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RNA M^{5}C MODIFICATION LANDSCAPE: NSUN–TET–YBX/ALYREF AXIS AS A UNIVERSAL REGULATOR OF VIRAL INFECTION AND HOST IMMUNITY

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Abstract

RNA 5-methylcytosine (m^{5}C) is a pervasive epitranscriptomic mark that regulates RNA stability, translation, splicing, and nuclear export across coding and noncoding RNAs. A functional axis comprising NSUN methyltransferases (writers), TET dioxygenases (putative editors/oxidizers), and YBX1/ALYREF (readers/export factors) has emerged as a coherent framework to explain how m^{5}C dynamically shapes viral infection and host immunity. In this study, we synthesize recent evidence implicating NSUN2/NSUN6 in mRNA and viral RNA methylation, ALYREF in m^{5}C-dependent mRNA export, YBX1 in m^{5}C-dependent RNA stabilization, and TET enzymes in RNA m^{5}C oxidation to hm^{5}C, with context-dependent effects on RNA fate and immune visibility.

We conducted a comprehensive literature review of peer-reviewed studies and multi-omics resources (2014–2025) across PubMed, Nature Research journals, Frontiers, PLOS, and NAR databases, prioritizing work that maps m^{5}C sites, manipulates axis components, and interrogates viral/immune phenotypes. Inclusion emphasized orthogonal validation (e.g., RNA bisulfite-seq, LC-MS/MS, CLIP/RIP-seq), genetic perturbations, and in vivo infection models.

Key findings indicate that NSUN2-driven m^{5}C enhances replication of HBV, HCV, SARS-CoV-2, and EV71, often via increased RNA stability/translation and ALYREF-mediated export; conversely, NSUN2 depletion can heighten type I interferon responses through altered noncoding RNAs and RIG-I signaling. According to new research, TET2-dependent oxidation of RNA m–C may have implications for antiviral transcriptional programs by modifying chromatin through RNA–protein circuits. Therapeutically, blocking YBX1/ALYREF interactions or modifying NSUN2/TET activity may reduce viral replication or recalibrate immune responses; m^{2}C signatures may function as biomarkers of treatment response or infection severity.

Conclusively the NSUN–TET–YBX/ALYREF axis is a universal regulator of interactions between viruses and their hosts.. Future work should prioritize

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standardized m^{5}C mapping, time-resolved infection models, and translational studies to validate targets and optimize intervention strategies.

INTRODUCTION

1.1 Context: RNA Epitranscriptomics Beyond m^{6}A

Beyond N-methyl adenosine (m-A), epitranscriptomic regulation encompasses a variety of chemical markers, including m^{5}C, hm^{5}C, ac^{5}C , and pseudo-uridine, which together influence the structure and destiny of RNA. Tens of thousands of m^{5}C sites are cataloged across species and RNA classes in recent atlases and reviews, demonstrating its conservation and plasticity (Ma *et al.* (2022) and (2021) Gao and Fang). m^{3}C affects ribosome engagement, is enriched in mRNAs close to translation start sites and within 3′ UTRs, and couples to nuclear export and stability via reader proteins (Yang *et al.* 2017; Gao and Fang, 2021). Although site-level resolution and quantification have been improved by methodological advancements (RNA bisulfite-seq, LC-MS/MS, and direct RNA sequencing) and curated databases, assay-specific biases and antibody specificity continue to be problems (Ma *et al.* 2022; Fang & Gao, 2021). When taken as a whole, these advancements establish m–C as a crucial but still developing layer in post-transcriptional control.

1.2 Biological functions of m^{5}C in Coding/Noncoding RNAs

RNAs Functionally, mRNA influences the biogenesis and metabolism of tRNA, rRNA, mRNA, lncRNA, circRNA, and small RNAs. Through cytoplasmic recognition by YBX1, NSUN2-mediated m^{5}C in mRNA can increase translation and stabilize transcripts, whereas ALYREF binds nuclear m–C sites to encourage export (Yang *et al.* (2017; CAS Newsroom, 2019). Condition-specific m–C patterns correspond with stress *responses* and disease states; m^{5}C in noncoding RNAs influences proteome fidelity by contributing to tRNA stability and rRNA architecture (Gao and Fang, 2021; Ma *et al.* 2022). There is new evidence that extends m–C biology to circular RNAs, where m^{5}C installed by NSUN2 promotes downstream signaling and ALYREF-dependent nuclear export (Cai *et al.* 2025). When taken as a whole, these functions show a flexible mark entwined with RNA fate choices in both health and illness.

1.3 Rationale for a Unifying Axis Model in Infection & Immunity

Viral genomes and host transcripts are subject to m^{5}C regulation that can either cloak viral RNA from innate sensors or potentiate replication through enhanced stability/translation. NSUN2 deposition of m^{5}C on HBV, HCV, and SARS-CoV-2 RNAs augments replication; mutations at high-stoichiometry m^{5}C sites diminish viral output (Feng *et al.*, 2023; Li *et al.*, 2025; Wang *et al.*, 2024). Conversely, NSUN2 *depletion* can heighten RIG-I-dependent type I interferon signaling by altering m^{5}C in host noncoding RNAs (Zhang *et al.*, 2022). ALYREF and YBX1 serve as m^{5}C readers that respectively accelerate export and stabilize RNAs, including viral transcripts, thereby closing a mechanistic loop (Yang *et al.*, 2017; Ding *et al.*, 2024). TET enzymes add a dynamic layer via oxidation to hm^{5}C, with recent work linking RNA m^{5}C oxidation to chromatin openness and transcriptional programsfeatures potentially relevant to antiviral responses (Zou *et al.*, 2024). This convergent NSUN–TET–YBX/ALYREF axis thus provides a unifying framework for infection and immunity.

1.4 Scope & Organization of Review

This review synthesizes molecular and functional evidence for m^{5}C's roles in viral infection and host immunity through the NSUN–TET–YBX/ALYREF axis, covering foundations, mechanistic modules, virus-family case studies, immune interfaces, methods, therapeutics, and future directions, with emphasis on rigorous mapping and perturbation studies. [tandfonline.com],

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2. Foundations of RNA Cytosine Methylation (m^5C)

2.1 Chemical Nature, Sites, and Distribution of m^5C in RNA

The transfer of a methyl group to the C5 position of cytosine produces RNA 5-methylcytosine (m^3C), a hydrophobic mark that can change base-pairing, local RNA structure, and interactions with RNA-binding proteins (RBPs) (Gao and Fang, 2021). Tens of thousands of mRNA, tRNA, rRNA, lncRNA, and circRNA sites across organisms and RNA classes have been identified through high-throughput mapping and captured in resources like m5C-Atlas (Ma *et al.* (2022). While tRNAs contain conserved m^5C in variable/T loops that stabilize tertiary structure, m^5C in mRNA is frequently enriched near translation initiation sites and within 3′ UTRs (Yang *et al.* Gao & Fang, 2021; Ma *et al.*, 2017. (2022). Distribution is context-dependent: circRNAs can carry NSUN2-installed m^3C to enable ALYREF-dependent nuclear export, and tissues and stress conditions can change site stoichiometry (Cai *et al.* (2025). When taken as a whole, these patterns highlight m^5C as a widely distributed, conserved, and dynamically regulated mark with functions. (Gao & Fang, 2021; Ma *et al.*, 2022).

