Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information for

Programmable RNA methylation and demethylation using PUF RNA binding proteins

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Supplementary materials and methods

Supplementary figures; Fig S1-S5

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 hybrid fragments showing below were purchased from Japan Bio Sciences. The PUF binding sequences are underlined and the MazF targeting sequences (unmethylated, or when demethylated) are shown in bold.

RNA[A^m] 5'-FAM –r(AUUGUAUAUAUCUAAG **^{m6}ACA**UUUUA) - 3'

RNA[A] 5'-FAM –r(AUUGUAUAUAUCUAAG**ACA**UUUUA) - 3'

RNA[B^m] 5'-TAMRA-r(AUAUCUCUUGGGGUUCUAUUAG **^{m6}ACA**UUUAG) - 3'

RNA[B] 5'-TAMRA-r(AUAUCUCUUGGGGUUCUAUUAG**ACA**UUUAG) - 3'

DNA/RNA chimera (Fig S2A); 5'-FAM - d(CAT) r(GG**^{m6}ACA**) d(TATGT) - 3' BHQ-1

-1RNA (Fig S2C); 5′FAM-AUUGUAUAU**^{m6}ACA**UUUUA-3′

+2RNA (Fig S2C); 5′FAM-AUUGUAUAUAAG**^{m6}ACA**UUUUA-3′

+10RNA(Fig S2C); 5′FAM-AUUGUAUAUAUCGGCUUUAG**^{m6}ACA**UUUUA-3′

Preparation of competitor RNAs

The 246 nt of the competitor RNA was synthesized using *in vitro* Transcription T7 Kit (Takara-bio). To prepare the DNA template, an ACA-less DNA fragment was amplified from pmirGLO plasmid (Promega) by PCR using a forward primer (5′-atggagcc TAATACGACTCACTATAGGG tcgaggaggatatcgccctgatc-3′, underline; T7 promoter) and a reverse primer (5′-agttgcggacaatctggacgacg-3′).

Total RNA was extracted from HeLa cells using NucleoSpin RNA Plus (Takara-bio).

Vector construction and protein preparation

The *E. coli* expression vector of mouse FTO (His_6-mFTO-strep/pET28b) was constructed by inserting cDNA of mouse FTO into pET28b with His-tag and strep-tag. The *E. coli* expression vectors of FTO-PUFs were created by constructing His_6-mFTO-[linker]-PUF(N-C)-strep/pET28b from His_6-mFTO-strep/pET28b and pET28-GG-PUF in PUF Assembly Kit (Addgene #1000000051). Then, DNA fragments encoding repeats 1-8 of PUF**a** and PUF**b** were assembled using the golden-gate assembly method using following units included in the PUF Assembly Kit; PUF**a**: 1SQ-2NQ-3CQ-4NQ-5CQ-6NQ-7SE-8NQ, PUF**b**: 1SR-2NQ-3NQ-4SE-5SE-6SE-7SE-8NQ. As linkers, $(GSS)_1$-, $(GSS)_2$-, $(GSS)_3$- and FXP-encoding

sequences were used. The amino acid sequences of these linkers are shown in Fig S2C.

The *E. coli* expression vector of the methyltransferase domains (MTDs) of METTL3 and METTL14 (MTD3/MTD14) was constructed by inserting the amplified fragments encoding human METTL14(111-456) into EcoRI/HindIII sites of pETDuet1 (Novagen) and those encoding human METTL3(255-580) with a Strep-tag at the N-terminal into NdeI/XhoI sites of pETDuet1 to co-express His_6-tagged MTD3 and Strep-tagged MTD14 in *E. coli*. The *E. coli* expression vector of MTD3 and MTD14d was constructed by deleting the METTL14(397-456) encoding sequence form the MTD3/MTD14 expression vector. The DNA sequence encoding PUF**a** or PUF**b** was inserted at the 3'-terminal of the MTD14d-encoding region, creating MTD3/MTD14d-PUF(**a** or **b**) in pETDuet1 vectors. Protein expression and purification were performed as described before (Imanishi, et al, *Chem Commun*, 2017, **53**, 12930-12933).

