Engineering and In Vivo Applications of Riboswitches

Zachary F. Hallberg,[1] Yichi Su,[1] Rebekah Z. Kitto,[1] and Ming C. Hammond[1,2]

[1]Department of Chemistry, University of California, Berkeley, California 94720; email: mingch@berkeley.edu

[2]Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

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Abstract

Riboswitches are common gene regulatory units mostly found in bacteria that are capable of altering gene expression in response to a small molecule. These structured RNA elements consist of two modular subunits: an aptamer domain that binds with high specificity and affinity to a target ligand and an expression platform that transduces ligand binding to a gene expression output. Significant progress has been made in engineering novel aptamer domains for new small molecule inducers of gene expression. Modified expression platforms have also been optimized to function when fused with both natural and synthetic aptamer domains. As this field expands, the use of these privileged scaffolds has permitted the development of tools such as RNA-based fluorescent biosensors. In this review, we summarize the methods that have been developed to engineer new riboswitches and highlight applications of natural and synthetic riboswitches in enzyme and strain engineering, in controlling gene expression and cellular physiology, and in real-time imaging of cellular metabolites and signals.

Contents

1. INTRODUCTION

The centrality of RNA in gene regulation is due not only to its role as messenger RNA but also to the regulatory roles of many new classes of noncoding RNAs that have been discovered. Some noncoding RNA classes function in the context of ribonucleoprotein complexes, including microRNAs, which typically target eukaryotic transcripts for translational repression (1), and clustered regularly interspaced short palindromic repeat (CRISPR) RNAs, which target phage DNA for cleavage as a bacterial defense mechanism (2). Other noncoding RNA classes function independently, without any protein partner. Riboswitches are a remarkable example class, as they are *cis*-regulatory structured RNA elements capable of controlling expression of downstream genes by direct response to a small molecule ligand. Riboswitches comprise a ligand-sensing domain, termed an aptamer, and a regulatory domain, termed the expression platform. Aptamer binding to the ligand compound stabilizes the aptamer structure and causes a conformational change or other activation mechanism in the expression platform domain, which mediates gene regulation.

In the past 2 decades, more than 20 distinct riboswitch–ligand pairs have been discovered (3) that showcase the capability of these natural aptamers for selective, tight molecular recognition of diverse compounds in cells. In addition, the study of natural expression platforms has revealed several different mechanisms for ligand-dependent gene regulation by riboswitches in bacteria, plants, and fungi. These findings reinforce that nature makes broad use of riboswitches for gene regulation (although mostly in bacteria and conspicuously absent in animals), which has fueled researchers' interest in engineering riboswitch-like systems for their own in vivo applications.

In this review, we present advances in riboswitch engineering and case studies for in vivo applications. Riboswitch engineering includes the design of new aptamer domains to recognize xenobiotic compounds, the development of new expression platforms, and the adaptation of riboswitches to make fluorescent biosensors. We have focused the scope of this part of the review on examples that have shown activity in cells. In vivo applications include the use of riboswitches for reporting on ligands, regulation of cellular function, control of metabolic flux, and real-time biosensing of ligands.

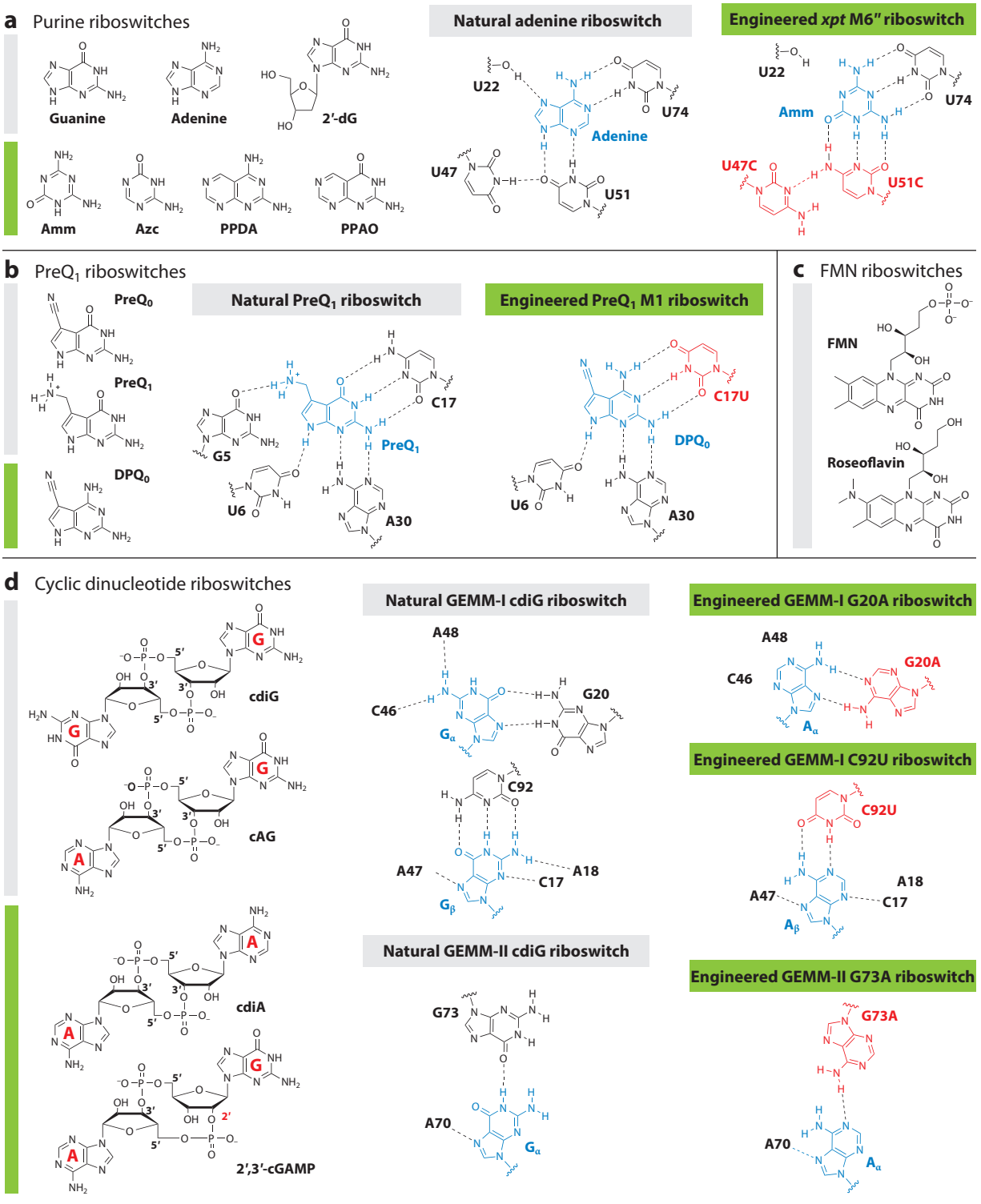
2. RIBOSWITCH ENGINEERING

2.1. Aptamer Domain Engineering

To achieve efficient and accurate gene regulation, aptamer domains in natural riboswitches must have biologically relevant specificity and binding affinity to respond to their ligands. For example, a representative of the *S*-adenosylhomocysteine (SAH) riboswitch class exhibits a dissociation constant (K_d) of 20 nM for SAH and discriminates more than 1,000-fold against *S*-adenosylmethionine (SAM), which differs by one small methyl group and is positively charged (4). From bioinformatics, structural, and biochemical studies, researchers have demonstrated that ligand-contacting nucleotides in natural riboswitches tend to be highly conserved across the evolutionary tree, whereas less conserved positions still can play roles in tuning the thermodynamics and/or kinetics of the binding event. Therefore, mutation of conserved nucleotides can result in changed ligand specificity, typically through altering the hydrogen-bonding pattern of ligand recognition. Here we present some case studies on natural riboswitch variants and corresponding engineering efforts to alter ligand specificity and sensitivity of riboswitch aptamers for gene regulation and biosensing applications (**Figure 1**).

