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 *MAT*α strains was scored by the *MAT*α-like faker assay (ALF), as previously described (1). Patches of *MAT*α 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.

1. Yuen KW, et al. (2007) Systematic genome instability screens in yeast and their potential relevance to cancer. *Proc Natl Acad Sci USA* 104(10):3925–3930.
2. Wellinger RE, Schär P, Sogo JM (2003) Rad52-independent accumulation of joint circular minichromosomes during S phase in *Saccharomyces cerevisiae*. *Mol Cell Biol* 23(18):6363–6372.
3. Visintin R, Hwang ES, Amon A (1999) Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus. *Nature* 398(6730):818–823.
4. Ide S, Miyazaki T, Maki H, Kobayashi T (2010) Abundance of ribosomal RNA gene copies maintains genome integrity. *Science* 327(5966):693–696.
5. Burkhalter MD, Sogo JM (2004) rDNA enhancer affects replication initiation and mitotic recombination: Fob1 mediates nucleolytic processing independently of replication. *Mol Cell* 15(3):409–421.

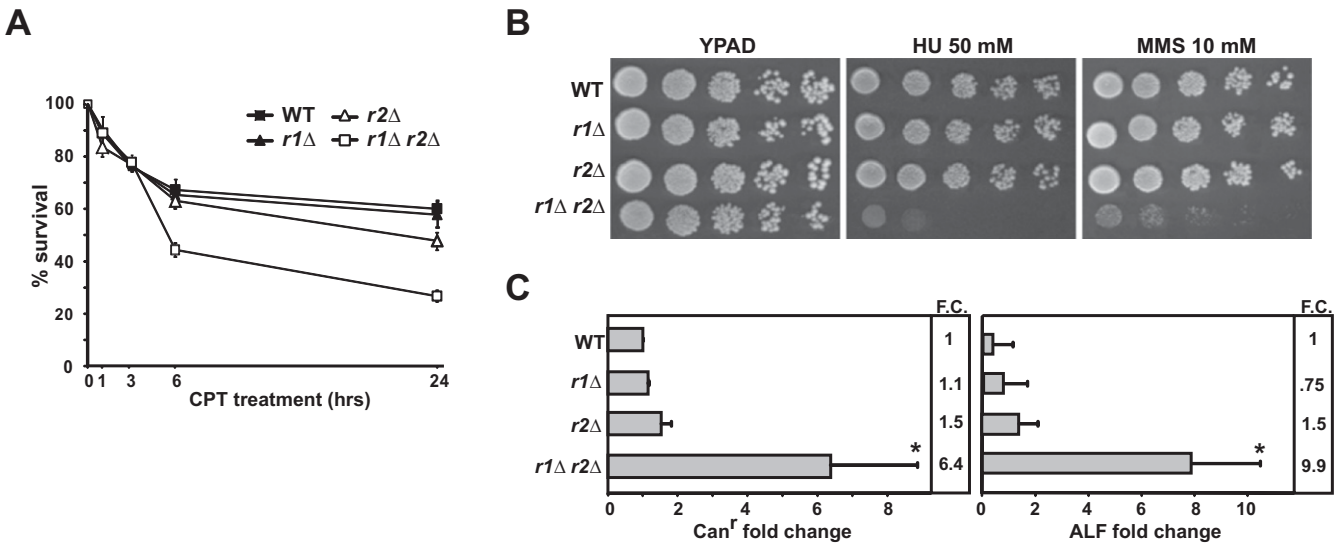

Fig. S1. The lack of RNaseH activities renders yeast sensitive to replication stress and increased genome instability. (*A*) Cell survival after prolonged incubation with CPT. Data are shown as the mean ± SD. (*B*) Analysis of sensitivity to genotoxic agents. Tenfold serial dilutions of cells grown for 3 d on YPAD or YPAD-containing HU (50 mM) or MMS (10 mM). (*C*) Mutation rates as determined by canavanine resistance (Can^R, *Left*). Rate of *MAT*α conversion to a-mating type (ALF; *Right*); 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 $*P < 0.05$.

