

In Vitro Selection for Small-Molecule-Triggered Strand Displacement and Riboswitch Activity

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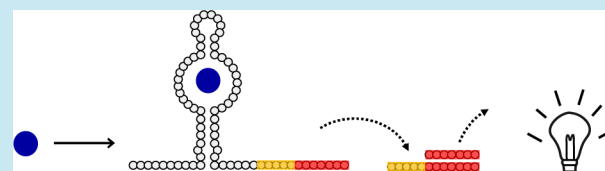
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S Supporting Information

ABSTRACT: An *in vitro* selection method for ligand-responsive RNA sensors was developed that exploited strand displacement reactions. The RNA library was based on the thiamine pyrophosphate (TPP) riboswitch, and RNA sequences capable of hybridizing to a target duplex DNA in a TPP regulated manner were identified. After three rounds of selection, RNA molecules that mediated a strand exchange reaction upon TPP binding were enriched. The enriched sequences also showed riboswitch activity. Our results demonstrated that small-molecule-responsive nucleic acid sensors can be selected to control the activity of target nucleic acid circuitry.

KEYWORDS: *in vitro* selection, strand displacement, riboswitch, aptamer, SELEX



Functional nucleic acid sequences often transition between different conformational states that correlate with different levels of activity. For example, naturally occurring attenuator sequences and riboswitches exploit conformational changes in response to metabolite availability to control gene expression. Similarly, non-natural nucleic acids that display conformationally dependent activity can be designed^{1,2} and selected^{3–6} by combining ligand-binding aptamer and catalytic ribozyme domains.

Nucleic acid sensors and transducers can potentially be adapted to more complex circuitry either *in vitro* or *in vivo* via strand exchange reactions. Strand exchange reactions exploit the ability of single-strand DNA or RNA to displace one strand of a preexisting duplex nucleic acid⁷ and are driven by the initial binding of an oligonucleotide to a toehold sequence. The rate of the reaction depends upon the length and sequence composition of the toehold,⁸ which is usually between 4 and 8 nucleotides long, with an optimum centered around 6 nucleotides in length.⁹ These reactions are widely used in DNA nanotechnology,¹⁰ including in structural DNA assemblies,¹¹ dynamic autonomous devices,^{12,13} and isothermal nucleic acid amplification methods.^{14,15} For analytical applications, the initial duplex nucleic acid is often modified with a fluorophore–quencher pair and thus functions as a reporter of the reaction.^{9,16}

In order to couple nucleic acid sensors and nucleic acid circuitry, it is necessary that the binding of an oligonucleotide sequence induces a conformational change that in turn modulates the activity of a target nucleic acid.^{17–20} However, the coupling of receptors and circuitry has been difficult in part because typical nucleic acid selections are designed to either enrich sequences that bind specific molecules^{21,22} or display

specific catalytic activity²³ without undergoing conformational changes that transduce ligand binding into strand exchange. We have therefore developed an *in vitro* selection strategy to identify RNAs in which a conformational change induced by ligand binding could be used to drive coupled strand displacement reactions²⁴ (Figure 1). A RNA library was constructed based on the thiamine pyrophosphate (TPP) riboswitch.²⁵ Ligand binding to the riboswitch liberated a single-strand region containing a ribosome binding site capable of hybridizing to and displacing a duplex reporter coupled to magnetic beads. Iterative rounds of selection showed improved strand displacement and riboswitch activity. This fully *in vitro* methodology should more generally allow for the selection of RNA circuitry based on ligand-induced conformational changes.

RESULTS AND DISCUSSION

Development of Strand Displacement Selections for Riboswitches. Nucleic acids can uniquely undergo programmed conformational changes via strand exchange and displacement. This property is both a key feature of nucleic acid biotechnologies, such as allosteric ribozymes, and nucleic acid regulatory elements, such as riboswitches. In this regard, shape-switching aptamer biosensors have been selected by immobilizing pools via hybridization to an oligonucleotide affinity column and then selecting for variants that undergo a strand displacement reaction that results in release from the column only in the presence of ligand.^{26,27} However, strand exchange

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selections have not been applied to the development of genetic regulatory elements. We therefore sought to select for riboswitches that could undergo strand exchange and shape switching in the presence of ligands.

The basis of our selection is shown in Figure 1. Ligand binding to the RNA induces a conformational change that

