

REVIEW

Looping forward: exploring R-loop processing and therapeutic potential

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Recently, there has been increasing interest in the complex relationship between transcription and genome stability, with specific attention directed toward the physiological significance of molecular structures known as R-loops. These structures arise when an RNA strand invades into the DNA duplex, and their formation is involved in a wide range of regulatory functions affecting gene expression, DNA repair processes or cell homeostasis. The persistent presence of R-loops, if not effectively removed, contributes to genome instability, underscoring the significance of the factors responsible for their resolution and modification. In this review, we provide a comprehensive overview of how R-loop processing can drive either a beneficial or a harmful outcome. Additionally, we explore the potential for manipulating such structures to devise rationalized therapeutic strategies targeting the aberrant accumulation of R-loops.

Keywords: DNA damage; DNA repair; genome instability; R-loops; transcription

Transcription is far from a neutral coordination of proteins toward the mechanical synthesis of RNA; the enzymatic activities involved and the mechanisms that ensure DNA accessibility paradoxically contribute to genome instability, making gene expression a risky endeavor. Besides the inherent mutagenesis associated with transcription [1,2], DNA breaks induced to relieve

RNA polymerase-associated torsional stress or the risk of transcription-replication conflicts, a necessary evil that accompanies mRNA synthesis arises from the formation of non-B DNA structures, such as R-loops. R-loops are generated when a transcribed RNA strand invades the DNA duplex and hybridizes with the template DNA, forming an RNA–DNA hybrid and, at

Abbreviations

AGS, Aicardi Goutières syndrome; AID, activation-induced cytidine deaminase; ALS, amyotrophic lateral sclerosis; ALS4, amyotrophic lateral sclerosis type 4; ALT, alternative telomere lengthening; Alt-NHEJ, alternative-non-homologous end joining; AML, acute myeloid leukemia; AOA2, ataxia with oculomotor apraxia 2; BER, base excision repair; BRD4, bromodomain-containing protein 4; CD, co-directional; cen R-loops, RNA–DNA hybrids at the centromere; cenRNAs, centromeric long non-coding RNAs; CPEO, chronic progressive external ophthalmoplegia; CPT, camptothecin; CSR, class-switch recombination; DDR, DNA damage response; DDRNAs, DNA damage response RNAs; dilncRNAs, damage-induced long non-coding RNAs; DSB, double-strand break; FTD, frontotemporal dementia; FXS, fragile X syndrome; G4, G-quadruplex; H3K27ac, H3 acetylation; H3K36me3, tri-methylation of lysine 36 of histone H3; H3K4me1, mono-methylation of lysine 4 of histone H3; H3K4me3, tri-methylation of lysine 4 of histone H3; HDR, homology-directed repair; HO, head-on; HR, homologous recombination; ICF, immunodeficiency-centromeric instability-facial anomalies syndrome; lncRNA, long non-coding RNA; m⁶A, N⁶-methyladenosine; MDS, myelodysplastic syndrome; mESC, mouse embryonic stem cell; MRN, MRE11-RAD50-NBS1; NER, nucleotide excision repair; NHEJ, non-homologous end joining; PRC2, polycomb repression complex 2; ROS, reactive oxygen species; RPA, replication protein A; SETX, senataxin; SMA, spinal muscular atrophy; SSB, single-strand break; ssDNA, single-stranded DNA; TC, transcription-coupled; TERRA, telomere repeat-containing RNA; TOP1, topoisomerase 1; TRCs, transcription-replication conflicts; TSSs, transcription start sites; *VIM*, *vimentin* gene; XP, xeroderma pigmentosum.

the same time, displacing the non-template DNA as a single-stranded DNA (ssDNA). Such three-stranded structures were originally thought to be merely by-products of transcription, generated exclusively *in cis* [3]; there is emerging evidence to suggest that they can be formed *in trans* [4,5], while they also exhibit regulatory functions. In addition, although R-loops are instinctively considered to form behind RNA polymerases, there is evidence to suggest that, during backtracking (the backward shift of RNA polymerase along DNA), R-loops are also generated anterior to the RNA polymerase complex [6]. While short, transient RNA–DNA hybrids form continuously during transcription or DNA synthesis of Okazaki fragments [7], R-loops are distinct structures that form throughout the cell cycle, span about 100–2000 base pairs and occupy 5–10% of the genome [8,9]. They have a tendency to form at sites with high GC-content or GC-skew [10–12], a preference which extends their influence beyond highly expressed genes, to encompass repetitive genomic regions, centromeres, telomeres and enhancers [12–14].

RNA–DNA hybrids and R-loops have recently attracted attention for having roles in multiple physiological processes, including gene expression regulation and DNA repair [15–18]. Nevertheless, the failure to promptly and effectively eliminate such structures results in DNA breaks leading to genome instability. For the detrimental effects of R-loops on genome integrity, we refer the readers to the following reviews [19,20]. Here, we discuss how processing of co-transcriptional R-loops affects the outcome of gene expression and cell homeostasis, with an emphasis on the factors involved in their recognition, modification or removal. We juxtapose the regulatory, beneficial roles of R-loops, against the detrimental consequences of their misregulation, while also considering the possibility of R-loop manipulation for therapeutic purposes.

Discerning the characteristics of physiological and harmful R-loops

Since the earliest evidence of R-loop formation *in vitro*, in 1976 [21], there have been numerous studies mapping R-loops and investigating their biological functions in different organisms [18,22]. Paradoxically, besides the multiple cases where R-loop formation is beneficial for fundamental cellular processes, they have also been proven to be deleterious to cells. The distinction between R-loops with physiological (regulatory) functions, formed either in specific genome *loci* or at scheduled time-points (programmed) and spontaneous

(unscheduled) R-loops, which can have detrimental consequences to the cell homeostasis, has raised many questions on their biology [17,20].

R-loops occur naturally across the genomes of bacteria, yeast and all higher eukaryotes and are now considered to be more than byproducts of transcription [9,12]. Comparison of results generated with different R-loop mapping strategies has revealed major discrepancies, especially in terms of size, sequence characteristics and distribution of R-loops in the genome. For example, depending on the technique [23–28], R-loops are either mapped predominantly along transcribed gene bodies and 3' gene terminal regions and at hot-spots of GC-skewed promoters, or identified at G-rich promoters and Transcription Start Sites (TSSs), possibly constricted by the presence of auxiliary endogenous protein factors, such as the ssDNA-binding protein Replication Protein A (RPA).

