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A versatile cis-blocking and trans-activation strategy for ribozyme characterization

SUPPLEMENTARY DATA

Supplementary Figure S1. Cis-blocked sTRSV HHRz constructs with varying stabilizing sequence lengths

Supplementary Figure S2. Development of additional cis-blocked HHRz constructs with type I and III topologies

Supplementary Figure S3. Quantification of activation efficiencies of the cis-blocked S. mansonii HHRz construct under varying assay conditions

Supplementary Figure S4. Cis-blocked constructs for synthetic ribozyme devices composed of different functional RNA components

Supplementary Figure S5. Gel analysis of the transcription products for the L2b8 construct without the cis-blocking sequence

Supplementary Figure S6. Representative gel-based cleavage assays for measuring cleavage rate constants ($k$) for RNA generated from the cis-blocking strategy

Supplementary Figure S7. Prediction of the dissociation rate constant of the 3’ cleaved fragment from the sTRSV HHRz duplex

Supplementary Figure S8. Representative SPR-based cleavage assays for measuring RNA dissociation rate constants ($kd$) for RNA generated from the cis-blocking strategy

Supplementary Figure S9. SPR-based characterization of cis-blocked ribozyme constructs under varying pH and MgCl₂ conditions

Supplementary Table S1. Sequences of primers, templates, and activators for all described cis-blocked constructs in this work
**Supplementary Figure S1.** Cis-blocked sTRSV HHRz constructs with varying stabilizing sequence lengths. The stabilizing sequence in the cis-blocked sTRSV HHRz construct (Figure 2) is increased in 2 nt increment to generate sTRSV-2, -4, -6, -8, and -10 HHRz constructs (Supplementary Table S1). The targeted ribozyme sequence, and the RNA blocking, activation, and stabilizing sequences are indicated in grey, red, yellow, and black, respectively. The additional 2, 4, 6, 8, and 10 nt to the stabilizing sequence in the original cis-blocked sTRSV HHRz construct results in a toehold length of 8, 6, 4, 2, and 0 nt, respectively. Secondary structures were predicted by RNAstructure folding software (32) and rendered using VARNA software (33).
(B) pLMVd HHRz

Blocked Conformation

DNA Activator

Activated Conformation
(C) *S. mansoni* HHRz
**Supplementary Figure S2.** Development of additional cis-blocked HHRz constructs with type I and III topologies. The cis-blocking and trans-activation strategy described in Figure 2 of the main text is applied to three additional HHRzs: (A) pLMVd, *S. mansoni*, and Smα1-CG HHRzs. Cleavage sites for each HHRz are indicated by the arrow. For each cis-blocked construct (B-D), an RNA blocking sequence (red) complementary to part of the ribozyme (targeted ribozyme sequence, grey) is directly incorporated into the 5’ end of the ribozyme transcript and can inhibit the ribozyme construct from folding into the active conformation during the transcription reaction. The activation sequence (yellow) provides a toehold for the DNA activator strand and the stabilizing sequence (black) can be modified to change the length of the toehold and the stability of the cis-blocked conformation. The stabilizing sequences are omitted from the initial cis-blocked (C) *S. mansoni* and (D) Smα1-CG HHRz constructs altogether because of the high predicted stability for the blocked conformations without the stabilizing sequence. The cis-blocked HHRz construct can be relieved by the addition of a DNA activator strand (brown),
which competes with the targeted ribozyme sequence for binding to the blocking sequence through a toehold-mediated strand-displacement reaction, thus allowing the HHRz to adopt an active conformation. Secondary structures were predicted by RNAstructure folding software (32) and rendered using VARNA software (33).
Supplementary Figure S3. Quantification of activation efficiencies of the cis-blocked *S. mansoni* HHRz construct under varying assay conditions. (A) The cis-blocked *S. mansoni* HHRz construct was incubated in HBS-N buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) in the absence or presence of 10 μM activator in 0.1, 1, 5, or 10 mM MgCl$_2$ for 1 or 2 hours at 25 or 37 °C. Denaturing PAGE was used to separate the uncleaved transcript and cleaved products, where the uncleaved and 5’ cleaved fragments are shown. A representative gel image is shown for the cis-blocked *S. mansoni* HHRz construct incubated without activator in 5 mM MgCl$_2$ for 2 hours at 37 °C (lane 1) and for all incubation conditions in the presence of 10 μM activator. A black vertical bar is used to denote samples run on different gels. (B) The uncleaved fragment fraction was quantified by autoradiography from at least three independent experiments. Incubations without activator are only presented for the 5 mM MgCl$_2$ experiments (black bars).
(B) L2b1

