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Review Article

# What causes an RNA-DNA hybrid to compromise genome integrity?⋆ ⋆⋆

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ABSTRACT

Transcription is a source of genome instability that stimulates mutation and recombination. Part of the damage produced by transcription is mediated by R-loops, non-B DNA structures that normally form by the re-annealing of the nascent RNA with the template DNA outside the catalytic center of the RNA polymerase, displacing the non-template strand. Recent discoveries have revealed that R-loops might not be harmful by themselves. Instead, chromatin compaction triggered by these structures seems necessary, as deduced from the histone modifications frequently found associated with harmful R-loops. Remarkably, hybrids may also become harmful if stabilized by specific RNA binding proteins, one example of which is the yeast Yra1. We discuss here the possible mechanisms by which cells may stabilize R-loops and the consequences on transcription-replication conflicts and telomere homeostasis.

## 1. Introduction

The initial step in gene expression is the synthesis of an RNA transcribing the information contained in the template DNA. Transcription is an important source of genetic instability, enhancing the rate of mutation and recombination from bacteria to human cells (reviewed in [1]). In principle, several mechanisms could account for transcription-associated genome instability: an increased accessibility to DNA due to the chromatin remodeling activities associated with transcription; transcription-replication conflicts caused by the replication fork approaching or encountering an RNA polymerase (RNAP); or formation of non-B DNA structures that generates DNA damage. A special mention in this last category should be done to R-loops, a byproduct of transcription formed by an RNA-DNA hybrid and the ssDNA displaced by the RNA. Although it is still unclear how an RNA could invade a ds-DNA, the proposed mechanism for R-loop formation is the thread back of the nascent transcript, once it exits the RNAP, into the template DNA strand, a situation favored by the accumulation of negative supercoiling behind the RNAP [2]. Accumulation of R-loops compromises genome integrity by mechanisms that are still being deciphered. Excellent reviews on the consequences R-loop formation and how this could be used in the treatment of diseases have been recently published [3–6]. Here, we will focus on the mechanisms and the implications of unbalancing R-loop dynamics in replication and telomere homeostasis.

## 2. R-loop-triggered chromatin modifications as a threat to genome integrity

R-loops as a source of DNA damage were initially detected as non-scheduled structures formed in the absence of factors that assembled the nascent RNA into a mature messenger ribonucleoparticle (mRNP) [7,8]. Yet, it was lately observed that RNA-DNA hybrids form naturally in the cell as a byproduct of transcription, but they are continuously counteracted by the combined action of helicases and RNase H, thus preventing DNA damage [6,9]. Then, the question raising is what causes an R-loop to be harmful.

In principle, an R-loop may be a source of DNA breaks and genome instability due to its potential to block replication fork progression or to the fact that the displaced ssDNA of an R-loop is more susceptible to damage caused by endogenous genotoxic metabolites such as ROS or DNA modifying enzymes. However, the recent identification of specific

*Abbreviation:* ALT, Alternative Telomere lengthening; dsDNA, double-stranded DNA; HB-GFP, Hybrid Binding Green Fluorescent Protein; ICF, Immunodeficiency, Centromeric instability and Facial anomalies; mRNP, messenger ribonucleoparticle; RNAP, RNA polymerase; ROS, reactive oxygen species; ssDNA, single-stranded DNA.

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histone mutants that accumulate R-loops without increasing genome instability suggests that R-loops may not generate DNA damage by themselves but that an additional step is required, at least in cycling cells [10]. One possibility is that the threatening element for the genome is not the R-loop itself but an additional molecular event triggered by the R-loop. In several organisms from yeast to nematodes and human cells, R-loop accumulation has been linked to an increase in chromatin compaction marks, especially histone H3S10 phosphorylation, a modification associated with chromosome condensation, and H3K9 di-methylation, a hallmark of heterochromatin [11–13]. If R-loops are formed in yeast histone mutants that are unable to undergo H3S10 phosphorylation, they do not induce genome instability as measured by recombination and Rad52 foci accumulation [10]. Therefore, R-loops do not seem to be deleterious by themselves; instead they require an additional step connected to chromatin compaction. Indeed, this may be related to the observation that naturally-formed R-loops, those detected in wild-type cells that do not cause genetic instability, are preferentially located in euchromatin rather than heterochromatin [9]. One appealing possibility is that only R-loops that are long-lived would trigger chromatin compaction. In this line, the reduced ability of cells depleted of RNaseH or RNA-DNA helicases to remove naturally formed R-loops would explain their high levels of DNA damage (reviewed in [2,4,6]).

