Versatile RNA-sensing transcriptional regulators for engineering genetic networks

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The widespread natural ability of RNA to sense small molecules and regulate genes has become an important tool for synthetic biology in applications as diverse as environmental sensing and metabolic engineering. Previous work in RNA synthetic biology has engineered RNA mechanisms that independently regulate multiple targets and integrate regulatory signals. However, intracellular regulatory networks built with these systems have required proteins to propagate regulatory signals. In this work, we remove this requirement and expand the RNA synthetic biology toolkit by engineering three unique features of the plasmid pT181 antisense-RNA-mediated transcription attenuation mechanism. First, because the antisense RNA mechanism relies on RNA-RNA interactions, we show how the specificity of the natural system can be engineered to create variants that independently regulate multiple targets in the same cell. Second, because the pT181 mechanism controls transcription, we show how independently acting variants can be configured in tandem to integrate regulatory signals and perform genetic logic. Finally, because both the input and output of the attenuator is RNA, we show how these variants can be configured to directly propagate RNA regulatory signals by constructing an RNA-meditated transcriptional cascade. The combination of these three features within a single RNA-based regulatory mechanism has the potential to simplify the design and construction of genetic networks by directly propagating signals as RNA molecules.

gene networks | regulatory systems | orthogonal regulators

Noncoding RNA has been found to play a central role in regulating gene expression in both prokaryotes and eukaryotes. Recently, the diverse roles of RNA-mediated regulation have become important tools for synthetic biology applications ranging from detecting metabolic state (1), balancing metabolic pathway expression (2), tightly regulating toxin genes (3), and detecting environmentally harmful chemicals (4). In particular, RNA-based genetic parts have been engineered that regulate transcription through RNA-mediated transcription factor recruitment (5, 6), transcript stability through small-molecule-mediated ribozyme cleavage (1, 7) and siRNA targeted degradation (8), and translation through *cis*-acting mRNA conformational changes (9) and *trans*-acting antisense RNA-mRNA interactions (10, 11).

This wide array of RNA function is beginning to be used to engineer programmable genetic circuitry required for the next level of synthetic biology applications (12). By interfacing with protein-based transcription factors and repressors, hybrid RNA/protein cascades have been made that perform sophisticated logic evaluation (8) and even count extracellular events (13). Much like previous work on protein-based cascades (14), protein regulators propagate the signal between different levels of the hybrid cascades. This makes the inner workings of these cascades complicated by the many interconversions between mRNA and protein that must take place. This not only increases the number of molecular species that must be accounted for in network designs, but also creates extra parameters such as the synthesis and decay rates of the intermediate species that further complicate network tuning (12) (Fig. 1*B*).

There is a potential to greatly simplify genetic networks by directly propagating signals as RNA molecules (Fig. 1*B*). One way to do this would be to implement network connections with RNA regulatory elements that sense an input RNA signal and regulate the synthesis of an output RNA signal. Antisense-RNA-mediated transcription attenuators are ideal candidate mechanisms to use as a starting point for engineering this capability. Much like riboswitches (15), they reside in 5′ untranslated regions of mRNA and regulate the synthesis of downstream protein-coding regions by terminating transcription through RNA structural changes. However, antisense-mediated attenuators are switched in the presence of antisense RNA rather than small molecule ligands (16, 17) (Fig. 1*A*). An essential challenge of creating pure RNA networks out of attenuators then is to engineer them to regulate the synthesis of antisense RNAs, which can then be propagated as signals.

In this work, we sought to combine a solution to this challenge with several advances from prior work in RNA synthetic biology to engineer RNA-based transcription regulators that could serve as a general platform for gene network engineering. Specifically, we focus on the internal network connections because there already exist numerous sources of initial input to the network through riboswitches (15), RNA transcriptional activators (5, 6), or general protein transcription factors. We start from the natural antisense-RNA-mediated transcriptional attenuator from the *Staphylococcus aureus* plasmid pT181 copy number control mechanism (18) (Fig. 1*A*). Building off of pioneering work on engineering orthogonal RNA-based translation regulators (10, 11, 19), we first show that orthogonally acting variants of the attenuator can be engineered through mutations of the wild-type system and can be used to regulate multiple targets in the same cell. We also show that our mutational strategy yields variants that have similar regulatory performance as measured by attenuation response to varying levels of antisense RNA. Next we show that transcription attenuators can be configured in tandem on the same transcript to perform genetic logic, much like logics constructed out of RNA mechanisms that regulate transcript stability (1, 8, 20). Finally, we show how these attenuators can be engineered to regulate the transcription of antisense signaling RNAs and use this to construct an RNA-mediated transcriptional cascade.