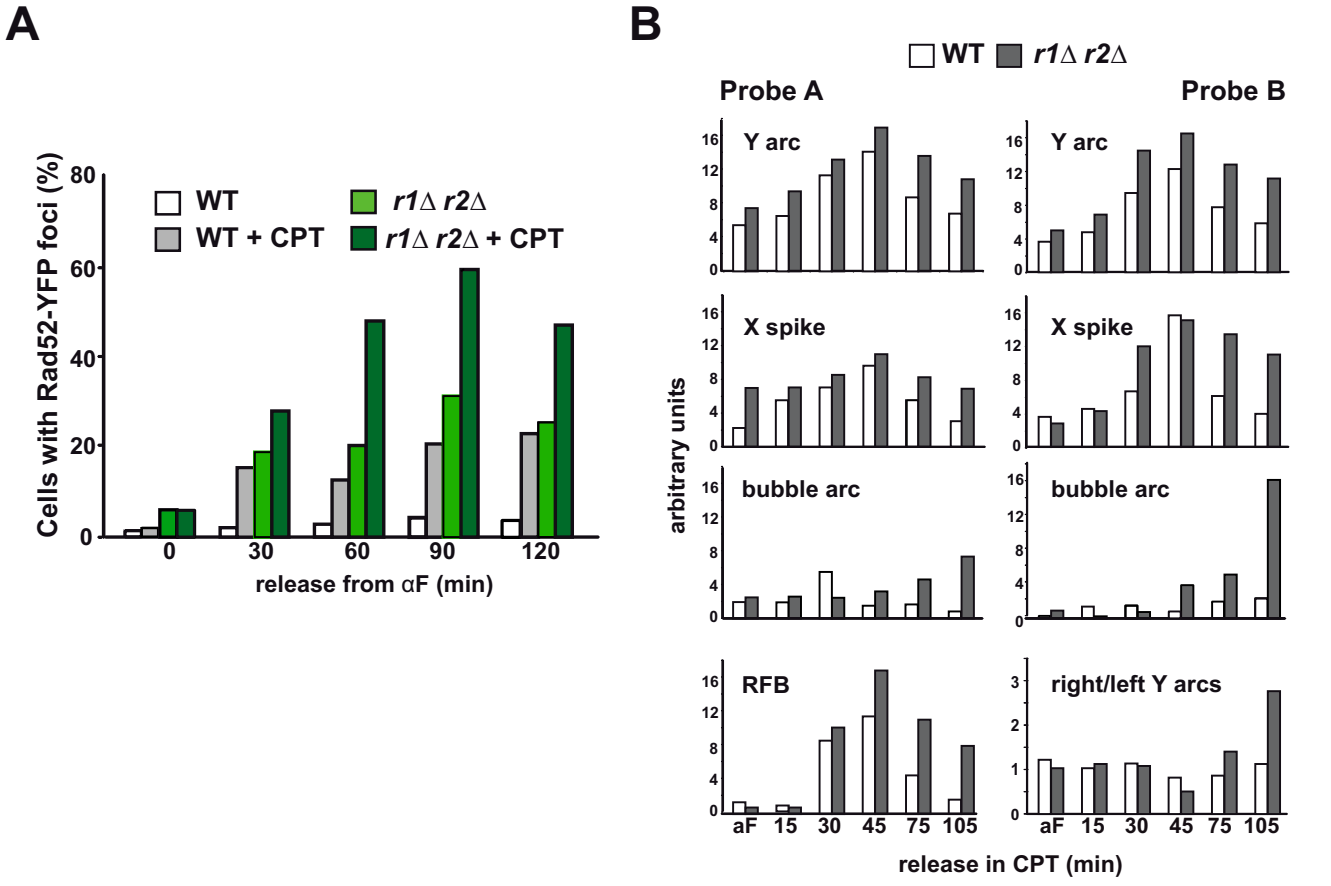

Fig. S2. The lack of RNase H activity and CPT treatment increase Rad52-YFP-monitored DNA damage during S-phase. (*A*) Time-course analysis of Rad52-YFP foci appearance in WT and *r1*Δ *r2*Δ cells following release from α-factor in the presence or absence of CPT. Data represent mean from two independent 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. 2*A*. Signal intensities relate to corresponding "1×" spot in each gel.

