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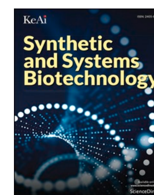

Review Article

Riboswitches, from cognition to transformation

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ABSTRACT

Riboswitches are functional RNA elements that regulate gene expression by directly detecting metabolites. Twenty years have passed since it was first discovered, researches on riboswitches are becoming increasingly standardized and refined, which could significantly promote people's cognition of RNA function as well. Here, we focus on some representative orphan riboswitches, enumerate the structural and functional transformation and artificial design of riboswitches including the coupling with ribozymes, hoping to attain a comprehensive understanding of riboswitch research.

1. Introduction

Ever since the Genetic Central Dogma came out, we've known what role does RNA play in the cellular world, while DNA carries the genetic information, protein performs specific biological functions, we had always thought that RNA was just the intermediate between DNA and protein, which existed as a communication tool for genetic information. Thoughts have been proved wrong as the multiple functions of RNA was found, earlier the discovery of RNA virus and ribozyme (catalytic RNA) [1] showed that RNA fulfilled the function of DNA and protein, which greatly increased the enthusiasm of scholars for RNA researches, more and more interesting functions of RNA were found including riboswitches.

In 2002, Ronald R. Breaker' team firstly discovered a special RNA structure in the 5′-untranslated region(5′-UTR) of the *btuB* (encoding cobalamin transport protein) gene in *Escherichia coli* was responded to adenosylcobalamin (AdoCbl, also called vitamin B12) [2] specifically, this binding leads to a structural change of the mRNA and thus controls downstream gene expression. At the same year, the RNA responded to thiamine pyrophosphate (TPP, derivative of vitamin B1) [3] and flavin mononucleotide (FMN, derivative of vitamin B2) [4] were found soon, these RNA sequences were called as riboswitches later, it declared that riboswitches had entered people's perspective formally.

Riboswitches are RNA elements mostly found in the 5′-UTR of bacterial mRNAs, where they sense concentration change of specific metabolites, usually called ligands, and regulate downstream gene expression in *cis* through tertiary structure changes of mRNAs, the controlled genes are always related to metabolic reaction of ligands [5]. The whole process does not involve protein to participate generally. However, exceptions are that we can find riboswitches exist in 3′-UTR of eukaryotic mRNAs [6], and sometimes riboswitches can act in *trans* [7,8] too.

Riboswitches are constituted of two structure domains: aptamer and gene expression platform, these two domains partially overlap each other. Aptamer is a highly conserved domain responsible for ligand binding, it can fold spontaneously and orderly into a unique tertiary structure soon after being transcribed, gene expression platform is a less conserved domain responsible for controlling gene expression. The binding between ligand and aptamer induces a conformational change which would be transmitted to expression platform, leading to the change of the gene expression levels and presenting gene inhibition or expression [3,9,10].

Riboswitch aptamers are one of the most highly conserved sequences among biological sequences, which embodies they only need four common nucleotides to form highly selective binding sites with ligand [11]. Aptamers' conserved features include primary structure nucleotide sequences [12], secondary structure such as base-paired stems and pseudoknots [13], super-secondary structure such as K-turn motif [14],

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special loop motif [15,16] and other structures. Plentiful riboswitches have been reported in bacteria, archaea, and eukaryotes to respond diverse molecules since its first discovery. Riboswitches are roughly divided into six categories according to the ligand which is sensed by the aptamer [11,17], the largest category is coenzymes, following are nucleotide derivatives, signaling molecules, ions, amino acids and other metabolites, most of them, you can tell, are RNA or RNA precursors derived compounds (Fig. 1). Recently, a representative and comprehensive map of riboswitch diversity and distribution was successfully drawn according to ligands [17], researchers have systematically analyzed the identified riboswitch classes using computational algorithms to generate refined consensus sequence and structural models, the phylogenetic distributions of each class were established subsequently. Furthermore, Infernal [18] and RNAMotif [19] were employed to perform homology searches to find more unknown riboswitches. The TPP, cobalamin and FMN riboswitches rank the top three positions based on the statistical results.

In bacteria the regulation of gene expression based on riboswitches is mainly accomplished through two mechanisms, termination of transcription and prevention of translation [20]. In the case of control at the transcriptional level, the binding of ligand affects the formation of terminator or anti-terminator structure downstream, mostly an intrinsic terminator works enough [4,21], but some riboswitches can utilize Rho-dependent terminators and require assistance of Rho factor protein to terminate transcription efficiently [22,23], this discovery reveals that protein may also be involved in the riboswitches. In the other case of control at the translational level, the binding of ligand affects the ribosome binding site (RBS) releasing or sequestering [4], so that the access and recognition of ribosome would be influenced (Fig. 2a). Some complex riboswitches such as *ribB* FMN riboswitch in *E. coli* was proved can operate at both transcriptional and translational levels [24]. Two rare and novel mechanisms besides above two were found with the deepening of researches, one is metabolite-induced self-cleaving ribozyme represented by glucosamine-6-phosphate (GlcN6P) riboswitches [25], located at 5′-UTR of *glmS* mRNA, which can be activated in the presence of GlcN6P (the metabolic product of GlmS enzyme) and cleaves itself. The other one is *trans*-acting inhibitory riboswitches [7,8], a *trans*-acting small RNA called *eutX* contains an AdoCbl riboswitch to regulate genes expression of ethanolamine utilization in *Enterococcus faecalis* (Fig. 2b). Different from prokaryotes, TPP riboswitch is the only riboswitch found in eukaryotes. In fungi such as *Neurospora crassa* [26] and algae such as *Chlamydomonas reinhardtii* [27], TPP riboswitch locates in the intron of relevant gene, and in plants such as *Arabidopsis thaliana* it locates upstream of polyA tail in 3′-UTR of mRNA [26], implying that the riboswitch is involved in mRNA splicing and processing [26,28]. In general, most of riboswitches regulate gene expression of enzymes and transport protein of metabolites through negative feedback control [20,29].

2. The diversity of riboswitches

Coenzymes are the most enormous family of all ligands, owning the largest number of ligands and the number of distributions, statistics shows that the five most common ligands are all coenzymes [17]. Reviews about them are massive [9,11,17,20,30,31] so below we focus on those who are less concluded or been called orphan riboswitches for a long time.

2.1. Coenzymes riboswitches

In the big family of coenzymes, nicotinamide adenine dinucleotide (NAD^+) is an extreme vital and ubiquitous existence, but the riboswitch responds to it is elusive, recently the *nadA* motif was finally been proved to bind NAD^+ [32], surprisingly the structural analysis showed that the natural *nadA* motif contained a tandem aptamer domain with high degree of similarity, the first aptamer was observed to bind the adenosine 5′-diphosphate (5′-ADP) moiety of NAD^+ while the second was not, the structure of binding was also revealed [33]. Besides, Aiming Ren verified that the second aptamer binds NAD^+ in a apparently higher concentration compared to aptamer 1 [34], while the apparent dissociation constant (K_d) of ADP and aptamer 1 or aptamer 2 were 94 μM and 3370 μM respectively, which displayed a novel putative mechanism that the *nadA* NAD^+ riboswitch aptamer 1 could be activated at low NAD^+ concentration but trigger translational repression at high NAD^+ concentration eventually. However, the author remained suspicion that it may have a stronger ligand in consideration of *ydaO* motif talked about later [35,36].

Tetrahydrofolate (THF) is a coenzyme of one-carbon metabolism. The THF riboswitches are tending to become active since the discovery of a second class of THF riboswitches (THF-II). Previous studies have shown that the first class of THF riboswitches (THF-I) possesses the ability to bind two THF molecules with a single aptamer at different sensitivity levels, structural analysis reveals that a three-way junction and a pseudoknot participate the recognition of the reduced pterin moiety [37]. The THF-II riboswitch was firstly reported four years ago, and it mostly exists in Gram-negative *Rhizobial* bacteria while THF-I mainly exists in Gram-positive bacteria [38]. The THF-II riboswitch was demonstrated to undergo only local conformational changes after ligand binding, which provides a unique viewpoint on the regulatory mechanism of riboswitches [39,40].

