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The Yin and Yang of R-loop Biology

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Abstract

RNA performs diverse functions in cells, directing translation, modulating transcription and catalyzing enzymatic reactions. Remarkably RNA can also anneal to its genomic template co- or post-transcriptionally to generate an RNA-DNA hybrid and a displaced single-stranded DNA. These unusual nucleic acid structures are called R-loops. Studies in the last decades concentrated on the detrimental effects of R-loop formation, particularly on genome stability. In fact, R-loops are thought to play a role in several human diseases like cancer and neurodegenerative syndromes. But recent data has revealed that R-loops can also have a positive impact on cell processes, like regulating gene expression, chromosome structure and DNA repair. Here we summarize our current understanding of the formation and dissolution of R-loops, and discuss their negative and positive impact on genome structure and function.

Introduction

For many years RNA-DNA hybrids were known to form during DNA replication (11bp hybrid of an Okazaki fragments), and transcription (8bp hybrid within the RNA polymerase active site). However, longer tracts of RNA-DNA hybrids, known as R-loops are also capable of forming in cells. R-loops form when an RNA molecule anneals to the antisense/template DNA strand after it exits the active site of RNA polymerase, generating a hybrid and a displaced sense ssDNA (Figure 1). R-loops were first identified decades ago, but the recent outburst of studies indicates how they represent an important, but poorly understood aspect of nucleic acid biology [1–3].

Formation of R-loops

In principle, R-loops can form whenever an RNA molecule is allowed to anneal with its template DNA strand. However, the template strand is normally occupied by the sense DNA strand. How then does RNA gain access to the template strand? One simple theory envisions the extension of the usual 8bp RNA-DNA hybrid formed during transcription, but this model could not be reconciled with the crystal structure of RNA polymerase showing that RNA and DNA exit from two distinct channels [4]. A second model, known as the thread back model, comes from the discovery that DNA behind the transcribing RNA polymerase

is negatively supercoiled [5]. This negatively supercoiled duplex DNA has a propensity to unwind, which may allow the template strand to anneal with nascent RNA. This model is supported by the observation that hybrids are elevated in mutants defective in transcription elongation, termination, splicing and relaxation of supercoiled DNA [6–9]. Defects in termination and elongation factors are thought to stall the RNA polymerase, prolonging both the proximity of nascent RNA and the negatively supercoiled (unwound) state of the DNA. Deficiencies in splicing may unmask the RNA, making it more accessible to hybridize with the DNA. Defects in topoisomerases enhance the unwound DNA by preventing relaxation of the negative supercoils. Based on these supporting observations, the thread back mechanism is the prevalent model for hybrid formation.

However, this co-transcriptional model for R-loop formation does not explain why hybrids are elevated in mutants that affect RNA post-transcriptionally, like RNA export and degradation. One potential explanation is that these mutants allow RNA to linger longer in the nucleus and hybridize to a homologous DNA template even after transcription is completed. Indeed, a recent observation revealed that R-loops can form *in trans*; when the RNA transcribed at one locus forms an R-loop with a homologous DNA sequence at another locus [10]. How do these RNAs get access to the antisense DNA? An important clue is that the formation of R-loops *in trans* is promoted by Rad51 and Rad52, proteins that are involved in DNA strand exchange during homologous recombination-mediated repair after DNA double-strand breaks (DSBs). These proteins allow ssDNA at a DSB to invade a homologous dsDNA and form a D-loop. Whether they promote R-loop formation by performing an analogous RNA-strand invasion reaction with dsDNA is unclear. Although there is evidence that RecA, the bacterial homolog of Rad51, promotes R-loop formation *in vitro* and *in vivo* [11]. Importantly, through R-loop formation *in cis* and *trans*, transcripts can form R-loops that act on the locus of origin and potentially many other loci across the genome with similar sequence composition, thereby constraining or amplifying their impact on genome structure and function.

Besides DNA supercoiling, other features of the RNA sequence or its DNA template likely contribute to R-loop formation. Indeed, in vitro high GC content and DNA nicks have also been shown to influence R-loop formation [12]. Recent studies in yeast mapping hybrids genome-wide, have reported a weak correlation between hybrid formation and GC content [13,14]. However, the causality of these features in hybrid formation *in vivo* has not been tested. Thus, the roles of the sequence and structure of nucleic acid are likely critical, but poorly explored aspects of R-loop formation.

