

Highly Sequence-specific, Timing-controllable m⁶A Demethylation by Modulating RNA-binding Affinity of m⁶A Erasers

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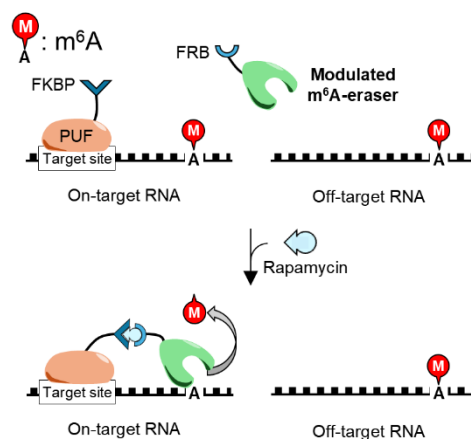
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Recent advancements in tools using programmable RNA binding proteins and m⁶A-erasers enable sequence-selective and timing-controllable m⁶A demethylation. However, off-target effects are still concern. This study addresses the problem by reducing the RNA-binding ability of m⁶A-erasers. The modulated m⁶A-erasers achieved sequence-specific and timing-controllable m⁶A demethylation with minimal off-target activity.

N⁶-methyladenosine (m⁶A) is one of the most abundant post-transcriptional modifications in eukaryotic RNA, regulating RNA metabolisms such as alternative splicing¹ and mRNA stability.² It is installed by a methyltransferase complex (m⁶A-writers) and removed by demethylases (m⁶A-erasers)^{3–9}. Studies manipulating the expression of the m⁶A-erasers, fat mass and obesity-associated protein (FTO) and AlkB homolog 5 (ALKBH5), revealed their crucial roles in processes like spermatogenesis¹⁰, tumorigenesis¹¹, adipogenesis¹², and differentiation¹³. However, identifying the m⁶As responsible for the phenotypes remains difficult because overexpression or knockdown of m⁶A-erasers impacts global m⁶A levels. To address this, programmable m⁶A demethylation tools have been developed utilizing sequence-specific RNA binding proteins to guide FTO or ALKBH5 to target m⁶As^{14–16}. Recent advancements in these tools enable temporal control of demethylation by associating the RNA-binding proteins and m⁶A-erasers in response to stimuli^{17,18}. These advancements allow for dynamic regulation of individual m⁶As in sequential or stepwise processes such as differentiation, reprogramming, and circadian clock. However, unintended background demethylation due to overexpressed m⁶A-erasers remains a concern, even though the global decrease in m⁶A levels by overexpressing m⁶A-erasers in living cells has been

reported^{6,19}. Therefore, engineered m⁶A-erasers that target specific sequences at a desired timing are highly needed.

m⁶A-erasers have intrinsic RNA-binding ability, causing off-target binding of the eraser and non-specific m⁶A demethylation. Reducing this intrinsic RNA-binding ability is a promising approach to mitigate these effects, allowing the m⁶A-eraser to act selectively through an RNA-binding protein segment that targets specific RNA sequences. A similar approach has been reported by Liu et al. using an m⁶A-“writer” consisting of catalytically dead Cas13 (dCas13), for targeting the specific RNA sequences, and the METTL3:METTL14 methyltransferase complex lacking the zinc finger RNA-binding domain of METTL3, to weaken its own RNA-binding ability²⁰. Unlike the METTL3:METTL14 complex, where RNA-binding domains are separate from the active site^{21–24}, the RNA-binding and active sites of m⁶A-erasers are structurally interrelated^{4,5,25}. While reducing RNA-binding ability is effective in minimizing off-target effects, maintaining catalytic activity while weakening RNA-binding ability in m⁶A-erasers is challenging.



Scheme 1. Concept of ligand-inducible sequence-specific m⁶A demethylation tools with minimal off-target effects. Modulated m⁶A-erasers do not demethylate in the absence of ligand but demethylate m⁶A only nearby the PUF-binding site in the presence of ligand.

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This study aimed to create m⁶A-erasers for highly sequence-specific m⁶A demethylation using two m⁶A-erasers, FTO and ALKBH5, as templates. Their RNA-binding abilities were modulated based on structural data to suppress nonspecific binding to undesirable off-target RNA sequences, resulting in “modulated” RNA-binding (mod) FTO and modALKBH5. For specific RNA segment recognition, they were fused with the RNA-binding protein PUF; a sequence-specific, monomeric RNA-binding protein that is programmable through arrangement of repeated motifs^{14,26,27}. Importantly, a switching system using FK-506 binding protein (FKBP) and FKBP-rapamycin-binding domain of FKBP12-rapamycin associated protein (FRB) was installed in the linker to connect the PUF domain and the mod-m⁶A-erasers, allowing for time-controllable, sequence-specific m⁶A demethylation (Scheme 1).

FTO interacts with RNA at a positively charged groove-like region, where basic residues (K88, K213) form a ‘pincer structure’ that holds substrate RNA strands^{7,25}. To reduce the RNA-binding affinity of FTO itself, alanine substitutions were introduced into FTO at these residues (Figure 1A). The FTO mutants were fused with PUF, which binds to the UGUUAUAU sequence¹⁴. The m⁶As near the PUF-binding site and those without a nearby PUF-binding site were designated as on-target and off-target, respectively. The demethylation activity of PUF-fused FTO mutants toward on-target and off-target m⁶As was examined using the m⁶A-sensitive endoribonuclease MazF²⁸ (Figure S1A, Table S1). Wild-type FTO-PUF (FP) showed on-target demethylation at concentrations below 500 nM but remarkable off-target demethylation at higher concentrations (Figure 1B, C, Figure S1B). FTO^{K88A}-PUF demethylated both on- and off-target m⁶As at high concentrations, similar to FP (Figure

1B, S1B, C); whereas FTO^{K213A}-PUF exhibited slightly reduced off-target demethylation while maintaining on-target RNA demethylation activity (Figure 1B, S1B, D).

