R Loops Are Linked to Histone H3 S10 Phosphorylation and Chromatin Condensation

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SUMMARY

R loops are transcription byproducts that constitute a threat to genome integrity. Here we show that R loops are tightly linked to histone H3 S10 phosphorylation (H3S10P), a mark of chromatin condensation. Chromatin immunoprecipitation (ChiP)-on-chip (ChiP-chip) analyses reveal H3S10P accumulation at centromeres, pericentromeric chromatin, and a large number of active open reading frames (ORFs) in R-loop-accumulating yeast cells, better observed in G1. Histone H3S10 plays a key role in maintaining genome stability, as scored by ectopic recombination and plasmid loss, Rad52 foci, and Rad53 checkpoint activation. H3S10P coincides with the presence of DNA-RNA hybrids, is suppressed by ribonuclease H overexpression, and causes reduced accessibility of restriction endonucleases, implying a tight connection between R loops, H3S10P, and chromatin compaction. Such histone modifications were also observed in R-loop-accumulating Caenorhabditis elegans and HeLa cells. We therefore provide a role of RNA in chromatin structure essential to understand how R loops modulate genome dynamics.

INTRODUCTION

R loops are essential in replication initiation of mitochondrial DNA, in an alternative mechanism of replication initiation in bacteria, and in class-switch recombination in vertebrate B cells (Aguilera and García-Muse, 2012) and have been suggested to play a role in transcription termination (Skourtis-Stathaki et al., 2011). However, R loops are potentially harmful structures that promote genome instability by impairing replication fork progression (Gan et al., 2011; Wellinger et al., 2006). Accordingly, cells use factors to remove R loops, such as ribonuclease H (RNase H) or the Sen1/SETX DNA-RNA helicase (Cerritelli and Crouch, 2009; Mischo et al., 2011), or prevent their formation, such as number of mRNA biogenesis and processing proteins (Huertas and Aguilera, 2003; Li and Manley, 2005; Paulsen et al., 2009; Stirling et al., 2012; Wahba et al., 2011), the THO complex being a paradigmatic example conserved from yeast to humans (Domínguez-Sánchez et al., 2011). Different mechanisms have been proposed to explain how R loops can contribute to genome instability. It is believed that R loops can either enhance the susceptibility of DNA to endogenous genotoxic agents or be an obstacle to the progression of replication fork that can lead to its collapse (Aguilera and García-Muse 2012). However, we do not yet know the mechanism and factors responsible for genome instability mediated by R loops or whether additional elements, such as R-loop-mediated chromatin modifications, could modulate genome dynamics. Here we investigated the possible connections of R loops with histone modifications as a first step to explore additional elements by which R loops can contribute to genome instability. Strikingly, we found that R loops are linked to histone H3 S10 phosphorylation, a mark of chromatin condensation that opens unexpected views to understand the role of R loops in genome dynamics.

RESULTS

High Levels of the H3S10P Chromatin Compaction Mark in R-Loop-Accumulating Strains during Mitosis and Meiosis

