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Supporting Information

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DECAL: Deoxyribozyme-Catalyzed Labeling of RNA

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General experimental considerations

DNA oligonucleotides were prepared at IDT (Coralville, IA). Short target RNA substrates for comprehensive sequence-dependence studies and the aminoallyl-modified tagging RNA were prepared by in vitro transcription with T7 RNA polymerase and a synthetic double-stranded DNA template that was prepared by annealing two DNA oligonucleotides.[1] The P4-P6 RNA and its mutant forms were prepared by in vitro transcription with T7 RNA polymerase and a linearized plasmid template.[2,3] DNA and RNA oligonucleotides and transcripts were purified by denaturing PAGE as described previously.[4,5]

Design and synthesis of tagging RNA

The sequence of the tagging RNA was designed on the basis of several considerations. We would like the biophysical label to be relatively close to the target RNA. Therefore, the 5-aminoallylcytidine nucleotide used for attaching the label to the tagging RNA was placed near the 5′-terminus of the transcript. Because T7 RNA polymerase requires G or A as the initiating nucleotide,[1,6] the closest possible position for the aminoallyl-nucleotide (which is commercially available only as the 5′-triphosphate of C or U) is the second position from the 5′-terminus. To ensure that the tagging RNA contains only a single label, the aminoallyl-nucleotide must be incorporated only once into the transcript. On the basis of these considerations and to avoid potential hybridization with any portion of the P4-P6 RNA sequence, the sequence of the unlabeled tagging RNA was 5′-GC^{aa}AAGAGAUGGUGAUGGGA-3′, where C^{aa} denotes 5-aminoallyl-C. 5-Aminoallyl-CTP was used instead of the UTP derivative because of higher transcription yield (data not shown). The two DNA template oligonucleotides were 5′-TCCCATCACCATCTCTTGCTATAGTGAGTCGTATTACAGCG-TGCGT-3′ and 5′-ACGCACGCTGTAATACGACTCACTATA-3′ (the coding sequence is underlined). Transcription conditions were as follows: 1 μM each DNA template, 40 mM Tris (pH 8.0), 30 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine, 4 mM each ATP, GTP, and UTP, and 2 mM 5-aminoallyl-CTP (TriLink BioTechnologies, San Diego, CA). After incubation of the 200–800-μL sample at 37 °C for 5 h, the transcript was purified by 20% denaturing PAGE. Typical yields after extraction and ethanol precipitation were 1.4–3.0 nmol of aminoallyl-modified RNA transcript per 100 μL of transcription reaction.

Derivatization of the aminoallyl-modified RNA transcript with biophysical labels

The aminoallyl-modified tagging RNA transcript was coupled with the amine-reactive NHS ester of biotin (ChemGenes, Wilmington, MA), DABCYL (AnaSpec, San Jose, CA), 5(6)-fluorescein (Pierce Biotechnology, Rockford, IL) or 5(6)-TAMRA (Molecular Probes, Eugene, OR). For biotin and TAMRA, 5 μM aminoallyl-RNA and 5 mM NHS ester were incubated with 0.2 mM EDTA in 100 mM sodium phosphate (pH 8.0) and 50% (v/v) DMSO at 37 °C for 24 h (biotin) or 3 h (TAMRA). For DABCYL, 5 μM aminoallyl-RNA and 100 mM NHS ester were incubated with 0.2 mM EDTA in 100 mM sodium phosphate (pH 8.0) and 50% (v/v) DMSO at 37 °C for 24 h. For fluorescein, 10 μM aminoallyl-RNA and 21 mM NHS ester were incubated with 0.2 mM EDTA in 70 mM sodium bicarbonate (pH 9.0) and 30% (v/v) DMSO at 37 °C for 3 h. Unreacted labeling reagent was removed by ethanol precipitation, and labeled transcripts were separated from unlabeled transcripts by 20% denaturing PAGE. Labeling reactions were performed on the 1 nmol scale. Isolated yields of labeled transcripts after gel extraction and ethanol precipitation were ~27% for biotin, 5–10% for DABCYL, ~20% for fluorescein, and ~15% for TAMRA.