Blocked Conformation

Activated Conformation
(C) L2b5
(D) L2b8-a1

Blocked Conformation

Activated Conformation
(E) L2b8-a14

Blocked Conformation

Activated Conformation
(F) L2b8-a1-t41
(G) L2bOFF1

Blocked Conformation

Activated Conformation
Supplementary Figure S4. Cis-blocked constructs for synthetic ribozyme devices composed of different functional RNA components. The same blocking (targeting the same sTRSV HHRz sequence), activation, and stabilizing sequences shown in Figure 2 were directly incorporated into several previously designed synthetic ribozyme devices to generate the corresponding cis-blocked ribozyme constructs (24) (Supplementary Table S1). The targeted ribozyme sequence, RNA blocking, activation, stabilizing sequences, and DNA activator are indicated in grey, red, yellow, black, and brown, respectively, for the (A) L2b8 construct. The sensor (RNA aptamer), transmitter, and actuator (sTRSV HHRz) in the L2b8 device are indicated in green, blue, and white, respectively. For the subsequent constructs, only components that are different from those in the L2b8 device are indicated in brown. Both the blocked (top) and activated (bottom)
conformations are shown for each construct. The (A) L2b8, (B) L2b1, and (C) L2b5 devices are theophylline-responsive ribozyme ON devices (upregulate gene expression in response to input ligand) that differ slightly in the transmitter sequences. The (D) L2b8-a1, (E) L2b8-a14, and (F) L2b8-a1-t41 devices were modified from the L2b8 device by incorporating a ribozyme variant that exhibits improved cleavage activity. The (G) L2bOFF1 device is a theophylline-responsive ribozyme OFF device (downregulate gene expression in response to input ligand) that is composed of a transmitter sequence that is different from those in the ribozyme ON devices. The (H) L2b8tc device is a tetracycline-responsive ribozyme ON device that was generated by directly replacing the theophylline aptamer in the L2b8 device with the tetracycline aptamer. Secondary structures were predicted by RNAstructure folding software (32) and rendered using VARNA software (33).
Supplementary Figure S5. Gel analysis of the transcription products for the L2b8 construct without the cis-blocking sequence. PCR products, which were used as DNA templates for in vitro T7 transcription reactions, were amplified using forward and reverse primers T7-L1-2-fwd (5'-TTCTAATACGACTCACTATAGGGACCTAGGAAACAAACAAAGCTGTCACC) and L1-2-rev (5'-GGCTCGAGTTTTTATTTTTCTTTTTGCTGTTTC), respectively. The transcription reactions were performed in the absence and presence of 5 mM theophylline at the same reaction conditions as described for the generation of cis-blocked RNA (see Materials and Methods). A previously described non-switch control, L2Theo (3), which lacks the transmitter sequence in the L2b8 device (thus only adopting the ribozyme-active conformation and cleaves) was transcribed, resulting in 5' (25 nt) and 3' (109 nt) cleaved fragments. In addition, a ribozyme-inactive control, L2Theo Contl, generated by randomizing the ribozyme catalytic core in the L2Theo construct (thus abolishing the ribozyme cleavage activity) was transcribed, resulting in a full-length fragment (134 nt). The transcription products were size-fractionated on a denaturing (7 M urea) 10% polyacrylamide gel at 25 W for 45 min and imaged by phosphorimaging analysis. The transcription of the L2b8 construct without the cis-blocking sequence resulted in 5’ (25 nt) and 3’ (116 nt) cleavage fragments, and little full-length (141 nt) fragment was observed.
(A) L2b8:

(B) L2b8-a1:

(C) L2b8-a1-t41
(D) L2b8-a14

![Cleavage rate constant graph](image)

(E) sTRSV HHRz

![Cleavage rate constant graph](image)

