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**Unexpected Versatility in Bacterial Riboswitches**

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1 **Abstract**

2 Bacterial riboswitches are elements present in the 5'untranslated regions  
3 of messenger RNA molecules that bind to ligands and regulate the expression of  
4 downstream genes. Riboswitches typically regulate the expression of protein  
5 coding genes. Mechanisms of riboswitch-mediated regulation, however, have  
6 recently been shown to be more diverse than originally thought, with reports  
7 showing riboswitches can regulate the expression of noncoding RNAs and  
8 control the access of proteins, such as the transcription termination factor Rho  
9 and the ribonuclease RNaseE, to a nascent RNA. Riboswitches are also  
10 increasingly used in biotechnology, with advances in the engineering of synthetic  
11 riboswitches and the development of riboswitch-based sensors. Herein, we  
12 review emerging roles and mechanisms of riboswitch-mediated regulation *in*  
13 *natura* and recent progress in the development of riboswitch-based technology.

14

15 **Introduction**

16 Twenty years ago it was first observed that cis elements named T-boxes,  
17 in the 5' UTRs of tRNA synthetase genes could bind uncharged tRNA molecules of  
18 the corresponding amino acid and modulate expression of the downstream gene  
19 [1,2]. Subsequently, cis-regulatory elements were identified in the leader regions  
20 of many other mRNAs and shown to bind a diverse set of ligands including  
21 metabolites such as glucosamine-6-phosphate, lysine and glycine [3-5],  
22 coenzymes such as vitamin B<sub>12</sub>, S-adenosylmethionine, thiamine pyrophosphate  
23 and flavin mononucleotide [6-10], and ions such as magnesium and fluoride  
24 [11,12]. These cis-encoded regulatory RNAs were termed "riboswitches," owing  
25 to their ability to adopt alternative conformations in the presence or absence of  
26 their cognate ligand [6,7,13], and at present, at least 19 distinct ligand binding  
27 riboswitch classes have been identified (Reviewed in [14]). Typically  
28 riboswitches control the expression of downstream genes by adopting a  
29 structural conformation that induces transcription termination or inhibits  
30 translation initiation in the presence of a cognate ligand, while adopting an  
31 alternative conformation, which enables transcription elongation or translation  
32 in the absence of ligand. In some cases, such as with T-boxes, S-  
33 adenosylhomocysteine (SAH) riboswitches [15] and some purine riboswitches

1 [16], binding of a riboswitch to its ligand can result in activation of expression of  
2 downstream genes. In either case, however, riboswitches are encoded in *cis*  
3 upstream of the genes to be regulated.

4

5 **Riboswitches regulate the transcription of antisense RNAs**

6 A first hint that riboswitches might regulate RNAs other than mRNAs  
7 derived from studies highlighting a number of riboswitches located at the 3' end  
8 of one gene and positioned antisense to the 5' adjacent open reading frame [17-  
9 19]. This suggested that these riboswitches were unable to regulate expression  
10 of the adjacent genes in a classical manner by controlling transcription or  
11 translation of the downstream ORF(s). Such riboswitches appeared to be  
12 "marooned" in genomes, without an associated gene to regulate, and led to the  
13 hypothesis that these riboswitches might regulate the expression of antisense  
14 RNAs (asRNAs).

