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Supporting Information

N^6-Methyladenosine-Sensitive RNA-Cleaving Deoxyribozymes

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General information

DNA oligonucleotides were purchased from Microsynth and purified by denaturing PAGE (15–20% polyacrylamide). RNA oligonucleotides were prepared by solid-phase synthesis using 5'-*O*-DMT-2'-*O*-TOM-protected 3'-β-cyanoethyl phosphoramidites of N^6-acetyladenosine, N^4-acetylcytidine, N^2-acetylguanosine, uridine (purchased from Chemgenes), N^6-methyladenosine (prepared according to the known procedure[1]), (2-cyanoethoxy)-5-hexyn-1-yl-*N*,*N*-diisopropylaminophosphine (prepared according to[2]), (2-cyanoethoxy)-2-(2'-*O*-4,4'-dimethoxytrityloxyethylsulfonyl)ethoxy-*N*,*N*-diisopropylaminophosphine (prepared according to [3]). Deoxyribo- and ribonucleotide triphosphates (dNTPs and NTPs including m^6ATP) were purchased from Jena Bioscience. T4 PNK, T4 DNA Ligase, and DreamTaq polymerase were purchased from Thermo Fisher Scientific. T7 RNA polymerase was prepared in house following a published procedure with minor modifications.[4] All DNA and RNA oligonucleotides were purified by denaturing PAGE (10-20% acrylamide/bis-acrylamide 19:1, 7 M urea) with running buffer 1x TBE (89 mM Tris, 89 mM boric acid and 2 mM EDTA, pH 8.3), extracted by crush & soak into TEN (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 300 mM NaCl) buffer and recovered by precipitation with ethanol. For preparative gels, oligonucleotides were visualized by UV shadowing on a TLC plate. Fluorescence gel images were recorded with a Biorad ChemiDoc MP using epi illumination. Analytical anion-exchange HPLC was carried out on a GE Healthcare Life Sciences ÄKTA™ micro system using DNAPac™ PA200 columns (4 x 250 mm, Thermo Scientific) at 60 °C. Solvent A: 25 mM Tris-HCl (pH 8.0), 6 M Urea. Solvent B: 25 mM Tris-HCl (pH 8.0), 6 M Urea, 0.5 M $NaClO_4$. Gradient: linear, 0–54% solvent B, 4% solvent B per 1 CV. The solvents were filtered through 0.2 µm cellulose acetate filters (Sartorius, Germany) prior to usage.

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RNA synthesis

Solid-phase RNA synthesis

RNA oligonucleotide syntheses were carried out at 0.7-1 µmol scale on Pharmacia LKB Gene Assembler Plus using standard cyanoethylphosphoramidite chemistry as described before.[1] The coupling efficiencies were determined using absorbance of released dimethoxytrityl (DMT) cation and exceeded 98% for all syntheses. Deprotection was performed in two steps under standard conditions using $MeNH_2$ in aqueous ethanol and TBAF in THF. 3'-Amino-modified RNA was synthesized on Amino-on-solid support (purchased from Sigma), and deprotected with an aqueous mixture of $MeNH_2$ and NH_3 incubating for 4 h at 55 °C and TBAF/THF. The crude oligonucleotides were desalted by size exclusion chromatography on GE Healthcare Life Sciences ÄKTA™ start system (3×5 ml HiTrap columns), purified by PAGE, and analyzed by anion exchange HPLC and ESI-MS.

In vitro transcription of SNORD RNAs and ACTB mRNA and MALAT1 fragments

In vitro transcription reactions were performed with T7 RNA polymerase using the corresponding dsDNA template (0.5–1 µM, prepared by PCR with synthetic DNA template strands for SNORDs (D31-D40) and ACTB (D44-D45); or amplified by cDNA from HeLa total RNA using primers to incorporate an upstream T7 promoter: template for 124 nt MALAT1 RNA fragment: D50, D53; for 189 nt RNA: D51, D54; for 299 nt RNA: D51, D52; for 403 nt RNA: D50, D52). Reactions were performed in an aqueous solution containing 40 mM Tris-HCl, pH 8.0, 30 mM $MgCl_2$, 10 mM DTT, 4 mM each NTP and 2 mM spermidine at 37 °C for 5 h. The transcription products were purified by denaturing PAGE (10% polyacrylamide). To allow for efficient transcription by T7 RNA polymerase, transcripts were initiated with 5'-GG.

Synthesis of SNORD2 by ligation

Splint-assisted ligation of short synthetic fragments of SNORD2 RNA (R21-R24) was performed with T4 RNA ligase and DNA splint D41. The RNA substrates (2.4 nmol each) were mixed with the DNA splint (2 nmol) in a total volume of 10 µl. The resulting solution was heated to 95 °C for 5 min for annealing and then allowed to cool down to r.t. Afterwards, 5 µl of 10x reaction buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM DTT, pH 7.5), 5 µl of 10 mM ATP, 50 U of T4 RNA ligase and water to a total volume of 50 µl were added. The reaction mixture was incubated for 12 h at 37°C and the ligation product was purified by denaturing PAGE (10% polyacrylamide) to yield 0.5-0.6 nmol full-length RNA.

RNA labeling

Labeling of 5'-alkyne functionalized oligonucleotides with 6-FAM-PEG3-azide

5'-Alkyne functionalized oligonucleotides were labeled with 6-FAM-PEG3-azide using copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC). In a typical procedure, a solution of the corresponding oligonucleotide (6 nmol) in water (5 µl) was mixed with a DMSO/*t*BuOH mixture (3:1 v/v, 3 µl) and with a solution of 6-FAM-PEG3-azide (0.5 µl, 50 mM) in DMSO/*t*BuOH (3:1 v/v). A freshly prepared solution of CuBr (0.5 µl, 100 mM) in DMSO/*t*BuOH (3:1 v/v) was combined with a solution of tris(benzyltriazolylmethyl)amine (1 µl, 100 mM) in DMSO/*t*BuOH (3:1 v/v) and then added to the reaction mixture. After 3 h incubation in the dark at 37 °C, the reaction mixture was ethanol-precipitated and the labeled RNA was purified by PAGE (20% polyacrylamide). Typical recovery was 50-60% labeled RNA.

Labeling of 3'-amino functionalized oligonucleotides with 5/6-carboxyfluorescein succinimidyl ester (NHS-fluorescein)

3'-Amino functionalized oligonucleotides were labeled with NHS-fluorescein as follows. Freeze-dried amino-modified RNA (typically 3 nmol) was dissolved in carbonate buffer (100 mM, 20 µl, pH 9.0) and a solution of NHS-fluorescein in DMF (30 mM, 2.5 µl) was added. The total volume was brought to 25 µl with water, and the resulting reaction mixture was incubated in the dark at 37 °C for 2 h. The crude product was purified by PAGE. Typical yields of the purified product were 60-70%.

3'-End labeling of RNA transcripts

RNA transcripts were labeled by periodate oxidation followed by a reaction with fluorescein-5-thiosemicarbazide. In a typical procedure, RNA (0.3–1 nmol) was dissolved in 7.5 µl water, followed by addition of 5x sodium phosphate buffer (2 µl, 250 mM, pH 7.4) and a freshly prepared aqueous solution of $NaIO_4$ (0.5 µl, 400 mM). The resulting mixture was incubated for 10–15 min at 37 °C. The excess of $NaIO_4$ was quenched by treatment with aqueous Na_2SO_3 (1 µl, 1 M) for 5–10 min at 37 °C. Afterwards, a solution of fluorescein-5-thiosemicarbazide (1 µl, 10 mM) in DMF was added, and the reaction mixture was incubated in the dark for additional 1 h at 37 °C. The labeled product was then purified by PAGE (10–20% polyacrylamide) and recovered by extraction and ethanol precipitation.

In vitro selection

Phosphorylation of RNA selection substrates

5'-End phosphorylation of RNA substrates R1, R2 was carried out with T4 PNK according to the protocol provided by the manufacturer. Briefly, a mixture of RNA (2.5–5 nmol), 10x PNK buffer A (5 µl, 500 mM Tris-HCl, 10 mM MgCl2, 50 mM DTT, 1 mM spermidine) and ATP (5 µl, 10 mM) was brought to a total volume of 50 µl with water, and the resulting mixture was incubated for 5 h at 37 °C. Afterwards, the reaction mixture was diluted to 300 µl with 1x TEN buffer, extracted by phenol-chloroform mixture and ethanol-precipitated.

Splinted ligation of the deoxyribozyme selection pool to RNA substrates

Splinted ligation was carried out with T4 DNA ligase as described earlier.[5] In a typical procedure, the deoxyribozyme pool, 5'-phosphorylated RNA and complementary DNA splint (1.82 nmol, 2.4 nmol and 2 nmol, respectively) were dissolved in water (9 µl), and 10x annealing buffer (1 µl; 40 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0) was added. After 2 min incubation at 95 °C, the solution was allowed to cool down to r.t. for 15 min and then further cooled down to 0 °C for 10 min. 10x DNA Ligase buffer (2 µl; 400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, pH 7.8), T4 DNA Ligase (2 µl; 5 U/µl) and water to a total volume of 20 µl were added, and the resulting mixture incubated for 2 h at 37 °C to yield a DNA-RNA hybrid product which was purified by PAGE.

Intramolecular cleavage of DNA-RNA hybrids (key selection step)

The DNA-RNA hybrid obtained by splinted ligation (150 pmol in the first selection round, ca 10-50 pmol in further rounds) was incubated in selection buffer (9.5 µl; 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 2 min at 95 °C, 15 min at 25 °C and finally for 10 min at 0 °C. To start the cleavage reaction, $MgCl_2$ was added to a final concentration of 5 mM, and the resulting reaction mixture was incubated for 12 h at 37 or 45 °C. Afterwards, the reaction mixture was subjected to PAGE, and the gel areas corresponding to the cleavage products of the desired length (determined by comparison with a size marker) were cut and extracted. Ethanol precipitation of the resulting extracts yielded products which were used for PCR amplification (see below).

