Supporting Information

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SI Materials and Methods

Synthesis of Peptides. Peptides were prepared by solid-phase synthesis using Fmoc Rink amide MBHA resin, with *N,N,N′,N′*-tetramethyl-*O*-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate (HATU) as coupling agent. Each synthesis was performed at 0.2 mmol scale, initiated using 308 mg of Rink amide resin with a loading capacity of 0.65 mmol/g (AAPPTec). All steps were monitored by ninhydrin test. For each coupling, 5 equivalents (1.0 mmol) of Fmoc-amino acid, 4.9 equivalents (373 mg, 0.98 mmol) of HATU, and 10 equivalents (350 μL, 2.0 mmol) of *N,N*-diisopropylethylamine (DIPEA) were mixed in 10 mL of anhydrous dimethylformamide (DMF). The coupling reaction was initiated by adding this mixture to the resin and agitating by bubbling with nitrogen for 45 min, followed by washing with 10 mL of DMF three times. The N-terminus of the newly installed amino acid was deprotected by agitating the resin in 20% piperidine in DMF (5 mL) under nitrogen for 5 min a total of three times, each time washing with 5 mL of DMF. Peptides with N-terminal cysteine were capped via acetylation of their N terminus by agitating the resin in 50 equivalents (924 μL, 10 mmol) of acetic anhydride and 25 equivalents of DIPEA (870 μL, 5 mmol) in 5 mL of DMF under nitrogen for 30 min. The peptide was cleaved from the solid support by stirring the resin in a separate vial with a solution containing 5 mL of TFA, 125 μL of water, 125 μL of ethanedithiol, and 50 μL of triisopropylsilane for 90 min. The liquid solution was separated from the resin by filtration. This solution was dried on a rotary evaporator, providing a solid (for hexapeptides) or an oily solid (for dipeptides). To this material 20 mL of cold diethyl ether was added, and the peptide was obtained as a white solid that was filtered and purified by HPLC.

Synthesis of DNA-Anchored Peptides. Each peptide was coupled to the DNA anchor oligonucleotide via either the N-terminal α-amino group (linkage created by reductive amination) or the N-terminal cysteine side chain (linkage created by disulfide formation).

Procedure for coupling to N-terminal α-amino group (Fig. S1A). The DNA anchor oligonucleotide was 5′-GGATAATACGACTCACTAT-rA-3′, where the 3′-terminal ribonucleotide was oxidized by $NaIO_4$. A 100-μL sample containing 1.0 nmol of DNA anchor oligonucleotide in 100 mM Hepes (pH 7.5) and 10 mM $NaIO_4$ was incubated at room temperature for 1 h. The oxidized product was precipitated to remove excess $NaIO_4$ by addition of 10 μL of 3 M NaCl and 300 μL of ethanol. The precipitated product was dissolved in 65 μL of water and used directly in the next step. A 100-μL sample containing the $NaIO_4$-oxidized DNA anchor oligonucleotide and 100 nmol (100 equivalents) of peptide in 100 mM NaOAc (pH 5.2), 50 mM $NiCl_2$, and 10 mM $NaCNBH_3$ was incubated at 37 °C for 14 h. The DNA-anchored hexapeptide was precipitated by addition of 10 μL of 3 M NaCl and 300 μL of ethanol and purified by 20% PAGE. A typical yield was 200–400 pmol. Table S1 gives MALDI MS values.

Procedure for coupling to N-terminal cysteine side chain (Fig. S1B). The DNA anchor oligonucleotide was 5′-GGATAATACGACTCAC-TAT-C_3-SS-C_3-OH-3′, where the 3′-disulfide linker was introduced via standard solid-phase DNA synthesis and unmasked to a 3′-thiol by DTT treatment. In some cases, 1 or 10 hexa(ethylene glycol), or HEG, spacer units were included immediately to the 5′-side of the disulfide linker; structures are shown in Fig. S5. A 50-μL sample containing 2 nmol of DNA anchor oligonucleotide in 50 mM Hepes (pH 7.5) and 50 mM DTT was incubated at 37 °C for 2 h. The reduced product was precipitated to remove excess DTT by addition of 50 μL of water, 10 μL of 3 M NaCl, and 300 μL of ethanol. The precipitated product (DNA-C_3-SH) was dissolved in 45 μL of water. Activation as the pyridyl disulfide was achieved by adding 5 μL of 100 mM 2,2′-dipyridyl disulfide in DMF and incubating at 37 °C for 2 h. The product (DNA-C_3-SSPy) was precipitated by addition of 50 μL of water, 10 μL of 3 M NaCl, and 300 μL of ethanol and dissolved in 25 μL of water. Conjugation to the peptide was performed by adding 20 μL of 50 mM triethylammonium acetate (pH 7.0) and 5 μL of 20 mM peptide (100 nmol, 100 equivalents). The sample was incubated at 37 °C for 2 h, and the DNA-anchored peptide was purified by 20% PAGE. A typical yield was 1.4–1.8 nmol. Table S1 gives MALDI MS values.

