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Supplemental Information

Toehold Switches: De-Novo-Designed Regulators of Gene Expression

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Extended Experimental Procedures

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S1. Detailed growth and toehold switch expression conditions

Unless otherwise mentioned, cells were all grown in LB media at 37°C. Antibiotics were used at the following concentrations: ampicillin (50 μg mL^{-1}), kanamycin (30 μg mL^{-1}), and chloramphenicol (34 μg mL^{-1}).

For first- and second-generation library testing, switch and trigger RNAs were expressed from separate plasmids with ColA and ColE1 origins, respectively. A GFP with an ASV degradation tag with a half-life of ~110 min (Andersen et al., 1998) was used as the reporter. In these experimental conditions, the copy number differences in the plasmids expressing switch and trigger RNAs led to a 6-8 fold excess of trigger compared to switch molecules as determined by fluorescence measurements of the GFP reporter expressed separately from each plasmid (see Figure S1A).

To characterize the toehold switches, chemically competent *E. coli* were transformed with the desired combination of switch and trigger plasmids, and spread onto LB/agar plates containing the appropriate antibiotics. For colony GFP fluorescence measurements, LB/agar plates were supplemented with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce RNA expression. For flow cytometry measurements, LB medium containing antibiotics was inoculated with cells picked from individual colonies and incubated overnight with shaking at 37°C. Cells were then diluted 100-fold into fresh selective LB medium and returned to shaking at 37°C and 900 rpm in 96-well plates. For T7 RNA polymerase driven expression in BL21 Star DE3 and BL21 DE3, cells were induced with 0.1 mM IPTG at 0.2-0.3 OD600 after 80 minutes of growth. Unless otherwise noted, measurements on cell cultures were taken 3 hours after addition of IPTG.

S2. Detailed plasmid construction

All DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. Double-stranded trigger and switch DNA was produced from either single > 100-nt oligonucleotides amplified using universal primers (see Table S1) or using gene assembly from short < 50-nt oligonucleotides segmented using the gene2oligo webserver (Rouillard et al., 2004). These PCR products were then inserted into vector backbones using Gibson assembly with 30-bp overlap regions (Gibson et al., 2009). Vector backbones were PCR amplified using the universal backbone primers shown in Table S1 and digested prior to assembly using DpnI (New England Biolabs, Inc.).

Backbones were generated from the T7-based expression plasmids pET15b, pCOLADuet, pCDFDuet and pACYCDuet (EMD Millipore). pET15b, pCOLADuet, pCDFDuet, and

pACYCDuet plasmids all contain a constitutively expressed lacI gene, a T7 RNA polymerase promoter and terminator pair, and the following respective resistance markers/replication origins: ampicillin/ColE1, kanamycin/ColA, spectinomycin/CDF and chloramphenicol/P15A. Reverse primers for the backbones were designed to bind to the region in the plasmid upstream of the T7 promoter. Forward primers for trigger backbones amplified from the beginning of the T7 terminator or after this terminator for triggers already containing a terminator. Forward primers for the switch backbones were designed to prime off the 5′ end of the desired output protein and add a 30-nt sequence containing the linker for Gibson assembly. Constructs were cloned inside DH5α and sequenced to ensure all toehold switch components were synthesized correctly. Plasmid transformations were performed using established chemical transformation protocols (Inoue et al., 1990), while linear DNA transformations were performed using electroporation.

S3. Flow cytometry data analysis

Flow cytometry datasets were analyzed using custom Matlab scripts. Data were first screened to remove any events with non-positive forward scatter (FSC), side scatter (SSC), or fluorescence intensity values. The remaining events were then used to generate a two-dimensional histogram with respect to FSC and SSC, each with bins defined on a logarithmic scale. The *E. coli* population had unimodal distributions in both FSC and SSC and thus provided a single peak in the two-dimensional histogram.

We used the maximum histogram value in this peak to define the gating area. This was done by first compiling a set of all coordinates in the two-dimensional histogram that had values of at least 10% of the maximum value in the peak. The gate was then defined by identifying the subset of these coordinates that formed a contiguous, closed area that contained the FSC and SSC coordinates of the maximum histogram value in the peak. This gate was then used to screen for acceptable events during subsequent analyses of cell fluorescence intensities.

Figure S1B shows representative GFP fluorescence histograms for cells in the toehold switch OFF state (expression of the switch RNA and a non-cognate trigger) and ON state (expression of the switch RNA and its cognate trigger). The mode fluorescence intensity from the histograms was used for calculations of ON/OFF fluorescence ratios. Since

fluorescence histograms from the cells was almost exclusively unimodal, mean, mode, and median fluorescence signals obtained from the cells had comparable values. We found that ON/OFF ratios based on the mode fluorescence levels had smaller colony-to-colony variations compared to those based on mean levels and yielded better correlations with thermodynamic parameters than median levels. In addition, mean fluorescence levels displayed greater sensitivity to low levels of contamination from the previous well sampled compared to mode and median fluorescence measures even under the most stringent cytometer washing conditions. In view of the above factors, we use the mode signal for all fluorescence levels reported in the main text and supplemental information.

S4. Crosstalk measurements using flow cytometry

For measurements of *in vivo* system crosstalk, single colonies of each of the 676 strains of transformed cells were measured using flow cytometry. To estimate colony-to-colony variations in GFP output for these strains, we measured a randomly selected subset of 18 transformants and assayed them in sextuplicate. The relative uncertainties for these measurements were 12% on average, which is comparable to uncertainties obtained for flow cytometry experiments used for determining ON/OFF fluorescence ratios for library components.

S5. Colony fluorescence imaging and image processing

Images of fluorescence from *E. coli* colonies were obtained using a Typhoon FLA 9000 biomolecular imaging system. All images were measured using the same PMT voltage, an imaging resolution of 0.1 mm, 473 nm laser excitation, and an LPB (>510 nm long pass) filter for detection of GFP. Induced cells were imaged ~18 hours after they were plated onto rectangular 8-well dishes and incubated at 37°C. Since IPTG exhibits low-level fluorescence in the same spectral region as GFP, variations in the thickness of the LB/agar in the plates result in variations in background fluorescence levels. In addition, the LB/agar in the plates is not perfectly level, which led to non-uniform background within each plate. Consequently, we used a plane subtraction algorithm to reduce the background fluorescence and to correct for variations in the flatness of LB/agar.

S6. *In silico* design and selection of toehold switches

This section describes the initial computer-based stages of the toehold switch generation process, namely design specification, *de novo* RNA sequence design, and *in silico* screening and selection.

S6.1. Design specification: Specification of RNA sequences and secondary structure

The first stage in the design process involves definition of the toehold switch secondary structure, conserved sequences, and interaction domain sizes. These parameters are detailed in Figure S1C for both the 168-component first-generation toehold switch library (Figure S1Ci) and the forward-engineered systems (Figure S1Cii). Colored regions in Figure 1 and Figure S1C represent regions with pre-determined sequences. These regions are the RBS of the mRNA (AGAGGAGA), the T7 RNA polymerase transcriptional terminator (see Table S1), the 21-nt linker region between the hairpin module and coding sequence of the regulated gene (see Table S1), and a GGG leader sequence that encourages efficient transcription by the T7 RNA polymerase.

S6.2. De novo RNA sequence design: NUPACK-based sequence generation

After definition of system parameters, the NUPACK software package (Zadeh et al., 2011a) was used to design toehold switch sequences satisfying the specified constraints. The NUPACK design algorithm computes candidate RNA sequences and progressively refines them until their deviation from the specified design constraints falls below a specified stop condition. In designing toehold switches, stop conditions were imposed on three different RNAs:

(1) the trigger RNA
(2) the switch RNA
(3) the trigger-switch complex that forms upon switch activation.

The complete trigger transcript, including a stabilizing 5′-hairpin and transcriptional terminator, was considered for the design algorithm. To reduce computational load for the switch RNA design, only the first 50-nts following the hairpin module were simulated. This region encompasses the conserved 21-nt linker and the first 29-nts of the GFPmut3b-ASV gene. Consequently, these switch designs are optimized for regulation of GFPmut3b-ASV;

however, they retain their functionality when regulating other output proteins (see Section S10.1).