(F)

| RNA Device     | Cleavage rate constant ($k$, min$^{-1}$) 0 mM theo | 5 mM theo |
|----------------|-----------------------------------------------|-----------|
| L2b8           | 0.063 ± 0.001                                 | 0.008 ± 0.002 |
| L2b8-a1        | 0.367 ± 0.007                                 | 0.045 ± 0.004 |
| L2b8-a1-t41    | 0.186 ± 0.008                                 | 0.0281 ± 0.003 |
| L2b8-a14       | 1.06 ± 0.08                                  | 0.33 ± 0.06 |
| sTRSV HHRz     | 4.0±1.6                                      | 3.0±0.7 |
Supplementary Figure S6. Representative gel-based cleavage assays for measuring cleavage rate constants ($k$) for RNA generated from the cis-blocking strategy. A representative assay is shown for each cis-blocked construct in the absence and presence (0 and 5 mM, respectively) of theophylline (theo): (A) L2b8, (B) L2b8-a1, (C) L2b8-a1-t41, (D) L2b8-a14 and (E) sTRSV HHRz. Bands for the full-length uncleaved substrate (UC) and longer cleaved product (3’C) are shown, the shorter 5’C product is omitted for clarity. Methods used to prepare full-length, activated RNA transcripts and conditions of the cleavage assays are detailed in the Materials and Methods section of the main text. Briefly, cis-blocked RNA was incubated with the DNA activator in a buffer (100 mM NaCl, 50 mM Tris-HCl (pH 7.5)) for 2 min to activate the blocked RNA. A zero time point aliquot was removed prior to initiating the reaction with addition of MgCl₂ to a final concentration of 500 μM. Reactions were quenched at the indicated time points. At least seven time points were taken in each cleavage assay to capture the cleavage dynamics of RNA devices exhibiting different cleavage kinetics. Phosphorimaging analysis of relative levels of the UC, 5’C, and 3’C bands was used to determine the fraction cleaved at each time point ($F_t$). The fraction cleaved at the beginning ($F_0$) and end of reaction ($F_\infty$) varied between assays, but all assays were well-fit to the single exponential equation ($R^2 > 0.95$):

$$F_t = F_0 + (F_\infty - F_0) \times (1 - e^{-kt})$$

The black and red fit lines represent assays performed at 0 and 5 mM theophylline, respectively. (F) The cleavage rate constant value ($k$) was determined for each assay. The reported $k$ for each device and theophylline assay condition is the mean and standard deviation of at least three independent experiments.
Supplementary Figure S7. Prediction of the dissociation rate constant of the 3’ cleaved fragment from the sTRSV HHRz duplex. The cleavage of sTRSV HHRz RNA results in 5’ and 3’ cleaved fragments, which are hybridized through five base pairs in the stem III of the sTRSV HHRz. The dissociation rate constant of the 3’ cleaved fragment (3C) from the 5’ cleaved fragment (5C) ($k_{PD}$) was predicted by a previously developed secondary structure nearest neighbor analysis (23). The analysis used the most recent parameters for the nucleic acid secondary structure prediction to calculate the thermal stability ($\Delta G = -6.65$ kcal/mol) of the secondary structure formed by the stem III of the sTRSV HHRz at 25°C (34). The equilibrium dissociation constant of the sTRSV HHRz duplex ($K_d = 1.33 \times 10^{-5}$ M, 25°C) was also determined similarly by calculating the thermal stability of the 5’ and 3’ cleaved fragments (5C, 3C), and the resultant ribozyme duplex. A previous study has shown that for short RNA duplexes the association kinetics between the two RNA strands is insensitive to either length or sequence composition (35). The association rate constant ($k_{PA} = 1.3 \times 10^8$ M$^{-1}$ min$^{-1}$, 25°C) has been previously determined for a well-characterized evolved HHRz (23,36) and was used as the association rate constant for the sTRSV HHRz duplex here. Finally, the dissociation rate constant ($k_{PD} = 1.73 \times 10^3$ min$^{-1}$, 25°C) was determined by calculating the ratio between $K_d$ and $k_{PA}$. This predicated rate constant is more than ~400 times faster than the fastest cleavage rate constant measured for the cis-blocked sTRSV HHRz RNA at 37°C (4.3 min$^{-1}$, Figure 4). It is anticipated that $k_{PD}$ will be faster at 37°C and thus the difference between $k_{PD}$ and $k$ is estimated conservatively here. Our analysis indicates that the dissociation of the 3’ cleaved fragment is not the rate-limiting step in the SPR-based assay.
(A) L2b8