Interestingly, with the combination of these alanine substitutions, FTO^{K88A/K213A}-PUF successfully reduced off-target demethylation even at high concentrations while maintaining on-target m⁶A demethylation activity (Figure 1B, C). K88 and K213 may cooperatively contribute to interactions with the substrate RNA. It is plausible that FTO^{K88A/K213A}-PUF relies on PUF for RNA binding and that FTO^{K88A/K213A} exhibits high sequence selectivity by being guided to its target m⁶A via PUF. We named the alanine substitution, FTO^{K88A/K213A} as ‘modulated-RNA-binding FTO (modFTO)’ (Figure 1A, S1E).

The two alanine substitutions were introduced to reduce the RNA-binding affinity, and then the K_d values of wild-type FTO and modFTO for ssRNA were measured by fluorescence polarization assay with FAM-labeled ssRNA (Table S1). Wild-type FTO and modFTO exhibited K_d values of $0.93 \pm 0.39 \mu\text{M}$ and $2.89 \pm 0.64 \mu\text{M}$, respectively (Figure 2A, S2).

ModFTO itself, without PUF, showed little demethylation activity towards both on- and off-target m⁶As, even at high concentrations (Figure S3). We validated the demethylation activity towards mRNA isolated from HEK293T cells. The ratios of m⁶A to A (m⁶A/A) was measured using liquid chromatography tandem mass spectrometry (LC-MS/MS). Wild-type FTO demethylated approximately 70% of m⁶A in mRNA, whereas modFTO did not show any significant demethylation (Figure 2B, S4A). FP also demethylated m⁶A at a similar level to wild-type FTO, reflecting PUF-independent demethylation; whereas, modFP showed significantly less demethylation than FP (Figure S4B). Given that modFTO alone rarely demethylates m⁶A, the observed demethylation by modFP is probably due to PUF-RNA interaction. Although the specificity of the RNA binding protein used should be high enough, the lower background activity of modFTO toward miscellaneous m⁶A may contribute to the reduced off-target effects of targeted m⁶A demethylation, even in living cells.

The generality of this design strategy for highly sequence-specific demethylation was verified using another m⁶A-eraser, ALKBH5. As expected, the fusion of wild-type ALKBH5 with PUF, ALKBH5-PUF (AP), showed on-target demethylation below 500 nM; however, at higher concentrations, it showed off-target activity owing to its innate recognition ability, similar to FP

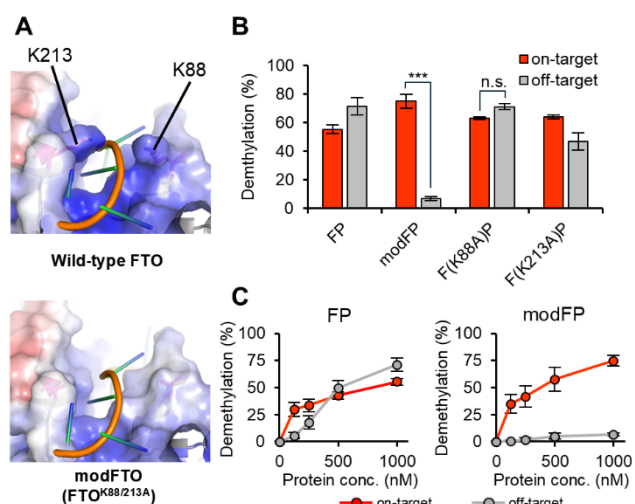


Figure 1. Alanine scanning on basic amino acids to lower off-target demethylation of FTO-PUF. (A) The two lysine residues (K88 and K213) in wild-type FTO which consist of the pincer structure interacting with the phosphate backbone of the ssRNA (upper; PDB ID: 3LFM) and in modFTO (lower; modified from 3LFM). (B, C) The sequence-specific demethylation activities to off-target RNA or on-target RNA of the alanine substitutions. (B) 1 μM or (C) indicated concentration of FTO-PUF or each substitutions were incubated with 50 nM of the two m⁶A-modified RNA oligos (on-target: 5'-FAM-AUUGUAUAUAUCUAAG(m⁶A)CAUUUUA-3', bold: PUF binding site, off-target: 5'-TAMRA-AUAUCUUCUUGGUUCUAUUAG(m⁶A)CAUUUUA-3') at 25 °C for 1 h. Values and error bars indicate mean $\pm$ SEM (n = 3 or 4, Tukey; ***: $p < 0.001$).

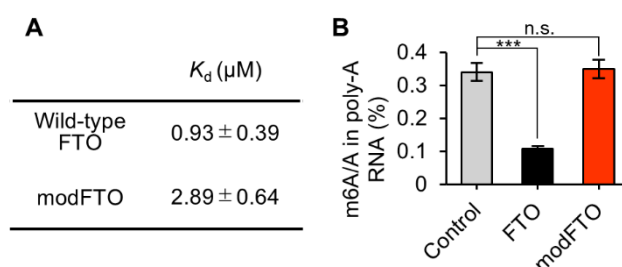


Figure 2. (A) K_d values of wild-type FTO and modFTO analysed by FP assay. The values indicate mean $\pm$ SEM (n=3). (B) Quantification of the m⁶A/A in poly-A RNA by LC-MS/MS. While wild-type FTO showed significant decrease of m⁶A/A compared to control, modFTO did not show any decrease. Values and error bars indicate mean $\pm$ SEM (n=3, Turkey; n.s.: not significant, ***: $p < 0.001$).

(Figure 3A, B). Therefore, we aimed to create a modulated RNA-binding ALKBH5 (modALKBH5) for higher sequence-specific m⁶A demethylation. Although both ALKBH5 and FTO belong to the AlkB subfamily, the pincer structure that was mutated in modFTO is unique to FTO among AlkB subfamily members²⁵ (Figure S5). In addition, the direction of the RNA phosphoribosyl backbone is reversed in ALKBH5 compared to that of the substrate structure of FTO⁵. Therefore, we explored the unique residues that contribute to RNA binding in ALKBH5.