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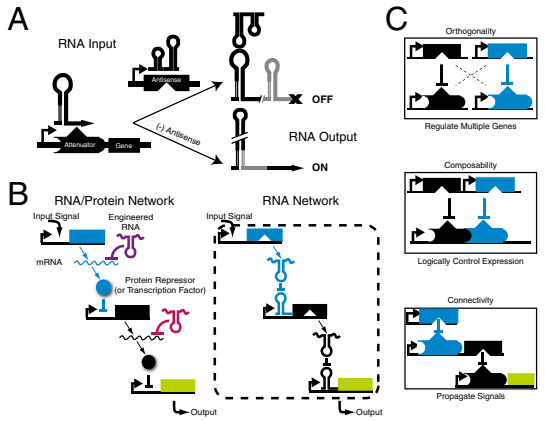

Fig. 1. RNA-mediated transcriptional attenuators can simplify gene networks. (*A*) Mechanism of the pT181 transcriptional attenuator (following ref. 16) showing how an RNA input governs the synthesis of an RNA output. Presence of antisense RNA biases the transcript fold to the OFF configuration through a complex, which exposes an intrinsic transcriptional terminator (gray). Absence of antisense RNA leads to sequestration of the intrinsic terminator and transcription of the downstream sequence in the ON configuration. (*B*) Cartoon of an example network (two-level transcriptional cascade) implemented as a hybrid RNA/protein network (*Left*), or an RNA-only network (*Right*). Both networks take a general input signal, propagate it through the cascade, and ultimately create an output RNA signal that can be any noncoding, coding, or engineered RNA regulator. In RNA/protein hybrid networks, signal propagation requires the interconversion between mRNA and protein at each step of the network (arrows). In contrast, RNA networks that use regulators such as the attenuator in this work greatly simplify network designs by propagating all signals as RNA molecules, which feed directly into the next regulatory decision. This work focuses on engineering RNA-based network connections (dashed box). (*C*) Schematic of three engineering goals (*Top*) for the attenuator in this work and the function (*Bottom*) that makes engineered RNA-mediated attenuators a versatile platform for engineering gene networks.

Results

Independently Acting Attenuator Variants Can Be Engineered Through Rational Mutagenesis. A prerequisite to building networks out of attenuators is to create orthogonal variants that can independently regulate different targets, but that are otherwise as similar as possible. The pT181 transcriptional attenuator resides in the 5′ untranslated portion of an mRNA that determines the fate of transcription elongation of the rest of the transcript (Fig. 1*A*). When the first part of the attenuator is transcribed, it folds into a hairpin structure. Watson–Crick base pairing of this hairpin with a complementary antisense RNA promotes the formation of a downstream intrinsic terminator hairpin that causes polymerase to fall off and stop transcription (16). Without antisense RNA, the terminator hairpin is sequestered and transcription elongation continues after the attenuator (21).

To engineer the attenuator, we started off by quantifying in vivo transcription attenuation in *Escherichia coli* using fluorescent reporter proteins as output for experimental convenience. We transcriptionally fused (22) the wild-type attenuator to the super folder green fluorescent protein (SFGFP) (23) on a medium-copy plasmid and measured average fluorescence of cells with and without antisense RNA on a high-copy plasmid (Fig. S2). Several designed mutations were introduced to the terminator stem of the wild-type system (WT-T4) to improve the dynamic range from 62% to 84% attenuation in the presence of WT antisense (Fig. S3).

Creating orthogonal variants requires changing the specificity of the antisense/attenuator base pairing. An additional goal was to do this with as few mutations as possible so that orthogonal variants would have near-identical response to their cognate antisense. Our design strategy centered on making mutations in two specific regions of the pT181 RNA structures based on the mechanism in the related CopA/CopT RNA translation regulator (24, 25) (Fig. 2): the loop regions of the antisense and attenuator structures that are known to be important for initial RNA-RNA recognition, and the hairpin collars that are involved in stable antisense-attenuator complexes.