To address whether R loops were connected to specific chromatin modifications in Saccharomyces cerevisiae, we assayed first by western blotting if posttranslational histone modifications were altered in R-loop-accumulating mutants. Notably, we found that histone H3 was highly phosphorylated at S10 (H3S10P), a marker of chromatin condensation (Hsu et al., 2000), both in mitotic and meiotic cells. In wild-type (WT) cells, H3S10P was either poorly or not detected in G1 during the mitotic cycle. Instead, H3S10 was clearly phosphorylated during G1 in R-loop-accumulating hpr1Δ mutants of the THO complex as well as at high levels in S/G2 (Figure 1A). In meiosis, H3S10P was observed only 1.5 hr after initiating synchronous meiosis in WT cells, whereas in hpr1Δ cells, H3S10P was clearly detected at all meiotic stages, as determined in synchronous meiosis of SK1 isogenic cells (Figure 1B), as the SK1 genetic background is able to undergo rapid and synchronized meiosis. Importantly, this mark is not specific to THO mutants, as we can also see a significant presence of H3S10P in G1-arrested cells of R-loop-accumulating mnh1Δ mnh2Δ strains (Figure S1A available online). Even though hpr1Δ cells remained longer in S/G2, the difference in H3S10P with respect to WT persists and even increases when cells are arrested in G1 for 3 hr (Figure S1B), confirming a true
phosphorylation of H3S10 in G1 hpr1Δ cells that does not occur in WT cells. Other histone H3 modifications, such as H3K14 acetylation (H3K14ac) and H3K4 trimethylation (H3K4me3) known to be associated with transcription, presented the same pattern as the wild-type, as determined by western blotting (Figure S1C) and by chromatin immunoprecipitation (ChIP) in the GAL1-FMP27 fusion construct (Figure S1D). Background levels of H3S10P were observed all over the FMP27 gene in wild-type and hpr1Δ cells (Figure S1D). The results indicate that the genome of R-loop-accumulating strains shows high levels of the H3S10P chromatin mark, known to associate with chromatin condensation, in mitosis and meiosis.

Deregulation of Histone H3S10 Phosphorylation Results in Genome Instability

We next determined whether such a chromatin mark had any effect on genome instability, a hallmark of R-loop-accumulating mutants. For this, we used specific histone H3 point mutations at S10 that cannot be phosphorylated (H3S10A) or that mimic constitutive phosphorylation (H3S10D). A slight, but significant, increase in direct-repeat recombination together with an increase in Rad52 foci and plasmid loss was observed in both H3S10 mutants (Figures 1C–1E). In addition, the S phase checkpoint was activated, as determined by Rad53 phosphorylation in both H3S10 mutants (Figure 1F). The results indicate that H3S10 is important for genome integrity, consistent with our prediction. However, the similar phenotypes observed in both H3S10 mutants indicate that the phosphorylation state of this residue, or even the S10 residue itself, may control genome dynamics by itself, whether or not R loops are involved. Indeed, Rad52 foci accumulation and hyperrecombination of hpr1Δ cells are partially suppressed in the double mutants hpr1Δ H3S10A and hpr1Δ H3S10D (Figures 1C and 1D), confirming the role of the S10 residue in genome instability in hpr1Δ cells regardless of its R-loop-dependent phosphorylation stage. It is possible that H3S10 itself, or its phosphorylation state, controls different DNA metabolic processes, including replication and repair, by helping recruit specific factors, but this is an open question. A similar situation can be that of other histone modifications like H3K56 acetylation (Muñoz-Galván et al., 2013).

Genome-wide Distribution of H3S10P in R-Loop-Accumulating Strains

To get further insight into the physiological meaning of the H3S10P mark, we determined the distribution of this mark along the genome. We used specific histone H3 point mutations...
the yeast genome of the R-loop-accumulating hpr1Δ strain compared to WT cells in G1 and S phase by ChIP-on-chip (ChIP-chip) analyses. Notably, in contrast to WT cells, there is a much more abundant and spread distribution of H3S10P along all chromosomes in the hpr1Δ strain that is clearly manifested in G1 cells (Figures 2 and S2). The H3S10P signal was clearly overabundant at the centromeres and the pericentromeric regions as well as all over the genome in G1-arrested hpr1Δ cells (Figures 2A, 2B, and S2). The number of genes located in the 40 kb pericentromeric region and all over the genome that contained H3S10P signals was significantly higher (p < 0.001) in G1-arrested hpr1Δ cells than in wild-type cells (Figure 2A). Interestingly, a detailed statistical analysis of all 40 kb pericentromeric regions of the genome revealed a significantly high H3S10P accumulation in hpr1Δ cells not observed in the wild-type, while the global amount of histone H3 was unchanged (Figures 2C and S3A). The accumulation profile of H3S10P at the pericentromeric genes was clearly higher along the whole extension of the open reading frames (ORFs) analyzed in hpr1Δ cells as compared to WT cells, which show a reduced signal (Figure 2D). Importantly, even though this higher H3S10P accumulation is better observed in the pericen-

