in the *rnh1Δ rnh201Δ* mutants, increasing GCR formation close to levels observed in the most severely affected mRNA biogenesis mutants. A similar effect on instability was also seen for LOH events in diploids (Figure 7b). Given that *rnh1Δ rnh201Δ* mutants have no detectable effect on transcription (Huertas and Aguilera, 2003), these results suggest that RNase H activity is an important endogenous inhibitor of GCRs and that at least a subset of the target substrates for the two complexes overlap.

The elevated rate of GCR formation in RNase H-compromised cells indicates that the RNase H machinery clears RNA-DNA hybrids that occur spontaneously while transcribing the

genome. To test this hypothesis we analyzed RNA-DNA hybrid formation in the *rnh1Δ rnh201Δ* mutant with our cytological assay. In the double mutant 97% of nuclei exhibit significant levels of RNA-DNA hybrids, with a median intensity 10-fold over WT (Figure 4a-b). Notably, the rDNA locus is not more enriched for staining in the *rnh1Δrnh201Δ* double, indicating that unlike the case in a *sin3Δ*, the rDNA locus is not typically more susceptible to hybrid formation (Figure 5a). Taken together our cytological and genetic assays suggest that RNase H acts as a surveillance system to remove RNA-DNA hybrids.

To test whether endogenous RNase H plays a role in removing RNA-DNA hybrids that may arise during errors in RNA biogenesis, we tested the effect of knocking out RNase H proteins in representative transcription mutants. In the three mutants tested, *bur2Δ*, *med13Δ* and *sin3Δ*, the rate of YAC instability in an *RNH1* background is roughly 4, 8 and 12-fold over WT, respectively (Figure 2a; Figure S1a). In an *rnh1Δ* background the rate further increases to 23, 29, and 32-fold over WT—significantly higher than can be accounted for by an additive effect for the double mutants on instability (Figure 7a; Figure S7a). This effect on genomic integrity also recapitulates on chromosome III and XII loci in diploids (Figure S7b; Figure 3b). Noteworthy, *sin3Δ rnh1Δ* double mutants also exhibit a lethal sectoring phenotype (Figure S4d). Instability in the *rnh201Δ* background was also high although still lower than that of the *rnh1Δ*, an indication the two complexes may have slightly different cellular functions (Figure S8). Again, the increased rates of YAC instability can be suppressed by over-expression of RNase H1 (Figure S7a). Therefore, RNase H plays an important role in removing RNA-DNA hybrids that may occur during errors in RNA biogenesis.

To our surprise, we found that roughly 95% of the instability events in *bur2Δ rnh1Δ*, *med13Δ rnh1Δ* and *sin3Δ rnh1Δ* were chromosome loss events; this is in contrast to the case in WT and single mutants where on average only 55-65% of events are loss events (Figure S9). One possibility is that the prevalence of loss may reflect the persistence of an RNA-DNA hybrid makes the DNA site refractory to repair (Klein, 2001).

Discussion

RNA biogenesis is a complex multi-step process mediated by an array of factors that ensure efficient and accurate gene expression (Perales and Bentley, 2009). Here we show that an additional function for many of these factors is to limit the formation of RNA-DNA hybrids to prevent them from altering genome structure. We find that in addition to elongation and splicing factors, the prevention of hybrid formation is a function of factors required for transcriptional repression, RNA export, and RNA degradation. Thus deleterious interactions between RNA and its template DNA are possible at multiple stages co- and post-transcriptionally. However, we find that not all factors involved in a particular step of RNA biogenesis or within a single complex are needed to prevent hybrid formation. This functional division of transcription factors infers mechanistic functions within RNA processes and complexes yet to be discovered.

How can defects in such diverse aspects of mRNA biogenesis lead to RNA-DNA hybrids? We propose that all these mutants make nascent transcripts more accessible to DNA, potentially by different underlying mechanisms. Mutants affecting the loading of RNA binding proteins onto RNA, may uncoat the RNA and allow for inappropriate interactions with target duplex DNA. Mutants in this class are the PAF complex (Cdc73p and Leo1p) and Spt2p, which are involved in transcriptional elongation and recruit cleavage/polyadenylation factors to RNA (Luna et al., 2005; Hershkovits et al., 2006). A similar model was proposed for the THO complex, which is required for co-transcriptional recruitment of the RNA binding proteins Yra1p and Sub2p (Rondón et al., 2010).

Alternatively, mutants defective in processes such as RNA degradation by Kem1p and the TRAMP complex (Air1p, Rrp6p and Trf4p), or subsequent export (Npl3p), may increase the half-life of RNA in the nucleus, hence increasing the time that hybrids can form.

Using our cytological assay we show that hybrids form at many genomic sites in cells compromised for RNAse H or RNA biogenesis factors. Thus, many sites in the genome must be prone to hybrid formation. In recent years, genome-wide studies have shown that widespread transcription occurs from coding and noncoding regions in yeast and higher eukaryotes, much of it originating from sites of cryptic transcription (Cheung et al., 2008; Wyers et al., 2005). Numerous transcription and degradation factors have been identified as required for the repression of cryptic transcription, including Bur2p, Cdc73p, Spt2p, Sin3p, Med13p, Cdk8p, Med12p, Rrp6p and Trf4p. We found these genes also suppress hybrid-mediated GCRs, and propose that expression from intergenic promoters may be regulated by RNA biogenesis factors because of the risk cryptic transcription poses to genomic integrity.