FRET detection of MazF cleavage of demethylated samples

The DNA/RNA hybrid oligo (500 nM) was mixed with FTO or FTO-PUFa (0.1, 0.25, 0.5, and 1 μM) in the demethylation buffer (150 μM alpha-ketoglutarate, 70 μM $Fe(NH_4)_2(SO_4)_2$, 1 mM ascorbic acid, 50 mM NaCl, 50 μg/mL BSA, 100 μg/mL yeast tRNA, 0.01% Tween 20, 25 mM Tris-HCl (pH 7.5)), incubated at 25 °C for 60 min and heat-inactivated (at 95 °C for 3 min). 3 μL of the samples was subjected to MazF reaction (total 30 μL) for FRET measurements as described before (Imanishi, et al, *Chem Commun*, 2017, **53**, 12930-12933).

PAGE analysis of MazF cleavage of demethylated samples

FTO or FTO-PUFa (0.1 μM) was incubated with -1RNA, +2RNA, +6RNA,or +10RNA (100 nM) in the demethylation buffer at 25 °C for 60 min and heat-inactivated (at 95 °C for 3 min). 1 μL of the samples was subjected to MazF reaction (total 10 μL) and loaded onto a urea-PAGE as described before (Imanishi, et al, *Chem Commun*, 2017, **53**, 12930-12933).

Detection of the sequence-specific demethylation

Either FTO, FTO-PUF**a** or FTO-PUF**b** (100 nM) was incubated with RNA[A^m] (FAM-labeled, 24 nt, 50 nM) and RNA[B^m] (TAMRA-labeled, 30 nt, 50 nM) in the presence or absence of competitor RNA (100 nM of in vitro transcribed 246 nt RNA or 50 ng/μL of total RNA extracted from HeLa cells) in the demethylation buffer at 25 °C for 60 min and heat-inactivated (at 95 °C for 3 min). 3 μL of the samples was subjected to MazF reaction (total 10 μL) and loaded onto a urea-PAGE as described before (Imanishi, et al, *Chem Commun*, 2017, **53**, 12930-12933). The fluorescently

labeled RNAs were visualized using Typhoon FLA9000 (GE Healthcare). For detection of FAM-labeled RNA, the settings of excitation and emission were 488 nm and 500-540 nm, respectively. For detection of TAMRA-labeled RNA, the settings of excitation and emission were 532 nm and 565-595 nm, respectively.

