R-Loop Physiology and Pathology: A Brief Review

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Physiological and pathological roles for R-loop structures continue to be discovered, and studies suggest that R-loops could contribute to human disease. R-loops are nucleic acid structures characterized by a DNA:RNA hybrid and displaced single-stranded DNA that occur in connection with transcription. R-loops form naturally and have been shown to be important for a number of physiological processes such as mitochondrial replication initiation, class switch recombination, DNA repair, modulating DNA topology, and regulation of gene expression. However, subsets of R-loops or persistent R-loops lead to DNA breaks, chromosome rearrangement, and genome instability. In addition, R-loops have been linked to human diseases, specifically neurological disorders and cancer. Of the large amount of research produced recently on R-loops, this review covers evidence for R-loop involvement in normal cellular physiology and pathophysiology, as well as describing factors that contribute to R-loop regulation.

Keywords: R-loop, cancer, genome instability, gene expression regulation

Background

R-LOOPS ARE DNA:RNA hybrids with a displaced single strand of DNA that occur in association with transcription. Newly transcribed RNA binds to its template DNA and prevents the reannealing of the nontemplate DNA. R-loops were overlooked for a while and considered to be insignificant by-products of transcription. Later studies have revealed roles for R-loops in normal cellular physiology, as well as connections between R-loops and human disease. R-loop biology is a rapidly expanding area of research, and R-loops have since been shown to be abundant nucleic acid structures that are enriched in specific sequences and areas of the genome. Specifically, R-loops form more often in GC or purine rich regions and are found most often near promoters or at the 3' end of genes (Ginno *et al.*, 2012, 2013; Skourti-Stathaki *et al.*, 2014; Sanz *et al.*, 2016; Chen *et al.*, 2017; Xu *et al.*, 2017; Hartono *et al.*, 2018).

In mammals, R-loops have been found in 3–5% of the genome (Ginno *et al.*, 2012; Lim *et al.*, 2015; Sanz *et al.*, 2016; Stork *et al.*, 2016) with lengths from 200 to 500 base pairs, roughly 10 times longer than other non-B DNA structures (Kouzine *et al.*, 2017). Although rare, kilo base length R-loops are not uncommon (Yu *et al.*, 2003; Huang *et al.*, 2006, 2007; Kao *et al.*, 2013; Malig *et al.*, 2020). R-loop formation in humans also occurs at specific regions, again surrounding genes at either end (Santos-Pereira and

Aguilera, 2015; Sanz *et al.*, 2016) and extending to various lengths (Huang *et al.*, 2007). Since R-loops are present in similar peri-gene regions in both prokaryotes and eukaryotes, these observations suggest that R-loops are evolutionarily conserved phenomena occurring at conserved sequences. Importantly, the close proximity of R-loops to gene sequences carries significant implications for gene expression.

R-Loop Physiologic Roles

R-loops in mitochondrial replication initiation

Significant roles have been discovered for R-loops as part of normal cellular physiology, including processes such as mitochondrial replication initiation. While examining mitochondrial replication intermediates, it was found that intermediates were duplex and contained extensive tracts of RNA (Pohjoismäki *et al.*, 2010). Other studies discovered that these DNA:RNA hybrids form at conserved sequence blocks in mitochondrial DNA (mtDNA) (Xu and Clayton, 1996), in both *Saccharomyces* and human cells. These stable DNA:RNA hybrids extended from conserved GC rich sequences to sites implicated in replication initiation, supporting the idea that the RNA in R-loops serves as a primer for mitochondrial replication. Recent studies have revealed factors responsible for processing R-loop 3' ends that can then be used by mtDNA polymerase γ , since R-loops by

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themselves cannot directly prime mtDNA synthesis (Posse *et al.*, 2019). A liver-specific RNase H1 knockout mouse model demonstrated reduced mtDNA levels, reduction in mitochondrial genes, and mitochondrial dysfunction, suggesting that RNase H1 is required for mtDNA replication (Lima *et al.*, 2016). This was later confirmed in human cells (Posse *et al.*, 2019), in a patient cell line with two heterozygous *RNASEH1* mutations and loss of RNase H1 activity.

The RNase H family of enzymes are the only RNases to degrade RNA in DNA:RNA hybrids, and there was no mtDNA synthesis observed in the absence of RNase H1 *in vitro* (Posse *et al.*, 2019). In the patient cell line deficient in RNase H1, researchers also noted mtDNA replication initiation from multiple sites in addition to the conserved origin of replication. It is possible that RNase H1 not only processes R-loop 3' ends for DNA polymerase γ but also removes all DNA:RNA hybrids except for in the conserved sequence blocks, restricting replication to a single origin (also known as origin of H-strand or OriH). Using sequencing, other studies have also placed R-loops near replication origins in human genomic DNA. In one such study (Ohsato *et al.*, 1999), R-loops colocalized to the same CpG islands as the origin recognition complex in some of the most efficient replication origins.