Our genetic and cytological data indicates that sites of hybrid formation vary depending upon cellular defect. This is exemplified by the difference in effect on GCR and direct-repeat recombination observed in PAF mutants versus THO mutants, respectively (this work, Gómez-González et al., 2009). Similarly, the *sin3Δ* mutant but not *med13Δ* enhances hybrid formation and instability of rDNA repeats. Evidence in yeast indicates that Sin3p may indirectly control polymerase II transcription within rDNA spacer regions (Ciocl et al., 2003 and Smith et al., 1999). While the functional consequence of this has been unclear, our work suggests it may be critical to prevent polymerase II transcription in the rDNA locus to maintain repeat homeostasis. It will be interesting to know whether other RNA biogenesis mutants also exhibit such locus-enriched formation of hybrids.

Despite their conservation, little is known about the function of nuclear-encoded RNAses H. Recently, RNase H2 has been shown to be involved in the removal of single ribonucleotides misincorporated in duplex DNA (Kim et al., 2011). We find that compromising RNase H function leads to the accumulation of RNA-DNA hybrids, indicating that RNase H must act as part of a surveillance system to remove hybrids that form as a result of spontaneous errors in RNA biogenesis in wild-type cells. However, it is likely that other mechanisms must exist to resolve RNA-DNA hybrids and/or any resulting DNA damage into non-deleterious outcomes, since the robust levels of hybrids in almost all *rnh1Δrnh201Δ* cells leads to GCRs in only 1% of cells.

Strikingly, our studies of the kinetics of hybrid-induced Rad52 foci indicate that hybrids can stimulate DNA damage outside of S phase. Rad52 foci are generally equated with DSBs, but we cannot rule out that these foci mark other types of damage or unusal DNA structures. A number of previous observations had suggested that all endogenous sources of DSBs were linked obligatorily to S phase progression. In WT cells, spontaneous Rad52-GFP foci appear almost exclusively during S phase (Lisby et al., 2001). In addition, all replication and DNA damage checkpoint mutants increase foci formation in S and G2/M, but not G1 cells. However, recent genetic evidence suggests that 40% of spontaneous mitotic recombination events reflect repair of a G1-inititated DSB (Lee et al., 2009). We suggest these G1 induced breaks may occur by RNA-DNA mediated hybrids that occur infrequently enough in WT cells so as not to be detected by foci. Induction of DSBs in G1 is a potentially potent cause of changes in the genome as it eliminates the ability to use the sister chromatid as a repair template.

The effects of perturbing RNA biogenesis and RNase H function on GCRs are comparable to those observed in mutants of DNA damage and repair processes, demonstrating the importance of regulating transcription and RNA processing for maintaining genome

structure. This finding presents an alternative model for the molecular mechanisms by which genome rearrangements associated with cancer may occur. First, because the number of RNA biogenesis genes that can be mutated to promote hybrids is so large, perturbations of RNA biogenesis are a potentially robust source for genome instability. Second, previous models have suggested that genome instability in cancer cells leads to the changes in transcription profiles necessary for cancer progression (Hanahan and Weinberg, 2000). However, our work suggests that changes in the transcriptional profile of cells, and ensuing hybrid formation, may precede and in fact be a causative factor in the generation of chromosomal rearrangements. Intriguingly, a recent study suggests that transcription is causative agent for recurrent translocations in prostate cancer cells (Lin et al., 2009). It will be interesting to see whether these translocations are associated with the formation of an RNA-DNA hybrid in the region. Analysis of rearrangements and transcriptional profiles in other cancer types will further elucidate potential contributions of the transcriptional machinery and RNA-DNA hybrids in promoting DNA rearrangements.

Materials and Methods

Yeast strains, media and reagents

Full genotypes for the strains used in this study are listed in table S1. All strains are derived from the S288C background, with the exception of the rDNA instability strains, which are SK1. Details for the RNase H1 over-expression plasmid used in this study are in Figure S10. For YAC strains, YACs were introduced by standard mating of knockouts with BY4732-YAC, sporulation and selection techniques. Full-length YAC size was confirmed by pulsed-field gel electrophoresis and Southern blot. Yeast strains were grown in YEP or minimal media supplemented with 2% glucose (Fisher Scientific) at 30°C according to standard yeast protocols (Guthrie and Fink, 1991). 5-Fluoroorotic (5-FOA) was purchased from Bio Vectra.

The AID degron was introduced at the C-terminus of *SIN3*, and was constructed using standard one-step PCR techniques, detailed in Nishimura et al., 2009.

