Published in final edited form as:
Curr Opin Biotechnol. 2012 October ; 23(5): 679–688. doi:10.1016/j.copbio.2012.01.005.

Synthetic RNA switches as a tool for temporal and spatial control over gene expression

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Abstract

The engineering of biological systems offers significant promise for advances in areas including health and medicine, chemical synthesis, energy production, and environmental sustainability. Realizing this potential requires tools that enable design of sophisticated genetic systems. The functional diversity of RNA makes it an attractive and versatile substrate for programming sensing, information processing, computation, and control functions. Recent advances in the design of synthetic RNA switches capable of detecting and responding to molecular and environmental signals enable dynamic modulation of gene expression through diverse mechanisms, including transcription, splicing, stability, RNA interference, and translation. Furthermore, implementation of these switches in genetic circuits highlights the potential for building synthetic cell systems targeted to applications in environmental remediation and next-generation therapeutics and diagnostics.

Introduction

Cells are dynamic systems that integrate intra- and extracellular signals to activate appropriate biological responses. Engineering synthetic cell systems that exhibit sophisticated and dynamic behaviors requires the ability to design synthetic gene networks that encode similar sensing, information processing, computation, and control capabilities. However, the construction of such genetic systems is generally limited by the availability of components encoding the desired functional activities [1]. As a result, new molecular platforms are needed to support the design of tailored information processing and control functions.

RNA is a biological macromolecule that plays diverse roles in controlling cellular behaviors. Natural RNAs can regulate multiple stages of gene expression, including transcription, splicing, mRNA stability, and translation, through varied mechanisms. RNA molecules are composed of four bases that form extensive intra- and intermolecular bonds through well-characterized base pairing interactions that determine the encoded regulatory functions. These interactions can be directly regulated in response to molecular and environmental inputs to modulate the controlled cellular processes. Tractable techniques for *in vitro* and *in*

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vivo experimental manipulation and computational methods that can predict structures and associated functions facilitate the creation of RNAs with new regulatory properties [2]. In particular, researchers have constructed a variety of RNA-based control devices that couple diverse gene-regulatory activities to molecular and environmental signals and demonstrate potential for advancing temporal and spatial control of gene expression. Here, we review recent advances in synthetic RNA switch design and the application of these synthetic controllers toward building more sophisticated synthetic cell systems.

RNA switches enable control of gene expression in response to molecular and environmental signals

Synthetic RNA switches are generally composed of a sensor domain that detects signals within a cell and an actuator domain that regulates gene expression. Ligand binding at the sensor domain typically modulates the activity of the actuator domain through directed conformational changes. These genetic devices may also include a distinct transmitter domain that serves to communicate the status of the sensor domain to the actuator domain. Sensors can respond to multiple classes of intracellular molecules, including small molecules, other RNAs, and proteins, and environmental cues such as temperature. For example, RNA structures known as aptamers recognize small molecule and protein ligands with high specificity and affinity. Aptamers can be harvested from natural biological systems [2], including protein binding sites in cellular RNAs [3], or generated *de novo* through selection processes to develop novel specificities [4]. RNA switches can also recognize intracellular RNAs through base pairing interactions. These sensing mechanisms have been integrated with natural RNA regulatory activities to engineer input-dependent control at multiple points of the gene expression pathway. We discuss several mechanisms to highlight the diversity of signal inputs and regulatory outputs accessible by synthetic RNA switches.

Transcription-modulation switches

Transcription represents the earliest control point in the regulation of gene expression. Synthetic RNA switches that regulate transcription in response to either small molecule or RNA signals have been demonstrated (Table 1). A recent study developed switches that terminate transcription in response to RNA signals (Figure 1a) [5•]. These switches were developed in *Escherichia coli* based on the *Staphylococcus aureus* pT181 antisense RNA-mediated transcriptional attenuation system. Researchers optimized attenuation of the wild-type system and engineered two orthogonal attenuator-antisense pairs to enable logic evaluation and signal propagation independent of protein factors.