Nevertheless, a general consensus emerging from R-loop mapping approaches is their preference for GC-rich or -skewed genic regions and their transient nature. This dynamic characteristic of R-loops, besides making the identification of their interacting factors and occupancy sites (e.g. enhancer RNAs have short half-lives) very challenging, also highlights detrimental implications for genome stability, in the case of other more stable or persistent hybrids, or R-loops occurring at non-scheduled times or at aberrant sites [29]. It has been suggested that the threat to genome stability is derived from a secondary event and not the R-loop itself [20]. This is supported by evidence that describe specific histone mutants that accumulate R-loops without concurrent DNA damage accumulation [30]. Current research only describes R-loops as harmful in the context of aberrant genetic modifications and disorders or in the presence of exogenously added agents that influence the transcription process. Although any discernible molecular features of such harmful R-loops have not been defined yet, a subset of partially RNase H-resistant RNA–DNA hybrids were recently identified, whose characteristics may well explain the secondary features of an intrinsically (un)stable R-loop [31].

In principle, the structure of an R-loop poses a significant threat for genome stability, as the displaced ssDNA strand is open to exogenous and endogenous DNA damaging agents and can be a substrate for nucleases and DNA modifying enzymes. A nick in the DNA can be further processed into a DNA double-strand break (DSB), making R-loops a source of DNA damage, while, at the same time, they have the potential to block replication fork progression, causing collisions with the transcription machinery. To this

end, cells have developed protective measures to secure the fine balance of R-loop formation and resolution; RNA export/processing factors [32] and DNA topoisomerases prevent their formation, RNA helicases [33] and RNases remove them, and nucleases [15] promote DNA repair (Table 1).

Intriguingly, the very same structure of R-loops that makes them a threat to genome integrity is also responsible for their regulatory functions. The first described example of biologically important regulatory R-loop formation highlights their transcription-induced contribution in immunoglobulin class-switch recombination (CSR), during B cell activation [34]. Indeed, it is accepted that high transcription rates increase the likelihood of co-transcriptional formation of R-loops [9]. In CSR, R-loops form co-transcriptionally at the G-rich switch region of the *IgH* locus. Cytosine residues in the displaced non-template ssDNA are then deaminated by Activation-Induced cytidine Deaminase (AID), converted to uracils and processed by factors of the Base Excision Repair (BER) and Mismatch Repair pathways, to generate DNA nicks and double-strand breaks (DSBs), respectively. The production of the different Ig types, vital for the immune response, is only possible through the consequent end joining of these DSBs. Yet, although the connection of RNA polymerase elongation rates with R-loops suggests that high gene expression levels promote R-loop formation, in line with rRNA and tRNA *loci* and multiple context-specific studies, it is widely established that R-loops are also potent inhibitors of RNA polymerases, leading to stalling and transcription inhibition [35].

This contradictory dual nature of R-loops is the focus of ongoing investigation [36], and a major challenge in the field is to dissect the phenotypes that result directly from R-loops, or from impaired transcription elongation-associated processes, like mRNA splicing, methylation or export. Besides the specific R-loop processing events described here, the possibility of a context-dependent R-loop biology and structure would be intriguing and could explain their different functions.

Physiological roles of R-loops during DNA-templated transactions

Linking R-loop processing to gene expression outcomes

In addition to their structure, the sequence specificity of RNA–DNA hybrids allows R-loops to either attract and precisely target proteins on particular

chromosomal regions or inhibit the binding of cofactors. For example, R-loops have been linked to the protection of CpG islands against DNA methylation, both by repelling DNA methyltransferases and by recruiting H3K4 methyltransferases, which results in chromatin decondensation and transcription activation [12,37,38]. This renders R-loops ideal candidates for gene expression and chromatin organization regulation. The fact that such structures occupy ~ 10% of the genome and are found primarily in genic regions, at promoters and termination sites, further supports this role.

R-loop-associated transcription activation has been linked to activating histone marks, such as mono- and tri-methylation of lysine 4 of histone H3 (H3K4me1/3) and H3 acetylation (H3K27ac), or to elongation marks, like the tri-methylation of lysine 36 of histone H3 (H3K36me3) [12,39]. Moreover, in the case of the *vimentin* (*VIM*) gene, transcription activation occurs when the antisense long non-coding RNA (lncRNA) *VIM-AS1* forms an R-loop downstream of the *VIM* TSS, which attracts activators of the NF- κ B pathway [40]. In a similar fashion, the lncRNA TARID-generated R-loop recruits the stress response protein GADD45A and the methylcytosine dioxygenase TET1 on the promoter of the *TCF21* tumor suppressor gene, for demethylation and transcription activation [41]. Additionally, R-loop-directed, CTCF-mediated DNA looping has recently been shown to induce transcription in mouse embryonic fibroblasts [42]. On the other hand, R-loops have also been shown to induce transcriptional repression. For instance, in *Arabidopsis thaliana*, the binding of the ATNDX transcription factor on the displaced ssDNA strand stabilizes an R-loop on the *COOLAIR* promoter, resulting in transcription silencing [43]. Similarly, transcription repression of the *RASSF1A* promoter and a subset of mouse embryonic stem cell (mESC) developmental genes is achieved by the R-loop-directed recruitment of the Polycomb Repression Complex 2 (PRC2) [44]. In contrast, a different study has shown that the formation of R-loops leads to the antagonistic recruitment of the histone acetyl-transferase complex TIP60-p400, over PRC2 binding, in promoter regions of mESCs [45]. These results strongly indicate that the role of R-loops as effectors of epigenetic and transcription regulation depends on the specific context and could be differentially modulated by their stabilization or resolution. Proteins that bind the displaced ssDNA strand of R-loops may either stabilize the R-loop structure, as in the case of ATNDX [43], or they can facilitate the consequent recruitment of chromatin-modifying enzymes or transcription factors. RPA, for example, is

Table 1. Protein factors involved in R-loop regulation and function.