PAGE analysis of MazF cleavage of methylated samples

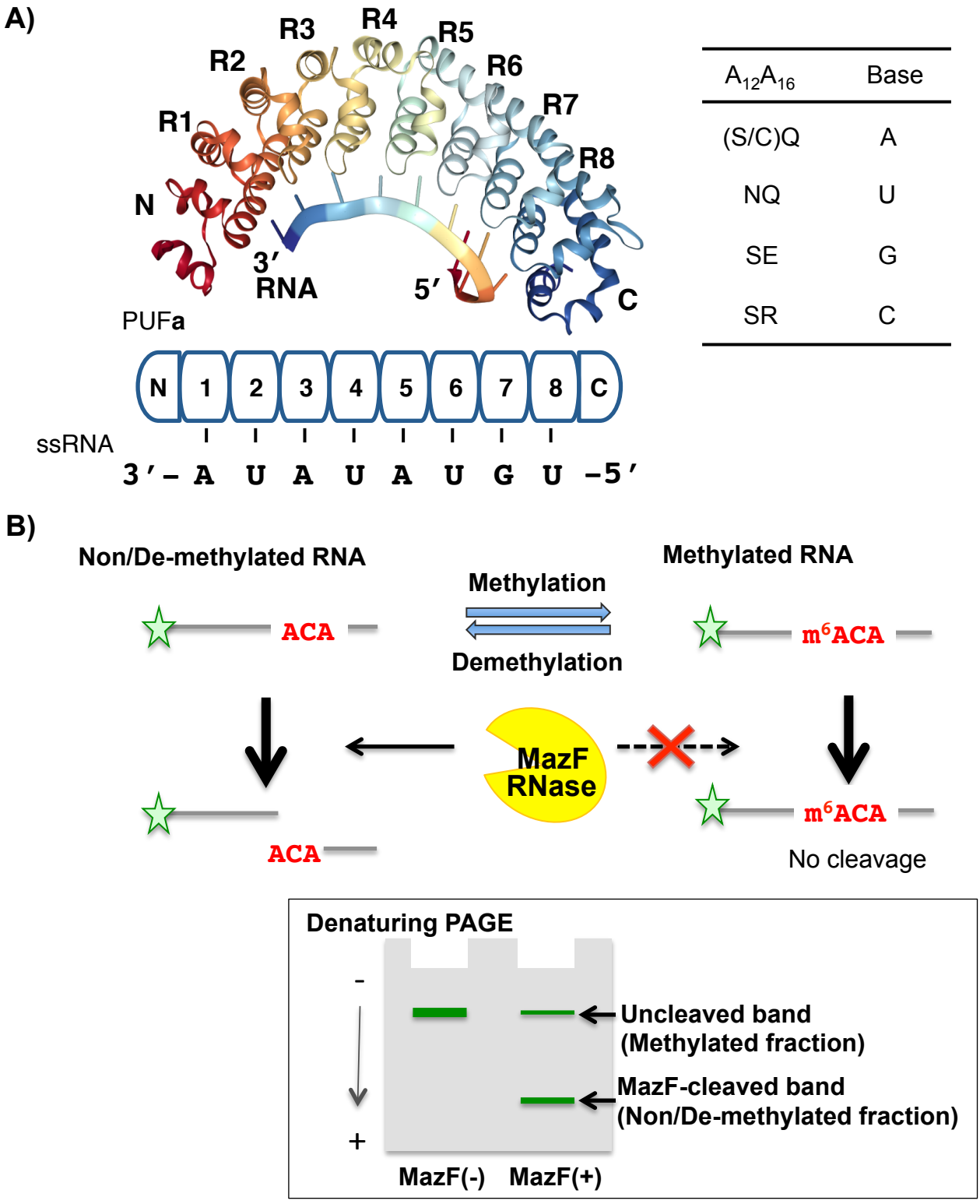
MTD3/MTD14 or MTD3/MTD14d-PUF**a** (100 nM) was mixed with RNA[A] (100 nM) in the methylation buffer (25 mM Tris-HCl (pH 7.5), 0.01% Tween-20, 1 mM DTT, 50 μM $ZnCl_2$, 0.1U/uL RNasin PLUS (Promega), 10 μM SAM) in the presence or absence of competitor RNA (700 nM of in vitro transcribed 246 nt RNA), incubated at 25 °C for 30 min and heat-inactivated (at 95 °C for 3 min). After demethylation reaction, 1 μL of the samples was subjected to MazF reaction (total 10 μL) and loaded onto urea-PAGE as described before (Imanishi, et al, *Chem Commun*, 2017, **53**, 12930-12933).

Detection of the sequence-specific methylation

Either MTD3/MTD14, MTD3/MTD14d-PUF**a** or MTD3/MTD14d-PUF**b** (200 nM) was incubated with RNA[A] (FAM-labeled, 24 nt, 100 nM) and RNA[B] (TAMRA-labeled, 30 nt, 100 nM) in the methylation buffer containing 50 ng/μL of total RNA extracted from HeLa cells at 25 °C for 30 min and heat-inactivated (at 95 °C for 3 min). 1 μL of the samples was subjected to MazF reaction (total 10 μL) and loaded onto a urea-PAGE as described before. The fluorescently labeled RNAs were visualized using Typhoon FLA9000 (GE Healthcare). For detection of FAM-labeled RNA, the settings of excitation and emission were 488 nm and 500-540 nm, respectively. For detection of TAMRA-labeled RNA, the settings of excitation and emission were 532 nm and 565-595 nm, respectively.

Electrophoretic mobility shift assay

After demethylation or methylation reaction without heat-inactivation, ficoll (2.5%) was added to the sample, and the mixture was loaded onto nondenaturing polyacrylamide gel and electrophoresed. The fluorescently labeled RNAs were visualized using Typhoon FLA9000 (GE Healthcare). For detection of FAM-labeled RNA, the settings of excitation and emission were 488 nm and 500-540 nm, respectively. For detection of TAMRA-labeled RNA, the settings of excitation and emission were 532 nm and 565-595 nm, respectively.