Quantitative assay for YAC terminal deletions and total loss

Cells were dilution streaked out on SC-URA plates to select for the YAC terminal marker (URA3). In cases where a plasmid was present, SC-URA-LEU plates were used instead to also select for the plasmid. Single colonies were then picked and resuspended in 0.5 mL of water, diluted, and10^5 cells were plated onto 5-FOA and –HIS (5-FOA) plates. Plating efficiency was monitored by plating 200 cells onto rich media plates. Plates were incubated at 30°C 2-3 days after which the number of colonies formed on each plate was counted. The number of colonies that grow on 5-FOA, normalized for plating efficiencies is a measure of the rate of events.

Quantitative assay for chromosome III LOH

The chromosome III assay is similar to the YAC assay described above, with the exception that LEU2 is used as a selectable marker in place of HIS3. Diploids were isolated by mating the haploids and pulling zygotes on YPD. Ploidy was confirmed with pt1*a* and pt2*α* tester strains.

Quantitative assay for chromosome XII LOH

Diploids were dilution streaked out onto SC-URA-HIS plates. Single colonies were then resuspended in 0.5 mL of water, diluted, and 200 colonies were plated on each YPD plate. Plates were incubated at 30C 2-3 days after which the total number of colonies formed, and the proportion with red color pigmentation—indicating loss of ADE2 were counted. The two

classes of events picked up are: terminal LOH (URA^+, ade^-) and chromosome loss (ura^-, ade^-). Determining the mechanism of LOH was done as described previously (McMurray and Gottschling, 2003). Briefly, half-sectored colonies were picked and restreaked onto YPD plates for single colonies. Resulting white and red colonies were patched to YPD plates, grown overnight at 30C, then replica plated to dropout plates for phenotypic analysis of other loci along the chromosome arm.

Quantitative assay for rDNA instability

Cells were dilution streaked out on SC-URA-LEU plates to select for presence of URA3 in the rDNA, as well as the RNase H or control vector. The rate of rDNA instability was calculated from 5-FOA plates as described above for YAC instability.

Chromosome spreads and microscopy

Chromosome spreads were performed as previously described (Hartman et al., 2000). Slides were incubated with the mouse monoclonal antibody S9.6 directed to RNA-DNA hybrids, and available in the hybridoma cell line HB-8730. The primary antibody was diluted 1:2000 in blocking buffer (5% BSA, 0.2% Milk, 1X PBS) for a final concentration 0.25 ug/mL. The secondary Cy3-conjugated goat anti-mouse antibody was obtained from Jackson labs (#115-165-003) and diluted 1:2000 in blocking buffer. For slides pre-treated with RNase H, there is an additional 15-minute incubation with 2U of RNase H (Roche) diluted in 400 uL blocking buffer prior to antibody incubations. Indirect immunofluorescence (IF) was observed using an Olympus IX-70 microscope with a 100x/NA 1.4 objective, and Orca II camera (Hamamatsu, Bridgewater, NJ).

Fluorescence intensity measurements

Measurements of IF intensity for chromosomes spreads were performed using NIH ImageJ software (version 1.43r). Total RNA-DNA hybrid signal—represented as arbitrary units—was measured for individual DAPI-staining DNA masses. A region was drawn around each DNA mass, and total signal was calculated as the mean IF intensity over the region minus mean background intensity, and multiplied by the regions' total area. The distribution of intensities is graphically represented as a histogram with eighteen bins, each encompassing 10^3 arbitrary units.

To measure the ratio of RNA-DNA hybrid staining at the rDNA versus the remaining bulk of DNA mass, regions were drawn around each, and separate total intensities measured as explained above. The ratio of staining represents the total intensity over rDNA divided by intensity of the remaining DNA mass. A ratio >1 indicates a higher level of RNA-DNA hybrid staining over the rDNA locus as compared to the remaining bulk DNA.

Rad52-GFP foci microscopy

Strains were grown in YEP + 2% glucose liquid media. Overnight cultures were diluted to OD ~0.2 and shaken at 30°C for 5-6 hours to an OD ~1.0. To synchronize cells in G1 or G2/M standard synchronization techniques were used (Guthrie and Fink, 2002). Alpha-factor was added to a final concentration of 3×10^{-6} M, and nocodazole added to a final concentration of 0.015 μg/ml in 1% DMSO. For imaging cells were fixed with 4% PFA and stored in KPO_4/Sorbitol at 4°C. To immobilize yeast cells, slides were pre-coated with 5 μl of 0.2mg/ml concanavalin A (Sigma).

Growth in auxin-containing media

Liquid cultures were grown as described above, except 500 μM indole-3-acetic acid (IAA) was added to the YEP/2% glucose media. 1-2 hours after addition cells were spun down,