| Molecular function | Protein factor | Associated disease | Proposed mechanism/process | References |
|-------------------------|--------------------|--|---|-----------------|
| Topoisomerases | | | | |
| Topoisomerase | TOP1, TOP2 | Cancer | Prevention of negative supercoiling, replication stress | [42,84,202–205] |
| Topoisomerase | TOP3B/TDRD3 | Multiple Myeloma Burkitt's Lymphoma | R-loop resolution | [179,206] |
| Nucleases | | | | |
| Ribonuclease | RNase H1 | Cancer, Mitochondrial Encephalomyopathy, Chronic Progressive External Ophthalmoplegia (CPEO) | R-loop resolution | [207–209] |
| Ribonuclease | RNase H2 | Aicardi-Goutières Syndrome (AGS) | R-loop resolution | [154,210–212] |
| Ribonuclease | DICER | Cancer, DICER1 syndrome | R-loop resolution | [57,213] |
| Ribonuclease | XRN2 | | R-loop resolution, transcription termination | [56,60,214,215] |
| Endonuclease | XPF, XPG | Xeroderma Pigmentosum (XP), chronic pancreatitis | R-loop processing | [42,51,53] |
| Endonuclease | FEN1 | Lung and gastrointestinal Cancer | Trinucleotide repeat deletion in R-loops | [216] |
| ssDNA nuclease | SAMHD1 | Aicardi-Goutières Syndrome (AGS) | R-loop resolution, tumor suppressor | [155,212] |
| ssDNA nuclease | TREX1 | Aicardi-Goutières Syndrome (AGS) | R-loop resolution | [212,217] |
| Helicases | | | | |
| Helicase | SETX | Amyotrophic Lateral Sclerosis type 4 (ALS4), Ataxia with Oculomotor Apraxia type 2 (AOA2) | R-loop resolution | [37,56,122,218] |
| Helicase/mRNA splicing | AQR | | R-loop resolution, DSB repair by HR | [53,219] |
| Helicase | DHX9 | | R-loop formation/resolution, transcription termination | [58,220] |
| Helicase | DDX5 | Cancer | R-loop resolution, transcription termination | [83,221] |
| Helicase | DDX21 | Spinal Muscular Atrophy (SMA) | R-loop resolution | [222,223] |
| Helicase | DDX17 | Cancer | R-loop formation/resolution | [33,224] |
| Helicase | DDX1 | Retinoblastoma, neuroblastoma | R-loop formation/resolution | [225,226] |
| Helicase | DDX41 | Myelodysplastic syndrome (MDS), Acute myeloid leukemia (AML) | R-loop suppression, tumor suppressor | [227] |
| Helicase, DNA repair | BLM | Bloom's syndrome, cancer | R-loop resolution | [228] |
| Helicase, transcription | RECQL5 | | Transcription elongation, R-loop suppression | [229] |
| Helicase, exonuclease | WRN | Werner syndrome | R-loop resolution | [230,231] |
| Translocase/DNA repair | FANCM | ALT-dependent cancer | R-loop resolution | [232] |
| Helicase | RTEL1 | | G4 binding on TERRA for R-loop resolution | [233,234] |
| Nucleic acid binding | | | | |
| ssDNA binding | AID | Immunodeficiency, cancer | Deaminates cytosine residues in the ssDNA | [235,236] |
| ssDNA binding | RPA | | R-loop formation/resolution | [46,47] |
| ssDNA binding | RAD51 | | R-loop formation | [237,238] |
| RNA binding | EWS-FLI1/ EWSR1 | Ewing's sarcoma | R-loop formation/suppression | [137] |
| DNA/RNA binding | TDP-43/FUS | Amyotrophic Lateral Sclerosis (ALS), Frontotemporal Dementia (FTD) | R-loop suppression, replication stress | [239–241] |

Table 1. (Continued).

| Molecular function | Protein factor | Associated disease | Proposed mechanism/process | References |
|--|--------------------|--|--|------------------|
| DNA binding | WASp | Wiskott-Aldrich syndrome | R-loop suppression, regulates RPA ssDNA binding | [242,243] |
| DNA binding, repair | RAD52 | | R-loop processing for DNA repair | [72] |
| DNA binding, repair | BRCA1/2 | Breast and ovarian Cancer | R-loop formation/suppression, recruited on R-loops for DNA repair | [244,245] |
| DNA binding, repair | FANCA-W | Fanconi Anemia | R-loop suppression by ssDNA/RNA binding | [196,246] |
| RNA processing | | | | |
| mRNA processing/export | THO/TREX-2 complex | Hyperrecombination, sarcoma | Transcription elongation, mRNA export | [35,244,247,248] |
| Splicing factor | U2AF1/2 | Myelodysplastic syndrome (MDS) | Recruited by R-loops for co-transcriptional pre-mRNA splicing | [64,249] |
| Splicing factor | SRSF1/2 | NASH, Myelodysplastic syndrome (MDS) | R-loop suppression | [250,251] |
| RNA editing | ADAR1 | Aicardi-Goutières syndrome (AGS), cancer | R-loop regulation by editing A-C mismatches | [252] |
| Telomere binding, RNA/DNA binding | Shelterin complex | | R-loop formation/suppression, G4 binding on TERRA | [253,254] |
| Chromatin remodeling | | | | |
| Chromatin remodeler, helicase | ATRX | ALT-dependent cancer, ATRX syndrome | R-loop suppression at telomeres | [47,255] |
| Chromatin remodeler, histone chaperone | FACT complex | Cancer | Nucleosome assembly at R-loop TRCs | [256] |
| Chromatin remodeler | ARID1A | Cancer | TOP2A-mediated R-loop regulation at TRCs | [257] |
| Chromatin remodeler | INO80 | Cancer | R-loop resolution, chromatin relaxation | [258,259] |
| Chromatin remodeler | BRG1 | Cancer | R-loop suppression, regulation of chromatin accessibility | [260] |
| Methyltransferases | | | | |
| RNA methyltransferase | METTL3/14, TonEBP | DDR, immunoregulation | R-loop formation/suppression by m6A methylation | [61,63] |
| RNA methyltransferase | TRDMT1 | Cancer | R-loop regulation by m ⁵ C methylation | [261] |
| DNA methyltransferase | DNMT3B | Immunodeficiency-centromeric instability-facial anomalies syndrome (ICF) | (peri)-centromeric R-loop suppression | [262] |
| Other | | | | |
| snRNP biogenesis | SMN1 | Spinal Muscular Atrophy (SMA) | Promotes splicing to prevent R-loops and instability | [263] |
| Poly [ADP-ribose] polymerase, DNA repair | PARP1 | Cancer | R-loop suppression | [160] |
| Transcription co-activator | BRD4/CDK9 | Cancer | R-loop suppression, transcription elongation at TSS, transcription termination | [164,213] |
| Helicase-exoribonuclease complex | SUV3-PolA-PNPase | | Mitochondrial R-loop resolution | [264] |

required for the stabilization of the ssDNA strand and also promotes R-loop formation [46], but can also recruit RNase H1 for their resolution [47]. Likewise,

AID initiates the resolution and repair of R-loops in CSR [34]. Interestingly, however, AID deficiency leads to genome-wide hypermethylation [48], suggesting

an additional role in R-loop-directed DNA demethylation.

