m^6A RNA methylation, a new hallmark in virus-host interactions

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ABSTRACT

The role of m^6A methylation of RNA has remained elusive for decades, however recent technological advances are now allowing the mapping of the m^6A methylation landscape at nucleotide level. This has spurred an explosion in our understanding of the role played by RNA epigenetics in RNA biology. m^6A modifications have been tied to almost every aspects of the mRNA life cycle and it is now clear that RNA virus genomes are subject to m^6A methylation. These modifications play various roles in the viral replication cycle. This review will summarize recent breakthroughs concerning m^6A RNA modification and their implications for cellular and viral RNAs.

INTRODUCTION

The concept of a modified RNA nucleoside emerged 60 years ago with the isolation of a fifth nucleotide following analysis of soluble RNA from yeast [1]. N6-methyladenosine, or m^6A, was then discovered in the 1970s in a wide range of cellular mRNAs but also in the vaccinia, Rous sarcoma and influenza viruses' mRNAs [2-8].

To date more than 100 distinct chemical modifications to RNA have been characterized, most of which are found to be abundant in non-coding RNA such as tRNA, rRNA and snRNA and paramount in keeping their functions in translation and splicing [9]. However, recent studies established that one of the most abundant modifications, m^6A, can also be detected internally in mRNAs, influencing the metabolism and function of mRNAs [9]. Overall, this multitude of RNA modifications highlights the importance of RNA epigenetics or the epitranscriptome.

m^6A RNA modifications are dynamically regulated by methyltransferases or writers, and demethylases or erasers, and exert their function by either directly being recognized by m^6A-binding proteins or readers, or indirectly by tuning the structure of the modified RNA to regulate RNA-reader protein interactions. Multiple m^6A-specific readers have been identified that affect the metabolism and function of m^6A-RNA in various ways, as discussed below [10-16]. In mammalian cells, the N6-methylation of adenosine is catalysed by a 200 kDa complex consisting of the methyltransferases Methyltransferase-Like Protein 3 (METTL3), METTL14 and the associated protein Wilms Tumor 1 Associated Protein (WTAP) [17-22]. Although it cannot be excluded that uncharacterized writers contribute to m^6A methylation, mouse embryonic stem cell lacking both METTL3 and METTL14 display up to 99% reduction in m^6A signals [23, 24]. Most of these mammalian m^6A sites are found within the consensus RRm^6ACH (R=Guanosine or Adenosine, H=Adenosine, Cytidine or Uridine), which is consistent with the binding motifs identified for METTL14, METTL3 and WTAP [19]. However, despite this strong consensus only a fraction all possible RRACH sites are found methylated *in vivo* which further highlights the dynamic nature of m^6A modification. m^6A modification can be removed by two demethylases, Fat mass and Obesity-associated protein (FTO) and AlkB Homolog 5 (ALKBH5), both of which use different mechanisms to revert m^6A to A [25, 26]. Finally, m^6A can also be detected on viral RNA and influences virus infection and production [27-31].

UNRAVELLING THE m^6A METHYLOME

The dissection of the m^{6}A landscape has been impaired by the fact that m^{6}A bases cannot be detected directly by sequencing, as they do not change the base pairing properties and are indistinguishable from regular bases by reverse transcription. Recently, new methods based on m^{6}A immunoprecipitation or modification selective RNA chemistry to isolate modified RNA fragments, coupled with high-throughput sequencing, namely m^{6}A-seq and MeRIP-seq have identified thousands of hundred-nucleotide fragments containing the modification in the transcriptomes of mammalian cells [11, 32]. A limitation of these antibody-based methods is the large amount of RNA material required, precluding experiments at single cell level or with low abundancy samples. The quality of the antibodies used is also critical with evidence for artefactual enrichment of mRNA fragments lacking m^{6}A. In addition, m^{6}A antibodies may have RNA sequence and structure bias [22].