Molybdenum cofactor (Moco) is a tricyclic pyranopterin containing a molybdenum atom, and is widely applied in key redox reactions in the metabolic cycles. The *moaA* motif was identified as Moco riboswitch in 2008 [41], however, few studies have been reported due to its highly oxygen sensitivity. The latest researches have revealed specific Mg^{2+}-binding pockets, indicating that the phosphate group of Moco is involved in ligand recognition [42]. Surprisingly, the absence of P3 element in Moco riboswitch causes it to bind Tungsten cofactor (Wco), which is almost identical in structure and function to Moco [9,41].

2.2. Nucleotide derivatives riboswitches

Nucleotide derivatives ligands includes theophylline, guanine, adenine, preQ1 and lately found xanthine etc. Barry Polisky has already identified a specific short oligonucleotide which showed great affinity to theophylline through SELEX (Systematic Evolution of Ligands by Exponential Enrichment) back in 1994 [43], today we know it is theophylline aptamer, which has been shown to have a 10000-fold stronger affinity for theophylline than caffeine, which only adds a methyl group to the theophylline chemical structure. The conserved RNA motif G box upstream of the *xpt-pbuX* operon in *Bacillus subtilis* was identified to be guanine riboswitch [44], it discriminates against adenine by 60000-fold. Soon after that they found adenine riboswitch which recognized adenine and discriminated against guanine by over 30-fold [21], however, it merely carries a C→U mutation in the core of the aptamer conserved sequence, implying that this residue is involved in ligand recognition. As can be seen from the above two specific examples, one group difference in ligand or one nucleotide difference in aptamer could cause huge functional divergence of riboswitches, this result greatly shows the high precision of riboswitches. Prequeuosine-1 (preQ-1) is an intermediate metabolite of rare nucleoside queuosine [45], the preQ-1 riboswitch was modified and verified successfully to shut down gene expression by induction in *Mycobacterium smegmatis* [46]. Researches are also developed on transcription-translation coupling mechanism using preQ-1 riboswitch [47], hoping to provide new inspiration to antibiotics design. An orphan riboswitch candidate previously reported named *NMT1* motif [48] was identified to bind xanthine and uric acid instead of TPP related metabolites [49], it recognized ligand through Mg^{2+} mediated and was used to detect oxidized purines intracellularly [50].

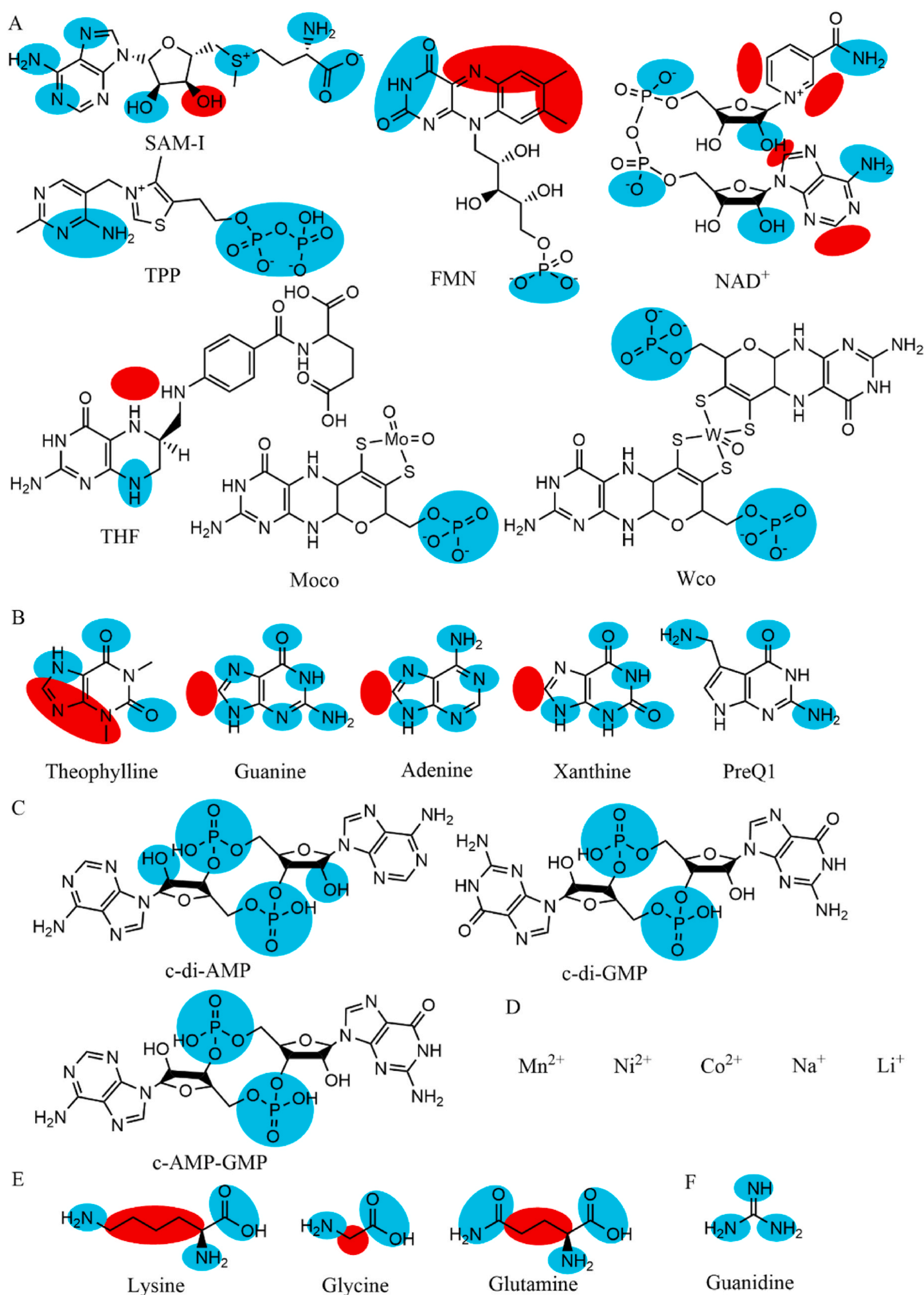

Fig. 1. Structure of ligands involved in this review. (A) coenzymes (B) nucleotide derivatives (C) signaling molecules (D) ions (E) amino acids (F) others. Colored shadow area represents proposed interaction sites with aptamers based on analysis of ligand analogues, red represents van der Waals force sites or steric clash, blue represents hydrogen bond or metal coordination sites. The coenzymes, nucleotide derivatives and signaling molecules could be regarded as RNA or RNA precursors derivatives. Information sources, SAM [146], TPP [3], FMN [144], NAD^+ [32], THF [38], Moco [42], Wco [42], theophylline [43], guanine [44], adenine [21], xanthine [49], preQ1 [45], c-di-AMP [36], c-di-GMP [54], c-AMP-GMP [56], lysine [64], glycine [67], glutamine [70], guanidine [72].

