Supplementary Information (SI) for ChemComm.

Highly Sequence-specific, Timing-controllable m^6A Demethylation by Modulating RNA-binding Affinity of m^6A Erasers

Kenko Otonari, Yuri Asami, Kosuke Ogata, Yasushi Ishihama, Shiroh Futaki, Miki Imanishi

SUPPORTING INFORMATION

Materials and Methods

◆ **Plasmid designing**

The *E. coli* expression vector of mouse FTO (His_6-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 His_6-FTO or ALKBH5-2GSS-FRB-Strep and His_6-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 ºC 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 m^6A-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 $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 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 $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 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 m^6A on mRNA *in vitro*, the mRNA was demethylated in the following buffer: 25 mM Tris-HCl (pH 7.5), 283 μM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 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 ºC for 1 hr followed by heating at 95 ºC for 3 min. The MazF-digested sample was mixed with the same amount of Hi-Di Formamide (Applied Biosynthesis, #4311320), heated at 95 ºC 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 m^6A-modified ssRNA was incubated with increasing concentrations (40 to 3×10^4 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 m^6A 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 NH_4Ac 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 tC_{18} 1 cc Vac

Cartridge (Waters, #WAT036820). The sample was diluted with SPE buffer A (0.1% heptafluorobutyric acid (HFBA) in H_2O) 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 H_2O (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**AG(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

