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Detection of N^6-methyladenosine based on the methyl-sensitivity of MazF RNA endonuclease

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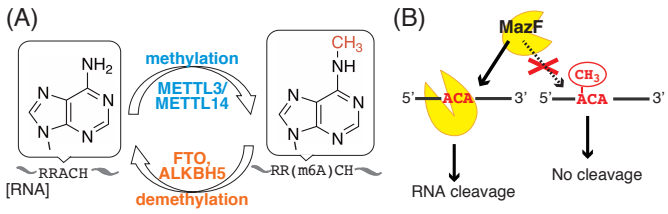
We found that *Escherichia coli* MazF toxin, an ACA-sequence-specific endoribonuclease, was sensitive to N^6-methyladenosine (m6A), representing the first m6A-sensitive RNA cleavage enzyme. The methyl-sensitivity of MazF allowed simple analyses of both m6A demethylase and methyltransferase activity. Furthermore, the approach could be used for inhibitor screening.

N^6-methyladenosine (m6A) is the most abundant internal modification in eukaryotic mRNA.[1, 2] Because of its crucial roles in various processes, including cell differentiation,[3] repair,[4] and the circadian clock,[5] m6A has been extensively studied in the fields of biology and chemical biology. m6A methylation and demethylation are catalyzed by the complex of methyltransferase-like (METTL)3/METTL14 methyltransferases,[6-9] and by fat mass and obesity-associated protein (FTO) or AlkB homolog 5 (ALKBH5) demethylases, respectively (Scheme 1A).[10-12] Recent reports have suggested that impairment of these m6A regulatory enzymes promotes tumorigenesis.[13-15] Despite increasing evidence of the importance of m6A RNA methylation, convenient methods to analyze the activities of RNA methyltransferases or demethylases and to screen their inhibitors are not available.

Conventional methods have been based on high-performance liquid chromatography (HPLC) analysis or the use of radioactive labeling of the methyl group.[16, 17] The former is laborious and time-consuming, and the latter requires radioactive materials. To conveniently detect demethylation activity of FTO or ALKBH5, a methylation-sensitive DNA restriction enzyme, DpnII, has been widely employed[16, 18] because DNA N^6-methyladenine can also be a substrate of FTO and ALKBH5. In this method, after demethylation of the single-stranded DNA fragment, the DNA is annealed with its complementary DNA and then reacted with DpnII. Demethylation can be detected as cleaved bands by polyacrylamide gel electrophoresis (PAGE), which is easy to perform without requiring any specific apparatus. However, it is impossible to evaluate the activity of demethylases against RNA substrates or those of METTL3/METTL14 RNA methyltransferases. More convenient methods are needed to assess the activities of these demethylases and methyltransferases using appropriate RNA substrates containing the 5′-RRACH-3′ (R = G/A; H = U, C, A) sequence, which is a consensus sequence highly methylated within eukaryotic mRNAs.[1, 2] RNA cleaving enzymes that discriminate between A and m6A would be useful for convenient determination of the activity of m6A regulatory enzymes on RNA methylation. However, no endoribonucleases with m6A sensitivity have been reported.



Scheme 1 (A) Reversible m6A RNA modification. Methylation occurs at the consensus RNA sequence 5′-RRACH-3′ (R=G/A, H=U/C/A) by the METTL3/METTL14 complex and demethylation is mediated by FTO or ALKBH5. (B) MazF cleaves only nonmethylated RNA, permitting specific detection of the methylation states of RNA.

The sequence-specific endoribonuclease MazF is an *Escherichia coli* toxin involved in growth regulation in response to stress.[19, 20] Under stress conditions, MazF dimers exhibit ribonuclease activity, leading to degradation of mRNA, reduction of protein synthesis, and promotion of growth arrest. The RNA cleavage reaction occurs at either the 5′ or 3′ phosphodiester linkage of the first A residue in the ACA sequence.[21,22] However, the sensitivity of MazF to methylated bases is unknown.