PCR amplification of the enriched DNA library

PCR amplification of the enriched DNA library was carried out as described previously[5] with slight modifications. A typical procedure consisted of two PCR reactions. For the first reaction, the gel extraction product from the previous step was used as a template. The template, forward primer D5 (60 pmol), reverse primer D6 (15 pmol), dNTP mixture (0.6 µl, 10 mM each dNTP), 10x DreamTaq Buffer (0.3 µl), DreamTaq DNA polymerase (0.3 µl, 5 U/µl) and water to a final volume of 30 µl were mixed, and the resulting solution was subjected to a 10-cycle PCR reaction (2 min at 95 °C, 10x [30 s at 95 °C, 30 s at 52 °C, 5 s at 72 °C], 5 min at 72 °C, 4 °C) using a Bio-Rad T100 thermal cycler. Afterwards, the reaction mixture was diluted with water to 100 µl and extracted with a phenol-chloroform mixture. The second PCR reaction was performed as follows. An aliquot from the first PCR reaction was mixed with fluorescently labeled forward primer D5F (100 pmol), reverse primer D6, dNTP mixture (0.5 µl, 10 mM each), 10x DreamTaq buffer (2.5 µl), DreamTaq DNA polymerase (0.3 µl, 5 U/µl) and water to a final volume of 25 µl. The resulting solution was subjected to a 30-cycle PCR reaction (2 min at 95 °C, 30x [30 s at 95 °C, 30 s at 52 °C, 5 s at 72 °C], 5 min at 72 °C, 4 °C), and the product was purified by PAGE. The fluorescent short strand was isolated and used for ligation to RNA substrate to initiate the next round of selection.

Kinetics characterization of deoxyribozymes

Kinetics characterization was carried out as described previously[6] with slight modifications. In a typical procedure, a deoxyribozyme or an active deoxyribozyme pool (125 pmol) was mixed with a fluorescently labeled substrate RNA (12.5 pmol) and water to a final

volume of 8.4 µl. The resulting solution was heated to 95 °C for 5 min and then allowed to cool down to r.t. for 10 min. To initiate the cleavage reaction, 10x kinetic buffer (1.2 µl; 500 mM Tris-HCl, 1.5 M NaCl, pH 7.5) and $MgCl_2$ (2.4 µl, 0.1 M) were added, and the reaction mixture incubated at 37 °C. Aliquots (2 µl) were taken at different time points, quenched with loading buffer (2 µl) and analyzed by PAGE. Cleavage yields were determined using fluorescent intensities of the corresponding bands. Values of k_{obs} and P_{max} were obtained by fitting of the cleavage yield versus time with the first-order kinetics equation $P = P_{max} * (1 - e^{-kobs*t})$, where P – yield at a particular time point, P_{max} – maximal yield, and k_{obs} – observed cleavage rate. In negative control experiments, the solution of deoxyribozyme was replaced with an equal volume of water. For reproducibility, each experiment was performed 2–3 times.
(for experiments shown in Fig S5 the final $MgCl_2$ concentration was varied from 5 to 50 mM).

Analysis of endogenous RNA

Mouse SNORD2 RNA

Mouse livers[8] were homogenized and total RNA was extracted using TRI reagent (Sigma). To enrich SNORD2, hydrophilic streptavidin beads (NEB) were coupled to a biotinylated DNA oligonucleotide complementary to SNORD2 (D42) in wash/binding buffer (500 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM EDTA). 200 µg total RNA diluted in wash/binding buffer was incubated at 65 °C for 5 min, stored on ice for 5 min and then incubated with the oligonucleotide-coupled streptavidin beads for 1 h at room temperature. After sequential washing steps in washing/binding buffer and low-salt buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM EDTA), bound RNAs were eluted in a buffer containing 20 mM Tris-HCl pH 7.4 and 1 mM EDTA at 90 °C for 2 min. Eluted RNAs were ethanol precipitated and subjected the DNA enzyme cleavage with 10–100-fold excess of DNA enzyme, in 50 mM Tris.HCl, pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$ at 37°C for 6-18 h. The products of cleavage reactions were separated by denaturing (7 M urea) polyacrylamide (10 %) gel electrophoresis and transferred by nylon membrane (GE Healthcare) by electroblotting. SNORD2 was detected by northern blotting using a 5' [^{32}P]-labelled DNA oligonucleotide probe (D43) as previously described[7] and signals were visualized using a Typhoon FLA9500 phosphorimager (GE Healthcare).

Human MALAT1 RNA

Enrichment of endogenous MALAT1

Total RNA was extracted from HeLa CCL2 cells using TRI reagent (Sigma). To enrich MALAT1, hydrophilic streptavadin beads (NEB) were coupled to a biotinylated DNA oligonucleotide complementary to MALAT1 (D49),[9] in Wash/binding buffer (500 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM EDTA). 200 µg total RNA diluted in Wash/binding buffer was incubated at 65 °C for 5 min, stored on ice for 5 min and then incubated with the oligonucleotide-coupled streptavadin beads for 1 h at room temperature. After sequential washing steps in Wash/binding buffer and Low-salt buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM EDTA), bound RNAs were eluted twice in a buffer containing 20 mM Tris-HCl pH 7.4 and 1 mM EDTA at 90 °C for 2 min. Eluted RNAs were ethanol precipitated and resuspended in water.

DNA enzyme cleavage and northern blot analysis

The enriched endogenous MALAT1 and the *in vitro* transcribed MALAT1 fragments were subjected to DNA enzyme-mediated cleavage using the corresponding DNA enzymes as described above. To disrupt MALAT1 secondary structures in close proximity to the DNA enzyme binding site, two oligonucleotides (D53 and D54) were included in the cleavage reactions. Reactions were performed for 6 h. The products of cleavage reactions were separated by denaturing (7 M urea) polyacrylamide (8 % or 10%) gel electrophoresis and transferred to a nylon membrane (GE Healthcare) by electroblotting. MALAT1 fragments were detected by northern blotting using a mixture of 5' [^{32}P]-labelled DNA oligonucleotide probes (D64-D66) as previously described[7] and signals were visualised using a Typhoon FLA9500 phosphorimager (GE Healthcare).

Quantitative PCR analysis of DNA enzyme cleavage reactions

The products of cleavage reactions performed on *in vitro* transcripts and endogenous MALAT1 were converted to cDNA using an oligonucleotide primer (D67) and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was performed as described in Warda et al. (2017) in a Light Cycler 480 using SYBR Green I Master (Roche) and primers upstream and downstream of A2577 (MALAT1_2577_F, D68 and MALAT1_2577_R, D69).

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Supporting Tables

Table S1a. Sequences of RNA oligonucleotides.

No	Description/motif	Sequence[a]	length	m^6A pos.
R1	GGACU	pAUAGACUGAAUGAAGGXCUUCCGUAACU	28	A17
R2	GGACU	pAUAGACUGAAUGAAGGACUUCCGUAACU	28	-
R1	GGACU	AUAGACUGAAUGAAGGXCUUCCGUAACU-NH_2	28	A17
R2	GGACU	AUAGACUGAAUGAAGGACUUCCGUAACU-NH_2	28	-
R3	UGACU	AUAGACUGAAUGAAUGXCUUCCGUAACU-NH_2	28	A17
R4	UGACU	AUAGACUGAAUGAAUGACUUCCGUAACU-NH_2	28	-
R5	AGACU	AUAGACUGAAUGAAAGXCUUCCGUAACU-NH_2	28	A17
R6	AGACU	AUAGACUGAAUGAAAGACUUCCGUAACU-NH_2	28	-
R7	GAACU	AUAGACUGAAUGAAGAXCUUCCGUAACU-NH_2	28	A17
R8	GAACU	AUAGACUGAAUGAAGAACUUCCGUAACU-NH_2	28	-
R9	U6 snRNA	UGGAACGAUACXGAGAGAUU-NH_2	20	A43(12)
R10	U6 snRNA	UGGAACGAUACAGAGAGAUU-NH_2	20	-
R11	Hs 18S rRNA	Alk-GUAAAAGUCGUAXCAAAGGU	20	A1832(13)
R12	Hs 18S rRNA	Alk-GUAAAAGUCGUAACAAAGGU	20	-
R13	HNRNPH1 5023	UGGGCUGGXCUGUUGGU-NH_2	17	A5023(9)
R14	HNRNPH1 5023	UGGGCUGGACUGUUGGU-NH_2	17	-
R15	MALAT 2577	UUUGCAUUGGXCUUUGAGUU-NH2	20	A2577(11)
R16	MALAT2577	UUUGCAUUGGACUUUGAGUU-NH2	20	-
R17	RSV RNA	UACGAGUCCUGGXCUGAAACGGACU-NH_2	25	A7414(13)
R18	RSV RNA	UACGAGUCCUGGACUGAAACGGACU-NH_2	25	-
R19	ACTB RNA	GGCUUCUAGGCGGXCUAUGACUUAG-NH2	25	A1216(14)
R20	ACTB RNA	GGCUUCUAGGCGGACUAUGACUUAG-NH2	25	-
R21	5'-SNORD2 1-18	Alk-AAGUGAAAUGAUGGCAAA	18	-
R22	SNORD2 19-42	pUCAUCUUUCGGGACUGXCCUGAAA	24	A35(17)
R23	SNORD2 19-42	pUCAUCUUUCGGGACUGACCUGAAA	24	-
R24	SNORD2 43-70	pUGAAGAGAAUACUCUUGCUGAUCACUp	26	-
R25	Mm SNORD2	GGAAGUGAAAUGAUGGCAAAUCAUCUUUCGGGACUGACCUGAAAUGAAGAG AAUACUCUUGCUGAUCACU	68/70*	A31/A35
R26	Hs SNORD29	GGUUUCUAUGAUGAAUCAAACUAGCUCACUAUGACCGACAGUGAAAAUACA UGAACACCUGAGAAAC	65/67*	A32
R27	Hs SNORD41	GGUGGGAAGUGAUGACACCUGUGACUGUUGAUGUGGAACUGAUUUAUCGCG UAUUCGUACUGGCUGAUCCUG	70/72*	A22
R28	Hs SNORD44	GGCCUGGAUGAUGAUAAGCAAAUGCUGACUGAACAUGAAGGUCUUAAUUAG CUCUAACUGACU	61/63*	A26

[a] X = m^6A, Alk = 5-hexynyl, NH_2 = 6-aminohexyl; [b] molecular mass of fluorescein-labeled RNA, * actual length with two preceding Gs.

