Synthetic feedback control using an RNAi-based control device
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Variable definitions:

\[ G_I = \text{nascent OFF control device transcript (without miRNA)} \]
\[ G_M = \text{full-length OFF control device transcript} \]
\[ G_{I-Bound} = \text{nascent OFF control device transcript (without miRNA) bound to input ligand} \]
\[ G_{M-Bound} = \text{full-length OFF control device transcript bound to input ligand} \]
\[ \text{miR} = \text{RISC-associated mature miRNA} \]
\[ P = \text{ligand that binds the ribozyme switch} \]
\[ TG = \text{target gene mRNA} \]
\[ TP = \text{target gene protein} \]
\[ k_{trs-OFF} = \text{transcription rate of the OFF control device transcript} \]
\[ k_{deg-OFF} = \text{degradation rate of the full-length OFF control device transcript} \]
\[ k_{cleave} = \text{cleavage rate of the unbound ribozyme switch} \]
\[ k_{cleave-bound} = \text{cleavage rate of the ligand-bound ribozyme switch} \]

\[ k_a = \text{association rate constant of ligand and ribozyme switch} \]
\[ k_d = \text{dissociation rate constant of ligand and ribozyme switch} \]
\[ k_{mature} = \text{rate of transition from } G_I \text{ to } G_M \]
\[ k_{miR} = \text{rate constant describing formation of mature miRNA from primary miRNA} \]
\[ k_{deg-miR} = \text{degradation rate constant of mature miRNA} \]
\[ K_m = \text{Concentration of } G \text{ at which miR degradation of the transcript is half of the maximum} \]
\[ k_{deg} = \text{degradation rate constant of target gene mRNA} \]
\[ k_{cat} = \text{catalytic constant for miR degradation of target gene transcript} \]

We developed a simple mathematical model describing the rate of microRNA formation resulting from our OFF control device. To accomplish this, we track the transcription of the OFF control device and divide it into two distinct steps. The first step is the transcription of the upstream ribozyme. The transcript in which the upstream ribozyme is transcribed and the downstream microRNA is not yet transcribed we call a nascent transcript, \( G_I \). A full-length transcript, \( G_M \), encodes the entire OFF control device, including the downstream microRNA(s), and is formed from a nascent transcript with a rate constant, \( k_{mature} \).

Both full-length and nascent transcripts include a ribozyme switch that is able to bind a ligand of interest, \( P \), with an association rate constant, \( k_a \), and a dissociation rate constant, \( k_d \). We define the transcripts bound to the ligand as new species, \( G_{I-Bound} \) and \( G_{M-Bound} \). The ribozyme switch cleaves at a rate \( k_{cleave} \) when unbound and at the rate \( k_{cleave-bound} \) when bound to the ligand. The model assumes that after ribozyme cleavage the transcript is rapidly degraded and no further transcription or microRNA formation can occur.

Mature miRNAs are formed from the pri-miRNA in the full-length transcripts at a rate \( k_{miR} \). We assume that once a pre-miRNA is formed from the pri-miRNA through microprocessor cleavage, the transcript that harbored the pri-miRNA is rapidly degraded.

These processes are described by the coupled ordinary differential equations provided below.
The level of target gene expression that results from different levels of miRNA within the cell is calculated from a previously described model\(^1\). Briefly, we describe miRNA-mediated transcript degradation using a Michaelis-Menten like term in which the RISC-associated mature miRNA complex (\(miR\)) acts as an enzyme and the target gene mRNA (\(TG\)) acts as the substrate.