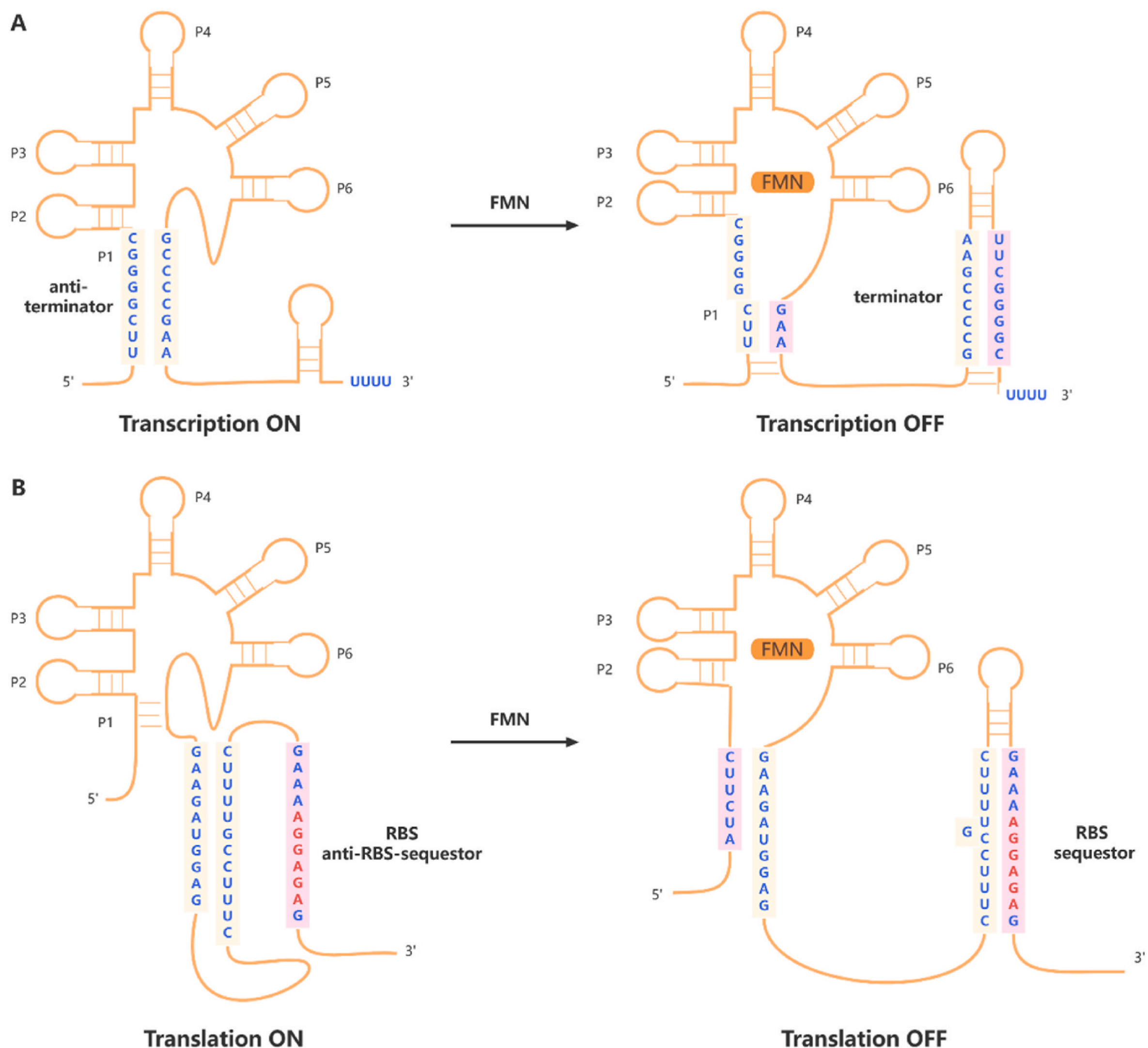

Fig. 2a. Two main regulatory mechanisms of riboswitches. (A) Transcriptional level, *ribD* FMN riboswitch of *Bacillus subtilis* [4], (B) Translational level, *ypaA* FMN riboswitch of *B. subtilis* [4]. Blue sequence in beige box represents anti-terminator sequence or anti-RBS-sequestor sequence, in pale pink box represents sequence paired with anti-terminator sequence or anti-RBS-sequestor sequence, red sequence represents RBS. Riboswitches regulate gene expression through influencing terminator or RBS structure when bound to ligands.

2.3. Signaling molecules riboswitches

As early as in 1993, Jack W. Szostak already found an RNA aptamer that bound ATP through selection from random RNA pools on a solid support *in vitro* [51], however, the *ydaO* motif was regarded as riboswitch candidate in 2010 [52] and was not identified to bind ATP until 2012 [35], when Martha J Fedor developed an alternative and efficient method to select native ATP aptamer from candidates of *B. subtilis*, which utilized the principal that the binding between purine and purine aptamers was more easily crosslinked through ultraviolet (UV) irradiation and became more stable, easier to analyze. Intriguingly, the *ydaO* motif has been elucidated to have a 1-million-fold stronger affinity to second messenger cyclic di-adenosine monophosphate (c-di-AMP) than ATP [36], it was proved that the c-di-AMP bound the *ydaO* riboswitch in two pseudo-symmetry-related pockets later soon [53].

Another second messenger cyclic di-guanosine monophosphate (c-di-GMP) also can bind the *GEMM* motif to regulate gene expression in pathogenic bacterium *Vibrio cholerae* [54], moreover, this binding is nearly 800-fold tighter than its binding with an *E. coli* c-di-GMP receptor PilZ protein [55]. Unexpectedly, the *GEMM-I* aptamer found in *Geobacter metallireducens* has an over 1000-fold selectivity for cyclic AMP-GMP over c-di-GMP [56]. To researchers' great delight, a natural triple-tandem c-di-GMP riboswitch was characterized in *Bacillus thuringiensis* to control gene expression in a more strict and digital way [57], it was also the first report on triple-tandem riboswitch structure ever, and was employed to verify putative diguanylate cyclase (DGCs) using a dual-fluorescence reporter.

2.4. Ions riboswitches

A widely distributed riboswitch candidate known as *yybP-ykoY* motif was confirmed as Mn^{2+} riboswitch controlling Mn^{2+} regulatory protein and transporter protein [58,59], the aptamer has two metal binding sites, one can recognize Mg^{2+} or Mn^{2+} while the other one strongly tends to bind Mn^{2+}. The same year another highly selective riboswitch for metal ions named CoNi riboswitch was found to maintain transition metal ions homeostasis intracellularly [60], whose high selectivity reflects in recognizing Co^{2+} and Ni^{2+} cooperatively and discriminating other metal ions. It is not similar with Mg^{2+} riboswitch which can also bind other divalent metal ions such as Ca^{2+}, Mn^{2+} [61].

Plenty of riboswitches in response to transition metal cations have been reported. In contrast, riboswitches which sense alkali metal cations

