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PERSPECTIVE

Evolutionary interplay between viruses and R-loops

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Viruses frequently interact with host transcriptional and epigenetic regulatory networks. A commonly overlooked element of these interactions is the formation of R-loops, three-stranded nucleic acid structures comprising an RNA–DNA hybrid and a displaced single DNA strand. Accumulating evidence implicates R-loops in viral integration site preferences, the regulation of latent viral genomes, epigenetic silencing, and even the genesis of small interfering RNAs (siRNAs) that modulate mobile viral elements. This perspective presents the potential connections among viral genes, transposons, and R-loops; examines the roles of R-loops in viral pathogenesis, latency, and reactivation; explores how viruses harness or evade R-loop–associated responses; and highlights future research directions—from mapping R-loop hotspots to exploiting R-loop modulation for antiviral therapy.

Keywords: chromatin; epigenetics; gene mimicry; host–virus interplay; R-loop; RNA–DNA hybrid; transposon; viral integration; virus

R-loops are three-stranded nucleic acid structures formed when a nascent RNA strand hybridizes with its template DNA, displacing the non-template DNA strand (Fig. 1). Although initially considered aberrant transcriptional byproducts, it is now evident that R-loops have diverse physiological and pathological functions [1–3]. These structures regulate gene expression, coordinate co-transcriptional RNA processing,

Abbreviations
BRCA1/2, BReast CAncer gene 1/2; Cas protein, CRISPR-associated protein; cccDNA, covalently closed circular DNA; cDNA, copy DNA; CMV, cytomegalovirus; ColE1, colicin E1; CRISPR-Cas9, clustered regularly interspersed short palindromic repeats/CRISPR-associated protein 9; crRNA, CRISPR RNAs; DAMP, damage-associated molecular patterns; DDX, DEAD-box helicases; DNMT, DNA methyltransferase; DRIP-seq, DNA–RNA immunoprecipitation sequencing; dsDNA, double-stranded DNA; dsRNA, double-stranded RNA; EBV, Epstein–Barr virus; G/C, guanine/cytosine; HBV, Hepatitis B virus; HBx protein, HBV-encoded oncogene X protein; HCMV, human cytomegalovirus; HCV, hepatitis C virus; HDAC, histone deacetylase; HERV, human endogenous retrovirus; HERV-K, human endogenous retrovirus K; het-siRNAs, heterochromatic siRNAs; HIV, human immunodeficiency virus; HIV-1, human immunodeficiency virus-1; HPV, human papillomavirus; HSV-1, herpes simplex virus-1; HTLV-1, human T-lymphotropic virus type 1; IL-10, interleukin-10; KSHV, Kaposi's sarcoma-associated herpesvirus; LANA, latent nuclear antigen; LEDGF/p75, lens epithelium-derived growth factor; LMP1, latent membrane protein 1; lncRNA4.9, long noncoding RNA4.9; LRA, latency-reversing agents; LTR, long terminal repeat; NSP14, non-structural protein 14; ORF57, open reading frame 57; ori, origin of replication; oriLyt, lytic origin of replication; PAMP, pathogen-associated molecular patterns; QDRIP, quantitative differential DNA–RNA immunoprecipitation; Rb/pRb, retinoblastoma protein; RdRp, RNA-dependent RNA polymerase; RISC, RNA-induced silencing complex; RNAi, RNA interference; S1-DRIP, S1 nuclease DNA:RNA immunoprecipitation; siRNA, small interfering RNA; Tat, trans-activator of transcription-1; TREX, transcription-export; vIL-6, viral interleukin-6.

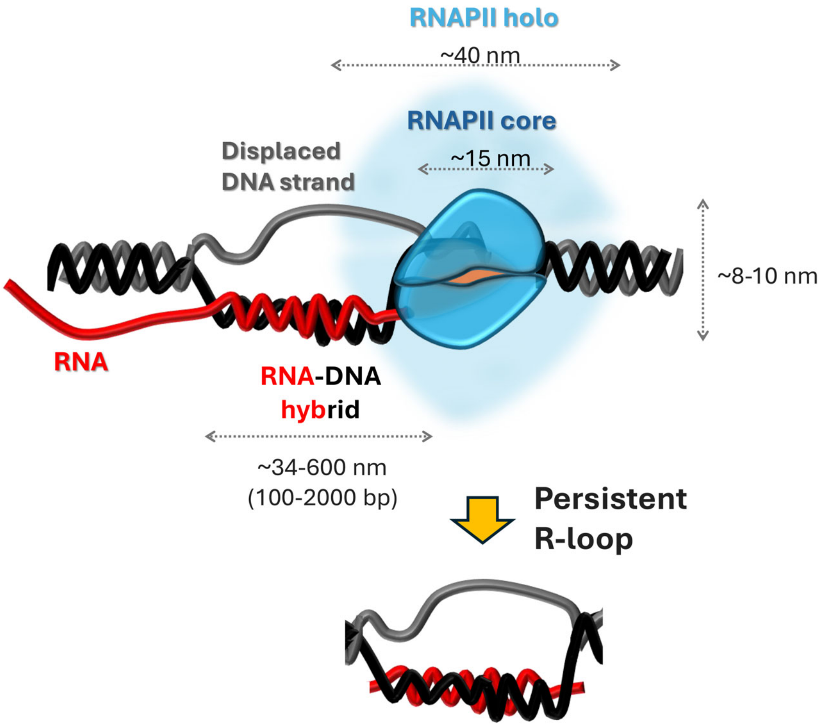

Fig. 1. Dimensions of an R-loop structure relative to RNA polymerase II (RNAPII), and its relevance to viral and host genomes. An R-loop forms when RNA hybridizes with the complementary DNA strand, typically spanning hundreds of base pairs, although in some genomic contexts, R-loops can extend up to 2 kilobases (kb) in length. This corresponds to a possible range from ~ 34 nanometers (nm) to over 600 nm. These structures can arise in both viral and host genomes and are characterized by similar biochemical features and dependence on comparable enzymatic machinery. For spatial context, the RNA Polymerase II (RNAPII) core enzyme has a compact structure with a length of around 15 nm and a height of 8 to 10 nm. The holoenzyme, which includes additional transcription factors such as Mediator and TFIIH, exceeds 40 nm in size and has a molecular mass greater than 2 megadaltons (MDa). Within the RNAPII complex, the transcription bubble spans ~ 10 nm, with DNA entry and exit channels measuring ~ 2 nm and the RNA exit channel slightly wider at ~ 3 nm. These structural comparisons highlight the physical scale of R-loops relative to the transcription machinery in both host and viral contexts.

facilitate DNA repair, and contribute to the alleviation of topological stress [2,4,5]. However, unresolved R-loops can compromise genomic integrity, leading to replication stress and DNA damage, underscoring their dual role in cellular homeostasis [2,6–9].