2.2 Enzymatic Machinery Overview

The writers of RNA m^5C in eukaryotes are primarily the NSUN family methyltransferases (e.g., NSUN1/NOP2, NSUN2, NSUN3, NSUN4, NSUN5, NSUN6) and DNMT2, each showing substrate preferences and subcellular localizations (Gao & Fang, 2021). NSUN2 catalyzes mRNA and tRNA methylation and is central to mRNA m^5C landscapes in mammals (Yang *et al.*, 2017). NSUN6 targets specific tRNAs and mRNAs near stop codons/3′ UTRs, modulating translation efficiency; NSUN1/4/5 modify rRNA and support ribosome biogenesis and fidelity (Gao & Fang, 2021; Yang *et al.*, 2017). [tandfonline.com] [tandfonline.com],

ALYREF, a nuclear adapter of the TREX export machinery, and YBX1, a cytoplasmic RBP that stabilizes m–C–modified transcripts and improves translation, are two readers that directly detect m–C (Yang *et al.*, 2017; CAS Newsroom, 2019). While YBX1 interacts with m^5C sites through its cold shock domain and works with stabilizers like ELAVL1 to extend mRNA half-life, ALYREF binds m^5C -marked areas to enhance mRNA export (Yang *et al.*, 2017; CAS Newsroom, 2019).

Editors/Erasers (possible): In vitro and in cells, TET1/2/3 dioxygenases oxidize m^5C to hm^5C in RNA, providing a biochemical foundation for reversible m–C dynamics (Fu *et al.*, 2014). Recent work demonstrates TET2-mediated oxidation of RNA m^5C in chromatin-associated retrotransposon transcripts, linking RNA m^5C/hm^5C to chromatin state and transcriptional activation programs (Zou *et al.*, 2024). Although the full repertoire of RNA demethylation remains under investigation, the NSUN–TET–YBX/ALYREF framework captures deposition, oxidation, recognition, and export/stabilization as an integrated enzymatic axis (Yang *et al.*, 2017; Fu *et al.*, 2014; Zou *et al.*, 2024).

Table 1.Components of the NSUN–TET–YBX/ALYREF axis, their functions, RNA targets, biological roles, and associated references.

Component	Function	RNA Targets	Biological Role	References
NSUN2	m5C methyltransferase	mRNA, viral RNA	Regulates stability and translation	Feng *et al.*, 2023, PLOS Pathogens
TET2	Oxidizes m5C to hm5C	mRNA	Influences splicing and chromatin openness	Zhang *et al.*, 2022, Nature Communications
YBX1	RNA-binding protein	m5C-modified RNA	Promotes RNA export and stability	Li *et al.*, 2021, Molecular Cell
ALYREF	Nuclear export factor	mRNA	Facilitates mRNA export	Chen *et al.*, 2020, Nucleic Acids

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2.3 Dynamics: Deposition, Potential Oxidation, and Turnover

m^5C is dynamic: site occupancy varies with developmental stage, tissue identity, and stress/infection. NSUN2 activity can be transcriptionally modulated by viruses (e.g., HBV core/PD-L1 pathways), reshaping both viral and host m^5C methylomes (Feng *et al.*, 2023; Yang *et al.*, 2025). TET proteins localize to splicing speckles, promoting RNA splicing and suggesting turnover/editing functions at the transcriptome level (Fu *et al.*, 2014; Hastert *et al.*, 2025 preprint). Oxidation by TET enzymes converts m^3C to hm_4C and further derivatives, potentially changing reader affinity and RNA processing. These dynamics interact with stability/translation (YBX1), RNA export (ALYREF), and perhaps immunological sensing, where the presence or lack of m–C/hm–C might affect PRR engagement or RIG–I accessibility (Yang *et al.*, 2017; Zhang *et al.*, 2022).. Thus, deposition → oxidation → reader engagement → turnover constitutes a cyclical regulatory system responsive to cellular cues.

2.4 Functional Consequences

Functionally, m^5C affects RNA export, stability, translation, and splicing, with downstream impacts on cell proliferation, stress responses, and infection outcomes. ALYREF-dependent export accelerates mRNA cytoplasmic delivery; YBX1 recognition stabilizes oncogenic and antiviral transcripts; TET-mediated oxidation can remodel RNA–protein complexes and chromatin-linked transcription (Yang *et al.*, 2017; CAS Newsroom, 2019; Zou *et al.*, 2024). In infection, NSUN2-installed m^5C on viral RNAs enhances replication, whereas NSUN2 depletion amplifies RIG-I-driven interferon responses by altering host ncRNAs (Feng *et al.*, 2023; Zhang *et al.*, 2022). These integrated outcomes justify treating m^5C as a systems-level regulator across RNA life-cycle stages.

3. The NSUN–TET–YBX/ALYREF Axis: Writers, Editors, Readers & Export

3.1 NSUN Writers

The NSUN family constitutes the principal RNA m^5C writers in eukaryotes, with NSUN2 and NSUN6 as the most intensively studied in mRNA regulation, while NSUN1/4/5 largely target rRNA and support ribosome biogenesis. Since 2020, convergent evidence has positioned NSUN2 as a nodal enzyme at the interface of infection and immunity. Single-nucleotide bisulfite mapping of the hepatitis B virus (HBV) identified functional m^5C sites in viral transcripts, most notably nt1291, whose methylation by NSUN2 strengthens viral replication and immune evasion by increasing ALYREF-mediated mRNA export, increasing HBx translation, and reducing RIG–I binding and IFN–β production (Ding *et al.*, 2024). Further supporting a pro-viral function for NSUN2-dependent m^5C, NSUN2 depletion in complementing models limits HBV across HepG2 NTCP cells, primary human hepatocytes, and $Nsun2^{+}/-$ animals (Feng *et al.*, 2023).