\[
\frac{dG_I}{dt} = k_{\text{trS-OFF}} - k_{\text{cleave}}G_I - k_a PG_I + k_d G_I - \text{Bound} - k_{\text{mR}}G_M - k_{\text{mature}}G_I
\]  
\(1\)

\[
\frac{dG_M}{dt} = k_{\text{mature}}G_I - k_{\text{cleave}}G_M - k_a PG_M + k_d G_M - \text{Bound} - k_{\text{process}}G_M - k_{\text{deg-OFF}}G_M
\]  
\(2\)

\[
\frac{dG_{I-\text{Bound}}}{dt} = k_d PG_{I-\text{Bound}} - k_d G_{I-\text{Bound}} - k_{\text{cleave-bound}}G_{I-\text{Bound}} - k_{\text{mature}}G_{I-\text{Bound}}
\]  
\(3\)

\[
\frac{dG_{M-\text{Bound}}}{dt} = k_d G_{M-\text{Bound}} - k_{\text{mR}}G_{M-\text{Bound}} - k_{\text{deg-OFF}}G_{M-\text{Bound}}
\]  
\(4\)

\[
\frac{dmiR}{dt} = k_{\text{mR}}(G_M + G_{M-\text{Bound}}) - k_{\text{deg-mR}}miR
\]  
\(5\)

\[
\frac{dTG}{dt} = k_{\text{trS}} - k_{\text{deg}}TG - k_{\text{cat}} \cdot miR \cdot \frac{TG}{K_m + TG}
\]  
\(6\)

From this, the equation for target protein formation is:

\[
\frac{dTP}{dt} = k_{\text{trp}} - k_{\text{deg}}TP
\]  
\(7\)

Both \(k_{\text{cat}}\) and \(K_m\) are fitted parameters and are unique to each miRNA-target gene pair. The parameters for both GFP- and DsRed-targeting miRNAs were determined previously\(^1\) using least-squares non-linear regression curve fit for the implicit equation using Graphpad prism software and bounding the \(K_m\) with a maximum value of 10,000 nM, in accordance with values reported in the literature\(^2\).

The series of differential equations used in the model (equations 1-7) was solved using an ordinary differential equation solver in MATLAB (Mathworks, Ipswich, MA).
Parameters that were not obtained experimentally were estimated using a non-linear least squares optimization method using the MATLAB Optimization toolbox. A summary of the model parameters and how they were derived is provided in Supplementary Table 1.

Using these parameters, we modified the model slightly to describe the case of the OFF control device used to exert negative feedback control. Rather than having two variables to represent both the ligand of interest, \( P \), and the target protein, \( TP \), in this case the target gene protein is the input ligand of interest, and thus these variables are represented by a single variable, \( TP \). The feedback system can be represented using the block diagram above, where the maximum protein level is the input, target protein level is the output, and the miRNA level is the control action. The series of differential equations was solved in the same way as described for the OFF control device case.

\[
\frac{dG_I}{dt} = k_{trs-\text{OFF}} - k_{\text{cleave}} G_I - k_a(TP)(G_I) + k_d G_{I-\text{Bound}} - k_{\text{miR}} G_M - k_{\text{mature}} G_I
\]

\[
\frac{dG_M}{dt} = k_{\text{mature}} G_I - k_{\text{cleave}} G_M - k_a(TP)(G_M) + k_d G_{M-\text{Bound}} - k_{\text{process}} G_M - k_{\text{deg-\text{OFF}}} G_M
\]

\[
\frac{dG_{I-\text{Bound}}}{dt} = k_a(TP)(G_I) - k_d G_{I-\text{Bound}} - k_{\text{cleave-bound}} G_{I-\text{Bound}} - k_{\text{mature}} G_{I-\text{Bound}}
\]

\[
\frac{dG_{M-\text{Bound}}}{dt} = k_{\text{mature}} G_{I-\text{Bound}} - k_{\text{cleave-bound}} G_M + k_a(TP)(G_M) - k_d G_{M-\text{Bound}} - k_{\text{miR}} G_{M-\text{Bound}} - k_{\text{deg-\text{OFF}}} G_{M-\text{Bound}}
\]