15 Evidence in support of this hypothesis came from *Clostridium*  
16 *acetobutylicum* in which a S-adenosylmethionine (SAM) riboswitch was  
17 identified in the antisense orientation to the three gene *ubiGmccBmccA* operon  
18 [17]. A subsequent study showed that this SAM riboswitch regulated  
19 transcription of an antisense RNA (asRNA) (Fig. 1A) [20]. The *ubiGmccBA* operon  
20 encodes enzymes that catalyze reactions required to convert SAM into cysteine.  
21 Interestingly the *ubiGmccBA* operon is itself preceded by a cysteine specific T-  
22 box riboswitch. In high cysteine concentrations, the riboswitch inhibits  
23 transcription of the operon. Conversely in low cysteine concentrations, the  
24 riboswitch bound to uncharged tRNA<sup>CYS</sup> enables transcription of the operon by  
25 forming an antiterminator structure (Fig. 1A, high cysteine, low SAM). However,  
26 if SAM concentrations are also low - conditions in which expression of enzymes  
27 to convert SAM to cysteine would be of no use - expression of the asRNA  
28 opposite the *ubiGmccBA* genes inhibits *ubiGmccBA* expression (Fig. 1A, low  
29 cysteine, & high SAM). Only when SAM concentrations are high, will SAM bind  
30 the riboswitch in the asRNA leader, producing a truncated asRNA transcript and  
31 allowing *ubiGmccBA* expression (Fig. 1A, low cysteine, high SAM). This  
32 regulatory system ensures that the enzymes that convert SAM into cysteine are

1 expressed only when concentrations of SAM are high and concentrations of  
2 cysteine are low.

3 A second report of a riboswitch-regulated asRNA arose from studies in  
4 *Listeria monocytogenes* [19]. Unlike the previous scenario, which ensures  
5 optimal expression of an operon in the absence of one signal (cysteine) and  
6 presence of another (SAM), here expression of the riboswitch-regulated asRNA  
7 controls expression of an operon only when two signals are simultaneously  
8 present. Specifically, a vitamin B<sub>12</sub> (B<sub>12</sub>) riboswitch was shown to regulate  
9 transcription of an asRNA (*aspocR*) opposite a gene encoding a transcriptional  
10 activator named *PocR* (Fig. 1B). In *Salmonella enterica*, *PocR* was known to  
11 activate transcription of genes encoding enzymes that carry out propanediol  
12 catabolism (*pdu* genes) and require B<sub>12</sub> as a cofactor [21-23]. It was shown that  
13 similarly, in *L. monocytogenes* expression of *pocR* is autoactivated in response to  
14 propanediol [19]. However expression of *aspocR* is simultaneously activated by  
15 propanediol, which prevents *pocR* from being highly expressed in the absence of  
16 B<sub>12</sub> and inhibits activation of *pdu* gene expression (Fig 1B, high propanediol, low  
17 B<sub>12</sub>). Conversely, when B<sub>12</sub> is present, B<sub>12</sub> binds the riboswitch, leading to a  
18 truncated *aspocR* transcript. This truncated *aspocR* transcript is unable to inhibit  
19 *pocR* expression, resulting in high levels of *PocR*, and activation of *pdu* gene  
20 expression (Fig 1B, high propanediol, high B<sub>12</sub>). This regulation of *pocR* by  
21 *aspocR* coordinates expression of *PocR* and the propanediol catabolic enzymes  
22 with the availability of both propanediol (substrate) and the B<sub>12</sub> cofactor  
23 required for propanediol degradation, ensuring that the operon is maximally  
24 expressed only when both molecules are present.

25 Interestingly, antisense-oriented riboswitches are not uncommon, but  
26 they seem particularly prevalent for a few classes of ligand-binding families  
27 including the B<sub>12</sub>, SAM and cyclic-di-GMP riboswitches [19]. For example, it was  
28 noted that in *Clostridium difficile*, three cyclic-di-GMP binding riboswitches are  
29 antisense to a transcriptional regulator, an array of prophage genes, and a  
30 CRISPR array, respectively, suggesting that *C. difficile* may use many cyclic-di-  
31 GMP riboswitch-regulated antisense RNAs to control expression of diverse types  
32 of genes [19]. The prevalence of antisense-oriented riboswitches of the B<sub>12</sub>, SAM  
33 and cyclic-di-GMP families suggests riboswitch-regulated antisense RNAs may

1 serve a specialized regulatory role for these molecules such as integrating a  
2 second signal into a gene expression program, as with the previously noted  
3 examples. It is tempting to speculate that this may be due the roles of B<sub>12</sub> and  
4 SAM as coenzymes and of cyclic-di-GMP as second messenger molecule, whereas  
5 the majority of other riboswitch ligands are metabolites.