The trigger-switch complex formed by the complete trigger RNA and the truncated switch RNA was considered for designs. The desired secondary structure for the complex was specified to be completely single-stranded in the bases following the **b** domain of the switch RNA. This single-stranded specification meant that the toehold switch designs were implicitly designed to favor lower magnitudes of the critical $\Delta G_{RBS\text{-}linker}$ term. Free energies specified in Serra and Turner 1995 (Serra and Turner, 1995), a temperature of 37°C, 1.0 M Na^+, and 0 M Mg^{2+} were used for the design algorithm. To preclude runs of the same nucleotide or pairs of nucleotides, the following sequence patterns were prevented by the design algorithm: AAAA, CCCC, GGGG, UUUU, KKKKKK, MMMMMM, RRRRRR, SSSSSS, WWWWWW, YYYYYY.

It is important to note that since NUPACK optimizes by considering the defect levels over the ensemble of RNAs, optimal designs can differ in their predicted minimum free energy (MFE) secondary structure from the intended design secondary structure. In some cases, this ensemble optimization can lead to additional base pairs in the stem of switch RNA at its base or in the AUG bulge region. The latter defect occurred often in the forward-engineered toehold switches likely as a means to compensate for weak base pairing imposed at the top of the stem. In other cases, this optimization led to toehold switches with strongly negative $\Delta G_{RBS\text{-}linker}$ values (see Figure 3D-E), which were correlated with lower switch dynamic range.

S6.3. In silico screening: Fast removal of unwanted designs

The resulting toehold switch designs were then screened to ensure they had no in-frame stop codons that would prematurely terminate translation of the output gene and to remove any duplicate sequences. A pool of 672 toehold switch designs with randomized sequences were generated for the first-generation library. Of the resulting designs, 25 were found to encode stop codons in the hairpin region after the start codon. In the remaining systems, one duplicate design was found leaving 646 unique toehold switch designs in the pool.

S6.4. In silico selection: Identification of designs with minimal crosstalk

The screened toehold switch designs were then analyzed for their orthogonality. We began this process by first computing the pairwise interactions between the trigger RNA and switch RNA sequences. Simulations were performed with a local implementation of NUPACK using the `complexes` and `concentrations` functions. The NUPACK functions were run with a specified temperature of 37°C, 1.0 M Na^+, and 0 M Mg^{2+} using Serra and Turner 1995 (Serra and Turner, 1995) free energy parameters, and assumed strand concentrations of 100 nM. The output of these functions provided the free energies of the individual RNA strands and the bimolecular trigger-switch complex, and the predicted concentrations and minimum free energy secondary structures of each of the three species in solution. In the case of the initial 144-component orthogonal toehold switch library, a total of 646 possible designs were considered, requiring 417,316 different pairwise interactions to be simulated. To reduce computational load, the switch RNA was truncated at the three bases following the hairpin module. Two different scoring terms from the large dataset of pairwise interactions were used to assess the orthogonality of groups of toehold switches.

S6.4.1. The stem integrity score

The stem integrity score reflects perturbations to the ideal stem region secondary structure caused by interactions of the switch RNA with trigger RNAs. It is calculated from the MFE structure of the trigger-switch complex computed by NUPACK. For instance, the stem of the initial library of switch RNAs has the following prescribed structure in dot-bracket notation, where "." specifies unpaired bases and "(" and ")" are used to denoted paired bases:

```
(((((((((...((((((..........))))))...)))))))))
```

If the corresponding bases in trigger-switch complex have the identical secondary structure, then the resulting stem integrity score is 0. However, for cognate trigger-switch interactions, stem unwinding by the trigger RNA exposes bases downstream of the RBS. This interaction can lead to a secondary structure of the following form:

```
(((((((((((((((((((.............................
```

A position-by-position comparison is made between the ideal and predicted stem secondary structures and the total number of bases with changes is computed, in this case yielding 18 for the string of 47 characters. The number of modified bases is normalized by the length of the ideal stem structure leading to a stem integrity score of 0.38. Consequently, higher stem integrity scores signify larger perturbations to the stem structure and in turn the degree of de-repression caused by the trigger-switch interaction. Although there are many other

potential ways of computing this stem weakening effect, for instance by using base pairing probabilities tables, we found this metric was simple to implement, fast to compute, and captured the critical features of potential crosstalk interactions.

S6.4.2. Scoring via the predicted trigger-switch complex concentration

The second scoring term we used for computing orthogonality was the predicted concentration of the trigger-switch complex. Simulated yields of the trigger-switch complex for cognate RNA interactions were uniformly 100% for the toehold switch systems, while non-cognate interactions had complexes with yields ranging between 0 to 100%. The majority of these strong non-cognate interactions arose from trigger binding to regions of the switch toehold domain and/or the downstream linker, and thus high yields of a non-cognate trigger-switch complex do not necessarily suggest a high level of crosstalk. As a result, we used the concentration of the trigger-switch complex as a more stringent metric for orthogonality after initial screening via stem integrity. In particular, this trigger-switch concentration metric is likely important for multiplexed operation of toehold switches *in vivo* since unwanted non-cognate interactions could interfere with cognate trigger-switch interactions.

S6.4.3. A Monte Carlo algorithm to select subsets of orthogonal switches

The scoring terms generated from pairwise interaction simulations were then used to generate libraries of orthogonal toehold switches using a Monte Carlo selection algorithm. The crosstalk between various toehold switches was determined using either one or a combination of the scoring terms discussed above, except scoring terms defined for cognate RNA interactions were set to zero. A target orthogonal library size M was defined for the complete set of N designs. To begin the algorithm, a random set of M designs was then selected and the resulting library assessed for orthogonality through a global orthogonality metric. This global orthogonality metric was defined either as the sum of the scoring terms for all pairwise interactions of the M selected toehold switches or as the maximum scoring term from the same set of all M^2 pairwise interactions.

After evaluation of global orthogonality, a random member of the M system library was selected for mutation and replaced sequentially with 10 (or $M - N$ if $10 > N - M$) different designs not present in the set of M and the global orthogonality metric calculated for the mutated library. From the resulting set of 11 libraries (including the original library), the one with the lowest global orthogonality score was passed into the next iteration of the Monte

Carlo algorithm whereupon another library slot was mutated. This selection process was repeated for a total of 10,000 iterations to return a set of *M* orthogonal toehold switches. In addition, the full algorithm was run 10 times, with the requisite 10,000 optimization rounds, but with different random initial libraries of *M* components to increase the probability that a true optimum combination of designs was selected.

S6.4.4. Selection of 144 orthogonal switches for first-generation library

Figure S1D demonstrates the outcome of this Monte Carlo selection algorithm when it was applied to 646 candidate designs to generate the library of 144 orthogonal toehold switches. These switches were selected using the stem integrity scoring function and global orthogonality was assessed from the sum of all pairwise interactions in the subset. Based on random selection of 144 components, the average global orthogonality metric for the library is 474.7 (Figure S1Di). In contrast, the Monte Carlo selection algorithm yielded a global orthogonality metric of 6.8 (Figure S1Dii), an improvement of nearly a factor of 70 compared to random selection. In Figure S1Dii, the 144 orthogonal members of the library are given indices 1 to 144 in the matrix of stem integrity scores and the remaining components are ordered in terms of increasing net crosstalk levels for indices greater than 144. The square defined by the (144,144) coordinate and the graph axes contains many fewer white pixels in Figure S1Dii compared to the corresponding square in Figure S1Di demonstrating the effectiveness of the algorithm at eliminating crosstalk interactions.

S6.4.5. Selection of 26 orthogonal switches for the in vivo crosstalk assay

The subset of 26 *in vivo* validated first-generation toehold switches assayed for orthogonality was also selected using the Monte Carlo algorithm. This subset was compiled from the top 35 switches identified from the initial orthogonal library of 144. The concentration of the trigger-switch complex was used as the scoring function for selection and the maximum value of the scoring function from the set of pairwise interactions was used for the global orthogonality metric.

S7. Design of positive controls for ON state expression

During initial testing of the toehold switches, we found that many of the highest activity switches provided GFP expression levels that exceeded that of a standard positive control *GFP* mRNA with the same 8-nt RBS sequence. This initial positive control mRNA had the sequence:

GGGACAGAACAATAGAACAAGAAC**AGAGGAGA**TATACC,

where the RBS is marked in bold. In the absence of the downstream mRNA, this sequence was designed to have zero secondary structure.