These approaches were therefore further refined, first by implementing the use of methyltransferase deficient cells and ligation-based strand specific library preparation [22]. While in yeast, around only 50% of the m^{6}A peaks are sensitive to depletion of the unique yeast methyltransferase [22], in human cells the genetic manipulation of methyltransferases and demethylases resulted in the expected changes in m^{6}A peaks [19, 22, 23, 25] and thousands of m^{6}A peaks overlap the binding sites for m^{6}A modifying enzymes *in vivo* [12, 21].

Finally, single nucleotide resolution m^{6}A mapping can now be achieved by crosslinking the antibody:RNA complexes through the incorporation of 4-thiouridine and determining the site of crosslink in enriched RNA fragments [33-35]. These methods have resulted in detection of over 10,000 m^{6}A sites in the mammalian transcriptome, revealing enrichment in the 3' untranslated region (UTR) and near stop codons and satisfyingly, the m^{6}A methylome of different human cell lines are highly overlapping [11, 23].

Therefore, high confidence mapping of m^{6}A sites can be achieved using these complementary approaches. However, most current approaches dissecting the m^{6}A methylome lack quantitative information. Indeed the impact of RNA modification depends both on function of individual m^{6}A sites and the fraction of transcripts that have been modified. To address these stoichiometry issues, a few methods have been developed to quantify the level of m^{6}A modification at one given loci or on a genome-wide level [36, 37].

m^{6}A RNA MODIFICATION AND RNA FUNCTION

Most post-transcriptional steps in gene expression are affected by m^6A modification with mechanistic studies linking it to mRNA stability, splicing, translational efficacy and pri-microRNA processing as summarized in Figure 1 [10, 13-15, 20, 24, 38]. m^6A modifications can exert their functions on mRNA metabolism by affecting interactions with regulatory m^6A-binding proteins, or m^6A readers, or impacting on RNA structure and acting as structural switches to alter the accessibility of m^6A readers.

m^6A RNA modifications impact on RNA structure:

The presence of m^6A reduces base pair stability and methylated transcripts are less structured both *in vitro* and *in vivo* than their demethylated counterparts [22, 39-41]. The accumulation of m^6A:U pairs can result in destabilizing AU-rich region and promoting the recruitment of Heterogeneous Nuclear Ribonucleoprotein C (hnRNPC) by exposing a Uridine-rich sequence, whose accessibility is otherwise hindered in the unmethylated transcripts [12]. Similarily, m^6A modifications also alter local RNA structure to allow the binding of hnRNPG [42]. By stimulating the binding of these two reader proteins, m^6A modifications therefore regulate alternative splicing [12, 42]. Furthermore, increased m^6A level in response to FTO depletion results in higher affinity for the splicing factor SRSF2, bolstering the links with RNA splicing [43].

m^6A RNA modifications impact on RNA stability:

Reader proteins from the YTH domain family, YTHDF1-3, can act as m^6A readers to regulate mRNA decay. Depending on the cellular context, YTHDF2 binding to m^6A transcripts results in their re-localisation from the pool of ribosome-associated translatable transcripts to cellular RNA decay sites such as P-bodies, where the decapping machinery and 5' to 3' exonucleases accumulate. YTHDF2 acts as an adaptor protein in this process as its carboxy-terminal domain binds to methylated mRNAs while its amino-terminal domain mediates the localisation to P-bodies [14, 38] (Figure 1). m^6A methylation adjacent to binding sites for Hu antigen R (HuR), an RNA-binding protein that binds AU-rich element to stabilise mRNAs, reduces HuR binding leading to increased RNA instability [20].

m^6A RNA modifications impact on mRNA translation:

m^6A moieties can promote translation when located within 5' UTRs or near stop codons and in the 3' UTR. In the 5' UTR, m^6A-induced ribosome engagement site (MIRES) can bind directly to the translation factor eIF3 to recruit 43S internally and initiate cap-independent translation [13] (Figure 1). In contrast, m^6A located near stop codons or in the 3' UTR are recognised by the reader protein YTHDF1 which then interacts with eIF3 and other ribosome-associated proteins to stimulate cap-dependent ribosome loading [15]. This has also led to the

exciting hypothesis that m^{6}A-driven translation could mediate specific stress-induced translational responses [13, 38]. Indeed upon heat shock the cytoplasmic reader YTHDF2 can relocate to nuclear speckles, where it protects the 5' UTR of stress-induced transcripts from demethylation by the eraser FTO, resulting in increased translation of these transcripts [38].