In Vitro Selection Procedures. The selection procedure, cloning, and initial analysis of individual clones were performed essentially as described previously (1, 2), but with a different ligation step as recently reported (3). An overview of the key selection and capture steps of each round is shown in Fig. 1 of the main text. The random deoxyribozyme pool was 5′-CGAAGTCCGCCATCTCTTC-N_{40}-ATAGTGAGTCGTATTAAGCTGATCCTGATGG-3′. PCR primers were 5′-CGAAGTCGCCATCTCTTC-3′ (forward primer) and 5′-$(AAC)_4$XCCATCAGATCAGCT-3′, where X is the HEG spacer to stop Taq polymerase (reverse primer). In each round, the ligation step to attach the deoxyribozyme pool at its 3′-end with the 5′-end of the DNA-anchored hexapeptide substrate was performed using a DNA splint and T4 DNA ligase. The splint sequence was 5′-ATAGTGAGTCGTATTATCCTCCATCAGGATCAGCTTA-ATACGACTCACTAT-3′, where the underlined T is included to account for the untemplated A nucleotide that is added at the 3′-end of each PCR product by Taq polymerase. This T nucleotide was omitted from the splint used for ligation of the initially random N_{40} pool, which was prepared by solid-phase synthesis without the untemplated A. Nucleotide sequences of the DNA anchor oligonucleotide, the deoxyribozyme binding arms, the 5′-triphosphorylated RNA substrate for the capture reaction, and the full 15MZ36 capture deoxyribozyme are shown in Fig. S2.

Procedure for ligation step in round 1. A 34-μL sample containing 1 nmol of DNA pool, 850 pmol of DNA splint, and 750 pmol of 5′-phosphorylated DNA-hexapeptide substrate 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 solution was added 4 μL of 10× T4 DNA ligase buffer (Fermentas) and 2 μL of 5 U/μL T4 DNA ligase (Fermentas). The sample was incubated at 37 °C for 12 h and purified by 8% PAGE.

Procedure for ligation step in subsequent rounds. A 16-μL sample containing the PCR-amplified DNA pool (~5–10 pmol), 20 pmol of DNA splint, and 30 pmol of 5′-phosphorylated DNA-hexapeptide substrate 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 solution was added 2 μL of 10× T4 DNA ligase buffer (Fermentas) and 2 μL of 1 U/μL T4 DNA ligase (Fermentas). The sample was incubated at 37 °C for 12 h and purified by 8% PAGE.

Procedure for selection step in round 1. Each selection experiment was initiated with 200 pmol of the ligated N_{40} pool. A 15-μL sample containing 200 pmol of ligated N_{40} pool 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 and cooling on ice for 5 min. The selection reaction was initiated by bringing the sample to a 30-μL total volume containing 70 mM Hepes (pH 7.5), 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl. The sample was incubated at 37 °C for 14 h.

Procedure for selection step in subsequent rounds. A 10-μL sample containing ligated pool 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 and cooling on ice for 5 min. The selection reaction was initiated by bringing the sample to a 20-μL total volume containing 70 mM Hepes (pH 7.5), 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl. The sample was incubated at 37 °C for 14 h.

Procedure for capture step in round 1. To the 30-μL selection sample was added 1.2 μL of 50 mM EDTA (pH 8.0) (60 nmol). The sample was then brought to a 45-μL total volume containing 300 pmol of 15MZ36 capture deoxyribozyme, 400 pmol of 5′-triphosphorylated RNA substrate, 20 mM $MnCl_2$, and 40 mM $MgCl_2$ and incubated at 37 °C for 14 h.

Procedure for capture step in subsequent rounds. To the 20-μL selection sample was added 0.8 μL of 50 mM EDTA (pH 8.0) (40 nmol). The sample was then brought to a 25-μL total volume containing 40 pmol of 15MZ36 capture deoxyribozyme, 80 pmol of 5′-triphosphorylated RNA substrate, 20 mM $MnCl_2$, and 40 mM $MgCl_2$ and incubated at 37 °C for 14 h.