The two structure-specific endonucleases involved in the transcription-coupled (TC) nucleotide excision repair (NER) pathway [49,50], XPF and XPG, have also been identified as R-loop modifiers [51–53]. Independently of their primary function in removing transcription-blocking DNA lesions, these factors are involved in R-loop processing, leaving single-strand breaks that can be converted into DSBs, through replication. Although the TC-NER pathway functions mainly in gene bodies, the two nucleases have been recently shown to be recruited preferentially on promoters, termination regions or imprinted genomic *loci*, where they facilitate R-loop-dependent, CTCF-mediated DNA looping for transcription activation or silencing [42,54,55]. Consistent with their enrichment in G-rich RNAPII termination sites, especially in gene-dense regions, R-loops have also been suggested to assist in transcription termination by stalling RNAPII at the poly-A sequence. Their resolution ensures the accurate processing and termination of transcripts, preventing transcriptional read-through [56]. This could be achieved by the recruitment of Senataxin (SETX), an RNA/DNA helicase involved both in transcription termination, through the recruitment of the XRN2 exoribonuclease, and R-loop resolution. DICER, the only other reported RNA–DNA hybrid resolvase, besides RNases H, has also been suggested to favor the release of RNAPII at termination sites, but it remains to be seen if this function is guided by replication [57]. Additionally, deficiency in the RNA helicases DHX9 and DDX5 has also been shown to impair termination of transcription by RNA polymerases [58–60]. Recently, the reversible RNA modification N⁶-methyladenosine (m⁶A), which is generated on R-loops by the METTL3 methyl-transferase, was shown to facilitate transcription termination [61]. The m⁶A reader YTHDF2 was additionally demonstrated to be involved in their resolution [62,63]. Thus, a unified model emerges, wherein R-loop binding factors may regulate gene expression while, at the same time, ensuring that R-loops are readily removed to avoid genome instability (Fig. 1A). However, it remains unclear in which genomic contexts these functions are exerted and how a balance in R-loop numbers is achieved.

R-loops influence DNA damage response and repair

Genome integrity is not only safeguarded by the timely dissolution of R-loops; a large body of evidence links

the presence of R-loops and RNA–DNA hybrids directly to DNA damage signaling and repair. By stalling replication forks or promoting DSB formation, R-loops activate the ATR and ATM protein kinases that orchestrate the response to DNA damage [64–67]. Additionally, as in CSR, R-loops act as DNA repair intermediates, activating a non-canonical ATM-induced DNA damage response (DDR), with the concurrent retrograde translocation of RNAPII, which allows accessibility for repair, even in the absence of DSBs [68].

Less is clear about the role of R-loop processing (recognition, editing or resolution) in genome maintenance (Fig. 1B). For instance, the ssDNA-binding proteins RPA and RAD51 can mediate R-loop-induced DNA repair. However, their role could be attributed both to R-loop binding and to end resection for DSB repair, after R-loop resolution [69–71]. Similarly, recruitment of RAD52 to DSBs was shown to be reduced in RNase H-overexpressing cells [72], while the depletion of SETX favored Non-Homologous End Joining (NHEJ)-associated 53BP1 accumulation, over RAD51 recruitment. Yet, evidence that R-loops enhance or favor Homologous Recombination (HR) is still missing. What seems critical, however, is the impact R-loops have on DSB end resection, the step which guides the repair choice of HR or NHEJ; contradicting evidence shows R-loops can either block [73] or enhance [74] DSB resection and, vice-versa, resection-associated proteins, such as CtIP, can promote their resolution [75]. Of note, HR is also selected over alternative (Alt-) NHEJ, when the methyltransferase TRDMT1 preferentially inserts RNA m⁵C modifications on R-loops at DSBs [76], while m⁶A-modified R-loops are stabilized to promote DNA repair upon UV irradiation or camptothecin treatment [63].

Another contradicting feature of the involvement of R-loops in DNA repair is the transcription repression that ensues as a response to DNA breaks. This transcriptional silencing seems at odds with the formation of R-loops that are generally correlated with high transcription rates. Nevertheless, there is ample evidence to suggest that pre-existing, or *de novo*-synthesized, RNA transcripts are involved in the transient formation of RNA–DNA hybrids, a key step in DSB repair (Fig. 1B). These RNAs (DDRNs, dilncRNAs) exploit their sequence-specificity to guide DNA repair factors, such as BRCA1, BRCA2, or RAD51, to the site of the damage [77–79]. Although some of these R-loops might be formed by the potential stalling of an elongating RNAPII complex near the site of the damage, it is clear that repair of DNA nicks or DSBs favors R-loop formation, especially at actively

