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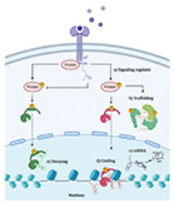

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REVIEW

An epigenetic 'extreme makeover': the methylation of flaviviral RNA (and beyond)

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ABSTRACT

Beyond their high clinical relevance worldwide, flaviviruses (comprising dengue and Zika viruses) are of particular interest to understand the spatiotemporal control of RNA metabolism. Indeed, their positive single-stranded viral RNA genome (vRNA) undergoes in the cytoplasm replication, translation and encapsidation, three steps of the flavivirus life cycle that are coordinated through a fine-tuned equilibrium. Over the last years, RNA methylation has emerged as a powerful mechanism to regulate messenger RNA metabolism at the posttranscriptional level. Not surprisingly, flaviviruses exploit RNA epigenetic strategies to control crucial steps of their replication cycle as well as to evade sensing by the innate immune system. This review summarizes the current knowledge about vRNA methylation events and their impacts on flavivirus replication and pathogenesis. We also address the important challenges that the field of epitranscriptomics faces in reliably and accurately identifying RNA methylation sites, which should be considered in future studies on viral RNA modifications.

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Introduction

Flaviviruses comprise more than 70 known viruses including several that are clinically relevant worldwide such as dengue virus (DENV), Zika virus (ZIKV), West Nile virus (WNV) and yellow fever virus (YFV). Belonging to the *Flavivirus* genus within the *Flaviviridae* virus family, these pathogens are enveloped positive strand RNA viruses. Following receptor-mediated endocytosis and envelop fusion, the non-segmented viral RNA genome (vRNA) is released into the cytosol. It is then translated in a cap-dependent manner into a unique large transmembrane polyprotein at the endoplasmic reticulum (ER), which is further cleaved by cellular and viral proteases. This generates ten mature viral proteins: 1- seven nonstructural (NS) proteins, namely NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 which are responsible for vRNA synthesis and amplification, and 2- Capsid (C), Envelop (E) and pre-membrane (prM) structural proteins which drive with vRNA the assembly of new virions at the ER. Viral particles egress through the secretory pathway and become infectious following cleavage of prM by cellular furin in the Golgi apparatus. Infectious particles are then released in the extracellular space and may infect other target cells [1].

Beyond the clinical relevance of flaviviruses due to the unavailability of vaccines or therapeutics for most of them, these pathogens are fascinating from a fundamental RNA biology perspective. With only one open reading frame in vRNA, the flavivirus replication cycle is relatively simple and occurs exclusively in the cytoplasm without any DNA intermediate. Yet, flavivirus replication is generally very efficient in cell culture, highlighting that all the genetic information required to fulfill a complete life cycle is condensed within one single cistron. More specifically, vRNA undergoes multiple fates in the cytoplasm, which cannot occur simultaneously and involve separate machineries. Indeed, vRNA can be: 1- translated by the ribosomes, 2- replicated by the viral RNA-dependent RNA polymerase (RdRp) NS5, and 3- selectively packaged into newly assembled virus particles (Fig. 1). These processes must be regulated in time and space to achieve an efficient viral life cycle. The equilibrium between those key steps is likely orchestrated by the viral replication factories, which derive from the remodelling of the ER and allow spatial segregation of the vRNA in the cytoplasm. In addition, flaviviruses have developed strategies to dampen sensing of vRNA by the pattern recognition receptors (PRR) of the innate immune system [2]. Finally, through XRN1 exonuclease-dependent 5′-3′ degradation of vRNA and partial resistance of its 3′ untranslated region (UTR), flaviviruses generate a non-coding RNA named subgenomic flaviviral RNA (sfRNA), which is involved in viral pathogenicity, antagonism of host innate immune and stress responses [3–7]. Hence, vRNA is an interesting tool to gain a better understanding of the fundamental aspects of the spatiotemporal regulation of RNA metabolism.

Such dynamic control involves RNA modifications. First, the 5′ end of the genome is methylated by NS5 during the capping process, which is required for translation initiation.

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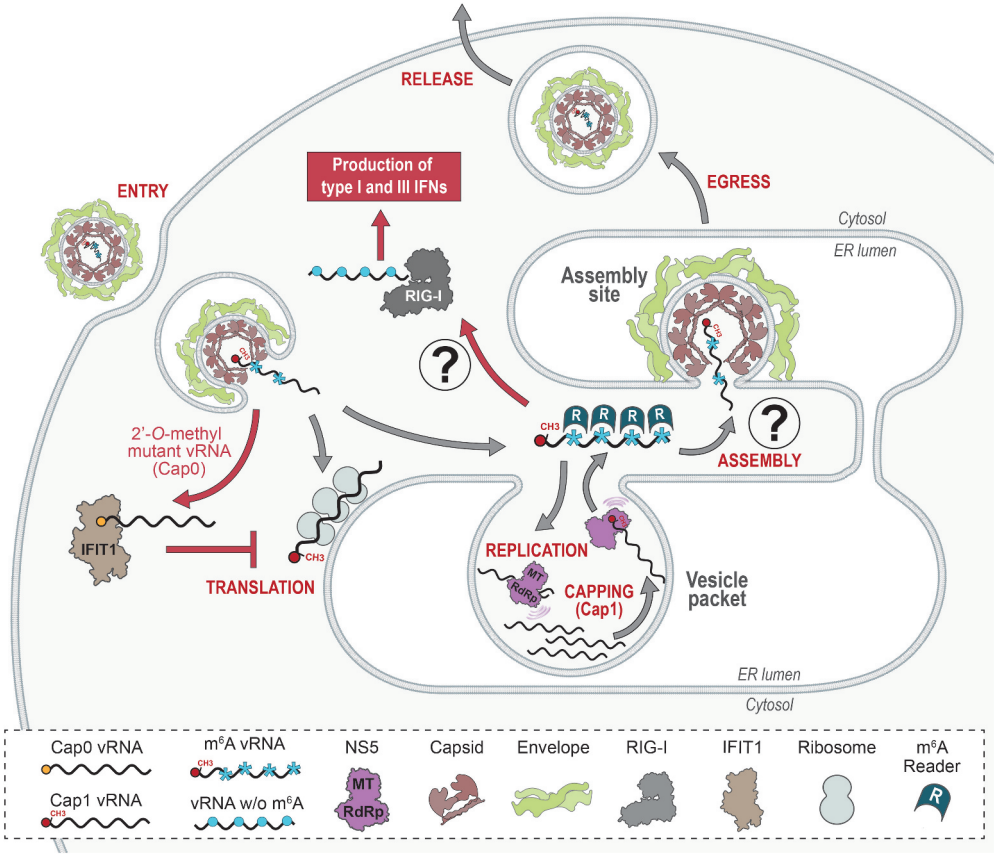

Figure 1. The fates of methylated and non-methylated vRNA species during flavivirus life cycle.