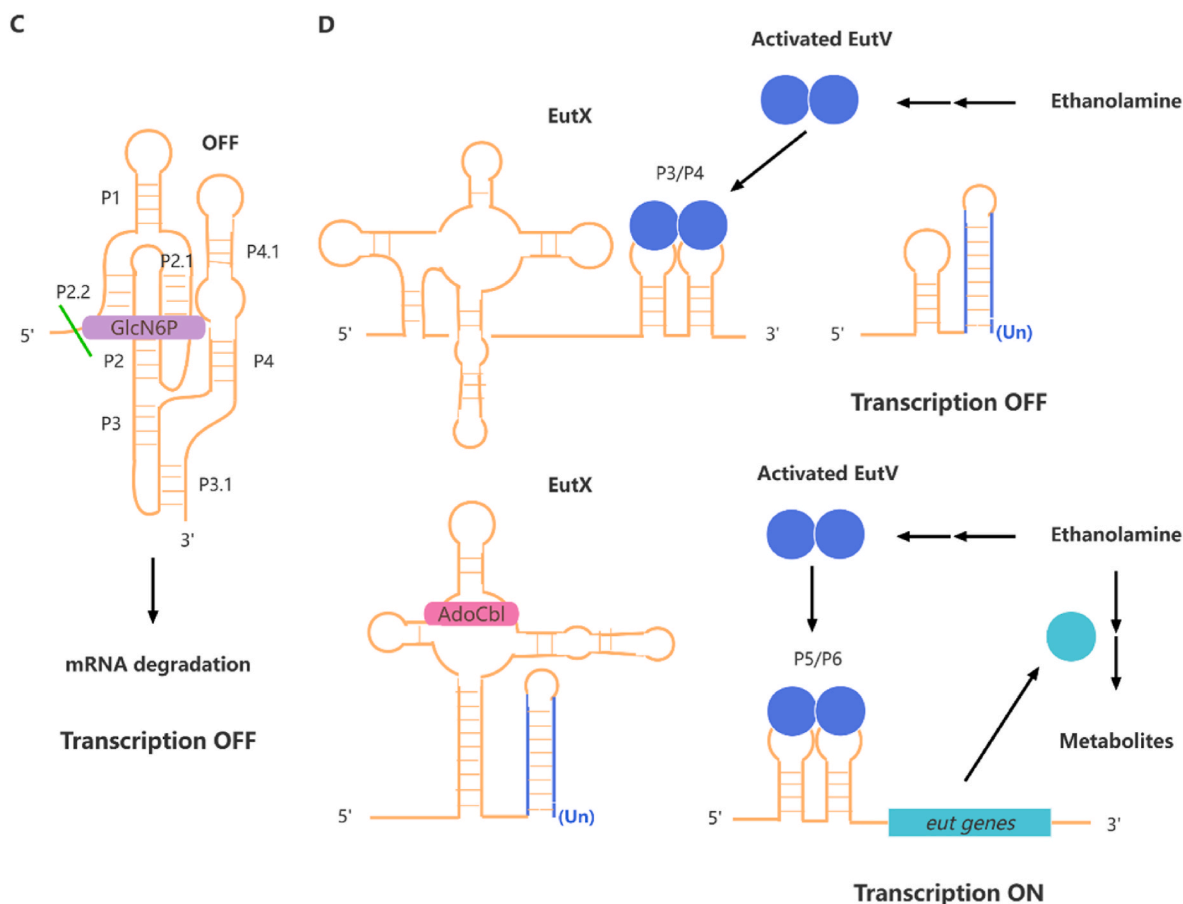

Fig. 2b. Two rare regulatory mechanisms of riboswitches. (C) *glmS* ribozyme (GlcN6P riboswitch) of *B. subtilis* [147], grass green line represents self-cleaving site of ribozyme. *glmS* ribozyme performs self-cleaving when bound to GlcN6P, causing mRNA degradation and transcription termination. (D) AdoCbl-riboswitch-containing regulatory RNA EutX of *Enterococcus faecalis* [7,8], blue line represents terminator stem sequence. The *eut* genes is only expressed when ethanolamine and AdoCbl are present simultaneously, AdoCbl riboswitch acts as part of *trans*-acting regulatory RNA EutX.

like Na^+ and K^+ are rarely discovered, although both ions exist in all cells at higher levels. This gap was not filled until the DUF1646 motif was confirmed as Na^+ riboswitch last year [62]. Most of the genes regulated by Na^+ riboswitch were noted as metal ion transporters. Interestingly, Na^+ riboswitch could form tandem structure with c-di-AMP riboswitch to regulate gene expression during osmotic stress. Almost at the same time, the *nahA*-I and *nahA*-II motifs were confirmed as Li^+ riboswitches, although Li^+ is far less abundant than Na^+ and K^+, the Li^+ riboswitch reveals the only known mechanism by which bacteria sense and respond to high concentration of toxic Li^+ [63].

2.5. Amino acids riboswitches

At present, only three kinds of amino acids ligands have been found. The lysine riboswitch is the first non-nucleotide metabolites riboswitch discovered, suggesting that RNA aptamer has the ability to recognize linear small molecular metabolites too [29,64]. It owns one of the most enormous structures demonstrated clearly [65,66]. Notably, the *lysC* lysine riboswitch in *E. coli* behaves an extra regulatory mode except prevention of translation [66], the binding of lysine would expose two RNase E cleavage sites (5′-UCUUCC-3′) in the expression platform to be targeted at RNase E recognition and accelerate mRNA degradation, conspicuously decreasing mRNA level. The second riboswitch glycine riboswitch located in 5′-UTR of *gcvT* operon (encoding required enzymes of glycine cleavage system) is a naturally rare ON type riboswitch containing a tandem aptamer structure [67], each aptamer could bind glycine and this binding is cooperative, ensuring its sensitivity to initiate gene expression in a narrow range of glycine concentration [68]. Researches have shown that the aptamer closer to the gene expression platform is more influential on downstream gene regulation [69]. Glutamine riboswitch is the third amino acids riboswitch, it only exists in *cyanobacteria* and marine metagenomic sequences to regulate nitrogen metabolism at present [70], interestingly, natural *glnA* glutamine riboswitches often form in tandem even triple structures. The binding between *glnA* aptamer and glutamine is less more tightly compared with other riboswitches while its K_d is up to 575 μM [4,44], but highly selective. A brand-new mechanism was proposed that the high selectivity was maintained in spite of low affinity through specific conformation for glutamine recognition at the expense of energy [71], corresponding the high intracellular concentration of glutamine naturally.

2.6. Other riboswitches

Guanidine has not always been regarded as a mainstream metabolite for a long time though its derivatives such as arginine is widely spread intracellularly. A skeptical RNA motif named *ykkC* motif without characterization across a long time was finally validated to regulate gene expression through binding guanidine [72], indicating that bacteria can synthesize, sense and respond to guanidine. It was thought once that there were three kinds of guanidine riboswitches found based on different binding pockets [73]. But interestingly, variants of *ykkC* motif were subdivided into at least 5 different types responded to guanosine tetraphosphate (ppGpp), phosphoribosyl pyrophosphate (PRPP) and nucleoside diphosphates [74] etc.

3. Structural and functional transformation

3.1. Functional reversal, dual selection based on tetA gene

The vast majority of natural riboswitches are OFF type riboswitches which means they inhibit gene expression when bound to ligands [4,66]. Nevertheless, it' s commonly required that genetic elements can activate gene expression when bound to ligands in researches, so here comes the problem that how to reverse natural OFF type riboswitches to needed ON type riboswitches? Justin P. Gallivan put forward a method concerning with randomizing the linker between aptamer and RBS region to construct screening library [75], through blue-white screening, superior riboswitches with higher activation ratios (ARs) were selected. The same year Yohei Yokobayashi firstly used a tetracycline resistance gene *tetA* as a positive and negative screening marker, the resistance to tetracycline acts as a positive marker, and its sensitivity to Ni^{2+}, Cd^{2+} which could kill the cells acts as a negative marker [76]. He successfully selected several TPP ON type riboswitches through repeated positive and negative screening from riboswitch mutant libraries [77,78], each screening could be regarded as an enrichment of targets.

This screening method with high efficiency was employed in many fields, such as screening of mutant enzymes. An example is superior mutant chimeric aspartate kinase BT screening with lysine riboswitch from evolutional strains [79], the best selected mutant showed 160% increased activity *in vitro* compared with wild-type enzyme, increased lysine production from 0 to 268 mg/L. Another example is evolution screening of alanine-glyoxylate aminotransferase with glycine riboswitch [80], they obtained mutant enzymes which increase the enzyme activity by 58% and 73%, besides, all applied to improve 5-aminolevulinic acid (5-ALA) titer effectively. Secondly is used in metabolic engineering regulation such as construction of lysine ON type riboswitch which was applied to enhance *lysE* (encoding lysine transport protein) gene expression, achieved a 21% increase in the yield of lysine [81], construction of glycine OFF type riboswitch which repressed gene expression up to 10.2-fold and was applied to downregulate *hemB* (encoding 5-ALA dehydratase) gene expression to improve 5-ALA titer by 11% [69], or even used to identify the best insertion site in genome for target genes through growth monitoring [82]. From above examples we can see its powerful function.

3.2. Functional reversal, Automated design

The above method involves repetitive screening which is time-consuming and labor-consuming, therefore several superior approaches using fully automated design were exploited and tested. The first declaimed method regards translational regulation process as a biophysical model analyzed by means of dynamics and thermodynamics [83]. Input aptamer, ligand-binding structure and output protein' s coding sequence, the algorithm would output the optimal pre- and post-aptamer sequence. Using this algorithm they successfully designed 62 synthetic riboswitches based on 6 RNA aptamers, including aptamers which respond to 3 non-ideal ligands dopamine, thyroxine and 2,4-dinitrotoluene, the tested best sample achieved a 383-fold activation of gene expression. It is capable to convert any RNA aptamers into synthetic riboswitches, making a big step in biosensors based on riboswitches.

Another novel tool called RiboLogic was characterized too [84], It requires an aptamer segment and design constraints as input, uses binding of MS2 RNA hairpin segment to a fluorescently tagged MS2 viral coat protein as an output. The computer would optimize the adjacent sequence of aptamer and MS2 RNA hairpin to simulate the binding with ligand and calculate scores, return the qualified sequence at last. The output RNA sequences were performed RNA-MaP (RNA on a massively parallel array) owing to its great amount. The tested riboswitches own ARs of up to 20, which provides a new design inspiration for riboswitch reformation. Nowadays many algorithms, websites and software are developed to predict and simulate RNA higher structure and the binding with ligands, providing great convenience to biological researches [85, 86].