### 6 7 **Riboswitches regulate the transcription of small RNAs (sRNAs) that** 8 **sequester proteins**

9 In addition to antisense oriented riboswitches, which upon first glance,  
10 are unlikely to regulate ORFs, many riboswitches are positioned a very long  
11 distance away from ORFs, suggesting they may not act as classical riboswitches.  
12 Inspection of 15 different riboswitch classes in over 800 bacterial genomes in  
13 fact showed many instances in which the 3' end of a riboswitch was more than  
14 250 nucleotides away from the 5' boundary of the downstream ORF (Fig. 2).  
15 Such a distance is significantly greater than for 90% of all riboswitches for each  
16 class, respectively, and hinted that these riboswitches might regulate noncoding  
17 RNAs. This type of analysis was used to identify three B<sub>12</sub> riboswitches in *L.*  
18 *monocytogenes*, *Enterococcus faecalis* and *Streptococcus sanguinis*, which were  
19 located greater than 200 nucleotides away from their adjacent ORFs. These  
20 riboswitches were subsequently shown to regulate trans-acting small RNAs  
21 (sRNAs) [24,25].

22 In *L. monocytogenes* and *E. faecalis*, B<sub>12</sub> riboswitches regulate  
23 transcription of the orthologous sRNAs, Rli55 and EutX, respectively. Rli55/EutX  
24 in turn control expression of the ethanolamine utilization (*eut*) locus in response  
25 to B<sub>12</sub>. Similar to propanediol catabolism, enzymes involved in ethanolamine  
26 catabolism require vitamin B<sub>12</sub> as an essential cofactor, and it was reasoned that  
27 bacteria might coordinate expression of the *eut* genes with both ethanolamine  
28 and B<sub>12</sub> availability. Studies in *E. faecalis* had previously shown that *eut* gene  
29 expression is activated in response to ethanolamine by an RNA-binding response  
30 regulator name, EutV [26]. In the absence of ethanolamine EutV is present as a  
31 monomer in the cytosol and unable to activate *eut* expression (Fig 3A, low  
32 ethanolamine, low B<sub>12</sub>). Conversely, in the presence of ethanolamine, EutV is  
33 phosphorylated by its cognate sensor kinase EutW, and is primed to activate *eut*

1 expression. However, if B<sub>12</sub> levels are low, Rli55/EutX is transcribed a long  
2 transcript containing a EutV binding site, which sequesters EutV and prevents it  
3 from activating *eut* gene expression (Fig 3A, high ethanolamine, low B<sub>12</sub>). In  
4 contrast, when B<sub>12</sub> is present, the B<sub>12</sub> riboswitch terminates transcription of  
5 Rli55/EutX, producing truncated sRNAs, which lack a EutV binding site. This in  
6 turn frees EutV, enabling it to activate *eut* expression by an antitermination  
7 mechanism (Fig 3A, high ethanolamine, high B<sub>12</sub>). Expression of Rli55/EutX thus  
8 coordinates activation of the *eut* operon with the availability of ethanolamine  
9 and B<sub>12</sub> in a manner similar to regulation of *pdu* expression by AspocR, but by an  
10 entirely different mechanism.

11 Combining riboswitches with noncoding RNAs, either asRNAs or sRNAs,  
12 represents a novel mechanism of signal integration in bacteria and one which is  
13 not entirely unexpected considering the prevalence of both noncoding RNAs and  
14 riboswitches. In fact, the occurrence of many “marooned” riboswitches in  
15 bacterial genomes, which do not appear to be associated with obvious genes,  
16 suggests that riboswitch-regulated noncoding RNAs may be common. To date, all  
17 riboswitch-regulated noncoding RNAs have been identified in Firmicutes, and  
18 are regulated by either SAM or B<sub>12</sub>-binding riboswitches [19,20,24,25]. It will be  
19 interesting, to see if such regulatory molecules are more widespread across  
20 prokaryotes and are regulated by additional ligand-binding riboswitches.