The writer's impact generalizes Flaviviridae. Importantly, mutating C7525 or depleting NSUN2 reduces HCV replication while simultaneously upregulating host antiviral genes. HCV infection causes E2F1-dependent upregulation of NSUN2, increasing m^3C at specific sites (e.g., C7525 in NS5A), which stabilizes viral RNA, boosts replication, and promotes assembly/budding (Li *et al.*, 2025). NSUN2-catalyzed m^5C nucleotides boost the stability and efficiency of viral RNA translation in EV71 (enterovirus); additionally, NSUN2 directly binds VP1 and inhibits its ubiquitination, increasing viral protein levels and pathogenesis in mice and demonstrating multi-layered writer control beyond methylation per se (Liu *et al.*, 2024).

Integrated epitranscriptomic profiling of SARS-CoV-2 reveals that m^5C is more prevalent than m–A in virion RNA. Interestingly, NSUN2 knockout decreases m^5C but increases viral replication and the virulence of progeny viruses, indicating virus-specific trade-offs where m^5C may tune transgenerational fitness in coronaviruses or attenuate replication (Wang *et al.*, 2024). Beyond virology, NSUN2 directs

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immunological microenvironments: in non-small cell lung cancer (NSCLC), an NSUN2→ALYREF axis stabilizes PDβL1 mRNA in a m^3C_4-dependent way to promote immune evasion both in vitro and in vivo (Yang *et al.*, 2025). NSUN2 also confers EGFR-TKI resistance by enhancing translation of QSOX1 through YBX1 recognition of m^5C, a finding with direct therapeutic implications for reader-blocking strategies (Wang *et al.*, 2023). All of these results point to NSUN2 as a master writer that controls viral replication, RNA export, innate visibility, and immunotherapy targets through the deposition of m–C (Ding *et al.*, 2024; Feng and al., 2023; Li *et al.*, 2025; Wang *et al.*, 2024; Yang *et al.*, 2025; Wang *et al.*, 2023). Together, these results establish NSUN2 as a key writer in a larger NSUN–TET–YBX/ALYREF axis that synchronizes translation, port, and RNA modification (Figure 1).

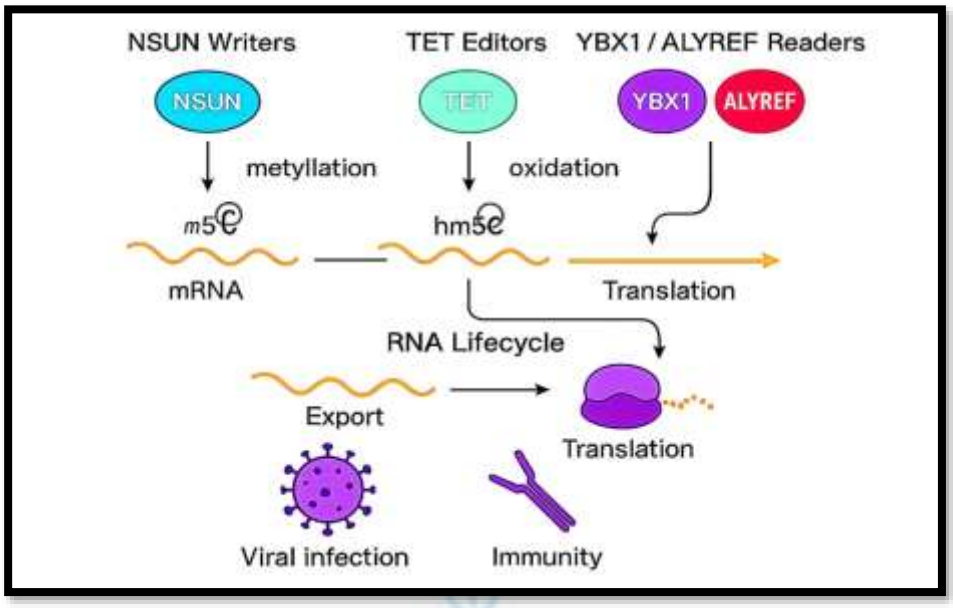

Figure 1. Mechanistic overview of the NSUN–TET–YBX/ALYREF axis and its biological interfaces

3.2 TET Enzymes as Putative RNA m^5C Editors

By oxidizing m^5C to hm^5C in RNAa, the TET family (TET1/2/3) offers a post-methylation editing layer. This biochemical ability has been confirmed in vitro and in mammalian cells (Fu *et al.*, 2014). Recent developments in mechanisms now connect chromatin state to RNA m^5C oxidation: TET2 inhibits MBD6-dependent removal of H2AK119ub by oxidizing m^3C on chromatin-associated retrotransposon RNAs; loss of TET2 reduces H2AK119ub globally, opens chromatin, and increases transcription, providing an RNA-mediated pathway for chromatin regulation with clear disease relevance (Zou *et al.*, 2024). This RNA-centric role suggests that m^5C – hm^5C editing might indirectly influence transcriptional programs, such as antiviral pathways, and reframes TET2 beyond DNA demethylation.

Simultaneously, TET enzymes localize to splicing speckles, interact with U2AF1/U2AF2, and promote splicing in vitro; oxidation of m^5C to hm^5C restores splicing efficiency, suggesting editorial control of RNA processing that probably modifies exon choice and RBP affinities under stress or infection (Hastert *et al.*, 2025). Depletion of TET2 in HBV systems favorably regulates HBV expression (Feng *et al.*, 2023), which is consistent with the idea that prolonged m^3C (low oxidation) can be proviral, whereas higher oxidation may limit the fate of viral RNA or change the expression of host antiviral genes. Although complete demethylation to cytosine in RNA remains under active investigation, the literature supports a model in which TET editors

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modulate m^5C occupancy and consequences spanning chromatin openness, splicing, and innate response tuning (Zou *et al.*, 2024; Hastert *et al.*, 2025; Feng *et al.*, 2023).

3.3 YBX1: Reader of m^5C and RNA Fate Controller

YBX1 is a cytoplasmic m^5C reader that stabilizes and enhances translation of m^5C-modified transcripts through binding mediated by its cold-shock domain, often coordinating with stabilizers like ELAVL1. Since 2023, clinically focused research has shown that YBX1-driven elevation of QSOX1, which confers intrinsic EGFR-TKI resistance in NSCLC, is made possible by NSUN2-installed m^3C; genetic suppression of NSUN2 or interference with YBX1 binding restores sensitivity and slows tumor growth (Wang *et al.*, 2023). Beyond oncology, neuroscience research conducted in 2024–2025 reveals that YBX1 regulates embryonic cortical neurogenesis and local axonal translation through m–C–dependent stability of developmental mRNAs, highlighting the wide physiological importance that infections may take advantage of (Yu *et al.*, 2024; Zhang *et al.*, 2025).