Table S1b. Sequences of DNA oligonucleotides.

No.	Description	Sequence[a]
D1	8-17NG for G15|G16 in R1/R2	GTTACGGAAGTTGTCAGCGACTCGAATTCATTCAG
D2	8-17NA for C11|A12 U6 snRNA fragment	AATCTCTCGATAGCATTCCCGAGTATCGTTCC
D3	8-17NA for U11|A12 hs rRNA fragment	ACCTTTGTGATAGCATTCCCGAGCGACTTTTA
D4	Selection pool	GTGACGCGACTAGTTACGGAAGN_{20}TTCATTCAGTTGGCGCCTCC
D5	Selection primer	Alk-GTGACGCGACTAGTTAC
D6	Tailed selection primer	$(AAC)_4$-$(E_3)_2$-GGAGGCGCCAACTGAATGAA
D7	Splint for selection pool (round1)	TTCATTCAGTCTATGGAGGCGCCAACTG
D8	Splint for other selection rounds	TTCATTCAGTCTATTGGAGGCGCCAACTG
D9	VMA8 for G15|G16 in R1-R8	TAGTTACGGAGGAGTCGGCCCCAGCTGGTTCGCTTCATTCAGT
D10	VMA15 for G16|A17 in R1-R8	TAGTTACGGAAGAAAGGGCGGGCAAACTCTGGTTCATTCAGT
D11	VMC10 for G16|A17 in R1-R8	TAGTTACGGAAGGGGTCTCCAGCTGGACGTTATTCATTCAGT
D12	VMA8 with restored binding arm for R1/R2	TAGTTACGGAAGAGTCGGCCCCAGCTGGTTCGCTTCATTCAGT
D13	VMC10 A20C for R1/R2	TAGTTACGGAAGGGGTCTCCAGCTGGACGTTCTTCATTCAGT
D14	VMA8 for HNRNPH1 RNA	CCACCAACGGAGTCGGCCCCAGCTGGTTCGCAGCCCA
D15	VMA15 for HNRNPH1RNA	CCACCAACAGAAAGGGCGGGCAAACTCTGGAGCCCA
D16	VMC10 for HNRNPH1 RNA	CCACCAACAGGGGTCTCCAGCTGGACGTTAAGCCCA
D17	VMA8 for RSV RNA	GTCCGTTTCGGAGTCGGCCCCAGCTGGTTCGCAGGACTCGT
D18	VMA15 for RSV RNA	GTCCGTTTCAGAAAGGGCGGGCAAACTCTGGAGGACTCGT
D19	VMC10 for RSV RNA	GTCCGTTTCAGGGGTCTCCAGCTGGACGTTAAGGACTCGT
D20	VMC10_A20C for RSV RNA	GTCCGTTTCAGGGGTCTCCAGCTGGACGTTCAGGACTCGT
D21	VMA15 for G30|A31 in SNORD2	TCAGGTCAGAAAGGGCGGGCAAACTCTGGCGAAAGATG
D22	VMC10 for G30|A31 in SNORD2	TTCAGGTCAGGGGTCTCCAGCTGGACGTTACGAAAGATG
D23	VMA15 for G34|A35 in SNORD2	TCATTTCAGGAAAGGGCGGGCAAACTCTGGGTCCCGAAA
D24	VMC10 for G34|A35 in SNORD2	TCATTTCAGGGGGTCTCCAGCTGGACGTTAGTCCCGAAA
D25	VMA15 SNORD29	CACTGTCGGAAAGGGCGGGCAAACTCTGGTAGTGAGCT
D26	VMC10 SNORD29	CACTGTCGGGGGTCTCCAGCTGGACGTTATAGTGAGCT
D27	VMA15 SNORD41	CATCAACAGAAAGGGCGGGCAAACTCTGGCAGGTGTCA
D28	VMC10 SNORD41	CATCAACAGGGGTCTCCAGCTGGACGTTACAGGTGTCA
D29	VMA15 SNORD44	CATGTTCAGAAAGGGCGGGCAAACTCTGGGCATTTGCT

D30	VMC10 SNORD44	CATGTTCAGGGGTCTCCAGCTGGACGTTAGCATTTGCT
D31	Transcription template SNORD29	GTTTCTCAGGTGTTCATGTATTTTCACTGTCGGtCATAGTGAGCTAGT TTGATTCATCATAGAAACCTATAGTGAGTCGTATTACAG
D32	Primer/disruptor SNORD29	GTTTCTCAGGTGTTCAT
D33	Transcription template SNORD41	CAGGATCAGCCAGTACGAATACGCGATAAATCAGTTCCACATCAACAG tCACAGGTGTCATCACTTCCCACCTATAGTGAGTCGTATTACAG
D34	Primer/disruptor SNORD41	CAGGATCAGCCAGTAC
D35	Transcription template SNORD44	AGTCAGTTAGAGCTAATTAAGACCTTCATGTTCAGtCAGCATTTGCTT ATCATCATCCAGGCCTATAGTGAGTCGTATTACAG
D36	Primer/disruptor SNORD44	AGTCAGTTAGAGCTAATTAA
D37	Forward primer T7 promotor	CTGTAATACGACTCACTATA
D38	Transcription template for SNORD2 RNA	AGTGATCAGCAAGAGTATTCTCTTCATTTCAGGTCAGTCCCGAAAGAT GATTTGCCATCATTTCACTTCCTATAGTGAGTCGTATTACAG
D39	Forward primer SNORD2	AGTGATCAGCAAGAGTA
D40	Reverse primer SNORD2	CTGTAATACGACTCACTATAGGAAGTGAAATGATGGCAAATC
D41	Splint for ligation of SNORD2	AGAGTATTCTCTTCATTTCAGGTCAGTCCCGAAAGATGATTTGCCATC ATTTC
D42	5'-biotinylated oligonucleotide for enrichment of SNORD2	Biotin-AGTGATCAGCAAGAGTATTCTC
D43	Probe for northern blotting of SNORD2	GAGTATTCTCTTCATTTCAGGTC
D44	Transcription template for ACTB mRNA	CTGTAATACGACTCACTATAGGAGTATGACGAGTCCGGCCCCTCCATC GTCCACCGCAAATG
D45	Transcription template for ACTB mRNA	GTCAAGAAAGGGTGTAACGCAACTAAGTCATAGTCCGCCTAGAAGCAT TTGCGGTGGACGA
D46	VMC10 A20C A1216 ACTB mRNA	TAAGTCATAGGGGTCTCCAGCTGGACGTTCGCCTAGAAGC
D47	VMA15 A1216 ACTB mRNA	TAAGTCATAGAAAGGGCGGGCAAACTCTGGGCCTAGAAGC
D48	VMA8 A1216 ACTB mRNA	TAAGTCATGGAGTCGGCCCCAGCTGGTTCGCGCCTAGAAGC
D49	Biotinylated oligonucleotide for enrichment of MALAT1	CTAAGCTACTATATTTAAGGCCTTCCAAATTCTTCTAACTCTTCCAAA A-TEG-Biotin
D50	T7 primer MALAT1 starting 2369	GTAATACGACTCACTATAGAAGGGGAAGTTGG
D51	T7 primer MALAT1 starting 2473	CTGTAATACGACTCACTATAGAAGGCCTTAAATATAGTAG
D52	Reverse primer MALAT1 starting 2771	GACTCTTTTCCTATCTTCAC
D53	Reverse primer/disruptor MALAT1 2492	CTACTATATTTAAGGCCTTC
D54	Reverse primer/disruptor MALAT1 2661	CTTCTGTGTAGCACCTGGGT
D55	VMC10 A20C A2414 MALAT1	ATTACACCAGGGGTCTCCAGCTGGACGTTCTTTTAGTAGC
D56	VMC10 A20C A2515 MALAT1	GTTACGAAAGGGGTCTCCAGCTGGACGTTCTTCACATTTT
D57	VMC10 A20C A2577 MALAT1	TAACTCAAAGGGGTCTCCAGCTGGACGTTCAATGCAAAAA
D58	VMC10 A20C A2611 MALAT1	TTAATGCTAGGGGTCTCCAGCTGGACGTTCTCAGGATTTA
D59	VMC10 A20C A2720 MALAT1	TGCTCCTCAGGGGTCTCCAGCTGGACGTTCTAGCTTCATC
D60	VMA15 A2577 MALAT1	TAACTCAAAGAAAGGGCGGGCAAACTCTGGAATGCAAAAA
D61	VMA8 A2577 MALAT1	TAACTCAAGGAGTCGGCCCCAGCTGGTTCGCAATGCAAAA
D62	VMC10 A20C short 2	AAGGGGTCTCCAGCTGGACGTTCAA
D63	VMC10 A20C short 3	CTCAAAGGGGTCTCCAGCTGGACGTTCAA
D64	Probe for northern blotting of MALAT1	CTGTGTAGCACCTGGGTCAGCTGTC
D65	Probe for northern blotting of MALAT1	CTAAATTGGTTCTGGTCTACG
D66	Probe for northern blotting of MALAT1	CTCTTGATCTTGAATTACTTCCG
D67	Primer for MALAT1 reverse transcription	ACTCTTTTCCTATCTTCACCACG
D68	Forward primer for qPCR of MALAT1	TAACGGAAGTAATTCAAGATCAAGAGTAAT
D69	Reverse primer for qPCR of MALAT1	CCTCAGGATTTAAAAAATAATCTTAACTCA

[a] Alk = 5-hexynyl, E_3 = triethylene glycol

Table S2. DNA enzymes identified by sequencing.

