Point of View

Human m^{6}A writers: Two subunits, two roles

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Abstract

Cellular RNAs with diverse chemical modifications have been observed, and *N*^{6}-methyladenosine (m^{6}A) is one of the most abundant internal modifications found on mRNA and non-coding RNAs, playing a vital role in diverse biological processes. In humans, m^{6}A modification is catalyzed by the METTL3-METTL14 methyltransferase

complex, which is regulated by WTAP and another factor. Three groups have recently and independently reported the structure of this complex with or without cofactors. Here, we focus on the detailed mechanism of the m^{6}A methyltransferase complex and the properties of each subunit. METTL3 is predominantly catalytic, with a function reminiscent of *N*^{6}-adenine DNA methyltransferase systems, whereas METTL14 appears to be a pseudomethyltransferase that stabilizes METTL3 and contributes to target RNA recognition. The structural and biochemical characterization of the METTL3-METTL14 complex is a major step toward understanding the function of m^{6}A modification and developing m^{6}A-related therapies.

Key Words

M^{6}A, Methyltransferase, METTL3, WTAP, Epigenetics

Main Text

To date, approximately 140 types of chemical modification have been identified in RNA.[1] Most modifications have been observed on rRNA and tRNA, whereas mRNA modification was considered rare. Nevertheless, several modifications have recently been identified on mRNA including N^6-methyladenosine (m^6A),[2, 3] N^1-methyladenosine (m^1A),[4] inosine (I),[5] 5-methylcytosine (m^5C),[6] and pseudouridine (ψ).[7, 8] Among these, m^6A is the most prevalent modification on mRNA and long noncoding RNA. In 2012, two groups independently identified thousands of m^6A sites on mammalian RNAs,[2, 3] driving research examining the epitranscriptome. Several studies have characterized the m^6A RNA landscape in organisms, including viruses,[9, 10] bacteria,[11] yeast,[12] and plants,[13] and these studies have identified the consensus sequence RRACH (R represents purine, A is m^6A and H is a non-guanine base), implying the functional importance of this modification. In fact, m^6A affects multiple cellular functions,[14, 15] including developmental regulation, the cell cycle, fate determination,[16, 17] and the heat-shock stress response[18] by affecting different stages of RNA metabolism such as RNA processing,[19, 20] stability,[21] and translation efficiency.[18, 22, 23]

Analogous to dynamic chemical modifications of DNA and protein, the m^6A RNA modification can be reversibly appended and removed by a methyltransferase and a demethylase (or "writer" and "eraser"), respectively (Fig. 1a). One m^6A writer, the METTL3 methyltransferase (formerly called MT-A70), was first identified as part of a ~200 kDa complex isolated from enzymatic mammalian cell nuclear extracts in 1997.[24] METTL3 was grouped into the DNA m^6A methyltransferase subfamily due to the conserved motif

[D/N/S/H]PP[Y/F/W] (Fig. 1b) and exhibits high sequence conservation among eukaryotes including yeasts, plants, *Drosophila* and mammals.[25] Importantly, disruption of METTL3 homologs causes severe developmental defects in yeasts and *Drosophila* and has a lethal phenotype in *Arabidopsis* and mice.[16, 26-28]

Recently, a second methyltransferase, METTL14, was identified as the other writer complex component.[29-32] Knockdown of *METTL14* leads to decreased m^{6}A levels in human cell lines,[30, 32] and the METTL14 methyltransferase domain is phylogenetically close to that of METTL3.[33] Interestingly, METTL3 and METTL14 were found to be associated in a global proteomic profiling and co-immunoprecipitation experiment.[31, 34] Consistent with *in vivo* observations, recombinant METTL3 and METTL14 form a stable heterodimer *in vitro*.[29, 32] Individually, METTL3 and METTL14 exhibit nearly undetectable methyltransferase activity, but the METTL3-METTL14 complex displays synergistic function. Why does the m^{6}A methyltransferase complex contain two conserved methyltransferase components, and what are the roles of each subunit in the complex? We recently reported crystal structures of the METTL3-METTL14 methyltransferase domains complex alone and bound to *S*-adenosylmethionine (AdoMet) or *S*-adenosylhomocysteine (AdoHcy).[35] Two other groups independently published nearly identical structures of the complex.[36, 37] On the basis of these structures, we suggest that METTL3 plays a catalytic role in the complex, whereas METTL14 is a pseudomethyltransferase that stabilizes METTL3 and contributes to RNA binding. Here, we focus on the detailed mechanism of the m^{6}A methyltransferase complex and the properties of each component.

