Electronic Supplementary Material (ESI) for ChemComm.

Detection of N^6-methyladenosine

based on the methyl-sensitivity of MazF RNA endonuclease

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SUPPORTING INFORMATION

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MATERIALS AND METHODS

Chemicals and oligonucleotides

Enzymes used for vector constructions were obtained from NEB. MazF (MazF mRNA intereferase) was obtained from TAKARA Bio. DNA primers for plasmid constructions were obtained from eurofin or thermofischer. Oligo RNA and DNA/RNA chimera fragments showing below were purchased from Japan Bio Sciences.

For PAGE analysis:

ACA (GGACA) oligo RNA; 5'-FAM- UUGGUUUUUUUUGGACAUGUAUAUAGU -3'

(m6A)CA oligo RNA; 5'-FAM- UUGGUUUUUUUUGG(m6A)CAUGUAUAUAGU -3'

UGACA oligo RNA; 5'-FAM- AUUGUAUAUAUUUGACAUUUGGGGUUCUU -3'

For FRET analysis (DNA/RNA chimera probes):

"ACA" Probe; 5'-FAM - d(CAT) r(GGACA) d(TATGT) - 3' BHQ-1

"(m6A)CA" Probe; 5'-FAM - d(CAT) r(GG(m6A)CA) d(TATGT) - 3' BHQ-1

"A(5mC)A" Probe; 5'-FAM - d(CAT) r(GGA(5mC)A) d(TATGT) - 3' BHQ-1

"AC(m1A)" Probe; 5'-FAM - d(CAT) r(GGAC(m1A)) d(TATGT) - 3' BHQ-1

Expression vectors for demethylases and MTases

The expression vector of human FTOdN31[1] FTOdN31/pET28b, was constructed by inserting the amplified fragments encoding human FTO with N-terminal 31 residues truncated into NdeI/XhoI sites of pET28b (Novagen) to express the His-tagged hFTOdN31 protein. The expression vector of ALKBH5(66-292)[2] was constructed by inserting the amplified fragments encoding human ALKBH5(66-292) with a Strep-tag at the C-terminal into NdeI/XhoI sites of pET28b (Novagen) to express the His tag-hALKBH5(66-292)-Strep tag protein. The expression vector of the

methyltransferase domains (MTD) of METTL3 and METTL14[3] was constructed by inserting the amplified fragments encoding human METTL14(111-456) into EcoRI/HindIII sites of pETDuet1 (Novagen) and those encoding human METTL3(272-580) with a Strep-tag at the N-terminal into NdeI/XhoI sites of pETDuet1 to co-express His-tagged METTL3 and Strep-tagged METTL14 in *E. coli*.

Protein expression and purification

Protein expression and purification were performed basically based on previous reports.[1-3] The protein expression vectors were transformed into *E. coli* BL21(DE3) competent cells. Protein expression was induced by adding 0.1 mM IPTG at the logarithmic growth phase and incubating at 18 °C for overnight. The soluble fraction containing ALKBH5 was purified by the StrepTrapHP column (GE healthcare). The soluble fraction containing FTO was purified by the HisTrapFF column followed by the anion exchange chromatography (Resource Q column; GE healthcare). The soluble fraction containing METTL3/METTL14 was purified by the HisTrapFF column followed by StrepTrapHP column (GE healthcarre). ALKBH5 and FTO were concentrated by ultrafiltration using 25 mM Tris-HCl (pH 7.5). METTL3/METTL14 proteins were concentrated by ultrafiltration using 25 mM Tris-HCl (pH 7.5) with 100 mM NaCl. The purities of these proteins were confirmed by SDS-PAGE. The concentrated proteins containing 30% glycerol were dispensed and kept at -80 °C.

