

REVIEW

A double-edged sword: R loops as threats to genome integrity and powerful regulators of gene expression

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R loops are three-stranded nucleic acid structures that comprise nascent RNA hybridized with the DNA template, leaving the nontemplate DNA single-stranded. R loops form naturally during transcription even though their persistent formation can be a risky outcome with deleterious effects on genome integrity. On the other hand, over the last few years, an increasingly strong case has been built for R loops as potential regulators of gene expression. Therefore, understanding their function and regulation under these opposite situations is essential to fully characterize the mechanisms that control genome integrity and gene expression. Here we review recent findings about these interesting structures that highlight their opposite roles in cellular fitness.

The R-loop structure was first characterized >38 years ago (Thomas et al. 1976), and the first demonstration that R loops exist in vivo came in 1995 with studies described by Crouch and colleagues (Drolet et al. 1995). They showed that R-loop formation occurs in a bacterial cell and is a consequence of the transcription process (Drolet et al. 1995). Since then and especially over the last decade, the R-loop field has become an increasingly expanded area of research, placing these structures as a potential regulator of gene expression but also as a major threat to genome stability.

Transcription-mediated R-loop formation

In general, where R loops have been described in vivo, the RNA strand is generated by RNA polymerase II (Pol II) transcribing a C-rich DNA template so that a G-rich transcript is generated. Interestingly, several studies have shown that R loops are formed preferentially when the nontemplate strand is G-rich (Reaban et al. 1994; Li and Manley 2005; Ginno et al. 2012, 2013). The increased thermodynamic stability of a G-rich RNA strand bound

to C-rich DNA could be a reason for this sequence specificity (Sugimoto et al. 1995). However, it is as yet unclear how R loops are generated. According to the “extended RNA/DNA hybrid” model, the RNA/DNA hybrid duplex could be the result of an extension of the usual 8-base-pair (bp) RNA/DNA hybrid (Westover et al. 2004) within the transcription bubble as Pol II elongates. This model, however, is inconsistent with the crystallographic structure of Pol II that demonstrates the exit of DNA and RNA molecules through different channels (Westover et al. 2004), strongly arguing against it (Aguilera and Garcia-Muse 2012). A more plausible model suggests that the RNA/DNA hybrid could arise by threading back the RNA before the two strands of the DNA duplex reanneal (called the “thread back model”). According to extensive in vitro studies from the Lieber laboratory (Roy and Lieber 2009), R loops depend on three features: high G density, negative supercoiling, and DNA nicks (Roy et al. 2010). Initial R-loop formation is favored by G clusters and DNA nicks downstream from the promoter on the nontemplate DNA strand, whereas subsequent RNA/DNA hybrid extension and stabilization are enhanced by high G density and negative supercoiling (Aguilera and Garcia-Muse 2012).

Once formed, R loops are particularly stable, as RNA/DNA associations are thermodynamically more stable than DNA/DNA interactions (Roberts and Crothers 1992). This may be due to the structure of the RNA/DNA hybrid, which is thought to adopt a conformation that is an intermediate between the A form of a dsRNA and the B form of a DNA duplex (Shaw and Arya 2008). Another possibility is that a G quadruplex (G4) formed on the single-stranded exposed strand, such as in the case of the immunoglobulin (Ig) class switch region (Duquette et al. 2004), stabilizes the R-loop structure.

R loops have been reported in vivo at prokaryotic origins of replication (Masukata and Tomizawa 1984, 1990; Baker and Kornberg 1988; Lee and Clayton 1996; Carles-Kinch and Kreuzer 1997), the mitochondrial origin of replication (Xu and Clayton 1996), and the mammalian Ig class

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switch region in activated B lymphocytes (Yu et al. 2003). In the latter case, R-loop formation is involved in facilitating class switch recombination (CSR) that generates diverse antibody isotypes.

R loops are not restricted to Pol II transcripts. Very highly transcribed Pol I rDNA repeats also form R loops (El Hage et al. 2010). Although Pol II transcripts were not thought to be associated with R loops, recent genomic analysis of R loops in *Saccharomyces cerevisiae* showed the presence of R loops over Pol III transcribed tRNA genes in various mutant backgrounds (Chan et al. 2014). This implies that Pol III transcripts can also form R loops in normal cells. In this review, we focus only on R loops formed in Pol II transcripts.

R loops and genomic instability

Transcription can be a “risky” process. R loops can lead to DNA damage by the exposure of ssDNA formed as a result of the RNA/DNA hybridization. Being more unstable, the ssDNA would then be susceptible to lesions and transcription-associated mutagenesis (TAM) or transcription-associated recombination (TAR) (see Fig. 1). However, the mechanism leading from an R loop to genomic instability still remains largely unknown. Possibly, the unpaired DNA strand resulting from R-loop formation is more susceptible to DNA damage such as spontaneous deamination of dC to dU, leading to double-strand breaks (DSBs) and recombination (Aguilera 2002; Li and Manley 2006; Aguilera and Garcia-Muse 2012). Thus, in one possible model, accumulation of R loops could make certain regions of the genome more prone to DNA-damaging agents by increasing the occurrence of single-stranded regions. In a second potential model, a protein recognizing R-loop structures could be involved in initiating the generation of mutagenesis. One possible candidate is the activation-induced cytidine deaminase (AID). AID is an enzyme that promotes Ig heavy chain CSR and hypermutation in B lymphocytes. It functions by deaminating cytosines into uracils on single-strand target DNA sequences (Muramatsu et al. 2000; Revy et al. 2000). R loops forming behind elongating Pol II can provide the ssDNA substrate for this enzyme (Yu et al. 2003). The generated U:G mismatch may then be replicated, creating two daughter species, one of which will undergo a C → T transition mutation. However, dU could also be processed by base excision repair (BER) components such as uracil-DNA glycosylase and abasic endonuclease (APE). These enzymes remove the uracil, creating a DNA nick or leaving an abasic site (a site of base loss). Replication past the abasic site will result in random incorporation of any of the four nucleotides, possibly leading to further mutations. DNA nicks could also be converted to DNA DSBs that are recognized by the recombination-mediated repair machinery, ultimately leading to CSR for antibody genes (Di Noia and Neuberger 2002) or, more generally, DNA translocations. However, AID is specifically expressed in activated B cells and in chicken DT40 cells (which are B-cell-derived). This raises the question of how R-loop