Assays of labeling generality using the 10DM24 deoxyribozyme: short target RNAs

The ability of the 10DM24 deoxyribozyme[7] to utilize the various tagging RNA transcripts was assayed with a systematic series of short target RNAs. In accordance with our previous nomenclature,[4] the target RNA serves as the left-hand substrate and is designated L, whereas the tagging RNA is the right-hand substrate and is designated R (Figure S1a). The initial target RNA (parent sequence 5′-GGAUAAUACGACUCACUAUA-3′ with branch-site adenosine underlined) was the L substrate originally used in the selection that led to identification of 10DM24.[7] We tested target L substrates that have systematic sequence changes relative to the parent sequence, except for the branch-site A, the 5′-GGA included for efficient transcription, and UAUA at the 3′-terminus (Figure S1b). The sequence changes were denoted as transitions (A↔G, U↔C), transversions-1 (A↔C, G↔U), and transversions-2 (A↔U, G↔C). The corresponding DNA changes were made at each Watson-Crick base-paired position of 10DM24. Each L substrate was tested with a series of tagging RNA transcripts. The tagging RNA was either entirely unmodified, unlabeled (i.e., 5-aminoallyl-C at the second nucleotide position), or labeled at the aminoallyl group with biotin, DABCYL, fluorescein, or TAMRA as described above. All assays with 10DM24 were performed in the previously described fashion,[4,8] in which the 5′-^{32}P-radiolabeled L substrate was the limiting reagent relative to 10DM24 (E) and the tagging RNA (R). The ratio L:E:R was 1:3:6, with E equal to 0.3 μM. Reactions were performed in 50 mM CHES (pH 9.0), 150 mM NaCl, 2 mM KCl, and 40 mM $MgCl_2$ at 37 °C for up to 2 h. At appropriate timepoints, 1.5 μL was removed from the sample and quenched into 8 μL stop solution (80% formamide, 1× TB [89 mM each Tris and boric acid, pH 8.3], and 50 mM EDTA, containing 0.025% bromophenol blue and xylene cyanol). Samples were separated by 20% denaturing PAGE and imaged with a PhosphorImager. The resulting data were fit to yield=Y•$(1-e^{-kt})$, where $k = k_{obs}$ and Y = final yield.

The 10DM24 deoxyribozyme successfully used the various tagging RNA substrates in many but not all target sequence contexts (Figure S1c–h). For all tagging RNAs, the parent L sequence had the highest ligation yield; L with either transversions-1 or transversions-2 as the sequence changes was slower but still generally high-yielding. In contrast, L with transitions as the sequence changes was a poorer target (this was also observed when either the P1 or P2 region alone was changed via transitions; data not shown). We have initiated new deoxyribozyme selection efforts that use other L sequences (particularly with transitions relative to the parent L sequence) and that use R substrates directly incorporating the biophysical labels. We anticipate that these new selection efforts will extend the scope of the deoxyribozyme-catalyzed labeling approach and increase the efficiency for a broad range of target RNA sequences.