Splicing-modulation switches

The programmed removal of intronic sequences is a critical regulatory step in the expression of many genes and offers an additional layer of control for synthetic RNA switches. By modulating accessibility of splicing components in response to molecular or temperature inputs, researchers have engineered temperature-, small molecule-, or protein-dependent splicing (Table 1). In particular, incorporating aptamers within key intronic locations can result in systems that regulate splicing in response to small molecule or protein ligands. A recent study described a modular RNA switch platform to regulate alternative splicing in HEK293 cells in response to changes in nuclear protein levels (Figure 1b) [6••]. This platform was demonstrated to increase or decrease exon inclusion in response to the heterologous protein MS2 depending on the location of the MS2 aptamer within the flanking introns and was expanded to respond to three endogenous proteins.

RNA stability-modulation switches

Modulating transcript stability provides another mechanism through which gene expression can be controlled. Internal cleavage exposes mRNAs to degradation by cellular ribonucleases (RNases), and small molecule-responsive RNA switches have been engineered that regulate this process through either ribozyme self-cleavage or programmed enzymatic processing by RNases (Table 1). Modular ribozyme-based devices were constructed in *Saccharomyces cerevisiae* by coupling aptamers that recognize the small molecules theophylline or tetracycline to a hammerhead ribozyme through a distinct transmitter domain, thereby linking ligand availability to ribozyme self-cleavage and subsequent mRNA degradation (Figure 1c) [7]. Since ribozyme activity is independent of cell-specific machinery, this class of RNA device has been shown to transport across different organisms, including mouse and human cells [8••].

RNA interference-modulation switches

The RNA interference (RNAi) pathway processes structured RNAs through a series of enzymatic steps to produce small regulatory RNAs that are incorporated into the RISC complex to silence gene expression. Target mRNAs undergo cleavage or translational repression determined by the degree of sequence complementarity with the small RNAs [9]. Synthetic RNA switches have been designed to modulate RNAi silencing of target genes by regulating Drosha processing of pri-miRNAs or Dicer processing of small hairpin RNAs (shRNAs) in response to small molecule or protein signals (Table 1). In a recent example, an aptamer to the archaeal ribosomal protein L7Ae was coupled to an shRNA and shown to inhibit Dicer processing and associated gene silencing when the protein was heterologously expressed in HeLa cells (Figure 1d) [10••]. An alternative design strategy for RNAi-based switches integrates information regarding small RNAs through target sites in the 3′ untranslated region (UTR) of genetic constructs. In one example, reporter constructs performed logic evaluations in HEK293 cells by assessing transfected small interfering RNAs (siRNAs) directly through 3′ UTR target site sensors and indirectly through regulation by co-transfected transcriptional regulators that were themselves regulated by the siRNAs [11].

Translation-modulation switches

RNA switches have been developed that regulate either prokaryotic or eukaryotic translation initiation in response to small molecules, RNAs, or temperature changes (Table 1). The predominant strategy to regulate translation initiation in prokaryotes is based on modulating ribosome binding site (RBS) accessibility, a mechanism identified in natural temperature- and metabolite-responsive RNA regulatory elements [12,13]. Thermal control in *E. coli* is achieved through temperature-controlled access of the RBS, where base pairing interactions masking the RBS can be liberated by local structural melting at elevated temperatures (Figure 1e) [14,15]. In addition, RNA switches termed riboregulators have been developed in *E. coli* to activate translation in response to RNA signals [16]. In this system, an upstream *cis*-repressor sequesters the RBS in an intramolecular hairpin, inhibiting translation of the target transcript. A separate, *trans*-activating RNA forms a more stable duplex with the *cis*-repressor, thereby unmasking the RBS to enable ribosome binding and translation initiation. Aptamer integration into the 5′ UTRs of eukaryotic mRNAs has also been demonstrated to regulate translation (Table 1), potentially by disrupting ribosome binding, scanning, or assembly in the presence of small molecule or protein ligands.

