Role for RNA:DNA hybrids in origin-independent replication priming in a eukaryotic system

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DNA replication initiates at defined replication origins along eukaryotic chromosomes, ensuring complete genome duplication within a single S-phase. A key feature of replication origins is their ability to control the onset of DNA synthesis mediated by DNA polymerase-α and its intrinsic RNA primase activity. Here, we describe a novel origin-independent replication process that is mediated by transcription. RNA polymerase I transcription constraints lead to persistent RNA:DNA hybrids (R-loops) that prime replication in the ribosomal DNA locus. Our results suggest that eukaryotic genomes have developed tools to prevent R-loop–mediated replication events that potentially contribute to copy number variation, particularly relevant to carcinogenesis.

RNA:DNA hybrids | RNase H | topoisomerase 1 | replication | ribosomal DNA

During transcription, RNA acts as a template for the synthesis of the nascent RNA. RNA synthesis is accompanied by the generation of positive and negative DNA supercoiling in front of and behind the transcription machinery, respectively (1). Unwinding of the DNA double helix by negative supercoiling may allow the RNA to hybridize to its DNA template behind the elongating RNA polymerase, leading to R-loops (2). Other elements that could potentiate R-loop accumulation include DNA:RNA hybrid-facilitating DNA sequences, such as G-quadruplex structures (3) or nicks in the nontemplate DNA strand (4).

Eukaryotic cells need to control R-loop formation to avoid replication impairment, genome instability, and life span shortening mediated by such intermediates (5–10; reviewed in ref. 11). To do so, cells catalyze the relaxation of supercoiled DNA by type I topoisomerases (12–15), thus preventing replication fork reversal (16), DNA overwinding with the potential to block replication fork progression (17), DNA unwinding (18), or R-loop–mediated blocks of ribosomal RNA synthesis (19). Other enzymatic activities involved in R-loop processing include ribonuclease H (RNase H) activities, DNA-RNA helicases, such as Sen1/senataxin (20, 21), or Ataxin-2 RNA-binding protein Pbp1 (10). The ribonuclease activity of Saccharomyces cerevisiae RNases H1 and H2 specifically cleaves the RNA moiety of the RNA:DNA hybrid structure (22), whereas RNase H2 and topoisomerase 1 (Top1) can also process ribonucleotides in duplex DNA (23, 24).

Notably, R-loops are required to initiate mitochondrial DNA replication (25) and pioneering studies connected R-loops to origin-independent replication in prokaryotic systems (26, 27). For example, DNA-dependent initiation of DNA replication at the Escherichia coli oriC replication origin can be overcome in the absence of RNase H1 (28, 29). As a consequence, mhlΔ mutants can survive complete inactivation of oriC by transcription-dependent activation of so-called oriK sites (30, 31), although candidate oriK sites have been identified only recently (32). Additional evidence for R-loop–primed replication was given by the observation that mhlΔ mutants are prone to an increase in mutation and DNA amplification events if origin activity is suppressed. These events required removal of the RNA polymerase (RNAP) to allow conversion of an R-loop into a replication fork (33). In summary, R-loops may act as the earliest known mutagenic intermediate in transcribed regions, and accelerate adaptation to genomic stress in prokaryotes. However, the possibility that R-loops mediate replication events in eukaryotic organisms still remains to be explored.

Highly transcribed ribosomal genes have been shown to favor R-loop formation in cells lacking both RNase H and Top1 activities (19). Here, by taking advantage of R-loop promoting conditions we potentiate the formation of DNA double-strand breaks (DSBs) and detect origin-independent replication intermediates (RI) within the transcribed 35S rRNA genes. A main finding in this work is the observation of “bubble-shaped” RIs by 2D agarose gels within the actively transcribed 35S rDNA when both Top1 and cellular RNases H are depleted. Importantly, in accordance with R-loop–mediated replication these “bubbles” are no longer observed when transcription by RNAPI is constrained. Our data suggest that R-loop–mediated replication contributes to stress-induced mutation, which is potentially relevant to eukaryotic genome evolution and disease formation.