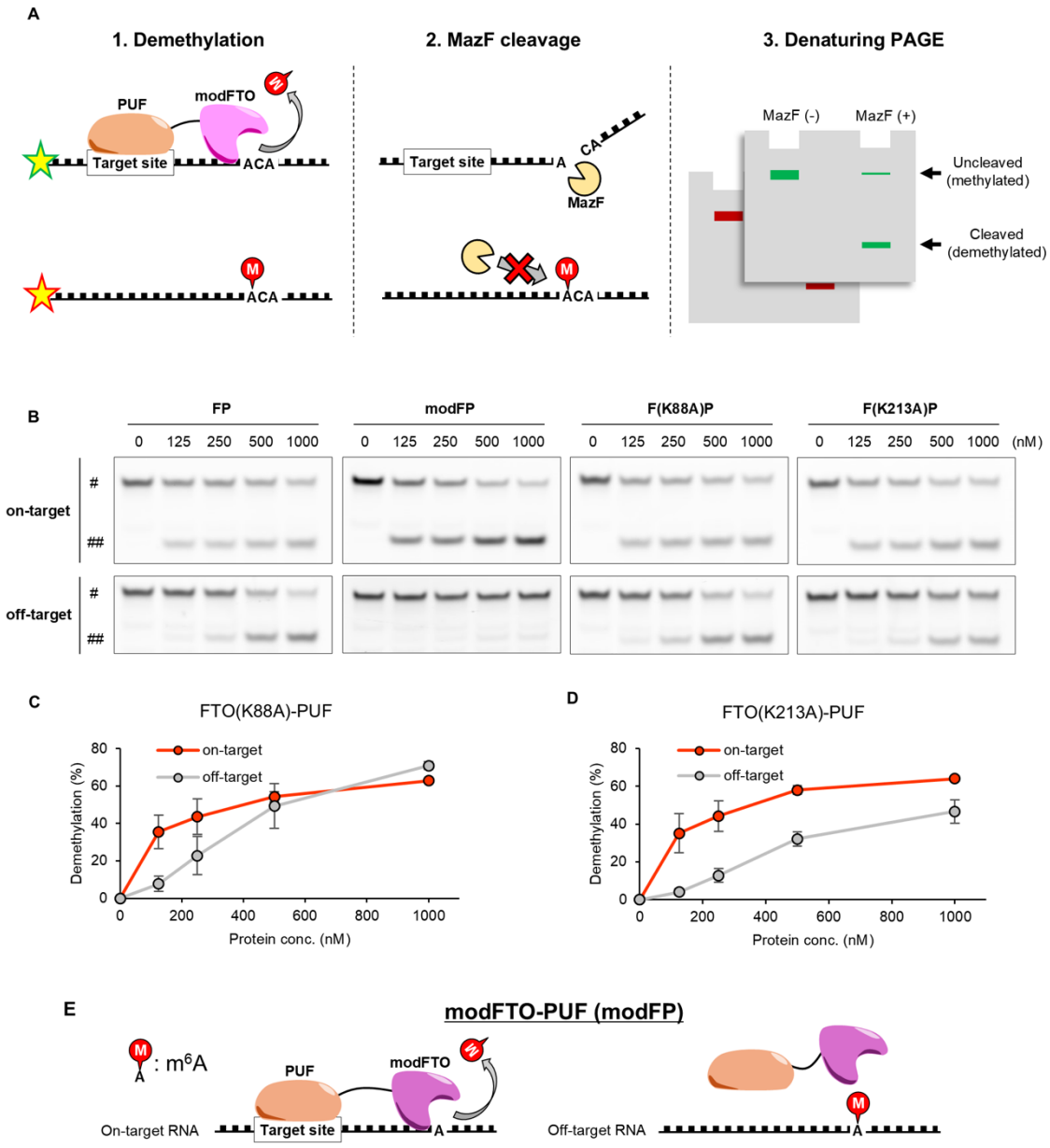

Figure S1. The demethylation activities of alanine substituted-FTO mutants fused with PUF to each m^{6}A-modified RNA oligos at each concentration. (**A**) Scheme to evaluate sequence-specific demethylation using MazF cleavage assay. MazF is an m^{6}A susceptive RNA endonuclease and cleaves only demethylated 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 ± SEM (n=3). **(E)** Schematic illustration about the sequence-specific m^{6}A demethylation by modFP.

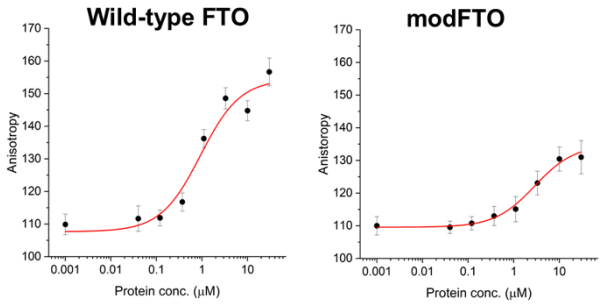

Figure S2. The plots of anisotropy at each concentration of proteins from 10 nM to 30 μM. Values and error bars indicate mean ± SEM (n=3).

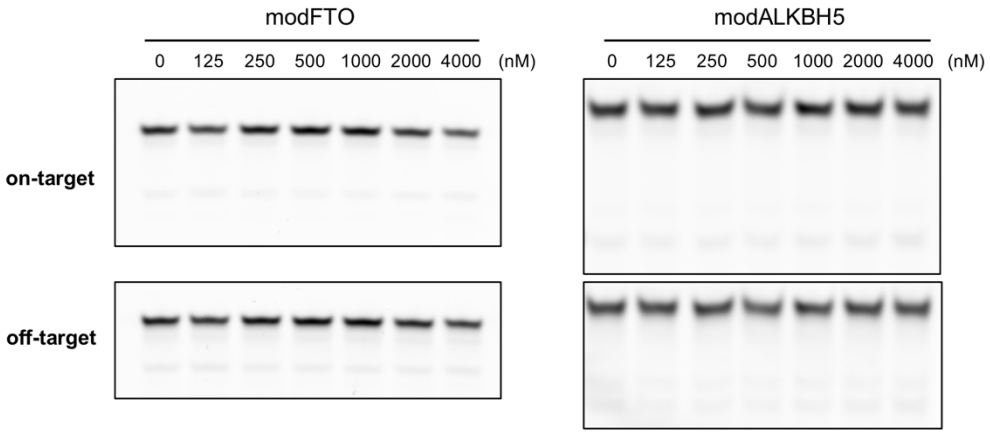

Figure S3. Demethylation activity of modFTO and modALKBH5 towards on-target RNA or off-target RNA evaluated by MazF cleavage assay. modFTO and modALKBH5 did not demethylate m^6A in either RNA oligo, even at higher concentration (~ 4000 nM).