2.1.1. Purine riboswitches. Purine riboswitches consist of structurally homologous subclasses that sense ligands including guanine, adenine, and 2′-deoxyguanosine (2′-dG), and therefore represent a great model for studying the relationship between aptamer sequence and ligand specificity (**Figure 1*a***). Within the purine riboswitch class, guanine riboswitches were first identified. Detailed characterization of the *xpt* guanine riboswitch in *Bacillus subtilis* revealed its tight binding affinity for guanine ($K_d < 5$ nM) and discrimination against many purine analogs, including adenine (5). A database search for sequences with secondary structure and sequence similarity to the *xpt* aptamer motif revealed a broad distribution of guanine riboswitches across the bacterial kingdom. Intriguingly, among these putative guanine riboswitches, only 3 out of 32 sequences were found to harbor a uracil instead of a cytosine at position 74 (numbering based on the *xpt* riboswitch) (5, 6). These U74 variants turned out to preferentially bind adenine instead of guanine (6). On the basis of this observation, it was hypothesized that the guanine and adenine riboswitches most likely recognize their respective ligands via a Watson-Crick base pair to nucleotide 74; thus, this nucleotide determines ligand selectivity (6). This base pair interaction, along with other important nucleotide–ligand interactions, was later validated by X-ray crystal structures and an NMR study of the purine riboswitch–ligand complexes (**Figure 1*a***) (7–10).

The single-base determinant of purine riboswitch selectivity offered an important precedent for rational engineering of riboswitch aptamers. However, these early studies also raised a cautionary note that riboswitch function hinges not only on engineering ligand specificity but also on binding affinity and regulatory function. For example, it was observed in vitro that the binding affinity of the C74U *xpt* riboswitch variant for adenine ($K_d \sim 100$ nM) is poorer than that of the wild-type riboswitch for guanine ($K_d \leq 5$ nM) (6). Additionally, guanine or adenine riboswitches incorporating the single mutation to nucleotide 74 that switched selectivity did not demonstrate the expected gene regulation in vivo (6). These observations suggest that other positions, although not systematically different between guanine and adenine riboswitches, contribute to ligand-binding affinities and in vivo function; thus, multiple mutations are required to engineer a useful riboswitch. This idea was supported by a systematic mutational analysis of nucleotides not directly contacting the ligand but involved in loop–loop interactions and the three-way junction in the riboswitch structure. These nucleotides were found to still play essential roles in riboswitch



function by promoting a binding-competent RNA fold or organizing a secondary structure switch with the expression platform (11, 12).

Similar principles were again demonstrated when 2′-dG riboswitches were discovered a few years later as a third subclass of purine riboswitches (13). Attempts to mutate the guanine-sensing *xpt* riboswitch to switch specificity toward 2′-dG showed that the pyrimidine at position 51 (numbering based on *xpt* riboswitch) was the principle determinant. However, the X-ray crystal structure showed that other positions, including base pairs proximal to the riboswitch binding pocket and nucleotides participating in loop–loop interactions, were also critical in organizing the tertiary structure to accommodate binding of 2′-dG (14), which was later corroborated by another structural study on a wild-type 2′-dG riboswitch (15).

The insights gained about natural purine riboswitches have led to engineered counterparts as orthogonal gene regulatory tools (16, 17). Structure-guided mutagenesis was performed on an adenine riboswitch by fully randomizing 2 residues (U47 and U51) around the ligand binding pocket to generate 15 candidate riboswitches (18, 19). These sequences were subjected to a chemical genetics screen in *Escherichia coli* involving nearly 80 heterocyclic analogs. The screen, along with further rational sequence optimization, resulted in three engineered riboswitches, termed M6, M6″, and M6C″. The first two are selective for ammeline (Amm) and the latter for azacytosine (Azc) (**Figure 1*a***) (18). These unnatural riboswitch–ligand pairs exhibited desirable orthogonality to the parent adenine riboswitch in vitro. This orthogonality was further demonstrated in *E. coli* by using the M6 and adenine riboswitches to independently control the expression of two reporter genes in response to Amm and 2-aminopurine, the latter of which binds to the adenine riboswitch (20). To improve the affinity of the engineered riboswitch–ligand pairs, a broader range of heterocyclic compounds were screened and second-generation ligands—pyrimido[4,5-*d*]pyrimidine-2,4-diamine (PPDA), and 2-aminopyrimido[4,5-*d*]pyrimidin-4(3*H*)-one (PPAO) (**Figure 1*a***)—with extended aromatic rings were developed for M6″ and M6C″ (21). These improved riboswitch–ligand pairs enabled construction of both turn-on and turn-off riboswitches for flexible gene regulation and application to orthogonal riboswitch-based control of bacterial cell behavior and physiology (21).

Figure 1

Ligands for natural and engineered aptamer domains and their binding pocket hydrogen-bonding network. Gray rectangles represent natural ligands for the indicated class of riboswitch, and green rectangles denote artificial ligands that require aptamer domain engineering. (*a*) Natural purine riboswitches have been found that respond to guanine, adenine, and 2′-deoxyguanosine (2′-dG). Mutants that instead interact with ammeline (Amm), azacytosine (Azc), pyrimido[4,5-*d*]pyrimidine-2,4-diamine (PPDA), and 2-aminopyrimido[4,5-*d*]pyrimidin-4(3*H*)-one (PPAO) have been engineered. The adenine ligand interacts with the riboswitch via a Watson-Crick interaction with U74, a Hoogsteen interaction with the hydroxyl group from the ribose of U22, and a sugar face interaction with U51 and U47 to impart specificity. In contrast, the engineered *xpt* M6″ riboswitch has U51C and U47C mutations, which allow specific interaction with Amm. (*b*) Natural $PreQ_1$ (7-aminomethyl-7-deazaguanine) riboswitches interact with $PreQ_0$ (7-cyano-7-deazaguanine) and $PreQ_1$. A C17U mutation in the riboswitch confers preferential binding to the diamino analog of $PreQ_0$ (DPQ_0). (*c*) Flavin mononucleotide (FMN) riboswitches naturally bind to both FMN and roseoflavin, which acts as an antibacterial compound by targeting FMN riboswitches. Natural riboswitches from species that biosynthesize roseoflavin, however, selectively respond to only FMN. (*d*) The GEMM-I and GEMM-II (genes for environment, membranes, and motility) riboswitch families are natural receptors for cyclic di-GMP (cdiG) and cyclic AMP-GMP (cAG), whereas engineered variants have been generated that bind to cyclic di-AMP (cdiA) and cyclic GMP-AMP with mixed phosphodiester linkages (2′,3′-cGAMP). Engineered and natural GEMM-I riboswitches that bind to cAG have an A20 instead of a G that interacts with the Hoogsteen face of the ligand A_α. In addition, cdiA-responsive GEMM-I riboswitches have a C92U mutation that interacts with the Watson-Crick face of A_β. Engineered GEMM-II riboswitches with a G73A mutation can bind cAG and 2′,3′-cGAMP, presumably due to favorable interaction with the endocyclic nitrogen on the Watson-Crick face of A_α.

2.1.2. $PreQ_1$ riboswitch. 7-Aminomethyl-7-deazaguanine ($PreQ_1$) is a queuosine precursor that is important for proper transfer RNA (tRNA) function in bacteria (**Figure 1*b***). The class I $PreQ_1$ riboswitch was first discovered and characterized in 2007 (22), followed by discovery of two other classes (23, 24). These riboswitches preferentially bind to $PreQ_1$ and exhibit a slightly poorer affinity for its metabolic precursor 7-cyano-7-deazaguanine ($PreQ_0$) (24).

