Physical proximity of chromatin to nuclear pores prevents harmful R loop accumulation contributing to maintain genome stability

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During transcription, the mRNA may hybridize with DNA, forming an R loop, which can be physiological or pathological, constituting in this case a source of genomic instability. To understand the mechanism by which eukaryotic cells prevent harmful R loops, we used human activation-induced cytidine deaminase (AID) to identify genes preventing R loops. A screening of 400 *Saccharomyces cerevisiae* selected strains deleted in nuclear genes revealed that cells lacking the Mlp1/2 nuclear basket proteins show AID-dependent genomic instability and replication defects that were suppressed by RNase H1 overexpression. Importantly, DNA–RNA hybrids accumulated at transcribed genes in *mlp1/2* mutants, indicating that Mlp1/2 prevents R loops. Consistent with the Mlp1/2 role in gene gating to nuclear pores, artificial tethering to the nuclear periphery of a transcribed locus suppressed R loops in *mlp1*Δ cells. The same occurred in THO-deficient *hpr1*Δ cells. We conclude that proximity of transcribed chromatin to the nuclear pore helps restrain pathological R loops.

nuclear pores | transcription | R loop | genome instability | Mpl1/2

During transcription elongation, the nascent mRNA may hybridize back with the DNA template forming a DNA–RNA hybrid and a displaced ssDNA, a structure called R loop. Although R loops can occur naturally as a physiologically relevant intermediate in specific processes, pathological R loops was revealed as a major determinant of genome instability (1, 2). Different protein complexes that act all along the path from the transcription site to the nuclear pore complex (NPC) enable the coordination of messenger ribonucleoprotein particle (mRNP) biogenesis and export. THO is an evolutionary conserved complex composed of the Tho2, Hpr1, Mft1, and Thp2 proteins in yeast (3), which associates with mRNA export factors such as Sub2/UAP56 (4). Mutations in THO components not only impair transcription but also lead to mRNA export defects and transcription-dependent genomic instability in yeast and metazoans (3, 5–7) that is a consequence of the accumulation of cotranscriptional R loops (8). Such unscheduled R loops interfere with DNA replication (9, 10), ultimately leading to genomic instability. Together with THO, a growing number of factors working at different stages of mRNP biogenesis and export have been shown to prevent R loops and contribute to maintaining genome stability (11–14), establishing mRNP biogenesis as one of the cellular mechanism that controls R loop formation in eukaryotes. Other mechanisms include removal of negative supercoiling by topoisomerase I; degradation of the RNA moiety by the catalytic activity of RNase H1; unwinding of the DNA–RNA hybrids by helicases such as Sen1/Senataxin, DDX19, DDX23, and Pif1; or nucleosomes as a barrier to R loop formation (15–23). In addition, a number of replisome-associated proteins contribute to the handling of R loops, such as Fanconi anemia/BRCA factors or ssDNA binding protein RPA (24–28).

With the aim at understanding the mechanisms that control R loop formation, we used the activation-induced cytidine deaminase (AID) that targets the ssDNA formed at R loops (29) to screen a selection of 400 viable yeast strains deleted of genes with nuclear functions for AID-dependent hyperrecombination. This allowed us to identify *MLP1* as a gene involved in preventing R loop accumulation. The yeast myosin-like protein 1 and 2 (Mlp1 and Mlp2) are structural components of the nuclear pore basket that form fibers anchored at the NPC (30). Mlp1 physically interacts with the mRNP component Nab2 (31), and Mlp1/2 have been proposed to be a docking site for mRNPs during export and to participate in mRNP quality control (32, 33). Gene gating, the transient localization of transcribed DNA in the proximity of the NPC has been proposed to facilitate the formation of an export-competent mRNP (34). Consistently, Mlp1/2 proteins preferentially associate with highly transcribed genes in an RNA-dependent manner (35), and Mlp1 is also required for the docking of actively transcribed DNA to the NPC (36). However, gene gating may also increase the torsional stress, enhancing the transcriptional barrier to replication, an effect counteracted by intra-S checkpoint activation by a mechanism involving phosphorylation of Mlp1 (37). Our study shows that loss of Mlp1 and/or Mlp2 leads to R loop accumulation, genome instability, and replication impairment, phenotypes that can be reverted by RNase H1 overexpression, pointing toward R loops as the causative event. Importantly, R loops are suppressed in *mlp1*Δ and *hpr1*Δ cells by artificially tethering a locus of interest to the NPC, indicating that physical proximity to the NPC is sufficient to prevent R loop accumulation. We conclude that gene gating prevents R loop formation.

Results

Screening of Yeast Null Mutants for AID-Mediated Hyperrecombination. We previously showed that expression of AID, a B-cell enzyme essential for somatic hypermutation and class switch recombination,

Significance

During transcription, the mRNA may hybridize back with its template DNA, forming a structure called R loop. These structures have been associated with genome instability and human disease. Using budding yeast as a model organism to screen for new genes preventing R loops, we identified *MLP1* and subsequently showed that the nuclear basket protein Mlp1/2 has a role in preventing R loop formation and genome instability in yeast. Our work indicates that R loops are formed in the nucleoplasm and that proximity of transcribed chromatin to the nuclear pore constrains R loop formation. Our study opens additional perspectives to understand the role of RNA in the control of genome integrity as a function of nuclear location.

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in yeast THO mutants exacerbate their transcription-dependent hyperrecombination phenotype (29). Here, we used AID overexpression as a tool to uncover mutations that lead to R loop accumulation in *Saccharomyces cerevisiae*. Using gene ontology (GO) term annotations, we selected 400 viable deletion strains lacking genes with nuclear functions from the yeast knockout collection (Table S1). The 400 strains were transformed with plasmid p313LZGAID, carrying a direct-repeat recombination construct with the *lacZ* gene between two *leu2* repeats and expressing the human *AID* gene under the control of the *GAL1* promoter. The transformed cells, including wild-type, *hpr1*Δ and *mft1*Δ, were grown in galactose to induce AID expression and plated on appropriate media to assess the amount of recombinants (Fig. S1*A*). The 123 candidates with high levels of recombination in AID-expression conditions in at least two of three independent experiments were selected for further analysis. Next, four fresh transformants were grown either in glucose (−AID) or galactose (+AID), and serial dilutions were plated on selective media for each candidate (Fig. S1*B*). Twenty-two strains showing high recombination by visual inspection only under AID expression were selected for further analysis, whereas those showing high recombination without AID were discarded. After performing fluctuation tests with the 22 selected strains (Fig. S1*C*), we selected *mpl1*Δ as the only mutation conferring significant hyperrecombination in the presence of AID.

