# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE *(DD-MM-YY)* | 2. REPORT TYPE | 3. DATES COVERED *(From - To)* |
|---|---|---|
| 25 05 18 | Book Chapter | |

**4. TITLE AND SUBTITLE**
Riboswitches as Sensor Entities

**5a. CONTRACT NUMBER**
In-House

**5b. GRANT NUMBER**

**5c. PROGRAM ELEMENT NUMBER**
61102F

**6. AUTHOR(S)**
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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**
H0E0

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
1Henry M. Jackson Foundation for the Advancement of Military Medicine, Dayton, OH

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
2Air Force Materiel Command
Air Force Research Laboratory
711th Human Performance Wing
Airman Systems Directorate
Human Centered ISR Division
Human Signatures Branch
Wright-Patterson AFB, OH 45433

**10. SPONSORING/MONITORING AGENCY ACRONYM(S)**
711 HPW/RHXBC

**11. SPONSORING/MONITORING AGENCY REPORT NUMBER(S)**
AFRL-RH-WP-JA-2018-0017

**12. DISTRIBUTION/AVAILABILITY STATEMENT**
Distribution A. Approved for public release. 88ABW-2018-2730, cleared 25 May 2018

**13. SUPPLEMENTARY NOTES**
G. Thouand (ed.), Handbook of Cell Biosensors, https://doi.org/10.1007/978-3-319-47405-2_121-1

**14. ABSTRACT**
Riboswitches are regulatory noncoding RNAs, predominantly located in the 50 untranslated region of mRNA, that can serve as molecular switches able to regulate the level of gene expression. This occurs through the conformational changes caused by binding to a specific metabolite. Riboswitches contain two structural domains: an aptamer domain that senses and binds to a metabolite and an expression platform that undergoes a conformational change in response to aptamer-ligand binding resulting in regulation of expression of downstream gene. In addition to natural riboswitches found in living organisms, a variety of synthetic riboswitches that respond to nonnatural small molecules have been developed. Synthetic riboswitches can be engineered to regulate expression of any gene in response to any nonnatural molecule that is capable of being bound by RNA and is not toxic to cells. This feature demonstrates a strong possibility for RNA switches to serve as sensor entities for design and development of cellbased biosensors with a variety of different applications. This chapter gives an overview of riboswitch selection techniques, describes reporter systems for monitoring riboswitch activation and approaches for riboswitch tuning and performance optimization in order to fulfill biosensor requirements, and discusses riboswitch applications as sensor entities.

**15. SUBJECT TERMS**
Synthetic riboswitch · Aptamer · Gene regulation · SELEX · In vivo selection ·Biosensor

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT: | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON (Monitor) |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | Michael Goodson |
| Unclassified | Unclassified | Unclassified | SAR | 31 | 19b. TELEPHONE NUMBER *(Include Area Code)* N/A |

**Standard Form 298 (Rev. 8-98)**
Prescribed by ANSI Std. Z39-18

# Riboswitches as Sensor Entities

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G. Thouand (ed.), *Handbook of Cell Biosensors*,
https://doi.org/10.1007/978-3-319-47405-2_121-1

**Abstract**

Riboswitches are regulatory noncoding RNAs, predominantly located in the 5′ untranslated region of mRNA, that can serve as molecular switches able to regulate the level of gene expression. This occurs through the conformational changes caused by binding to a specific metabolite. Riboswitches contain two structural domains: an aptamer domain that senses and binds to a metabolite and an expression platform that undergoes a conformational change in response to aptamer-ligand binding resulting in regulation of expression of downstream gene. In addition to natural riboswitches found in living organisms, a variety of synthetic riboswitches that respond to nonnatural small molecules have been developed. Synthetic riboswitches can be engineered to regulate expression of any gene in response to any nonnatural molecule that is capable of being bound by RNA and is not toxic to cells. This feature demonstrates a strong possibility for RNA switches to serve as sensor entities for design and development of cell-based biosensors with a variety of different applications. This chapter gives an overview of riboswitch selection techniques, describes reporter systems for monitoring riboswitch activation and approaches for riboswitch tuning and performance optimization in order to fulfill biosensor requirements, and discusses riboswitch applications as sensor entities.

**Keywords**

Synthetic riboswitch · Aptamer · Gene regulation · SELEX · *In vivo* selection · Biosensor

## Introduction

Living systems have developed the ability to control their gene expression patterns in response to changes in the extracellular and intracellular environment. While the majority of regulatory mechanisms involve protein action, genetic regulation by noncoding RNAs, or riboswitches, is widespread in bacteria (Mandal and Breaker 2004; Nudler and Mironov 2004; Winkler and Breaker 2005; Serganov and Nudler 2013) and is also found in some eukaryotes (Cheah et al. 2007; Wachter 2010; Serganov and Nudler 2013). Riboswitches are predominantly located in the 5′untranslated region (5′-UTR) of mRNA; however, they can be present in the 3′-UTR in some eukaryotic mRNA. Similar to regulatory proteins, riboswitches act as molecular sensors able to regulate the level of gene expression through the conformational changes caused by binding to a specific ligand.

Structurally, riboswitches are composed of two functional domains: an aptamer domain that senses and binds to a ligand and an expression platform that controls the expression of a downstream gene by changing its conformation in response to the ligand-induced changes in the aptamer domain (Tucker and Breaker 2005; Nudler 2006; Edwards and Batey 2010). Riboswitches can up- or downregulate gene expression employing a variety of regulatory mechanisms (Fig. 1). The mechanism

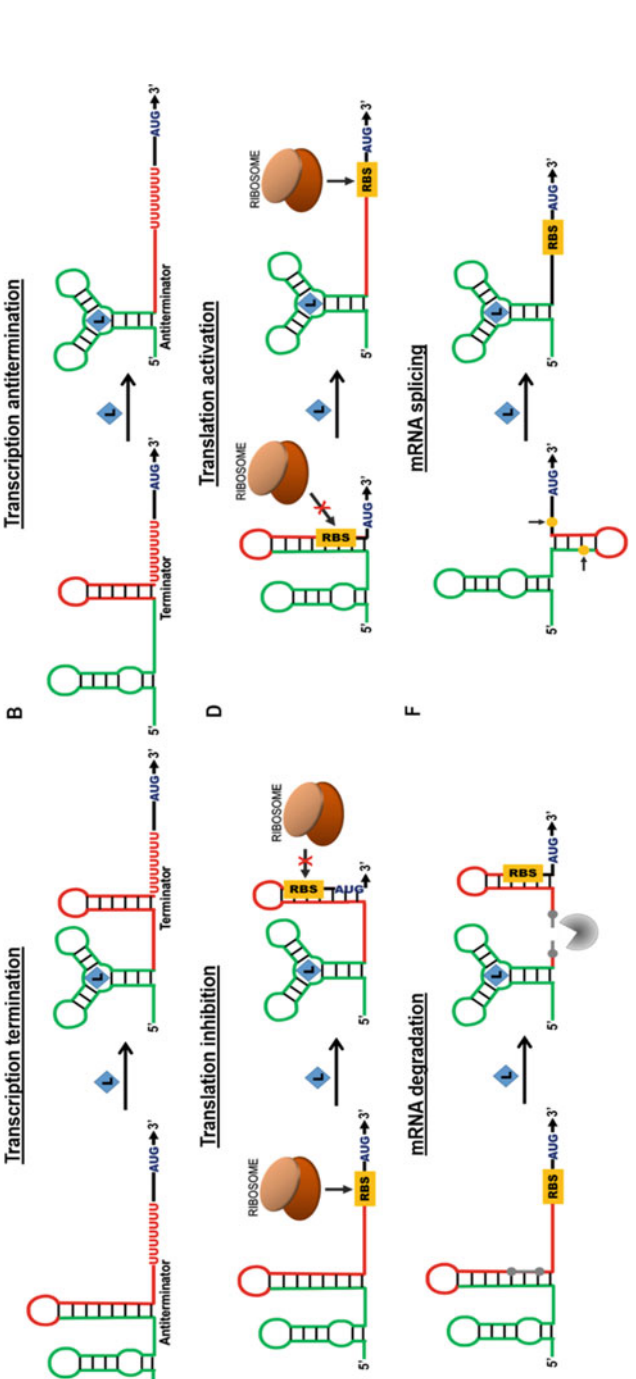


**Fig. 1** Examples of riboswitch mechanisms. Bacterial riboswitches upregulate or downregulate gene expression operating at level of transcription, translation, mRNA degradation, or splicing. Aptamer is colored green. Terminator hairpin is colored red. RNAse cleavage site is colored grey. (**a**) Ligand (L) binding to the aptamer causes formation of a terminator hairpin leading to transcription termination. (**b**) In the absence of ligand, an intrinsic terminator stops transcription. Ligand binding to the aptamer induces a conformational shift that forms an antiterminator, enabling expression of downstream gene. (**c**) Ligand binding to the aptamer causes an alternative structure to form, blocking the ribosome binding site (RBS) and preventing initiation of translation. (**d**) The RBS is blocked in the absence of ligand. Upon ligand binding to the aptamer, the RNA undergoes a conformational shift, revealing the RBS and enabling translation. (**e**) Upon ligand binding to the aptamer, the riboswitch adopts a conformation that exposes RNase cleavage site leading to mRNA degradation. (**f**) Ligand binding to the aptamer leads to mRNA self-cleavage and splicing that brings together two halves of the RBS, and the resulting mRNA is efficiently translated