In this study, we focused on MazF because its target sequence, the ACA triplet, fulfills the criteria of a substrate for RNA methyltransferases. We found that MazF cleaved RNAs with a 5′-ACA-3′ sequence but not those with a 5′-(m6A)CA-3′

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sequence (Scheme 1B). To the best of our knowledge, this is the first example of an m6A-sensitive RNA cleavage enzyme. The cleavage of RNA by MazF can be detected by PAGE and by a fluorescence-resonance energy transfer (FRET)-based plate assays.[23] Based on these properties, we developed a novel high-throughput detection strategy for the activity of m6A demethylases and RNA methyltransferases.

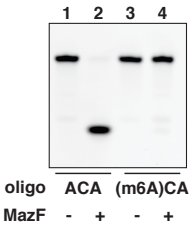

Fig. 1 Methylation sensitivity of MazF. 5′-FAM-labeled RNA fragments were reacted with MazF and loaded onto a denaturing polyacrylamide gel. The sequences of oligos "ACA" and "(m6A)CA" were 5′-FAM/UUGGUUUUUUUUGGACAUGUAUAUAGU-3′ and 5′-FAM/UUGGUUUUUUUUGG(m6A)CAUGUAUAUAGU-3′, respectively.

First, we explored whether MazF was a methylation-sensitive endoribonuclease. 5′-Fluorescently labeled RNA fragments containing 5′-ACA-3′ or 5′-(m6A)CA-3′ sequences were digested with MazF, which is commercially available, and electrophoresed in a denaturing urea polyacrylamide gel (Fig. 1). As a result, the nonmethylated RNA was almost completely cleaved by MazF, whereas no digested band was observed with the (m6A)CA-containing RNA. This clearly showed that MazF was m6A-sensitive.

FRET-based MazF cleavage assays also showed the m6A sensitivity of MazF. A 13-mer RNA/DNA chimeric oligonucleotide with a donor fluorophore (6-FAM) at the 5′ end and a fluorescence quencher (BHQ1) at the 3′ end was digested with MazF in a 96-well microplate, and fluorescence was monitored according to a previous report (Fig. 2A).[23] Without MazF, no increase in the intensity of the fluorescent signal was observed due to FRET between FAM and BHQ1 at each end of the probes. Increased fluorescence intensity was promptly observed by reaction of the nonmethylated probe with MazF, indicating the separation of the fluorophore from the quencher by MazF cleavage. In contrast, the (m6A)CA-containing probe did not show increased fluorescence even with MazF (Fig. 2B). This result again indicated that MazF was m6A-sensitive and that the methylation status at the N6 position of adenine in the sequence of 5′-ACA-3′ was detectable by MazF cleavage.

Quantitative accuracy of FRET-based MazF cleavage assays was assessed using mixtures of nonmethylated and methylated FRET probes in various ratios (Fig. 2C, S1). As shown in Figure 2C, the intensity of fluorescence was proportional to the content of the nonmethylated probe. Even a 5% content (0.75 pmol) of the nonmethylated probe was significantly detectable (Fig. 2C, inset). These findings demonstrate that the intensity of the fluorescence reflected the ratio of the nonmethylated probe.

The sensitivity of MazF to other RNA modifications within the MazF target sequence, 5′-ACA-3′, was also examined. 5-Methylcytosine (5mC) and N^1-methyladenosine (m1A) have also been identified as epigenetic modifications in RNA.[11, 24, 25] FRET-based MazF cleavage assays using dually labeled probes containing 5′-A(5mC)A-3′ or 5′-AC(m1A)-3′ sequences were performed. As shown in Figure 1D, MazF cleaved RNA with 5′-A(5mC)A-3′ but barely cleaved RNA with 5′-AC(m1A)-3′, indicating the sensitivity of MazF to m1A (Fig. 2D).