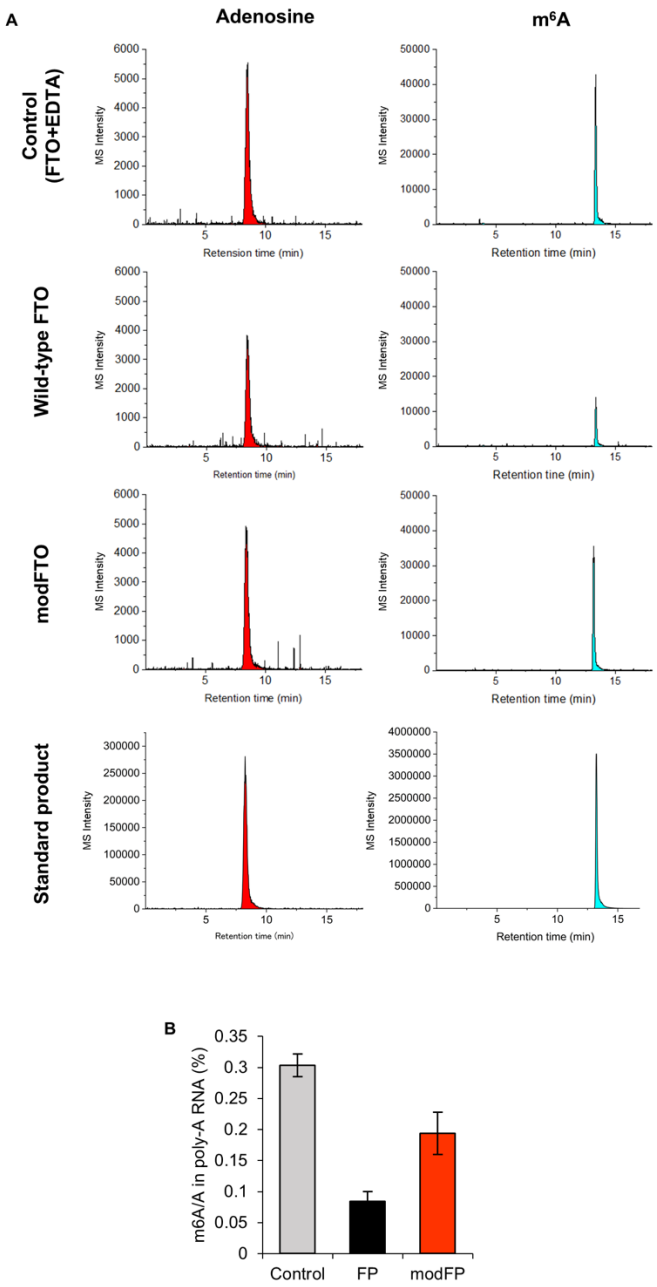

Figure S4. (**A**) Representative LC-MS/MS spectrometry of standard products (adenosine, m^6A) and mRNA purified from HEK293T cells treated with wild-type FTO or modFTO *in vitro*. Standard products were diluted at 1 g/L and injected 10 μL into HPLC. (**B**) Quantification of the m^6A/A in poly-A RNA treated with FP or modFP by LC-MS/MS. Values and error bars indicate mean ± SEM (n=3, Turkey; n.s.: not significant, ***: $p < 0.001$). The data were analyzed by OriginPro 2024 (ver. 10.1.0.170).