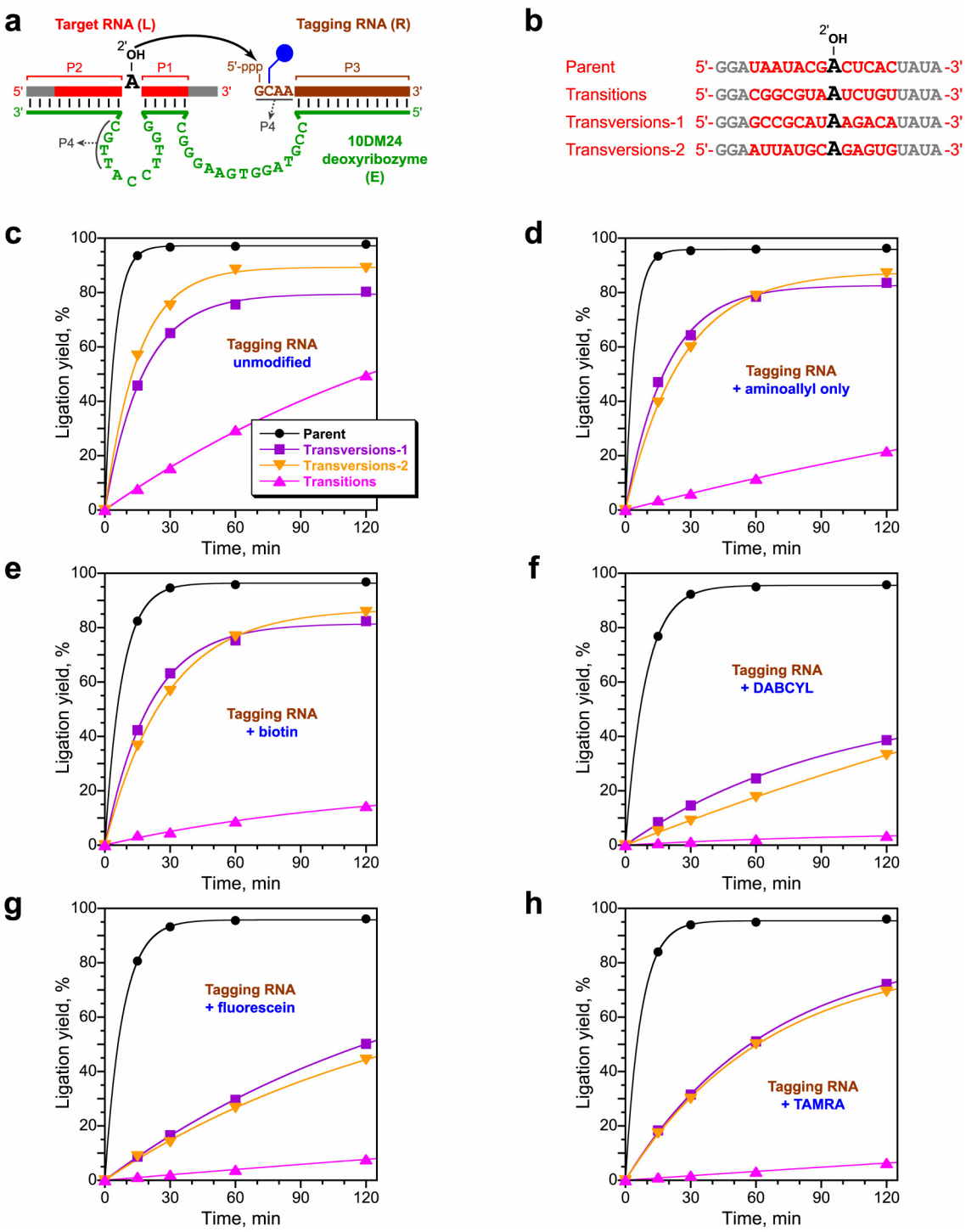

Figure S1. Assaying the generality of deoxyribozyme-catalyzed RNA labeling using the 10DM24 deoxyribozyme and short target RNA substrates. a) The overall reaction. b) The systematic changes to the target RNA (L substrate). c) Testing the four L substrates with the unmodified tagging RNA. d–h) Testing the four L substrates with the tagging RNA modified either with a 5-aminoallyl-C at the second position or with the indicated biophysical probe appended to the aminoallyl group.