The antisense and attenuator loops both have YUNR [Y-pyrimidine (C,U), R-purine (A,G), N-Y, or R] motifs, which are ubiquitous in recognition loops of natural antisense gene regulation systems (26, 27), and have been used as a design element in synthetic RNA regulators (10). We therefore searched for mutations that preserved these motifs, while otherwise disrupting interac-

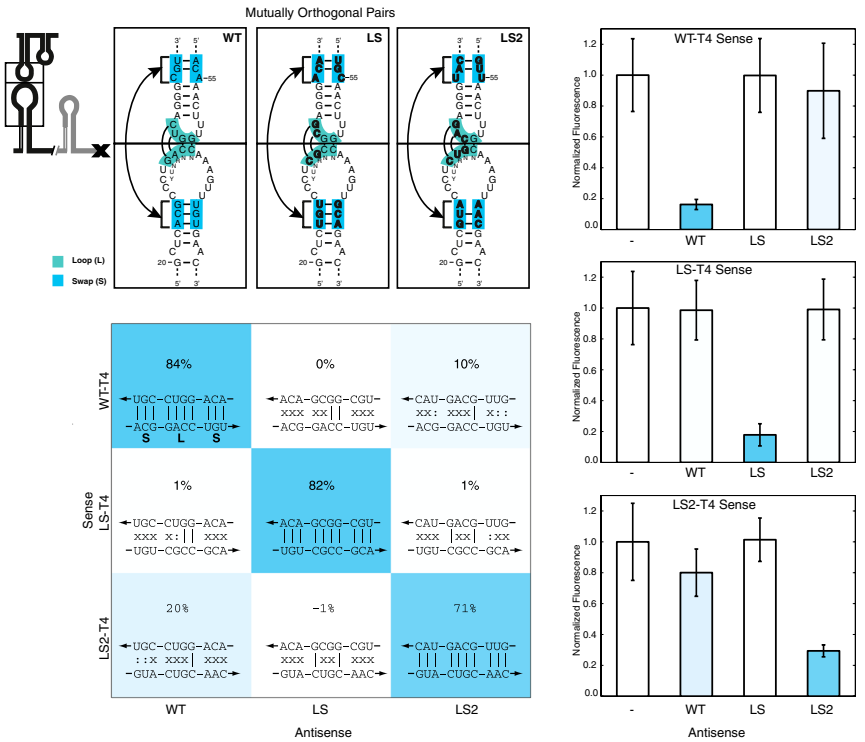

Fig. 2. Engineering mutual orthogonal attenuators through rational mutagenesis. (*Top Left*) Schematic secondary structures of the three cognate antisense/attenuator pairs highlighting the loop and swap mutational regions and their binding interactions (solid arcs) (structures after ref. 16). Mutated bases in bold. (*Bottom Left*) A heat map of the percent of repression of the nine different antisense/attenuator combinations. Nucleotide sequences for the antisense (*Top*) and attenuator (*Bottom*) molecules are shown for the swap (S) and loop (L) regions. Between the sequences, |, a standard Watson–Crick base pair; :, a G-U pair; and *x*, a base pair that has been disrupted due to mutations. (*Right*) Average fluorescence plots used to calculate the heat map, normalized by the fluorescence observed without antisense RNA (left bar). Error bars represent the standard deviation from measurements on at least three independent transformants.

tions between noncognate antisense/attenuator pairs. We found as few as a two-nucleotide change could decrease attenuation between noncognate WT and mutant (MT) (antisense/attenuator) pairs to between 19% (WT/MT) and 34% (MT/WT) (Fig. S4).

In the CopA/CopT system, it was found that simply swapping base pairs of the hairpin stems disrupted noncognate antisense/attenuator complexes, even if they had complementary hairpin loops (24, 25). Swapping bases also has the advantage of preserving the RNA structures of the individual molecules, thus causing minimal disruption to the functioning of the mutant systems. We found that swapping three base pairs on the hairpin collar of either the pT181 antisense or attenuator substantially reduced cross-talk attenuation to between 13% (WT/MT) and 22% (MT/WT) (Fig. S4).

More significantly, when we combined the loop (L) and swap (S) mutations, they acted synergistically to completely remove cross-talk between noncognate antisense/attenuator molecules (Fig. 2). To demonstrate the effectiveness of this design strategy, we constructed another orthogonal variant, LS2, by mutating bases in these two regions (Fig. 2). This mutant shows near-identical ON and OFF levels to the WT and LS attenuators and is completely orthogonal to the LS variant. We note that there is some cross-talk with the WT system, which is discussed below.

To verify that designed orthogonal antisense/attenuator pairs could regulate multiple genes in the same cell independently, we performed two-color assays using monomeric red fluorescent protein (mRFP) and SFGFP regulated by separate attenuator variants (Fig. 3). Two-color flow cytometry (Fig. 3) and microscopy (Fig. S5) both demonstrate that there is only minor cross-talk (6%-WT antisense, 12%-LS antisense—Fig. S5) when the attenuators are simultaneously used in the same cell.

