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N^6-methyl-Adenosine modification in messenger and long non-coding RNA

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Abstract

N^6-methyl-Adenosine (m^6A) is the most abundant modification in mammalian mRNA and long noncoding RNA. First discovered in 1970s, m^6A modification has been proposed to function in mRNA splicing, export, stability and immune tolerance. Interest and excitement in m^6A modification has recently been revived based on the discovery of a mammalian enzyme that removes m^6A and the application of deep sequencing to localize modification sites. The m^6A demethylase FTO controls cellular energy homeostasis and is the first enzyme discovered that reverses an RNA modification. m^6A sequencing demonstrates cell type and cell state-dependent m^6A patterns, indicating that m^6A modifications are highly regulated. This review describes the current knowledge of mammalian m^6A modifications and future perspectives on how to push the field forward.

Modifications in messenger and long non-coding RNA

Over one hundred types of RNA modifications have been identified in all three kingdoms of life (http://rna-mdb.cas.albany.edu/RNAmods/). The most chemically diverse modifications are present in ribosomal RNA (rRNA) and transfer RNA (tRNA); rRNA and tRNA modifications are commonly proposed to find-tune their structure and function [1]. Most modification studies have focused on rRNA and tRNA due to their high cellular abundance, large chemical diversity and functional importance in translation.

Modifications are also present in messenger RNA (mRNA) and long non-coding RNA (lncRNA) in eukaryotes (Figure 1). Additional chemical changes of specific RNA residues include adenosine to inosine (A-to-I) and cytosine to uridine (C-to-U), commonly referred to as “RNA editing” (Figure 1). The 5′ cap of mRNA, some lncRNA, and spliceosomal RNA (snRNA) is the best known example of such modifications. The 5′cap of mRNA is created by both 7-methyl-G addition to the 5′ triphosphate as well as 2′OMe modification of the first and second mRNA residues. The 5′ cap in mRNA recruits the cap-binding protein eIF4E, other initiation factors and the 40S ribosomal subunit to initiate mRNA scanning and translation at the first codon of the coding sequence. The 5′ cap is also required for mRNA stability; its removal is often a necessary step for complete mRNA degradation [2, 3]. Up to three types of mRNA/lncRNA modifications are known to be present away from the 5′ cap. Two types, N^6-methyl-adenosine (m^6A) and 5-methyl-cytosine (m^5C), have been shown to occur in significant amounts [4–7]. One other type, pseudouridine (Ψ) has not been shown

conclusively to be present in mRNA/lncRNA, although Ψ-modification of codons can have a profound effect on decoding [8, 9]. A-to-I and C-to-U editing changes the base pairing property of the edited bases, therefore resulting in recoding of the edited codons. Biological functions of the m^6A and m^5C modifications in mRNA/lncRNA, however, are much less well understood. This review discusses the current knowledge and future perspectives of mRNA/lncRNA modification, focusing on m^6A modification in mammals.

N^6-methyl-A, discovered in the 1970s [5], is the most abundant modification in eukaryotic mRNA/lncRNA. N^6-methyl-A modifications in polyadenylated RNA have been found in mammals, plants and yeast [10]. In mammals, m^6A occurs on average in 3–5 sites per mRNA molecule, and up to 15 sites in viral RNA. Since a mammalian cell contains over 10,000 mRNA species, tens of thousands of m^6A sites may exist in the mRNA population in each cell. Two recent developments have revived the interest and excitement in understanding the biological function of m^6A modification. The first is the discovery in 2011 that m^6A modification can be reversed by a mammalian enzyme involved in diabetes and obesity [11]. The second is the transcriptome-wide mapping of tens of thousands of m^6A-containing segments in mRNA and long non-coding RNA in 2012 [12, 13]. These developments have elevated m^6A modification to a new level in understanding RNA modification in biology.

