DNA-catalyzed sequence-specific hydrolysis of DNA

Madhavaiah Chandra, Amit Sachdeva, and Scott K. Silverman*

Department of Chemistry, University of Illinois at Urbana-Champaign, 600 South Mathews Avenue, Urbana, Illinois 61801

Supplementary Methods

Substrates and in vitro selection. DNA-tripeptide conjugates were synthesized using the protocols described previously[1]. The in vitro selection experiments were performed essentially as described previously[2,3]. The cleavage substrate used during selection had sequence 5′-AAAGTCTCATGTACTT-GHB-Ala-Ser-Ala-ATGTTCTAGCGCGGA-3′, with embedded tripeptide as shown in **Fig. 1b**. Both this sequence and its all-DNA variant (lacking both the GHB linker and the Ala-Ser-Ala tripeptide) were used as appropriate in cleavage assays. The substrates were purified by denaturing 20% PAGE. Due to the Zn^{2+} concentration sensitivity discovered for 10MD5, all substrates used in kinetic assays were extracted from gels using TN buffer (10 mM Tris, pH 8.0, 300 mM NaCl) lacking EDTA and precipitated with ethanol.

Single-turnover cleavage assays. For single-turnover analytical-scale cleavage of the DNA substrate, the 5′-^{32}P-radiolabeled substrate (S) was the limiting reagent relative to the deoxyribozyme (E). A 10 μL sample containing 0.4 pmol of S and 20 pmol of E was annealed in 5 mM buffer, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min, cooling on ice for 5 min, and heating at 37 °C for 2 min (buffer was HEPES or Tris as appropriate). The cleavage reaction was initiated by addition of stock solutions to a final volume of 20 μL containing 70 mM buffer, pH 7.5, 150 mM NaCl, 20 mM $MnCl_2$, and 1 mM $ZnCl_2$ (defined as the standard incubation conditions when the sample was incubated at 37 °C). Final concentrations used for all single-turnover experiments were 20 nM S and 1 μM E. The metal Mn^{2+} was added from a 10× stock solution containing 200 mM $MnCl^2$. The metal Zn^{2+} was added from a 10× stock solution containing 10 mM $ZnCl_2$, 20 mM HNO_3, and 200 mM buffer at pH 7.5; this stock solution was freshly prepared from a 100× stock of 100 mM $ZnCl_2$ in 200 mM HNO_3. The metal ion stocks were added last to the final sample, which was divided into 2 μL aliquots that were all incubated at 37 °C. At appropriate times, aliquots were quenched with 5 μL of stop solution (80% formamide, 1× TBE [89 mM each Tris and boric acid and 2 mM EDTA, pH 8.3], 50 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol). Samples were separated by 20% PAGE and quantified with a PhosphorImager. Values of k_{obs} and final yield were obtained by fitting the yield versus time data directly to first-order kinetics; i.e., yield = $Y(1 - e^{-kt})$, where $k = k_{obs}$ and Y = final yield. When k_{obs} was sufficiently small that an exponential fit was not meaningful, the initial points were fit to a straight line, and k_{obs} was taken as the slope of the line.

A substantial excess of deoxyribozyme relative to substrate should be used to ensure maximal single-turnover cleavage activity. For assays of 10MD5 as shown in **Fig. 2a**, similar rates and cleavage yields were observed when the deoxyribozyme concentration was 0.5, 1, or 2 μM (i.e., 25×, 50×, or 100× relative to substrate). However, when the deoxyribozyme concentration was only 0.1 or 0.2 μM (5× or 10× relative to substrate), much lower cleavage yields were observed. When the Na^+ concentration was increased to 1 M (from 150 mM in the standard incubation conditions), higher cleavage yields were observed with the more modest excesses of deoxyribozyme relative to substrate.

Multiple-turnover cleavage assays. For multiple-turnover cleavage assays (**Supplementary Fig. 15**), the procedure was similar to that used for the single-turnover assays. In some multiple-turnover assays, 5′-^{32}P-radiolabeled substrate (S) was used. Alternatively, a DNA substrate of identical sequence

Nature Chemical Biology: doi:10.1038/nchembio.201

but without a 5′-phosphate was prepared with an internal ^{32}P-radiolabel by ligation using T4 DNA ligase. The deoxyribozyme (E) was present in an amount equal to 0.5–10% of S (0.1–2 pmol of E and 20 pmol of S, of which 0.4 pmol was ^{32}P-radiolabeled). In initial assays (not shown), the binding arm lengths were optimized for multiple turnover by testing a wide range of lengths on both sides of the cleavage site, including asymmetrical binding arms. These assays were performed with E present as 10% of S. The specific binding arm lengths that led to the greatest number of turnovers by 10MD5 are depicted in **Supplementary Fig. 15** for each of the two tested substrate variants. The multiple-turnover assays were performed in the standard incubation conditions also including 40 mM Mg^{2+}. Turnover was also observed in the absence of Mg^{2+}, but the optimal binding arm lengths were slightly different than in the presence of Mg^{2+}.

Metal ion sources. In most experiments, Mn^{2+} was in the form of $MnCl_2$•$4H_2O$ (Aldrich), 98+% ACS reagent grade [≤0.005% Zn, ≤5 ppm Fe, and ≤5 ppm heavy metals (as Pb)], and Zn^{2+} was in the form of $ZnCl_2$ (Alfa Aesar), 99.999%. To test whether trace impurities in the Mn^{2+} contributed to the observed catalytic activity, the 10MD5 deoxyribozyme was assayed under both single-turnover and multiple-turnover conditions (as in **Fig. 2a** and **Supplementary Fig. 15**, respectively) using Mn^{2+} in the form of $MnCl_2$•$4H_2O$ (Alfa Aesar), 99.999%. Under both conditions, the observed catalytic activity was essentially identical to that observed with the 98+% $MnCl_2$•$4H_2O$ (e.g., <10% difference in k_{obs} and <2% difference in cleavage yield for the single-turnover experiment; data not shown). These results demonstrate that any trace impurities in the Mn^{2+} do not contribute significantly to the catalytic activity of 10MD5.

Larger-scale substrate cleavage. Larger-scale cleavage of the substrate provided the products for subsequent MALDI mass spectrometry (**Supplementary Fig. 2** and **Supplementary Table 2**) as well as the biochemical ligation assays (**Supplementary Fig. 4**). A 20 μL sample containing 500 pmol of S and 750 pmol of E was annealed in 5 mM HEPES, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min, cooling on ice for 5 min, and heating at 37 °C for 2 min. The cleavage reaction was initiated by addition of stock solutions to a final volume of 50 μL containing 70 mM buffer, pH 7.5, 150 mM NaCl, 20 mM $MnCl_2$, and 1 mM $ZnCl_2$ (buffer was HEPES for 10MD5 and Tris for the other deoxyribozymes). Metal ion stocks were prepared as for the analytical-scale assays. The metal ion stocks were added last to the final sample, which was incubated at 37 °C. After 12 h, the reaction was quenched with 10 μL of 0.5 M EDTA. The nucleic acids were precipitated by adding 100 μL of water, 10 μL of 3 M NaCl, and 330 μL of ethanol and dissolved in 20 μL water. A portion of the crude product was used directly for MALDI mass spectrometry analysis. The remaining portion was purified by 20% PAGE for use in the biochemical ligation assays.

For the ^{18}O incorporation experiment using ^{18}O-water (**Supplementary Fig. 13**), the procedure was modified to ensure maximal removal of ^{16}O-water. The natural-abundance (^{16}O) water control experiment was performed the same way in parallel. The DNA substrate (500 pmol), 10MD5 deoxyribozyme (750 pmol), and appropriate buffer components were evaporated from ^{18}O-water three times (20 μL, 15 μL, and 15 μL) using a SpeedVac, then redissolved in 25 μL of ^{18}O-water. The sample, which at this point contained 100 mM HEPES, pH 7.5, 300 mM NaCl, and 0.1 mM EDTA, was annealed by heating at 95 °C for 3 min, cooling on ice for 5 min, and heating at 37 °C for 2 min. Separately, portions of $MnCl_2$ and $ZnCl_2$ (the latter freshly dissolved for this experiment in ^{16}O-water without HNO_3 or HEPES) were combined and evaporated from ^{18}O-water three times as above, then redissolved in 25 μL of ^{18}O-water. The two 25 μL samples were combined and incubated at 37 °C for 12 h, then separated by 20% PAGE.

Biochemical ligation assays of cleaved products. The biochemical ligation assays (**Supplementary Fig. 4**) were performed using left-hand (L) or right-hand (R) product PAGE-purified as described above. The L or R product was additionally treated with an appropriate combination of PNK and CIP as described in **Supplementary Fig. 4**. For assays of L, the L product was 5′-^{32}P-radiolabeled and tested with an unradiolabeled R oligonucleotide prepared by solid-phase synthesis. For assays of R, the unradiolabeled R product was tested with a 5′-^{32}P-radiolabeled L oligonucleotide prepared by solid-phase synthesis. When included, the 33-mer DNA splint was fully complementary to L+R. A 5 μL sample containing 1.0 pmol of L, 1.5 pmol of DNA splint, and 2.0 pmol of R was annealed in 5 mM Tris, 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. To this sample was added 3.0 μL of water, 1.0 μL of 10× T4 DNA ligase buffer (1× is 40 mM Tris, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, pH 7.8 at 25 °C), and 1.0 μL of T4 DNA ligase (1 U/μL, Fermentas), and the 10 μL sample was incubated at 37 °C. Aliquots of 1 μL were removed, quenched onto 5 μL of stop solution, separated by 20% PAGE, and quantified with a PhosphorImager.