Lack of Mlp1/2 Causes R Loop-Dependent Genome Instability and R Loop Accumulation. Because *MLP1* and its paralog *MLP2* encode proteins of the nuclear basket with distinct but overlapping functions, we pursued our analysis in *mlp1*Δ, *mlp2*Δ, and *mlp1*Δ *mlp2*Δ double mutants in the W303 strain background. First, we showed that AID expression leads to hyperrecombination in *mlp1*Δ, *mlp2*Δ, and *mlp1*Δ *mlp2*Δ using the *L-lacZ* system under the control of the *LEU2* promoter (Fig. 1*A* and Table S2). In the double mutant, the basal recombination frequency is slightly increased compared with the wild type (4.6-fold), whereas the levels of AID-induced recombination are similar to those of either single mutant. Importantly, the AID-dependent increase in recombination was suppressed by RNase H1 overexpression, suggesting that the action of AID is enabled by the presence of R loops in the three strains. Analysis of the transcription dependency of AID-mediated recombination in the *L-lacZ* system under control of the *GAL1* promoter under conditions of low (glucose) or high (galactose) transcription (Fig. 1*B*, Fig. S2, and Table S2) revealed that hyperrecombination in *mlp1/2* mutants depended on both AID expression and transcription, consistent with its dependency on R loops. Recombination was also analyzed in a direct-repeat chromosomal system based on two mutated copies of the *HIS3* gene on chromosome XV (38). AID expression substantially increased recombination in an RNase H1-sensitive manner in the three mutants (Fig. 1*C* and Table S2), consistent with the plasmid-born system results. In this case, recombination frequencies were also slightly increased in the absence of AID expression compared with the wild type, indicating that mutation of Mlp1/2 leads to a weak increase in recombination.

Next, Rad52-foci were monitored as a mark of double-strand breaks (DSBs) by fluorescence microscopy in cells expressing a Rad52–YFP fusion protein. AID expression led to significantly higher percentages of S/G2 cells with Rad52-foci in *mlp1*Δ, *mlp2*Δ, and *mlp1*Δ *mlp2*Δ mutants compared with the wild type (Fig. 1*D* and Table S2). This AID-dependent increase in Rad52 foci was also suppressed by RNase H1 overexpression. The percentage of S/G2 cells with Rad52 foci showed a slight but significant increase in the three mutants strains also in the absence of AID expression, consistent with the idea that *mlp1/2* mutants increase genomic instability, in agreement with the implication of the Mlp1/2-bound nucleoporin Nup60 in DSB appearance (39), and as further supported by the analysis of plasmid loss, which increases over twofold in *mlp1*Δ, *mlp2*Δ, and *mlp1*Δ *mlp2*Δ compared with the wild type (Fig. 1*E* and Table S2).

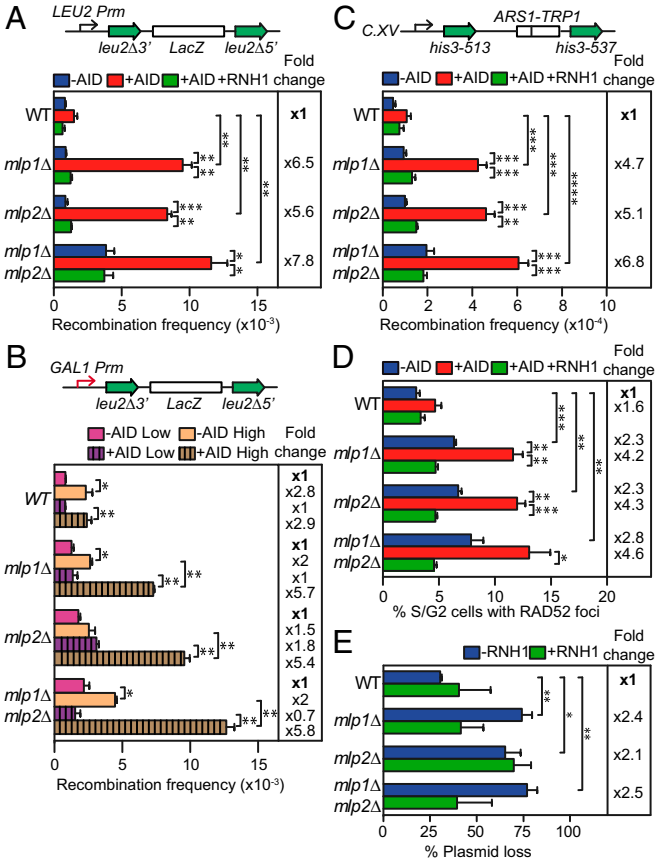

Fig. 1. Lack of Mlp1/2 leads to AID-dependent genome instability that is suppressed by RNase H1 overexpression. (*A*) Recombination analysis using the *L-lacZ* direct-repeat plasmid-borne system under the control of the *LEU2* promoter in wild-type, *mlp1*Δ, *mlp2*Δ, and *mlp1*Δ *mlp2*Δ cells that do not express AID (−AID), express AID (+AID), or express both AID and RNase H1 (+AID +RNH1). A scheme of the system is shown (*Upper*). Average and SEM of independent fluctuation tests are shown ($n \geq 3$). Fold increases vs. the wild type with AID are shown on the left. Statistical analyses using a two-tailed unpaired Student *t* test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. All values are provided in Table S2. (*B*) Recombination analysis using the *L-lacZ* system under the control of the *GAL1* promoter in cells that do not express AID (−AID) or express AID (+AID) in conditions of low or high transcription. Fold increases with respect to levels under low transcription without AID are shown on the left. Expression levels of the recombination system under high transcription in the different strains were not significantly different (Fig. S2). Details as in *A*. (*C*) Recombination analysis using the chromosomal *his3*-based direct-repeat recombination system. $****P < 0.0001$. Details as in *A*. (*D*) Percentage of S/G2 cells containing Rad52-YFP foci. Fold increases with respect to the wild type without AID expression are shown on the left. Average and SEM of independent experiments are shown ($n \geq 3$). Details as in *A*. (*E*) Plasmid loss in cells that do not express RNase H1 (−RNH1) or do express it (+RNH1). Average and SEM of independent experiments are plotted ($n \geq 3$). Details as in *A*.