Dissolution of R-loops

The elevation of R-loops in cells defect for RNA biogenesis factors and topoisomerase suggests that these proteins act to suppress R-loop formation in wild type cells. However, to remove R-loops when they do form, cells have a number of potential hybrid-dissolution mechanisms [15]. RNase H1 and RNase H2 are enzymes conserved from bacteria to human that specifically degrade the RNA in hybrids. They contain a HBD (hybrid-binding domain) that binds the RNA-DNA substrate and an RNase H domain that catalyzes the cleavage of RNA via a hydrolytic mechanism. RNase H2 contains two auxiliary subunits, important for

protein-protein interaction and complex recruitment [16]. RNase H2 is also able to remove single ribonucleotides mis-incorporated into DNA during replication [17] and Okazaki primer during lagging strand synthesis [18]. Prokaryotic and eukaryotic cells also encode many RNA-DNA helicases that are able to unwind the RNA from R-loops, like Rho, DHX9 and Senataxin [19–21]. Thus all cells have multiple mechanisms for R-loop dissolution.

Why do so many mechanisms for R-loop dissolution exist? The presence of two distinct, but evolutionary conserved RNase H proteins suggests they have some non-overlapping function. So far this distinction has eluded detection. In yeast, an increase in R-loops is observed only when both RNases H are inactivated [9], implying they can substitute for each other to degrade the same R-loops. None-the-less, the increased R-loop formation in cells lacking RNase H activity is surprising given the presence of numerous RNA-DNA helicases. Similarly, the increase in R-loop formation by defects in RNA biogenesis factors in bacteria, yeast and mammalian cells can be suppressed by overexpression of just RNase H1 [8,9] This result begs the question why overexpression is needed, given the cells already have the two RNases H and RNA-DNA helicases. In summary, the presence of multiple hybrid dissolution factors with limited potency infers that each factor is regulated spatially or temporally to constrain its ability to remove R-loops.

The regulation of hybrid dissolution factors may be dictated by their coupling to specific processes of hybrid formation, like RNA transcription and DNA replication [22,23], presumably by protein-protein interaction or their tethering to specific loci. In fact, the discovery that R-loops have positive biological functions necessitates that hybrid dissolution must occur through regulated mechanisms, to ensure that positively acting R-loops are protected. Elucidating the distinct mechanistic and biological roles of the different hybrid dissolution mechanisms is another important challenge to the field.

Detrimental effects of R-loop formation

The first identifications of R-loops came from the discovery that transcribing from a model locus in yeast cells defective for the THO transcription elongation complex caused hyper-recombination in a R-loop dependent manner [6]. Subsequent studies in yeast and mammalian cells showed that R-loops can also induce cell cycle checkpoint activation, DNA damage and gross chromosome rearrangements [24].

In principle, R-loops can cause DNA damage by multiple mechanisms. R-loops result in stretches of exposed ssDNA, which are chemically more unstable than dsDNA, and likely more prone to transcription associated mutagenesis (TAM), recombination (TAR) and double strand breaks (DSBs) [25,26]. In fact, mutations during transcription are mainly found in the non-transcribed DNA strand (the ssDNA in the R-loop), over the transcribed strand (the DNA hybridized with RNA) [27]. Mammalian cells express a variety of DNA and RNA modifying-enzyme such as the activation-induced cytidine deaminase (AID) [28] and members of the ApolipoproteinB mRNA-editing catalytic polypeptide (APOBEC) [29]. These protein complexes can generate abasic sites which can lead to base substitutions or ssDNA nicks that are then processed to DSBs upon arrival of the replication fork [2]. R-loops may also be directly blocking progression of the DNA replication machinery, causing

fork collapse and subsequently DSBs [1,30]. Finally, the RNA in an R-loop may serve as a primer to trigger unscheduled replication, or possibly re-replication. In fact, in bacteria R-loop can be used as sites of replication initiation [31]. Most recently, work in human and yeast cells has revealed the involvement of nucleotide excision repair (NER) endonucleases in processing R-loops into DSBs [32]. The exact mechanism by which NER converts R-loops to DSBs, the resulting lesions, and whether it is the only pathway driving the mutagenic potential R-loops remains to be elucidated.