Results

R-Loops Promote Genome Instability and Noncanonical Replication Events. We maximized R-loop accumulation by treatment of mutants devoid of both RNase H1 and H2 (mhlΔ mh201Δ, referred to herein as r1Δ r2Δ) with the Top1 inhibitor camptothecin (CPT). CPT causes the accumulation of a covalent Top1–DNA complex that prevents religation of a nicked DNA duplex (15). Sensitivity to CPT and replicative stress generated by hydroxyurea (HU) and methyl methanesulfonate (MMS) was dependent on the removal of both RNase H activities, suggesting that both enzymes can substitute for each other (Fig. L4 and Fig. S1 A and B). Similarly, the lack of both RNase H activities was necessary to induce genomic instability. Double mutants r1Δ r2Δ showed a sixfold increase in CAN mutation rates (Fig. SIC), a 10-fold increase in loss of heterozygosity at the MAT locus (Fig. SIC), and a sevenfold increase (WT 4.5%, r1Δ r2Δ 31.6%) in S/G2 phase DNA repair centers as monitored by Rad52-YFP foci (Fig. 1B). More than half of the Rad52-YFP foci appeared to be associated with nucleolar

Significance

R-loop formation has been related to genome instability and human disease, yet the role of R-loops in replication priming remains to be explored in the eukaryotic genome. This investigation discloses that transcription-dependent R-loops have the potential to initiate origin-independent replication events in ribosomal DNA. Taken together, our data suggest that R-loops contribute to transcription-driven endoreplication events and alterations in genome copy number.


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DNA in rΔ rΔ cells (61% in the absence and 65% in the presence of CPT), indicating an increased susceptibility of ribosomal DNA (rDNA) to DNA damage caused by impaired processing of RNA:DNA hybrids.

The rDNA, which is hosted in the nucleolus, is localized on chromosome XII and consists of ~150 rDNA copies per haploid that are susceptible to stress-induced changes in repeat number and the formation of extrachromosomal rDNA circles (ERCs) caused by the excision of rDNA repeats (34, 35). Consistent with an increase in replicative stress upon CPT treatment, the number of Rad52 YFP foci-containing cells doubled 30–90 min after release from α-factor (Fig. S2A) and CPT-treated rΔ rΔ mutant cells accumulated in late S or G2 phase (Fig. 2A). To further investigate the impact of CPT on rDNA maintenance, we monitored the fate of replication forks in S-phase cells. Following G1-synchronization by α-factor and release into CPT-containing medium, RIs were isolated and analyzed by 2D agarose gel electrophoresis (for interpretation of the results, see Fig. 2B). Although the S-phase specific pattern of replication (Y-arc) and recombination (X-spike) intermediates in WT and rΔ rΔ strains were similar, a clear difference was observed at late S/G2 phase. Whereas replication in the WT had finished, Y-arc and X-spike RIs remained in the rΔ rΔ mutants, and replication fork pausing sites (RFPs) (Fig. 2C; open arrowheads) appeared in the nontranscribed spacer region (Fig. 2C; probe Δ). These pausing sites may be detected in RIs isolated from untreated rΔ rΔ mutants (Fig. S3A). The majority of these RFP sites overlapped with those previously described in cells lacking the Rrm3 DNA helicase (36) (Fig. 3). The observed pausing sites may be enriched at hot spots of Top1–DNA interaction sites and correspond to CPT-trapped Top1–DNA complexes, yet they correlated with potential sites of R-loop formation (37, 38), such as the 3′ end of 35S genes (d in Fig. 3) or 5S genes (c in Fig. 3), but also included sites of protein barriers such as the ribosomal autonomously replicating sequence (ARS) or 35S promoter (a/b and c in Fig. 3).