Figure 2. Genome-wide Accumulation of H3S10P in R-Loop-Accumulating hpr1Δ Cells during G1

(A) Statistical analysis of H3S10P hits (p value < 0.01) obtained in the ChIP-chip analysis according to the different annotation categories of the Stanford Genome Database for wild-type and hpr1Δ strains. *Values significantly different from wild-type (p < 0.001).

(B) ChIP-chip distribution of the H3S10P signal along chromosomes IV, VI, and VIII. The y axis shows the signal log2 ratio significantly enriched in the immunoprecipitated fraction of wild-type and hpr1Δ G1-arrested cells.

(C) ChIP-chip enrichment of H3S10P at centromeric and 40 kb pericentromeric regions. The y axis shows the median of H3S10P-IP (log2 ratio) signal per segment of all the chromosomes for wild-type and hpr1Δ G1-arrested cells.

(D) Profile of H3S10P enrichment across the ORFs of either the 16 pericentromeric regions of 40 kb or the whole genome plotted as the average signal (log2 ratio) significantly enriched (p < 0.01) in the immunoprecipitated fraction per segment in wild-type and hpr1Δ G1-arrested cells. The length of the ORFs was divided in 10 segments and analyzed together with the 500 bp immediately upstream and downstream of the segments, as previously described (Gómez-González et al., 2011). See also Figures S2 and S3.

H3S10P Is Linked to R Loops and Chromatin Compaction

The results suggest that the high levels of H3S10P are linked to R loops. To determine whether this was the case, we performed ChIP and DNA-RNA immunoprecipitation (DRIP) analysis of the pericentromeric region in chromosome VI with anti-H3S10P and anti-RNA-DNA hybrid (S9.6) antibodies, respectively (Figures 3A and 3B). G1 cells clearly accumulated the H3S10P signal in hpr1Δ, but not in WT cells, validating the ChIP-chip results. This signal was abolished by RNH1 overexpression (Figure 3A), indicating a tight link between R loops and H3S10P. Importantly, RNH1 overexpression does not affect cell-cycle progression in either WT or in hpr1Δ cells, excluding any possible effect of
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The Link between R Loops and H3S10P Also Occurs in C. elegans and Human Cells

In parallel with our yeast study, we wondered whether this phenomenon was conserved in multicellular eukaryotes. Given that R loops also accumulate in C. elegans thoc-2 null mutants of the THO complex, causing replication defects linked to genome instability (Castellano-Pozo et al., 2012), we determined by immunostaining whether chromatin compaction also occurred in these C. elegans strains. Notably, histone H3 was not only highly phosphorylated at S10 (H3S10P), but it was also dimethylated at K9 (H3K9me2), a marker of heterochromatin not conserved in yeast (Hsu et al., 2000; Reddy and Villeneuve, 2004), both at mitotic and pachytenic meiotic regions of the germline of the R-loop-accumulating thoc-2 strain (Figure 4A). Such modifications are not found in N2(WT) worms during mitosis, whereas in meiosis they are observed only after pachytene, consistent with them being marks of condensation. Indeed, DAPI staining shows bright and condensed nuclei in the germline of thoc-2 worms (Figure 4D), in agreement with the yeast data, we detect S9.6 foci in thoc-2 germlines, but not in N2(WT) worms (Figure S4E). No difference between N2(WT) and thoc-2 was observed for the H4 tetra-acetylation pattern used as a marker of euchromatin (Figure 4A) (Reddy and Villeneuve, 2004).