R-loops in human diseases

The genomic instability mediated by R-loops might contribute to the mutations and gross chromosome rearrangements typical of cancer cells [33]. Common fragile sites (CFSs) are regions in the human genome that contain long genes, replicate late in S-phase, and are hotspots for DNA breaks [34]. R-loops have been found to form at CFSs, where collisions between the DNA replication fork and the transcription machinery contribute to the instability of these loci [35]. Removal of the R-loop trough RNase H overexpression suppresses the genomic instability associated with CFSs. Translocations between the immunoglobulin (Ig) locus and oncogene containing loci are often found in lymphoma cells [36]. A likely cause of these translocations is the improper processing/repair of R-loop-induced genomic rearrangements that are normally part of the immunoglobulin class switch.

Notably, the tumor suppressor BRCA2 is often mutated and inactivated in cancer, in particular breast cancer [37]. BRCA2 depletion has also been shown to cause R-loop accumulation and DNA damage checkpoint activation. These results indicate that BRCA2 might be involved in processing/removal of RNA-DNA hybrids, otherwise a source of replication stress and genomic instability [38]. Finally some cancers have elevated levels of RAD51[39] or altered FIP1L1 activity [40], two proteins that promote hybrid formation in yeast [10]. These examples may be the tip of the iceberg in the links between R-loop formation and cancer development.

Unscheduled R-loop formation may play an important role in several other human diseases (reviewed in [41]). Mutations in the Setx gene (Sen1 in yeast), is associated with ataxia oculomotor apraxia type 2 (AOA2), a neurodegenerative disease characterized by progressive degeneration of the motor neurons in the brain and spinal cord, combined with muscle weakness and atrophy [42]. However, a direct link between the regulation of R-loops and disease etiology is still missing. In fact mouse models that try to recapitulate the human disease show elevated R-loop formation, DNA damage and apoptosis in proliferating cells, but not in post-mitotic cells, like the brain neurons [43]. It is still possible that neurons are particularly sensitive to low levels of R-loop, or R-loop formation in other tissues indirectly impacts neuron function.

A well-characterized example of R-loops involvement in human pathologies comes from studies on fragile X syndrome, the most common genetic form of mental retardation [44,45]. Expansion and contraction of short repetitive DNA sequences has been linked to fragile X syndrome and several other disorders [46]. R-loop formation in these loci might play a key role in transcription regulation and copy number variations. In fact, transcription of

sequences rich in small repeats results in R-loop accumulation mediated by ssDNA prone to form secondary structures, notably G-quartets and triplex strand [47]. DSBs and DNA replication problems due to R-loops may induce the aberrant recombination events that cause nucleotide-repeats expansion.

In summary, R-loops are correlated with a number of cancers and human disease conditions. It will be important to demonstrate a causative role between R-loops and disease etiology. Whether causative or not, the presence of elevated R-loops, at least in cancer cells, may potentially be exploited through synthetic lethal strategies as cancer therapy.

Positive side of R-loops

The first positive function exploited how R-loop formation drives the programmed genomic rearrangement associated with immunoglobulin class switch in activated B-cells. Transcription through the switch immunoglobulin locus drives R-loop formation and provides the ssDNA substrate for the AID enzyme. Accumulation of R-loops initiates the cascade of events that lead to the genomic rearrangements needed to change class of antibody produced [48].

However, a number of recent studies have shown that R-loops play important roles in gene expression. In *Arabidopsis*, the formation of R-loops in the promoter region silences the expression of the long noncoding RNA COOLAIR. R-loops can modulate COOLAIR transcription, which in turns regulates the flowering locus in response to cold temperatures [49]. This is the first reported example on how R-loop formation can regulate expression of a specific gene and modulate physiological processes, like plant flowering. R-loops may also control global gene expression. CpG islands are present in approximately 60% of human genes and regulate their transcription. Also methylated CpG island is a prevalent epigenetic mark of transcriptional silencing [50]. The Chedin group discovered that R-loops form in a subset of transcriptionally active, unmethylated CpG islands [51]. R-loops appear to shield the promoter from the de-novo methyltransferase DNMT3B1, keeping genes transcriptionally active.