3.3. Cascade structure

Considering some riboswitches have low multiple responsiveness and undetectable signal output, a 2-aminopurine (2-AP) riboswitch was coupled with quorum sensing system [87], the 2-AP riboswitch activates *rhlI* (encoding acyl homoserine lactone synthetase, AHL synthetase) gene expression to synthesize signal molecules AHL of quorum sensing system when bound to ligand, AHL binds cognate transcription factor RhlR and together initiate reporter gene expression with Rhl promoter. The signal was amplifying conspicuously through cascade system, the AR was lifted from 3.7 to 15.5. Similarly, the strategy was adopted merely with a special exogenous SP6 RNA polymerase and SP6 promoter instead of AHL synthetase and Rhl promoter [88], increasing AR by 4-fold.

Another innovative signal amplification approach was established by controlling plasmid copy number [89]. Two theophylline ON type riboswitches controlled *nluc* (encoding NanoLuc luciferase) and *repL* gene expression located in the same plasmid, *repL* encodes a phage-derived replication protein which could promote plasmid replication. The AR of cascade system reached up to 3900, displaying huge success (Fig. 3).

3.4. Tandem structure

In general cognition, riboswitch functions depending on an aptamer to identify ligand and a gene expression platform to regulate gene expression, whereas the discovery of natural tandem glycine riboswitch [67] prominently enriched the form and complexity of riboswitch regulation, the structure that two aptamers binding to the same ligand cooperatively could upgrade the sensitivity and performance of riboswitch. Subsequently the second tandem riboswitch was found could bind two ligands, SAM and AdoCbl with two separated gene expression platform located in 5′-UTR of *metE* mRNA in *Bacillus clausii* [90], the whole riboswitch works similar to a Boolean NOR logic gate which means each ligand binding could inhibit gene expression, plus, their binding does not impact each other. The regulation mechanism is described in Fig. 4b, the SAM synthetic pathway involves three enzymes encoded by *metE*, *metH* and *metK* gene, which SAM has consistent negative feedback inhibition on, the *metE* and *metH* encode a group of isoenzymes while MetH needs coenzyme methylcobalamin (MeCbl, works as AdoCbl), the high concentration of AdoCbl represses *metE* gene expression so that MetH could catalyze reaction more effectively. Differing from the tandem glycine riboswitch, the third found TPP tandem riboswitch in *Bacillus anthracis* has two intact riboswitch units which can bind and respond to TPP independently [91]. Recently researchers found another natural tandem guanine and phosphoribosyl pyrophosphate (PRPP) riboswitch works as a Boolean IMPLY logic gate in *Bacillus megaterium* [92]. And the tandem NAD^+ riboswitch is described before (Fig. 4a).

The natural tandem riboswitches' discoveries inspire researchers' enthusiasm on synthetic complex riboswitches. In fact, as early as in 2001, a FMN aptamer and theophylline aptamer were attached to a hammerhead ribozyme through modular rational design [93]. Yohei Yokobayashi used dual selection based on *tetA* gene to separate two synthetic tandem riboswitches containing theophylline and TPP aptamers function as Boolean logics AND and NAND gates [94], Mario Morl developed a computational design method on the basis of secondary structure and folding prediction to functionalize tetracycline and streptomycin riboswitches [95], he combined a tetracycline riboswitch with a theophylline riboswitch into a Boolean logic AND gate with 10.4-fold and 7.7-fold intact AR (Fig. 4a).

A tandem strategy with band-pass effect deserves attention, it was original came up with a combination of TPP ON type and TPP OFF type

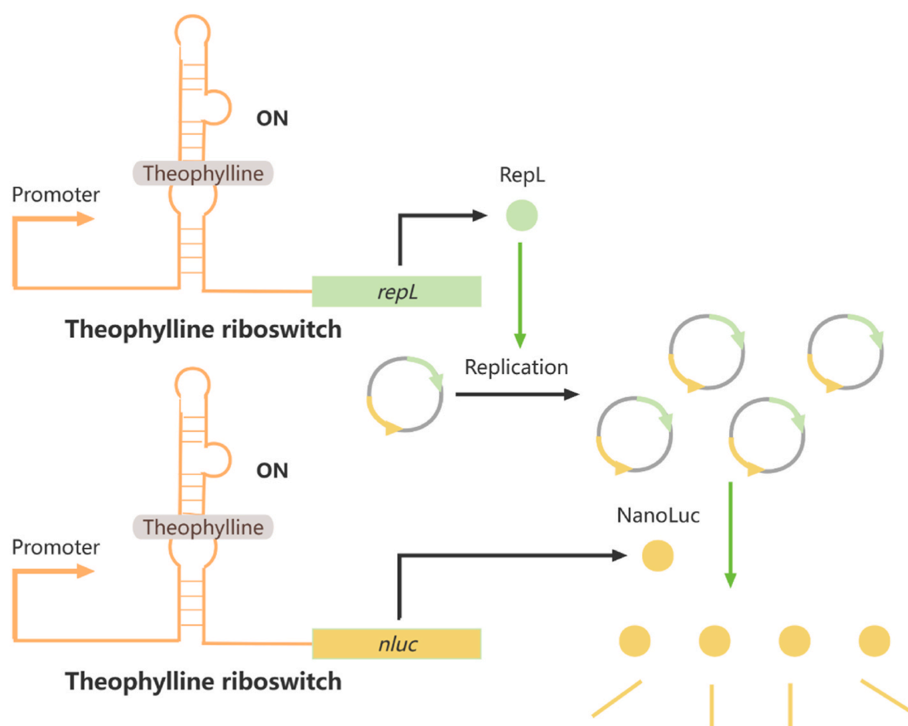

Fig. 3. Cascade structure of riboswitches. Theophylline riboswitch controlling signal amplification in *Escherichia coli* [89]. The *repL* gene encodes a plasmid replication protein RepL, *nluC* encodes NanoLuc luciferase, the activation ratio (AR) of this system achieves up to 3900.

riboswitches [96], making the system only responds to TPP in a suitable concentration range while silences in higher or lower concentration. Another method chose to split reporter green fluorescent protein (GFP) into two fragments [97], one was attached to maltose binding protein (MBP), the protein complementation of two GFP fragments could make cells fluorescence regulated by two opposite TPP riboswitches, this structure was seemed as a differential Boolean logic AND gate.

3.5. Other structural transformation

Most of riboswitches are *cis*-acting gene regulatory elements, but they can be redesigned to perform *trans*-acting regulation through rational design. In a specific example, a theophylline aptamer was linked to an antisense stem and an aptamer stem, once the aptamer binds the ligand, the formation of aptamer stem compels the antisense stem to become a single strand which pairs with the region near the start code of regulated gene mRNA and hinders translation [98].

The secondary structure of riboswitches folding is essential for regulatory function. Some researches focused on the intrinsic terminator structures of transcriptional controlling riboswitches. For example, a theophylline aptamer was linked to a computer-designed spacer sequence followed by an aptamer-complementary sequence [99], the partial sequence of aptamer and complementary sequence form the terminator while spacer serves as loop area, a series of synthetic riboswitches with differentiated sensitivities and responsiveness were constructed, declaring success of the spacer design strategy based on computer design. Researchers even concluded two influential parameters, terminator hairpin stability and folding traps, and gave a regular procedure [100], other design principles were also come up with from dynamics perspective [101],

For translational controlling riboswitches, the RBS sequestor structures were also paid attention to, even 1 nucleotide slide with RBS region matching could cause gene expression changes and was validated in construction of FMN-aptamer allosteric ribozymes [102]. According to the principle, a kindred riboswitch was constituted of a theophylline aptamer and a communication module which executed helix slipping [103], the communication module was inserted next to RBS to produce 1 nucleotide shift of critical distance for ribosome binding through helix slipping. It turned out the first synthetic riboswitches using slipping mechanism. Moreover, evidence have shown that the RBS complementary to ribosome and the spacing between RBS and start codon also influence gene expression [104], the ARs of modified riboswitches ranged from almost 0 to 450-fold through induction time and dose optimization.