In view of this effect, we chose to specifically design new positive controls that approximated the secondary structure of the activated trigger-switch complex and encoded a protein using identical codons as the toehold switch in its ON state (Figure S1E). We decided to avoid direct recapitulations of the trigger-switch complex in which the complete trigger sequence is inserted upstream of the switch sequence. Encoding both trigger and switch in a single strand of RNA in this way would result in a transcript with a 30-bp hairpin stem with strong potential for rho-independent terminator activity. Instead, we employed a dummy hairpin with the same secondary structure as the hairpin of the corresponding switch, including a 12-nt toehold. This dummy hairpin was inserted immediately upstream of the loop region of a given switch and its toehold was designed not to interact with any of the downstream bases. The resulting positive control mRNAs thus retained the 5′ hairpin stabilizing effects, if any, of the repressed switch RNAs; encoded a reporter protein using identical codons; and would exhibit similar transcriptional efficiency as the corresponding switch RNA. These positive control constructs exhibited similar levels of expression as their toehold switch counterparts in their activated state suggesting approximately complete activation of switches by the trigger RNAs (Figure 1C-D). The RNA sequences of the positive controls are provided in Table S1.

S8. Comparison of toehold switches and engineered riboregulators

We measured the performance of the engineered riboregulators reported by Isaacs et al. (Isaacs et al., 2004) in identical experimental conditions as the toehold switches. In these experiments, the taRNA and crRNA transcripts were both expressed using T7 RNA polymerase with the same vector backbones used for the toehold switches. The crRNA module was inserted upstream of the GFPmut3b-ASV reporter. Experiments were also conducted in BL21 Star DE3 with 0.1 mM IPTG induction and flow cytometry measurements were taken 3 hours after induction. Figure S1F presents the ON/OFF GFP fluorescence values obtained from crRNA10 and crRNA12 engineered riboregulators. Both systems provide dynamic range in the 10- to 15-fold range and displayed OFF state fluorescence that was similar to the toehold switches. Thus the main cause of the relatively lower dynamic range

of the engineered riboregulators is their reduced ON state protein output. For comparison, Figure S1F also provides the mean ON/OFF GFP fluorescence measured for the two libraries of toehold switches. The first-generation switches provide 3.4-fold increase in typical dynamic range compared to the older system, while the forward-engineered switches provide 33-fold improvement on average.

S9. Sequence dependence of first-generation switch performance

The ON/OFF ratios of the toehold switches exhibit a strong dependence on the sequences at the top and bottom of the stem of the switch RNA (Figure S1Gi). Prior to delving into these sequence-dependent effects, we hypothesized that the strength of the base pairing at the bottom of the stem in the switch RNA would modulate its repression strength, as these base pairs are essential to sequestering the start codon. Furthermore, the bases at both the top and bottom of the stem also would also affect the secondary structure of the RBS and mRNA region once the switch is activated and thereby influence translational efficiency (Kudla et al., 2009).

Analysis of the top and bottom three base pairs in the stem of the switch RNA revealed significant variations in the ON/OFF ratio of the devices as a function of the G-C base pair content in these regions. Figure S1Gii displays the average ON/OFF fluorescence obtained for all 16 possible permutations of G-C content in the two stem regions, as well as the ON/OFF values obtained for each toehold switch that satisfied the specified G-C conditions. Based on the size of the library and secondary structure constraints imposed during *in silico* design, a number of G-C permutations had only one or two representative toehold switches. As a result, it is difficult to make definitive statements regarding switch performance under these G-C conditions. However, we can conclude toehold switches containing zero and two G-C base pairs at the top and bottom regions of the stem, respectively, displayed an average ON/OFF fluorescence ratio of 154, over three times higher than the next highest permutation. Mean ON/OFF levels also tended to decrease as G-C combinations deviated further from this optimum combination.

S10. Toehold switch performance in different contexts

To assess their behavior in different contexts, we investigated the performance of the toehold switches in regulating new proteins, in different strains of *E. coli*, and using

transcription from the endogenous *E. coli* RNA polymerase. The toehold switches preserved their regulatory capacity in all these experiments.

S10.1. Toehold switches successfully regulate different output proteins

We performed experiments regulating proteins other than the GFPmut3b-ASV reporter used for most of the experiments in the main text. The new output proteins were superfolder GFP (sfGFP) (Pedelacq et al., 2006), venus, cerulean, and mCherry, which provided four different emission wavelengths for facile characterization via flow cytometry. A degradation tag was not added to the four proteins. To construct plasmids with these new reporters and enable future multiplexing, we used pACYCDuet (P15A origin) as the backbone for these systems. This enabled existing GFPmut3b-ASV toehold switches based on pCOLADuet (ColA origin) to be co-expressed with the new reporters in the same cell if desired.

New reporter plasmids were produced using Gibson assembly with the reporter gene inserted after the switch hairpin module and before the T7 RNA polymerase terminator. Since the switch sequences were optimized for regulation of GFPmut3b, we attempted to minimize disruption to the regulator by preserving the sequence of the first 30-nts immediately following the hairpin module. This 30-nt region contains the 21-nt linker and the first three codons of *GFPmut3b*. Downstream of this region, the complete sequence of each reporter gene was preserved. Three different toehold switches were used for testing the different reporters. Of these switches, two were high-performance switches (numbers 1 and 3) from the first-generation 144-component orthogonal library and the third was a loop variant switch that featured a 27-nt loop and was derived from toehold switch number 80 (see Section S12.1 and Table S1).

Figure S1H displays the ON/OFF fluorescence ratios obtained for the set of 12 different switches. Flow cytometry was performed on cells after three hours of induction with IPTG. All systems provide greater than 10-fold activation upon expression of the trigger RNA; however, their ON/OFF levels are not as high as those observed for regulation of GFPmut3b-ASV. We attribute much of this lowered dynamic range to the properties of the reporters themselves.

S10.1.1. Effects of reporter sequence and fluorescence on switch dynamic range

In control expression experiments, GFPmut3b-ASV provided much stronger fluorescence than all four of the new reporters relative to the background fluorescence levels measured for induced cells not expressing any reporters. Since our ON/OFF metric does not subtract this background fluorescence level from the OFF state nor the ON state fluorescence, the relatively higher background fluorescence for these reporters leads to a decrease in reporter ON/OFF ratio.

Differences in the secondary structure of the switch RNAs caused by the new downstream gene could also have an effect on the output of the switches. Although we found that the $\Delta G_{RBS\text{-}linker}$ term was the best descriptor for switch output, other mRNA secondary structure related terms, in particular $\Delta G_{RBS\text{-}GFP}$ which considers a region that spans 29-nt into *GFPmut3b*, also displayed a significant correlation with switch performance for several subsets in the first-generation library (see Section S13 and Figure S2D). Finally, differences in the relative copy numbers of the P15A switch plasmid and ColE1 trigger plasmid may have caused some changes in the dynamic range with the new reporters. Experiments measuring GFPmut3b-ASV expressed from either plasmid indicated a 5-7 fold excess of trigger plasmid copy number compared to the switch plasmid, which was slightly lower than the 6-8 fold difference observed for the ColE1/ColA combination (Figure S1A).

S10.2. Toehold switches retain their activity in non-RNase-deficient E. coli

The BL21 Star DE3 strain used for most of the toehold switch experiments contains a truncated form of RNase E that lacks the portion of the native enzyme responsible for mRNA degradation. We measured the performance of several forward-engineered toehold switches in BL21 DE3, a relative of BL21 Star DE3 with the fully functional wild-type RNase E. Figure S2Ci shows the ON/OFF GFP fluorescence levels obtained for five different toehold switches measured in both strains 3 hours after induction with IPTG. The dynamic range for the switches is similar in both strains, which indicates that RNase-deficient conditions are not essential for toehold switch activity.

S10.3. Toehold switches exhibit >100-fold dynamic range when transcribed using the endogenous E. coli RNA polymerase

We also investigated the performance of the switches upon transcription using the endogenous *E. coli* RNA polymerase, as opposed to the T7 RNA polymerase used for earlier

experiments. We employed the strong phage-derived PN25 constitutive *E. coli* promoter (Brunner and Bujard, 1987) for these experiments and used it to express both trigger and switch RNAs. We made a few modifications to the toehold switch components to adjust for expression from the endogenous polymerase. Transcription of the trigger strand was terminated using the his [min] (S) terminator (Cambray et al., 2013), and a hairpin was added to the 5′ end of the switch RNA in an effort to increase its stability (Carrier and Keasling, 1999) and insulate it from any earlier bases transcribed from the promoter (see Table S3 for RNA sequences). Using these updated designs, we characterized a pair of forward-engineered toehold switches in four different *E. coli* strains: BL21 Star DE3, BL21 DE3, DH5・ and MG1655Pro.

