Supporting Information

Tag-Free Internal RNA Labeling and Photocaging Based on mRNA Methyltransferases

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anie_202013936_sm_miscellaneous_information.pdf
Abstract: The mRNA modification \( N^6 \)-methyladenosine (m\(^6\)A) is associated with multiple roles in cell function and disease. The methyltransferases METTL3-METTL14 and METTL16 act as "writers" for different target transcripts and sequence motifs. The modification is perceived by dedicated "reader" and "eraser" proteins, but not by polymerases. We report that METTL3-14 shows remarkable cosubstrate promiscuity, enabling sequence-specific internal labeling of RNA without additional guide RNAs. The transfer of ortho-nitrobenzyl and 6-nitropiperonyl groups allowed enzymatic photocaging of RNA in the consensus motif, which impaired polymerase-catalyzed primer extension in a reversible manner. METTL16 was less promiscuous but suitable for chemoenzymatic labeling using different types of click chemistry. Since both enzymes act on distinct sequence motifs, their combination allowed orthogonal chemo-enzymatic modification of different sites in a single RNA.

DOI: 10.1002/anie.202013936
Experimental Procedures

All chemicals were purchased from Alfa Aesar, Applichem, Fluorochem, Sigma Aldrich or TCI unless otherwise noted. All commercially available chemicals were used without further purification. The HPLC grade acetonitrile was purchased from VWR. Fluorescence images of polyacrylamide (PAA)-gels and the phosphorimaging of BAS-MS 3543 imaging plate (FUJIFILM) were recorded on a Typhoon FLA 9500 (GE Healthcare). Preparative HPLC purification of AdoMet analogs was carried out on the Agilent 1260 Infinity HPLC equipped with a diode array detector (DAD) (190-640 nm) using a Nucleodur® C18 Pyramid reversed-phase column (5 µm, 125 x 10 mm, 10 mm ID). Electroporation was performed on an Eppendorf Eporator® (Eppendorf, Hamburg). Sonication was carried out using a Sonoplus GM3100 (Bandelin, Berlin). MTAN was recombinantly expressed and purified as previously described.\(^1\)

\(^1\)H and \(^13\)C NMR spectra were measured at 299 K on a Bruker Avance II 300, an Agilent DD2 500 or Agilent DD2 600 spectrometer. The chemical shifts (δ) were reported in ppm relative to deuterated solvents as internal standard (δH: CDCl₃ = 7.26, DMSO-d₆ = 62.50 ppm, D₂O = 4.79 and δC: CDCl₃ = 77.16, DMSO-d₆ = 39.52 ppm). Multiplicities are indicated as s (singlet), d (doublet), dd (doublet of doublets), dt (doublet of triplet) t (triplet), q (quartet) and m (multiplet).

HPLC analysis of AdoMet analogues were performed on an Agilent 1260 Infinity HPLC equipped with a diode array detector (DAD) (190-640 nm) using a Nucleodur® C18 Pyramid reversed-phase column (5 µm, 125 x 4 mm) from Macherey-Nagel. Elution was performed at a flow rate of 1 mL/min applying a linear gradient for buffer A (50 mM ammonium acetate, pH = 6.0) and buffer B (1:1 buffer A: acetonitrile).

Oligonucleotides

Sequence, purchase and synthesis information of all the used oligonucleotides is listed in Table S1 for RNA and Table S2 for DNA. T7 transcription was performed as previously described.\(^2\)

LC-MS analysis of nucleosides
The digested and dephosphorylated nucleosides were analyzed either by LC-QQQ-MS or by LC-QTOF-MS, as indicated for each figure.

**LC-QQQ-MS analysis of nucleosides**

LC-QQQ-MS measurements were performed on an Agilent Ulivo Triple Quadrupole LC/MS (Agilent Technologies). The nucleosides were separated by reverse phase ultra-performance liquid chromatography on a Poroshell 120 EC-C18 (3 × 150 mm) column (Agilent Technologies) with mass spectrometric detection in positive Agilent Jet Stream (AJS) electrospray ionization mode. Buffer A: 20 mM NH₄OAc (pH = 6.0), buffer B: ACN (LC-MS grade). Gradient: 1 min 100 % A, 1-7 min to 60 % B, 7-7.20 min to 100 % B, 7.20-8.80 min 100 % B, 8.80-9 min to 100 % A, 9-12 min 100 % A. Flow rate: 0.8 mL/min. Scan mode parameters: Start mass 100 m/z, end mass 999 m/z, fragmentor 100 V, CAV 9 V. SIM mode parameters: fragmentor 121 V, CAV 9 V.