R loops: gene regulators and genome threats

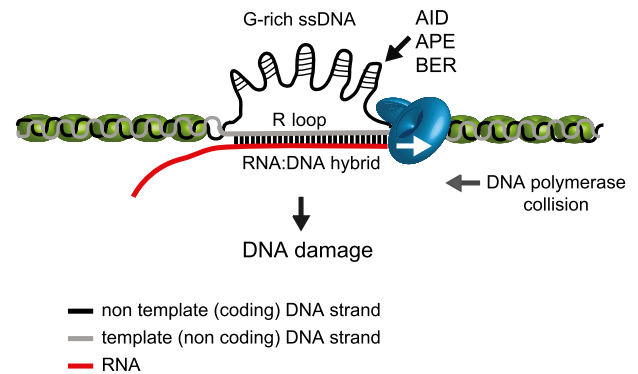


Figure 1. R loops as a source of DNA damage. Nascent transcripts behind elongating Pol II can invade the DNA duplex and hybridize with the DNA template strand. The RNA/DNA hybrid so formed displaces the nontemplate strand, and this three-stranded structure constitutes an R loop. R loops can cause genomic instability in different ways. First, the displaced ssDNA can act as a substrate to DNA-damaging agents, deaminases (AID), and repair enzymes (APE and BER), leading to DNA lesions and nicks. Second, G4 structures forming on the G-rich nontemplate strand can generate susceptible sites for nucleases. Finally, transcription elongation machinery impeded by stable R loops can cause replication–transcription collisions, leading to DNA recombination and DSBs. Points of contact between the DNA strand and nascent RNA indicate R-loop formation, whereas points of contact within the ssDNA indicate G4 structures. Pol II is shown as a blue icon, with an arrow indicating transcription direction. Nucleosomes are shown in green. The diagram is not drawn to scale.

formation leads to genomic instability in cells that lack AID. DSBs have also been observed in HeLa cells upon depletion of SRSF1 (Li and Manley 2005), suggesting that other proteins could function analogously to AID in different cell types to initiate R-loop-induced genomic instability. Alternatively, spontaneous dC → dU mutation may occur at low levels.

An additional possible scenario suggests that transcriptional R loops induce genomic instability by interfering with DNA replication (Aguilera 2002; Gan et al. 2011; Houlard et al. 2011; Aguilera and Garcia-Muse 2012). Thus, replication fork collisions with blocked Pol II have been shown to induce TAR or DNA breaks in budding yeast and mammals (Prado and Aguilera 2005; Gottipati et al. 2008; Boubakri et al. 2010). Unrepaired DNA lesions formed on the ssDNA of the R loop or the RNA/DNA hybrids themselves forming behind elongating Pol II may somehow restrict transcription, which in turn may block replication forks. Such blocked replication forks could then generate DNA lesions and DSBs in the newly synthesized DNA. These would induce recombination-mediated repair, which in turn could lead to chromosome rearrangements and genomic instability (Aguilera and Garcia-Muse 2012). R loops are prevalent in meiosis, at least in *S. cerevisiae* and *Caenorhabditis elegans*, and their accumulation leads to replication impairment and genomic instability (Castellano-Pozo et al. 2012). In mammalian cells, replication–transcription collisions in long human genes (≥ 800 kb) have been shown to be

associated with R-loop accumulation, which in turn causes instability at so-called common fragile sites (CFSs) (Helmrich et al. 2011). Instability at CFSs may also relate to slower or incomplete replication in areas of chromatin compaction (Debatisse et al. 2012).

Additional factors leading from a transient R-loop structure to deleterious genomic instability have only recently been identified. R-loop accumulation in *S. cerevisiae*, *C. elegans*, and human cells has been linked to histone 3 Ser10 phosphorylation (H3S10P), a mark of chromatin compaction (Castellano-Pozo et al. 2013). It is proposed that R loops trigger formation of the H3S10P mark, which in turn could cause replication fork stalling, transcription–replication collisions, and, ultimately, DSBs in the newly synthesized DNA (Castellano-Pozo et al. 2013).

Surveillance mechanisms: What protects us from R loops?

The deleterious effects of R loops formed during transcription on genome integrity have been variously documented (Aguilera 2002). Given that formation of R loops is an evolutionarily conserved mechanism (Li and Manley 2005; Aguilera and Garcia-Muse 2012), it is perhaps not surprising that different organisms have used diverse mechanisms to protect their genomes (Li and Manley 2006; Aguilera and Garcia-Muse 2012). So far, five different mechanisms are thought to regulate R-loop formation (see Fig. 2): (1) RNase H enzyme, which specifically degrades the RNA in RNA/DNA hybrid (for review, see Cerritelli and Crouch 2009); (2) RNA/DNA helicases such as the yeast Sen1 or homologous human Senataxin (Mischo et al. 2011; Skourti-Stathaki et al. 2011) and the human DHX9 helicase, which also acts on G4 structures (Chakraborty and Grosse 2011); (3) topoisomerases, which relax DNA-negative supercoiling that otherwise causes persistent R-loop formation (Drolet et al. 1994, 1995; Tuduri et al. 2009; El Hage et al. 2010; Yang et al. 2014); (4) proteins that prevent R-loop formation, such as mRNA biogenesis (Huertas and Aguilera 2003; Dominguez-Sanchez et al. 2011; Castellano-Pozo et al. 2012) and processing proteins (Li and Manley 2005; Paulsen et al. 2009; Wahba et al. 2011; Stirling et al. 2012; Santos-Pereira et al. 2013); and (5) suppressors of proteins that promote R-loop formation (i.e., Rad51 and AtNDX) (Sun et al. 2013; Wahba et al. 2013).