References for Supplementary Methods

1. Pradeepkumar, P.I., Höbartner, C., Baum, D.A. & Silverman, S.K. DNA-Catalyzed Formation of Nucleopeptide Linkages. *Angew. Chem. Int. Ed.* **47**, 1753-1757 (2008).
2. Flynn-Charlebois, A. et al. Deoxyribozymes with 2′-5′ RNA Ligase Activity. *J. Am. Chem. Soc.* **125**, 2444-2454 (2003).
3. Kost, D.M., Gerdt, J.P., Pradeepkumar, P.I. & Silverman, S.K. Controlling regioselectivity and site-selectivity in RNA ligation by Zn^{2+}-dependent deoxyribozymes that use 2′,3′-cyclic phosphate RNA substrates. *Org. Biomol. Chem.* **6**, 4391-4398 (2008).

deoxyribozyme	enzyme region sequence
10MD1	5′-CCAGCGTCAACTTGTCCACGATTTGTGATAGGTTCAAGTT-3′
10MD4	5′-AGTGCACACGTCAGTCTTTTGTGATATTTTCAGCGTATCA-3′
10MD5	5′-CGCTAGATAAGTGGGTGCGTTTGCTATAGCTGTCCCTCAA-3′
10MD9	5′-CCCACTCGAGCTCACGGGTTAGACGACCGTCTTTTCCGC -3′
10MD13	5′-TAAGCGTTCGGGGGAACATTCCCTTTGTGATATTGTTCCG-3′
10MD14	5′-GGCCTCGTGCTGGGAGTGCGCGGCAAGACACTAACGCCGA-3′
10MD30	5′-GTGGTTGCGTTCGGTCTGGTAAGAACGGTATGGCCCGAT -3′
10MD36	5′-GTGGTTGCGTTCAAATGTTGGAGGAACATTTAGGCCCGAT-3′
10MD41	5′-GTGGTTGGTTGGCTCATATTAGATGCCTTACGGGCCCGAT-3′

Supplementary Table 1. Sequences of the DNA-cleaving deoxyribozymes. Only the enzyme region sequences are shown. For single-turnover experiments, the sequence to the 5′-side of the enzyme region was CCGCGCTAGAACAT, and the sequence to the 3′-side of the enzyme region was AGTACATGAGACTT. For 10MD5, these flanking sequences are fully complementary to the DNA substrate nucleotides as depicted in **Fig. 2b**. The requirement for base pairing with the recognition site (ATG^T) has not been established, but providing deoxyribozyme nucleotides that can form these base pairs does support high cleavage activity.

deoxyribozyme	substrate	mass L calcd.	mass L found	L error, % (found – calcd.)	mass R calcd.	mass R found	R error, % (found – calcd.)
10MD5	**D**	6418.9	6420.9	+0.03	3741.3	3742.6	+0.03
10MD5	**P**	6420.8	6421.6	+0.01	3741.3	3741.7	+0.01
10MD9 [a]	**P**	4934.9	4935.5	+0.01	5227.4	5227.3	0.00
					5141.3	5141.0	–0.01
10MD30 [a]	**P**	4934.9	4935.6	+0.01	5227.4	5227.2	0.00
					5141.3	5141.0	–0.01
10MD36 [a]	**P**	4934.9	4935.4	+0.01	5227.4	5227.2	0.00
					5141.3	5140.5	–0.02
10MD41 [a]	**P**	4934.9	4935.1	0.00	5227.4	5227.1	–0.01
					5141.3	5140.7	–0.01
10MD1	**P**	5473.5	5473.6	0.00	4687.8	4687.9	0.00
10MD4	**P**	5473.5	5476.1	+0.05	4687.8	4690	+0.05
10MD13	**P**	5473.5	5474.5	+0.02	4687.8	4688.7	+0.02
10MD14	**P**	5551.3	5552.3	+0.02	4607.9	4606.7	–0.03
round 7 pool [b]	**P** (no GHB)	4934.9	4935.2	+0.01	5189.2	5188.8	–0.01

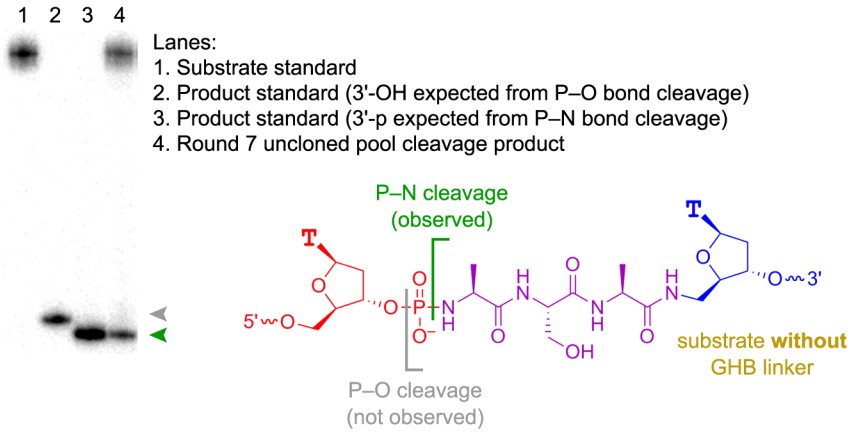
Supplementary Table 2. MALDI mass spectrometry analysis of the cleavage products formed by the nine new deoxyribozymes. L and R respectively denote the left-hand (5′) and right-hand (3′) cleavage products. Substrate **P** = 5′-AAAGTCTCATGTACTT-GHB-Ala-Ser-Ala-ATGTTCTAGCGCGGA-3′, with embedded tripeptide as shown in **Fig. 1b**. Substrate **D** = 5′-AAAGTCTCATGTACTTATATGTTCTAGCGCGGA-3′, which is all DNA; the underlined A replaces the GHB-tripeptide unit in substrate **P**. Cleavage sites for 10MD9, 10MD1, 10MD14, and 10MD5 are marked in **Fig. 1c**. Cleavage sites for 10MD30, 10MD36, and 10MD41 are the same as for 10MD9. Cleavage sites for 10MD4 and 10MD13 are the same as for 10MD1. All MALDI mass spectra were obtained in the mass spectrometry laboratory of the UIUC School of Chemical Sciences.

[a] For 10MD9 and the other three deoxyribozymes that have the same cleavage site, a secondary reaction of the R product is loss of the GHB linker, likely as γ-butyrolactone via lactonization (see **Supplementary Fig. 7**). Another possible secondary reaction is simple amide hydrolysis that removes the GHB, although lactonization seems more likely because no primary amide hydrolysis product is observed. The secondary R product was observed in all mass spectra (second data line for each deoxyribozyme). The primary product before loss of GHB (i.e., 5′-GHB-Ala-Ser-Ala-ATGTTCTAGCGCGGA-3′) was separately synthesized and provided to 10MD9 under the standard incubation conditions, either with or without a DNA strand corresponding to the L product (i.e., 5′-AAAGTCTCATGTACTT-p-3′). Under these conditions no loss of GHB was detected, suggesting that the secondary reaction is catalyzed by 10MD9 only if the deoxyribozyme-substrate complex has proceeded through the primary phosphodiester bond cleavage reaction.

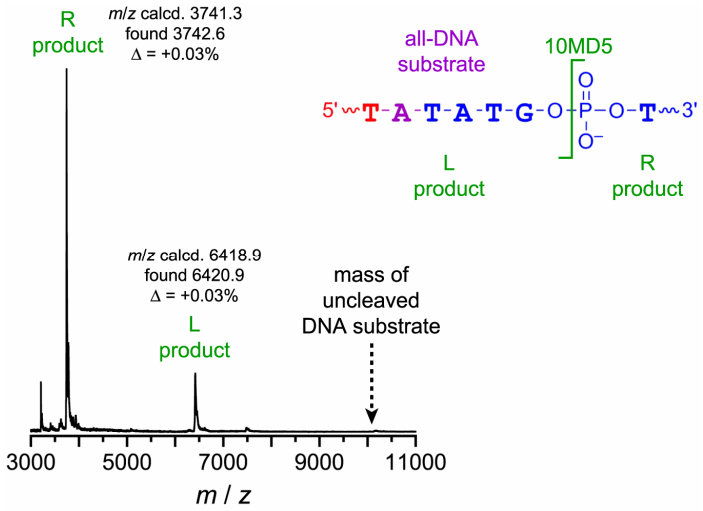
[b] The final line in the table shows the cleavage products from the uncloned round 7 pool, obtained during in vitro selection using substrate **P** that lacks the GHB linkage during each round (see **Supplementary Fig. 1**).

deoxyribozyme	k_{obs} , h^{-1} (substrate **D**)	k_{obs} , h^{-1} (substrate **P**)
10MD9	$<3 \times 10^{-4}$	0.26
10MD1	$<3 \times 10^{-4}$	0.14
10MD14	3.6×10^{-3}	0.14
10MD5	2.3	2.0

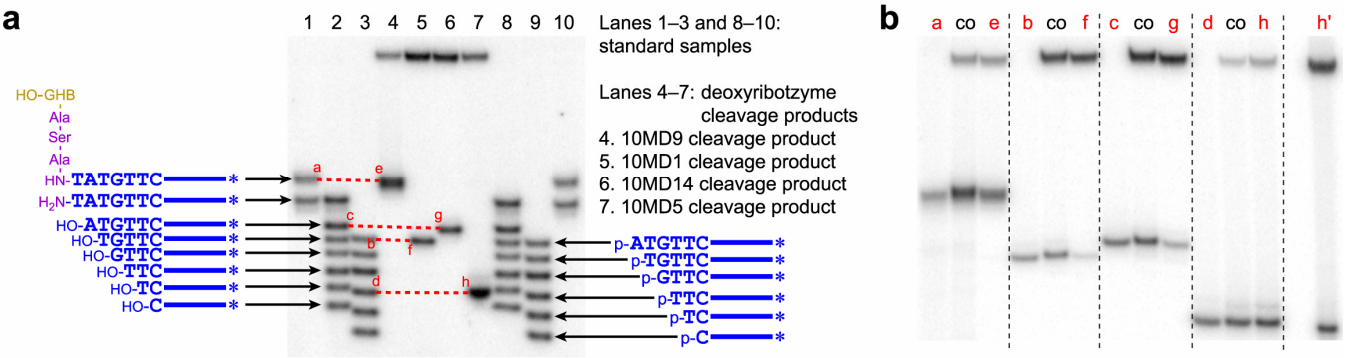
Supplementary Table 3. k_{obs} values for the 10MD9, 10MD1, 10MD14, and 10MD5 deoxyribozymes. Data from **Fig. 2a**. obtained under the standard incubation conditions. Substrate **D** = 5′-AAAGTCTCATGTACTTATATGTTCTAGCGCGGA-3′ (all-DNA; the underlined A replaces the GHB-tripeptide unit in substrate **P**). Substrate **P** = 5′-AAAGTCTCATGTACTT-GHB-Ala-Ser-Ala-ATGTTCTAGCGCGGA-3′, with embedded tripeptide as shown in **Fig. 1b**. For 10MD5, several experiments analogous to that shown in Fig. 2a led with substrate **D** to k_{obs} of 2.7 ± 0.3 h^{-1} and final cleavage yield of 66 ± 4% (n = 11; average ± standard deviation).