(B) L2b8-a1

(C) L2b8-a14

(D) L2b8-a1-t41

(E) sTRSV-2 HHRz
**Supplementary Figure S8.** Representative SPR-based cleavage assays for measuring RNA dissociation rate constants ($kd$, min$^{-1}$) for RNA generated from the cis-blocking strategy. (A) A representative Biacore sensorgram, showing the complete experimental run (capture, reaction, and regeneration steps) is presented for L2b8. Subsequently, representative Biacore sensorgrams showing only the reaction period is shown for each cis-blocked construct: (B) L2b8-a1, (C) L2b8-a14, (D) L2b8-a1-t41, and (E) sTRSV-2 HHRz. The assays were performed in reactions buffer (100 mM NaCl, 50 mM Tris-HCl (pH 8.5)) containing: (grey line) no MgCl$_2$; (black line) 500 µM MgCl$_2$; and (red line) 500 µM MgCl$_2$ and 5 mM theophylline (theo) at 37 °C. Methods used to prepare full-length RNA transcripts and conditions of the cleavage assays are detailed in the Materials and Methods section of the main text. The reaction step of the sensorgram was well-fit to the single exponential equation ($R^2 > 0.95$):

\[
R_t = (R_0 - R_\infty) \times (e^{-kd t}) + R_\infty
\]

(F) The RNA dissociation rate constant ($kd$) was determined for each assay. The reported $kd$ for each construct are the mean and standard deviation of at least three independent experiments.
Supplementary Figure S9. SPR-based characterization of cis-blocked ribozyme constructs under varying pH and MgCl₂ conditions. The SPR-based cleavage assay was performed on two synthetic ribozyme devices, L2b8-a1 and L2b8-a14, to measure dissociation rate constants (kd) under varying (A) MgCl₂ concentrations (0-1 mM; fixed pH 7.3) and (B) pH (7.3-8.5; fixed 500 μM MgCl₂) in buffer containing 100 mM NaCl, 50 mM Tris-HCl at 37 °C. Dashed lines indicate assays performed in the presence of 5 mM theophylline. The dissociation rate constants increase as a function of pH and MgCl₂ concentration in agreement with trends observed in previous studies measuring cleavage rates through gel-based assays (21, 23, 26-28).
**Supplementary Table S1.** Sequences of primers, templates, and activators for all described cis-blocked constructs in this work. The T7 promoter region in the template sequence is indicated in bold. Unless listed, the activator sequence for the cis-blocked constructs is the same as the sTRSV activator sequence.

| Primer | Sequence |
|--------|----------|
| T7-fwd | TTCTAATACGACTCACTATAGGG |
| sTRSV-rev | CAAAGCTGTTTCGTCCTCAC |
| sTRSV-2-rev | AACAAAGCTGTTCGTCCTCAC |
| sTRSV-4-rev | CAAACAAAGCTGTTCGTCCTCAC |
| sTRSV-6-rev | AACAAACAAAGCTGTTCGTCCTCAC |
| sTRSV-8-rev | GAAACAACAAAGCTGTTCGTCCTCAC |
| sTRSV-10-rev | GGGAAACAACAAAGCTGTTCGTCCTCAC |
| pLMVd-rev | CAAATCATAAGTTTCGTCGCAT |
| S. mansoni-rev | GTGGATAGCAGTGGAATCCA |
| Smα1-CG-rev | AGATCCAGGACGCCG |
| L2b1-rev | CAAAGCTGTTTCGTCCTGTCCTCGTC |
| L2b5-rev | Same as b-L2b1-rev |
| L2b8-rev | CAAAGCTGTTTCGTCCTGTCCTCGTC |
| L2b8-a1-rev | Same as b-L2b8-rev |
| L2b8-a14-rev | Same as b-L2b8-rev |
| L2b8-a1-t41-rev | Same as b-L2b8-rev |
| L2bOFF1-rev | CAAAGCTGTTTCGTCCTGTCCTCGTC |
| L2b8tc-rev | Same as b-L2b8-rev |