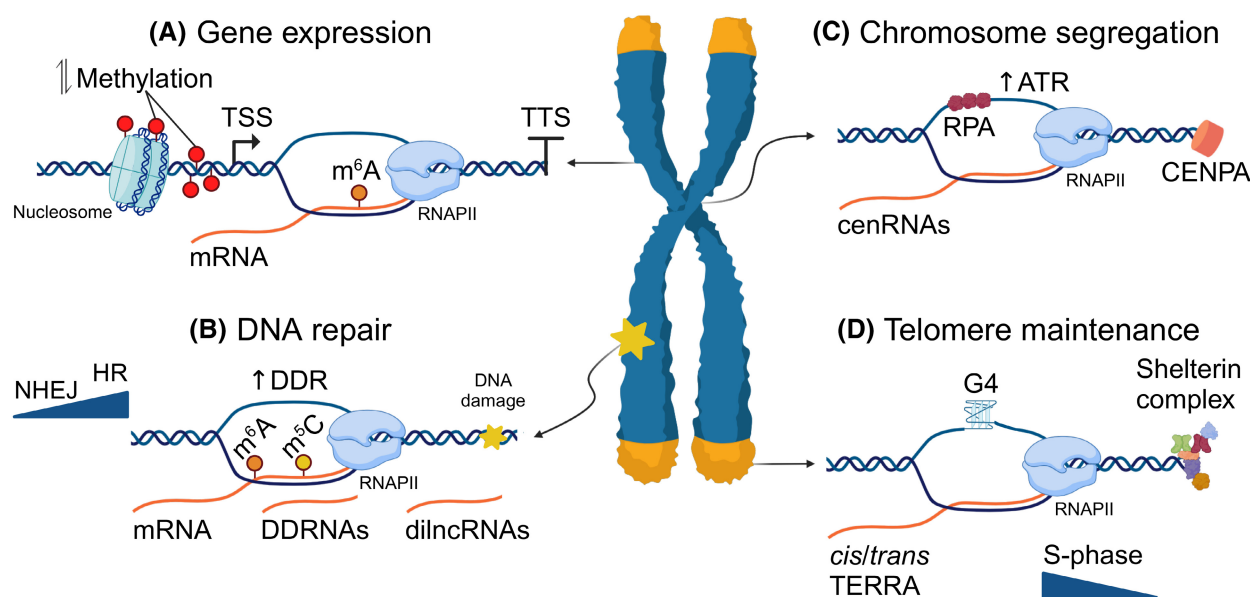


Fig. 1. Functions of regulatory R-loops. (A) R-loops form at promoters and transcription termination sites and affect transcriptional programs by altering the histone/DNA methylation status or by recruiting chromatin remodelers or transcription factors. (B) R-loops promote DNA repair by activating the DNA Damage Response, by recruiting protein factors that facilitate repair or by acting as repair intermediates. Pre-existing or *de novo* synthesized RNAs (DDRNs, dilncRNAs) form transient hybrids to guide DNA repair factors to the lesion, while RNA methyl-modifications either direct the choice of repair pathway or stabilize them to promote repair. (C) Centromeric R-loops are formed by long non-coding RNAs transcribed from centromeres (cenRNAs) and facilitate chromosome segregation through an RPA-dependent activation of ATR. Precise fine-tuning of cen R-loops is required for proper centromere assembly (CENPA recruitment) and DSB repair. (D) Telomeres are transcribed into telomere repeat-containing RNA (TERRA), a G-rich repetitive long non-coding RNA which has the propensity for R-loop formation, necessary for telomere maintenance. TERRA R-loops are transient, cell cycle-controlled and are regulated by the shelterin complex. Created with [BioRender.com](https://www.biorender.com).

transcribed regions [80]. This seems counter-intuitive, as one would expect that R-loop resolution would be required to allow repair. Indeed, RNase H overexpression enhances resection [70,77], while the helicase activity of several R-loop processing factors, such as DDX5 or DHX9, has been shown to be promoted in HR [81–83].

R-loops in telomere and centromere maintenance

RNA–DNA hybrids preferentially accumulate in telomeres and centromeres [9,10,12,23,84]. Despite their heterochromatinized nature, centromeres are transcribed by RNAPII into long non-coding RNAs (cenRNAs) [85–87]. CenRNAs are synthesized at low levels, in a cell cycle-dependent manner and can be found in single-, double-stranded RNA forms, or in RNA–DNA hybrids at the centromere (cen R-loops) [39,88,89]. The main function of centromeres, the interaction of microtubules with the kinetochore during cell division, is facilitated by cen R-loops, through the recruitment of RPA and the consequent ATR-dependent activation of Aurora B [90]. Although cen

R-loops are necessary for chromosome segregation, they are involved in CENP-A localization [91] and seem to promote end-resection for DSB repair [92]; their accumulation can be detrimental to genome stability [89] (Fig. 1C). It is tempting to speculate that, from an evolutionary perspective, cen R-loops offer a significant advantage to proper centromere functionality, especially if one considers that centromeres do not favor their formation; centromeric DNA sequences are mainly AT-rich [93,94], while CENP-A, the centromere-specific histone H3 variant, introduces positive DNA supercoiling, not suitable to promote R-loop formation [95–97].

Similar to centromeres, telomeres are transcribed into telomere repeat-containing RNA (TERRA), a G-rich repetitive long non-coding RNA which has the propensity for R-loop formation, *in cis* or *in trans* (reviewed in [98,99]). TERRA hybrids are degraded by RNase H enzymes in the S phase, to avoid replication conflicts, while they promote telomere maintenance by driving DNA repair and, as all R-loops, they are unstable and dynamic. Their transient nature becomes more evident by the different conditions that either

increase, reduce or stabilize them, especially considering that TERRA levels are also subject to multiple modifications (Fig. 1D). For example, co-transcriptional R-loop accumulation leads to telomere dysfunction [100], while in critically short telomeres, R-loops are stable throughout the cell cycle and accumulate due to impaired RNase H degradation [101–103]. This, in turn, promotes HR to prevent premature senescence. Similarly, R-loops promote telomere length in the tumor cells that maintain their telomeres by the alternative telomere lengthening (ALT) pathway. In fact, homology-directed repair (HDR) can only be achieved with strictly regulated R-loop levels, to avoid replication stress or insufficient HDR factor recruitment [13,104–106]. The formation of TERRA R-loops can be counteracted by proteins like the RNA processing THO complex, members of the shelterin complex that determine how TERRA interacts with the DNA, the SWI/SNF2 chromatin remodeler ATRX, or DNA repair factors, such as RAD51, BRCA1 and RAD27.

R-loops as drivers of genome instability

R-loops processing in transcription and replication stress

Within transcriptionally active regions, R-loops can interfere with the DNA repair process by forming physical obstacles to the lesion [107,108]. The contribution of R-loop formation on genome instability has been extensively covered in reviews and shall not be discussed here [15,19,20,29,109]. It is noteworthy that persistent R-loops can affect nearby gene transcription, altering the chromatin landscape, inducing global changes in transcription programs and contributing to multiple disorders, including neurodegenerative disease and cancer [18,110,111]. RNAPII stalling, due to unresolved R-loops, can also lead to truncated transcripts which, if translated, can yield aberrant proteins [112]. Since RNase H overexpression can suppress these effects, it has been presumed that the source of DNA damage is the R-loop structure. However, R-loop formation might simply be an indicator of transcription stress, prompting the argument that stalled/arrested RNAPII compromises genome integrity. In line with this, R-loops have been shown to be involved in the XRN2-dependent premature termination of RNAPII, under conditions of transcription stress. This suggests that unresolved R-loops might represent the actual cause of genome instability (Fig. 2A) [113,114].

Transcription and replication share the same DNA template and, regardless of the mechanisms to separate

them, a progressing replication fork can encounter R-loops during the S phase [115]. Chromatin-retained RNAPII due to transcription elongation defects can hinder replication [116], and the impact of R-loops on replication stress and genome stability has been highlighted either as a source, or a consequence of transcription-replication conflicts (TRCs) [117]. Evidence from depletion or overexpression of RNase H experiments suggests that R-loops can interfere with replication fork progression, yet the mechanistic details remain obscure [118–120]. In line, SETX, the FANCM helicase and RTEL1 have also been associated with R-loop resolution at TRCs [31,56,73,121–125]. The complexity arises from the varying effects of replication fork orientation in relation to an R-loop. It is suggested that conflicts of R-loops associated with head-on (HO)-oriented genes are more harmful, while the co-directional (CD) encounter of the transcription machinery by the replisome constitutes a smaller impediment [65]. When the replication fork encounters an R-loop, molecular events diverge depending on the length of the R-loop, the presence of secondary structures, like G4-quadruplexes, on the ssDNA strand, or even due to the presence of RNAPII (Fig. 2B) [126–128]. For example, the convergence of transcription and replication machineries in the HO orientation may induce topological stress, hindering further progression, whereas CD encounters can pose issues if RNA polymerase is stabilized by backtracking. Of note, the RECQL5 helicase mitigates backtracking, thereby reducing TRCs and genome instability [129,130].