Name	Frequency	Cleavage site	Sequence
VMA8	**3(1A+2B)**	G|GACU	TAGTTACGGAGG AGTCGGCCCCAGCTGGTTCGCTTCATTCAGT
VMA11	2	G|GACU	TAGTTACGGAAG GAGGGTTTCTAGGGGACGTG TTCATTCAGT
VMA15	**11 (4A+7B)**	GG|ACU	TAGTTACGGAAG AAAGGGCGGGCAAACTCTGG TTCATTCAGT
VMA14	1	GG|ACU	TAGTTACGGAAG GGTCTAGTGGGTTCCTGGCTCTTCATTCAGT
VMA1	1	GG|ACU	TAGTTACGGAAG AAGGATTGCCGGAACTGGGG TTCATTCAGT
VMA2	1	GGAC|U	TAGTTACGGAGG TTGCGTAGCGCCTGGGCACC TTCATTCAGT
VMA5	1	GGA|CU	TAGTTACGGAAG GGGGTATCGGGGGGTGTAC TTCATTCAGT
VMB1	3	GG|ACU	TAGTTACGGAGG GGTCTCGCGGGGCCTGGCTC TTCATTCAGT
VMB39	1	GG|ACU	TAGTTACGGAGG GGTCTTGCGGGTCCTGGCTC TTCATTCAGT
VMB15	3	GG|ACU	TAGTTACGGAAG AAGGATCCGAGCAACTTCGGCTTCATTCAGT
VMB10	1	n.d.	TAGTTACGGAAG GATTCCAGGGCTTGAGGAGG TTCATTCAGT
VMB33	1	n.d.	TAGTTACGGAAG GGAGCGCAGTCTGTTGGGGG TTCATTCAGT
VMC10	**15(5C+8D)**	GG|ACU	TAGTTACGGAAG GGGTCTCCAGCTGGACGTTA TTCATTCAGT
VMC6	1	GG|ACU	TAGTTACGGAAG GGTCTCGCGGGTCCTGGCTC TTCATTCAGT
VMC17	1	G|GACU	TAGTTACGGAAG GGTTCGGTTGAGTGGGGCGA TTCATTCAGT
VMC1	1	n.d.	TAGTTACGGAAG GCGTGGCGTGGGACCGATGG TTCATTCAGT
VMC4	1	n.d.	TAGTTACGGAAG AGATCGGTGACGTCGTTGTG TTCATTCAGT
VMD1	3	GG|ACU	TAGTTACGGAAG CAGGACCCGAGCAACTTCGGCTTCATTCAGT
VMD35	1	GG|ACU	TAGTTACGGAAG GGGATTCCAGCTGGACGTTG TTCATTCAGT
VMD3	2	n.d.	TAGTTACGGAAG CGGCAGGGTCGGCACACGCG TTCATTCAGT
VMD13	1	n.d.	TAGTTACGGAGG ACAGGAGCGGGTGGCCATGG TTCATTCAGT
VMD32	1	n.d.	TAGTTACGGAGC GGCGGTGTGATCCAGGCAG TTCATTCAGT
VMD43	2	n.d.	TAGTTACGGAAG GGAGCCAGTCTAGTGGGGGG TTCATTCAGT

Table S3a. Summary of cleavage kinetics. k_{obs} (h^{-1}) for VMA DNA enzymes on various RNA substrates.

RNA	motif	VMA8		ratio	VMA15		ratio
		unmod (k_1/h)	m^{6}A (k_2/h)	k_2/k_1	unmod (k_1/h)	m^{6}A (k_2/h)	k_2/k_1
R1/R2	GGACU	0.038±0.001	0.27±0.01	6.9	0.19±0.01	0.60±0.02	3.2
R3/R4	UGACU	<0.001	<0.001	-	0.40±0.03	0.85±0.03	2.1
R5/R6	AGACU	<0.001	<0.001	-	0.007±0.001	0.023±0.001	3.2
HNRNPH1A5023	GGACU	0.018±0.001	0.14±0.01	7.8	0.12±0.02	0.27±0.02	2.2
RSV A7414	GGACU	0.16±0.02	0.85±0.06	5.3	0.28±0.02	0.48±0.06	1.8
SNORD 29	UGACC	n.d.	n.d	-	0.16±0.03	0.24±0.03	1.3
SNORD 41	UGACU	n.d	n.d	-	0.40±0.07	1.06±0.12	2.6
SNORD 44	UGACU	n.d.	n.d	-	0.015±0.001	0.038±0.002	3.1

Table S3b. Summary of cleavage kinetics. k_{obs} (h^{-1}) for VMC10 DNA enzymes on various RNA substrates.

RNA sequence	motif	VMC10		ratio	VMC10 _A20C		Ratio
		unmod (k_1/h)	m^{6}A (k_2/h)	k_1/k_2	unmod (k_1/h)	m^{6}A (k_2/h)	k_1/k_2
R1/R2	GGACU	0.26±0.03	0.003±0.0005	85	0.36±0.03	0.005±0.0005	72
R3/R4	UGACU	0.34±0.03	0.006±0.0005	56	n.d.	n.d.	-
R5/R6	AGACU	0.15±0.03	0.0016±0.0001	95	n.d.	n.d.	-
HNRNPH1A5023	GGACU	0.021±0.003	<10e-4	>100	n.d	n.d	-
RSV A7414	GGACU	0.36±0.03	0.003±0.0005	118	1.0±0.1	0.013±0.002	77
SNORD 29	UGACC	1.12±0.08	0.021±0.001	81	n.d.	n.d.	-
SNORD 41	UGACU	1.20±0.06	0.013±0.001	147	n.d.	n.d.	-
SNORD44	UGACU	0.74±0.03	0.008±0.001	115	n.d.	n.d.	-

n.d. = not determined

SUPPORTING INFORMATION

Supporting Figures

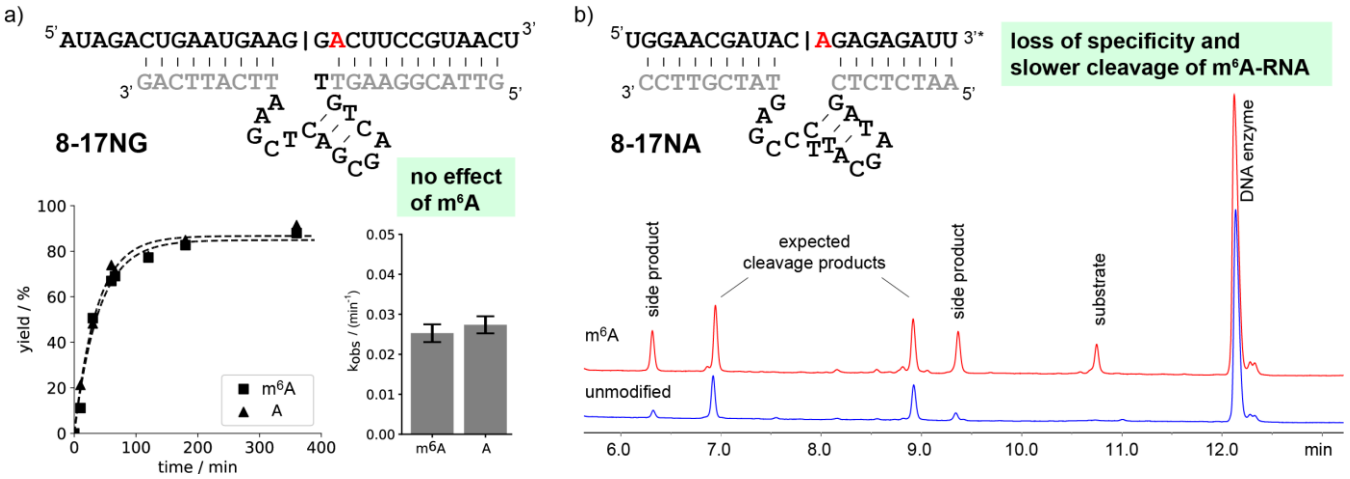

Figure S1. (a) Cleavage of R1 and R2 with 8-17NG DNA enzyme (D1). Incubation conditions: 1 µM substrate RNA, 1 µM deoxyribozyme, 20 mM $MgCl_2$, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 37 °C. Both methylated and unmethylated RNA are cleaved efficiently, with no difference in k_{obs}. For the kinetic plot, each data point represents the mean form three experiments. Fitted k_{obs} plus/minus standard error of the mean. (b) Cleavage of a U6 snRNA fragment (R9/R10) with the 8-17NA deoxyribozyme (D2). The known m^6A site at position 43 of human snRNA is shown in red. Incubation conditions: 5 µM RNA, 5 µM deoxyribozyme, 20 mM $MgCl_2$, 20 mM $MnCl_2$, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 37 °C, 26 h. Analysis by anion exchange HPLC (Dionex DNA Pac PA200, 2x250 mm) monitored by A^{260} shows complete cleavage of unmodified RNA (blue trace) to give two fragments of expected size (5'-fragment is 11 nt, 3' fragment is 9 nt with 3'-aminohexyl modification). In contrast, cleavage of m^6A RNA is incomplete, and shows significant amounts of miscleavage (the side products are assigned to cleavage on the 3'-side of m^6A, resulting in 12 nt and 8 nt fragments).