Similar to the purine riboswitch, the $PreQ_1$ riboswitch has been structurally and biochemically characterized (25, 26), and structural analogs of the ligand are synthetically accessible and cell permeable, which makes the system promising for development of additional orthogonal riboswitch–ligand pairs. Structure-guided mutagenesis of nucleotides involved in hydrogen bonding to the ligand (**Figure 1*b***) was performed on a class I $PreQ_1$ riboswitch to generate seven candidate riboswitches (27, 28). After first ruling out candidates that responded to endogenous $PreQ_1$/$PreQ_0$, the remaining riboswitches were screened against six synthetic $PreQ_0$ analogs to identify ones that displayed ligand-dependent gene repression in *B. subtilis*. The resulting artificial riboswitch, M1 (C17U), responded only to the diamino analog of $PreQ_0$ (DPQ_0) and effectively repressed transcription of *mreB*, a gene for cell morphology in *B. subtilis*, upon addition of 2 mM DPQ_0 (**Figure 1*b***).

2.1.3. Flavin mononucleotide riboswitches. Roseoflavin is a rare but interesting example of a natural product whose antibacterial activity involves targeting riboswitches, and studying the mechanism of natural orthogonality in this system has led to some lessons for riboswitch aptamer engineering (**Figure 1*c***). Flavin mononucleotide (FMN) riboswitches are found across a broad spectrum of bacteria and regulate a number of essential genes involved in biosynthesis and transport of riboflavin, the nonphosphorylated precursor of FMN (29, 30). Similarly, roseoflavin is phosphorylated in vivo to make roseoflavin mononucleotide (RoFMN), which competes with FMN for riboswitch binding and downregulates FMN biosynthesis, resulting in growth defects and bacterial cell death (31–33).

Intriguingly, the bacterium that produces roseoflavin, *Streptomyces davawensis*, has an FMN riboswitch located upstream of the *ribB* gene, but evolutionary pressure on this producer organism apparently led to mutations that conferred response to FMN but insensitivity to RoFMN in vivo (34). However, in vitro characterization of this FMN riboswitch revealed that its aptamer domain does not selectively discriminate against roseoflavin under equilibrium binding conditions (31) and the complete riboswitch (aptamer plus expression domain) is unable to bind either FMN or RoFMN (34). The likely explanation for these observations is that the *ribB* riboswitch is kinetically rather than thermodynamically controlled, as was shown for other FMN riboswitches (35). In cotranscriptional regulation, kinetically driven riboswitches do not reach thermodynamic equilibrium with the ligand before RNA polymerase continues or terminates transcription. In other words, the requirement is for the ligand to bind fast enough to trigger downstream gene regulation, so the key parameter is the rate constant k_{on}, rather than the equilibrium K_d. Therefore, on the basis of these observations, it was proposed that a key nucleotide A61 within the *ribB* riboswitch alters the ligand specificity by slowing down RoFMN binding (34).

Kinetic analysis of FMN binding to several natural FMN riboswitch variants revealed that sequence variation in the stem regions, which are conserved only in length and pairing pattern but not in sequence composition, resulted in different dissociation rates and affinities (up to 100-fold difference among 8 variants) (36). On the basis of this observation for natural variants, a wild-type FMN riboswitch was engineered by systematically replacing A–U/G–U pairs with more stable G–C pairs in the stem regions, leading to 10-fold variance in both binding affinity and dissociation rates (36). Thus, this case study demonstrates the lesson that instead of focusing on conserved nucleotides for engineering ligand specificity, systematic analysis of stem regions

separated from the ligand binding pocket can also tune the binding kinetics and affinity of a riboswitch. Another important lesson from FMN riboswitches is that kinetically driven riboswitches complicate engineering efforts.

2.1.4. Cyclic dinucleotide riboswitches. We have already reviewed several cases of engineering inspired by nature. Conversely, this next case study shows how riboswitch aptamer engineering efforts led to the discovery of novel natural riboswitches. Cyclic di-GMP (cdiG) riboswitches were first identified by bioinformatics analysis as a highly conserved RNA structured element termed the GEMM motif (genes for environment, membranes, and motility) (37). Some GEMM motifs were located upstream of genes responsible for synthesis and degradation of cdiG or genes controlled by cdiG, leading to the hypothesis that they are cdiG riboswitches. This was later confirmed by in vitro biochemical probing that showed binding of the Vc2 GEMM-I riboswitch to cdiG with high selectivity and affinity as well as by in vivo reporter assays (38).

For the GEMM-I riboswitch, structural analysis revealed an asymmetrical mode of recognition for cdiG, as the two guanine bases in the ligand are recognized by a guanosine at position 20 via a Hoogsteen interaction and a cytosine at position 92 via a Watson-Crick interaction, respectively (numbering based on Vc2 riboswitch) (**Figure 1*d***) (39, 40). Structure-based mutation of these residues, G20A and/or C92U, generated artificial variants that preferentially bound to cyclic di-AMP (cdiA) or cyclic AMP-GMP (cAG), as expected from the nucleobase interactions (**Figure 1*d***) (40, 41). This was an intriguing finding, because around the same time, these other cyclic dinucleotides were in fact discovered as second messengers in bacteria.

The prior study showed that only C92U conferred binding to cAG, but we showed in the context of a riboswitch–Spinach biosensor fusion that the C92U variant did not bind cAG, whereas the G20A variant was promiscuous for both cdiG and cAG (42). The result that A20 could support cAG binding was exciting because a prior bioinformatics analysis had revealed that although the majority of GEMM-I riboswitch sequences had G20 like Vc2, 23% instead harbored A20 (43). By applying our ability to engineer riboswitch–Spinach biosensor fusions, we performed a phylogenetic screen for natural A20 GEMM-I riboswitches with altered ligand specificity, leading to the discovery of a subclass of GEMM-I riboswitches (named GEMM-1b) that selectively respond to cAG (44). Independent research based on the observation of unusual gene associations for a subset of GEMM-I riboswitches also led to the discovery of the same cAG riboswitches in Deltaproteobacteria (45). The identification of GEMM-Ib riboswitches by our group and others presents an inspiring example of riboswitch engineering facilitating biological discovery (40–42, 44).

Like other riboswitch classes, detailed characterization including structural elucidation of natural variants offers valuable lessons for engineering. In the phylogenetic screen, the majority of A20 sequences from GEMM-I class riboswitches displayed indiscriminate binding to cdiG and cAG. This observation indicated that, similar to position 74 in the purine riboswitch class, residue 20 in GEMM-I riboswitches represents a primary but not sole determinant for ligand specificity. Other residues located at the peripheral region of the ligand binding pocket also affect ligand recognition. It was found that the first two base pairs that stack above the ligand and residue 20 in GEMM-I riboswitches could affect ligand discrimination (46). This result again reinforces that nucleotides directly interacting with the ligand act as primary determinants, but subsequent optimization of peripheral positions might be necessary to generate artificial riboswitches or riboswitch-based biosensors with the desired selectivity.

We recently engineered another class of cdiG riboswitches for novel ligand response. The GEMM-II riboswitch class recognizes the guanines of cdiG via hydrogen bonding but makes fewer interactions with the ligand phosphate backbone relative to the GEMM-I class (41, 47, 48).

Mutation of key residues responsible for hydrogen bonding with cdiG resulted in increased affinity for cAG (49). Furthermore, this engineered riboswitch responds to cyclic GMP-AMP with mixed phosphodiester linkages (2′,3′-cGAMP), which is involved in the mammalian innate immune response (**Figure 1*d***) (49).

Together, the above examples demonstrate the potential for riboswitch aptamer domains to be engineered for sensing novel ligands. A general strategy that has been employed is one of semirational structure-based design: to mutate conserved nucleotides around the ligand binding pocket and sometimes to also mutate less conserved positions to fine-tune binding properties. However, one critical assumption made in structure-based design is that point mutations will not destabilize the RNA fold. Many examples of outlier mutants in the above studies confirm that this assumption does not always hold. Furthermore, these studies also show that regulatory function may require more extensive screening of riboswitch libraries to optimize the dynamic range for gene regulation, in order to tune ligand-binding affinities to physiologically relevant ranges or ligand on-rates to complement transcriptional rates for kinetically driven riboswitches.