R-loops are widely distributed across the genome, forming preferentially in G/C-rich regions, at telomeres, centromeres, and within mitochondrial DNA [10–13]. They serve as critical regulators of transcription, influencing initiation, elongation, termination, and chromatin modifications [2,11,14]. Notably, during transcription of the polyA signal, endonucleolytic cleavage of the nascent RNA induces a kinetic pause that facilitates re-hybridization of the upstream RNA fragment with the DNA template, generating a stable RNA–DNA hybrid [7,15,16]. These R-loops act as physical barriers that promote Pol II dissociation, reinforcing transcription termination. Furthermore, they recruit termination factors such as the helicase senataxin (SETX), which resolves RNA–DNA hybrids and prevents transcriptional readthrough, preserving genomic stability.

R-loop dynamics is further controlled by a number of R-loop-resolving enzymes (resolvases) that safeguard genome integrity. These include helicases such as SETX, DDX5, DHX9, and Aquarius, which recognize and unwind RNA–DNA hybrids at gene bodies, terminators, and repetitive sequences [17]. Dysfunction or depletion of these enzymes results in R-loop accumulation, altered transcription, replication stress, and genomic instability [18].

Recent studies have highlighted a critical, noncanonical function of the tumor suppressor p53 in maintaining R-loop homeostasis through transcriptional regulation of key R-loop resolving enzymes. p53 has been shown to transcriptionally upregulate the RNA helicase DDX5 along with its stabilizing circular RNA partner, circASCC3, thereby promoting the resolution of R-loops under stress conditions [19]. Furthermore, p53 represses R-loop formation at retrotransposon-rich loci, including LINE1 elements, which are otherwise prone to inducing genomic instability [20]. The metabolic role of p53 also contributes to R-loop regulation by maintaining high levels of S-adenosylmethionine (SAM), a methyl donor required for histone and DNA methylation that influences R-loop stability [21]. Disruption of p53 function—either through mutation or viral targeting—can therefore lead to widespread R-loop accumulation and transcriptional dysregulation, as demonstrated in HPV-transformed cells where the viral E6 protein promotes R-loop formation by degrading p53 [22]. This loss of p53 not only impairs its canonical tumor-suppressive activities but also disrupts its regulation of R-loop-resolving pathways. As a result, R-loops accumulate particularly at innate immune gene loci and polyadenylation sites of the viral genome, contributing to immune suppression and sustaining a chromatin environment favorable for viral persistence and replication. These findings reinforce the concept of p53 as a central node in the cellular machinery that prevents pathological R-loop accumulation and preserves genome integrity.

Beyond their role in gene expression, R-loops mediate immunoglobulin class-switch recombination, centromere cohesion, mitochondrial genome maintenance [2,23], and even CRISPR-Cas9 targeting [24]. However, their persistence or misregulation is associated with transcriptional interference, replication stress, and genome instability—features underlying several human diseases, including neurodegeneration and cancer [6–8,25–27].

While the roles of R-loops in physiological gene regulation are increasingly understood, their involvement in viral infection and pathogenesis remains to be elucidated. Viral infections, particularly those involving retroviruses and DNA viruses that integrate into or interact with the host genome, are known to alter transcriptional and epigenetic landscapes [28]. Endogenous retroviral sequences, remnants of ancient infections, have been repurposed as regulatory elements within the host genome [29]. Some viruses manipulate host R-loop resolution mechanisms to enhance their replication or evade immune detection. Recent evidence suggests that R-loops may form preferentially at viral promoters and integration sites, influencing viral gene expression and latency [13,22].

Furthermore, viruses may actively manipulate host R-loop-resolving enzymes such as SETX and DDX5 to suppress antiviral gene expression and redirect DNA repair pathways to facilitate their replication [30]. These interactions point to a new paradigm in which viruses harness or reshape R-loop dynamics for their own evolutionary advantage.

Herein, we synthesize recent advances in understanding how viruses interact with R-loop biology. While some ideas remain hypothesis-driven, they are grounded in emerging experimental evidence. Specifically, we draw parallels between viral strategies across Baltimore groups, viral groups classified by genome type and replication method, and propose new avenues to investigate this interface of host-pathogen coevolution. As R-loops lie at the intersection of transcriptional control, genome maintenance, and innate immunity, understanding how viruses engage with R-loop structures may reveal novel targets for antiviral intervention.

R-loops in viral genomes

The Baltimore classification distinguishes seven viral groups based on genome type and replication strategy (Fig. 2), ranging from double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), double-stranded RNA (dsRNA), and single-stranded RNA (ssRNA) viruses with either positive- or negative-sense genomes. Despite their distinct replication mechanisms, all viral classes inherently involve the formation of R-loops at some stage in their life cycle. These structures arise naturally during viral transcription, when newly synthesized RNA strands hybridize with their complementary DNA templates. In some cases, transcription-generated R-loops play an active role in viral replication, particularly at viral origins of replication. Furthermore, group 6 and group 7 viruses transiently possess RNA–DNA hybrid genomes as an obligatory intermediate in their replication cycle, such as retroviruses that undergo reverse transcription and hepadnaviruses that rely on RNA templates for DNA synthesis. These observations suggest that RNA–DNA hybrids are often essential intermediates that viruses exploit for efficient genome replication and persistence.