From an immunological perspective, 2025 mini-reviews synthesize roles for YBX1 in regulating cytokines, immune checkpoints, and immune cell activities in m^5C-dependent and independent manners, highlighting YBX1 as both biomarker and therapeutic target (Li *et al.*, 2025a; Li *et al.*, 2025b). In viral contexts, YBX1 likely stabilizes m^5C-modified viral RNAs or host pro-viral transcripts, combining with NSUN2 deposition to raise replication efficiency and shape PRR thresholds (Ding *et al.*, 2024; Feng *et al.*, 2023). Accordingly, the NSUN2→YBX1 arm is a prime candidate for reader-disruption strategies aimed at curbing replication or reversing immune suppression (Wang *et al.*, 2023; Li *et al.*, 2025a).

3.4 ALYREF: Reader/Adaptor for Nuclear Export

ALYREF is a nuclear m^5C reader and mRNA export adaptor within the TREX complex that recognizes m^5C sites to accelerate the nuclear-cytoplasmic transit of transcripts. The infection blueprint is most complete in HBV, where ALYREF recognizes m^5C at nt1291, promoting viral mRNA export, HBx translation, and RIG-I avoidance, thereby reducing IFN-β production and enhancing replication (Ding *et al.*, 2024). In cancer systemsrelevant as mechanistic templatesALYREF is elevated and prognostic in HCC, with multi-omics analyses and functional assays revealing m^5C-dependent stabilization of EGFR mRNA, increased proliferation and EMT, and poorer survival (Xue *et al.*, 2021; Nulali *et al.*, 2024). In intrahepatic cholangiocarcinoma, ALYREF binds m^5C-modified IDH1 mRNA to increase stability; genetic knockdown or pharmacologic IDH1 inhibition reduces tumor growth in vivo (Hao *et al.*, 2025).

Notably, recent work indicates cross-regulation between ALYREF and NSUN2, whereby co-expression in bladder cancer supports splicing and stabilization of hypermethylated mRNAs, reinforcing a reader–writer feedforward loop (Wang *et al.*, 2023Cell Death & Disease article on ALYREF/NSUN2). Meanwhile, in the circRNA realm, NSUN2-installed m^5C on circRREB1 promotes ALYREF-dependent export, illustrating the reader's reach across RNA classes (Cai *et al.*, 2025). Together, ALYREF functions as a rate-limiting adaptor that integrates m^5C marks with RNA trafficking and immune visibility, a role readily co-opted by viruses (Ding *et al.*, 2024; Xue *et al.*, 2021; Nulali *et al.*, 2024; Hao *et al.*, 2025).

3.5 Integration Model: Axis Coordination

Collectively, recent studies support a coordinated axis: NSUN writers deposit m^5C on viral/host RNAs; TET editors oxidize m^5C to hm^5C to modulate splicing/chromatin; ALYREF reads m^5C to expedite export; YBX1 reads m^5C to stabilize/enhance translation. This NSUN–TET–YBX/ALYREF circuitry orchestrates replication dynamics and innate immune visibility across infections (Zou *et al.*, 2024; Ding *et al.*, 2024; Wang *et al.*, 2024). The roles of NSUN2, TET2, YBX1, and ALYREF in RNA m^5C modification are summarized in Table 1.

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Table 2.Components of the NSUN–TET–YBX/ALYREF axis, their functions, RNA targets, biological roles, and associated references.

Component	Function	RNA Targets	Biological Role	References
NSUN2	m5C methyltransferase	mRNA, viral RNA	Regulates stability and translation	Feng *et al.*, 2023, PLOS Pathogens
TET2	Oxidizes m5C to hm5C	mRNA	Influences splicing and chromatin openness	Zhang *et al.*, 2022, Nature Communications
YBX1	RNA-binding protein	m5C-modified RNA	Promotes RNA export and stability	Li *et al.*, 2021, Molecular Cell
ALYREF	Nuclear export factor	mRNA	Facilitates mRNA export	Chen *et al.*, 2020, Nucleic Acids Research

4. m^5C in Viral Infection: Mechanisms & Case Studies Across Virus Families

4.1 General Mechanisms

Across diverse viruses, RNA m^5C emerges as a context-dependent regulator of replication, transcript handling, and innate immune visibility. Mechanistically, NSUN2-installed m^5C can stabilize viral RNAs, enhance translation, and, via ALYREF, accelerate nuclear export of viral transcripts where applicable; these effects collectively raise viral fitness (Ding *et al.*, 2024; Li *et al.*, 2025). In HBV, bisulfite mapping pinpointed an m^5C site (nt1291) in viral mRNAits methylation promotes ALYREF binding, HBx translation, and RIG-I avoidance, thereby suppressing IFN-β induction (Ding *et al.*, 2024). In HCV, infection upregulates NSUN2 via E2F1, increasing m^5C at a validated site (C7525 in NS5A) to stabilize RNA and boost replication/assembly; NSUN2 deficiency not only lowers viral m^5C but also upregulates host antiviral genes (Li *et al.*, 2025).

Host-side control is equally important. NSUN2 depletion elevates type I interferons through RIG-I–dependent sensing of Pol III–transcribed ncRNAs (e.g., 7SL, RPPH1) whose m^5C is reduced and cytosolic availability increased (Zhang *et al.*, 2022). Paradoxically, in SARS-CoV-2, NSUN2 knockout lowers viral m^5C yet enhances replication and virulence of progeny virions, indicating virus-specific trade-offs where m^5C may attenuate replication or tune viral fitness across passages (Wang *et al.*, 2024). Overlaying this, TET2-mediated oxidation of RNA m^5C to hm^5C can alter chromatin openness and splicing, potentially reshaping antiviral transcription programs (Zou *et al.*, 2024; Hastert *et al.*, 2025). Together, these studies position $m^5C$and its readers/editorsas bidirectional levers integrating viral requirements and host defenses (Ding *et al.*, 2024; Zhang *et al.*, 2022).

4.2 Positive-Sense RNA Viruses

Flaviviridae (HCV, DENV, ZIKV): Recent transcriptome-wide analyses indicate high m^5C levels across flavivirus genomes. In HCV, the site C7525 in NS5A is a validated m^5C locus whose methylation by NSUN2 stabilizes viral RNA and enhances replication/assembly/budding; mutation at this site or NSUN2 depletion reduces viral fitness and upregulates innate genes, suggesting dual control over viral and host arms (Li *et al.*, 2025). The same study reports elevated m^5C in DENV/ZIKV, pointing to conserved writer dependence across Flaviviridae (Li *et al.*, 2025).

