Recent Discoveries in the Use of R-loops to Regulate the Lifecycles of DNA Tumor Viruses

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Abstract

R-loops are triple-stranded nucleic acid structures that occur normally as a part of transcription, when single-stranded RNA anneals with duplex DNA. It has been established that R-loops are important for mammalian gene regulation and genome stability. New evidence has shown that R-loops are key contributors to the lifecycles of DNA tumor viruses (e.g. Human papillomavirus (HPV), Epstein-Barr virus (EBV), and Kaposi sarcoma-associated herpesvirus (KSHV)). DNA tumor viruses carefully maintain their genomes as episomes, balancing gene expression for viral replication while avoiding immune activation or host cell death. Recent studies have demonstrated that disruption of R-loop homeostasis can lead to replication stress, genomic instability, and increased oncogenic signaling. This review summarizes recent discoveries about DNA tumor viruses and R-loops, emphasizing their mechanistic roles in viral persistence and cancer development. We also explore how insights gained from DNA viruses can be applied to extrachromosomal DNA (ecDNA), circular DNA elements that exist outside of chromosomal DNA to drive transcriptional DNA in mammalian cells.

Introduction

DNA tumor viruses are unique in that they encode oncogenes that disrupt normal host processes that can lead to cancer. These viruses hijack host proteins to support viral transcription and replication. However, oncogenesis is generally not advantageous for viral persistence, as productive viral lifecycles typically rely on episomal genome maintenance. These episomes are double-stranded DNA plasmids that persist independently of host chromosomes. Cancer development usually arises when viral genomes integrate into host DNA and viral oncogene expression is dependent on transcription regulation from the host; sometimes resulting in elevated viral oncogene expression. DNA tumor viruses balance persistence by driving viral gene transcription and replication, while avoiding excessive expression that would trigger immune detection or kill the host cell.

R-loops have emerged as key regulators of viral transcription and origin firing for DNA tumor viruses.[1] Over the past decade, these structures have been recognized as important for tightly regulating mammalian gene expression and genome stability.[2] R-loops form during transcription when nascent RNA hybridizes back to its DNA template, displacing the complementary DNA strand and creating a three-stranded structure. R-loop resolution on the Human Papillomavirus (HPV) genome is required for episome maintenance and to prevent viral integration.[3,4] In Epstein–Barr virus (EBV), R-loops form at the origin of lytic replication (oriLyt) to promote lytic replication and are recognized by the SMC5/6 complex to restrict reactivation.[5] Additional studies have identified herpesviruses, including KSHV, as having R-loop–forming sequences linked to the regulation of their lifecycle.[6] A detailed review of these foundational studies has been described previously.[1]

As a rapidly expanding topic in mammalian and

DNA tumor virus transcriptional biology, several important studies have been published in the past year alone. These discoveries continue to highlight the importance of R-loops in maintaining viral persistence for HPV, EBV, and KSHV. Additionally, new evidence supports the role of R-loops as essential contributors to DNA tumor virus induced transformation, immunosuppression, and carcinogenesis. R-loops influence key steps in DNA tumor virus replication and can promote conditions that favor long-term viral persistence.

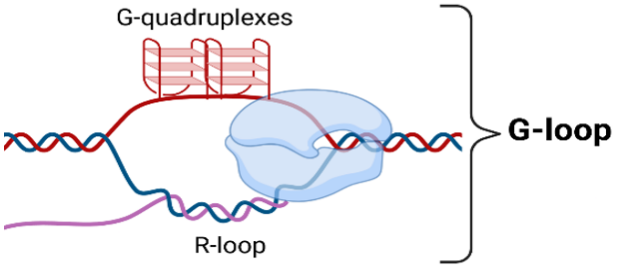

Figure 1: Structure of a G-loop. A G-loop forms when a G-quadruplexes form on the displaced DNA strand of an R-loop. Figure made on BioRender.com

Many DNA tumor viruses establish latency by replicating and maintaining their genomes as episomes in infected cells, a strategy that supports persistent infection while avoiding host genome integration, immune detection, and DNA-damage responses. Recent studies have demonstrated that R-loops are integral to these episomal maintenance mechanisms, underscoring their regulated role in viral genome stability and persistence. The physical structure of R-loops and their capacity to direct viral transcriptional regulators are described below.