Overnight cultures of cells transformed with different combinations of the trigger and switch strands were diluted 100-fold into fresh media. The cells were left shaking over 4 hours at 37°C and then characterized using flow cytometry. The ON/OFF GFP fluorescence levels from these experiments are shown in Figure S2Cii. Both forward-engineered switches provide at least 100-fold activation in all four *E. coli* strains. We found that the dynamic range for the toehold switches was lower than that observed using T7 RNA polymerase-based transcription. While some of this effect could be due to the different polymerase, we found that growth of cells was slowed significantly for activated cells when using the strong *PN25* constitutive promoter. In contrast, growth rates were normal when a switch was co-expressed with a non-cognate trigger. This difference in growth rates likely decreased the rate of GFP production for the ON state cells and also led to relatively greater leakage of GFP from OFF state cells expressing comparatively larger amounts of switch RNAs. As a result, these two effects led to decreases in the observed dynamic range for the switches.

S11. Identification of orthogonal sets of toehold switches

Sets of orthogonal toehold switches were determined as a function threshold crosstalk level. Crosstalk levels were first calculated from flow cytometry data by taking the mode GFP fluorescence intensity of a trigger-switch combination and dividing it by the mode GFP fluorescence intensity obtained for the same switch with its cognate trigger RNA. The resulting crosstalk matrix was then converted into a scoring matrix **S** defined by the specified threshold crosstalk level δ as:

$$S_{ij} = \begin{cases} 0, & \text{if } C_{ij} < \delta \text{ or if } i = j \\ 1, & \text{if } C_{ij} \geq \delta \end{cases} \quad (1)$$

We then input this scoring matrix into the Monte Carlo selection algorithm described in Section S6.4.3. Since this algorithm was designed to select optimal orthogonal subsets of a specified size M using the scoring matrix, we progressively lowered the value of M until the algorithm returned a subset of switches for which all the corresponding elements in **S** were zero. Hence with this process, we were able to identify sets of toehold switches that all had crosstalk levels below δ across the entire set. Table S2 provides a conversion table to convert the orthogonal index used in Figure 2 to a toehold switch number. Table S2 lists the elements in the orthogonal toehold switch sets for multiple threshold crosstalk levels.

S12. Systematic studies of toehold switch design parameters

S12.1. Effects of switch RNA loop size

After observing increased activation for toehold switches having weak base pairs at the top of their stems (Figure S1G), we undertook a study of toehold switch behavior as a function of the size of the loop in the hairpin module. Previous studies have found that A/U rich regions in front of the RBS can enhance the rate of translation (Vimberg et al., 2007). Furthermore, footprinting studies of prokaryotic ribosome-mRNA complexes have shown that the 30S subunit can protect bases up to 35-nts upstream of the start codon (Huttenhofer and Noller, 1994). These sequence-specific and steric factors suggest that it is possible to tune the ON state expression of the toehold switches simply by engineering the so-called pre-RBS sequence between the trigger binding site and the RBS of the switch RNA (Figure S2Ai). We found the least perturbative approach to studying this effect was by increasing the size of switch RNA loop, specifically by adding bases in front of the RBS domain. This enabled us to study a series of loop-engineered toehold switch variants activated by the same trigger RNA and maintain a constant distance between the RBS and the start codon.

S12.1.1. Design of loop-variant switches

We first selected a toehold switch from the library that exhibited medium range activation, as this would enable us to probe changes in switch dynamic range without pushing the limits of our characterization methods. The particular system was toehold switch number 80 from the first-generation library, which had an ON/OFF fluorescence of 19 ± 4 and an A-U base pair at the top of its stem. Next we used NUPACK to design a number of 27-nt-long candidate loops, each candidate containing the same 11-nts at the 3′ end. These 11-nts

were taken from the original loop sequence of switch number 80. We then calculated the ensemble defect of the resulting hairpin modules as nucleotides were removed from the 5′ end of the loop. We selected the 27-nt domain that displayed the optimal overall ensemble defect from the set of loop-variant hairpins, and constructed a series of switches with loops of 27-, 24-, 21-, 18-, 15-, 12-, and 9-nts, with the loop sequence of shorter loops defined by removing 3-nts from the 5′ end of the next shortest loop (see Table S1 for RNA sequences). Since the loop sequence was designed to have low internal binding, it generally has 50% or more A sequence content (the one exception is the 9-nt loop) and could provide translational enhancement via its high A/U content (Vimberg et al., 2007).

S12.1.2. Behavior of loop-variant switches

The loop-engineered toehold switches were evaluated using flow cytometry and yielded the ON and OFF state fluorescence levels shown in S2Aii. Increasing loop size led to dramatic increases in the ON state GFP fluorescence with the 27-nt loop displaying a 45-fold increase in fluorescence compared to the original 11-nt loop system. The loop size effect appears to be nearing saturation at 27-nts, this corresponds to a pre-RBS region beginning 33-nts upstream of the start codon and roughly agrees with the observed 30S subunit footprint on mRNA (Huttenhofer and Noller, 1994).

Nevertheless, increasing loop sizes had a detrimental effect on system OFF state. 21- and 27-nt loops exhibited ~5-fold increase in fluorescence leakage and fluorescence histograms for the 27-nt system in particular showed a broader distribution of intensities. We attribute this effect to two factors: the increase in entropy caused by the longer loop may decrease the probability of proper hairpin module folding, thereby reducing repression; and the longer single-stranded region nearby the RBS may encourage stable docking by the ribosome, which increases background translation. The combined effects of loop size on the ON and OFF state fluorescence yielded optimum ON/OFF levels for loops 15- to 18-nts.

S12.1.3. Fitting of experimental ON state data

To facilitate modeling and design of future toehold switches, we used a sigmoidal function to fit the measurements of the loop-engineered toehold switch ON state fluorescence. A sigmoid was used for fitting since we expected that translational enhancement achieved by increasing the length of the switch loop should eventually saturate for sufficiently large loops. The sigmoid function is given by the following function:

$$\log_{10} ON(L) = \frac{\beta}{1+\exp[-\kappa(L-L_0)]} \quad (2)$$

where ON is the ON state mode fluorescence, L is loop size of the switch RNA, and β = 4.88, κ = 0.44, and L_0 = 9.95 are the fitting parameters. The resulting fit is shown as the black curve in Figure S2Aii.

S12.2. Effects of trigger RNA length and binding site

We carried out studies of toehold switch behavior as a function of the length of the trigger RNA and the region over which it bound to the switch RNA. The sequences of the switch and set of trigger RNAs used for these experiments are included in Table S1. The switch RNA had bulges in its stem at locations 9-nts and 14-nts above the stem base, in contrast to the standard first-generation toehold switches with bulges at positions 10 to 12, corresponding to the start codon. Although the findings described in this section should be understood to apply only to this particular toehold switch, they provide useful information for understanding switch behavior and could aid in the design of future riboregulator systems.

S12.2.1. Toehold switch ON/OFF level increases with toehold length

Systematic measurements of ON/OFF GFP fluorescence for the toehold switch as a function of the length of the toehold domain were carried out. Experiments were performed using a series of triggers programmed to bind starting at different positions along the toehold domain of the switch (Figure S2Aiii). While the toehold-binding 3′ end of the triggers varied, the 5′ bases were left constant and designed to unwind up to the top of the switch RNA stem. As shown in Figure S2Aiv, systems with toehold lengths of -2-nts (i.e. unwinding of the top 16 base pairs of the 18-nt stem) to 6-nts demonstrated little activation of protein translation. The transition between a 6- and 8-nt toehold leads to a strong increase protein expression and ON/OFF fluorescence continues to increase strongly up to the complete 12-nt toehold domain.

