Unexpected Versatility in Bacterial Riboswitches

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Abstract

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

Introduction

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

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

Riboswitches regulate the transcription of antisense RNAs

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

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

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

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

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

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

Riboswitches regulate the transcription of small RNAs (sRNAs) that sequester proteins

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

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

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

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

Riboswitches control access of proteins to RNAs

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

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

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

Synthetic Riboswitches

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

Riboswitch-based sensors

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

Concluding Remarks

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

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Figure 1. Regulation by riboswitch-regulated asRNAs. (A) In high cysteine & low S-adenosylmethionine (SAM) conditions the T-Box riboswitch (dark blue) binds charged $tRNA^{CYS}$ resulting in termination of transcription of the *ubiGmccBA* operon. In low cysteine & low SAM conditions, the T-Box riboswitch binds uncharged $tRNA^{CYS}$ (red) resulting in formation of an antiterminor and enabling transcription of *ubiGmccBA.* However in low SAM conditions, transcription of an asRNA inhibits *ubiGmccBA* expression. Conversely, when cysteine concentrations are low but SAM concentrations are high, SAM (purple ball) binds the SAM riboswitch (purple) resulting in transcription termination of the asRNA and maximal *ubiGmccBA* expression. **(B)** An asRNA *aspocR* (green arrow) is encoded opposite the *pocR* gene (orange arrow). In low propanediol & low Vitamin B_{12} (B_{12}) conditions, *aspocR* is lowly expressed and *pocR* and the *pdu* genes are not expressed. In high propanediol & low B_{12} conditions expression of both *pocR* (orange transcript) and *aspocR* (green transcript) is activated by propanediol, but transcription of *aspocR* inhibits *pocR* expression and subsequently *pdu* gene expression. In high propanediol & high B_{12} conditions, B_{12} binds the B_{12} riboswitch (green) resulting in transcription termination of *aspocR* and maximal *pocR* and *pdu* gene expression.

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

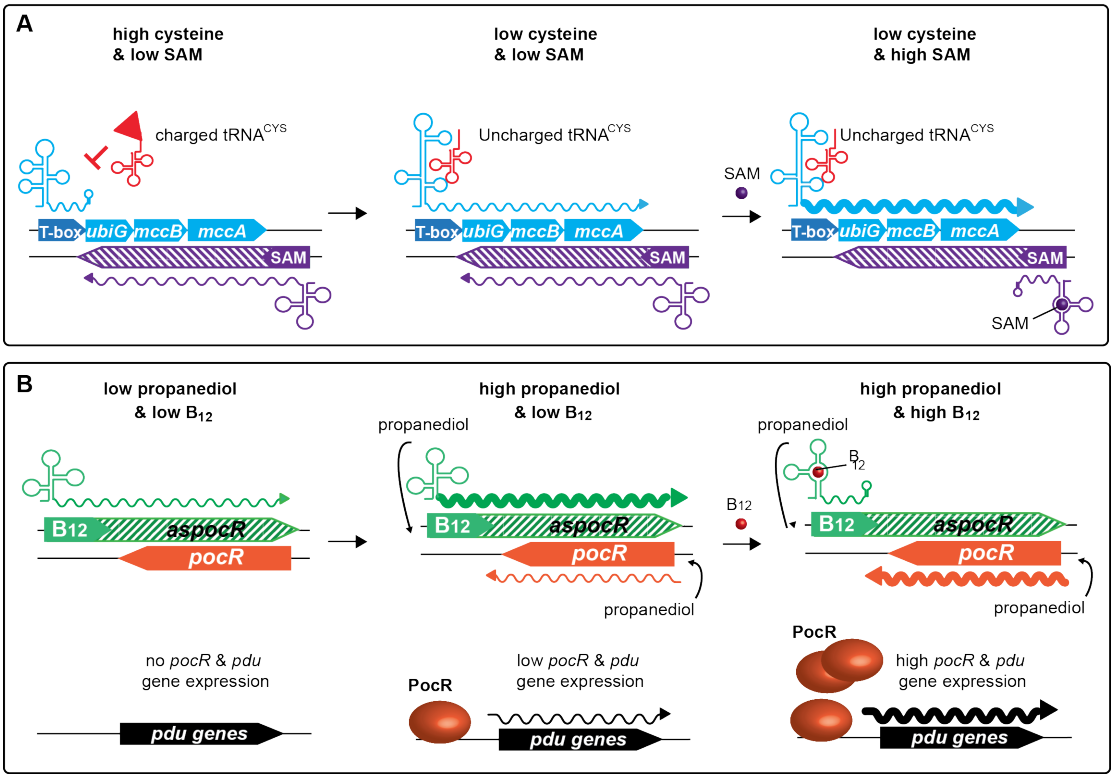
Figure 3. Riboswitches control access to functional domains. (A) In low ethanolamine & low B_{12} conditions EutV proteins are monomers in the cytoplasm unable to bind RNA and *eut* genes are not expressed. In such low B_{12} conditions the riboswitch (green box) permits transcription of a long isoform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