2.2. Expression Platform Engineering

Although natural riboswitch aptamer domains are conserved in secondary structure across broad bacterial phyla, the associated expression platforms can vary greatly in both sequence and mode of action between species and even between examples within the same species (47). Therefore, engineering expression platforms requires choice of regulatory mode and fine-tuning of allosteric regulation, which often cannot be done rationally. In part, the difficulty in design arises from having limited information on ligand-free structures of riboswitches and also from having few full riboswitch structures that include the expression platform, as opposed to just the aptamer domain. In this section, we summarize the mechanisms of natural expression platforms as well as alternative expression platforms that have been invented for use in bacteria and eukaryotes (**Figure 2**). We then describe the three major approaches that have been taken toward engineering new expression platforms: rational design, screening and selection methods, and computational design.

2.2.1. Mechanisms of natural expression platforms. Natural riboswitches are typically found in the 5′-UTR (untranslated region) of bacterial mRNAs. One primary mechanism for bacterial riboswitch function in vivo is cotranscriptional regulation (**Figure 2*a***) (50). Ligand-dependent premature termination of transcription can turn off gene expression by preventing RNA polymerase from transcribing the coding region. Ligand binding typically destabilizes the antiterminator and permits formation of a terminator hairpin that disrupts the active transcription complex and leads to RNA polymerase dissociation from the mRNA. Alternatively, ligand binding may affect rho-dependent transcription termination (51). Although most riboswitches turn off genes, the inverse to these mechanisms (e.g., the ligand stabilizes the antiterminator) is used by riboswitches that turn on gene expression.

Another common mechanism in bacteria is translational regulation (**Figure 2*b***). Protein translation in bacteria initiates with binding of the 30S ribosome to the ribosome binding site (RBS), so blocking the RBS prevents translation. Ligand binding to a riboswitch aptamer can influence stem formation either to block the RBS or, conversely, to reveal the RBS (50). A rarer mechanism involves ligand-induced ribozyme activation (**Figure 2*c***), which has been observed for the glucosamine-6-phosphate riboswitch class. It was found that ligand binding activates self-cleavage of the *glmS* RNA, leading to mRNA degradation (52). The glucosamine-6-phosphate riboswitch is a natural example of an aptazyme—an aptamer coupled to a ribozyme as the expression platform—that initiates phosphodiester cleavage in response to ligand binding.

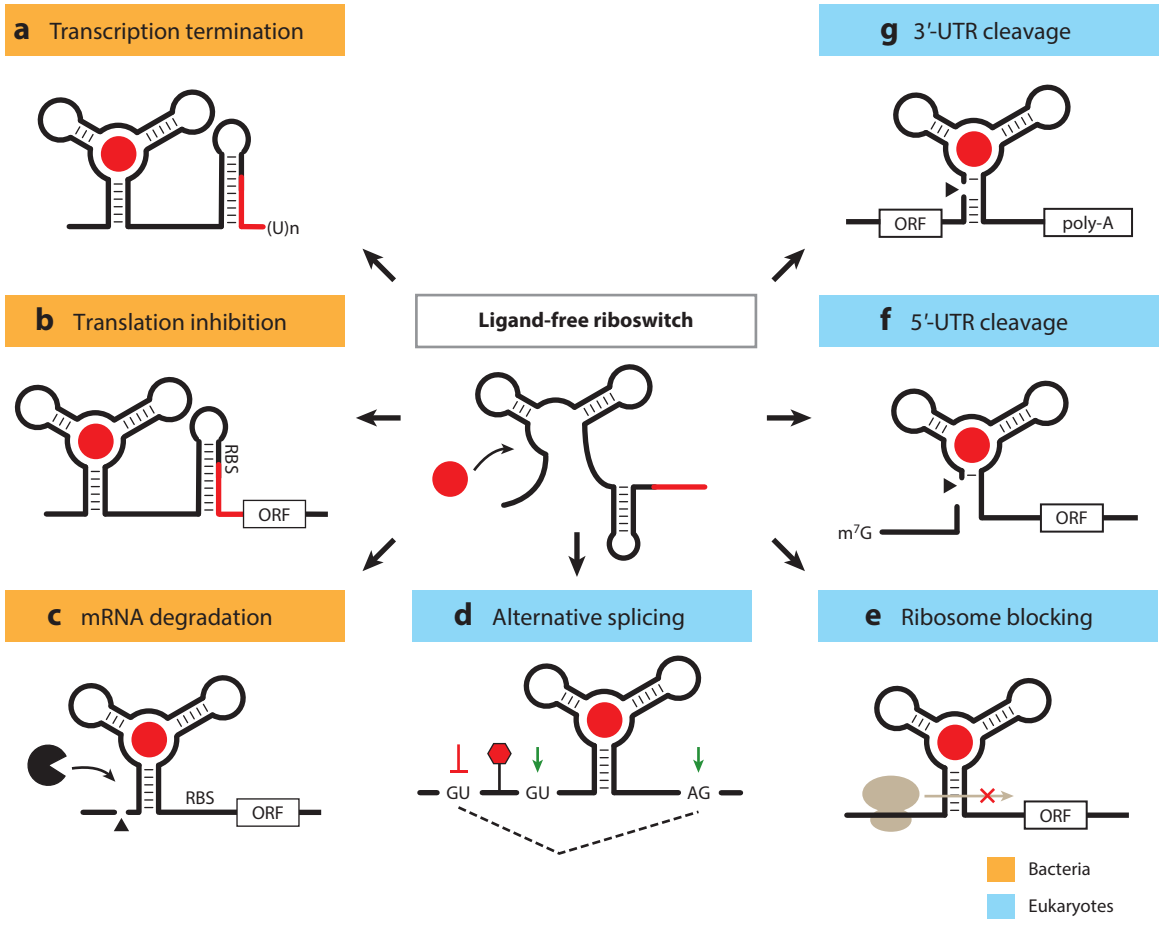


Figure 2

Mechanisms for natural and synthetic riboswitch regulation. Orange mechanisms are functional in bacteria, whereas blue mechanisms function in eukaryotes. The riboswitch ligand is denoted as a red circle. (*a*) Transcriptional attenuation: In the ligand-bound form, a terminator hairpin is formed, causing transcription termination. (*b*) Translation inhibition: Ligand binding causes an alternative structure to form, occluding the ribosome-binding site (RBS) and preventing initiation of translation. (*c*) Messenger RNA (mRNA) degradation caused by ribozyme activity: Ligand-induced self-cleavage by ribozyme activity destabilizes the mRNA, leading to rapid degradation. (*d*) Alternative splicing: Ligand binding leads to an alternate mature mRNA forming through changes in splice site selection. Depending on the organism, this leads to addition of a small upstream open reading frame (ORF), inclusion of a premature stop codon, or exclusion of the polyadenosine (poly-A) tail and mRNA degradation. (*e*) Ribosome blocking: In yeast, aptamer–ligand interactions can inhibit the ribosome from scanning from the 5′ 7-methylguanosine cap to the translation start site, preventing translation. (*f*) 5′-UTR (untranslated region) cleavage: Removal of the 5′ 7-methylguanosine cap by aptazyme activity prevents ribosome progression and destabilizes the mRNA. (*g*) 3′-UTR cleavage: Removal of the poly-A tail by aptazyme activity leads to rapid mRNA degradation.

The only natural riboswitches currently found in eukaryotes are thiamine pyrophosphate (TPP) riboswitches in plants and filamentous fungi. Eukaryotic riboswitches regulate gene expression via alternative splicing (**Figure 2*d***). In filamentous fungi and algae, the ligand-free aptamer masks a 5′-splice site, causing an alternative 5′-splice site to be utilized. The ligand-bound state unmasks this splice site, permitting inclusion of the exon located between the two alternative 5′-splice sites. This state leads to either inclusion of an upstream open reading frame, which is translated instead

of the main open reading frame (ORF) (in fungi), or inclusion of a premature stop codon (in algae) (53, 54). In higher plants, the ligand-free form in the 3′-UTR sequesters both 5′- and 3′-splice sites, leading to inclusion of a polyadenylation sequence and a mature RNA transcript. The ligand-bound form unveils both splice sites, leading to removal of the polyadenylation sequence and a transcript that is quickly degraded (55, 56).