**LC-QTOF-MS analysis of nucleosides**

LC-QTOF-MS measurements were performed on a maXis II ultra-high-resolution QTOF (Bruker, Bremen) coupled to an UltiMate 3000 UHPLC (Thermo Scientific, Waltham, USA). The nucleosides were separated by reverse phase ultra-performance liquid chromatography on a NUCLEODUR C18 Pyramid (125 × 2 mm) column (Macherey-Nagel) with mass spectrometric detection in positive electrospray ionization mode. Buffer A: 20 mM HCO₂NH₄ (pH = 3.5), buffer B: CH₃OH (LC-MS grade); gradient: 5 min 100 % A, 5-30 min to 50 % A. Flow rate: 0.6 mL/min. For separation of biotin-modified nucleosides, the following gradient was used: 5 min 100 % A, 5-65 min to 100 % B.

The quantification of the modification yield was performed via integration of the peaks of the UV absorbance trace calculated as

\[
\frac{\text{AUC}(x\text{A})}{\text{AUC}(x\text{A}) + \text{AUC}(A)}
\]

where \( \text{AUC} \) is area under curve, \( x\text{A} \) – modified adenosine, \( A \) - adenosine. For the \( o \)-nitrobenzyl and 6-nitropiperonyl modifications, the yield was determined as the difference between the adenosine \( \text{AUCs} \) in the analyzed sample and the control.

**Primer extension assays**

The RT primer was radioactively labeled with \([\gamma^{32}\text{P}]-\text{ATP}\) as described previously.\(^{2}\) Enzymatically propargylated RNA (0.5 \( \mu \)M) was incubated with 2 \( \mu \)M \([\gamma^{32}\text{P}]-\text{ATP}\) and 1 U/\( \mu \)L reverse transcriptase (Maxima H Minus, SuperScriptII or SuperScriptIV) for 30 min, 50 °C. The RNA was digested via alkaline hydrolysis by addition of 200 mM NaOH (80 °C, 10 min), neutralized with HCl and analyzed via PAGE (10-15 % denat. PAA gel, 1× TBE). The resulting termination bands were quantified using ImageJ and normalized to the total amount of primer extension products. Positions 18-19 (+1 and +2 from the primer) were excluded from quantification. If bioconjugation via CuAAC was performed, the biotinylated RNA was bound to 400 \( \mu \)g Dynabeads™ M-280 Streptavidin (Thermo Fisher Scientific) according to the manufacturer’s protocol.

**CuAAC on enzymatically modified RNA**

Biotin azide was purchased from Carl Roth (#7803.2). Picolyl-azide-PEG₄-biotin and picolyl-azide-sulfo-Cy5 were purchased from Jena Bioscience (#CLK-1167-5 and #CLK-1177-1). The reactions were performed according to the instructions of the CuAAC Reaction Ligand Test Kit (Jena Bioscience, #CLK-075) with the following adjustments. The CuAAC with picolyl-azide-sulfo-Cy5 was performed in 100 \( \mu \)L with the following end concentrations: 0.0015 mM propargylated RNA, 0.05 mM picolyl-azide-sulfo-Cy5, 2 mM Cu-BTTAA mix, 100 mM sodium ascorbate. The mixture was incubated for 30 min followed by ethanol precipitation and additional desalting with RNA Clean & Concentrator™-5 kit (Zymo #R1016). The CuAAC with picolyl-azide-PEG₄-biotin for primer extension assays was performed in 50 \( \mu \)L with the following end concentrations: 0.009 mM propargylated RNA, 0.05 mM picolyl-azide-PEG₄-biotin, 5 mM Cu-THPTA mix, 100 mM sodium ascorbate. The mixture was incubated for 30 min followed by ethanol precipitation.

**SPAAC on enzymatically modified RNA**

For SPAAC, the in vitro methyltransferase assay was performed in 20 \( \mu \)L reaction volume as described below. The RNA was purified by phenol-chloroform extraction and ethanol precipitation. The pellet was dissolved in 7.5 \( \mu \)L ddH₂O. Then, 2.5 \( \mu \)L of DBCO-Sulfo-Cy5 (200 \( \mu \)M) were added. The reaction was incubated (1 h, 37 °C) and the RNA was purified by phenol-chloroform extraction and ethanol precipitation. The pellet was dissolved in 10 \( \mu \)L ddH₂O and analyzed on denat. PAGE.
Cloning

The plasmid encoding His10-METTL16 was a gift from Prof. Dr. Markus Bohnsack (Göttingen university, Germany, [3]). The METTL16 gene was cloned via Gibson assembly into pFastBac_HTA (Invitrogen, # 10584027), simultaneously the GST-tag was introduced. The Gibson assembly was designed with SnapGene software. The used primers are listed in Table S2. The pFastBacDual vector encoding METTL3 and METTL14 genes with the GST-His8-tag and TEV cleavage site fused to the METTL3 N-terminus was kindly provided by Prof. Dr. Gunter Meister (University of Regensburg, Germany, [4]).