Epstein-Barr Virus

The Epstein-Barr Virus (EBV) is characterized by its ability to cycle between latent and lytic phases, which requires tight transcriptional regulation for viral persistence. It was recently proposed that this regulation is mediated through R-loops. Wang et al.[7] identified a non-coding EBV transcript, ebv- sisRNA-3 that binds upstream of the viral lytic origin, OriLyt. This transcript mediates the formation of a quadruplex–R-loop complex (Figure 1), resulting in the formation of a G-loop.[8] The authors predict that this structure is responsible for suppression of lytic replication. However, the host and viral factors that generate and regulate these G-loops, as well as their biological consequences for EBV, remain unclear.

Kaposi Sarcoma-Associated Herpesvirus

In a study by Asghar et al., R-loops are proposed to play a deliberate and programmed role in maintaining viral genome integrity.[9] Kaposi Sarcoma-Associated Herpesvirus (KSHV), like other oncogenic viruses, sustains its episome through latency. During this mechanism, Latency-Associated Nuclear Antigen (LANA) binds to the terminal repeats (TRs) of the KSHV genome, initiating a coordinated replication transcription process that preserves viral survival. Disrupting these R-loops with treatment of a RNAPII inhibitor resulted in reduced histone modifications H3K4me3 and H3K27ac that impaired LANA binding. The N-terminal region of LANA, known for its protein-protein interactions with the host, induces R-loops formation at these TRs, but removal of R-loops with RNAse H treatment reduced LANA binding to these TR R-loop associated regions. LANA association to the KSHV genome is essential for viral genome maintenance and viral gene expression regulation.[10,11] This study highlights the importance of R-loops to modulate LANA binding and therefore viral persistence.

Human Papillomavirus

High-risk HPV-31 infection markedly increased R-loops across the host genes of infected keratinocytes,[3] and this finding was recently confirmed using DRIP-sequencing analysis.[12] These host R-loops were associated with γH2AX and H3K36me3 enrichment and are linked to repression of genes involved in the host innate immune response. Specifically, interferon-α/γ responses, complement signaling, and inflammatory pathways were downregulated. At the same time, R-loops were associated with activation of genes involved in the DNA damage

pathway.[13-16] This is particularly important as HPV replication and genome maintenance are dependent on these repair pathways. This suggests a connection between DNA damage repair pathways and innate regulatory pathways that contribute to HPV pathogenesis and subsequent oncogenesis.[12]

R-loops form on the HPV genome, and failure to resolve these structures results in impaired viral episome maintenance and promotes viral integration into the host genome.[3,4] The HPV E2 protein interacts with the R-loop helicase, Senataxin (SETX) likely for recruitment to E2 binding sites on the viral genome to mediate R-loop resolution.[4] Moffitt et al. recently discovered that ZPR1, a protein necessary for SETX recruitment to mammalian R-loop foci, was not important for HPV R-loops resolution.[17] Instead, the authors propose a mechanism where E2 sequesters away SETX from ZPR1 on host chromatin to preferentially resolve viral R-loops and prevent viral genome integration, causing aberrant R-loops on the infected mammalian genome.

Topoisomerases have emerged as key regulators of R-loop homeostasis.[18] This is not surprising as these enzymes are required to relax supercoiled DNA. Type I topoisomerases (TOP1α and TOP3β) are upregulated in HPV-positive cells, playing a central role in R-loop regulation and viral gene expression.[19] Vats et al. conducted a knockdown study of these topoisomerases, which resulted in impaired HPV replication and transcription, decreased cellular proliferation, and alteration of DNA repair signaling pathways (ATM/ATR). Interestingly, TOP1α and TOP3β were found to regulate different R-loop mechanisms. TOP1α primarily acts on HPV specific R-loops near the early promoter and E2 regions of the genome, and depletion leads to p53 upregulation. In contrast, TOP3β was seen at both viral and cellular genes where it assists in recruitment of R-loop resolving enzymes like DHX9 and POLR3H. TOP3β depletion increased R-loop accumulation at EGR3 to promote cell proliferation, reducing its expression. HPV oncoproteins E6 and E7 drive the increased topoisomerase I levels, which promotes DNA breaks that activate repair pathways that the virus exploits for replication. Top1α recruitment to the viral LCR is likely mediated through HPV E2 protein. Previous studies identified that acetylation of E2 at K111 was required for Top1 association to the viral upper regulatory region 20 and this lysine is important for genome maintenance.[21]