The crystal structure of *E. coli* MazF in complex with a 7-nucleotide substrate mimic showed three hydrogen bonds through the Hoogsteen edge of the first adenine base with backbone carbonyl and amide atoms of Gly22, Glu24, and Ala26.[26] The involvement of the N^6-amino group of the first adenine in the above interaction may result in inhibition of the interaction between MazF and the 5′-(m6A)CA-3′ RNA. The crystal structure also showed that the side chain of Ser23 formed a hydrophobic interaction with the pyrimidine ring of the second adenine, but that no interaction existed between MazF and the fifth position of cytosine. Such properties can also explain the sensitivity of MazF to 5′-AC(m1A)-3′ but not to 5′-A(5mC)A-3′.

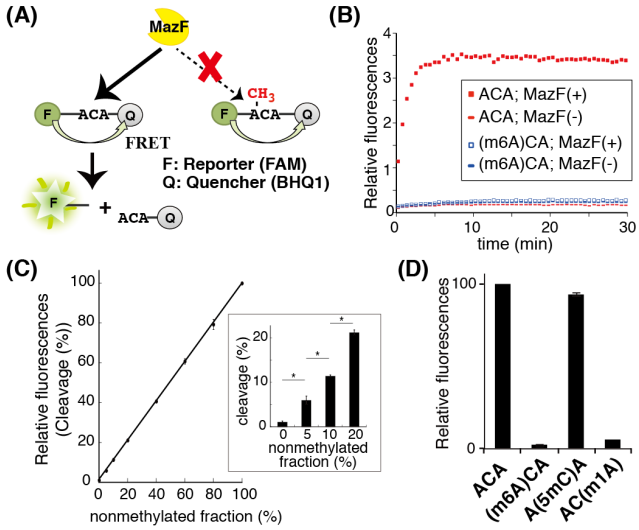

Fig. 2 Determination of the methylation status of RNA by FRET-based MazF assays. (A) Schematic representation of FRET-based m6A detection by MazF. RNA/DNA chimera probes with a FRET pair at the ends (5′-FAM/3′-BHQ1) were mixed with MazF, and the fluorescent signals from the cleaved probe were monitored. (B) Discrimination of ACA and (m6A)CA by FRET-based MazF assays. The sequences of the "ACA" and "(m6A)CA" probes were 5′-FAM/d(CAT)r(GGACA)d(TATGT)/BHQ1-3′ and 5′-FAM/d(CAT)r(GG(m6A)CA)d(TATGT)/BHQ1-3′, respectively. (C) Quantitative measurements of nonmethylated probes. The "ACA" and "(m6A)CA" FRET probes were mixed to a final concentration of 500 nM and subjected to MazF in 30 μL reactions. The inset shows significant differences in cleavage ratio among samples with low contents of ACA oligo (0–20%). Values represent mean ± SD. (n = 3) *P < 0.001 (Student's t-test). (D) Dually labeled FRET probes containing "ACA", "(m6A)CA", "A(5mC)A", or "AC(m1A)" sequences were reacted with MazF and the fluorescent signals from the cleaved probe were monitored. Fluorescence intensities from each probe were normalized to the "ACA" probe.

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Next, RNA demethylation activities of FTO and ALKBH5 were examined by MazF cleavage assays. The MazF reaction following treatment of (m6A)CA RNA with FTO or ALKBH5 gave cleaved bands with the same mobility as a cleaved band of the nonmethylated oligo in a polyacrylamide gel (Fig. 3A, S2). The FRET-based MazF assay also demonstrated an increase in the intensity of fluorescence depending on the concentration of FTO and ALKBH5 (Fig. 3B, C). These results indicate that the (m6A)CA RNA fragments were demethylated by FTO and ALKBH5, resulting in cleavage by MazF.