washed and fixed with 4% PFA as above. For experiments where Sin3p is depleted in arrested cells, cells were synchronized for 2 hours before adding 500 μM IAA; to maintain arrest cultures were spiked with either alpha-factor or nocodazole.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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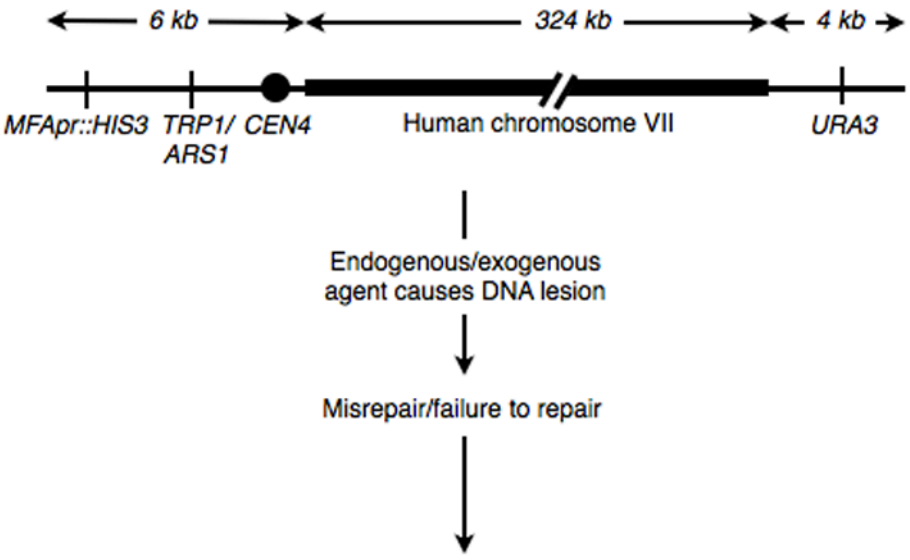
Highlights

- Defects in many aspects of RNA biogenesis cause RNA-DNA hybrids to accumulate
- Endogenous RNases H are part of a surveillance mechanism that removes RNA-DNA hybrids
- Hybrids form at many loci, both spontaneously and through RNA biogenesis mutants
- RNA-DNA hybrids induce Rad52 foci in G1, S and G2/M of the cell cycle



GCR Event	Rate of Instability (% of total)
Terminal Deletion (His^+,Ura^-)	0.23×10^{-3} (36%)
Chromosome Loss (His^-,Ura^-)	0.41×10^{-3} (64%)
	Total: 0.64×10^{-3} (100%)

FIGURE 1. Monitoring GCRs on the YAC

Assay for gross chromosomal rearrangements. Rates are determined by plating 10^5 cells and determining the number of cells that retained the YAC (His+) but lost the telomeric marker (Ura-), or lost the YAC completely (His- and Ura-).

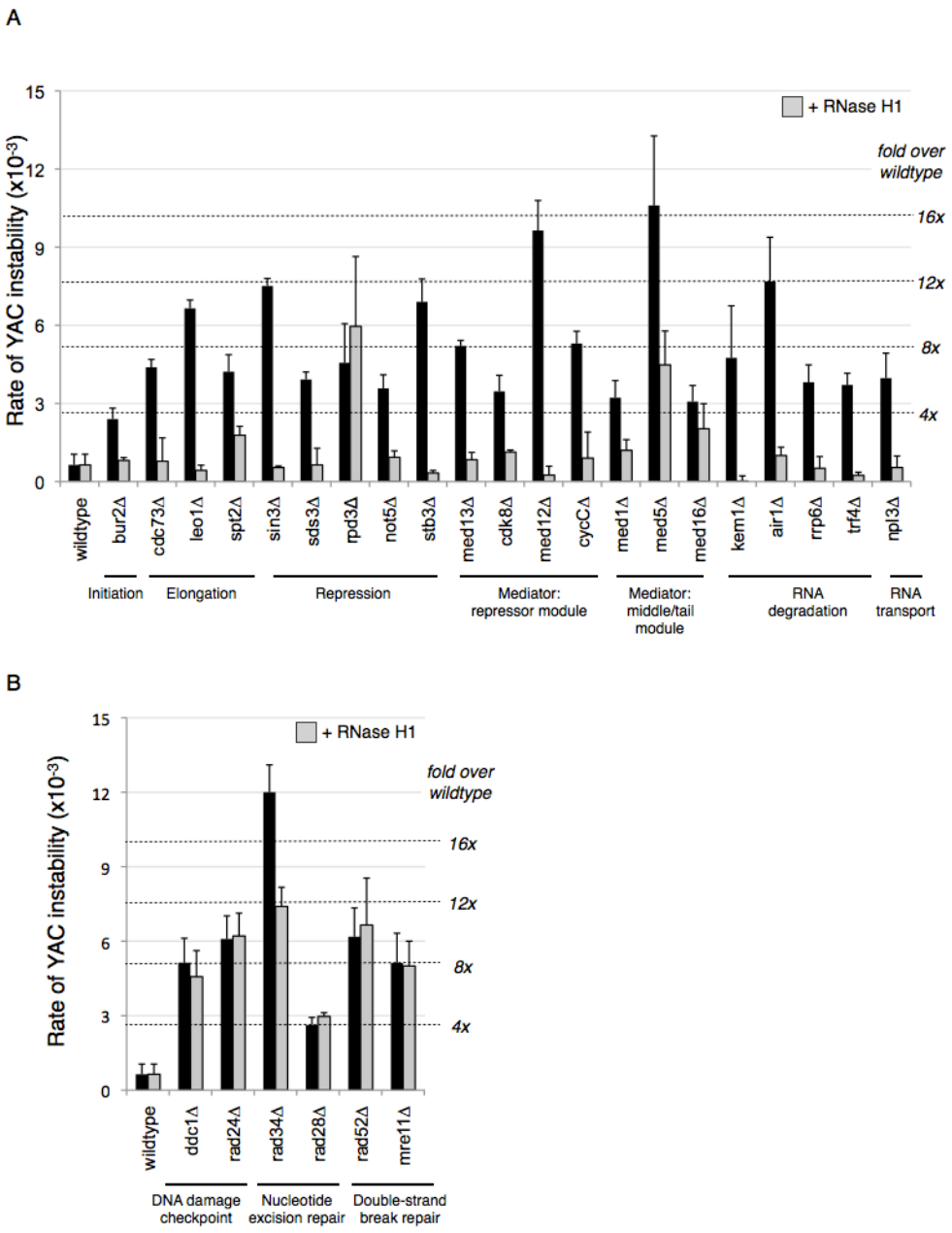
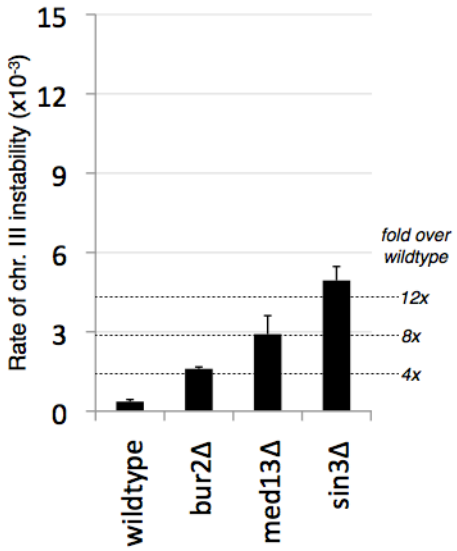