Recent analysis of the crystal structure of ALKBH5 in complex with an m⁶A-containing ssRNA⁵ suggested that nucleotide recognition lids (NRL) 2 and β IV-V loop facilitate substrate ssRNA recognition via electrostatic and π - π stacking interactions (Figure 3C). Among the PUF-fused ALKBH5 mutants with alanine substitutions in these regions, a mutant with six substitutions, ALKBH5^{K147A/R148A/F232A/Q233A/F234A/K235A}-PUF, overcame the off-target activity (Figure 3A, B, S6A) even at 1 μ M and was designated modALKBH5. Partial substitutions at K147/R148/K235, which contribute to electrostatic interactions with RNA, still showed off-target demethylation at 1 μ M (Figure 3A, S6A, B). This result is partly in accordance with a previous report demonstrating that ALKBH5^{K147A} retained its demethylation activity⁴. ALKBH5^{F232A/Q233A/F234A}-PUF, which has mutations at the π - π stacking interaction sites of ALKBH5, largely reduced off-target demethylation (~40%) while retaining the demethylation activity against on-target RNA (Figure 3A, S6A, B). ModALKBH5, with six alanine substitutions, showed minimal m⁶A demethylation activity on its own (Figure S3), whereas modALKBH5-PUF (modAP) achieved highly sequence-selective demethylation dependent on the PUF binding site. This suggests that ALKBH5 recognizes its substrate RNAs through multiple residues, including basic and aromatic amino

acids. Furthermore, the results for modAP, as well as modFP, demonstrated the effectiveness of the strategy to reduce the RNA-binding ability of the m⁶A-erasers for highly sequence-specific m⁶A demethylation with low off-target activity.

Ligand-responsive m⁶A demethylation of a specific sequence is useful for switching of the methylation state of target sites. A rapamycin-inducible modFTO-PUF (imodFP) was designed using modFTO, which does not actively demethylate m⁶A alone owing to reduced RNA-binding ability (Figure S3). FK-506 binding protein (FKBP) and FKBP-rapamycin-binding domain of FKBP12-rapamycin associated protein (FRB) are known to dimerize rapidly in the presence of rapamycin²⁹. In imodFP, modFTO and PUF were fused to FRB and FKBP, forming modFTO-FRB and FKBP-PUF, respectively (Scheme 1). As a result, imodFP (modFTO-FRB and FKBP-PUF) successfully demethylated on-target m⁶A in the presence of rapamycin but not off-target m⁶A (Figure 4A, S7A). By contrast, iFP (wild-type FTO-FRB and FKBP-PUF) demethylated both on- and off-target m⁶As, independent of rapamycin (Figure S7A, C). This may be because the wild-type FTO-FRB can demethylate any m⁶As.

Using the same strategy, we created imodAP (modALKBH5-FRB and FKBP-PUF). imodAP showed on-target demethylation only in the presence of rapamycin (Figure 4B, S7B), whereas iAP (wild-type ALKBH5-FRB and FKBP-PUF) lacked sequence-specificity or rapamycin-responsiveness (Figure S7B, D). The high sequence- and ligand-dependent demethylation ability of imodFP and imodAP appears to rely on the properties of modFTO and modALKBH5, that do not demethylate on its own unless coupled with PUF (Figure S3). These results suggest that imodFP and imodAP can demethylate a specific m⁶A at a particular time with high accuracy.

In summary, we created a novel system that regulates the timing of sequence-specific m⁶A demethylation with minimal off-target effects by fine-tuning the RNA-binding affinity of m⁶A-erasers. To reduce RNA-binding affinity, residues on the RNA recognition surface, but not in its active site, were substituted with alanine so that they exerted their demethylation ability only when in proximity to RNA. Ultimately, the ligand-inducible imodFP and imodAP were designed using modFTO and modALKBH5, which were activated by rapamycin and demonstrated highly sequence-specific demethylation.

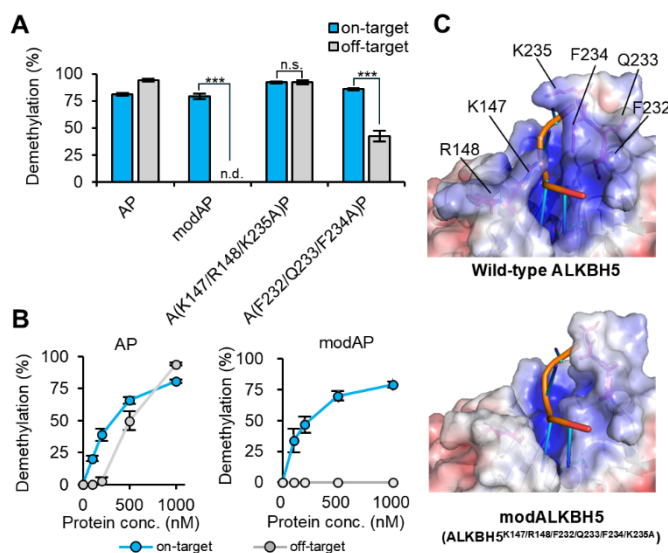


Figure 4. Creation of modAP based on RNA recognition domains. (A, B) The sequence-specific demethylation activities of the alanine-substitutions to on- or off-target RNA. (A) 1 μ M or (B) indicated concentration of AP, modA(K147/R148/F232/Q233/F234/K235A)P or each substitution were incubated with two m⁶A-modified RNA oligos at 25 °C for 1 h. (C) The electrostatic surface of wild type ALKBH5 (PDB ID: 7WLO) and modALKBH5 (Modified from 7WLO). The six amino acids substituted with alanine in creating modALKBH5 are indicated. Values and error bars indicate mean $\pm$ SEM (n = 4, Tukey; n.s., not significant; ***: p < 0.001).

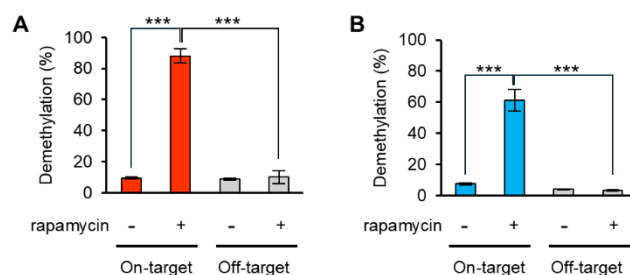


Figure 3. Rapamycin-inducible sequence-specific demethylations. (A, B) Rapamycin-responsiveness of (A) imodFP or (B) imodAP. 1 μ M of modFTO-FRB or modALKBH5-FRB and FKBP-PUF were incubated with 50 nM of m⁶A-modified RNA oligos. Although imodFP and imodAP did not show demethylation activities in the absence of rapamycin, both showed the activities to only on-target RNA in the presence of rapamycin. Values and error bars indicate means $\pm$ SEM (n=3, Turkey's; ***: p < 0.001)

To date, approaches to reduce non-specific substrate recognition of enzymes have been employed in the target RNA methylation system using an m⁶A-writer lacking the zinc finger domain²⁰ and in highly target-specific genome-editing systems using Cas9 mutants with substituted residues in the substrate recognition or Rec3 domains^{30–32}. In contrast to these approaches, which use enzymes with independent substrate-recognition and catalytic domains, our strategy could be applied to other epitranscriptomic and epigenetic enzymes; however, optimization is required for each enzyme.