In several nuclear-replicating DNA viruses, notably herpesviruses, R-loop structures are increasingly appreciated as functional replication intermediates. Genome-wide analyses have identified R-loop-forming sequences in Epstein–Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV), and human cytomegalovirus (HCMV) [31,32]. In EBV, stable R-loops form at the lytic origin of replication (OriLyt) [33] enabling recruitment of the viral single-stranded DNA-binding protein BALF2 to initiate lytic DNA synthesis [8]. Disruption of these R-loops by RNase H1 impairs replication, underscoring their critical importance. Host defenses target these R-loops: the SMC5/6 complex binds OriLyt R-loops and represses replication and late gene expression, which EBV circumvents by expressing BNRF1, which degrades

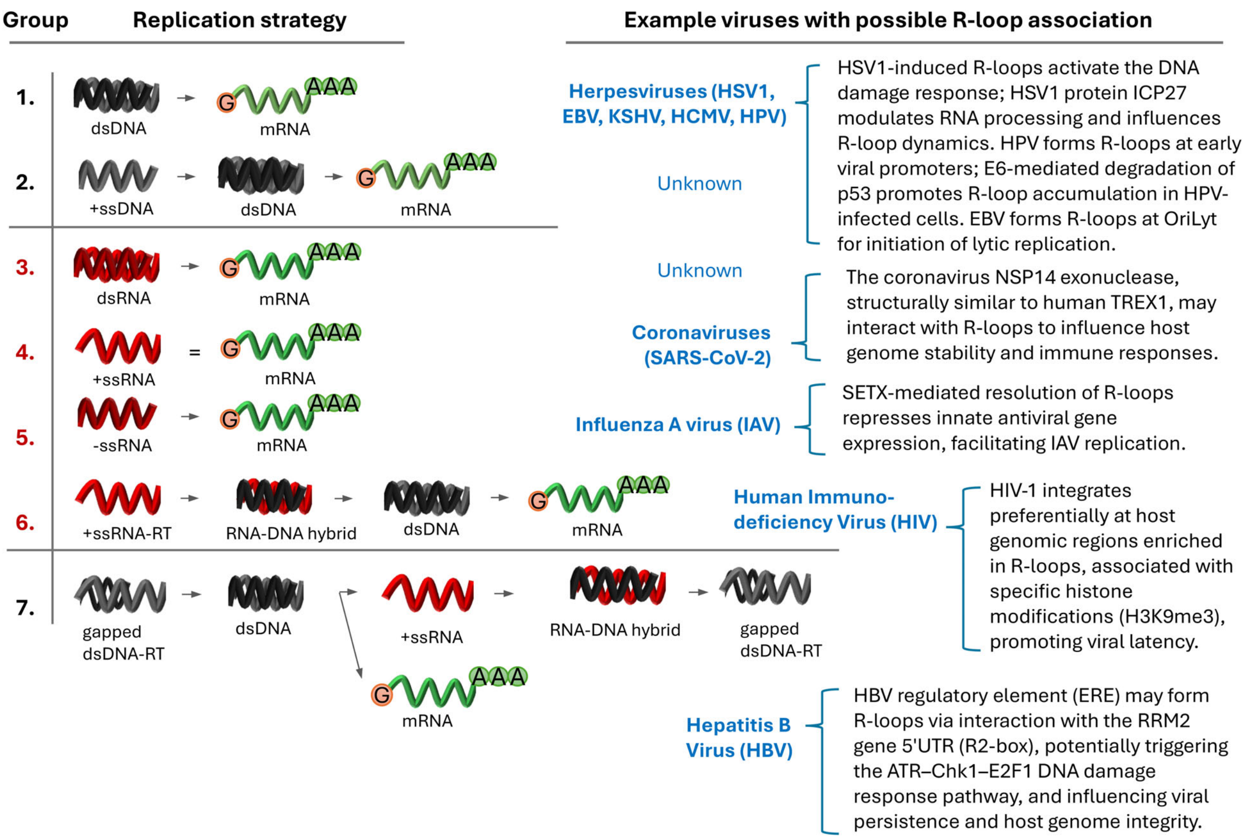

Fig. 2. The Baltimore classification of viruses and examples with R-loop associations. The Baltimore classification categorizes viruses into seven groups based on their genome type and replication strategy, specifically how they produce mRNA. This system highlights the mechanistic diversity of viral gene expression. *Group 1* viruses possess a double-stranded DNA (dsDNA) genome. Transcription of mRNA is carried out using the host cell's RNA polymerase, exemplified by herpesviruses and adenoviruses. Alternatively, the virus has its own DNA-dependent RNA polymerase, such as poxviruses. *Group 2* viruses have a single-stranded DNA (ssDNA) genome that must first be converted into dsDNA form before transcription can occur, as seen in parvoviruses. *Group 3* includes double-stranded RNA (dsRNA) viruses, which require their own RNA-dependent RNA polymerase to transcribe mRNA from their genome, as observed in reoviruses like rotaviruses. *Group 4* consists of positive-sense single-stranded RNA (+ssRNA) viruses, whose genome can be directly translated by host ribosomes, though they rely on their own RNA-dependent RNA polymerase for replication, as in coronaviruses, picornaviruses, and flaviviruses. *Group 5* includes negative-sense single-stranded RNA (−ssRNA) viruses that first convert their genomes into a +ssRNA before translation, a process facilitated by their own RNA polymerase, characteristic of orthomyxoviruses and rhabdoviruses. *Group 6*, the retroviruses, such as HIV-1, use reverse transcriptase to convert their +ssRNA genome into double-stranded DNA, which then integrates into the host genome and serves as a template for transcription. *Group 7* viruses, including hepadnaviruses like Hepatitis B, have a gapped (partially double-stranded) DNA genome and also rely on reverse transcription (from the progenomic +ssRNA) as part of their replication cycle. The viruses highlighted in blue have known or proposed associations to R-loop structures. Key findings related to their connection to R-loops are briefly summarized and further detailed in the main text.

SMC5/6, freeing the OriLyt for productive replication. Similarly, in HCMV, R-loops are stabilized at OriLyt by the lncRNA4.9 and the viral replication factor UL84, which binds RNA secondary structures to coordinate origin activation [34–36]. These structures not only facilitate replication but likely also shield viral genomes from host surveillance. In human papillomavirus (HPV), R-loops form at the early promoter region and must be resolved to preserve the episomal status of the viral genome. SETX is required for this resolution, highlighting a conserved interplay between host R-loop metabolism and viral persistence [22,37]. Other herpesviruses, such as HSV1 and KSHV, encode proteins that modulate R-loop dynamics indirectly [38]. The HSV1 ICP27 and KSHV ORF57 proteins sequester components of the TREX (TRanscription-Export) mRNA export complex, leading to transcriptional stress, R-loop accumulation, and genome instability—a phenomenon thought to enhance viral gene expression or bypass host defenses [39].

Retroviruses (Group VI), such as HIV-1 and HTLV-1, inherently involve RNA–DNA hybrids during reverse transcription, a step critical for integration into the host genome [40]. Once integrated, strong viral promoters in the long terminal repeats (LTRs) promote high RNA polymerase II activity, creating a transcriptionally intense environment conducive to R-loop formation. In HIV-1, the viral protein Tat (Trans-Activator of Transcription-1) amplifies Pol II elongation and R-loop accumulation near the LTRs [41]. Importantly, HIV-1 integration frequently occurs within transcriptionally active genes that are enriched in R-loops, with Tat further amplifying transcription and promoting R-loop accumulation around the LTRs [42].