of riboswitch action is based on the existence of two thermodynamically stable conformations ("off" and "on" states), separated by the energy barrier which inhibits spontaneous switching. In general, when the concentration of a recognized molecule reaches the binding threshold value, the interaction with the aptamer domain takes place. This promotes the switching to the alternative conformation by stabilization of the intermediate and final states (Edwards and Batey 2010; Machtel et al. 2016). The most frequently riboswitch-mediated changes in gene expression occur either transcriptionally or translationally. The expression platform for a riboswitch that acts during transcription typically involves the ligand-dependent formation of an intrinsic terminator or antiterminator structure (Blouin et al. 2009; Peselis and Serganov 2014; Topp and Gallivan 2010; Lemay et al. 2011; Gong et al. 2017b); Hallberg et al. 2017). In contrast, riboswitches that operate at a translational level most often function by masking or releasing the Shine-Dalgarno (SD) sequence (also known as the ribosome binding site; RBS) in a ligand-dependent fashion. When the SD sequence is released, the ribosome can bind to the mRNA and permit translation; masking the SD sequence represses translation (Blouin et al. 2009; Peselis and Serganov 2014; Topp and Gallivan 2010; Lemay et al. 2011; Hallberg et al. 2017; Antunes et al. 2018).

To date, numerous examples of naturally occurring riboswitches responding to a variety of ligands (enzyme cofactors, nucleotide precursors, amino acids, and metal ions) as well as changes in temperature and pH-value have been discovered (Coppins et al. 2007; Barrick and Breaker 2007; Roth and Breaker 2009; Serganov and Nudler 2013; Peselis and Serganov 2014; Hallberg et al. 2017; Pham et al. 2017). The size and structure of riboswitch ligands vary significantly, an indication that riboswitches could be used to detect a wide range of targets. In fact, some natural riboswitches found applications in enzyme and strain engineering, in controlling gene expression and cellular physiology, and in real-time imaging of cellular metabolites and signals (Topp and Gallivan 2010; Machtel et al. 2016; Hallberg et al. 2017). This apparent versatility of riboswitches in nature is being exploited by researchers in order to develop synthetic riboswitches that regulate gene expression in response to a desired target molecule. The advantage of such engineered riboswitches is that they offer a way to control gene expression via nonnatural molecules, for example, drugs or explosives. Moreover, synthetic riboswitches are of particular interest and demand since the majority of natural riboswitches respond to essential metabolites and, therefore, their use as sensing elements can be compromised by fluctuations in intracellular metabolite concentrations, and the exogenous addition of these compounds could negatively impact normal cellular function.

This chapter gives an overview of riboswitch selection techniques, describes reporter systems for monitoring riboswitch activation and approaches for riboswitch tuning and performance optimization in order to fulfill biosensor requirements, and discusses riboswitch applications as sensor entities.

## Development of Riboswitches Responsive to Small Molecule Analytes

Different methods have been applied to develop synthetic riboswitches that respond to a variety of different target analytes (Wittmann and Suess 2012; Groher and Suess 2014; Berens and Suess 2015; Gong et al. 2017a; Etzel and Mörl 2017; Findeiß et al. 2017). Similar to their natural counterparts, synthetic riboswitches are composed of the same structural domains and can be designed to downregulate or upregulate gene expression at transcriptional or translational levels. To create new riboswitches, some researchers follow a bottom-up approach, in which individual riboswitch building blocks are designed de novo and assembled into functional regulators. Another approach to generate synthetic riboswitches is reengineering natural riboswitches by altering the specificity of the aptamer domain or modifying the expression platform. Both approaches require time consuming *in vivo* screening with analysis of large number of riboswitch clones before a functional riboswitch can be obtained. In order to speed up the selection process, researchers employ new strategies based on rational and computational design for synthetic riboswitches.

### Aptamer Selection

Typically, synthetic riboswitch development starts with selection of an analyte sensing domain, the aptamer. Synthetic riboswitches can be designed to respond to different targets by integrating different aptamers as recognition elements. Suitable aptamers must bind a molecule of interest (that, in most cases, does not resemble a natural cell compound) with high affinity and specificity, and undergo sufficient changes in structure and/or stability upon an analyte binding. Such aptamers can be generated using a method known as Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (Ellington and Szostak 1990; Tuerk and Gold 1990) depicted in Fig. 2. In a typical SELEX experiment, the target molecules are usually immobilized on a solid surface to enable an efficient separation of target-binding and nonbinding oligonucleotides. The procedure begins with $10^{13}$–$10^{15}$ unique sequences from a random oligonucleotide pool that compete to bind a target. This library consists of sequences designed with two PCR primer regions flanking a random region of, typically, 30–50 nucleotides. After incubation with the immobilized target, nonbinding oligonucleotides are removed by washing, whereas bound oligonucleotides are specifically eluted with free target. The binding sequences are reverse transcribed, amplified, and subjected to the next round of selection. The process is repeated in cyclical fashion until the final pool is enriched for sequences binding to the target. Typically, after the first round, researchers may institute a counter-selection step in which sequences binding to a control (such as the support matrix alone or a molecule similar in structure to the target) are removed from solution, and those that do not bind to the control are retained for future SELEX rounds. Gradually increasing the stringency during the selection cycles can lead to aptamers that bind a desired target with high affinity. A small portion of the pool