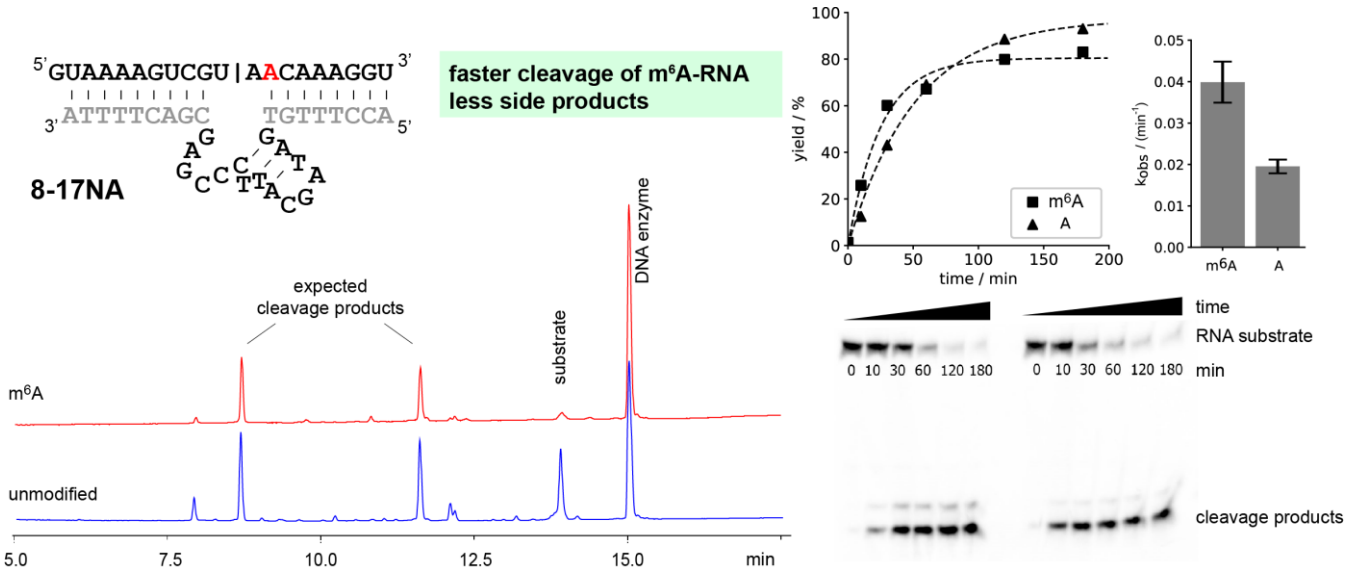

Figure S2. Cleavage of hs 18S rRNA fragement (nt 1820-1838, R11/R12)) with 8-17NA (D3) one position upstream of A1832. Incubation conditions: 5 µM substrate RNA, 5 µM deoxyribozyme, 20 mM $MnCl_2$, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 37°C. Anion exchange HPLC traces shown after 30 min, representative images of denaturing PAGE gels show the extent of substrate cleavage over a time course of 3 hours. The results demonstrate slightly faster cleavage of the m^6A RNA compared to unmodified RNA, by 8-17NA at the indicated position. For the kinetic plot, each data point represents mean from three experiments. Fitted k_{obs} plus/minus standard error of the mean.

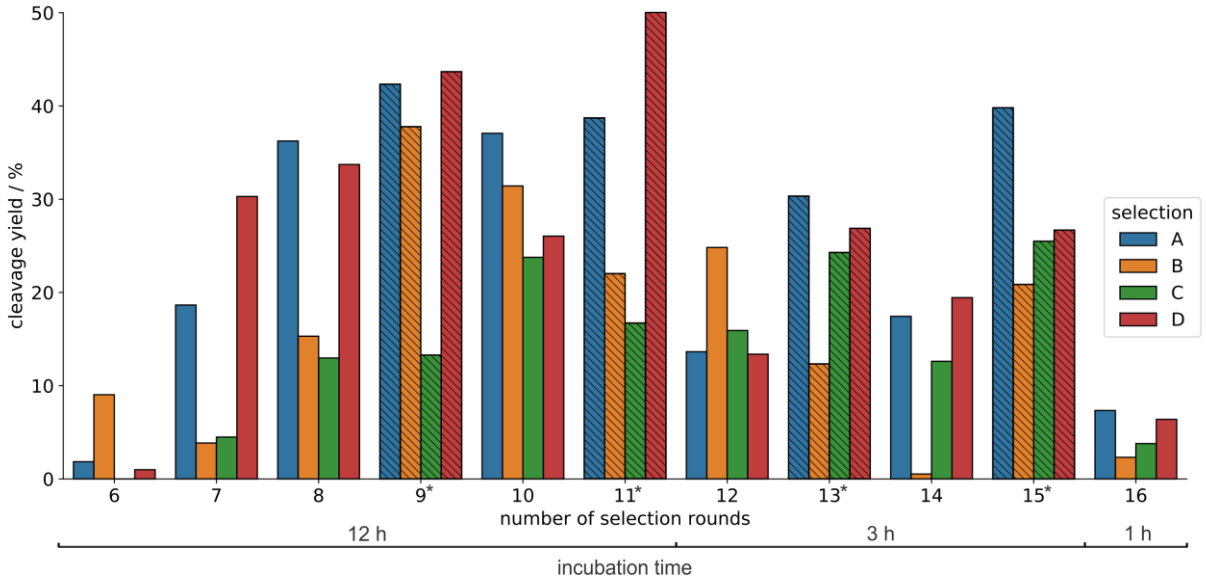

Figure S3. Progress of the *in vitro* selections. Starting in round 6, cleavage activity was visible and cleavage yield was determined by quantification of fluorescent bands on the PAGE gels of the selection step. Counter-selection rounds are marked by asterisks and hatching, and were always performed using 12 h incubation steps.

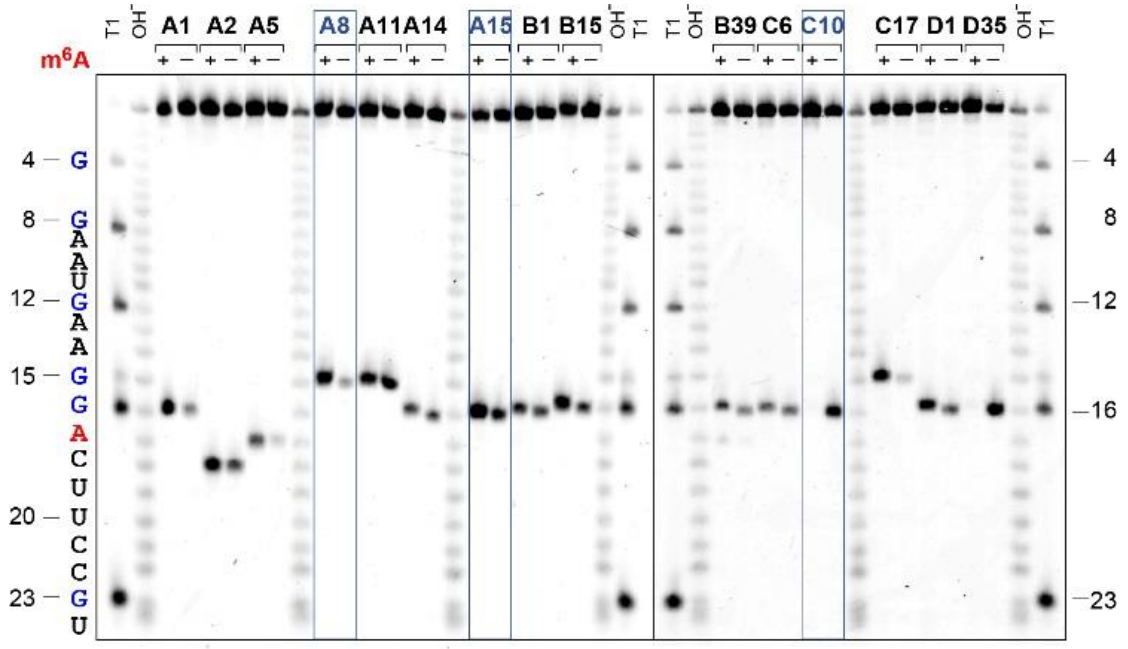

Figure S4. PAGE analysis of the cleavage products for selected VMA/B/C/D DNA enzymes and comparison of cleavage activity for m^{6}A-modified and unmodified RNA. The results for VMA8, VMA15 and VMC10 are boxed to indicate the enzymes chosen for further characterization. 3'-Fluorescein-labeled RNA were incubated with DNA enzymes with 20 mM Mg^{2+} at 37°C (VMA and VMC) or 45°C (VMB and VMD) for 5 h.