m^6A studies before the advent of deep sequencing

The classical phase of m^6A modification studies in mRNA and viral RNA has been comprehensively described in a 2005 review [10]. In contrast to abundant rRNA and tRNA, the low abundance of mRNA presents a huge challenge in the identification of m^6A sites in mRNA. By 2005, specific sites for m^6A modification were determined in only two individual mRNAs. The m^6A site in bovine prolactin mRNA is present in the 3′ UTR and near a consensus 3′ polyadenylation sequence. Interestingly, the extent of modification appeared to be only ~20%, suggesting that m^6A modifications in mRNA may be incomplete at other sites as well. Over ten m^6A sites have been found in the Rous sarcoma virus (RSV) RNA. Again, modification fractions of the RSV sites are incomplete, ranging between 20–90%. Through mutational analysis of m^6A sites *in vivo* and *in vitro*, the consensus sequence of m^6A modification has been defined as RRm^6ACH, where R is A/G and H is A/C/U [14–17]. This consensus sequence can occur once every 85 nucleotides, so that an average mRNA can have more than 30 consensus sites. Since only ~3–5 m^6A sites were estimated to be present in an mRNA, the majority of the consensus sequences are either not modified, or many of them are modified but at substoichiometic amounts.

A major advance in m^6A study was the identification of a human m^6A methyl-transferase subunit of a nuclear-localized complex [18, 19]. This methyltransferase, MTA70 (or METTL3), is a homolog of yeast IME4, which is known to introduce m^6A modification in yeast mRNA during sporulation [20, 21]. MTA70 is a subunit of a nearly one mega-Dalton complex. RNA interference of this methyltransferase in HeLa cells resulted in cell death through apoptosis, indicating that m^6A modifications perform crucial regulatory functions. The MTA-homologs in plant and in Drosophila are essential for viability and are particularly important in certain developmental pathways [22, 23].

Proposed functions that m^6A modification affects include mRNA splicing, nuclear export, stability and translational efficiency [10]. However, it has been extremely difficult to test m^6A function for an individual RNA. Unexpectedly, mutating the modified adenosine or the 3′ cytidine residue in the consensus sequence often resulted in the appearance of new m^6A modification at a nearby consensus site in the same RNA [10]. Further, because many sites are potentially modified in each mRNA, one may not expect a very large effect upon mutating a single modification site.

An exciting development in the studies of m^6A modification function was the discovery that it can prevent recognition of the modified RNA by cellular innate immunity [24]. The innate immune system recognizes self versus foreign molecules and triggers a cascade of cellular activities upon activation. Known components in the innate immune system that directly recognize RNA include toll-like receptor 3 (TLR3), which recognizes double-stranded RNA, and TLR7, which recognizes single-stranded RNA [25]. The innate immune system is activated upon transfection of unmodified RNA oligonucleotides, but remains dormant upon transfection of the same RNA containing m^6A modification. Interestingly, transfection of modified RNA containing m^5C or Ψ also markedly reduced innate immune activation, although m^6A modification was most effective in preventing activation. By contrast, compared to unmodified mRNA transcripts, complete substitution of A to m^6A was highly detrimental to translation, whereas substitution of C/U to m^5C/Ψ increased the level of translation [26]. However, substituting ~5% of A to m^6A did not affect translation levels; this low level of A-to- m^6A substitution more closely resembles the m^6A levels observed in natural mRNAs [26]. These results suggest that one function of m^6A (and potentially m^5C or Ψ) modification in mRNA is to tag cellular mRNA to be "self" when presented to the innate immune system [24, 26]. This insight led to the remarkable success in efficiently generating induced pluripotent stem cells (iPS) simply by transfecting m^5C/Ψmodified mRNAs of the four key factors in iPS formation [27].