21

## 22 **Riboswitches control access of proteins to RNAs**

23 Recently, studies have shown riboswitches can regulate the interaction of  
24 a nascent RNA with other proteins or protein complexes in addition RNA  
25 polymerase and the ribosome. In *Salmonella enterica*, an Mg<sup>2+</sup>-binding  
26 riboswitch in the 5' UTR of the *mgtA* gene regulates expression of a magnesium  
27 transporter by controlling transcription termination in response to magnesium.  
28 Interestingly, although the Mg<sup>2+</sup> binding aptamer was clearly identified, a  
29 potential intrinsic Rho-independent terminator was not found associated with  
30 the riboswitch, raising the question of how the riboswitch might induce Rho-  
31 independent transcription termination. Surprisingly, it was shown that the  
32 riboswitch, when bound to magnesium, promotes the interaction of the nascent  
33 RNA with the bacterial termination factor Rho (Fig. 3B) [27]. The interaction

1 with Rho was required for magnesium-dependent termination of *mgtA*  
2 transcription, and binding of the riboswitch to magnesium alone did not result in  
3 termination of *mgtA* transcription. This interaction with Rho was stimulated by  
4 the riboswitch stabilizing a RNA polymerase pause site in the *mgtA* leader, which  
5 appears to be conserved in other enterobacteria and suggests the mechanism is  
6 widely conserved [28]. Similarly, a flavin mononucleotide (FMN) binding  
7 riboswitch preceding the *ribB* gene in *E. coli* was shown to regulate *ribB*  
8 transcription by controlling access of Rho to the nascent transcript in response  
9 to FMN [27]. Many riboswitches lack obvious Rho-independent terminator  
10 sequences [27,29,30], suggesting that riboswitches mediating access of a  
11 transcript to Rho may prove to be a widespread mechanism of riboswitch-  
12 mediated regulation.

13 Riboswitches can also mediate interactions of a transcript with the  
14 ribonuclease RNaseE [31]. In *E. coli*, a lysine-binding riboswitch modulates  
15 access to the ribosome-binding site of the *lysC* transcript in response to lysine  
16 levels, thus controlling translation initiation. However, the lysine riboswitch  
17 simultaneously sequesters RNaseE cleavage sites in the *lysC* riboswitch when not  
18 bound to lysine (Fig. 3C). Upon lysine binding, these cleavage sites are exposed.  
19 Exposure of these sites leads to RNaseE-dependent degradation of the *lysC*  
20 transcript [32]. While the lysine riboswitch also controls translation of the *lysC*  
21 message in a classical manner, by mediating access to the ribosome-binding site,  
22 cleavage of *lysC* by RNaseE provides an irreversible regulatory outcome, that  
23 sequestration of the ribosome-binding site alone does not accomplish.  
24 Interestingly the aforementioned riboswitch-regulated *mgtA* transcript in *S.*  
25 *enterica* was also shown to be targeted for degradation by RNaseE in a Mg<sup>2+</sup>-  
26 dependent manner suggesting similar riboswitch mediated interactions with  
27 RNaseE [33]. Although no other instances of riboswitches mediating interactions  
28 with RNases are currently known, it is reasonable to think that such a  
29 mechanism could mediate interactions with other ribonucleases and would  
30 provide a means for riboswitches controlling translation initiation to ensure the  
31 finality of such a regulatory decision.