m^{6}A RNA modifications impact on cellular functions:

All these effects suggest the existence of previously unknown RNA regulons involved in combinatorial control of gene expression [14, 15, 38]. This further impacts on several cellular functions, with a strong body of evidence connecting m^{6}A modification with mammalian embryonic stem cell fate and the regulation of pluripotency factors, and also with X chromosome inactivation, the response to UV-induced DNA damage, metabolic diseases and the stability of the mammalian circadian clock [20, 24, 25, 44-47].

m^{6}A RNA MODIFICATION DURING VIRAL INFECTIONS

While viral RNAs have been known to contain m^{6}A modifications, for example influenza virus [6], Rous sarcoma virus [8] or simian virus 40 [48], the function of these modifications and whether the viruses alter the cellular m^{6}A landscape have long remained unanswered questions. With the development of genome-wide methods to map m^{6}A residues, researchers are now only beginning to unravel the links between m^{6}A modification and viruses. The following sections will review five recent studies addressing the role of functional m^{6}A modifications of transcripts and genomic RNA of human viruses in the context of replicative infection with human immunodefiency virus type 1 (HIV-1) or *Flaviviridae* [27-31] (Figure 2).

m^{6}A RNA MODIFICATION OF HIV-1 gRNA AND mRNA

By using MeRIP-seq, Lichinchi *et al*, Kennedy *et al* and Tirumuru *et al* all report the identification of m^{6}A sites on HIV-1 RNA in the cytoplasm of infected cells between 48 and 72hrs post infection, conserved within the three studies [28, 29, 31]. These sites mostly cluster within three regions of the genome, the 3' UTR defining the U3/NF-κB and TAR clusters, the nef and U3/nef overlap cluster and the env/rev overlap cluster. In addition, the occurrence of m^{6}A modifications on some specific loci in individual studies may reflect a dependency of the m^{6}A landscape to the experimental approach. For instance, Kennedy *et al* identified two m^{6}A

sites in the HIV-1 primary isolate JR-CSF but not in the laboratory isolate NL4-3 or the primary isolate BaL [28]. Interestingly one of these coincides with a novel adenosine residue present only in JR-CSF, and further studies should aim at identifying this unique residue as the exact site of m^6A modification. In addition, Tirumuru *et al* identified several m^6A peaks in the 5' UTR leader sequence, the *gag* gene, the Rev-response element (RRE) and in the *rev* gene [31].

Addressing the role of those m^6A modifications by suppressing or increasing the activity of the cellular writers and erasers and measuring the viral replication efficiency, both Tirumuru *et al* and Lichinchi *et al* confirmed the potential for regulatory control of this modification on viral fitness, ruling out the presence of a viral m^6A-modifying enzymatic machinery [29, 31]. Furthermore, Tirumuru *et al* also showed a functional interaction between the m^6A modified viral RNA and the cellular YTHDF proteins by cross-linking immunoprecipitation (CLIP)-RNAseq and silencing or overexpression assays [31].