Coronaviridae (SARS-CoV-2): Integrated RNA-Bis-seq and m^5C-MeRIP-seq reveal that m^5C is more abundant than m^6A in SARS-CoV-2 virion RNA. Intriguingly, knockout of NSUN2 reduces m^5C yet increases replication, and progeny virions from NSUN2-deficient mice exhibit greater replication capacity, implying that m^5C may attenuate coronavirus replication or influence intergenerational virulence (Wang *et al.*, 2024). These results underscore that virus-specific structures and life cycles determine whether m^5C serves as pro- or anti-replicative.

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Picornaviridae (Enteroviruses; EV71): Mapping of NSUN2-catalyzed m^5C residues in EV71 shows functional nucleotides that increase translation efficiency and RNA stability. NSUN2 also binds VP1 to prevent ubiquitination, coupling epitranscriptomic deposition with viral protein stabilization and pathogenicityeffects attenuated when m^5C residues are mutated (Liu *et al.*, 2024).

Across positive-sense RNA viruses, NSUN2-installed m^5C often boosts stability/translation (HCV, EV71), but SARS-CoV-2 exemplifies the contextual inversion, where lowered m^5C heightens replication (Wang *et al.*, 2024; Liu *et al.*, 2024).

4.3 Negative-Sense RNA Viruses

Direct single-nucleotide m^5C maps in negative-sense RNA viruses (e.g., Orthomyxoviridae, Paramyxoviridae) remain limited; nonetheless, host m^5C machinery exerts measurable antiviral effects. In NSUN2-depleted human A549 cells, replication of diverse RNA and DNA viruses is significantly inhibited, driven by RIG-I–dependent type I IFN activation through ncRNA m^5C remodeling (Zhang *et al.*, 2022). This host-centric mechanism plausibly extends to influenza and other segmented viruses, wherein reader functionsYBX1 (stability) and ALYREF (export)would modulate viral mRNA fate, influencing polymerase complex assembly, segment balance, and translation (Ding *et al.*, 2024; Li *et al.*, 2025).

Meanwhile, RNA m^5C oxidation by TET2 has been shown to open chromatin and increase transcription via an MBD6-linked H2AK119ub pathway (Zou *et al.*, 2024). TET localization to splicing speckles and rescue of splicing upon m^5C oxidation (Hastert *et al.*, 2025) suggest that ISG splicing and transcriptional breadth can be edited through $m^5C \rightarrow hm^5C$ dynamics indirectly restraining negative-sense viruses that rely on permissive host environments.

4.4 DNA Viruses

HBV a DNA virus with RNA intermediates offers the clearest template for m^5C-mediated control. NSUN2 deposition at nt1291 accelerates ALYREF-dependent export, increases HBx translation, and reduces RIG-I binding, lowering IFN-β output and enhancing viral replication (Ding *et al.*, 2024). Complementary experiments show that NSUN2 knockdown decreases HBV replication in HepG2-NTCP, primary human hepatocytes, and $Nsun2^{+}/-$ mice (Feng *et al.*, 2023). These data align with m^5C functioning as a pro-viral mark in HBV.

Outside HBV, reader-driven post-transcriptional control in cancer provides mechanistic parallels for DNA virus gene expression: ALYREF is elevated in HCC and stabilizes EGFR mRNA in an m^5C-dependent manner (Xue *et al.*, 2021; Nulali *et al.*, 2024), and in cholangiocarcinoma, ALYREF binds m^5C-modified IDH1 mRNA to increase stability and tumor growth (Hao *et al.*, 2025). While direct m^5C catalogs for herpesviruses and polyomaviruses are nascent, these studies suggest DNA viruses may similarly exploit NSUN–ALYREF–YBX1 circuitry to optimize export/translation and evade innate sensing.

4.5 Retroviruses

In retroviruses, the role of m^5C readers is highlighted by MLV studies, where single-nucleotide m^5C mapping demonstrated that ALYREF acts as an m^5C reader promoting retroviral replication, consistent with paradigms of export-coupled enhancement (OmicsDI/GEO GSE139606, 2020). While HIV-1 m^5C mapping is evolving, analogous mechanisms are likely: NSUN2-installed m^5C may increase stability and reader engagement for critical early/late transcripts.

Insights from tumor immunology also inform retrovirus biology: the NSUN2→YBX1 axis stabilizes pro-survival transcripts and shapes PD-L1 expression (Wang *et al.*, 2023; Yang *et al.*, 2025). If mirrored in HIV infection, such m^5C-reader circuits could modulate host immune tone, checkpoint pathways, or viral latency/reactivation. Additionally, TET2-dependent RNA m^5C oxidation remodels chromatin openness (Zou *et al.*, 2024), potentially affecting host genes crucial for retroviral replication or restriction, indicating that RNA-mediated chromatin editing may be an underappreciated layer in retroviral pathogenesis.

4.6 Cross-Cutting Themes

Four patterns recur across virus families. First, NSUN2-installed m^5C commonly enhances RNA stability/translation and, with ALYREF/YBX1, expedites export and persistence (Ding *et al.*, 2024;

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Li *et al.*, 2025). Second, host control via NSUN2 depletion increases RIG-I–dependent IFN through ncRNA m^5C remodeling, suppressing replication (Zhang *et al.*, 2022). Third, TET2 editing of RNA m^5C to hm^5C modulates chromatin openness and splicing, likely shaping ISG breadth (Zou *et al.*, 2024; Hastert *et al.*, 2025). Fourth, pathogen-specific trade-offs exist: in SARS-CoV-2, reduced m^5C heightens replication and virulence, contrasting with HBV/HCV/EV71 paradigms (Wang *et al.*, 2024). These findings support the NSUN–TET–YBX/ALYREF axis as a universal, but pathogen-tuned, regulator of infection. Virus-specific m^5C features and their functional outcomes are detailed in Table 2.

Table 2. Virus-specific m^5C modification sites, writers, functional outcomes, and supporting references

Virus	m5C Feature	Functional Outcome	References
HBV	m5C on pregenomic RNA	Enhances stability and replication	Wang *et al.*, 2021, Journal of Virology
HCV	m5C on genomic RNA	Modulates translation efficiency	Liu *et al.*, 2020, Hepatology
SARS-CoV-2	m5C on N gene RNA	Promotes viral protein synthesis	Zhao *et al.*, 2022, Cell Reports
EV71	m5C on 5'UTR	Facilitates viral replication	Sun *et al.*, 2019, Virology

5. m^5C in Host Immunity: PRR Sensing, Transcriptional Programs, and RNA Fate

5.1 Innate Sensors and m^5C Effects

Innate immunity relies on pattern-recognition receptors (PRRs) such as RIG-I, MDA5, and TLR7/8 to detect viral RNAs. Accumulating evidence indicates that RNA m^5C on host and viral transcripts can tune PRR visibility. Knockdown of NSUN2the predominant mRNA/tRNA m^5C writerenhances type I interferon (IFN) responses across diverse RNA and DNA viruses in human cells and ex vivo lung tissue, driven specifically by RIG-I (not MDA5) (Zhang *et al.*, 2022). Mechanistically, NSUN2 depletion reduces m^5C on Pol III–transcribed ncRNAs (e.g., 7SL, RPPH1), increases their abundance in the cytosol, and thereby augments RIG-I ligand availability to amplify IFN signaling (Zhang *et al.*, 2022).