R-loops in class switch recombination

Class switch recombination (CSR) happens in immunoglobulin genes of activated B cells, changing the isotype of the immunoglobulin constant region from μ (which corresponds to IgM) to other isotypes such as γ , α , or ϵ (which generate various IgG subtypes, IgA, or IgE). Certain regions, known as switch regions, are required for CSR to occur. Although these repeated switch regions are important for recombination, no consensus sequences have been identified. Instead, these switch regions produce GC-rich RNA transcripts that do not encode protein. Without these “sterile transcripts,” class switching is reduced²¹. It is now known that these RNA transcripts lead to the formation of stable long (more than 1 kb) R-loops (Yu *et al.*, 2003).

In one theory, stable R-loop structures provide a single-stranded DNA substrate for activation-induced cytidine deaminase (AID) to begin the CSR process. AID converts cytosine to uracil, which leads to a DNA nick when processed by base excision repair machinery. This DNA nick results in a double-strand DNA break when acted on by mismatch repair proteins. Finally, end joining DNA repair results in the Ig class switch.

Another study using RNase H1 overexpression confirms a role for R-loops in CSR, where decreased R-loop levels result in inefficient CSR and also suggest an alternative mechanism to explain the involvement of R-loops in CSR (Wiedemann *et al.*, 2016). Investigators in this study show that entry into S phase and active DNA replication are necessary for efficient CSR. Decreasing R-loops by overexpressing RNase H1 led to decreased loading of the origin replication complex and Mcm complex (a replicative helicase). In addition, reducing R-loop levels interfered with the long-range interactions between donor and acceptor switch regions. As a result, the researchers concluded that R-loops were important for topological conformations allowing synapsis between donor and acceptor switch regions. This process depends on S-phase entry and active DNA replica-

tion, possibly by activation of origins within the *IgH* locus that then become associated in origin clusters, where multiple activated origins within a replication domain congregate to form a focus of ongoing replication activity.

R-loops in DNA repair

R-loops have also been associated with DNA repair, although many aspects of this relationship remain unclear. It has been shown that when R-loop levels are lower than normal, the efficiency of homologous recombination and nonhomologous end joining repair is decreased (Ohle *et al.*, 2016; Lu *et al.*, 2018; Yasuhara *et al.*, 2018). R-loops have also been shown to recruit the nucleotide excision repair factor CSB, which results in the recruitment of Rad52 to promote homologous recombination at sites of reactive oxygen species (ROS)-induced DNA damage (Teng *et al.*, 2018). Intriguingly, R-loops are also directly involved in a form of homologous recombination called transcription-associated homologous recombination repair, functioning near double-strand DNA breaks to recruit Rad52 (Yasuhara *et al.*, 2018). Rad52 then recruits XPG to resect DNA and initiate homologous recombination. In addition, an alternative form of homologous recombination actually makes use of RNA instead of DNA as a template for double-strand break repair (Keskin *et al.*, 2014; Mazina *et al.*, 2017).

R-loops modulate gene expression and chromatin state

Due to their enrichment at gene promoters and at gene 3' ends (Ginno *et al.*, 2012; Nadel *et al.*, 2015; Sanz *et al.*, 2016; Chen *et al.*, 2017; Dumelie and Jaffrey, 2017), R-loops unsurprisingly affect transcription machinery and could serve as a regulatory mechanism for gene expression. Some researchers propose that R-loops located at the 3' end of mammalian genes help terminate transcription by stalling the RNA polymerase past the poly A sequence (Skourti-Stathaki *et al.*, 2011, 2014; Yanling Zhao *et al.*, 2016). It is believed that R-loops on the 3' end of genes facilitate efficient transcription termination by recruiting the helicase senataxin. In turn, senataxin recruits the 5'-3' exoribonuclease XRN2 to promote transcription termination (Skourti-Stathaki *et al.*, 2011; Morales *et al.*, 2016).

In contrast, R-loops positioned at upstream promoter sequences have been shown to block transcription (Bonnet *et al.*, 2017; Hamperl *et al.*, 2017; Lang *et al.*, 2017) and can possibly interfere with transcription factor binding (Boque-Sastre *et al.*, 2015). One study showed that the presence of R-loops led to antisense transcription, formation of double-stranded RNA, and activation of RNA interference machinery (Skourti-Stathaki *et al.*, 2014). In addition, this process was linked to the formation of repressive chromatin marker H3K9me2.

This last observation of altered chromatin state represents another mechanism by which R loops could modulate gene expression, with transient R-loop formation leading to long-term epigenetic changes. Since DNA methyltransferases bind poorly to DNA:RNA hybrids (Ginno *et al.*, 2012; Grunseich *et al.*, 2018), some groups suggest that R-loops serve as positive transcription regulators at the epigenetic level by protecting CpG islands in promoters from methylation. Interestingly, R-loops have been associated with both

open (Powell *et al.*, 2013; Boque-Sastre *et al.*, 2015; Sanz *et al.*, 2016) and condensed (Castellano-Pozo *et al.*, 2013; Skourti-Stathaki *et al.*, 2014) chromatin. Specifically, R-loops have been linked to repressive histone modifications such as H3S10 phosphorylation (Castellano-Pozo *et al.*, 2013; García-Pichardo *et al.*, 2017) and H3K9me2 (Zeller *et al.*, 2016). Similar to the previously mentioned study (Skourti-Stathaki *et al.*, 2014) describing an association between R-loop formation and heterochromatin formation, R-loops implicated in trinucleotide repeat neurologic diseases lead to trinucleotide repeat expansion and the establishment of repressive histone modifications (Colak *et al.*, 2014; Groh *et al.*, 2014; Skourti-Stathaki *et al.*, 2014).