\[
\frac{dm_iR}{dt} = k_{\text{miR}}(G_M + G_{M-\text{Bound}}) - k_{\text{deg-miR}} m_iR
\]
Supplemental Figure 1. Overview of ribozyme switch design. Ribozyme switches were designed by combining 3 distinct components: the sTRSV hammerhead ribozyme (grey), an aptamer (blue), and a transmitter (red) that joins the aptamer to loop II of the hammerhead ribozyme. With no ligand present, the ribozyme switch primarily adopts a conformation in which the ribozyme component is catalytically active and will cleave. The ligand will bind to a conformation in which the aptamer is formed, which shifts the conformational distribution to this ribozyme-inactive conformation such that cleavage is reduced. The sequences used in each ribozyme switch are listed in Supplementary Table 3 using the same color scheme to indicate the individual components.
Supplemental Figure 2. Non-cleaving ribozyme controls for different spacer lengths. GFP expression for OFF control devices with a non-cleaving ribozyme placed at various distances upstream of a GFP-targeting miRNA.
Supplementary Figure 3. Schematic representation of the computational model.
Schematic representation of the rates and variables used in the series of differential equations representing the OFF control devices studied in this work.
Supplementary Figure 4. Characterization of the E2F1-A1 ribozyme switch. (a) Representative sensorgrams associated with the surface plasmon resonance (SPR)-based \textit{in vitro} binding assay for the E2F1-A1 ribozyme across a range of ligand concentrations. Binding and dissociation constants were determined as $k_a$: $3.80 \times 10^4$ M$^{-1}$ min$^{-1}$, $k_d$: $1.88 \times 10^{-2}$ min$^{-1}$, $K_D$: $548.2$ nM. b) Fraction of the ribozyme switch RNA uncleaved after a 20 min incubation in presence and absence of E2F1 protein. Phosphorimaging analysis of relative levels of the uncleaved, 5’ cleaved, and 3’ cleaved bands was used to determine the fraction cleaved. Error bars represent the standard deviation of two independent experiments. (c) Representative radiogel images from an end-point ribozyme cleavage assay for the E2F1-A1 ribozyme switch. Bands for the full-length uncleaved substrate and longer 3’ cleaved product are shown, the shorter 5’ cleaved product is omitted for clarity.
Supplementary Figure 5. Base plasmids used to create all plasmids stably integrated into HEK293 Flp-In cells in this work. pCS2722 and pCS2934 were used to create the vectors that were subsequently stably integrated into HEK293 Flp-In cells. Each of the plasmids contained a hygromycin resistance gene flanked by FRT recombination sites. miRNAs were cloned behind the spacer region using the AgeI and ClaI restriction sites and ribozymes were cloned between the BstBI and Xmal restriction sites.
Supplementary Figure 6. Representative flow cytometry plots for HEK293 Flp-In cells stably expressing OFF control device constructs. (a) Plot showing side scatter vs. electronic volume for HEK293 cells, which was used to initially screen for viability. (b) A cell line lacking GFP was used to set the gate between the GFP negative population and GFP positive population. The GFP positive population was used in further data analysis. (c, d) Representative histograms depicting GFP expression from HEK293 Flp-In cell lines in the absence (c) and presence (d) of theophylline used in theophylline responsive control device experiments. Black: cell population expressing a theophylline OFF control device; red: cell population expressing no miRNA.
**Supplementary Table 1. Description of parameters used in model**

| Parameter          | value                | Determination                                      |
|--------------------|----------------------|---------------------------------------------------|
| $k_{trs-\text{OFF}}$ | 7.012 / min          | fit to data with bounds of $1 < k_{trs-\text{OFF}} < 10$ |
| $k_{\text{deg-\text{OFF}}}$ | 0.434 /M-min        | fit to data with bounds of $.1 < k_{\text{deg-\text{OFF}}} < 10$ |
| $k_{\text{transcribe}}$ | 0.511 /min          | fit to data                                       |
| $k_{\text{miR}}$     | 0.198 /min           | fit to data                                       |
| $k_{\text{deg-miR}}$ | 0.014 /min           | fit to data                                       |
| MS2-A3 $k_{\text{cleave-ribo}}$ | 0.228 /min     | calculated using gel-cleavage assay$^3$            |
| MS2-A3 $k_{\text{cleave-bound}}$ | 0.0563 /min | calculated using gel-cleavage assay$^3$            |
| L2B8-theo $k_{\text{cleave-ribo}}$ | .140 /min       | calculated using gel-cleavage assay$^4$            |
| L2B8-theo $k_{\text{cleave-bound}}$ | .025 / min      | calculated using gel-cleavage assay$^4$            |
| L2B8-theo $k_a$     | 9.0E6 /M-min         | calculated using SPR-based assay$^5$               |
| L2B8-theo $k_d$     | 3.78 /min            | calculated using SPR-based assay$^5$               |
| MS2-A3 $k_a$       | 4.52E4 /M-min        | calculated using SPR-based assay$^3$               |
| MS2-A3 $k_d$       | 1.34E-2 /min         | calculated using SPR-based assay$^3$               |
### Supplementary Table 2. List of plasmids used in this work