$A_{12}A_{16}$	Base
(S/C)Q	A
NQ	U
SE	G
SR	C

Fig. S1 (A) Crystal structure of Pumilio 1 (PUM1; PUFa in this study) bound to RNA, adapted from PDB ID: 1M8Y. 12^{th} and 16^{th} amino acids (A_{12} and A_{16}) in each repeat of PUF interact with one RNA base. The combinations of $A_{12}A_{16}$ and RNA bases are shown in the right. (B) Detection of RNA methylation or demethylation by MazF cleavage assay. MazF cleaves non-methylated ACA sequences but not m^6ACA sequences in RNA, which enables the detection of methylation and demethylation activities by the denaturing gel electrophoresis.

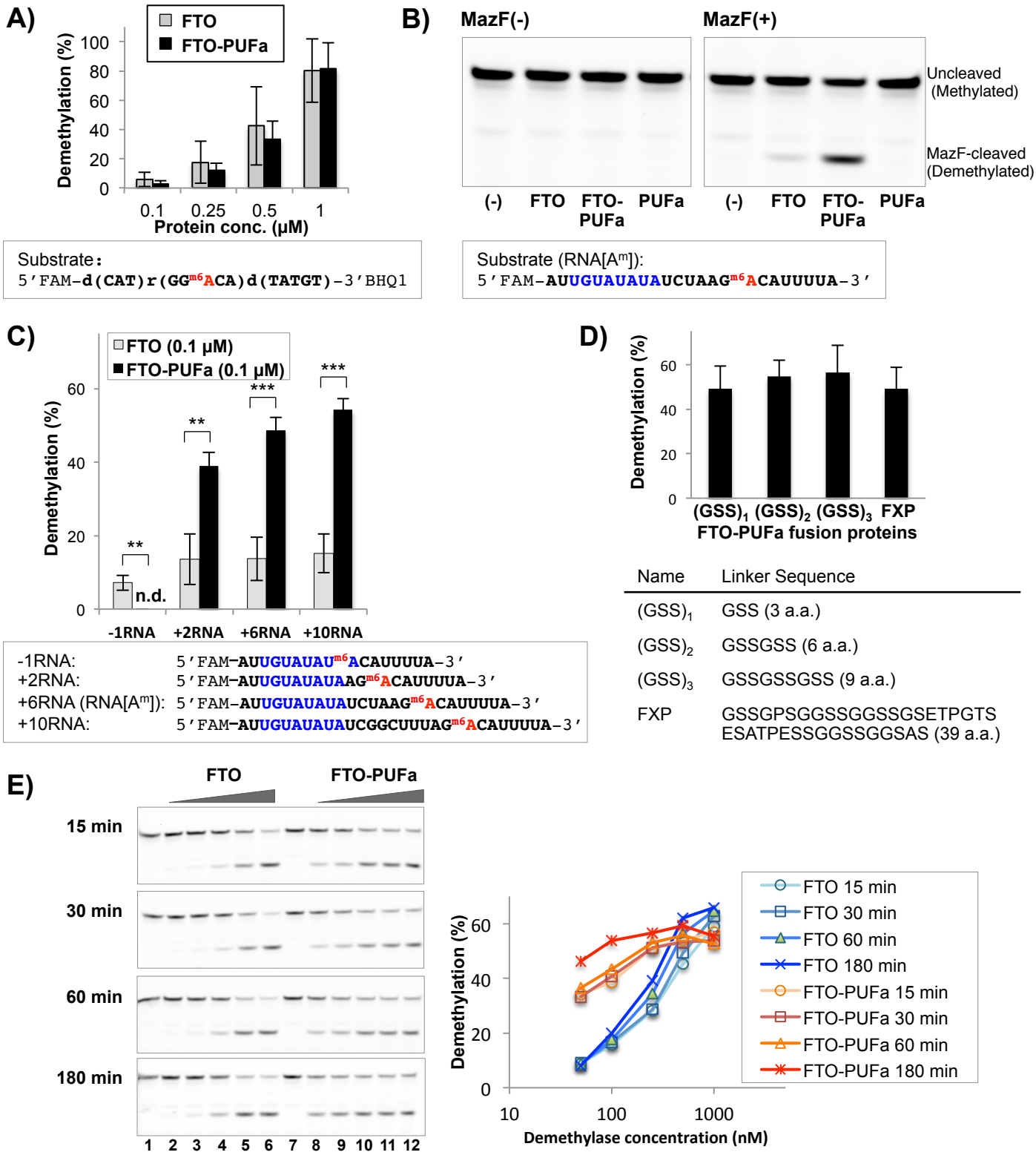


Fig. S2 FTO-PUFa fusion proteins showed sequence-specific RNA demethylation activities. (A) Demethylation ratio of a substrate RNA without any PUF**a** binding sequences by FTO and FTO-PUF**a**. Data shown are the mean ± S.D. (B) Control experiments of Fig. 1A. Note that PUFa domain alone (0.1 μM) did not show any demethylation activity. (C) Demethylation activity of FTO (0.1 μM) and FTO-PUFa (0.1 μM) against RNA substrates containing a PUFa binding sequence and ^{m6}ACA sequence. Among the substrate RNAs, the distances between the PUF**a** binding site and the ^{m6}A are different (2 nt, 6 nt, and 10 nt). The "-1RNA" contains an ^{m6}A within the PUF**a** binding sequence. Data shown are the mean ± S.D. **; p<0.01, ***; p<0.001 (one-way ANOVA). n.d.; not detected. (D) Demethylation activity of the FTO-PUF**a** fusion proteins (0.1 μM) with different linkers against oligo RNA containing ^{m6}A at 6 nt separated from the PUF**a** binding site (RNA[A^m]) . The linker names of the fusion proteins and their sequences are shown in a table. Data shown are the mean ± S.D. (E) Time and concentration dependency of the demethylase activity of FTO and FTO-PUF**a** for RNA[A^m] (100 nM). 0, 50, 100, 250, 500, 1000 nM of FTO (lanes 1-6), or FTO-PUFa (lanes 7-12). Times indicate the incubation time of the demethylation reactions. The gel images (left) show MazF-cleavage patterns of RNA[A^m]. The graph (right) indicates demethylated RNA ratios.