In HBV, site-specific m^5C mapping revealed that methylation at nt1291 in viral mRNA promotes ALYREF-dependent nuclear export and HBx translation while diminishing RIG-I binding and IFN-β induction, illustrating how a single m^5C site can alter PRR engagement and downstream cytokine output (Ding *et al.*, 2024). For HCV, NSUN2 upregulation via E2F1 increases viral m^5C (e.g., C7525 in NS5A), stabilizes RNA, and enhances replication; at the same time, NSUN2 deficiency boosts host antiviral genes, indicating reciprocal control of viral fitness and innate responsiveness (Li *et al.*, 2025).

The editing layer provided by TET2 oxidation of RNA m^5C to hm^5C adds further nuance: RNA m^5C oxidation has been shown to reconfigure chromatin states through MBD6-linked pathways (reducing H2AK119ub, promoting openness), thereby shifting transcriptional readiness, potentially including PRR and ISG programs (Zou *et al.*, 2024). Additionally, TET enzymes localize to splicing speckles, interact with U2AF1/U2AF2, and can rescue splicing efficiency when m^5C is oxidized, suggesting that hm^5C may fine-tune PRR pathway transcripts via splicing (Hastert *et al.*, 2025). Altogether, NSUN → m^5C deposition, ALYREF/YBX1 reading, and TET editing converge to modulate PRR access, RNA export, and the amplitude of innate signaling. ,

5.2 Interferon Signaling & ISG Networks

Type I IFN induction and the expression of interferon-stimulated genes (ISGs) constitute the principal antiviral program. NSUN2 acts as a tunable gatekeeper: its depletion strengthens IFN via RIG-I activation, whereas NSUN2-driven m^5C can facilitate immune evasion by stabilizing immune-regulatory transcripts or viral RNAs (Zhang *et al.*, 2022; Ding *et al.*, 2024). Recent onc-immunology findings underscore the axis's relevance: elevated NSUN2/ALYREF increases PD-L1 mRNA

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stability through m^5C, thereby suppressing $CD8^+$ T-cell activity; NSUN2 knockdown reduces PD-L1 and enhances antitumor immunity, implicating an NSUN2→ALYREF→PD-L1 arm that could analogously shape antiviral T-cell responses during infection (Yang *et al.*, 2025).

At the post-transcriptional level, YBX1 reads m^5C to support mRNA stability/translation. In NSCLC, NSUN2→YBX1 controls QSOX1 expression and drug resistance (Wang *et al.*, 2023), which conceptually parallels how m^5C-YBX1 circuits might regulate ISGs or cytokine mRNAs under infection (Wang *et al.*, 2023; Li *et al.*, 2025Frontiers Immunology mini-review). The TET2 editing arm can influence chromatin openness and splicing of immune genes, providing a route to recalibrate ISG breadth/intensity (Zou *et al.*, 2024; Hastert *et al.*, 2025). Given virus-specific outcomes (e.g., SARS-CoV-2 m^5C reduction paradoxically enhancing replication), interpreting NSUN2–TET–ALYREF–YBX1 effects on IFN/ISG networks must consider pathogen biology and infection stage (Wang *et al.*, 2024).

5.3 Adaptive Immunity Interfaces

Beyond innate sensing, m^5C impacts adaptive immunity via antigen presentation and T/B cell transcript regulation. NSUN2/ALYREF elevation increases PD-L1 stability and expression in tumor cells, attenuating T-cell effector functions; while studied in cancer, the same m^5C-dependent stabilization/export logic could influence PD-L1 and co-stimulatory molecules during chronic viral infection (Yang *et al.*, 2025). YBX1, as a reader, modulates mRNA stability and translation of immune-relevant transcripts, with 2025 syntheses outlining m^5C-dependent regulation of cytokines and immune checkpoints that may alter T-cell priming and exhaustion (Li *et al.*, 2025; Li *et al.*, 2025Frontiers Immunology mini-review).

In tissues, TET2-mediated RNA m^5C oxidation reshapes chromatin access and transcriptional programs, potentially impacting antigen presentation machinery, chemokine networks, and lymphocyte recruitment to infected sites (Zou *et al.*, 2024). These cross-layer effects position the NSUN–TET–YBX/ALYREF axis as a bridge between innate triggering and adaptive effector calibration.

5.4 Cellular Stress & RNA Quality Control

Viral infection imposes proteostatic and translational stress, prompting stress granule (SG) and P-body assembly, RNA triage, and selective translation. YBX1 participates in phase-separation phenomena and stabilizes m^5C-bearing mRNAs, potentially shielding them within SGs to ensure rapid redeployment post-stress (Wang *et al.*, 2023; Yu *et al.*, 2024). ALYREF accelerates export of m^5C-modified RNAs, biasing the nuclear-cytoplasmic flux toward transcripts prioritized for translation during antiviral states (Ding *et al.*, 2024; Xue *et al.*, 2021).

The editing function of TET enzymesobserved in splicing speckles and linked to recovery of splicing when m^5C is oxidizedsuggests a role in RNA quality control, preventing accumulation of aberrantly processed transcripts under infection stress (Hastert *et al.*, 2025). Meanwhile, NSUN2 dynamically adjusts m^5C to balance host defense and viral replication; its depletion enhances IFN via RIG-I ligands (ncRNAs), whereas its activity on viral RNAs or immune checkpoints (e.g., PD-L1) can dampen responses, highlighting a bidirectional rheostat (Zhang *et al.*, 2022; Yang *et al.*, 2025). Overall, the axis integrates stress signaling, RNA surveillance, export, and stabilization to fine-tune the translational landscape during infection.

6. Multi-Omics and Methodological Landscape for RNA m^5C

6.1 Detection Technologies

Mapping m^5C across transcriptomes relies on complementary chemistries and sequencing workflows, each with distinct resolution, sensitivity, and bias profiles. RNA bisulfite sequencing (RNA-BisSeq) remains the workhorse for single-nucleotide detection, converting unmethylated cytosines to uracil while retaining m^5C; modern pipelines correct for secondary-structure artifacts and incomplete conversion, enabling robust stoichiometry estimates across coding and noncoding RNAs (Gao & Fang, 2021; Ma *et al.*, 2022). Antibody-based enrichment (MeRIP-seq/miCLIP-like approaches) can profile m^5C at scale but often trade locus precision for breadth and may suffer antibody specificity issues; consequently, many studies integrate immunoprecipitation with bisulfite

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validation and LC-MS/MS for global quantification (Gao & Fang, 2021; Chen *et al.*, 2025).