Posttranslational activity-modulation switches

RNA switches that posttranslationally control protein functions rather than protein levels will enable systems that provide rapid and conditional control over protein activities.

Aptamers have been shown to bind functional protein domains to modify protein activities [17]. In one example, an aptamer that inhibits Tet repressor (TetR) was selected and used to induce transcription in *E. coli* in a manner analogous to the repressor's natural ligand tetracycline [18]. This protein-inhibiting aptamer was coupled to the theophylline aptamer to engineer a switch that regulated the function of a TetR fusion protein in a theophylline-dependent manner in mammalian HEK293 and CHO cell lines (Figure 1f) [19]. This recent work highlights the potential of building RNA switches that allow rapid and dynamic control over protein activities.

Applications of switch technology

With increasing options for regulating gene expression using synthetic RNA switches responsive to molecular and environmental inputs, recent efforts have focused on the integration of these synthetic controllers within diverse genetic systems. The ability to tailor the input processing and output regulatory activities of these synthetic devices allow for genetic control systems that can be reconfigured for a number of different applications.

Environmental remediation

Natural microorganisms play significant roles in environmental bioremediation, such as microbial communities that degrade hydrocarbons released in oil spills [20]. One recent example highlighted the ability to engineer *E. coli* to seek and destroy an environmental pollutant, the herbicide atrazine (Figure 2a) [21••]. An atrazine-responsive, RBS-based RNA switch was coupled to the *cheZ* gene to control cell motility, allowing cells to move along a gradient of the pollutant. Addition of an atrazine-catabolizing enzyme to the genetic system enabled degradation of atrazine into a nontoxic product. Similar strategies could be applied to engineer organisms suitable for release into the environment to remediate toxic contaminants.

Managing engineered microbial population growth and expansion is a critical consideration in environmental release. Genetic systems that activate a kill switch in response to specific inputs or input frequencies could help contain engineered organisms. Recent systems engineered with synthetic RNA switches take steps towards this goal. A genetic circuit in *E. coli* based on nesting polymerase-promoter pairs controlled by riboregulators was used to count up to three exposure events to a small molecule inducer [22], where the riboregulators were responsive to a *trans*-acting RNA under the control of an inducible promoter. A second genetic circuit demonstrated independent regulation of multiple genes required for cell lysis [23•]. This system regulated two genes required in combination for lysis with independent *trans*-acting RNAs expressed in response to separate small molecule inducers such that lysis occurred only in the presence of both inputs. Extended to additional inputs and outputs, these types of regulatory systems may provide safeguards for controlled release of engineered microorganisms.

Health and medicine

Recent progress has been made in the translational application of RNA switches to health and medicine. One study explored the application of drug-responsive RNA switches to controlling the proliferation and activation of engineered T cells (Figure 2b) [8••], addressing a major challenge to the efficacy and safety of immunotherapy strategies. A ribozyme-based system was implemented to regulate production of the proliferative cytokines interleukin-2 (IL-2) and IL-15 in response to the small molecule drugs theophylline and tetracycline and was demonstrated to function in a rapid, reversible, and dose-dependent manner in a mouse T cell line. The RNA-based control system was further

extended to primary human T cells and demonstrated to regulate T cell populations *in vivo* in mice, suggesting potential for therapeutic applications in humans.

Other applications of synthetic RNA switches have been directed to building control systems to regulate apoptosis in defined cell populations. For example, protein-responsive RNA switches have been used to regulate apoptosis in HeLa cells in response to heterologously expressed L7Ae [10••]. L7Ae was shown to either increase or decrease apoptosis depending on the RNA switch configuration used to regulate co-transfected Bim, a proapoptotic gene, and Bcl-xL, an antiapoptotic gene. L7Ae-mediated repression of Bcl-xL through a translation-modulation switch increased cell death, whereas either L7Ae-mediated inhibition of shRNA processing to small RNAs that silence Bcl-xL or repression of a translation-modulation switch regulating Bim decreased cell death.