To assess whether Mlp1/2 inactivation increases R loops, DNA–RNA hybrid immunoprecipitation (DRIP) with the S9.6 antibody were done in *mlp1*Δ, *mlp2*Δ, and *mlp1*Δ *mlp2*Δ strains at genes previously shown to form DNA–RNA hybrids or to generate transcription-dependent replication defects in R loop-accumulating THO mutants (10, 40, 41). The results show that R loops accumulate at the tested genes (*GCN4*, *PDC1*, *SPF1*, and *PDR5*) in the three strains (Fig. 2*B*). RNase H treatment decreased the R loop levels and a very low S9.6 signal was obtained at *GAL1* when not transcribed, confirming that the signal was DNA–RNA hybrid specific. In addition, DRIP

analysis at the transcribed *TRP1* gene located in the intervening sequence of the *his3* direct-repeat recombination system (Fig. 1*C*) showed also R loop accumulation in three mutants (Fig. 2*C*), consistent with the results of the recombination assays. Altogether, these results indicate that the lack of Mlp1/2 increases cotranscriptional R loops that may potentially cause genome instability. Because similar results were obtained in single and double mutants, we hereafter focus on *mlp1*Δ strains.

R Loop Accumulation Interferes with Replication in *mlp1Δ* Cells. Interference with the replication process has emerged as one of the major causes of R loop-mediated genome instability (42, 43). Consequently, we assayed replication fork (RF) progression in *mlp1*Δ cells by different means. First, we analyzed replication by FACS and pulse-field gel electrophoresis (PFGE) in *mlp1*Δ cells (Fig. S3). No significant differences could be appreciated between the *mlp1*Δ and wild-type strains, independently of AID expression or the presence of hydroxyurea (HU), which slows down replication. Because this method might not be sufficiently sensitive, we analyzed replication by monitoring BrdU incorporation in cells upon release from α-factor–mediated G1 arrest in the vicinity of the early firing origins *ARS508* and *ARS1211*. These origins were chosen because they are situated in close vicinity to the *SPF1* and *PDC1* genes, respectively, in which R loops accumulate in *mlp1/2* mutants (Fig. 2*B*) and in head-on orientation in respect to replication. DNA was immunoprecipitated with anti-BrdU antibody and subjected to real-time qPCR at the 5′ and 3′ end of the transcription units and the ARS sequences. *mlp1*Δ cells showed mild defects in replication compared with wild-type cells (Fig. 3*A* and Fig. S4), which are also seen in the absence of HU (Fig. S5). Importantly, the difference in BrdU incorporation observed between wild-type and *mlp1*Δ cells was completely lost in cells overexpressing RNase H1 at all analyzed regions, indicating that R loop accumulation is likely responsible for the replication delay (Fig. 3*A* and Fig. S4). To appraise whether the replication defects might be due to the head-on orientation of replication–transcription of the systems tested, we analyzed BrdU incorporation at *ARS1021*, an early firing ARS located next to the codirectionally orientated gene *ECM17* (Fig. 3*B* and Fig. S4). RNase H1-sensitive replication defects were observed at this locus as well, supporting the conclusion that R loop accumulation leads to general replication defects in *mlp1*Δ cells.

Gene Tethering to the NPC Prevents R Loop Accumulation. Recent work has shown that Mlp1 is a target for intra-S checkpoint kinase Rad53, and that Mlp1 phosphorylation results in the release of DNA gated at the NPC (37). So, we took advantage of reported mutants carrying a phosphomimetic or a nonphosphorylatable *mlp1* allele (*mlp1-S1710D* and *mlp1-S1710A*, respectively) (37) and analyzed AID-mediated recombination and Rad52-foci (Fig. 4 *A* and *B* and Table S3). The results are consistent with our hypothesis that gene gating may prevent the formation of R loops and their associated genomic instability because the *mlp1-S1710D* mutant that mimics constitutive checkpoint activation—that in turn supposes release of the damaged DNA from the NPC—behaved similarly to the gene gating-deficient *mlp1*Δ strain in both assays. On the contrary, the *mlp1-S1710A* mutant that does not allow checkpoint-dependent release from the NPC did not appear to show R loop-mediated increase in recombination or Rad52 foci accumulation. Consistently, plasmid loss was also exacerbated in the *D* allele compared with the *A* allele (Fig. 4*C* and Table S3). Direct assessment by DRIP with the S9.6 antibody confirmed R loop accumulation in the *D* mutant (Fig. 4*D*). In the *A* mutant, the S9.6 signal was also increased, but it was RNase H-resistant, indicating that it does not correspond to R loops.

Next, we asked whether R loops in *mlp1*Δ cells could be suppressed by artificially tethering the DNA to the NPC. For this we used the reported artificial anchoring system of the *GAL1* locus to the NPC (44). Artificial tethering is achieved through the insertion of LexA binding sites downstream of *GAL1* and expression of a LexA–Nup60 fusion protein containing a nuclear localization signal (NLS). An NLS-containing LexA is used as a control with no NPC anchoring. Samples were collected at different time points upon transcriptional activation of the locus in galactose medium. Transcription was followed by northern of *GAL1* and ChIP of RNA polymerase II (RNAP II) occupancy, and R loops where detected by DRIP (Fig. 5 *B–E*). In the control experiment in which the *GAL1* locus was not tethered to the NPC, transcriptional activation occurred less efficiently in *mlp1*Δ than in the wild type, and R loops were detected from the first time-point after transcriptional activation, in agreement with the idea that R loops are concomitant with transcription. When the LexA–Nup60 fusion protein was expressed in *mlp1*Δ cells, transcription activation was improved and R loops did not accumulate. These results indicate that artificial tethering of the *GAL1* gene abolishes both the transcriptional defects and the accumulation of R loops at this locus in *mlp1*Δ cells. This role of gene gating in preventing R loops is not specific to cells lacking the nuclear basket Mlp1 protein, because analysis of R loops with and without NPC tethering in *mlp1*Δ and THO-deficient *hpr1*Δ cells revealed that artificial anchoring of the *GAL1* locus to the NPC did suppress R loop formation in both *mlp1*Δ and *hpr1*Δ cells (Fig. 5*F*). LexA–Nup60 expression per se did not have a global effect on R loop formation, as shown by analysis of the unrelated and R loop-rich rDNA locus that showed equal R loop levels in wild-type, *mlp1*Δ, and *hpr1*Δ cells expressing LexA and LexA–Nup60 at the rDNA (Fig. S6). These results demonstrate that gene gating plays a determinant role in the prevention of R loop accumulation in yeast.