In contrast, one study found no correlation between R-loops and repressive chromatin markers after performing genome-wide R-loop mapping and instead found an association between R-loops and H3K4 methylation, which is associated with active transcription (Sanz *et al.*, 2016). Similarly, R-loops in mouse cells inhibited repressive histone modifications and heterochromatin formation, facilitating a more open and transcriptionally active chromatin state (Chen *et al.*, 2015). A few studies have described a context-specific ability of R-loops to alter chromatin structure, for either gene activation or inhibition. This is evidenced by the recruitment of polycomb repressive complex 2 (PRC2) to repressed developmental genes while simultaneously inhibiting the recruitment of PRC2 at active genes (Chen *et al.*, 2015; Skourti-Stathaki *et al.*, 2019). It is also interesting to consider that the DNA:RNA hybrid in R-loops hinders nucleosome formation (Dunn and Griffith, 1980), making it less likely for R-loops and histones to be closely associated spatially. The direct links between R-loops and epigenetic changes remain unclear. Since many questions remain to be answered, it appears that uncovering these connections could be a fruitful area of research in years to come.

R-loops and DNA topology

In addition to nucleotide sequence, R-loop formation is also determined by another major parameter: topology of the DNA template (Drolet, 2006). As a consequence, interplay among transcription, R-loop formation, and supercoiling can result in significant perturbations in cell physiology under stress conditions. The relationship between R-loop formation and DNA topology was described in *topA* (a type 1 topoisomerase) *Escherichia coli* mutants with hypernegatively supercoiled plasmid DNA (Drolet *et al.*, 1994), with supercoiling of plasmid DNA being tightly linked to transcription elongation and R-loop formation *in vitro*. Later *in vivo* studies showed that growth defects in *topA E. coli* mutants could be overcome by loss-of-function mutations in DNA gyrase (which generates negative supercoils) or with overexpression of RNase H, confirming that R-loops are linked to growth restriction in the absence of DNA topoisomerase I (Drolet *et al.*, 1994). Finally, it was shown that R-loops in bacteria could only form with negative supercoiling through DNA gyrase, and transcription-translation coupling prevented R-loop formation, presumably by translation machinery that captures nascent RNA before it hybridizes with the template DNA strand (Massé and Drolet, 1999).

Given the relationship between R-loops and DNA topology, some groups have suggested that R-loops could act as topological “stress relief valves” by relieving the higher energy of supercoiled DNA (Stolz *et al.*, 2019). In the relaxed Watson–Crick B form of DNA, strands cross every 10.5 base pairs. Supercoiling, positive or negative, happens when this ratio is altered and is possible in circular DNA or in linear DNA with semirigid attachments such as lamins (Dekker and Mirny, 2016; Yáñez-Cuna and van Steensel, 2017; Rowley and Corces, 2018) or chromatin (Mondal, 2003; Mondal and Parvin, 2001). Supercoiling represents a higher energy state than relaxed DNA. In this way, supercoiling favors the formation of non-B DNA structures as long as the resulting structure has an energy low enough to facilitate the transition state. *In vitro*, negative supercoiling is enough to drive the formation of alternative structures such as melted DNA, Z-form DNA, cruciform DNA, H-form DNA, and R-loops (Kouzine, 2007; Levens and Benham, 2011; Baranello *et al.*, 2012).

In this theory of “release valves,” R-loops would accumulate wherever there is excessive supercoiling, specifically negative supercoiling. RNA in R-loops would be able to absorb one negative superhelical turn every 10.5 base pairs, and the displaced single strand of DNA would be allowed to wrap around the DNA:RNA hybrid and absorb additional supercoiling. One group showed that one R-loop could relax plasmids with ~18 negative supercoils (Stolz *et al.*, 2019). R-loops in this study were 80 to 175 base pairs in length, while plasmids were 3.5 kilobase pairs, revealing that R-loops can absorb a surprising amount of supercoiling. In human genomes, R-loops form in thousands of conserved regions (Santos-Pereira and Aguilera, 2015; Sanz *et al.*, 2016) and are more abundant than previously thought (Ginno *et al.*, 2012; Lim *et al.*, 2015; Sanz *et al.*, 2016; Stork *et al.*, 2016). Since R-loops in human genomes range from 200 to 500 base pairs (Kouzine *et al.*, 2017) and occasionally reach into the thousands (Yu *et al.*, 2003; Huang *et al.*, 2006, 2007; Kao *et al.*, 2013; Malig *et al.*, 2020), they could be responsible for resolving a huge amount of supercoiling, all without changing the actual linking number of DNA. This could also explain why R-loops, which originate at conserved sequences that are GC or purine rich (Sanz *et al.*, 2016; Chen *et al.*, 2017; Xu *et al.*, 2017; Hartono *et al.*, 2018), extend to a variety of lengths (Huang *et al.*, 2007)—R-loops would expand until the supercoiling that favors their formation has been relieved. This theory could also partly account for the transcription regulation reported in other studies. Some amount of negative supercoiling is required for strand separation before promoter or replication origin firing.