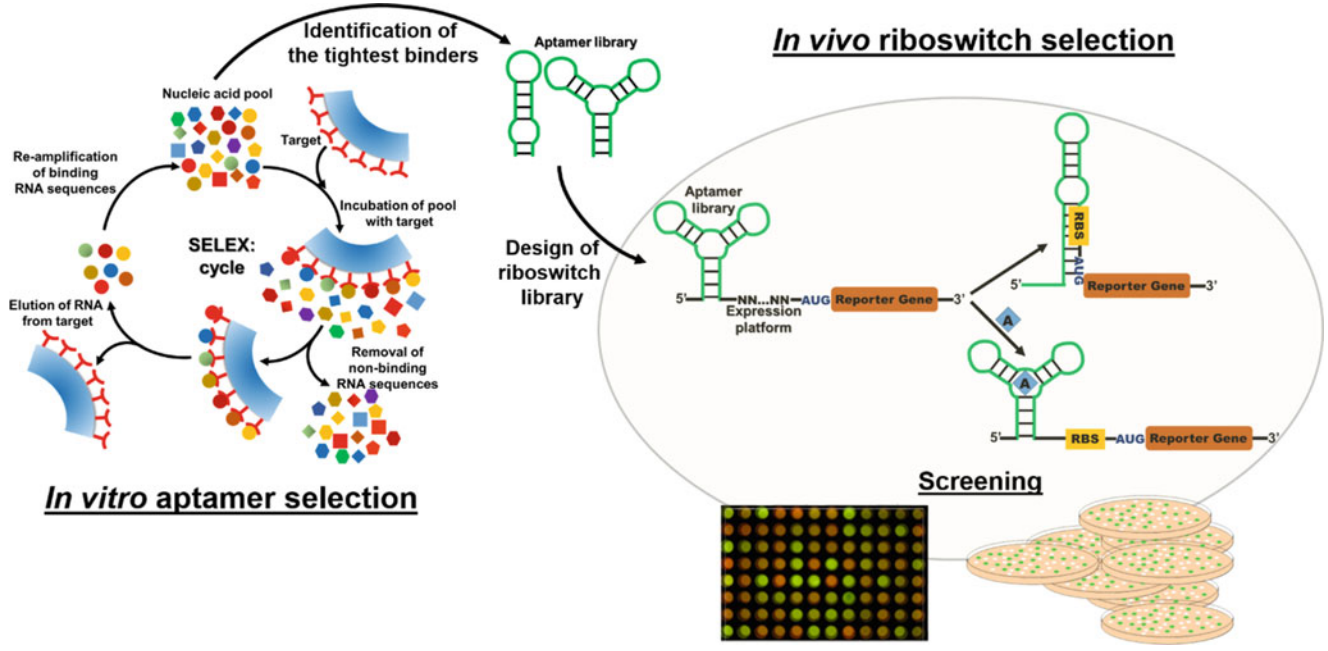


**Fig. 2** Example of selection strategy to generate synthetic riboswitches functional in *E. coli* cells using a combination of *in vitro* and *in vivo* selections. Riboswitch development begins with aptamer selection. RNA aptamers are isolated from large pools of randomized sequences using an *in vitro* selection technique called SELEX. After incubation with the immobilized target, nonbinding RNA oligonucleotides are removed by washing whereas bound oligonucleotides are specifically eluted with free target. The binding RNA oligonucleotide sequences are reverse transcribed, amplified, and subjected to the next round of selection. The process is repeated in cyclical fashion until the final pool is enriched for sequences binding to the target with high affinity. To select for synthetic riboswitches, a library of aptamers is cloned upstream of a randomized sequence (expression platform) in the 5′-untranslated region (UTR) of a reporter gene. The created riboswitch plasmid library is transformed in *E. coli* cells and screened without and in the presence of target analyte. Colonies of *E. coli* cells showing an increase in the reporter gene expression (increase in the fluorescence intensity or fluorescence color change are shown as examples) are selected, riboswitch containing plasmids are isolated and sequenced

obtained in the last selection round is sequenced and the binders are identified. While SELEX has been tremendously successful in attaining high affinity aptamers for a variety of targets (Jenison et al. 1994; Berens et al. 2001; Weigand et al. 2008; Ehrentreich-Förster et al. 2008; Carothers et al. 2010; Filonov et al. 2014; Han et al. 2014; Xu et al. 2016; Xiu et al. 2017; Groher et al. 2018), it has some disadvantages. First of all, the standard SELEX process tends to be time consuming and uses a large amount of reagents making aptamer selection costly. Also, immobilization of the target may change its original conformation; steric hindrance of the immobilized target may block the binding site, and nonspecific binding to a solid substrate may lead to the selection of nonspecific sequences. To overcome these disadvantages, several modified SELEX methods have been set up. Combinations of traditional SELEX with a microfluidic system (Hybarger et al. 2006), and later integration of the magnetic bead-based SELEX with microfluidic technology and a continuous-flow magnetic activated chip-based separation device (Lou et al. 2009), resulted in a more efficient, rapid, and automatic aptamer selection system.

To reduce time and reagent consumption, Szeto et al. developed a modified version of SELEX called RNA Aptamer Isolation via Dual-cycles (RAPID) (Szeto et al. 2013). This method incorporates nonamplification cycles in which the eluted RNA is purified and used in a second binding cycle without prior amplification of the material. To compare the techniques, RAPID was performed in parallel with traditional SELEX using the same targets, and many of the same top aptamer candidates (~10% identical aptamers in the top 10,000 enriched sequences) emerged. Both selections using RAPID and traditional SELEX started with a library of $5 \times 10^{15}$ unique sequences, yet the RAPID selection took approximately one third the time as the SELEX process and significantly reduced the amount of reagents used (Szeto et al. 2013). To perform a multiplexed RNA aptamer selection, the same group developed a microcolumn-based device, MEDUSA (Microplate-based Enrichment Device Used for the Selection of Aptamers) (Szeto and Craighead 2014). This device is designed around a 96-well microplate format, consisting of 96 microcolumns packed with a resin. Various targets are individually immobilized on a resin in each column and the nucleic acid library is pumped through the columns in serial and/or parallel mode. Combination of the multi-well format MEDUSA device with the RAPID approach allowed simultaneous multiplexed aptamer selection to 19 different targets to be performed, that significantly reduced the time and reagents needed for selection (Reinholt et al. 2016).

Another modified version of SELEX is Structure-Switching or Capture SELEX (Morse 2007). This technique is the opposite of above mentioned SELEX methods with regards to target immobilization, and relies on a conformational change in the aptamer sequence upon target binding, coined as "structure-switching." Rather than immobilizing the target to the solid support matrix of choice, an oligonucleotide pool is tethered and the target is free in solution. To tether the pool to the matrix (often magnetic spherical polymer beads), a short oligonucleotide strand complementary to the 5′-region of the RNA library is conjugated to the matrix. The library is hybridized to the short oligonucleotide and the pool-labeled beads are exposed to the target. Only the sequences that change structure conformation upon binding to the target

will effectively release themselves from the immobilized short oligonucleotide. Sequences found in solution following incubation with the target are sequestered, converted to cDNA, and amplified to produce the transcription template for the next round of selection. Capture SELEX is ideal for molecules that are difficult to immobilize, such as small molecules, and does not require modification of the analyte. Modifying a small molecule can block groups that are critical for binding, which can affect the selectivity of identified aptamers.

Although some limitations are still present, the considerable contributions to improving SELEX allow for better selections, thus yielding high affinity binders.

## Riboswitch Selection *In Vivo*

The first synthetic riboswitches were generated using a simple and straight forward approach in which *in vitro*-selected small molecule-binding aptamers were inserted into the 5′-UTR of mRNAs, without being fused to an expression platform domain. Werstuck and Green inserted aptamers specific to Hoechst dye 33258, and the closely related drug H33342, into the 5′-UTR of a mammalian β-galactosidase gene (Werstuck and Green 1998). When bound to their ligands, the aptamers formed a stable structure that resulted in blocking either the scanning ribosomal small subunit or the ribosome-mRNA interaction leading to down-regulation of gene expression *in vivo*. Later, Harvey et al. (2002) demonstrated that insertion of theophylline or biotin binding aptamers into the 5′-UTR of a eukaryotic chloramphenicol acetyltransferase gene led to inhibition of mRNA translation in the presence of the corresponding ligands. It was found that small molecule ligand–RNA interactions were sufficiently stable and prevented 80S ribosome assembly on the mRNA template. Similarly, Suess et al. (2003) and Hanson et al. (2003) introduced aptamers selected against tetracycline that behaved as highly efficient ribosome-blocking elements into the 5′-UTRs of reporter genes in yeast. To demonstrate that synthetic riboswitches can be used to perform genetic screens and selections for the presence of small molecules in *Escherichia coli*, Desai and Gallivan subcloned the theophylline aptamer sequence at a location five base pairs upstream of the ribosome binding site (RBS) of the β-galactosidase reporter gene (Desai and Gallivan 2004). The aptamer insertion led to a theophylline-dependent upregulation of β-galactosidase gene expression. The riboswitch performance was further optimized by increasing the distance between the aptamer and RBS to eight nucleotides, resulting in a ten-fold increase in β-galactosidase expression in the presence of theophylline compared to the system without the analyte. It was determined that the created riboswitch activated gene expression at translational level. The theophylline aptamer was also used to engineer a riboswitch for regulation of pre-mRNA splicing in HeLa nuclear extracts (Kim et al. 2005). Insertion of this aptamer into the 3′ splice site region of a model pre-mRNA enabled its splicing to be repressed by theophylline addition. Weigand and Suess (2007) applied the synthetic tetracycline binding riboswitch to establish a gene expression system for conditional tetracycline-dependent control of pre-mRNA splicing in yeast. Efficient regulation was obtained when

the aptamer was inserted close to the 5′ splice site (SS) with the consensus sequence of the SS located within the aptamer stem. Structural probing indicated limited spontaneous cleavage within this stem in the absence of the ligand. Addition of tetracycline led to tightening of the stem and the whole aptamer structure which prevented recognition of the 5′ SS (Weigand and Suess 2007).

Although the above mentioned riboswitch constructs demonstrated regulation of gene expression in response to specific analytes, they actually did not correspond to the structural composition of natural riboswitches (aptamer-expression platform), and as a result they did not completely repress gene expression and could be further optimized. To achieve more sufficient gene regulation with synthetic riboswitches, more sophisticated design and selection strategies engaging in vivo screening for riboswitch functionality were developed.

Combination of *in vitro* and *in vivo* selection is a useful strategy to identify riboswitches with desired functionalities (Fig. 2). It should be noted that, traditionally, SELEX experiments have been designed to find the tightest-binding RNAs. However, using a single aptamer with high affinity to a specific analyte in a riboswitch selection does not always result in functional riboswitches (Suess et al. 2003). To control gene expression, riboswitches must not only bind the ligand, they must also undergo a conformational change on a physiologically relevant timescale. The enriched library of aptamers incorporated into the riboswitch architecture will help to reduce the sequence search space by offering a higher percentage of potential analyte binders. To generate functional riboswitches, an expression platform is generally added to an *in vitro*-selected aptamer or a library of aptamers. An expression platform is usually a fully randomized region of 12–30 bases introduced between the aptamer and a region of 5–7 constant bases located immediately before the start codon. The introduction of a completely randomized nucleotide region allows an *in vivo* selection to identify the functional expression platform, and optimization of the strength of the ribosome-binding site to achieve a high level of reporter protein expression. The resultant aptamer-expression platform library is cloned upstream of a reporter gene to generate a signal output for selection (Fig. 2).