METTL3 primarily serves a catalytic role

The overall structures of both the METTL3 and METTL14 methyltransferase domains resemble dozens of class I DNA N^6 methyltransferase via DALI analysis.[38] Most of these contain one catalytic motif [D/N/S/H]PP[Y/F/W] located centrally in the methyltransferase domain.[33] Interestingly, in the AdoMet-bound METTL3-METTL14 complex structure, a single AdoMet molecule was positioned in the catalytic pocket of METTL3 but was not observed in METTL14. The AdoMet molecule is adjacent to the most conserved DPPW motif and coordinated by Asp in this motif via a hydrogen-bonding interaction. The mutation that substitutes Asp with Ala completely abolished AdoMet binding and enzyme activity *in vitro*, reinforcing *in vivo* mutagenesis studies, in which a mutated METTL3 (DPPW replaced by APPA) altered circadian clock speed in mammalian cells.[39] Additionally, an IME4 (METTL3 homolog in *Saccharomyces cerevisiae*) mutant encoding a D384A mutation displayed meiotic defects.[28] Together with these data, we speculate that METTL3 primarily plays a catalytic role in the complex using a similar mechanism to DNA N^6-methyladenine transfer of a methyl group to a target adenosine: the aromatic residue [Y/F/W] stacks with the target base via π–π interactions, and the side chain of the polar residue [D/N/S/H] and the carbonyl oxygen of the proline donate two hydrogen bonds to the 6-amino group of adenine, priming the S_N2 chemical reaction by increasing its negative charge.

Sequence analysis indicated that the N-terminus of METTL3 contains two Cys-Cys-Cys-His (CCCH)-type zinc finger (ZnF) motifs common in RNA-binding proteins (Fig. 1b).[33] The crystal structures of the METTL3-METTL14 complex contain only methyltransferase

domains without the ZnF motifs, highlighting the flexibility of these regions. However, the crystallized truncation showed no detectable methyltransferase activity, whereas truncations containing ZnF motifs exhibited comparable activity to the full-length complex, suggesting a critical role for ZnF motifs (Fig. 1c). In contrast, deleting the N-terminal and C-terminal motifs of METTL14 had negligible effects. Several CCCH-type ZnF protein structures in complex with target RNAs have exhibited direct interactions between the ZnF motif and RNA.[40, 41] Accordingly, we hypothesized that the METTL3 ZnF motifs are necessary for methylation because they enhance interaction with substrate RNA. In the future, the appearance of these motifs and how they precisely recognize RNA sequences await to be investigated.

Is METTL14 a pseudomethyltransferase?

Although the methyltransferase domains of METTL3 and METTL14 share approximately 22% sequence identity and an almost identical topological structure, three pieces of data suggested that METTL14 is a pseudomethyltransferase in the complex. First, in the crystal structures, neither AdoMet nor AdoHcy is present in the METTL14 pocket. Second, the METTL3-METTL14 complex binds to the ligand in a 1:1 stoichiometric ratio as measured by isothermal titration calorimetry (ITC). Finally, there are moderately conserved EPPL sequences in METTL14 corresponding to the catalytic motif of METTL3, but the substitution of Glu to Ala had little effect on ligand binding and enzymatic activity.[35-37] The structure of METTL14 offers a possible explanation. METTL14 superimposes well onto METTL3 except for three loops with distinct conformations, which are referred to as gate loop1 (residues

192-211 in METTL14), interface loop (residues 265-284 in METTL14) and gateloop2 (residues 318-328 in METTL14).(Fig. 2a) Gate loop1 and gateloop2 of METTL3 contribute to coordination with AdoMet, constituting part of the catalytic center. Interestingly, both METT14 gate loops are longer than those of METTL3. For METTL14, seven residues of gate loop1 adopt a short helical conformation, resulting in a possible obstacle to ligand entry. (Fig. 2a) Gate loop 2 shows significant inward movement, which likely leads to closure of the ligand-binding pocket.