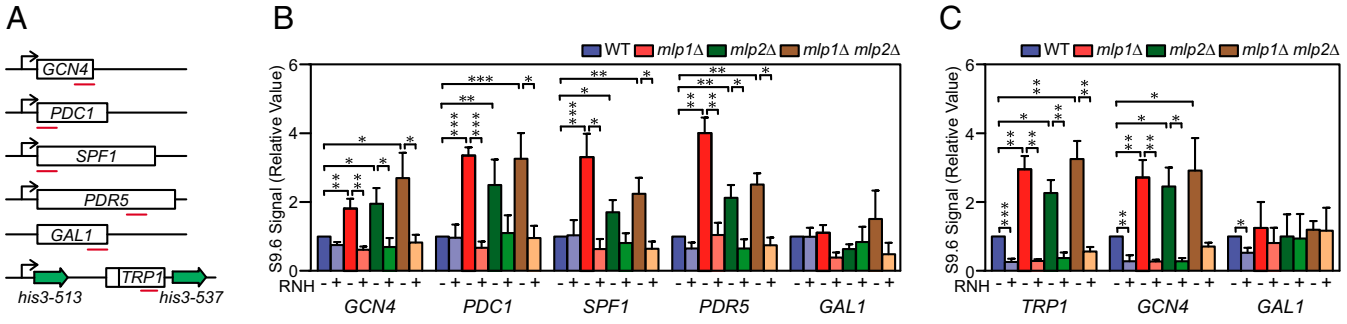

Fig. 2. R loops accumulate at transcribed genes in *mlp1/2* mutants. (*A*) Schematic view of the analyzed genes and amplicons. (*B*) DRIP using the S9.6 antibody in wild-type, *mlp1*Δ, *mlp2*Δ, and *mlp1*Δ *mlp2*Δ asynchronously growing cells. Where indicated, samples were treated with RNase H (RNH). S9.6 signal were normalized to the wild-type value in each experiment. Average and SEM of independent experiments are shown ($n \geq 3$). Statistical analyses as in Fig. 1*A*. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (*C*) DRIP in the *his3*-based direct-repeat recombination system. The S9.6 signal at the *GNC4* and *GAL1* genes were used as controls. Details as in *B*.

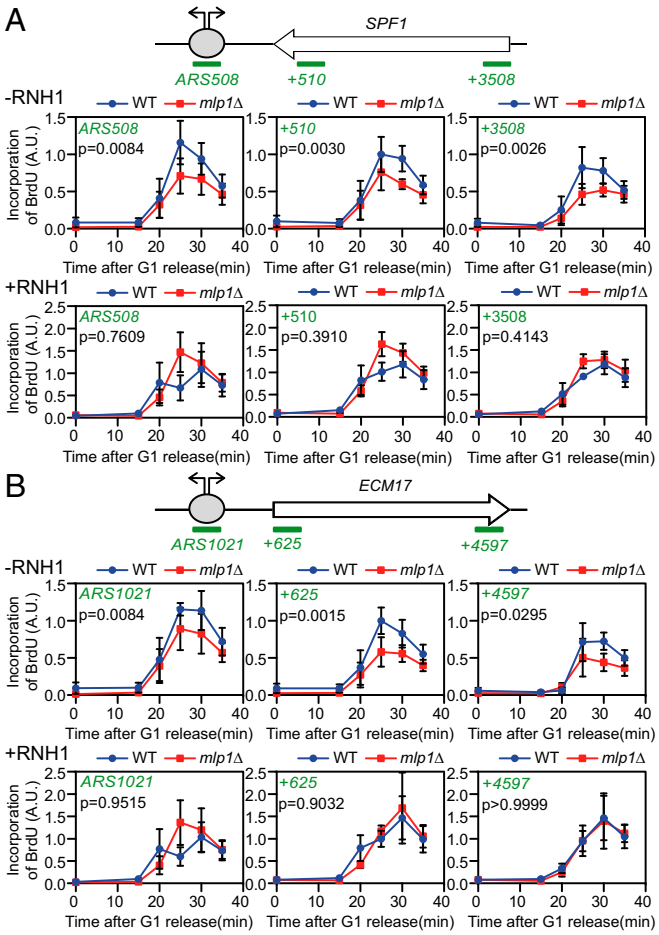

Fig. 3. Replication progression is impaired in *mlp1*Δ cells. (*A*) BrdU incorporation upon release of G1-arrested cells was analyzed at early replication origin *ARS508* by immunoprecipitation and real-time-qPCR in wild-type and *mlp1*Δ cells. A schematic drawing of the genomic region and amplicons are depicted (*Top*). Experiments were done in cells treated with 20 mM hydroxyurea (HU) not overexpressing RNase H1 (−RNH1) or overexpressing RNase H1 (+RNH1). Quantification of BrdU incorporation relative to a late replication locus is plotted for each region. Average and SEM of independent experiments are shown ($n = 3$). The *P* values calculated by the Wilcoxon signed-rank test are shown for each condition. Plotted values are normalized to the average signal obtained for the wild type at the 25-min time-point in the +510 region for each experiment. The graphs obtained without normalization to the wild-type value are shown in Fig. S4. (*B*) BrdU incorporation upon release of G1-arrested cells at *ARS1021*. Signal in the +625 region was used for normalization. Details as in *A*.