Interestingly, Tirumuru *et al* demonstrated a remarkable post-entry modulation role for the m^6A modifications located in the U5/TAR and *gag* region of the HIV-1 NL4-3 genomic RNA (gRNA) in infected Jurkat and primary CD4+ T cells which is not addressed in the two other studies. Mechanistic analysis further linked the recognition of this site by the cellular YTHDF1-3 proteins to a repression of the reverse transcription of the viral gRNA, pointing to a cellular anti-viral mechanism which could explain differences in infectivity levels between laboratory strains and cultivable models. This observation may be of particular importance as m^6A modification of viral RNA had been previously described as a cellular innate immune response escape mechanism, suggesting a wider impact on viral fitness [23]. Furthermore, m^6A modifications have been shown to prevent RNA recognition by the viral sensors TLR3 and TLR7, and an RNA fragment derived from hepatitis C virus (HCV) 5' UTR was shown to evade RIG-I sensing *in vitro* when it contained modified nucleotides, including m^6A [49, 50]. However, further evidence for a role of the m^6A methylation pathway in controlling the innate response against viruses is lacking. Kennedy *et al* also describe how m^6A motifs within the viral mRNAs 3' UTRs interacting with YTDH1-3, and in particular YTDH2, contribute to the stabilisation of the viral mRNAs and are thus linked to strong expression of the viral proteins in the CD4+ T CEM-SS cell line [28].

Finally, Lichinchi *et al* proposed another mechanism involving m^6A modification of two adenosine residues in the stem-loop II region of HIV-1 RRE enhancing the binding of Rev protein to the HIV-1 RNA, its nuclear export and subsequently increasing viral replication. Site-directed mutagenesis of those adenosines in the HIV-1 LAI strain showed a dramatic

decrease in viral replication, confirming that the effect seen by the inhibition of the cellular methyltransferases was mainly due to an effect on the viral RNA rather than on cellular RNAs. The authors also showed the evolutionary benefit of this mechanism by highlighting the conservation of those two residues across 2,501 HIV-1 sequences isolated from infected humans [29].

m^6A RNA MODIFICATION OF *FLAVIVIRIDAE* GENOMIC RNA

Perhaps counter-intuitively given the previously described activity of the m^6A host machinery being restricted to the nucleus, similar studies investigating the role of m^6A have been conducted for the single-stranded positive RNA viruses HCV and Zika virus (ZIKV), whose life cycles remain cytoplasmic [27, 30].

Both studies characterized m^6A-modified viral RNAs, leading to the identification of 19 peaks across the HCV genomic RNA during infection, with the exception of the 3' UTR, at a global m^6A/A ratio of 0.16%, all adenosine residues included. As for ZIKV, 12 m^6A peaks had been shown, spanning the full length of the genomic RNA packaged into virions, with the exception of the 5' UTR, at a striking m6A/A ratio of 3%. Interestingly, further m^6A mapping for different members of the *Flaviviridae* family highlighted conserved m^6A modifications within the regions coding for the non-structural proteins NS3 and NS5, and within 3' UTR sequences, that could suggest conserved functions.

In addition, while the writers METTL3 and 14 activity is associated with a restriction of viral replication, the erasers FTO and ALKBH5 exert an opposite effect in infected cell culture, suggesting that a negative regulation of viral replication by m^6A modification. Further mechanistic studies identified that (i) the YTHDF proteins mediate this m^6A-associated regulation of the viral life cycle and (ii) this effect is due to modification of the viral genomic RNAs rather than to changes in the m^6A cellular methylome as discussed in more detail below.

Unlike the effect described on cellular mRNAs, Gokhale *et al* propose that m^6A modification does not seem to have any effect on the stability or on the translation efficiency of HCV RNA [27]. However, the authors identified a direct interaction between the m^6A modified HCV RNA and YTHDF proteins, and showed an inverse correlation with its packaging into newly produced virions in the cytoplasm of infected cells (Figure 2). The comparison between the methylome of the HCV RNA and YTHDF binding sites identified by PAR-CLIP analysis, revealed a unique region consisting of m^6A sites clustered within the envelope E1 coding

region, both containing m^{6}A modifications and bound by YTHDF proteins, as responsible for this molecular switch from one step of the viral life cycle to the other. This E1 coding region cluster displays a greater affinity for all YTDH when methylated, which in turn prevents the binding of HCV Core protein to the viral RNA and results in impaired virion packaging. Corroborating these results, the authors also showed a very low occurrence of m^{6}A modifications in virions from the supernatant of infected cells compared to cytoplasmic viral RNA. Furthermore, confocal microscopy analysis suggested that YTHDF1-3 relocates to the lipids droplets associated with sites of virion assembly in infected cells, compared to their more diffuse cellular distribution in uninfected cells, suggesting an active recruitment to the viral replication sites in infected cells. The conservation of several m^{6}A sites within this region between different HCV strains further supports the role that m^{6}A modification could play in controlling the fate of the viral RNA and packaging into virions.