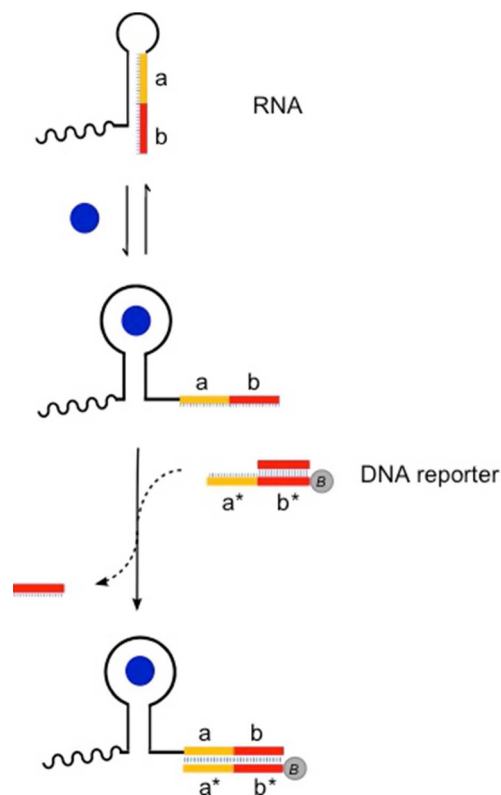


Figure 1. Ligand binding to the RNA triggers a strand displacement reaction. When domain a of the RNA becomes accessible due to the binding of TPP (circle), the strand displacement reaction can proceed. The interaction between the RNA and the DNA reporter is based on sequence complementarity. The reporter is partly double stranded with a single strand toehold region (a*) that interacts with the complementary sequence of domain a of the RNA. Upon toehold hybridization, RNA domain b binds to the complementary domain b* of the reporter, displacing the shorter strand. The DNA reporter is either modified with a fluorophore–quencher pair or tagged with a biotin molecule at the 5' end of b*. A version of this figure showing sequence information is provided in the Supporting Information, Figure S1.

renders a previously inaccessible segment of the RNA accessible. This liberated sequence contains a RBS and is complementary to one strand of a biotinylated duplex DNA. Biotinylation allows for isolation with streptavidin beads. Hybridization between the RNA and the DNA is mediated by a toehold sequence and results in the displacement of the shorter of the two DNA strands. Note that in the absence of ligand the RNA is not in a conformation capable of hybridizing with the biotinylated nucleic acid and thus should not be retained for further rounds of selection.

Model System for Selection. To construct a ligand-responsive strand displacement reaction, we started with the thiamine pyrophosphate riboswitch (ThiM) that regulates translation by modulating the accessibility of a ribosome binding site (RBS) in a manner dependent on ligand binding.²⁸

While the natural *Escherichia coli* ThiM thiamine pyrophosphate riboswitch conceals the RBS upon ligand binding, Nomura and Yokobayashi selected for a variant (+ThiM#2) in which the RBS is liberated to activate translation when bound to TPP. We opted to use this version, +ThiM#2, that is activated by TPP as a starting point for selection.²⁵

To test if changes in accessibility of the RBS induced by TPP binding could also induce strand displacement, a reporter with a toehold sequence complementary to the RBS was designed. The reporter contained a 5' fluorophore (fluorescein) on the longer strand and a 3' quencher (Iowa black) on the shorter strand so that strand displacement would result in an increase of fluorescence. Two test constructs with the same RBS sequence as that of the +ThiM#2 TPP riboswitch were prepared that displayed RBS sites either in an accessible (ON) or an inaccessible (OFF) conformation. The two constructs reacted differently with the reporter, with the ON construct resulting in 4.20 ± 0.03 -fold greater fluorescence than that of the OFF construct (Figure 2).

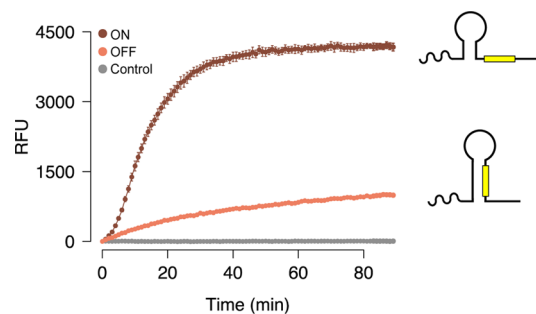


Figure 2. A FRET-modified reporter can distinguish between RNA sequences with an accessible (ON) and inaccessible (OFF) RBS. 100 nM ON or OFF RNA was incubated with 50 nM of a FRET-modified reporter (Rep Q/Rep F, Table S1) in TNaK buffer. Strand displacement occurred when the RBS sequence of either ON or OFF construct hybridized with the complementary sequence of the reporter. The control reaction did not include ON or OFF RNA. The RBS is shown as a yellow rectangle. The sequences of all of the nucleic acids can be found in Table S1.

Since the reporter was capable of distinguishing between fixed conformations, we next sought to determine if it would also discriminate between a construct that adopted different conformational states in response to ligand binding. The +ThiM#2 riboswitch selected by Nomura and Yokobayashi²⁵ was incubated with the fluorescent reporter tested above and increasing concentrations of TPP (Figure 3b). Fluorescence increased with increasing TPP concentration, with 100 μ M TPP resulting in 4.6 ± 0.2 -fold greater fluorescence than that in the absence of TPP after 4 h. To confirm that the differences in strand displacement activity were due to TPP-induced conformational changes of the riboswitch, two mutant sequences of +ThiM#2 were evaluated. The helix of the riboswitch that blocks the RBS in the absence of TPP was destabilized in +ThiM#2 mut1, and the residues required for TPP binding were removed in +ThiM#2 mut2 (Table S1).²⁵ Both mutant constructs had previously been characterized *in vivo* through assays that monitored the control of gene expression in response to TPP.²⁵ Consistent with the *in vivo* results, *in vitro* strand displacement activity increased in the presence and absence of TPP for +ThiM#2 mut1, and strand displacement activity was no longer distinguishable between the