32

### 33 **Synthetic Riboswitches**

1           The utility of riboswitches to conditionally control gene expression has  
2 not escaped the attention of the biotechnology community. Significant effort has  
3 been made to develop synthetic riboswitches, which respond to ligands of choice  
4 and in turn control transcription, translation, splicing, or even the activity of  
5 regulatory RNAs (Reviewed in [34]). The vast majority of synthetic riboswitches  
6 have utilized aptamers from natural riboswitches, such as those for the purine-  
7 [35], thiamine pyrophosphate-binding [36] families *in vivo* and those of the SAM-,  
8 FMN- and lysine-binding families *in vitro* [35,37]. Significant work has also  
9 focused on the development of synthetic riboswitches using a small number of *in*  
10 *vitro* selected aptamers, which bind to molecules such as theophylline [38],  
11 tetracycline [39] and neomycin [40]. Theophylline and tetracycline-based  
12 synthetic riboswitches in particular, have proven to be robust conditional  
13 expression systems and have been deployed in a wide range of prokaryotes and  
14 eukaryotes where they have been used in diverse applications to both activate  
15 and inhibit transcription and translation [38,39,41-47], trigger ribozyme-  
16 mediated RNA cleavage reactions, [48-51] and mediate the interactions of  
17 regulatory RNAs with target RNAs [52-55]. The range of genes these  
18 riboswitches can control has been shown to be diverse, from simple reporter  
19 genes such as GFP and *lacZ*, to the expression of flagellar genes controlling  
20 cellular motility [56]. Importantly, conditional expression systems based on  
21 synthetic riboswitches have been proposed to have the advantage of being more  
22 easily engineered than protein based systems and to impose a low metabolic  
23 burden on the host organism. Additionally many of the first synthetic  
24 riboswitches appear to function well in both prokaryotes and eukaryotes,  
25 supporting the notion that synthetic riboswitches represent robust conditional  
26 expression systems. Nonetheless, despite the existence of *in vitro* selected  
27 aptamers to many different ligands, challenges remain in reliably coupling a  
28 given sensor domain to an expression domain of choice, and achieving a large  
29 dynamic range of output signals. As additional synthetic riboswitches are  
30 developed, we envision that the principles guiding synthetic riboswitch design  
31 will become clearer and synthetic riboswitches for many additional ligands will  
32 become more common.

33

## 1 **Riboswitch-based sensors**

2           The ability of aptamers to sense metabolites *in vivo* with high specificity  
3 has led many groups to propose the development of aptamer based sensors for  
4 the detection and study of metabolites in real-time [57-59]. One approach has  
5 been to combine a riboswitch with a reporter gene as in a recent study of B<sub>12</sub>  
6 metabolism and transport, where a B<sub>12</sub> riboswitch was fused to a  
7 betagalactosidase, luciferase or red fluorescent protein reporter gene [60].  
8 However, fusion of riboswitches to reporter genes is currently limited to the  
9 study of ligands for which there are naturally occurring riboswitches, or the few  
10 ligands for which synthetic riboswitches have been developed. A potentially  
11 more promising approach has been developed whereby two aptamers are  
12 combined in a single RNA sensor [61]. The sensor is comprised of two linked  
13 aptamers; one, which binds a ligand of interest, and one which binds a  
14 fluorophore (Fig. 4). The strategy utilizes an *in vitro* developed aptamer named  
15 Spinach, which binds a fluorophore 3,5-difluoro- 4-hydroxybenzylidene  
16 imidazolinone (DFHBI). When DFHBI is bound to the RNA aptamer it fluoresces  
17 whereas the fluorophore does not fluoresce when unbound to the RNA aptamer  
18 [62]. The Spinach aptamer stabilizes DFHBI and induces fluorescence in the  
19 same way the GFP beta barrel stabilizes the inner GFP fluorophore moiety and is  
20 required for GFP fluorescence. By linking a second aptamer to the Spinach  
21 aptamer via a small transducing hairpin essential to both aptamers (Fig. 4, red  
22 nucleotides), Paige and colleagues were able to design sensors where the  
23 Spinach aptamer bound to DFHBI only in the presence of the second ligand. In  
24 essence, this produced a fluorescence sensor for the second ligand. Such an  
25 approach was shown to be extendable to the design of sensors for adenosine,  
26 adenosine diphosphate (ADP), SAM, guanine, and guanosine 5'-triphosphate  
27 (GTP) [61], and a subsequent study has utilized the same strategy to create  
28 Spinach based sensors for cyclic-di-AMP and cyclic-di-GMP [63]. The potential to  
29 create genetically-encoded aptamer based sensors with the ability to monitor  
30 cellular metabolites *in vivo*, in real-time, has broad applications in the study of  
31 metabolism in both basic science and industrial domains.