Next, we monitored replication intermediates present within the 35S gene (Fig. 2C; probe B). Although the 35S gene lacks common characteristics of replication origins that allow the binding of the prereplication complex (39), intermediates migrating as expected for bubble-shaped molecules were detected (indicated by a black arrowhead at the 105-min timepoint in Fig. 2C; see ref. 40 for a more detailed explanation of RI characteristics), indicative of replication initiation events within the 35S gene. We analyzed the bubble-shaped RIs by several means to determine the structure of these molecules (Fig. S3B). Previously it had been shown that in vitro RNase H treatment of mtDNA replication intermediates removes RNA:DNA hybrids and leads to the appearance of simple-Y structures in 2D agarose gels (41, 42). In accordance with a replicon-like structure, the bubble-shaped molecules were resistant to in vitro RNase H treatment and heat-induced branch migration. Furthermore, as expected for replicating molecules having an extendable 3′ end, in vitro DNA synthesis by Klenow/gp32 treatment could destroy the bubble-shaped molecules by strand displacement (43). These analyses rule out the presence of long stretches of R-loops, which could have a bubble-like appearance, and suggest a rapid conversion of R-loops into noncanonical replicons.
Depletion of Top1 in the Absence of RNase H Leads to Unscheduled Replication Within the 35S rDNA. Next we generated an indole acetic acid (IAA)-inducible Top1-degron mutant (TOPIAID*) and assayed the impact of Top1 degradation on RNA:DNA hybrid formation. Top1 levels rapidly decreased upon IAA addition (Fig. 4A, Left and Fig. S4A), and consistent with previous observations (19), growth of r1Δ r2Δ mutants was inhibited in the absence of Top1 (Fig. S4A). Although 30% of r1Δ r2Δ mutant cells cross-reacted with the R-loop specific S9.6 antibody, 61% of cells were S9.6+ upon CPT addition, and similar values (39% vs. 76%) were obtained for the IAA-stimulated Top1 degradation in r1Δ r2Δ mutants, respectively (Fig. 4A, Right and Fig. S4B). This result supports the idea that the concomitant loss of RNase H and Top1 activities has an additive effect on RNA:DNA hybrid formation (13, 33).

Top1 depletion in r1Δ r2Δ mutants was accompanied by an increase in Rad52 foci, indicative of an increase in DSB accumulation in the absence of both Top1 and RNase H activities (Fig. S4C) and a cell cycle arrest in S/G2 (Fig. S4D). Some of the CPT-mediated pausing sites characterized within the intergenic space region in r1Δ r2Δ mutants (Fig. 3) were barely detectable in the triple mutant (Fig. 4B, probe A). However, a strong pausing site at the 3’ end of the 35S gene (Fig. 4B, open arrowhead) may indicate a failure of the replication machinery to bypass torsional stress generated ahead of the transcribing RNA Pol I (RNAPI). Interestingly, the appearance of this strong RFP correlates with a decrease of RF accumulation at the Fob1-dependent replication fork barrier (RFB) (Fig. 4B, asterisk). A cease in ribosomal ARS firing, increased torsional stress, or weakened Fob1 binding in the absence of Top1 (44) could contribute to the loss of the Fob1-dependent RFB signal. Nevertheless, the presence of replication-like structures within the 35S gene (Fig. 4B, probe B, black arrowhead) upon Top1 depletion in cells lacking RNase H strengthens the idea that R-loops could mediate origin-independent replication initiation events.

Unscheduled Replication Events Are Transcription-Dependent. Our model predicts that RNAPI transcription would be a prerequisite for R-loop–initiated replication. To slow down rDNA transcription rates, we made use of the rpa190-3 mutant of the largest RNAPI subunit Rpa190 and the rm3-8 mutant of Rnr3, which recruits RNAPI to the promoter of 35S rRNA genes (45). Strikingly, both rpa190-3 and rm3-8 mutants alleviated the CPT-sensitivity of r1Δ r2Δ mutants at semipermissive temperature (Fig. 5A and Fig. S4A), reduced the formation of nucleosome-associated Rad52-foci formation and suppressed S/G2-phase cell cycle arrest (Fig. 5B and Fig. S5B). These observations suggest that the majority of the CPT-induced Top1-dependent DNA lesions are linked to rDNA transcription, as well as a potential link between rDNA damage, checkpoint activation, and growth rate. Next, we examined the fate of replication in rpa190-3 r1Δ r2Δ mutants by 2D gel electrophoresis. Cells were grown at 26 °C, before α-factor synchronization and released in CPT-containing media at permissive (23 °C) or semipermissive (30 °C) temperature. The absence of bubble-shaped replication intermediates in cells released from α-factor synchronization at semipermissive temperature (Fig. 5C and Fig. S5C) confirms a mechanism where transcription-mediated R-loops initiate replication at late S/G2 phase within the 35S rDNA.

Discussion

Decades ago it became evident that R-loops take part in replication initiation of prokaryotic cells (46, 47). Here we present evidence that this is also the case for eukaryotic cells based on the observation that persistent R-loops can mediate unscheduled, origin-independent replication initiation in yeast chromosomal DNA. These replication events were observed in the highly transcribed 35S rRNA gene, and occurred spatially and temporally outside of the regular replication schedule. Unscheduled replication was not linked to a defined replication origin, and it was observed in late S/G2 phase of the cell cycle where replication termination and completion is expected to take place.