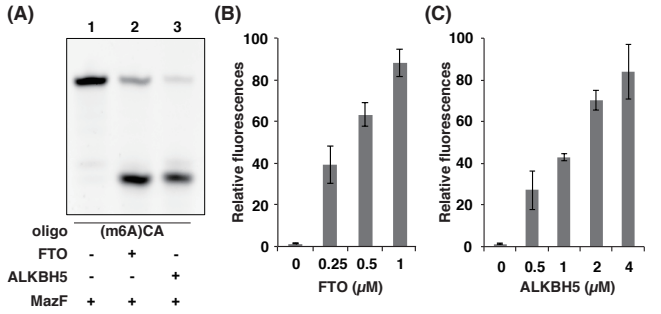

Fig. 3 Detection of the demethylation activity of FTO and ALKBH5 by MazF cleavage assays. (A) PAGE analysis of RNA cleavage by MazF following the demethylation reaction with FTO (lane 2) or ALKBH5 (lane 3). The 5′-FAM-labeled "(m6A)CA" RNA was used. (B, C) Results of FRET-based MazF cleavage assays after the demethylation reaction with various concentrations of FTO (B) or ALKBH5 (C). The "(m6A)CA" FRET probe was used. The fluorescence intensity obtained from MazF-based cleavage of the nonmethylated "ACA" FRET probe was set to 100.

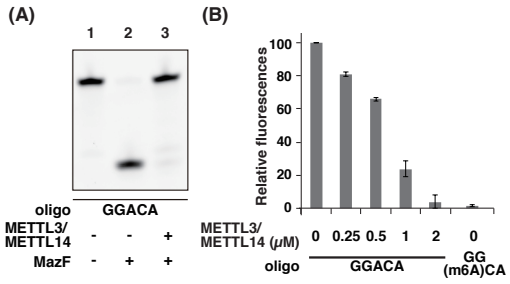

Fig. 4 Detection of the methylation activity of METTL3/METTL14 by MazF cleavage assays. (A) PAGE analysis of RNA cleavage by MazF following the methylation reaction. The 5′-FAM-labeled RNA containing 5′-GGACA-3′ was used. (B) The FRET-based MazF cleavage assay after methylation by various concentration of METTL3/METTL14. The "ACA" FRET probe was used.

Furthermore, RNA methylation at the N6 position of adenine mediated by the RNA methyltransferases METTL3/METTL14 was also detected by the MazF cleavage assay (Fig. 4). A nonmethylated RNA with the 5′-GGACA-3′ sequence was reacted with METTL3/METTL14 and S-adenosyl methionine (SAM), a methyl donor, and then subjected to MazF. In the absence of METTL3/METTL14, the RNA probe was almost completely cleaved by MazF (Fig. 4A, lane 2). After reaction with METTL3/METTL14, no cleaved band was observed, indicating that the RNA probe was methylated by METTL3/METTL14 (Fig. 4A, lane 3). To confirm that the lack of cleavage did not result from interference between MazF and METTL3/METTL14, we showed that an RNA probe containing the 5′-UGACA-3′ sequence, which is not the consensus target sequence (5′-RRACH-3′) for METTL3/METTL14, was mostly cleaved by MazF even after reacting with METTL3/METTL14 (Fig. S3). FRET-based MazF assays using the nonmethylated FRET probe with the 5′-GGACA-3′ sequence showed that the intensity of fluorescence decreased depending on the concentration of METTL3/METTL14 (Fig. 4B). These results also suggested the feasibility of this method to detect m6A RNA methylation.

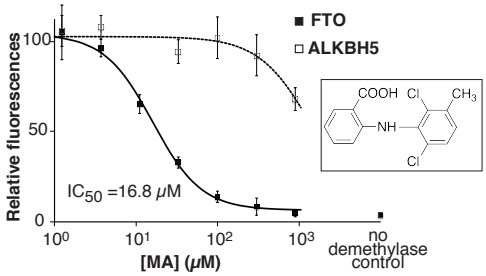

Fig. 5 Inhibition of FTO by meclofenamic acid (MA; inset) detected by FRET-based MazF cleavage assays. MA (0–900 μM) was added to the demethylation samples containing 250 nM FTO (filled square) or 1 μM ALKBH5 (open square), followed by MazF cleavage. The "(m6A)CA" FRET probe was used. The fluorescence intensity obtained from the sample without MA was set to 100. Data are mean ± SD (n = 3).