S12.2.2. Partial unwinding of switch RNA stem by the trigger can increase ON/OFF level

A similar set of trigger RNAs was used to probe the effects of the stem unwinding length on the output of the toehold switch as illustrated in Figure S2Av. For these experiments, the 3′ toehold binding end of the trigger was held constant, corresponding to a toehold length of 12. However, the 5′ end was varied to unwind different numbers of bases from the base pairs of the switch RNA stem. Very little system leakage is observed when only the bottom

4- to 8-nts of the stem are unwound as shown in Figure S2Avi. (Note this behavior is drastically different for toehold switch number 1 as described in Section S14.1.) Between 8- to 14-nts, a ~50-fold increase in activation is observed as repression of the start codon region is relieved. Maximum ON/OFF fluorescence ratios are observed for triggers that unwind 14- to 16-nts of the stem and decrease by ~40% for the complete 18-nt branch migration process. We posit this optimum trigger length is related to the switch RNA loop size effects described in Section S12.1. The 4- to 2-nts in the stem that remain unwound for these shortened triggers are likely to breath open spontaneously at 37°C and thus yield similar effects to those observed with switches having increased loop sizes.

S13. Thermodynamic analysis of toehold switch performance

In order to develop an understanding of the important thermodynamic parameters governing toehold switch performance, we used NUPACK to rapidly evaluate 48 thermodynamic parameters described in detail in Table S3. These parameters were divided into six general categories:

1. **Free energies of individual RNAs:**
   These terms are potentially useful for determining the strength of repression for the switch RNA and for evaluating the single-strandedness for the trigger RNA binding region and the activated trigger-switch complex.
2. **Deviations from ideal secondary structures*:***
   These parameters are calculated from the difference between the free energy of the RNA sequence in its predicted secondary structure compared to its free energy in its ideal, programmed secondary structure. These parameters therefore reflect the deviation of the actual RNA structure from its intended state.
3. **Net reaction free energies:**
   These terms reflect energy changes that occur upon binding for different toehold switch components. For instance, the free energy of binding for the toehold domain to its reverse complement was calculated. In addition, the net free energy change of trigger-switch binding was also calculated for this category.
4. **RBS/mRNA secondary structure:**
   These parameters take into consideration the secondary structure of the RBS and mRNA coding regions for the toehold switch in its activated state. More negative

free energies imply stronger secondary structures, which are well known impediments to efficient translation (Bentele et al., 2013; Kudla et al., 2009).

5. **Stability of top of toehold switch stem region:**
   These parameters are focused on the free energy of different sub-sections of the top of the toehold switch stem. They provide a means of converting the heuristic design rules used for the forward-engineered switches into a quantitative metric that can be used for modeling.
6. **Stability of bottom of toehold switch stem region:**
   These terms are the counterparts of the previous category but focus on bases at the bottom of switch stem region.

Each of these parameters returns an output measured in units of free energy (kcal mol^{-1}), simplifying regressions over multiple parameters, unlike other potentially useful metrics such as GC content or ensemble defect levels. The 48 thermodynamic parameters were calculated for all members of the 168-component first-generation library and the 13 forward-engineered switches.

Following the treatment by Salis et al (Salis et al., 2009), we screened these thermodynamic parameters using the relation $p \propto \exp(-k\Delta G)$, where p is the amount of expressed protein and k is a fitting parameter. ON/OFF ratios as opposed to fluorescence output in the ON and OFF states alone were used for quantitative analysis since fluorescence OFF levels varied relatively little over the library compared to ON levels, leaving ON/OFF ratios essentially a measure of ON state fluorescence. A custom Matlab script was used to evaluate linear regressions between $\log_{10}$(ON/OFF GFP fluorescence) and each of the thermodynamic parameters. Although strong correlations were not observed for the first-generation switch library when analyzed as a whole, significant correlations began to emerge as we began to analyze subsets of the library that satisfied different sequence criteria. In particular, we found that base pair composition near the top of the switch RNA stem provided a useful means through which to categorize different classes of switches. We found that thermodynamic terms produced using Mathews et al., 1999 (Mathews et al., 1999) energy parameters produced stronger correlations than those returned using Serra and Turner, 1995 (Serra and Turner, 1995). Thus the former set of energy parameters were used for thermodynamic analyses in this study.

Figure S2D provides a map of the coefficients of determination R^2 obtained from linear regressions of single thermodynamic parameters versus toehold switch ON/OFF performance. Each column in the map was generated from subsets of the first-generation library that satisfied base pairing identities at the top of their stems specified above each column. Weak A-U base pairs are denoted "W", while strong G-C base pairs are denoted "S". Inspection of the R^2 map reveals that the RBS/mRNA secondary structure terms constitute the category of parameters with the strongest overall correlation with the observed toehold behavior. Correlations are particularly strong for those subsets in which there is a weak base pair at the top of the stem, with $\Delta G_{RBS\text{-}linker}$ consistently providing better R^2 values than the rest of the RBS/mRNA secondary structure terms.

In a few cases, some correlation is observed with stem stability terms, for instance ΔG stem bot. 12 for the WSS subset. Also, $\Delta G_{RBS\text{-}GFP}$ provides a very high R^2 value for the SSS subset. However, due to the limited size of both subsets (11 members each), it is difficult to draw strong conclusions regarding these effects. Occasional hot spots in the R^2 map are also observed for ΔG terms linked to the RNA-RNA complex, in particular Dev. ΔG complex and Dev. ΔG min. complex. These terms are affected by the secondary structure of the RBS/mRNA region, which suggests that these correlations are another manifestation of the RBS/mRNA translational efficiency effects generally better described by $\Delta G_{RBS\text{-}linker}$.

S14. Design rationale and selection of toehold switch mRNA sensors

The toehold switch mRNA sensors are all derived from toehold switch number 1. In this section, we will describe the properties of toehold switch number 1 that facilitated its use as a system for detecting active mRNAs, and describe the rationale and specifics behind the mRNA sensor design. We will also detail the selection process used to identify optimal binding sites for the mRNA sensors.

S14.1. Properties of toehold switch number 1

Out of the initial library of 168 systems, toehold switch number 1 provided the highest observed ON/OFF GFP fluorescence ratio at 290 ± 20. The stem of its switch RNA contains an additional base pair at its base predicted by MFE calculations. This base pair forms between the U at the 3′ end of its intended toehold that binds to the first A at the 5′ end of the linker. This additional base pair likely compensates for the unusually low GC content of

the stem, which places the switch RNA in the 96th percentile in terms of low stem GC content out of the 168 switches in the first-generation library. Of the six G-C base pairs in this stem, half are found in the bottom five base pairs (Figure S3Ai). Consequently, disruption of these five base pairs leaves a weak stem containing 11 base pairs of which only three were G-C bonds.

S14.1.1. Toehold switch number 1 exhibits extreme sensitivity to truncated triggers

While studying toehold switch number 1, we unexpectedly discovered that a truncated trigger RNA containing only the latter 15-nts of the trigger sequence (i.e. the 3′ half of the 30-nt trigger) provided an extremely high ON/OFF ratio exceeding 1000, despite the MFE-predicted 11-nt toehold of the switch RNA. We proceeded to investigate this phenomenon in greater detail, probing the switch response with trigger RNAs 9-, 10-, 11-, 12-, 13-, 14-, 15-, 16-, 17-, 18-, 19-, 20-, 21-, 24-, and 27-nts in length, each truncated by removing bases from the 5′ end of the base trigger (Figure S3Ai; see Table S4 for sequences). Figure S3Aii presents the ON/OFF fluorescence obtained for each of these triggers as a function of the intended number of base pairs of the switch RNA unwound and the length of the trigger programmed for binding to the switch. Between two and five base pairs unwound (corresponding to 13- and 16-nt trigger RNAs), there is striking change in the ON/OFF ratio, which increases from 3.1 ± 0.5 to 1900 ± 200. NUPACK simulations of the trigger-switch complexes for each of these situations are shown in Figure S3Aiii-iv and suggest a potential explanation for this effect. The 13-nt trigger RNA shown in Figure S3Aiii binds to the 11-nt toehold and displaces one stem base pair, but it is not capable of unwinding the second base pair of the stem. In contrast, the 16-nt trigger RNA in Figure S3Aiv unwinds one more base than expected as a result of RNA refolding induced by interaction between newly exposed bases and the downstream linker. The remaining 10 intact base pairs in the stem have 7 A-U bonds, which are ostensibly weak enough to be disrupted by the ribosome and/or stem breathing.