The discovery of two m^6A demethylases

A major breakthrough was the discovery in 2011 of a mammalian enzyme that specifically removes m^6A modification in polyadenylated RNA, which are primarily composed of mRNA and lncRNA [11]. The fat mass and obesity associated protein (FTO) belongs to a human family of AlkB-homologues that can remove N-methylated RNA or DNA bases. *Escherichia coli* AlkB is a repair enzyme capable of demethylating N^1-modified A or N^3-modified C both in DNA and RNA [28, 29]. Many AlkB-homologues or closely related proteins have been found in mammalian genomes including AlkBH1–8, FTO and Tet1–3. AlkBH8 was found to be a tRNA modification enzyme containing two catalytic domains: one catalyzes methylation of several wobble uridine modifications [30, 31] and the other catalyzes hydroxylation of the hypermodified U34 wobble base of $tRNA^{Gly}$(UCC) [32, 33]. The Tet enzymes were found to catalyze the oxidation of m^5C in DNA to 5-hydroxymethyl-C, which is a new form of DNA modification and may represent the first step in the reversal of m^5C modification in chromosomal DNA [34, 35]. The FTO enzyme is one of the key factors in regulating mammalian energy homeostasis; the FTO gene was identified in genome-wide association studies to be strongly linked to diabetes and obesity [36], and loss of FTO function in mice leads to a significant reduction in body mass [37]. On a mechanistic level, FTO was found to selectively remove m^6A in polyadenylated RNAs both *in vitro* and *in vivo. In vivo*, siRNA knockdown or overexpression of FTO increased or decreased total m^6A content by ~15–20%, respectively. The FTO protein partially co-localizes with markers of nuclear speckles, which are sites of mRNA splicing. This localization of FTO activity is consistent with previous suggestions that m^6A is involved in mRNA splicing. These results indicate that m^6A modifications in mRNA/lncRNA play a significant role in cellular energy homeostasis and metabolism through their influences on the spliced mRNA/lncRNA populations.

FTO is the first enzyme discovered that can reverse an endogenous RNA modification. Base methylation is a dominant form of RNA modification in all cellular RNA types. Aside from m^6A, which occurs in polyadenylated RNA as well as in rRNA/tRNA and snRNA, other known N-methylations in rRNA/tRNA include N^1-methyl-A (m^1A), N^3-methyl-C (m^3C), N^4-methyl-C (m^4C), N^1-methyl-G (m^1G), N^2-methyl-G (m^2G), N^3-methyl-U (m^3U), and N^3-methyl-pseudoU ($m^3Ψ$), all of which can potentially be subjected to reversal by a similar

or other mechanisms. The discovery of m^6A as a cellular substrate of FTO indicates that other cellular enzymes may exist to catalyze the reversal of other base methylations [38, 39].

A second human m^6A-demethylase has just been described [40]. Like FTO, AlkBH5 is another member of the AlkB-homologous gene family of dioxygenases. Perturbation of AlkBH5 significantly affects nuclear mRNA export and RNA metabolism. Furthermore, AlkBH5 appears to be involved in the assembly of mRNA processing factors in nuclear speckles. The physiological effect of AlkBH5 deficient mice includes impaired male fertility. The discovery of a second m^6A-demethylase strongly indicates that reversible m^6A modification likely has broad functions in mammalian biology.

Both mRNA m^6A methyltransferase (MTA70) and mRNA m^6A demethylase (FTO and AlkBH5) are catalytic subunits of much larger complexes in the cell that presumably confer specificity of m^6A addition and removal. These accessory factors need to be identified using modern proteomic techniques to further advance studies of m^6A function.

Transcriptome-wide profiling of m⁶A in mRNA and lncRNA

A new wave of breakthroughs in 2012 took advantage of recent advances in high throughput sequencing [12, 13]. Reverse transcriptase does not discriminate m^6A from A in bulk cDNA synthesis, therefore, m^6A cannot be currently detected by standard high throughput sequencing methods. Using m^6A-specific antibodies originally generated for m^6A modification studies of spliceosomal RNA, two independent studies developed similar methods that enabled the identification of ten thousands of m^6A-containing mRNA/lncRNA segments in different tissues and stress conditions (Figure 2). In these studies, polyadenylated RNAs are first fragmented to ~50–100 nucleotides then pulled down with an m^6A-antibody, followed by sequencing of the immunoprecipitated RNA fragments. Analysis of the high throughput sequences reveals ~200-residue-wide peaks; many, although not all contain the known m^6A consensus sequences. These m^6A profiling studies showed that m^6A modifications are enriched around the stop codon of the coding regions of mRNA, although the function of this enrichment is currently unknown. Further, m^6A modifications have distinct distributions among tissue types and stress conditions, thus indicating that each cell type and cell state likely have unique m^6A modification patterns. Another very exciting finding from these studies was the discovery of m^6A modification in many long non-coding RNAs, indicating that m^6A modifications are not confined to mRNA. The transcriptome-wide m^6A profiles are similar to the profiles for m^5C modifications [7]. Both modifications are non-randomly distributed in mRNA with enrichment in and near the 3′UTR, and both are also found in non-coding RNAs.