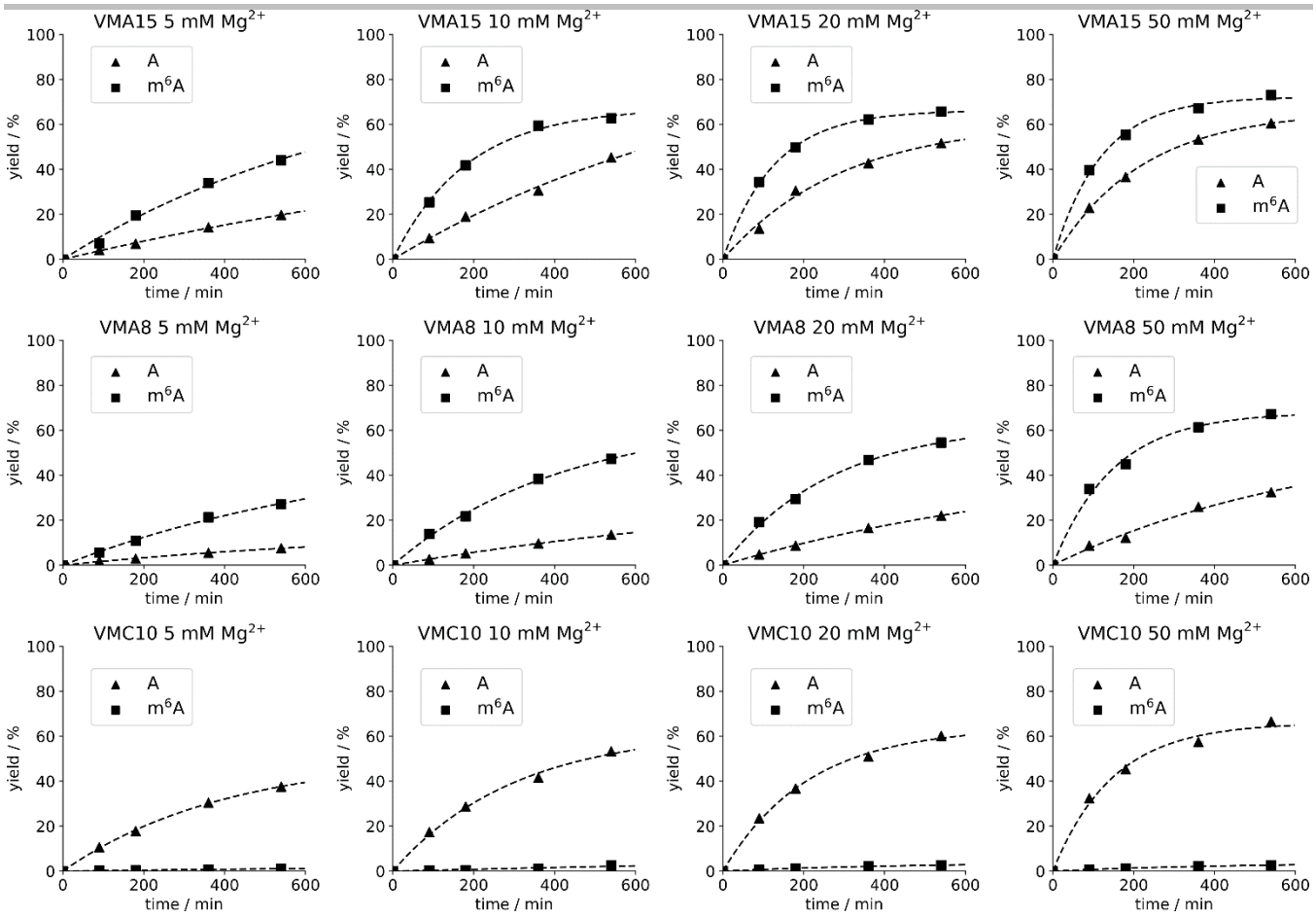

Figure S5. Dependence of cleavage yield on concentration of Mg^{2+} for the reactions of the selection substrate (R1 and R2) with deoxyribozymes VMA15, VMA8 and VMC10 (D9, D10 and D11, correspondingly). Incubation conditions: 1 µM substrate RNA, 1 µM deoxyribozyme, indicated concentration of Mg^{2+}, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 37 °C.

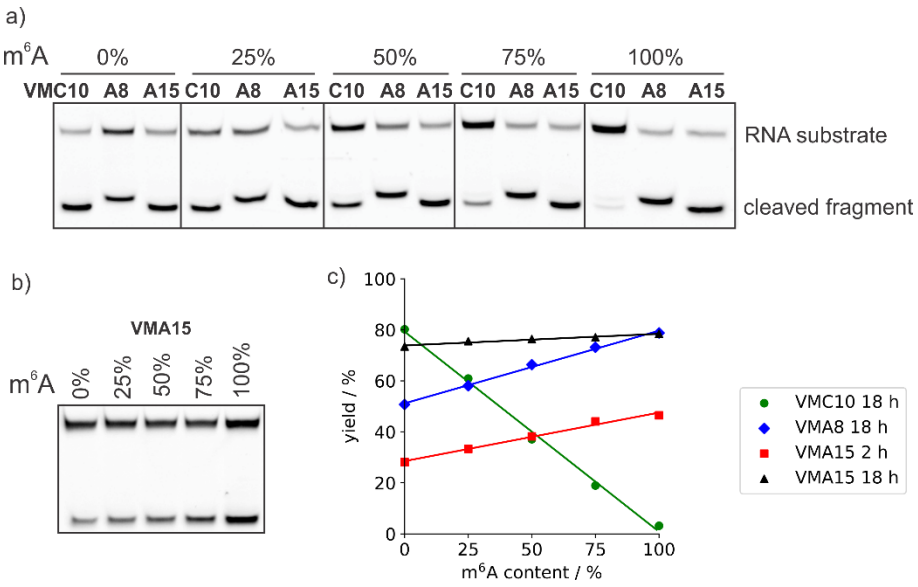

Figure S6. Dependence of cleavage yield on m^{6}A content for the reactions of the selection substrates (defined mixtures of R1 and R2) with deoxyribozymes VMA15, VMA8 and VMC10 (D9, D10, D11, respectively). a) Incubation conditions: 1 µM substrate RNA, 10 µM deoxyribozyme, 20 mM Mg^{2+}, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 37 °C. PAGE image for aliquots taken after 18 h. b) PAGE image for VMA15 samples after 2 h. c) Cleavage yields of samples shown in a) and b) plotted as function of m^{6}A content.

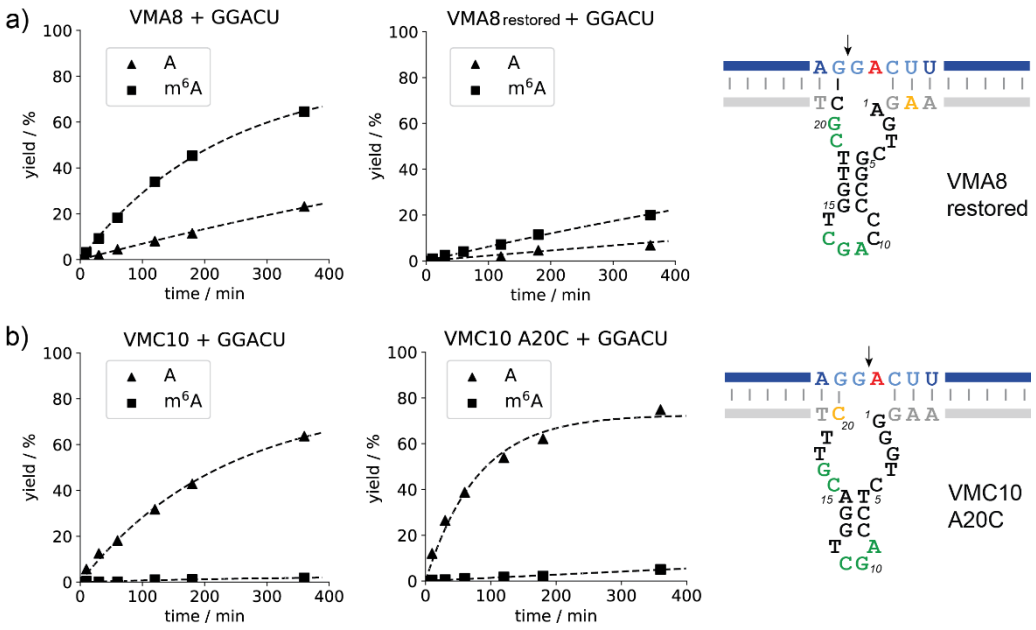

Figure S7. Influence of mutations in the binding arms on reactivity of VMA8 (a), left: D9; right: D12) and VMC10 (b), left: D11, right: D13). Incubation conditions: 1 µM substrate RNA (R1/R2), 10 µM deoxyribozyme, 20 mM $MgCl_2$, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 37 °C. Each data point represents the mean from three experiments.

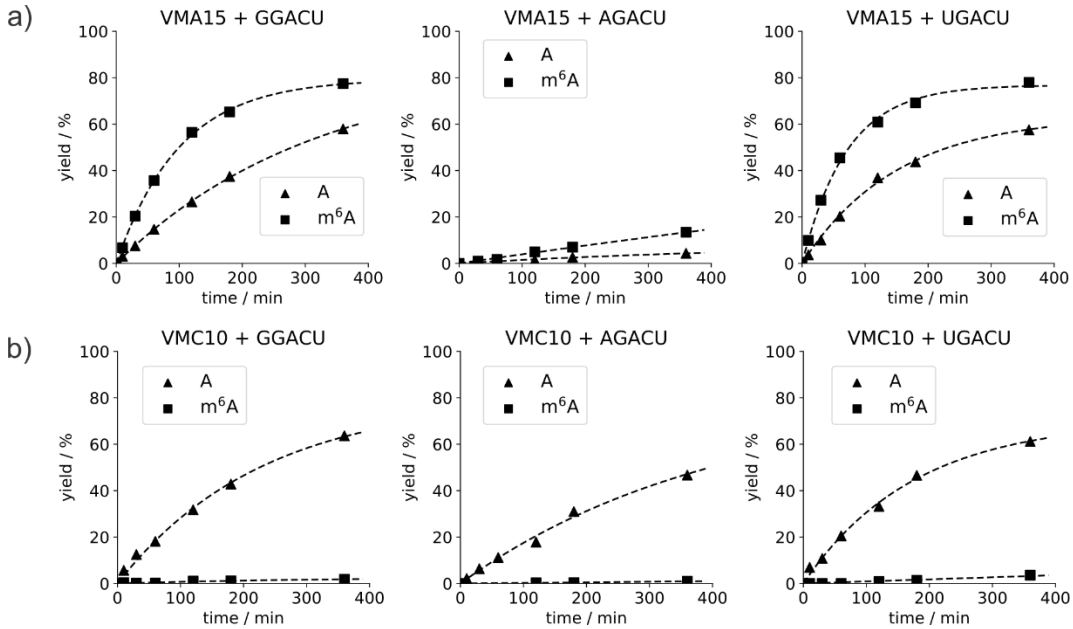

Figure S8. Dependence of the cleavage yield on the sequence context at the cleavage site for deoxyribozymes VMA15 (a), left: R1/R2, middle: R3/R4, right: R5/R6; all with D10) and VMC10 (b), left: R1/R2, middle: R3/R4, right: R5/R6; all with D11). Incubation conditions: 1 µM substrate RNA, 10 µM deoxyribozyme, 20 mM $MgCl_2$, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 37 °C. Each data point represents the mean from three experiments.