R-loops hinder DNA repair

As briefly mentioned before, the mechanisms that link R-loops to DNA repair remain obscure. Nonetheless, there is evidence to suggest a role for R-loops in DSB end resection. After recognition by the MRE11-RAD50-NBS1 (MRN) complex, together with CtIP [131], EXO1/DNA2-mediated long-range resection follows, a process requiring the BLM helicase [132,133]. End resection at induced DSBs can be hindered by improper R-loop resolution, in the absence of crucial resolution factors (e.g. DDX5, DHX9) that lead to R-loop accumulation. This results in reduced RPA and RAD51 loading and nuclease recruitment [70,77,134], resulting in additional DNA damage [81–83,135]. In support of the idea that unscheduled R-loops inhibit end resection, RNase H/Sen1-depleted cells accumulated DSBs inhibiting resection at the end of the R-loop [73,136]. Hybrid clearance appears crucial at DSB sites, and deregulated hybrids may interfere with resection, causing repair defects and genome instability (Fig. 2C).

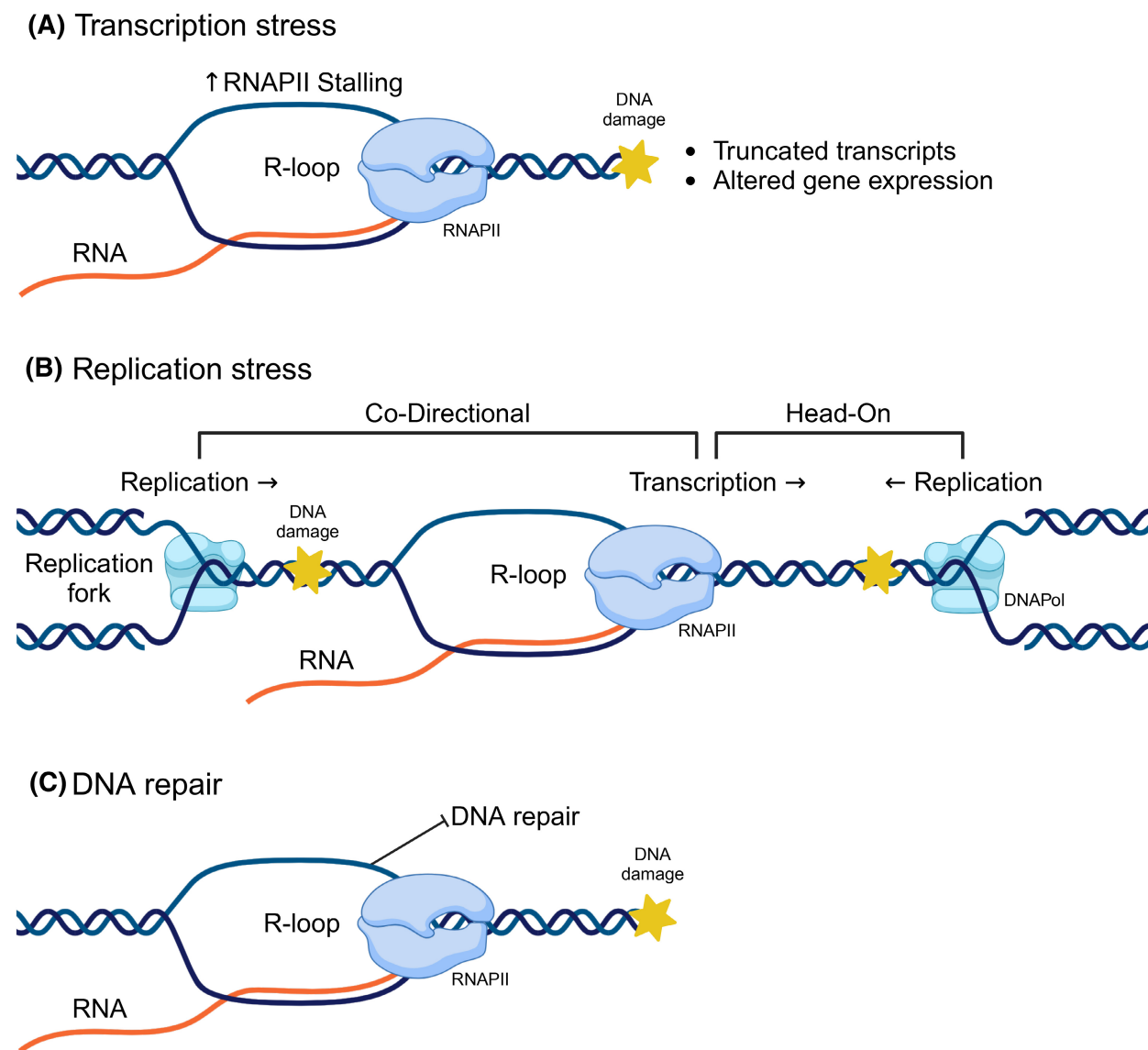


Fig. 2. R-loops as a source of genome stability. R-loops, if left unresolved, can be processed into single-strand or double-strand breaks, yet their impact extends to multiple DNA-templated processes, further contributing to genome instability. (A) R-loop formation leads to RNAPII stalling, DNA repair inhibition or replication stress. Persistent R-loop accumulation introduces global gene expression changes and leads to truncated transcripts and the aggregation of aberrant proteins, further adding to transcription stress. (B) Replication forks encountering the transcription machinery in a head-on (HO) or co-directional (CD) orientation lead to distinct outcomes. HO conflicts, being more damaging than CD, create favorable conditions for R-loop formation and DNA damage. (C) R-loops impede DNA repair and inhibit end resection at DSBs, by physically blocking access to or by interfering with recruitment of repair factors. Created with [BioRender.com](https://www.biorender.com).

Several factors that are implicated in R-loop resolution also interact with HR proteins, indicating a connection between HR and R-loop processing. For example, BRCA2 stimulates the helicase activity of DDX5, affecting R-loop dissolution [82], while the USP42 deubiquitinase facilitates DHX9 and recruits BRCA1 at R-loops located near DSBs, reinforcing the association between HR and hybrid resolution [81]. Additionally, R-loops could indirectly hinder DNA

repair by titrating out repair proteins, as observed in cells expressing EWS-FLI, where unscheduled R-loops prevent BRCA1 relocalization to DSB sites [137].