For timing control, targeted m⁶A demethylation systems that respond to stimuli, such as abscisic acid and light irradiation, have been reported but they utilize wild-type m⁶A-erasers and do not consider background demethylation^{17,18}. Our mod-m⁶A-erasers with reduced RNA-binding ability can be integrated with various systems that respond to stimuli, such as light and chemicals, along with various RNA binding domains, including CRISPR/dCas13, to achieve more accurate timing- and sequence-specific m⁶A demethylation than wild-type m⁶A-erasers. Thus, they will be powerful tools to reveal the period-dependent roles of individual m⁶A modifications in dynamic physiological processes. In cellulo experiments for biological application are underway.

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Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the ESI.†

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Highly Sequence-specific, Timing-controllable m⁶A Demethylation by Modulating RNA-binding Affinity of m⁶A Erasers

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SUPPORTING INFORMATION

1. Materials and methods
2. Supporting figures

Materials and Methods

◆ Plasmid designing

The *E. coli* expression vector of mouse FTO (His₆-mFTO-Strep/pET28b) and FTO-PUF have been described in our previous report. cDNA of mutants was synthesized by PCR using mutagenesis primers and subcloned into the above plasmids. For designing modALKBH5-PUF, the same method was used as FTO. For construction of imodFP and imodAP, FRB and FKBR were subcloned as fused by 2GSS linker in pET28b vector, resulted in His₆-FTO or ALKBH5-2GSS-FRB-Strep and His₆-FKBP-2GSS-PUF-Strep.

◆ Recombinant proteins preparation

Protein expression and purification were performed basically based on previous research (ref). The plasmids were transformed into *E. coli* BL21 (DE3) and grown on LB-agar plates containing 25 mg/L kanamycin. Protein expression was induced by adding 0.1 mM IPTG at logarithmic growth phase and incubated overnight at 18 °C and 100 rpm. The cells were fractionated to soluble fraction by sonication and centrifugation, and the fraction was purified by the HisTrap FF (Cytiva, #17531901) followed by StrepTrap HP (Cytiva, #29048653) or StrepTrap XT (Cytiva, #29401317). FTO, FTO-PUF, ALKBH5-PUF and their mutants were concentrated with Amicon Ultra – 0.5 mL 30kDa (Millipore, #UFC503024) using 25 mM Tris-HCl (pH 7.5). FTO-FRB, modFTO-FRB, ALKBH5-FRB, modALKBH5-FRB and FKBP-PUF were concentrated using 25 mM Tris-HCl (pH 7.5) with 100 mM NaCl.

◆ 3D structure prediction of mutated proteins

The 3D structure and its surface potential of wild-type FTO or wild-type ALKBH5 with single strand DNA were obtained by PDB: 3LFM or 7LW0 using PyMOL 2.5.4. The 3D structures of the mutated proteins were created by mutagenesis mode based on the wild-type m⁶A-erasers and those surface potential were predicted.

◆ RNA isolation

Total RNA was extracted from the cultured HEK293T cells using NucleoSpin RNA Plus (MACHEREY-NAGEL, #740984). mRNA was isolated using Dynabeads Oligo(dT)₂₅ (Invitrogen, #61005) following the manufacture's protocols. The concentrations of mRNA were measured by Qubit 4 Fluorometer (Invitrogen).

◆ In vitro demethylation and its validation by MazF cleavage assay

50 nM of on-target RNA and off-target RNA (Table S1) were demethylated by the indicated concentrations of demethylases in demethylation buffer (total 10 μL). The buffer composition for validating the demethylation activities of FTO-PUF and its mutants was 25 mM Tris-HCl (pH 7.5), 35 μM Fe(NH₄)₂(SO₄)₂·6H₂O, 50 μM α-ketoglutarate, 500 μM L-ascorbate, 50 mM NaCl, 50 ng/μL BSA, 0.01%

Tween20, 50 ng/μL total RNA from HeLa cells, and for ALKBH5-PUF and mutants, imodFP and imodAP was 25 mM Tris-HCl (pH 7.5), 283 μM Fe(NH₄)₂(SO₄)₂·6H₂O, 300 μM α-ketoglutarate, 2 mM L-ascorbate, 50 mM NaCl, 50 ng/μL BSA, 0.01% Tween20, 50 ng/μL total RNA from HeLa cells, at 25 °C for 1hr followed by heating at 95 °C for 3 min to stop reaction. For validating the demethylation activity of wild-type FTO or modFTO towards m⁶A on mRNA *in vitro*, the mRNA was demethylated in the following buffer: 25 mM Tris-HCl (pH 7.5), 283 μM Fe(NH₄)₂(SO₄)₂·6H₂O, 300 μM α-ketoglutarate, 2 mM L-ascorbate, 50 mM NaCl, 0.5 U/μL RNasin Plus (Promega, #N261A) with 1 μM protein, at 37 °C for 5 or 10 min, then reaction was quenched by adding 5 mM EDTA.

For MazF assays, 2 μL of the demethylated RNA was subjected to 8 μL of MazF buffer: 40 mM PBS (pH 7.5), 5 mM EDTA, 250 nM MazF, and incubated at 37 °C for 1 hr followed by heating at 95 °C for 3 min. The MazF-digested sample was mixed with the same amount of Hi-Di Formamide (Applied Biosynthesis, #4311320), heated at 95 °C for 3 min, and immediately cooled on ice. 8-μL of the sample was loaded onto 20% urea-polyacrylamide gel and electrophoresed in 0.5 × TBE buffer (Nippon Gene, #318-90041). The fluorescently labeled RNAs were visualized using Amersham Typhoon (GE healthcare).