Recombinant expression and purification of METTL3-METTL14

Protein expression in Sf21 cells via Bac-to-Bac expression system was performed as described earlier.[2] The cells were homogenized in ice-cold lysis buffer containing 1×PBS (pH 7.4), 1.5 M NaCl, 2 mM DTT, 0.08 mM phenylmethanesulfonyl-fluoride (PMSF), a spatula tip of DNase I and 2.4 mM MgCl2. The mixture was sonicated (3 × 3 min, 30 % intensity). The insoluble fraction was precipitated by centrifugation (11,000 rpm) for 30 min at 4 °C. The supernatant was sterile-filtered (0.45 µM syringe filter, Thermo Scientific). Protein purification was performed by affinity chromatography (GSTrap 4B, GE Healthcare) on an ÄKTA Purifier system (GE Healthcare). After the protein binding, it was additionally washed with a buffer containing 1×PBS, 50 mM Tris (pH 8.0), 2 mM DTT. The elution was performed with a wash buffer supplemented with 10 mM reduced L-glutathione. For RNase-free preparation, the protein complex was subjected to size exclusion chromatography (Superdex 200 Increase, GE Healthcare), with a buffer containing 20 mM HEPES (pH 7.5), 200 mM NaCl, 2 mM DTT. Finally, the protein complex was concentrated to approximately 60 µM (Amicon Ultra-15, 100 kDa cutoff, Millipore), and the buffer was supplemented with glycerol (to 40 %) for storage. The aliquots were flash-frozen in liquid N2 and stored at −80 °C.

Recombinant expression and purification of GST-METTL16

Protein expression in Sf21 cells via Bac-to-Bac expression system, cell lysis and protein binding to GSTrap column were performed as described for METTL3-METTL14. The elution buffer contained 50 mM Tris (pH 8), 200 mM NaCl and 10 mM reduced L-glutathione. For RNase-free preparation, the protein complex was subjected to size exclusion chromatography (Superdex 200 Increase, GE Healthcare), with a buffer containing 25 mM HEPES (pH 7.50), 50 mM NaCl, 1 mM TCEP. Finally, the protein complex was concentrated to approximately 100 µM (Amicon Ultra-15, 10 kDa cutoff, Millipore), and the buffer was supplemented with glycerol (to 40 %) for storage. The aliquots were flash-frozen in liquid N2 and stored at −80 °C.

In vitro methyltransferase assays

The assays were performed in 50 µL volume. The reactions for the primer extension assays were performed in 30 µL volume, and the reactions for the kinetics measurements in 20 µL volume. Reaction components listed in their pipetting order: 1× activity buffer (see below), 40 U RiboLock, 4 µM MTAN, 1 mM AdoMet or AdoMet analog, 3 µM methyltransferase and 3 µM RNA. Activity buffer for METTL3-METTL14 (10×): 150 mM HEPES (pH 7.9), 800 mM KCl, 15 mM MgCl2, 100 mM DTT, 4 % glycerol. Activity buffer for METTL16 (10×): 100 mM HEPES-KOH (pH 7.4), 1 M NaCl. The 5′-methylthioadenosine nucleosidase (MTAN) was added to all reactions to enzymatically degrade the by-product 5′-methylthioadenosine, which is a strong inhibitor of methyltransferases. For the control reactions the methyltransferase was heat-deactivated (90 °C, 5 min). The reactions were incubated for 1 h at 37 °C followed by the deactivation of proteins (90 °C, 2 min). The RNA was recovered by phenol-chloroform (5:1) extraction and precipitated with ethanol after addition of 0.5 M NH4OAc and glycogen. After centrifugation the pellet was washed with 70 % EtOH and dissolved in 30 µL ddH2O. The RNA was digested with P1 nuclease (0.16 U) in a buffer containing 20 mM NH4OAc (pH = 5.3) for 4 h at 50 °C and FastAP alkaline phosphatase (1 U) for 1 h at 37 °C. Proteins were precipitated with HClO4 (final concentration 0.1 M) and centrifuged. The supernatant (30 µL) was directly injected into LC-QTOF-MS. Alternatively, 25 µL were injected into LC-QQQ-MS/MS for the measurement in the Scan mode and 2 µL were measured in the SIM mode.

UV irradiation experiments

UV irradiation experiments were conducted using a 365 nm/ 405 nm/ 420 nm UV LED LZ1-00UV00 (700 mA, 3.5 V) from LED Engin.