Third-generation, direct RNA sequencing (e.g., Nanopore) offers label-free detection of modified bases by current-signal deviations; although m^5C classifiers are improving, best practices still recommend orthogonal confirmation (Gao & Fang, 2021; Ma *et al.*, 2022). Recent infection studies illustrate balanced assay selection: HBV and HCV investigations combined bisulfite mapping with functional genetics to verify site function (Feng *et al.*, 2023; Li *et al.*, 2025), whereas SARS-CoV-2 work integrated RNA-BisSeq and m^5C-MeRIP-seq to produce a comprehensive viral m^5C landscape (Wank *et al.*, 2024).

Curated resources such as m5C-Atlas aggregate base-resolution sites (166,540 loci across 13 species), conservation metrics, and condition-specific methylation levels from hundreds of RNA-BisSeq datasets, supporting reproducibility checks and meta-analysis (Ma *et al.*, 2022). Collectively, the detection landscape favors multi-assay corroborationsite discovery by bisulfite/DRS, global LC-MS/MS quantification, and biological validation through perturbation and reader binding assays. (Gao & Fang, 2021; Ma *et al.*, 2022).

Together, these approaches form the foundation of a broader experimental and translational pipeline, as illustrated in Figure 2 .

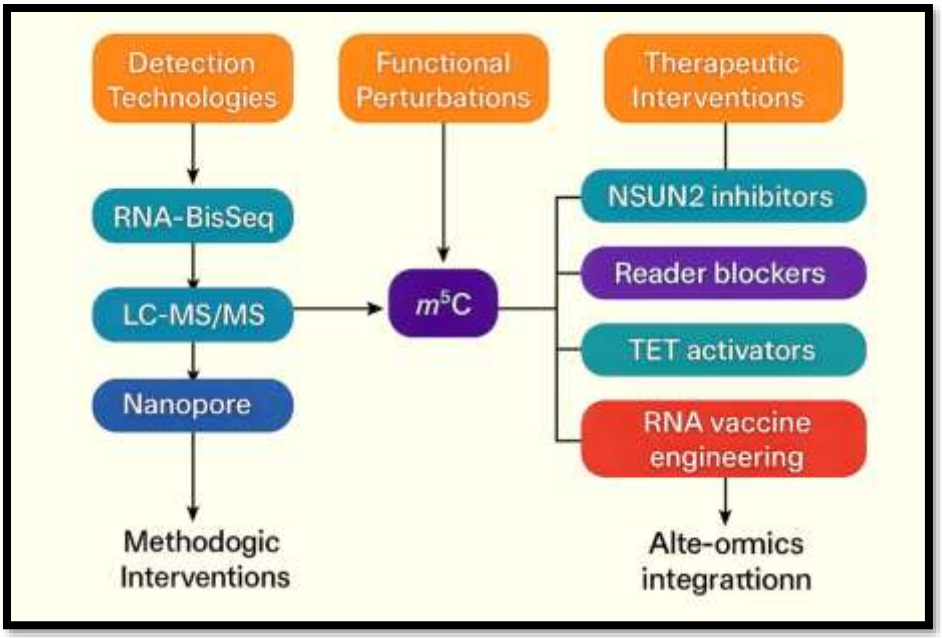

Figure 2.Integrated pipeline for m^5C research and therapeutic interventions.

6.2 Functional Perturbations

Functional assignment of m^5C sites and regulators increasingly relies on CRISPR/Cas9-mediated knockouts/knockdowns, RNAi, degron, and rescue experiments in infection models. For HBV, NSUN2 depletion reduced replication in HepG2-NTCP, primary human hepatocytes, and Nsun2$^{+/-}$ mice, while site-directed mutations at bisulfite-validated loci (e.g., nt1291) impaired ALYREF-dependent export and HBx translation (Feng *et al.*, 2023; Ding *et al.*, 2024). In HCV and flaviviruses, NSUN2 manipulation altered viral m^5C stoichiometry, RNA stability, and budding (Li *et al.*, 2025), and in EV71 mapping plus mutagenesis revealed m^5C residues controlling translation efficiency and pathogenicity (Liu *et al.*, 2024).

On the editing side, TET2 loss or gain-of-function experiments uncovered RNA m^5C oxidation effects on chromatin openness and transcription (Zou *et al.*, 2024), while TET localization to splicing speckles and in vitro splicing rescue upon $m^5C \rightarrow hm^5C$ oxidation implicated editors in RNA processing (Hastert *et al.*, 2025). Reader perturbationsALYREF for export and YBX1 for stability/translationhave

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been validated via RIP/CLIP, mutational reader domains, and co-expression/rescue (Xue *et al.*, 2021; Nulali *et al.*, 2024).

6.3 Integrative Omics & Modeling

State-of-the-art studies combine methylome maps (RNA-BisSeq / MeRIP / DRS) with RBP interactomes (CLIP/RIP-seq for ALYREF/YBX1), ribosome profiling (translation), RNA stability assays (4sU-seq), and export interactomes (TREX components) to resolve causal circuits (Xue *et al.*, 2021; Nulali *et al.*, 2024). In infection models, multi-omics designs integrated site-specific mutagenesis, writer/editor/reader perturbations, and innate signaling readouts (IFN/ISGs), enabling mechanism-anchored conclusions (Feng *et al.*, 2023; Li *et al.*, 2025; Wang *et al.*, 2024). Databases like m5C-Atlas further support meta-analysis, conservation, and structure-aware modeling, accelerating hypothesis generation and cross-study validation (Ma *et al.*, 2022).

7. Therapeutic & Translational Implications

7.1 Targeting NSUN/TET Readers & Export

The NSUN–TET–YBX/ALYREF axis offers multiple intervention points for antiviral and immunomodulatory strategies. NSUN2 inhibition has shown promise in reducing replication of HBV, HCV, and EV71, and in enhancing type I interferon responses by restoring PRR accessibility (Feng *et al.*, 2023; Li *et al.*, 2025; Liu *et al.*, 2024). Small-molecule inhibitors or CRISPR-based epitranscriptomic editing could selectively downregulate NSUN2 activity, though specificity remains a challenge given NSUN2's essential roles in tRNA and mRNA metabolism. Similarly, TET2 activation or mimetics may promote m^5C oxidation, potentially reversing pro-viral methylation and reprogramming chromatin toward antiviral states (Zou *et al.*, 2024; Hastert *et al.*, 2025).

