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Modulation of N-terminal Methyltransferase 1 by an N^6-methyladenosine-based Epitranscriptomic Mechanism

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Abstract

Protein α-N-methylation is an evolutionarily conserved type of post-translational modification; however, little is known about the regulatory mechanisms for this modification. Methylation at the N^6 position of adenosine in mRNAs is dynamic and modulates their stability, splicing, and translational efficiency. Here, we found that the expression of N-terminal methyltransferase 1 (NTMT1) protein is altered by depletion of those genes encoding the reader/writer/eraser proteins of N^6-methyladenosine (m^6A). We also observed that MRG15 is N-terminally methylated by NTMT1, and this methylation could also be modulated by reader/writer/eraser proteins of m^6A. Together, these results revealed a novel m^6A-based epitranscriptomic mechanism in regulating protein N-terminal methylation.

1. Introduction

Post-transcriptional modifications of mRNAs and post-translational modifications (PTMs) of proteins constitute evolutionarily conserved mechanisms of gene regulation. α-N-methylation of proteins was initially observed several decades ago [1, 2]. N-terminal methyltransferase 1 (NTMT1) was the first discovered enzyme for protein α-N-methylation [3], and a number of substrate proteins have been identified for NTMT1, including regulator of chromatin condensation 1 (RCC1), centromere proteins A and B (CENP-A and CENP-B), damage DNA-binding protein 2 (DDB2), and poly(ADP-ribose) polymerase 3 [3–8]. In this vein, N-terminal methylations of CENP-A and CENP-B promote the recruitment of kinetochore and binding of CENP-B to α-satellite DNA, respectively [6, 8, 9], and loss of function of NTMT1 could result in diminished DNA repair and elevated sensitivity to DNA damaging agents [5, 10, 11]. In addition, NTMT2 forms a heterodimer with NTMT1 and enhances its function in protein α-N-methylation [12].

NTMT1 is down-regulated in breast cancer tissues [10] and mutations disrupting the catalytic functions of NTMT1 were found in different types of cancer [13], suggesting its potential as a therapeutic target. Moreover, loss of function of NTMT1 was shown to elicit premature aging in mice [11]. Despite these discoveries, N-terminal methylation is still a

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

poorly understood form of PTM, where little is known about the regulatory mechanisms of this modification.

Recent studies revealed that the N^6 position of adenosine in RNA can be dynamically methylated and this methylation assumes important functions in normal development [14]; dysregulation of this methylation is implicated in many human diseases, including cancer and neurological disorders [15]. Methyltransferase-like 3 (METTL3), together with other regulatory subunits, including METTL14, RBM15, RBM15B, WTAP, VIRMA, and ZC3H13, were shown to constitute the major methyltransferase complex for m^6A generation [14, 16, 17]. m^6A in RNA can be recognized by a number of proteins, including the YTH domain-containing family proteins 1/2/3 (YTHDF1/2/3) [18–21]. In addition, m^6A in mRNA can be demethylated by FTO and ALKBH5 [22, 23].

In this study, we found that NTMT1 protein expression is subjected to regulation by m^6A reader, writer and eraser proteins. We also showed that these m^6A regulators could modulate the N-terminal methylation of MRG15 protein.

2. Materials and Methods

2.1. Plasmid preparation

The coding sequence of human *MRG15* gene was amplified from a cDNA library prepared from mRNAs isolated from HEK293T cells and cloned into the pRK7 plasmid between the XbaI and BamHI restriction sites, in which three tandem repeats of Flag epitope tag (DYKDDDDK) were fused at the carboxyl terminus. MRG15-K4Q mutant plasmid was amplified from the MRG15 plasmid with primers designed to contain the mutation.