Integration-dependent R-loop formation is also relevant for HPV, where viral integration disrupts the E2 repressor, creating chimeric transcripts and R-loops at integration breakpoints [22,43]. In hepadnaviruses like hepatitis B virus (HBV) (Group VII), integration of partially double-stranded DNA into host chromosomes is common in chronic infections. HBV's oncogenic HBx protein alters host replication and repair pathways, which may intersect with R-loop metabolism [44,45]. The presence of R-loops in HBV integration breakpoints or covalently closed circular DNA (cccDNA) remains uncertain, but their potential impact on viral persistence warrants further investigation. Another proposed mechanism suggests that a viral ERE (Enhancer Regulatory Element) may engage a region called the R2-box to promote R-loop formation [46]. These R-loops, in turn, are known to trigger a DNA damage response through the ATR (ataxia telangiectasia and Rad3-related) pathway, ultimately activating downstream factors such as the checkpoint kinase Chk1 and the transcription factor E2F1 [47]. Together, this ATR–Chk1–E2F1 signaling cascade helps coordinate DNA repair and maintain genomic integrity. Although still hypothetical, this mechanism proposes that HBV may exploit R-loop-mediated signaling to manipulate the host cell cycle and repair machinery.

Beyond eukaryotic viruses, bacteriophages also provide compelling examples of R-loop exploitation [48,49]. In phage T4, R-loops form at multiple replication origins (oriA, oriE, oriF, oriG) and are essential for initiating DNA replication [49]. These structures create single-stranded DNA regions for the recruitment of host or phage-encoded replication machinery [50]. Similarly, phage ColE1 (colicin E1) uses R-loops as a critical intermediate during DNA replication. The initiation process involves R-loop formation at the replication origin, where a persistent unwound region is stabilized, allowing for efficient synthesis of the nascent DNA strand [51]. In the case of phages T3 and T7, *in vitro* studies have demonstrated that their respective RNA polymerases can generate stable RNA–DNA hybrids during transcription [48]. However, it is important to note that the formation of such hybrids is not unique to these viral polymerases. Rather, RNA–DNA hybrid formation is dictated by the sequence and structural features of the DNA template, such as GC content and the presence of specific motifs. This highlights that R-loop formation is a general property of transcription under conducive sequence contexts, rather than a specialized function of specific RNA polymerases.

Even more striking is the role of R-loops in bacterial adaptive immunity. In Type I and III CRISPR-Cas systems, crRNAs guide Cas proteins to form R-loops at phage DNA targets, enabling sequence-specific cleavage [52]. The presence of R-loops in phage genomes may render specific gene regions more susceptible to CRISPR-mediated targeting [53]. Some type III CRISPR systems extend this mechanism by degrading both viral RNA and DNA simultaneously, leveraging R-loops for dual interference [54,55]. In response, phages have evolved anti-CRISPR proteins that block R-loop formation or degrade crRNAs, effectively evading host defenses [56]. The evolutionary interplay between host immune systems and viral genomes has shaped phage genome architecture, with phages continuously adapting to minimize R-loop accumulation in critical protospacer regions. This arms race continues to drive bacterial and phage coevolution, reinforcing the role of R-loops in genome defense mechanisms [57,58].

Viral integration and R-loop hotspots

Genome-wide mapping techniques such as DNA–RNA immunoprecipitation sequencing (DRIP-seq) [11,59] have revealed that R-loops accumulate predominantly in genomic regions that are often highly transcriptionally active. Since HIV integrase and other retroviral integrases tend to target transcriptionally open chromatin, integration hotspots may overlap with R-loop-prone sites [41,42]. R-loops may facilitate viral integration through various mechanisms, including their ability to alter chromatin accessibility [42], recruit protein complexes [60,61], and alleviate topological stress [5]. By opening chromatin and influencing local epigenetic states [4], R-loops create an environment that may enhance integration efficiency [61]. Additionally, viral integrases or their tethering factors, such as LEDGF/p75 (lens epithelium-derived growth factor), may colocalize with R-loop-associated protein complexes, further guiding integration into specific genomic locations [42,62]. Furthermore, the presence of R-loops can relieve supercoiling stress [5], making DNA more accessible to viral integrases. Conflicts between transcription and replication, which often coincide with R-loop accumulation [63], may also provide a favorable environment for viral integration, as these sites are prone to transient strand breaks or paused replication forks. Certain viruses, such as HTLV-1, can actively interfere with DNA repair mechanisms, increasing the likelihood of DNA breaks that facilitate stable viral insertion [64].

Viruses and retrotransposons share several structural and functional similarities, including LTRs and integrases [65]. Once integrated into the host genome, these viral-derived elements can be repurposed for host gene regulation, functioning as enhancers or promoters. Human endogenous retroviruses (HERVs) are remnants of ancient retroviral infections embedded in our genome. Although most HERV sequences are silenced or defective, some retain transcriptional activity [66], and their LTRs may be prone to R-loop formation. Transcription through an LTR could produce an RNA that hybridizes back to the complementary DNA strand, generating an R-loop. In general, repetitive and transposable elements like HERVs pose a challenge to the cell – improper R-loop resolution at these repetitive regions can cause replication-transcription collisions, DNA damage, and genome instability [67]. Thus, if a HERV sequence is transcribed without proper R-loop management, it may compromise local genome stability. This could lead to insertions, deletions, or recombination at the HERV locus or neighboring genes, potentially contributing to disease or evolution of the genome. As integration sites for HERVs become epigenetically modified through DNA methylation or histone remodeling, the accumulation of R-loops can further reshape these epigenetic landscapes, leading to shifts in HERV expression patterns [29,68]. This suggests that R-loops at endogenous retroviral sequences [69] may play a broader role in regulating gene expression in mammalian genomes.