End-point kinetics measurements

The reactions were done as described in “In vitro methyltransferase assays” with the AdoMet/AdoMet analog concentration was varied from 0 to 1 mM. The UV absorbance peaks (260 nm) were integrated, and the Michaelis-Menten curve was fitted to data with OriginPro 2020b software.
**Synthesis of AdoMet analogs**

Synthesis, purification and analysis of 5′-[(R/S)-(3S)-3-amino-3-carboxypropyl]-5′-deoxyadenosine (SeAdoYn), 5′-[(R/S)-(3S)-3-amino-3-carboxypropyl]-4-[(2-nitrobenzylsulfonyl)]-5′-deoxyadenosine (AdoONB), 5′-[(R/S)-(3S)-3-amino-3-carboxypropyl]-4-[(4-nitrobenzylsulfonyl)]-5′-deoxyadenosine (AdoPNB), 5′-[(R/S)-(3S)-3-amino-3-carboxypropyl]-4-azidobut-2-ene-1-ylselenio]-5′-deoxyadenosine (AbSAM) and 5′-[(R/S)-(3S)-3-amino-3-carboxypropyl]-4-vinylbenzylsulfonio]-5′-deoxyadenosine (AdoViBe) was carried out as described earlier [2, 5].

**6-Nitropiperonol:** 6-nitropiperonal (1.2 g, 6.2 mmol, 1 eq.) was dissolved in THF (35 mL) and ddH2O (2 mL), sodium tetrahydridoborate (164 mg, 4.34 mmol, 0.7 eq.) was added and the reaction mixture was stirred at room temperature for 8 h. After extraction with ethyl acetate (3×100 mL) the solvent was removed under reduced pressure and the residue was purified by flash chromatography (cyclohexane : ethyl acetate 80:20 to 50:50). The product was obtained as yellow solid (1.1 g, 5.6 mmol, 90%). Rf =0.27 (cyclohexane/ethyl acetate 6:4).

1H NMR (500 MHz, DMSO-d6) δ [ppm] = 7.65 (s, 1H, H-3), 7.29 (d, J = 0.9 Hz, 1H, H-6), 6.22 (s, 2H, H-1), 5.56 (t, J = 5.4 Hz, 1H, OH), 4.77 – 4.75 (m, 2H, H-8).

13C NMR (126 MHz, DMSO-d6) δ [ppm] = 152.81, 146.76, 140.51, 137.34, 107.04, 105.33, 103.73, 60.68.

**Orbitrap XL (ESI-positive):** calculated for [C7H8NO5Na]+: 220.02164, measured: 220.02141.

**5-(Bromomethyl)-6-nitrobenzo[1,3]dioxol:** 6-nitropiperonol (1.1 g, 5.6 mmol, 1 eq.) was dissolved in diethylether (50 mL) at 0 °C, phosphorus tribromide (1.06 mL, 11.2 mmol, 2 eq.) was added dropwise and the reaction mixture was stirred at room temperature for 28 h. Ethyl acetate (100 mL) was added and extracted with saturated sodium bicarbonate (1×100 mL). The solvent was removed under reduced pressure and the remaining residue was purified by flash chromatography (cyclohexane : ethyl acetate 85:15 to 60:40). The product was obtained as orange oily solid (435 mg, 1.67 mmol 30 %). Rf =0.46 (cyclohexane/ethyl acetate 6:4).

1H NMR (599 MHz, DMSO-d6) δ [ppm] = 7.64 (s, 1H, H-3), 7.29 (d, J = 0.9 Hz, 1H, H-6), 6.22 (s, 2H, H-1), 4.75 (d, J = 0.9 Hz, 2H, H-8).

13C NMR (151 MHz, DMSO-d6) δ [ppm] = 152.37, 146.31, 140.06, 136.93, 106.64, 104.88, 103.30, 60.19.

**5′-[(R/S)(3S)-3-Amino-3-carboxypropyl]-4-[(6-nitrobenzo[d][1,3]dioxol-5-yl)methyl)sulfonio]-5′-deoxyadenosine (AdoNP):** 5′-(Bromomethyl)-6-nitrobenzo[d][1,3]dioxol (203 mg, 0.78 mmol, 29 eq.) and S-Adenosyl-L-homocysteine (10.5 mg, 27 µmol, 1 eq.) were dissolved in acetic acid (750 µL) and formic acid (750 µL) at 0 °C. Silver perchlorate (4.2 mg, 0.78 mmol, 29 eq.) was added and the solution was stirred at room temperature for 19 h. The reaction mixture was diluted with 5 mL ddH2O and extracted with diethyl ether.
The aqueous layer was freeze-dried and the remaining residue was dissolved in 0.01% trifluoroacetic acid (1.5 mL) and purified by C18-chromatography. The product was obtained as light yellow solid (1.6 nmol, 6%).