2.2.2. Mechanisms of artificial expression platforms. Artificial riboswitches have been designed to regulate gene expression at the translational level in eukaryotes. Protein translation in eukaryotes begins with the 43S initiation complex binding to the 5′ 7-methylguanosine cap of the mRNA followed by scanning to the start codon. Insertion of aptamers downstream of the 5′ cap, but before the start codon, can block ribosome scanning and translation of the downstream gene (**Figure 2*e***). This mechanism appears to only work in yeast, presumably because the mammalian ribosome is able to efficiently scan through structured RNAs before the start codon (57).

Artificial aptazymes have been used to modulate gene expression in eukaryotes. Aptazymes located in either the 5′- or 3′-UTR can prevent translation. In the former case, aptazyme cleavage leads to loss of the 5′-cap, preventing ribosome initiation (**Figure 2*f***) (58). Aptazyme activity at the 3′-end leads to loss of the poly-A tail, favoring degradation of the mRNA transcript (**Figure 2*g***) (59). These aptazymes can act to activate or repress gene expression, depending upon whether the small molecule ligand turns the ribozyme on or off (59).

Besides these *cis*-regulatory mechanisms, aptamers and aptazymes can also regulate genes in *trans*. In principle, any RNA structure that can be unmasked by alternate stem formation or in response to ribozyme activity has the potential to be regulated by small molecules. Aptamer or aptazyme fusion to precursor microRNAs, for instance, permits their processing and subsequent activity only in response to small molecule addition (60, 61). This engineering effort resulted in ligand-inducible RNA interference. In two other examples, orthogonal tRNAs for unnatural amino acid incorporation and ribosome function have been successfully regulated by aptazymes (62, 63). We expect that, given the diversity already seen in nature and in engineered examples, new mechanisms for riboswitch-mediated gene regulation will continue to be discovered and developed.

2.2.3. Rational and semirational design. Careful study of riboswitch mechanisms has allowed the rational design of new riboswitches. In some transcriptionally regulated riboswitches, expression platforms can be decoupled from the activity of the natural aptamer domain and fused to foreign aptamers, which can yield riboswitches responsive to other ligands. The *B. subtilis metE*, *yitJ*, and *lysC* expression platforms were amenable to exchange with a number of aptamer domains that bind lysine, guanine, FMN, or theophylline to generate riboswitches that work in *E. coli* (64). Although these fusions exhibited lower dynamic ranges than the parent riboswitches, it was found that the dynamic range of a chimeric theophylline–*lysC* riboswitch could be restored to match the natural *lysC* riboswitch through optimization of P1 stem length. A similar aptamer swap design strategy has been used to generate a turn-off switch for cdiG in *B. subtilis* with the magnesium-sensing M-box riboswitch expression platform (65).

Natural turn-on riboswitches, such as the purine and cyclic di-GMP riboswitches, often contain significant overlap between the aptamer domain and expression platform, and this overlap prevents the generation of a simple, modular platform. To circumvent this issue, so-called decoupler sequences were engineered to create portable expression platforms of the *B. subtilis pbuE* adenine and *Dechloromonas aromatica metH* SAH riboswitches (66). Chimeric turn-on riboswitches were generated for SAM, other purines, and theophylline with higher dynamic ranges than the parent riboswitches (66).

Translationally regulated riboswitches have required different approaches to rational design, depending on the organism. In *B. subtilis*, the minimal length of accessible RNA required for stable binding of the ribosome to the RBS was found to be 14 nucleotides (67). Thus, inserting the theophylline aptamer (68) with a transducer element designed to act by a so-called helix-slipping mechanism (69) resulted in theophylline-induced gene expression via changing the accessible length of the RBS. Additional efforts were based on the hypothesis that inserting an aptamer upstream of the start codon would affect translation. This strategy worked in the case of the theophylline aptamer for *E. coli* and the tetracycline aptamer in *Saccharomyces cerevisiae*. The highest fold changes in gene expression upon ligand addition were obtained when the aptamer was inserted eight base pairs from the RBS or five base pairs upstream of the start codon (70, 71).

2.2.4. Screens and selections. One challenge with rational riboswitch design is that the aptamer must function in the context of the expression platform in vivo for gene regulation to occur (72). Although highly specific aptamers can be readily generated through in vitro systematic evolution of ligands by exponential enrichment (SELEX) (73), SELEX-generated aptamer/expression platform hybrids do not always function in vivo (74). As a result, most methods for generating riboswitches require in vivo screening for regulatory function before a desirable riboswitch can be obtained.

In a typical screen, the riboswitch library is cloned in front of a reporter gene to generate a signal output for selection. For colorimetric reporter genes such as *LacZ*, plate-based screening is used (71, 75). One improved screening method uses the *CheZ* gene as a reporter to modulate cell motility, which requires only basic molecular biology reagents and can screen libraries with $\sim 10^5$ clones (76). Employing a green fluorescent protein (GFP) reporter for riboswitch function allows the use of fluorescence-activated cell sorting (FACS) to screen much larger libraries for both basal and activated levels of gene expression (77). However, these studies also revealed a possible pitfall for in vivo screens: Individual cells in a population often exhibit heterogeneous expression of the reporter gene, which can complicate analysis and selection. This problem has been overcome by using an additional noise reporter system wherein the riboswitch gene is expressed along with a normalization control, such as *mCherry* (78).

Although these screening techniques offer significant improvements in throughput, the development of an antibiotic resistance–based selection process would permit analysis of even larger riboswitch libraries. Construct analysis would require only multiple rounds of growth under increasingly stringent selection conditions until a desired level of riboswitch activity was obtained. However, antibiotic resistance cassettes have several problems, including the inability to select for low cassette expression in the absence of a ligand and to select against mutations rendering constitutive expression of the resistance cassette, thwarting the selection process. These problems have been overcome through the development of a dual selection system with the tetracycline resistance cassette *tetA*. Under high levels of *tetA* expression, cells become resistant to tetracycline but sensitive to Ni^{2+}. In this way, riboswitches can be selected for both turn-on and turn-off functions by selecting for low expression with Ni^{2+} and high expression with tetracycline (79–82).

Similar selection methods have been developed recently for use in *S. cerevisiae* (83). On the basis of the two-hybrid assay used commonly to study protein interactions, the riboswitch is placed on the 3′-end of the *GAL4* transcription factor gene. High Gal4 levels then lead to increased expression of the three reporter genes: *HIS3*, *URA3*, and *LacZ*. Under high levels of expression, cells are able to survive on histidine-deficient media. A negative selection can be employed with the addition of 5-fluoroorotic acid to the medium: The *URA3* gene product, orotidine 5-phosphate decarboxylase, converts 5-fluoroorotic acid to the toxic 5-fluorouracil. This system allowed screening of an aptazyme library of 10^6 members. Because only a small percentage of riboswitches that

function in yeast can also function in mammalian systems, a similar dual selection method for use in mammalian cells would be highly desirable (74, 84). Until then, candidate riboswitches will have to be screened individually in mammalian cells (85).

2.2.5. Computational design. Although combinatorial libraries are useful for many ligands, high-throughput screening in vivo is difficult with ligands that have poor solubility, are cytotoxic, or are impermeable. Thus, drawing on the rich body of literature on the thermodynamics and kinetics of RNA function, some groups have turned to computational modeling to generate functional riboswitches for use in vivo. Initial attempts involved the generation of a hypothetical model for riboswitch function based on changes in rate constants; however, although modeling allowed for predictive validation of known riboswitches, it did not allow de novo prediction of riboswitch functionality from sequence and thermodynamic constraints alone (86).

One computational approach toward transcriptionally regulated riboswitches analyzed potential sequences based on three energetic criteria: (*a*) no secondary structure formation could occur between the aptamer and spacer; (*b*) the full-length transcript must contain a single hairpin formed from part of the aptamer, linker, and terminator regions; and (*c*) the mean free energy of the folded sequence must be more stable than the average free energy of a random sequence containing the same number and types of bases (87). Using these criteria, an artificial transcriptionally regulated riboswitch was designed that possessed similar fold regulation to that of natural riboswitches in response to theophylline. Further computational studies revealed that two parameters are likely important for transcriptionally regulated riboswitches: the difference in free energy between the terminator hairpin and the ligand-bound structure, and the kinetic barrier to terminator formation (88).