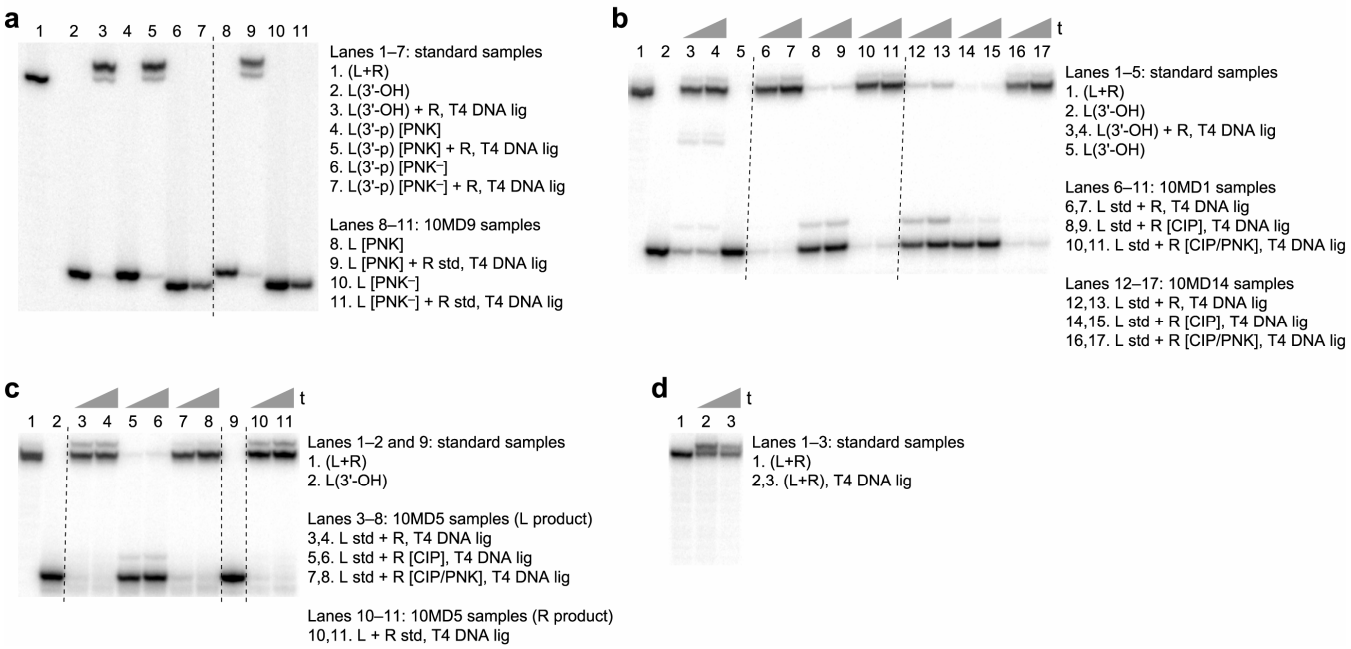
Supplementary Fig. 1. Data from a parallel selection experiment in which the GHB linker was omitted from the substrate, and the N-terminal Ala was connected directly to the 3′-phosphorus of the DNA binding arm via a phosphoramidate (P–N) bond. The emergent deoxyribozymes cleaved only this phosphoramidate linkage, rather than any amide or phosphodiester bond of the substrate. This outcome is sensible because the phosphoramidate bond is expected to be the most labile linkage within the substrate [Wada, T., Moriguchi, T. & Sekine, M. *J. Am. Chem. Soc.* **116**, 9901-9911 (1994)]. See last line of **Supplementary Table 2** for MALDI mass spectrometry data for the two cleavage fragments.



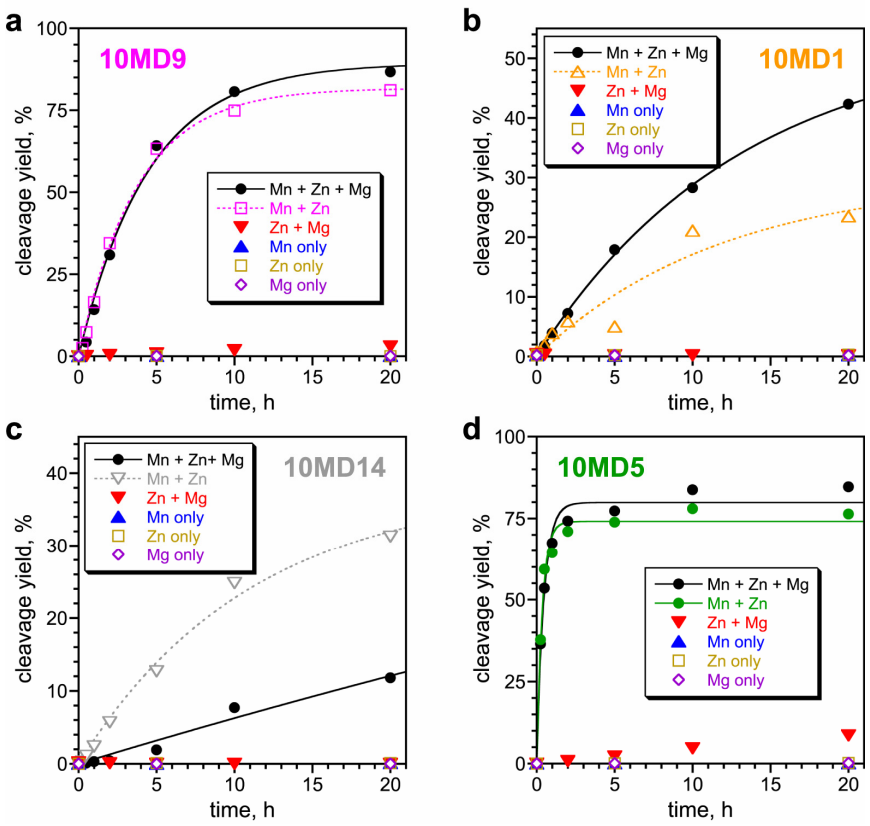
Supplementary Fig. 2. MALDI mass spectrometry of the 10MD5 deoxyribozyme cleavage products, using an all-DNA substrate. Mass spectrometry data for the cleavage products of all new deoxyribozymes are collected in **Supplementary Table 2**. See **Supplementary Methods** for experimental procedure.