Factors that prevent R-loop formation

In *S. cerevisiae*, transcription-induced R loops were first documented with the characterization of the THO complex, suggested to be involved in transcriptional elongation (Aguilera 2002). This complex consists of four nuclear proteins (Hrp1, Tho2, Mft1, and Thp2) and is associated with the transcription–export complex (TREX) containing Tex1 and the mRNA export factors Sub2 and Yra1 (Chavez et al. 2000; Strasser et al. 2002). The physical interaction of the THO complex with TREX directly links mRNA packaging with RNA export. Mutations affecting THO/TREX have been shown to affect transcriptional elongation, proper

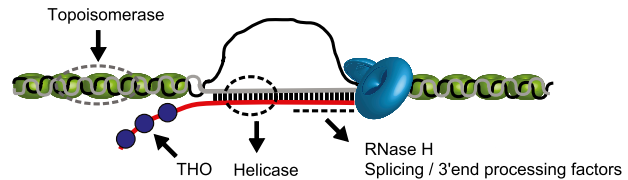


Figure 2. Diverse protection mechanisms against R-loop formation. Two types of surveillance factors have been identified: factors that prevent formation of R loops and factors that actively remove them. DNA topoisomerase enzymes suppress R-loop formation by relaxing the negative supercoiling behind elongating Pol II. The THO complex (blue circle) facilitates efficient packaging of nascent RNA into messenger ribonucleotide proteins (mRNPs), preventing R-loop formation. Splicing and 3' end processing factors associate with nascent RNA and prevent R loops. RNA/DNA helicases and RNase H enzymes remove R loops once formed. DNA is shown as gray and black lines, and RNA is shown as a red line. Dotted lines indicate the site of action of different factors. The diagram is not drawn to scale.

mRNA export, and recombination (Strasser et al. 2002; Rondon et al. 2003). A distinctive phenotype of yeast THO mutants (first identified in the *HRP1* and *THO2* genes) is their transcription-associated hyperrecombination phenotype (Aguilera and Klein 1990; Piruat and Aguilera 1998), and this is directly associated with R-loop formation (Huertas and Aguilera 2003).

The THO/TREX complex is responsible for packaging of pre-mRNA with RNA-binding proteins. Recently, another factor involved in mRNA export and processing, the yeast Npl3, also prevented R-loop induced transcription–replication collisions and genome instability (Santos-Pereira et al. 2013), suggesting a functional link between RNA metabolism and R-loop-associated genomic instability. As the nascent transcript emerges from elongating Pol II, it may have two immediate fates. It either is cotranscriptionally packaged into messenger ribonucleotide proteins (mRNPs) and exported through the nuclear pore or may invade the DNA duplex behind the elongating Pol II to form R-loop structures. R loops could then interfere with DNA replication, induce ssDNA breaks, or become recombination intermediates. Consequently, it could be argued that efficient mRNA packaging into mRNPs prevents R-loop formation and in turn restricts TAR and DNA damage (Huertas and Aguilera 2003; Moore and Proudfoot 2009; Mischo et al. 2011; Aguilera and Garcia-Muse 2012; Santos-Pereira et al. 2013). However, it has been reported that even with normal mRNP biogenesis, accumulation of R loops can still occur (Mischo et al. 2011), suggesting that R loops form at a much higher frequency than once thought.

DNA TOP1 is an evolutionarily conserved factor that suppresses R-loop formation (Drolet et al. 1995; Tuduri et al. 2009; El Hage et al. 2010). This is possibly due to the ability of TOP1 to relax negative DNA supercoiling. In the absence of TOP1, negative supercoils accumulate behind elongating Pol II to promote opening of DNA, which in turn facilitates annealing between the nascent RNA and DNA template strand with subsequent R-loop formation in bacteria (Drolet et al. 1995), *S. cerevisiae*

(El Hage et al. 2010), and human cells (Tuduri et al. 2009). Very recently, TOP3B, another member of this subfamily, was found to reduce negative supercoiling and R-loop formation (Yang et al. 2014). The interesting point here is that TOP3B is recruited to human and mouse gene loci by recognizing arginine methylation histone marks through its interaction with the methyl-arginine effector tudor domain-containing protein 3 (TDRD3). According to the proposed model, the TDRD3–TOP3B complex is recruited to regions of active transcription; TDRD3 recognizes the methyl-arginine histone marks and the methylated C-terminal domain (CTD) of Pol II, whereas TOP3B resolves negative supercoiling that forms behind elongating Pol II and by doing so restricts R-loop formation (Yang et al. 2014). Interestingly, in this study, formation of R loops is controlled by the presence of a chromatin mark as opposed to the alternative scenario suggested by the Aguilera laboratory (Castellano-Pozo et al. 2013), where accumulated R loops cause formation of H3S10P. In the future, it will be intriguing to test under which conditions R loops regulate chromatin structure (and consequently genome dynamics). Indeed, are they a cause or consequence of the epigenetic microenvironment?

Genes of higher vertebrates are significantly longer, and the presence of introns is almost ubiquitous; therefore, they have adapted some additional mechanisms to protect their genomes. The SRSF1, a serine–arginine-rich (SR) protein that regulates the first steps of splicing, appears to interconnect pre-mRNA processing and genomic instability (Li and Manley 2005). Li and Manley (2005) demonstrated in chicken DT40 cells and human HeLa cells that depletion of SRSF1 causes the nascent transcript to form R loops, which in turn promote DNA rearrangements mediated by DSBs. In essence, the model proposed is that SRSF1 is cotranscriptionally loaded onto the nascent pre-mRNA via the phosphorylated CTD of Pol II to not only promote splicing but also prevent R-loop formation and subsequent genomic instability (Aguilera 2005; Li and Manley 2005). This is a clear example of the connections between transcription-induced R loops, pre-mRNA processing, the Pol II CTD, and transcription-associated genomic instability.

The cotranscriptional R-loop-induced genomic instability observed in DT40 cells with the loss of SRSF1 factor (Li and Manley 2005, 2006) resembles the phenotype of yeast THO mutants (Huertas and Aguilera 2003). Until recently, the involvement of the human THO/TREX complex in genomic instability was not clearly identified. A study from the Aguilera laboratory (Dominguez-Sanchez et al. 2011), however, revealed that the interplay between mRNP biogenesis and genomic instability is indeed conserved from yeast to humans. In essence, depletion of the human THO complex results in accumulated DNA breaks and consequent genomic instability, which is dependent on R-loop formation (Dominguez-Sanchez et al. 2011). In addition to this, genome-wide data suggest that RNA processing factors help prevent genomic instability (Paulsen et al. 2009), further pointing toward a powerful interplay between pre-mRNA processing and R-loop-dependent genomic instability.

Factors that remove R loops

As mentioned above, apart from the active prevention of R loops, cells also use a range of dedicated factors to actively remove them once formed. First of all, the RNase H enzymes act to cleave the RNA of RNA/DNA hybrids (Stein and Hausen 1969; Hausen and Stein 1970; Cerritelli and Crouch 2009). In most organisms, there are two types of RNase H. Eukaryotic RNase H1 consists of a single polypeptide, with the N-terminal domain being responsible for binding to the RNA/DNA hybrid (hybrid-binding domain or HBD), and the CTD containing the RNase H active site (Cerritelli and Crouch 2009). RNase H2 is composed of three different polypeptides, with the RNase 2A being the catalytic subunit. RNase H1 and RNase H2 endonucleolytically cleave the RNA within the RNA/DNA hybrid in a sequence-independent manner. However, they may have different *in vivo* substrates due to their differences in hybrid hydrolysis.