Negative supercoiling is also necessary to generate the DNA conformations necessary for long-range interactions between promoters and enhancers (Benedetti *et al.*, 2014). R-loop formation would absorb any negative supercoiling, decreasing promoter and replication origin firing, as well as impairing enhancer–promoter interaction. Indeed, R-loops have been shown to have negative effects on transcription elongation (Hraiky *et al.*, 2000; Baaklini *et al.*, 2004; Belotserkovskii *et al.*, 2010, 2017; El Hage *et al.*, 2010; Belotserkovskii and Hanawalt, 2011) and replication (Santos-Pereira and Aguilera, 2015; Crossley *et al.*, 2019).

Topoisomerases are enzymes that manipulate DNA structure to relieve supercoiling that occurs during transcription and replication, as well as participating in

decatenation and chromosome untangling. This is achieved by breaking and rejoining the DNA double helix, and topoisomerases are classified as type I or type 2 depending on if they cause a single- or double-strand break, respectively. Topoisomerase activity is necessary for efficient transcription, and mammalian type 1 topoisomerase has been shown to directly associate with transcription machinery (Baranello *et al.*, 2016). Type 2 topoisomerases have also shown to be active at transcription start sites, especially in highly expressed genes (Kouzine *et al.*, 2013; Teves and Henikoff, 2014). Consequently, studies have shown that decreasing topoisomerase 1 levels or inhibiting its activity with camptothecin results in the accumulation of R-loops. Loss of topoisomerase 1 in yeast (El Hage *et al.*, 2010) or *E. coli* (Drolet *et al.*, 1994) leads to R-loop accumulation and genome instability (Usongo *et al.*, 2016; Brochu *et al.*, 2018; Drolet and Brochu, 2019) which is made worse with concomitant loss of RNaseH (El Hage *et al.*, 2010). RNaseH is the only RNase to degrade RNA in DNA:RNA hybrids and is commonly used to confirm or detect the presence of R-loops. Studies in human cells have yielded similar results, where increased R-loop levels and genome instability are seen with loss of topoisomerase 1 (Manzo *et al.*, 2018). Other topoisomerases have also been shown to prevent R-loop buildup, including topoisomerases 3a (Oh *et al.*, 2007) and 3b (Wilson-Sali and Hsieh, 2002; Stoll *et al.*, 2013; Xu *et al.*, 2013; Yang *et al.*, 2014). Topoisomerase 3b, which also has RNA topoisomerase activity in the cytoplasm, acts with its partner TDRD3 to reduce R-loops through its relaxation activity (Yang *et al.*, 2014) and possibly through cleavage activity (Wilson-Sali and Hsieh, 2002).

Transcription and replication machinery generate supercoiling on both sides—positive supercoiling in front of forks and negative supercoiling behind (Liu and Wang, 1987; Ma and Wang, 2016). It is estimated that for an average human gene of 30 kilobases, one transcription event would generate 150 turns of supercoiling (Kouzine *et al.*, 2013). Classically, negative supercoiling allows for DNA strand separation necessary for transcription and replication. As a result, negative supercoiling improves transcription elongation, while positive supercoiling inhibits it (Gilbert and Allan, 2014). DNA gyrase in *E. coli* generates negative supercoiling, and cells exhibit negative supercoiling at baseline, presumably to allow for transcription. In this way, DNA gyrase activity favors R-loop formation (Drolet *et al.*, 1994), while topoisomerase 1 activity decreases R-loop levels (Drolet *et al.*, 1994; Massé *et al.*, 1997; Phoenix *et al.*, 1997; Massé and Drolet, 1999). Consequently, gene expression responds to changes in supercoiling (Pruss and Drlica, 1989; Hatfield and Benham, 2002; Peter *et al.*, 2004), and negatively supercoiled domains are generally associated with higher gene activity (Pope *et al.*, 2014; Dekker and Heard, 2015). In addition, it has been shown in *E. coli* and *Drosophila* that transcription factors and replication initiating proteins preferentially bind negatively supercoiled DNA (Fuller and Kornberg, 1983; Mizutani *et al.*, 1991; Kobryn *et al.*, 1999; Remus *et al.*, 2004; Donczew *et al.*, 2014; Kasho *et al.*, 2017).

Nucleosomes, which incorporate about 147 base pairs of DNA, have been shown to relax one turn of negative supercoiling (Finch *et al.*, 1977; Luger *et al.*, 1997; Richmond

and Davey, 2003). In fact, negative supercoiling is favorable for nucleosome formation (Luger *et al.*, 1997; Forth *et al.*, 2013), while positive supercoiling or even relaxed DNA reduces nucleosome formation (Pfaffle and Jackson, 1990; Pfaffle *et al.*, 1990; Patterton and von Holt, 1993). Since nucleosomes are forced off DNA when transcription machinery passes through, any stored negative supercoiling will be released. R-loops could interfere with nucleosome formation in two ways, by (1) relieving the negative supercoiling that drives nucleosome formation and (2) since the DNA:RNA hybrid in R-loops makes nucleosome formation less favorable (Dunn and Griffith, 1980). In this way, transcription and transcription-associated R-loops could be a link to the establishment of chromatin domains (Naughton *et al.*, 2013).