The demonstration that m^6A modification in mRNA/lncRNA has distinct patterns in response to developmental or environmental cues is akin to m^5C modification in chromosomal DNA where distinct patterns are present depending on cell type and cell state in order to establish epigenetic maintenance and regulation. It is likely that m^6A modification patterns also follow a well defined but distinct pattern depending on cell type and cell state. The identification of m^6A patterns in mRNA/lncRNA that correlate to cell type and states could therefore establish the concept of “RNA epigenetics” as another layer of epigenetic regulation [38, 39].

A major challenge will be the development of a high throughput technique that identifies m^6A modification in mRNA/lncRNA at single nucleotide resolution. The current technique of using an m^6A antibody generates m^6A candidate sites within ~200 nucleotide segments, but there is no simple method to actually validate the presence of m^6A modification there. Furthermore, the known m^6A consensus sequence cannot explain many sequencing peaks, suggesting that more than one m^6A modification enzyme is present in cells, and each

enzyme may have distinct requirements for m^6A modification. The development of an m^6A sequencing technique with single nucleotide resolution may involve chemical reactions that are specific for m^6A, but not for unmodified A. Chemically reacted m^6A-containing RNA fragments can then be enriched by affinity pull-down followed by sequencing. Another potential method involves single molecule sequencing which has been shown to be capable of detecting m^6A modification in DNA [41]. Another highly desirable feature of new m^6A sequencing technologies would be the determination of the m^6A modification fraction at each site. All m^6A modifications in cellular mRNA or viral RNA that are mapped to single nucleotide resolution have 20–90% methylation for each site. Why cells maintain fractional m^6A modification at these sites is unclear; it is difficult to derive functional hypotheses given the paucity of such experimental data. Fractional modification can be anywhere from 0–100%, so multiple regulatory states may be available depending on the extent of modification at each site.

Several proteins that seem to prefer binding to m^6A modified RNA have also been identified from cell lysates using a known viral m^6A-containing RNA oligonucleotide as *in vitro* bait [12]. Some of these m^6A binding proteins have been shown previously to bind RNA, although a few had no previously known association with RNA. At this time, it remains to be determined whether these proteins actually bind m^6A-containing RNA *in vivo* and whether m^6A binding of these proteins is functional. In any case, identification of such proteins strongly suggests that an important function of m^6A modification is to recruit specific proteins to modified mRNA or lncRNA. Thus, m^6A modification has potential to introduce a new layer of biological regulation through m^6A binding proteins and beyond.

Concluding remarks

N^6-methyl-A modification can exert its function in at least two ways: alteration of RNA structure or recruitment of specific m^6A binding proteins (Figure 3). The addition of a single methyl-group to the N^6-position of adenosine does not perturb Watson-Crick base pairing. However, m^6A modification is known to weaken RNA secondary structure [42], and can affect RNA tertiary structure, in particular tertiary interactions involving base triples or Hoogsteen pairs that rely on the N^6 proton as a Hydrogen bond donor. N^6-methyl-A modification may also prevent RNA folding into alternate structures, as demonstrated for certain base methylations in tRNA [43]. That N^6-methylation might create new binding sites for specific proteins is an attractive and readily identifiable function for specific m^6A modification sites. Recruitment of m^6A binding proteins to specific sites in the RNA would be a way to concurrently recruit additional complexes that bind m^6A, such as the spliceosome, the mRNA export machinery, or chromatin remodeling enzymes.