Which Factors and Mechanism Would Participate in Transcription-Initiated Replication Events? Various, nonexclusive mechanisms could cooperate to trigger such transcription-initiated replication (TIR) (Fig. 5D). At present we do not know whether the presence of single-stranded DNA within an R-loop may permit strand invasion-dependent replication events, favored by the repetitive nature of the rDNA array. DSBs seem to be particularly frequent in the rDNA locus. Thus, R-loops and DSBs could stimulate recombination-driven replication events as observed in Candida albicans mtDNA (42) or break-induced replication (48, 49) and involve the transient formation of simple Y-like replication intermediates.

The synthetic lethality observed in the absence of Top1 and RNase H activities (19, 50, and present work) complement previous notions that R-loops have an evolutionary conserved impact on transcription. E. coli Top1 mutants suffer from impaired
growth and rDNA transcription (51–53), and that RNase H1 overexpression can partially compensate for the absence of Top1 (54). Other factors that possibly contribute to synthetic lethality include the accumulation of positive supercoiling generated ahead of RNAPI and in front of an advancing replication fork in convergent orientation. Such supercoiling can promote DNA extrusions and secondary structures that can be substrates for specific DNA nucleases (55).

Consistent with the observation that R-loops block replication fork progression (6), replication fork collapse at the site of nicked DNA may result in the physical presence of a “truncated” replication machinery in close vicinity to the R-loop and also explain the observed increase in DSB formation during S-phase (Fig. S2A). A truncated replication machinery potentially restarts replication from an R-loop, given that elegant in vitro experiments demonstrated that replication can restart from a purified E. coli replisome–RNAP complex, and that the replisome uses mRNA as a primer to reinitiate leading-strand synthesis after displacing a codirectional RNAP from DNA (56). One can speculate that RNAPI is no longer associated with the R-loop, a scenario that facilitates TIR without the need for factors that drive the displacement of RNAPs being head-on to a replisome (57).

In contrast to replication restart from a colliding replisome–RNAP complex, TIR events may be driven by the de novo replisome assembly at an R-loop. Assembly of the replication factor A protein complex to single-stranded DNA opposite a RNA:DNA hybrid could promote interaction with DNA replicases that are available at the end of chromosomal DNA synthesis at late S/G2 (58). There is evidence that replicases remain replication competent at S/G2 (58), and by doing so they may be able to initiate DNA synthesis within an R-loop. The DNA pol-primase subunit Pol12 is essential for replication initiation and has been suggested to act as a molecular tether during DNA replication (59). Pol12 is an essential but stable protein and its phosphorylated form appears to be required for the initial stages of DNA synthesis before the HU-sensitive elongation step (60). Pol12 remains in active and phosphorylated form in S/G2 and its inactivation by dephosphorylation only occurs while cells exit mitosis (61). Our observations suggest that RNAPI-associated R-loops and replication-competent DNA pol-primase complexes could drive S/G2-dependent TIR events.

Are TIR Events Limited to rDNA? At a much lower frequency, TIR events may happen throughout the whole genome, and hot-spots for R-loop formation, such as highly transcribed genes, might be more prone to TIR events. Numerous enzymatic activities linked to the suppression of RNA:DNA hybrids including the THO-complex (37), the mRNA polyadenylation factor Pp1l, G4-quadruplex binding proteins Stem1 and Pif1 (10, 62), or the Sen1 subunit of the Nrd1 complex (20, 21, 63) may also be required to avoid TIR events. Mutations in the yeast Sen1 ortholog senataxin have been shown to be associated with human AOA2/ALS4 neurodegenerative disorders (64, 65). Indeed, Senataxin is needed to maintain genome integrity because of its function in the coordination of transcription, DNA replication, and the DNA damage response (reviewed in ref. 66). Thus, it would be interesting to see if TIR events are stimulated by the absence of Sen1 or other factors involved in mRNA biogenesis.