RNase H1 is present in the nucleus and mitochondria and is essential for mitochondrial replication (Cerritelli et al. 2003). Overexpression of nuclear RNase H1 (Cerritelli et al. 2003) has been widely used to experimentally remove R loops and so far is perhaps the only well-studied and efficient way to diminish the cellular levels of R loops. RNase H2 is not as well defined due to its multisubunit composition and low abundance. It is believed to be mostly a repair enzyme due to its substrate specificity. Unlike RNase H1, RNase H2 can recognize and cleave a single ribonucleotide inserted in a DNA duplex, and therefore it was suggested that RNase H2 removes ribonucleotides misincorporated into DNA (Eder et al. 1993; for review, see Cerritelli and Crouch 2009). RNase H2 is also responsible for removing the Okazaki fragment RNA primers from the newly synthesized lagging strand during DNA replication (Murante et al. 1998; for review, see Cerritelli and Crouch 2009). Recently, another very interesting function of RNase H2 was shown: It can uniquely process R loops that are generated during DNA replication/repair (Chon et al. 2013). It was also suggested in this same study that RNase H1 and RNase H2 have some overlapping specificities in R-loop resolution; however, RNase H1 is mainly responsible for the resolution of transcription-associated R loops (Chon et al. 2013).

Second, the yeast Sen1, a superfamily I RNA/DNA helicase (Kim et al. 1999), acts to remove R loops and prevent genomic instability by R-loop-mediated DNA damage (Mischo et al. 2011). R loops accumulate in a *sen1-1* strain that carries a mutation in the helicase domain of Sen1. Furthermore, *SEN1* genetically interacts with genes involved in homologous recombination (HR). Sen1 is also a termination factor for coding and noncoding genes (Ursic et al. 1997; Steinmetz et al. 2006; Kawauchi et al. 2008). Sen1, Nrd1, and Nab3 proteins comprise the NRD complex, the major factor in promoting sno/snRNA termination (Ursic et al. 1997; Steinmetz et al. 2001). Sen1 binds to the CTD phosphorylated on Ser2 (Ursic et al. 2004; Chinchilla et al. 2012), which possibly facilitates its recruitment to multiple coding as well as noncoding genes,

where it tends to accumulate toward the 3' end (Chinchilla et al. 2012). Sen1 may also play a role in coordinating transcription and replication, since it is associated with replication forks across Pol II active genes (Alzu et al. 2012).

Senataxin is the human homolog of Sen1 and has also been implicated in transcriptional termination (Suraweera et al. 2009; Skourti-Stathaki et al. 2011; Padmanabhan et al. 2012). Senataxin was initially identified when mutations causing ataxia oculomotor apraxia 2 (AOA2) and amyotrophic lateral sclerosis type 4 (ALS4) were mapped to the *SETX* gene (James and Talbot 2006; Palau and Espinos 2006). AOA2 mutations include both missense and nonsense mutations leading to senataxin loss of function, whereas mutations linked to ALS4 appear to be missense, dominant mutations resulting in gain of function (Chen et al. 2004; Arning et al. 2013). These diseases are associated with the progressive degeneration of motor neurons in the brain and spinal cord, progressive muscle weakness, and atrophy. *SETX* encodes a 302.8-kD widely expressed protein containing an N-terminal putative protein–protein interaction domain and a C-terminal DEAD-box helicase domain followed by a nuclear localization signal (NLS) (Chen et al. 2004). Most senataxin mutations found in AOA2/ALS4 families either cause premature translational termination or interfere with the function of the helicase or N-terminal protein interaction domains (Chen et al. 2004; Moreira et al. 2004; Duquette et al. 2005; Criscuolo et al. 2006; Fogel and Perlman 2006). However, the precise mechanism of toxicity caused by these mutations and manifested in AOA2/ALS4 patients remains to be elucidated. Similar to its yeast counterpart, senataxin interacts with Pol II and other RNA processing factors, such as poly(A)-binding proteins 1 and 2 (PABP1/2), hnRNPs, SAP155, and SMN, pointing toward a role for senataxin in pre-mRNA processing as well as transcriptional termination (Suraweera et al. 2009).

Increasing evidence identifies senataxin as a DNA repair enzyme (Becherel et al. 2013; Yüce and West 2013) in addition to its role in the resolution of R loops arising at G-rich termination pause sites (Skourti-Stathaki et al. 2011). Yüce and West (2013) revealed that senataxin forms increased nuclear foci in S/G2 phase in response to DNA damage and impaired DNA replication. Importantly, these foci decreased significantly after R-loop resolution or transcriptional inhibition (Yüce and West 2013). The role of senataxin in DNA damage response, particularly during mouse male meiosis (spermatogenesis), has been highlighted by analysis of mouse strains with *SETX* gene knockouts. This study led to a model in which senataxin is proposed to resolve R loops to ensure genome integrity during meiotic recombination (Becherel et al. 2013). THO mutants from *C. elegans* and *S. cerevisiae* also show defective meiosis and increased DNA damage (Castellano-Pozo et al. 2012), pointing toward an evolutionarily conserved mechanism to maintain genome stability during meiosis by preventing formation of these potentially harmful structures.

It is of note that defects in DNA repair enzymes are strongly linked with neurodegenerative disorders

(McKinnon 2009). In the future, it will be of vital importance to understand why defects in senataxin, a ubiquitously expressed protein, particularly affect neuronal cells. AOA2 disorder manifests primarily in post-replication neurons where DNA repair could rely mostly on non-homologous end-joining (NHEJ) rather than HR. This could possibly increase genomic instability in these neurons. A recent study suggests a novel role for the exosome in senataxin-mediated DNA damage response (Richard et al. 2013). In essence, the exosome interacts with sumoylated senataxin, which is in turn targeted to DNA damage regions. Importantly, both sumoylation and the interaction are disrupted in AOA2, but not ALS4, disorder. In agreement, it has also been shown that deletion of *TRF4* in *S. cerevisiae*, a component of the TRAMP complex that activates the exosome, leads to accumulation of R loops and genomic instability (Gavalda et al. 2013). Significantly, the exosome also associates with AID in the R-loop-enriched switch regions of B cells (Basu et al. 2011). The physiological role of the exosome–senataxin interaction has yet to be established. Future research is necessary to understand why and how this mechanism is disrupted in AOA2 disorder.