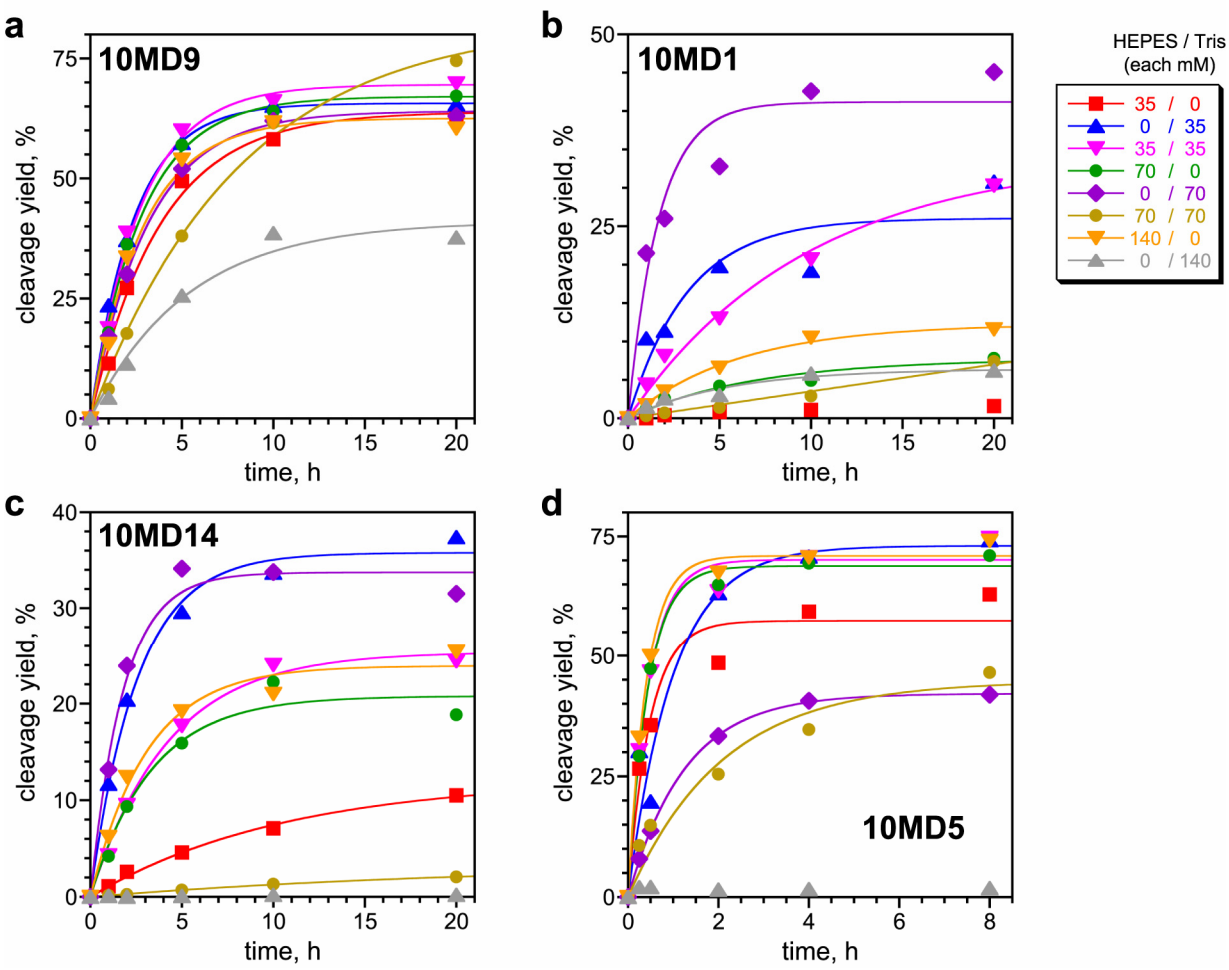
Supplementary Fig. 3. Comparisons with PAGE standards to assign cleavage-site locations within the substrate for the 10MD9, 10MD1, 10MD14, and 10MD5 deoxyribozymes. In all cases, the DNA substrate had the embedded tripeptide and was 3′-^{32}P-radiolabeled (α-^{32}P-dCTP and terminal transferase, NEB; purified twice on 20% PAGE to improve homogeneity of the labeled substrate). (**a**) The single-turnover analytical-scale assay protocol from **Supplementary Methods** was followed. Samples for loading were denatured by heating at 95 °C for 3 min in the presence of 100 pmol of unradiolabeled substrate to ensure displacement of the deoxyribozyme from the cleaved products. Gel migration standards were prepared by solid-phase synthesis, 5′-phosphorylated with unradiolabeled ATP and T4 PNK if required, and 3′-^{32}P-radiolabeled. On the gel image, horizontal red dashed lines connect each deoxyribozyme's cleavage product to the appropriate standard band. Bands marked a–h were used in panel **b**. (**b**) Validation of the equivalence of the indicated bands of panel **a**. The bands marked a–h from panel **a** were electrophoresed as shown, demonstrating equivalence of migration rate between each cleavage product band (e, f, g, or h) and the corresponding standard band (a, b, c, or d). Each pair of bands surrounds a lane marked "co" in which the two individual samples were co-loaded in equal amounts. The lane marked h′ is equivalent to the lane marked h except that in lane h′ the DNA substrate was terminated with three ribonucleotides (3′-rGGA), which allowed the substrate to be homogeneously 3′-^{32}P-radiolabeled using ^{32}P-pCp and T4 RNA ligase. As shown in lane h′, cleavage of this substrate by 10MD5 clearly leads to just one product band.



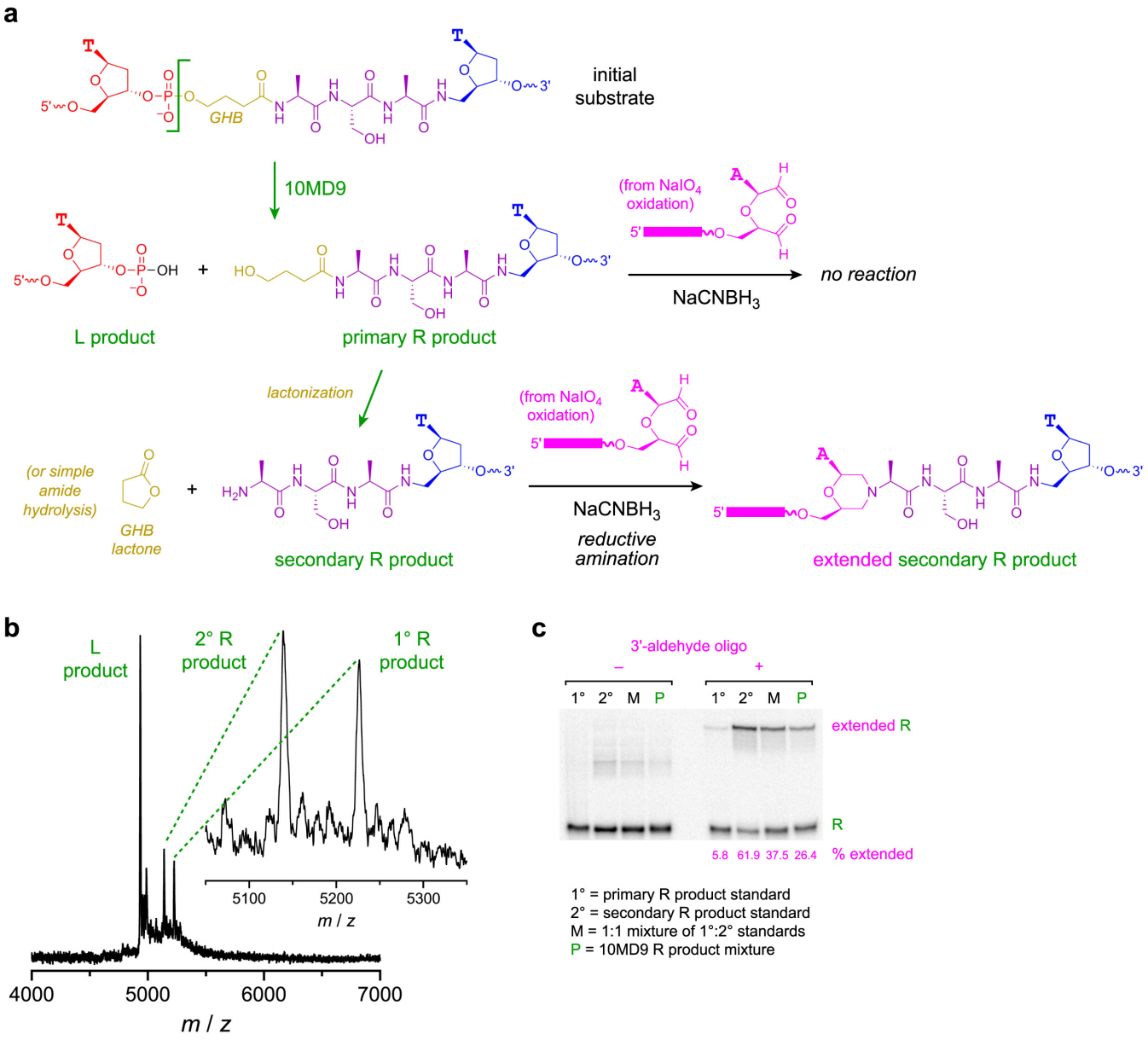
Supplementary Fig. 4. Biochemical ligation assays with T4 DNA ligase to assign cleavage-site termini for the 10MD9, 10MD1, 10MD14, and 10MD5 deoxyribozymes. In all captions, L denotes the left-hand deoxyribozyme product (or standard DNA oligonucleotide); R denotes the right-hand deoxyribozyme product (or standard DNA oligonucleotide); [PNK] denotes treatment with T4 PNK (Fermentas) in the absence of ATP to remove a 3′-phosphate; [PNK⁻] denotes use of T4 PNK devoid of 3′-phosphatase activity (NEB); "T4 DNA lig" denotes splint ligation using a DNA oligonucleotide splint complementary to L+R and T4 DNA ligase; [CIP] denotes treatment with CIP to remove 5′-phosphate; [CIP/PNK] denotes treatment with CIP to remove 5′-phosphate followed by treatment with T4 PNK and ATP to restore 5′-phosphate. Timepoints at 0.5 and 2 h where indicated. See **Supplementary Methods** for procedures. (**a**) Assay of the left-hand cleavage product from 10MD9. (**b**) Assay of the right-hand cleavage product from 10MD1 and 10MD14. (**c**) Assay of the left-hand and right-hand cleavage products from 10MD5. (d) Incubation of L+R ligation standard and DNA splint with T4 DNA ligase, showing the appearance of secondary product under these conditions [perhaps related to the findings reported in Kuhn, R. & Frank-Kamenetskii, M. D. *FEBS J.* **272**, 5991-6000 (2005)].



Supplementary Fig. 5. Analysis of Mn^{2+}, Zn^{2+}, and Mg^{2+} requirements for the 10MD9, 10MD1, 10MD14, and 10MD5 deoxyribozymes. Assays were performed under the standard incubation conditions (HEPES for 10MD5; Tris for the other deoxyribozymes), except the indicated ions were included at concentrations of 20 mM Mn^{2+}, 1 mM Zn^{2+}, and 40 mM Mg^{2+} as appropriate. For 10MD9, 10MD1, and 10MD14, the substrate contained the Ala-Ser-Ala tripeptide. For 10MD5, the substrate was entirely DNA.