A second emerging and complex function of R-loops is as a regulator of chromosome structure. R-loops are associated with a heterochromatin marker, H3S10 phosphorylation both in yeast and human cells [52]. H3S10 is known to be associated with changes in chromosome structure. Overexpression of RNase H not only reduces R-loop accumulation, but also H3S10 phosphorylation and accessibility of chromatin to restriction enzyme digestion, suggesting a link between R-loops, epigenetic modifications and chromatin compaction. It is worth mentioning, that H3S10 has been reported to also mark actively transcribed genes, usually characterized by open chromatin, in mouse and drosophila. The Proudfoot lab has shown that R-loops form on G-rich transcription-termination regions, promoting RNA polymerase pausing prior to termination [53]. Moreover, R-loop formation drives transcription of antisense RNA, which in turn recruits epigenetic protein complexes. The accumulation of specific epigenetic marks drives the formation of heterochromatin DNA in this regions, helping an efficient transcription termination [54]. The formation of R-loops might drive local chromatin changes in the promoter, gene body or terminator region,

to fine tune gene expression levels. These data may explain why the overexpression of RNase H1 is toxic to cells [55], potentially because the unconstrained RNase H1 removes critical R-loops needed for modulating chromosome structure and gene expression.

R-loops also form at telomeres where the non-coding RNA TERRA hybridizes on the telomeric DNA. Formation of R-loops at telomeres in cells lacking the telomerase enzyme mediates telomeres elongation and delays cell senescence [56]. This observation is intriguing given the role of cell senescence in preventing cancer proliferation [33].

RNA-DNA hybrids may also act as structural element in an alternative pathway for DSBs repair. Storici and colleagues showed that yeast, *S.Cerevisiae*, is able to repair a restriction enzyme-mediated break using an mRNA as a template [57].

Finally, Prader–Willi syndrome (PWS) and Angelman syndrome (AS) are oppositely imprinted autism-spectrum disorders with a known genomic locus regulated by complex epigenetic mechanism. Topoisomerase inhibitor topotecan has been shown to stabilize formation of R-loop in the PWS/AS locus restoring physiological gene expression and chromatin decondensation [58,59]. Thus as in cancer, R-loop biology might provide new targets for therapeutic strategies.

Concluding remarks

The bloom of studies published in the last decades concentrated on the detrimental effects of R-loops, shedding some light on the proteins and mechanisms that cells have evolved to prevent R-loops formation, or to remove them once present. R-loops were considered byproducts of transcription and a potential threat to genomic stability [1]. But the real impact of this “dark side” of R-loops still needs to be fully addressed: we still do not know if R-loops are directly involved in the etiology of human diseases like cancer and neurodegenerative syndromes.

On the other end, recent data suggest that R-loops also have a “bright side”, and contribute in regulating different physiological cell processes, like chromosomal structure and gene expression (Figure 2). A big challenge in the field is to understand the mechanisms that regulate R-loops and create the balance between the positive and negative effects.