◆ Fluorescence polarization assay

20 nM of FAM-labeled at 5' of m⁶A-modified ssRNA was incubated with increasing concentrations (40 to 3 × 10⁴ nM) of wild-type FTO or modFTO in FP buffer (25 mM Tris-HCl, 0.01% Tween20, pH 7.5) for 1hr at 25°C in 96-well half-area microplate (Corning, #CLS3694). Fluorescence anisotropy was measured on an Infinite F Plex (TECAN) with a 485 nm excitation light and a 535 nm fluorescence wavelength filter. The results were analyzed by Kaleida graph (Synergy software, version 4.5.2.), and the binding dissociation constant was calculated by the following equation.

$$A = \left(\frac{([P] + [R] + K_d) - \sqrt{([P] + [R] + K_d)^2 - 4[R]K_d}}{2[R]} \right) \times (A_{\infty} - A_0) + A_0$$

[P]: Protein concentration, [R]: FAM-labeled ssRNA concentration, A: Anisotropy

◆ Measurement of m⁶A level of mRNA using LC-MS/MS

The demethylated mRNA was purified using TRIzol LS Reagent (Invitrogen, #10296010) following to manufacture's protocol and measured the concentration by Nanodrop. 400 ng of the purified mRNA was decapped with 20 units of RppH (NEB, #M0356) in 1 × Thermopol buffer (TritonX-100 concentration was reduced to 0.01%) at 37°C for 6hr. Decapped mRNA was digested to single nucleotides with 50 units Nuclease P1 (NEB, #M0660) in 20 mM NH₄Ac at 37°C for 12hr, and then 5' phosphates of the nucleotide sample were removed with 0.5 units of quick-CIP (NEB, #M0525) at 37 °C for 2hr with agitation at 800 rpm for 1 min every 10 min.

Nucleoside sample was desalted by solid phase extraction (SPE) using Sep-Pak tC₁₈ 1 cc Vac

Cartridge (Waters, #WAT036820). The sample was diluted with SPE buffer A (0.1% heptafluorobutyric acid (HFBA) in H₂O) to 1 mL. The cartridge was activated with 1-mL MeOH and SPE buffer B (0.1% HFBA, 80% acetonitrile (ACN)), and then equilibrated with 1-mL SPE buffer A. The diluted nucleoside was bound to the cartridge, washed with 1-mL SPE buffer A, and subsequently eluted by 1-mL SPE buffer B to 1.5-mL tube. The purified sample was evaporated using SpeedVac and dissolved in 20-μL mobile phase A and 10 μL was injected into LCMS-8060 (SHIMADZU). The mobile phases consisted of 0.1% formic acid in H₂O (A) and in ACN (B) were used. The sample was separated on C18 column.

Table S1 ssRNA oligo used for demethylation reactions and FP assay

Oligo name	Sequence (5'→3')
on-target RNA	FAM-AU UGUAUAUA UCUAAG(m ⁶ A)CAUUUUA
off-target RNA	TAMRA-AUAUCUCUUGGGUUCUAUUAG(m ⁶ A)CAUUUAG
on-target MazF control	FAM-AU UGUAUAUA UCUAAGACAUUUUA
off-target MazF control	TAMRA-AUAUCUCUUGGGUUCUAUUAGACAUUUAG
2nt-RNA	FAM-AU UGUAUAUA AAG(m ⁶ A)CAUUUUA
4nt-RNA	FAM-AU UGUAUAUA UUUG(m ⁶ A)CAUUUGGGGUUCUU
6nt-RNA	FAM-AU UGUAUAUA UCUAAG(m ⁶ A)CAUUUUA
8nt-RNA	FAM-AU UGUAUAUA UCCUUUAG(m ⁶ A)CAUUUUA
10nt-RNA	FAM-AU UGUAUAUA UCGGCUUUAG(m ⁶ A)CAUUUUA
RNA for FP assay	FAM-AUUGUAUAU(m ⁶ A)CAUUUA