Second, despite well characterized for several decades in the case of transfer RNAs (tRNA) and ribosomal RNAs (rRNA), RNA internal modification has emerged in the recent years as an important process for the posttranscriptional regulation of messenger RNA (mRNA). Notably, mRNA methylation can regulate RNA translation, stability and interactions with proteins sometimes by conferring specific structural conformations [8–10]. Not so surprisingly, vRNA internal methylation has been described for viruses from different families and reported to regulate specific steps of their life cycle as well as to mimic cellular RNA to avoid sensing as non-self RNA by the innate immune system [11–21]. Flaviviruses make no exception and this review explores the current understanding of how flaviviral RNA methylation affects vRNA cytoplasmic fate, life cycle and pathogenesis. We also elaborate on the technical challenges related to confidently identifying and precisely mapping methylation sites in RNAs to consider in future studies.

Methylations of the flavivirus capped vRNA

Capping is likely the first RNA modification event for flaviviral vRNA since it occurs during genome replication. First evidence was obtained in the late 1970s by Wengler *et al.* who reported the presence of a 2′-*O* methylated cap1 structure on WNV vRNA molecules purified from hamster and mosquito cells [22]. Similar cap structures were thereafter identified on DENV2 vRNA [23]. One notable feature, however, distinguished flaviviral RNAs from cellular mRNAs, namely the absence of cap2 structures [24].

NS5 MTase, the swiss army knife of flavivirus genome capping

Due to their entirely cytoplasmic life cycle, flaviviruses did not evolve to co-opt the canonical cellular capping machinery of the nucleus but rather incorporated the necessary enzymatic activities into their own replication enzymes. While in eukaryotes cap methylation reactions involve at least five sequential steps and are usually carried out by multiple cellular enzymes [25], vRNA capping is achieved exclusively in the cytoplasm by only two viral proteins, namely NS5 and NS3, reflecting their optimized enzymatic capability. Thus, in addition to its protease activity, NS3 encodes both RNA helicase and RNA 5′ triphosphatase (RTPase) activities, first identified in WNV and DENV [26,27]. The former is responsible for unwinding the viral double-stranded RNA involving the neo-synthesized vRNA and the negative strand RNA template, while the latter removes vRNA 5′ phosphates. Both activities are required for subsequent cap addition by the RdRp NS5, which remarkably combines three capping enzymatic activities: an atypical RNA guanylyltransferase (GTase) [28–31] and a dual *N7*- and 2′-*O* methyltransferase (MTase) [32–34] responsible for the formation of the cap1 structure (Fig. 1, Fig. 2A).

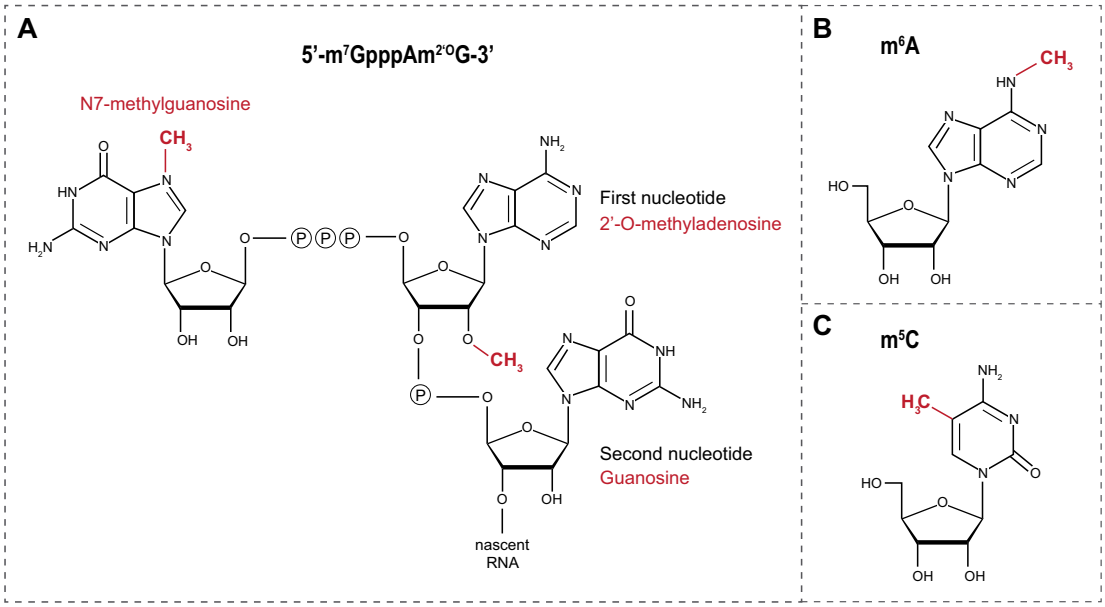

Figure 2. Schematic representation of (A) the flavivirus cap1 structure, (B) N^6-methyladenosine (m^6A) and (C) 5-methylcytosine (m^5C).

Flaviviral NS5 MTases specifically methylate RNAs of viral origin. This selectivity occurs through the recognition of both distinct nucleotides and structural elements in the 5′ UTR [32,35]. Thus, the first two transcribed nucleotides after the cap structure are conserved among flaviviruses and consist in an adenine in position +1 and a guanine in position +2 [36,37]. Critical for the *N7*-methylation and formation of the cap0 structure (m^7GpppApGpUp) are the guanine at position +2, the uracil at position +3 as well as the presence of a stem loop structure whose sequence is not relevant for this activity. Subsequently, NS5 modifies vRNA cap0 structure by methylation at the 2′-*O* position of the ribose of the first adenosine of vRNA to produce the cap1 structure (m^7GpppAm$^{2'O}$pGp) (Fig. 2A). This 2′-*O*-methylation activity requires, in addition to the first adenine and guanine nucleotides, the vRNA to be at least 20 nucleotides long [35]. On the structural level, NS5 substrate preference for flaviviral RNAs is determined by a pocket (constituted by residues I147, G148, E149, and S150), which optimally accommodates the adenine at position +1, and by the presence of a glutamate residue E111, which creates an essential polar interaction with the guanine at position +2 [38]. This nucleotide specificity also influences the subsequent methylations steps [30,32,35,39,40].