PAGE analysis of MazF cleavage

5'-FAM-labeled oligo RNA fragments (100 nM) were incubated with MazF mRNA interferase (0.05 ~ 0.1 U/μL) in the MazF reaction buffer (40 mM sodium phosphate (pH 7.5), 0.01% Tween 20, 0 or 5 mM EDTA) at 37 °C for 30 min. It was reported that divalent cation could inhibit the

enzymatic activity of MazF.[4] Therefore, 5 mM EDTA was added to MazF reaction buffer when treating the RNA solution after demethylation and methylation reactions containing 140 μM ferrous and 50 μM Zn(II), respectively. After MazF reaction, 1 μL of the solution was mixed with Hi-Di formamide (Thermofisher) and heated. The samples were loaded onto a 15% urea-polyacrylamide gel and electrophoresed in 0.5 × TBE buffer. The fluorescently labeled RNAs were visualized using Typhoon FLA9000 (GE Healthcare).

FRET detection of MazF cleavage

5'-FAM and 3'-BHQ1 dually-labeled oligo DNA/RNA chimera probes (100 or 500 nM) were mixed with MazF mRNA interferase (0.05 ~ 0.1 U/μL) in the MazF reaction buffer (40 mM sodium phosphate (pH 7.5), 0.01% Tween 20, 0 or 5 mM EDTA) in a Corning nonbinding surface (NBS) 96 well half area microplate (Corning) and fluorescences (Ex. filter: F485, Em. filter: F535) were measured using Arvo sx 1420 multilabel counter (Perkin Elmer) at 37 °C every 20-30 seconds for 20-60 min. The reaction volume in each well was 30 μL. As negative controls, each sample was treated with the MazF reaction buffer without MazF mRNA interferase. The intensities of fluorescences were plotted against time and averaged fluorescence levels at the saturated time points were used for further analysis.

Demethylation reaction

Labeled oligo RNA or DNA/RNA chimera fragments (1 or 5 μM) were mixed with FTO or ALKBH5 in the demethylation buffer (300 μM alpha-ketoglutarate, 140 μM $Fe(NH_4)_2(SO_4)_2$, 2 mM ascorbic acid, 50 mM Tris-HCl (pH 7.5)), incubated at 25 °C for 60 min and heat-inactivated (at 95 °C for 3 min). After demethylation reaction, 1/10 ~ 1/5 volume of the samples was subjected to MazF reaction for PAGE analysis or FRET measurements.

Methylation reaction

Labeled oligo RNA or DNA/RNA chimera fragments (0.5 or 1 μM) were mixed with METTL3/METTL14 in the methylation buffer (25 mM Tris-HCl (pH 7.5), 0.01% Tween-20, 1 mM DTT, 50 μM $ZnCl_2$, 0.1 U/uL RNasin PLUS (Promega), 10 μM SAM), incubated at 25 °C for 30 min and heat-inactivated (at 95 °C for 3 min). After methylation reaction, 1/10 ~ 1/5 volume of the samples was subjected to MazF reaction for PAGE analysis or FRET measurements.

Inhibition assays

Dually-labeled FRET probes (5 μM) were mixed with FTO or ALKBH5 in the demethylation buffer containing various concentrations (3-fold dilution from 900 μM) of meclofenamic acid (Sigma-aldrich), incubated at 25 °C for 30 min and heat inactivated (90 °C for 3 min). 3 μL of the samples was subjected to MazF reaction (30 μL) for FRET measurements.

REFERENCES

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3. P. Wang, K. A. Doxtader and Y. Nam, *Mol Cell*, 2016, **63**, 306-317.
4. Y. L. Zhang, J. J. Zhang, H. Hara, I. Kato and M. Inouye, *J Biol Chem*, 2005, **280**, 3143-3150.