DNA breaks as a result of R-loop processing

The NER endonucleases XPF and XPG contribute to DSBs induced by R-loops, and their depletion results in an increase in cellular R-loop levels

[42,51,53,72,138]. The mechanism by which XPF and XPG act on R-loops is not fully understood; they might cleave opposite strands, generating a DSB, or cleave the same strand, generating a single-strand break (SSB), that is then processed to form a DSB. XPG and XPF can also be recruited to R-loops by XAB2, indicating a potential role beyond classical NER [52]. Yet, one could consider these XPF/XPG-induced DSBs not to be a mark of genome instability, but an R-loop processing intermediate that escapes the repair control. Lastly, it has been described that XPF-XPG are also involved in the generation of RNA–DNA hybrids or ssDNA fragments, that are actively exported to the cytoplasm of SETX/BRCA1/SAMHD1-challenged or ERCC1-deficient cells, respectively, where they induce an inflammatory response [51,139].

Topoisomerase 1 (TOP1) inhibition also results in R-loop-associated DSBs. For instance, the TOP1 inhibitor camptothecin (CPT) induces R-loops containing trapped TOP1 cleavage complexes, and the endonucleases XPF, XPG, FEN1, and MRE11 that contribute to break formation [138]. Additionally, the suppression of R-loops by Sae2/CtIP leads to reduced DNA break formation in human cells, while the absence of CtIP and XPG leads to R-loop accumulation, which is further exacerbated with the addition of CPT [53,75].

Lastly, cellular metabolic byproducts, such as reactive oxygen species (ROS), and enzymatic activities can cause damage to the exposed single-stranded DNA (ssDNA) of an R-loop structure.

R-loop formation or stabilization is increased specifically in actively transcribed regions, due to ROS-induced DNA nicks at these sites, consequently resulting in DNA damage [71,140,141]. Additionally, cytidine deaminases like APOBEC proteins or AID can contribute to damage of the exposed ssDNA in R-loops [142–144], resulting in uracil formation and subsequent single-strand break formation, as observed in immunoglobulin CSR [143].

R-loops as putative therapeutic targets

It becomes evident, from the above that R-loops are an integral part of normal cellular processes. Yet, the need for nuanced regulation of R-loop formation and resolution, for the efficient execution of their functions, as opposed to driving genome instability, is a recurrent theme in the study of their (patho-) physiology. It thus seems imperative for cells, for example, to separate the role of R-loops in facilitating

the recruitment of transcription factors and chromatin remodelers, from their involvement in transcription inhibition through RNAPII stalling. Likewise, cells need to fine-tune the timing of R-loop formation and subsequent removal. This is in line, for example, with the cell cycle-dependent regulation of telomere or centromere R-loops, which fits the DNA replication schedule to avoid transcription-replication collisions. To do so, cells must be able to determine the exact location or the chromatin environment that favors R-loop formation, or select which processing factors will be guided to the structures and when. Harnessing this information would provide us with insights on R-loop manipulation toward rationalized therapeutic interventions.

Indeed, R-loops have been documented to be associated with multiple diseases, including cancer, neurological syndromes and autoimmune disorders, which have been extensively described in multiple reviews [19,145–150]. In cancer, the abnormal accumulation of R-loops arises from global perturbations in transcription, replication, or RNA processing. The complex interplay between R-loops and oncogenes or tumor suppressor genes further drives tumorigenesis. Disease progression in several neurodegenerative disorders, such as amyotrophic lateral sclerosis type 4 (ALS4) or ataxia with oculomotor apraxia 2 (AOA2), is also associated with alterations in R-loop levels, while they also contribute to repeat instability, either through expansion or contraction [37,110,124,151–153]. Lastly, an immunogenic role has been attributed to R-loops, especially those associated with the Aicardi Goutières Syndrome (AGS), an autoimmune disorder that leads to progressive neurological damage driven by increased immune signaling [154,155]. AGS is caused by mutations in a number of genes involved in RNA/DNA processing, including RNase H2, TREX1, SAMHD1 and ADAR1, which eventually lead to the cytoplasmic accumulation of nucleic acid fragments.

As an approach toward combination therapy in oncology, R-loops have been targeted in order to sensitize cancer cells to chemotherapeutic treatments. Intercalating molecules, such as ethidium bromide [156] or actinomycin D [157] that directly interact with the RNA–DNA hybrids, have been used to inhibit telomerase activity or cell growth [158,159]. So far, there have been several attempts to exploit R-loop-driven DNA damage, toward the induction of cancer cell death. ATR inhibition, for example, sensitizes cells to R-loop-induced DNA breaks [66], while PARP inhibition leads to synthetic lethality in cells with impaired HR repair [160]. Moreover, reduced levels of RNF168, the E3 ubiquitin ligase of the R-loop helicase DHX9,

are correlated with a lower tumor incidence and better survival outcomes in patients [161]. The inhibition of RPA has also been shown to induce cytotoxicity and increase the sensitivity of cancer cells to chemotherapy [162]. By impeding the transcription process, Bromodomain-containing protein 4 (BRD4) inhibition leads to R-loop-dependent transcription-replication collisions, DSBs and cell death [163,164], while inhibition of pre-mRNA release leads to elevated R-loop levels and, interestingly, to the induction of apoptosis [165,166]. Similarly, G-quadruplex (G4) stabilizers [167–175], histone deacetylase inhibitors [176] or compounds that alter R-loop homeostasis [64,177–180] induce R-loop-dependent DNA damage and are efficient anticancer treatments. Similarly, selective inhibitory molecules that affect R-loop homeostasis (e.g. the CARM1 histone arginine methylase inhibitor EZM2302 [181], the PRMT1 histone arginine methyltransferase inhibitor GSK3368715 [179] or spliceosome inhibitors [182]) have been shown to induce cytotoxicity. Putative anticancer drug targets also include topoisomerase 1 [183], RNase H2 [184], SETX [185] or

splicing factors [64,186,187] whose selective inhibition increases R-loop levels. In neurodegenerative syndromes like fragile X syndrome (FXS), the therapeutic target is to inhibit R-loop formation and trinucleotide-repeat expansion that would lead to gene silencing [188]. Upregulation of R-loop-specific helicases, such as SETX, or endonucleases like RNase H1/H2, represents an efficient strategy to reduce R-loop levels. Alternatively, targeting the resolution of G4 structures that stabilize R-loops (e.g. in C9ORF72) or promoting the DSB repair processes to counteract the downstream R-loop effects could prevent the pathology of disease. The RNA–DNA hybrid present in the polypurine tract of HIV-1 has also been targeted with triple-helix-forming oligonucleotides [189] or small intercalating molecules [190], in order to inhibit reverse transcription of the virus RNA. Accordingly, G4 ligands have been used as antiviral therapy or to target telomeric R-loops [191–193].