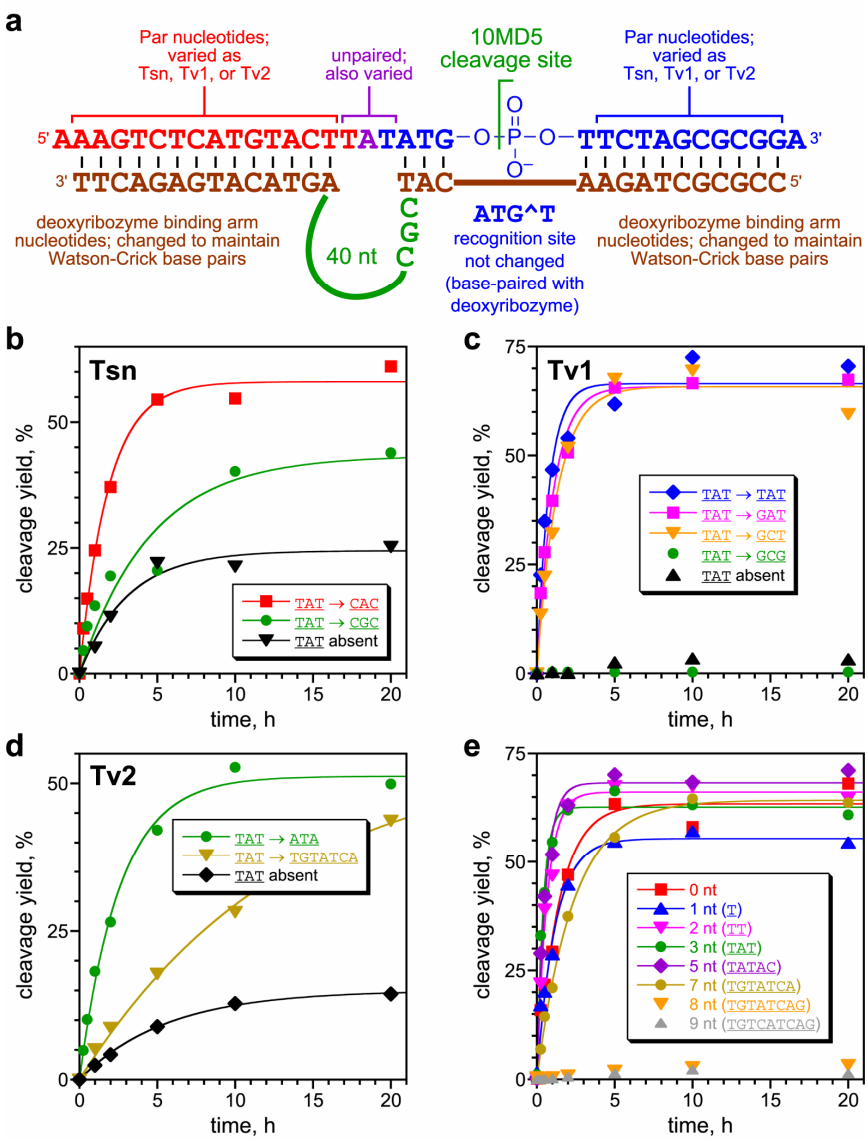
Supplementary Fig. 6. Analysis of Tris and HEPES buffer dependence for the 10MD9, 10MD1, 10MD14, and 10MD5 deoxyribozymes. Assays were performed under the standard incubation conditions, except for the variation of buffer identity and concentration.



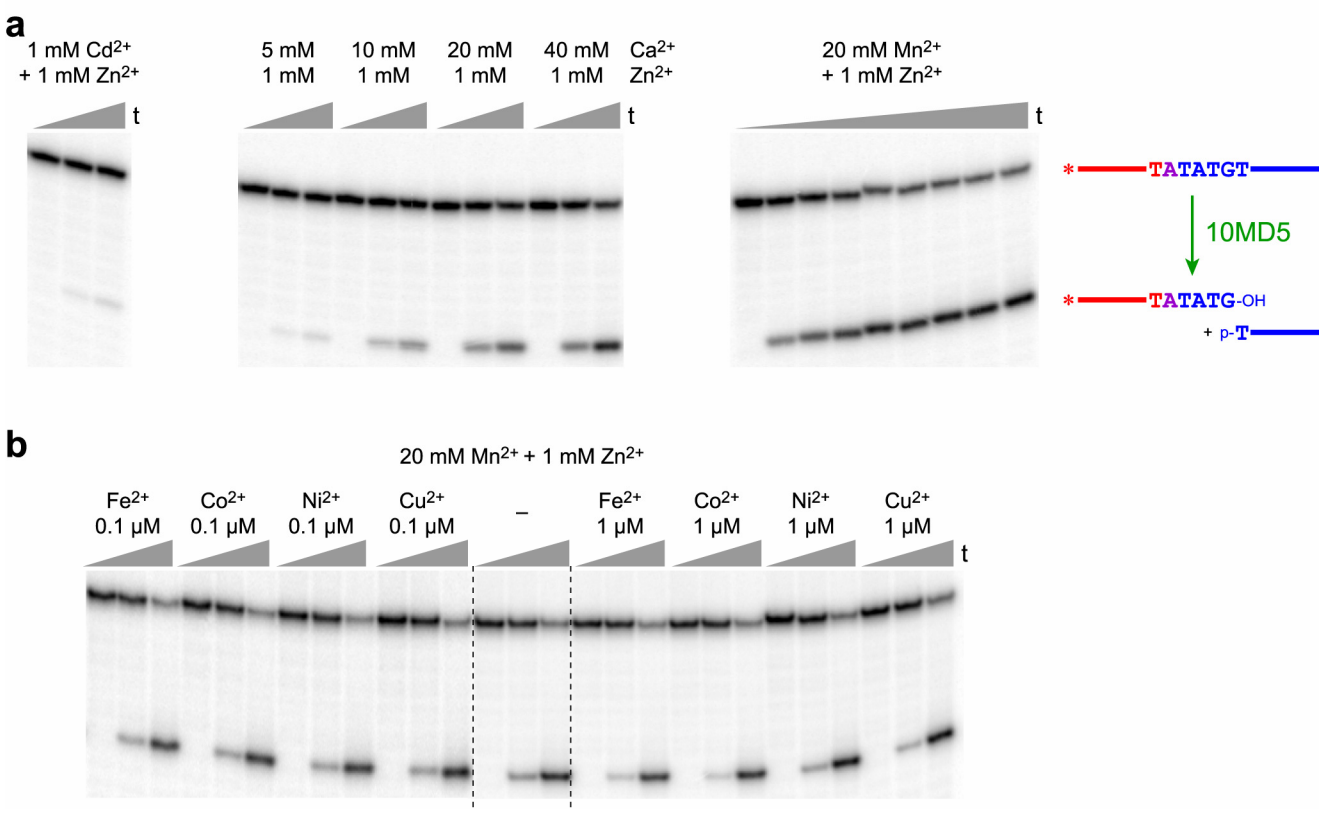
Supplementary Fig. 7. Reductive amination assay to validate the occurrence of the secondary reaction of the primary cleavage product from the 10MD9 deoxyribozyme. (**a**) 10MD9 catalyzes a primary phosphodiester bond cleavage reaction, which results in an R product that includes the GHB linker. This reaction appears to be followed by a secondary reaction, which removes the GHB moiety and provides an R product that has a free N-terminus. The secondary reaction is depicted here as a lactonization, although simple amide hydrolysis is an alternative possibility. In either case, only the secondary R product should be joined efficiently by reductive amination with a 3′-aldehyde DNA oligonucleotide, which is prepared by $NaIO_4$ oxidation of a 3′-rA DNA oligonucleotide. (**b**) MALDI mass spectrometry of the 10MD9 products. A nonradiolabeled sample of substrate **P** (i.e., DNA with embedded GHB-tripeptide; see **Supplementary Table 1**) was incubated with 10MD9. The comigrating mixture of primary and secondary products (see **Supplementary Fig. 3**) was isolated by PAGE (not shown) and analyzed by MALDI-MS, revealing a mixture of the primary and secondary R products. See **Supplementary Table 1** for quantitative mass values. In addition, an ESI mass spectrum of the mixture was acquired as described in **Supplementary Fig. 13**. The mass values were as expected, with much higher precision than available via MALDI-MS. *m/z* for $[M–3H]^{3–}$, with all calculations for the lowest *m/z* isotope combination (i.e., the monoisotopic peak): primary R product calcd. 1740.334, found 1740.31 ± 0.03; secondary R product calcd. 1711.655, found 1711.63 ± 0.03 (ca. 2:1 ratio of primary:secondary peaks). It should be noted that a standard corresponding to the primary R product shows a single, clean

MALDI-MS peak, demonstrating that the secondary product is not formed in uncatalyzed fashion from the primary product under the MALDI conditions. (**c**) Reductive amination reactions of the R products. The 3′-aldehyde DNA oligonucleotide was prepared by oxidation of 3′-rA DNA with 10 mM $NaIO_4$ in 100 mM HEPES, pH 7.5 at 25 °C for 1 h and purified by ethanol precipitation. The sample from panel **b** was 3′-^{32}P-radiolabeled (α-^{32}P-dCTP and terminal transferase, NEB) and incubated in 100 mM sodium acetate, pH 5.2, at 37 °C for 12 h with 10 mM $NaCNBH_3$, 50 mM $NiCl_2$, the 3′-aldehyde DNA, and a DNA splint complementary to the R product and the 3′-aldehyde DNA. In parallel, a 1:1 mixture of standards corresponding to the primary and secondary products was treated in the same way. The observed reductive amination yields indicate a ca. 2:1 ratio of primary:secondary R products in the 10MD9 product mixture. Note that the primary R product, although lacking an aliphatic amine, still has detectable reductive amination yield. This is likely due to slow reactivity of 3′-aldehyde DNA with exocyclic amines on the nucleobases, analogous to slow reactivity of *N*-hydroxysuccinimide (NHS) esters with unmodified DNA/RNA.