Rad51 and *trans*-induced R loops

So far, we described here what is known about the factors that prevent or resolve R loops. However, do some factors actively promote R-loop formation? One particular factor has been shown to promote *in vivo* R-loop formation in *S. cerevisiae*: the Rad51 protein (see Fig. 3; Wahba et al. 2013). Eukaryotic Rad51 protein that is homologous to the bacterial RecA plays a major role in HR during DNA repair of DSBs. Its loading on “damaged” DNA is thought to be stimulated by the ssDNA protein RPA. Rad51 then promotes strand exchange (invasion of ssDNA into duplex DNA) by forming nucleoprotein filaments (Benson et al. 1994; Baumann et al. 1996). The bacterial RecA was also shown to promote RNA/DNA hybrid formation *in vitro* (Kasahara et al. 2000; Zaitsev and Kowalczykowski 2000). The Koshland laboratory (Wahba et al. 2013) demonstrated that in *S. cerevisiae*, deletion of Rad51 results in reduced formation of R loops and subsequent genomic instability, especially when R loops are enhanced by inactivation of RNA processing activities. Furthermore, Rad51 colocalizes with R loops prior to formation of any DSBs. Paradoxically, Rad51 is not only a repair factor but also promotes R-loop-mediated DNA damage and genomic instability. This is particularly important in cancer cells where Rad51 could directly promote tumor biogenesis. Two regulators of Rad51 have been described: Rad52, which is required for binding of Rad51 to ssDNA, and Srs2, which acts as an antagonist and consequently prevents R-loop formation (Wahba et al. 2013). Therefore, Srs2 can be added to the list of proteins that prevent R-loop formation and subsequent induced genomic instability.

Interestingly, in the absence of Rad51, transcription itself fails to lead to accumulation of R loops and genomic instability (Wahba et al. 2013). A yeast strain was generated with two copies of a particular human DNA

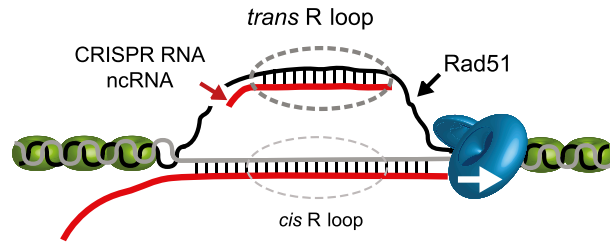


Figure 3. Rad51 can promote *cis* and *trans* R loops. The HR factor Rad51 can promote strand exchange, ultimately leading to cotranscriptional R-loop formation (*cis* R-loop). *Trans* R loops can also be mediated by Rad51. As shown in the diagram, *trans* RNA may target the ssDNA as part of a pre-existing R loop. Alternatively, *trans* RNA could target dsDNA if local unwinding of the DNA duplex occurs by mechanisms such as DNA replication. Such *trans* R loops are associated with the popular CRISPR–Cas9 system in which CRISPR guide RNA hybridizes with target DNA loci generating targeted DNA breaks. *Trans* R loops can also occur between ncRNAs and homologous DNA, ultimately leading to transcriptional gene silencing. DNA is shown as gray and black lines, and RNA is shown as a red line. The diagram is not drawn to scale.

sequence: one in a yeast artificial chromosome (YAC) and the other in one of yeast's own chromosomes but under induced transcription regulation. When transcription was activated, the induced transcript invaded the homologous DNA in the YAC, resulting in the formation of an R loop in *trans* (away from the initial transcription point). The formation of this *trans* R loop was promoted by Rad51 (Wahba et al. 2013). This finding challenges for the first time the dogma in the field that R loops form only cotranscriptionally (in *cis*). It also raises important questions of R-loop-induced genomic instability. *Trans*-induced R loops could be a bigger threat to genome integrity than those formed in *cis*. Upon transcription of highly repetitive elements, in *cis* R loops would only occur at that region, whereas in *trans*-induced R loops could occur in many places across the genome, creating multiple “hot spots” for genomic instability.

Trans-induced R loops have also recently been shown to enable a broad range of applications, including fast generation of genetically modified cells and animals (Gratz et al. 2013; Hwang et al. 2013; Wang et al. 2013; Yang et al. 2013) and genetic screening at a genomic level (Shalem et al. 2014; Wang et al. 2014). The so-called CRISPR (clustered regularly interspaced short palindromic repeat)–Cas9 system is a naturally occurring microbial immune system for protection against phage and other genetic elements. (for review, see Terns and Terns 2011). Short DNA fragments from infecting phage genomes are incorporated into the host genome within the CRISPR locus. Transcription of this CRISPR locus then gives rise to CRISPR RNA, which in turn is processed into short RNAs consisting of phage sequence and repeat elements from the CRISPR locus. These small RNAs complexed with Cas9 act as guides to target the homologous DNA locus, creating a *trans* R loop. Cas9 then cuts the target locus on each strand and ultimately silences the target DNA (Jinek et al. 2012). Very recently, the crystal structure of Cas9 in

complex with guide RNA and target DNA was reported, revealing the key functional interactions, including a bona fide RNA/DNA hybrid (Nishimasu et al. 2014). By analogy to CRISPR–Cas9, it is tempting to speculate that *trans* R loops could explain how noncoding RNAs (ncRNAs) generally target homologous DNA, which is an underlying principle of transcriptional gene silencing by the RNAi machinery.

Even though the precise targeting of CRISPR guide RNA to target loci (Jinek et al. 2012) has made this system very popular for genome editing, little is known about the actual molecular mechanism. Given that the target DNA is in a duplex, how does guide RNA hybridize with DNA? Does Cas9 itself or another factor first denature the DNA to allow RNA/DNA hybridization? An alternative, interesting scenario could be that *cis* R loops must form prior to CRISPR targeting and so enable hybridization of the now single-stranded target DNA with the CRISPR guide RNA. In this case, targeting would be dependent on the formation of *cis* R loops and consequently on active transcription.

So far, we discussed here R loops as precursors of chromosomal rearrangements in yeast and mammalian cells with deleterious consequences to cell integrity. But is the formation of R loops always unprogrammed and potentially harmful?