**LC-MS (ESI-positive):** calculated for \([\text{C}_{22}\text{H}_{18}\text{N}_{7}\text{O}_{9}\text{S}]^+\): 564.15, found: 564.1.

\(^1\text{H NMR spectrum (500 MHz, DMSO-d}_6, 299 \text{ K)} of 3.\)

\(^{13}\text{C NMR spectrum (126 MHz, DMSO-d}_6, 299 \text{ K)} of 3.\)
$^1$H NMR spectrum (599 MHz, DMSO-\textit{d}_6, 299 K) of 4.

$^{13}$C NMR spectrum (151 MHz, DMSO-\textit{d}_6, 299 K) of 4.
**Table S1.** Ribo-oligonucleotides. Adenosines targeted by METTL3-METTL14 are underlined. Adenosines targeted by METTL16 are shown in bold. IVT: *in vitro* transcription.

| Name                                           | Sequence (5′-3′)                                                                 | Length (nt) | Source | Purification |
|------------------------------------------------|---------------------------------------------------------------------------------|-------------|--------|--------------|
| METTL3–14 substrate (RNA I)                    | GGACUGGACUGGACUGGACU                                                           | 20          | Biomers | HPLC         |
| METTL16 substrate (RNA II)                     | GGGAGACGAGCUUUCUGAAGAGCUUGAGCUAGAAGGAACACCGCUUCCUAGAAGC                         | 61          | IVT    | PAGE         |
| Chimeric METTL3-METTL16 substrate (RNA III)    | GGCUGAGCCUACAGAGAGAGCCAGAGCAAGC                                               | 26          | Biomers | HPLC         |
| Control chimera 1                              | GGCGUAGGCUACAGAAGAGCCGAGU                                                     | 26          | Biomers | HPLC         |
| Control chimera 2                              | GGCGUAGGCUACAGAAGAGCCGAGU                                                     | 26          | Biomers | HPLC         |
| Primer extension RNA (RNA IV)                  | GGGAGACGAGGCAAGAGAGGCAAGGCAAGGCAAGGCAAGGCAAGGCAAGGCAAGGCAAGGCAAGGCAAGGCAAGGCA | 56          | IVT    | PAGE         |
| GGCCU-Primer extension RNA (RNA V)             | GGGAGACGAGGCAAGAGAGGCAAGGCAAGGCAAGGCAAGGCAAGGCAAGGCAAGGCAAGGCAAGGCAAGGCAAGGCA | 56          | IVT    | PAGE         |
## Table S2. DNA Oligonucleotides. RC: reverse complement

| Name | Sequence (5′-3′) | Length (nt) | Source | Purification |
|------|------------------|-------------|--------|--------------|
| **Templates for in vitro methyltransferase assays** |
| Template for RNA II | TAATACGACTCACTATAGGGAGACTAAATGGACTATAATC ATACAATGCTCCTGGCTGCTCGTCGATTA | 78 | Biolegio | - |
| Template for RNA II, RC | GCTCTCTGAAGGAAGCTGGTTTCTCTGAGCTACACCAGCTGTTCAG AAAGCTCGTCTCCCTATAGTGAGTCGATTA | 78 | Biolegio | - |
| **Templates for the primer extension assay** |
| Template for RNA IV | TAATACGACTCACTATAGGGAGACTAAATGGACTATAATC ATACAATGGCTCCTGGCTGCTCGTCGATTA | 73 | Biolegio | - |
| Template for RNA IV, RC | GCACAGACTCAAGGGGCATGGCTTTAATGGCTGAGTCGATTA | 73 | Biolegio | - |
| Template for RNA V | TAATACGACTCACTATAGGGAGACTAAATGGCTGAGTCGATTA | 73 | Biolegio | - |
| Template for RNA IV, RC | GCACAGACTCAAGGGGCATGGCTTTAATGGCTGAGTCGATTA | 73 | Biolegio | - |
| RT primer | GCACAGACTCAAGGGGCATGGCTTTAATGGCTGAGTCGATTA | 17 | Biolegio | PAGE |
| **Primers for Gibson assembly cloning of METTL16 into pFastBac HTA** |
| GA_GST_His_TEV.FOR | GCGGATCTCGTGGAGTTAACATGCTCCCTATAGTTATGGGAAA ATAAAGGG | 55 | Biolegio | - |
| GA_pFBHTA.REV | TAACCTATAGGAGAATTCCTGGTGGCATGCCGCTGATCC | 38 | Biolegio | - |
| GA_GST_His_TEV.REV | ATGGGTTTATCTTAGGCACATGGCTTTTTCGACGCTGATCC | 57 | Biolegio | - |
| GA_Mett16.FOR | AAAACCTGATTTTTCAGGGAATGGACTCGAGAATCAATGCATG | 45 | Biolegio | - |
| GA_Mett16.REV | ATCTCTTATGACTTCTGACCTAGTAAAATCTGCAACAGAACTGGAATCC | 46 | Biolegio | - |
| GA_pFBHTA.FOR | GGCTTGTTCAGTAAATCTAGGAGGCAGATAGTGCGTATGATGG | 50 | Biolegio | - |
## Results and Discussion