What Could be the Consequences of TIR Events? R-loop primed, unlicensed genome replication would provide a new threat to eukaryotic genome stability. TIR carried out by a noncanonical replisome is likely to be inaccurate, and hence may have serious repercussions for genome instability. Unscheduled replication, particularly in regions of repetitive sequences, such as the rDNA array, could lead to the deletion of repetitive sequences or gene-amplification events, respectively. The rDNA repeat is located on chromosome XII and we find by pulse-field gel-electrophoresis (PFGE) analysis that the migration of chromosome XII is retarded (Fig. S6A). These initial findings indicate that loss of RNase H activity leads to rDNA expansion or intermediate structures that hamper electrophoretic separation as shown for
replicating chromosomes (67). The observation that ERC formation is enhanced in r\( \Delta \) r2a mutants (Fig. S6B) confirms that the loss of RNase H activities causes genetic instability (Fig. S1). It would be particularly interesting to determine to which extent impaired RNA:DNA hybrid processing contributes to rereplication, gene amplification, and alterations in chromosome copy number in human cells. These events would have disastrous consequences for eukaryotic genome function and are particularly relevant to carcinogenesis (68).

Selective gene amplification is frequently observed in differentiating eukaryotic cells and results in transcript number and gene product increases in a dosage-dependent manner (69). The mechanisms by which gene amplification is achieved include DSB-dependent sister chromatid fusion and repeated breakage-fusion-bridge cycles evident in the dihydrofolate reductase locus of CHO cells (70), endoreduplication of diptera chorion genes by multiple activation of replication origins within the same S-phase (71), RNA-template derived nanochromosome amplification in Stylonychia lemnae (72), or rolling circle amplification of extrachromosomal rDNA circles in Xenopus oocytes (73). Although in our study we could not distinguish if ERCs are more prone to TIR events, our results provide the possibility that ERC replication can be driven by impaired RNA transcript-processing suggesting that R-loops could have a physiological role in the control of gene amplification linked to nuclear differentiation events.

Experimental Procedures

Yeast Strains and Growth Conditions. Yeast strains used in this study are listed in Table S1. Gene deletions were constructed by PCR-based methods (74). If not generated by a direct knock-out in the YKL background, mutant strains were backcrossed at least twice to the YKL83 strain background. Yeast strains were grown in YPAD, or synthetic complete (SC) minimal medium supplemented with 2% (v/v) glucose and amino acids. IAA (Sigma) was added at 500 \( \mu \)M for solid and 1 mM for liquid YPAD media.

Viability Assays. To test for sensitivity to genotoxic agents, 10-fold serial dilutions of cells were grown on 3 \( \times \) YP plates or YP-plates containing HU (50 mM; USBiological), MMS (10 mM; Fluka), or CPT (5 \( \mu \)g/mL; Santa Cruz Biotechnology), unless otherwise specified.

Quantification and Colocalization of Rad52-YFP Foci. For colocalization of Rad52-YFP foci with the nucleolar marker Not1, cells were cotransformed with the RAD52-YFP expressing plasmid (F. Prado, Sevilla, Spain) and a plasmid expressing MOPT1-mRFP (75). Transformants were grown in exponential phase in SC synthetic medium supplemented with 1 \( \mu \)g/mL CPT for 3 h, and fixed using 2.5% (vol/vol) formaldehyde. YFP fluorescence (480-nm excitation/527-nm emission) and RFP fluorescence (584-nm excitation/607-nm emission) were detected by wide-field fluorescence microscopy (DM-6000B, Leica) at 100× magnification. Images were taken using LAS AF software (Leica). For each sample, 600 cells were counted from three independent experiments. For time-course analysis of Rad52-YFP foci, cells expressing the RAD52-YFP plasmid were synchronized with the \( \alpha \) factor and released in the presence or absence of 10 \( \mu \)g/mL CPT. Samples were retrieved at the specified time points following release and arrested by adding sodium azide (to a final concentration of 0.1%). DNA was extracted according to ref. 6.

Two Dimensional Agarose Gel Electrophoresis. In a total volume of 100 \( \mu \)L, about 5 \( \mu \)g of genomic DNA was digested with 40 units BglII for 6 h, iso-propanol precipitated, and resuspended in 10 \( \mu \)L loading buffer. First, dimension electrophoresis was performed in 0.4% TBE-buffered agarose gels at 40 V for 20 h. A gel slice containing DNA fragments between 3 and 12 kb was cut out for second dimension resolution in 1.1% TBE-buffered agarose at 140 V for 6 h. Denatured DNA was transferred to a Hybond XL membrane (Amersham) by standard procedures. Replication intermediates were detected by hybridization with specific \( \beta \)-P-labeled DNA probes, matching to nucleotides 452691-453344 (probe a) and 453834-454699 (probe b) on chromosome XII. Signals were quantified using a PhosphorImager with ImageGauge software (Fuji). The relative intensity of replication intermediates was normalized to the signal intensity obtained in the 1x-control (nonsaturating exposure).