Similarly to other viral and cellular RNA MTases [41,42], flaviviral MTases exhibit a conserved, highly positively charged, groove constituted by a K-D-K-E tetrad motif (K61-D146-K182 -E218 for WNV and K61-D146-K181-E217 for DENV2) that sequentially orchestrates the cap methylation events [30,32]. Within the tetrad, and likely because of its proximity to the S-adenosylmethionine (SAM) donor site, the aspartate residue D146 is essential to *N7*-methylation and therefore to virus growth. All other residues of the tetrad are involved in the 2′-*O*-methylation process and substitutions attenuate or delay viral replication to varying degrees, as measured by the formation of smaller plaques and lower viral titres [30,43].

2′-O-methylation of the flaviviral cap, a mimicry to escape innate immune recognition by IFIT1

Cap1 structures, commonly found in mRNAs, play a crucial role in discerning self from non-self RNAs and preventing the undesired activation the innate immune system. RNAs terminated by a 5′ triphosphate usually carry the molecular signature of viral RNAs whose translation is driven by an internal ribosome entry site. These are therefore specifically and efficiently recognized by cytosolic sentinels such as retinoic acid inducible gene I (RIG-I) that initiates the antiviral signalling cascade leading to interferon (IFN) production [44–46]. RIG-I preference for uncapped RNAs is provided by the presence of a histidine residue at position 830 in the triphosphate-RNA-binding pocket, which contributes to the weak binding affinity of methylated RNAs, notably cap1 mRNAs, thereby ensuring tolerance to self-RNAs [47,48].

Flaviviruses have developed a mechanism of 2′-*O*-methylation of the cap as a strategy to camouflage their genome among cellular mRNAs and escape host antiviral responses. The work of several independent groups confirmed the key function of the 2′-*O*-methylation on flaviviral cap structures in this regard by characterizing 2′-*O*-MTase mutant viruses *e.g.* WNV E218A, WNV K182A, DENV E217A and JEV K61A viruses. In immune-deficient cells such as Vero and BHK-21 cells, the replication competence of the 2′-*O* mutant viruses is almost similar to that of wild type virus, yet delayed of several hours in some cases [30,49–53]. In contrast, their replication is attenuated in immune-competent cell lines such as A549 cells, primary cells (e.g. mouse embryonic fibroblasts (MEFs), neurons, dendritic cells and macrophages) [50,52–56] and insect cells [30,43,49]. Interestingly, 2′-*O*-MTase mutant viruses retain their ability to induce an IFN response at levels similar to those of the wild type viruses, however, earlier after infection [52–54]. *In vivo*, the attenuation of replication [30,50,51,54] correlates with the

absence of the virulence and pathogenicity normally observed for wild type viruses, both being restored in the context of IFN-deficient mice lacking the type I IFN receptor (*Ifnar1*-/-) [49–51,54,55]. Interestingly, 2′-*O*-MTase mutant viruses showed an increased sensitivity to IFN pretreatment in mice suggesting that non-methylated vRNAs are targeted by IFN-induced effectors [49,50,54].

The IFN-induced protein with tetratricopeptide repeats 1 (IFIT1) [57], also known as p56 and ISG56, is one of the effectors most highly induced in response to IFN and virus infections [57–60]. In their study, Daffis *et al.* elucidated the role of IFIT proteins in restricting 2′-*O*-MTase mutant viruses *in vivo* and showed that ectopic expression of IFIT1 and IFIT2 (ISG54) in MEFs reduced the replication of wild type WNV and abrogated that of WNV E218A mutant virus [54]. While it is clear that IFIT1 senses vRNAs lacking 2′-*O*-methyls, its restriction mechanism is yet unclear and may involve interactions at two different steps of the translation initiation process. First, IFIT1 and IFIT2 directly interact with the eukaryotic translation initiation factors eIF3E and eIF3C, thereby affecting the stability of the ternary pre-initiation complex and inhibiting cap-dependent translation [61–64]. Second, IFIT1 binds with high affinity to cap0 and 5′ triphosphate-terminated RNAs *in vitro* while this interaction is abrogated by the presence of the cap1 structure. Thus, IFIT1 substrate recognition sorts out uncapped and cap0 RNAs thereby sustaining cap1 RNA translation. In addition, IFIT1 inhibits translation initiation of cap0 RNAs by efficiently outcompeting eIF4E and the cap binding complex eIF4F [65–68] (Fig. 1). Using mobility-shift assays, Kimura *et al.* confirmed that IFIT1 binds preferentially to uncapped full-length JEV vRNA and selectively inhibits the translation of 2′-*O* non-methylated (cap0) JEV vRNA [56].

Internal 2′-O-methylation: an additional role for NS5?

Whilst its ability to methylate the cap is more effective, flaviviral NS5 is able to specifically methylate vRNA internal adenosines, in a sequence-unspecific manner [69]. Indeed, *in vitro* WNV and DENV4 MTases indiscriminately methylate RNAs exclusively composed of adenosines as well as host ribosomal RNAs. With regard to the life cycle, *in vitro* 2′-*O*-methylation of internal adenosines does not affect vRNA stability but rather attenuates its translation efficiency and reduces the capacity of the RdRp to elongate vRNA during replication. Liquid chromatography-mass spectrometry (LC-MS) analyses confirmed the existence of internal 2′-*O*-methyladenosine on genomes purified from DENV1 virions, despite a very low frequency. Most importantly, these internal modifications were not detected in genomes purified from 2′-*O*-MTase mutant viruses confirming the involvement of NS5 methylation activity [69]. Recently, a large-scale epigenetic analysis by McIntyre *et al.* reported consistent results for DENV2 and ZIVK genomes [18].