Next, we asked whether this phenomenon was also conserved in human cells. For this, we assayed H3S10P accumulation in R-loop-accumulating THOC1- and senataxin (SETX)-depleted human cells (Domínguez-Sánchez et al., 2011; Skourtis-Stathaki...
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et al., 2011). We included senataxin-depleted cells to assay whether the R-loop-associated H3S10 phosphorylation occurred regardless of the system we used to enhance R loops. We performed immunofluorescence with anti-H3S10P antibody in HeLa cells grown in ethynyldeoxyuridine (EdU)-containing media to differentiate nonreplicating G1 cells from actively replicating S phase cells. Consistent with the yeast and worm results, G1 HeLa cells with H3S10P foci were only observed in the absence of THOC1 or SETX (Figures 4B and 4C). Moreover, RNase H1 overexpression decreases H3S10P foci formation, supporting the connection between H3S10P and R loops (Figures 4B and 4C). The results suggest that the link between R loops and chromatin condensation marks such as H3S10P is conserved in all eukaryotes, including yeast, nematodes, and humans.

DISCUSSION

The link between different histone modifications and RNA molecules has been shown in eukaryotes. A well-known example is the X-inactivation in mammals by RNA Xist (Lee and Bartolomei, 2013) or gene silencing linked to long noncoding RNAs (ncRNA) that, in most cases, is mediated by siRNAs (Bernstein and Allis, 2005) or via chromatin deacetylation by an antisense ncRNA, as shown in S. cerevisiae (Camblong et al., 2007). Interestingly, RNA-DNA hybrids prevent methylation and transcriptional silencing at CpG island promoters (Ginno et al., 2012) and induce transcriptional silencing, likely through siRNA, in S. pombe (Nakama et al., 2012). Furthermore, centromeres and pericentromeric regions are known to be transcribed, and transcription of pericentromeric DNA repeats is...
required for pericentromeric heterochromatin formation in S. pombe (Chan and Wong, 2012).

Histone H3S10P and chromatin condensation have never been connected to RNA metabolism, even though H3S10P also controls transcription elongation by releasing the RNA polymerase II (RNAPII) from promoter-proximal pausing sites in Drosophila (Ivaldi et al., 2007). It is believed that H3S10P may modulate the access of different proteins to chromatin, establishing a hierarchy of subsequent events that affect chromatin structure and function (Liokatis et al., 2012). Our study shows that R loops are linked to H3S10P and chromatin compaction. The H3S10P mark is scattered at transcribed genes all over the genome, even though it is better detected at the pericentromeric and centromeric regions. This phenomenon is independent of siRNA, provided that S. cerevisiae does not have siRNA processes. According to our results, R loops can either trigger phosphorylation of H3S10 or inhibit its dephosphorylation. This can be achieved by either enhancement of the recruitment of the responsible kinase or inhibition of the activity of the responsible phosphatase. As more than one enzyme may be involved in this process (Baek, 2011), further extensive work will be needed to decipher the molecular mechanism of this control. Nevertheless, it has been shown that H3S10P levels in yeast depend on the Ipl1 kinase and the Gic7 phosphatase and that Ipl1 and Gic7 interact physically or genetically with factors, such as Set1 and Sen1 (Breitkreutz et al., 2010; Nedea et al., 2008; Zhang et al., 2005), that have been implicated in the metabolism of R loops (Mischo et al., 2011). These data open the possibility of a functional link between R loops and H3S10P that would need to be explored in the future.

Although our study does not establish whether R loops help chromatin condensation or vice versa, we favor a model in which R loops trigger the formation of highly condensed chromatin patches that would appear throughout the genome, in particular at centromere and pericentromeric regions due to its higher capacity to nucleate chromatin condensation (Figure 4D). This model is supported by the reduction of H3S10P levels along the yeast genome of hpr1Δ cells by RNase H1 overexpression. Thus, it is possible that condensation spreads from the R loop. Importantly, such condensed regions would interfere with replication and/or transcription (Figure 4D), causing transcription-associated genome instability and gene silencing. This could contribute to the previously reported effect of R loops on both processes in eukaryotes (Huertas and Aguilera 2003). Interestingly, the observations that common fragile sites (CFSs) can be explained by retarded or incomplete replication at chromatin-condensed regions (Debatistse et al., 2012) and that CFSs can be related to cotranscriptional R loops (Helmrich et al., 2011) open the possibility that local chromatin compaction contributes to the replication fork progression impairment or to a delayed or less-efficient replication initiation as possible factors that cause genome instability. This unexpected link between R loops, histone H3S10P, and chromatin compaction or condensation therefore opens unexpected perspectives with which to understand the mechanism by which RNA controls genome dynamics and function.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth**