FIGURE 2. Rates of YAC instability in various mutant classes without and with RNAse H over-expression

(a) RNA biogenesis mutants carrying the YAC were assayed for GCRs with either an empty vector (black bars) or a *2μ* plasmid expressing RNase H1 (grey bars). Error bars represent standard deviation calculated from at least 4 independent colonies.

(b) Repair mutants carrying the YAC were assayed for GCRs with either an empty vector (black bars) or a *2μ* plasmid expressing RNase H1 (grey bars). Error bars represent standard deviation calculated from at least 4 independent colonies.

A



B

	Percent of cells with instability *(fold over wt)*	95% Confidence Interval
wildtype	0.18 *(1.0)*	0.15-0.22
rnh1Δ	0.26 *(1.4)*	0.21-0.29
sin3Δ	0.89 *(4.9)*	0.76-1.01
sin3Δ rnh1Δ	2.48 *(13.7)*	2.21-2.75

FIGURE 3. Rates of chromosome III and XII instability in transcription mutants

(a) RNA biogenesis mutants with a modified chromosome III were assayed for GCRs. Error bars represent standard deviation calculated from at least 4 independent colonies

(b) Percent of chromosome XII instability in *sin3Δ* mutants. Percent instability is determined by plating on YPD plates and determining the number of colonies with red sectors. A total of 13,000 to 26,000 cells originating from 20 independent colonies were scored for each genotype. Confidence intervals (CI) are calculated from the standard deviation of the 20 independent colonies.