Procedure for PCR. In each selection round, two PCR reactions were performed, 10-cycle PCR followed by 30-cycle PCR. First, a 100-μL sample was prepared containing the PAGE-purified selection product, 200 pmol of forward primer, 50 pmol of reverse primer, 20 nmol of each dNTP, and 10 μL of 10× Taq polymerase buffer [1× Taq polymerase buffer consists of 20 mM Tris·HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, and 0.1% Triton X-100]. This sample was cycled 10 times according to the following PCR program: 94 °C for 2 min, 10× (94 °C for 30 s, 47 °C for 30 s, 72 °C for 30 s), and 72 °C for 5 min. Taq polymerase was removed by phenol/chloroform extraction. Second, a 50-μL sample was prepared containing 1 μL of the 10-cycle PCR product, 100 pmol of forward primer, 25 pmol of reverse primer, 10 nmol of each dNTP, 20 μCi of α-^{32}P-dCTP (800 Ci/mmol), and 5 μL of 10× Taq polymerase buffer. This sample was cycled 30 times according to the following PCR program: 94 °C for 2 min, 30× (94 °C for 30 s, 47 °C for 30 s, 72 °C for 30 s), and 72 °C for 5 min. Samples were separated by 8% PAGE.

Overexpression and Purification of ProcA2.8 Thioester. The procedure was followed essentially as reported (4), with minor modifications. *Escherichia coli* BL21(DE3) cells harboring the pTXB1 plasmid that encodes the His_6-ProcA2.8(1–66)-intein-CBD fusion protein were grown in 2 L of LB with 100 μg/mL ampicillin at 37 °C until an OD_{600} of 0.6–0.7 was reached. Protein expression was induced by the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside, and the cells were grown for an additional 12 h at 18 °C. Cells were harvested by centrifugation at 5,500 × *g* for 20 min at 4 °C, and the cell pellet was resuspended in 30 mL of column buffer containing 100 mM sodium phosphate (pH 7.0), 500 mM NaCl, 1 mM EDTA, and 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP). Cells were lysed by sonication on ice at an amplitude of 65% (3 s on, 9 s off) for 16 min. The lysate was centrifuged at 20,000 × *g* for 20 min, and the supernatant containing the fusion protein was loaded onto a chitin column (20 mL) that was pre-equilibrated with column buffer. The fusion protein was bound to the chitin column by shaking gently at 4 °C for 2 h. The column was mounted upright and washed with 200 mL (10 column volumes) of the column buffer. The column was washed with 40 mL of buffer containing 100 mM sodium phosphate (pH 7.4), 500 mM NaCl, 1 mM EDTA, and 1 mM TCEP. To the column was added 20 mL of cleavage buffer containing 100 mM sodium phosphate (pH 7.4), 500 mM NaCl, 1 mM EDTA, 1 mM TCEP, and 100 mM mercaptoethanesulfonic acid. Intein-mediated cleavage of the 85-mer His_6-ProcA2.8(1–66) thioester was performed by gentle shaking of the chitin resin for 12 h at 4 °C. The His_6-ProcA2.8(1–66) thioester was eluted, concentrated using a 15-mL Amicon 3-kDa filter (Millipore), and lyophilized to dryness.

1. Flynn-Charlebois A, et al. (2003) Deoxyribozymes with 2′-5′ RNA ligase activity. *J Am Chem Soc* 125(9):2444–2454.
2. Kost DM, Gerdt JP, Pradeepkumar PI, Silverman SK (2008) Controlling the direction of site-selectivity and regioselectivity in RNA ligation by Zn^{2+}-dependent deoxyribozymes that use 2′,3′-cyclic phosphate RNA substrates. *Org Biomol Chem* 6(23):4391–4398.
3. Sachdeva A, Chandra M, Chandrasekar J, Silverman SK (2012) Covalent tagging of phosphorylated peptides by phosphate-specific deoxyribozymes. *ChemBioChem* 13(5): 654–657.
4. Levengood MR, Kerwood CC, Chatterjee C, van der Donk WA (2009) Investigation of the substrate specificity of lacticin 481 synthetase by using nonproteinogenic amino acids. *ChemBioChem* 10(5):911–919.

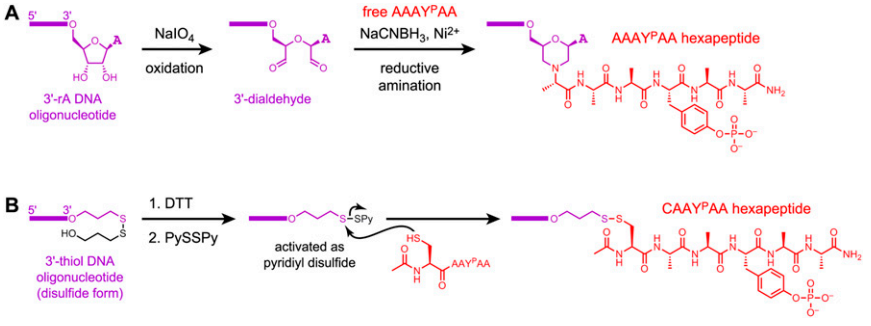

Fig. S1. Synthesis of DNA-anchored hexapeptides. (*A*) Synthesis by attachment of the DNA anchor to the N-terminal α-amino group, with the linkage created by reductive amination. (*B*) Synthesis by attachment of the DNA anchor to the N-terminal cysteine side chain, with the linkage created by disulfide formation.