In addition to mutant screening *in vivo*, a kinetic trapping mechanism was proposed during transcription of theophylline riboswitch which showed the potential of screening for novel synthetic riboswitches *in vitro* [105], the greatest advantage of screening *in vitro* is that it relieves the limitation of extensive libraries screening in bacterial transformation. It is worth celebrating that screening in cell-free protein expression systems (CFPS) eventually realized six years later [106], researchers selected two kinds of CFPS, extract of *E. coli* made by themselves and commercial PURE CFPS, displaying a similar and feasible result, signing initial success of screening *in vitro*.

4. Regulation of riboswitches coupled with ribozymes

Ribozymes are small RNA molecular with catalytic power firstly found in *tetrahymena* by Thomas R. Cech [1], this discovery changed the biologists' understanding of RNA function. At present ribozymes are mainly divided into two major categories, splicing ribozymes and cleaving ribozymes [107], which can be subdivided into hammerhead ribozyme (HHRz) [108–110], *glmS* GlcN6P riboswitch [25], and lately found twister ribozyme [111], hatchet ribozyme and pistol ribozyme [112] and so on. The self-cleaving HHRz is researched most thoroughly and applied most widely [113]. Ribozymes are often introduced to fuse aptamers of riboswitches, this kind of aptamers-based ribozymes are also called aptazymes or allosteric ribozymes for synthetic riboswitches study, such as theophylline aptazymes [88,114,115], TPP aptazymes [116], FMN aptazymes [102,117].

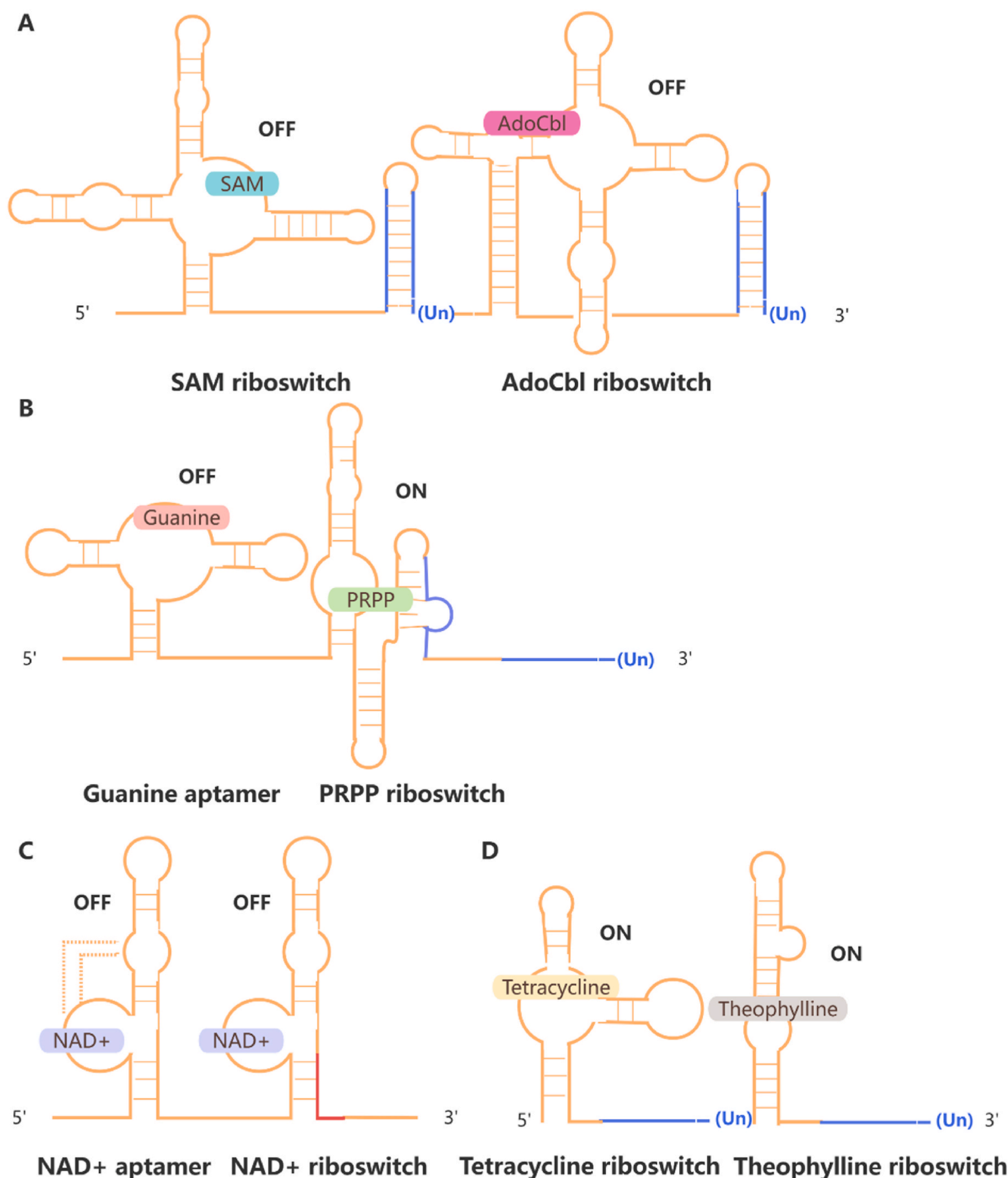

Fig. 4a. Tandem structure of riboswitches. (A) *metE* tandem SAM-AdoCbl riboswitches of *Bacillus clausii* [90] (B) *codA* tandem guanine-PRPP riboswitches of *Bacillus megaterium* [92] (C) *nadA* tandem NAD^+ riboswitches of *Acidobacterium capsulatum* [34] (D) artificial synthetic tandem tetracycline-theophylline riboswitches through computational design in *E. coli* [95]. Blue line represents terminator stem sequence, red line represents RBS, dashed line represents interaction between two loops.

4.1. Aptazymes based on hammerhead ribozyme (HHRz)

Similar to regular riboswitches, the aptazymes are used in many same fields. In eukaryotic cells, aptazymes are mainly inserted in 3′-UTR of mRNA due to the insertion in 5′-UTR would hinder normal gene expression without ligands [109], the mRNA of eukaryotic cells carries a polyA tail which is requisite for translation, the activated ribozymes components perform self-cleaving to prevent translation. For example, a theophylline aptazyme was applied in superior mutant caffeine demethylase screening in *Saccharomyces cerevisiae* [118], the best mutant had an increase product selectivity by 22-fold and enzyme activity *in vivo* by 33-fold. And it was reported that a tetracycline-dependent aptazyme was testified to promote gene expression by 8.8-fold in human cells with great advantages such as performing independently, low background activity etc [108] (Fig. 5).

Most natural ribozymes are *cis*-acting elements performing self-cleaving process. However, self-cleaving ribozymes could act in *trans* through artificial designs [110,119]. In *E. coli*, HHRz was designed to display *trans*-cleaving to silence gene expression [110]. The HHRz was fused into the anticodon loop of tRNA to prevent digestion, an enhanced

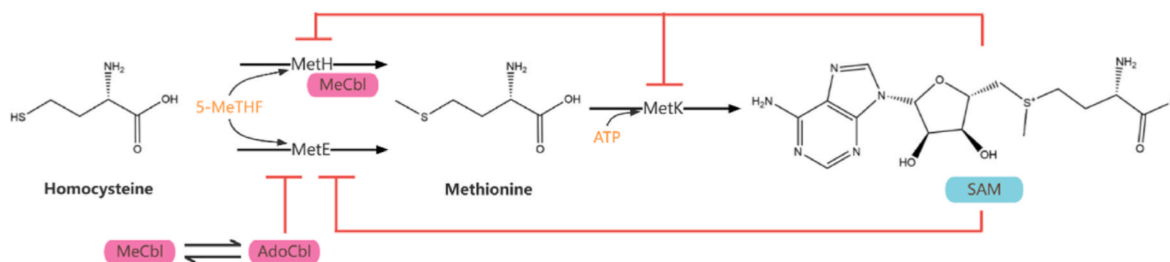

Fig. 4b. Metabolic principle of *metE* tandem SAM-AdoCbl riboswitches in *B. clausii* [90]. Red line represents inhibition effects. Product SAM has consistent negative feedback inhibition on *metH*, *metE* and *metK* through riboswitches, meanwhile adequate AdoCbl inhibits *metE* expression due to MetH is available for catalysis.

green fluorescent protein gene *egfp* was fused to a toxin protein gene *ibsC*, the high activated HHRz cleaved the mRNA of *egfp* plus *ibsC* to inhibit translation. The selected superior variants were evaluated quantitatively and the best candidate was testified to silence gene expression in cancer cells and zebrafish efficiently.