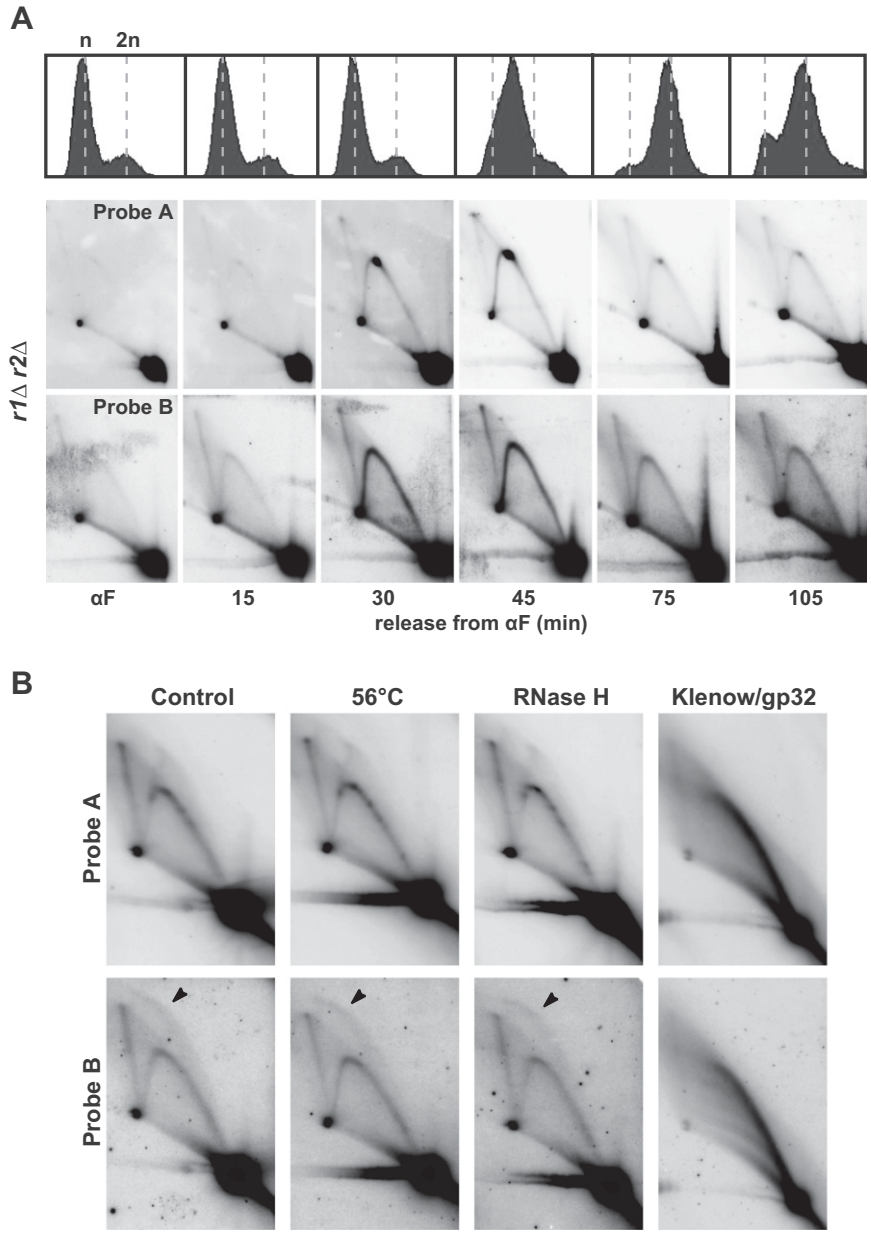

Fig. S3. Characterization of unscheduled replication intermediates in the rDNA. (*A*) CPT treatment and the lack of RNase H activities contribute to unscheduled replication events. FACS analysis (*Upper*) and 2D-gel electrophoresis of RIs derived from *r1*Δ *r2*Δ mutant cells released from G1 phase α-factor 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×.