Exploring the therapeutic potential of R-loop manipulation, while conceptually straightforward, faces certain pitfalls. Similarly to their regulatory

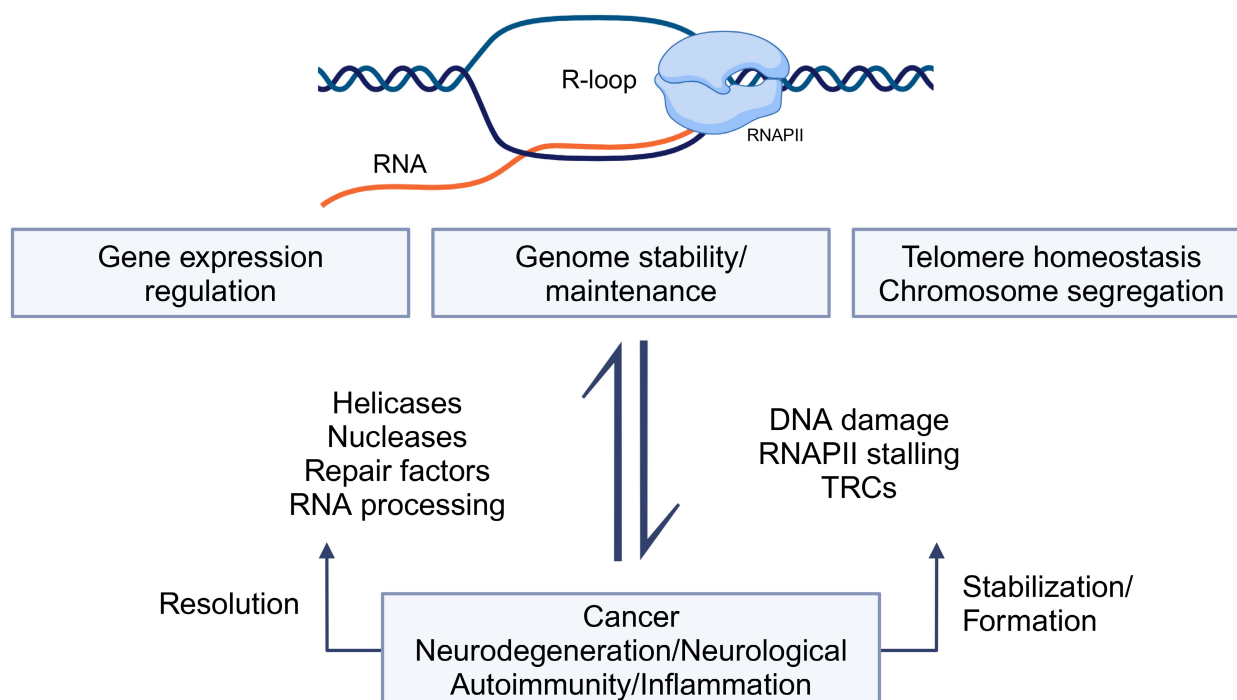


Fig. 3. Unlocking the dual nature of R-loops. R-loops function as both regulators of gene expression, DNA repair, telomere maintenance or chromosome segregation, and as drivers of genome instability. This inherent dual nature of these structures is coordinated by RNA/DNA/chromatin processing factors that safeguard the genome from detrimental R-loop-instigated DNA damage or cellular dysfunction. The association of R-loops with multiple diseases, including cancer, neurodegenerative or autoimmune disorders, underscores their clinical relevance. Therapeutic strategies targeting R-loop manipulation show promise, with approaches ranging from promoting (e.g. fragile X syndrome) to inhibiting their resolution (e.g. cancer). However, the context-dependent nature of R-loops necessitates careful consideration to avoid disrupting cellular homeostasis. Future research aimed at understanding the intricacies of R-loop biology and developing context-specific interventions will be crucial for realizing their therapeutic potential while minimizing adverse effects. Created with BioRender.com.

functions, the adverse effects of R-loops also seem context-dependent. In this sense, any efforts to forcefully eliminate them would disrupt the tight balance of their context-specific functions and lead to perturbations of cellular homeostasis. Of note, the indirect targeting of R-loops through their associated factors brings more complexity to the end result. For example, RNase H1 overexpression, besides being toxic [194–199], can alter the stability of a number of DDR factors [199], or impact the outcome of transcription programs [200,201]. Presumably, the unwinding capacity of helicases could prove more beneficial over the loss of nascent RNA transcripts. Moreover, the cell-cycle-regulated delivery of RPA-independent RNase H1 molecules could enhance efficiency. Utilizing the sequence specificity of R-loops in specific contexts, such as employing guide RNAs, would offer a more targeted approach. Overall, the therapeutic potential of targeting R-loops is promising, with strategies including inhibition of R-loop formation and promotion of their resolution offering avenues for intervention (Fig. 3). However, the complex nature of R-loop biology presents challenges, as their dual role as both regulators and sources of genome instability must be carefully considered. Context-specific approaches will be essential to minimize off-target effects and maintain cellular homeostasis while harnessing the therapeutic potential of R-loop manipulation.

Concluding remarks

Research on R-loops, the three-stranded nucleic acid structures formed by the hybridization of RNA to the DNA template, has seen significant interest due to the multitude of cellular processes that are affected by their presence. Inspiring work in the past years highlights their inherent duality as both regulators of gene expression, DNA repair, telomere maintenance or chromosome segregation, and as a potent source of DNA damage and genome instability (Fig. 3). Each role depends heavily on the genomic context and time of formation, but also, equally, on the distinct protein factors that mediate their resolution. Although our knowledge of the R-loops' impact on genome stability is increasing, so much so that they could even be brazenly considered as a DNA damage marker, many questions remain on what makes an R-loop to be harmful. Their DNA content/sequence definitely adds to this complexity, especially when it modifies their accessibility potential, while the surrounding torsional or epigenetic status of chromatin seems key to their functional outcome. Yet, from an evolutionary perspective, one can't help but ponder the causality of R-loop formation. For example, is their

transcription termination function general for all, or at least a specific subset of genes? Is the choice of HR over NHEJ repair affected by R-loop presence and could this be a characteristic of actively transcribed *loci*? And, vice-versa, could we extrapolate information from R-loop features (e.g., RNA–DNA hybridization or secondary structures) to be necessary for all DNA looping events? Continuing research over such questions will introduce an exciting number of opportunities toward the understanding of R-loop physiology and regulation. By navigating the complexities of R-loop biology, we can unlock new possibilities for developing targeted therapeutic interventions, to address a wide range of diseases and improve human health.

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Author contributions

KS and AS wrote the manuscript; GAG edited the manuscript.

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