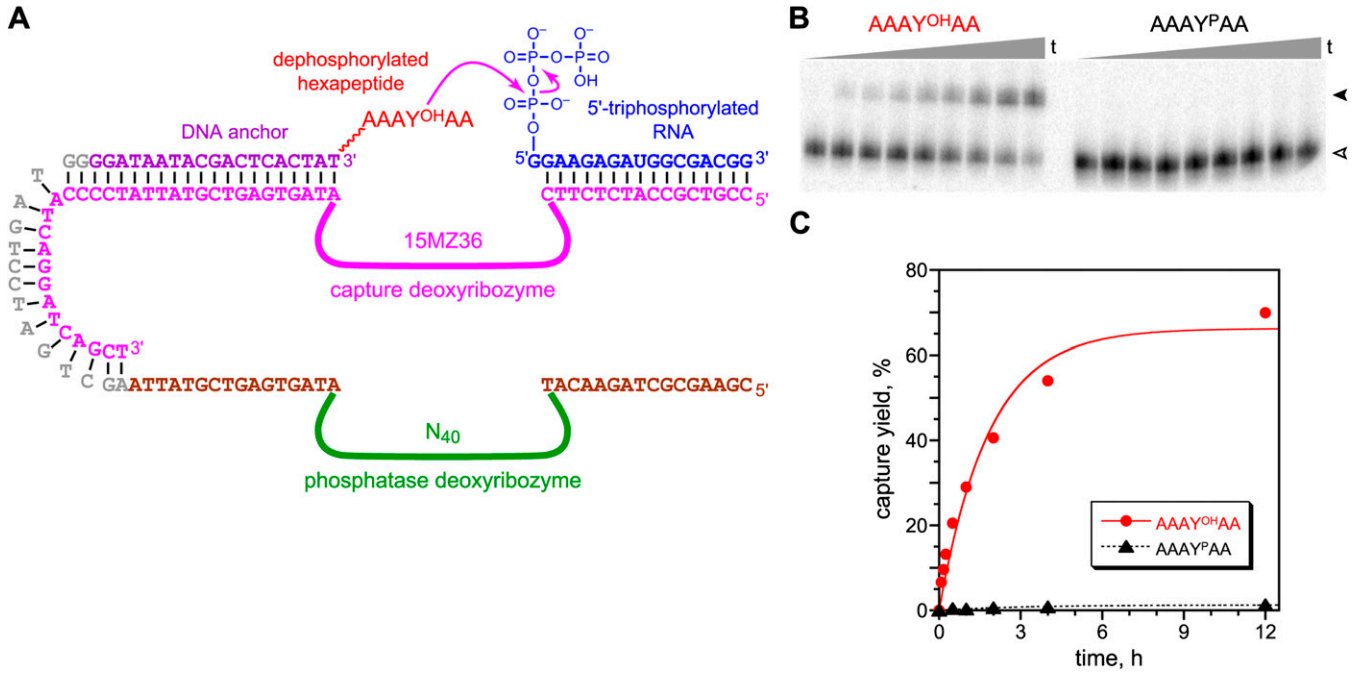

Fig. S2. Validating use of the 15MZ36 deoxyribozyme for capture of catalytically active DNA sequences that have dephosphorylated the Y^P residue of their covalently attached $AAAY^PAA$ hexapeptide substrate. (*A*) Illustration of the 15MZ36-catalyzed capture reaction to attach the 5′-triphosphorylated RNA to the dephosphorylated $AAAY^{OH}AA$ substrate. Note that the substrate molecule is physically connected to the deoxyribozyme sequence that catalyzed its dephosphorylation (denoted as N_{40}). The 15MZ36 originally random region (41 nt) is 5′-CAAGGAGAGTTGTACAAGCTCGGGTCGTGTTCAAAGGGATC-3′ (1). (*B*) PAGE image, illustrating selectivity for $AAAY^{OH}AA$ over $AAAY^PAA$ of the 15MZ36-catalyzed capture reaction. t = 30 s, 5 min, 10 min, 15 min, 30 min, 1 h, 2 h, 4 h, and 12 h. The open arrowhead marks the reactant, and the filled arrowhead marks the product. Incubation conditions: 50 mM Hepes (pH 7.5), 20 mM $MnCl_2$, 40 mM $MgCl_2$, 100 mM NaCl, 37 °C. (*C*) Kinetic plot to quantify the PAGE image.