A variety of methods for screening and selection of robust-performing synthetic riboswitches from RNA libraries in different cell types have been reported in the literature (Lynch et al. 2007; Topp and Gallivan 2008a; Wieland and Hartig 2008; Weigand et al. 2008; Lynch and Gallivan 2009). Using a screening method based on green fluorescent protein expression, Suess and coworkers screened libraries of up to 50,000 members and isolated riboswitches that reduce gene expression 7.5-fold in the presence of the antibiotic neomycin in *Saccharomyces cerevisiae* yeast cells (Weigand et al. 2008). Gallivan and coworkers developed a high-throughput screen (Lynch et al. 2007) for theophylline-dependent riboswitches in *E. coli* cells. In their screening method, the theophylline binding aptamer, followed by randomized sequence of nucleotides, was placed upstream of the β-galactosidase reporter gene. The riboswitch libraries of up to 65,000 members were screened without and in the presence of theophylline using a β-galactosidase assay on solid media and in cellular lysates. Using this screening assay, they identified a new riboswitch that could activate the expression of β-galactosidase by 36-fold in the presence of theophylline

in *E. coli* cells. The same screening strategy also allowed for the identification of a theophylline-dependent riboswitch that could repress the expression of β-galactosidase by 27-fold in the presence of analyte (Topp and Gallivan 2008b). The high-throughput screen was applied to develop a series of synthetic theophylline-sensitive riboswitches that functioned as genetic control elements in a diverse set of gram-negative and gram-positive bacteria (Topp et al. 2010). An alternative method for identification of functional riboswitches is a high-throughput selection, which is based on ligand-induced changes in cell motility. Utilizing *cheZ* as a reporter gene, responsible for migration of *E. coli* cells on semisolid media, Gallivan and coworkers performed positive and negative selections for isolation of new theophylline responsive riboswitches from libraries of more than $10^5$ *E. coli* clones (Topp and Gallivan 2008a).

Another example of utilizing an *in vitro*-selected aptamer to generate a synthetic riboswitch is the development of 2,4-dinitrotoluene (DNT)-responsive riboswitch in *E. coli* cells (Davidson et al. 2013). A riboswitch library was constructed by incorporation of 30 degenerate bases between an in vitro-selected 2,4,6-trinitrotoluene (TNT) aptamer (Ehrentreich-Förster et al. 2008) and the RBS. Screening was performed by placing the riboswitch library upstream of the Tobacco Etch Virus (TEV) protease coding sequence in one plasmid; a second plasmid encoded a FRET-based construct linked with a peptide containing the TEV protease cleavage site. Although the aptamer was selected for efficient TNT binding, it turned out to also bind DNT. Addition of DNT to bacterial cell culture activated the riboswitch, initiating translation of TEV protease. The produced protease cleaved the linker in the FRET-based fusion protein, causing a change in fluorescence. The DNT-responsive riboswitch exhibited a 10-fold increase in fluorescence in the presence of 0.5 mM DNT compared to the system without target.

Using an in vitro-selected ciprofloxacin binding aptamer, Suess and coworkers developed a ciprofloxacin-responsive riboswitch by next-generation sequencing (NGS)-guided cellular screening (Groher et al. 2018). The application of NGS allows the collection of detailed information for the individual selection rounds. Thus, it was possible to choose selection rounds that showed a certain degree of enrichment, yet maintained maximum diversity. This approach allows for a substantial acceleration of the transition between *in vitro* and *in vivo*, while simultaneously reducing screening efforts.

Advances in the development of fluorescence-activated cell sorting (FACS) facilitates application of this technique for quick and efficient screening of large (~$10^8$ members) libraries of riboswitch-like sequences to identify those with desired activity. Because FACS can readily distinguish small differences in fluorescence emission intensity, it was successfully used for discovering synthetic riboswitches that display low background levels of gene expression in the absence of a ligand and robust increases in gene expression in its presence (Fowler et al. 2008; Lynch and Gallivan 2009; Ghazi et al. 2014). Using FACS assay, Lynch and Gallivan identified

a theophylline synthetic riboswitch that activates protein translation to 96-fold in *E. coli*.

## Reengineering Natural Riboswitches

An alternative strategy to generate synthetic riboswitches is to reengineer natural riboswitches. Despite advances in the development of SELEX methods for aptamer selection, high-throughput genetic screens, and selection strategies for creation of novel riboswitches with desired gene regulatory functions, only a very limited range of *in vitro*-selected aptamers have been successfully exploited in artificial riboswitch applications (Berens and Suess 2015; Findeiß et al. 2017; Hallberg et al. 2017). A variety of existing natural riboswitches offers a huge possibility to use these regulatory elements for biosensing applications. However, as was mentioned above, the major problem with using natural riboswitches is that their ligands are typically metabolites ordinarily present at varying levels in the cell, and, therefore, any activation due to binding the target molecule would result in unwanted background activity. To overcome this problem, Micklefield and coworkers have reengineered natural riboswitches to bind nonnatural synthetic small molecules, while the original natural target molecules were no longer recognized (Dixon et al. 2010; Robinson et al. 2014; Wu et al. 2015). Using randomization by site-directed mutagenesis with genetic selection, they reprogrammed two natural riboswitches, an adenine-sensing *add* A-riboswitch from *Vibrio vulnificus* and a queuosine precursor-responsive $PreQ_1$ class I riboswitch from *Bacillus subtilis*, to respond to synthetic triazine-based and pyrimidopyrimidine ligands and diamino-faced analogs of $preQ_1$, respectively.

A majority of naturally found riboswitches downregulate gene expression upon metabolite binding, probably because of their roles in negative feedback regulation within the metabolic pathways. However, for sensing applications, riboswitch activation is more desirable to produce a positive signal when the aptamer binds the target. A reengineering approach was applied to reverse a translational expression platform of a natural thiamine pyrophosphate (TPP)-responsive riboswitch which originally downregulates gene expression in *E. coli* cells (Nomura and Yokobayashi 2007). To identify riboswitches that upregulate gene expression upon TPP binding, a dual selection strategy based on antibiotic resistance was developed. A randomized region of up to 30 nucleotides was inserted between the TPP aptamer and the RBS, and the resultant riboswitch library was placed upstream of the tetracycline resistance gene *tetA*. In a dual-selection strategy, *E. coli* cells harboring functional riboswitches were able to grow in medium containing tetracycline and TPP but couldn't survive in the presence of $Ni^{+2}$ and without TPP. The dual selection strategy permitted the analysis of large riboswitch libraries and allowed sufficient elimination of false positives. Besides the reprogramming of a natural expression platform for the generation of upregulating riboswitches, the reciprocal redesign of upregulating riboswitches to downregulating was also successful (Muranaka et al. 2009a).

## Riboswitch Selection *In Vitro*

A promising variation of a SELEX-type approach for riboswitch selection *in vitro* with nonimmobilized small molecule target was performed by Martini et al. (2015). The researchers utilized a library version of a synthetic riboswitch for thiamine pyrophosphate (TPP). This library was modified from the original riboswitch, such that four regions responsible for riboswitch activity were randomized, and the RNA pool and TPP were free in solution. Upon binding of a sequence to the target, a change in conformation occured, exposing an area for a short double stranded biotin-labeled DNA reporter with a complementary sequence to bind. Upon binding to the RNA sequence, a strand on the reporter was displaced. Sequences which have bound to the reporter were isolated with streptavidin beads whereas those that did not change conformation couldn't bind the biotin-labeled reporter and thus were discarded. Using this approach, only three rounds were required to obtain a number of functional TPP-responsive riboswitches.

## Rational and Computational Design of Synthetic Riboswitches

To rationally design new synthetic riboswitches, it is crucial to understand the fundamental mechanisms of riboswitch functionality. As was mentioned above, there are two main classes of riboswitches: transcriptional riboswitches that regulate production of full-length mRNA by controlling the formation of transcription terminators and translational riboswitches that alter protein expression by changing the initiation of mRNA translation upon the ligand binding. Furthermore, increasing the ligand concentration can increase protein synthesis (where the riboswitch upregulates gene expression and performs as an ON switch) or decrease it (the riboswitch downregulates gene expression and performs as an OFF switch). For biosensing applications the ON switches are preferred. Although, transcriptional and translational riboswitches function by different specific mechanisms, they have the same global mechanism in which the mRNA molecule can fold reversibly into two distinct conformations that are associated with a different level of protein expression and different ligand-binding affinities. Mathematically, riboswitches can be described by a simple three-state population-shift model where binding of the target ligand shifts a preexisting equilibrium between the OFF and ON conformations (Lynch et al. 2007; Vallée-Bélisle et al. 2009; Beisel and Smolke 2009).