N6-methyladenosine (m6A) modification of R-loops was recently identified to be linked to R-loop dynamics in high-risk HPV infections.[22] m6A binds to R-loops on their RNA moiety and can destabilize them, leading to decreased R-loops. When compared to normal keratinocytes, HPV cell lines showed reduced m6A levels, which resulted in R-loop stabilization and persistence. The HPV oncoprotein E6, which degrades p53, drives degradation of m6A methyltransferase complex components Mettl3 and

Mettl14, lowering m6A levels and increasing R-loop accumulation. The helicase protein SETX can counter-regulate this process by facilitating R-loop resolution and promoting expression of m6A regulators. This study also reported elevated SETX and m6A levels during HPV epithelial differentiation, an essential stage for viral replication and late gene expression. This indicates that m6A not only regulates R-loop levels but also is required for efficient viral gene expression of HPV.

Interestingly, Templeton and colleagues iden tified a unique HPV regulator that functions differently from its role in other oncogenic DNA viruses. APOBEC3B (A3B), a cytosine deaminase in the APOBEC family, binds viral chromatin and is highly enriched at R-loop–rich regions of the HPV genome, including the URR and the early polyadenylation site. Using HPV-31 and CIN612 episomal cell models, the study found that A3B is required for efficient HPV transcription and replication, suggesting it acts as a positive regulator of viral pathogenesis in high-risk HPVs. Additionally, the viral proteins E6 and E7 increase A3B expression, further supporting its role in HPV-induced carcinogenesis.[23]

R-Loops as Key Mediators of Oncogenic Signaling in DNA Tumor Virus Infection

When R-loop homeostasis is disrupted, RNA–DNA hybrids accumulate and trigger oncogenic signaling pathways and activated DNA damage