Therapeutic and prophylactic strategies around vRNA cap methylation

NS5 GTase and MTase activities have been the scope of several antiviral therapeutics approaches during the last decades, as well as more recently because of the ZIKV outbreak in the Americas. Compounds developed to block these enzymatic activities include (i) GTP analogues such as BG-323, a thioxothiazolidin-derived inhibitor [70,71], as well as ribavirin 5′ triphosphate whose impact on replication [33,72] might rather result from its ability to induce RdRp errors [73–76], (ii) SAM analogues such as sinefungin [31,77–79] and the compounds NSC 12,155 and NSC 12,590 [80], F3043-0013 and F0922-0796 [81], and (iii) non-nucleoside inhibitors [82,83]. However, although the ‘straightforward’ aspect of this therapeutic approach is very appealing, the development of effective and selective methylation inhibitors seems to be very challenging since most of these compounds exhibit half maximal inhibitory concentration (IC_{50}) values in the micromolar range and a relatively low impact on viral replication, notably when replication is already established.

2′-*O*-MTase mutant viruses have emerged as promising candidates for the development of live-attenuated vaccines with several advantages over conventional live-attenuated viruses, which exhibit reduced replication without fully precluding pathogenic effects (*e.g.* YFV-17D vaccine [84,85]). This idea emerged from the work by Zhou and colleagues who demonstrated that the WNV E218A 2′-*O*-MTase mutant virus is attenuated in mice and protects animals from a subsequent challenge with pathogenic wild type WNV [30]. This concept was subsequently applied to the design of JEV, DENV1 and DENV2 live-attenuated vaccines [49,50] and extended more recently to the design of a tetravalent non-chimeric vaccine against all four DENV serotypes [51]. For all approaches, a single dose of 2′-*O*-MTase mutant viruses induced a protective IFN-based immune response and elicited a $CD8^+$ T cell-specific response against peptides of the non-structural protein that protected both mice and non-human primates from a challenge with the wild type virus [49–51]. With regard to pathogenic effects, JEV 2′-*O*-MTase mutant virus exhibited a reduced neuroinvasiveness and did not induce any neurological symptoms [49]. Whether these live-attenuated vaccines provide protection in humans without a risk of antibody-dependent enhancement is yet to be addressed.

N^6-adenosine methylation of flavivirus vRNA

A methyl group can be added to the nitrogen linked to the position 6 of adenosines (N^6) within mRNA (Fig. 2B, m^6A), hence conferring several new properties to the modified RNA. This reversible N^6-adenosine methylation is the most prevalent internal RNA modification in the cell [86,87]. It primarily occurs in the nucleus within DRA^mCH consensus motifs (where D = G, A or U; R = G or A; A^m is the methylated adenosine; and H = U, A or C) and is mediated by the MTase-like (METTL) 3 and METTL14, generically called ‘writers’, with the contribution of the cofactors Wilms tumour 1-associated protein (WTAP) and KIAA1429 [88–93]. To a lesser extent, MGA^mCK (where M = A or C and K = G or U) and UGA^mC sequences are also methylated. The presence m^6A in mRNAs confers increased affinity to several cellular RNA-binding proteins referred to as ‘readers’ that regulate the fate of the methylated mRNA [10]. For instance, the most characterized

'readers', YTH Domain Family 1 (YTHDF1) and YTHDF2, regulate translation and stability of m^{6}A-containing mRNAs, respectively [94,95]. m^{6}A modification is reversible and can be removed by 'erasers', namely demethylases of the AlkB homologues family FTO (ALKBH9) and ALKBH5 [96,97]. Such dynamics suggests the existence of a fine-tuned equilibrium between methylation and demethylation, which may allow a quick and local posttranscriptional response of the cell to environmental and/or metabolic changes. Changes in the m^{6}A-containg mRNA profiles are associated with diseases such as obesity, cancer, neurodevelopment defects and plasticity in brain development [98–104].

Impact of m^{6}A modification on flavivirus life cycle

Taking advantage of m^{6}A-specific antibody-based purification and next-generation RNA sequencing methods (MeRIP-Seq) [86,105], two groups have independently reported the presence of m^{6}A in ZIKV vRNA [14,21]. This observation was expanded to other flaviviruses (DENV, YFV and WNV) as well as to hepatitis C virus (HCV), another *Flaviviridae* belonging to the *Hepacivirus* genus [14], suggesting a conserved mechanism across this virus family. The existence of m^{6}A in DENV and ZIKV vRNAs was recently confirmed by oligonucleotide-based purification of vRNA combined with LC-MS-based m^{6}A detection [18].

METTL3/14 knockdown reduced the level of ZIKV vRNA immunopurified with anti-m^{6}A antibodies while the opposite phenotype was observed when the expression of the demethylases was decreased [21]. This demonstrated that m^{6}A modification of ZIKV vRNA is mediated at least partly by cellular proteins in contrast to NS5-mediated 2′-*O*-methylation. Importantly, the 'readers' YTHDF1, YTHDF2 and YTHDF3 are all associated with ZIKV vRNA in a m^{6}A-dependent manner (Fig. 1). Nevertheless, none of these studies mapped the precise methylation sites. Indeed, MeRIP-Seq approaches identify rather large regions of vRNA that are modified (> 100 nucleotides) and potential vRNA methylation sites are inferred by the presence of DRACH consensus motifs. Noteworthy, the regions identified in different flaviviruses or in different strains of the same flavivirus do not always fully overlap. For example, regions differ between ZIKV Dakar 41525-DAK strain (African lineage) and Puerto Rican PRVABC59 strain (Asian lineage) [14], the latter being associated with severe symptoms (*e.g.* neonate microcephaly) observed during the 2015 pandemic. It is thus tempting to hypothesize a link between vRNA N^6A methylomic profile and pathogenic features. In contrast, the methylated regions of DENV and YFV vRNAs overlap relatively well suggesting some conservation of methylated regions. Further studies using techniques that allow accurately mapping m^{6}A modifications in vRNA at the single nucleotide resolution will determine in which extent these are conserved across the *Flavivirus* genus.