Supporting Information

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**SI Experimental Procedures**

**Survival Assays.** For the survival assay, a sample of exponentially growing cells was taken from a liquid YPAD culture and plated onto YPAD. Next, 10 μg/mL CPT was added to the liquid culture and cells were incubated for 24 h, with appropriate dilutions to maintain cells in the exponential phase of growth. At determined time points, cells were taken from the population and plated onto YPAD plates. Cells were counted after 3-d growth and presented as the percentage of surviving cells with respect to time 0, set as 100% survival.

**Genome Instability Assays.** The formation of a-mating cells from MATα strains was scored by the MATα-like faker assay (ALF), as previously described (1). Patches of MATα strains were replica-plated onto a mating tester lawn of F15 strain, and mated products were scored by growth on SD plates [0.17% YNB, 0.5% ammonium sulfate, 2% (wt/vol) glucose, and 2% (wt/vol) agar]. ALF frequency values (mated products per total cells) were obtained from the mean of three fluctuation tests of four independent colonies each.

Forward mutation frequencies were obtained by comparing the number of colonies growing on SC-Arg plates (SC media lacking arginine) containing 60 mg/mL canavanine to the total number of colonies obtained on SC. Each mutation value was obtained as the mean value of three different fluctuation tests, and each test represents the median value of six independent colonies. The fold-change numbers represent the rate relative to WT, which was expressed as 1. Data are shown as the mean ± SD. Differences between groups were examined by Student’s t test and were considered statistically significant for P values < 0.05.

**In Vitro Characterization of RIs.** Following BglII digestion and isopropanol precipitation, DNA was resuspended in 2 mM Tris-HCl pH 8.0 and subjected to heat or enzymatic treatments as previously described (2). In brief, to induce branch migration, DNA was incubated at 56 °C for 1 h in the presence of 10 mM EDTA. For the strand displacement reaction, Klenow polymerase was used. Stepwise, 1 μL of nucleotide mix (5 mM each dATP, dCTP, dGTP, dTTP; Pharmacia), 1 μL of gp32 protein (Biolabs; 4 μg/μL), and 1 μL of Klenow (Takara; 5 U/μL) were added to 17 μL of restriction enzyme-digested DNA in 1× restriction buffer and were incubated for 1 h at 37 °C. For R-loop removal, 1 μL RNase H (Biolabs; 5 U/μL) was added to 19 μL of restriction enzyme-digested DNA in 1× restriction buffer and incubated for 1 h at 37 °C.

**RNA:DNA Hybrid Detection.** For RNA:DNA hybrid detection, 1 mM IAA was added to YPAD medium for the last 30 min of α-factor incubation, and cells were fixed for immunofluorescence as previously described (3). To analyze the formation of RNA:DNA hybrids, the S9.6 antibody (ATCC) and anti-mouse Alexa 546 (Invitrogen) antibodies were used at 1:200 and 1:500 diluted in PBS-5% (wt/vol) BSA, respectively.

**PFGE and ERC Detection.** For PFGE, 10^8 cells per low-melting agarose plug were prepared as described in Ide et al. (4). Electrophoresis was performed in a 0.8% agarose gel at 14 °C in a Bio-Rad CHEF Mapper under the following condition: a voltage gradient of 34 V/cm, switch times of 300 to 900 s, a switch angle of 120°, 0.5× TBE for 68 h. Detection of ERCs was done as previously described (5). Ribosomal DNA was detected by Southern blot analysis using probe A.


Fig. S1. The lack of RNaseH activities renders yeast sensitive to replication stress and increased genome instability. (A) Cell survival after prolonged incubation with CPT. Data are shown as the mean ± SD. (B) Analysis of sensitivity to genotoxic agents. Tenfold serial dilutions of cells grown for 3 d on YPAD or YPAD-containing HU (50 mM) or MMS (10 mM). (C) Mutation rates as determined by canavanine resistance (CanR, Left); frequency values (mated products per total cells) shown as the mean ± SD obtained from the mean of three fluctuation tests of four independent colonies each. Fold-change (F.C.) relative to WT indicated. Differences between mutants and the WT determined by Student’s t test and considered statistically significant for *P < 0.05.