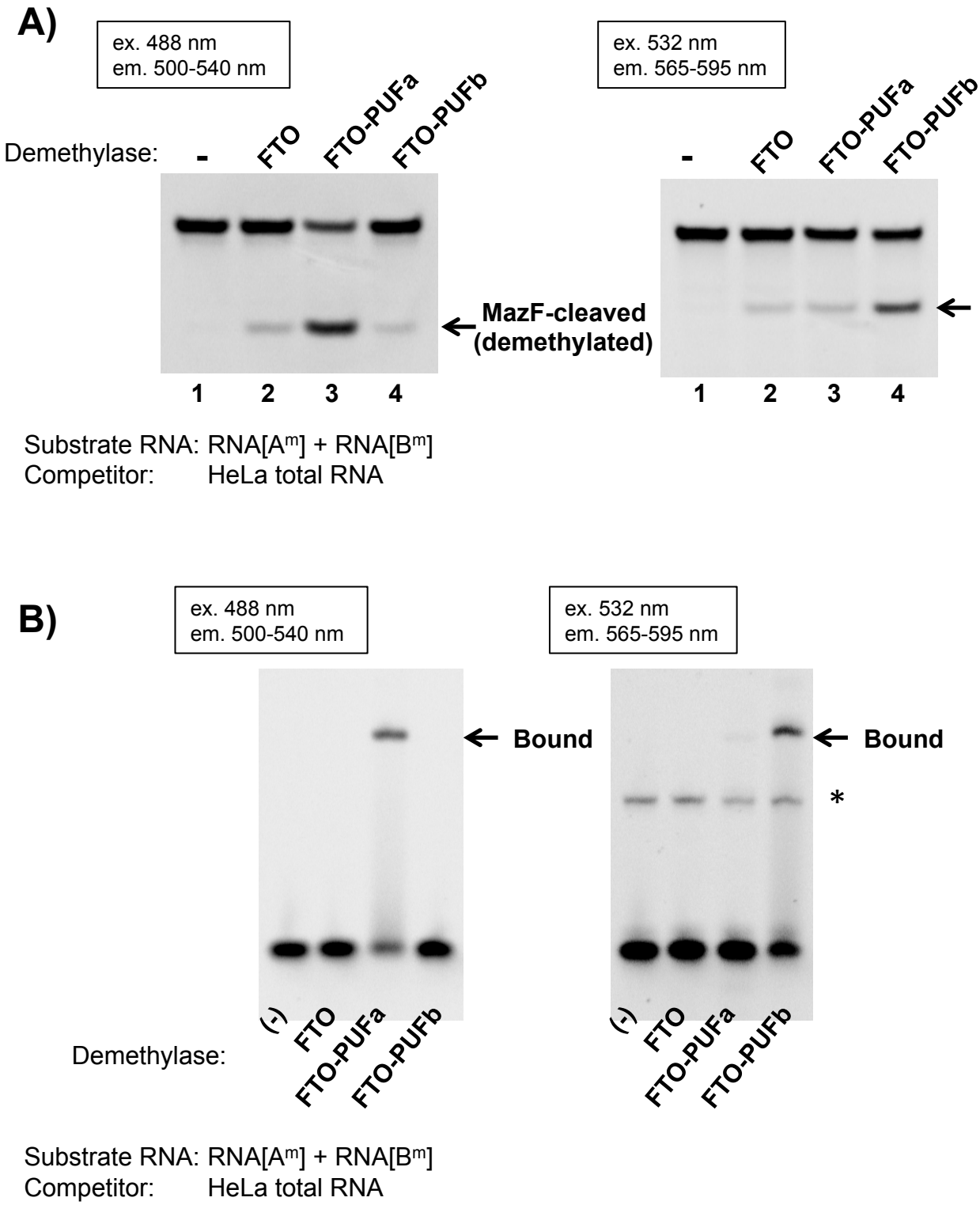


Fig. S3 Sequence-specific demethylation by FTO-PUF**a** and FTO-PUF**b** in the presence of total RNA extracted from HeLa cells. (A) MazF-cleavage patterns after demethylation reactions. RNA[A^m] and RNA[B^m] were mixed (lane 1) and demethylated by FTO (lane 2), FTO-PUF**a** (lane 3), or FTO-PUF**b** (lane 4) in the presence of HeLa total RNA. After demethylation reaction, each sample was treated with MazF and loaded onto a denaturing gel. FAM-labeled RNA[A^m] and TAMRA-labeled RNA[B^m] were visualized by excitation with 488 nm (left) and 532 nm (right), respectively. The arrows indicate cleaved RNA by MazF, namely demethylated RNA. (B) RNA binding patterns after demethylation reactions. A part of the demethylation reaction mixture was loaded onto a non-denaturing gel. The arrows indicate protein-bound RNA. The RNA band marked as "*" seems a conformational isomer of RNA[B^m].