## 32 **Concluding Remarks**

1 Riboswitches represent a versatile means of sensing and modulating gene  
2 expression in response to environmental cues. Not surprisingly nature has found  
3 uses for riboswitches in the regulation of noncoding RNA expression in addition  
4 to their more common role in controlling the expression of open reading frames.  
5 Similarly, the ability of riboswitches to mediate interactions of a nascent RNA  
6 with proteins has been exploited in unexpected ways to control interactions with  
7 proteins or protein complexes other than RNA polymerase and the ribosome. It  
8 is this same versatility that has caused riboswitches to be co-opted for  
9 engineering purposes in synthetic biology. As our understanding of riboswitches  
10 increases, we expect the roles of riboswitches, in both natural and applied  
11 environments to continue to expand.

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19

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1 **Figure 1. Regulation by riboswitch-regulated asRNAs. (A)** In high cysteine &  
2 low S-adenosylmethionine (SAM) conditions the T-Box riboswitch (dark blue)  
3 binds charged tRNA<sup>CYS</sup> resulting in termination of transcription of the *ubiGmccBA*  
4 operon. In low cysteine & low SAM conditions, the T-Box riboswitch binds  
5 uncharged tRNA<sup>CYS</sup> (red) resulting in formation of an antiterminator and enabling  
6 transcription of *ubiGmccBA*. However in low SAM conditions, transcription of an  
7 asRNA inhibits *ubiGmccBA* expression. Conversely, when cysteine concentrations  
8 are low but SAM concentrations are high, SAM (purple ball) binds the SAM  
9 riboswitch (purple) resulting in transcription termination of the asRNA and  
10 maximal *ubiGmccBA* expression. **(B)** An asRNA *aspocR* (green arrow) is encoded  
11 opposite the *pocR* gene (orange arrow). In low propanediol & low Vitamin B<sub>12</sub>  
12 (B<sub>12</sub>) conditions, *aspocR* is lowly expressed and *pocR* and the *pdu* genes are not  
13 expressed. In high propanediol & low B<sub>12</sub> conditions expression of both *pocR*  
14 (orange transcript) and *aspocR* (green transcript) is activated by propanediol,  
15 but transcription of *aspocR* inhibits *pocR* expression and subsequently *pdu* gene  
16 expression. In high propanediol & high B<sub>12</sub> conditions, B<sub>12</sub> binds the B<sub>12</sub>  
17 riboswitch (green) resulting in transcription termination of *aspocR* and maximal  
18 *pocR* and *pdu* gene expression.

19

20 **Figure 2. “Marooned” riboswitches.** Gene and riboswitch annotations were  
21 obtained from the NCBI RefSeq and Rfam databases for 834 bacterial genomes.  
22 The intervening distance between the 3' boundary of a riboswitch and an  
23 adjacent ORF was plotted. Each dot represents a single riboswitch. Boxes  
24 denote the interval containing all riboswitches within each class excluding the  
25 bottom and top 10% and black bars mark the average. Dotted horizontal lines  
26 denote 0 and 250 nt.

27

28 **Figure 3. Riboswitches control access to functional domains. (A)** In low  
29 ethanolamine & low B<sub>12</sub> conditions EutV proteins are monomers in the  
30 cytoplasm unable to bind RNA and *eut* genes are not expressed. In such low B<sub>12</sub>  
31 conditions the riboswitch (green box) permits transcription of a long isoform of  
32 the Rli55 or EutX sRNA, which contains a binding site (solid green balls) for the  
33 response regulator EutV. In high ethanolamine & low B<sub>12</sub> conditions, EutV is