In the unicellular eukaryote budding yeast, R-loops have been identified as significant features of Ty LTR retrotransposons [70,71]. While functionally analogous to retroviral proviruses, Ty elements replicate entirely intracellularly without an extracellular phase. These Ty R-loops raise intriguing questions about whether these noncanonical structures serve a conserved role in regulating retroelements across species. One possibility is that R-loops function as suppressive agents, forming physical barriers that impede transcriptional machinery and reduce transposon expression. Additionally, their accumulation may promote the establishment of a heterochromatic state, leading to chromatin compaction and transcriptional silencing, a mechanism reminiscent of retrotransposon repression strategies observed throughout eukaryotic genomes to safeguard genomic stability. Conversely, R-loops may actively enhance Ty element activity by inducing localized topological stress that facilitates strand invasion events, a crucial step in cDNA synthesis and retrotransposon integration. In this capacity, R-loops may not be incidental byproducts of high transcriptional activity but rather dynamic intermediates that contribute to the life cycle of Ty elements. Their influence may function as a regulatory switch, where the cellular context dictates whether R-loops act as barriers to transposition or as facilitators of Ty mobility. This dual role suggests that, through evolution, R-loop formation may have been co-opted to fine-tune the balance between transposon propagation and genome integrity, thereby shaping the genomic landscape in response to selective pressures.

Viral sequences that impact host genes and regulatory elements

Viruses and hosts have exchanged genetic material throughout evolutionary history, leading to instances where viral sequences bear homology to host genes or *vice versa* [72,73]. This genetic interplay has contributed to various biological phenomena, such as the integration of HPV into the host genome, where viral RNA can fuse with human transcripts, potentially activating oncogenes or generating chimeric transcripts [74]. Herpesviruses have similarly evolved proteins that resemble host molecules, enabling them to modulate immune responses. In the case of KSHV, the expression of a viral interleukin-6 (vIL-6) homolog allows the virus to mimic a host cytokine, altering immune signaling and contributing to viral persistence [75,76]. Beyond direct genetic integration, viral elements have been co-opted by the host genome over evolutionary time, a phenomenon exemplified by retrotransposons of viral origin that have acquired novel functions, such as regulating immune responses and developmental processes [29].

The integration of viral DNA and the expression of viral oncogenes can induce genomic instability, in part by promoting the accumulation of R-loops [77]. This R-loop accumulation is largely attributed to the action of viral oncoproteins. For instance, papillomaviruses, such as HPV, exploit host genomic mechanisms to establish persistent infection and facilitate cellular transformation. An emerging mechanism by which HPV suppresses host innate immune responses involves the targeted induction of R-loops at specific innate immune gene loci, predominantly in HPV-transformed cells. Recent studies have linked this phenomenon to the activity of HPV oncoproteins E6 and E7, which degrade tumor suppressors such as p53, disrupting R-loop homeostasis. Templeton and Laimins [22] demonstrated that loss of p53 function in HPV-transformed cells correlates with widespread R-loop accumulation, implicating p53 as a key regulator of RNA–DNA hybrid stability during infection. In a follow-up study, Templeton and Laimins [78] further showed that HPV-induced R-loops at innate immune gene loci suppress their transcriptional activation, thereby blunting antiviral signaling. Concurrently, these structures activate DNA damage repair pathways—co-opted by the virus to facilitate its replication and persistence. Interestingly, SETX plays a dual role in HPV biology; Jose *et al.* [37] reported that SETX acts directly on HPV episomes to resolve transcription-associated R-loops, maintaining the viral genome in its episomal state. However, in HPV-transformed cells, viral interference with SETX function may lead to selective stabilization of R-loops at host immune loci, promoting sustained transcriptional repression. This uncoupling of SETX function between the viral and host genomes suggests a viral strategy to protect its own episome while silencing host defenses. Notably, HPV's impact on host DNA damage repair pathways further reinforces this dual regulatory strategy. As shown by Vats and Laimins [79], HPV oncoproteins rewire the host DNA repair network, enabling viral genome maintenance while tolerating or even promoting R-loop-induced genome instability in host cells. In addition to these chromatin-associated mechanisms, recent findings reveal that HPV can generate human–viral hybrid extrachromosomal DNA (ecDNA) structures that act as potent enhancers to drive the expression of the viral oncogenes E6 and E7. These hybrid ecDNAs are enriched in oropharyngeal squamous cell carcinomas and can promote tumor growth. Functional experiments show that targeting these enhancer elements—either through CRISPR interference or BET inhibitors—leads to repression of E6/E7 and a marked reduction in tumor proliferation [80]. Although R-loops were not directly studied in this context, the intense transcriptional activity associated with ecDNA enhancers and their capacity to engage with the host transcriptional machinery raise the possibility that R-loop formation may also occur at these regulatory sites. Together, these findings reveal a sophisticated mechanism by which HPV manipulates multiple layers of genome biology—R-loop formation, chromatin regulation, and extrachromosomal enhancer activity—to transform cellular defense pathways into viral replication platforms and drivers of oncogenic progression [77,80,81].

EBV and KSHV similarly modulate host signaling through proteins such as latent membrane protein 1 (LMP1) and latent nuclear antigen (LANA), which interfere with cellular regulatory mechanisms [82–84]. These viral oncogenes can also affect R-loop resolution pathways, increasing the likelihood of double-strand breaks and mutations [9]. In addition to direct effects on genome stability, some viruses have evolved strategies of molecular mimicry, producing proteins that structurally or functionally resemble host ligands [75,85]. By adopting these host-like features, viruses can suppress immune detection, manipulate cytokine signaling networks, and subvert cellular defense mechanisms [86]. KSHV's vIL-6, for instance, closely resembles its cellular counterpart, promoting cell proliferation and immune evasion [87,88]. Similarly, cytomegalovirus (CMV) encodes a viral interleukin-10 (IL-10) homolog that dampens inflammatory responses, allowing the virus to persist in the host [89,90].

Molecular mimicry extends beyond individual cytokines, encompassing a range of viral strategies that exploit host regulatory pathways [91,92]. Some viruses encode proteins that bind directly to host DNA or transcriptional co-regulators, thereby reprogramming transcription networks. Beta- and gamma-herpesviruses, for example, produce homologs of interleukins and chemokines that influence immune cell recruitment and activation [68]. Additionally, RNA viruses such as influenza A virus (IAV) have evolved sophisticated mechanisms to interfere with host cellular signaling, including the expression of viral peptides that mimic post-translational modifications such as phosphorylation or ubiquitylation, thereby disrupting pathways that regulate cell survival and apoptosis [86]. In a seminal study, Miller *et al.* [30] demonstrated that during IAV infection, SETX plays a pivotal role in modulating the antiviral response. Specifically, SETX suppresses the expression of interferon-stimulated genes (ISGs) by resolving R-loops at their transcriptional terminators, facilitating proper RNA polymerase II disengagement and preventing inappropriate immune gene activation. This activity not only attenuates antiviral signaling but also contributes to efficient viral replication, suggesting that influenza viruses may co-opt host R-loop resolution machinery to optimize their intracellular environment. Supporting this model, Rogan *et al.* [93] proposed that the accumulation of replicating influenza viral RNA can sequester host

RNA-binding proteins (RBPs), impairing R-loop resolution and leading to the persistence of RNA–DNA hybrids. This unresolved R-loop burden may trigger DNA damage responses and apoptosis, potentially exacerbating lung pathology during severe infections. Together, these studies highlight R-loop metabolism as a crucial interface in influenza virus pathogenesis, shaping both immune evasion and viral propagation.