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

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

1

Figure 1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& high SAM
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Uncharged tRNA^CYS
Uncharged tRNA^CYS
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ubiG
mccB
mccA
SAM
SAM
B
low propanediol
& low B12
high propanediol
& low B12
high propanediol
& high B12
propanediol
propanediol
propanediol
B12
B12
aspocR
pocR
no pocR & pdu
gene expression
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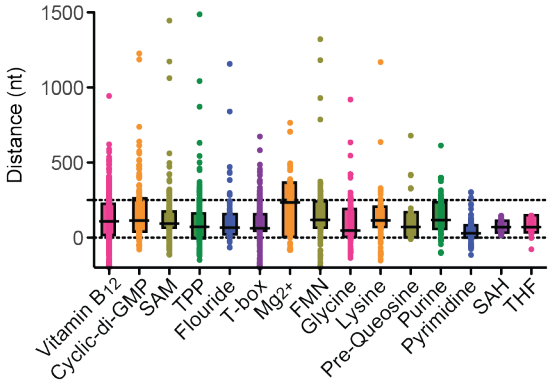
high pocR & pdu
gene expression
PocR
PocR
pdu genes
pdu genes
pdu genes

1

Figure 2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1000
500
0
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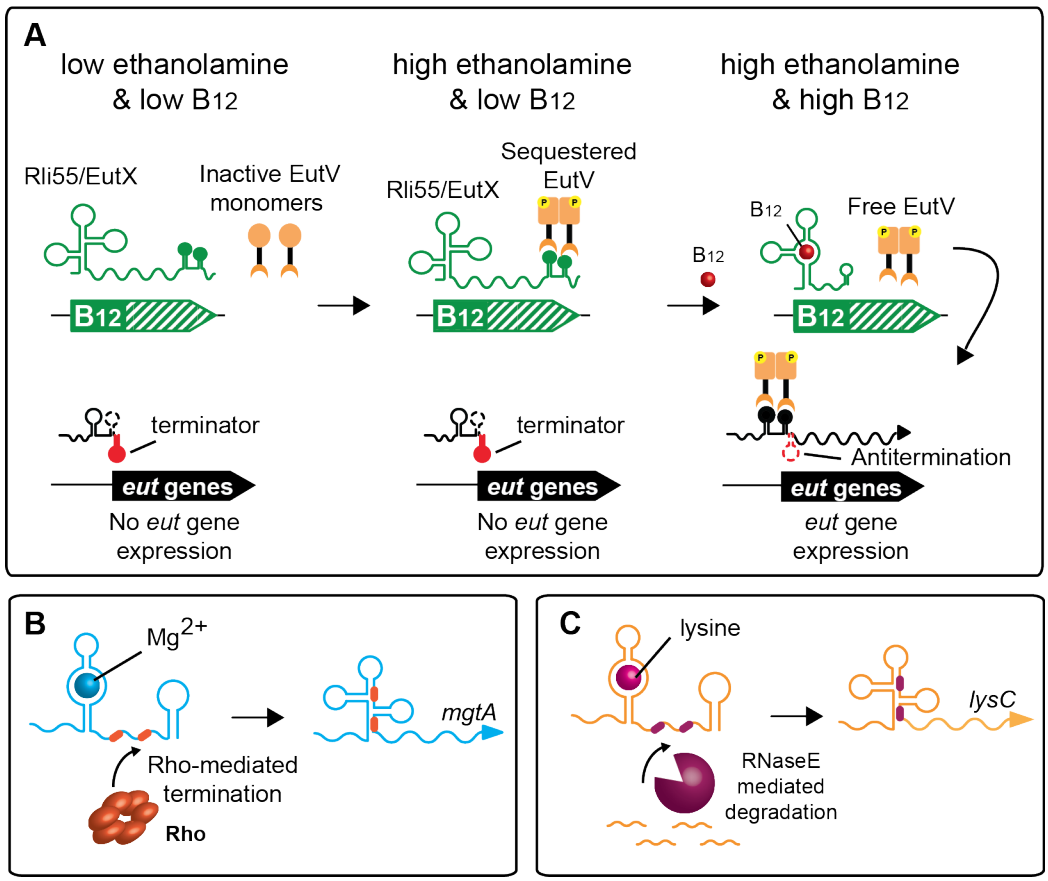
Cyclic-di-GMP
SAM
TPP
Flouride
T-box
Mg2+
FMN
Glycine
Lysine
Pre-Queosine
Purine
Pyrimidine
SAH
THF

1

Figure 3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high ethanolamine & low B12
high ethanolamine & high B12
Rli55/EutX
Inactive EutV monomers
Sequestered EutV
B12
Free EutV
terminator
eut genes
Antitermination
No eut gene expression
eut gene expression
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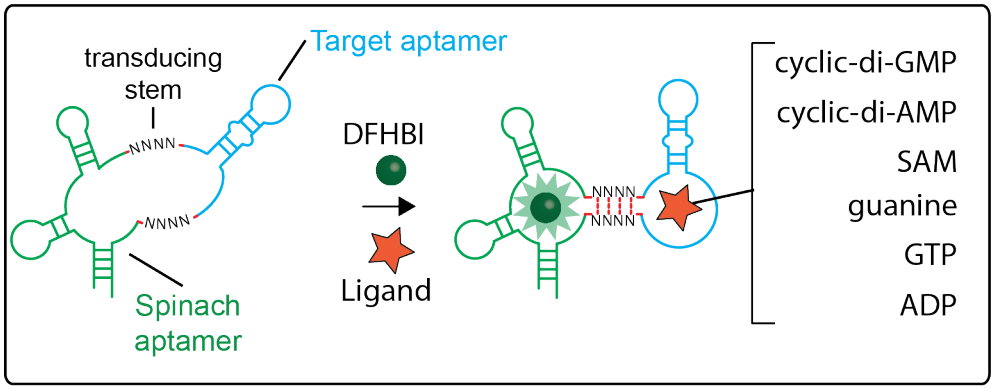
Mg2+
mgtA
Rho-mediated termination
Rho
C
lysine
lysC
RNaseE mediated degradation

1

Figure 4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cyclic-di-AMP
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guanine
GTP
ADP