Assays of labeling generality using the 10DM24 deoxyribozyme: P4-P6 as target RNA

As a more realistic test of deoxyribozyme-catalyzed labeling of RNA, we assayed 10DM24 for the ability to label nucleotides in the 160-nt *Tetrahymena* group I intron P4-P6 domain.[9,10] The labeling sites in P4-P6 were chosen on the basis of the X-ray crystal structure.[10] Only nucleotides with accessible 2′-hydroxyl groups were chosen, and we restricted our choices to adenosines on the basis of the 10DM24 branch-site preference.[7] Approximately 24 adenosines were identified as accessible. The ten tested adenosines were scattered throughout P4-P6, including within the central region of the RNA (where modifications that require subsequent ligations are challenging). There were no obvious similarities among the RNA sequences surrounding the adenosines.

The labeling assays were performed as described above. We included 100 equivalents relative to L of a disruptor (D) DNA oligonucleotide that interferes with the local RNA secondary structure, thereby allowing 10DM24 to bind the target site within P4-P6. Samples were annealed in 7 µL of 5 mM HEPES (pH 7.5), 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. The reaction buffer was added and the samples were incubated at 37 °C for 2 min, then $MgCl_2$ was added. The final incubation conditions were 50 mM CHES (pH 9.0), 150 mM NaCl, 2 mM KCl, and 40 mM $MgCl_2$ in a volume of 10 µL. Reactions were incubated at 37 °C for 2 h and quenched with stop solution. The products were analyzed by 6% denaturing PAGE.

The data are shown in Figure S2 (see Figure 2 for representative gel images). Eight of the ten tested nucleotide locations were readily derivatized (>50%) with the entirely unmodified R transcript, which contains cytidine instead of 5-aminoallyl-C at the second position. Six nucleotide locations were readily derivatized using R that has a 5-aminoallyl-C at the second position. Although inclusion of the tested biophysical labels (biotin, fluorescein, or TAMRA) within the tagging RNA generally led to a decrease in yield, multiple sites were successfully labeled in preparatively useful yield (>40%) with biotin (six sites), fluorescein (five sites including A231) and TAMRA (one site, A146).