Viral adaptations often confer advantages by disrupting immune surveillance, modifying cell cycle checkpoints, and rewiring metabolic functions. HIV-1 exemplifies this strategy *via* Tat, which interacts with host transcription factors to enhance transcription elongation [94]. This interaction inadvertently increases R-loop formation near the viral LTR, contributing to the regulation of viral gene expression and latency [95].

Endogenous retroviral LTRs have been co-opted to drive gene expression in immune cells and the placenta [68]. Viruses, in turn, often acquire host-like regulatory motifs that enhance their own replication. Over evolutionary time, transposons have contributed to genome evolution [96] by introducing transcription factor binding sites and viral origin-like sequences, which have subsequently been adopted for tissue-specific gene regulation or stress responses [29]. This continuous interaction between viruses, LTRs, transposons [96], and host genomes has led to the emergence of mosaic genomes, where 'virus-like transposons' are capable of crossing species boundaries [97].

The consequences of viral mimicry extend beyond immune evasion and transcriptional control, as viral proteins that closely resemble host antigens can provoke cross-reactive immune responses. Such interactions have been implicated in autoimmune diseases, as seen in hepatitis C virus (HCV) infections, where viral mimotopes trigger aberrant immune activation [85]. Coronaviruses have similarly been hypothesized to encode molecular mimics that contribute to autoimmune pathologies [27]. The presence of R-loops may further exacerbate these effects by acting as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), leading to chronic inflammation and immune dysregulation. If R-loops contribute to aberrant gene expression, their accumulation in virus-infected cells could further amplify immune dysfunction, strengthening the link between viral infections and inflammatory or autoimmune diseases [27,98].

The coronavirus SARS-CoV-2 non-structural protein 14 (NSP14) is a crucial exonuclease that ensures the fidelity (proofreading activity) of coronavirus RNA replication [99]. It belongs to the DEDDh family of exonucleases, characterized by the conserved DEEDh motif, which coordinates metal ions essential for catalytic activity. This structural feature establishes an evolutionary link between NSP14 and the human exonuclease TREX1 [100], suggesting a possible functional overlap in nucleic acid metabolism. TREX1, a key component of the THO/TREX (Transcription and Export) complex, plays a fundamental role in R-loop resolution [101–103]. The THO/TREX complex regulates transcriptional integrity by facilitating RNA processing and export, preventing the accumulation of RNA–DNA hybrids that can lead to genomic instability. TREX1 contributes to this process by degrading excess nucleic acids, including nuclear-derived cytosolic RNA–DNA hybrids [104], thereby ensuring proper R-loop homeostasis and protecting against immune activation. The structural homology between TREX1 and NSP14 raises the question of whether NSP14 might engage with R-loop structures, either to facilitate viral replication or to modulate host nucleic acid metabolism. As viruses often hijack host cellular processes to optimize their replication cycle, it is conceivable that NSP14 could interact with RNA–DNA hybrids in a manner that influences viral genome stability or transcriptional regulation. Moreover, NSP14 could potentially interfere with R-loop processing to subvert host antiviral pathways. By mimicking aspects of TREX1 function, NSP14 might alter R-loop regulation, affecting the expression of host genes or modulating immune signaling in a manner that favors viral persistence. Although direct evidence for NSP14 involvement in R-loop metabolism remains to be established, its evolutionary relationship with TREX1 and its critical role in viral nucleic acid processing provide a compelling rationale for further investigation.

R-loops as epigenetic regulators of viral and host gene expression

Beyond their role in genome instability, R-loops can act as dynamic epigenetic regulators that shape chromatin architecture and gene expression. A growing number of studies have demonstrated that R-loops can either promote or repress transcription depending on their genomic context and associated chromatin features. For example, Sanz *et al.* [105] showed that R-loops are preferentially enriched at gene promoters and correlate with active histone marks such as H3K4me3, suggesting a role in maintaining transcriptionally competent chromatin. Similarly, Grunseich *et al.* [106] reported that loss of SETX results in aberrant DNA methylation at gene promoters, indicating that R-loops can prevent silencing by blocking DNA methyltransferase access.

Mechanistically, R-loops can act as scaffolds for chromatin-modifying complexes. Arab *et al.* [107] revealed that the stress-response factor GADD45A binds R-loops and recruits TET1 to demethylate CpG islands, modulating gene expression programs. Moreover, histone demethylation, such as H3K9me2 loss, can itself induce R-loop accumulation and is linked to chromatin disorganization, as shown by Zhou *et al.* [108]. In pluripotent cells, R-loops have been shown to coordinate with key transcription factors like SOX2 to regulate reprogramming [109] emphasizing their role in developmental gene regulation. These structures are also linked to transcription termination through interactions with m6A-modified RNA and GC-skewed DNA motifs that favor hybrid formation [110,111].

Recent work further supports a model in which R-loops are sensitive to post-translational modifications of R-loop regulatory proteins. For instance, PRMT5-mediated methylation of DDX5 influences hybrid resolution and chromatin accessibility [112]. Notably, Rakshit *et al.* [113] demonstrated that dysregulation of R-loop-associated epigenetic programs contributes to disease phenotypes such as cancer and cardiovascular disorders, highlighting the functional relevance of these structures in physiological and pathological contexts.

Incorporating these findings into the framework of virus–host interactions, it becomes evident that viruses exploiting R-loop dynamics may also manipulate host epigenetic states. For example, virally induced R-loop accumulation at immune gene loci—such as those documented in HPV-infected cells [78]—can reinforce transcriptional silencing through epigenetic pathways, contributing to immune evasion.

