## Supplemental Information

A Trigger and switch plasmid copy number comparison


C Detailed toehold switch design schematics


D In silico selection of orthogonal toehold switches


E Toehold switch positive control RNA design F


F Riboregulator performance comparison


G Sequence analysis of first-generation switches



H Toehold switch performance with different output proteins


Figure S1. Toehold Switch Design and First-Generation In Vivo Characterization, Related to Figure 1
(A) Trigger and switch plasmid copy number comparison. GFP fluorescence histograms obtained from expression of GFPmut3b-ASV from either a medium copy CoIA origin plasmid used for switch RNAs (blue curve) or a high copy ColE1 origin plasmid used for trigger RNAs (green curve).
(B) Illustration of ON/OFF fluorescence ratio calculation. ON/OFF fluorescence ratios were calculated from flow cytometry data by taking the mode fluorescence values from the ON state (switch with cognate trigger) and OFF state (switch with a non-cognate trigger) measurements. Autofluorescence was not subtracted from either fluorescence value prior to calculating the ON/OFF ratio.
(C) Detailed toehold switch design schematics. (i) Design specifics for the first-generation toehold switch library with 168 components. (ii) Design specifics for the forward-engineered toehold switches.
(D) In silico identification of orthogonal toehold switches. Visual representation of the Monte Carlo selection algorithm displaying values of the switch RNA stem integrity score for all pairwise interactions of a 646-component library. (i) A library with a randomly selected subset of 144 systems has many unwanted high scoring (high crosstalk) interactions signifying disruption of the switch stem region. (ii) A library selected through the Monte Carlo algorithm provides 144 systems (marked by the lower left square) with very low cross-interaction levels.
(E) Toehold switch positive control RNA design. Positive control constructs were designed to closely reflect the characteristics of the corresponding triggerswitch complex. A dummy hairpin was inserted upstream to mimic the RNA duplex of the trigger-switch complex and to minimize differences in transcription between the switch RNA and its positive control RNA. The active mRNA sequence of the ON state toehold switch was appended immediately after the dummy hairpin.
(F) Riboregulator performance comparison. All riboregulators were characterized in identical conditions. The mean ON/OFF levels of the first-generation and second-generation (forward-engineered) toehold switches are 3.4 -fold and 33 -fold higher, respectively, than the average ON/OFF measured for the older engineered riboregulators.
(G) Sequence analysis of first-generation switches. (i) Schematic showing the location of critical bases of the toehold switch in its repressed and active state. (ii) Evaluation of the 168-member first-generation toehold switch library as a function of the number of G-C base pairs in the top and bottom three base pairs in the switch RNA stem. Color of the background squares in the figure corresponds to the mean ON/OFF GFP fluorescence for the set of riboregulators that satisfy the specified G-C base pairing constraints. Color of the circles within each square corresponds to the actual ON/OFF ratio obtained for each of the components that satisfy the constraints.
(H) Toehold switch performance with different output proteins. Regulation of three different toehold switches from the first-generation library was tested using four additional fluorescent reporters: (i) cerulean, (ii) sfGFP, (iii) venus, and (iv) mCherry.

A Systematic in vivo studies of toehold switch design parameters



C Forward-engineered switch performance in different contexts


B Flow cytometry GFP fluorescence histograms of forward-engineered switches






D Thermodynamic analysis of first-generation toehold switches


Figure S2. Forward-Engineered Toehold Switches and Thermodynamic Analyses, Related to Figure 3
(A) Systematic in vivo studies of toehold switch design parameters. (i) Schematic showing the location of the region of the switch RNA loop being modified in the loop studies (left) and the effect of these modifications on the activated trigger-switch complex (right). (ii) Mode GFP output for the loop-variant switch RNA systems. ON and OFF state levels are given by the blue and red markers, respectively. Dashed lines are provided to guide the eye. Black curve is a fitting curve for the ON state fluorescence obtained from a sigmoid function. The standard switch RNAs from the first-generation library all had 11-nt loop domains. (iii) Schematic showing trigger RNAs with variable $3^{\prime}$ ends that bind starting at different positions along the toehold of the switch RNA. (iv) ON/OFF levels obtained from different switch RNA toehold lengths probed by the trigger. (v) Schematic showing trigger RNAs with variable $5^{\prime}$ ends that unwind different lengths of the switch stem. (vi) ON/OFF levels obtained for triggers that unwind different numbers of base pairs in the switch RNA stem.
(B) Flow cytometry GFP fluorescence histograms of forward-engineered switches. Fluorescence histograms obtained from flow cytometry for the switch in its OFF state are shown in blue and its ON state are shown in green. Cell cultures for each histogram were initially inoculated from a single $E$. coli colony. Histograms are provided for the top three and bottom four forward-engineered designs. Curves are offset for clarity.
(C) Forward-engineered switch performance in different contexts. (i) RNase deficient and wild-type RNase cells. Blue bars correspond to ON/OFF levels of forward-engineered switches in BL21 Star DE3, a strain with a mutated version of RNase E with decreased activity on mRNAs. Green bars correspond to values from BL21 DE3, a strain with the wild-type RNase E. (ii) Four strains using expression via the endogenous RNA polymerase. Forward-engineered switches were tested in BL21 Star DE3, BL21 DE3, DH5 $\alpha$, and MG1655Pro. Expression was driven by the strong PN25 constitutive promoter using the endogenous E. coli RNA polymerase.
(D) Linear regression map of thermodynamic terms. Map of $R^{2}$ values for a single parameter linear regression between one of 48 thermodynamic parameters and $\log _{10}$ (ON/OFF GFP fluorescence) of the first-generation toehold switches. y axis of matrix corresponds to different thermodynamic parameters each grouped into one of six main categories. $x$ axis corresponds to different subsets of the toehold switch library that were used for the linear regression. Subsets were defined by the strength of the base pair at the top of the toehold switch stem, with strong G-C base pairs denoted with " $S$ " and weak A-U base pairs denoted by "W." The number of switches in each subset is listed at the bottom of each column.