The FRET-based MazF cleavage assay is a homogeneous assay and can be performed using microplates. Therefore, the applicability of the assay for screening of demethylases inhibitors was explored. In the presence of meclofenamic acid, which selectively inhibits FTO demethylation of m6A over ALKBH5,[27] the intensity of fluorescent signals from FTO-treated samples decreased in response to the concentration of meclofenamic acid (Fig. 5). The half maximal inhibitory concentration (IC_{50}) value was 16.8 ± 1.1 μM, which was similar to the reported value (8 μM).[27] In contrast, there was little decrease in the intensity of fluorescence in ALKBH5-treated samples, even in the presence of 300 μM meclofenamic acid, and the IC_{50} value was higher than 1 mM (Fig. 5). The result, i.e., that meclofenamic acid inhibited FTO but not ALKBH5, was consistent with a previous report,[27] indicating the applicability of our method for the screening of demethylase or methyltransferase inhibitors.

Inhibitors or activators of m6A regulatory enzymes have great potential for both basic research and therapeutic application. To date, FTO and ALKBH5 demethylase inhibitors have been screened using analogues of 2-oxoglutarate (2OG) and substrate nucleotides through two independent assays: binding assays of compounds to enzymes and laborious demethylation assays using each hit.[28] The strategy is not very effective for screening large pools of compounds to identify FTO- or ALKBH5-specific inhibitors. Structure-based modeling and screening strategies contribute to the exploration of subfamily-selective inhibitors,[18] but also require step-by-step evaluation processes. Huang *et al*. developed a high-throughput fluorescence polarization assay for identifying compounds inhibiting the interaction between FTO and m6A-

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containing single-stranded DNA and found an FTO-selective inhibitor from various compounds.[27] However, it is still difficult to obtain inhibitors with no effects on m6A binding. Li *et al*. reported a radioactivity-based high-throughput screening strategy that could directly detect the effects of compounds on demethylation activities.[17] Our FRET-based MazF cleavage assay should be an alternative strategy to directly detect methylation states without using radioactive materials.

In this study, we found that the *E. coli* toxin MazF, an RNA endonuclease specific to the 5′-ACA-3′ sequence, was sensitive to N^6-methylation of the first adenine. Based on the ability of MazF to discriminate between 5′-ACA-3′ and 5′-(m6A)CA-3′, new strategies to detect the enzymatic activities of both demethylases and methyltransferases on substrate RNA were developed. The applicability of the MazF cleavage methods is limited to *in vitro* evaluation of m6A in this stage, because MazF cleaves single stranded RNA at 5′-ACA-3′ sequences frequently shown in endogenous RNAs. In addition, MazF dose not cleave 5′-ACA-3′ in double stranded RNA,[20] prohibiting accurate judgment of the existence of m6A in a structured RNA. Nonetheless, the FRET-based homogeneous assay enabled simple, rapid detection of the activities of m6A regulatory enzymes on substrate RNA, providing a means for screening of their inhibitors. In addition to m6A, we showed that MazF was sensitive to m1A at the second adenine. Our strategy can be applied to analyze the m1A regulatory enzymes.

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Conflicts of interest

There are no conflicts to declare.