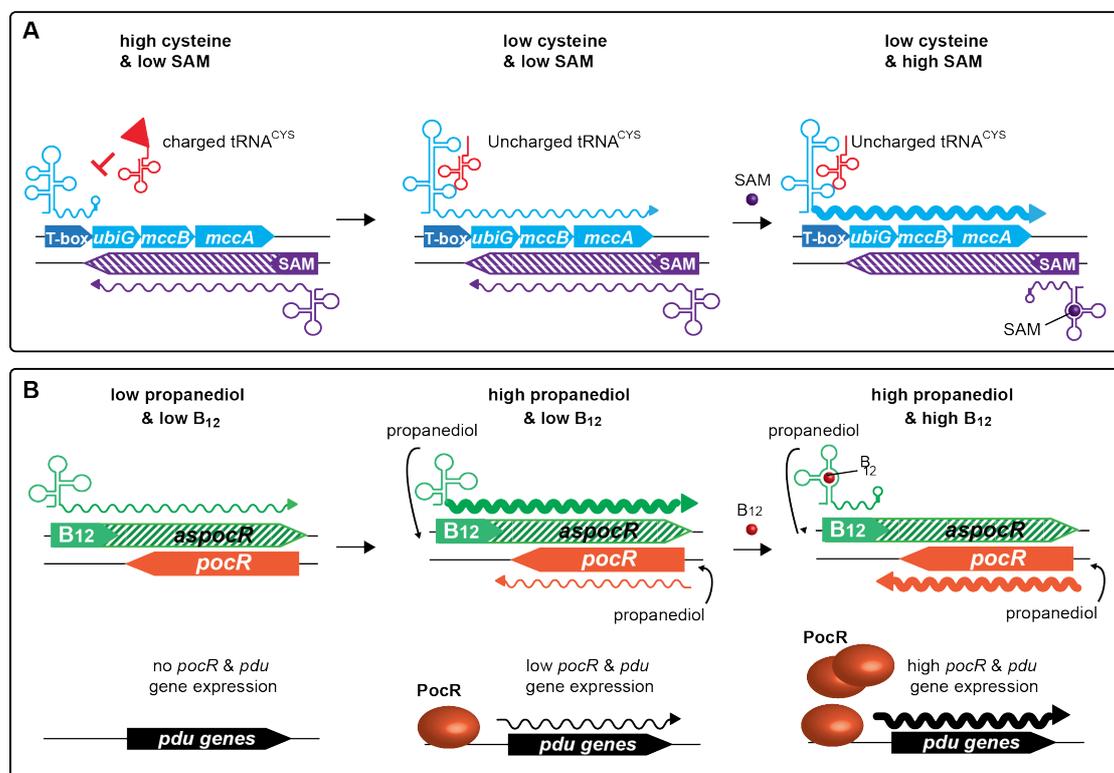
1 phosphorylated by EutW enabling it to bind RNA and making it competent to  
2 activate *eut* gene expression. However, production of the long isoform of the  
3 Rli55/EutX sRNA results in sequestration of EutV and no activation of *eut* gene  
4 expression. Conversely, in high ethanolamine & high B<sub>12</sub> conditions, B<sub>12</sub> (red ball)  
5 binds the B<sub>12</sub> riboswitch, producing a truncated Rli55/EutX transcript, which  
6 lacks a EutV binding site and thereby frees EutV to activate transcription of the  
7 *eut* genes by an antitermination mechanism. **(B)** In the presence Mg<sup>2+</sup> (blue ball)  
8 a magnesium riboswitch (blue) adopts a conformation exposing binding sites  
9 (orange) for the transcription termination factor Rho leading to transcription  
10 termination. In the absence of Mg<sup>2+</sup> the Rho binding sites are sequestered in the  
11 riboswitch resulting in transcription elongation. **(C)** In the presence of lysine  
12 (burgandy ball) a lysine riboswitch (orange) adopts a conformation exposing  
13 cleavage sites (purple) for the ribonuclease RNaseE, resulting in transcript  
14 degradation. In the absence of lysine, the RNaseE cleavage sites are sequestered  
15 in the riboswitch, preventing transcript degradation by RNaseE.

16

17 **Figure 4. Dual Aptamer-based sensors.** General design strategy of dual  
18 aptamer based sensors. Binding of a metabolite (star) by aptamer 1 (blue line)  
19 stabilizes a Spinach aptamer (green line) through a variable shared stem  
20 structure (red line), enabling it to bind to a flourophore DFHBI (green ball) and  
21 produce fluorescence. Sensors have been validated for the molecules shown.