Discussion

R loops cause genome instability and have been linked with a number of neurodegenerative diseases and with tumorigenesis (42, 45, 46). Using AID expression as a tool, we identified *MLP1* in a screen for genes involved in preventing R loop accumulation in budding yeast. Our analyses of *mlp1*, *mlp2*, and *mlp1 mlp2* mutants revealed that R loops accumulate at transcribed genes in either of these strains, leading to increased genome instability as seen by AID-dependent hyperrecombination, increased Rad52 foci, and plasmid loss. In agreement with the idea that interference with the replication process is one of the major causes of R loop-mediated genome stability (42, 43), deletion of *MLP1* causes mild replication defects that are suppressed by RNase H1 overexpression. Such replication defects were observed without AID expression, consistent with the moderate increase in recombination, Rad52 foci, and plasmid loss observed in untreated *mlp1/2* mutants, suggesting that R loops accumulated in the absence of the Mlp1/2 moderately challenge genome stability.

In yeast, Mlp1 associates with highly transcribed genes and is required for gene gating at the NPC (35, 36). Our results demonstrate that tethering of the *GAL* locus to the NPC is sufficient to suppress R loops at this locus in *mlp1* cells, as well as in the *hpr1* THO mutant, suggesting that gene gating does generally prevent R loop formation. Formation of export-competent mRNPs is important in preventing R loops and their accompanying genome instability (2), whereas gene gating facilitates mRNA export (34). Covering of the nascent RNA with mRNP biogenesis factors and physical transport through nuclear pores would prevent the nascent RNA from forming a DNA–RNA hybrid. In support of this view, overexpression of RNA binding factors such as Tho1, Nab2, or Sub2/UAP56 suppresses the transcription-dependent hyperrecombination of THO mutants (5, 47, 48). Noteworthy, Nab2 physically interacts with Mlp1 and is believed to be required for proper docking of the mRNP to the Mlp1/2 platform (31, 32). Consistently, *mlp1/2* or *nab2* mutations lead to frequent release of the mRNP into the nucleoplasm (49). Because the THSC/TREX-2 mRNP biogenesis complex is required for gene gating (50), it would be interesting to investigate whether Nab2 overexpression restores gene gating in THSC/TREX-2 mutants.

Our results show that *mlp1* and *hpr1* cells share a similar behavior with respect to their R loops being suppressed by artificial tethering of a chromosomal locus, whereas their impact on genome stability is far apart, *hpr1* being extremely hyperrecombinant in a transcription-dependent manner (51) and *mlp1* showing moderately increased transcription-dependent recombination only after

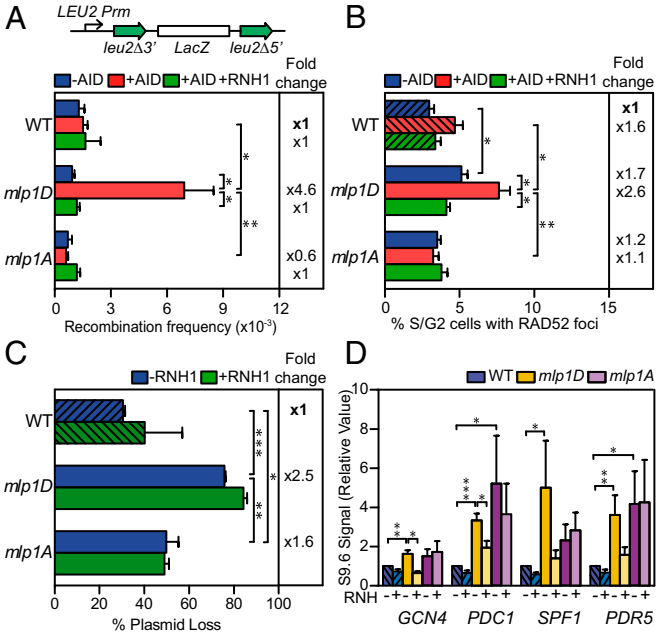

Fig. 4. R loop-dependent genome instability in *mlp1* mutants mimicking constitutive intra-S checkpoint activation or blind to checkpoint activation. (*A*) Recombination analysis using the *L-lacZ* system under the control of the *LEU2* promoter in *mlp1S1710D* (*mlp1D*) and *mlp1S1710A* (*mlp1A*) cells that do not express AID (−AID), express AID (+AID), or express both AID and RNase H1 (+AID +RNH1). *$P < 0.05$; **$P < 0.01$. Details as in Fig. 1. (*B*) Percentage of S/G2 cells containing Rad52-YFP foci. Details as in *A*. (*C*) Plasmid loss in cells that do not express RNase H1 (−RNH1) or do express RNase H1 (+RNH1). ***$P < 0.001$. Details as in *A*. (*D*) DRIP using the S9.6 antibody. Where indicated, samples were pretreated with RNase H (RNH). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Details as in Fig. 2. Experiments of B–*D* were performed concomitantly with those shown in Figs. 1 and 2, so values for wild-type cells are the same.