However, the MeRIP-seq analysis of different viruses belonging to the *Flaviviridae* family, dengue virus, yellow fever virus, West Nile virus, and ZIKV strains DAK and PR2015 did not reveal any conservation of the E1 coding region region m^{6}A modification. Yet, all the flavivirus RNAs contain m^{6}A sites within the NS5 coding regions and the 3' UTRs. The functional significance of this observation remains to be dissected as the analysis was carried out in infected human hepatoma-derived Huh7 cells at different time-point (24hrs to 48hrs), without evidence of conducting the analysis at the relevant time point for each virus [27]. Furthermore, the use of poly(A)+ RNA selection for the MeRIP experiment whilst the viral RNAs studied do not contain poly(A) tails may have resulted in some bias.

In contrast with these results, Lichinchi *et al* identified 12 sites of m^{6}A modifications in the ZIKV strain MR766 RNA in HEK 293T cells and suggested that these modifications result in the destabilisation of the viral RNA and impact on viral replication. The binding of YTHDF2 to m^{6}A sites in the ZIKV 3' UTR results in the relocalisation of the m^{6}A modified viral RNA with the cellular mRNA decay machinery. Furthermore, the absence of m^{6}A modification in YTHDF2 knock-out cells results in increased viral protein expression and viral replication [27, 30].

Interestingly, as shown for HIV-1, the locations of m^{6}A modifications identified for ZIKV RNA in the two studies do not perfectly overlap, potentially reflecting the use of different viral strains and cellular models. Bolstering this observation, Gokhale *et al* showed important differences between the two ZIKV strains investigated despite the use of the same cell line [27]. The existence of such variability in the topology of the m^{6}A modifications of viral RNA strongly could reflect its importance as a dynamic, given its potential to be reverted, versatile

tool of adaption to the host. However, this strain-related variability of m^6A topology also demonstrates how much those studies have to be primly initially comparative rather than mechanistic, and perhaps most of the divergent m^6A modifications are unlikely to be of biological relevance in these simple *ex vivo in vitro* models. Ideally, this analysis of the viral RNA m^6A landscape may rather be carried out on material isolated from clinical samples rather than viral RNA synthesised by replication in permissive cell lines. The quintessential principle of epigenetics markings, as a tool of finely tuned adaptation to a defined environment -or host when in view of viruses-, thus resides both in its vertical conservation and its versatility.

CHANGES IN CELLULAR m^6A LANDSCAPE UPON INFECTION

The main outcomes of the studies discussed above is the dissection of how m^6A modification of viral RNAs regulate HIV-1 and *Flaviviridae* replication in cell cultures, however, Lichinchi *et al* a and b, and Tirumuru *et al* also provide a comprehensive analysis of the changes in the m^6A cellular epitranscriptome. In particular, the identification of modified transcripts involved in immune and viral infection evasion pathways may have important consequences *in vivo* [27-31].

While HIV-1 infection in Jurkat cells and primary CD4+ T cells does not result in significant differences in the abundance of total m^6A peaks mapped to the human transcriptome compared to uninfected cells, the analysis of the preferred motifs showed a slight increase of the frequency of the consensus RRACH motif and a corresponding decrease of the GGACU motif frequency [31]. Further gene ontology analysis of the m^6A-modified cellular genes associate this redistribution of m^6A sites with functional clustering in pathways involved in viral infection and in wider processes such as metabolism, the immune system, multicellular organismal processes and development. In contrast, using a different analytical approach, Lichinchi *et al* identified 56 cellular gene transcripts specifically methylated upon HIV-1 infection of another T cell line, MT4 cells [29]. Interestingly, 19 of these have previously been associated with HIV replication and promote viral replication or directly interact with viral proteins [29].