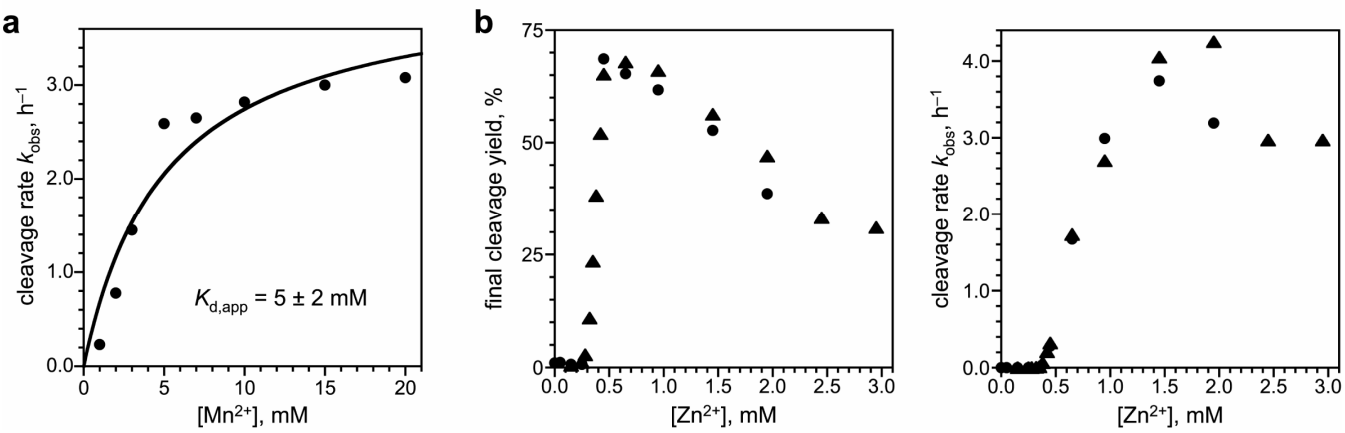
Nature Chemical Biology: doi:10.1038/nchembio.201



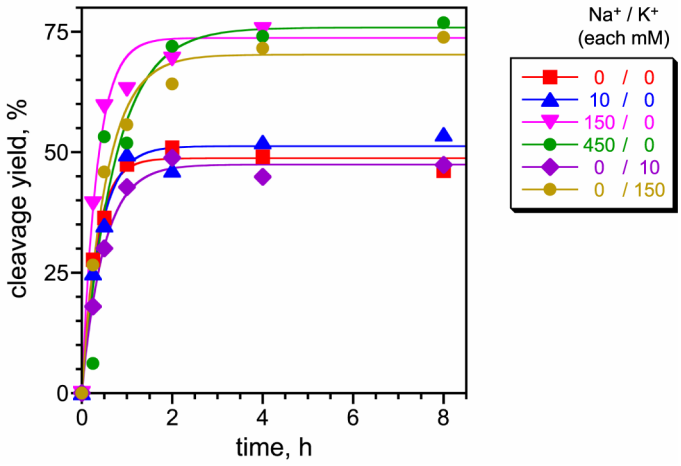
Supplementary Fig. 8. Assaying 10MD5 for its DNA substrate sequence requirements. Assays were performed under the standard incubation conditions (HEPES). (**a**) Depiction of the assays. See **Supplementary Table 1** for the complete 40 nt of the 10MD5 enzyme region. From the data in the other panels, the two key conclusions are that (i) all DNA nucleotides outside of the recognition-site ATG^T can be changed while retaining high cleavage activity, as long as the 10MD5 binding arm nucleotides are changed to maintain Watson-Crick base pairing; and (ii) the unpaired TAT nucleotides may be removed or extended while retaining significant cleavage activity, but optimal generality for the surrounding sequences requires three unpaired nucleotides at these positions. In the assays, substrate nucleotides except ATG^T were changed, with corresponding changes to the 10MD5 binding arms to maintain Watson-Crick base pairing. (**b**) Nucleotide changes by transitions (abbreviated Tsn; A↔G and T↔C). The TAT nucleotides (mutated by Tsn to CGC) were changed or absent as noted. The Tsn substrate variant with TAT changed to CAC (i.e., retaining the middle A from parent TAT) was tested because the first two nucleotides of CGC could base-pair deleteriously with the first two nucleotides (CG) of the 10MD5 40 nt enzyme region. The higher activity for the CAC variant supports the occurrence of this deleterious base-pairing. (**c**) Nucleotide changes by transversions of type 1 (abbreviated Tv1; A↔C and G↔T). The TAT nucleotides (mutated by Tv1 to GCG) were unchanged or absent as noted. In addition, the GCT variant was tested (i.e., not changing the final T), because the three GCG nucleotides could base-pair deleteriously with the first three nucleotides (CGC) of the 10MD5 40 nt enzyme region. (**d**) Nucleotide changes by transversions of type 2 (abbreviated Tv2; A↔T and G↔C). (**e**) Changes to the length of the unpaired TAT nucleotides. The legend shows the sequence of the unpaired nucleotides.



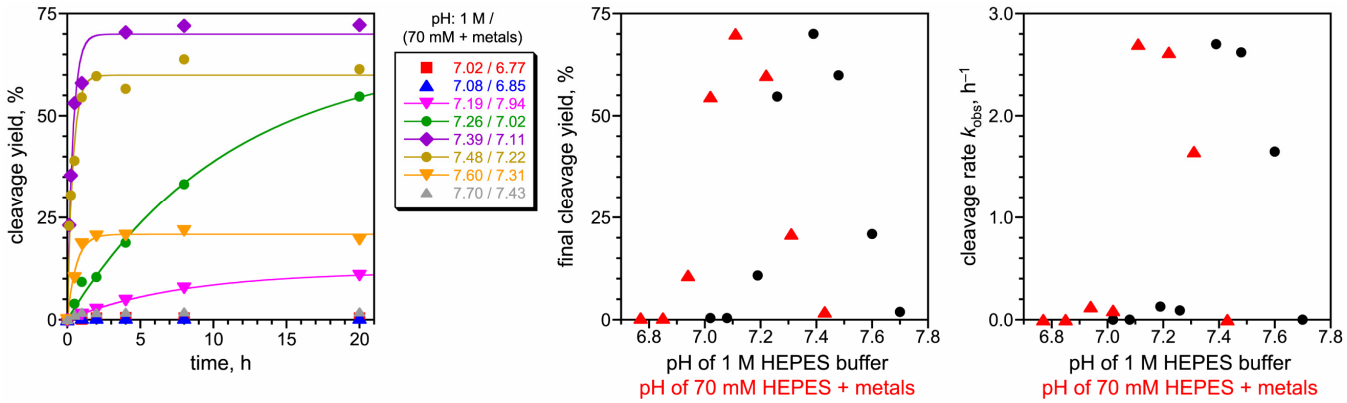
Supplementary Fig. 9. Analysis of 10MD5 with divalent metal ions other than Mn^{2+} and Zn^{2+}. The standard incubation conditions are 70 mM HEPES, pH 7.5, 20 mM $MnCl_2$, 1 mM $ZnCl_2$, and 150 mM NaCl at 37 °C. (**a**) Each of Mn^{2+} and Zn^{2+} was separately replaced with one of Ca^{2+}, Cd^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, or Cu^{2+}. Ca^{2+} was tested at 5 mM, 10 mM, 20 mM, or 40 mM, whereas each of the other ions was tested at 0.1 µM, 1 µM, 10 µM, 100 µM, 1 mM, or 10 mM. All of the assays showed no detectable cleavage activity (<0.5%) in 20 h, except for replacement of Mn^{2+} with either Ca^{2+} or Cd^{2+} (at 1 mM or 10 mM Fe^{2+}, substantial nonspecific DNA degradation was observed) Shown here are the PAGE images from assays with Ca^{2+} or Cd^{2+} in which cleavage activity was observed. At 40 mM Ca^{2+}, the cleavage yield was 24% in 5 h, which is ~0.048 h^{-1} (~2.4% of 2 h^{-1}, or ~40× slower than with 20 mM Mn^{2+}). With Cd^{2+} at 1 mM, the cleavage yield was ~2% in 20 h, which is ~0.001 h^{-1} (~5 × 10^{-4} of 2 h^{-1}). Timepoints: Cd^{2+} or Ca^{2+} with Zn^{2+}, 0, 5, and 20 h; Mn^{2+} with Zn^{2+}, 0, 8, 15, 30 min; 1, 2, 5, 10, and 20 h. (**b**) Assays in which the standard incubation conditions (including both Mn^{2+} and Zn^{2+}) were supplemented with either 0.1 µM or 1 µM of Fe^{2+}, Co^{2+}, Ni^{2+}, or Cu^{2+}. In all cases, the results of the cleavage assay were indistinguishable from the results observed in the absence of the added metal ion. The samples of $MnCl_2$ and $ZnCl_2$ were both 99.999%. In the case of 20 mM Mn^{2+}, this means that the maximal total impurity was 0.001% of 20 mM, or 0.2 µM, which would not necessarily be contributed by only one chemical compound. Timepoints: 0, 8 min, 4 h.