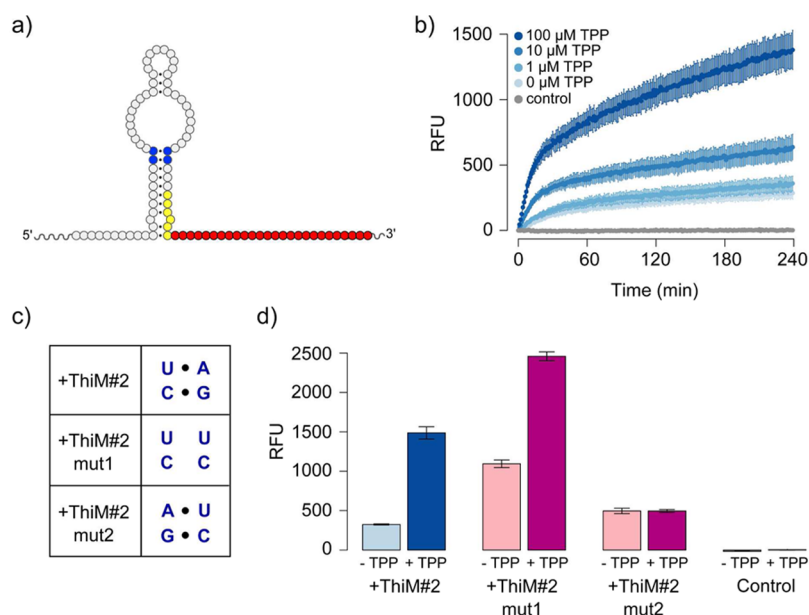


Figure 3. TPP binding to the riboswitch triggers a strand displacement reaction. (a) Schematic view of the +ThiM#2 riboswitch highlighting the positions involved in mediating strand displacement. The RBS (yellow) is complementary to the toehold sequence of the reporter. +ThiM#2 mut1 and +ThiM#2 mut2 mutation positions are shown in blue. The sequence that hybridizes with the reporter is shown in red. (b) The +ThiM#2 riboswitch was characterized by strand displacement with the FRET-based DNA reporter. 100 nM +ThiM#2 RNA was incubated with 50 nM FRET reporter, 1 μM oligo (dT)₂₁, 5 mM MgCl₂ in TNaK buffer. Reactions were carried out in the presence of increasing concentration of TPP for 4 h at 37 °C. In the control reaction, RNA and TPP were not included. (c) Nucleotides that were mutated in the +ThiM#2, +ThiM#2 mut1, and +ThiM#2 mut2 constructs are shown. (d) +ThiM#2 mutant activity in performing strand displacement was compared to the +ThiM#2 riboswitch. The reaction conditions were 100 nM RNA, 50 nM FRET reporter, 1 μM oligo (dT)₂₁, 5 mM MgCl₂ in TNaK buffer either in the presence or absence of 100 μM TPP for 4 h at 37 °C. Fluorescence was recorded with a TECAN Safire plate reader. All reported results are the average of triplicate measurements.

presence or absence of TPP for +ThiM#2 mut2 (Figure 3d). These experiments established the groundwork for a successful selection.

Strand Displacement Can Be Used To Select RNA-Based Sensors. To select riboswitches that would activate strand displacement, we generated a library consisting of sequences based on the +ThiM#2 TPP riboswitch²⁵ in which four positions required for activity were randomized. The randomized positions were the same as the mutant positions found in +ThiM#2 mut1 and mut2 constructs, as the identity of these positions should definitively impact conformational change and strand displacement.²⁵

Active sequences were enriched through binding to the same reporter described above except that the reporter was tagged with a biotin molecule, allowing immobilization on streptavidin magnetic beads in the presence of TPP. Conversely, in the absence of TPP, active sequences should not hybridize with the reporter and the sequences would be lost from the pool. As shown in Figure 1, iterative selection cycles designed to deplete sequences that bound the reporter in the absence of TPP and enriched sequences that bound in the presence of TPP were used to isolate sequences with TPP-responsive strand displacement activity. The experimental details for each round are illustrated in Figure S2.

Three rounds of selection were performed, and the overall activity of the pool was monitored by strand displacement. An aliquot of the RNA output from each round of selection was incubated with the FRET-modified reporter and TPP, and strand displacement ability was monitored by fluorescence spectroscopy. After 4 h at 37 °C, the fluorescence was not significantly changed after one round of selection and increased

by 64 and 120% for rounds two and three, respectively, with respect to the starting pool (Figures 4b and S3). The