In terms of impact on the viral cycle, N^6A methylation of ZIKV vRNA limits the production of infectious particles. Indeed, the knockdown of METLL3/14 'writers' as well as YTHDF1-3 'readers' increased extracellular infectious titres [21]. In contrast, decreasing demethylases expression or overexpressing YTHDF proteins altered virus production. Whether the presence of m^{6}A in ZIKV vRNA restricts replication or particle assembly and release is yet unknown. Interestingly, N^6A methylation similarly affects HCV production [14]. In contrast to known functions for mRNAs [94,95], m-[95], m6A modification does not regulate HCV vRNA translation or stability, nor its synthesis but rather acts on virus particle assembly together with YTHDF proteins. Consistently, in HCV-infected cells, YTHDF proteins relocalized around the lipid droplets, where virus assembly takes place [14]. The combined introduction of synonymous mutations into four high confidence DRACH motifs identified in HCV envelope E1 coding sequence increased virus production without altering RNA replication and correlated with a significant enrichment of vRNA in complexes containing HCV structural protein core [14]. Altogether, this suggests that the methylation of this region acts as a negative regulator that is important for genome packaging into assembling virions (Fig. 1). Further studies will have to demonstrate how conserved is this function across the *Flaviviridae* family and whether it applies to flaviviruses as well. It is though tantalizing to speculate that alterations of vRNA m^{6}A modification profile could influence the equilibrium between the synthesis of vRNA and its packaging into assembling virus particles. Flaviviral vRNA is likely replicated insides vesicular substructure of replication factories, which originate from ER invaginations [106–110]. These so-called vesicle packets (VPs) exhibit an opening that would allow neosynthesized vRNAs to exit and to be targeted either to ribosomes for translation or to assembly complexes for encapsidation. 3D reconstruction of DENV and ZIKV replication factories has revealed that virus particle budding events are juxtaposed to VP pores [106,108]. This suggested that vRNAs exiting VPs might be directly targeted to budding viruses. It will be interesting to assess whether m^{6}A-containing vRNAs are enriched in VPs and whether their demethylation or YTHDF loss-of-binding contribute to the selective packaging of vRNA into assembling particles (Fig. 1). m^{6}A moieties might 'flag' vRNAs and contribute to the spatial segregation of the different steps of the life cycle in the cytoplasm.

Several studies have revealed other m^{6}A readers as regulators of flavivirus replication. These include candidates identified in a large-scale analysis of ZIKV and DENV vRNA riboproteomic e.g. IGF2BP1-3, FMR1, hnRNPC, hnRNPG and hnRNPA2/B1 that associate with the vRNA and in some cases modulate viral replication [111–114]. It will be relevant to evaluate whether the regulation of flavivirus replication by these m^{6}A readers occurs in a methylation-dependent manner.

m^{6}A, a possible structural switch regulator?

While these discoveries have broadened our perspective on the regulation of flavivirus life cycle by vRNA m^{6}A modifications, much remains to be explored and understood about the molecular mechanisms involved. Internal N^6A methylation alters RNA duplexes with impacts on molecular switches [115–117]. The NH2 group linked to position 6 of adenosine is engaged in pairwise interactions through a hydrogen bond with the neighbouring uridine. Thus, the addition of a methyl to this nitrogen may influence adenosine-uridine base pairing, disturb the hybridization of two strands and hence, local secondary or

tertiary structures of the RNA. Alternatively, the secondary structure of the RNA might govern the accessibility of specific regions to 'writer', 'eraser' and/or 'reader' proteins, thus indirectly influencing their posttranscriptional actions. Such alterations are highly relevant for flaviviruses whose vRNA forms long distance intramolecular interactions for its circularization, which is essential for RNA synthesis, and undergoes important structural switches that regulate the equilibrium between the different steps of the life cycle [2,118–120]. While much focus has been paid to vRNA UTRs, there is since recently strong evidence that secondary and tertiary structures (including novel long-range interactions) in the coding region also play important roles in the life cycle of DENV and ZIKV both in cells or in the virions [121–123].

With this in mind, we have compared for the purpose of this review these published structural models with the corresponding epitranscriptomic data described above in order to highlight possible structure/m^{6}A relationships (Fig. 3). The three examples highlighted below reveal interesting features that may merit further investigation. In their MeRIP-Seq analysis mentioned above, Lichinchi *et al.* identified two contiguous m^{6}A-containing regions in the NS5 coding sequence of ZIKV African strain MR766 vRNA purified from virions (Fig. 3A, nt 8651–8800 in yellow and nt 8904–9073 in light pink) [21]. According to the structural map of the full-length ZIKV MR766 vRNA *in cellulo* reported by Li *et al.*, these regions are partly located in a highly structured region of the genome with a ~90 nucleotide-long imperfect stem (Fig. 3A, upper part of the structure) [123]. Most notably, one strand of this stem (highlighted in grey), which is located right before an unstructured region, makes a long distance interaction with a sequence located ~8,000 nucleotides upstream, in the prM coding sequence. Closer analysis of the sequence of the whole region identified seven m^{6}A consensus motifs. This includes two DRACH motifs in the long stem mentioned above (nucleotides circled in red). In addition, one MGACK motif (circled in black) was identified in the unstructured region directly upstream the strand, which hybridizes with the region involved in the NS5/prM RNA interaction. If one considers that the presence of m^{6}A influences local hybridization properties, this suggests that the methylation status of this region influences whether or not this strand makes the stem in NS5 depicted in Fig. 3A or is rather engaged in NS5/prM long-range interaction.