In case of the transcriptional ON switch (Fig. 3), mRNA folds into a stable conformation where the aptamer domain forms a terminator hairpin with the complementary expression platform, thus leading to a termination of transcription ("off" state). This conformation also has a low ligand-binding affinity $K_2 = 0$. The ON conformation, in which terminator is disrupted, has a high mRNA translational rate $K_P$ and ligand-binding affinity $K_2$, but it is unstable without the presence of ligand. The equilibrium between these two conformations is characterized by the equilibrium constant $K_1$. In the presence of the ligand, conformation "on" +L becomes stable leading to a large increase in the level of protein expression. Therefore, the

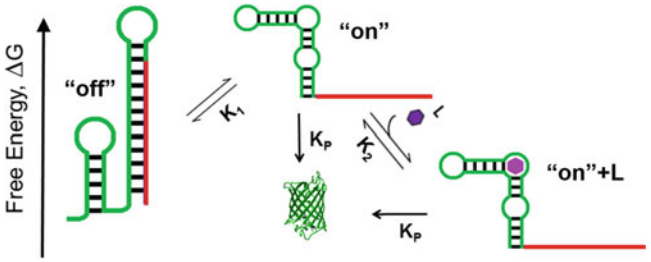


**Fig. 3** Schematic illustration of the ligand-activated transcriptional riboswitch. The aptamer domain is shown in green and expression platform is shown in red. Conformation "off" is a stable confirmation that terminate transcription. This conformation can spontaneously switch into the "on" state which is unstable without the presence of a ligand L. Addition of ligand stabilizes the conformation "on," leading to a large increase in the level of protein expression

ligand thermodynamically shifts the equilibrium from conformation "off" to conformation "on."

Translational riboswitch functions in a similar way (Fig. 4). In the absence of a ligand, the aptamer extensively binds with the expression platform that includes the RBS, thus making it inaccessible for the ribosome and preventing protein expression. Addition of the ligand stabilizes the "on" conformation in which the RBS is unpaired and available for ribosome binding leading to a large increase in the level of protein expression.

It was shown (Vallée-Bélisle et al. 2009) that the performance of biomolecular switches can be modulated by changing the equilibrium constant $K_1$. For example, changing the equilibrium constant $K_1$ toward a nonbinding, nonactive "off" conformation ensures a large signal change. However, it also reduces the affinity since the riboswitch now must overcome more significant conformational changes. On the other hand, changing the equilibrium constant toward the active "on" conformation increases the background signal without the presence of the ligand. Therefore, the optimal performance of the riboswitch is achieved at the intermediate values of $K_1$.

Folding of mRNA molecules and switching between the conformations plays a key role in the functioning of riboswitches. The three-dimensional structure of RNA molecules is dominated by the formation of their secondary structure via the Watson-Crick and GU base pairs. There are several software tools that predict optimal and suboptimal secondary structure of RNA molecules based on their sequences such as Mfold (Zuker 2003), ViennaRNA (Lorenz et al. 2011), NUPACK (Zadeh et al. 2011), and RNAStructure (Reuter and Mathews 2010). However, readers should be aware that different software sometimes can produce different results due to different algorithms used, as well as using different thermodynamic parameters for RNA folding. For example, Mfold and NUPACK use thermodynamic parameters derived in Mathews et al. (1999), while the ViennaRNA package uses revised parameters that take into account experimental results (Mathews et al. 2004). Software tools that predict folding of RNA molecules, together with the knowledge

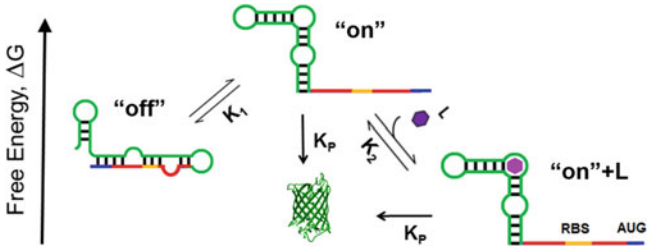


**Fig. 4** Schematic illustration of the ligand-activated translational riboswitch. The aptamer domain is shown in green, expression platform is shown in red as in Fig. 1, yellow is a ribosome binding site (RBS) and blue is a gene start codon AUG. In the absence of the ligand, the aptamer extensively binds with the expression platform that includes RBS thus making it inaccessible for ribosome and preventing protein expression. Addition of the ligand stabilizes the "on" conformation, in which the RBS is unpaired and available for ribosome binding leading to a large increase in the level of protein expression

of mechanisms that govern riboswitch functions, make it possible to develop computational approaches for the design of synthetic riboswitches.

Wachsmuth et al. (2012) used the ViennaRNA package to generate and select short spacer sequences, which can fold into functional terminators with a sequence complementary to the 3′-part of the theophylline aptamer, to design theophylline-dependent transcriptional ON switches. The stability of the terminator hairpin was considered as the main factor in the selection of designed riboswitches for experimental verification. Three of their designed constructs were functional *in vivo* and regulated the expression of the reporter, β-galactosidase gene. Further sequence optimization of the best performing riboswitch resulted in a 6.5-fold activation of gene expression in the presence of theophylline (Wachsmuth et al. 2012). Further investigation found that riboswitch functionality is strongly affected not only by the stability of the terminator hairpin, but also by the folding pathway and the existence of potential folding traps (Wachsmuth et al. 2015). The revised in silico strategy was applied to design ligand-dependent riboswitches for tetracycline and streptomycin aptamers (Domin et al. 2017). The resulting tetracycline riboswitches were functional *in vivo* and showed a fold change up to 3.4. However, none of the four tested candidates for the streptomycin sensing riboswitches showed a clear switching behavior. Further investigations of the streptomycin aptamer by in-line probing showed that the actual probed secondary structure is different from the predicted lowest energy secondary structure (Domin et al. 2017). The conclusion was that further improvement to the design strategy is required to include aptamer structures that are not the minimal free energy structures as predicted by ViennaRNA software.

In a different approach, the Batey group developed transcriptional riboswitches in a modular "mix-and-match" fashion (Ceres et al. 2013). From a set of natural riboswitches, they derived expression platforms that can be uncoupled structurally from their aptamer domain. These expression platforms were then combined with a variety of natural and synthetic aptamers. Two sets of modular transcriptional ON switches were developed: one based on the expression platform of *pbuE* riboswitch

from *B. subtilis* and another one based on the expression platform of *metH* riboswitch from *Dechloromonas aromatica.* Initially, only one of the aptamers connected to an expression platform showed switching functionality in vitro. A series of alterations in the wild-type expression platforms were required to optimize the performance of designed riboswitches (Ceres et al. 2013).

Rational design of translational riboswitches functional in bacteria was presented by Suess et al. (2004). In *B. subtilis*, a translational control element was created by combining the theophylline aptamer with a helical communication module for which a ligand-dependent one-nucleotide slipping mechanism has been proposed. This structural element was inserted close to the RBS so that its nonbound conformation interfered with ribosome accessibility. Binding of theophylline then induced a structural transition in the helix leading to a one nucleotide shift which moved the inhibitory element away by exactly the critical distance to allow ribosome binding.

Synthetic riboswitches acting at translational level were also designed for internal ribosomal entry sites (IRES) in an eukaryotic cell-free translation system (wheat germ extract) (Ogawa 2011). In these riboswitches, the specific aptamer is coupled with short sequences competing for base pairing with essential IRES parts (anti-IRES as well as anti-anti-IRES sequences) and a folding-modulating element (MS). Translation of a reporter gene was promoted only in the presence of a specific ligand due to rearrangement and refolding of the IRES and anti-IRES sequences. When the order of individual parts of the riboswitch was changed, the system was reprogrammed into functional OFF switches, where ligand interaction suppressed IRES function (Ogawa 2012).