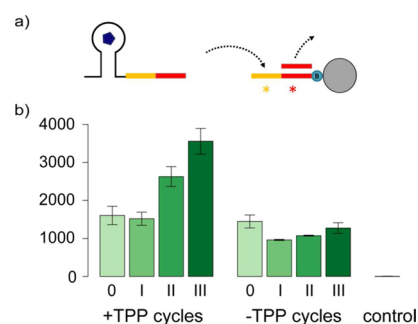


Figure 4. Successive rounds of selection improved TPP-responsive strand displacement activity. (a) During the selection, the reporter contained a biotin molecule so that active sequence could be isolated from the pool with streptavidin beads. (b) After each cycle of selection, the activity of the RNA pool was assessed by incubating an aliquot with the reporter modified with a FRET pair. Reactions were run either in the presence or absence of 100 μM TPP for 4 h at 37 °C. RNA and TPP were not added to the control reaction. The average of three measurements is shown.

fluorescence of the library before the first round of selection was similar in the presence and absence of TPP, consistent with the starting pool not containing detectable levels of ligand responsive activity. Furthermore, fluorescence in the absence of TPP was lower after each round of selection than that in the starting RNA pool (Figures 4 and S3).

To gain more insight into the progression of the selection, aliquots after each round of selection were sequenced. The

starting pool had the highest diversity of sequences (all 10 sequences were different). Conversely, after three rounds of selection, one sequence (Seq8) represented 40% of 20 sequences (Table S2). Seq8 contained the same sequence as the +ThiM#2 riboswitch at the first two randomized positions (CU), but it contained CC in place of the AG found in the +ThiM#2 riboswitch for the last two randomized positions (Figure 5). The full +ThiM#2 sequence did not appear in any



Library	...GAT NN GGA...//...AGA NN ATC...	
Selection results		
Seq8	CT	CC
+ThiM#2	CT	AG

Figure 5. Three rounds of selection resulted in an enrichment of sequences different from the +ThiM#2 riboswitch. Twenty clones were sequenced to determine the enrichment at the four randomized positions of the library after the third round of selection. Seq8 was the most highly represented sequence. The +ThiM#2 riboswitch sequence is shown for comparison. Additional sequence information for the other rounds of selection is provided in Figure S4 and Table S2.

of the sequenced samples. Similarly, the nonfunctional +ThiM#2 mut2 sequence was not observed, consistent with a selection that enriched for TPP binding RNA sequences. However, the +ThiM#2 mut1 sequence (Seq6) was identified in samples taken after each round of selection with a frequency of 0.1 (Table S2). In addition to Seq6 and Seq8, Seq1 and Seq7 were enriched to a frequency of 0.15 each after three rounds of selection.

The most abundant sequence, Seq8, showed the strongest strand displacement activity of all of the sequences tested. The addition of 100 μM TPP to a solution containing Seq8 and the FRET-modified reporter resulted in a 7.0 ± 0.3 -fold increase of fluorescence over that of the same reaction in the absence of TPP (Figure 6). Seq8 was more active in strand displacement than the original +ThiM#2 riboswitch, from which the library was based, which showed a 4.6 ± 0.2 -fold increase in fluorescence upon the addition of 100 μM TPP. The strand

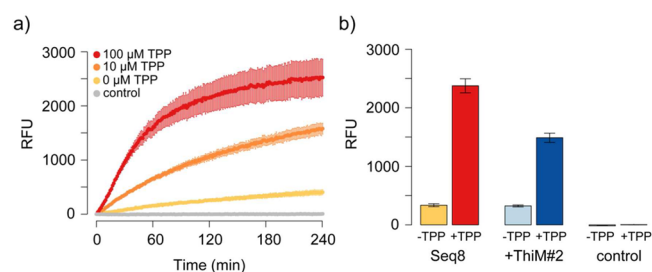


Figure 6. Seq8 is optimized for strand displacement. (a) Seq8 showed TPP-dependent strand displacement activity. The reactions contained 100 nM RNA Seq8, 50 nM FRET reporter, 1 μM oligo (dT)₂₁, 5 mM MgCl₂, TNaK buffer, and varying concentrations of TPP (0, 10, and 100 μM). In the control reaction, the RNA was omitted. Fluorescence was recorded with TECAN Safire plate reader for 4 h at 37 $^{\circ}\text{C}$. (b) Seq8 is more effective at strand displacement than +ThiM#2. 100 nM Seq 8 RNA and +ThiM#2 RNA were added, respectively, to reactions (20 μL) containing 50 nM FRET reporter, 1 μM oligo (dT)₂₁, 5 mM MgCl₂ in TNaK buffer. Data were collected in the presence and absence of 100 μM TPP for 4 h at 37 $^{\circ}\text{C}$ with a TECAN Safire plate reader. Control reactions lacked RNA. The average of three measurements is displayed.

displacement activity of Seq1, Seq6 (+ThiM#2 mut1), and Seq7 was similar to changes in fluorescence of 2.5 ± 0.1 -fold, 2.4 ± 0.2 -fold, and 1.8 ± 0.1 -fold, respectively, upon the addition of TPP (Figure S5). The fact that the selection gave rise to a sequence with better strand displacement activity than that of +ThiM#2 showed that the selection method was well-designed and can likely yield improved variants from even larger libraries.