1. Wong OY, Pradeepkumar PI, Silverman SK (2011) DNA-catalyzed covalent modification of amino acid side chains in tethered and free peptide substrates. *Biochemistry* 50(21): 4741–4749.

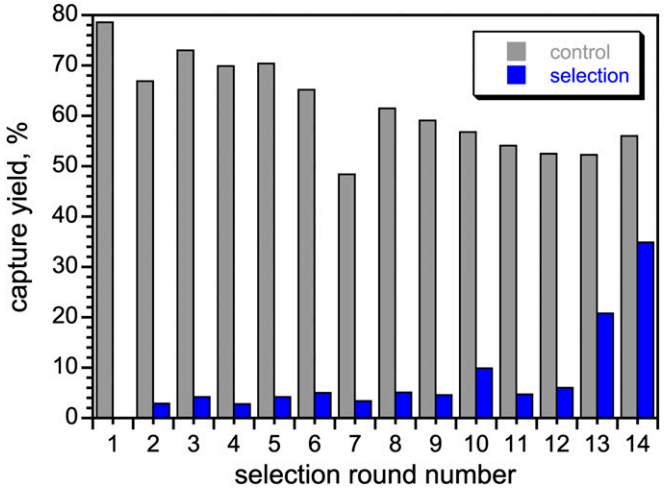

Fig. S3. Progression of the in vitro selection experiment. In each round, "control" refers to the yield for the 15MZ36-catalyzed capture reaction using the DNA-anchored dephosphorylated $AAAY^{OH}AA$ substrate, and "selection" refers to the yield for the 15MZ36-catalyzed capture reaction using the deoxyribozyme pool for that round. *SI Materials and Methods* gives experimental procedures.

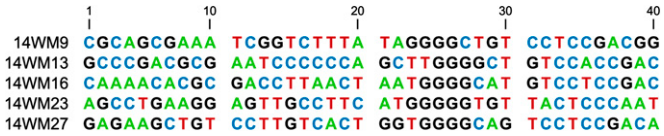

Fig. S4. Sequences of the phosphatase deoxyribozymes described in this study. Only the originally random (N_{40}) sequences are shown. For single-turnover intramolecular (*in cis*) reactions, each originally random region was surrounded by additional DNA nucleotides as shown in Fig. S2*A*. For multiple-turnover intermolecular (*in trans*) reactions, each originally random region was surrounded by the two single-stranded constant regions (each 16 nt) as shown in Fig. S2*A*.