Notes and references

1. D. Dominissini, S. Moshitch-Moshkovitz, S. Schwartz, M. Salmon-Divon, L. Ungar, S. Osenberg, K. Cesarkas, J. Jacob-Hirsch, N. Amariglio, M. Kupiec, R. Sorek and G. Rechavi, *Nature*, 2012, **485**, 201-206.
2. K. D. Meyer, Y. Saletore, P. Zumbo, O. Elemento, C. E. Mason and S. R. Jaffrey, *Cell*, 2012, **149**, 1635-1646.
3. P. J. Batista, B. Molinie, J. K. Wang, K. Qu, J. J. Zhang, L. J. Li, D. M. Bouley, E. Lujan, B. Haddad, K. Daneshvar, A. C. Carter, R. A. Flynn, C. Zhou, K. S. Lim, P. Dedon, M. Wernig, A. C. Mullen, Y. Xing, C. C. Giallourakis and H. Y. Chang, *Cell Stem Cell*, 2014, **15**, 707-719.
4. Y. Xiang, B. Laurent, C. H. Hsu, S. Nachtergaele, Z. K. Lu, W. Q. Sheng, C. Y. Xu, H. C. Hen, O. Y. Jian, S. Q. Wang, D. Ling, P. H. Hsu, L. Zou, A. Jambhekar, C. Iie and Y. Shi, *Nature*, 2017, **543**, 573-576.
5. J. M. Fustin, M. Doi, Y. Yamaguchi, H. Hida, S. Nishimura, M. Yoshida, T. Isagawa, M. S. Morioka, H. Kakeya, I. Manabe and H. Okamura, *Cell*, 2013, **155**, 793-806.
6. J. Z. Liu, Y. N. Yue, D. L. Han, X. Wang, Y. Fu, L. Zhang, G. F. Jia, M. Yu, Z. K. Lu, X. Deng, Q. Dai, W. Z. Chen and C. He, *Nat Chem Biol*, 2014, **10**, 93-95.
7. X. Wang, J. Feng, Y. Xue, Z. Guan, D. Zhang, Z. Liu, Z. Gong, Q. Wang, J. Huang, C. Tang, T. Zou and P. Yin, *Nature*, 2016, **534**, 575-578.
8. P. Wang, K. A. Doxtader and Y. Nam, *Mol Cell*, 2016, **63**, 306-317.
9. P. Sledz and M. Jinek, *Elife*, 2016, **5**, e18434.
10. G. F. Jia, Y. Fu, X. Zhao, Q. Dai, G. Q. Zheng, Y. Yang, C. Q. Yi, T. Lindahl, T. Pan, Y. G. Yang and C. He, *Nat Chem Biol*, 2011, **7**, 885-887.
11. B. X. S. Zhao, I. A. Roundtree and C. He, *Nat Rev Mol Cell Bio*, 2017, **18**, 31-42.
12. G. Q. Zheng, J. A. Dahl, Y. M. Niu, P. Fedorcsak, C. M. Huang, C. J. Li, C. B. Vagbo, Y. Shi, W. L. Wang, S. H. Song, Z. K. Lu, R. P. G. Bosmans, Q. Dai, Y. J. Hao, X. Yang, W. M. Zhao, W. M. Tong, X. J. Wang, F. Bogdan, K. Furu, Y. Fu, G. F. Jia, X. Zhao, J. Liu, H. E. Krokan, A. Klungland, Y. G. Yang and C. He, *Mol Cell*, 2013, **49**, 18-29.