Strand Displacement Selection Yields Riboswitches That Are Active *in Vitro*. Since +ThiM#2 has both strand displacement and riboswitch activity, we next probed whether the sequences identified through a selection based on strand displacement could also function as riboswitches. The same sequences tested for strand displacement activity were placed in the 5'-untranslated region of a gene encoding yellow fluorescent protein (YFP), and gene expression was evaluated. All of the sequences enriched after three rounds of selection showed between 2- and 3-fold increased YFP synthesis in the presence of TPP (Figure 7). For comparison, YFP synthesis

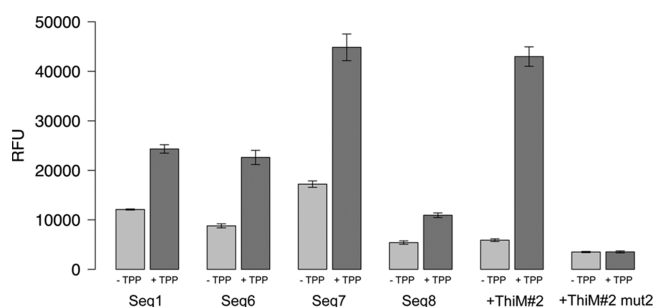


Figure 7. Sequences identified after the third round of selection assessed for their ability to control translation *in vitro*. Each sequence was inserted into the 5'-untranslated region of a gene coding for yellow fluorescent protein. The fluorescence of the reaction was recorded after *in vitro* transcription–translation at 37 $^{\circ}\text{C}$ for 3 h. Seq6 is identical to the +ThiM#2 mut1 sequence.

increased 7.3 ± 0.3 -fold in the presence of TPP when under the control of the +ThiM#2 riboswitch. Conversely, the previously characterized inactive riboswitch +ThiM#2 mut2 showed no difference in protein expression in the presence or absence of TPP.

Strand Displacement Selections for the Engineering of Nucleic Acid Circuitry. Ligand-induced strand exchange reactions can potentially form the basis for a wide variety of applications, especially in diagnostics and sensing. Small-molecule-triggered strand displacement can be coupled with a nucleic acid circuit, catalytic hairpin assembly (CHA), for analyte detection and quantitation.¹⁶ Designed toehold-mediated RNA switches that can respond to mRNAs have now formed the basis of 4-input logic gates in cells,¹⁹ expanding upon previous efforts with small-molecule-triggered riboswitches that yielded 2-input logic gates.^{29,30}

In order to further potentiate such applications, we have now developed a generalizable method for selecting simple nucleic acid subcircuits that undergo ligand binding, leading to conformational changes that in turn lead to strand displacement. Our selected riboswitches showed a smaller dynamic range than that of previously selected, artificial riboswitches,^{31–33} but they were in line with natural riboswitches that regulate protein production in cells.^{34,35} While we have shown that the method works with a TPP riboswitch, it should

be possible to select for strand displacement reactions controlled by small molecules other than TPP.

Our results extend the possibilities for engineered nucleic acid circuitry in two ways. First, most strand displacement reactions reported thus far are built solely with DNA and are not responsive to small molecules. Exceptions include the incorporation of DNA aptamers to control strand displacement in response to ATP and arginine amide ligands.²⁴ While RNA-based strand displacement systems have been developed that are triggered by specific oligonucleotide sequences,^{17–19} ours is the first example of a fully RNA-based subcircuit.

Second, while there are some examples of direct selection for conformational change,^{36,37} these have been focused on the generation of isolated switches, rather than more complex subcircuits. By selecting for riboswitch-based strand exchanges that involve particular sequences, we can potentially engineer complex nucleic acid circuits *in vitro*, prior to introducing them into cells. As this method is more fully vetted, synthetic riboswitches that transduce small molecule recognition to sequence-specific strand exchange and ultimately changes in the expression of particular mRNA sequences should better enable the rational engineering of both living^{38,39} and artificial cellular systems.^{31,40}

METHODS

Chemicals, Oligonucleotides, DNA, and RNA Production. All chemicals were purchased from Sigma-Aldrich, unless otherwise indicated. Oligonucleotides were from Integrated DNA Technologies. DNA templates for transcription were prepared by PCR of overlapping oligonucleotides with AccuPrime Pfx DNA polymerase (Life Technologies). PCR products were purified by extraction from an agarose gel consisting of 1:1 SeaKem GTG and NuSieve GTG agaroses (Lonza) with the Wizard SV gel and PCR clean-up system (Promega). A list of all constructs is included in the Supporting Information, Table S1.

Transcription buffers were prepared with diethyl pyrocarbonate (DEPC)-treated water. Transcription reactions were in a final volume of 50 μL and contained 10 pmol DNA, T7 RNA polymerase buffer (35 mM MgCl_2 , 2 mM spermidine, 200 mM HEPES, pH 7.5), 40 mM DTT, 5 μg of BSA (New England Biolabs), 5 mM each ribonucleotide (NEB), 20 U of human placenta RNase inhibitor (NEB), 50 U of yeast inorganic pyrophosphatase (NEB), and 150 U of T7 RNA polymerase (NEB). Reactions were incubated at 37 $^\circ\text{C}$ for at least 4 h. Samples were then treated with RNase-free DNase I (NEB) for 1 h at 37 $^\circ\text{C}$. Next, RNA was purified from a 6% denaturing (7 M Urea) PAGE. The acrylamide–bis acrylamide solution (19:1) was from Bio-Rad. The RNA was visualized by UV shadowing and isolated by crush-soak. Briefly, the excised RNA band was crushed and left overnight at 37 $^\circ\text{C}$ with tumbling in 500 μL TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.5). The samples were centrifuged, and the supernatants were filtered through 0.45 μm ultrafree-MC centrifugal filters (Millipore). Finally, the samples were ethanol-precipitated and resuspended in 30 μL of DEPC-treated water. Concentrations were determined by UV absorbance with a NanoDrop 1000 spectrophotometer (Thermo Scientific). RNA molecules were stored at $-20\text{ }^\circ\text{C}$.