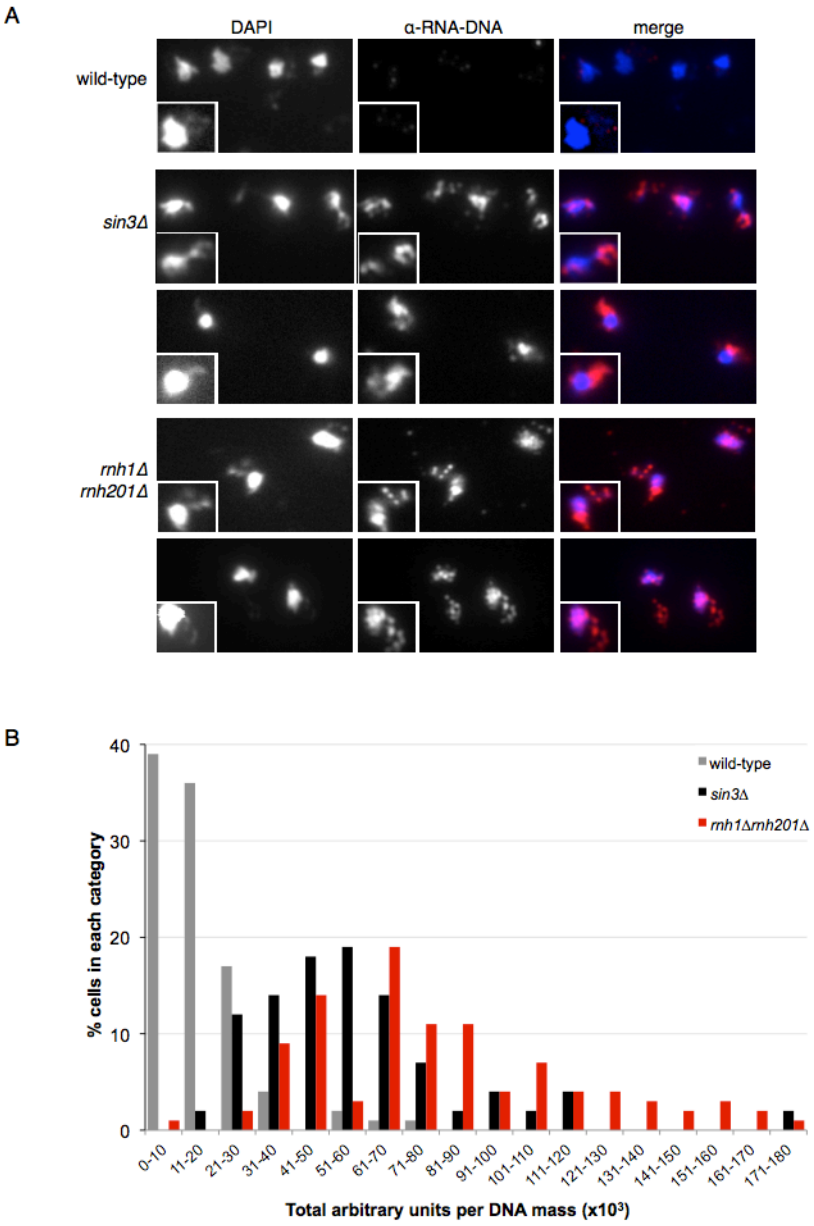


FIGURE 4. Cytological detection of RNA-DNA hybrids in transcription and RNase H mutants

(a) Representative images of chromatin spreads stained with S9.6 antibody, recognizing RNA-DNA hybrids.

(b) Measurement distribution of staining intensity in WT, sin3Δ, and rnh1Δrnh201Δ. A total of 100 cells were analyzed for each genotypic class.

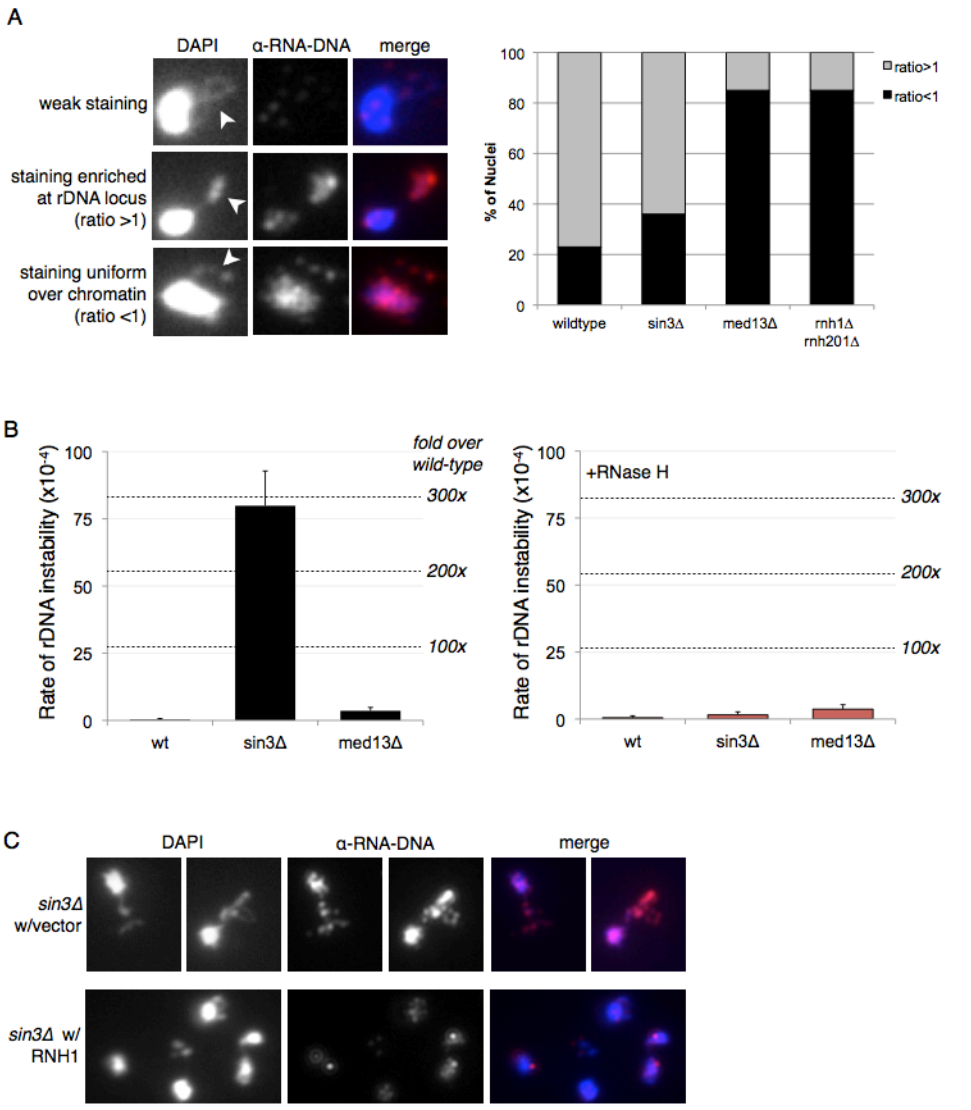


FIGURE 5. RNA-DNA hybrids and genome instability at the rDNA locus in *sin3Δ*

(a) *Left panel*- Representative images for patterns of staining observed with the RNA-DNA hybrid antibody. Arrow heads point to the nucleolus.