13. C. Zhang, W. I. Zhi, H. Lu, D. Samanta, I. Chen, E. Gabrielson and G. L. Semenza, *Oncotarget*, 2016, **7**, 64527-64542.
14. S. R. Jaffrey and M. G. Kharas, *Genome Med*, 2017, **9**, 2.
15. C. T. Kwok, A. D. Marshall, J. E. Rasko and J. J. Wong, *J Hematol Oncol*, 2017, **10**, 39.
16. G. F. Jia, C. G. Yang, S. D. Yang, X. Jian, C. Q. Yi, Z. Q. Zhou and C. He, *Febs Lett*, 2008, **582**, 3313-3319.
17. F. Li, S. Kennedy, T. Hajian, E. Gibson, A. Seitova, C. Xu, C. H. Arrowsmith and M. Vedadi, *J Biomol Screen*, 2016, **21**, 290-297.
18. B. E. Chen, F. Ye, L. Yu, G. F. Jia, X. T. Huang, X. J. Zhang, S. Y. Peng, K. Chen, M. N. Wang, S. Z. Gong, R. H. Zhang, J. Y. Yin, H. Y. Li, Y. M. Yang, H. Liu, J. W. Zhang, H. Y. Zhang, A. Zhang, H. L. Jiang, C. Luo and C. G. Yang, *J Am Chem Soc*, 2012, **134**, 17963-17971.
19. H. Engelberg-Kulka, R. Hazan and S. Amitai, *J Cell Sci*, 2005, **118**, 4327-4332.
20. M. Inouye, *J Cell Physiol*, 2006, **209**, 670-676.
21. Y. L. Zhang, J. J. Zhang, H. Hara, I. Kato and M. Inouye, *J Biol Chem*, 2005, **280**, 3143-3150.
22. Y. L. Zhang, J. J. Zhang, K. P. Hoeflich, M. Ikura, G. L. Qing and M. Inouye, *Mol Cell*, 2003, **12**, 913-923.
23. N. R. Wang and P. J. Hergenrother, *Anal Biochem*, 2007, **371**, 173-183.
24. D. Dominissini, S. Nachtergaele, S. Moshitch-Moshkovitz, E. Peer, N. Kol, M. S. Ben-Haim, Q. Dai, A. Di Segni, M. Salmon-Divon, W. C. Clark, G. Zheng, T. Pan, O. Solomon, E. Eyal, V. Hershkovitz, D. Han, L. C. Dore, N. Amariglio, G. Rechavi and C. He, *Nature*, 2016, **530**, 441-446.
25. X. Li, X. Xiong, K. Wang, L. Wang, X. Shu, S. Ma and C. Yi, *Nat Chem Biol*, 2016, **12**, 311-316.
26. V. Zorzini, A. Mernik, J. Lah, Y. G. J. Sterckx, N. De Jonge, A. Garcia-Pino, H. De Greve, W. Versees and R. Loris, *J Biol Chem*, 2016, **291**, 10950-10960.
27. Y. Huang, J. L. Yan, Q. Li, J. F. Li, S. Z. Gong, H. Zhou, J. H. Gan, H. L. Jiang, G. F. Jia, C. Luo and C. G. Yang, *Nucleic Acids Res*, 2015, **43**, 373-384.
28. W. Aik, M. Demetriades, M. K. Hamdan, E. A. Bagg, K. K. Yeoh, C. Lejeune, Z. Zhang, M. A. McDonough and C. J. Schofield, *J Med Chem*, 2013, **56**, 3680-3688.