UGUAUAUA: PUF binding sequence

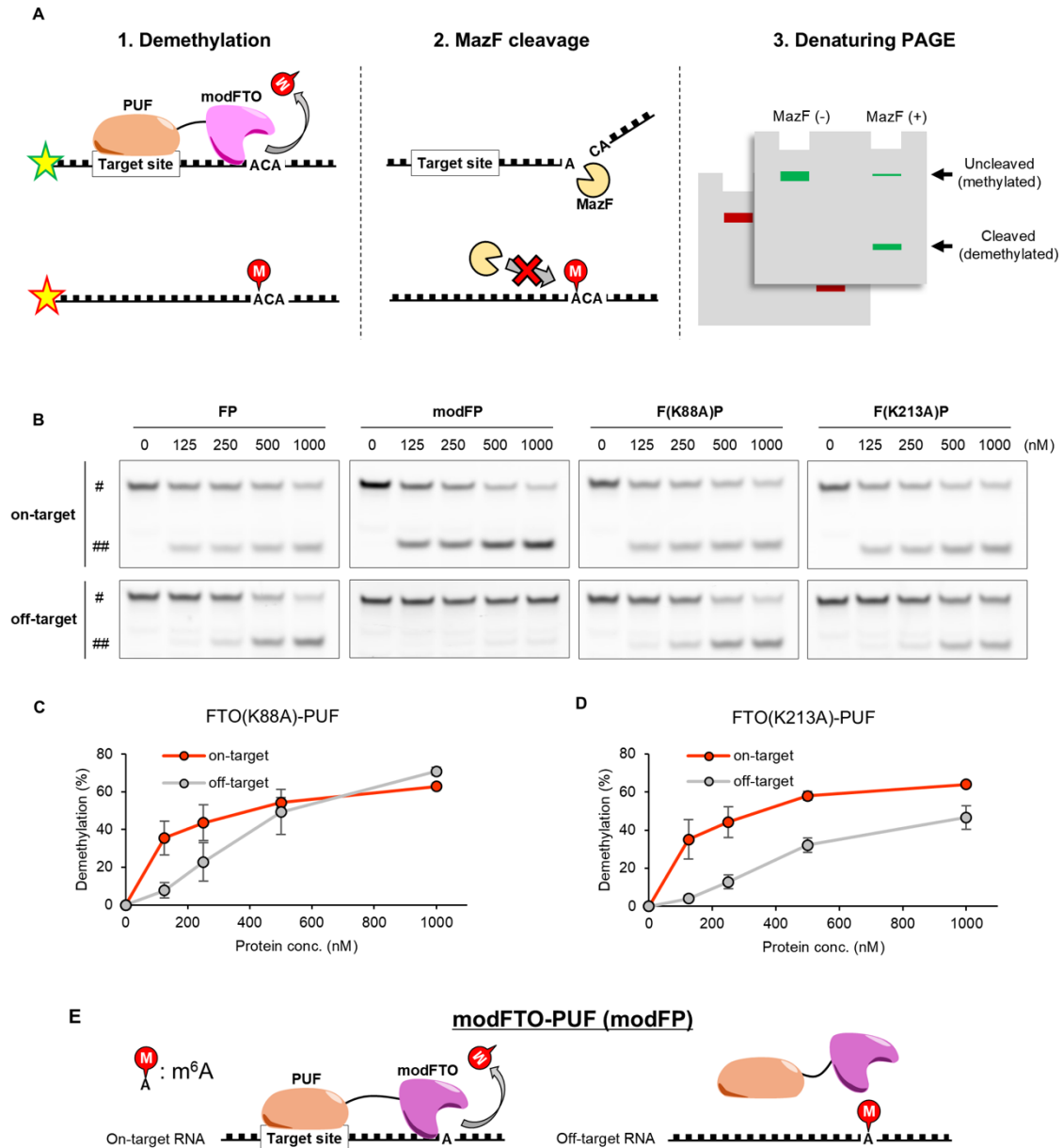


Figure S1. The demethylation activities of alanine substituted-FTO mutants fused with PUF to each m^6A -modified RNA oligos at each concentration. **(A)** Scheme to evaluate sequence-specific demethylation using MazF cleavage assay. MazF is an m^6A susceptible RNA endonuclease and cleaves only demethylated RNA oligo. **(B)** PAGE images of MazF assay after demethylation reaction. On-target RNA labeled with FAM and off-target RNA labeled with TAMRA were visualized by excitation with 488 nm (upper) and 532 nm (lower). #: un-cleaved RNA (methylated RNA), ##: cleaved RNA (demethylated RNA). **(C, D)** The plot of concentration-dependent demethylation activities of FTO(K88A)-PUF (C) and FTO(K213A)-PUF (D). Values and error bars indicate mean $\pm$ SEM ($n=3$). **(E)** Schematic illustration about the sequence-specific m^6A demethylation by modFP.

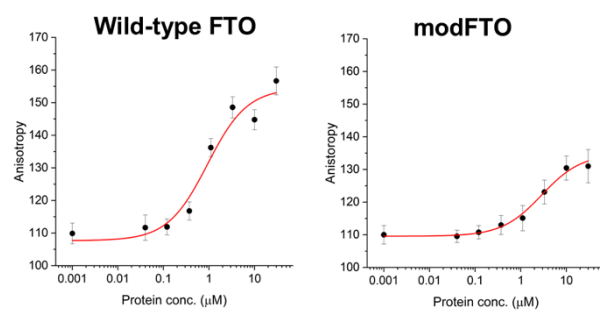


Figure S2. The plots of anisotropy at each concentration of proteins from 10 nM to 30 μM. Values and error bars indicate mean $\pm$ SEM (n=3).

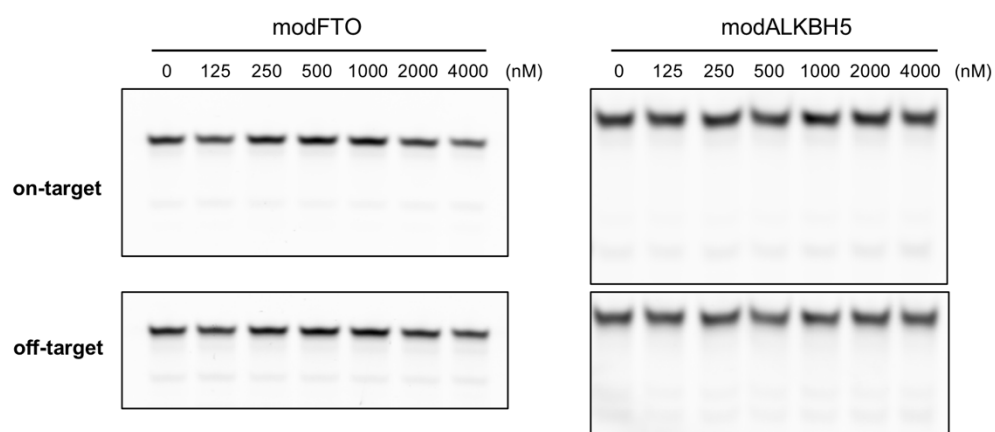


Figure S3. Demethylation activity of modFTO and modALKBH5 towards on-target RNA or off-target RNA evaluated by MazF cleavage assay. modFTO and modALKBH5 did not demethylate m⁶A in either RNA oligo, even at higher concentration (~ 4000 nM).