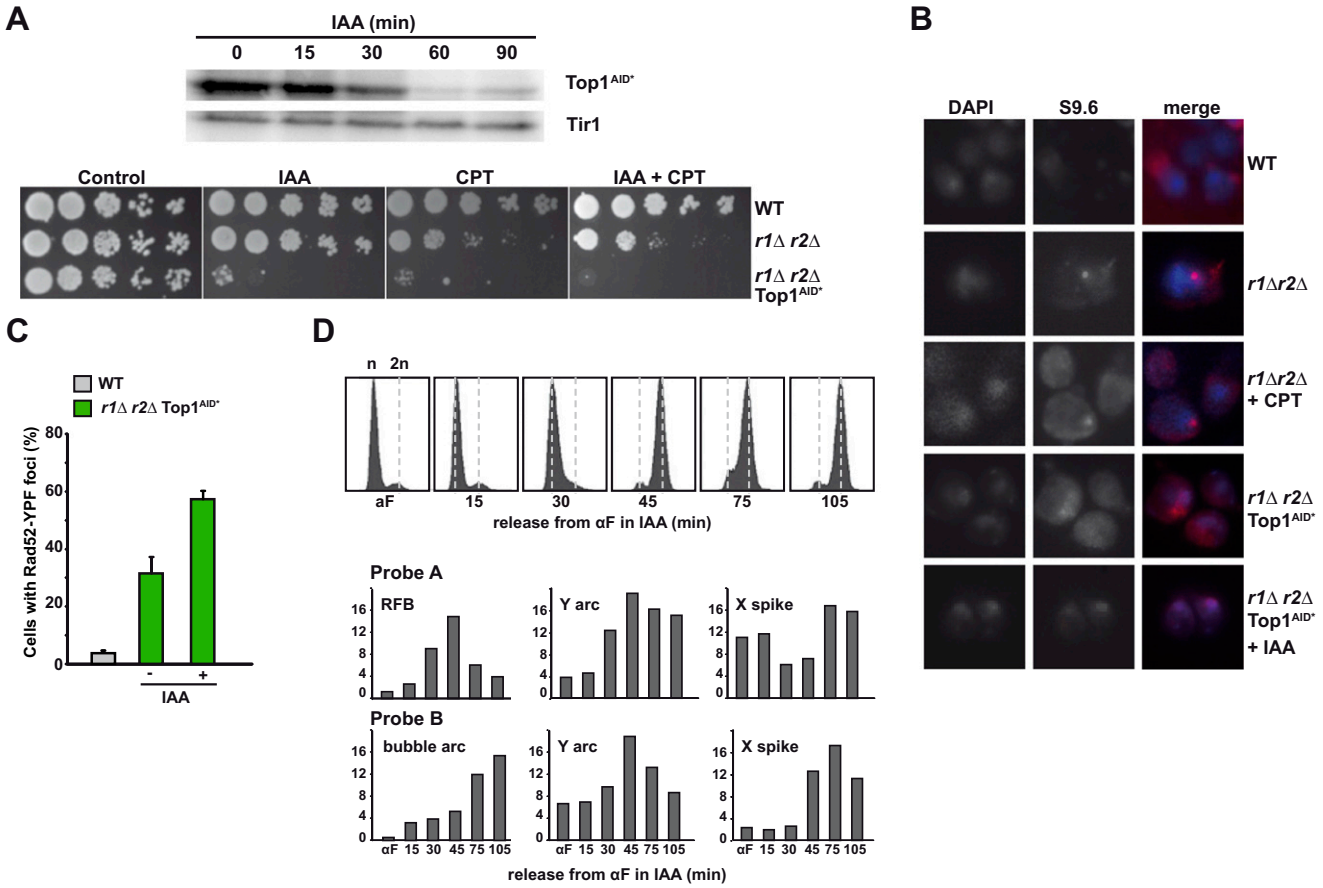

Fig. S4. The stimulation of R-loop formation in the absence of RNase H or Top1 activities contributes to DNA damage and unscheduled replication events. (*A*) Kinetics of Top1^{AID*} degradation by Western blot analysis, and drop test analysis of cell viability upon Top1^{AID*} degradation in *r1*Δ *r2*Δ 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, *r1*Δ *r2*Δ and *r1*Δ *r2*Δ *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 *r1*Δ *r2*Δ *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. 3*B*. For details see Fig. 2*C*.