The yeast strains used in this work are listed in Table S1. Cells were arrested in G1 with α factor (0.14 μg/ml) and in S phase with 200 mM hydroxyurea (HU). For meiotic analysis, synchronous meiotic cultures were prepared as previously described (Aliani et al., 1990).

**Nematode Strains and Growth**

The nematode strain Bristol N2 used as wild-type was provided by the Caenorhabditis Genetics Center. The thoc-2 (tm1310) mutant was previously characterized (Castellano-Pozo et al., 2012). C. elegans strains were grown at 20°C, and standard methods were used for their maintenance and manipulation (Brenner, 1974).

**Human Cell Culture and Transfection**

HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum at 37°C (5% CO2). Transient transfection of siRNA (10 nM) was performed using DharmaFECT (Dharmacon) according to the manufacturer’s instructions. Then, 96 hr after transfection, cells were incubated with 20 μM EdU (Click-IT EdU Imaging Kit, Invitrogen) for 20 min to label replicating cells.

**ChiP and DIP/DRIP Assays**

For ChiP experiments at the pericentromeric region of CEN6, cells harboring the empty vector pCM189 or the pETRHN1 plasmid were grown in synthetic complete media without uracil (SC-ura), and samples were treated as described (Duch et al., 2013). Antibodies used are listed in Table S2. For real-time PCR, oligonucleotides for the CEN6 pericentromeric region previously described (Choy et al., 2011) and for other loci (Table S3) were used. Means ± SD of three independent experiments are shown.

DRIP experiments were carried out essentially as originally described like DNA IP (DIP) (Mischo et al., 2011). Means ± SD of three independent experiments are shown.

**ChiP-Chip Assays**

Cells were grown in YEPD medium and arrested in G1 with α factor (0.14 μg/ml) for 2 hr (G1 phase) and then released in new rich medium with 200 mM HU for another hour (S phase). ChiP-Chip was carried out as previously described (Bermejo et al., 2007; Bermejo et al., 2009; Katou et al., 2003; 2006). For details and bioinformatics analysis, see Supplemental Information.

**Chromatin Accessibility Assays**

Nuclei preparations from G1-arrested cells with α factor (0.14 μg/ml) were made as previously described (Crottì and Basrai, 2004). Similar quantities of nuclei were incubated with 0, 5, 15, 50, and 150 units/ml of DraI at 37°C for 30 min followed by digestion with 250 U/ml EcoRI. Southern blotting was performed, and filters were probed with a 32P-deoxycytidine triphosphate (dCTP)-labeled CEN3 0.9 kb HindIII-BamHI fragment following standard procedures.

**Miscellanea**

Immunofluorescence analyses using the indicated antibodies were performed in 1 day post-L4 C. elegans adult gonads treated as described (Castellano-Pozo et al., 2012) and in human cells cultured on glass coverslips and fixed as described previously (Domínguez-Sánchez et al., 2011). EdU detection was performed using a Click-IT EdU Imaging Kit (Invitrogen) following manufacturer’s instructions. DNA was stained with DAPI. Recombination and plasmid-loss assays and Rad52 foci are described in Gómez-González et al. (2009) and the Supplemental Information.

**ACCESSION NUMBERS**

The Gene Expression Omnibus accession number for the set of ChiP-chip data reported in this paper is GSE46627.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2013.10.006.

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