The comparison between the m^{6}A-methylome and the structure of DENV2 vRNA from two independent studies also revealed interesting features [14,121]. Indeed, a methylated region in NS3 coding sequence of the New Guinea C strain partially overlaps with a structured region identified in the genome of the closely related S16803 strain

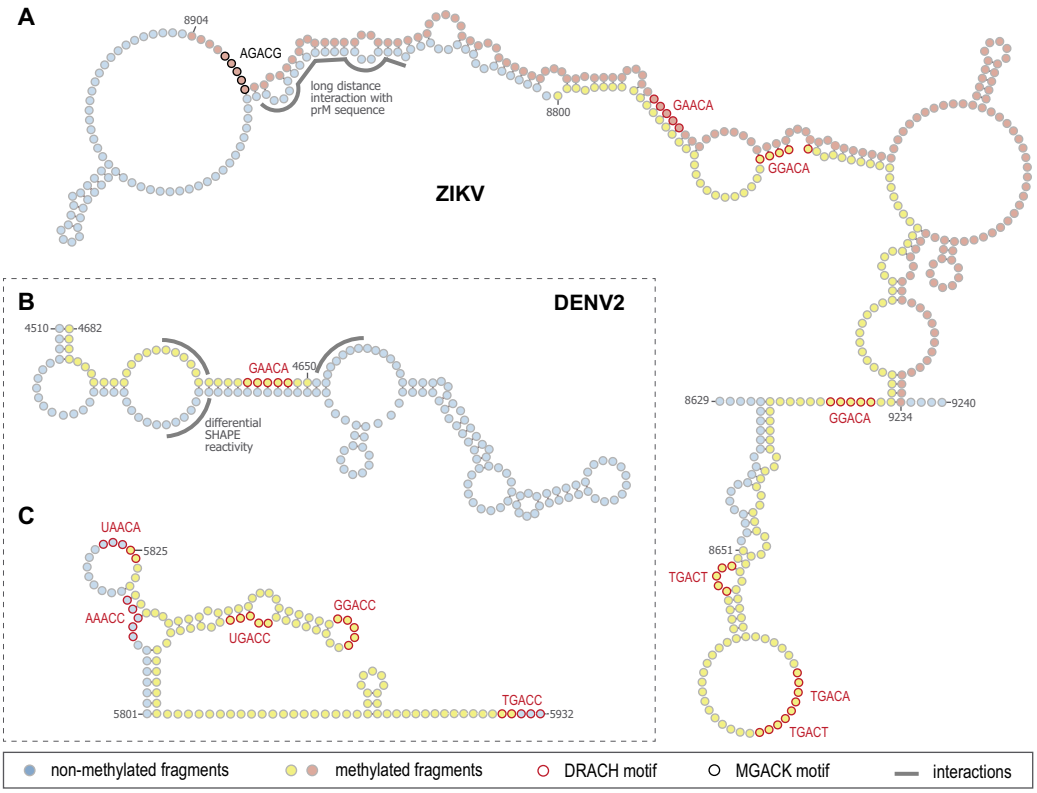

Figure 3. Examples of ZIKV and DENV structural elements with m^{6}A consensus motifs. (A) Two contiguous m^{6}A-containing regions in the NS5 coding sequence of ZIKV MR766 genome (yellow and light pink circles) were identified in the reported secondary structure [14,21,121,123]. The nucleosides in predicted DRACH and MGACK m^{6}A consensus motifs are circled in red and black, respectively. The RNA strand making intramolecular long-range interaction with prM coding region is indicated in grey. (B, C) Two different methylated regions of DENV2 NGC strain (yellow circles) were mapped within two independent reported structures in the NS3 coding sequence of the closely related strain DENV2 S16803 [14,21,121,123]. The nucleosides in predicted DRACH m^{6}A consensus motifs are circled in red. In B, the DRACH motif is located in a 14 nucleotide-long stem flanked by unstructured regions whose SHAPE reactivity (indicated in grey) changed if vRNA was gently extracted from virions.

(element 10 in [121]; Fig. 3B, nt 4650–4682 in yellow). This shared region contains a 14 nucleotide-long stem, which includes a consensus DRACH motif, and is flanked by three unstructured sequences whose reactivity changes depending on whether the vRNA structure is analysed in authentic virions (*i.e.* capsid associated) or following gentle extraction (*i.e.* protein-free). These differences might reflect a structural switch of this region that would occur during vRNA uncoating following virus entry or during genome encapsidation into assembling particles. In cells, methylation of this stem may affect its stability hence, structurally rearranging the whole region, modifying its interactome and targeting the RNA to specific steps of the life cycle.

Finally, another methylated region is found in a structural element located in NS3 coding sequence (element 14 in [121]; Fig. 3C, nt 5825–5932) and contains 4 DRACH motifs, which all completely or partially overlap with loops of bulges. Another motif is located right downstream this element in an unstructured sequence. Despite still unclear, this suggests that m^{6}A modifications primarily occur in unstructured regions or destabilize neighbouring base pairing. In the case of HCV described above, the four predicted m^{6}A sites in E1 coding sequence which regulates virus production, are located in rather unstructured regions [14,124]. If *N*^{6}A methylation of vRNA indeed influences its structure, it is conceivable that this will result in direct impacts on structural switches as well as long-range interactions. Whether the three selected examples of a hypothetical methylation/structure relationship for flaviviruses actually exist as m^{6}A switches and are functionally relevant to viral replication needs to be addressed experimentally. It is noteworthy that this comparative exercise did not take into consideration that the reference studies used different cell lines *(e.g.* Vero vs. Huh7 cells) and vRNAs of different origins (intracellular vRNA *vs* virion vRNA).

Possible additional functions of flavivirus vRNA *N*^{6}A methylation

N^{6}A methylation of vRNA may also allow flaviviruses to escape the sensing by the innate immune system. Indeed, for HCV (as well as for human metapneumovirus, a negative strand RNA virus), the presence of internal m^{6}A in vRNA was recently reported to decrease the production of type I IFN in infected cells [17,125]. This correlated with a decreased binding efficiency of the vRNA to RIG-I. While the molecular mechanisms behind this interference remain to be fully elucidated, internal m^{6}A seems to constitute an additional strategy to the cap 2′-*O* methylation to 'disguise' vRNAs as self-RNAs (Fig. 1). Future studies will have to confirm or invalidate this hypothesis.

The fact that flavivirus vRNA does not transit through the nucleus during its life cycle implies that m^{6}A modifications occur in the cytoplasm, in contrast to the co-transcriptional methylation of host mRNAs. While some studies have reported this methylation activity in the cytoplasm, it will be interesting to determine how flaviviruses hijack this cellular activity in the vicinity of replication factories. Interestingly, besides exploiting the *N*^{6}A-methylation-related machinery to regulate their own genomes, DENV and ZIKV also alter the host *N*^{6}A methylome [21,126,127]. Notably, infection increased and reduced m^{6}A content in RIO Kinase 3 (*RIOK3*) and Cold Inducible RNA Binding Protein (*CIRBP*) mRNAs, respectively, changing the expression profile of the corresponding proteins, which were shown to regulate replication efficiency [126]. These changes in the mRNA methylation profile, likely occurring in the nucleus, were indirect since attributed to the virus-induced ER stress or immune response. Nevertheless, this suggests that flaviviruses hijack the m^{6}A machinery to generate a cellular environment favourable to viral replication.