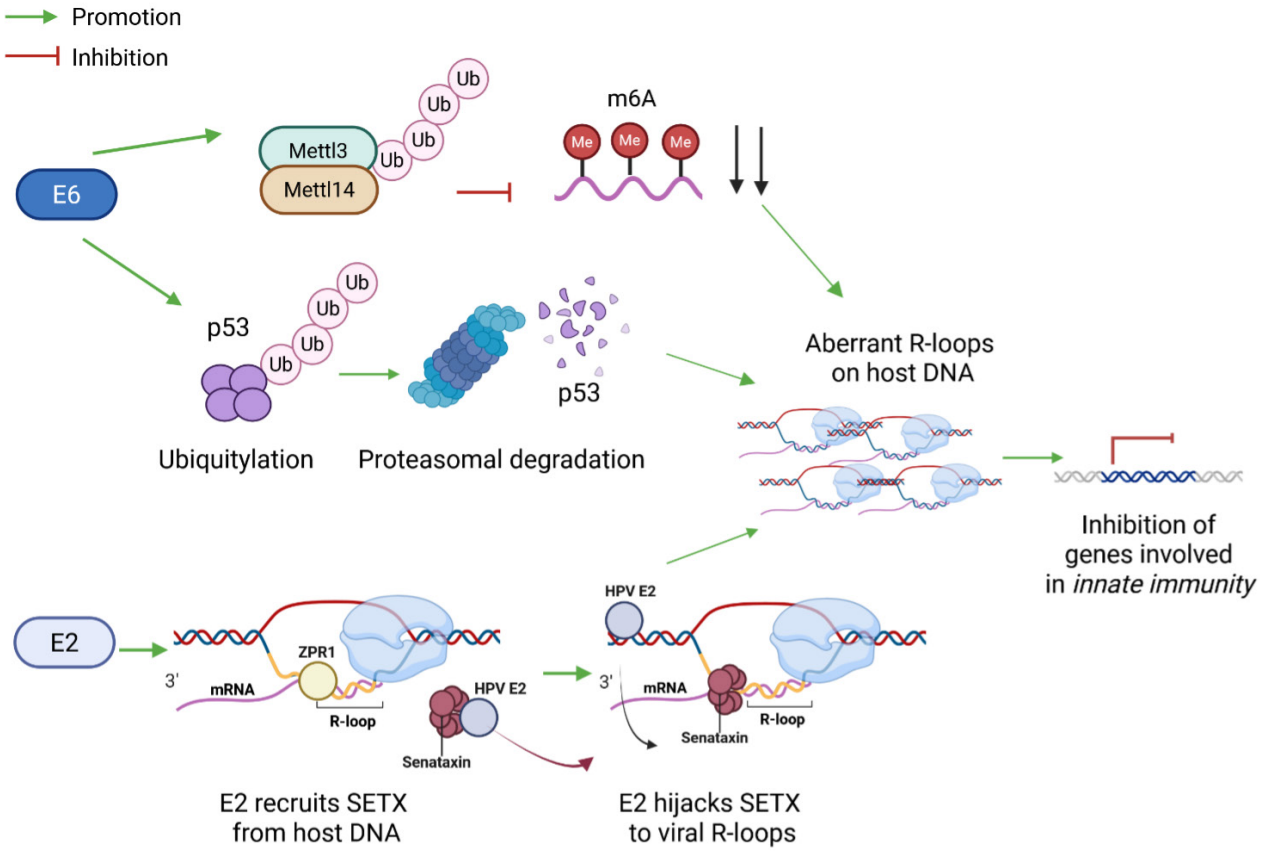

Figure 2: HPV increases mammalian R-loops to support viral maintenance and persistence. (Top) The viral oncogene E6 promotes R-loops through different mechanisms. First, E6 degrades the Mettl3/Mettl14 complex through the host ubiquitin ligase E6AP to increase N6-methyladenosine (m6A)-modified RNAs. These reduced m6A levels stabilize R-loops. However, SETX activates expression of m6A regulators (not shown). E6 also mediates ubiquitin-mediated degradation of p53. This mechanism increases genomic R-loops by an unknown mechanism. Lastly, APOBEC3B increases E6 expression and R-loop formation within the host and circular genonme (not shown). (Bottom) ZPR1 recruits the R-loop helicase SETX to mammalian R-loops. E2 sequesters away SETX on host chromatin to preferentially resolve viral R-loops and prevent viral genome integration. Together, p53 degradation and SETX hijacking result in aberrant R-loop accumulation on host DNA and suppression of innate immune gene expression (e.g., IL6, STAT1, IFNB1). Figure made with BioRender.com.

responses. Although DNA tumor viruses have been classically understood to drive carcinogenesis primarily through the action of their viral oncogenes, emerging evidence demonstrates that viral persistence and oncogenic progression are far more dynamic. Recent studies show that both mammalian and viral R-loops actively modulate replication, transcription, genome stability, and host antiviral responses by placing R-loop regulation at the center of DNA tumor virus–associated oncogenesis. These recent studies are highlighted below.

Epstein-Barr Virus

EBV establishes lifelong latent infection in nearly all adults and contributes to lymphoid/epithelial tumors and several autoimmune or inflammatory disorders. Cai et al.,[24] identified recurrent loss-of-function mutations in the RNA helicase DDX3X in EBV-associated malignancies. Mutant DDX3X fails to resolve R-loops, leading to replication stress and genomic instability. In this mechanism, DDX3X impairs antiviral STING–TBK1–IRF7 signaling which weakens innate control of EBV. These events lead to overexpression of the EBV lytic gene BNLF2b the driver of

R-loops and malignant progression. Together, helicase dysfunction and defective antiviral signaling create an environment in which R-loop driven genomic instability and EBV persistence synergize to drive oncogenesis.

Human Papillomavirus

Crane et al.[25] developed cisplatin resistant HPV+ and HPV- Oropharyngeal Squamous Cell Carcinoma (OPSCC) cell lines to identify R-loop regulators involved in chemotherapy resistance. It was found that cisplatin-resistant cells had elevated R-loop levels, and HPV+ OPSCC strains also had an upregulation in the SETX protein. In HPV+ OPSCC cell lines, knockdown of SETX increased tumor cell susceptibility to cisplatin by increasing R-loops and associated DNA damage. These findings suggest that SETX-mediated R-loop resolution contributes to cisplatin resistance in HPV+ OPSCC tumor cells and may serve as a future therapeutic target.