| Cell Line     | Expression Cassettes                                                                 | Figure       |
|---------------|--------------------------------------------------------------------------------------|--------------|
| Trex+pCS2407  | $\text{PCMV-d2EGFP-T}_{\text{bghpa}}$, $\text{PCMV-strsv-cntrl} - 500\text{bp spacer} - T_{\text{bghpa}}$ | 1b, 1c, S1   |
| Trex+pCS2873  | $\text{PCMV-d2EGFP-T}_{\text{bghpa}}$, $\text{PCMV-strsv-cntrl} - 1000\text{bp spacer} - T_{\text{bghpa}}$ | 1b, 1c, S1   |
| Trex+pCS2874  | $\text{PCMV-d2EGFP-T}_{\text{bghpa}}$, $\text{PCMV-strsv-cntrl} - 2500\text{bp spacer} - T_{\text{bghpa}}$ | 1b, 1c, 4b, 4c, 4e, S1 |
| Trex+pCS2875  | $\text{PCMV-d2EGFP-T}_{\text{bghpa}}$, $\text{PCMV-strsv-cntrl} - 500\text{bp spacer} - \text{GFP-miR-T}_{\text{bghpa}}$ | S1           |
| Trex+pCS2876  | $\text{PCMV-d2EGFP-T}_{\text{bghpa}}$, $\text{PCMV-strsv-cntrl} - 1000\text{bp spacer} - \text{GFP-miR-T}_{\text{bghpa}}$ | S1           |
| Trex+pCS2877  | $\text{PCMV-d2EGFP-T}_{\text{bghpa}}$, $\text{PCMV-strsv-cntrl} - 2500\text{bp spacer} - \text{GFP-miR-T}_{\text{bghpa}}$ | 1b, 1c, S1   |
| Trex+pCS2878  | $\text{PCMV-d2EGFP-T}_{\text{bghpa}}$, $\text{PCMV-L2B8-A1} - 500\text{bp spacer} - \text{GFP-miR-T}_{\text{bghpa}}$ | 1b, 1c       |
| Trex+pCS2879  | $\text{PCMV-d2EGFP-T}_{\text{bghpa}}$, $\text{PCMV-L2B8-A1} - 1000\text{bp spacer} - \text{GFP-miR-T}_{\text{bghpa}}$ | 1b, 1c       |
| Trex+pCS2880  | $\text{PCMV-d2EGFP-T}_{\text{bghpa}}$, $\text{PCMV-L2B8-A1} - 2500\text{bp spacer} - \text{GFP-miR-T}_{\text{bghpa}}$ | 1b, 1c       |
| Trex+pCS2881  | $\text{PCMV-d2EGFP-T}_{\text{bghpa}}$, $\text{PCMV-MS2-D5} - 2500\text{bp spacer} - \text{GFP-miR-T}_{\text{bghpa}}$ | 4b, 4e       |
| Trex+pCS2882  | $\text{PCMV-d2EGFP-T}_{\text{bghpa}}$, $\text{PCMV-E2F1-A1} - 2500\text{bp spacer} - \text{GFP-miR-T}_{\text{bghpa}}$ | 4c, 4e       |
| Trex+pCS2883  | $\text{PCMV-MS2-DsRed-T}_{\text{bghpa}}$, $\text{P}_{\text{TetO2}}$ - MS2-C3 - 2500bp spacer - $T_{\text{bghpa}}$ | 5d, 5e       |
| Trex+pCS2884  | $\text{PCMV-MS2-DsRed-T}_{\text{bghpa}}$, $\text{P}_{\text{TetO2}}$ - MS2-C3 - 2500bp spacer - GFP-miR - $T_{\text{bghpa}}$ | 5d, 5e       |
**Supplementary Table 3. Sequences of ribozymes and miRNAs used in this work**