2.2. Cell Culture

HEK293T human embryonic kidney cells (ATCC), and the isogenic *METTL3*$^{-/-}$, *FTO*$^{-/-}$, *ALKBH5*$^{-/-}$, *YTHDF1*$^{-/-}$, *YTHDF2*$^{-/-}$, and *YTHDF3*$^{-/-}$ cells, which were previously generated by CRISPR-Cas9 [24, 25], were cultured in Dulbecco's Modified Eagle Medium (DMEM; Fisher Scientific, Hampton, NH). All culture media were supplemented with 10% fetal bovine serum (FBS; Fisher Scientific, Hampton, NH), and 100 IU/mL penicillin/streptomycin (GE Healthcare, Chicago, IL). The cells were maintained at 37°C in a humidified environment containing 5% CO_2.

2.3. Preparation of Flag-tagged MRG15 protein and subsequent LC-MS/MS analysis

HEK293T cells and the aforementioned knockout cells were cultured in antibiotic-free media at a density of 5×10^6 cells/well. Plasmid for ectopic expression of Flag-tagged MRG15 (1 μg) was transfected into cells cultured in 6-well plates using Lipofectamine 2000 (Life Technologies, Carlsbad, CA). After a 24-hr incubation, the cells were harvested and lysed in CelLytic M (Sigma-Aldrich, St. Louis, MO), supplemented with a protease inhibitor cocktail (Sigma-Aldrich). C-terminally Flag-tagged MRG15 protein was isolated from the resultant lysates by affinity purification with anti-FLAG M2 beads (Sigma-Aldrich), and digested with Glu-C (NEB) at a protein/enzyme ratio of 10:1.

The Glu-C-produced peptides were analyzed by LC-MS/MS on an LTQ XL linear ion trap mass spectrometer equipped with a nanoelectrospray ionization source (Thermo Scientific, San Jose, CA) and with an Easy-nLC II. Separation was conducted by using a home-made trapping column (150 μm × 50 mm) and a separation column (75 μm × 120 mm), both of which were packed with ReproSil-Pur C18-AQ resin (3 μm in diameter, Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany). Peptide samples were initially loaded onto the trapping column with a mixture of 0.1% formic acid in CH_3CN/H_2O (2:98, v/v) at a flow rate of 5.0 μl/min. The peptides were separated using at 40-min linear gradient of 2–40% acetonitrile in 0.1% formic acid, and the flow rate was 300 μl/min. The mass spectrometer was operated in the positive-ion mode with a spray voltage of 1.8 kV. The full-scan mass spectra were acquired in the *m/z* range of 150–1000. MS/MS were recorded in a selected-ion monitoring mode, where the doubly protonated ions of unmodified, mono-, di-, and tri-methylated forms of N-terminal peptide of MRG15 were chosen for fragmentation. All data were analyzed manually.

2.4. siRNA knockdown of NTMT1

The control siRNA and human NTMT1 SMARTpool siRNA were obtained from Thermo Scientific. Sequences of NTMT1 SMARTpool siRNA were GCGAGGUGAUAGAAGACGA, AGGUGGAUAUGGUCGACAU, UGAGGGAAGGCCCGAACAA and GGACUGUGGAGCUGGCAUU. HEK293T cells were cultured in 6-well plates in antibiotic-free medium at a density of 5×10^5 cells per well for 24 hr, and each well of cells were transfected with 100 pmol siRNA using Lipofectamine 2000 (Invitrogen).