Other types of RNA methylations potentially involved in flavivirus life cycle

A recent epitranscriptome analysis of affinity-purified vRNAs from several positive strand RNA viruses (including DENV and ZIKV) opened the possibility that flaviviral RNA might actually be decorated by modifications with over 40 types of methylation (including m^{6}A and A$^{2'O}$m) detected by mass spectrometry [18]. While this study made clear that vRNA is modified, the fact that so many types of methylations were detected does not make them directly relevant for replication. Indeed, some were found at very low stoichiometry, and it is conceptually challenging to envision how the viral genome would have evolved to co-opt in the cytoplasm all the cellular machineries required to fulfill this massive epigenetic task, especially considering that vRNA is mostly confined in membranous replication factories. To reconcile this, further accurate mapping and functional studies for each type of nucleoside methylation are needed. Very interestingly, the presence of some methylated nucleotides was specific to either flavivirus. For instance, methylated uridines m^{3}Um and m^{5}Um were detected in DENV vRNA but not in ZIKV vRNA. In contrast, dimethylcytosines m^{5}Cm and m$^4{}_4$C were present only within ZIKV vRNA inside virions. This suggests that this modification is not only ZIKV-specific but may also be a marker of encapsidated vRNA since it was absent in genomes purified from infected cells. Whether the methylation landscape of flavivirus influence the different steps of the life cycle or specific aspects of ZIKV pathogenesis need to be fully explored. Furthermore, the authors identified in this study DEAD box protein 6 (DDX6) as a host factor modulating the abundance of m^{5}Cm and m$^4{}_4$C as well as ZIKV replication. Future studies will have to evaluate the contribution of m^{5}Cm and m$^4{}_4$C to the ZIKV life cycle and whether DDX6 role in viral replication is related to these modifications or rather to its implication in decapping activity, RNA stability in P-bodies and sfRNA metabolism [128,129]. Finally, since m^{5}C (Fig. 2C) was shown to play important roles in viral RNAs of murine leukaemia virus (MLV), human immunodeficiency virus (HIV-1) and Epstein-Barr virus (EBV) [12,15,130,131] and was detected in DENV and ZIKV vRNAs, it will be interesting to further investigate the potential function of this modification in the flavivirus replication cycle.

Technical challenges in flaviviral RNA epigenetics

In relation to the recent attention received by RNA modifications in the RNA field, molecular virology on RNA modifications is still relatively sparse. This is likely due to a number of important technical limitations concerning analytics of RNA modifications and the isolation of pure vRNA in high amounts that we will address in this section. We also highlight additional approaches that have not yet been applied and could be considered for flaviviral RNAs.

Analytical techniques of RNA modifications can be roughly divided into two categories [132,133]. In brief, biophysical methods for the characterization of modified nucleosides such as chromatography, mass spectrometry, or combinations thereof, can identify and quantify RNA modifications in small samples only at the expense of sequence information. A combination of modification and sequence information, commonly obtained from proteomics approaches, is available only for samples of double-digit microgram amounts, *i.e.* typically for tRNA and rRNA [134,135]. Moreover, as described below, sample purity must be considered in the analysis in order to reliably assign a proper modification to the RNA of interest.

In contrast, there exists an ever-growing array of RNAseq-derived methods that make use of various chemical or biochemical properties of RNA modifications to place them in a sequence context [132]. Results from this so-called 'modification mapping', or 'modification calling' form the basis for the vast majority of reported viral RNA modification sites. However, these approaches come with their own set of experimental problems. The large diversity of mapping methods makes discussions about advantages and drawbacks impractical at this point. It should be made clear, though, that the different methods result in modification calling data of variegated quality, and that it is typically at the authors' discretion to set an arbitrary threshold above which a signal is 'called' a modification.

Antibody-based detection of viral RNA modifications

Among the various reagents used in modification mapping, antibodies hold a special place, given that the first m^6A mapping was performed using antibody affinity enrichment in the 1980s [13,136] before the current surge was triggered by combination with RNAseq [86,87]. However, antibodies recently emerged as problematic [137,138]. Similar to applications in epigenetics, where *e.g.* single methylations on either nucleobases or amino acids must be discriminated, certain commercial antibodies were found to have been insufficiently validated [139,140]. Most antibody-based mapping reports employ modification calling based on simple enrichment calculations, which identify regions or sequence stretches (generally hundreds nucleotide-long) rather than single nucleotides as mapping data [127]. Hence, the modification calling is necessarily vague. Authors generally look afterwards for consensus methylation motifs to identify putative modified sites and to elaborate validating directed mutagenesis-based experiments. More advanced techniques include a crosslinking step that allows more stringent washing conditions to remove non-specific binders [141].

In the particular case of m^6A, it is often assumed (tacitly or explicitly) that signals only originate from activity of the METTL3/14 complex, which is known to depose methylation marks at the DRACH consensus motif, and other potential sites could be ignored. A smart method to validate such presumed m^6A sites has been developed already in the 1980s [142]. Within the central consensus, Kane and Beemon mutated the pyrimidine downstream of the methylation target adenosine to a uridine, and found that a DRAUH was no longer methylated. This 'silent' mutagenesis approach has been used in several more recent publications on m^6A in vRNAs, including *Flaviviridae* vRNAs [12,14,21] as described above.