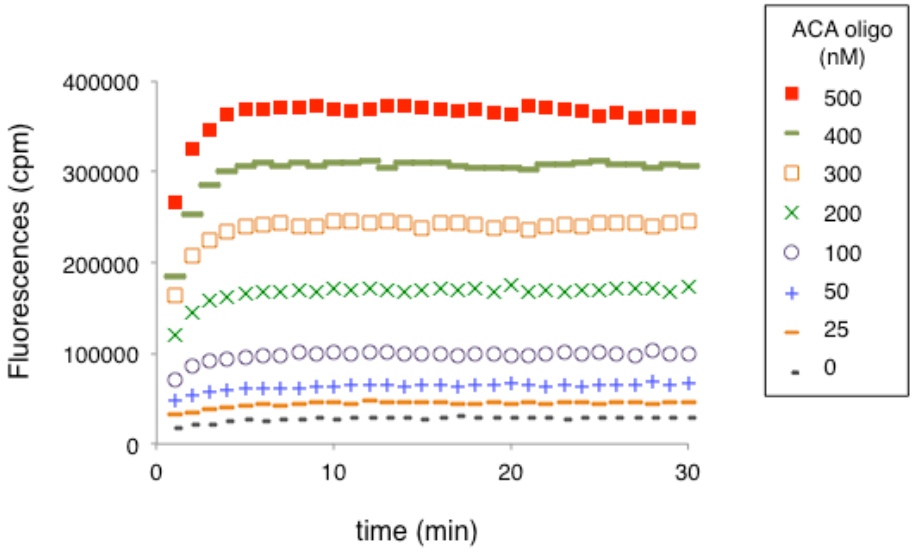

Figure S1. Raw data of Figure 1C, measuring fluorescence signals from the mixtures of "ACA" and "(m6A)CA" probes after addition of MazF. The "ACA" and "(m6A)CA" probes were mixed to a final concentration of 500 nM in total.

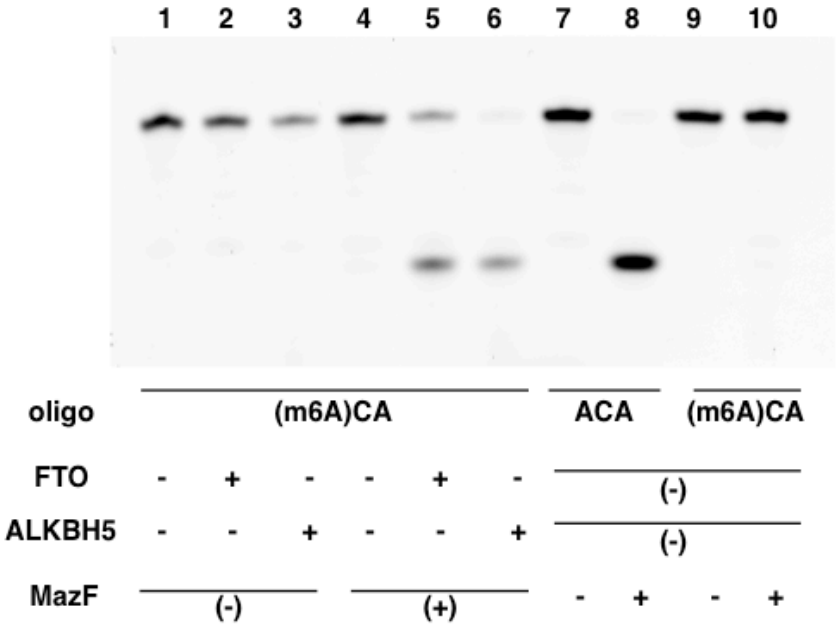

Figure S2. PAGE analysis of RNA cleavage by MazF. After reacting with FTO or ALKBH5, the "(m6A)CA" oligo gave cleaved bands (lanes 5 and 6) with the same mobility as the cleaved band of "ACA" oligo (lane 8).

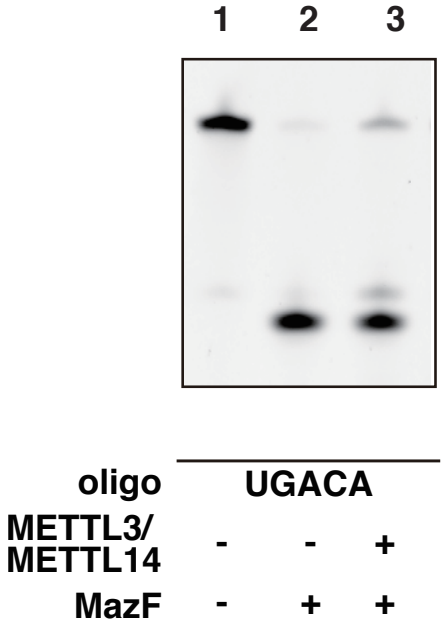

Figure S3. PAGE analysis of RNA cleavage by MazF following the methylation reaction. The 5'-FAM-labeled RNA containing 5'-UGACA-3' instead of 5'-GGACA-3' was used.