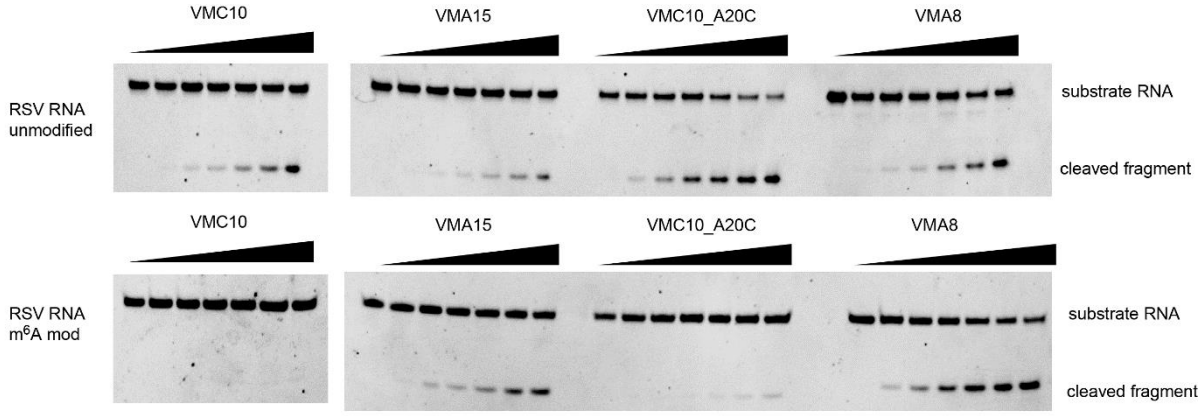

Figure S9. Cleavage of unmodified and m^{6}A-modified RSV RNA fragments (R17/R18) with VMA8 (D17), VMA15 (D18), VMC10 (D19), and VMC10_A20C (D20). Images of denaturing PAGE show time course of the cleavage up to 5 hours. Incubation conditions: 0.5 µM RNA substrate, 10 µM deoxyribozyme, 20 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 37°C. Aliquots taken at 0, 10, 25, 45, 90, 180 and 300 min.

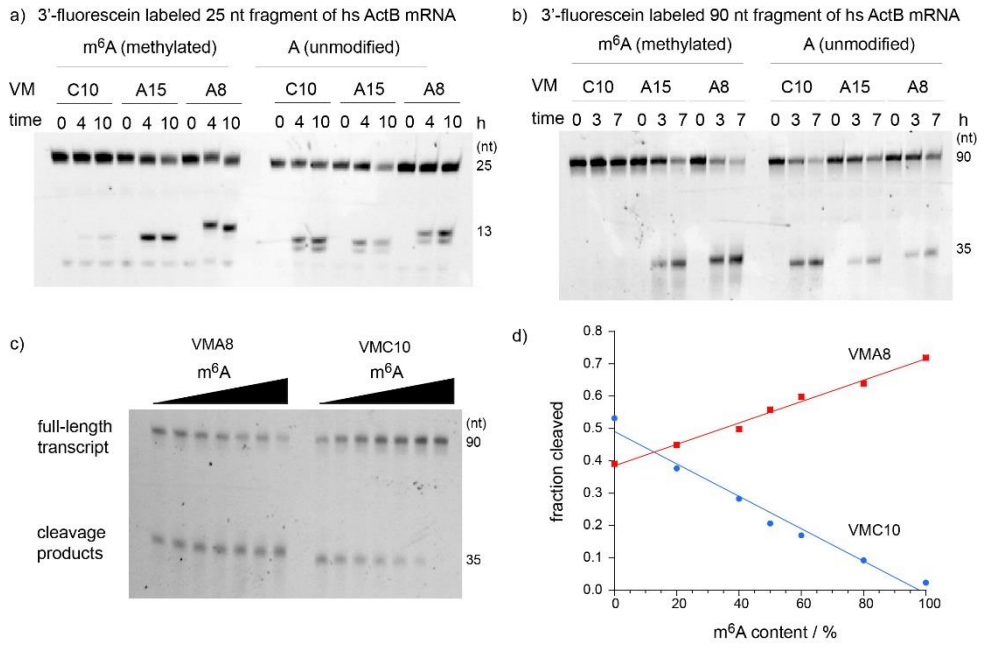

Figure S10. Analysis of m^{6}A in synthetic fragments of human ACTB mRNA by m^{6}A-sensitive deoxyribozymes. A 3'-fluorescein-labeled 25 nt (a) and *in vitro* transcribed 90 nt (b) fragments with or without m^{6}A at A1216 were treated with VMC10, VMA15 and VMA8 and cleavage yield was analyzed by PAGE after the indicated time. Incubation conditions: 1 µM RNA, 10 µM deoxyribozyme, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 37°C. c) Mixtures of methylated and unmethylated transcripts with known m^{6}A content were cleaved by VMA8 and VMC10. Incubation for 6 h at 37°C. Band intensities were quantified and fraction of cleaved product plotted as function of m^{6}A content (d).

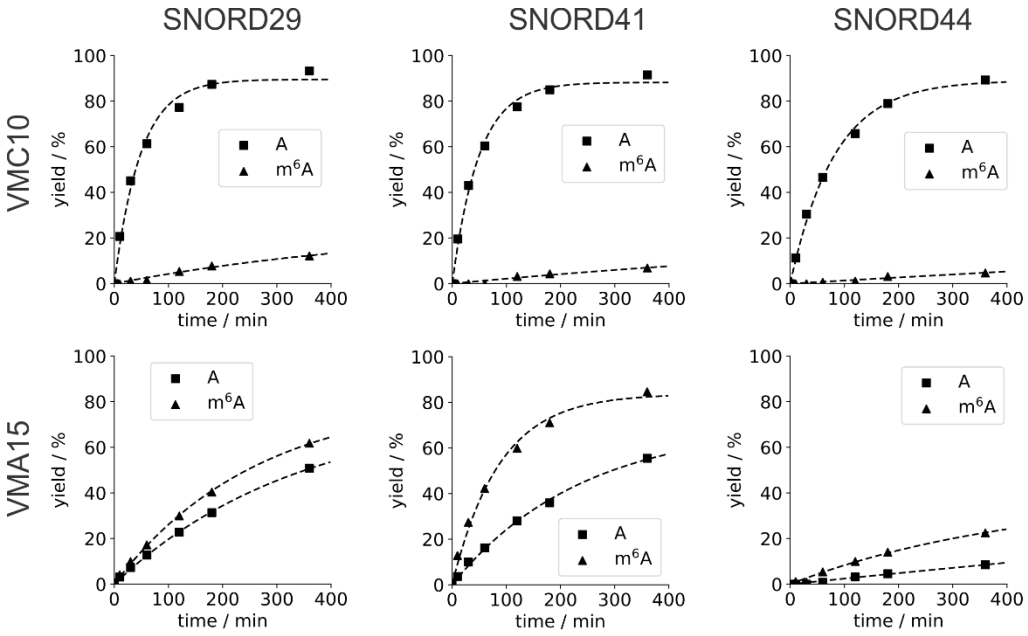

Figure S11. Kinetics of unmodified and methylated transcripts of SNORD29, SNORD41, and SNORD44 with corresponding VMC10 and VMA15 DNA enzymes. Incubation conditions: 1 µM substrate RNA, 10 µM deoxyribozyme, 20 mM $MgCl_2$, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 37 °C. Each data point represents the mean from three experiments.

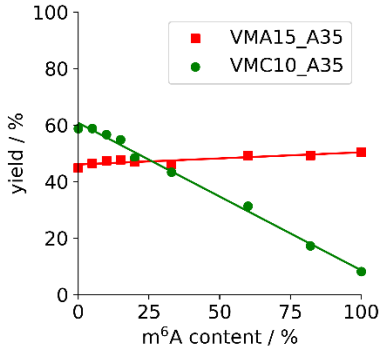

Figure S12. Calibration curves prepared with synthetic single m^6A35-modified RNA, mixed in defined ratios with unmodified ligation product, and cut with VMA15 (D23) / VMC10 (D24) DNA enzymes. Incubation conditions: 1 µM substrate RNA, 10 µM deoxyribozyme, 20 mM $MgCl_2$, 50 mM Tris- HCl, 150 mM NaCl, pH 7.5.