A more generalizable computational pipeline was developed for translationally regulated riboswitches (89). The model for riboswitch activity takes into account the folding energy of the riboswitch combined with the free energy change resulting from ribosome binding to the RBS and the start of translation (89). Using this computational model, a theophylline-responsive riboswitch was generated that activated gene expression in *E. coli* by 383-fold. More broadly, an automated riboswitch design system based upon this model generated computationally designed riboswitches that responded in vivo to fluoride or dinitrotoluene. This automated pipeline can be used to generate new riboswitches from known RNA aptamers for which ligand-bound structures and binding free energies are known.

2.3. Biosensor Engineering

Natural riboswitch aptamers exhibit high affinity for and dynamic response to a target ligand, making them privileged scaffolds for biosensor development. A biosensor binds specifically to a biological molecule of interest to generate a signal that can be detected by a noninvasive method such as fluorescence. Biosensors allow for the real-time imaging of biologically relevant molecules in vivo. General features of RNA-based fluorescent biosensors include: (*a*) a recognition domain for binding the target ligand (e.g., riboswitch aptamer), which is fused to (*b*) a transducer module that communicates ligand binding to activate (*c*) a signaling domain that binds the dye to produce a fluorescent output (90). The modular design of these sensors makes them easily adaptable for sensing a wide range of metabolites. Here we confine our discussion to riboswitch-based sensors shown to function in vivo; for in vitro assay applications, we refer readers to reviews on alternative strategies to modulate fluorescence in aptamer-based systems (91, 92).

The signaling domain for RNA-based biosensors consists of an in vitro selected aptamer sequence with high affinity to a profluorescent dye molecule. The first aptamer used as a signaling domain was the malachite green (MG) aptamer, which exhibits >2,000-fold fluorescence turn-on

when bound to its target fluorophore (93). Fusing the MG aptamer to ligand-binding aptamer domains led to development of theophylline, adenosine triphosphate (ATP), and FMN sensors that induced up to 8-fold fluorescent turn-on upon binding to the target ligand (90). However, because MG is a nonspecific DNA intercalator, these biosensors cannot be used in vivo. More recently, the Spinach aptamer was selected to bind 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), a small molecule mimic of the GFP chromophore (94). Similar to MG, the DFHBI molecule is nonfluorescent in dilute solution and exhibits 1,000-fold increase in fluorescence quantum yield upon binding, but in contrast has little to no cellular toxicity. Rational modifications to the Spinach scaffold have generated the Spinach2 aptamer, a variant with improved thermostability and enhanced fluorescent signal in vivo (95). High-throughput screening approaches in vivo have yielded the Broccoli aptamer (96).

Spinach, Spinach2, and Broccoli aptamers have been fused to riboswitch aptamers to generate ligand-responsive biosensors for live-cell imaging of target metabolites. The key challenge to biosensor engineering is the design of the transducer module, which must give allosteric regulation of the signaling domain by the recognition domain. An RNA-based biosensor that exhibited 25-fold fluorescence turn-on with SAM was constructed using the SAM-III riboswitch aptamer fused to 10 candidate transducer modules, which were stem sequences predicted to have weak thermodynamic stabilities (97). The same strategy was applied to generate an adenosine diphosphate (ADP) biosensor (97).

For natural riboswitches, their regulatory mechanisms often involve ligand binding affecting the P1 stem, so we expected that a transducer module involving the natural P1 stem would be effective for allosteric regulation. This P1–P2′ strategy (**Figure 3*a***) was used to construct an RNA-based fluorescent biosensor capable of visualizing the signaling molecule cdiG in live bacterial cells (42). With the P1–P2′ strategy, it was possible to screen a library of riboswitch aptamers fused to Spinach, in which the natural riboswitch sequences were identified by bioinformatics analysis of bacterial genomes. The screen resulted in a suite of four biosensors for cdiG that exhibited a broad range of affinities, including one with $K_d < 5$ nM, and improved fluorescent turn-on, with a maximal fluorescence signal that was brighter than the parent Spinach2 aptamer in vivo and useful in flow cytometry assays (98). The P1–P2′ strategy also has been employed to generate biosensors for cdiA (99) and 2′,3′-cGAMP (49).

Although the conventional riboswitch–Spinach fusion design is effective for appending most riboswitch aptamers to a Spinach aptamer, it is problematic for sequences that contain a terminal pseudoknot structure, such as the one found in the 3′ portion of the SAH riboswitch aptamer. To solve this problem and generate a biosensor that exhibits >11-fold fluorescence turn-on in response to SAH, we instead fused the riboswitch through the natural P2 stem to a circularly permuted Spinach2 (cpSpinach2) aptamer (**Figure 3*b***) (100). The design of cpSpinach2 was necessary to keep the biosensor as one contiguous RNA sequence. Similar to the second-generation cdiG biosensors, highly functional SAH biosensors were identified in one round of phylogenetic screening. We also have preliminary data suggesting that this cpSpinach2 strategy is generalizable to other terminal pseudoknot-containing riboswitches (P. Sander & M.C. Hammond, unpublished data).

An alternate strategy to engineering Spinach-based biosensors involves the incorporation of Spinach as an expression platform–like element, separate from the riboswitch aptamer (**Figure 3*c***). Similar to the antiterminator–terminator structure switch in transcriptionally regulated riboswitches, the ligand-free riboswitch aptamer interacts with part of the Spinach aptamer and blocks DFHBI binding, but upon ligand binding, Spinach is restored to its functional state. These Spinach fusions provide fluorescence turn-on for detection of guanine, adenine, TPP, and SAM (101). Similar construction principles were used to produce a turn-off glycine biosensor, which assumes the functional Spinach conformation in the absence of ligand (102).

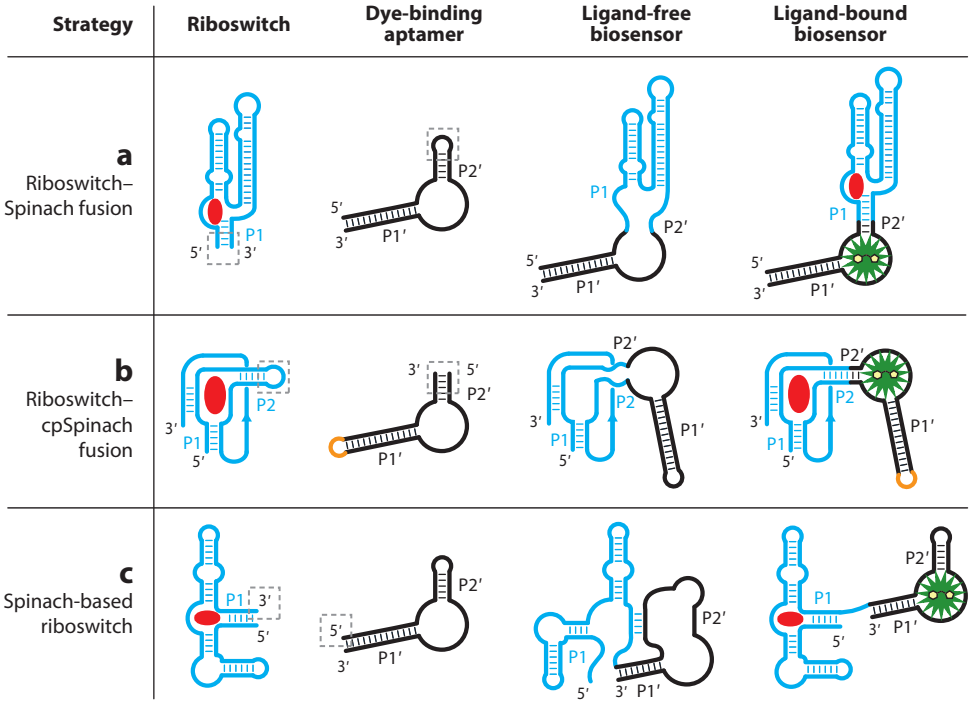


Figure 3

Structures of the three main classes of riboswitch-based fluorescent biosensors. Riboswitch domains and their corresponding metabolites are depicted in blue and red, and the Spinach/Spinach2 aptamer and 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) are shown in black and green. (*a*) The riboswitch–Spinach fusion strategy for biosensor development involves grafting of the P1 stem of a riboswitch sensor domain to the P2′ stem of Spinach. (*b*) To accommodate pseudoknot-containing riboswitches with noninteracting 5′- and 3′-ends, circularly permuted Spinach (cpSpinach), which has an engineered P2 open stem, can be fused to the P2′ stem of a riboswitch. (*c*) A Spinach riboswitch can be generated by linking the Spinach aptamer to a sensor domain through a transmitter sequence that allows fluorophore binding only in the presence of the metabolite.