To design synthetic riboswitches that regulate translation initiation in bacteria, Salis and coworkers developed the riboswitch calculator for automated physics-based design of synthetic riboswitches (Espah Borujeni et al. 2015). Using statistical thermodynamics, it calculates the energy $\Delta G_{\text{total}}$ for mRNA molecules in the "off" and "on" states. This energy can be converted into the translation initiation rate $r = \exp(-\beta \Delta G_{\text{total}})$. The riboswitch activation ratio can be then calculated as $AR = r_{\text{ON}}/r_{\text{OFF}} = \exp[-\beta(\Delta G_{\text{ON}} - \Delta G_{\text{OFF}})]$. To design new riboswitches, the developed algorithm searches about $10^{36}$ sequences to identify sequences that maximize a selected objective function. This approach was applied to design 62 synthetic riboswitches for six different aptamers that were tested *in vitro* and/or *in vivo*. The authors demonstrated the versatility of their design method as the performances of 55% of the tested riboswitches were correctly modeled.

## Riboswitch Detection Systems

A readable detection of an output signal that is either suppressed or produced due to riboswitch-mediated gene regulation is an important factor which not only reflects the synthetic riboswitch functionality but also determines further riboswitch applications.

## Monitoring Functionality of Synthetic Riboswitches

A variety of different reporter systems were applied to monitor the functionality of synthetic riboswitches during and after the selection process. Most frequently used reporter systems are based on expression of green fluorescent protein (GFP) or its variants in prokaryotic and eukaryotic cells (Fowler et al. 2008; Weigand et al. 2008; Lynch and Gallivan 2009; Dixon et al. 2010). The relative fluorescence of produced GFP or variants can be directly measured for quantitative analysis of riboswitch activation. Another very common reporter system is based on bacterial *lacZ* gene, encoding for β-galactosidase (Lynch et al. 2007). The β-galactosidase enzymatic system allows a qualitative evaluation of riboswitch clones that relies on color-based screening of individual colonies and quantitative analysis of riboswitch functionality by measuring enzymatic activity in cellular extracts using the chromogenic substrate, o-nitrophenyl-β-D-galactopyranoside (ONPG) (Lynch et al. 2007).

In some cases, enzymatic reporters coupled with colorimetric or fluorescent detection systems can be more advantageous for monitoring riboswitch activation, since only a small amount of enzyme is required to catalyze an enzyme-specific reaction and to achieve a measurable output signal. For example, Harbaugh et al. (2009) have developed a fluorescence resonance energy transfer (FRET)-based enzymatic assay for monitoring riboswitch activation in *E. coli* cells. This assay is based on the expression of two genes, tobacco etch virus (TEV) protease and its optical engineered protein substrate, within the same cell. The protein substrate is a FRET construct composed of a donor, an enhanced green fluorescent protein (eGFP), and an acceptor, a nonfluorescent mutant of yellow fluorescent protein called resonance energy-accepting chromoprotein (REACh), connected with a peptide linker containing a TEV protease cleavage site. Use of this type of FRET pair eliminates acceptor fluorescence, and therefore little to no fluorescence is observed prior to cleavage. Using the TEV protease-based reporter system coupled with a theophylline synthetic riboswitch, the authors were able to observe a detectable increase in fluorescence intensity as early as 30 min post analyte addition. Moreover, the superiority of TEV protease–FRET substrate system over direct coupling of the riboswitch with fluorescent protein in terms of sensitivity was demonstrated. When the eGFP gene was placed downstream of theophylline riboswitch, only a very modest increase (~1.4-fold) in fluorescence intensity of cells in response to analyte was observed. In contrast, riboswitch activation of TEV protease gene expression followed by cleavage of FRET protein resulted in an 11.3-fold increase in fluorescence (Harbaugh et al. 2009).

Another example of an enzymatic reporter, that was used to monitor synthetic riboswitch activation and could amplify the output of the riboswitch and improve its efficiency, is a T7 polymerase-based system. To boost riboswitch functionality in plastids and to develop a generally applicable tool for high-level transgene expression under riboswitch control, Emadpour et al. (2015) placed the T7 RNA polymerase under the control of theophylline-responsive riboswitch. The transgene of interest (which, for the purpose of establishing the system, was *gfp* encoding the green fluorescent protein) was driven by the T7 promoter. Thus, treatment of plants

with theophylline resulted in induction of T7 RNA polymerase expression at relatively low levels, which however was sufficient to trigger strong transcriptional activation of the *gfp* transgene (Emadpour et al. 2015).

Some reporter systems are highly efficient for riboswitch selection but cannot be directly used to quantitatively monitor riboswitch performance. Using reporter systems based on genes conferring resistance against antibiotics allows a growth-dependent analysis of riboswitch-mediated regulation. As was mentioned above, the possibility to use the tetracycline-resistance mediating *tetA* gene for positive as well as negative selection renders this reporter a very versatile selection system (Nomura and Yokobayashi 2007). However, the lack of a quantitative readout of the expression level of *tetA* necessitated a design of the TetA-GFPuv translational fusion to enable quantitative fluorescence-based screening of the selected riboswitch clones (Muranaka et al. 2009a, b)). A different reporter system, based on *cheZ* gene that controls *E. coli* motility, also can be used to perform both positive and negative selections resulting in riboswitches with down- and upregulating functions. However, similar to *tetA* this system lacks a quantitative readout and was fused with *lacZ* gene to estimate the efficiency of selected riboswitches (Topp and Gallivan 2008a).

## Coupling Synthetic Riboswitches with New Reporter Systems

Although the reporter systems utilized for riboswitch selection can be used as readout assays for riboswitch-based biosensing, in some cases coupling previously selected synthetic riboswitches with new reporters are highly desirable. Moreover, to be truly useful as sensor entities, riboswitches should be compatible with a variety of different reporters, i.e., they should be modular "plug and play" platforms. However, this is not always the case. For many riboswitches, selective binding of a small molecule of interest and conformational change in RNA secondary structure is influenced not only by its own sequence, but also by the surrounding genetic context including the proximal open reading frame (ORF) under the control of the riboswitch. Thus, substituting the original ORF with a new one on a "start codon for start codon" basis can affect the ability of the riboswitch to regulate gene expression in response to a given ligand (Caron et al. 2012; Folliard et al. 2017). To overcome this lack of modularity, many studies have created fusions comprised of a desired riboswitch, the first few hundred base pairs of its working ORF, and a new reporter gene (Dixon et al. 2012). A short N-terminal peptide fusion can also be coupled with a reporter gene utilized for riboswitch selection (Sinha et al. 2011). In this case, the incorporation of a short peptide fusion ensures that the sequence immediately 3′ to the expression platform is constant throughout the selection and the riboswitch functionality will be preserved even if the original reporter gene will be replaced with a new one. However, these approaches can fail in some circumstances since the inclusion of 5′ fusions can alter the new reporter gene's functionality (Folliard et al. 2017).

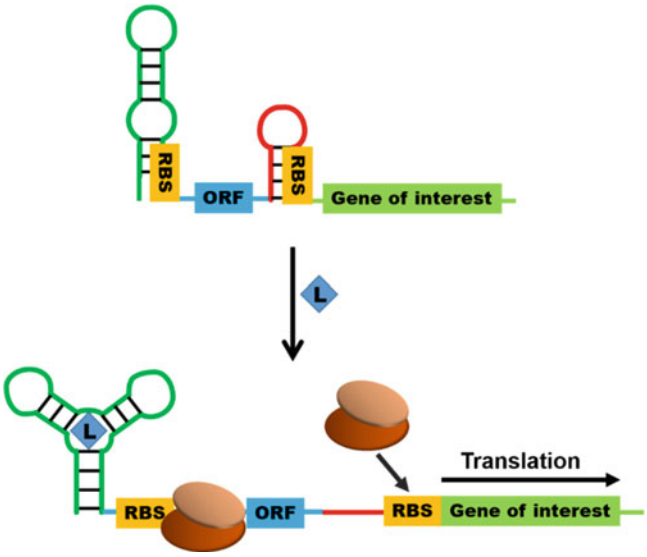


**Fig. 5** Riboswitch activation and ribo-attenuator context. A ribosome binding site (RBS, yellow) is sequestered within a riboswitch preventing ribosome recruitment. A ribo-attenuator adds a second RBS, sequestered away by a local hairpin. Binding of a ligand (L) causes a conformational change exposing the riboswitch RBS. The hairpin can be opened by a ribosome travelling from the riboswitch RBS, exposing the attenuator RBS and allowing translation of the gene of interest (Folliard et al. 2017)

To solve the problem associated with 5′ fusions, Folliard et al. (2017) have designed and introduced a novel genetic element called the ribo-attenuator (Fig. 5). The ribo-attenuator is placed after 150 base pairs of a riboswitch's original ORF. It consists of a hairpin containing a ribosome binding site (RBS) on the downstream portion of a stem in order to silence translation independent of the upstream riboswitch activity. This is followed by a negative one base pair shifted transcriptionally fused stop and start codon (TAATG). The passage of ribosomes recruited by the riboswitch opens up the introduced hairpin, before the ribosome dissociates at the proximal in-frame stop codon (TAA) in the junction between the original ORF and the ORF containing the gene of interest. Additional ribosomes can then assemble at the ribo-attenuator RBS and initiate translation at the first start codon of the introduced gene of interest (Fig. 5). Therefore, instead of directly controlling the translation of a gene, the riboswitch controls the translational initiation from the downstream attenuator RBS. Thus, the developed ribo-attenuator system enabled riboswitch controlled expression of a new gene of interest without the inclusion of a 5′ fusion (Folliard et al. 2017).