SUPPORTING INFORMATION

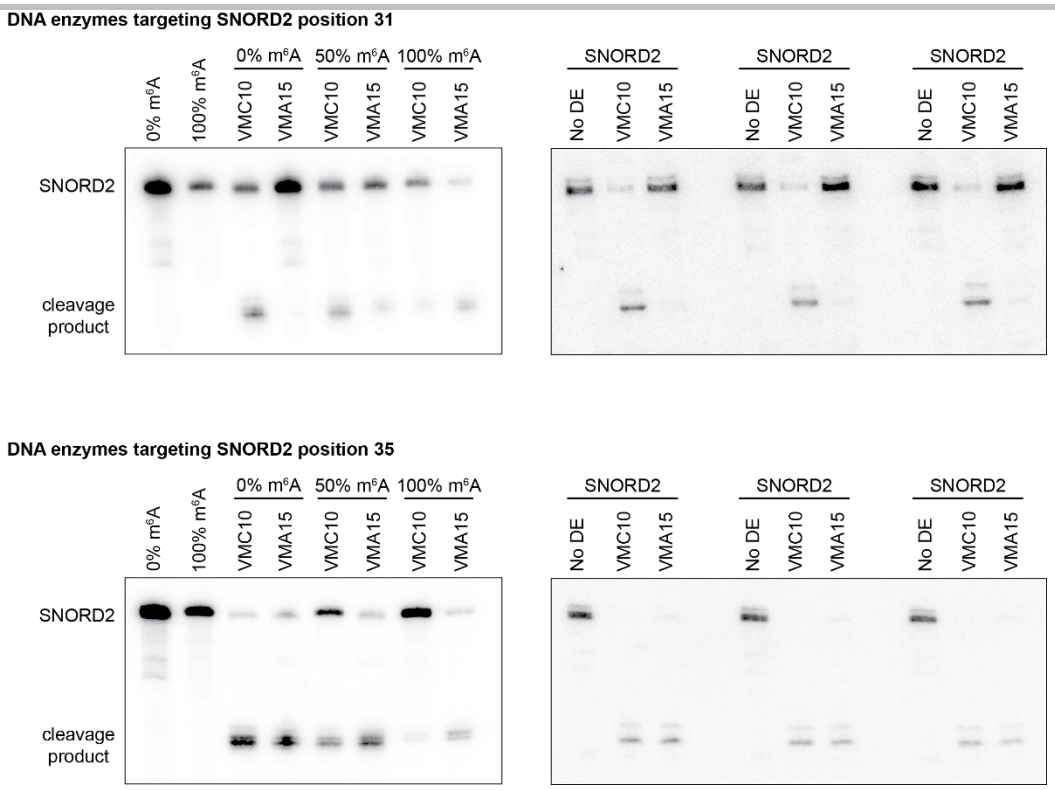

Figure S13. Full figure corresponding to Figure 5d for endogenous mouse SNORD2. Left: control experiments with modified and unmodified transcripts and an equimolar mixture thereof. Right: Three replicate experiments with RNAs isolated by pulldown with 5'-biotinylated oligonucleotides complementary to the 3'-end of SNORD2 RNA.

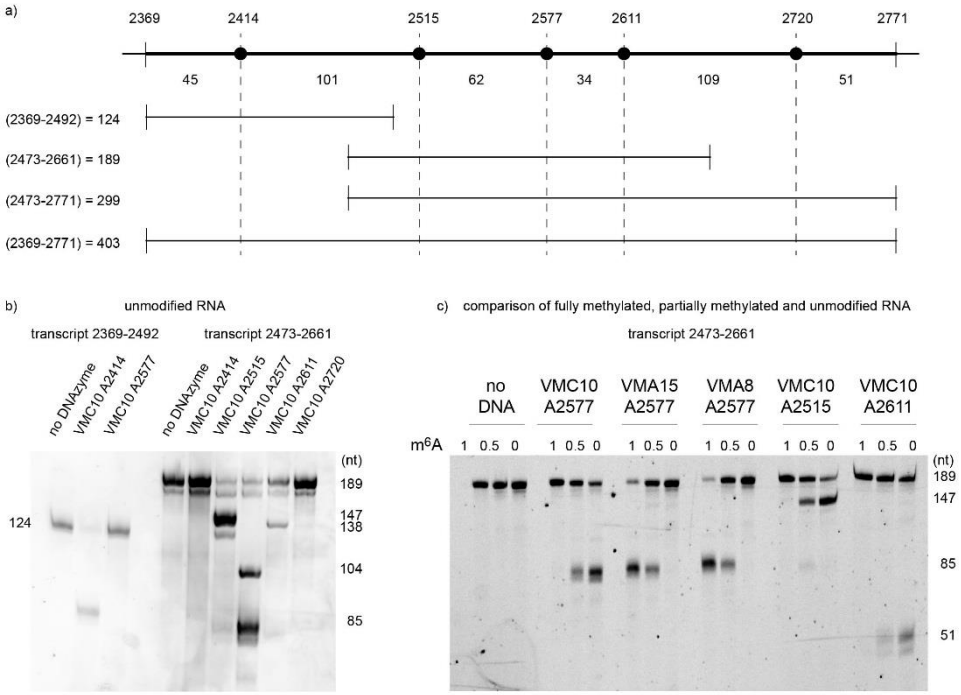

Figure S14. DNA-catalzyed cleavage of hs MALAT1 lncRNA fragments. a) Schematic depiction of synthetic constructs of MALAT1 RNA, spanning five GGACU sites within 400nt. b) Specific cleavage by VMC10 occurs only at target sites, as demonstrated for the 124 and 189 nt fragments. This gel was stained with Sybr gold. The short fragments <50 nt overlap with the DNA enzymes at the bottom of the gel (not shown). c) Comparison of cleaving fully or partially methylated RNA with unmodified RNA, using VMC10, VMA15 and VMA8 for A2577, and also VMC10 at A2515 and A2611. Here, 3'-fluorescently labeled RNA was used, therefore only one cleavage fragment is visible. Incubation conditions for: ca 3 pmol RNA, 50 pmol DNAzyme, 2 x 25 pmol disruptor, 20 mM $MgCl_2$, 37°C, 6 h (samples in b), and 3 h (samples in c).

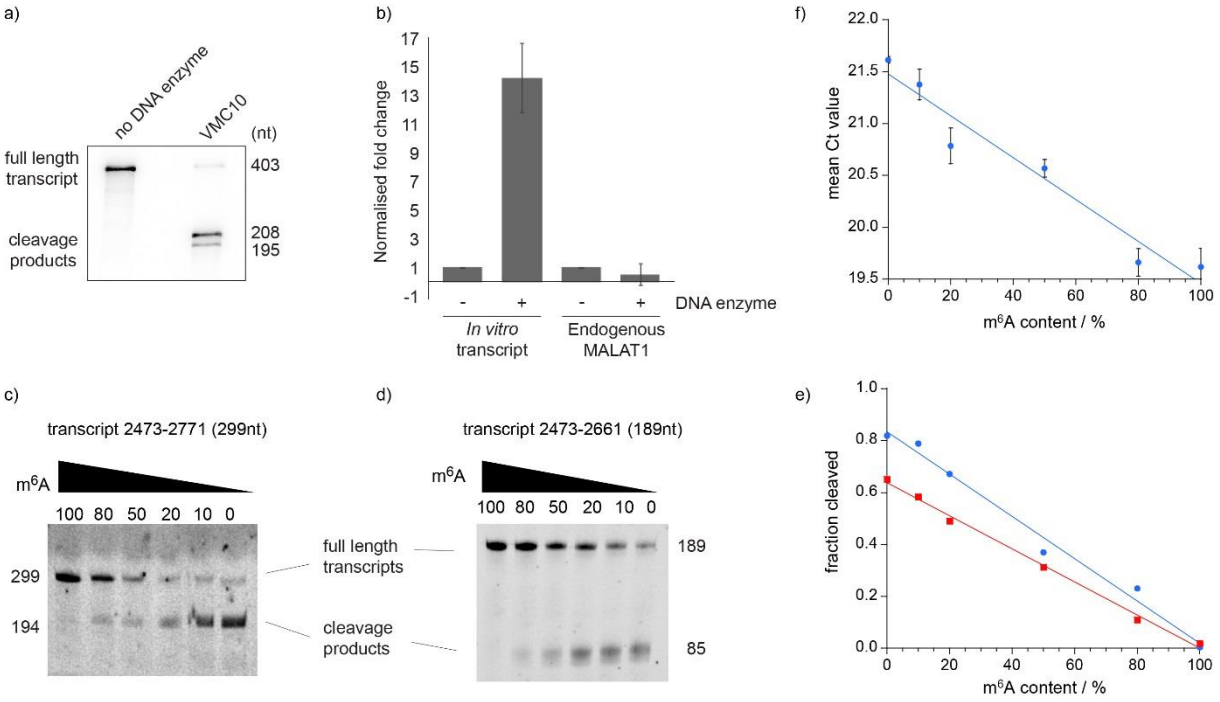

Figure S15. Analysis of hs MALAT1 lncRNA at A2577 by VMC10. a) Northern blot showing almost complete cleavage of a 403 nt long unmodified *in vitro* transcript (6 h incubation). b) The result of quantitative PCR for *in vitro* transcript and endogenous MALAT1 RNA, represented as fold change normalized to the non-treated control samples. c,d) Mixtures of 3'-fluorescein-labeled unmodified and methylated 299 nt and 189 nt long transcripts, respectively were cleaved by VMC10 at A2577 and analyzed by PAGE. e) The band intensities were analyzed and cleavage fraction from c) and d) is plotted as a function of m^6A content. The blue data points are for c) 299 nt RNA, the red symbols represent the data from d). f) The samples of the 189 nt RNA used in d) were also analyzed by qPCR, and the mean Ct values from three experiments are plotted as a function of m^6A content in the RNA. Error bars represent the standard deviation.

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