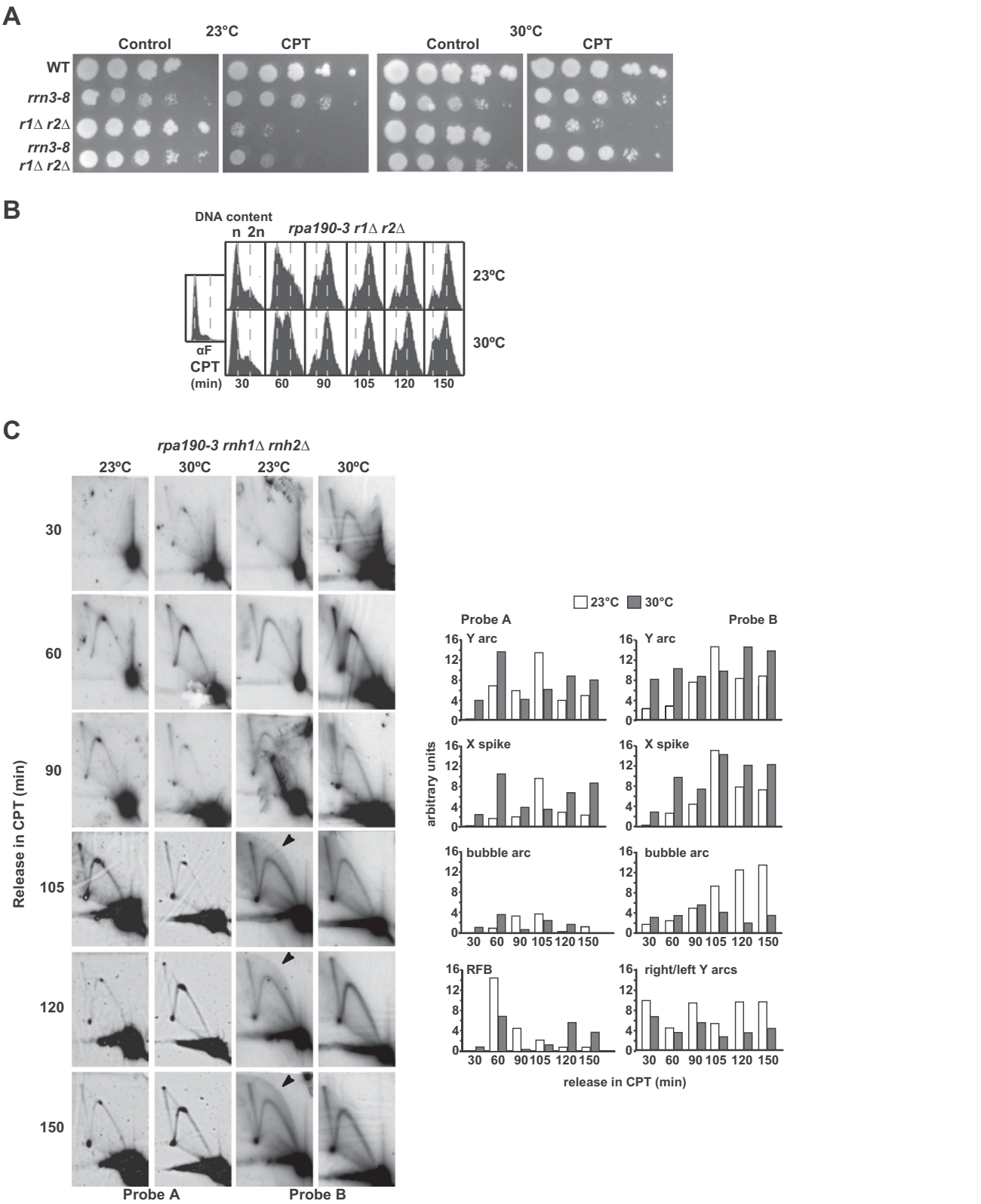

Fig. S5. CPT sensitivity and unscheduled replication depend on RNAPI transcription. (*A*) Tenfold serial dilutions of the temperature-sensitive, conditional *rrn3-8* (Rrn3 is an essential RNAPI transcription factor) mutants grown on YPAD (control) or YPAD-containing 5 μg/mL CPT for 3 d at permissive (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. 2*B*. Note that the 105-min timepoint 2D gels correspond to the ones shown in Fig. 5*C*. 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×.

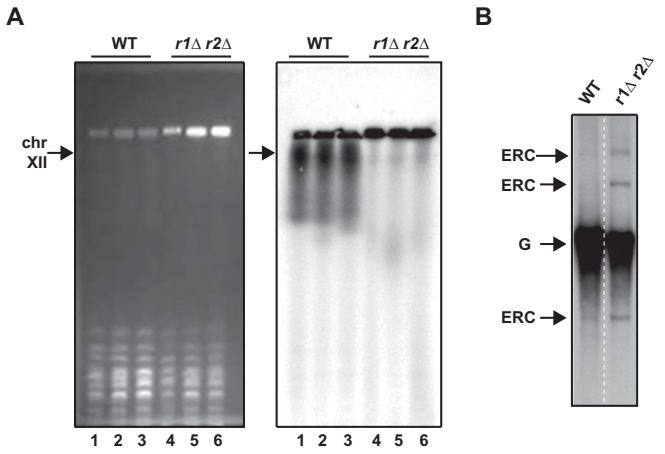

Fig. S6. Chromosome XII migration and ERC formation is affected in *r1Δ r2Δ* mutants. (*A*) PFGE analysis of chromosomes derived from independent colonies of WT (lanes 1–3) and *r1Δ r2Δ* cells (lanes 4–6). The gel was stained with EtBr (*Left*) and subjected to Southern blot analysis using probe A (*Right*). 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

Strain or plasmid	Genotype or feature	Source
Strain		
YKL83	MATa *ubr1Δ::GAL-UBR1::HIS3 ade2-1 trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100*	(1)