In my opinion, the current biggest challenge for the field is to develop methods that can perturb m^6A modification at specific sites in order to directly assess m^6A function in specific genes. RNA interference or over-expression of an mRNA may simply decrease or increase both modified and unmodified RNA alike. In a few cases (summarized in [10]), mutation of a known m^6A site in an mRNA resulted in additional modification at a nearby consensus site, so that one cannot simply assume that mutation of a known site would not lead to the modification of cryptic sites nearby that may perform the same function. Further, functional understanding of a specific site should also take into account that all currently known m^6A sites in mRNA and viral RNA are incompletely modified, so that one may need to explain why cells simultaneously maintain two RNA species that differ only at the site of m^6A modification.

In summary, since the discovery of m^6A modification in mRNA in 1970s, the field has come a long way to establish this modification as a wide spread biological process in many

branches of eukaryotes. The m^6A modifications have been proposed to play significant roles in cellular RNA functions including mRNA splicing, nuclear export, stability, and immune tolerance. The presence of appreciable amounts of m^6A in lncRNAs suggests that m^6A can also be important in cellular processes other than protein synthesis. Despite these advances, many technical, conceptual and biological challenges remain, and the functional significance of m^6A modification in specific RNAs remains to be determined. The field is wide open for exploration.

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Abbreviations

m^6A	N^6-methyl-adenosine
m^5C	5-methyl-cytosine
Ψ	pseudouridine

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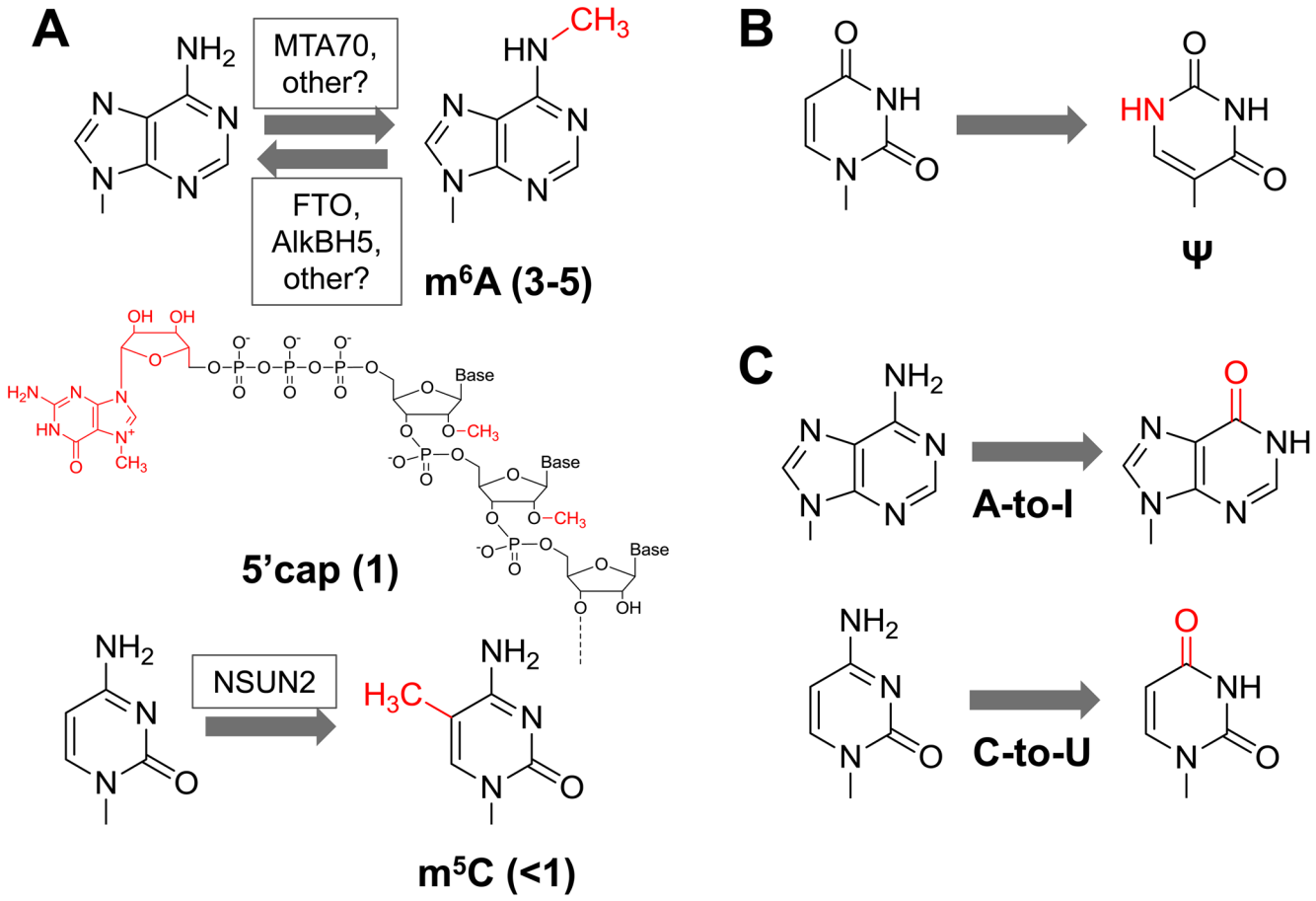

