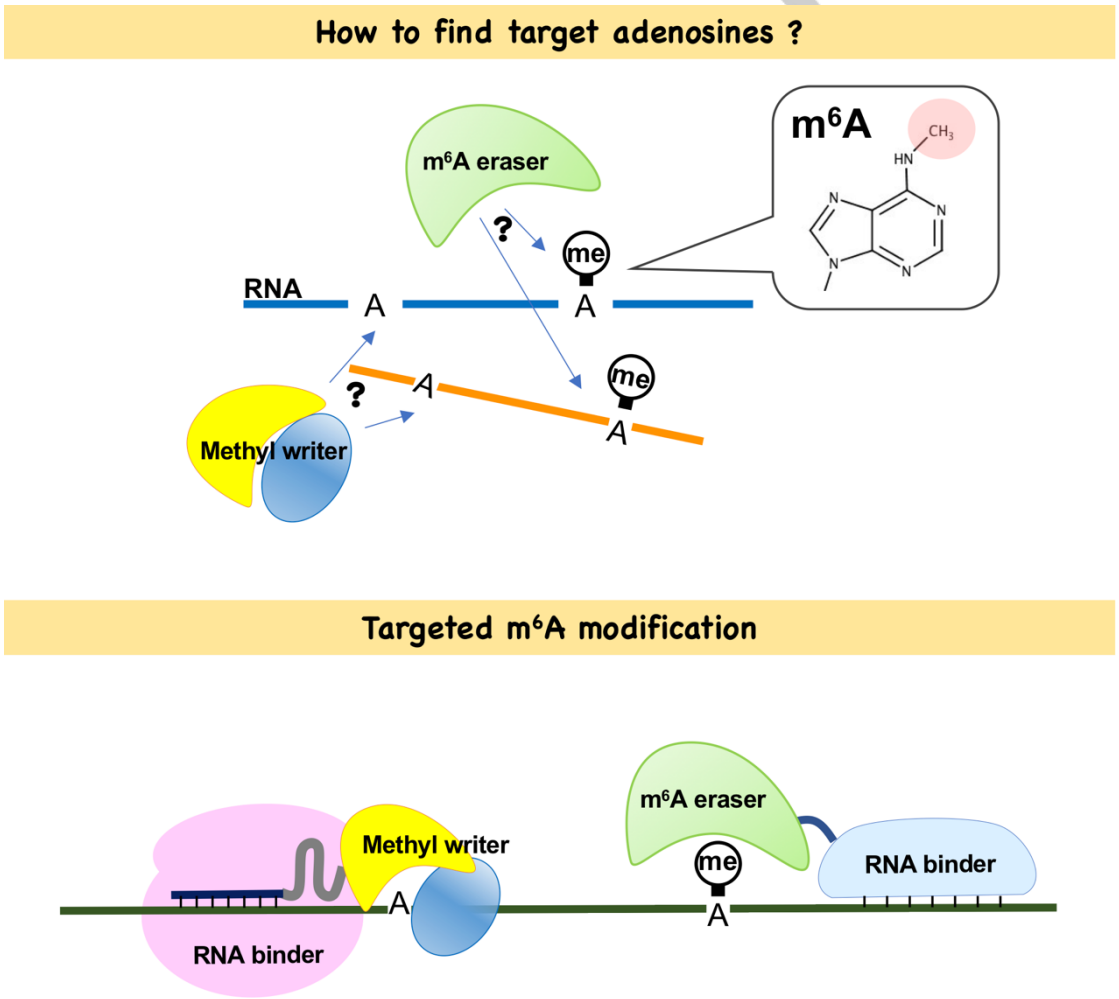
REVIEW

Mechanisms and strategies for determining m^{6}A RNA modification sites by natural and engineered m^{6}A effector proteins

Miki Imanishi*[a]



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Abstract: *N*^{6}-methyladenosine (m^{6}A) is the most common internal RNA modification in the consensus sequence of 5′-RRACH-3′. The methyl mark is added by writer proteins (METTL3/METTL14 metyltransferase complex) and removed by eraser proteins (m^{6}A demethylases; FTO and ALKBH5). Recognition of this methyl mark by m^{6}A reader proteins leads to changes in RNA metabolism. How the writer and eraser proteins determine their targets is not well-understood, despite the importance of this information in understanding the regulatory mechanisms and physiological roles of m^{6}A. However, approaches for targeted manipulation of the methylation state at specific sites are being developed. In this review, I summarize the recent findings on the mechanisms of target identification of m^{6}A regulatory proteins, as well as recent approaches for targeted m^{6}A modifications.

1. Introduction

More than 170 types of modified nucleotides in cellular RNA have been identified to date[1]. Among them, *N*^{6}-methyladenosine (m^{6}A), which was first reported approximately 50 years ago[2], is the most common internal RNA modification in mRNA and non-coding RNA. Adenosine *N*^{6}-methylation is catalyzed by the m^{6}A writer complex, which contains methyltransferase-like (METTL)3/METTL14 as the core. And m^{6}A eraser proteins, alkB homolog 5 (ALKBH5) or fat mass and obesity-associated protein (FTO), catalyze demethylation (Figure 1). m^{6}A reader proteins, including the YT521-B homology (YTH) domain family proteins, recognize m^{6}A and affect mRNA metabolism, such as pre-mRNA processing, mRNA stability, translation efficiency, and mRNA localization. Thus, m^{6}A modification is important in various biological phenomena, including differentiation[3, 4], tumorigenesis[5-7], the circadian clock[8], and viral infection[9].