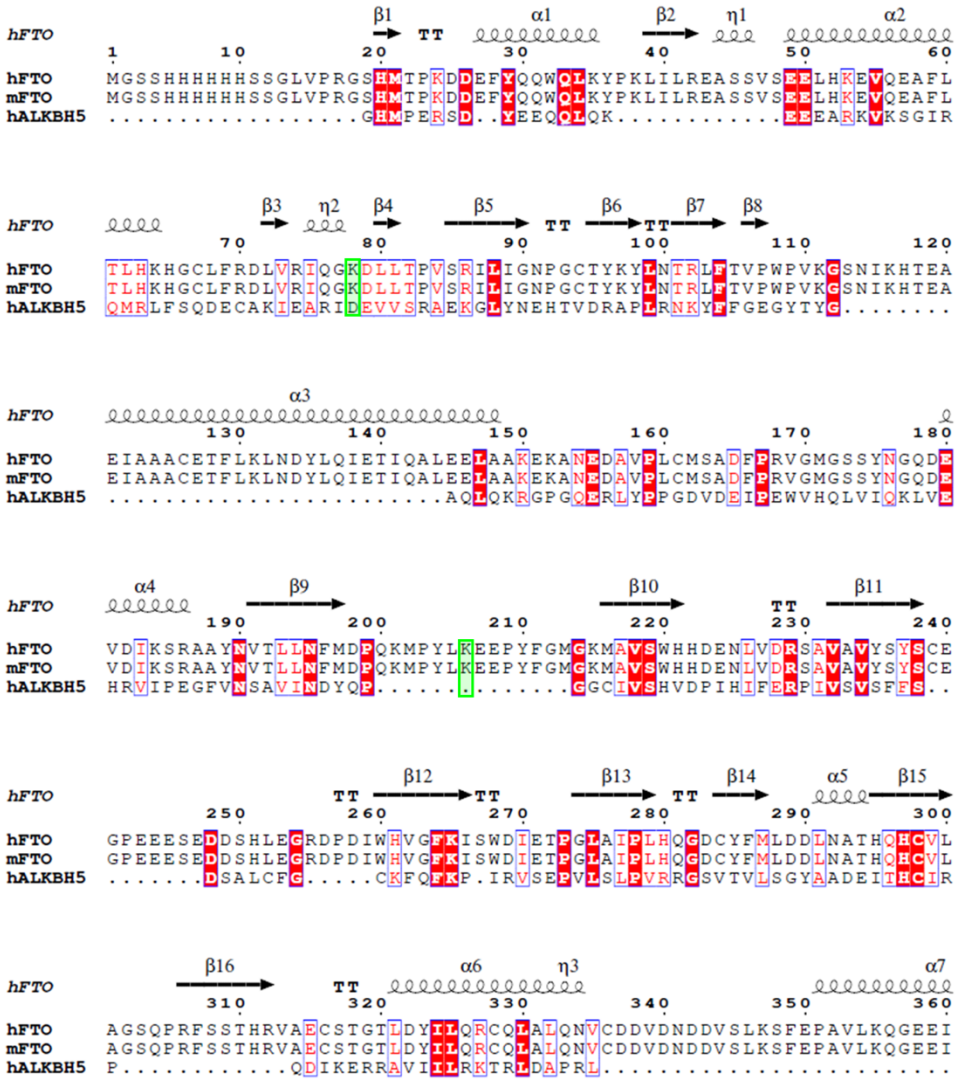

Figure S5. Structure-based sequence alignment of m^{6}A-erasers by STRAP. Residues added alanine substitutions for generating modFTO are colored in light-green. The secondary structure was obtained from PDB file (ID: 3lfm) and is displayed using ESPript 3.0.