Fig. 1. mRNA and lncRNA modifications

The functional groups introduced through modifications are shown in red. (A) The three abundant modifications are m^6A, 5′ cap and m^5C. The 5′ cap contains both modifications at the 5′ triphosphate and 2′O-methylation of the first and sometimes the second nucleotide. The estimated number of modifications per type, per RNA molecule, is shown in parentheses [4, 6, 10]. Some known human enzymes that catalyze the forward and reverse reactions are also shown [7, 10, 11, 40]. MTA70: METTL3 methyltransferase like 3; FTO: fat mass and obesity associated; AlkBH5: alkB alkylation repair homolog 5; NSUN2: NOP2/Sun RNA methyltransferase family, member 2. (B) Pseudouridine modification (Ψ) may also be present, although the extent of Ψ is currently unknown. (C) RNA editing commonly refers to deamination of A to inosine and C to uridine at specific sites in mRNA.

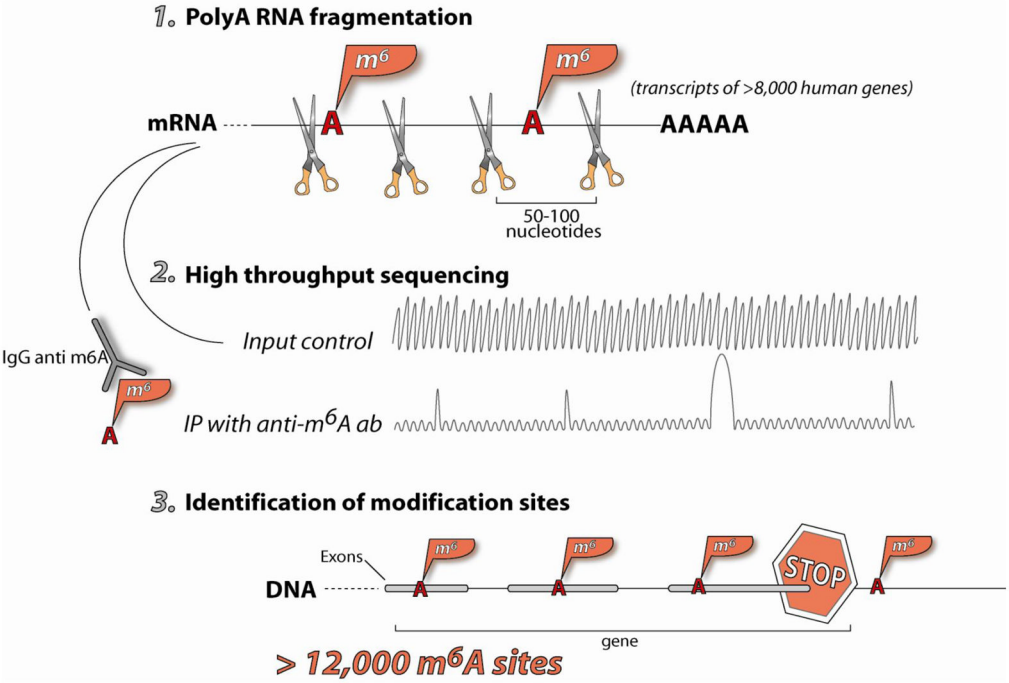

Fig. 2. m^{6}A sequencing method and results [12, 13]