R-Loop Pathology

R-loops in DNA damage and genome instability

Although R-loops form naturally and appear to function as part of normal cellular physiology with roles in mitochondrial replication initiation, immunoglobulin CSR, transcription regulation, and chromatin marker regulation, R-loops have also been strongly associated with pathological DNA damage such as double strand DNA breaks and genome instability (Sollier *et al.*, 2014). One of the first descriptions of this was shown in yeast with *hpr1* deletion (Huertas and Aguilera, 2003). *hpr1* is part of the THO/TREX complex, which is involved in RNA processing and nuclear export. These cells displayed increased DNA damage and a hyper-recombinant phenotype, which was shown to be due to R-loop formation. Similar results involving other members of the THO complex were later provided in chicken (Li and Manley, 2005; Wahba *et al.*, 2011) and then mammalian cells (Domínguez-Sánchez *et al.*, 2011).

Specifically, R-loop formation is often linked to genome instability through replication fork stalling and transcription-replication collision. For instance, R-loop mediated DNA damage seems to happen more in S phase, when origins of replication are firing and replication machinery is active (Wellinger *et al.*, 2006; Gan *et al.*, 2011). This is supported by observations that RNaseH overexpression decreases this DNA damage (Kotsantis *et al.*, 2016; Stork *et al.*, 2016) and rescues cells from stalled replication forks (Kotsantis *et al.*, 2016). It remains unclear which comes first—whether R-loop formation precedes collision or if transcription-replication collision leads to transcription stalling and increased probability of R-loop formation. In either case, genomic rearrangement seems to be the common outcome (Wellinger *et al.*, 2006; Gan *et al.*, 2011; Castellano-Pozo *et al.*, 2012; Herrera-Moyano *et al.*, 2014). As mentioned previously, decreased topoisomerase 1 levels or activity causes increased R-loops. These results are also associated with transcription and replication stalling, leading to recombination and genome instability (Huertas and Aguilera, 2003; Tuduri *et al.*, 2009; El Hage *et al.*, 2010).

Studies examining genome instability in trinucleotide repeat expansion neurologic disorders (Lin and Wilson, 2012; Reddy *et al.*, 2014), such as Fragile X syndrome (Helmrich *et al.*, 2011; Lang *et al.*, 2017), have also demonstrated strong links between genome instability and R-loops. Members of the Fanconi Anemia pathway such as

FANCA, FANCD2, and FANCM, which have classical roles to resolve replication forks that have stalled due to cross linking, have also been shown to control R-loop levels and prevent genome instability (García-Rubio *et al.*, 2015; Schwab *et al.*, 2015).

One theory to explain the connection between R-loops and DNA damage suggests that, although R-loops are normal biological structures, excessive buildup or persistence of R-loops overwhelms the factors involved in R-loop resolution and leads to DNA damage. This idea is supported by global increases in R-loops accompanied by RNaseH-sensitive DNA damage (Stork *et al.*, 2016; Gorthi *et al.*, 2018). Another theory suggests that only certain subsets of R-loops are truly pathologic and lead to replication fork stalling and genome instability. One study that supports this idea found that R-loop-mediated genome instability only occurred with simultaneous histone H3 phosphorylation at serine 10 (García-Pichardo *et al.*, 2017), a sign of chromatin condensation. In contrast, chromatin markers such as H3K9me2 or H3K9me3 have been shown to suppress R-loop-mediated replication stress (Zeller *et al.*, 2016). In other studies using bacterial (Lang *et al.*, 2017) and mammalian episome (Hamperl *et al.*, 2017) models, researchers show that transcription-replication collisions in the head-on orientation increase R-loop formation, while collisions that have codirectional orientation reduce R-loops. Furthermore, R-loops result in genome instability only when transcription-replication collision occurs in the head-on orientation. Interestingly, in mammalian cells (Hamperl *et al.*, 2017) head-on collisions activate the ATR DNA damage pathway, which is classically involved with stalled replication forks and activated by single-stranded DNA to recruit replication protein A. Codirectional collisions, on the other hand, lead to specific activation of the ATM DNA damage pathway, which is associated with double strand DNA breaks.

R-loops in neurologic disease

Noteworthy advances in R-loop biology have recently tied R-loops to human disease. Although these relationships require more exploration to uncover precise mechanisms, R-loops are most strongly linked to neurologic diseases and cancer. Of the neurologic diseases, R-loops have been strongly implicated in the repeat expansion disorders such as Fragile X, Huntington's, and myotonic dystrophy. In these diseases, trinucleotide repeats extend for various lengths in genes. R-loop involvement in these diseases is unsurprising since many of these repeats are GC rich—CGG repeats in *fragile X mental retardation 1* (*FMR1*) gene in Fragile X syndrome, CAG repeats in *huntingtin* (*HTT*) gene of Huntington's disease, CTG repeats in *dystrophin myotonic protein kinase* (*DMPK*) gene of myotonic dystrophy type 1, and CAG repeats in *ataxin 1* (*ATXN1*) gene of spinocerebellar ataxia type 1. Inserting these repeats into bacteria is enough to cause R-loop formation (Reddy *et al.*, 2011) and transcription arrest (Grabczyk *et al.*, 2007). Specifically in these diseases, R-loop formation is thought to be detrimental by contributing to genome instability (Lin and Wilson, 2012; Reddy *et al.*, 2014) and has been shown to lead to expansion of these repeats (Nakamori *et al.*, 2011). In addition to repeat expansion, R-loops can also lead to the establishment of repressive histone modifications (Colak

et al., 2014; Groh *et al.*, 2014; Skourti-Stathaki *et al.*, 2014), as mentioned previously. This is seen in Friedreich ataxia, another trinucleotide repeat expansion disease, where R-loop formation leads to repressive histone modification H3K9me2 (Groh *et al.*, 2014).