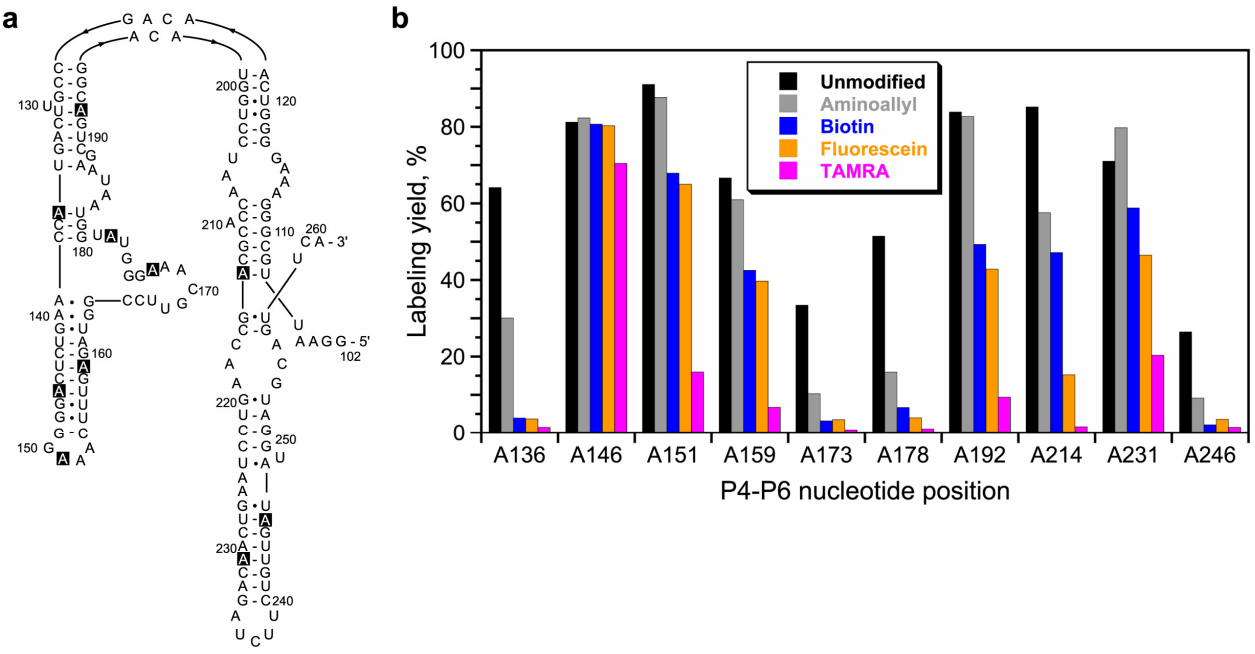

Figure S2. Assaying the generality of deoxyribozyme-catalyzed RNA labeling using the 10DM24 deoxyribozyme and the P4-P6 RNA. a) Secondary structure of P4-P6. The ten tested adenosines are marked. b) Labeling yields after 2 h. The modifications to each tagging RNA are indicated in the legend.

Preparative double-labeling of P4-P6 to enable FRET studies

Preparative double-labeling of P4-P6 with fluorescein and TAMRA was achieved in two steps. The first tag (either with or without attached fluorescein) was attached at nucleotide A231. After PAGE purification, the second tag (with or without attached TAMRA) was attached at nucleotide A146. The RNAs without chromophores were synthesized as controls and to facilitate FRET analysis (see below).

For ligation at A231, the ratio R:E:L:D was 1.0:1.1:1.2:2.0, where R was equal to 12.5–25 μM. Samples were annealed in 28 μL of 5 mM HEPES (pH 7.5), 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. The reaction buffer was added and the samples were incubated at 37 °C for 2 min, then $MgCl_2$ was added. The final conditions were 50 mM CHES (pH 9.0), 150 mM NaCl, 2 mM KCl, and 40 mM $MgCl_2$ in a volume of 40 μL. Reactions were incubated at 37 °C for 2 h and quenched with 40 μL stop solution. The A231-modified RNAs were separated from unmodified P4-P6 by 6% denaturing PAGE.

For ligation at A146, the ratio L:E:R:D was 1.0:1.1:1.2:2.0, where L was equal to ~1–3.4 μM. Reactions were performed under the same reaction conditions as used for the ligation at A231. The doubly modified P4-P6 RNA was readily separated from the singly modified P4-P6 RNA by 6% denaturing PAGE.

Nondenaturing gel electrophoresis (native PAGE) analysis of labeled P4-P6

The native PAGE experiments were performed at 35 °C as previously described,[2,11-13] except each RNA sample included 10 pmol of U_{13} carrier RNA.[12] The titration curves for unmodified P4-P6 and for doubly tagged P4-P6 (A231-fluorescein and A146-TAMRA) are shown in Figure S3. From these data, $[Mg^{2+}]_{1/2}$ is 0.72 mM for unmodified P4-P6 and 0.88 mM for doubly tagged P4-P6 ($\Delta\Delta G° = 0.5$ kcal/mol).