Orthogonal Variants Have Similar Function. A central aspect of our mutational strategy was to use a minimal number of mutations that change specificity without altering attenuation characteristics. To investigate the similarity of attenuation under various antisense RNA concentrations, we measured and compared the induction curves for both wild-type (WT-T4) and mutant (LS-T4) attenuators using an IPTG-inducible $P_{Llac0\text{-}1}$ promoter to express a range of cognate antisense RNA (28) (Fig. 4*A*, solid curves). We observed a wide range of attenuation, which combined with the mutations to the terminator stem (Fig. S3) illustrate our flexibility in tuning attenuator strength. Furthermore, the high degree of similarity between each corresponding point of the wild-type and mutant attenuator induction curves further demonstrates the uniformity in attenuation response over a wide range of antisense concentrations.

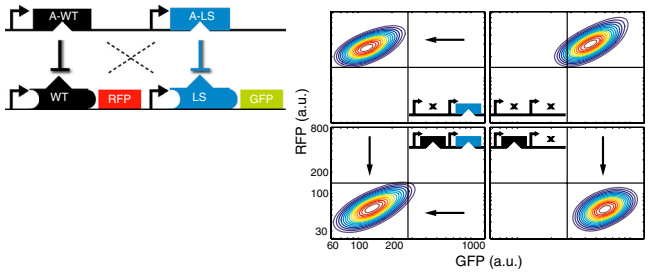

Fig. 3. Independent regulation of two fluorescent reporters in the same cell. mRFP and SFGFP were separately controlled by the WT and LS-mutated attenuators through transcriptional fusions on the same plasmid. Representative two-color flow cytometry percentile contours (darkest blue—75% cells; red—5% cells) for the four combinations of WT and LS antisense (inset cartoons—X represents no antisense sequence included at that position). Arrows denote changes of the cellular density location in the RFP versus GFP plane that indicate orthogonal changes in gene expression when different combinations of antisense are expressed. Fluorescence intensity is indicated as a.u. as determined by flow cytometry.

Tandem Attenuators Function Independently. We next tested whether we could compose two attenuators in series to integrate multiple antisense RNA signals. Inspired by naturally occurring tandem riboswitches (29) and engineered tandem arrays of ribozymes (20) that integrate small molecule signals, we physically fused two attenuators in series upstream of SFGFP and measured the attenuation due to cognate antisense RNA expressed from an inducible promoter (Fig. 4*A*). When we composed two identical attenuators, we observed increased relative attenuation and steeper normalized induction curves. To explain this effect, we hypothesized that attenuators in series function independently, much like the case for engineered tandem ribozyme devices (20). This implies that the overall attenuation of a tandem composite attenuator is a multiplicative function of the individual outputs. Fig. 4*A*, *Insets*, plots this multiplication rule versus the observed double attenuation for each tested induction point. The fact that each value falls on a line of slope one shows that this simple multiplication rule is remarkably accurate for both WT (WT-T4) and MT (LS-T4) attenuators, reiterating their similarity of function. Interestingly, these tandem repeated attenuators operated reliably over a number of generations, showing less susceptibility to genetic recombination than might be expected (see Fig. S6).

Genetic Logics Can Be Constructed with Tandem Orthogonal Attenuators. Because of this remarkable degree of independence of two repressible attenuators in tandem, we expect two composed orthogonal attenuators to integrate two antisense signals and allow gene expression only when neither wild-type nor mutant antisense is present. This not-or-like (NOR) gene expression logic is indeed what we observed (Fig. 4*B*). This is an important feature of our system because NOR logics can be chained together to theoretically construct any other type of logic (30, 31), which has important implications for higher-order synthetic biology devices (32).

Attenuators Can Be Engineered to Propagate Antisense RNA Signals in Transcriptional Cascades. Finally, we demonstrated the ability to propagate signals between these orthogonal attenuators by constructing a three-level transcriptional cascade (Fig. 5). The cascade was designed such that expression of SFGFP is controlled by attenuator-1's (Att-1, WT-T4) interaction with antisense-1 (Anti-1,WT), whose transcription is in turn controlled by attenuator-2's (Att-2,LS-T4) interaction with antisense-2 (LS). In this way the antisense regulatory signal is propagated through a double inversion, which should produce a net activation of SFGFP expression.