R-loops have been implicated in modulating repressive DNA methylation through two opposing mechanisms. On the one hand, they can inhibit methylation by physically preventing DNA methyltransferases (DNMTs) from binding to CpG islands, thereby maintaining promoters in an active or poised state [11,106]. On the other hand, persistent R-loops can induce DNA damage, triggering repair pathways that inadvertently lead to hypermethylation of nearby genomic regions [26]. Many viruses, including EBV, HPV, and HBV, are subject to host-driven methylation of both integrated and episomal viral DNA, which plays a key role in regulating gene expression during latent and chronic infection states [44,114]. The presence of R-loops near viral promoters or enhancers could interfere with DNMT recruitment, allowing viral genes to remain transcriptionally active or facilitating reactivation under specific cellular conditions [115]. Conversely, some viruses actively recruit or manipulate DNMTs to silence their own gene expression, a strategy that enables them to evade immune detection [116].

While direct evidence linking histone modifications to R-loop regulation in virally infected cells remains limited, some studies provide insights into potential mechanisms. For example, in HIV-1 infection, the virus preferentially integrates its genome into regions of the host genome enriched with R-loops, which are also associated with repressive histone marks, facilitating proviral latency [117]. In the context of herpes simplex virus (HSV), the incoming viral genome is rapidly chromatinized through interactions with promyelocytic leukemia nuclear bodies (PML-NBs), where host histone chaperones such as DAXX and ATRX deposit histone H3.3 onto viral DNA, promoting heterochromatin formation and silencing of immediate-early genes [118]. This process is associated with repressive histone modifications and likely intersects with R-loop regulation at latency-associated loci.

In uninfected cells, histone modifications are closely linked to R-loop dynamics. For instance, histone acetylation by PCAF enhances R-loop resolution by promoting the recruitment or activity of RNA–DNA helicases; loss of PCAF leads to increased R-loop accumulation and genome instability [119]. These findings suggest that viruses may indirectly exploit or disrupt host histone modification pathways to regulate R-loop formation and resolution, thereby influencing viral replication, gene expression, and latency.

The relationship between RNA interference (RNAi) pathways and R-loop biology remains an emerging area of study. While canonical antiviral siRNAs are typically derived from double-stranded RNA precursors [120], the possibility that RNA–DNA hybrids may interface with RNAi machinery is still under investigation. Notably, our previous work demonstrated that certain heterochromatic siRNAs (het-siRNAs) can guide sequence-specific recruitment of RNA to chromatin, where they promote R-loop formation and transcriptional silencing through homologous pairing with genomic loci [12]. This mechanism suggests that small RNAs may, under specific chromatin conditions, reinforce R-loop stability and contribute to epigenetic regulation.

The role of RNAi in antiviral defense varies across species. In plants, viral replication intermediates can be amplified by RNA-dependent RNA polymerase (RdRp), enhancing siRNA-mediated viral suppression [121]. In insects, robust RNAi responses contribute to antiviral immunity, with some viral transcripts forming R-loops that serve as precursors for siRNA production [122]. Although mammalian cells rely more on interferon responses than RNAi for antiviral defense, certain viruses, such as HCV and influenza, remain susceptible to exogenous siRNA-mediated inhibition, which may stem from partial R-loop-like structures within infected cells [123].

To counteract RNA silencing, viruses have evolved diverse RNAi suppressors that interfere with host antiviral responses. For example, CMV encodes the 2b protein, while coronaviruses express the N protein, both of which bind to dsRNA or R-loop-like structures to block the activity of Dicer or RISC [124,125]. This ongoing molecular conflict illustrates the necessity for viruses to manipulate or neutralize R-loop-associated immune strategies in order to establish successful infections and maintain latency.

Therapeutic potential of virus-R-loop interactions

The therapeutic potential of targeting R-loops in the context of viral infections and latency presents an area of research with considerable clinical implications. If R-loops contribute to viral silencing by acting as physical barriers or by recruiting repressive chromatin modifiers at integrated retroviral LTRs, then resolving these structures could potentially reverse latency and reactivate dormant viral transcription. Strategies aimed at modulating R-loop formation, such as RNase H enhancers or small-molecule helicase activators, may serve as a novel class of latency-reversing agents (LRAs) capable of disrupting viral dormancy and rendering infected cells susceptible to immune clearance [126]. However, a critical challenge in developing these therapeutic strategies is distinguishing viral-derived R-loops from host R-loops to avoid unintended genomic instability or disruption of essential cellular processes.

One potential approach to achieve specificity is to exploit sequence-specific recognition of viral integration sites or viral genome sequences. Viral genomes, including integrated retroviral sequences, frequently contain unique nucleotide motifs or structural contexts distinct from host genomic regions. Precise targeting of viral R-loops could therefore be achieved by designing CRISPR-based tools, such as dCas9 coupled with RNase H domains or helicase enzymes, guided specifically to viral integration loci. Additionally, recent advances in genome-wide R-loop profiling technologies, such as DRIP-seq and single-molecule sequencing approaches, facilitate the identification of unique viral R-loop signatures that differ in length, stability, and nucleotide composition from host R-loops.

Moreover, novel CRISPR systems capable of sensing R-loop formation in a sequence- and context-dependent manner offer further opportunities for specificity. For instance, Hu *et al*. identified a Type I-A CRISPR-Cas3 variant from *Thermococcus siculi* that is selectively activated by R-loop formation, contingent upon specific protospacer adjacent motif (PAM) and protospacer recognition sequences [127]. This property could be harnessed to selectively target viral R-loops by engineering guide RNAs recognizing virus-specific sequences, thereby minimizing off-target effects on host chromatin structures. Their system exhibits expanded (PAM) recognition, stringent mismatch intolerance, and dual activation modes responsive to both DNA and RNA targets. Leveraging these characteristics, the researchers developed the Hyper-Active-Verification Establishment (HAVE), enabling rapid and precise diagnosis of HPV in clinical samples.

HERVs, while non-infectious, further illustrate the therapeutic complexity. HERV-derived R-loops can threaten genome stability and potentially engage immune pathways, underscoring the importance of tightly silencing these elements under normal conditions. Reactivation of HERV expression (for instance by demethylation in cancer therapy or in autoimmune disease) produces viral-like RNAs and proteins that can trigger innate immunity. Double-stranded RNAs from convergent HERV transcription can activate immune sensors like MDA5 or TLR3. R-loops from HERV transcripts might similarly be sensed upon accumulation. Although direct evidence of R-loops triggering immune responses in the context of HERVs is limited, by analogy, any cytosolic RNA–DNA hybrids originating from HERV DNA could be detected by pattern recognition receptors. Unresolved R-loops can lead to single-stranded DNA fragments or RNA–DNA hybrids in the cytoplasm, which are immunogenic [104]. Pharmacological agents such as epigenetic modulators or CRISPR interference techniques may be employed to modulate these elements, thereby either stimulating antiviral defenses or exposing viral proteins for immune recognition and clearance [68]. Selective upregulation of HERV-K expression has been explored as a means to enhance antiviral immune pathways [128]; however, these approaches require careful optimization to balance the potential benefits of viral reactivation and immune clearance against the risks associated with increased genome instability.