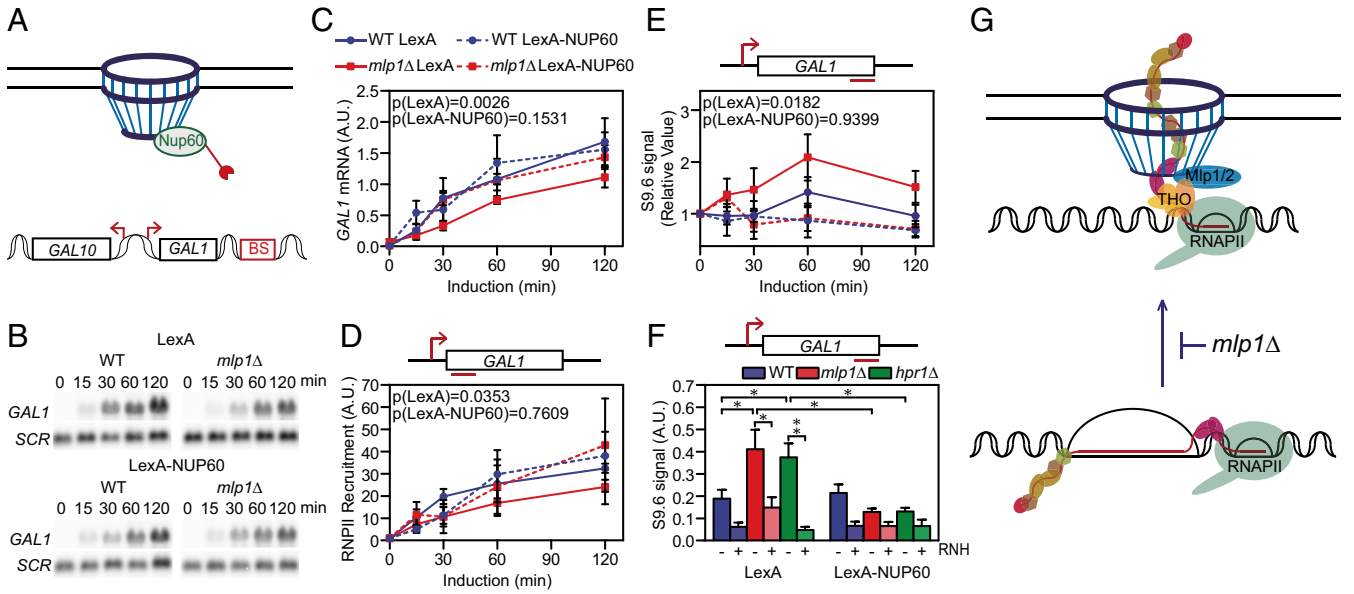

Fig. 5. Artificial tethering to the nuclear pore is sufficient to suppress cotranscriptional R loops in *mlp1*Δ and *hpr1*Δ cells. (*A*) Scheme of the artificial tethering system. Binding of a LexA–Nup60 fusion protein to LexA binding sites (BS) inserted downstream of the *GAL1* gene anchors the *GAL1* to the nuclear pore. (*B*) Time-course analysis of *GAL1* mRNA upon transcription induction in wild-type and *mlp1*Δ cells expressing either LexA or LexA–Nup60. Representative results are shown. (*C*) Quantification of *GAL1* mRNA induction as shown in *B*. *GAL1* signal was normalized to the *SCR1* levels of each sample. Average and SEM of independent experiments are plotted ($n \geq 3$). The *P* values were calculated by the Wilcoxon signed-rank test. (*D*) Time-course ChIP of the RNAP II large subunit Rpb1. In each experiment, ChIP values were normalized to the 0 time-point of the wild type. Location of the amplicons relative to *GAL1* gene is indicated (*Upper*). Details as in *C*. (*E*) Time-course DRIP analysis with the S9.6 antibody. DRIP values were normalized to the 0-min time-point in each strain. Details as in *D*. (*F*) DRIP using the S9.6 antibody in wild-type, *mlp1*Δ, and *hpr1*Δ cells grown to midlog phase in galactose medium and expressing either LexA or LexA–Nup60. $*P < 0.05$; $**P < 0.01$. Details as in Fig. 2. DRIP data of the rDNA locus is shown in Fig. S6. (*G*) Model for R loop formation in the nucleoplasm. Gating of a transcribed locus to the NPC prevents formation of R loops. This likely occurs thanks to the prompt export of the nascent mRNP that minimizes the probability of back-hybridization to the template DNA. In gene gating defective mutants such as *mlp1*Δ, transcribed genes remain in the nucleoplasm and R loops accumulate, leading to moderate genome stability.

AID expression (Fig. 1). These differences might rely on the fact that, unlike Mlp1, THO is recruited to active genes by the elongating RNAP II and participate in transcription (10, 52, 53). In addition, altered chromatin structure has been associated with R loops in THO mutants both in *Caenorhabditis elegans* and yeast (54), which could represent a serious threat to RF progression. It is thus possible that THO mutants accumulate genome-threatening situations, which may interfere with the cellular processes that normally deal with pathological R loops or stalled forks, whereas these situations are not produced in *mlp1* cells. However, according to the recent evidence that a second step of R loop stabilization is required for R loop-mediated genome instability, as would occur in *hpr1* mutants but not in specific histone H3 and H4 mutants (23), it is also possible that R loops in *mlp1/2* mutants might not be efficiently stabilized.

Altogether, our data supports a model in which physical proximity to the NPC would protect transcribed genes from R loop accumulation (Fig. 5*G*). In NPC-gated genes, the nascent mRNP would readily get exported out of the nucleus, reducing the probability of back-hybridization with the DNA. In addition, a more efficient mRNP assembly or the NPC itself may contribute to prevent R loop formation at gated genes. Consequently, R loop accumulation would occur in DNA transcribed in the nucleoplasm, as seen in *mlp1/2* gene-gating defective cells. This model is consistent with the facts that both transcription elongation and RNA export are impaired in THO mutants (5, 55) and that R loops in THO- and Mlp1-depleted cells are suppressed by artificial DNA tethering to the NPC (Fig. 5*F*).

Finally, our study suggests that replication stress-induced release of transcribed genes from nuclear pores (37) would lead to R loop formation in the nucleoplasm, which may serve to further amplify the checkpoint activation cascade and signal the sites of RF stalling. Consistent with this idea, different studies suggest that R loops may accumulate in cells undergoing replicative stress (20, 25, 27) and participate in DNA damage signaling to activate ATM in human cells (56). Interestingly, the NPC-associated helicase DDX19 relocates to the core of the nucleus in response to replication stress or DNA damage to remove R loops (20). Our study thus opens new perspectives to understand the role of RNA in genome dynamics and the control of genome integrity as a function of nuclear location and raises the question of whether the human Mlp1/2 homolog TPR may fulfill the same functions in replication checkpoint and R loop prevention as in yeast.

Materials and Methods

DRIP Assays. Genomic DNA was carefully extracted and enzymatically digested. DNA–RNA hybrids were immunoprecipitated with the S9.6 antibody, with or without RNase H pretreatment. S9.6 signals were determined by dividing the immunoprecipitated S9.6 signal between the input for each sample as quantified by real-time-qPCR. See *SI Materials and Methods* for details.

Replication Analysis. BrdU ChIPs were performed in cells released from G1 arrest in the presence of 200 μg/mL BrdU. Immunoprecipitation was performed using monoclonal anti-BrdU antibody. See *SI Materials and Methods* for details.