In 1977, the methylation consensus sequence context was identified as (G/A)(m^{6}A)C[10]. Recent transcriptome-wide mapping of m^{6}A sites using m^{6}A-sequencing or methylated RNA immunoprecipitation-sequencing (MeRIP-seq) verified the consensus sequence as RR(m^{6}A)CH (R = A/G, H = A/C/U)[11, 12]. However, only ~5% of the consensus sequences are methylated within cells. m^{6}A is enriched around transcription start sites and stop codons, and at the 3′ untranslated region (UTR)[11, 12]. Although a co-transcriptional RNA methylation model has been suggested, it cannot fully explain the biased distribution of the RR(m^{6}A)CH sites. There may be multiple determinants in the selection of adenosine for methylation, including RNA methylation guided by intermolecular interactions and the intrinsic RNA binding preferences of the m^{6}A writer complex. The balance of methylation and demethylation is also reflected in the amount of m^{6}A sites detected. Interactions between the m^{6}A writer complex or eraser proteins with various proteins have been reported in various cell types, but how the installation and demethylation sites of m^{6}A modification are determined remains unclear. Identifying the determinants of target specificity is important for understanding the fundamental mechanism and physiological roles of m^{6}A modification. The target-finding mechanisms would also be helpful for designing targeted m^{6}A modification systems, as described below, to manipulate RNA modification in a site-specific and/or cellular state-specific manner.

In contrast to the poorly understood target site determination mechanisms of endogenous m^{6}A writers and erasers, tremendous progress has been made in developing artificial systems to control site-specific m^{6}A modifications, particularly along with the advancement of molecular tools using clustered regularly interspersed short palindromic repeat-CRISPR-associated (CRISPR-Cas). Multiple m^{6}As are often present in a single transcript, with each m^{6}A exerting an independent role. However, knockdown or overexpression of writer or eraser proteins has been performed in biological research to evaluate the involvement of m^{6}A in biological phenomena. Such treatment alters the overall methylation pattern of the transcript, and thus the contribution of individual m^{6}A modifications to the biological phenotype is unclear. Sequence-specific m^{6}A manipulation may clarify the role of a single m^{6}A residue in biological events and shows potential as a therapeutic strategy.

In this review, I focus on the methylation- or demethylation-targeting mechanisms of m^{6}A writer or eraser proteins. In addition, recent progress in designing molecular targeting tools for manipulating RNA methylation or demethylation is summarized.

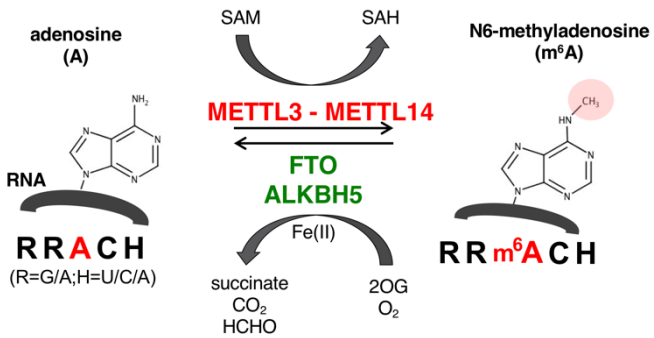

Figure 1. Reversible regulation of m^{6}A methylation in mRNA. The m^{6}A writer protein complex, with the METTL3/METTL14 methyltransferase heterodimer as a core, installs m^{6}A in the RRACH (R=A or G, H=A, C, or U) sequence. The Fe(II) and alpha-ketoglutarate (2-oxoglutarate, 2OG)-dependent dioxygenases FTO and ALKBH5, function as m^{6}A eraser proteins. SAM: *S*-adenosylmethionine; SAH: *S*-adenosylhomocysteine.

2. How do endogenous demethylases and methyltransferases find their target sites?

One of the most fundamental questions regarding m^{6}A regulation is how m^{6}A regulatory enzymes identify the target adenosine residues to be methylated or demethylated among the huge transcriptome. Extrinsic and intrinsic factors are thought to act as determinants of adenosine targeting [13]. Extrinsic factors include proteins and non-coding RNAs (ncRNAs) that recruit m^{6}A regulatory enzymes to the target sites as a complex. These mediators not only determine the basal level of m^{6}A, but also cause transient or localized changes in m^{6}A modifications dependent on the cellular state. Intrinsic factors include the RNA sequence preference of the m^{6}A regulatory enzymes themselves, as they are fundamentally nucleic acid-interacting proteins. The intrinsic factors contribute to regulating overall m^{6}A levels dependent on enzyme levels. Although the m^{6}A demethylases FTO and ALKBH5 contain RNA-binding surfaces with multiple positively charged amino acid residues, they do not contain independent RNA-binding domains. In contrast, METTL14, an essential component of the methyltransferase complex, has an independent RNA-binding domain.

2.1. Extrinsic factor-guided regulation of m^{6}A demethylation and methylation.

2.1.1. m^{6}A writers: METTL3/METTL14 heterodimer

The process of m^{6}A modification is catalyzed by the m^{6}A writer complex composed of the METTL3/METTL14 heterodimer and additional adaptor proteins, such as Wilms' tumor 1-associating protein (WTAP), Vir-like m^{6}A methyltransferase associated (VIRMA), and RNA-binding motif protein 15/15B (RBM15/15B) (Figure 2a). METTL3 functions as a catalytic core, whereas METTL14 is catalytically inactive but contributes to the maintenance of complex integrity and RNA binding[14-16]. WTAP is important for the localization of the METTL3/METTL14 heterodimer to the nuclear speckle[17, 18]. VIRMA is critical for recruiting the m^{6}A writer complex to catalytic sites near the stop codon at the 3′UTR, where m^{6}A is enriched, by associating with polyadenylation cleavage factors CPSF5 and CPSF6[19]. RBM15/15B binds to U-rich sequences and recruits the m^{6}A writer complex to U-rich regions close to target adenosines[20]. Furthermore, direct interactions of the methyltransferase complex with H3K36me3 and RNA polymerase II have been reported, indicating co-transcriptional RNA methylation[21-23]. These interactions contribute to the installation of m^{6}A in nascent transcripts in the nucleus (Figure 2a). However, not all methylated RRACH sites are in the 3′-UTR or near U-rich regions. Other mechanisms for determining methylation sites may also exist.