One of the key design aspects of this cascade was controlling expression of Anti-1 with Att-2, which is particularly challenging because they share 91% sequence complementarity. In fact, using the secondary structure prediction algorithm RNAStructure (33), we predict that a direct fusion of the attenuator followed by antisense will fold into a stable structure that diminishes the activity of Anti-1 (Fig. S10). This is consistent with in vivo attenuation experiments that show that these fusions cause a significant reduction in attenuation, which is not improved by adding an RNA linker sequence in between Att-2 and Anti-1 to increase their distance on the transcript (Fig. S9). We therefore explored an alternative insulation strategy to physically separate the two regions once the transcript was made using self-cleaving ribozymes. A hammerhead ribozyme from small tobacco ring spot virus (sTRSV) (34) was inserted into the region between Att-2 and Anti-1, which showed improved attenuation and preserved orthogonality (Figs. S11 and S12). This suggested a general strategy to tune and amplify the antisense signal on this level by tandemly duplicating the sTRSV-Anti-1 module on the transcript, which was found to further increase attenuation (Fig. S12). With these innovations, the sophisticated three-level cascade was found to activate the expression of SFGFP to 94% of its theore-

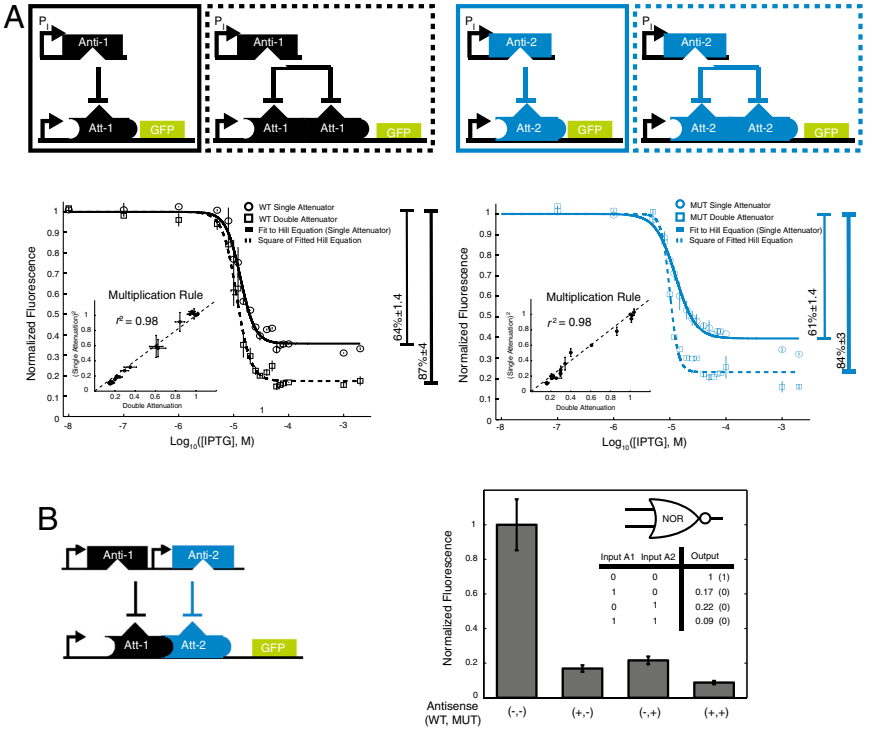

Fig. 4. Increasing attenuation and logically controlling expression with attenuators in tandem. (*A*) Induction curves using an IPTG-inducible $P_{Llac0-1}$ promoter (28) (P_I) for single (circles; solid lines) and double (squares; dashed lines) attenuators in series. The wild-type (black) and mutant (blue) composite attenuators show similar attenuation over the full range of induction. Measured fluorescence was normalized to the case with [IPTG] = 0 mM for each attenuator (MEFL: single WT 12725, double WT 5744, single MT 14561, double MT 5348). Hill equation fits to the single attenuator data are in solid curves. The dotted lines were calculated by squaring the fitted Hill functions (see *SI Appendix*). (*Insets*) Plots show the comparison between the measured double attenuation and the square of measured single attenuation (calculated as 1—normalized fluorescence) to verify the multiplication model. Error bars represent the difference between measurements on two independent transformants. (*B*) Composed orthogonal attenuators have a NOR logical expression pattern. Data were normalized to 1 for the no antisense condition. Error bars represent the standard deviation from measurements on at least three independent transformants. (*Inset*) Table shows the measured performance of the NOR logical gate, and the expected values for a perfect digital NOR gate (parentheses).

tical maximum. Although the functioning of the full three-level cascade is near optimal, the repression caused by the attenuator-ribozyme-antisense molecule is less than that caused by bare antisense. This could be due to the effects of attenuator autotermination as discussed in *SI Appendix* (Fig. S13). It should be noted that this three-level RNA regulatory cascade was constructed simply by connecting our basic attenuators together.