| Template | Sequence |
|----------|----------|
| sTRSV | TTCTAATACGACTCACTATAGGGAAAAAACAAACAAAGTTGTTTGGT |
| sTRSV-2 | TTCTAATACGACTCACTATAGGGAAAAAACAAACAAAGTTGTTTGGT |
| sTRSV-4 | TTCTAATACGACTCACTATAGGGAAAAAACAAACAAAGTTGTTTGGT |
| sTRSV-6 | TTCTAATACGACTCACTATAGGGAAAAAACAAACAAAGTTGTTTGGT |
| sTRSV-8 | TTCTAATACGACTCACTATAGGGAAAAAACAAACAAAGTTGTTTGGT |
| sTRSV-10 | TTCTAATACGACTCACTATAGGGAAAAAACAAACAAAGTTGTTTGGT |
GTCACCCGGATGTGCTATTCCGGTCTGATGAGTCCGTGAGGACGAAACAGCTTTTGTTTGTTTCC

**pLMVd**
TTCTAATACGACTCATAATTAGGAACAAACAAACATATAAGTTCATTAAGTTCATTAAGTTCATTAAGG
TCATAATGCTGGCTATAAGCCCCTATGATGCTGCTGAAATAGCGACGAGAACAAACTATAGT

**S. mansoni**
TTCTAATACGACTCATAATAGGCCGACCGACTCGTACGCTAGGTGCTGAGGACGAAACAGCTTTTTCC
TTGTTTCC

**Smα1-CG**
TTCTAATACGACTCATAATAGGCACCGGACTCATCAGCTGGCCGACCGGCTACGATCCAGTCACTCGAGCTGACG
TCGCTGACGAGGACGAAACAGCTTTGTTTGCTGACCGGACTCATCAGCTGGCC

**L2b1**
TTCTAATACGACTCATAATAGGAACAAACAAACAAAGTTGTTTGCTGACCGGACTCATCAGCTGGCC
TCGCTGACGAGGACGAAACAGCTTTTTCC

**L2b5**
TTCTAATACGACTCATAATAGGAACAAACAAACAAAGTTGTTTGCTGACCGGACTCATCAGCTGGCC
TCGCTGACGAGGACGAAACAGCTTTTTCC

**L2b8**
TTCTAATACGACTCATAATAGGAACAAACAAACAAAGTTGTTTGCTGACCGGACTCATCAGCTGGCC
TCGCTGACGAGGACGAAACAGCTTTTTCC

**L2b8-a1**
TTCTAATACGACTCATAATAGGAACAAACAAACAAAGTTGTTTGCTGACCGGACTCATCAGCTGGCC
TCGCTGACGAGGACGAAACAGCTTTTTCC

**L2b8-a14**
TTCTAATACGACTCATAATAGGAACAAACAAACAAAGTTGTTTGCTGACCGGACTCATCAGCTGGCC
TCGCTGACGAGGACGAAACAGCTTTTTCC

**L2b8-a1-t41**
TTCTAATACGACTCATAATAGGAACAAACAAACAAAGTTGTTTGCTGACCGGACTCATCAGCTGGCC
TCGCTGACGAGGACGAAACAGCTTTTTCC

**L2bOFF1**
TTCTAATACGACTCATAATAGGAACAAACAAACAAAGTTGTTTGCTGACCGGACTCATCAGCTGGCC
TCGCTGACGAGGACGAAACAGCTTTTTCC

**L2b8tc**
TTCTAATACGACTCATAATAGGAACAAACAAACAAAGTTGTTTGCTGACCGGACTCATCAGCTGGCC
TCGCTGACGAGGACGAAACAGCTTTTTCC
| Activator       | Sequence                        |
|----------------|--------------------------------|
| sTRSV          | AAACAGCTTTGTTTGTTCCTCC         |
| pLMVd          | Same as sTRSV                  |
| S.mansonii     | CCAGCTGACGAGTCCGTGCGG          |
| Smα1-CG        | CCAGCTGATGAGTCCGTGCGCG         |