In addition to trinucleotide repeat expansion diseases, R-loops have also been associated with other neurologic diseases. In Aicardi-Goutières syndrome, mutations were discovered in *TREX1* or in *RNASEH2* along with global increases in R-loops (Lim *et al.*, 2015). *TREX1* encodes an exonuclease that is a member of the THO/TREX complex and has an implicated role in R-loop resolution. Yeast Pbp1 is homologous to human ATXN2, which is linked to amyotrophic lateral sclerosis and spinocerebellar ataxia type 2. Loss of Pbp1 results in R-loop accumulation in spacer regions of DNA encoding ribosomal repeats and at telomeres (Salvi *et al.*, 2014). Senataxin is a DNA:RNA helicase that has been shown to suppress R-loop formation and transcription-associated recombination in yeast (Mischo *et al.*, 2011) and in human cells (Skourti-Stathaki *et al.*, 2011). Mutations in senataxin can be found in ataxia with oculomotor apraxia type 2 and in a type of amyotrophic lateral sclerosis known as ALS4.

R-loops in cancer

R-loops are also thought to be involved in cancer and are believed to contribute to the genome instability that is a feature of cancer. It is believed that the proliferative and transcriptional activity of cancer cells predisposes them to R-loop accumulation (Kotsantis *et al.*, 2016) due to nucleotide depletion or replication-transcription collision (Helmrich *et al.*, 2011). These are made more likely due to the uncoordinated nature of cancer cell cycle progression and proliferation.

In estrogen receptor positive breast cancer cells, strong proliferative stimulation in the form of applied estrogen was enough to increase R-loop levels (Stork *et al.*, 2016). As a result, R-loop-mediated DNA damage was seen at genes downstream of estrogen signaling. Increased proliferative signaling through oncogene mutations, such as in *HRAS* (Kotsantis *et al.*, 2016), can similarly drive R-loop formation and subsequent DNA damage and replication stress. Since R-loops form more often in and closely surround gene-encoding regions (Ginno *et al.*, 2012, 2013; Skourti-Stathaki *et al.*, 2014; Sanz *et al.*, 2016; Chen *et al.*, 2017; Xu *et al.*, 2017; Hartono *et al.*, 2018), any R-loop-mediated DNA damage is more likely to affect genes compared to noncoding DNA.

A number of factors with links to cancer have also been implicated in R-loop regulation. Among other roles, BRCA1 and BRCA2 function as important intermediates for DNA repair and have strong ties to cancers, including breast cancer. These factors are also important for maintaining genome stability, and loss of either leads to increased R-loop levels (Bhatia *et al.*, 2014; Hill *et al.*, 2014; Savage *et al.*, 2014). BRCA1 and BRCA2 are part of the Fanconi Anemia pathway, and patients with Fanconi Anemia are at increased risk for a variety of cancers, including acute myelogenous leukemia, head and neck, gastrointestinal, and esophageal cancers among others. The FANCD1 helicase, for example, has been shown to interact with BRCA1 and has

been linked to development of breast cancer (Wu *et al.*, 2008). Likewise, loss of other members of the Fanconi Anemia pathway such as FANCA and FANCD2 result in R-loop buildup, transcription-replication collision DNA damage, and genome instability (García-Rubio *et al.*, 2015; Schwab *et al.*, 2015). The Fanconi Anemia factor FANCM is possibly involved in direct R-loop resolution through its translocase activity. It is also interesting to consider that R-loops are a transcription-associated phenomenon, and the BRCA proteins directly interact with RNA polymerase II. In this way and in light of their roles in DNA repair, the BRCA proteins could serve as mediators between R-loops and DNA repair (Hatchi *et al.*, 2015) or as a primary signal between transcription arrest and R-loop resolution.

R-loops might also play a role in telomere elongation to prevent senescence and is another way R-loops could be involved as part of cancer pathophysiology. In S phase in yeast cells, short telomeres cause the formation of stable R-loops. Transcription-replication collisions at these sites promote recombination and serve to maintain telomere length (Graf *et al.*, 2017). Other studies in yeast and mammalian cells support these findings and emphasize the importance of DNA:RNA hybrids at telomeres to promote recombination-mediated elongation, possibly through senataxin (De Amicis *et al.*, 2011; Balk *et al.*, 2013).