Right panel- Distribution of rDNA locus enrichment in WT, sin3Δ, rnh1Δrnh201Δ and med13Δ. Only nuclei with an intensity of 20,000 or more were scored. n= 25, 100, 100, and 50 for each genotype, respectively.

(b) Transcription mutants with URA3 integrated at the rDNA were assayed for loss of the URA3 marker. Error bars represent standard deviation calculated from at least 8 independent colonies.

(c) Representative images of chromatin spreads stained with S9.6 antibody, recognizing RNA-DNA hybrids. Strains were harboring either an empty control *2μ* plasmid or one expressing RNase H1.

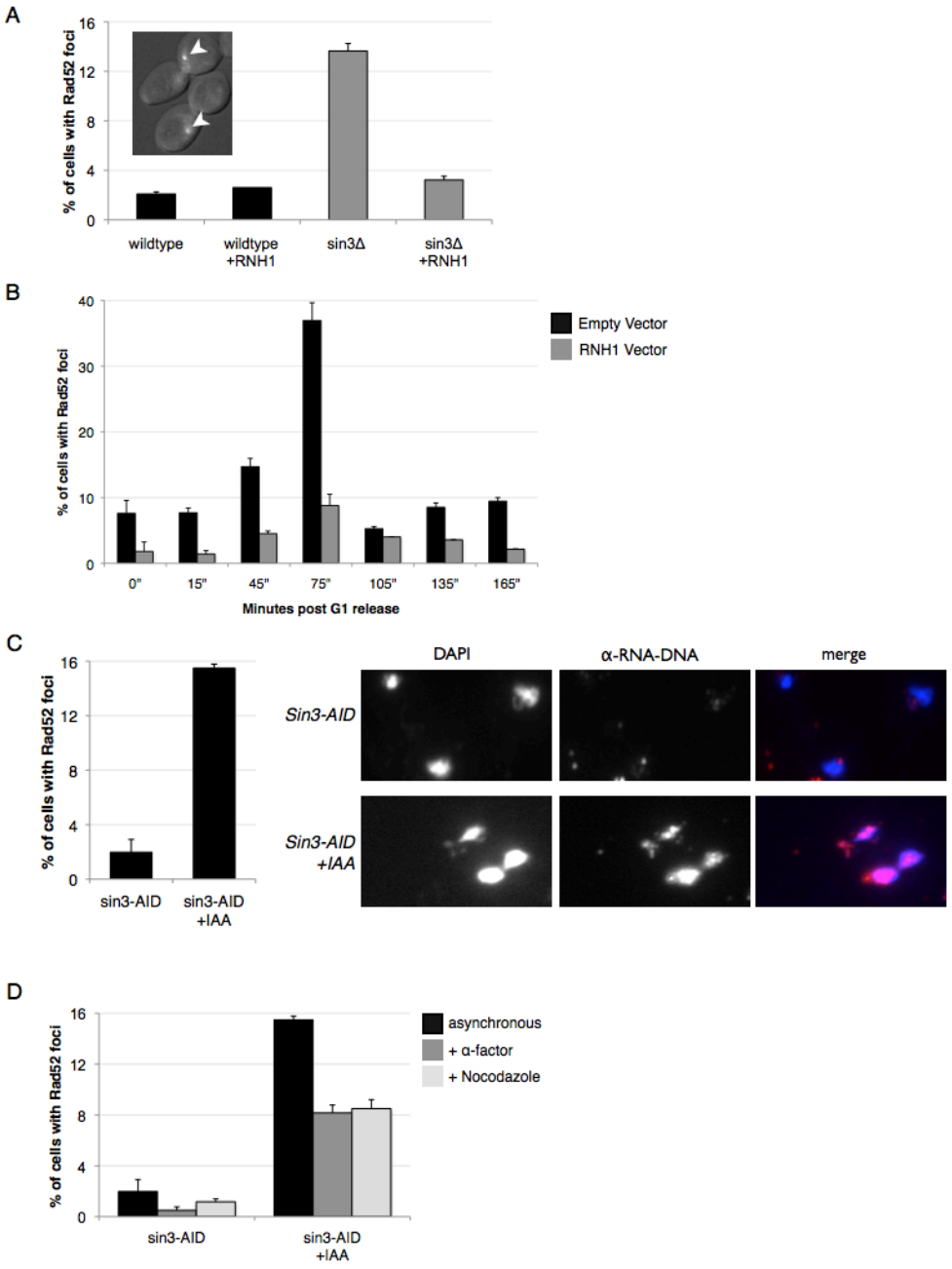


FIGURE 6. The absence of Sin3p increases the number of cells with Rad52-GFP foci

(a) The number of cells with Rad52-GFP foci was quantified in asynchronous log phase cells of WT and *sin3Δ* mutant carrying either an empty control *2μ* plasmid or one expressing RNase H1. A total of approximately 1000 cells were scored for each of the two WT genotypes and 3000 for each *sin3Δ* genotype, spanning three or more experiments of two independently isolated colonies. For all graphs, error bars represent standard deviation between experiments.