S14.1.2. Correlation between switch number 1 output and $\Delta G_{RBS\text{-}linker}$

Since uncovering the importance of $\Delta G_{RBS\text{-}linker}$, we have also examined the behavior of toehold switch number 1 with respect to this thermodynamic parameter. We calculated $\Delta G_{RBS\text{-}linker}$ by evaluating the free energy of a subsequence of the switch RNA starting immediately after the expected binding site of the trigger through to the last base of the 21-nt linker. Figure S3Bi presents the relationship between toehold switch ON/OFF ratio as function of $\Delta G_{RBS\text{-}linker}$. The resulting linear regression has an R^2 = 0.878, but does not

perform well in predicting the behavior of the highest dynamic range triggers with corresponding free energies above -15 kcal mol^{-1} (marked by a red box in Figure S3Bi).

S14.1.3. Explaining switch number 1 behavior using $\Delta G_{RBS\text{-}linker}$ and translational enhancement effects

To better describe these outliers, we posited that translational enhancement effects studied for the loop-engineered switches (see Section S12.1) could also play in behavior of switch number 1. If translation is occurring in a switch whose trigger only partially unwinds its stem, it follows that base pairs in switch RNA downstream of the RBS are broken by the active ribosome (Figure S3C). Consequently, bases upstream of the RBS are also unpaired and can contribute to enhancing translational output. We derived an effective ON/OFF GFP fluorescence level that compensated for these translational enhancement effects using the following equation:

$$\text{Effective ON/OFF} = \frac{\text{ON/OFF}}{ON(L)} \quad (3)$$

with $ON(L)$ calculated using equation (2) and the fitting parameters provided in Section S12.1.3. The values of L for equation (2) were defined as the length of the subsequence of the switch RNA starting immediately after the intended binding site of the trigger through to the end of the RBS. The resulting effective ON/OFF GFP fluorescence, normalized by dividing the terms by the lowest effective ON/OFF value, is plotted against $\Delta G_{RBS\text{-}linker}$ in Figure S3Bii. The effective ON/OFF values yield a significantly improved correlation with R^2 = 0.962. This result indicates that while $\Delta G_{RBS\text{-}linker}$ is useful for describing the probability of translation as it corresponds to the energy required for the ribosome to begin translation, additional terms that describe the local environment of the ribosome when it docks must also be considered to place limits on the maximum level of expression should translation occur.

S14.2. Toehold switch mRNA sensor design parameters

The capacity for toehold switch number 1 to activate strongly after unwinding only the bottom five base pairs of its stem made it an attractive system for detecting mRNAs with strong secondary structure as described in the main text. We adapted toehold switch number 1 to detecting mRNAs as shown in Figure S3D. To retain much of the high ON state expression from the system, the top 12-nts of the switch RNA number 1 stem were preserved. The loop of the hairpin module was increased from 11- to 18-nts to enhance ON state signal. To facilitate binding to mRNA regions with potentially high secondary structure,

we increased the toehold domain of the switch from 12-nts to 24- or 30-nts depending on the specific mRNA sensor. The bottom 6 base pairs of the sensor stem were then programmed to be unwound upon trigger mRNA binding. Consequently, the length of the trigger mRNA bound to by the sensor was 30- to 36-nts depending on the toehold length.

Inspired by the mechanism shown in Figure S3Aiv, we explicitly programmed RNA refolding into the sensors to decrease the energetic barrier for switch activation. Refolding was incorporated into the systems by adding domains **a*** and **x*** downstream of the hairpin module along with a 3-nt **y** spacer domain (Figure S3D). Upon the trigger mRNA binding to the first 6 base pairs of the stem, the RNA refolding mechanism leads to a total of 9 base pairs being unwound from the stem. This effect results in an activated complex with similar secondary structure to the 7-nts of stem unwound case shown in Figure S3Aii with an ON/OFF ratio of 1300 ± 100.

Several of the modifications made to generate an mRNA sensor with high dynamic range could increase system leakage. These changes include: reduction in hairpin stem length by 1-bp, removal of some G-C base pairs at the bottom of the stem, increase in the loop size of the switch, and potential decrease in stem stability caused by the RNA refolding module. Lastly, the stem-loop region created by RNA refolding can have a detrimental effect on $\Delta G_{RBS\text{-}linker}$, in turn lowering system ON state. The length of this stem, however, can be decreased to potentially increase sensor translation rate.

S14.3. Selection of toehold switch mRNA sensor designs

Putative mRNA sensors were simulated using a local implementation of NUPACK and assessed using custom Matlab scripts. For a specified trigger mRNA, the NUPACK pairs function was used to compute base pairing probabilities for all pairs of bases over the entire mRNA. The resulting pair probability matrix was used to compute the local single-strandedness for all possible sensor binding sites along the trigger mRNA. This metric is calculated using the following equation:

$$l = \frac{\sum_i P_{ii}}{b} \tag{4}$$

where l is the local single-strandedness, P_{ii} is the probability that the base at position i is unpaired, and b is the length of the mRNA sensor binding site (either 30- or 36-nts depending on the design). The local single-strandedness is equivalent to the normalized

ensemble defect (Zadeh et al., 2011b) metric implemented in NUPACK, except it is applied to only a portion of the RNA rather than the entire RNA sequence.

For each mRNA binding site, the corresponding mRNA sensor was defined and scanned for the presence of stop codons in the coding region. The quality of its secondary structure assessed using the ensemble defect. Local single-strandedness was also calculated over the sensor toehold region to score its availability for binding to the trigger mRNA.

After computing defect metrics for the sensors over all possible mRNA binding sites, the resulting designs were scored using the following function:

$$\phi(i) = \beta_1 l_{mRNA} + \beta_2 l_{toehold} + \beta_3 n_{sensor} \quad (5)$$

where $\phi(i)$ is the design score for the sensor at location i of the mRNA, l_{mRNA} is the local single-strandedness of the mRNA at the sensor binding site, l_{toehold} is the local single-strandedness of the toehold of the sensor, and n_{sensor} is the normalized ensemble defect of the sensor. The score weight factors used were β_1 = 5, β_2 = 4, and β_3 = 3. The resulting scores were sorted from lowest to highest and used to select optimal mRNA sensor designs. In cases where more than one sensor was tested for a particular mRNA, an additional design criterion was added to prevent selection of two designs having toehold switch binding regions within 15-nts of each other. Since nearby binding sites tended to have very similar scores, this criterion ensured that sensors did not cluster around the same binding region on the mRNA. The sequences for the resulting mRNA sensors and the subsequences of the mRNAs used for triggering are listed in Table S4.

S15. Characterization of toehold switch RyhB sRNA sensor

The toehold switch RyhB sRNA sensor (see Table S4 for sequences) was constructed using the same design and *in silico* validation process as the mRNA sensors described in Section S14.3. The sensor was inserted into a pET15b-derived vector containing the ColE1 origin of replication and ampicillin resistance. An ASV-tagged GFP was used as the reporter and expression of the sensor-*GFP* module was driven by the *proD* constitutive promoter (Davis et al., 2011). The resulting plasmid was transformed into MG1655Pro for sensing experiments.

Output from the sensor was obtained using flow cytometry. An overnight culture of the MG1655Pro/RyhB-sensor strain was diluted 100-fold into fresh LB/ampicillin media supplemented with 100 μM $FeSO_4$ and shaken at 37°C and 900 rpm in a 96-well plate. After 80 minutes, aliquots of the early log phase culture were supplemented with an aqueous solution of 2,2′-bipyridyl to achieve the desired concentration of the iron chelator. GFP expression was evaluated 1 hr after addition of the inducer. Comparison control measurements using a constitutively expressed GFP were performed in identical conditions. We also conducted control experiments using exogenously expressed RyhB sRNA and an off-target RNA (data not shown). The RyhB sensor activated GFP expression in response to the exogenously-expressed RyhB, but remained inactive for the off-target RNA confirming its specificity for the intended target RNA.

S16. Regulation of endogenous genes using toehold switches

S16.1. Design and construction of template insertion plasmids

Template plasmids used for generating the linear DNA fragments for λ Red recombination were derived from the plasmid pKD13 (Datsenko and Wanner, 2000). Like pKD13, the template plasmids contain the conditionally replicative *oriRγ* origin that requires *pir+* hosts such as BW25141 in order to replicate. PCR and Gibson assembly were used to insert the switch module downstream of priming site 4 (primer P4) of pKD13 as shown in Figure S4. The sequences of the switch modules used in the template plasmids are shown in Table S5. Primers listed in Table S5 were used to amplify the region of the template running from priming site 1 (primer P1) up to the conserved linker sequence of the switch module at priming site 5 (primer P5). Each of these primers has 50-nts of homology with the desired insertion site in the chromosome. Homology regions were designed to insert the switch module in frame with the target endogenous gene and to replace the endogenous RBS site with that of the switch RNA.