Factors Involved in R-Loop Prevention/Resolution

Although usually considered to be short lived, R-loop structures can be considerably stable, and DNA:RNA hybrids can even be more stable than the DNA:DNA complex especially if GC or purine rich (Huppert, 2008). Since R-loops can lead to DNA damage and genome instability, it is believed that cells tightly control R-loop levels by producing factors to remove pathologic R-loops or to maintain a beneficial “balance” of R-loops. A number of factors have been identified for the prevention or resolution of R-loops. These include members of the RNaseH family of enzymes (Wahba *et al.*, 2011), RNaseH1 and RNaseH2, which are the only RNases that are specific for and capable of degrading RNA in DNA:RNA hybrids. With this unique activity, RNaseH is often used to confirm presence or involvement of R-loops in a variety of methods, including rescue from R-loop-mediated DNA damage, replication fork stalling, and genome instability (Wahba *et al.*, 2011; Chernikova *et al.*, 2012; Kotsantis *et al.*, 2016; Stork *et al.*, 2016; Nguyen *et al.*, 2017). Catalytically inactive versions of RNaseH1 are also used in DNA/RNA immunoprecipitation assays in combination with sequencing to measure R-loop levels and location. Interestingly, replication protein A, which acts classically as part of the ATR DNA damage response to resolve stalled replication forks by recruiting the ATR-ATRIP complex to single-stranded DNA, also recruits RNaseH (Nguyen *et al.*, 2017).

A number of RNA binding and processing factors have been shown to prevent R-loop formation (Wahba *et al.*, 2011; Stirling *et al.*, 2012) such as the ribonucleoproteins that bind newly transcribed RNA and facilitate its transport to the cytosol. These include RNA binding protein RNPS1 (Li *et al.*, 2007), yeast polyadenylation factor FIP1 (Stirling *et al.*, 2012), and splicing factor ASF/SF2 (Li and Manley, 2005) among others. The THO/TREX complex has evolu-

tionarily conserved components for RNA processing and export, and one of the first significant descriptions of pathologic R-loop formation was in yeast with *hpr1* deletion, a member of the THO complex, which exhibited a hyper-recombination phenotype (Huertas and Aguilera, 2003). Loss of other members of the THO/TREX complex also result in transcription impairment and genome instability that are rescued with RNaseH overexpression (Huertas and Aguilera, 2003). Similar results have been described in other model organisms (Li and Manley, 2005; Wahba *et al.*, 2011) and with the mammalian THO complex (Domínguez-Sánchez *et al.*, 2011). The THSC/TREX-2 complex is a group of factors that functions at a different step of RNA processing and export. Similar to THO complex mutants, loss of THSC/TREX-2 factors results in transcription defects and hyper-recombination that is rescued by RNaseH (Gallardo *et al.*, 2003; González-Aguilera *et al.*, 2008). BRCA2, which has shown to prevent R-loop accumulation, is known to associate with TREX-2 factor PCID2 (Bhatia *et al.*, 2014).

As mentioned previously, loss of members of the Fanconi Anemia pathway (FANCA, FANCD2, FANCM) results in R-loop-mediated DNA damage and genome instability (García-Rubio *et al.*, 2015; Schwab *et al.*, 2015). BRCA1 and BRCA2 also act as part of the Fanconi Anemia pathway and have important roles in DNA repair. Loss of either BRCA1 (Hatchi *et al.*, 2015; Gorthi *et al.*, 2018) or BRCA2 (Shivji *et al.*, 2018) results in R-loop accumulation and DNA damage. While BRCA2 depletion results in R-loop accumulation at the 5' end of genes, BRCA1 depletion causes R-loops to form at both 5' and 3' end of genes. Interestingly, both BRCA1 and BRCA2 associate with RNA polymerase II and appear to be important for RNA elongation, as well as R-loop resolution (Zhang *et al.*, 2017; Shivji *et al.*, 2018). BRCA1 is also known to interact with senataxin (Becherel *et al.*, 2013; Hatchi *et al.*, 2015; Shivji *et al.*, 2018) and could promote R-loop resolution, at least in part, by recruiting this DNA:RNA helicase.

Senataxin is a DNA:RNA helicase with a known role in R-loop processing (Alzu *et al.*, 2012) and has been shown to suppress R-loop formation and transcription-replication-collision-mediated genome instability in yeast (Mischo *et al.*, 2011) and in human cells (Hatchi *et al.*, 2015), often in connection to BRCA1 (Becherel *et al.*, 2013; Hatchi *et al.*, 2015). In its classic role, senataxin facilitates efficient transcription termination by recruiting the 5'-3' exoribonuclease XRN2 (Skourti-Stathaki *et al.*, 2011; Morales *et al.*, 2016). Senataxin has also been shown to associate with a 3'-5' exoribonuclease complex known as the exosome by interacting with subunit Rrp45 (Richard *et al.*, 2013; Yuce and West, 2013). In this way, it is believed that senataxin contributes to R-loop resolution by directly unwinding DNA:RNA hybrids in R-loops and recruiting the exosome (Richard *et al.*, 2013; Yuce and West, 2013) or XRN2 (Skourti-Stathaki *et al.*, 2011; Morales *et al.*, 2016) to degrade released RNA. As mentioned before, senataxin and R-loops have also been connected to lengthening of telomeres (De Amicis *et al.*, 2011; Balk *et al.*, 2013). Other helicases such as DXH9 (Cristini *et al.*, 2018), Pif1 (Tran *et al.*, 2017), DDX5 (Mersaoui *et al.*, 2019), and aquarius (Sollier *et al.*, 2014) have also been shown to play roles in R-loop processing.

