CellPress

Turning It Up to 11: Modular Proteins Amplify RNA Sensors for Sophisticated Circuitry

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http://dx.doi.org/10.1016/j.cels.2016.12.004

Ligand-sensing RNA switches can be enhanced using protein-based amplifiers to deliver sophisticated signal-processing genetic circuitry.

Since its inception, achieving programmable control of cellular functions has been one of the central quests of synthetic biology. Systems biology has taught us that many natural cellular functions are controlled by genetic networks that use sensing and signal propagation to turn the right genes on at the right times and in the right amounts. As such, our quest for biological control has boiled down to our ability to create synthetic regulators that can be composed into synthetic genetic networks with predictable function. While RNAs play diverse and ubiquitous regulatory roles in natural systems, historically protein regulators have been favored in synthetic biology due to their high dynamic range of regulation and a belief that the challenges of engineering RNAs that are functionally equivalent are too difficult. In this issue of *Cell Systems*, Wang et al. (2016) address this challenge head on by innovating a circuit-level design approach that harnesses modular protein-based transcriptional amplifiers to enhance the performance of ligand-sensing RNA regulators. By combining the best of the RNA and protein worlds, they establish a potentially generalizable approach for making RNA the molecule of choice for programming cellular systems.

The creation of synthetic RNA-based gene regulators was one of the early successes of synthetic biology (Isaacs et al., 2004), spawning a sub-field built on the promise that RNAs offer unparalleled designability compared to their protein counterparts. Much of this promise is due to the predictable relationship between RNA structure and function. Regulation occurs through the formation of specific structures within mRNAs, which is constrained by the biophysics of interaction between the nucleotides that comprise the genetic code. When these structures are simple—for example, RNA hairpins that are governed by canonical base pairing (A-U, G-C, and G-U)—RNA folding and design algorithms (Zadeh et al., 2011) can be leveraged for rational (Chappell et al., 2015) and computational (Green et al., 2014) design approaches that result in RNA regulators with protein-like dynamic ranges. However, the computational design of RNA regulators that require more sophisticated structural motifs is currently not possible due to limitations in design algorithms. For example, self-cleaving ribozymes that regulate mRNA degradation have to form complex tertiary structures that are currently out of reach due to the computational burden of modeling the large repertoire of potential complex interactions, such as pseudoknots and non-canonical base pairing. Until now, this posed a serious problem for utilizing ribozymes in sophisticated networks; although their mode of genetic control is highly desirable, improving their function using RNA-level design approaches remains challenging.

This was the challenge facing Wang et al. (2016), who sought to create a generalizable strategy to optimize ligand-switchable versions of these self-cleaving ribozymes. In early implementations, these RNA switches, composed of an RNA aptamer fused to a ribozyme catalytic core, were fused directly to the transcript of a gene of interest (GOI) (Figure 1A). In the absence of the ligand, these ribozymes are able to functionally fold, cleave the mRNA, and induce degradation of the mRNA encoding the GOI. Conversely, binding of the ligand to the aptamer domain causes structural rearrangements that result in an inactive ribozyme conformation, preventing degradation of the mRNA and ultimately activating GOI expression. While functional, the utility of this direct design is severely limited by its dynamic range (the ratio of gene expression in the "ON" over the "OFF" state), which is not large enough to produce clear downstream cellular responses.

To overcome the dynamic range bottleneck, Wang et al. (2016) innovate on the direct design by using a modular multistage circuit architecture that has a transcriptional amplifier as an intermediate between the RNA switch and the GOI (Figure 1B). In this architecture, the RNA switch regulates an mRNA encoding a protein transcription factor, which once translated, binds to the GOI DNA to activate its transcription. As the production of a single transcription factor mRNA will result in the production of multiple GOI mRNAs, this architecture effectively acts as a transcriptional amplifier. Thus a *weak* transcriptional output from the RNA switch can be amplified to create a *strong* activation of transcription of the GOI—all without having to engineer either the RNA switch or the GOI.