As examples in which the cellular states impact m^{6}A installation on selective transcripts, the interaction between METTL3/METTL14-WTAP and SMAD family member 2/3 (SMAD2/3) in response to transforming growth factor β (TGFβ) was suggested in human embryonic stem cells and human induced pluripotent stem cells[24]. In acute myeloid leukemia cells, METTL3 was shown to interact with a transcription factor CCAAT/enhancer-binding protein zeta (CEBPZ)[25]. These interactions contribute to co-transcriptional m^{6}A installation to cell-type specific transcripts.

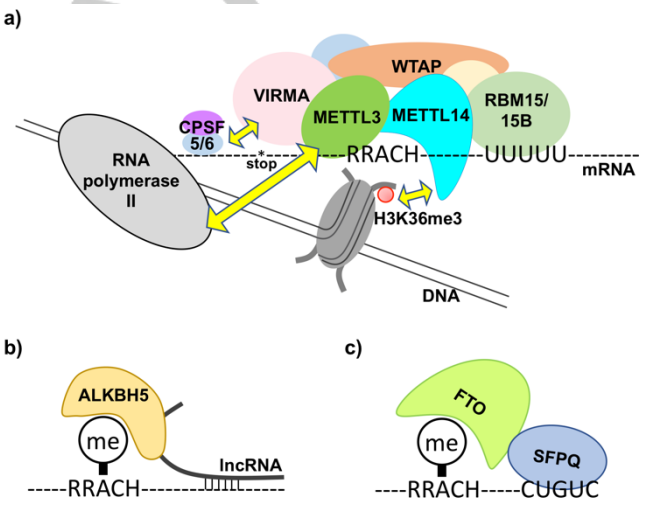

Figure 2. Mediator-guided regulation of m^{6}A demethylation and methylation. (a) METTL3/METTL14 heterodimer interacts with multiple proteins including RBM15/15B, which binds to U-rich sequences, and VIRMA, which interact with polyadenylation cleavage factors CPSF5 and CPSF6, resulting in methylation close to the U-rich sequences and close to the 3′ untranslated region (UTR) and stop codon. Direct interaction of the METTL3/METTL14 heterodimer with RNA polymerase II along with H3K36me3 histone modification comprise the transcription-coupled m^{6}A RNA methylation model. (b) ALKBH5 interacts with lncRNAs to demethylate m^{6}A near lncRNA-binding sequences. (c) FTO interacts with SFPQ, an RNA-binding protein targeting CUGUC sequences, and demethylates bases close to its binding sequences.

2.1.2. m^{6}A erasers: FTO and ALKBH5

To date, two demethylases of m^{6}A, FTO and ALKBH5, have been identified. Both of these enzymes belong to Fe(II)- and alpha-ketoglutarate-dependent dioxygenases and demethylate m^{6}A, although FTO also demethylates *N*^{6},2′-O-dimethyladenosine (m^{6}A$_m$) in mRNA and small nuclear RNA[26, 27] and *N*^{1}-

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methyladenosine (m^1A) in tRNA[28]. Both ALKBH5 and the catalytic N-terminal domain of FTO contain the conserved jelly roll core of the AlkB family members[29, 30], although the C-terminal domain of FTO, which is indispensable for FTO activity but catalytically inactive, is unique[31, 32]. Despite their similar catalytic structures, the biological functions and target m^6A of ALKBH5 and FTO are different. The expression levels of FTO and ALKBH5 also differ among tissues and are most abundant in the brain[33] and testis[34], respectively. Although differences in the distribution of demethylases may be related to their different target sites, the target-finding mechanisms at the molecular level are largely unknown.

As an example of the target-finding mechanisms of ALKBH5, it was reported that ALKBH5 interacts with the long non-coding RNA (lncRNA) antisense to FOXM1, which guides ALKBH5 to target m^6A sites on nascent transcripts of the transcription factor FOXM1 in glioblastoma stem-like cells (Figure 2b)[35]. This finding is physiologically meaningful because FOXM1 is among the most important molecules in glioblastoma stem-like cells. Another lncRNA, SOX2OT, recruits ALKBH5 to demethylate the SOX2 transcript, leading to enhanced SOX2 expression and resulting in poor prognosis in glioblastoma[36]. Although the relationship between the interaction and demethylation activity remains unclear, ALKBH5 was shown to interact with circular RNAs [37], which are generated through pre-mRNA back-splicing and contain covalently bonded loops[38]. How ALKBH5 recognizes specific non-coding RNAs requires further analysis, but various types of non-coding RNAs may be key for recruiting ALKBH5 to specific m^6A sites for demethylation.

During vesicular stomatitis virus infection, ALKBH5 interacts with DEAD-box helicase 46 (DDX46) between the nucleotide-recognition NRL domain of ALKBH5 and DDX46 DEAD helicase domain [39]. This interaction is increased after viral infection, and innate immunity is inhibited. It has been suggested that DDX46 recruits ALKBH5 to demethylate m^6A of the DDX46-targeted antiviral transcripts, including *Mavs*, *Traf3*, and *Traf6* mRNA, which contain the DDX46 binding sequence CCGGUU. These demethylated antiviral transcripts were entrapped in the nucleus, resulting in attenuation of interferon production. The same group reported that FTO associates with heterogeneous nuclear ribonucleoprotein A2/B1; this interaction dissociates after herpes simplex virus-1 infection, resulting in a strong antiviral response [40]. Because m^6A modification is important in virus infection and host cells immune responses[41, 42], the interaction between RNA-binding proteins and m^6A effector proteins after virus infection may be a general model for identifying the locus of substrate m^6A.