Finally, synthetic RNA switches have been recently applied to trigger cell death in response to endogenous signals of disease, moving toward targeted therapeutic applications. In one example, an alternative splicing platform that responds to activation of two signaling pathways associated with human disease, Wnt and NF-κB, was recently described in HEK293 cells [6••]. These protein-responsive switches respond to nuclear accumulation of β-catenin or p65 to regulate the alternative splicing and functional production of a HSV-TK transcript, which encodes a proapoptotic protein and promotes cell death. miRNA profiles indicative of a disease state have also been used to activate cell death. Building on the success of previous genetic circuits that performed logic evaluations on transfected siRNAs [11], researchers developed a genetic classifier circuit that evaluates endogenous miRNAs (Figure 2c) [24••]. Six miRNAs were identified from public datasets whose combined expression profile distinguishes HeLa cancer cells from most healthy human cell types. Regulation of proapoptotic hBax by a genetic classifier that integrates expression levels of these miRNAs was shown to selectively kill HeLa cells within a panel of seven immortalized cell lines, suggesting the potential for selective killing of cancer cells with custom classifier circuits.

Future directions and addressing persistent needs

Synthetic RNA switches have demonstrated capacities for engineering cell systems; however, in order to maximize the potential of this emerging technology, many challenges must be addressed. The limited diversity of available RNA parts is a major constraint in the application of RNA switches. Switches that operate through diverse regulatory mechanisms have been developed, yet additional regulatory mechanisms, including bacterial small RNAs [25,26], CRISPR RNAs [27], long noncoding RNAs [28], and internal ribosome entry sites [29] remain undeveloped resources for constructing ligand-responsive switches that function *in vivo*. Techniques for generating RNA components *de novo* that exhibit novel sensing and catalytic activities have been developed [4,30]. However, scalable strategies are needed to accelerate the generation of diverse functional RNA activities, particularly sensing activities. Methods that couple *in vitro* and *in vivo* screens and selections will enable broad exploration of sequence space and optimization of functional activities within the context of desired genetic circuits and cellular environments. A second limiting factor is the lack of computational tools to guide the design of RNA components and devices. Computational models have been developed to support RNA device design [31,32], but enhanced modeling and predictive capacities are needed to further elucidate relationships between RNA sequence, structure, folding, and function.

Enhanced switch design will drive higher-order information processing capabilities and advanced applications. Synthetic RNA switches have already been demonstrated that encode band-pass filters [33–35], programmed cooperativity [33], and logic operations [5•,24••,

33,36]. Interfacing devices at multiple points in a network and integrating several levels of control will enable more advanced systems behaviors. Future advances in the design of synthetic RNA switches may extend their application beyond control of protein levels to control of protein activities through posttranslational regulatory strategies or alternative splicing of functional protein domains. Synthetic RNA switches are also promising candidates for encoding spatial and temporal control within biosynthetic and signaling pathways. RNA switches can spatially orient a cell within its environment, directing cell migration along a concentration gradient and inducing spatial patterning [21••,37]. RNA can also serve as a physical scaffold within cells, self-assembling into nanostructures that spatially organize biosynthetic enzymes [38]. Temporal control is achieved through rapid and reversible regulation of gene expression [8••], enabling coordinated gene expression [39] and construction of dynamic feedback loops [39] and genetic counters [22]. Integration of spatial organization and temporal control strategies will aid the design of more complex and sophisticated biological systems, furthering applications in next-generation therapeutic strategies and biomanufacturing platforms.

Acknowledgments

C.D.S. is supported by funds from the National Institutes of Health, National Science Foundation, and Defense Advanced Research Projects Agency. J.J.W. is supported by a Stanford Dean's Postdoctoral Fellowship.