Another way to preserve the riboswitch functionality and expand the applicability of synthetic riboswitches is to use recombinase-based reporter system. Recently, Harbaugh et al. have developed a dual-color detection system comprising of the *E. coli* FimE recombinase controlled by a synthetic riboswitch and an invertible DNA

segment containing a constitutively active promoter placed between two fluorescent protein genes (Harbaugh et al. 2017). Utilizing the recombinase-based system as a means of riboswitch selection negates the requirement of an insertion of a 5′ fusion when a different reporter is necessary because a new reporter can be placed on either side of invertible promoter, keeping the riboswitch-FimE construct intact. The T7 polymerase reporter system mentioned above also can be advantageous for riboswitch selection and further riboswitch applications since it can initiate expression of any reporter gene.

## Riboswitch Optimization toward Sensing Requirements

### Selectivity, but at a Cost

As it was discussed, riboswitches offer the capability to design and develop specific sensors to potentially any ligand, but often require sensitive, laboratory-based equipment to detect their reporter output. Once a specific riboswitch has been optimized, it is often constrained to the context it was optimized for. For example, altering riboswitch output by switching out the RBS sequence with one that would increase reporter gene expression is likely to disrupt the function of the riboswitch, through changes in its secondary and tertiary structure. Similarly, if a riboswitch is used to control expression of a reporter gene that it was not designed and optimized for, the conformation of the riboswitch may be altered, rendering it unswitchable. Further, synthetic riboswitches are often optimized within a cellular chassis that has been developed for enhanced transcript or protein expression (Lynch and Gallivan 2009; Davidson et al. 2013). These cell types do not generally survive well outside of controlled laboratory conditions. Overcoming these hurdles would enhance the utility of riboswitches in fieldable sensors.

### Biological Circuits

Recent advances in synthetic biology have allowed different genetic "components" to be connected into biological circuits via signaling molecules. The signaling molecules that connect these components are often those involved in bacterial quorum sensing (Brenner et al. 2007; Tabor et al. 2009; Tamsir et al. 2011). These molecules freely cross cell membranes and, when their concentration surpasses a threshold level, they activate transcription of genes that have the cognate quorum sensing promoter. In nature, this process allows appropriate genes to be activated only in a high cell density environment (Pesci et al. 1997). In engineered biological circuits, using quorum molecules that are orthologous to the cellular chassis, this system allows the output from an upstream component to be biologically "wired" to the input of a downstream component, generating genetic logic gates and forming genetic programs within and between cells (Brenner et al. 2007; Tabor et al. 2009; Tamsir et al. 2011; Friedland et al. 2009; Lou et al. 2010; Moon et al. 2012).

Similarly, this method has also been used to produce a signal amplification circuit that increases the dynamic range of responses from promoters to a specific input (Karig and Weiss 2005).

## Riboswitch Circuitry

Wiring riboswitches into biological circuits using quorum signaling molecules overcomes many of the hurdles discussed earlier in section "Selectivity, but at a cost". By separating the riboswitch-induced quorum signaling molecule production and the quorum sensing promoter into different cell types, Goodson et al. (2017) demonstrated tunable riboswitch signal amplification even in a chassis that was not optimized for enhanced protein expression. The riboswitch specifically controlled quorum sensing molecule production in one cell type. Meanwhile, the reporter cell type contained a quorum sensing promoter upstream of a RBS and reporter gene. This architecture resulted from the design-build-test paradigm of synthetic biology, since initial designs of amplification circuits as positive feedback loops on a single plasmid resulted in nonspecific activation because of localized buildup of the quorum signal (Goodson et al. 2015). This was also evident when the riboswitch and reporter were separated into two plasmids but were located in same cell. Separating the riboswitch and reporter into different cell types insulated the function of each plasmid (Tamsir et al. 2011), thus reducing the likelihood of false positives. This arrangement also negated the sometimes "leaky" nature of riboswitches since degradation tags could be used to reduce the production of quorum sensing signal producing proteins, even when the riboswitch is "off," thus keeping the signaling molecule concentration below threshold levels until riboswitch activation. Concomitantly, this had the effect of converting the analog nature of the riboswitch to a digital output, dependent upon the activation threshold concentration of the quorum sensing signal being used.

## Corollary Advantages of Signal Amplification

Using the amplification circuit increases riboswitch sensitivity and response time compared to designs where the riboswitch is upstream of the reporter gene (Goodson et al. 2017), since only a small increase in signaling molecule production can activate the quorum sensing promoter upstream of the reporter gene, the strength of which can be modified by optimizing RBS strength and rate of reporter degradation. This increases the utility of riboswitches that have low activation ratios (i.e., a small difference in gene expression between the inactivated and activated states), and, similarly, it can aid in riboswitch screening and selection by ensuring even weak riboswitches are identified. Indeed, identifying riboswitches in this way facilitates their inclusion into existing sensing systems that contain reporter cell types that respond to the signaling molecule produced by the riboswitch, and separating the

reporter from the riboswitch enables reporter genes to be exchanged without the associated context-related disruption of riboswitch function.

Thus separating the riboswitch and the reporter, but biologically wiring them together, increases the modularity of the system and streamlines the inclusion of riboswitches into fieldable sensors for practical applications.

## Tandem Riboswitches

Another promising approach for improving riboswitch response to its analyte is to combine several repeats of the riboswitch construct 5′ of the gene to be regulated. Tandem riboswitches arranged in different configurations have been found in nature (Breaker 2012; Roßmanith and Narberhaus 2017). For example, a riboswitch for the amino acid glycine from *Vibrio cholerae* carries two aptamers and only a single expression platform (Mandal et al. 2004). These aptamers function cooperatively, such that glycine binding by one aptamer increases the affinity for glycine binding to the adjacent aptamer. Other natural tandem architectures consist of two complete riboswitch repeats, as observed, for instance for some TPP riboswitches, where each element acts independently of the other (Rodionov et al. 2004; Welz and Breaker 2007).

Similar to their natural analogues, synthetic riboswitches can be toggled in series that, in some cases, helps to reduce background activity and increase the dynamic range. Using a tetracycline binding aptamer to regulate translation initiation in yeast, Kötter et al. (2009) demonstrated that addition of a second or third copy of the aptamer presented in the 5′-UTR of a reporter gene strongly increases the dynamic range of regulation.

Muranaka and Yokobayashi (2010) have developed a system with two individual riboswitches by inserting previously engineered TPP riboswitches into the 5′-UTR of a GFP-encoding mRNA in *E. coli* cells. In this tandem, the first riboswitch represented a transcriptional OFF switch, where TPP binding terminates mRNA transcription. The second riboswitch, placed downstream of the first element, was a translational ON switch, where ligand binding initiates mRNA translation. In the designed system, transcription termination could be achieved only at high thiamine concentrations resulting in a repression of a reporter gene expression. Low thiamine concentrations were not sufficient to activate the transcriptional OFF switch and terminate mRNA transcription, however the translational ON switch couldn't be activated blocking mRNA translation. Only an intermediate amount of thiamine resulted in GFP production, as both riboswitches were not completely repressed at such concentrations. Hence, this tandem riboswitches functioned as a chemical band-pass filter circuit allowing gene expression only in certain concentration range of the ligand, where the OFF switch acts as a low-pass filter and the ON switch as a high-pass filter (Muranaka and Yokobayashi 2010).

The Mörl group (Wachsmuth et al. 2015) inserted two or three copies of theophylline-responsive identical transcriptional ON switches into the 5′-UTR of a β-galactosidase reporter gene. While a single-copy riboswitch had activation ratios

of 3–6.5-fold, the serial repeats showed increased response ratios from 17-fold (tandem) to 23-fold (tridem). The authors suggested that a possible reason for this improved ligand-dependent response is the presence of several terminator elements within one transcriptional unit. The enhanced transcription termination leads to a very low background activity in the riboswitch-mediated OFF state, resulting in a dramatically increased difference in reporter gene expression without and in the presence of the ligand.