S16.2. Chromosomal integration of switch modules

Electrocompetent MG1655Pro containing the λ Red recombinase plasmid pKD46 (Datsenko and Wanner, 2000) was transformed with the linear DNA produced from the template switch plasmids. After several hours of recovery with shaking at 37°C, cells were spread on kanamycin (25 μg mL^{-1}) plates and incubated overnight at 37°C to select cells that had

integrated the switch/kanamycin-resistance cassette and to encourage loss of the pKD46 plasmid with its temperature sensitive origin of replication. Transformants were then screened for phenotype (see Section S16.3) to ensure the correct gene had been disrupted. Confirmed transformants were then grown overnight at 37°C and transformed with the FLP recombinase expressing plasmid pE-FLP (St-Pierre et al., 2013). We found that pE-FLP excised the kanamycin marker via its flanking FRT sites with high efficiency and was successfully cured during growth at 37°C. The resulting switch-edited strains of MG1655Pro were then transformed with different plasmids expressing switch RNAs under the control of either $P_{LtetO-1}$ or $P_{LlacO-1}$ inducible promoters from pET15b-derived plasmids (ColE1 origin, ampicillin resistance).

S16.3. Characterization of switch-edited strains

Plate-based assays were used to characterize the switch-edited MG1655Pro strains. Plates were imaged using a flatbed scanner and images adjusted for brightness and contrast.

S16.3.1. uidA::Switch A and uidA::Switch B

Cells containing disrupted *uidA* genes were assayed on plates containing the substrate X-Gluc (5-Bromo-4-chloro-3-indolyl-β-D-glucuronide sodium salt) at 25 μg mL^{-1}, the inducer IPTG at 0.2 mM, and ampicillin. Cells were spread onto the plates and incubated overnight at 37°C.

S16.3.2. lacZ::Switch C

lacZ::Switch C cells were assayed on plates containing ampicillin and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 40 μg mL^{-1}. The chemical inputs IPTG and aTc were added to the plates as required at concentrations of 0.2 mM and 500 ng mL^{-1}, respectively. Cells were spread onto plates and incubated overnight at 37°C.

S16.3.3. cheY::Switch D

cheY::Switch D cells were assayed for motility on ampicillin/LB/0.25% agar plates. IPTG was added to the plates at a concentration of 0.2 mM. Cells were inoculated at the center of the plates and incubated for ~24 hours at 37°C. While cells with disrupted *uidA* and *lacZ* genes regained the full phenotype of the wild-type genes upon trigger RNA expression, wild-type *cheY* showed approximately 5-fold higher motility than activated *cheY*::Switch D cells.

S17. Multiplexed regulation using toehold switches

S17.1. Design and construction of multiplexing plasmids

Construction of the plasmids for the multiplexing system required multiple cloning stages. First, we synthesized new codon optimized versions of GFP, venus, and cerulean that had significantly decreased homology. GFP, venus, and cerulean have very similar amino acid sequences, with GFP deviating from venus and cerulean at only 8 positions out of 236, and venus and cerulean differing from one another at only 10 sites out of 236. As result, insertion of all three proteins into a single vector could lead to genetic instability in the plasmid as a result of homologous recombination. Accordingly, we employed a Monte-Carlo-based algorithm that randomly inserted synonymous codons into each of the proteins to select new RNA sequences for the three proteins that simultaneously displayed low overall homology and made use of commonly used *E. coli* codons. We then used gene assembly (Rouillard et al., 2004) to construct plasmids bearing the new codon-optimized proteins (see Table S6 for sequences) and confirmed successful assembly using DNA sequencing. Next, we extracted switch modules from existing toehold switch plasmids and inserted them upstream of the new proteins and also mCherry. Following this step, we had a complete set of source plasmids for expressing trigger RNAs and switch RNAs coupled to the four output fluorescent proteins.

Trigger and switch-protein sequences were then amplified from the source plasmids using PCR. A set of orthogonal, 30-bp domains were used in this PCR stage to create 30-nt homology domains flanking the amplicons to facilitate Gibson assembly of plasmids for expressing up to four different toehold switch components. Plasmids for expressing multiple triggers were derived from the pET15b vector (ColE1 origin, ampicillin resistance). These plasmids expressed each trigger from its own T7 promoter and each trigger also had its own T7 terminator (see Table S6 for trigger construct DNA sequences). Multi-trigger plasmid construction was verified using DNA sequencing. Plasmids expressing multiple switch RNAs employed only a single T7 promoter and, consequently, transcribed four switch-protein modules as a ~3.4-kb polycistronic mRNA accompanied by a single T7 terminator (see Table S6 for switches used for each mRNA). Successful assembly of these multi-switch plasmids was confirmed using PCR and by transforming cells with the switch plasmids and cognate trigger plasmids to screen by phenotype. Three different plasmids were used for testing: a

pCDFduet-derived plasmid with spectinomycin resistance, a pCOLAduet-derived plasmid with kanamycin resistance, and a pACYC-derived plasmid with chloramphenicol resistance.

Notably, the switch RNA sequences in the multiplexing system were not modified in any way to improve their secondary structure in their new context within the polycistronic mRNAs. For instance, the 30-nt Gibson assembly sequences used for cloning the constructs were not screened to eliminate interactions with the nearby toehold domains of the switch RNAs, neither were toehold interactions with new codon-optimized proteins. As a result, our success in regulating twelve components in a cell using these toehold switch sequences suggests they are quite robust to changes in surrounding sequences and can be used in a "plug-and-play" fashion.

S17.2. Characterization of the multiplexing system

The multiplexing system was evaluated using BL21 Star DE3 cells. Cells were first transformed with the trio of multi-switch plasmids. Chemically competent cells from this strain were then transformed with the set of 24 trigger/multi-trigger plasmids shown in Figure 6 and incubated on spectinomycin (25 μg mL^{-1}), ampicillin (50 μg mL^{-1}), chloramphenicol (17 μg mL^{-1}), and kanamycin (30 μg mL^{-1}) plates.

For flow cytometry measurements, six colonies from each of the 24 transformants were inoculated into LB media with the same antibiotic concentrations used for plating. These cells were grown overnight and diluted 100-fold into fresh LB media. After 80 minutes of shaking at 37°C and 900 rpm in 96-well plates, the cultures were induced with 0.1 mM IPTG. Measurements were taken 6 hours after induction. We found that the onset of detectable reporter expression was delayed for the multiplexing system compared to single toehold switch experiments, most likely as a result of its increased metabolic burden on the cells.

S17.3. Analysis of multiplexing data

We acquired data from the multiplexing cells via flow cytometry using 16 different fluorescence channels. To compensate for spectral overlap between reporters, we first generated a set of standard curves representing the typical spectral profile of each reporter. These standard curves were obtained from positive control cells transformed with plasmids for constitutive expression of each of the fluorophores. Data from these control cells were first gated by forward- and side-scatter as described in Section S3. These gated events were

then filtered by intensity to eliminate outliers. An overall intensity factor R was calculated using the following equation:

$$R = \sqrt{\sum_i x_i^2}, \quad (5)$$

where x_i is the fluorescence measured in channel i. The distribution of R-values followed a log-normal distribution. We calculated the standard deviation of this log-normal distribution and filtered out those events whose R-values were more than two standard deviations away from the mean value of the distribution. We then normalized each of the events by their R-value. The median from each channel in this filtered, normalized data set was used to generate the standard curve for each reporter. These standard curves are shown in Figure S5A. Notably, there is substantial overlap between the curves of GFP, venus, and cerulean, which makes compensation for each of these reporters challenging.

We used the standard curves as a basis set for compensation of the flow cytometry data from cells expressing the multiplexing system. Compensation was performed on each event after forward- and side-scatter gating using the Matlab `lsqnonneg` function, a linear least squares fitting algorithm with non-negativity constraints. This function converted the 16-channel flow cytometer data into a set of four intensity values for each of the reporters. Figure S5B-E shows representative flow cytometry fluorescence data compared to fitting curves consisting of a superposition of the standard curves. Agreement between experimental data and the fits is quite good for single-color excitation experiments, but becomes more challenging for multi-color excitation.