RS310	YKL83; *rnh1Δ::hphMX4*	Present study
RW027.17	YKL83; *rnh201Δ::natMX4*	Present study
RW025.C	YKL83; *rnh1Δ:: hphMX4 rnh201Δ:: natMX4*	Present study
RS072	MATα YKL83	Present study
RS075	MATα YKL83; *rnh1Δ:: hphMX4 rnh201Δ:: natMX4*	Present study
RS182	YKL83; *fob1Δ::kanMX6 rrm3Δ:: kanMX6*	Present study
RS364	*TOP1$^{AID*\text{-}9myc}$::KanMX6 rnh1Δ::hphMX4 rnh201Δ::natMX4 bar1Δ URA3:: ADH1-AtTIR1^{9myc}*	Present study
RS284	YKL83; *rpa190-3*	Present study
RS230	YKL83; *rpa190-3 rnh1Δ:: hphMX4 rnh201Δ:: natMX4*	Present study
RS266	YKL83; *rrn3-8*	Present study
RS267	YKL83; *rrn3-8 rnh1Δ:: hphMX4 rnh201Δ:: natMX4*	Present study
Plasmid		
p316-R52-YFP	CEN, *URA3, RAD52-YFP*	F. Prado, Seville, Spain
3317	CEN, *LEU2, RPL25-GFP, mRFP-Nop1*	(2)
pFA6a-kanMX6		Euroscarf
pAG32-hphMX4		Euroscarf
pAG25-natMX4		Euroscarf

1. Labib K, Tercero JA, Diffley JF (2000) Uninterrupted MCM2-7 function required for DNA replication fork progression. *Science* 288(5471):1643–1647.
2. Ulbrich C, et al. (2009) Mechanochemical removal of ribosome biogenesis factors from nascent 60S ribosomal subunits. *Cell* 138(5):911–922.