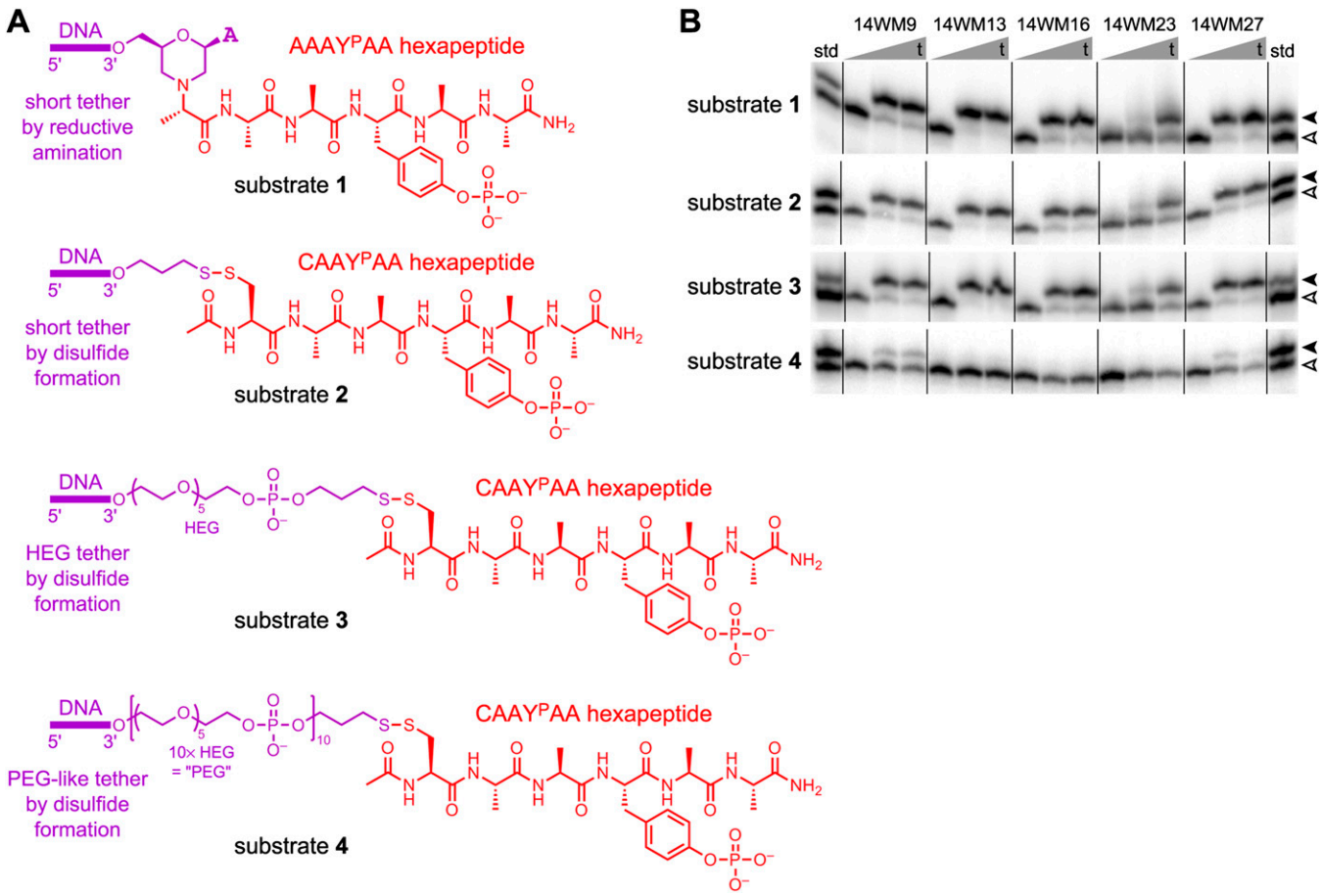

Fig. S5. Evaluation by single-turnover intramolecular assays of the dependence of the activity of the five 14WM deoxyribozymes on the structure and connectivity of the Y^P-containing hexapeptide substrate. (*A*) Chemical structures of the four evaluated DNA-anchored hexapeptide substrates. Substrate 1 was used during the in vitro selection process and has the N-terminal amino group of AAAY^{P}AA joined by reductive amination ($NaCNBH_3$) with the $NaIO_4$-oxidized 3′-rA terminus of the anchor. Substrate 2 is directly analogous to substrate 1, except the hexapeptide is CAAY^{P}AA rather than AAAY^{P}AA and is attached to the DNA anchor oligonucleotide by disulfide bond formation of the Cys thiol side chain of CAAY^{P}AA to the 3′-thiol linker of the anchor. Substrate 3 is the same as substrate 1, except a hexa(ethylene glycol) (HEG) spacer is included immediately to the 5′-side of the 3′-terminal thiol linker. Substrate 4 is the PEG-like analog of substrate 3, in which 10 HEG units were concatenated to provide the covalent tether. (*B*) PAGE assays with each of the four substrates. t = 30 s, 2 h, and 12 h. Incubation conditions: 70 mM Hepes (pH 7.5), 1 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C. Each standard lane is a mixture of the Y^P substrate (open arrowhead) and Y^{OH} dephosphorylation product (filled arrowhead). For the assays with substrate 4, the 14WM9 yield was 20% at 2 h and 29% at 12 h, and the 14WM27 yield was 20% at 2 h and 16% at 12 h. The 12-h yield for 14WM27 may be artifactually low due to nonspecific loss of signal.

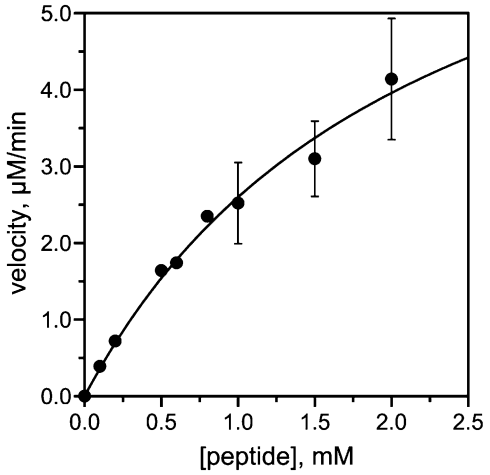

Fig. S6. Determination of K_m for the 14WM9 deoxyribozyme. Data points are $n = 1$ for peptide concentrations below 1.0 mM, $n = 3$ for 1.0 mM, $n = 5$ for 1.5 mM, and $n = 6$ for 2.0 mM (error bars for $n > 1$ are SD). Each velocity determination was derived from a linear fit of product formation (micromolar) versus time (minutes) by HPLC. The fit value to the standard equation $V = V_{max} \bullet [K_m/([\text{peptide}] + K_m)]$ gave $V_{max} = 8.3 \pm 1.1$ μM/min and $K_m = 2.2 \pm 0.5$ mM. However, because the data points do not turn over at high peptide concentration (and >2 mM peptide was not practical), the safest assessment is that $K_m \geq 1$ mM.