To accomplish this, the first task for Wang et al. (2016) was to design the transcriptional amplifier, which they chose to implement using a hybrid transcription factor composed of a DNA binding domain fused to an activation domain. This protein fusion is designed to bind to a corresponding DNA operator site to activate transcription by locally recruiting other transcription factors and chromatin-modifying proteins to an adjacent promoter. While such regulators exist, to expand the dynamic range and achieve a transcriptional amplifier, Wang et al. (2016) undertook a series of genetic optimizations. This included optimizing the coding sequence, peptide linker, nuclear localization sequences, activation domain, and enhancer sequences that resulted in 184-fold amplification gain

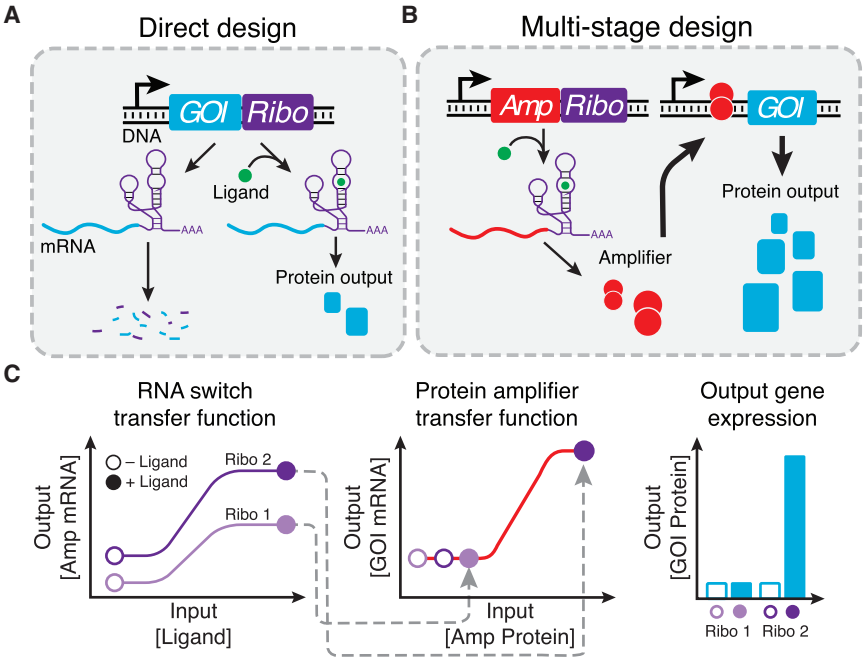

Figure 1. Enhancing Ligand-Switchable Ribozymes Using Protein-Based Transcriptional Amplifiers

(A) Schematic of a direct design circuit architecture, whereby ligand-switchable ribozymes (Ribo) are directly fused to a gene of interest (GOI). In the absence of ligand, the ribozyme folds into an active conformation and the GOI mRNA is degraded. In the presence of ligand, the ribozyme folds into an inactive conformation, preventing GOI mRNA degradation and thus activating GOI expression.

(B) Schematic of a multi-stage design circuit architecture in which a protein-based amplifier serves as an intermediate between the Ribo and the GOI. A transcription factor is used as an amplifier, which when expressed binds to its target promoter and activates transcription of the GOI. Through this mechanism, the transcriptional output of the Ribo is amplified to increase production of the GOI.

(C) Level matching between the output of RNA switches and the input of protein amplifiers. Two different ligand-switchable ribozymes (Ribo 1 and Ribo 2) will have unique relationships between the input of ligand required to produce specific outputs of amplifier mRNA (Amp mRNA), defined as their transfer functions. To achieve amplification, the output level of Amp mRNA from the ligand-switchable ribozymes needs to *match* the input level of amplifier protein (Amp Protein) required to activate transcription of GOI mRNA. Improper level matching will result in switches that appear broken, while proper level matching will result in switches with large dynamic range.

(the ratio of ON state gene expression between the presence and absence of amplifier) from a constitutive promoter. While analogous protein-based transcriptional amplifiers have been demonstrated in prokaryotes (Wang et al., 2014), this work provides the first eukaryotic version, which showed even greater amplification gain. Moreover, due to the inherent modularity of the hybrid transcription factor used, orthogonal amplifiers were simply created by switching the DNA binding domain, opening the door for further expansion by leveraging existing libraries of orthogonal transcription factors (Stanton et al., 2014).

While successfully amplifying the outputs of constitutive promoters, the next challenge was to properly interface the amplifier with the outputs generated from ligand-switchable ribozymes. Key to achieving this is the concept of level matching, whereby the amount of the transcriptional output from the first stage (the RNA switch) must match the transcriptional input required for the second stage (the amplifier) to activate expression of the GOI. Such level matching is non-trivial, as different gene regulators with unique biochemical properties have distinct relationships between the molecular inputs required to produce outputs of gene expression, often referred to as their transfer functions. For example, two different theophylline switchable ribozymes will have unique relationships between the theophylline input and mRNA stability, determining the resulting mRNA levels in the absence (OFF state) and presence (ON state) of theophylline (Figure 1C). Likewise, the transcriptional amplifier has a transfer function of its own, defining how much of the protein-based transcription factor is required to activate the target promoter and ultimately GOI expression (Figure 1C). When interfaced together, if the ON state of the RNA switch is insufficient to make enough transcription factor to activate the ON state of the amplifier, the system will be broken (Figure 1C). In order to create a simple framework for level matching, Wang et al. (2016) developed a mathematical model that captured enough of the underlying biochemical processes to be able to both predict and optimize level matching of different ligand-switchable ribozymes with impressive accuracy. This level-matching strategy was ultimately the key to constructing large dynamic range multi-stage RNA-protein sensors.