The new era of R loops: from threats of genomic instability to powerful regulators of gene expression

Over the last decade and particularly the last 3 years, a new era has emerged for the R-loop field, identifying these structures as powerful regulators of gene expression. So far, we documented the unprogrammed formation of R loops as a rare outcome of the transcriptional process with potential harmful consequences—in effect, an enemy to cellular fitness (Aguilera and Garcia-Muse 2012). However, the “other side of the coin” is more positive. An ever-increasing body of evidence has shed light on a number of biological processes controlled by the programmed formation of R loops.

The first beneficial function of R loops to be uncovered was CSR at the Ig heavy chain locus in activated B cells (Yu et al. 2003), as mentioned above. This study is of particular importance, as it firstly demonstrated the *in vivo* formation of R loops over switch regions that undergo CSR and revealed that these R-loop structures facilitate CSR only under specific conditions. R loops forming at the CSR locus are quite long (>1 kb) and very stable, as opposed to R loops at replication origins. The length of the observed stable R loop led to the view that the DNA sequence itself might play a vital role in R-loop formation and stabilization. Indeed, these switch regions are highly repetitive, GC-rich regions. Formation of transcription-dependent R loops over these regions leaves the G-rich nontemplate DNA strand displaced. Based on an analysis using bisulfite treatment to target ssDNA, it was also shown that stable R loops occur only in the physiological orientation (with G-rich transcripts) (Yu et al. 2003).

Controlling gene expression requires the definition of gene boundaries: the 5' end promoter and 3' end terminator. R loops have been recently shown to form at both gene ends (see Fig. 4; Skourti-Stathaki et al. 2011; Ginno et al. 2012, 2013). However, do they control gene expression in these cases? The Chedin laboratory (Ginno et al. 2012, 2013) has recently presented evidence for widespread R-loop formation over the 5' regions downstream from CpG promoters in the human genome. These genomic regions are GC-rich and have a strong positive GC skew (template strand having an excess of C vs. G residues). Even though a direct association of R loops in the maintenance of the unmethylated state of CpG promoters has not been established, this study suggested that R loops forming at promoter regions could potentially lead to the activation of genes by recruiting either the protective histone 3 Lys4 trimethylation (H3K4me3) mark or the DNA demethylation complex (Ginno et al. 2012). Consistent with this model, the AID complex is also found at H3K4me3-enriched promoter-proximal chromatin, including CpG islands (Yamane et al. 2011). The key factor here could be the displaced ssDNA in the R loops, which may directly recruit histone methyltransferases or DNA demethylases. Paradoxically, the ssDNA is also suggested to link R loops and DNA damage events. How cells sense these two opposite situations and act accordingly remains an important but still unanswered question.

R loops are directly implicated in transcriptional termination of some human genes by studies from our laboratory (Skourti-Stathaki et al. 2011). R loops formed over G-rich termination regions facilitate Pol II pausing downstream from the poly(A) signal prior to transcriptional termination. However, in this situation, a very fine balance of R loops is required for efficient termination. Once formed, these R loops must then be resolved by the helicase senataxin to release the nascent RNA and so allow its Xrn2-mediated degradation, which ultimately leads to efficient Pol II transcriptional termination (Skourti-Stathaki et al. 2011). This is a situation in which, even in the same gene, R loops can have a dual role: They are required for efficient termination, but their accumulation (followed by senataxin knockdown) inhibits this process. In the future, it will be interesting to investigate how, within one gene, cells can prevent the deleterious effects of R loops (resolved by senataxin) but at the same time allow their positive function (in this case, efficient termination).

Another interesting observation further supports the connection of R loops with Pol II termination. Genome-wide analysis has previously revealed that G-rich sequences immediately downstream from the poly(A) signal are relatively common in mammalian genes (Salisbury et al. 2006). Recent genome-wide bioinformatic analysis has also shown that promoters and 3' regions of genes are enriched in G4-forming sequences (Huppert et al. 2008). Interestingly, 3' untranslated region (UTR) G4s are particularly prevalent in cases in which a second gene is placed in close proximity, suggesting that G4s may be involved in transcriptional termination (Huppert et al. 2008). However, it still remains to be established

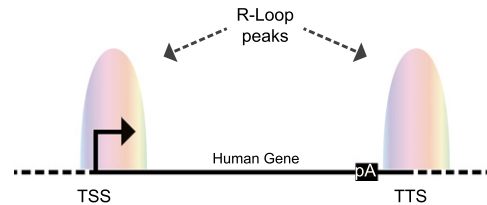


Figure 4. R loops are enriched at both gene ends. In human protein-coding genes, R loops form over unmethylated CpG island promoters with positive GC skew and G-rich termination regions. Promoter-enriched R loops could activate gene expression, whereas terminator-enriched R loops promote transcriptional termination by facilitating Pol II pausing downstream from the poly(A) signal. Transcription start site (TSS), transcription termination site (TTS), and poly(A) (pA) signal are shown. Colored shading indicates peaks of R loops over 5' and 3' gene ends. The diagram is not drawn to scale.

whether genes that form R loops at their G-rich 3' ends or at CpG island promoters also form G4 structures. Finally, a second R-loop genomic analysis strikingly suggested that a subset of Pol II terminators with a positive GC skew corresponds to R-loop regions genome-wide (Ginno et al. 2013). As in G4s (Huppert et al. 2008), genes with R loops at their 3' ends are located in gene-dense regions, further reinforcing the role of R loops in efficient termination.

Altogether, we suggest that R loops are a common feature of G-rich pause terminator elements in human genes (Skourti-Stathaki et al. 2011; Ginno et al. 2013). How do they promote termination? Stopping Pol II is not an easy task. Once in a processive elongation mode, Pol II elongates at 4.3 kb/min (70 bp/sec) (Darzacq et al. 2007) over a diverse sequence landscape that may extend to >1 Mb in vertebrates. Also, given the fact that Pol II is a very large protein, one could argue that such transient structures such as R loops are inadequate to anchor Pol II. Identifying the molecular mechanism by which R loops promote termination is likely to provide new insights into the regulation of gene expression at the level of transcription.