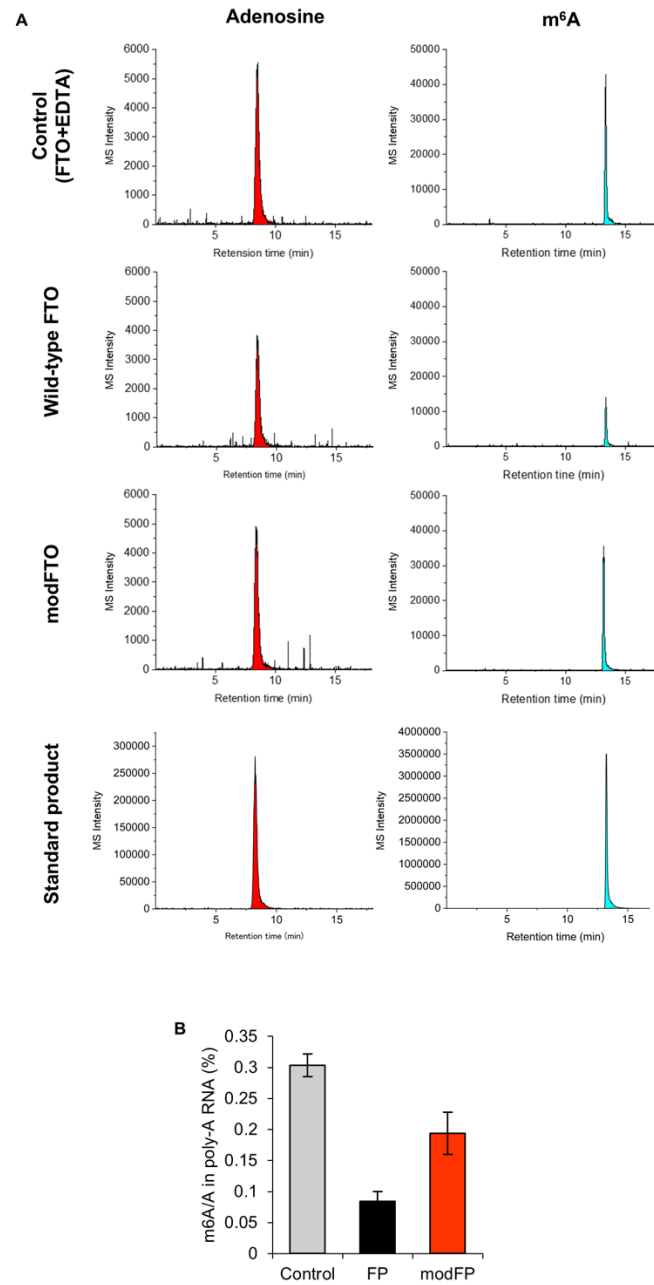


Figure S4. (A) Representative LC-MS/MS spectrometry of standard products (adenosine, m⁶A) and mRNA purified from HEK293T cells treated with wild-type FTO or modFTO *in vitro*. Standard products were diluted at 1 g/L and injected 10 μ L into HPLC. (B) Quantification of the m⁶A/A in poly-A RNA treated with FP or modFP by LC-MS/MS. Values and error bars indicate mean $\pm$ SEM (n=3, Turkey; n.s.: not significant, ***: p < 0.001). The data were analyzed by OriginPro 2024 (ver. 10.1.0.170).

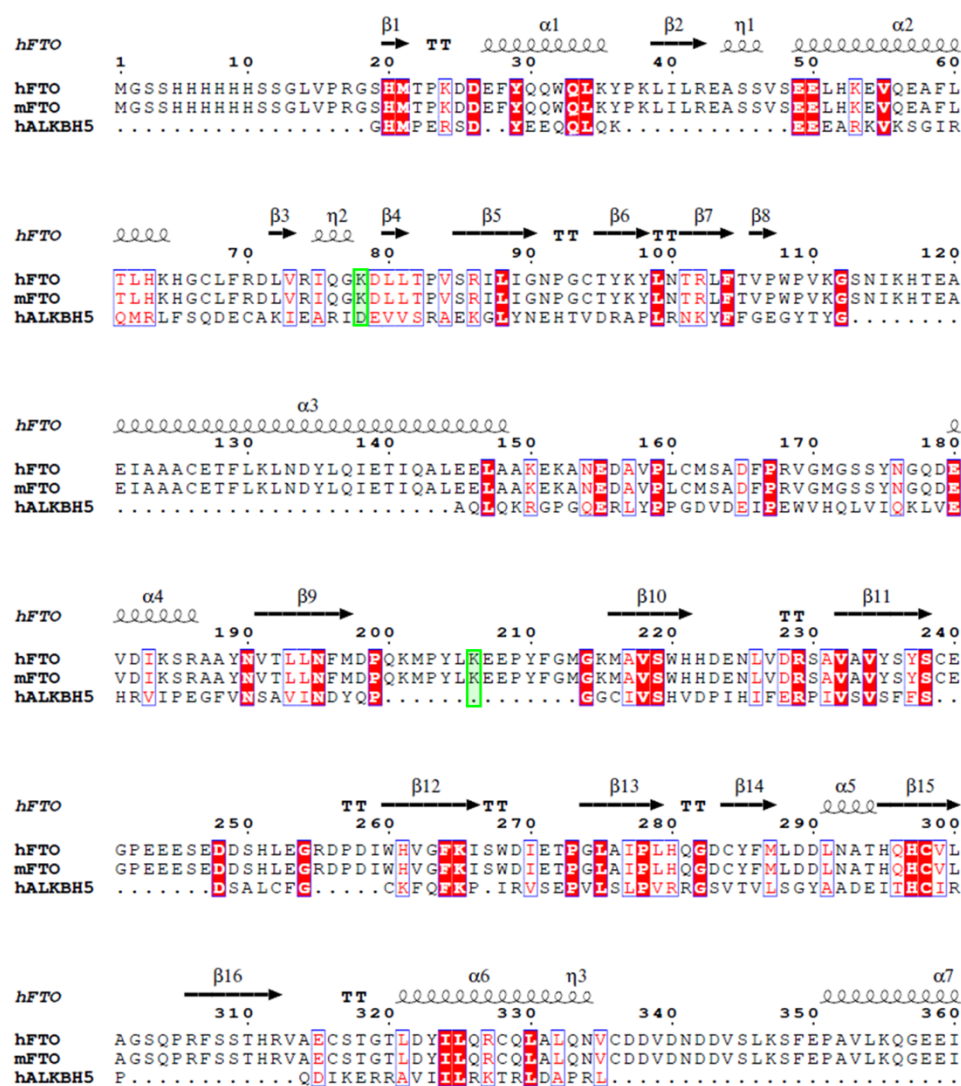


Figure S5. Structure-based sequence alignment of m⁶A-erasers by STRAP. Residues added alanine substitutions for generating modFTO are colored in light-green. The secondary structure was obtained from PDB file (ID: 3lfm) and is displayed using ESPrnt 3.0.