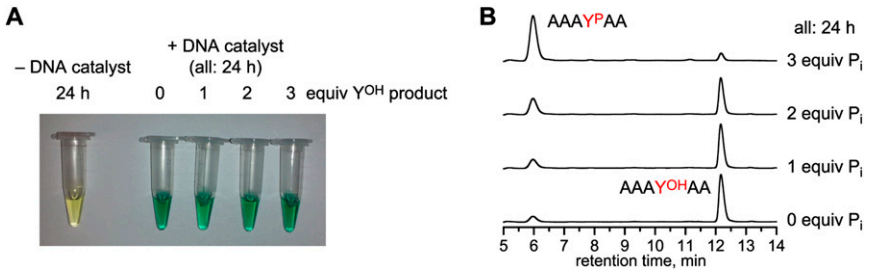

Fig. S7. Evaluating inhibition of the 14WM9 deoxyribozyme by the dephosphorylated AAAY^{OH}AA peptide product and by inorganic phosphate (P_i). (*A*) Evaluating inhibition by AAAY^{OH}AA, as determined by the malachite green dye assay for detection of released P_i. Each assay included 100 μM 14WM9, 125 μM DNA anchor oligonucleotide, 500 μM AAAY^{P}AA substrate, and up to 1.5 mM AAAY^{OH}AA product in 70 mM Hepes (pH 7.5), 6 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C. No inhibition was observed, even with 3 equivalents of product relative to substrate. (*B*) Evaluating inhibition by P_i, as determined by HPLC assay. Each assay included 100 μM 14WM9, 125 μM DNA anchor oligonucleotide, 500 μM AAAY^{P}AA substrate, and up to 1.5 mM P_i in 70 mM Hepes (pH 7.5), 6 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C. Substantial inhibition was reproducibly observed, especially above 2 equivalents of P_i relative to substrate.

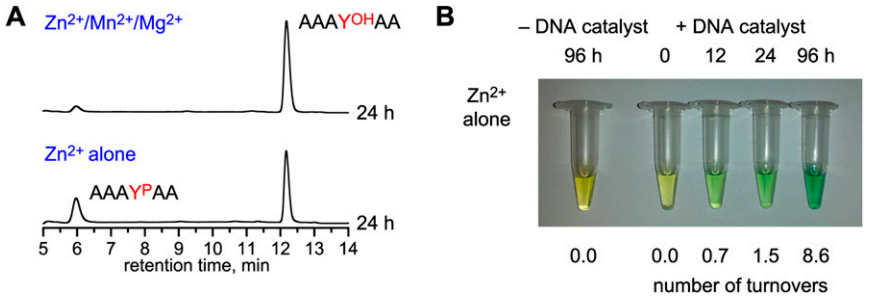

Fig. S8. Activity of the 14WM27 deoxyribozyme with the free AAAY^{P}AA substrate. (*A*) HPLC assay, under conditions of Fig. 3*A* (either 6 mM Zn^{2+} alone or 6 mM Zn^{2+} with 20 mM Mn^{2+} and 40 mM Mg^{2+}). (*B*) Malachite green assay for released P_i, under conditions of Fig. 3*B*.

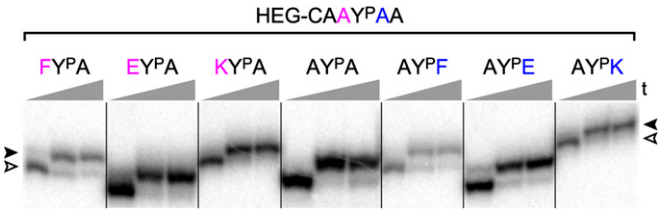

Fig. S9. Assays of 14WM9 with various hexapeptide substrate sequences in which one amino acid adjacent to the Y^P was altered, under conditions of Fig. 2 (Zn^{2+}/Mn^{2+}/Mg^{2+}). t = 0, 2, and 12 h. Data were obtained in parallel with Fig. 4.

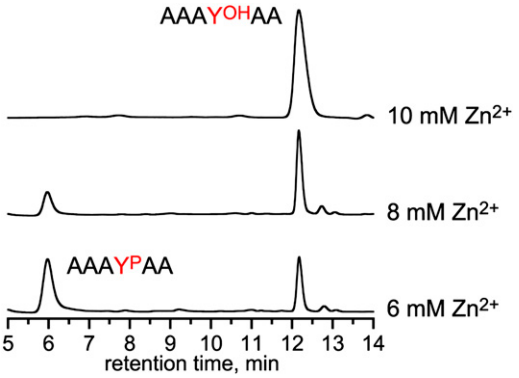

Fig. S10. 14WM9 multiple-turnover activity with free peptide substrate assayed in the presence of 20 mg/mL BSA and different Zn^{2+} concentrations. Data were acquired as in Fig. 5*B* (t = 24 h).