R loops and ncRNAs

R loops have also been shown recently to play a role in the regulation of ncRNA (see Fig. 5; Powell et al. 2013; Sun et al. 2013). In *Arabidopsis thaliana*, *COOLAIR* is the antisense long ncRNA (lncRNA) that regulates the expression of the *FLC* gene, a key repressor of flower development. Upon prolonged cold conditions, *COOLAIR* becomes transcriptionally active and represses *FLC* transcription. Until recently, the transcriptional regulation of *COOLAIR* itself remained uncharacterized. However, compelling new evidence on the role of R loops in this process recently came to light (Sun et al. 2013). In essence, R loops are shown to form over the promoter region of *COOLAIR*, and a ssDNA-binding homeodomain protein, AtNDX, binds and stabilizes these R loops. This ultimately leads to *COOLAIR* transcriptional repression (Sun et al. 2013). This study provides a clear example of how regula-

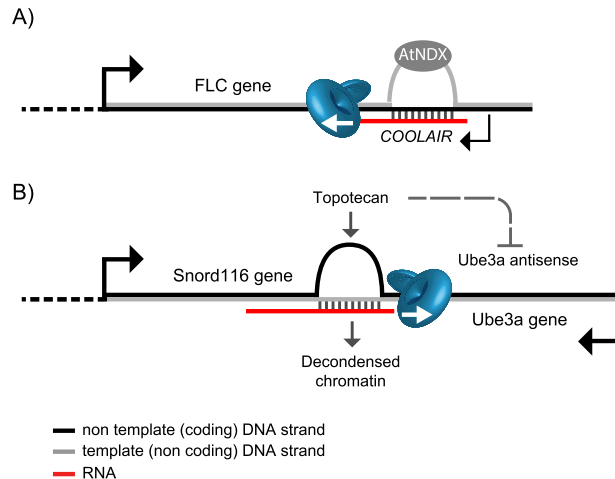


Figure 5. R loops transcriptionally regulate ncRNAs. (A) In plants, *COOLAIR* antisense lncRNA controls the expression of the *FLC* gene. R loops form over the promoter region of *COOLAIR* and are stabilized by the ssDNA-binding protein *AtNDX*. This causes transcriptional repression of *COOLAIR* and, ultimately, activation of the *FLC* gene. (B) In human neuronal cells, topoisomerase inhibitor topotecan causes accumulation of R loops in the G-rich termination region of the *Snord116* gene. This causes chromatin decondensation and blocks read-through transcription that otherwise forms the *Ube3a* antisense transcript. This activates the expression of the *Ube3a* sense transcript. Arrows indicate the direction of transcription. For simplicity, nucleosomes are omitted. The diagram is not drawn to scale.

tory lncRNAs can themselves be regulated but also raises intriguing questions. How does *AtNDX* maintain R-loop formation in the presence of surveillance mechanisms? Does it act faster than helicases and RNase H enzymes, or do these cells somehow protect R loops and ensure *COOLAIR* repression and proper flowering patterns? A comparison with Rad51 may be relevant, as this protein also binds ssDNA and promotes R-loop formation in vivo in *S. cerevisiae* (Wahba et al. 2013). In this case, however, Rad51 promotes “unprogrammed” R-loop formation and potential genomic instability. Given the fact that both *AtNDX* and Rad51 recognize unpaired ssDNA derived from an R loop, how does Rad51 on the one hand restrict potentially “deleterious” R loops, while *AtNDX* promotes “regulatory” R loops? Regardless of the exact mechanism, it is clear that discovering how *AtNDX* and Rad51 regulation occurs will provide a powerful new tool to understand how cells distinguish between the two “types” of R loops.

R loops have recently been linked with the molecular mechanism of a cancer drug, topotecan, that reactivates the expression of the imprinted silenced gene *Ube3a* (Powell et al. 2013). Angelman syndrome (AS) is an autism-related disorder that is caused by mutations or deletions of the maternal copy of the *Ube3a* gene (Kishino et al. 1997; Matsuura et al. 1997). Normally, neurons express only the maternal copy of this gene and silence the paternal copy via the *Ube3a* antisense transcript. So, *Ube3a* mutations in the maternal copy result in a com-

plete loss of the protein, a brain-specific ubiquitin E3 ligase. *Ube3a* antisense is located immediately downstream from the *Snord116* gene, mutations of which cause a second disorder, Prader-Willi syndrome. The cancer drug topotecan was found to reactivate *Ube3a* transcript in neurons and therefore could be potentially used to treat AS (Huang et al. 2011). Even though topotecan holds promise for AS treatment, it still remains unknown how it targets specifically *Ube3a* and no other genes within this locus. Importantly, topotecan is an inhibitor of topoisomerase, which, as mentioned above, relaxes negative supercoiling. It is now revealed that R-loop formation plays a role in the topotecan effect (Powell et al. 2013). In essence, R loops form over the G-rich *Snord116* gene, which in turn causes nucleosome depletion and chromatin decondensation in the paternal allele.

Under physiological conditions, *Ube3a* antisense transcription silences *Ube3a* in *cis*. Upon topotecan treatment, these R loops are stabilized and so accumulate. According to this model, this R-loop accumulation causes excessive chromatin decondensation, stalling of the transcriptional machinery, and inhibition of *Ube3a* antisense expression. This in turn activates paternal *Ube3a* expression (Powell et al. 2013). This suggests that topotecan can also be used as a powerful regulator of R-loop formation in other contexts and so provides a new tool in the characterization of R-loop biology.

Two important points arise from this study: First, in this case, R loops are shown to induce nucleosome depletion and chromatin decondensation, although it is not clear how this occurs. It could also be argued that chromatin decondensation/nucleosome depletion facilitates R-loop formation. Cause cannot easily be distinguished from the effect in this case as in other R-loop-associated processes. As mentioned above, the accumulation of R loops can also induce chromatin condensation (Castellano-Pozo et al. 2013) in contrast to the *Snord116* gene. Even though, in these two studies, R-loop formation may have opposite outcomes on chromatin structure, it is tempting to speculate that a more general link exists between R loops and chromatin dynamics. Second, topotecan is the first R-loop targeting drug used as a therapeutic agent in genetic disorders. Camptothecin (CPT), another topoisomerase inhibitor that is used as an anti-cancer drug, has also been shown to stabilize R loops. Interestingly, CPT causes accumulation of Pol II antisense transcripts over CpG island promoters (Marinello et al. 2013). It is evident that, more than ever, research on R loops is vital to understand how these structures could be disrupted in cancer and other diseases. This would also strengthen the likelihood that R-loop formation is tightly controlled, as its dysregulation and/or accumulation can compromise genome dynamics and function.