Additionally, epigenetic modulators, including DNMT inhibitors (e.g., 5-azacytidine) or histone deacetylase (HDAC) inhibitors, have been evaluated viral latency reversal strategies, for example, in the context of HIV 'shock and kill' therapies, where reactivation of latent viral promoters is induced to expose infected cells to immune clearance [129]. Future therapeutic strategies may explore ways to effectively exploit virus-R-loop interactions as therapeutic vulnerabilities.

Conclusions and perspectives

Our growing understanding of R-loop biology is reshaping perspectives in virology, immunology, and genome structure. Rather than being mere transcriptional byproducts, R-loops play crucial roles in regulating viral gene expression by modulating promoter and enhancer accessibility, particularly in retroviral LTRs. Their presence influences viral integration patterns, as viral integrases often prefer open, transcriptionally active chromatin enriched in R-loops [42]. Additionally, R-loops intersect with host antiviral defenses, serving as substrates for CRISPR interference in prokaryotes and as triggers for RNAi in eukaryotic cells [54,130,131]. Beyond transcriptional control, these structures contribute to epigenetic regulation by interacting with DNA methylation, histone modifications, and DNA damage repair mechanisms [106,107,132]. Evolutionary interactions between viruses, transposons, and host genes are also shaped by R-loops, as transposable elements and endogenous retroviruses frequently become incorporated into host regulatory networks.

The interplay between viruses and host R-loop formation presents a dual challenge. Viral genomes often exploit R-loop-induced chromatin relaxation for integration or enhanced transcription [42], yet host immune mechanisms can leverage R-loops to detect and degrade viral RNAs [130]. Furthermore, some viruses mimic host proteins involved in R-loop resolution, hijacking helicases and RNases to facilitate their replication or evade immune detection. The clinical implications of these findings are profound, suggesting potential applications in refining HIV latency reversal strategies [133–136], enhancing immune recognition of latent herpesviruses or oncogenic viruses, and manipulating transposon- or virus-derived enhancers to bolster antiviral immunity. However, several questions remain regarding the cell-type specificity of R-loop dynamics and how different viruses modulate R-loop resolution or contribute to genome instability.

While the connection between R-loops and genomic instability is well established, recent research suggests that viruses may exploit these structures as regulatory nodes to reprogram host gene expression and DNA damage responses. Viruses appear to manipulate both the formation and resolution of R-loops at host gene loci, particularly those involved in antiviral immunity and genome maintenance, providing a selective advantage that promotes their survival and propagation. One key mechanism through which viruses achieve this control is by targeting the cellular machinery responsible for R-loop resolution, which is highly dependent on host cellular machinery, including RNase H, helicases such as SETX or the DDX family (DEAD-box helicases), topoisomerases, and DNA repair pathways involving Fanconi anemia proteins and the BRCA1/2 axis [60]. Viruses often interact with or manipulate these same pathways, sometimes co-opting them for replication, genome integration, or immune evasion [137]. For instance, SETX, which normally resolves R-loops to maintain transcriptional and genomic stability, is hijacked during influenza A virus infection to limit the host's antiviral gene expression by repressing interferon-stimulated genes [30].

Additionally, viruses appear to manipulate chromatin-associated factors that influence R-loop formation. HIV-1, for instance, integrates into genomic regions enriched in R-loop-forming sequences and repressive histone modifications, favoring the establishment of a latent, transcriptionally silent state that enables long-term persistence. In the case of herpesviruses, R-loop-associated heterochromatin marks deposited by DAXX and ATRX contribute to the silencing of immediate-early genes during latency [117,118]. These findings suggest that R-loops may be strategically targeted by viruses not only to silence antiviral responses but also to modulate epigenetic states conducive to latency and reactivation.

The evolutionary relationship between endogenous retroviruses, transposons, and exogenous viruses highlights a long history of genetic exchange between hosts and viral sequences. These interactions have led to repeated cycles of viral integration, partial domestication through endogenous retrovirus (ERV) activation, and host adaptations aimed at countering viral threats. The ability of R-loop structures to promote genomic rearrangements and influence regulatory networks suggests that they may serve as catalysts in these evolutionary processes [68].

Altogether, the above evidence supports a model in which viruses exploit R-loops as both a threat and a tool: inducing them to destabilize specific genomic regions or silence key genes, while recruiting resolution factors to prevent deleterious genome-wide instability. The dynamic regulation of R-loops thus represents a crucial interface in virus–host coevolution, one that enables precise reprogramming of host cell function to serve viral needs.

The growing body of knowledge surrounding R-loops offers promising new directions for therapeutic interventions. Although combining R-loop modulators with epigenetic drugs, such as HDAC or DNMT inhibitors, has shown preliminary effectiveness in experimental settings, additional research is needed to fully understand the scope and limitations of these strategies [22,136,138]. For instance, HDAC inhibitors have demonstrated initial success in exposing latent viruses, such as HIV and EBV, to immune clearance *in vitro*; however, clinical efficacy remains variable, emphasizing the necessity for careful validation and optimization before considering these approaches for clinical translation.

Additionally, harnessing R-loop-derived double-stranded RNA for antiviral RNAi amplification in plants, insects, or even mammalian cells could offer a novel antiviral strategy [98]. The potential of CRISPR interference in targeting R-loop-prone regions of viral genomes may also be explored, drawing inspiration from bacterial defense mechanisms that leverage R-loops to guide nuclease targeting [139].

In summary, from bacteriophage-bacterium interactions to retroviral integration in human T cells, R-loops emerge as fundamental molecular interfaces that regulate transcription, influence viral burden, and shape host immunity. While they can function as regulatory elements, unresolved R-loops also pose significant risks by contributing to genome instability and DNA damage. Understanding how viruses form, exploit, or neutralize R-loops will be critical for both basic virology and the development of next-generation antiviral therapies, improving the ability to control viral latency, enhance immune clearance, and prevent virus-driven genomic instability.

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Author contributions

All authors contributed to manuscript drafting.

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