Real-Time Detection of Strand Displacement. Strand displacement reactions were assembled as previously reported.¹⁶ Briefly, a dsDNA reporter consisting of two unequal length oligonucleotides was designed to allow for real-time

fluorescence measurements. The smaller oligonucleotide (Rep Q) was tagged with an Iowa black quencher molecule, whereas the longer oligonucleotide (Rep F) contained a 5' fluorescein molecule (Table S1). Rep Q was hybridized to Rep F by an annealing step before final reaction assembly. Initially, a 10 μM stock containing 2:1 Rep Q/Rep F was prepared. The reporter stock was diluted 10-fold into the annealing reaction (1 μM reporter final concentration). The annealing was performed in TNaK buffer (140 mM NaCl, 5 mM KCl, 20 mM Tris-HCl, pH 7.5) containing 1 μM oligo (dT)₂₁ to decrease interactions with the walls of the tube.¹⁶ The solution was subjected to 5 min at 90 $^\circ\text{C}$ and slowly cooled to 25 $^\circ\text{C}$ at a rate of 0.1 $^\circ\text{C}/\text{s}$. The annealed reporter was then ready for the strand displacement reaction.

The strand displacement reaction was initiated by the addition of 100 nM RNA in a total volume of 20 μL . Strand displacement was performed in TNaK buffer, 1 μM oligo (dT)₂₁, 5 mM MgCl_2 , and 50 nM annealed reporter. TPP, when present, was added to the reaction mixture at 100 μM , unless otherwise indicated. Reactions were performed in a 384-well black plate (NUNC), and fluorescence was recorded with a TECAN Safire plate reader at 37 $^\circ\text{C}$ for at least 2 h. Plates were covered with a thin sealing foil (Lightcycler, Roche). Excitation and emission wavelengths were 485 and 520 nm, respectively.

Selection by Strand Displacement. The DNA library was assembled by PCR of overlapping oligonucleotides. The sequence of the library was of the +ThiM#2 TPP-responsive riboswitch²⁵ with four randomized positions (Table S1) and was purified as described above for template DNA constructs. Transcription reactions used 2 pmol of DNA template and were run and purified as described in Chemicals, Oligonucleotides, DNA, and RNA Production. Twenty picomoles of library RNA was used for each round of selection. Each round consisted of four steps, including three negative selections in the absence of TPP and a final positive selection in the presence of TPP (Figure S1). The reporter used for the selection was the same as described above for the real-time fluorescence measurements except that the reporter did not contain a fluorophore–quencher pair and the longer oligonucleotide contained a biotin molecule to allow separation via streptavidin magnetic beads (Life Technologies). For each round, the first negative step was performed by incubating the strand displacement reaction at 37 $^\circ\text{C}$ for 2 h. The reaction volume was 100 μL in TNaK buffer, 1 μM oligo (dT)₂₁, 5 mM MgCl_2 , and 50 nM reporter. Twenty microliters of streptavidin magnetic beads was washed three times with 150 μL of BWBT buffer (0.2 M NaCl, 1 mM EDTA, 0.1% (v/v) Tween 20, 10 mM Tris-HCl, pH 7.4) and once with the selection buffer (TNaK buffer supplemented with 5 mM MgCl_2). Finally, the beads were resuspended in the strand displacement reaction and incubated at room temperature for 20 min. After magnetic separation, the supernatant was retained and the magnetic beads were discarded. Twenty microliters of fresh reporter solution (1 μM) and 5 mM MgCl_2 were then added to the retained supernatant, and the negative selection was repeated in the same way except that the incubation of the strand displacement reaction was carried out at 37 $^\circ\text{C}$ for 1 h. Isolation of the supernatant from the beads was performed as indicated above. The third negative selection followed the same procedure as the second. Next, 100 μM TPP and 10 μL of the reporter solution were added to the supernatant and incubated for 2 h at 37 $^\circ\text{C}$. Ten microliters of washed streptavidin magnetic beads was added and incubated at room temperature

for 20 min. To enrich for sequences that bound the reporter, the supernatant was discarded after magnetic separation. The beads were washed four times with 150 μL of selection buffer. The beads were then resuspended directly in the reverse transcription PCR reaction (Life Technology) that additionally contained the primers necessary for amplification. Ten cycles of PCR were performed with a Bio-Rad thermocycler. Two picomoles of the resulting DNA was then transcribed, and 20 pmol of this RNA was used for the next round of selection.