A link between R-loops and DNA damage could come in the form of nucleotide excision repair factors. Nucleotide excision repair factors XPG and XPF were shown to be important for the increased DNA damage seen following R-loop formation (Sollier *et al.*, 2014; Yasuhara *et al.*, 2018). Although the precise mechanism is unclear, these factors could remove R-loops that are responsible for transcription stalling and leave a gap of single-stranded DNA which gets converted to a double-strand DNA break during replication.

As mentioned previously, a number of topoisomerases have been implicated in R-loop formation, which include topoisomerases Top1 (Tuduri *et al.*, 2009; El Hage *et al.*, 2010; Manzo *et al.*, 2018), Top3a (Oh *et al.*, 2007), and Top3b (Wilson-Sali and Hsieh, 2002; Stoll *et al.*, 2013; Xu *et al.*, 2013; Yang *et al.*, 2014). Conceivably, these enzymes act to prevent R-loop formation by decreasing high-energy negative supercoiling (Stolz *et al.*, 2019) that favors conversion to non-B forms of DNA (Kouzine, 2007; Levens and Benham, 2011; Baranello *et al.*, 2012). It is likely that other topoisomerases are also involved in this process, specifically the type 2 topoisomerases given their presence at transcription start sites of highly expressed genes (Kouzine *et al.*, 2013; Teves and Henikoff, 2014). It is also possible that topoisomerases have other activity that is necessary to control R-loop levels. For instance, topoisomerase I directly interacts with transcription machinery and is important for transcription elongation (Baranello *et al.*, 2016). In addition to relaxation activity, topoisomerase 3b was also shown to cleave R-loops in other model organisms (Wilson-Sali and Hsieh, 2002).

Conclusion

In conclusion, R-loops are a biological phenomenon with major implications in normal cellular physiology, as well as pathology, with extensions to human disease. R-loops are no longer considered by-products of transcription and have significant roles in processes such as mitochondrial replication. R-loops also form stable structures in conserved switch regions to allow for action of CSR machinery. One of the most significant ways R-loops could be involved in cell physiology is through regulation of gene expression. Since R-loops form most often at promoters and at the 3' end of genes, they can regulate transcription at either end. Acting near the promoter, the R-loop DNA:RNA hybrid could directly inhibit transcription factor binding or R-loops could absorb supercoiling necessary to open the DNA double helix and the supercoiling required for DNA conformational changes that allow for enhancer-promoter interaction. Acting with other factors downstream of genes, R-loops could affect transcription termination efficiency. Finally, R-loops could affect gene expression at the epigenetic level, modifying histones and chromatin states.

Some studies reveal pathological consequences of R-loop formation. There are strong connections between R-loops and DNA damage, as well as findings that suggest a role for R-loops in human disease. This is an exciting and young area of research, with many opportunities for further research. It is unknown whether R-loops play a causative or consequential role in these diseases. In addition, it is unknown as to what impact these novel findings will have on our understanding of these disease processes. R-loops seem to contribute to DNA damage and genome rearrangements in trinucleotide repeat neurologic disorders and in cancer,

and perhaps targeting R-loops represents an alternative therapeutic approach. In theory, decreasing R-loop formation could protect against genome instability in these neurologic disorders. In contrast, highly proliferative cancer cells, which are predisposed to R-loop formation, could be susceptible to fatal DNA damage if the balance was tipped even further toward R-loop accumulation.

An evolving subject, many avenues of research should be explored to explain and resolve the range of findings in this area, as well as evaluate applications of this newfound knowledge. For instance, what differentiates normal or beneficial R-loops with physiologic functions from R-loops that lead to DNA damage and genome instability? Some suggest that the abnormal buildup or persistence of R-loops overwhelms resolution pathways with negative consequences since global R-loop increases have been measured and linked to DNA damage and RNaseH rescue (Stork *et al.*, 2016; Gorthi *et al.*, 2018). Others propose that only subsets of R-loops are responsible for these harmful effects, depending on the surrounding environment (García-Pichardo *et al.*, 2017) or characteristics of the R-loop itself such as orientation (Hamperl *et al.*, 2017).

It is yet to be discovered how R-loops can have seemingly contradictory influences on transcription or chromatin state—how can R-loops promote successful transcription in some instances and impair it in others? How do R-loops protect against repressive epigenetic modifications in some places and favor it in others? Another example would be the effect of R-loops on DNA repair. In many cases, R-loops hinder DNA repair (Amon and Koshland, 2016; Cohen *et al.*, 2018) while proving essential in other instances and in specific types of double-strand break repair (Keskin *et al.*, 2014; Ohle *et al.*, 2016; Mazina *et al.*, 2017; Lu *et al.*, 2018; Teng *et al.*, 2018; Yasuhara *et al.*, 2018). A number of opportunities for R-loop research exist, including the ones just mentioned, and it is likely that R-loop biology will be an exciting area of research for many years.

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