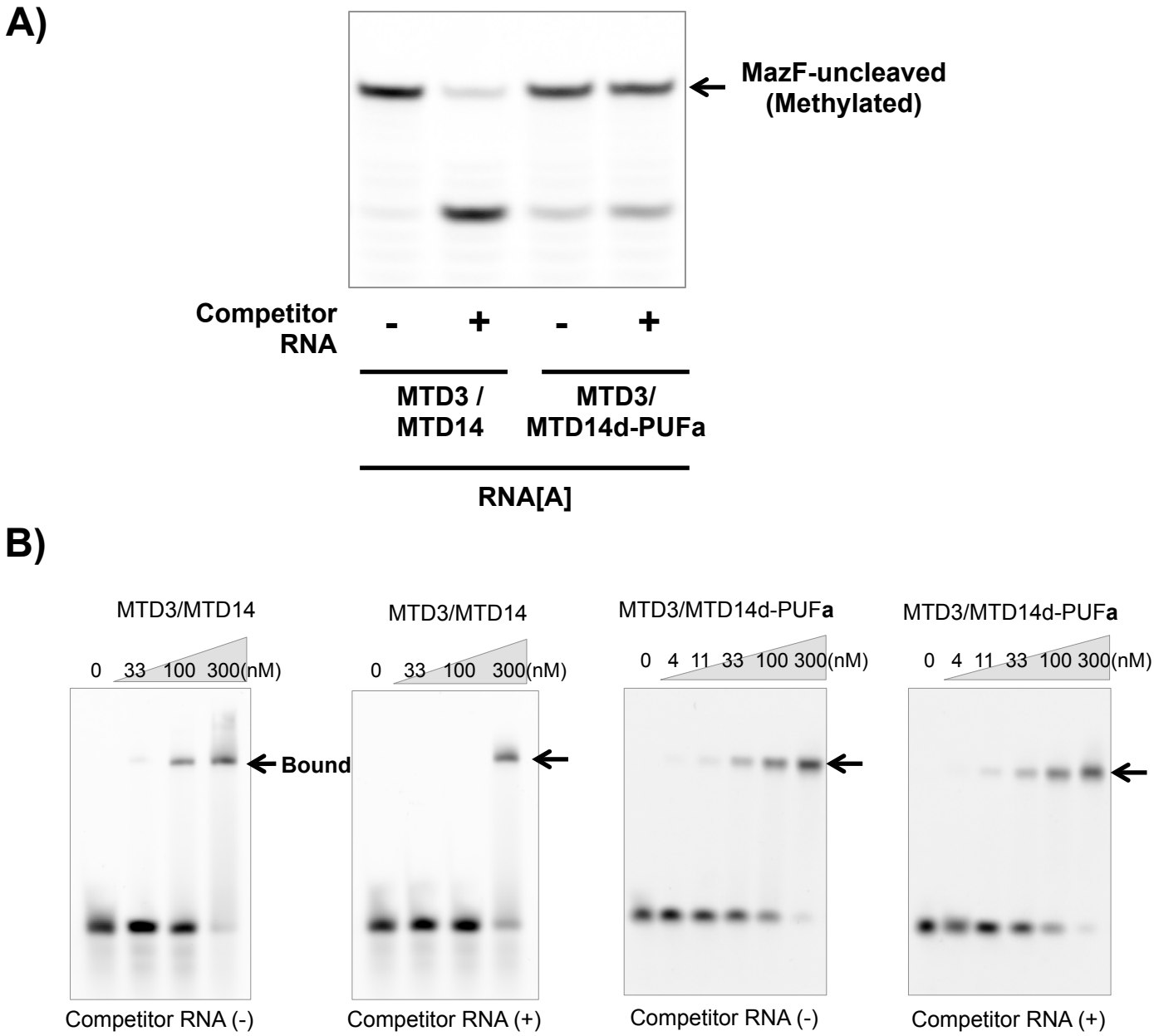


Fig. S4 The effect of non-specific (competitor) RNA on the interaction between methyltransferases and labeled substrate RNAs. (A) MazF-cleavage patterns after methylation reactions of RNA[A] (100 nM) by MTD3/MTD14 (100 nM) or MTD3/MTD14d-PUFa (100 nM) in the presence or absence of competitor RNA (246 nt, 700 nM). (B) Gel mobility shift assay of MTD3/MTD14 and MTD3/MTD14d-PUF**a** with RNA[A] (100 nM) in the absence or presence of non-specific RNA (246-nt, 50 nM) in reaction buffer (25 mM Tris-HCl (pH7.5), 0.01% Tween-20, 1 mM DTT, 50 μM $ZnCl_2$, 0.1U/μL RNasin, 10 μM SAM). Samples were incubated at 25 °C for 30 min and 2.5% ficoll was added before loading onto non-denaturing gels. The arrows indicate protein-bound RNA.