A protein-mediated target-finding model of FTO was proposed by Song et al.[43]. The direct interaction partner proteins of FTO were identified using co-immunoprecipitation assays and a genetically encoded site-specific photo-crosslinking strategy [44, 45]. Among the candidate proteins, an RNA-binding protein, splicing factor, proline- and glutamine-rich (SFPQ), was suggested to interact with the C-terminal domain of FTO, which is unique to FTO compared to ALKBH5. Cross-linking and immunoprecipitation sequencing (CLIP-seq) data for FTO and SFPQ indicated that approximately 20% of FTO target sites were within 200-nucleotide (nt) of SFPQ target sites. In addition, overexpression of SFPQ in HeLa cells decreased the methylation levels of adenosines near the SFPQ-binding sequence CUGUC. Thus, SFPQ-mediated m^6A demethylation by FTO is a possible mechanism for determining the FTO demethylation sites (Figure 2c). Other RNA-binding proteins can also help FTO to locate the substrate m^6A sites.

2.2. Intrinsic sequence preference of METTL3/METTL14 methyltransferase

The above-mentioned protein-protein interaction-mediated RNA methylation occurs co-transcriptionally in the nucleus. In contrast, the RNA genomes of *Flaviviridae*, including the Zika virus (ZIKV), dengue virus, West Nile virus, yellow fever virus, and hepatitis C virus, contain m^6A modifications mediated by METTL3/METTL14[46, 47]. Because flaviviruses are positive-sense single-stranded RNA viruses replicated in the cytoplasm, the m^6A machinery also functions in the cytoplasm via different mechanisms from co-transcriptional RNA methylation in the nucleus. These results suggest the existence of unknown factors that determine the specificity of particular RRACH sites.

a)

	K_D (nM)	
RNA	K^+ buffer G4-folded condition	Li^+ buffer G4-nonstabilized condition
G4 RNA*	35 ± 12	131 ± 30
Non-G4 RNA**	209 ± 94	149 ± 11

*: 5′-UUA**GGG**UUA**GGG**UUUUAGACAUUUUUUA**GGG**UUA**GGG**-3′
**: 5′-UGAGUGUGAGUGUUUUAGACAUUUUUGAGUGUGAGUG-3′

b)

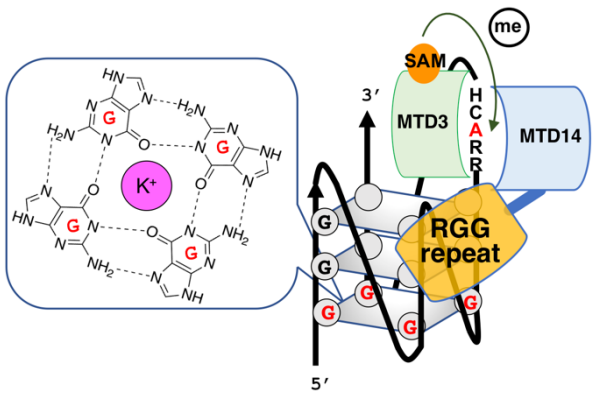

Figure 3. Intrinsic sequence preference of METTL3/METTL14 heterodimer to G4 structure RNA. (a) Dissociation constants of MTD3 and MTD14 heterodimer with the RGG domain[58]. Note that the G4 RNA oligonucleotide forms the G4 structure in K^+ buffer but not in Li^+ buffer. (b) Schematic representation of preferential m^6A methylation close to the RNA G4-structure. The inset shows a G-tetrad plane. In G4 structures, multiple G-tetrad planes are stacked and stabilized by K^+.

METTL14 contains an arginine (R)-glycine (G)-rich RNA-binding domain, RGG-repeat motif, at the C-terminus [48]. Mutants of the RGG domain of METTL14 greatly reduced the RNA-binding affinity of the METTL3/METTL14 heterodimer, indicating that the RGG domain of METTL14 contributes to the direct interaction between the methyltransferase complex and RNA[18, 23]. The

RGG-repeat motif is a typical RNA G-quadruplex (rG4)-binding motif, as exemplified by fragile X mental retardation protein and FUS/TLS[49-53]. rG4s are non-canonical four-stranded structures generally formed by G-rich sequences. In rG4 structures, two or more planar G-tetrads formed via Hoogsteen base pairing are stacked and stabilized through coordination with potassium ions[54, 55]. Bioinformatic analyses suggested that m^{6}A and potential G4-forming sequences are well-colocalized in viral RNA (ZIKV and HIV) and at human pre-mRNA intron splice sites [56, 57]. Therefore, the intrinsic RNA-binding preference of the RGG domain of METTL14 was recently examined, which revealed a binding preference for rG4 structures[58]. The affinity of the methyltransferase domain (MTD) heterodimer derived from METTL3 and METTL14, MTD3/MTD14-RGG, was higher for G4-forming RNAs than for non-G4-forming RNAs in the presence of potassium ions that stabilize the rG4 structure of G4-forming RNAs (Figure 3a). Preferential RNA methylation near rG4 structures was also demonstrated *in vitro* in the presence of nonspecific miscellaneous RNA extracted from HeLa cells. The results suggest that METTL3/METTL14 are recruited to specific methylation sites, specifically those close to G4-forming regions, within many RRACH sequences (Figure 3b). In contrast, arginine residues in the RGG domain of METTL14 were methylated by the protein arginine methyltransferase 1, enhancing METTL14-RNA interactions within cells[23]. Further studies are needed to clarify the mechanism of m^{6}A installation by the m^{6}A writer complex composed of the METTL3/METTL14 heterodimer and adaptor proteins.