Heterochromatinized regions tend to replicate lately in S-phase and they require additional activities like chromatin remodelers. Indeed, highly compacted structures like telomeres and centromeres are intrinsically difficult to replicate. Therefore, an R-loop-induced compacted chromatin would impose a barrier for replication progression and could potentially stall the fork, ultimately generating DNA breaks. If that were the case, we would expect that R-loops formed in histone mutants that do not undergo histone H3S10 phosphorylation and that do not generate genome instability would not affect replication.

## 3. Converting transient harmless R-loops into harmful persistent structures

Although chromatin modification may be an important natural mechanism to convert an innocuous R-loop into a pathological one, this may not be the only way to do so. In principle, this could also occur by mechanisms that lead to R-loop persistency, regardless of chromatin modifications. Artificial R-loop stabilization could be achieved by an RNA-DNA hybrid-binding protein that when bound to the hybrid, would block access of RNA-DNA helicases or RNaseH to this structure or could make an innocuous R-loop a strong obstacle to replication fork progression. Indeed, expressing an R-loop-binding protein constructed by fusing the RNA-DNA hybrid-binding domain of RNase H to the Green Fluorescent Protein (HB:GFP) increases genome instability [14]. Recent work on R-loop metabolism uncovered an alternative R-loop-stabilizing factor in the budding yeast *Saccharomyces cerevisiae*: Yra1 [15,16].

## 4. Yeast Yra1 as an RNA-DNA hybrid stabilizer

Yra1 was initially identified as a RNA-binding protein that is recruited to transcribed genes by interacting with RNA polymerase II and loaded into the nascent RNA. Yra1 interacts with multiple components of the mRNP including Sub2 and the THO complex, finally recruiting the mRNA export factor Mex67 and thus promoting mRNP exit to the cytoplasm [17]. Mutation of Yra1 causes transcription-dependent genome instability and defects in transcription similar to those observed in the absence of other mRNP assembly factors that prevent R-loop accumulation e.g. the THO complex, Sub2 or Npl3 [18–20]. Importantly, Yra1 stoichiometry in the cell is tightly regulated so that excess or deficit of this protein causes lethality. The expression is autoregulated at the level of mRNA processing by Yra1 ability to bind to its own pre-mRNA and to inhibit splicing. Intron-containing pre-mRNAs are exported and degraded in the cytoplasm [21]. A negative feedback-loop guarantees low levels of Yra1 in the cells. Indeed, artificially removing YRA1 intron bypasses this control and causes Yra1 over-expression with drastic consequences for the cell ranging from defects in mRNA export and replication to increased DNA damage and recombination [16,22].

A close look at the origin of Yra1-mediated genome instability revealed that when it is in excess, Yra1 not only binds to RNA but also to RNA-DNA hybrids accumulating at naturally R-loop-forming regions, increasing the amount of these structures in the cell, probably by preventing their resolution [15]. Stable R-loops would block replication fork progression and transcription, inducing genome instability (Fig. 1). It seems that Yra1 overexpression in yeast causes a similar effect than expressing HB:GFP fusion in human cells. It would be interesting