Discussion

The General Utility of Versatile RNA-Based Transcription Attenuators. In this work, we have demonstrated the ability to design independently acting RNA-mediated transcriptional attenuators that can be configured to regulate multiple genes in the same cell, logically control gene expression, and directly propagate RNA regulatory signals. Each of these different functions was achieved by a simple reconfiguration of the attenuators. There has been substantial work on other RNA-based regulatory mechanisms that can each perform some of these functions (1, 8, 10, 11). However, the attenuators in this study provide the simplest route to achieving all of the functions within a single regulatory mechanism. The advantages of this are highlighted by considering two different implementations of transcriptional cascades as either hybrid protein/RNA networks or purely RNA networks (Fig. 1*B*). In the case of hybrid networks, any protein regulators must be translated from intermediate mRNAs, whereas in RNA networks this intermolecular conversion process is not required. This eliminates one molecular species (with associated gene expression parameters such as half-life, maturation time, etc.), and one interconversion process *for each network connection*. Such benefits will be compounded as the number of network connections increase, and we believe the attenuators used in this study could become important components for designing large gene networks.

In addition to simplicity, the particular combination of functions displayed by the attenuators have recently been shown to be important for constructing complex genetic logics. NOR logic gates are universal in that they can be connected together to construct any logical circuit (30). This property was recently demonstrated using combinations of transcription factor logic gates inside different cells, and quorum sensing circuits to propagate the signals among them (32). Because NOR logic and signal propagation are two of the features of the attenuators, we anticipate our system to be useful when constructing computational circuits inside cells.

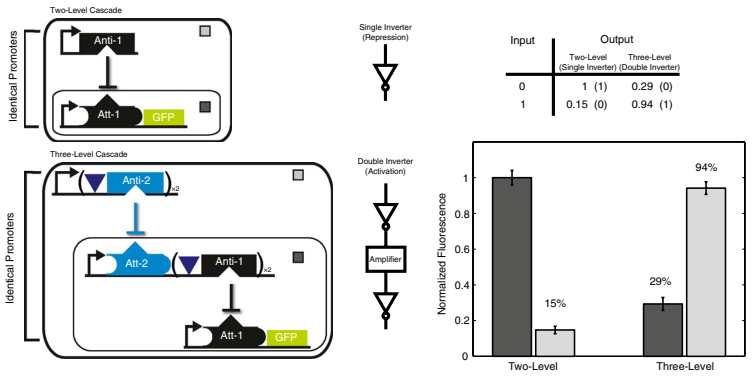

Fig. 5. Engineered three-level RNA-mediated cascade using two pairs of orthogonal attenuators and a hammerhead ribozyme (inverted triangle). Cartoons show two- and three-level cascade architectures. Subnetworks of each cascade are labeled with dark and light gray boxes, which correspond to the observed SFGFP expression from each subnetwork (bar plot). All fluorescence data was normalized to the one-level cascade value (Att-1-SFGFP, left black bar). The two-level cascade with one attenuator shows an 85% repression (middle gray bar), whereas with two attenuators shows 71% repression (middle black bar). The full three-level cascade activates SFGFP expression to 94% of its maximum (right gray bar). In this way the three-level cascade represents a double inverter (electrical circuit symbol). (*Inset*) Table shows the expected (parenthesis) idealized inverter circuit outputs versus the measured values. The ×2 symbol represents a tandem repeat of the enclosed module. The same synthetic promoter was used at each level. Error bars represent the standard deviation from measurements on six independent transformants.

There may be other important applications of this system where the speed of signal propagation is a critical design requirement. The rate-limiting step in signal propagation through a cascade is the time required to degrade intermediate signaling molecules (35). Because protein regulators often have half-lives greater than the doubling time, this is achieved by dilution through division, and protein cascades can propagate only as fast as one cell-cycle per step. Because the half-lives of the antisense RNAs that propagate the signals in this work are around 5 min (22), networks built out of the attenuators should propagate signals faster. Faster transcriptional cascades may allow different considerations when designing gene networks (3). These could be particularly useful if coupled to appropriate sensing mechanisms, such as two-component systems or riboswitches to control antisense production, to design fast responses to environmental signals. More work is needed to understand how other aspects of gene expression such as noise propagation is affected by the attenuators, and how this compares with protein transcription factors and other small RNA regulators (36).