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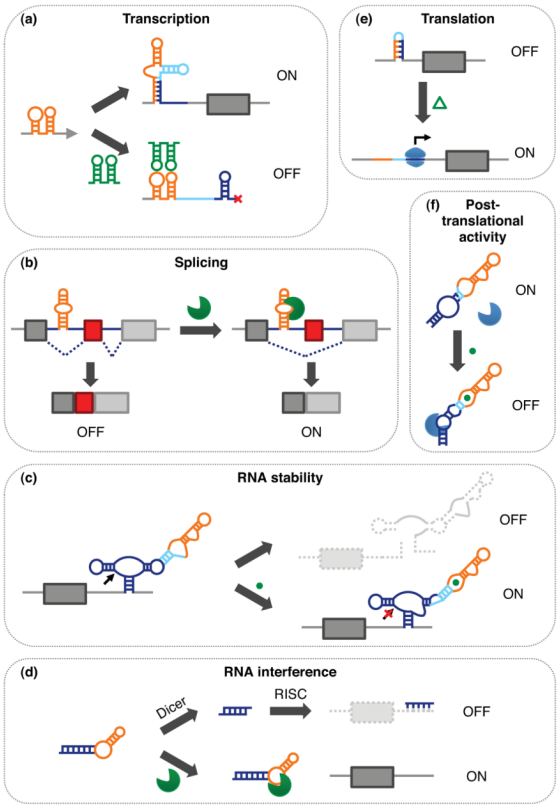

Figure 1.

Regulation of gene expression by synthetic RNA switches. One representative synthetic RNA switch is depicted for each stage of gene expression described in the text. Switch components are indicated as follows: sensors are colored orange, actuators are dark blue, and transmitters are light blue. Inputs are colored green, coding regions are represented as rectangular boxes, and degraded transcripts are indicated with gray dotted lines. **(a)** Transcriptional control is achieved using an antisense-mediated transcriptional attenuator. In the absence of antisense RNA, transcription proceeds through the coding region. Antisense RNA binding promotes formation of an intrinsic terminator hairpin. **(b)** Insertion of protein binding aptamers within introns can modulate splicing patterns (blue dotted lines) in response to ligand. The three-exon, two-intron system contains a stop codon in the middle exon (red). Ligand binding favors exclusion of the middle exon, leading to high gene expression. **(c)** Ribozyme-based switches regulate RNA stability. Ribozyme self-cleavage leads to transcript inactivation and degradation. Ligand binding stabilizes an inactive ribozyme conformation. Black arrow indicates site of ribozyme self-cleavage. **(d)** Aptamer integration into small hairpin RNAs can modulate enzymatic processing required for RNA interference. Ligand binding interferes with Dicer processing, preventing gene silencing associated with RISC activity. **(e)** Translation initiation in prokaryotes can be regulated by limiting ribosome access to the RBS. Base pairing interactions in temperature-sensitive switches sequester the RBS, which is freed upon addition of heat (triangle). **(f)** Protein activity can be regulated posttranslationally using inhibitory aptamers. Coupling a small molecule binding aptamer to a protein inhibiting aptamer allows for ligand-dependent control of protein activity. Small molecule addition promotes conformational switching to form the properly folded ligand binding and protein inhibiting structure.