What is the function of METTL14 in the complex? We observed a positively charged groove between METTL3 and METTL14 adjacent to AdoMet using surface electrostatic potential analysis. At least six positively charged residues from METTL14—R245, R249, R254, R255, K297 and R298—are located in this groove. A complex with double mutations (K297E and R298E) showed moderately reduced RNA binding affinity and methyltransferase activity. [35]Consistent with this observation, other groups have biochemically shown that R298P in METTL14 alters the sequence specificity for the RNA substrate.[37] We further modeled the RNA-binding state of the complex using SAXS data. In the model, the RNA substrate appeared in the positively charged groove, indicating that METTL14 might contribute to RNA interaction.

Moreover, METTL3 and METTL14 are closely associated through an extensive buried interface, which explains the high stability of the complex. Indeed, without METTL14, METTL3 binds to AdoMet alone with a dissociation constant of approximately 47μM, 20-fold weaker than the wild-type complex (Fig. 2b). A crystallized truncation of the METTL3-METTL14 complex had a slight effect on AdoMet binding activity, suggesting that

METTL14 stabilizes METTL3 to enhance AdoMet binding activity. We hypothesize that METTL14 is a pseudomethyltransferase in the complex that primarily plays a stabilization role and provides a platform with METTL3 for RNA recognition. However, we cannot exclude the possibility that METTL14 exhibits methyltransferase activity after binding additional factors. METTL14 is not well conserved; in plants, METTL14 homologues contain the DPPW motif instead of the EPPL motif.[33] To date, METTL14 in plants is not known to associate with METTL3, leading us to speculate that there is a different m^6A writer system in plants.

WTAP and other regulators

Wilms' tumor 1-associating protein (WTAP) was identified as another component of the human m^6A methyltransferase complex. WTAP plays an important role in METTL3-METTL14 complex localization to the nuclear speckle.[31] WTAP knockdown significantly decreased global m^6A levels in human cell lines, indicating its importance in generating the distinct landscape of mRNA methylation at 5′ and internal sites.[30] In zebrafish, depletion of the WTAP homologue caused apoptosis and tissue differentiation defects.[31] Similar results were observed in *Arabidopsis*. The 37 kDa FKBP12-intereaction protein (FIP37), a homologue of WTAP, mediates m^6A modification to control shoot stem cell fate.[42]However, WTAP has no effect on enzymatic activity *in vitro* other than to change the preferred RNA substrate of the METTL3-METTL14 complex. Because domain information is not available, how WTAP regulates m^6A modification cannot be easily elucidated. Furthermore, additional regulators such as KIAA1429 involved in mRNA methylation have

been identified by proteomic screening.[30] Further investigation into their regulation of the m^6A writer complex will shed light on RNA epigenetics.

Future perspectives

These structures and biochemical data provide new insights into the m^6A mechanism in which METTL3 primarily serves as a catalytic subunit and METTL14 functions as a stabilizer. Together, the subunits provide an RNA binding platform. However, the absence of an RNA-bound structure precludes our understanding of how the writer complex specifically targets the RRACH sequence and of why only a small portion of RRACH motifs contain an m^6A modification.[43] Therefore, determination of the METTL3-METTL14 complex structure in the presence of RNA substrate and/or including ZnF motifs is a major goal. Some m^6A modifications are relevant to cancer and infectious diseases: METTL3 deletion leads to significantly reduced cell invasion in cancer cells andHIV-1 virus infectivity in T cells.[10, 44-46] Thus, on the basis of these structures, the development of an inhibitor-specific target for m^6A writers is one of the next important tasks.