Identification of viral RNA modifications from cDNA-affinity purified samples

Sample purity is an often-overlooked problem for such analyses. Indeed, LC-MS-based analyses of mRNA populations purified by oligo-dT affinity frequently pick up marker modifications of contaminating rRNA [143]. Given that viral particles of any kind typically package other host RNAs in a rather unspecific manner [144], an LC-MS analysis of total RNA isolates of viral particles can be expected to contain standard modifications encountered in host tRNA and rRNA fragments [145]. Hence, further highly specific purification approaches *e.g.* by RNA size fractionation [12] or cDNA affinity are required to reduce non-specific signals [15]. Early work in the 1980s on the detection of modifications in coding sequences relied on *in vivo* labelling of RNA with ^{32}P and/or L-[methyl-^{3}H]-methionine, respectively. Thus, labelled RNA was affinity-purified *e.g.* using oligo-dT or cDNA, digested to nucleotides and separated by multi-dimensional chromatography after which autoradiography of the label allowed quantification [136,146,147]. Many of the RNAs thus investigated were indeed viral coding RNA containing mainly m^6A in viruses replicating in the nucleus, *e.g.* in simian virus 40 [148], adenoviruses [149], herpes virus [150], influenza virus [151] and Rous sarcoma virus [147]. For the latter retrovirus, 13 sites have been individually mapped, constituting a major early contribution to the establishment of the DRACH consensus [146,147]. Other modifications detected in viral RNA using radioactive label included cap structures, ribose methylations, and m^5C in low quantities [22,23,152,153].

More examples of cDNA purification-based analysis include the above-mentioned LC-MS analysis of various viral RNAs, including those of ZIKV and DENV [18] and more recently, EBV. Two small RNAs from EBV, namely EBER1 and EBER2, were isolated by cDNA affinity, and m^5C was detected by LC-MS in EBER1 at near quantitative stoichiometry [15]. As mentioned above, special attention must be given to the purity of the analysed samples as well as to subsequent validation since heavily modified rRNAs and/or tRNAs, which may be unspecifically co-purified are expected to introduce undesired methylation signals during the detection. In case of EBER1, the presence of m^5C was confirmed by

bisulfite-seq, which allowed to place the modification at position C145 in this small structured RNA. Of note, the presence of m^5C 145 was contingent upon the expression of NSUN2, a tRNA m^5C MTase known to act on highly structured tRNA [15].

Other approaches for the identification of viral RNA modifications

The following methods have not yet been applied to study flavivirus epitranscriptomics: (i) Ribomethseq is a mapping method based on protection against alkaline degradation conferred by ribose methylation [154]. Using this approach Ringeard *et al.* identified 17 distinct 2′-*O*-methylated residues in the HIV RNA genome and showed that HIV recruits the host methyltransferase FTSJ3, via a complex including the TAR RNA-binding protein to execute internal ribose methylations [155]. Interestingly, absence of ribose methylation resulted in recognition by the PRR melanoma differentiation-associated protein 5 (MDA5) and subsequent induction of IFNs. (ii) Direct RNA sequencing using the nanopore technology was applied to unravel the epitranscriptome of severe acute respiratory syndrome coronaviruses SARS-CoV and most recently SARS-CoV-2 allowing the detection of 41 potential modification sites in the latter [156,157]. (iii) Other methods for modification mapping that yield single nucleotide resolution frequently rely on chemical treatment that achieves a certain discrimination in its reaction with a modified versus unmodified nucleoside [132,133,158,159]. Fortunately for the field of viral RNA modification, these methods perform better on short transcriptomes such as viral RNA genomes than they do on cellular transcriptomes of a complexity in the order of 10^7. Thus, while many transcriptome-wide studies on modification mapping are subject to controversial findings because the large sequence space gives rise to signal-to-noise problems, short viral RNA genomes can be expected to be suitable and it will be interesting to explore such approach in the case of flaviviral RNA.

Perspectives and conclusions

In addition to being critical for initiating the cap-dependent translation, vRNA methylation has emerged during the last years as a mechanism exploited by flaviviruses to control their life cycle as well as to evade sensing as non-self by the cellular antiviral machinery. While much progress was recently made, several important hypotheses will be scientifically very exciting to address thanks to the advent of new technologies and/or approaches. Regions of the genomes with m^6A have been identified but they are generally over 100 nucleotide-long and contain several consensus methylation motifs. Moreover, determination of the m^6A methylome mostly relied on the binding of vRNA to anti-m^6A antibodies, which may not be completely specific to this modification. Hence, even if functional validation must be made by directed mutagenesis of the consensus motifs, it is not indicative that these sites are actually methylated and it will be critical to accurately identify the modified positions at the single nucleotide resolution. This also applies to 2′-*O*-methylated internal adenosines as well as to any other modifications for which no role was described at all for flaviviruses although their presence was experimentally evidenced. Deeper characterization of these newly identified modified sites is expected to reveal specific roles in vRNA structure dynamics, notably in riboswitches regulating transitions between vRNA translation, replication and packaging. In the case of m^6A in flavivirus vRNA, the step(s) of the life cycle impacted by this modification is unknown. By analogy with HCV, one should anticipate that it is important for the production of virus particles. Future studies will need to address whether unmethylated vRNA is specifically selected for packaging into assembling virions and whether this is related to the architecture of the viral replication compartment or involves specific RNA packaging signals. It will be interesting to also evaluate the regulation by cellular 'readers' of methylated vRNA. It is noteworthy that the notion of a 'reader' function, seeped into the field from the epigenetic DNA methylation field, implies that the principle function of a modification lies within the selective interaction with a dedicated binding protein. While this has turned out to be a guiding principle in m^6A research, the situation is very different for other modifications. Indeed, the examples of tRNA and EBV EBER1/2 modifications [15] pinpoint that given 'readers' and 'writers' recognize aspects of RNA secondary and/or tertiary structure rather than a sequence motif *per se*.

Interestingly, methylation of specific vRNA regions may contribute to the evasion of the innate immune response synergistically with the cap1 structure. It will be essential to address whether such molecular mechanisms are conserved across the *Flavivirus* genus. Alternatively, some structure/activity relationships specific to a given flavivirus may hypothetically contribute to unique aspects of viral tropism and/or pathogenesis (*e.g.* neurovirulence in ZIKV-infected foetuses). Finally, the methylation status of the noncoding sfRNA, which is abundantly produced in the course of flavivirus infection is completely unknown. Since fragments corresponding to vRNA 3′ UTR contained m^6A, it is tempting to speculate that the sfRNA is differentially modified as compared to the flaviviral genome. If that is the case, it could be envisioned that specific sfRNA modifications contribute to its biogenesis and/or stability as well as to its functions in virus-induced cell death and antiviral response. Overall, a complete and highly resolved functional landscape of flavivirus RNA methylome, which may be extended to other epigenetic modifications, will hopefully unveil novel molecular mechanisms governing the viral replication cycle and pathogenesis.

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