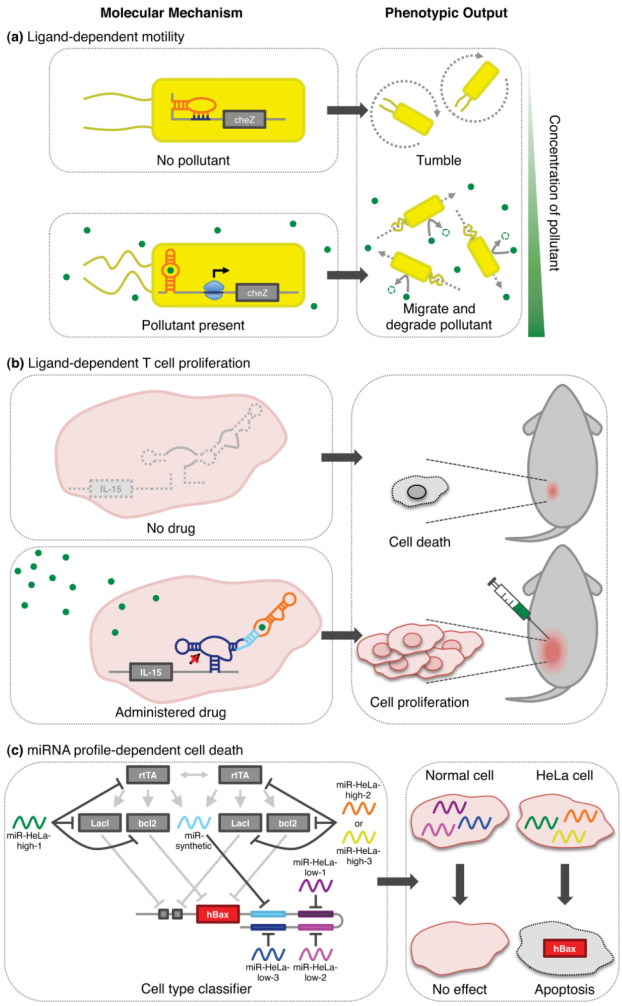

Figure 2.

Application of synthetic RNA switches to environmental remediation and health and medicine. **(a)** Regulation of the motility gene *cheZ* by a translation-modulation switch responsive to the herbicide atrazine causes engineered *E. coli* cells to move along an atrazine gradient. Expression of an atrazine-catabolizing enzyme enables cells to degrade atrazine into a nontoxic product. **(b)** Drug-responsive, ribozyme-based switches control T cell proliferation. Small molecule input prevents ribozyme self-cleavage, leading to cytokine expression and proliferation of cells implanted into mice. **(c)** An RNAi-based classifier circuit integrates signals from six endogenous miRNAs to regulate expression of proapoptotic hBax. The HeLa miRNA expression profile uniquely allows hBax expression and subsequent apoptosis. A representative miRNA profile that does not produce apoptosis is also shown. rtTA activates transcription of LacI, bcl2, and a synthetic miRNA; LacI represses hBax transcription; and bcl2 is an hBax inhibitor.

Table 1

Synthetic RNA switches respond to molecular and environmental inputs and operate through diverse regulatory mechanisms.

Regulated Process	Host Cell	Regulatory Mechanism	Input Signal(s)	Effect of Input Signal(s)	Reference(s)
Transcription	*E. coli*	Transcription termination	antisense RNA	Inhibits transcription as a function of single and multiple input signals	[5•]
	E. coli	Transcription termination	thiamine pyrophosphate	Inhibits transcription	[35]
	S. cerevisiae	RNA Polymerase recruitment	tetramethylrosamine	Promotes transcription	[40]
Splicing	*E. coli*	Splice site accessibility	theophylline, 3-methylxanthine	Promotes splicing	[41]
	S. cerevisiae	Position-dependent splicing	tetracycline	Inhibits splicing	[42]
	S. cerevisiae	Splice site accessibility	heat	Promotes splicing	[43]
	HeLa	Branch point sequence accessibility	theophylline	Inhibits splicing	[44]
	HEK293	Position-dependent splicing	β-catenin, MS2, NF-κB	Promotes or inhibits splicing as a function of single and multiple input signals	[6••]
mRNA Stability	*S. cerevisiae*	Ribozyme self-cleavage	tetracycline, theophylline, xanthine	Promotes or inhibits mRNA stability	[7]
	S. cerevisiae	Ribozyme self-cleavage	tetracycline, theophylline	Promotes or inhibits mRNA stability as a function of single and multiple input signals	[33]
	S. cerevisiae	Rnt1p enzymatic processing	theophylline	Promotes mRNA stability	[45]
	CTLL-2, primary human T_{CM}	Ribozyme self-cleavage	tetracycline, theophylline	Promotes mRNA stability	[8••]
	HEK293, HeLa	Ribozyme self-cleavage	theophylline	Inhibits mRNA stability	[46]
RNAi	HEK293	Drosha pri-miRNA processing	hypoxanthine, tetracycline, theophylline	Inhibits RNAi	[47]
	HEK293	Ribozyme-mediated pri-miRNA folding	theophylline	Promotes RNAi	[48]
	HEK293	Dicer shRNA processing	theophylline	Inhibits RNAi	[49]
	HepG2	Dicer shRNA processing	theophylline	Inhibits RNAi	[50]
	HeLa	Dicer shRNA processing	L7Ae	Inhibits RNAi	[10••]
	HEK293	Dicer shRNA processing	hypoxanthine, tetracycline, theophylline	Inhibits RNAi as a function of single and multiple inputs	[31]
	HEK293	RNAi computation	transfected siRNAs	Promotes or inhibits RNAi as a function of multiple input signals	[11]
	HEK293	RNAi computation	transfected heterologous transcription factors	Promotes or inhibits RNAi as a function of multiple input signals	[51]
	DAOY, HEK293, HeLa, MCF-7, SH-SY5Y, SKBR3, T47D	RNAi computation	endogenous miRNAs	Promotes or inhibits RNAi as a function of multiple input signals	[24••]