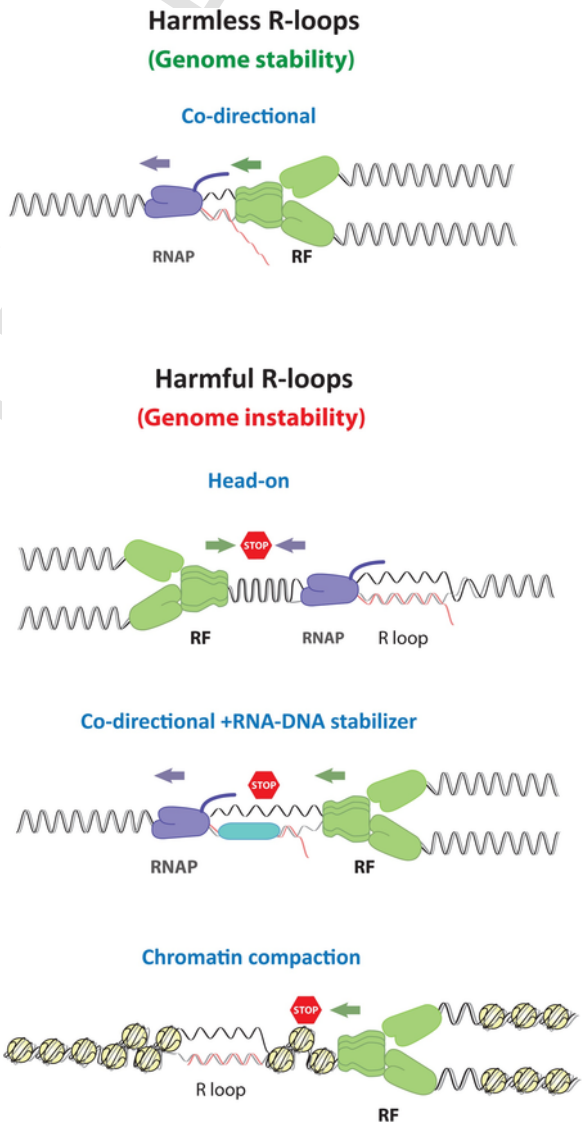


**Fig. 1. Harmless and harmful R-loops.** R-loops by themselves may not be a major cause of genome instability in cycling cells. An R-loop generated by an RNA polymerase elongating co-directionally with respect to the replication fork would be removed by the replicative helicases (harmless R-loops). Instead, R-loops generated by an RNA polymerase elongating head-on with respect to the replication fork and R-loops either stabilized by an RNA-DNA hybrid-binding protein or associated with histone modifications leading to chromatin compaction would cause genome instability regardless of the orientation of the transcription-replication conflict (harmful R-loops).

to know whether R-loops artificially fixed by Yra1 or HB:GFP trigger H3S10 phosphorylation and chromatin compaction, but in principle they may be unrelated phenomena. In the case of an artificial stabilization of R-loops, chromatin compaction would not be a pre-requisite for causing DNA damage as a protein stably-bound to an RNA-DNA hybrid could be harmful by itself if it impedes the progression of replication forks (Fig. 1).

## 5. From R-loops to replication stress

When transcription and replication take place at the same time on the same template, a situation that although avoided is sometime inevitable, conflicts between these two processes appear (reviewed in [23–25]). Considering the directionally of both processes, the conflicts may occur co-directionally or head-on. Initial studies in budding yeast suggested that only head-on transcription replication encounters pause the replisome and induce recombination while co-directional transcription has no effect on fork progression or genome stability [26]. Recent work on bacteria, yeast and human cells suggests that the different outcome of head-on and co-directional conflicts might be related to the presence of stable R-loops [15,27,28].

R-loop formation is one possible cause of interference between transcription and replication (review in [2–4]). Recent studies demonstrate that naturally formed R-loops are partly responsible of the increase in plasmid instability, mutagenesis and recombination observed in head-on transcription replication conflicts [15,27,28]. When the presence of R-loops is analyzed in plasmids designed to confront transcription replication in head-on or co-directional orientation, RNA-DNA hybrids are only detected in head-on orientation in asynchronous cultures of bacteria or human cells [27,28]. However, if R-loops are artificially stabilized in yeast by overexpressing Yra1 they are also detected in codirectional orientation, they generate DNA damage and increase recombination [15]. This suggests that R-loops are formed previous to and not as a consequence of replication stalling. Therefore, it appears that R-loops formed codirectionally are not stable enough to threaten replication and thus induce genetic instability unless they are artificially stabilized (Fig. 1). In agreement with this idea, in human cells synchronized in G1 R-loops are detected at both orientations suggesting that codirectional replication eliminates RNA-DNA hybrids [28]. If R-loops are stabilized by Yra1 interaction, the replication fork stalls at them even if it is approaching co-directionally (Fig. 1). Consistently, Yra1 overexpression reduces the global level of replication and increases recruitment to chromatin of the accessory helicase Rrm3 that helps to overcome replication obstacles [16]. It is still unclear how does an R-loop block replication fork progression. One possibility is that the RNA-DNA hybrid by retaining the RNAP or by recruiting RNA-DNA interacting proteins creates a road-block itself. An alternative explanation could be that R-loops promote positive superhelicity accumulation between head-on transcription-replication conflicts. Finally, it is also possible that the impediment is not the RNA-DNA hybrid but the compacted chromatin structure induced by it (Fig. 1).

From the mechanistic point of view, the difference in stability between a co-directional and a head-on R-loop might rely on the access that helicases may have to each of them. Although the fate of the RNAP that generates the R-loop is not known, it is possible that it remains attached to the template (reviewed in [3]). If that is the case, the RNAP might preclude the access of the replicative helicases, either MCMs or other accessory helicases, to the R-loop, specifically at head-on collisions. On the contrary, a replisome approaching codirectionally would reach the RNA-DNA hybrid instead of the RNAP and could potentially eliminate it releasing the RNAP and resolving the conflict (Fig. 1).

## 6. Consequences of R-loop stabilization in telomere homeostasis

R-loop stabilization could be a critical issue at regions difficult to replicate and/or regions prone to form R-loops. In this category are the rDNA repeats, fragile sites and telomeres. At highly transcribed rDNA R-loops accumulate naturally [29], and cause genomic instability if they are stabilized by mutations in the topoisomerase Top1 or RNaseH [30–32]. Fragile sites are regions intrinsically difficult to replicate where chromosomal rearrangements occur preferentially [33]. Interestingly, RNA-DNA hybrids have been detected at common and rare fragile sites [12,34], contributing to the replication problems observed in these regions [35]. Hence, any change in the cell that stabilizes R-loops would probably impede replication of these regions enhancing genome instability.