Figure S3. Design Rationale and Schematics of Toehold Switch mRNA Sensors, Related to Figure 4
(A) Detailed study of switch number 1 behavior. (i) Schematic illustration of the truncated triggers with variable $5^{\prime}$ ends studied with switch RNA number 1 . Switch RNA number 1 has an extra base pair at the bottom of its stem. G-C and A-U base pairs are denoted by black and green lines, respectively, in panels (i), (iii), and (iv). (ii) The ON/OFF GFP fluorescence of the toehold switch number 1 RNA upon activation by triggers designed to unwind different numbers of base pairs of the switch stem. Optimal dynamic range is observed when only the bottom 5 base pairs of the stem are programmed to be disrupted by the trigger. (iii) Interaction of the switch with a 13-nt trigger RNA. (Left) Schematic showing intended binding site of the trigger. (Right) MFE structure of trigger-switch complex. Trigger and its binding region are shaded blue and red, respectively, and emphasize the base of the switch stem not disrupted by the trigger. (iv) Interaction of the switch with a 16-nt trigger. (Left) Schematic showing binding site of trigger. (Right) MFE structure of trigger switch complex showing RNA refolding domain in the dashed red box and the additional stem base pair unwound.
(B) Thermodynamic analysis of switch number 1 behavior. (i) Correlation between ON/OFF GFP fluorescence and $\Delta G_{\text {RBS-linker }}$ for switch number 1 when interrogated by different trigger RNA lengths. (ii) Correlation between effective ON/OFF GFP fluorescence and $\Delta G_{\text {RBS-linker }}$ for the same data set. Effective ON/ OFF GFP fluorescence compensates for the effect of the different pre-RBS regions established by changes in trigger RNA lengths.
(C) Schematic of putative mechanism for switch number 1 translational enhancement. Depiction of the effect of a bound ribosome on the switch RNA as it begins translation and disrupts base pairs in the switch RNA stem. The overall effect the base pairs broken by the ribosome is an increase in the size of the pre-RBS region, which is associated with enhanced translation rates.
(D) Detailed mRNA sensor design schematic and putative detection mechanism. Secondary structure and domain sizes for the toehold switch mRNA sensors. The middle two rows of the schematic show the intermediate complex formed immediately after displacement of the sensor stem by the trigger mRNA. The newly exposed a domain refolds by binding to the downstream $\mathrm{a}^{*}$ domain and in turn triggers disruption of the $\mathrm{x}^{\star}$-x base pairs in the sensor stem. The depleted secondary structure surrounding the RBS is sufficiently weakened to enable the ribosome to begin translation of the reporter.


Figure S4. Endogenous Regulation Template Plasmid, Related to Figure 5
Map of the template endogenous regulation plasmid derived from pKD13. Priming sites 1, 4, and 5 are labeled P1, P4, and P5, respectively. Primer regions homologous to the target site in the chromosome are marked H 1 and H 2 .


Figure S5. Multiplexed Regulation Using Toehold Switches, Related to Figure 6
(A) Fluorescent protein standard curves used for compensation of flow cytometer data. Curves were taken from measurements on cells expressing a single fluorophore.
(B) Experimental data for cells activating mRNA 1 GFP switch compared to compensation fit.
(C) Experimental data for cells activating mRNA 1 venus switch compared to compensation fit.
(D) Experimental data for cells activating mRNA 1 cerulean switch compared to compensation fit.
(E) Experimental data for cells activating mRNA 1 mCherry switch compared to compensation fit.
(F) Compensated GFP fluorescence data for mRNA 1 GFP switch used for calculating percentage of cells expressing GFP.
(G) Compensated venus fluorescence data for mRNA 1 venus switch used for calculating percentage of cells expressing venus.
(H) Compensated cerulean fluorescence data for mRNA 1 cerulean switch used for calculating percentage of cells expressing cerulean.
(I) Compensated mCherry fluorescence data for mRNA 1 mCherry switch used for calculating percentage of cells expressing mCherry.


Layer 1 Plasmids


Figure S6. Layered 4-Input AND Logic Gate Plasmids, Related to Figure 7
Four different types of plasmids used for the AND gate circuit. There are four versions of each layer 1 plasmid to express all 16 permutations of the RNA inputs.