Time-Course Analysis of *GAL1* Induction. Cells were grown at 30 °C to midlog phase in SC medium with 2% raffinose. A total of 2% galactose was added to the medium and samples taken at the indicated time. Isolation and Northern analysis of *GAL1* and *SCR1* mRNAs were performed following standard procedures. RNAP II ChIP was performed with the monoclonal anti-Rpb1 antibody (8WG16; Covance) and signals were quantified by real-time-qPCR. See *SI Materials and Methods* for details.

Miscellanea. Yeast strains, plasmids, and primers used are listed in Tables S4 and S5. Recombination and plasmid loss assays, Rad52-YPF foci, FACS, and PFGE were performed using standard procedures. See *SI Materials and Methods* for details.

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1. Aguilera A, García-Muse T (2012) R loops: From transcription byproducts to threats to genome stability. *Mol Cell* 46:115–124.
2. Hamperl S, Cimprich KA (2014) The contribution of co-transcriptional RNA:DNA hybrid structures to DNA damage and genome instability. *DNA Repair (Amst)* 19:84–94.
3. Chávez S, et al. (2000) A protein complex containing Tho2, Hpr1, Mft1 and a novel protein, Thp2, connects transcription elongation with mitotic recombination in *Saccharomyces cerevisiae*. *EMBO J* 19:5824–5834.
4. Strässer K, et al. (2002) TREX is a conserved complex coupling transcription with messenger RNA export. *Nature* 417:304–308.
5. Jimeno S, Rondón AG, Luna R, Aguilera A (2002) The yeast THO complex and mRNA export factors link RNA metabolism with transcription and genome instability. *EMBO J* 21:3526–3535.
6. Zenklusen D, Vinciguerra P, Wyss JC, Stutz F (2002) Stable mRNP formation and export require cotranscriptional recruitment of the mRNA export factors Yra1p and Sub2p by Hpr1p. *Mol Cell Biol* 22:8241–8253.
7. Domínguez-Sánchez MS, Barroso S, Gómez-González B, Luna R, Aguilera A (2011) Genome instability and transcription elongation impairment in human cells depleted of THO/TREX. *PLoS Genet* 7:e1002386.
8. Huertas P, Aguilera A (2003) Cotranscriptionally formed DNA:RNA hybrids mediate transcription elongation impairment and transcription-associated recombination. *Mol Cell* 12:711–721.
9. Wellinger RE, Prado F, Aguilera A (2006) Replication fork progression is impaired by transcription in hyperrecombinant yeast cells lacking a functional THO complex. *Mol Cell Biol* 26:3327–3334.
10. Gómez-González B, et al. (2011) Genome-wide function of THO/TREX in active genes prevents R-loop-dependent replication obstacles. *EMBO J* 30:3106–3119.
11. Li X, Manley JL (2005) Inactivation of the SR protein splicing factor ASF/SF2 results in genomic instability. *Cell* 122:365–378.
12. Paulsen RD, et al. (2009) A genome-wide siRNA screen reveals diverse cellular processes and pathways that mediate genome stability. *Mol Cell* 35:228–239.
13. Stirling PC, et al. (2012) R-loop-mediated genome instability in mRNA cleavage and polyadenylation mutants. *Genes Dev* 26:163–175.
14. Wahba L, Amon JD, Koshland D, Vuica-Ross M (2011) RNase H and multiple RNA biogenesis factors cooperate to prevent RNA:DNA hybrids from generating genome instability. *Mol Cell* 44:978–988.
15. Tuduri S, et al. (2009) Topoisomerase I suppresses genomic instability by preventing interference between replication and transcription. *Nat Cell Biol* 11:1315–1324.
16. El Hage A, French SL, Beyer AL, Tollervey D (2010) Loss of Topoisomerase I leads to R-loop-mediated transcriptional blocks during ribosomal RNA synthesis. *Genes Dev* 24: 1546–1558.
17. Cerritelli SM, Crouch RJ (2009) Ribonuclease H: The enzymes in eukaryotes. *FEBS J* 276: 1494–1505.
18. Mischo HE, et al. (2011) Yeast Sen1 helicase protects the genome from transcription-associated instability. *Mol Cell* 41:21–32.
19. Skourti-Stathaki K, Proudfoot NJ, Gromak N (2011) Human senataxin resolves RNA/DNA hybrids formed at transcriptional pause sites to promote Xrn2-dependent termination. *Mol Cell* 42:794–805.
20. Hodroj D, et al. (2017) An ATR-dependent function for the Ddx19 RNA helicase in nuclear R-loop metabolism. *EMBO J* 36:1182–1198.
21. Sridhara SC, et al. (2017) Transcription dynamics prevent RNA-mediated genomic instability through SRPK2-dependent DDX23 phosphorylation. *Cell Rep* 18:334–343.
22. Tran PLT, et al. (2017) PIF1 family DNA helicases suppress R-loop mediated genome instability at tRNA genes. *Nat Commun* 8:15025.
23. Garcia-Pichardo D, et al. (2017) Histone mutants separate R loop formation from genome instability induction. *Mol Cell* 66:597–609.e5.
24. Bhatia V, et al. (2014) BRCA2 prevents R-loop accumulation and associates with TREX-2 mRNA export factor PCID2. *Nature* 511:362–365.
25. Schwab RA, et al. (2015) The Fanconi anemia pathway maintains genome stability by coordinating replication and transcription. *Mol Cell* 60:351–361.
26. Hatchi E, et al. (2015) BRCA1 recruitment to transcriptional pause sites is required for R-loop-driven DNA damage repair. *Mol Cell* 57:636–647.
27. García-Rubio ML, et al. (2015) The Fanconi anemia pathway protects genome integrity from R-loops. *PLoS Genet* 11:e1005674.
28. Nguyen HD, et al. (2017) Functions of replication protein A as a sensor of R loops and a regulator of RNaseH1. *Mol Cell* 65:832–847.e4.
29. Gómez-González B, Aguilera A (2007) Activation-induced cytidine deaminase action is strongly stimulated by mutations of the THO complex. *Proc Natl Acad Sci USA* 104: 8409–8414.
30. Strambio-de-Castillia C, Blobel G, Rout MP (1999) Proteins connecting the nuclear pore complex with the nuclear interior. *J Cell Biol* 144:839–855.