### Table S3. Products of enzymatic digestion of enzymatically modified RNAs

| Number | Name                                             | Formula       | [M+H]+ m/z   | m/z   |
|--------|--------------------------------------------------|---------------|--------------|-------|
| 2a     | N6-methyladenosine                               | C11H15N5O4    | 282.120±0.005| 282.1 |
| 2b     | N6-propargyladenosine                            | C13H15N5O4    | 306.120±0.005| 306.1 |
| 2c     | N6-4-azido-but-2-ene-1-yladenosine               | C14H18N8O4    | 363.152±0.005| 363.1 |
| 2d     | N6-hexenynyladenosine                            | C16H19N5O4    | 346.151±0.005| 346.1 |
| 2e     | N6-4-vinylbenzyladenosine                        | C19H21N5O4    | 384.167±0.005| 384.1 |
| 2f     | N6-(o-nitrobenzyl)adenosine                      | C17H18N6O6    | 403.136±0.005| 403.1 |
| 2g     | N6-(p-nitrobenzyl)adenosine                      | C17H18N6O6    | 403.136±0.005| 403.1 |
| 2h     | N6-(6-nitropiperonyl)adenosine                   | C20H19N5O5    | 447.126±0.005| 447.1 |
| 2i     | Biotin-conjugated adenosine                      | C26H37N11O6S  | 632.272±0.005| 632.3 |
| 2j     | Picoly-PEG4-biotin-conjugated adenosine          | C40H57N13O11S | 928.409±0.005| 928.4 |

### Table S4. Km values measured for different methyltransferase/cosubstrate combinations

| Enzyme | Cosubstrate | Km       |
|--------|-------------|----------|
| METTL3-14 | 1a          | (7.9 ± 1.6) µM |
| METTL3-14 | 1f          | (51.3 ± 12.8) µM |
| METTL3-14 | 1h          | (54.8 ± 9.4) µM |
| METTL3-14 | Se-1b       | (390 ± 215) µM |
| METTL16  | 1a          | (14.1 ± 1.6) µM |
| METTL16  | Se-1b       | (67.4 ± 28) µM |
Figure S1. Crystal structures of the catalytic domains of METTL3-METTL14 and METTL16. A. AdoMet-binding domain of METTL3-METTL14 in surface representation. AdoMet is shown in sticks in blue. Left: PDB ID: 5IL1. Right: PDB ID: 5K7U. B. AdoMet-binding domain of METTL16 in surface representation. SAH (3-adenosyl-L-homocysteine) is shown as sticks in blue. Left: PDB ID: 6B92. Right: PDB ID: 6GFN.
Figure S2. Evaluation of the substrate scope of METTL3-METTL14. LC-QQQ-MS/MS or LC-QTOF-MS analyses of ribonucleosides after enzymatic degradation is shown. The UV traces (260 nm) are shown in black when the corresponding peak was visible, the extracted ion chromatograms (EICs) are shown in red. The names, formulas and masses of the analytes are listed in Table S3. The scheme of each reaction is shown on top of the chromatogram. The control reactions containing heat-deactivated enzyme are shown in Figure S3.
Figure S3. Analysis of the control reactions containing heat-deactivated METTL3-METTL14. The EICs of samples are shown next to control reactions containing heat-deactivated enzymes. The names, formulas and masses of the analytes are listed in Table S3.
Figure S4. Michaelis-Menten curves for the end-point kinetics measured for different methyltransferase/cosubstrate combinations. A. Kinetics measurements for METTL3-METTL14. B. Kinetics measurements for METTL16. Each point was measured in triplicate.
Figure S5. Purification of GST-METTL16 expressed in Sf-21 insect cells. A. Purification of GST-METTL16 via a GSTrap 4B column and UV detection at 280 nm. Collected fractions are shown in red. B. Purification of the fractions collected in (A) via size exclusion chromatography (Superdex 200 column) and UV detection at 280 nm. Collected fractions are shown in red. C. Analysis of the fractions collected in (B) on 12% SDS gel. Expected molecular weight for GST-METTL16: 90.4 kDa. SEC: size exclusion chromatography.
Figure S6. Evaluation of the substrate scope of m^6A methyltransferase METTL16. LC-QQQ-MS/MS or LC-QTOF-MS analyses of ribonucleosides after enzymatic degradation is shown. The UV traces (260 nm) are shown in black when the corresponding peak was visible, the extracted ion chromatograms (EICs) are shown in red. The names, formulas and masses of the analytes are listed in Table S3. The scheme of each reaction is shown on top of the chromatogram. The control reactions containing heat-deactivated enzyme are shown in Figure S7.
Figure S7. Analysis of the control reactions containing heat-deactivated METTL16. The EICs of samples are shown next to control reactions containing heat-deactivated enzymes. The scheme of each reaction is shown on top of the chromatogram. The names, formulas and masses of the analytes are listed in Table S3.
Figure S8. CuAAC reaction of RNAs bearing propargyl groups installed by METTL3-METTL14. LC-QTOF-MS analyses of ribonucleosides after enzymatic degradation. A. RNA I clicked to biotin azide. EIC for biotin-conjugated adenosine is shown in red (2i, C_{26}H_{37}N_{11}O_{6}S [M+H]^+ 632.272 ± 0.005. The structural formula is shown on the right). B. RNA IV clicked to picolyl-azide-PEG4-biotin: EIC for biotin-conjugated adenosine is shown in red (C_{40}H_{57}N_{13}O_{11}S [M+H]^+ 928.409 ± 0.005. The structural formula is shown on the right). C. Structural formula of the picolyl-azide-sulfo-Cy5 (see Figure 1C).