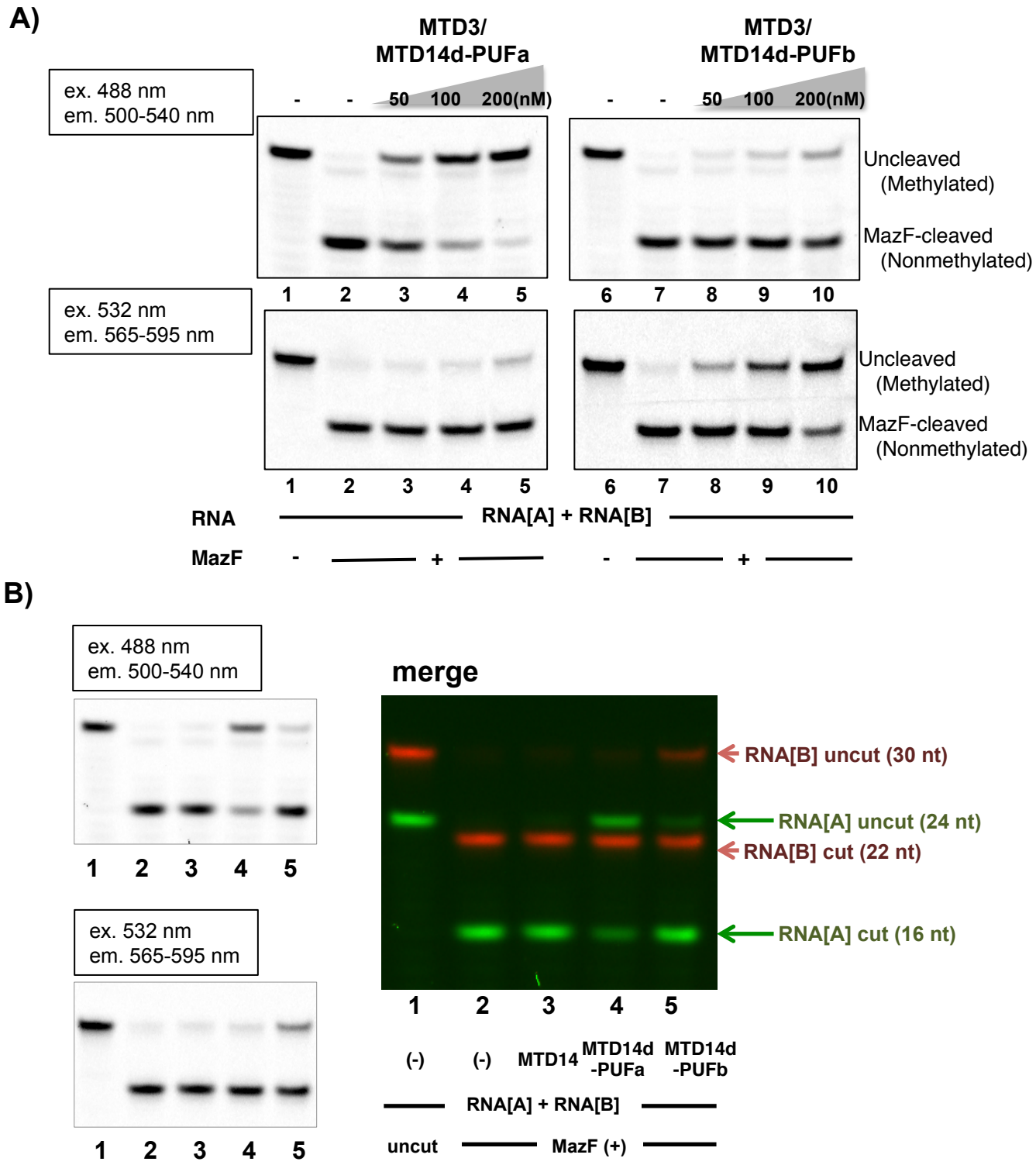


Fig. S5 Sequence-specific methylation by MTD14d-PUF**a** and MTD14d-PUF**b**. (A) RNA[A] and RNA[B] (100 nM each) were mixed and methylated by MTD3/MTD14d-PUF**a** (left), or MTD3/MTD14d-PUF**b** (right) in the presence of non-specific RNA (246-nt, 300 nM). After methylation reaction, a part of samples were treated with MazF and loaded onto a denaturing gel. FAM-labeled RNA[A] and TAMRA-labeled RNA[B] were visualized by excitation with 488 nm (upper) and 532 nm (lower), respectively. (B) RNA[A] and RNA[B] (100 nM each) were mixed (lanes 1, 2) and methylated by 200 nM of MTD3/MTD14 (lane 3), MTD3/MTD14d-PUF**a** (lane 4), or MTD3/MTD14d-PUF**b** (lane 5) in the presence of 50 ng/μL of HeLa total RNA. After methylation reaction, a part of samples were treated with MazF and loaded onto a denaturing gel. FAM-labeled RNA[A] and TAMRA-labeled RNA[B] were visualized by excitation with 488 nm (upper) and 532 nm (lower), respectively. The merged image was shown in the right. Arrows indicate each RNA band.