Evaluation of Selection Cycles. Aliquots from each round of selection were assessed for strand displacement activity by real-time fluorescence following the same procedure as that described above. Additionally, the DNA pool after each round was cloned and sequenced. Ten nanograms of DNA was amplified with Taq DNA polymerase (NEB) to add a deoxyadenosine to the 3' end of the fragments. The products were gel-extracted and column-purified with the Wizard SV gel and PCR clean-up system (Promega). Next, the DNA was ligated into pCR 2.1 according to the TA cloning kit protocol (Life Technologies). Colonies were chosen by blue–white screening with LB supplemented with 156 μM X-gal and sent for sequencing at University of Texas ICMB Core Facilities—DNA Sequencing. Enriched sequences after three rounds of selection were further evaluated by their ability to control protein expression. Selected sequences were inserted into the 5'-untranslated region of a gene coding for the yellow fluorescent protein mYPet.^{34,41} Each construct was assembled by overlapping PCR, gel extracted, and purified with the Wizard SV gel and PCR clean-up kit. *In vitro* transcription–translation was performed using the PURE system (NEB). The reactions (20 μL) contained 250 ng of double-strand linear template, 16 U of human placenta RNase inhibitor (NEB), and, when present, 1 mM TPP. Fluorescence was recorded over time with a TECAN Infinite 200 plate reader with excitation and emission wavelengths of 510 and 540 nm, respectively. Reactions were monitored for 3 h at 37 °C in a 384-well black plate (NUNC) covered by a thin adhesive foil.

■ ASSOCIATED CONTENT

■ Supporting Information

Table S1: DNA sequences used in this work. Table S2: Frequencies of DNA molecules after each round of selection. Figure S1: Riboswitch-mediated strand displacement. Figure S2: The strand displacement based selection procedure. Figure S3: The RNA pool after each round of selection was tested for strand displacement activity with the FRET reporter. Figure S4: Calculated nucleotide frequencies for every randomized position in the pool after each round of selection. Figure S5: Strand displacement activity of sequences after three round of selections. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.5b00054.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

- (1) Win, M. N., and Smolke, C. D. (2008) Higher-order cellular information processing with synthetic RNA devices. *Science* 322, 456.
- (2) Ogawa, A., and Maeda, M. (2008) An artificial aptazyme-based riboswitch and its cascading system in *E. coli*. *ChemBioChem* 9, 206.
- (3) Soukup, G. A., and Breaker, R. R. (1999) Engineering precision RNA molecular switches. *Proc. Natl. Acad. Sci. U.S.A.* 96, 3584.
- (4) Robertson, M. P., and Ellington, A. D. (2000) Design and optimization of effector-activated ribozyme ligases. *Nucleic Acids Res.* 28, 1751.
- (5) Soukup, G. A., Emilsson, G. A., and Breaker, R. R. (2000) Altering molecular recognition of RNA aptamers by allosteric selection. *J. Mol. Biol.* 298, 623.
- (6) Wieland, M., Benz, A., Klausner, B., and Hartig, J. S. (2009) Artificial ribozyme switches containing natural riboswitch aptamer domains. *Angew. Chem., Int. Ed.* 48, 2715.
- (7) Yurke, B., Turberfield, A. J., Mills, A. P., Jr., Simmel, F. C., and Neumann, J. L. (2000) A DNA-fuelled molecular machine made of DNA. *Nature* 406, 605.
- (8) Zhang, D. Y., and Winfree, E. (2009) Control of DNA strand displacement kinetics using toehold exchange. *J. Am. Chem. Soc.* 131, 17303.
- (9) Li, Q., Luan, G., Guo, Q., and Liang, J. (2002) A new class of homogeneous nucleic acid probes based on specific displacement hybridization. *Nucleic Acids Res.* 30, E5.
- (10) Zhang, D. Y., and Seelig, G. (2011) Dynamic DNA nanotechnology using strand-displacement reactions. *Nat. Chem.* 3, 103.
- (11) Seeman, N. C. (2010) Structural DNA nanotechnology: growing along with Nano Letters. *Nano Lett.* 10, 1971.
- (12) Yan, H., Zhang, X., Shen, Z., and Seeman, N. C. (2002) A robust DNA mechanical device controlled by hybridization topology. *Nature* 415, 62.
- (13) Yin, P., Choi, H. M., Calvert, C. R., and Pierce, N. A. (2008) Programming biomolecular self-assembly pathways. *Nature* 451, 318.
- (14) Jiang, Y. S., Li, B., Milligan, J. N., Bhadra, S., and Ellington, A. D. (2013) Real-time detection of isothermal amplification reactions with thermostable catalytic hairpin assembly. *J. Am. Chem. Soc.* 135, 7430.
- (15) Compton, J. (1991) Nucleic acid sequence-based amplification. *Nature* 350, 91.
- (16) Li, B., Ellington, A. D., and Chen, X. (2011) Rational, modular adaptation of enzyme-free DNA circuits to multiple detection methods. *Nucleic Acids Res.* 39, e110.
- (17) Bhadra, S., and Ellington, A. D. (2014) A spinach molecular beacon triggered by strand displacement. *RNA* 20, 1183.
- (18) Bhadra, S., and Ellington, A. D. (2014) Design and application of cotranscriptional non-enzymatic RNA circuits and signal transducers. *Nucleic Acids Res.* 42, e58.
- (19) Green, A. A., Silver, P. A., Collins, J. J., and Yin, P. (2014) Toehold switches: de-novo-designed regulators of gene expression. *Cell* 159, 925.
- (20) Akter, F., and Yokobayashi, Y. (2015) RNA signal amplifier circuit with integrated fluorescence output. *ACS Synth. Biol.* 4, 655.
- (21) Ellington, A. D., and Szostak, J. W. (1990) *In vitro* selection of RNA molecules that bind specific ligands. *Nature* 346, 818.
- (22) Tuerk, C., and Gold, L. (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* 249, 505.