With the modular ligand-switchable RNA-protein hybrid activators established, the ground was set for demonstrating its use in an array of genetic circuitry. Transcriptional inverters were created by expressing a "decoy" to sequester the DNA operator site, AND gates were made by fusing two ligand-switchable ribozymes in tandem, and OR gates were built by fusing two DNA operator sites in tandem. These add to an increasing repertoire of RNA-based logic gates in both prokaryotes (Chappell et al., 2015; Green et al., 2014) and eukaryotes, which offer distinct advantages compared to their protein counterparts. For example, RNA-based logic gates employ a simple architecture, often achieved by simply placing regulators in tandem. They also potentially offer smaller genetic footprints and less cellular burden and can propagate signals much faster than protein-based circuits (Takahashi et al., 2015). Wang et al. (2016) also implemented closed-loop feedback control, whereby the GOI is an enzyme that catalyzes synthesis of the ligand input for the RNA switch. Such enzymatic feedback has long been theorized as an effective strategy to provide dynamic feedback for metabolic pathways, helping to prevent cellular burdening or to prevent accumulation of toxic pathway intermediates. For example, Stevens et al. recently published a theoretical study of the benefits of using ligand-sensing ribozymes for dynamic control of the *p*-aminostyrene pathway to control expression

of enzymes and pumps to maximize production while minimizing toxicity (Stevens and Carothers, 2015).

With the addition of the work by Wang et al. (2016), RNA-based circuits are quickly matching the capabilities of protein-based circuitry. However, there are still challenges to overcome. One of the major challenges is the creation of ligand-switchable RNA regulators for ligands relevant to real-world applications such as metabolic engineering, diagnostics, and intracellular sensing. RNA aptamers are abundant in natural systems and can now be reliably evolved to sense a myriad of metabolites, cofactors, metals, ions, and macromolecules, with stunning specificity and sensitivity. Yet the vast majority of synthetic RNA switches have utilized only a handful of well-characterized aptamers, partly because like ribozymes, aptamers often make use of complex RNA-ligand interactions that are hard to measure and model computationally. While this challenge can be circumvented by high-throughput screening of large variant libraries (Townshend et al., 2015), significant progress is being made in the quest for RNA design. For example, incorporating the thermodynamics of ligand binding into RNA design algorithms (Espah Borujeni et al., 2016) and using more versatile ribozymes (Felletti et al., 2016) hold promise for improving the reliability and efficacy of ligand-switchable RNA regulators. Moreover, given the inherent modularity of the innovations described by Wang et al. (2016), even if new RNA switches suffer from a low dynamic range, they can now be significantly improved, helping deliver on the promise of RNA circuitry.

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Cell Lineage Trees Bear Fruit

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http://dx.doi.org/10.1016/j.cels.2016.12.006

Two inference approaches harness the information present in cell lineage trees to better understand the dynamic transitions between cell states.

Living cells are dynamic entities, with proteins and other biomolecules being produced and decaying constantly at rates that frequently vary in time. Many biological processes even involve cells switching between distinct cellular states as time goes by. This dynamic character, together with the unavoidable heterogeneity of cellular populations, requires monitoring the activity of gene regulatory circuits in time and at the level of single cells. Time-lapse microscopy of cells carrying fluorescent reporters is routinely used for this purpose (Locke and Elowitz, 2009), but this method suffers from several shortcomings that limit its usefulness.

First, only a handful of species (at most three or four) can be monitored simultaneously due to spectral overlap, which leads to a partial view of the process under study. Second, sampling frequency is limited due to photobleaching and phototoxicity of the fluorescent reporters, leaving time gaps during which the behavior of the system is unknown. Third, the combination of measurement errors and biological noise leads to significant uncertainty in the observations.

The situation would seem to worsen in populations of proliferating cells due to the amplification in the number of variables to measure, resulting from the exponential increase in cell number. However, as shown in the November issue of *Cell Systems* (Feigelman et al., 2016; Hormoz et al., 2016), the genealogical structure of proliferating cell populations, reflected in their lineage trees, provides constraints that can in fact relieve some of the limitations of time-lapse fluorescence microscopy, substantially enhancing its ability to shed light on the molecular and functional