2.5. Western Blot Analysis

HEK293T cells and the isogenic CRISPR knockout cells were lysed at 50–80% confluency with CelLytic M, supplemented with a protease inhibitor cocktail (Sigma-Aldrich). The protein concentrations of the resultant lysates were determined with Bradford Reagent (Bio-Rad, Hercules, CA), and the whole cell lysate (12 μg) was denatured by boiling in Laemmli loading buffer (Bio-Rad). The lysate was resolved with SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad) at 4°C overnight. The resultant membrane was blocked with PBS-T (PBS with 0.1% Tween 20) supplemented with 5% powdered milk (Bio-Rad) at room temperature for 45 min, and subsequently incubated with primary antibody at room temperature for 2 hr, and then with secondary antibody at room temperature for 1 hr. The HRP signal from Amersham ECL Select western blotting detection reagent was then recorded (GE Healthcare, Chicago, IL). Antibodies recognizing human NTMT1 (Abcam, Cambridge, United Kingdom, ab72660, 1:2,500 dilution) and GAPDH (Santa Cruz Biotechnology, Dallas, TX, sc-32233, 1:5,000) were used as primary antibodies for Western blot analyses. Anti-rabbit IgG peroxidase antibody (Sigma-Aldrich, A0545, 1:10,000 dilution), and anti-mouse IgG kappa binding peroxidase antibody (Santa Cruz Biotechnology, sc-516102, 1:5,000 dilution) were employed as secondary antibodies.

3. Results and Discussion

3.1. The expression of NTMT1 protein is subjected to regulation by m^{6}A reader, writer and eraser proteins

We set out to investigate if the expression of NTMT1 protein can be altered upon genetic depletion of m^{6}A reader, writer, and eraser proteins. We first analyzed the expression level of NTMT1 protein in HEK293T cells or the isogenic cells with genetic ablation of *METTL3*, which encodes the catalytic subunit of the major m^{6}A writer complex [16], or either of the two m^{6}A eraser proteins, FTO and ALKBH5 [22, 23]. Our Western blot results revealed that NTMT1 protein level was increased in HEK293T cells upon genetic depletion of METTL3, but decreased upon genetic depletion of FTO (Figure 1). Genetic depletion of ALKBH5, nevertheless, did not induce any appreciable changes in the expression level of NTMT1 protein (Figure 1).

We also assessed how genetic knockout of YTHDF1/2/3 proteins affects the expression level of NTMT1 protein in HEK293T cells. We observed elevated expression of NTMT1 protein in HEK293T cells upon genetic ablation of YTHDF1 or YTHDF2, whereas depletion of YTHDF3 resulted in diminished expression of NTMT1 protein (Figure 1). Together, the above results demonstrated that NTMT1 protein expression can be dynamically regulated by m^{6}A reader/writer/eraser proteins.

3.2. MRG15 is N-terminally methylated by NTMT1

Previous studies revealed a number of substrates for NTMT1, and many of them carry an N-terminal XPK motif (‘X’ represents any amino acid) after the removal of the initial methionine [3]. The N-terminus of MRG15 possesses such a motif. Hence, we next asked whether MRG15 is α-N-methylated. To this end, we constructed a plasmid for ectopic expression of MRG15 protein, which is fused with 3 tandem repeats of Flag epitope tag on its C-terminus, in HEK293T cells. We subsequently isolated the Flag-tagged protein from the whole cell lysate with anti-Flag M2 affinity gel, digested the protein with Glu-C, and analyzed the ensuing mixture with LC-MS and MS/MS. Our results revealed that MRG15 is methylated on the N-terminus (Figure 2a–b).

Next we sought to determine whether NTMT1 is responsible for the N-terminal methylation of MRG15. Our results revealed that siRNA-mediated knockdown of NTMT1 led to nearly complete loss of α-N-methylation of MRG15 (Figure 2c–d). Previous studies showed that the 4th lysine is important for the NTMT1-mediated protein α-N-methylation [3]. We found that the α-N-methylation of MRG15 is entirely abolished in its variant with the 4th lysine residue being mutated to a glutamine (MRG15-K4Q) (Figure 2a). These results support that MRG15 is a substrate for NTMT1.

3.3. N-terminal methylation on MRG15 is modulated through an m^{6}A-based epitranscriptomic mechanism

Our above results revealed that NTMT1 protein is subjected to regulation by reader, writer and eraser proteins of m^{6}A. We subsequently asked whether α-N-methylation of MRG15 is also subjected to regulation by these m^{6}A modulators. In line with the findings made from

NTMT1 protein, we observed a notable increase in N-terminal trimethylation of MRG15, which is accompanied with a concomitant decrease in unmethylated N-terminal peptide upon genetic ablation of METTL3, whereas an opposite trend was found for MRG15 isolated from the isogenic HEK293T cells depleted of FTO (Figure 3).