Regulated Process	Host Cell	Regulatory Mechanism	Input Signal(s)	Effect of Input Signal(s)	Reference(s)
Translation	*E. coli*	RBS accessibility	theophylline	Promotes translation	[52–54]
	E. coli	RBS accessibility	theophylline	Inhibits translation	[55]
	A. baumannii, A. baylyi, A. tumefaciens, B. subtilis, E. coli, M. magneticum, M. smegmatis, S. pyogenes	RBS accessibility	theophylline	Promotes translation	[56]
	E. coli	RBS accessibility	atrazine	Promotes translation	[21••]
	B. subtilis	RBS accessibility	theophylline	Promotes translation	[57]
	E. coli, N. tabacum chloroplasts	RBS accessibility	theophylline	Promotes translation	[58]
	E. coli	RBS accessibility	thiamine pyrophosphate	Promotes translation	[59,60]
	E. coli	RBS accessibility	thiamine pyrophosphate, theophylline	Promotes or inhibits translation as a function of multiple input signals	[36]
	E. coli	RBS accessibility	ammeline, azacytosine	Promotes translation	[61]
	E. coli	RBS accessibility	heat	Promotes translation	[14,15]
	E. coli	RBS accessibility	antisense RNA	Promotes translation	[16,22,23•]
	E. coli	Ribozyme-mediated RBS accessibility	theophylline	Promotes translation	[62,63]
	E. coli	Ribozyme-mediated RBS accessibility	thiamine pyrophosphate	Promotes or inhibits translation	[64]
	S. cerevisiae	Ribosome binding, scanning, or assembly	tetramethylrosamine	Inhibits translation	[65]
	S. cerevisiae	Ribosome binding, scanning, or assembly	tetracycline	Inhibits translation	[66]
	S. cerevisiae	Ribosome binding, scanning, or assembly	neomycin	Inhibits translation	[67]
	S. cerevisiae	Ribosome binding, scanning, or assembly	MS2, U1A	Inhibits translation	[68]
	S. cerevisiae	Antisense RNA binding to start codon	tetracycline, theophylline	Promotes or inhibits translation	[69]
	X. laevis	Ribosome binding, scanning, or assembly	biotin	Inhibits translation	[70]
	CHO	Ribosome binding, scanning, or assembly	Hoechst 33342	Inhibits translation	[71]
	HeLa	Ribosome binding, scanning, or assembly	MS2	Inhibits translation	[68]
	HeLa	Ribosome binding, scanning, or assembly	L7Ae	Inhibits translation	[3]
Posttranslation	CHO, HEK293	TetR activity	theophylline	Inhibits posttranslational activity	[19]