Aptazymes are also used as biosensors. Martin Held developed a high-throughput screening method using mixed cultivation in nanolitre reactors (nLRs) [120]. The mixed cultivation contains riboflavin producer cells of *B. subtilis* and engineered sensor cells of *E. coli* in gel capsules, the riboflavin secreted by producer cells would be transported into sensor cells and converted into FMN *in vivo*, FMN triggered FMN aptazyme and activated GFP gene expression. The high-yield strain was selected through GFP fluorescence. It also provided design principles for application of microcompartmentalization (Fig. 5).

For the Boolean logic gates concerned with in the construction of riboswitch regulatory system in eukaryotic cell, Christina D. Smolke summarized and elaborated a universal approach to execute higher-order cellular information processing in detail [121], they proposed a general framework including three basic components, a sensor derived from an aptamer, an actuator derived from a hammerhead ribozyme and a transmitter for connection between sensor and actuator. The whole system locates in 3′-UTR of mRNA, the sensor binds specific ligand, and causes a conformational change of actuator through transmitter, this change influences the activity of actuator, the activated actuator performs self-cleavage and inactivates mRNA therefore prevent translation. Higher-order and complex devices could assembly from general frameworks, and represent multiple processing effects analogy to Boolean logic gates.

4.2. Aptazymes based on other ribozymes

Other ribozymes are also applied in artificial riboswitches design. For example, the previously uncharacterized active site buried in a double-pseudoknot structure of twister ribozyme offered a novel insight into catalytic mechanism of ribozymes [111]. Twister ribozyme was employed as an expression platform to regulate gene expression in *E. coli* and yeast [122], it is unique that twister ribozyme could fuse regulated mRNA with two different independent positions, and aptamers too. Three aptamers responded to theophylline, TPP and neomycin were linked to display gene expression regulation, besides, two-input twister ribozyme riboswitches acted as various Boolean logic gates were screened by communication module randomization, displaying great vitality. There were also guanine aptazyme based on twister ribozyme reported to activate gene expression in mammalian cells and optimize its performance by fine-tuning the key stem structure [123], yielding an AR of greater than 6 (Fig. 5).

The hatchet ribozyme and pistol ribozyme were discovered through comparative genome analysis [112]. Researchers once failed in aptazymes construction of natural pistol ribozyme, which promoted they to develop artificial circularly permuted pistol ribozyme as a novel qualified expression platform and testify in mammalian cells [124], the AR achieved up to 8.6 and this work added a new member to limited ribozyme scaffold toolbox.

5. Novel regulation mechanisms mediated by riboswitches

Strictly speaking, riboswitches are metabolite-responsive RNA sequence to regulate gene expression without protein participation [29]. However, discoveries of some fresh RNA regulatory elements blurred the boundary, among which were RNA thermosensors located in 5′-UTR of *prfA* mRNA in *Listeria monocytogenes* [125], and T-box RNAs found upstream of amino acids related genes in Gram-positive bacteria [126]. The T-box RNAs could recognize specific cognate tRNA through anticodon and other features, detect the aminoacylation status of tRNA to regulate downstream gene expression, this interaction is far more complex than regular riboswitches, which greatly broadened people' s understanding of RNA interaction (Fig. 6).

Based on its high sensitivity to ligands, riboswitches are often applied in conjunction with other regulatory elements in artificial design. A widely researched structure in retroviruses called −1 programmed ribosomal frameshifting (−1 PRF) was combined with aptamers in tandem [127], −1 PRF allows translation complex to slip back by one nucleotide and continue translation in a new reading order [128], the binding of ligand would decide the downstream gene reading order directly. Other combined examples such as RNA interference was also reported earlier [129].

6. Applications and challenges

Riboswitches are simple and efficient gene regulatory elements, so far researchers have been discovered almost 40 kinds of riboswitches widely distributed in three domains of biology [17,20], it was reported that at least 2% of genes were controlled by riboswitches in *B. subtilis* [44]. After rapid development in twenty years, riboswitches are becoming an essential constituent in RNA study, its modularization and programmability endows infinite potential of application. Here we focus on some representative application fields.

6.1. Biosensors based on riboswitches

Riboswitches are inborn metabolite sensors owning the potential of detecting various chemical substance. The primary application must be biosensors. Riboswitches biosensors have more extensive detecting range and higher accuracy compared with traditional biosensors, therefore achieve rapid development in recent years. First, riboswitches have broad applications in metabolic engineering [69,81,82,130]. Riboswitches are essential for proper expression of downstream regulated genes, study have shown that the titer of a *B. subtilis* riboflavin producing strain reduces because of the deletion of *rib* operon FMN riboswitch, due to the transcriptional level of *rib* genes declines [131], indicating that the genes are strictly regulated. In order to make great use of riboswitches for metabolic engineering serving, a rational design approach of deleting a key nucleotide sequence participates in transcriptional terminator was employed in transcriptional *xpt-pbuX* purine