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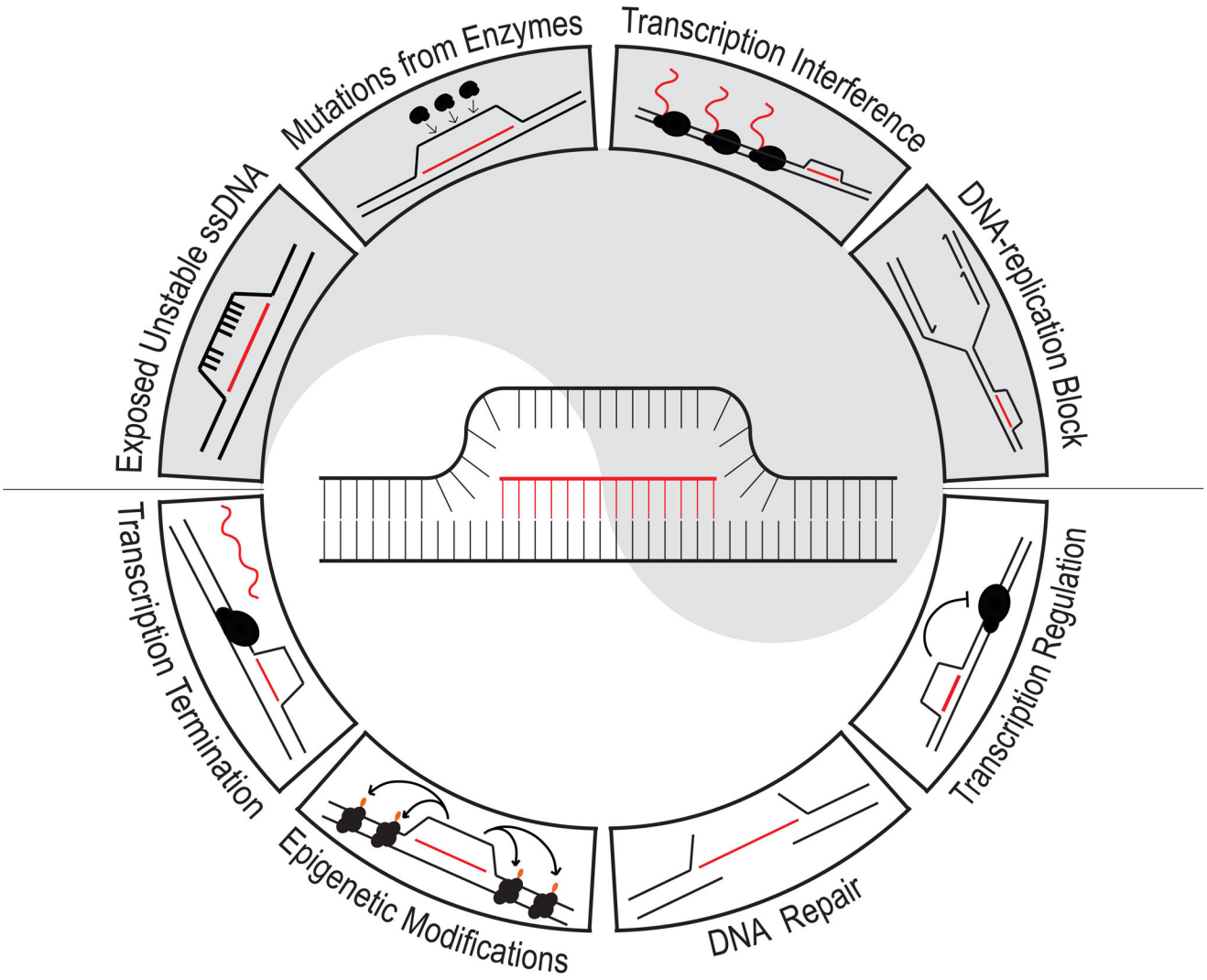

Figure 1. R-loop formation and dissolution

The R-loop can form co-transcriptionally (*in cis*) or post-transcriptionally (*in trans*), a process mediated by Rad51. R-loop formation is elevated by defects in RNA processing factors that coat, splice, export and degrade the nascent RNA. R-loop dissolution is promoted by RNase H enzymes that recognize and degrade the RNA in the R-loop, and by RNA-DNA helicases that unwind the RNA molecule.

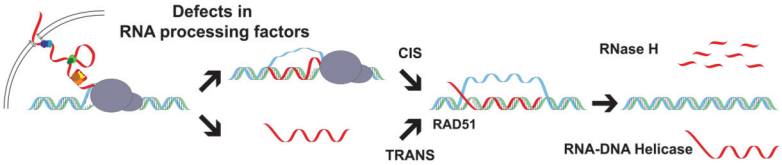

Figure 2. The Yin and Yang of R-loop Biology

R-loop formation can have a detrimental impact on the genome stability, but also contributes positively in regulating cellular processes. Several factors can contribute to R-loop mediated genomic instability: exposure of unstable ssDNA, proteins that modify the ssDNA, R-loop interference with the transcription and replication machinery. On the other side, R-loops can contribute to efficiently terminate transcription, recruit epigenetic regulators to modulate gene expression and chromosome compaction, contribute to DNA repair and regulate transcription of specific genes.