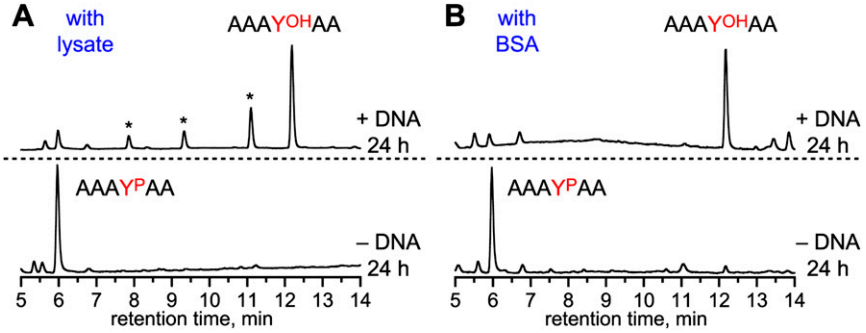

Fig. S11. 14WM27 multiple-turnover activity assayed in the presence of human cell lysate and BSA. (*A*) HPLC assay in the presence of human cell lysate (160 μg/mL lysate protein). Conditions: 100 μM 14WM9, 125 μM DNA anchor oligonucleotide, 500 μM free $AAAY^PAA$, 70 mM Hepes (pH 7.5), 6 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C. The asterisks denote shorter peptide fragments formed by nonspecific peptidase activity in the lysate. (*B*) HPLC assay in the presence of 20 mg/mL BSA. Conditions: 10 μM 14WM9, 12.5 μM DNA anchor oligonucleotide, 500 μM free $AAAY^PAA$, 70 mM Hepes (pH 7.5), 10 mM $ZnCl_2$, 20 mM $MnCl_2$, 40 mM $MgCl_2$, and 150 mM NaCl at 37 °C.

Table S1. MALDI MS data for DNA-anchored peptide standards

DNA-anchored peptide	Mass calculated	Mass found	Error, % (found – calculated)
DNA-AAAY^{P}AA	6,705.6	6,703.7*	−0.03
DNA-AAAY^{OH}AA	6,625.6	6,625.3*	−0.01
DNA-AAAS^{P}AA	6,629.5	6,627.2*	−0.03
DNA-AAAS^{OH}AA	6,549.5	6,547.6*	−0.03
DNA-C_3-CAAY^{P}AA	6,636.7	6,637.2	+0.01
DNA-HEG-CAAY^{P}AA	6,980.9	6,977.9	−0.04
DNA-HEG-CAAY^{OH}AA	6,901.0	6,902.5	+0.02
DNA-HEG-CAAS^{P}AA	6,904.8	6,897.5	−0.11
DNA-HEG-CAAS^{OH}AA	6,824.9	6,825.5	+0.01
DNA-HEG-CY^{P}A	6,767.7	6,771.3	+0.05
DNA-HEG-CS^{P}A	6,691.6	6,693.8	+0.03
DNA-HEG-CY^{P}	6,696.6	6,697.5	+0.01
DNA-HEG-CS^{P}	6,620.5	6,618.9	−0.02
DNA-HEG-CAFY^{P}AA	7,057.0	7,059.9	+0.04
DNA-HEG-CAEY^{P}AA	7,039.0	7,040.6	+0.02
DNA-HEG-CAKY^{P}AA	7,038.0	7,039.2	+0.02
DNA-HEG-CAAY^{P}FA	7,057.0	7,057.7	+0.01
DNA-HEG-CAAY^{P}EA	7,039.0	7,039.8	+0.01
DNA-HEG-CAAY^{P}KA	7,038.0	7,038.5	+0.01
DNA-PEG-CAAY^{P}AA	10,079.8	10,080.4	+0.01

*The found values for these DNA-anchored peptides as reported in the main text were observed by MALDI MS analysis directly from the DNA-catalyzed reaction samples, rather than by using separately synthesized standard samples as tabulated here.

The DNA was either 5′-GGATAATACGACTCACTAT-rA-3′ (for peptides with N-terminal Ala anchored by reductive amination; the rA was oxidized by $NaIO_4$) or 5′-GGATAATACGACTCACTAT-C_3-SS-C_3-OH-3′ (for peptides with N-terminal Cys anchored by disulfide bond formation; the disulfide linker was reduced by DTT). The HEG linker was hexa(ethylene glycol) as illustrated in Fig. 4. The PEG linker was 10 consecutive HEG linkers.