In addition, changes in the distribution of m^6A peaks across the 5' UTR, CDS, 3' UTR and intronic sequences for these HIV-1 specific cellular methylated transcripts, when compared with total cellular mRNA, are reported as 17.5%, 66%, 14.4% and 2.1% compared to 13.4%, 41%, 31.6% and 14%, respectively. This suggests that these m^6A modifications may exert

functions in RNA splicing and translation rather than in RNA stabilisation. Furthermore, HIV-1 infection results in the enrichment of MGACK m^6A motifs in cellular motifs (where M=Adenosine or Cytidine and K=Guanosine or Uridine), mimicking the changes observed for the viral RNA.

ZIKV infection also results in changes in the distribution of m^6A modification with an increase within the 5' UTR of the host transcripts and a corresponding decrease within the 3' UTR regions suggesting an effect on cellular mRNA splicing, stabilisation and translation [30]. Analysis of the consensus sequence further showed a switch in motif usage from RAACH over RGACH upon ZIKV infection. Gene ontology analysis revealed that both the gained and lost m^6A modifications are linked to immune–related pathways, which may lead to the suppression of immune surveillance systems or disturb other cellular processes to ensure viral replication [30].

CONCLUSIONS AND PRACTICAL CONSIDERATIONS

Taken together, these studies demonstrate that m^6A RNA modification of viral RNA is likely to illustrate a broad range of adaptive host-pathogen interactions throughout all steps of the viral life cycle. The first 3 studies describe 3 different mechanisms involved in HIV-1 viral fitness that are described as modulated by m^6A modification of the viral mRNA and genomic RNA, respectively [29], [28] and [31]. This illustrates how promising the study of this mechanism could be in terms of both broadening and elaborating our understanding of host-pathogen interactions during viral infection. But beyond the renewed excitement for RNA epigenetics in microbiology, the converse effects of the m^6A modifications of the HIV-1 RNAs at different steps of the viral life cycle point out the need for more systemic and dynamic approaches in physiologically relevant cellular context if we were to gain a comprehensive and translatable knowledge of the functionality of this epitranscriptomics event. Two additional studies focussing on *Flaviviridae* are remarkably providing examples of absolute cytoplasmic m^6A modifications of the viral genomic RNAs [27] and [30]. While reporting novel and unique mechanisms of regulation of the viral replication of HCV and ZIKV, those publications also highlight the obvious need for a standardised dynamic approach to the investigation of viral RNA m^6A modification during infection. This should also prompt further studies to dissect the functional relationships between different viral systems and the m^6A epitranscriptome. This is relevant as well for viruses such as influenza

virus or SV40 that were found to contain m^6A modification in the first studies discovering m^6A residues.

The recent advances in RNA sequencing and commercialisation of the m^6A antibody have made the study of the modifications of the m^6A landscape of viral and cellular genomes at the nucleotide level a readily available and straightforward process. The non-exhaustive list suggested in table 1 recapitulates the practical approaches that could be used for the standard investigation of this event, also relevant in many types of biological processes. For technical reasons and perhaps financial impediments, complete investigations cannot be carried out in a systematic manner in different cellular models or using a variety of viral strains. However, the functional mechanisms discovered should be validated and addressed from a dynamic point of view, bearing in mind that comparison with non-replicative models may also broaden our general understanding. Finally, key technological improvements in the near future should enable a move from mapping the epitranscriptome to understanding the biological function of individual modifications. The implementation of CRISPR-based genome editing will enable to specifically alter single m^6A sites to interrogate the biological function of specific modifications, helping to unravel new functions for m^6A modification in viral replication.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICAL STATEMENT

Not applicable.