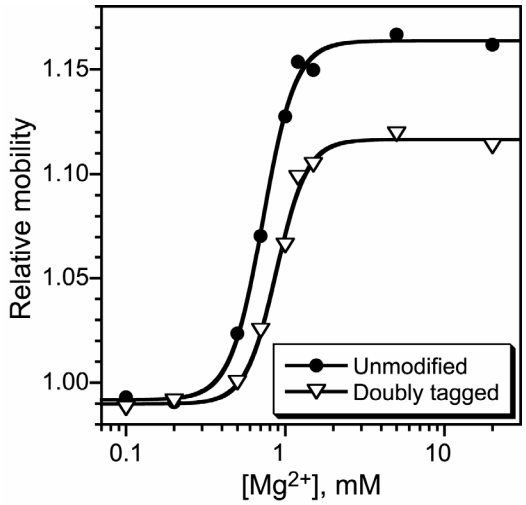

Figure S3. Native PAGE data for unmodified and doubly tagged P4-P6 RNA, showing almost no shift in $[Mg^{2+}]_{1/2}$ due to appending the tags. The slight reduction in the limiting high-Mg^{2+} relative mobility is as expected from our experiments with DNA-modified P4-P6 (in particular, the control experiments in which two noncomplementary DNA strands were attached to P4-P6; see Supporting Information Figure S5 of the reference).[12]

Truncation by a 10–23 deoxyribozyme of the tagging RNA after target labeling

Ligation of a single tagging RNA to the target RNA adds 19 nucleotides to the target. Although appending these single-stranded nucleotides is not anticipated to affect the folding of a large RNA target,[12] as demonstrated directly by native PAGE for P4-P6 (Figure S3), the additional nucleotides could be problematic on certain RNA targets. Therefore, we developed a method for removing some of the added nucleotides using the 10-23 deoxyribozyme,[14] leaving only eight nucleotides of each truncated tagging RNA (Figure S4a).

The truncation reactions were performed with P4-P6 doubly tagged at A231 with fluorescein and at A146 with TAMRA, along with an excess of the 10-23 deoxyribozyme. The ratio of doubly tagged P4-P6 to 10-23 deoxyribozyme was 1:6 (0.1 and 0.6 μM) or 1:600 (0.03 and 18 μM). 5′-^{32}P-Radiolabeled doubly tagged P4-P6 was included in a trace amount in each sample, with the remainder of the RNA as 5′-unradiolabeled (no disruptor oligonucleotides were included). Samples were annealed in 5 mM HEPES (pH 7.5), 15 mM NaCl and 0.1 mM EDTA by heating at 95 °C for 3 min and cooling on ice for 5 min. The reaction buffer was added and the samples were incubated at 37 °C for 2 min, then $MgCl_2$ or $MnCl_2$ was added. The final incubation conditions were 50 mM HEPES (pH 7.5), 150 mM NaCl, and either 10 mM $MgCl_2$, 40 mM $MgCl_2$, 5 mM $MnCl_2$, or 20 mM $MnCl_2$ at 37 °C in a volume of 10 μL. At appropriate timepoints, 1.5 μL was removed from the sample and quenched into 8 μL stop solution. Samples were separated by 6% denaturing PAGE and imaged with a PhosphorImager.

As shown in Figure S4b, the 10-23 deoxyribozyme readily truncates both tagging RNAs when they are attached to P4-P6. The presence of fluorescein and TAMRA on the tags does not inhibit truncation. Increasing the excess of 10-23 from 6-fold to 600-fold had little effect, and both Mg^{2+} and Mn^{2+} were effective. This successful truncation suggests that the tagging strands are freely accessible to the 10-23 deoxyribozyme.