We then established threshold levels defining cells actively expressing each of the fluorescent proteins using compensated data obtained from the system upon transcription of non-cognate trigger RNA 1. The median intensity level from these cells was first calculated for each fluorophore. The threshold levels were set by multiplying each of these median values by 10. The resulting thresholds were used for all of the plots in Figure 6B. A cell was deemed to be expressing a given protein if the fluorescence intensity measured for that protein was greater than or equal to the specified threshold level. Using this thresholding procedure, a 10-fold increase in reporter expression is equivalent to 50% of the cells expressing the reporter. Figure S5F-I shows the histograms used for computing the percentage of cells expressing for the same triggers used in Figure S5B-E.

S18. A layered 4-input AND circuit using toehold switches

S18.1. Design and construction of AND circuit plasmids

AND circuit plasmids were designed and constructed using the same PCR/Gibson assembly methods used for generating the multiplexing system (Section S17.1). Transcription factor genes *ECF42_4454* and *ECF41_491* (see Table S7 for sequences) were amplified from plasmids pVRa42_4454 and pVRa41_491, respectively (Rhodius et al., 2013). The cognate promoters for ECF42_4454 and ECF41_491 are *P42_up4062* and *P41_up1141*, respectively, and were constructed from gene assembly.

Each of the RNA inputs in the first layer of the circuit was expressed from their own IPTG-inducible $P_{LlacO-1}$ promoter (see Figure S6 for plasmid maps). First layer trigger RNAs were expressed from a ColE1/ampicillin plasmid, while first layer switch/transcription factor RNAs were expressed from a ColA/kanamycin plasmid. The second layer trigger C RNA was transcribed with *P41_up1141* from a CDF/spectinomycin plasmid. The second layer switch C RNA was transcribed with *P42_up4062* from a P15A/chloramphenicol plasmid and regulated an ASV-tagged GFP.

Sets of four different first layer trigger and switch plasmids were constructed to evaluate the full truth table for the 4-input circuit (see Table S7 for trigger and switch construct DNA sequences). Conditions in which an input RNA is not present were implemented *in vivo* by using non-cognate trigger RNAs from orthogonal toehold switches not used in the circuit. OFF states for trigger RNAs A and B used trigger RNAs D and E, respectively. OFF states for switch RNAs A and B used switch RNAs F and G, respectively, and still regulated the two transcription factors. The use of decoy trigger and switch RNAs to represent OFF states of the input species ensured that cells were subject to similar metabolic loads for all entries in the AND gate truth table.

S18.2. Characterization of AND circuit

AND circuits were tested in MG1655Pro. The four plasmids required for the circuit were transformed into cells in two stages. The second layer CDF/spectinomycin and P15A/chloramphenicol plasmids were first co-transformed as these plasmids are the same for all input RNA combinations. Competent cells prepared from the resulting spectinomycin-

and chloramphenicol-resistant strain were then transformed with 16 different combinations of the trigger and switch RNA plasmids.

AND circuit strains were measured in triplicate using flow cytometry. Cells were incubated overnight at 37°C and 900 rpm in LB media supplemented with spectinomycin (25 μg mL^{-1}), ampicillin (50 μg mL^{-1}), chloramphenicol (17 μg mL^{-1}), and kanamycin (30 μg mL^{-1}). Stationary phase cultures were diluted 100-fold into fresh media and shaken at 37°C for 80 minutes. These early log phase cultures were then induced with 0.1 mM IPTG and left to grow for 8 hours, at which point aliquots were taken for flow cytometry. ON/OFF GFP fluorescence values were calculated from mode GFP values with the same procedures used for measuring toehold switch library ON/OFF levels.

Supplemental References

Andersen, J.B., Sternberg, C., Poulsen, L.K., Bjorn, S.P., Givskov, M., and Molin, S. (1998). New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. Appl. Environ. Microbiol. *64*, 2240-2246.

Bentele, K., Saffert, P., Rauscher, R., Ignatova, Z., and Bluethgen, N. (2013). Efficient translation initiation dictates codon usage at gene start. Mol. Syst. Biol. *9*, 675.

Brunner, M., and Bujard, H. (1987). Promoter recognition and promoter strength in the Escherichia coli system. The EMBO journal *6*, 3139-3144.

Cambray, G., Guimaraes, J.C., Mutalik, V.K., Lam, C., Mai, Q.-A., Thimmaiah, T., Carothers, J.M., Arkin, A.P., and Endy, D. (2013). Measurement and modeling of intrinsic transcription terminators. Nucleic Acids Res. *41*, 5139-5148.

Carrier, T.A., and Keasling, J.D. (1999). Library of synthetic 5' secondary structures to manipulate mRNA stability in Escherichia coli. Biotechnology progress *15*, 58-64.

Datsenko, K.A., and Wanner, B.L. (2000). One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc. Natl. Acad. Sci. USA *97*, 6640-6645.

Davis, J.H., Rubin, A.J., and Sauer, R.T. (2011). Design, construction and characterization of a set of insulated bacterial promoters. Nucleic Acids Res. *39*, 1131-1141.

Gibson, D.G., Young, L., Chuang, R.-Y., Venter, J.C., Hutchison, C.A., and Smith, H.O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods *6*, 343-345.

Huttenhofer, A., and Noller, H.F. (1994). Footprinting mRNA-ribosome complexes with chemical probes. EMBO J. *13*, 3892-3901.

Inoue, H., Nojima, H., and Okayama, H. (1990). High efficiency transformation of Escherichia coli with plasmids. Gene *96*, 23-28.

Isaacs, F.J., Dwyer, D.J., Ding, C.M., Pervouchine, D.D., Cantor, C.R., and Collins, J.J. (2004). Engineered riboregulators enable post-transcriptional control of gene expression. Nat. Biotechnol. *22*, 841-847.

Kudla, G., Murray, A.W., Tollervey, D., and Plotkin, J.B. (2009). Coding-Sequence Determinants of Gene Expression in Escherichia coli. Science *324*, 255-258.

Mathews, D.H., Sabina, J., Zuker, M., and Turner, D.H. (1999). Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J. Mol. Biol. *288*, 911-940.

Pedelacq, J.-D., Cabantous, S., Tran, T., Terwilliger, T.C., and Waldo, G.S. (2006). Engineering and characterization of a superfolder green fluorescent protein. Nat. Biotechnol. *24*, 79-88.

Qian, L., and Winfree, E. (2011). Scaling Up Digital Circuit Computation with DNA Strand Displacement Cascades. Science *332*, 1196-1201.

Rhodius, V.A., Segall-Shapiro, T.H., Sharon, B.D., Ghodasara, A., Orlova, E., Tabakh, H., Burkhardt, D.H., Clancy, K., Peterson, T.C., Gross, C.A.*, et al.* (2013). Design of orthogonal genetic switches based on a crosstalk map of σs, anti-σs, and promoters. Mol. Syst. Biol. *9*.

Rouillard, J.M., Lee, W., Truan, G., Gao, X., Zhou, X., and Gulari, E. (2004). Gene2Oligo: oligonucleotide design for in vitro gene synthesis. Nucleic Acids Res. *32*, W176-180.

Salis, H.M., Mirsky, E.A., and Voigt, C.A. (2009). Automated design of synthetic ribosome binding sites to control protein expression. Nat. Biotechnol. *27*, 946-950.

Serra, M.J., and Turner, D.H. (1995). Predicting thermodynamic properties of RNA. Methods in enzymology *259*, 242-261.

St-Pierre, F., Cui, L., Priest, D.G., Endy, D., Dodd, I.B., and Shearwin, K.E. (2013). One-Step Cloning and Chromosomal Integration of DNA. ACS Synthetic Biology *2*, 537-541.

Vimberg, V., Tats, A., Remm, M., and Tenson, T. (2007). Translation initiation region sequence preferences in Escherichia coli. BMC Mol. Biol. *8*, 1-13.

Zadeh, J.N., Steenberg, C.D., Bois, J.S., Wolfe, B.R., Pierce, M.B., Khan, A.R., Dirks, R.M., and Pierce, N.A. (2011a). NUPACK: Analysis and design of nucleic acid systems. J. Comput. Chem. *32*, 170-173.

Zadeh, J.N., Wolfe, B.R., and Pierce, N.A. (2011b). Nucleic acid sequence design via efficient ensemble defect optimization. J. Comput. Chem. *32*, 439-452.