We also examined how genetic ablation of YTHDF1/2/3 affects N-terminal methylation of MRG15. We found that individual depletion of YTHDF1 or YTHDF2 led to a significant increase in trimethylation, which is associated with a concomitant decrease in monomethylation of the N-terminus of MRG15 (Figure 3b, c & f). Conversely, genetic depletion of YTHDF3 led to a significant decrease in α-N-methylation of MRG15 (Figure 3d & f).

4. Conclusions

To summarize, we found that NTMT1, the major methyltransferase for protein α-N-methylation, could be subjected to regulation by m^6A reader, writer, erase proteins. To our knowledge, this is the first report about the regulatory mechanism for this evolutionarily conserved type of post-translational modification. We also uncovered that MRG15 is N-terminally methylated by NTMT1 and demonstrated that this methylation is modulated in a similar way as NTMT1 by the m^6A reader, writer, and eraser proteins.

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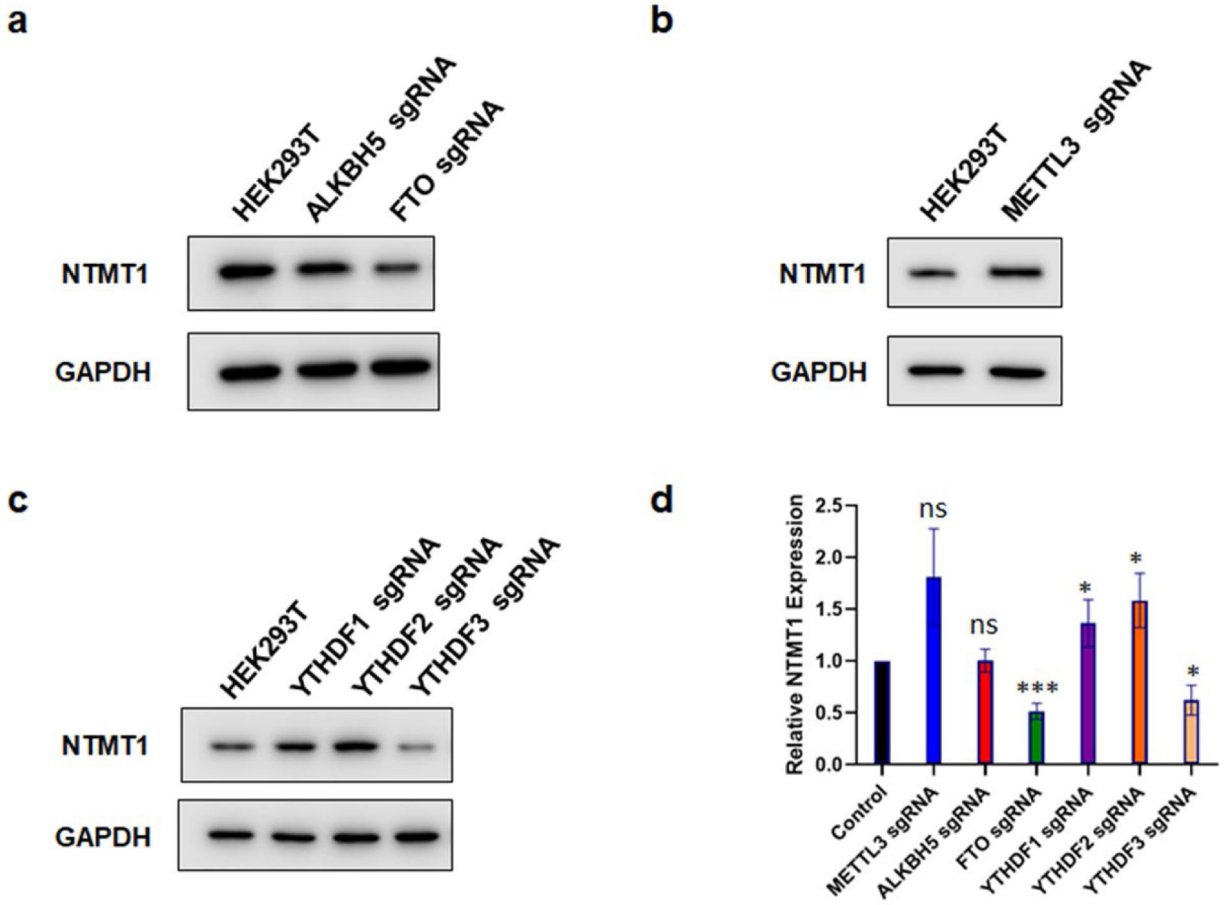