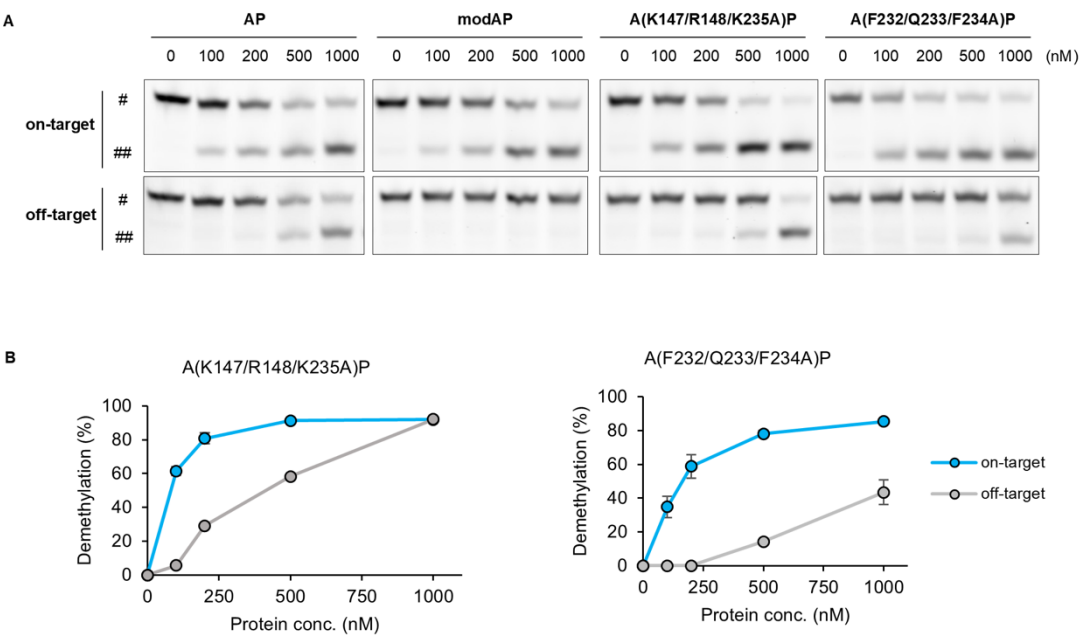

Figure S6. The demethylation activities of AP and its alanine substitutions to each m^{6}A-modified RNA oligos at each protein concentration. (**A**) PAGE images of MazF assay. On-target RNA and off-target RNA were visualized by excitation with 488 nm (upper) and 532 nm (lower). #: un-cleaved RNA (methylated 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 ± SEM (n=3).

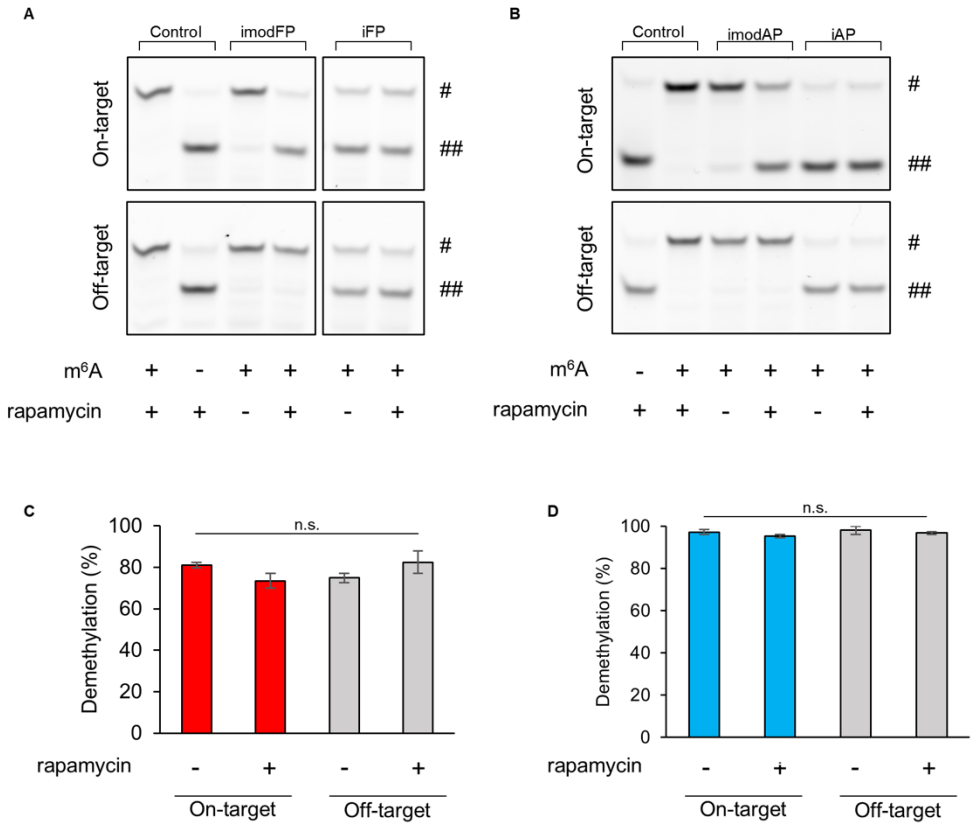

Figure S7. Sequence-specific demethylation activities and responsiveness to rapamycin of iFP, imodFP, iAP and imodAP. (**A, B**) PAGE images of MazF assay. The concentrations of (A) iFP and imodFP or (B) iAP and imodAP were 1 μM and control contained no protein. On-target RNA and off-target RNA were 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 ± SEM (n=3, Turkey's; n.s.: not significant).