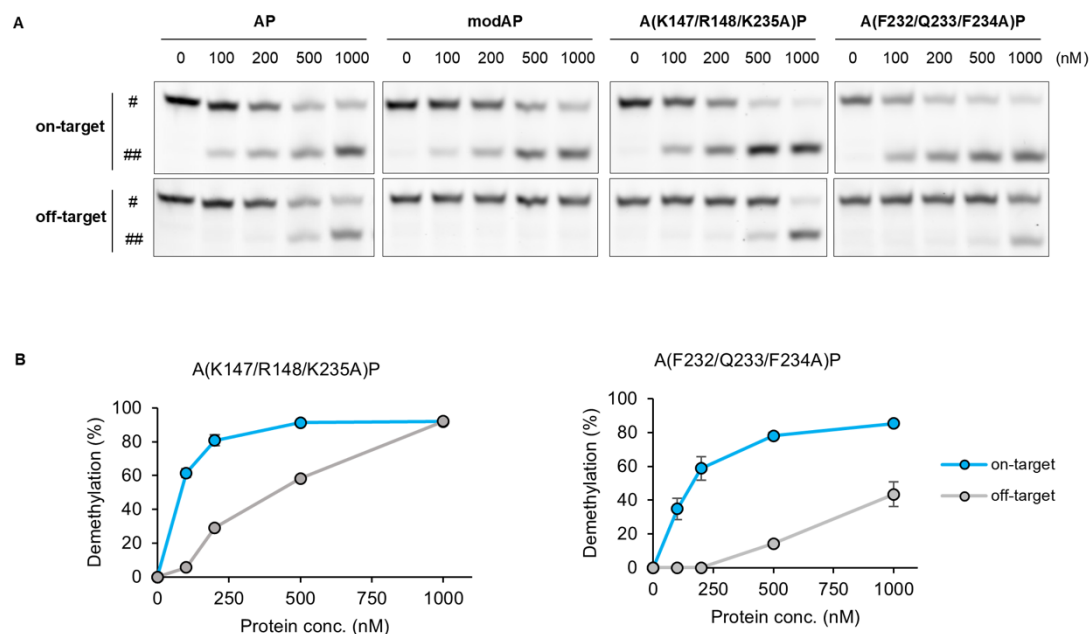


Figure S6. The demethylation activities of AP and its alanine substitutions to each m⁶A-modified RNA oligos at each protein concentration. **(A)** PAGE images of MazF assay. On-target RNA and off-target RNA were visualized by excitation with 488 nm (upper) and 532 nm (lower). #: un-cleaved RNA (methylated RNA), ##: cleaved RNA (demethylated RNA). **(B)** The plot of concentration-dependent demethylation activities of A(K147/R148/K235A)P and A(F232/Q232/F234A)P. Values and error bars indicate mean $\pm$ SEM (n=3).

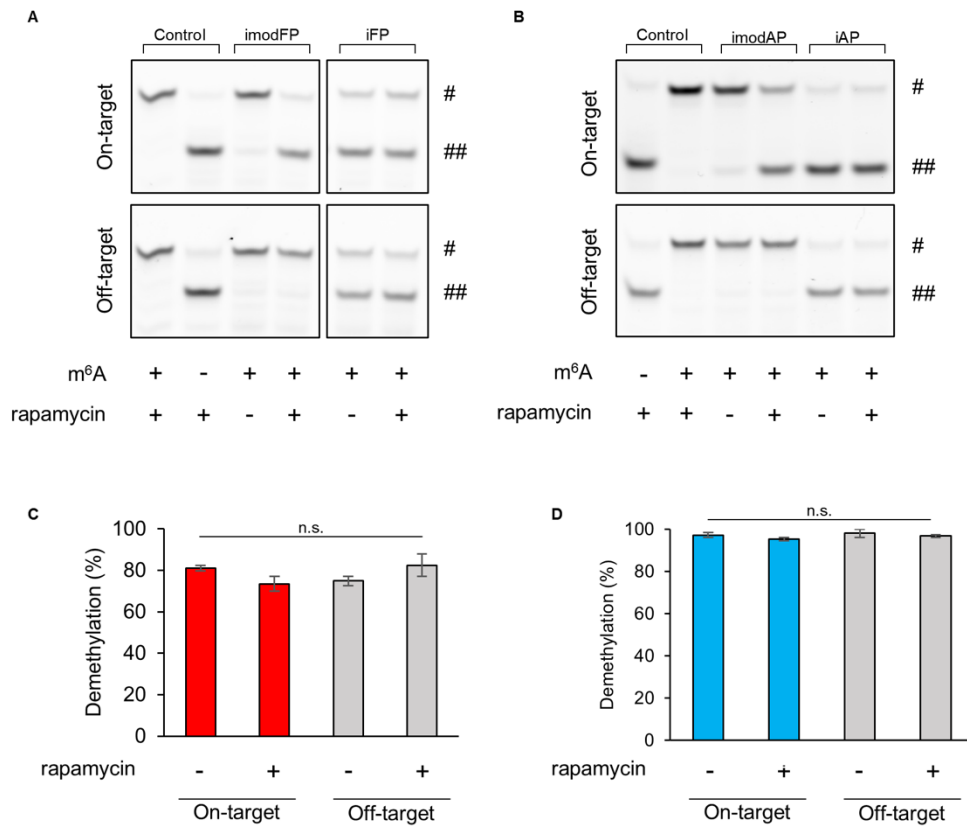


Figure S7. Sequence-specific demethylation activities and responsiveness to rapamycin of iFP, imodFP, iAP and imodAP. **(A, B)** PAGE images of MazF assay. The concentrations of **(A)** iFP and imodFP or **(B)** iAP and imodAP were 1 μ M and control contained no protein. On-target RNA and off-target RNA were visualized by excitation with 488 nm (upper) and 532 nm (lower). #: un-cleaved RNA (methylated RNA), ##: cleaved RNA (demethylated RNA). **(C, D)** Demethylation efficiencies of **(C)** iFP and **(D)** iAP towards on-target RNA or off-target RNA. For validation of iAP's demethylation activity 2-nt RNA (Table S1) was used as on-target RNA. Values and error bars indicate mean $\pm$ SEM (n=3, Turkey's; n.s.: not significant).