3. How to manipulate m^{6}A modifications at specific target sites?

The importance of m^{6}A RNA methylation in various physiological processes, including differentiation[3, 4], tumorigenesis[5-7], and viral infection[9] has been demonstrated through down- or upregulation of the expression levels of m^{6}A regulatory enzymes. However, the functions of locus-specific m^{6}A are unclear following treatments that change overall RNA methylation states within the huge transcriptome. New approaches were recently reported to regulate mRNA methylation states at specific sites. Modular RNA-binding proteins and CRISPR-Cas-based RNA-targeting technologies have been developed to target a specific mRNA site to achieve programmable post-transcriptional regulation (Figure 4).

3.1. Pumilio and FBF homology protein

Naturally occurring Pumilio and FBF homology protein (PUF) family proteins regulate mRNA stability and translation by binding to the 3′UTR of mRNAs. The RNA-binding domain of PUF proteins consists of 8-tandemly repeated structural modules and binds to 8 nt of RNA[59, 60]. Because each repeat consisting of approximately 36 amino acid residues recognizes a single nucleotide at specific amino acid residues, artificial RNA-binding proteins targeting various 8-nt sequences can be designed by simply substituting the amino acid residues[61-66]. Furthermore, by connecting multiple RNA-binding repeats, engineered PUF proteins can be designed to bind to more than 8 nt, which would satisfy the requirement for specificity to the target locus within the transcriptome[64, 67-69]. This programmable RNA-binding mode has been used for sequence-specific control of RNA metabolism (Figure 4a) [70]. Fusion proteins of PUFs with the RNA decay factor, tristetraprolin, significantly repressed protein production from mRNA containing PUF-binding sites at the 3′UTR[69, 71, 72]. The combination of translational activators or repressors resulted in PUF-dependent translational activation of endogenous mRNA[61, 73]. The control of mRNA-specific splicing has also been achieved using fusion proteins of PUFs and splicing factors[63, 74]. Fluorescent protein-fused PUFs enabled visualization of endogenous RNA[75-80].

The construction of recombinant vectors expressing engineered PUF proteins requires more time than constructing CRISPR-Cas systems, which will be discussed later. However, the advantages of using PUFs include the small size of the PUF expression vector compared to genes of large Cas-fused proteins that are difficult to pack into adeno-associated virus vectors and the simplicity of the system without additional guide RNA. In addition, although CRISPR-Cas-based systems cannot be applied in mitochondria because of the difficulty of importing exogenous guide RNAs into mitochondria[81], PUF-based RNA manipulation can be achieved in mitochondria[80, 82, 83].

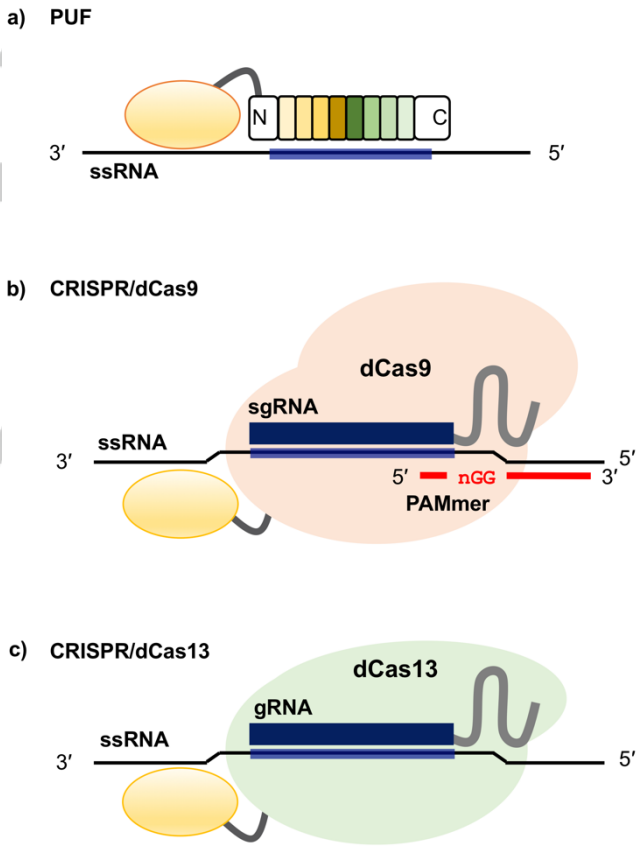

Figure 4. Sequence-specific manipulation of RNA metabolism using various functional fusion proteins with guide molecules to the desired specific sequences. Programmable RNA-binding proteins, PUFs, fused with functional proteins (a), dCas9-fused functional proteins together with sgRNA and PAMmer (b), and dCas13-fused functional proteins together with gRNA (c) can regulate RNA metabolism in a sequence-specific manner.