These different design strategies offer a generalizable approach for the engineering of fluorescent biosensors to visualize a variety of small molecule ligands in vivo. Our natural stem approach has proven effective for generating sensors that exhibit greater sensitivity and fluorescence turn-on in response to natural targets. This strategy has also proven useful when engineering aptamers selective for new target ligands, as evidenced by the development of our cGAMP- and cAG-responsive biosensors (44, 49). The development of new fluorophore–aptamer pairs in vitro bodes well for the expansion of RNA-based biosensors into further wavelengths for orthogonal imaging (103–105). The future is bright for RNA-based fluorescent biosensors.

3. IN VIVO APPLICATIONS OF RIBOSWITCHES

The ability of both natural and synthetic riboswitches to respond to levels of a particular small molecule in a concentration-dependent manner has made them valuable for a wide range of applications in living systems, which we review in this section. These applications include the use of riboswitches in reporter systems for biochemically important ligands, the generation of riboswitches

responsive to xenobiotic molecules for control of gene expression and cellular behaviors, and the use of riboswitches in metabolic engineering to optimize small molecule production. We also discuss recent developments in the use of riboswitch-based biosensors for in vivo imaging of small molecules.

3.1. Riboswitches as Small Molecule Reporters

A common experiment to validate function of newly discovered riboswitches fuses 5′-UTRs containing potential riboswitches to the coding sequence of β-galactosidase, and these reporter constructs are used to demonstrate modulation of recombinant protein levels in response to added ligand (50, 106, 107). Furthermore, once the riboswitch ligand is known, similar reporter constructs can be used to monitor ligand levels in vivo. In this section, we review case studies for how riboswitches have been used to report in vivo levels of small molecules for enzyme screening, protein engineering, and strain engineering.

Vitamin B_{12} is an important corrinoid cofactor involved in C-C bond isomerization reactions; however, much remains unknown about regulation of its levels in the cell. A B_{12} reporter was constructed by fusing the promoter and 5′-UTR of the *btuB* operon from *E. coli* to the GFP coding sequence, which leads to B_{12}-dependent repression of GFP expression in *E. coli* (108). This reporter was used to validate the corrinoid salvage pathway by analyzing the fluorescence signal from genetic knockout strains upon addition of an exogenous precursor to B_{12}, cobinamide. The reporter–knockout strategy was also applied to evaluate the role of various transporters in maintaining B_{12} homeostasis (108). In a follow-up study on the $BtuC_2D_2F$ transporter, the same riboswitch reporter enabled rapid in vivo screening of transporter activity of more than 100 mutants of the substrate binding pocket to identify the amino acids important for specific interactions with B_{12} (109).

cdiG is the master regulator of the transition between the motile planktonic and sessile biofilm-forming states of bacteria, and multiple reporters and sensors have been developed for its detection. Genomic insertion of a cdiG reporter into *B. subtilis* allowed a high-throughput screen of 37 predicted cdiG metabolizing enzymes from *Clostridium difficile*, a pathogen associated with colitis, to be performed through flow cytometry analysis (65). Similar validation of two membrane-bound diguanylate cyclases from *Bacillus thuringiensis* and *Xanthomonas oryzae* was possible through adaptation of a naturally occurring triple-tandem GEMM-I riboswitch from *B. thuringiensis* into a fluorescent reporter for use in *E. coli* (110).

Riboswitch reporters can also be used in screens or selections to engineer or tune enzyme activities. One proof-of-principle study in yeast developed an enzyme capable of specifically removing a methyl group from caffeine to yield theophylline (111). A tandem plate/FACS-based screen of a mutant library constructed from a monooxygenase with low theophylline synthase activity used a theophylline aptazyme fused to a GFP coding sequence to successfully identify variants with higher theophylline synthase activity (111). Similarly, a lysine riboswitch controlling a tetracycline resistance cassette facilitated selection of a chimeric aspartate kinase optimized for lysine fermentation (112). An analogous riboswitch-resistance cassette was used to select the appropriate promoter from a plasmid-based library to drive production of the TCA cycle enzyme phosphoenolpyruvate carboxylase, leading to more than 10-fold increase in lysine titers with little decrease in cellular growth rate (113). These rapid optimizations of enzyme mutants and expression levels were performed without having to engineer a novel theophylline- or lysine-responsive transcription factor.

Riboswitch reporters can also be used to select for cell strains producing a desired cell-permeable product. To avoid the need to transform strain libraries and have reporter expression

take resources away from production, riboswitch reporters can be used in reporter cells separate from the producer cell to be optimized. To improve riboflavin (vitamin B_2) production, an *E. coli* reporter strain was engineered that lacked B_2 synthesis machinery but was able to convert riboflavin to FMN and carried a *ribB* riboswitch-based aptazyme controlling GFP production (114). Thus, the reporter *E. coli* generate fluorescence in the presence of B_2. Using nanoliter reactors, in which individual members of a *Bacillus* production strain library were grown with *E. coli* reporter cells and analyzed by FACS, strains possessing up to 150% of the B_2 synthesis capabilities of the parent industrial strain were isolated (114).

3.2. Riboswitches for Conditional Gene Regulation

Although riboswitches can be used to understand and engineer biological processes involving natural metabolites, it is often desirable to induce gene expression by the addition of a xenobiotic small molecule. Riboswitch-based gene regulation has been utilized in a variety of bacterial and eukaryotic species. This ability to precisely regulate gene expression has made higher-level applications, such as controlling cellular behaviors and developing logic gates, possible.

Riboswitch-based regulation of protein production has been demonstrated in a wide array of bacterial phyla. Modern riboswitches optimized through screens or computational methods have induction levels more than 300-fold in *E. coli* (89). Riboswitches can be incorporated as part of the transgene component, in contrast to other regulatory systems that require both the regulatory protein and transgene to be introduced. A set of engineered theophylline-inducible riboswitches function in a wide range of bacterial phyla, including both gram-positive and gram-negative species (115, 116). Optimized riboswitches activate gene expression 80-fold in *Acinetobacter baumannii*, 70-fold in *B. subtilis*, and 60-fold in *Mycobacterium smegmatis*. Although the optimized system resulted in only an 8.2-fold activation in the pathogen *Mycobacterium tuberculosis*, it robustly regulated protein production in a macrophage infection model (117). Similar systems of theophylline-responsive riboswitches have been demonstrated in streptomyces and cyanobacterial species (118–121).

Riboswitch engineering has also been applied to eukaryotic gene regulation: Tetracycline and neomycin riboswitches have been developed that block ribosome scanning in yeast (70, 122, 123), and the tetracycline and theophylline aptamers have been used to regulate alternative splicing, similar to the natural fungal and plant TPP riboswitches described earlier (124, 125). The development of aptazyme systems based primarily on the theophylline and tetracycline aptamers has added options for control of gene expression (59). Other recent reports have demonstrated theophylline riboswitch-based control of genes in plant plastids and in viral replication in mammalian cell hosts (126–130).