Telomeres are formed by G-rich repeats added by the telomerase to counteract erosion caused by replication. Despite their compacted heterochromatin structure telomeres and subtelomeric regions are transcribed into long non-coding RNAs termed TERRAs [36,37] that hybridize with the DNA forming R-loops [38–40]. Telomeric R-loop levels are maintained low in the cell by the concerted action of the THO complex and RNases H among others [38,39]. To avoid conflicts with replication, TERRA R-loops are degraded prior to the entry into S phase [41]. The importance of the dynamic nature of TERRA R-loops is appreciated under conditions that either increase or stabilize them. One example is the increased expression of Yra1 in yeast. In this artificial situation, Yra1 is recruited to telomeres increasing the level of R-loops and causing telomere shortening [15,16] what might be the consequence of fixing TERRA R-loops throughout the cell cycle avoiding their resolution prior to replication. Indeed, cells overexpressing Yra1 present premature senescent as a result of an accelerated telomere loss [16].

Although initially R-loops would decrease telomere length, once they pass a critical size R-loops could reverse the process favoring telomere extension though recombination. When telomeres reach a minimal length, they trigger senescence and cell death, a process that is though to safeguard the organism from uncontrolled cellular division. In yeast, as telomeres decrease their size, they increase TERRA transcription and R-loop formation [41]. In addition, in short telomeres R-loops are stable throughout the cell cycle not being removed at S phase a situation that hamper replication and generates DNA damage [41]. Although most of the cells enter into crisis and die, a few of them survive by extending the telomeres through homologous recombination between the telomeric repeats, a mechanism similar to the human alternative telomere lengthening (ALT) pathway employed by tumor cells. In agreement with this, in yeast *rnh1 rnh2* mutants, accumulation of TERRA R-loops induces telomere elongation by increasing homologous recombination at chromosome ends, delaying senescence [38] and, in cancer cell lines that maintain their telomeres by the ALT mechanism, a high level of TERRA R-loops is required to keep telomere length [42].

In human cancer cells, stabilization of TERRA R-loop might be achieved by depleting the chromatin remodeler ATRX. ATRX is a chromatin remodeler of the SWI/SNF family that is mutated in most of the cell lines that rely on ALT to survive and it inhibits ALT when expressed ectopically [43,44]. Moreover, ATRX is naturally recruited to telomeres where it counteracts the formation of G-quadruplex and R-loops [43,45]. Therefore, it is possible that cancer cells develop strategies to stabilize R-loops to facilitate ALT, strengthening the importance of the dynamic nature of RNA-DNA hybrids for telomere maintenance to prevent senescent and cancer. Human telomere homeostasis in the context of R-loop stabilization is clearly an interesting topic that will surely shed light on the process of tumor development.

## 7. Conclusions and future directions

Although in recent years R-loops are the focus of research from various fields including genome instability, replication, transcription or telomere homeostasis, there are still plenty of questions unanswered. We know little about any possible mechanism by which R-loops can modulate chromatin as a feature associated with R-loop-dependent genome instability. On the other hand, in relation to possible mechanisms of R-loop stabilization, it would be important to know whether other RNA binding factors, including yeast Npl3 or Nab2 that are also autoregulated at low levels in the cell [46,47], interact with and stabilize R-loops, or whether this is a particularity of Yra1, observed when it is in excess in the cells. In the context of cancer, knowing whether ALY/REF, the human ortholog of Yra1 that is overexpressed in numerous tumors, shares the ability to bind and stabilize R-loops would be interesting [48]. This could open the possibility of exploring a putative implication of R-loops in cancer cells from a different angle, by elucidating the specific consequences of TERRA R-loop stabilization in ALT.

The recent finding of the different behavior of R-loops when they are encountered by a replication fork in co-directional or head-on orientation opens numerous questions on how they are specifically eliminated in one situation and how are they blocking replication in the other. Getting to understand how R-loops damage the DNA in the cells will allow us not only to prevent R-loop accumulation and the subsequent genome instability but also to develop potential strategies based on R-loop stabilization to selectively remove cancer cells (see [3]). Certainly, the more we know about the nature of R-loops the better we will understand how they are connected to diseases like Immunodeficiency, Centromeric instability and Facial anomalies (ICF) syndrome or cancer and hopefully in a better position we would be to control them and their harmful consequences.

## Declaration of Competing Interest

The authors declare no conflicts of interest.

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