Figure 1. The alterations in expression levels of NTMT1 protein following knockout of m^{6}A reader/writer/eraser genes.

(a-c) Western blot images showing the relative levels of NTMT1 protein in HEK293T cells and the isogenic cells with m^{6}A erasers (a), writer (b) and readers (c) being genetically depleted by CRISPR-Cas9. (d) Quantification results for the relative expression levels of NTMT1 protein in HEK293T cells vs. the isogenic cells with ALKBH5, FTO, METTL3 and YTHDF1/2/3 genes being individually depleted. "ns", $p > 0.05$; "*", $0.01 \leq p < 0.05$; "***", $p < 0.001$. The data represent the mean ± S.D. of results from 3 or 4 independent experiments. All p values were calculated using the unpaired, two-tailed t-test.

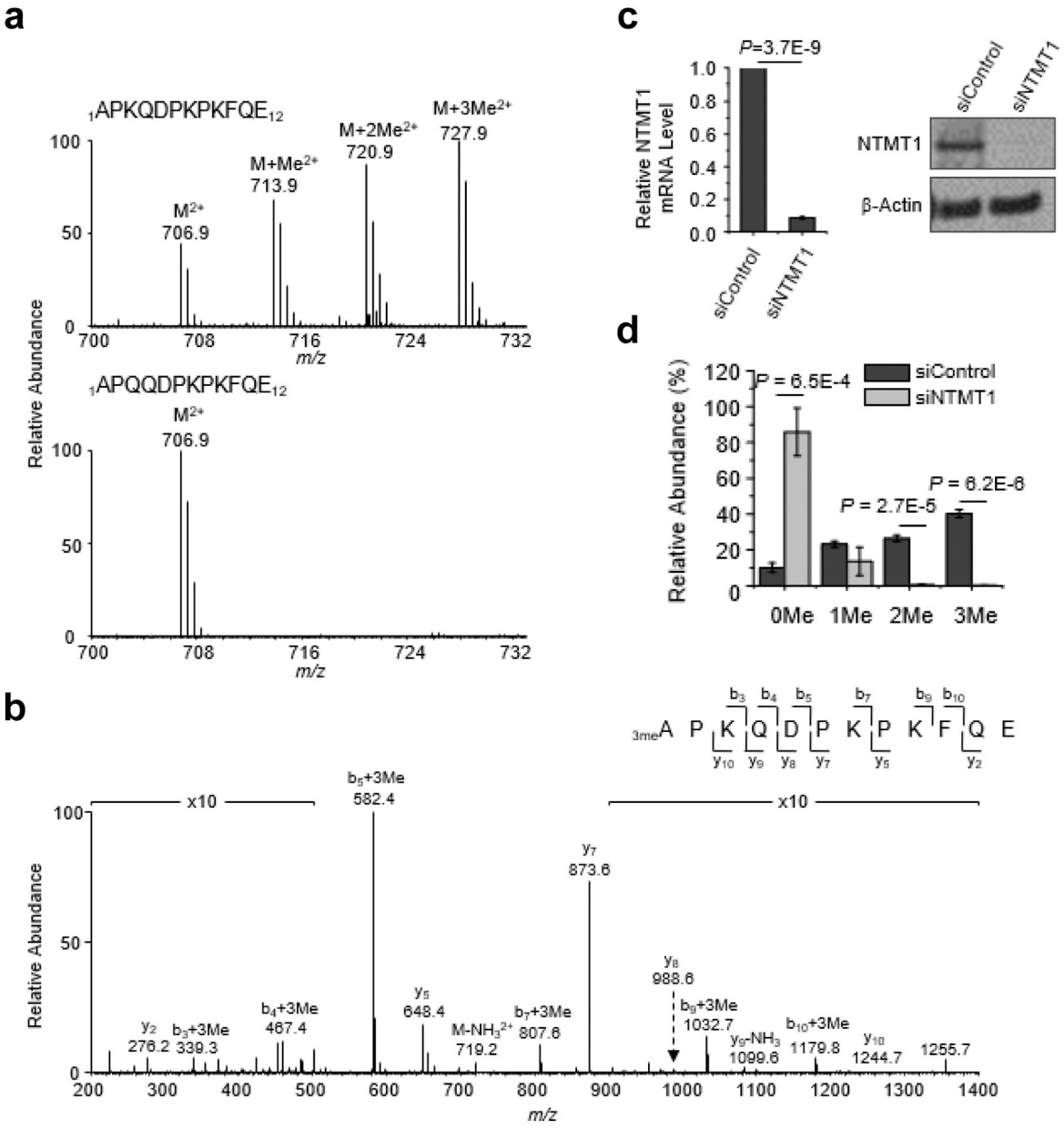

Figure 2. Identification and characterization of α-N-terminal methylation of MRG15.

(a) 'Ultra-zoom' scan ESI-MS showing the $[M+2H]^{2+}$ ions of the Glu-C-produced N-terminal peptide of MRG15 and its K4Q mutant, i.e. APKQDPKPKFQE and APQQPKPKFQE, isolated from HEK293T cells. (b) MS/MS of tri-methylated N-terminal peptide of MRG15. Displayed in the inset is a scheme summarizing the observed fragment ions. (c) RT-qPCR and Western blot showing the efficient knockdown of *NTMT1* gene in HEK293T cells. (d) Knockdown of NTMT1 led to marked attenuation of α-N-methylation of MRG15. The methylation levels were quantified based on the relative abundances of the $[M+2H]^{2+}$ ions of the unmethylated and the mono-, di- and tri-methylated forms of the N-terminal peptide of MRG15. The *p* values were calculated using the unpaired, two-tailed *t*-test.