Figure S9. SPAAC clicking of azido-but-2-ene-1-yl modified RNA to DBCO-sulfo-Cy5. A. The RNA was and analyzed via 15 % denat. PAGE and stained with SYBR Gold. 1: RNA III was modified with METTL16 followed by SPAAC, 2: control containing heat-deactivated METTL16, 3: RNA I was modified with METTL3-METTL14 followed by SPAAC, 2: control containing heat-deactivated METTL3-METTL14. Left side shows the Cy5 channel, the right side shows overlay of SybrGold channel (green) and Cy5 channel (green). B. Structural formula of the DBO-Sulfo-Cy5 used for the experiment.
Figure S10. METTL3-14-mediated modification of FLuc mRNA (~1900 nt). dMETTL3-14 corresponds to the control containing heat-deactivated enzyme. No enzyme ctrl corresponds to the sample containing no enzyme. The RNA was propargylated with METTL3-METTL14 or METTL16, clicked to picoly-sulfo-Cy5 azide and separated on 7.5% denat. PAGE. M: Riboruler LR. Left: SYBR Gold staining. Right: Cy5 channel.
Figure S11. Primer extension assays with RNA propargylated via METTL3-METTL14-. A. Scheme of the T7-transcribed RNA IV. METTL3 consensus motives are shown in blue, the targeted adenosines are underlined and distances from them to the 3' end are shown. The RT primer is shown in green. B. LC-QTOF-MS analysis of the modified RNA (56-mer) prior to RT. Each panel shows the absorbance at 260 nm (in black) and extracted ion chromatogram (in red) for the expected product. Upper panel: \( \text{N}^6\)-propargyladenosine (2b, \([\text{M}+\text{H}]^+ = 306.120 \pm 0.005\)), lower panel: picolyl-biotin modified adenosine (2j, \([\text{M}+\text{H}]^+ = 928.409 \pm 0.005\)). C. Autoradiograph of the primer extension assay with MaximaH, SuperScriptIII and SuperscriptIV after separation on 15% TBE gel. Used dNTP concentration: 1 mM. Ctrl: negative control, the other lanes are labeled in accordance with the transferred groups.
Figure S12. Photocleavage of o-nitrobenzyl and 6-nitropiperonyl groups by irradiation. LC-QTOF-MS/ LC-QQQ-MS analysis of ribonucleosides after enzymatic degradation of RNA I before irradiation (upper panel) and after irradiation (lower panel). A. Irradiation of o-nitrobenzyl group at 365 nm, 2 min. B. Irradiation of o-nitrobenzyl group at 405 nm, 2 min. UV traces (260 nm) are shown in black, EICs for N6-(o-nitrobenzyl)adenosine are shown in red (2f, C17H18N6O6 [M+H]+ 403.136 ± 0.005, m/z 403.1). C. Irradiation of 6-nitropiperonyl group at 420 nm, 1 min. EICs for N6-(6-nitropiperonyl)adenosine are shown in red (2h, C18H18N6O8, m/z 447.1). D. Gel analysis of samples from (B) before and after irradiation (15% denat. PAGE). E. Gel analysis of samples from (C) before and after irradiation (15% denat. PAGE).
Figure S13. Remodification of RNA after photocaging and uncaging. A, C show the schemes of the experiments. B. Extracted ion chromatograms for N\textsuperscript{6}-methyladenosine (2a, m/z = 282.1) and N\textsuperscript{6}-(6-nitropiperonyl)adenosine (2h, m/z = 447.1). Chromatograms are shown for samples I-IV from (A) and the expected retention times for 2a and 2h are indicated with dashed lines. D. Extracted ion chromatograms for N\textsuperscript{6}-methyladenosine (2a, m/z = 282.1) and N\textsuperscript{6}-(o-nitrobenzyl)adenosine (2f, m/z = 403.1). Chromatograms are shown for samples I-II from (C) and the expected retention times for 2a and 2f are indicated with dashed lines.
Figure S14. Primer extension assays with METTL3-METTL14-modified RNA. A. LC-QQQ-MS analysis of RNA IV. Each panel shows the absorbance at 260 nm (in black) and chromatograms generated in single ion monitoring mode (SIM) (in red) for N6-\((\text{o}-\text{nitrobenzyl})\)adenosine (\(2f\), m/z 403.1). Upper panel: before irradiation, lower panel: after 2 min, 365 nm. B. Autoradiograph of the primer extension assay with SuperScriptIII after separation on 20 % TBE gel. Used dNTP concentration: 1 mM. This is a full-length image of Figure 2D. Ctrl: negative control with non-modified RNA, the other lanes are labeled in accordance with the transferred groups. A representative gel of three independent experiments is shown.