The described tandem and tridem riboswitch arrangements, consisting of aptamers that recognize the same ligand, allow a better activation ratio in gene regulation. The combination of riboswitches interacting with different target molecules, however, leads to construction of Boolean logics allowing possible detection of multiple analytes. Using the previously described *tetA* based dual genetic selection method, Yokobayashi and coworkers combined the theophylline aptamer with reengineered or natural TPP riboswitches (Sharma et al. 2008). Two libraries were generated by incorporation of 20 randomized nucleotides between the theophylline aptamer and TPP riboswitch. Combining the theophylline aptamer and the reengineered TPP-responsive translational ON switch resulted in selection of several AND gates, activating gene expression in the presence of both theophylline and TPP. On the other hand, coupling the theophylline riboswitch with the natural TPP sensing translational OFF switch allowed identification of several NAND gates, repressing gene expression only when both theophylline and TPP are present. Thus, the riboswitch regulatory mechanism and the sequence of the linker connecting two sensing domains can determine the type of created logic gates.

Other logic gates switches were designed on the basis of transcriptional riboswitches. Mörl and coworkers constructed AND gates consisting of theophylline and tetracycline riboswitches. When the theophylline switch was placed upstream of the tetracycline switch only the addition of both ligands induced gene expression by 10.4 fold as expected for a logic AND gate (Domin et al. 2017).

The described examples of tandem constructs clearly show that it is possible to generate Boolean logic gates from synthetic translational and transcriptional riboswitch elements.

## Applications of Synthetic Riboswitches

As was described above, a number of small molecule analytes sensing riboswitches were developed in the past couple of decades. Like their natural counterparts, synthetic riboswitches have the ability to regulate gene expression in response to levels of a specific small molecule in a concentration-dependent manner and, as a result, can be valuable for a range of applications. To date, synthetic riboswitches responsive to nonnatural small molecules have been used for regulation of gene expression, to control cellular behavior, and to optimize small molecule production in metabolic engineering.

Riboswitch-based regulation of protein production has been demonstrated in a wide range of gram-positive and gram-negative bacterial species. Topp et al. (2010)

have developed a set of five theophylline synthetic riboswitches that enable inducible gene expression in eight diverse bacterial species, including organisms that currently have few or no tools, which have been used to titrate gene expression in the laboratory. Developed riboswitches showed 25-fold increase in gene expression in all species tested and a greater-than-50-fold increase in two human pathogens, *Acinetobacter baumannii* and *Streptococcus pyogenes*. These riboswitches can be useful tools to enable studies on the mechanisms of *A. baumannii* and *S. pyogenes* pathogenesis that have been hindered by the inability to induce gene expression in conditional knockouts in these organisms (Topp et al. 2010). Reynoso et al. (2012) have applied theophylline-sensitive synthetic riboswitches to induce protein expression in the intracellular pathogen *Francisella tularensis*. It was demonstrated that riboswitches can be used to functionally control a bacterial gene that is critical to the ability of a pathogen to cause disease during intracellular infection. Since this system can be adapted to diverse bacteria, riboswitches will likely facilitate the in-depth study of the virulence mechanisms of numerous difficult-to-study intracellular pathogens, as well as future emerging pathogens (Reynoso et al. 2012). Seeliger et al. (2012) have applied a synthetic riboswitch-based system for translational control of gene expression in the human pathogen *Mycobacterium tuberculosis*. Although the optimized system resulted in only an 8.2-fold activation, it robustly regulated protein production in a macrophage infection model (Seeliger et al. 2012). Similar systems of theophylline-responsive riboswitches have been demonstrated in streptomyces and cyanobacterial species (Nakahira et al. 2013; Rudolph et al. 2013; Ma et al. 2014; Ohbayashi et al. 2016).

In other studies, theophylline riboswitch-mediated regulation has been applied to control genes in viral replication and in plant plastids (Wang and White 2007; Verhounig et al. 2010). Coupling a theophylline-binding aptamer with a viral regulatory RNA element (RE) resulted in a theophylline-dependent induction of viral replication. Analysis of this engineered viral genome revealed that this RE, located in the 5′-UTR, specifically mediates efficient accumulation of plus-strands of the virus genome. Therefore, in addition to allowing for modulation of virus reproduction, the RE riboswitch system also provided insight into RE function (Wang and White 2007). Verhounig et al. (2010) have identified a theophylline-responsive synthetic riboswitch that functions as an efficient translational regulator of gene expression in plastids. This riboswitch provides a novel tool for plastid genome engineering that facilitates the tightly regulated inducible expression of chloroplast genes and transgenes and provides opportunities for plastid biotechnology (Verhounig et al. 2010).

Another application of riboswitch-mediated 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 (Kötter et al. 2009). 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 (Jin et al. 2009). Both studies suggest that synthetic riboswitches may find broad use in investigating genetics of microorganisms.

The ability of riboswitches to control gene expression has been applied to reprogram bacterial behaviors such as cell motility. Gallivan and coworkers have reprogrammed *E. coli* chemotaxis system by placing a key chemotaxis signaling protein (CheZ) under the control of theophylline-sensitive riboswitch (Topp and Gallivan 2007; Mishler et al. 2010). Reprogrammed cells could migrate up gradients if this analyte and autonomously localize to regions of high theophylline concentration, which is a behavior that cannot be accomplished by the natural *E. coli* chemotaxis system. In a different study, Micklefield and coworkers coupled *cheZ* gene with a synthetic riboswitch responsive to pyrimido[4,5-d]pyrimidine-2,4-diamine (PPDA) (Robinson et al. 2014). *E. coli* cells harboring a riboswitch-*cheZ* construct exhibited dose-dependent cell migration in response to PPDA. The ability to modulate bacterial motility in response to arbitrary chemical signals can provide new tools for bioremediation and drug delivery. Another riboswitch application is a control of cell morphology. PPDA-sensitive transcriptional OFF switch was used to chemically control a production of an actin homologue, MreB protein, in *B. subtilis* (Robinson et al. 2014). MreB has a critical role in morphogenesis in rod-shaped bacterial species and is an important new target for the development of antimicrobial agents. Dose-dependent regulation of antimicrobial targets, such as *mreB*, can find use in sensitive and specific antimicrobial screening systems or mechanism-of-action studies.

Riboswitch reporters can also be used to monitor and optimize *in vivo* production of small molecules. For instance, Xiu et al. (2017) have developed RNA riboswitch-based biosensor for identification of naringenin over producing *E. coli* strains. Naringenin is a key flavonoid precursor, and its production is in a particular interest since it is being researched as a potential treatment for Alzheimer's disease.

Riboswitch-mediated regulation of gene expression in response to explosive compounds can be applied for cell-based environmental biosensing. Kelley-Loughnane and coworkers have demonstrated that 2,4-dinitrotoluene (DNT)- and 2,4,6-trinitrotoluene (TNT)-sensitive synthetic riboswitches activate expression of downstream reporter genes at concentrations of DNT and TNT that are in a range of previously reported environmental concentrations of these compounds found in contaminated water and soil (Davidson et al. 2013; Harbaugh et al. 2017).

Although application of riboswitches as sensor entities is still limited due to a low number of selected functional switches responsive to small molecule analytes, the development of new approaches for fast and modular riboswitch design and discovery will allow to expand their utilization in a nearest future.

## Conclusions

Synthetic riboswitches can be engineered to regulate expression of any gene in response to any nonnatural molecule that is capable of being bound by RNA and is nontoxic to cells. This feature demonstrates a strong possibility for RNA switches

to serve as sensor entities for design and development of cell-based biosensors with a variety of different applications. Riboswitches have passed the proof-of-concept state and, due to the current mechanistic understanding of their function, now are being developed to address specific applications. The advantage of using riboswitches is that they do not require protein cofactors and give access to directly alter protein expression at an early stage, which saves resources and is potentially faster than regulation by proteins such as transcription factors, which need to be produced on demand. They consist solely of RNA and therefore they are easy to implement, because they involve the transfer of only a single genetic control element into an organism. Finally, they control gene expression in dose dependent manner resulting in the ability not only to sense a desired target but also to determine its concentration. Synthetic tandem riboswitches demonstrate that multiunit riboswitches can achieve complex functions similar to those of the more elaborate circuits consisting of multiple genes and regulatory proteins.

Despite their useful features, synthetic riboswitches still remain outnumbered by other genetic regulatory elements (such as promoters and transcriptional factors) in the field of biosensors, mainly due to the low number of available aptamers that can be converted into functional switches. Another reason for limited riboswitch applications is their incompatibility with a variety of different reporters and low sensitivity. Advances in the development of aptamer and riboswitch selection and screening techniques, improvements in rational structure-based designs and computational modeling, and progress in development of sophisticated approaches for riboswitch tuning and performance optimization will guide the design of increased number of better riboswitches to harness the full regulatory and sensing potential of these RNA switches in sensing devices.

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