Starting from total polyadenylated RNA, step 1 is to chemically fragment RNA to ~50–100 nucleotides. This sample is split in two; one is used as input control, and the other immunoprecipitated with anti-m^{6}A antibody to isolate RNA fragments containing m^{6}A modification. Both samples are deep sequenced (step 2), and the sequencing results compared to identify m^{6}A-containing RNA segments (step 3). The m^{6}A modification is markedly enriched around the stop codon of mRNA, although the reason for this enrichment is unclear.

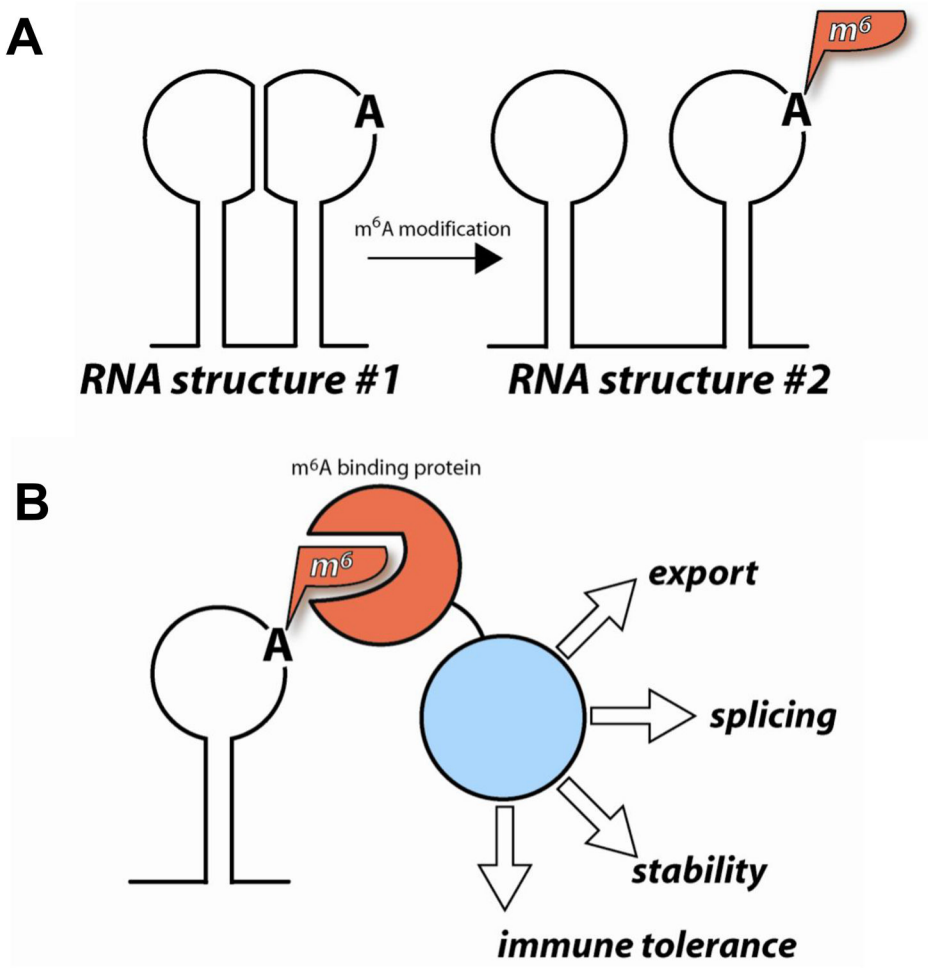

Fig. 3. Functional effects of m^{6}A modification

(A) m^{6}A modification could alter RNA structure either through weakening of base pairing or through the loss of one of the two hydrogen bond donors at the N^6-position of A. This H-bond donor could be involved in RNA tertiary structure. (B) m^{6}A modification could generate a new binding site for proteins that recognize m^{6}A modified RNA. The m^{6}A binding proteins can recruit additional complexes involved in cellular processes such as mRNA splicing, export, stability and immune tolerance.