Given the hypothesis that HPV E2 sequesters SETX from ZPR1 and thereby disrupts normal mammalian R-loop resolution, a recent study examined ZPR1 expression in HPV+ cancers.[17] Using TCGA datasets, ZPR1 expression was found to be significantly reduced in HPV+ cervical and head and neck cancers compared to HPV-negative tumors. Notably, E2 transcripts are retained in most HPV+ tumors, with 69 percent of HPV+ cervical cancers and 80 percent of HPV+ head and neck cancers expressing detectable E2.[26] These findings indicate that a substantial proportion of HPV-associated cancers maintain E2 expression, supporting the biological relevance of investigating E2–SETX–ZPR1 interactions in tumor contexts where E2 is present.

Hepatitis B Virus

Hepatocellular carcinoma (HCC), the most common type of primary liver cancer, is strongly linked to chronic liver injury, particularly from Hepatitis B Virus (HBV) infection and is characterized by dysfunctional lipid metabolism. Using scRNA-seq data, Chen et al.,[38] developed an R-loop scoring signature based on R-loop regulator genes (RLRGs) associated with HBV infection and constructed a prognostic risk model centered on fatty acid metabolism related RLRGs. Among these RLRGs, Clathrin Heavy Chain 1 (CLTC) was significantly upregulated in HCC. As an R-loop regulator involved in lipid metabolism, CLTC emerged as a key driver of R-loop-mediated metabolic dysregulation. Targeting CLTC effectively suppressed abnormal lipid metabolism, reducing HCC tumor growth and migration. Overall, this study identifies CLTC as a potential prognostic biomarker and therapeutic target for HBV-associated HCC. This manuscript highlights the importance of identifying R-loop signatures associated with DNA tumor virus infections.

FUTURE DIRECTIONS

Integrating extrachromosomal DNAs (ecDNAs) and R-Loop Biology in DNA Tumor Virus Oncogenesis

R-loops are important structures that regulated the lifecycle and oncogenesis of DNA tumor viruses. In a new breakthrough in cancer progression, extrachromosomal DNAs (ecDNAs) were discovered to contribute to oncogenic signaling. These circular DNA molecules exist outside the chromosome and originate from chromosomal fragments that have been excised and circularized from host chromosomal fragments. ecDNAs often contain oncogenes that navigate clonal selection and expansion.[27,28] It is not surprising that these mini circular DNAs, like the genomes of DNA tumor viruses, contain aberrant R-loops.[29] Consistently, these ecDNAs have been identified in HPV+ head and neck cancers. HPV+ oropharyngeal cancers (HPV-OPC) frequently harbor integrated or extrachromosomal DNA composed of oncogenic host DNA and or episomal HPV DNA.[30-37] In contrast, HPV genomes in cervical cancers are typically integrated into the host chromosome. It remains unclear whether the formation or maintenance of these ecDNAs depends on R-loops and their transcriptional machinery.

We hypothesize that this field represents a key direction for the R-loop and DNA tumor virus field. DNA tumor viruses offer powerful model systems for dissecting transcriptional regulation and oncogenic mechanisms: their genomes are small, genetically tractable, and their DNA machinery and promoters have already been identified and are well characterized. Importantly, viral episomes function as natural models of ecDNAs. They not only mimic the circular architecture and transcriptional state of ecDNAs but also experience the same R-loop biology (Figure 3). DNA tumor viruses are excellent models to discover how ecDNAs and their own genomes drive oncogenesis through R-loop transcriptional machinery. Together, these findings elucidate the importance of R-loops in DNA tumor viruses and position these viruses as excellent model