Fig. S2. The lack of RNase H activity and CPT treatment increase Rad52-YFP-monitored DNA damage during S-phase. (A) Time-course analysis of Rad52-YFP foci appearance in WT and r1Δ r2Δ cells following release from α-factor in the presence or absence of CPT. Data represent mean from two independent experiments. (B) Quantification of the RFB, bubble- (bubble-arc), simple Y- (Y-arc), and X- (X-spike) shaped replication intermediates from representative 2D-gels displayed in Fig. 2A. Signal intensities relate to corresponding “1×” spot in each gel.
Fig. S3. Characterization of unscheduled replication intermediates in the rDNA. (A) CPT treatment and the lack of RNase H activities contribute to unscheduled replication events. FACS analysis (Upper) and 2D-gel electrophoresis of RIs derived from r1Δ r2Δ mutant cells released from G1 phase α-factor synchronization in the absence of CPT (Lower). For description, see Fig. 2 B and C. Note the absence of additional replication pausing sites (probe A) and the bubble-arc (probe B). The images are reduced 3.5×. (B) Two-dimensional gel analysis of in vitro treated RIs. RIs were either incubated at 56 °C to promote branch migration, treated with RNase H to remove RNA:DNA hybrids or subjected to strand-displacement by concurrent addition of Klenow, gp32 and dNTPs. RIs were detected by probe A or probe B. The bubble-arc is indicated (black arrowhead). The images are reduced 2.5×.
**Fig. S4.** The stimulation of R-loop formation in the absence of RNase H or Top1 activities contributes to DNA damage and unscheduled replication events. (A) Kinetics of Top1<sup>ADP</sup>* degradation by Western blot analysis, and drop test analysis of cell viability upon Top1<sup>ADP</sup>* degradation in r1Δ r2Δ mutant cells. Indicated strains were grown on YPAD, or plates containing 1 mM IAA in the presence or absence of 5 μg/mL CPT. (B) Representative examples of R-loop detection by S9.6 antibody cross-reaction in cells grown in the presence of CPT or IAA. Only WT, r1Δ r2Δ and r1Δ r2Δ TOP1AID* mutant cells with overlapping S9.6 (red) and DAPI staining (blue) were considered R-loop-positive. Cells were detected at 100× magnification. (C) Rad52-YFP foci formation in exponentially growing r1Δ r2Δ TOP1AID* mutant cells in the presence of absence of IAA. Data represent mean ± SD from two independent experiments. (D) FACS analysis and quantification of 2D gels corresponding to the representative experiment shown in Fig. 3B. For details see Fig. 2C.
**Fig. S5.** CPT sensitivity and unscheduled replication depend on RNAPI transcription. (A) Tenfold serial dilutions of the temperature-sensitive, conditional rm3-8 (Rrn3 is an essential RNAPI transcription factor) mutants grown on YPAD (control) or YPAD-containing 5 μg/mL CPT for 3 d at permissive (23 °C) or semipermissive (30 °C) temperature. (B) FACS and (C) 2D-gel analysis of RIs from rpa190-3 r1Δ r2Δ cells upon α-factor release in the presence of 10 μg/mL CPT at permissive or semipermissive temperatures. For details on RI quantification, see Fig. 2B. Note that the 105-min timepoint 2D gels correspond to the ones shown in Fig. 5C. The blank area (60 min, 30 °C condition) is due to a physical damage of the PhosphorImager screen. Bubble-shaped molecules are indicated (black arrowheads). The images are reduced 4.5×.
Fig. S6. Chromosome XII migration and ERC formation is affected in r1Δ r2Δ mutants. (A) PFGE analysis of chromosomes derived from independent colonies of WT (lanes 1–3) and r1Δ r2Δ cells (lanes 4–6). The gel was stained with EtBr (Left) and subjected to Southern blot analysis using probe A (Right). Migration of the rDNA containing chromosome XII (chr. XII) is indicated. (B) Determination of ERC formation in r1Δ r2Δ mutants. Genomic rDNA (G) and ERCs (arrows) detected by hybridization against probe A are indicated.

Table S1. Yeast strains and plasmids used in this study

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