31. Green DM, Johnson CP, Hagan H, Corbett AH (2003) The C-terminal domain of myosin-like protein 1 (Mlp1p) is a docking site for heterogeneous nuclear ribonucleoproteins that are required for mRNA export. *Proc Natl Acad Sci USA* 100: 1010–1015.
32. Vinciguerra P, Iglesias N, Camblong J, Zenklusen D, Stutz F (2005) Perinuclear Mlp proteins downregulate gene expression in response to a defect in mRNA export. *EMBO J* 24:813–823.
33. Galy V, et al. (2004) Nuclear retention of unspliced mRNAs in yeast is mediated by perinuclear Mlp1. *Cell* 116:63–73.
34. Blobel G (1985) Gene gating: A hypothesis. *Proc Natl Acad Sci USA* 82:8527–8529.
35. Casolari JM, Brown CR, Drubin DA, Rando OJ, Silver PA (2005) Developmentally induced changes in transcriptional program alter spatial organization across chromosomes. *Genes Dev* 19:1188–1198.
36. Dieppois G, Iglesias N, Stutz F (2006) Cotranscriptional recruitment to the mRNA export receptor Mex67p contributes to nuclear pore anchoring of activated genes. *Mol Cell Biol* 26:7858–7870.
37. Bermejo R, et al. (2011) The replication checkpoint protects fork stability by releasing transcribed genes from nuclear pores. *Cell* 146:233–246.
38. Aguilera A, Klein HL (1988) Genetic control of intrachromosomal recombination in *Saccharomyces cerevisiae*. I. Isolation and genetic characterization of hyper-recombination mutations. *Genetics* 119:779–790.
39. Palancade B, et al. (2007) Nucleoporins prevent DNA damage accumulation by modulating Ulp1-dependent sumoylation processes. *Mol Biol Cell* 18:2912–2923.
40. Herrera-Moyano E, Mergui X, García-Rubio ML, Barroso S, Aguilera A (2014) The yeast and human FACT chromatin-reorganizing complexes solve R-loop-mediated transcription-replication conflicts. *Genes Dev* 28:735–748.
41. Chan YA, et al. (2014) Genome-wide profiling of yeast DNA:RNA hybrid prone sites with DRIP-chip. *PLoS Genet* 10:e1004288.
42. Santos-Pereira JM, Aguilera A (2015) R loops: New modulators of genome dynamics and function. *Nat Rev Genet* 16:583–597.
43. Chang EY, Stirling PC (2017) Replication fork protection factors controlling R-loop bypass and suppression. *Genes (Basel)* 8:E33.
44. Texari L, et al. (2013) The nuclear pore regulates GAL1 gene transcription by controlling the localization of the SUMO protease Ulp1. *Mol Cell* 51:807–818.
45. Richard P, Manley JL (2016) R loops and links to human disease. *J Mol Biol*, 10.1016/j.jmb.2016.08.031.
46. Sollier J, Cimprich KA (2015) Breaking bad: R-loops and genome integrity. *Trends Cell Biol* 25:514–522.
47. Fan HY, Merker RJ, Klein HL (2001) High-copy-number expression of Sub2p, a member of the RNA helicase superfamily, suppresses hpr1-mediated genomic instability. *Mol Cell Biol* 21:5459–5470.
48. Jimeno S, Luna R, García-Rubio M, Aguilera A (2006) Tho1, a novel hnRNP, and Sub2 provide alternative pathways for mRNP biogenesis in yeast THO mutants. *Mol Cell Biol* 26:4387–4398.
49. Saroufim MA, et al. (2015) The nuclear basket mediates perinuclear mRNA scanning in budding yeast. *J Cell Biol* 211:1131–1140.
50. Drubin DA, Garakani AM, Silver PA (2006) Motion as a phenotype: The use of live-cell imaging and machine visual screening to characterize transcription-dependent chromosome dynamics. *BMC Cell Biol* 7:19.
51. Prado F, Piruat JI, Aguilera A (1997) Recombination between DNA repeats in yeast hpr1delta cells is linked to transcription elongation. *EMBO J* 16:2826–2835.
52. Rondón AG, Jimeno S, García-Rubio M, Aguilera A (2003) Molecular evidence that the eukaryotic THO/TREX complex is required for efficient transcription elongation. *J Biol Chem* 278:39037–39043.
53. Mason PB, Struhl K (2005) Distinction and relationship between elongation rate and processivity of RNA polymerase II in vivo. *Mol Cell* 17:831–840.
54. Castellano-Pozo M, et al. (2013) R loops are linked to histone H3 S10 phosphorylation and chromatin condensation. *Mol Cell* 52:583–590.
55. Chávez S, Aguilera A (1997) The yeast HPR1 gene has a functional role in transcriptional elongation that uncovers a novel source of genome instability. *Genes Dev* 11: 3459–3470.
56. Tresini M, et al. (2015) The core spliceosome as target and effector of non-canonical ATM signalling. *Nature* 523:53–58.
57. Gómez-González B, Ruiz JF, Aguilera A (2011) Genetic and molecular analysis of mitotic recombination in Saccharomyces cerevisiae. *Methods Mol Biol* 745:151–172.
58. Lisby M, Rothstein R, Mortensen UH (2001) Rad52 forms DNA repair and recombination centers during S phase. *Proc Natl Acad Sci USA* 98:8276–8282.
59. Gaillard H, Wellinger RE, Aguilera A (2015) Methods to study transcription-coupled repair in chromatin. *Methods Mol Biol* 1288:273–288.
60. Sikorski RS, Hieter P (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122:19–27.
61. Piruat JI, Aguilera A (1998) A novel yeast gene, THO2, is involved in RNA pol II transcription and provides new evidence for transcriptional elongation-associated recombination. *EMBO J* 17:4859–4872.
62. Garí E, Piedrafita L, Aldea M, Herrero E (1997) A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in *Saccharomyces cerevisiae*. *Yeast* 13:837–848.
63. Santos-Pereira JM, et al. (2013) The Npl3 hnRNP prevents R-loop-mediated transcription-replication conflicts and genome instability. *Genes Dev* 27:2445–2458.
64. Feng Q, et al. (2007) Rad52 and Rad59 exhibit both overlapping and distinct functions. *DNA Repair (Amst)* 6:27–37.
65. Mumberg D, Müller R, Funk M (1994) Regulatable promoters of *Saccharomyces cerevisiae*: Comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res* 22:5767–5768.