Finally, R loops formed over the centromeric repeats in *S. pombe* have been shown to mediate RNAi-dependent heterochromatin formation (Nakama et al. 2012). This study is of particular interest, since it shows that R loops are potentially involved in silencing centromeric DNA.

Heterochromatic ncRNA have been suggested to remain on chromatin and function as a binding platform for the RNAi apparatus (Cam et al. 2009). Following this study, two important questions remain unanswered. How do ncRNAs remain bound to chromatin? Do RNAi factors (especially the RITS complex) target the gene transcript or the DNA strand? Nakama et al. (2012) suggested that ncRNA transcribed from heterochromatin remains bound to chromatin via the formation of an R loop and that the so-formed RNA/DNA hybrid itself is involved in the heterochromatin formation. In essence, RNA/DNA hybrid foci colocalize with centromeric heterochromatic regions, and overexpression of RNase H decreases these foci, which in turn disrupts heterochromatin formation. Given that, upon R-loop formation, the single-strand nontemplate DNA remains unpaired, Nakama et al. (2012) predicted that the RITS complex could target the single-stranded “unprotected” DNA or, alternatively, the chromatin-associated RNA to generate heterochromatin formation.

Even though this study did not discuss whether R-loop formation is the trigger of heterochromatin formation rather than its consequence, these observations again tightly connected the fields of transcription and heterochromatin, raising intriguing questions for further investigation. Do R loops facilitate transcriptional gene silencing in other regions of the genome? Is this phenomenon conserved in mammalian cells? Could R-loop “hot spots” be regions of RNAi-dependent heterochromatin assembly? In any case, this study suggested that R loops control an epigenetic mark directly or indirectly. Future studies on this potential connection will shed light on the “RNA-guided pathway for the epigenome” (Jenuwein 2002) and might place R loops as key players of the critical interrelationship between transcription and chromatin.

R loops and neurodegenerative disorders

The mechanistic connection between R loops and neurodegenerative disorders remains unclear even though examples and associations are growing. Apart from the connections presented for the *Snord116* study, R loops are often associated with neurodegenerative disease caused by abnormal expansion of repeated DNA sequences (the so-called repeat expansion disorders) (Lin et al. 2010; McIvor et al. 2010; Reddy et al. 2011; Wongsurawat et al. 2012). Very recently, R loops were shown to form over the promoter of the *fragile X mental retardation 1* (*Fmr1*) gene and coincide with its epigenetic silencing in fragile X syndrome (Colak et al. 2014). A similar mechanism has also recently been shown to occur in another trinucleotide repeat expansion disease, Friedreich's ataxia (Groh et al. 2014). In this study, expanded disease alleles were shown to accumulate R loops, resulting in a transcriptional block and heterochromatin formation. G4 structures were also found to form in a hexanucleotide repeat expansion of the *C9orf72* gene, which causes ALS and frontotemporal dementia (FTD). G4 in *C9orf72* DNA promotes the formation of stable R loops, which in turn impedes transcriptional elongation and leads to production of short, abortive transcripts (Haeusler et al. 2014).

Furthermore, it was recently shown that topoisomerases promote transcription of human and mouse long genes linked to autism (King et al. 2013). Interestingly, *Snord116-Ube3a* antisense is an extremely long transcription unit, implying that topotecan might reduce the expression of other long genes. Indeed, topotecan has also been shown to reduce the expression of other long genes in human and mouse neurons in a dose-dependent manner (King et al. 2013). Transcription of very long genes has also been shown to cause replication/transcription collisions and accumulation of R loops at the CFSs (Helmrich et al. 2011). Some of these genes have been shown to be down-regulated in neurological diseases, such as Alzheimer's disease (Sze et al. 2004). Additionally, defects in DNA repair proteins (which, as mentioned above, are connected to R loops) cause neurodegenerative syndromes (McKinnon 2009). Defective DNA repair in mature neuronal tissues has also been linked to aging and neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease. Senataxin has recently been suggested to act as a DNA repair protein (Yüce and West 2013) by resolving R loops at human genes (Skourti-Stathaki et al. 2011). Given that mutations in senataxin cause specific neurodegenerative disorders (James and Talbot 2006; Palau and Espinos 2006), perhaps senataxin, despite being a ubiquitously expressed protein, has a special role in neuronal genes by controlling the transcription of some fragile sites present in long genes. Altogether, these studies reveal a complex and coordinated network in neurons between transcription, DNA repair, and R loops. Indeed, the general physiological relevance of R loops as transcriptional regulators seems more and more likely.

Conclusions and perspectives

The last decade has seen a significant expansion of our knowledge of R-loop biology and function. For years, R loops were considered a threat to cells as a rare transcriptional by-product (Aguilera and Garcia-Muse 2012). It is only now that we start to realize that they may have a major regulatory role in gene function. They can be the “two sides of a coin,” deleterious structures but also fine-tuners of gene expression. Given their involvement in multiple cellular processes, understanding how cells prevent the negative functions of R loops yet allow their positive ones is a challenge for the years to come. Perhaps the key to this question is the unpaired ssDNA derived from these structures. It can be the trigger for genomic instability but can also provide base-pairing for *trans* RNAs or act as a binding scaffold for enzymes that control the transcription cycle.

A great deal has been learned in recent years about factors that prevent or resolve R loops. Research should now aim to discover more factors (in addition to Rad51 and AtNDX) that actively promote R-loop formation. Is there an evolutionarily conserved protein that is generally responsible for R-loop formation? Answering this fundamental question will perhaps allow us to better understand the dual functions of R loops and also link R loops to hitherto unanticipated cellular processes.

As mentioned above, R loops are thought to play a role in neurodegenerative disorders even though strong evidence for this association has yet to be established. R loops could offer a novel angle on regulation of transcription, and it is now the time to unravel their possible links with cancer and neurodegenerative disease. From the examples mentioned in this review, it is evident that R loops lie at the interphase of different fields: transcription, RNA processing, DNA damage, and chromatin. More than ever, we need to interconnect these fields to fully understand how R loops modulate genome dynamics.

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A double-edged sword: R loops as threats to genome integrity and powerful regulators of gene expression

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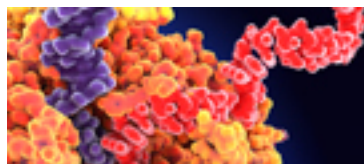
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