Figure S15. Analysis of the N6-\((\text{o}-\text{nitrobenzyl})\)adenosine (f) (A) or N6-\((6\text{-nitropiperonyl})\)adenosine (h) (B) -modified RNA IV. The samples are separated on 10 % denat. PAGE before and after irradiation at indicated wavelengths. M: IDT 20/100 Ladder (#51-05-15-02).
Figure S16. Primer extension assays with METTL3-METTL14-modified RNAs IV, V. A. Autoradiograph of the primer extension assay with SuperScriptIII after separation on 15 % TBE gel. Used dNTP concentration: 1 mM. Ctrl: negative control with non-modified RNA, the other lanes are labeled in accordance with the transferred groups (h and f). B. LC-QQQ-MS analysis of RNA IV from (A), lanes h. Each panel shows the absorbance at 260 nm (in black) and extracted ion chromatograms (EIC) (in red) for N^6-(6-nitropiperonyl)adenosine (2h, m/z 447.1). Upper panel: before irradiation, lower panel: after 1 min, 420 nm. C. LC-QQQ-MS analysis of RNA IV from (A), lanes f. Each panel shows the absorbance at 260 nm (in black) and extracted ion chromatograms (EIC) (in red) for N^6-(o-nitrobenzyl)adenosine (2f, m/z 403.1). Upper panel: before irradiation, lower panel: after 2 min, 405 nm.
Figure S17. Orthogonal RNA labeling approach. A. Experiment scheme with METTL16 (M16) depicted as a red drop and METTL3-METTL14 (M3-14) as a grey circle. The propargyl and o-nitrobenzyl groups are shown as blue and violet ovals, respectively. B. Extracted ion chromatograms recorded in SIM (single ion monitoring) mode for N<sup>6</sup>-propargyladenosine (2b, m/z = 306.1) and N<sup>6</sup>-o-nitrobenzyladenosine (2f, m/z = 403.1). Chromatograms are shown for samples I-V from (A) and the expected retention times for 2b and 2f are indicated with dashed lines. C. Analysis of the modified RNA on 15 % TBE gel.

References

[1] J. M. Holstein, L. Anhauser and A. Rentmeister, *Angew Chem Int Ed Engl* 2016, 55, 10899-10903.
[2] K. Hartstock, B. S. Nilges, A. Ovcharenko, N. V. Cornelissen, N. Pullen, A. M. Lawrence-Dorner, S. A. Leidel and A. Rentmeister, *Angew Chem Int Ed Engl* 2018, 57, 6342-6346.
[3] A. S. Warda, J. Kretschmer, P. Hackert, C. Lenz, H. Urlaub, C. Hobartner, K. E. Sloan and M. T. Bohnsack, *EMBO Rep* 2017.
[4] E. Scholler, F. Weichmann, T. Treiber, S. Ringle, N. Treiber, A. Flatley, R. Feederle, A. Bruckmann and G. Meister, *Rna* 2018.
[5] a) L. Anhauser, F. Muttach and A. Rentmeister, *Chem Commun (Camb)* 2018, 54, 449-451; b) J. M. Holstein, F. Muttach, S. H. H. Schiefelbein and A. Rentmeister, *Chemistry* 2017, 23, 6165-6173; c) J. M. Holstein, D. Stummer and A. Rentmeister, *Chemical science* 2015, 6, 1362-1369.
[6] L.-H. Yeh, H.-K. Wang, G. Pallikonda, Y.-L. Ciou and J.-C. Hsieh, *Organic Letters* 2019, 21, 1730-1734.