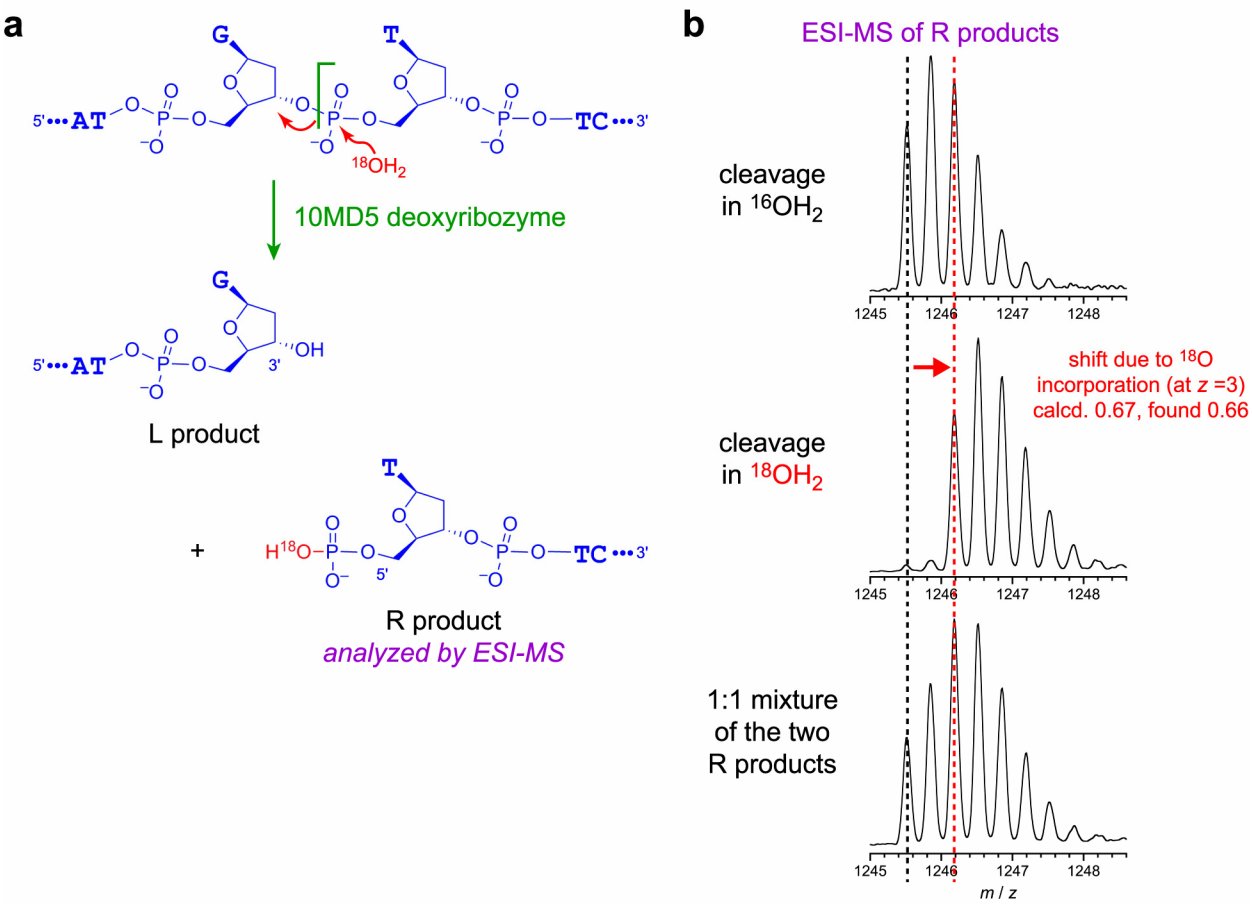
Supplementary Fig. 10. Determining $K_{d,app}$ for Mn^{2+} and the optimal Zn^{2+} concentration for the 10MD5 deoxyribozyme. (**a**) $K_{d,app}$ for Mn^{2+}. The k_{obs} value was determined under the standard incubation conditions, except for variation of the Mn^{2+} concentration. Above 20 mM Mn^{2+}, suppression of cleavage activity was observed. The cleavage yield was 72–77% for all Mn^{2+} concentrations between 1–15 mM but 68% at 20 mM, 59% at 30 mM, 43% at 40 mM, 5% at 80 mM, and <2% at 160 mM Mn^{2+}. (**b**) Zn^{2+} concentration dependence. The [Zn^{2+}] was varied between 0 and 3 mM under otherwise-standard incubation conditions, including 20 mM Mn^{2+} and fixed HEPES concentration of 70 mM. Final cleavage yields were computed from the first-order kinetic fit except when k_{obs} was <0.1 h^{-1}, in which case the cleavage yield is reported for the 20 h time point. The two symbols represent two different data sets. The Zn^{2+} concentrations were corrected for the small amount of EDTA in the annealing buffer, which chelates 0.05 mM of the Zn^{2+} in the final sample.



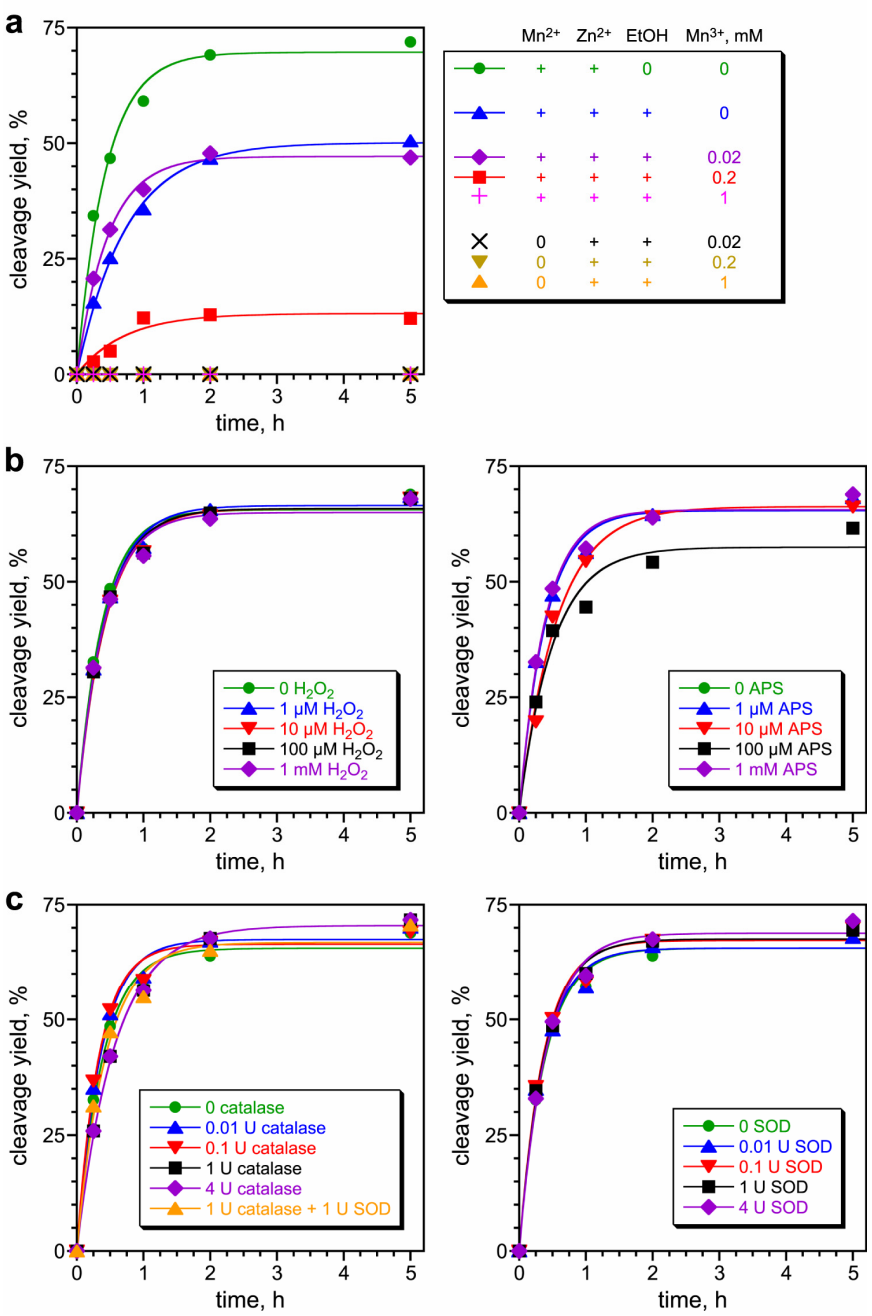
Supplementary Fig. 11. Analysis of 10MD5 for its monovalent ion (Na^+ and K^+) requirements. Assays were performed under the standard incubation conditions (HEPES), except for the variation of NaCl/KCl concentrations.