22

Figure 1



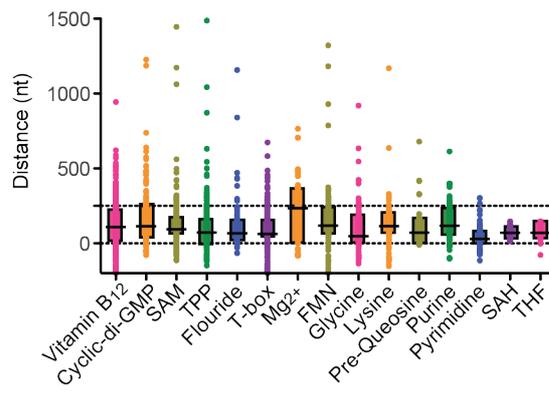
**Figure 2**

Figure 3

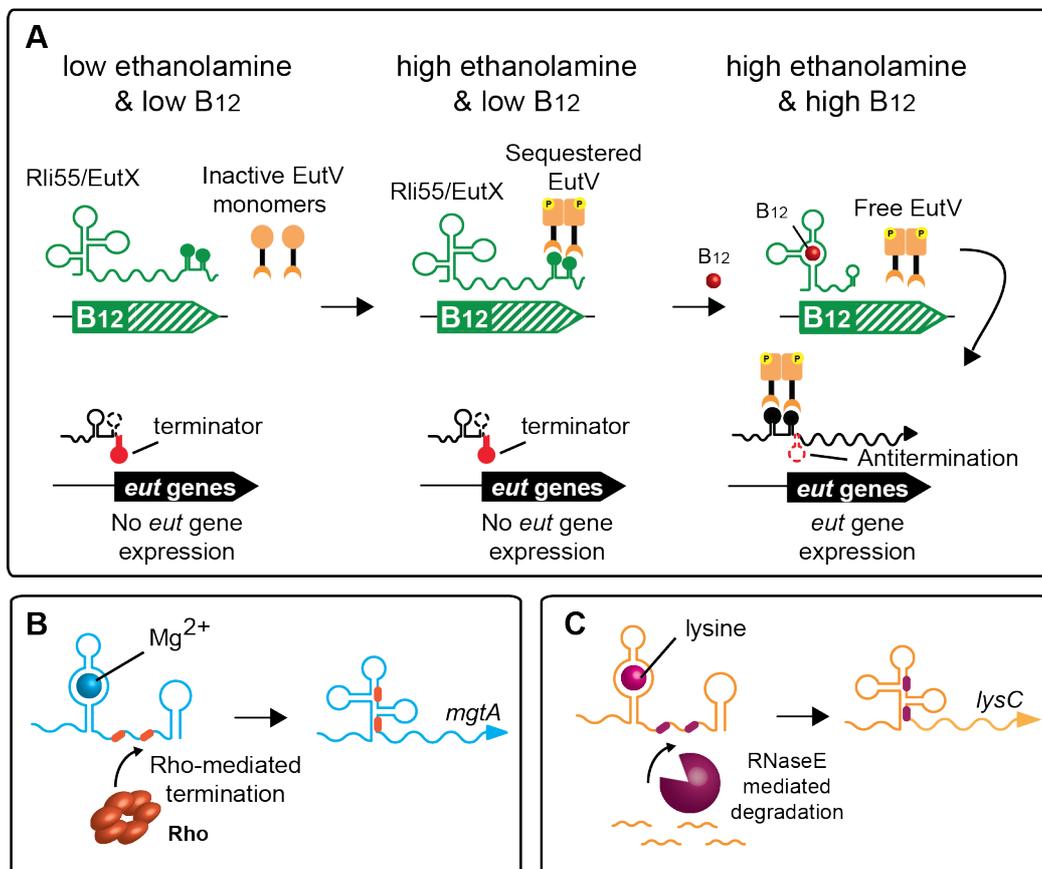


Figure 4