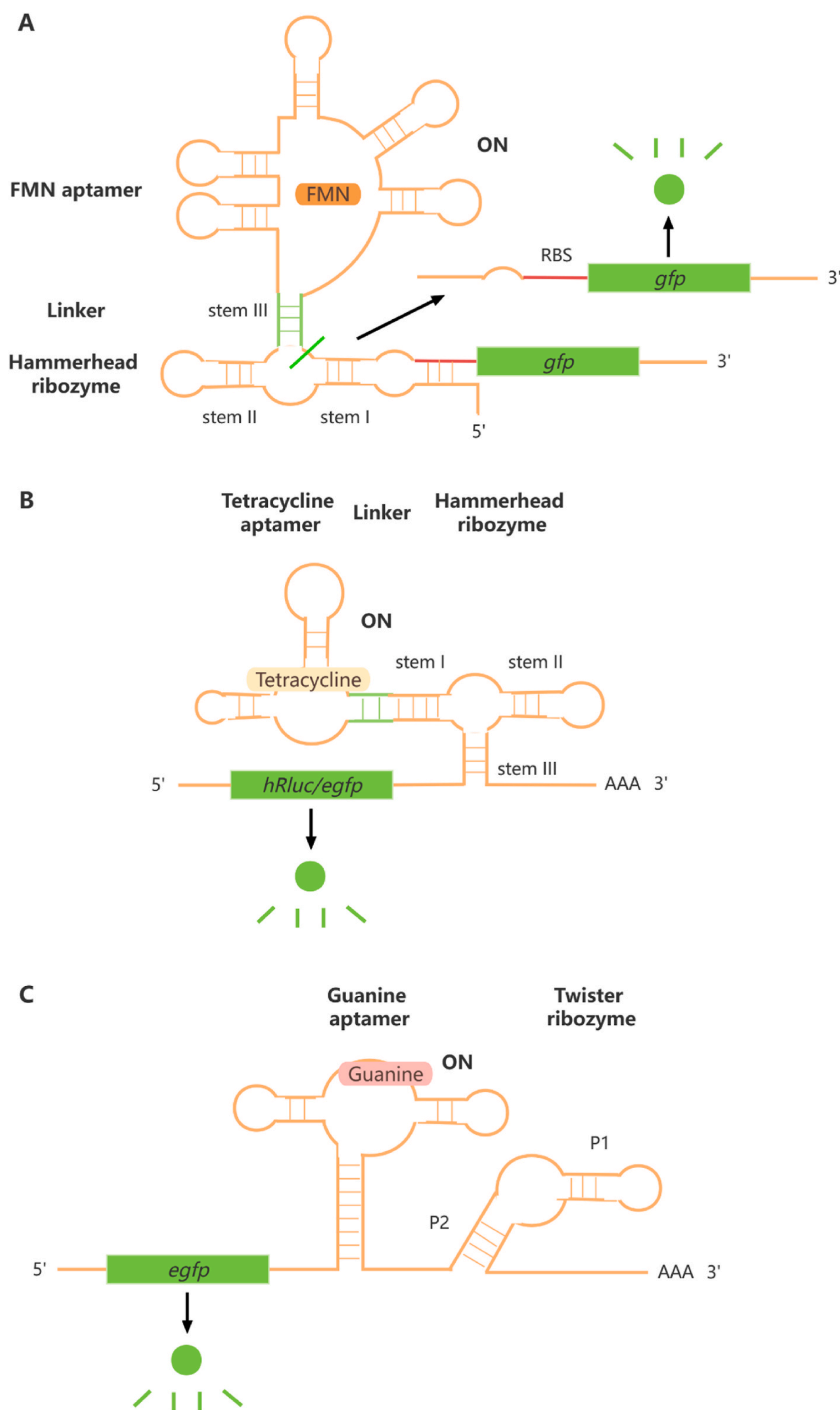

Fig. 5. Aptazymes structure. (A) FMN aptazyme construction in *E. coli* [120], light green line represents linker between aptamer and ribozyme, grass green line represents self-cleaving site, red line represents RBS. FMN induces ribozyme to cleave itself and promotes gene expression. (B) Tetracycline aptazyme construction in mammalian cells [108]. *hRluc* encodes *Renilla* luciferase. Tetracycline stables ribozyme structure and the intact mRNA with polyA could proceed smoothly. (C) Guanine aptazyme construction in mammalian cells [123]. Same as (B).

riboswitch and *rib* operon FMN riboswitch in *B. subtilis* [132], the secondary structures of riboswitches were depicted by NUPACK program [133], resulting the growth, gene expression and product synthesis of strains get a significant increase. The lysine riboswitch has been applied in many aspects in metabolic engineering. For example, to enhance product synthetic pathway reflux, two lysine OFF type riboswitches were introduced to downregulate *gltA* (encoding citrate synthase) gene expression to reduce tricarboxylic acid (TCA) cycle reflux in *Corynebacterium glutamicum*, the better one caused lysine production to increase by 63% [134].

Lately the CoNi riboswitch was applied in detecting Co^{2+}/Ni^{2+} transport protein in *E. coli* [135], the CoNi riboswitch promotes *mCherry* gene expression when bound to Co^{2+}/Ni^{2+}, the deletion of assumed gene encoding Co^{2+}/Ni^{2+} transport protein will elevate the intracellular concentration of Co^{2+}/Ni^{2+} and make the cell fluoresce. It shows that riboswitches have great potential in detecting metal ions.

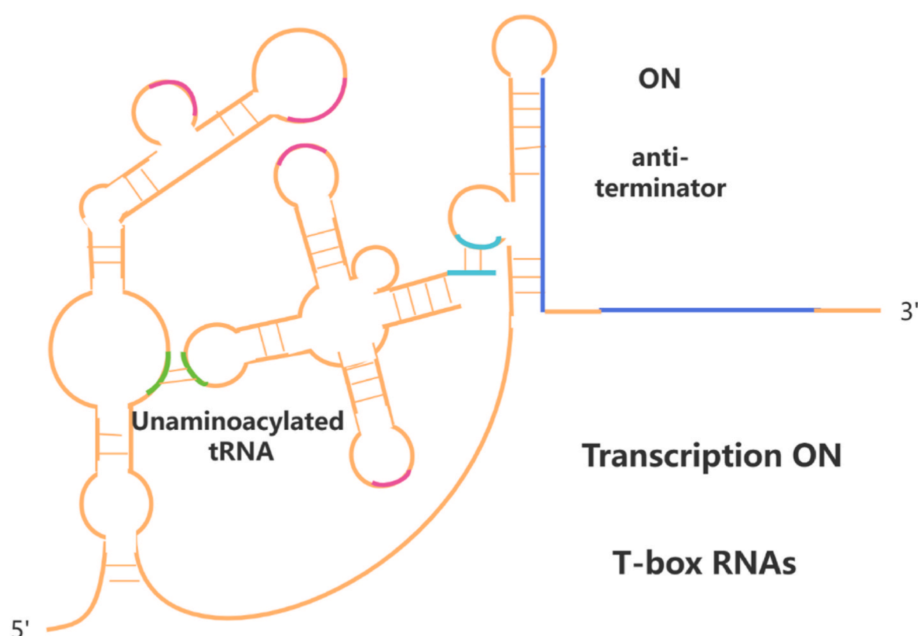

Fig. 6. Novel regulatory mechanism of riboswitches. *tyrS* T-box RNAs of *B. subtilis* [126]. Blue line represents terminator stem sequence, other colored line represents interaction sites. The unaminoacylated tRNA induces formation of anti-termination to promote gene expression and improves synthesis of matching amino acids.

Based on biosensors, riboswitches are applied in cell activity control [136] and biological imaging [10,137,138] etc. A theophylline riboswitch was used to regulate flagellar sigma factor *fliA* to show a faster predation kinetics in *Bdellovibrio bacteriovorus* [136]. And riboswitch biosensors using fluorescence signals are usually applied in biological imaging to track cells real-time quantitatively [138]. For example, a biosensor consisting of a c-di-GMP riboswitch with a spinach aptamer could respond to c-di-GMP and produce fluorescent signals in both aerobic and anaerobic conditions [137]. Moreover, biosensors respond to theophylline [139], lysine [79,81,134] and tetracycline [108] have already developed in food sample detection [140], mutant enzymes screening [79,80] and many other fields.

6.2. Targets of antibacterial drugs

The second major application is to be used as the targets of antibacterial drugs in drug design. Former researches have been elucidated that thiamine analog pyrithiamine exerts efficacy mediated by interfering with the normal function of TPP riboswitches in microbes [141]. Ronald R. Breaker investigated the phylogenetic distributions of riboswitches in 2017 and listed the whole riboswitch classes in overall bacterial groups [17], from which we can see riboswitches are broadly distributed among various bacterial groups. The idea of antibacterial drug design was discovered in nature too. A natural riboflavin analog roseoflavin produced by *Streptomyces davawensis* was proved to own the potential as antibacterial drugs aiming at FMN riboswitch [142], later researchers studied ribocil [143] and 5FDQD (5-(3-(4-fluorophenyl) butyl)-7,8-dimethylpyrido[3,4-b]- quinoxaline-1,3(2H,5H)-dione, a roseoflavin analog) [144], trying to find superior antibacterial drugs. There was a breakthrough that GlcN6P analog carba-GlcN6P was proposed as potential medicine targeting *glmS* GlcN6P riboswitch for *Staphylococcus aureus* because of its multiple drugs resistance [145]. The above examples all show the great potential of riboswitches in drug design.

6.3. Challenges of applications

Based on a study on riboswitch diversity and distribution from bioinformatics searching and analysis, Ronald R. Breaker and his team predicted there were massive unknown different riboswitches remaining to be discovered [17], unluckily most of them were extremely rare. Besides for those known riboswitch study as mentioned before, one nucleotide difference could affect riboswitch function, variants of one riboswitch could have thousands of differences in structure and function. So the urgent challenge is to find a more rational approach which could match sequence, structure and function according to existing researches [138].

The second challenge to limit its application is riboswitches are specific ligand-dependent biosensors, indicating we must consider whether there are such aptamers to respond to the detected metabolites, and how to find, optimize them [140]. At this present time this work still depends a lot of labor time with low effectiveness.

7. Conclusion

Riboswitches are powerful gene regulatory elements. In this review, starting from the discovery history and structure, we have introduced the diversity, structural and functional transformation including coupling with ribozymes and applications in detail. We summarized all the regulatory mechanisms, adopted the latest and authoritative classification methods, each category covered some representative and newly discovered riboswitches. Following we displayed researchers' transformation, enabling riboswitches to achieve finer control and simpler artificial design. In the end, we discussed current applications and challenges of riboswitches.

The researches of riboswitches show how powerful RNA can be, simple RNA motif possess the ability to detect metabolites, achieve gene expression regulation in the absence of protein, this ability would change even one nucleotide change in primary structure, displaying amazing potential in detection. And artificial design could create many aptamers in response to uncharacterized metabolites, endowing infinite possibilities of metabolites detection. As new biological and bioinformatical strategies are applied, researches on riboswitches will become more sophisticated and automated, we believe that more and more novel synthetic riboswitches will be developed and widely used in lives.

Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the content reported in this review.

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