Reader disruption represents another tractable strategy. YBX1 blockadevia peptide inhibitors or PROTACscould destabilize m^5C-marked viral RNAs and oncogenic transcripts, reducing replication and immune evasion (Wang *et al.*, 2023; Li *et al.*, 2025a). Likewise, targeting ALYREF–TREX interactions may impair nuclear export of viral mRNAs, as demonstrated in HBV models where ALYREF knockdown curtailed replication and restored IFN-β signaling (Ding *et al.*, 2024). Rational design of reader antagonists thus holds potential for broad-spectrum antivirals and cancer immunotherapy.

7.2 RNA Therapeutics & Vaccines

Synthetic RNA platformsmRNA vaccines and gene therapiescan exploit m^5C engineering to optimize stability, translation, and innate sensing. Incorporating m^5C modifications into therapeutic RNAs enhances half-life and reduces immunogenicity, improving protein yield (Chen *et al.*, 2025). Conversely, strategic omission or site-specific editing of m^5C may amplify PRR activation, boosting adjuvanticity for vaccines targeting chronic infections or tumors. Emerging CRISPR–Cas13 tethering systems enable programmable m^5C deposition or removal, offering precision control over RNA fate in vivo (Li *et al.*, 2025b). These approaches underscore the translational versatility of epitranscriptomic marks in next-generation therapeutics.

7.3 Biomarkers & Diagnostics

m^5C signaturesquantified via RNA-BisSeq, LC-MS/MS, or Nanopore direct RNA sequencingare emerging as diagnostic biomarkers for infection severity, treatment response, and cancer prognosis. Elevated NSUN2/ALYREF expression correlates with poor survival in hepatocellular carcinoma and cholangiocarcinoma (Xue *et al.*, 2021; Hao *et al.*, 2025), while YBX1-driven m^5C circuits predict drug resistance in NSCLC (Wang *et al.*, 2023). In virology, viral m^5C stoichiometry may stratify pathogenicity, as shown for SARS-CoV-2 variants (Wang *et al.*, 2024). Integration of m^5C profiles into multi-omic panels could refine prognostic models and guide personalized antiviral or immunotherapy regimens.

8. Open Questions, Limitations & Future Directions

8.1 Evidence Strength & Controversies

Despite substantial progress, several methodological and biological uncertainties remain. First, assay-specific biases complicate confident site calling and stoichiometry for m^5C: RNA-bisulfite can be confounded by secondary structure and incomplete

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conversion; antibody-based approaches face specificity and cross-reactivity concerns; and direct RNA sequencing still requires orthogonal validation for robust m^5C calls (Ma *et al.*, 2022; Gao & Fang, 2021). Second, the editorial role of TET enzymes on RNAthough biochemically plausible and functionally supported in recent workneeds broader site-resolved, orthogonal evidence across infection models to clarify where hm^5C is produced and what it does at scale (Zou *et al.*, 2024; Hastert *et al.*, 2025). Third, pathogen-specific inversionssuch as SARS-CoV-2, where reduced m^5C increases replicationraise questions about structure-encoded trade-offs, selective pressures, and the generality of pro-viral m^5C (Wang *et al.*, 2024). Finally, the extent to which ALYREF/YBX1 directly bind and regulate viral RNAs versus host RNAs that indirectly favor replication remains incompletely charted at single-nucleotide resolution (Ding *et al.*, 2024; Li *et al.*, 2025).

8.2 Key Experiments to Validate the Axis

We propose four priority lines of experimentation.
(i) **Multi-assay mapping:** Combine RNA BisSeq, Nanopore DRS, and LC-MS/MS in matched infection time-course studies, employing site-specific mutants in viral genomes to resolve causal m^5C–phenotype relationships (Ma *et al.*, 2022; Wang *et al.*, 2024).
(ii) **Genetic perturbations with rescue:** Implement CRISPR/dCas13 writer/editor/reader manipulations (NSUN2, TET2, ALYREF, YBX1) in both human and animal models, paired with reader-domain mutants and chemogenetic degrons, to elucidate the functional wiring of this regulatory axis across diverse virus families (Feng *et al.*, 2023; Hastert *et al.*, 2025).
(iii) **Innate sensing interfaces:** Apply cross-linking immunoprecipitation (CLIP) and toeprinting/SHAPE-MA assays to quantify how m^5C or hm^5C modifications alter RIG-I/TLR7-8 accessibility on viral and host RNAs. Integrate ISG transcriptomics and chromatin profiling to capture TET2-dependent remodeling events (Zhang *et al.*, 2022; Zou *et al.*, 2024).
(iv) **Translational pipelines:** Assess NSUN2/ALYREF/YBX1 inhibitors or TET2 activators in HBV, HCV, EV71, and SARS-CoV-2 infection models. Track viral replication, RNA export, interferon signaling, and safety outcomes, and develop m^5C-aware RNA vaccine designs to optimize RNA stability and immunogenicity (Ding *et al.*, 2024; Li *et al.*, 2025).

9. Conclusion

The emerging picture from recent studies (2020–2025) positions RNA m^5C modification as a pivotal epitranscriptomic mark that integrates viral replication strategies with host immune regulation. The NSUN–TET–YBX/ALYREF axis provides a mechanistic framework for this integration: NSUN writers install m^5C on viral and host RNAs, influencing stability, translation, and export; TET enzymes introduce an editing layer through m^5C oxidation to hm^5C, potentially reshaping splicing and chromatin accessibility; and readersYBX1 and ALYREFtranslate these chemical marks into functional outcomes by stabilizing transcripts and accelerating nuclear export.

Across virus families, m^5C exhibits context-dependent roles: it generally promotes replication in HBV, HCV, and EV71, while paradoxically attenuating replication in SARS-CoV-2, underscoring pathogen-specific trade-offs. On the host side, NSUN2 depletion amplifies RIG-I–dependent interferon responses, revealing m^5C as a rheostat for innate immunity. These findings highlight the axis as a universal regulator, yet one that operates with nuanced, virus-specific logic.

Therapeutically, targeting this axisvia NSUN2 inhibitors, reader antagonists, or TET activatorsoffers promising avenues for antiviral intervention and cancer immunotherapy. Likewise, m^5C engineering in RNA vaccines and therapeutics can optimize stability or immunogenicity, bridging fundamental biology with translational innovation.

Future research should prioritize multi-omics mapping, genetic perturbation, and time-resolved infection models to clarify causal relationships and validate druggable nodes. Ultimately, decoding the NSUN–TET–YBX/ALYREF axis will not only advance our understanding of RNA biology but also unlock new strategies for combating viral diseases

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and immune dysregulation (Ding *et al.*, 2024; Zou *et al.*, 2024).

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