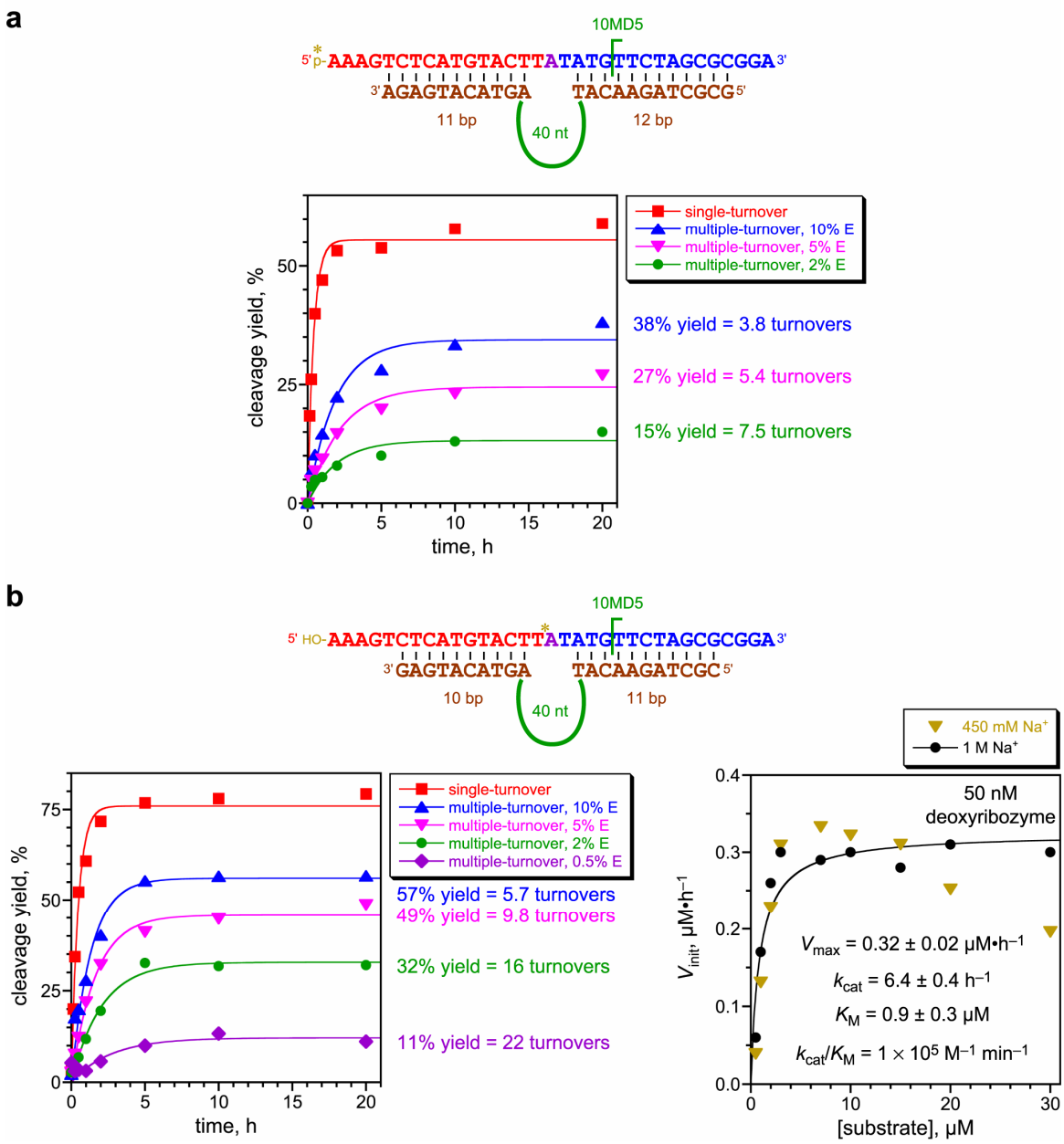
Supplementary Fig. 12. Analysis of 10MD5 for pH dependence, revealing a sharp pH dependence of DNA cleavage activity. Assays were performed under the standard incubation conditions (HEPES), except for the variation of pH. Stock solutions of 1 M HEPES were prepared with measured pH values at room temperature (~23 °C) ranging from 7.02 to 7.70. These 1 M stock solutions were used to prepare the incubation buffers for the DNA cleavage assays. The pH values of these 1 M stock solutions after dilution to 70 mM were measured at room temperature to be 0.12 to 0.20 units lower than at 1 M. The pH values measured after dilution to 70 mM and with inclusion of 20 mM Mn^{2+}, 1 mM Zn^{2+}, and 150 mM NaCl were measured at room temperature to be 0.23 to 0.29 units lower than at 1 M without dilution or inclusion of any of the metal ions. k_{obs} values (top to bottom for the five data sets with curve fits in legend): 0.13, 0.09, 2.7, 2.6, 1.7 h^{-1}. Final cleavage yields were computed from the first-order kinetic fit except when k_{obs} was <0.1 h^{-1}, in which case the cleavage yield is reported for the 20 h time point. The optimal final cleavage yield and k_{obs} values were observed at pH of 7.1–7.2, as measured at 70 mM HEPES with the metal ions present.



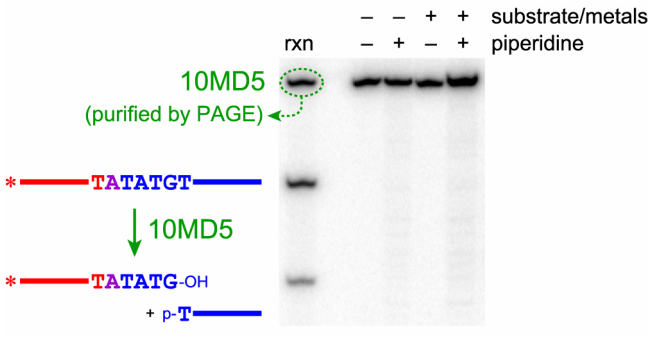
Supplementary Fig. 13. Experimental demonstration that the oxygen atom newly incorporated into the 5′-phosphate group of the 10MD5 cleaved R product originates from H_2O via hydrolytic cleavage and not dissolved O_2 via oxidative cleavage. (**a**) Cleavage reaction depicting incorporation of ^{18}O from ^{18}O-water into the R product. (**b**) The large-scale DNA substrate cleavage procedure was performed either in ^{18}O-water (98% ^{18}O; Olinax) or in natural-abundance (^{16}O) water as described in **Supplementary Methods**. The R products were each isolated by PAGE and analyzed directly by ESI mass spectrometry (Waters Synapt HDMS ESI Q-TOF; samples were desalted using three Millipore ZipTip ZTC18S tips, one tip per 20 pmol of sample, eluted using 1:1 water:acetonitrile; the combined 30 µL elutions were diluted with 10 µL of water and 40 µL of an aqueous solution of 30 mM triethylamine and 800 mM hexafluoroisopropyl alcohol, pH 7.9). The mass spectra clearly reveal that ^{18}O from water is incorporated into the R cleavage product. This finding supports the hydrolytic cleavage pathway illustrated in panel **a** but does not support an oxidative cleavage pathway. The illustrated peaks are for m/z where $z = 3$. Peaks for other values of z also showed the expected shift due to ^{18}O incorporation into the R product. m/z for $[M–3H]^{3–}$, with all calculations for the lowest m/z isotope combination (i.e., the monoisotopic peak): ^{16}O-water calcd. 1245.528, found 1245.52 ± 0.02; ^{18}O-water calcd. 1246.195, found 1246.18 ± 0.02.



Supplementary Fig. 14. Additional single-turnover experiments to validate the lack of participation of various oxidant species in the DNA hydrolysis reaction catalyzed by 10MD5. (**a**) Assays with Mn^{3+}, which could potentially be formed by oxidation of Mn^{2+}. A 20 mM stock solution of $Mn(OAc)_3 \cdot 2H_2O$ in ethanol was prepared. Assays were performed under the standard incubation conditions of 20 mM Mn^{2+} and 1 mM Zn^{2+} supplemented with Mn^{3+} (final ethanol concentration 5% v/v). The assay without any added Mn^{3+} but with 5% ethanol revealed a modest decrease in rate and yield due solely to the ethanol, which is required for Mn^{3+} solubility. Inclusion of Mn^{3+} (0.02, 0.2, or 1 mM) as well as ethanol did not enhance the DNA cleavage activity; in contrast, the higher tested concentrations of Mn^{3+} suppressed the activity. (**b**) Assays with H_2O_2 and $(NH_4)_2S_2O_8$ (ammonium persulfate). No enhancement of DNA cleavage activity (e.g., increase in k_{obs}) was observed for either oxidant at any tested concentration. (**c**) Assays (20 μL) with catalase (Sigma C9322, from bovine liver, 5000 units/mg) and superoxide dismutase (Sigma S7571, from bovine erythrocytes, 3000 units/mg). No suppression of DNA cleavage activity was observed for either enzyme at any tested concentration, or with both enzymes present.



Supplementary Fig. 15. Analysis of 10MD5 for multiple turnover. See **Supplementary Methods** for experimental procedure. Shown are the slightly different optimal deoxyribozyme binding arm lengths for each of the two tested substrate variants (**a**, 5′-^{32}P-radiolabeled; **b**, internally ^{32}P-radiolabeled). These substrates differ only in the location of the ^{32}P radiolabel and the presence or absence of a 5′-phosphate; the latter presumably affects the binding energies and changes the precise lengths that are optimal for achieving efficient turnover. The numbers of turnovers were calculated from the % cleavage at the final (20 h) timepoint. No corrections were made for the <100% yield observed in the corresponding single-turnover experiments; therefore, the indicated numbers of turnovers are lower limits. In panel **b** is shown determination of k_{cat} and K_M for 10MD5. Assays were performed with 50 nM deoxyribozyme under the standard incubation conditions (20 mM Mn^{2+} and 1 mM Zn^{2+}) except including either 450 mM or 1 M Na^+, because nearly complete inhibition of cleavage activity was observed at >10 μM substrate with only 150 mM Na^+. We attribute this to sequestration of Zn^{2+}, noting that 10 μM of a ca. 30-mer substrate is 0.3 mM nucleotide monomers, and the cleavage rate and yield are very sensitive to the Zn^{2+} concentration as shown in **Supplementary Fig. 10**. V_{init} values were determined from linear fits to plots of product concentration versus time (0–2 h). Note that 40 turnovers were observed with 0.5% deoxyribozyme in the experiment of **Supplementary Fig. 16**.



Supplementary Fig. 16. Piperidine treatment of the 10MD5 deoxyribozyme to assay for nonspecific oxidative damage. After multiple-turnover incubation, the deoxyribozyme was separated by PAGE and treated with piperidine. No oxidative damage was evident, either immediately after the multiple-turnover incubation or upon subsequent piperidine treatment. A mixture of 0.1 pmol of 5′-^{32}P-radiolabeled 10MD5, 0.1 pmol of internally ^{32}P-radiolabeled DNA substrate, and 20 pmol of nonradiolabeled substrate was incubated in 20 μL of 70 mM HEPES, pH 7.5, 150 mM NaCl, 40 mM $MgCl_2$, 20 mM $MnCl_2$, and 1 mM $ZnCl_2$ for 12 h. The products were separated by 8% PAGE. A small portion of the crude sample is shown in the "rxn" lane; the product yield was 20% with 0.5% deoxyribozyme, revealing 40 turnovers in this particular assay. The PAGE-purified deoxyribozyme was treated with piperidine alongside a control sample for which the deoxyribozyme was incubated in the reaction buffer in the absence of DNA substrate and divalent metal ions. For each lane, ca. 0.025 pmol of deoxyribozyme was incubated in 50 μL of 10% (v/v) piperidine at 95 °C for 30 min; the sample was dried in a SpeedVac and analyzed by 20% PAGE.