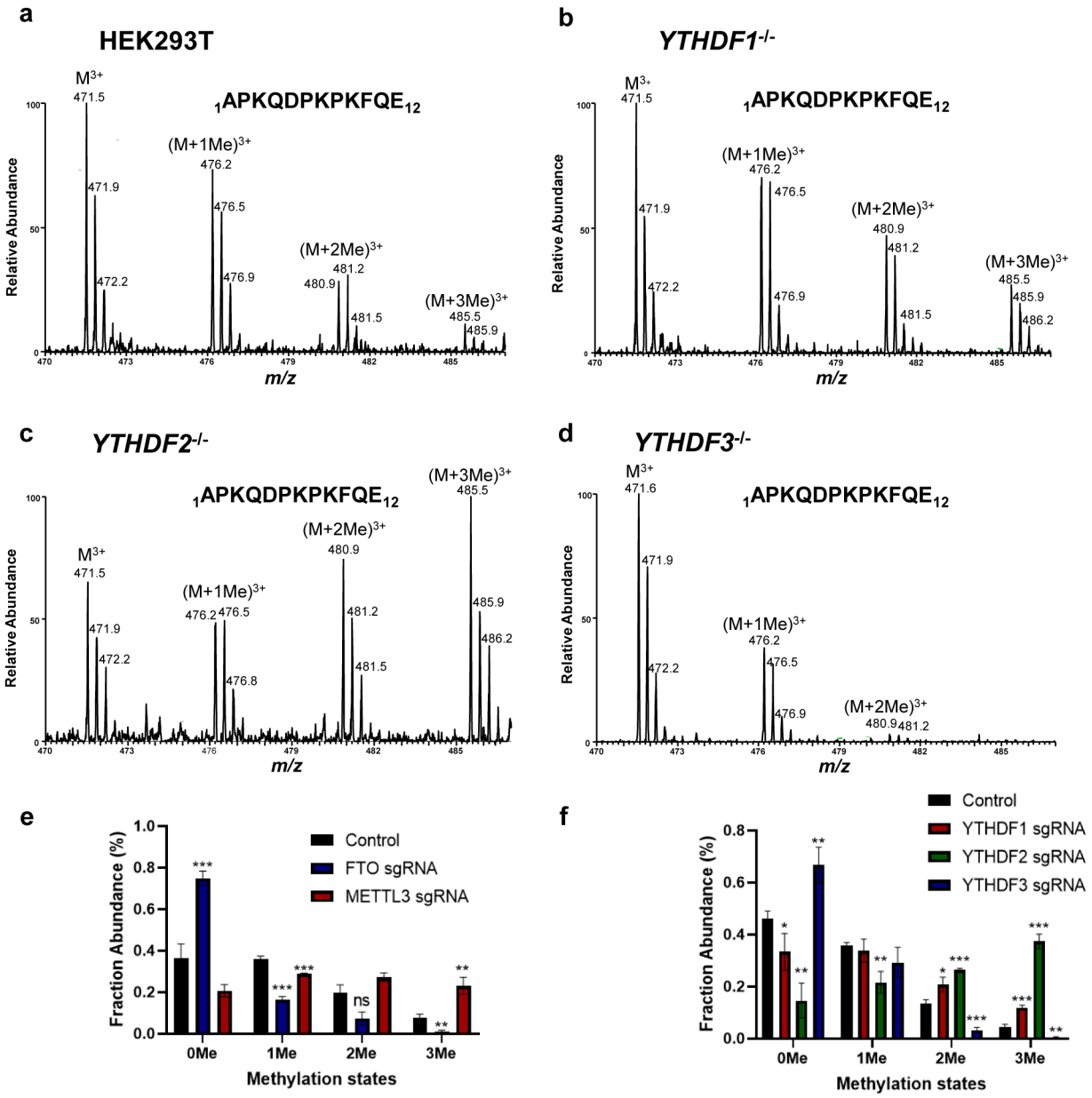

Figure 3. N-terminal methylation levels of MRG15 are modulated by m^{6}A reader/writer/eraser genes.

(a-d) 'Ultra-zoom' scan ESI-MS showing the $[M+3H]^{3+}$ ions of the Glu-C-produced N-terminal peptide of C-terminally Flag-tagged MRG15 isolated from HEK293T cells or the isogenic cells with YTHDF1/2/3 genes being individually ablated by CRISPR-Cas9. (e-f) Genetic ablations of FTO, METTL3, YTHDF1, YTHDF2, and YTHDF3 alter the α-N-methylation of MRG15. The methylation levels were quantified based on the relative abundances of the $[M+3H]^{3+}$ ions of the unmethylated and the mono-, di- and tri-methylated forms of the N-terminal peptide of MRG15. The *p* values were calculated using unpaired, two-tailed *t*-test: "ns", $p > 0.05$; "*", $0.01 \leq p < 0.05$; "**", $0.001 \leq p < 0.01$; "***", $p < 0.001$.