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FIGURE LEGENDS

Figure 1. Regulation of m^6A RNA modifications. Methylation (CH3) of adenosine (A) on position N6 to form N6-methylated A (m^6A) is catalysed by writers such as the methyltransferase complex composed of METTL3, METTL14 and WTAP. This process is reversible and methylation is removed by demethylases or erasers such as FTO and ALKBH5. m^6A RNA modifications are recognized and bound by readers such as among others proteins of the YTHDF family, but also SRSF2, hnRNPC, and hnRNPG (i.e other readers). Depending on the cellular context and their position in mRNAs, m^6A RNA modifications can (i) result in the recruitment of splicing factors favouring alternative splicing, (ii) promote mRNA destabilisation and degradation in P-bodies by the decay machinery, and (iii) promote translation.

Figure 2. m^6A RNA modifications affect viral cycles by different mechanisms. HIV-1 genomic RNA is methylated (m^6A) in a post-entry step. Recognition and binding of these

537 m^6A sites by the readers YTHDF1-3 represses reverse-transcription of the genomic RNA into
538 the proviral dsDNA. In the nucleus, m^6A modification in the Rev-responsive element (RRE)
539 region of HIV-1 genomic RNA favours Rev binding, its nuclear export, gag protein synthesis
540 and viral replication (lower panel). Although *Flaviviridae* life cycle remains cytosolic, HCV
541 RNA genome can be methylated at several positions. Interestingly, methylation sites
542 clustering in the E1 ORF region are recognized by YTHDF proteins which localize to lipid
543 droplets upon infection, thereby prevent binding of the genome with Core proteins and its
544 packaging into viral particles (upper panel).
545
546 TABLES
547

Table 1: Suggested workflow for studying the role of m^6A epitranscriptomics during viral infection.

Rapid assessment of m^6A involvement during viral infection.	Poly(A)+ and total RNA Dot-Blots of cellular and viral RNA using m^6A antibodies.
Dynamic synopsis of the m^6A epitranscriptome.	m^6A RNA immunoprecipitation (RIP) and qPCR on RNA contained in virions, post entry, at replication, translation and egress (or latent against lytic) steps.
Genome-wide mapping of m^6A modified transcripts.	(i) MeRIP-Seq experiments to map m^6A sites on cellular and viral RNAs. (ii) Metagene analysis of normalized m^6A peaks to determine the gene ontology distribution, topology and motif preferences of the m^6A modification and its evolution upon viral infection. (iii) In silico sequence analysis of different viral strains to assess the level of conservation of identified motifs, indicating a degree of causality with roles in viral fitness.
Assigning biological functions to specific m^6A sites.	(i) RIP-Seq analysis for viral or cellular readers to generate correlation maps between unique m^6A modifications and sites of interactions with m^6A readers. (ii) Site-directed mutagenesis of viral m6A sites identified by MeRIP-Seq and refined by readers RIP-Seq. Care should be taken to ensure particular mutations do not affect the function of the encoded viral proteins or RNA structures (this may require a thorough *in vitro* and *in vivo* analysis or the use of reporter models).
Assessing regulatory roles for m^6A modifications.	(i) Viral replication assays in cells silenced for components of the m^6A editing machinery or overexpressing these components. (ii) Subcellular localization analysis of viral proteins, replication complexes and identified m^6A readers to illustrate the spatio-

	temporal dynamic of these interactions, contributing to an integrated understanding of this new layer of virus-host interactions.

548

549

Nucleus

Cytoplasm

Splicing
mRNA processing

Writers

MTTL3/MTTL14
WTAP

Erasers

FTO
ALKBH5

P-body

mRNA decay

Translation

A

m^{6}A

mRNA

eIF3

YTHDF1-3

Ribosome

Other readers

HCV RNA

Replication Site

Lipid Droplet

Particle Release

HCV

Integration

cDNA

Reverse Transcription

HIV RNA

Transcription

Rev

HIV RNA

RRE

Rev

Rev

Rev

Gag synthesis

Nucleus

Cytoplasm

HIV-1

HCV particle

HIV-1 particle

HCV Core

HIV-1 Gag

m^{6}A

YTHDF1-3

RRE Rev-responsive Element

Ribosome