It is important to note that in some cases, the added complexity of protein-mediated networks may have advantages. For example, small-molecule-sensing protein intermediates could be used to control signal propagation at intermediate positions in the network, though future work may leverage the natural ability of RNA to do this as well (15). Extra intermediates, either proteins or RNAs, could also be used specifically to change the timing of signal propagation, and as a means of amplifying signals between steps (14). As synthetic biology aims to scale network designs to higher levels of sophistication, it will become an important area of research to determine when the particular advantages of RNA-protein-mediated and RNA-only-mediated designs can be most effectively utilized.

Expanding Families of Orthogonal Regulators. Our fundamental approach to finding orthogonal regulators was to engineer them through rational mutagenesis of a carefully selected natural regulator (37). Previous studies that established the broad features of the pT181 attenuation mechanism (16, 22) placed great emphasis on the series of RNA structures responsible for antisense recognition and attenuation. Therefore, as a design principle, we specifically focused on mutational strategies that would minimize disruption of the antisense and attenuator hairpin structures. In particular, the mutations of attenuator LS both conserve the YUNR motif in the loop of the hairpins, as well as the overall base-pairing pattern in the stems of the hairpins.

To test whether we could extend this mutational strategy, we constructed 29 antisense/attenuator pairs that were mutated in the loop and swap regions, but relaxed the requirement to preserve the YUNR motif and stem base pairs (Fig. S14). Attenuator LS2 was identified from this pool and found to be mutually orthogonal to the WT and LS attenuators, indicating that at least small perturbations in the sequence and structures of the antisense and attenuator RNAs can be tolerated, and that perhaps a larger sequence space could be sampled to find more orthogonal pairs. However, the fact that other RNA-mediated transcriptional attenuators have largely similar RNA structures (17) suggests that there is a deeper structural principle to this type of gene regulation that is at the core of making quick decisions with RNA hairpin–hairpin interactions and that completely arbitrary sequences would not yield functional attenuators.

In this work we have begun to develop design rules for constructing more orthogonal attenuator/antisense pairs. Although minimizing mutations is desired, we found that both loop and swap mutations are required for orthogonality (Fig. S4). However, not every mutant that we created was found to be orthogonal, or even functional (Fig. S14), and there is still some degree of cross-talk observed between the LS2 and WT pairs (Fig. 2). In previous work on translational regulation with engineered RNAs, it was found that lower thermodynamic RNA–RNA binding free energies were positively correlated with stronger repression (10). However, in this work, we found only a loose correlation between lower calculated binding free energy and attenuation (Fig. S14). Furthermore, there are many mutant cognate pairs that are predicted to have a low free energy of interaction but show very little attenuation. This suggests that these mutants may be misfolding and that thermodynamic free energies can be used as a secondary design principle after the overall attenuator and antisense structures conform to the requirements of this system. More work is needed to uncover all the design principles behind orthogonality in this system.

Assuming that we consider only the loop and swap regions identified in this work, there are still 4^{10}, or over a million potential antisense/attenuator pairs. Given that LS2 was found out of a set of 29 of these, an upper estimate of the number of these that would be orthogonal is still 4^8, although finding mutually orthogonal groups of regulators becomes more difficult as the size of the group grows.

Remaining Challenges. In addition to orthogonality, there are two specific remaining challenges associated with optimizing the attenuator ON and OFF levels, respectively. The attenuator ON level is determined by the strength of the promoter and the propensity for the attenuator to autoterminate in the absence of antisense. Autotermination manifests itself as the drop in fluorescence observed when two attenuators are placed in series (Fig. S13), which can be used to estimate the amount of autotermination due to a single attenuator to be 59%. When attenuators are used to control protein-coding regions that are later translated, these deficiencies can be compensated by tuning the strength of the ribosome binding site (RBS) (Fig. S15). However, in RNA-based circuits created by wiring together attenuators such as the cascade, autotermination reduces the amount of antisense that can propagate the signal and was found to be the likely cause for imperfect cascade performance (see *SI Appendix* and Figs. S12 and S13). Autotermination is likely a property of the dynamic refolding the attenuator undergoes as it is being transcribed, and decreasing it will likely require a deeper understanding of cotranscriptional RNA folding pathways.