We created programmable RNA demethylation and methylation systems by combining PUF RNA-binding proteins with m^{6}A erasers or writer proteins[84] (Figure 5). PUFa, derived from the Pumillio 1 RNA-binding domain that binds to 5′-UGUAUAUA-3′, and PUFb, an engineered PUF protein that binds to 5′-UGGGGUUC-3′, were fused with m^{6}A regulatory enzymes. Target RNA oligonucleotides contain the PUFa- or PUFb-binding sequence close to the substrate m^{6}A or A nucleotide. PUF-dependent RNA demethylation and methylation were evaluated *in vitro* using a MazF-based m^{6}A detection assay[85]. The *Escherichia coli* MazF toxin is a 5′-ACA-3′ sequence-specific endoribonuclease[86, 87] that cleaves RNA containing 5′-ACA-3′ but not 5′-(m^{6}A)CA-3′ [85]. Based on this property, the demethylation or methylation activity of m^{6}A demethylases or methyltransferases can be evaluated by determining the ratio of cleaved or uncleaved RNA bands by MazF. FTO-PUFa and FTO-PUFb demethylate m^{6}A close to the PUFa- and PUFb-binding sequences, respectively, even at low concentrations; FTO alone did not perform demethylation at these concentrations (Figure 5a). Although FTO-PUFa efficiently demethylated m^{6}A at 6–10 nt from the PUF-binding site, it did not demethylate the m^{6}A overlapping with the PUFa-binding sequence, 5′-UGUAUAU(m^{6}A)-3′. This indicates that the fusion protein binds to the PUF-binding sequence and then demethylates m^{6}A close to the binding site. Sequence-specific methylation was achieved using the PUF-fused METTL14 methyltransferase domain (MTD14ΔRGG) and METTL3 (Figure 5b). Notably, the construct in which a PUF protein was fused with a METTL14 deletion mutant of the RGG domain showed higher sequence specificity to the target adenosine, compared to the construct with RGG-containing METTL14. Replacing the RNA-binding domain from the RGG motif with an artificial PUF protein altered the methylation target sequences.

PUFs have not been used to control RNA modification in cells. To achieve selectivity for specific endogenous mRNAs, it is desirable to use modified PUFs that can recognize longer sequences[64, 67-69]. Notably, PUFs can target RNAs in mitochondria, which cannot be achieved using CRISPR-Cas[83]. Although the presence of m^{6}A has been reported in plant mitochondrial transcripts [88, 89], its presence in mammalian cells has not been observed [90], but PUF has the potential to control mitochondrial RNA modifications.

3.2. CRISPR-dCas9

CRISPR-Cas9-based technology is an innovative gene-editing tool for binding and cleaving specific DNA sequences within the genome [91]. In addition to the original Cas9 nuclease, catalytically dead Cas9 (dCas9), which contains mutated key amino acid residues in RuvC and HNH nucleases, has been used to control the transcription and epigenomics of specific positions in the genome by fusing with transcriptional activators and histone-modifying enzymes. Based on these properties, the CRISPR-Cas9 system has become a genome editing tool that can manipulate DNA freely; however, CRISPR-Cas-based tools that can target RNA at will were not initially developed. Therefore, an innovative tool was developed for targeting RNA by utilizing the DNA-binding mechanism of CRISPR-Cas9. To bind the target DNA sequence corresponding to the designed single-guide RNA (sgRNA), Cas9 or dCas9 requires the protospacer adjacent motif (PAM) adjacent to the target DNA complementary sequence. *Streptococcus pyogenes* Cas9 complex with sgRNA can target specific RNA sequences by supplying an antisense oligonucleotide containing the PAM sequence, PAMmer (Figure 4b) [92, 93]. In principle, base pairing in two regions by sgRNA and PAMmer can minimize off-targeting of dCas9 and improve the specificity of RNA targeting. RNA-targeting Cas9 or “RCas9” has achieved sequence-specific RNA cleavage and visualization of specific RNA in live cells[92, 93].

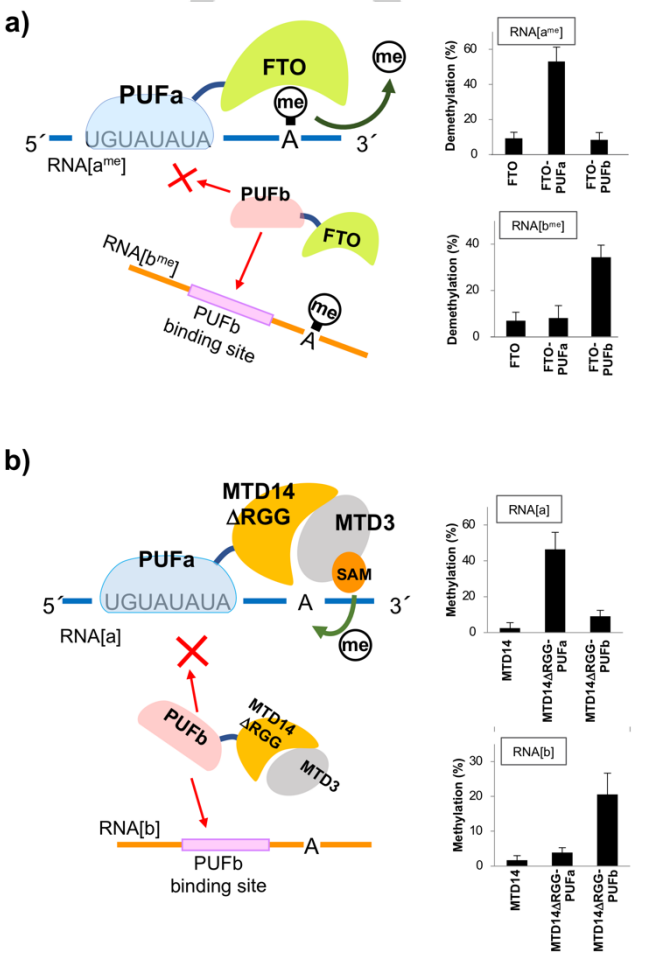

Figure 5. Sequence-specific m^{6}A manipulation using PUF RNA-binding domains. PUFa and PUFb, which bind different RNA sequences with each other, were fused with FTO (a) or MTD14ΔRGG (b). Demethylation of the RNA oligonucleotides, RNA[a^{me}] and RNA[b^{me}], containing m^{6}A close to the PUFa- and PUFb-binding sites, respectively, occurred by FTO-PUFa and FTO-PUFb, respectively[84] (a). Methylation of RNA oligonucleotides, RNA[a] and RNA[b], containing a GGACA methylation consensus sequence close to the PUFa- and PUFb-binding sites, respectively, occurred by the methyltransferase complex used with PUFa and PUFb, respectively[84] (b). Graphs are modified from reference 84.