The ability to induce gene expression has been applied to control important cellular behaviors such as cell motility. Control of the CheZ motility protein in *E. coli* through riboswitch-related activity has generated bacteria that preferentially move toward theophylline and Amm (21, 131). Another desirable trait to control is cell proliferation. Modular theophylline and tetracycline aptazymes were used to chemically control proliferation of different cytokines in murine and human T cells, leading to 2-fold regulation of cell viability (132). Aptazyme-based regulation of cellular behaviors also has been demonstrated through the modulation of mating efficiency in yeast and control of the cell cycle in the U2-OS cell line (133, 134).

Another application of riboswitch-based protein regulation is to generate conditional knockouts of essential genes for basic biological studies. Using a tetracycline-controlled expression system, a conditional knockdown system for five essential genes in *S. cerevisiae* was created by varying the promoter sequence to change the expression strength (135). Notably, two of these genes,

NOP8 and *SEC1*, were not successfully knocked down with previously established conditional expression systems. Additionally, riboswitches can enable the selective expression of dominant negative mutations, as shown with theophylline-responsive *csrA* in *E. coli* to demonstrate its role in autoaggregation and cell cycle control (136).

In addition to simple on/off functionalities that respond to one small molecule, riboswitches can be engineered to engage in more complex modes of regulation through implementation of biochemical logic gates and feedback systems. Riboswitch-based logic gates exist in nature, with tandem riboswitches controlling pathways involving multiple ligands or allowing more digital control over gene expression (137, 138). Similar logic gates have been implemented through the use of synthetic riboswitches and aptazymes (80, 139, 140). Additionally, a tandem riboswitch that only generates a signal within a tight range of ligand concentration was developed by combining a translationally controlled ON riboswitch with a less sensitive transcriptionally controlled OFF TPP riboswitch (141).

3.3. Riboswitches to Engineer Metabolic Flux

One primary goal of metabolic engineering is to create organisms that produce industrial and fuel compounds at higher yields than can be obtained from a naturally occurring organism. A major associated challenge is diverting biochemical building blocks to produce the desired compound without compromising cell viability. Because riboswitches can regulate gene expression in response to a particular compound, they hold promise as tools for metabolic engineering. As described earlier, riboswitch reporters have been applied to screen or select for mutant enzymes or strains with higher production capacities. In addition, riboswitches have been used to directly control central metabolism enzymes to improve yields.

The lysine riboswitch was used to regulate the flux of carbon-containing precursors through the citric acid (TCA) cycle. Lysine is produced from the TCA intermediate oxaloacetate (OAA), which alternatively is converted to citrate by citrate synthase as part of the TCA cycle. Inserting an *E. coli* lysine-OFF riboswitch upstream of the citrate synthase gene in the industrially relevant lysine fermenting gram-positive bacterium *Corynebacterium glutamicum* led to 63% higher lysine yields (142). Addition of an engineered lysine-ON switch upstream of the natural lysine transporter *lysE* gene further increased total lysine yield (143).

3.4. Riboswitch-Based Fluorescent Biosensors

Quantitative, real-time detection and tracking of small molecules in living cells have traditionally relied upon the use of protein-based biosensors (144). Riboswitch-based biosensors present an appealing alternative for several reasons. First, unlike proteins, these RNAs can be synthesized in vitro from commercial DNA templates using a single enzyme, T7 RNA polymerase, which accelerates high-throughput screening for biosensor development. Second, riboswitch aptamers are naturally evolved for in vivo function with high ligand specificity, affinity, and allosteric regulation in response to ligand. These qualities enable RNA biosensors to produce a high fluorescence turn-on in the presence of their target molecule, with excellent discrimination against other cellular metabolites. Third, Spinach-based biosensors employ a fluorophore dye molecule that enables cellular imaging under anaerobic conditions (98). In contrast, protein-based sensors generally require extensive engineering to optimize signal output and affinity, and fluorescent proteins in the GFP family require oxygen for chromophore maturation (145). In this section, we review the applications of riboswitch-based fluorescent biosensors for monitoring metabolites and small molecule signals in bacteria.

To date, riboswitch-based biosensors have been developed for in vivo fluorescent imaging of metabolites SAM, ADP, TPP, and SAH in bacteria (97, 100, 101). For example, the TPP biosensor was used to monitor TPP biosynthesis in *E. coli* after addition of thiamine, detecting different rates of accumulation within the cell population (101). A more stringent example for in vivo metabolite detection is the SAH biosensor, which must detect SAH selectively because the related SAM cofactor has been measured to be in ~300-fold excess in *E. coli* (146). We showed that the SAH biosensor has higher selectivity than commercial monoclonal antibodies and further demonstrated fluorescence monitoring of the chemical inhibition of 5′-methylthioadenosine nucleosidase (MTAN), an endogenous enzyme involved in SAH turnover, in live *E. coli* cells using flow cytometry (100). Another potential application of SAH biosensors is to detect methyltransferase activity in vivo, although product inhibition of this enzyme class by SAH is common and will require a more sensitive biosensor to detect low turnover enzyme activity. These initial applications do illustrate the potential for using riboswitch-based biosensors to study real-time dynamics of specific metabolic pathways in bacteria.

To date, riboswitch-based biosensors also have been developed for in vivo fluorescent imaging of small molecule signals cdiG, cdiA, and cAG in bacteria (42, 44, 98, 99). The biosensors have been applied toward studying the activity of the corresponding signaling enzymes within cells. We employed the cdiA biosensor in a flow cytometry assay to validate the activity of a putative archaeal diadenylate cyclase, providing the first experimental evidence for cdiA as a signaling molecule in this domain of life (99). A high-throughput flow cytometry screen of 29 candidate GGDEF enzymes (named after five conserved residues in the catalytic domain) from *Geobacter sulfurreducens* using our cdiG and cAG biosensors recently revealed a novel subclass of hybrid promiscuous (Hypr) GGDEF enzymes that produce cAG (147). This discovery breaks the long-standing paradigm that GGDEF enzymes make only cdiG, and the distribution of Hypr GGDEFs shows that cAG signaling is more widespread in bacteria than previously understood. These initial applications demonstrate the power of riboswitch-based biosensors to study in vivo enzyme activity and signaling activity.

4. CONCLUSIONS

Among the diverse roles and functions of RNA, natural riboswitches stand out as small molecule induced regulators of gene expression and privileged scaffolds for future engineering. These roles have been facilitated by the multiple strengths possessed by riboswitch aptamers, including their modular nature, small genetic footprint, and amenability toward structural characterization.

Riboswitches can be engineered through their aptamer domains to sense unnatural small molecules for orthogonal gene regulation. Furthermore, diverse expression platforms have been engineered to modulate genetic outputs in a broad spectrum of organisms. Beyond traditional roles in gene regulation, the portable nature of riboswitch aptamer domains has allowed their import to new signal outputs such as fluorescence in RNA-based biosensors. Engineered riboswitches have already demonstrated multiple applications in biological discovery and the development of synthetic biological systems. As this field continues to expand, new engineering efforts must meet the growing demand for more stringent applications.

Riboswitch engineering has been enabled by multiple strategies, including rational structure-based design, computational models, and screens/selections. To meet the rising need, more strategies must be developed to overcome limitations inherent in these methods. The current strategy coupling structure-based design and screening can lead to engineered aptamer domains, but this method is laborious and low-yielding, making higher-throughput selection strategies desirable. SELEX-based methods offer a possible solution, but in vitro activity does not always correlate

with in vivo function, especially if function requires allosteric changes or tuning of kinetic parameters. This limitation implies that selection methods must have an in vivo component. Although a general strategy for expression platform engineering is desirable, the best mammalian riboswitches possess lower efficiency in gene regulation than their bacterial counterparts, necessitating the development of selection methods specific to these types of cells. In the context of riboswitch-based biosensors, a broadened palette of fluorescent output wavelengths would permit multiplexed imaging of metabolites. We envision that with these new developments, engineered riboswitches will attain an even more powerful place in the toolbox of synthetic and chemical biologists.

DISCLOSURE STATEMENT

M.C.H. and Y.S. are co-inventors on patent applications for RNA-based fluorescent biosensors.

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