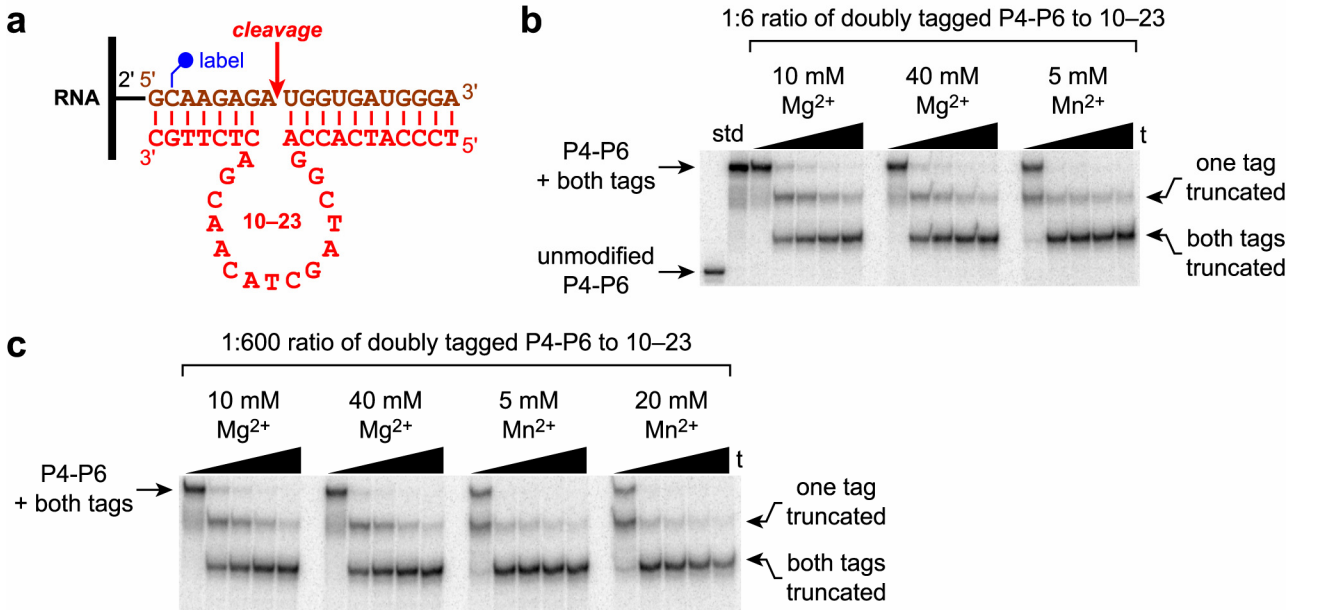

Figure S4. Truncation of the tagging RNA by the 10–23 deoxyribozyme. a) Cleavage reaction. b) Assays using a 6-fold excess of 10–23. c) Assays using a 600-fold excess of 10–23. t = 0, 15, 30, 60, and 120 min. At the final time points, the yields of doubly truncated products were 82%, 86% and 87% for panel b and 86%, 89%, 91%, and 86% for panel c.

Steady-state fluorescence resonance energy transfer (FRET) experiments

The Mg^{2+}-dependent folding of doubly tagged P4-P6 was analyzed by steady-state FRET using a Thermo AB2 spectrometer. The sample temperature was maintained at 25 °C with a recirculating water bath. For all scans, the excitation and emission bandpass settings were 4 nm and 8 nm with a resolution of 1 nm. Three different versions of the P4-P6 sequence were doubly tagged for FRET studies: wild-type P4-P6 (P4-P6-wt), non-foldable P4-P6 (P4-P6-bp), and a P4-P6 mutant with two adenosine nucleotides inserted within the GAAA tetraloop.[11] The latter mutant was previously shown by native PAGE to have a significantly higher $[Mg^{2+}]_{1/2}$ value (by approximately ten-fold) than wild-type P4-P6.[11] Each doubly tagged P4-P6 variant had fluorescein at A231 and TAMRA at A146. We also prepared donor-only control samples with fluorescein at A231 and an unlabeled aminoallyl tag at A146.

For each titration, the initial sample was 14 nM RNA in 1× TB buffer in a volume of 70 μL. During the titration, aliquots of $MgCl_2$ in 1× TB were added to the sample, which was mixed manually in the cuvette by pipetting and re-equilibrated at 25 °C prior to starting the scan. The titrations were performed from 0 to 200 mM Mg^{2+}. For measurements of donor (fluorescein) fluorescence in the presence and absence of acceptor and measurements of acceptor (TAMRA) fluorescence due to FRET, samples were excited at 494 nm and the emission spectra were obtained from 505-650 nm. For measurements of acceptor fluorescence due to direct excitation, samples were excited at 565 nm and the emission spectra were obtained over the range 575-650 nm. The scan rate was set at 4 nm/s to minimize fluorescein photobleaching, which we estimate to be <2% during the course of a complete FRET experiment. All spectra were corrected for dilution due to $MgCl_2$ addition and for buffer background fluorescence. Representative fluorescence spectra for P4-P6-wt are shown in Figure S5.