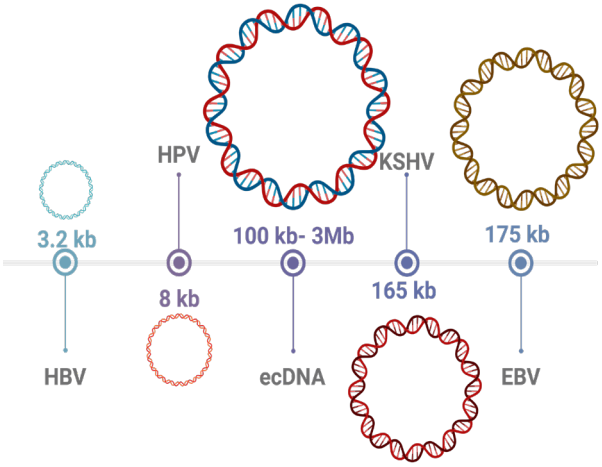

Figure 3: Comparison of genome sizes and architecture of DNA tumor viruses and oncogenic ecDNAs. Schematic representation of circular DNA elements found in infection and cancer. Sizes are shown in kilobases (kb) and megabases (Mb). ecDNAs range from 100kb to 3MB in size. Figure made with BioRender.com. Abbreviations: ecDNA, extrachromosomal DNA; HBV, Hepatitis B virus; HPV, Human papillomavirus; KSHV, Kapsi sarcoma-associated herpesvirus; EBV, Epstein-Barr virus.

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References

1. Crowner A, et al. Pathogens. 2024;13(10).
2. Niehrs C, Luke B. Nat Rev Mol Cell Biol. 2020.
3. Templeton CW, Laimins LA. Proc Natl Acad Sci U S A. 2023;120(35):e2305907120.
4. Jose L, et al. J Virol. 2024:e0100324.
5. Yiu SPT, et al. Cell Rep. 2022;38(10):110411.
6. Wongsurawat T, et al. Sci Rep. 2020;10(1):6389.
7. Wang B, et al. Cell Biosci. 2025;15(1):91.
8. Sato K, et al. Science. 2025;388(6752):1225-31.
9. Asghar A, et al. PLoS Pathog. 2025;21(8):e1013029.
10. Kumar A, et al. Cell Rep. 2022;39(6):110788.
11. Barbera AJ, et al. Cell Cycle. 2006;5(10):1048-52.
12. Templeton CW, Laimins LA. PLoS Pathog. 2024;20(8):e1012454.
13. Kono T, Laimins L. Viruses. 2021;13(9).
14. Spriggs CC, Laimins LA. Viruses. 2017;9(8).
15. Gillespie KA, et al. J Virol. 2012;86(17):9520-6.
16. Moody CA, Laimins LA. PLoS Pathog. 2009;5(10):e1000605.
17. Moffitt R, et al. Viruses. 2025;17(11).
18. Patel PS, et al. Mutat Res Genet Toxicol Environ Mutagen. 2022;876-877:503450.
19. Vats A, et al. Proc Natl Acad Sci U S A. 2026;123(1):e2526296123.
20. Thomas Y, Androphy EJ. J Virol. 2019;93(7).
21. Thomas Y, Androphy EJ. J Virol. 2018;92(3).
22. Templeton CW, Laimins LA. Cell Rep. 2026;45(1):116828.
23. Templeton CW, et al. PLoS Pathog. 2026;22(3):e1014088.
24. Cai HM, et al. Cell Rep. 2025;44(9):116237.
25. Crane H, et al. iScience. 2025;28(9):113348.
26. Ren S, et al. Oncogene. 2020;39(40):6327-39.
27. Haughey M, et al. Trends Cancer. 2025;11(9):901-16.
28. Bailey C, et al. Nature. 2024;635(8037):193-200.
29. Tang J, et al. Nature. 2024;635(8037):210-8.
30. Pang J, et al. Clin Cancer Res. 2021.
31. Nulton TJ, et al. Oral Oncol. 2018;80:52-5.
32. Vojtechova Z, et al. Int J Cancer. 2016;138(2):386-95.
33. Olthof NC, et al. PLoS One. 2014;9(2):e88718.
34. Nulton TJ, et al. Oncotarget. 2017;8(11):17684-99.
35. Morgan IM, et al. Viruses. 2017;9(8).
36. Akagi K, et al. Cancer Discov. 2023;13(4):910-27.
37. Nakagawa T, et al. Nat Commun. 2025;16(1):2964.
38. Chen X, et al. Front Immunol. 2025; 15:1487372.