(b) The number of cells with Rad52-GFP foci was quantified in *sin3Δ* cells carrying the empty of RNase H1 plasmid at indicated time points post-release from G1 arrest. A total of 600-1000 cells were scored for each time point over two experiments.

(c) Left panel- The number of cells with Rad52-GFP foci was quantified in Sin3-aid cells prior to and 2 hours after addition of 500 μM IAA. 600 cells were scored for each condition. Right panel- Representative images of chromatin spreads from Sin3-aid cells prior to and 2 hours after addition of 500 μM IAA.
(d) The number of cells with Rad52-GFP foci was quantified in Sin3-aid cells not arrested, or arrested with alpha-factor or Nocodazole prior to and 1 hour after addition of 500 μM IAA.

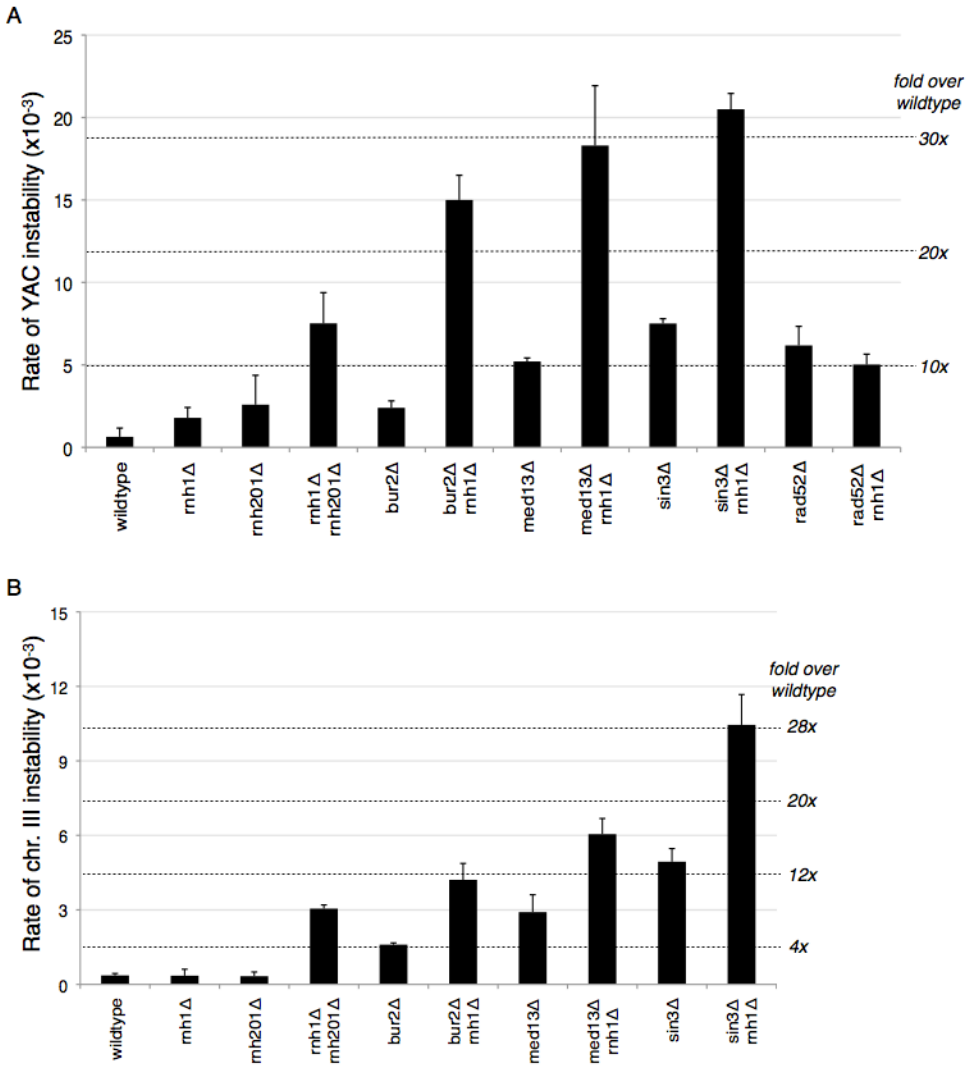


FIGURE 7. Deleting *RNH1* in transcription mutants synergistically increases YAC and chromosome III instability

(a) RNA biogenesis-RNase H double mutants carrying the YAC were assayed for instability. Error bars represent standard deviation calculated from at least 4 independent colonies.

(b) RNA biogenesis and RNase H mutants with a modified chromosome III were assayed for instability. Error bars represent standard deviation calculated from at least 4 independent colonies.