The FRET efficiency (E_{FRET}) was determined by the $(ratio)_A$ method.[15] The spectrum of the donor-only P4-P6 was normalized to the donor emission peak (521 nm) of the doubly tagged P4-P6 spectrum. The normalized donor-only spectrum was then subtracted from the doubly tagged P4-P6 spectrum, providing the extracted acceptor spectrum. The extracted acceptor spectrum, which is a measure of the fluorescence (F) of the acceptor from excitation at $\nu' = 494$ nm via both direct excitation and energy transfer with emission at $\nu_1 = 575$-650 nm, was then divided by the acceptor spectrum from excitation at $\nu'' = 565$ nm with emission at $\nu_2 = 575$-650 nm to give $(ratio)_A$ as follows:

$$(\text{ratio})_A = \frac{F(\nu_1, \nu')}{F(\nu_2, \nu'')}$$

E_{FRET} was then calculated from $(ratio)_A$ as follows:

$$(\text{ratio})_A = \left\{ E_{FRET} d^+ \left[\frac{\varepsilon^D(\nu')}{\varepsilon^A(\nu'')} \right] + \frac{\varepsilon^A(\nu')}{\varepsilon^A(\nu'')} \right\} \frac{\Phi^A(\nu_1)}{\Phi^A(\nu_2)}$$

In our experiments, because the samples are 100% labeled with donor, $d^+ = 1$. Because $\nu_1 = \nu_2$, the final term in the equation is also equal to 1. $\varepsilon^D(\nu')/\varepsilon^A(\nu'')$ was calculated using extinction coefficients of 83,000 cm^{-1} M^{-1} for fluorescein and 91,000 M^{-1} cm^{-1} for TAMRA (values according to IDT). $\varepsilon^A(\nu')/\varepsilon^A(\nu'')$ was determined from the excitation spectrum of P4-P6 labeled with TAMRA at A146 and an aminoallyl tag at A231 (i.e., acceptor-only sample). The reported E_{FRET} values are the average of two Mg^{2+} titrations. The data were fit in a similar fashion as the native PAGE data by using the equation $(E_{FRET})_{obs} = ((E_{FRET})_{low} + (E_{FRET})_{high} \bullet K \bullet [Mg^{2+}]^n)/(1 + K \bullet [Mg^{2+}]^n)$, where $(E_{FRET})_{obs}$ is the observed E_{FRET} as a function of $[Mg^{2+}]$; $(E_{FRET})_{low}$ and $(E_{FRET})_{high}$ are the limiting low and high values of E_{FRET}; and K, n and $[Mg^{2+}]_{1/2}$ are defined as described.[2] From the curve fits in Figure 3, the $[Mg^{2+}]_{1/2}$ values for P4-P6-wt, P4-P6 tetraloop mutant, and P4-P6-bp were 1.57 mM, 5.5 mM, and 33 mM, respectively.

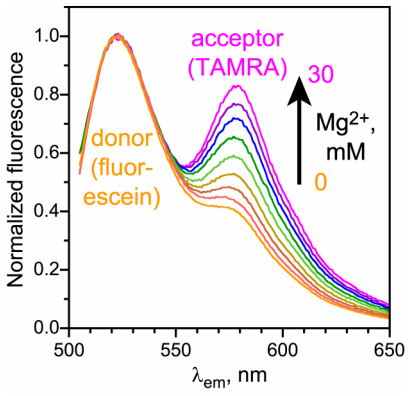

Figure S5. Representative fluorescence spectra for doubly tagged P4-P6-wt.

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