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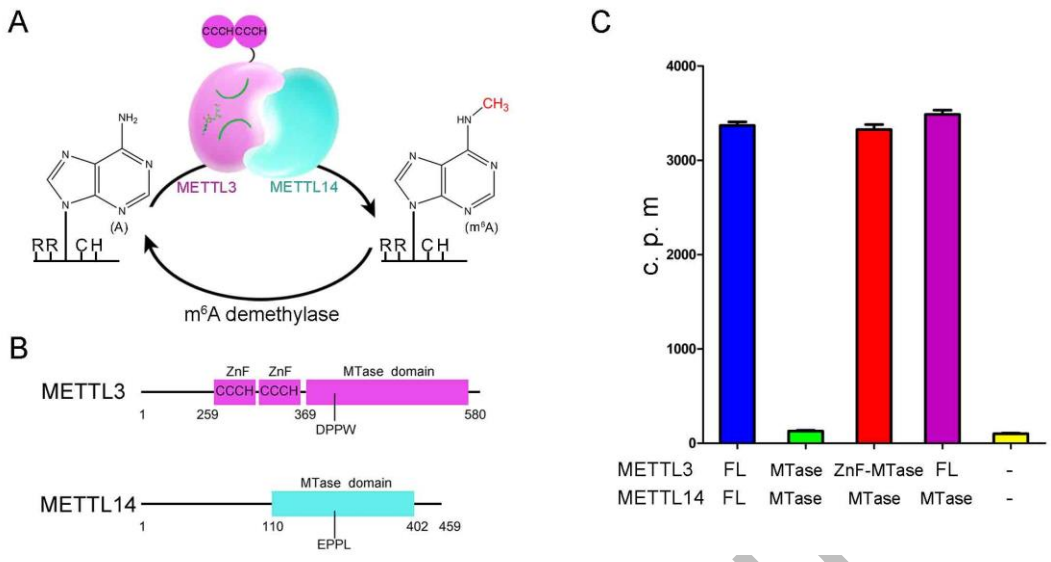

Figure 1

(**a**) Proposed model for reversible m^6A methylation. m^6A on RRACH is mainly appended by the METTL3-METTL14 complex, and oxidative demethylation is performed by m^6A demethylase. CCCH: Cys-Cys-Cys-His type zinc finger motif (ZnF). (**b**) Schematic illustration of METTL3 and METTL14 domain structures. The METTL3 ZnF and METTL3 methyltransferase (abbreviation as MTase) domains are colored in magenta, and the METTL14 methyltransferase domain is colored in cyan. Detailed domain boundaries are labeled beneath the structures. (**c**) Comparison of the methyltransferase activity of full-length (abbreviation as FL) and truncated methyltransferase complexes. The c.p.m. of the extracted RNA was measured in a scintillation counter and averaged (± s.e.m.); the c.p.m. was determined from three replicates.

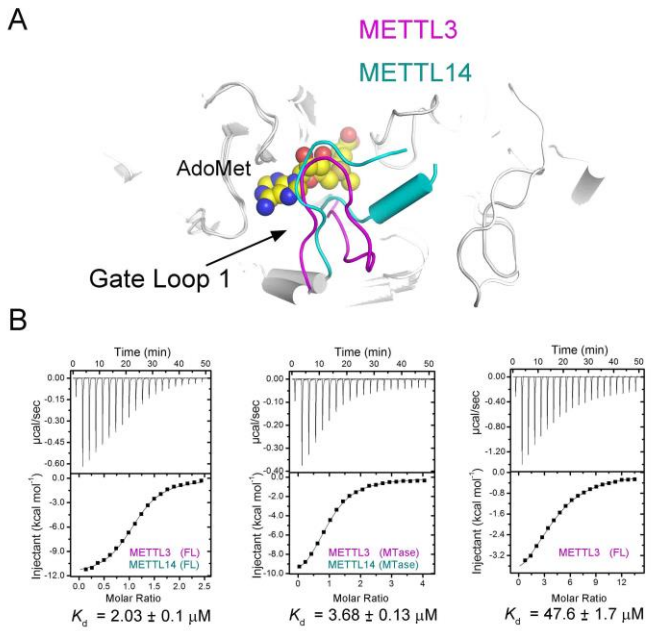

Figure 2

(**a**) Superposition of AdoMet-bound METTL3 and METTL14. AdoMet is shown in space-filling view. Gate loop 1 in METTL3 and METTL14 is colored in magenta and cyan, respectively. (**b**) Measurement of binding affinity between AdoMet and the METTL3–METTL14 complex using ITC. The first data point was removed from the analysis. The dissociation constant (K_d) of the wild type was approximately 2.0 µM. The AdoMet binding affinity of the methyltransferase domain complex was comparable to wild type. METTL3 alone exhibited significantly reduced AdoMet binding activity.