Green indicates miRNA targeting sequence. Blue represents the aptamer sequence. Red indicates the transmitter. Bold indicates restriction sites used for cloning.

| Construct                  | Sequence                                                                                                                                 |
|----------------------------|------------------------------------------------------------------------------------------------------------------------------------------|
| miR-GFP                    | **ACCGGTGC** **GATC** **GGC** **GAACGGTGC** **CTGATACCAGCGTGAGCGAG** **CACAAGCTGGAGTACAACTATAGTGAAGCCACAGATGTA** **TAGTT** **GTACTCCAGCTTTGTGCC** **CGCCTACGCCCTTTGGCAGCGAGGCCCCG** **T** **TTAAATTAAATCGAT** |
| miR-DsRed                  | **ACCGGTGCGATCGC** **GCGCCTGGAGGCTTGCTGAAGGCTGTATGC** **TGTTGGAAGTCCACGTATGAATG** **TTTTTGGCCAC** **ACTGACCTA** **CTACTGTGGA** **ACTCCA** **AACAGGAC** **ACAAG** **GCT** **GTAGATGACTACACT** **ACATGGAAC** **AAATGGCC** **CGCG** **TTAATTAAATCGAT** |
| Non-targeting miRNA        | **ACCGGTGCGATCGC** **GAACGGTGC** **CTGCTGATACCAGCGTGAGCGAG** **CACAAGCTGGAGTACAACTATAGTGAAGCCACAGATGTA** **TAGTT** **GTACTCCAGCTTTGTGCC** **CGCCTACGCCCTTTGGCAGCGAGGCCCCG** **T** **TTAAATTAAATCGAT** |
| L2B8 theo (ribozyme switch) | **CAATTGG** **CTGTCACCCGG** **ATG** **TCTTCCGG** **GCTGATGAG** **TCCGTT** **GTC** **CCACCATCAGG** **GAG** **GAGGACGGAGGACGGAGG** |
| MS2-A3 (ribozyme switch)   | **CAATTGG** **CTGTCACCCGG** **ATG** **TCTTCCGG** **GCTGATGAG** **TCCGTT** **GTC** **CCACCATCAGG** **GAG** **GAGGACGGAGG** **GACGGGACGG** |
| E2F1-A1 (ribozyme switch)  | **CAATTGG** **CTGTCACCCGG** **ATG** **TCTTCCGG** **GCTGATGAG** **TCCGTT** **GTC** **CCACCATCAGG** **GAG** **GAGGACGGAGGACGG** |
| miRNA only                 | **CAATTGG** **CTGTCACCCGG** **ATG** **TCTTCCGG** **GCTGATGAG** **TCCGTT** **GTC** **CCACCATCAGG** **GAG** **GAGGACGGAGG** **GACGGGACGG** |
References

1 Bloom, R. J., WInkler, S. M., Smolke, C. D. A quantitative framework for the forward design of synthetic miRNA circuits. *Nature methods in press* (2014).

2 Haley, B. & Zamore, P. D. Kinetic analysis of the RNAi enzyme complex. *Nat Struct Mol Biol* 11, 599-606, doi:10.1038/nsmb780 (2004).

3 Kennedy, A. B. e. a. Protein responsive ribozyme switches in eukaryotic cells. *in review*.

4 Kennedy, A. B., Liang, J. C. & Smolke, C. D. A versatile cis-blocking and trans-activation strategy for ribozyme characterization. *Nucleic Acids Res* 41, e41, doi:10.1093/nar/gks1036 (2013).

5 Chang, A. L., McKeague, M., Liang, J. C. & Smolke, C. D. Kinetic and equilibrium binding characterization of aptamers to small molecules using a label-free, sensitive, and scalable platform. *Anal Chem* 86, 3273-3278, doi:10.1021/ac5001527 (2014).