Rau et al. reported the concept of sequence-specific RNA demethylation *in vitro*. They designed an FTO and dCas9 fusion protein for use with a PAMmer and sgRNA complementary to RNA sequences close to the targeted m^{6}A [94]. Sequence-specific demethylation by RCas9-FTO was confirmed using site-specific cleavage and radioactive labeling followed by ligation-assisted extraction and thin-layer chromatography (SCARLET) analysis [95].

Shu-Bing Qian's group succeeded in controlling sequence-specific RNA methylation and demethylation in living cells [96]. The engineered m^{6}A writer, M13M14-dCas9, was prepared by fusing dCas9 with tandemly connected single-chain MTDs comprised of METTL3 (369–580) and METTL14 (116–402) (Figure 6a). Because METTL3 and METTL14 MTDs form a tight heterodimer, the single-chain M13M14 was expected to interact intramolecularly and exert methyltransferase activity. Site-specific m^{6}A modification of endogenous mRNA was achieved via cotransfection of sgRNA and PAMmer with M13M14-dCas9. The authors also achieved site-specific m^{6}A demethylation using ALKBH5-dCas9 and FTO-dCas9 fusion proteins.

Because of the large size of the Cas9 protein (1367 aa) and need for synthetic 2′-OMe PAMmer oligonucleotides with short lifetimes, RNA targeting tools are shifting from Cas9-PAMmer-systems to Cas13-based systems. However, CRISPR-dCas9-based m^{6}A editing tools were innovative during the budding phase of m^{6}A editing and had important impacts in the field, as described below.

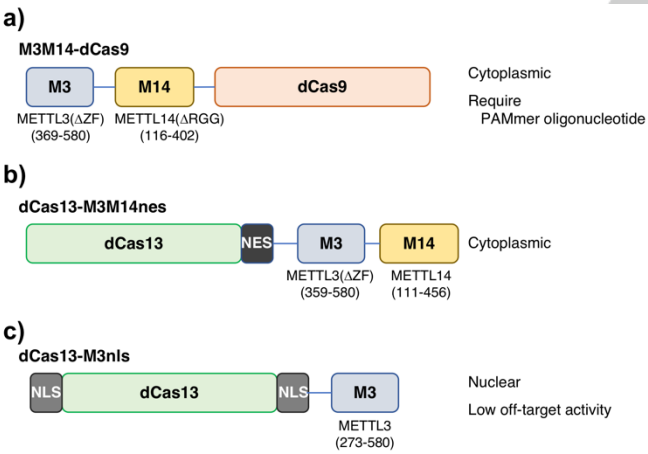

Figure 6. Targeted RNA methylation systems. M3M14-dCas9[96] (a), dCas13-M3M14nes[106] (b), and dCas13-M3nls[106] (c).

3.3. CRISPR-dCas13

Cas13 belongs to the type VI CRISPR-Cas system, which is an RNA-guided RNA-targeting CRISPR-Cas [97, 98]. Engineered CRISPR-Cas13 systems can cleave specific transcripts [99-102]. In contrast to the RCas9 system, which requires a synthetic PAMmer oligonucleotide in addition to sgRNA, Cas13 only requires a guide RNA (gRNA) (Figure 4c). Similar to dCas9, dCas13 has been used as an RNA-targeting tool fused with various functional proteins that regulate gene expression. *Prevotella* sp. (Psp)dCas13 fused with engineered RNA-editing enzymes achieved sequence-specific A-to-I and C-to-U RNA editing [101, 103, 104]. CasRx, an engineered CRISPR-Cas13d effector derived from *Ruminococcus flavefaciens* strain XPD3002, which has the smallest size (~930 aa) among Cas13 effectors described to date [105], was also applied for targeted RNA regulation. Combining dCasRx with heterogeneous nuclear ribonucleoproteins (hnRNPs), a negative splice factor, resulted in targeted exon skipping [105]. m^{6}A effector enzymes have also been combined with dCas13s to manipulate methylation and demethylation in a sequence-specific manner, as described below.

A targeted RNA methylation system (TRM) was reported by David Liu's group [106]. dPspCas13b was fused with single-chain truncated METTL3/14 (M3M14) or truncated METTL3 (M3), and with two nuclear localization signal (nls) or a nuclear export signal (nes) sequences (Figure 6b, c). To minimize off-target methylation derived from the intrinsic RNA-binding ability of the writer complex, M3 (METTL3 (359–580)) lacking a zinc finger domain was used in dCas13-M3M14 constructs. In contrast, M3 of dCas13-M3 -nls or -nes contains a zinc finger domain; however, instead, METTL14, which is primarily involved in RNA binding in the METTL3/METTL14 complex, is excluded from the dCas13-M3 constructs. Among the four constructs with different combinations of the methyltransferase domain and localization signal sequences, dCas13-M3nls and dCas13-M3M14nes achieved a marked increase in m^{6}A abundance located at 8–15 nt 3′ to the guide RNA-binding site in endogenous mRNAs in HEK293T cells compared to in samples with coexpression with non-target guide RNA. M3M14-dCas9 also showed a similar on-target methylation efficiency as dCas13-M3nls and dCas13-M3M14nes. However, compared to methyltransferase-inactive controls, off-target methylation significantly differed between the three TRM editors in transcriptome-wide analysis by MeRIP-seq. Among them, dCas13-M3nls induced the least off-target methylation (~3% of >21,000 m^{6}A peaks), whereas dCas13-M3M14nes and M3M14-dCas9, localized in the cytoplasm, induced a substantial degree of nonspecific methylation (10–20%). Because of the different components and localization of these TRM editors, it is difficult to identify the key factors that enhance on-target specificity. Nonetheless, dCas13-M3nls is the most promising candidate tool for targeted RNA methylation.