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Detection of N^6-methyladenosine

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SUPPORTING INFORMATION

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

1. Z. Han, T. Niu, J. Chang, X. Lei, M. Zhao, Q. Wang, W. Cheng, J. Wang, Y. Feng and J. Chai, *Nature*, 2010, **464**, 1205-1209.
2. W. Aik, J. S. Scotti, H. Choi, L. Z. Gong, M. Demetriades, C. J. Schofield and M. A. McDonough, *Nucleic Acids Res*, 2014, **42**, 4741-4754.
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.

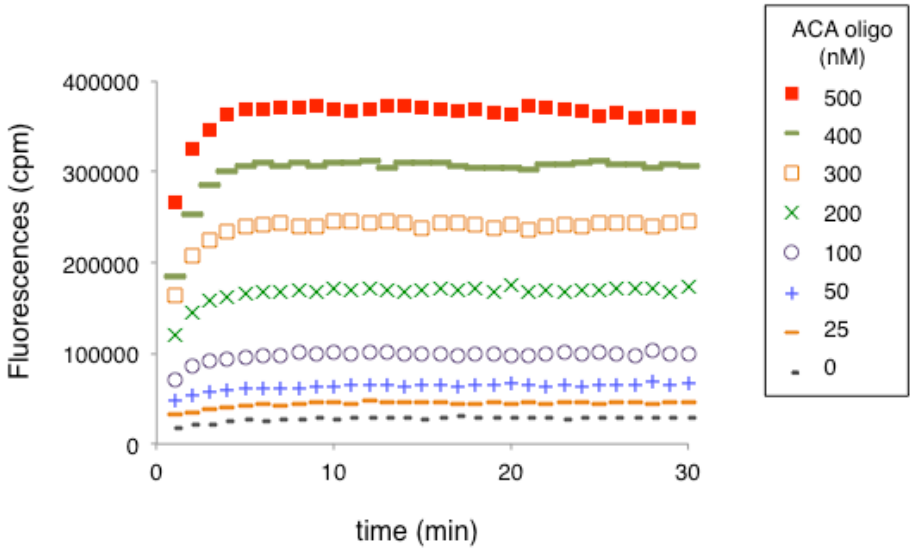

Figure S1. Raw data of Figure 1C, measuring fluorescence signals from the mixtures of "ACA" and "(m6A)CA" probes after addition of MazF. The "ACA" and "(m6A)CA" probes were mixed to a final concentration of 500 nM in total.

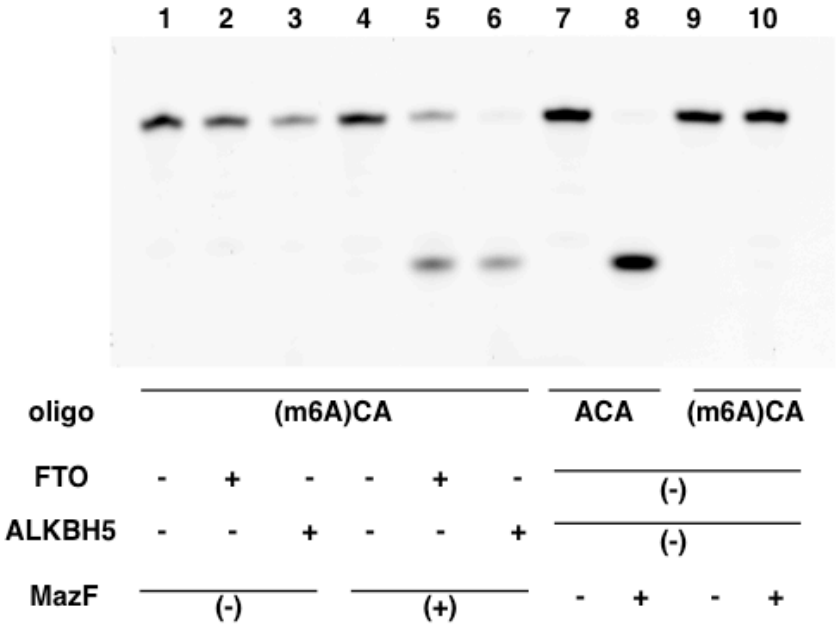

Figure S2. PAGE analysis of RNA cleavage by MazF. After reacting with FTO or ALKBH5, the "(m6A)CA" oligo gave cleaved bands (lanes 5 and 6) with the same mobility as the cleaved band of "ACA" oligo (lane 8).

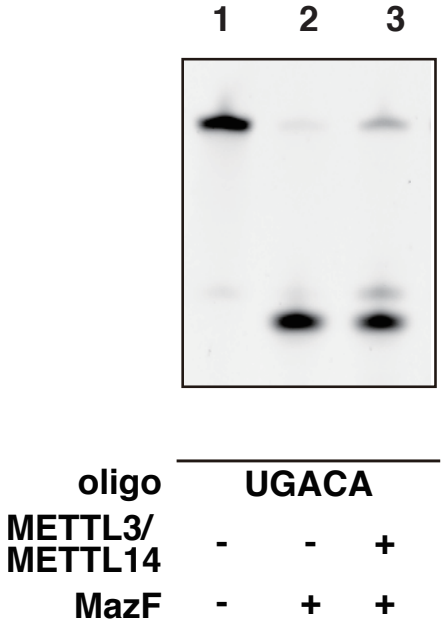

Figure S3. PAGE analysis of RNA cleavage by MazF following the methylation reaction. The 5'-FAM-labeled RNA containing 5'-UGACA-3' instead of 5'-GGACA-3' was used.