Equally important is the OFF level attainable by the attenuator in the presence of antisense. The fact that we could improve this level by almost 100-fold (Fig. S3) with only four mutations suggests that the WT system is far from optimal and more mutations along these lines could reduce the level further. Another limiting factor in the attenuator OFF level is due to the amount of antisense expressed. RNA-level measurements for this system show that the [antisense]/[attenuator] ratio is in the range of 3.8–9.7, confirming earlier work showing that antisense needs to be in abundance of sense for efficient attenuation (see *SI Appendix*). This has also been observed in previous work on engineering antisense-mediated translation control (10) and may be a general feature of RNA-based regulation.

Titration experiments also show that increasing the ratio increases attenuation (Fig. 4 and Fig. S7). Indeed, one way of improving the leakiness of attenuation, and thereby potentially some of the cell-to-cell variation that gives rise to the error bars in our experiment, would be to increase the strength of the promoter driving antisense transcription. We note that in our current configuration the [antisense]/[sense] ratio is achieved by the difference in plasmid copy number, and integrating our attenuation system into the chromosome or lower copy plasmids may require promoter tuning. Improving the attenuator OFF level in the presence of lower concentrations of antisense represents an important challenge in optimizing the system and may be addressed by increasing the thermodynamic binding free energy between the attenuator and antisense (Fig. S14).

Summary. This work adds to the growing repertoire of RNA synthetic biology by providing a versatile set of RNA-based transcriptional regulators that could change the way we think about designing and constructing gene networks. Our engineering strategy—constructing orthogonal variants of a natural RNA system with minimal changes so as to preserve overall function—should be applicable to other gene regulatory mechanisms to further expand the diversity of genetic building blocks available. Furthermore, we anticipate that the ribozyme-mediated insulation strategy used in this work can be used as a general technique to compose diverse RNA regulatory elements on a single transcript (38), which could substantially increase the sophistication of RNA-based gene regulatory networks.

Materials and Methods

Plasmid Construction. A table of all plasmids used in this study can be found in Table S2. Fig. S2 outlines the two-plasmid architecture of the attenuation system used in all experiments. The basic attenuator consisted of the 191-nt pT181 transcriptional attenuator followed by a 96-nt fragment of the pT181 repC gene with an introduced TAA stop codon at the end. This was obtained by PCR amplification from plasmid pT181 (a gift from R. Novick, Department of Microbiology, New York University School of Medicine, New York) and was transcriptionally fused to a RBS and a fluorescent reporter protein-coding sequence (either SFGFP or mRFP) with BglBrick (39) restriction sites. All sense plasmids had the p15A origin and chloramphenicol resistance. The antisense plasmid consisted of the 91-nt pT181 antisense RNAI followed by the TrrnB terminator, using the ColE1 origin and ampicillin resistance. The J23119 *E. coli* consensus promoter (http://partsregistry.org/Part:BBa_J23119), modified to include a SpeI site right before the start of transcription, was used for all sense and antisense in vivo transcription, except for induction curve measurements where $P_{Llac0\text{-}1}$ (28) was used. All mutations were done following the New England Biolabs site-directed mutagenesis protocol (see *SI Methods* for mutation details and a list of plasmids used in this study.)

In Vivo Gene Expression Reporter Assays. All experiments were performed in *E. coli* strain TG1. Plasmid combinations were transformed into chemically competent *E. coli* TG1 cells (Zymo Research), plated on Difco LB+Agar plates containing 100 μg/mL carbenicillin and 34 μg/m chloramphenicol, and incubated overnight at 37 °C. At least three colonies were picked into 300 μL of Difco LB containing 100 μg/mL carbenicillin and 34 μg/mL chloramphenicol in a 2-mL 96-well block (Costar 3960) and grown approximately 15 h overnight at 37 °C and 1,000 rpm in a Labnet Vortemp 56 bench top shaker. Three microliters of this overnight culture was then added to 147 μL (1:50 dilution) of supplemented M9 Minimal Media and grown for 3 h at the same conditions to an absorbance (600 nm) of 0.2–0.4. The culture was then analyzed by flow cytometry using a Partec CyFlow Space (details in *SI Appendix*). In the case of induction curves, duplicate overnight cultures were initially diluted 1:20 in supplemented M9 with inducer, grown for 3 h, diluted 1:10 again, and then measured after 2.5 h. Flow cytometry data analysis is described in *SI Appendix*. All data are normalized to the case of no antisense present unless otherwise indicated.

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