The fusion between dCas13b and METTL14, dCas13b-M14, also achieves site-specific adenosine methylation *in vitro* and in HEK293T cells [107]. The enzyme-assisted chemical labeling assay, in which allyl-Se-adenosylmethionine is used rather than *S*-adenosylmethionine, clarified the site-specific methylation corresponding to each gRNA at single-base resolution. dCas13b-M14 used with METTL3 *in vitro* showed a significant increase in the methylation level of adenosine located at 1 nt 3′ to the gRNA binding site.

A targeted RNA demethylation system fusing ALKBH5 to the N- or C-terminus of dPspCas13b (ALKBH5-dCas13, dCas13-ALKBH5) was also reported [108]. These proteins, together with gRNAs, decreased the m^{6}A level at the target sites. In this system, demethylation occurred using gRNAs at relatively long distances (100–3000 nt) from the targeted m^{6}A.

Leptotrichia wadei (Lwa)dCas13a [99] was also used for site-specific m^{6}A demethylation combined with the catalytic domain of ALKBH5, creating dCas13a-ALK [109]. Furthermore, dCasRx was used for site-specific m^{6}A modification in combination with METTL3 or ALKBH5 [110]. A limitation of the CRISPR-Cas system is the large size (>1000 aa) of the Cas proteins, which complicates its packaging into viral vehicles to deliver the dCas-fusion gene

into cells. Using dCasRx facilitates *in vivo* application of the m^{6}A modification systems.

In addition, the site-specific m^{6}A regulatory system shows further progress, including innovations to increase demethylation efficiency by employing the SunTag system [111, 112], and stimulation-responsive methylation or demethylation systems [109, 113, 114]. In the former, dCas13b was fused to ten copies of the GCN peptide. Site-specific demethylation was achieved by recruiting ALKBH5 or FTO fused with the scFv-GCN4 antibody [112]. In the latter, *Porphyromonas gulae* (Pgu)dCas13a and M3M14 or FTO were fused with CIBN and CRY2PHR, respectively, to achieve photoactivatable and reversible regulation of site-specific m^{6}A modification [113], as CIBN and CRY2PHR dimerize upon light stimulation [115, 116]. Integration of abscisic acid (ABA)-based chemically induced proximity technologies and dCas13-based m^{6}A regulatory system also enabled inducible and reversible control of m^{6}A modification[114].

The contribution of these systems to biology is also increasing. A targeted RNA m^{6}A erasure system controlled by a Tet-On promoter suggested that a single m^{6}A site of SOX2 mRNA is involved in human embryonic stem cell differentiation [109]. Demethylation of m^{6}A-modificed chromosome-associated regulatory RNAs (carRNAs) by dCas13b-FTO indicated a role for carRNAs m^{6}A in the tuning of the global chromatin state [117]. The importance of m^{6}A in class switch recombination in B lymphocytes has also been suggested using dCas13b-FTO and dCas13b-ALKBH5 [118]. Targeted demethylation of PLOD2 mRNA and MYC mRNA m^{6}A has been shown to inhibit the progression of renal cell carcinoma and bladder cancer, respectively, providing a novel strategy for cancer therapy [119, 120].

4. Summary and outlook

The importance of m^{6}A modification in various biological events has been reported following epitranscriptome analysis of the m^{6}A position using anti-m^{6}A antibodies [11, 12]. This review summarizes recent advances in the target finding mechanisms of endogenous m^{6}A effector proteins, writers and effectors, and targeting strategies to manipulate RNA methylation using artificial m^{6}A editors. Although various partner proteins and ncRNAs have been discovered as guides, one of the most fundamental questions, “how do m^{6}A writers and erasers determine the modification sites?”, remains unclear. Identification of the interactive partners with m^{6}A writers and erasers or direct targets of the enzymes would lead to the discovery of potential drug targets, which should be further examined.

In contrast, targeted m^{6}A regulation systems have undergone dramatic development in recent years. However, because most systems employ fully active m^{6}A writers or erasers as fusion partners of sequence-specific RNA guide molecules such as PUF and dCas13, there is concern of potential off-target effects, particularly when the expression levels of m^{6}A editors are high. Intentionally reducing the RNA-binding properties of m^{6}A writers and erasers and relying on sequence-specific RNA-targeting guide molecules for the RNA-binding capacity of the m^{6}A editors is one strategy for increasing the specificity of m^{6}A editors to their on-target sites. In addition, although the on-target specificity of the m^{6}A editor has been demonstrated compared with limited non-specific sites, the entire transcriptome, as demonstrated in the evaluation of the TMR system [106], should be analyzed for reliable application in biology and medicine. Furthermore, to flexibly apply m^{6}A editors to various target sites, it is necessary to establish design guidelines for optimal guide RNAs in individual systems, considering the distance to the target adenosine and structure of the RNA. These engineered systems will play an important role not only in biomedical research but also as novel therapeutic tools.

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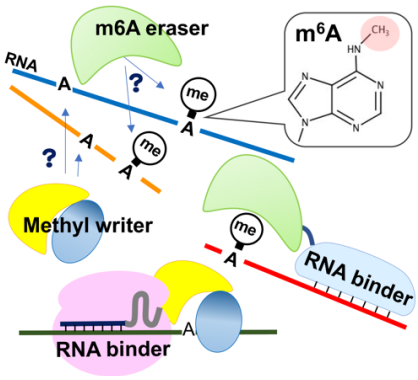

How do the writer and eraser proteins determine the targets adenosine? How are site-specific m^6A modifications manipulated? In this review, the mechanisms and strategies for targeted m^6A modification by natural and engineered proteins are summarized.

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