- (23) Bartel, D. P., and Szostak, J. W. (1993) Isolation of new ribozymes from a large pool of random sequences [see comment]. *Science* 261, 1411.
- (24) Wieland, M., Benz, A., Haar, J., Halder, K., and Hartig, J. S. (2010) Small molecule-triggered assembly of DNA nanoarchitectures. *Chem. Commun.* 46, 1866.
- (25) Nomura, Y., and Yokobayashi, Y. (2007) Reengineering a natural riboswitch by dual genetic selection. *J. Am. Chem. Soc.* 129, 13814.
- (26) Nutiu, R., and Li, Y. (2005) In vitro selection of structure-switching signaling aptamers. *Angew. Chem., Int. Ed.* 44, 1061.
- (27) Stoltenburg, R., Nikolaus, N., and Strehlitz, B. (2012) Capture-SELEX: selection of DNA aptamers for aminoglycoside antibiotics. *J. Anal. Methods Chem.* 2012, 415697.
- (28) Winkler, W. C., and Breaker, R. R. (2003) Genetic control by metabolite-binding riboswitches. *ChemBioChem* 4, 1024.
- (29) Sudarsan, N., Hammond, M. C., Block, K. F., Welz, R., Barrick, J. E., Roth, A., and Breaker, R. R. (2006) Tandem riboswitch architectures exhibit complex gene control functions. *Science* 314, 300.
- (30) Goodson, M. S., Harbaugh, S. V., Chushak, Y. G., and Kelley-Loughnane, N. (2015) Integrating and amplifying signal from riboswitch biosensors. *Methods Enzymol.* 550, 73.
- (31) Lentini, R., Santero, S. P., Chizzolini, F., Cecchi, D., Fontana, J., Marchiorretto, M., Del Bianco, C., Terrell, J. L., Spencer, A. C., Martini, L., Forlin, M., Assfalg, M., Dalla Serra, M., Bentley, W. E., and Mansy, S. S. (2014) Integrating artificial with natural cells to translate chemical messages that direct *E. coli* behaviour. *Nat. Commun.* 5, 4012.
- (32) Lynch, S. A., and Gallivan, J. P. (2009) A flow cytometry-based screen for synthetic riboswitches. *Nucleic Acids Res.* 37, 184.
- (33) Mishler, D. M., and Gallivan, J. P. (2014) A family of synthetic riboswitches adopts a kinetic trapping mechanism. *Nucleic Acids Res.* 42, 6753.
- (34) Martini, L., and Mansy, S. S. (2011) Cell-like systems with riboswitch controlled gene expression. *Chem. Commun.* 47, 10734.
- (35) Lemay, J. F., Desnoyers, G., Blouin, S., Heppell, B., Bastet, L., St-Pierre, P., Masse, E., and Lafontaine, D. A. (2011) Comparative study between transcriptionally- and translationally-acting adenine riboswitches reveals key differences in riboswitch regulatory mechanisms. *PLoS Genet.* 7, e1001278.
- (36) Rajendran, M., and Ellington, A. D. (2008) Selection of fluorescent aptamer beacons that light up in the presence of zinc. *Anal. Bioanal. Chem.* 390, 1067.
- (37) Rajendran, M., and Ellington, A. D. (2003) In vitro selection of molecular beacons. *Nucleic Acids Res.* 31, 5700.
- (38) Culler, S. J., Hoff, K. G., and Smolke, C. D. (2010) Reprogramming cellular behavior with RNA controllers responsive to endogenous proteins. *Science* 330, 1251.
- (39) Topp, S., and Gallivan, J. P. (2007) Guiding bacteria with small molecules and RNA. *J. Am. Chem. Soc.* 129, 6807.
- (40) Ichihashi, N., Usui, K., Kazuta, Y., Sunami, T., Matsuura, T., and Yomo, T. (2013) Darwinian evolution in a translation-coupled RNA replication system within a cell-like compartment. *Nat. Commun.* 4, 2494.
- (41) Lentini, R., Forlin, M., Martini, L., Del Bianco, C., Spencer, A. C., Torino, D., and Mansy, S. S. (2013) Fluorescent proteins and in vitro genetic organization for cell-free synthetic biology. *ACS Synth. Biol.* 2, 482.