Article

Riboswitch Dynamics and Their Expanding Biotechnological Applications

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Abstract: Riboswitches are natural RNA regulatory elements that control gene expression through ligand-induced conformational changes. These dynamic RNA sensors modulate downstream gene activity by influencing transcription or translation. Recent advances in riboswitch engineering have expanded their utility in biotechnology and synthetic biology. This review highlights key insights into the structural and functional dynamics of riboswitches and discusses emerging strategies for their application as programmable biological tools.

Keywords: RNA; riboswitch; dynamics; applications

1. Introduction

RNAs play central roles in cells not only as messenger RNA (mRNA) but also through their regulatory functions as noncoding RNAs [1]. Regulatory RNAs are broadly classified into two categories: *cis*-acting and *trans*-acting elements [2–4]. *Trans*-acting regulators, such as small RNAs (sRNAs), typically bind to target mRNAs to inhibit translation or promote mRNA degradation [5–7]. In contrast, *cis*-acting RNA elements directly sense intracellular stimuli—including ions, metabolites, and temperature—to modulate downstream gene expression [7–10]. These regulated genes are involved in essential cellular processes, such as metabolism, signal transduction, and stress response [11–13].

Among *cis*-acting regulators, riboswitches are highly conserved RNA motifs located in the 5′-untranslated region (5′-UTR) of mRNAs, where they control fundamental metabolic pathways (e.g., coenzyme and amino acid biosynthesis) [14–16]. Structurally, riboswitches comprise two functional domains: an aptamer for ligand sensing and an expression platform for gene regulation [17,18]. Upon ligand binding, the aptamer undergoes conformational changes that alter the expression platform's activity, enabling precise modulation of downstream genes. Advances in dynamic studies have enabled the clarification of the intricate mechanisms underlying riboswitch-mediated ligand response and gene regulation, facilitating their engineering for in vitro and in vivo applications [19,20]. Given their programmable gene-regulatory capabilities, riboswitches hold significant promise as tools in synthetic biology and as potential antibiotic targets [21–23]. This review highlights recent insights into riboswitch dynamics and explores their emerging applications in biotechnology.

2. Dynamic Studies of Riboswitches

Structural studies of riboswitches have primarily focused on determining high-resolution structures of aptamer-ligand complexes to elucidate their atomic-level architecture [24–26]. Key structural motifs—such as kissing loops (KL), pseudoknots (PK), and helical regions—play pivotal roles in the global folding of riboswitches, ultimately governing their gene regulatory functions. These structural insights are essential for identifying the precise interactions between riboswitches and their cognate ligands [27]. However, riboswitches are highly dynamic molecules that undergo conformational switching upon ligand binding. As depicted in Figure 1a, ligand binding can induce terminator formation, prompting RNA polymerase (RNAP) dissociation and transcription termination [28]. Alternatively, riboswitches may regulate gene expression at the translational level by modulating ribosome binding site (RBS) accessibility, thereby activating or suppressing translation [29] (Figure 1a). A unique example is the glucosamine-6-phosphate riboswitch, where ligand binding triggers ribozyme activity to influence RNA stability [30].

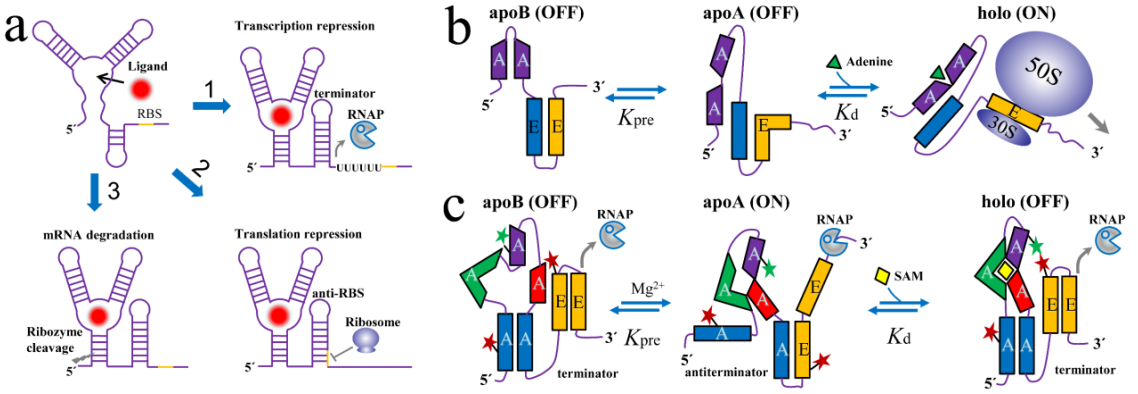

Figure 1. Dynamic structure folding of riboswitches. (**a**) riboswitch adopts three approaches to influence the transcriptional level, the translational level, and the mRNA stability to modulate gene expression under ligands binding. NMR (Nuclear magnetic resonance spectroscopy) and FRET (Förster resonance energy transfer) show the powerful ability to reveal the multiple states of adenine riboswitch [31]. (**b**) and SAM-I riboswitch [32]. (**c**) respectively. Abbreviation: RNAP (RNA polymerase), RBS (Ribosome binding site), A (Aptamer domain), and E (Expression platform).

Traditionally, riboswitch dynamics were described using a simplified two-state model [33]. Yet, advanced structural techniques have revealed far greater complexity in their conformational landscapes. For instance, the *Vibrio vulnificus* adenine riboswitch exhibits three distinct states: an inactive *apoB* conformation, an active *apoA* state, and a ligand-bound *holo* state (Figure 1b). In this system, RBS refolding into a single-stranded form enables ribosome engagement for translation initiation. Further evidence from X-ray free electron laser (XFEL) studies identified a third conformation—a ligand-bound intermediate—supporting a three-state mechanistic model for metabolite sensing and signal transmission [34] (Figure 2b).

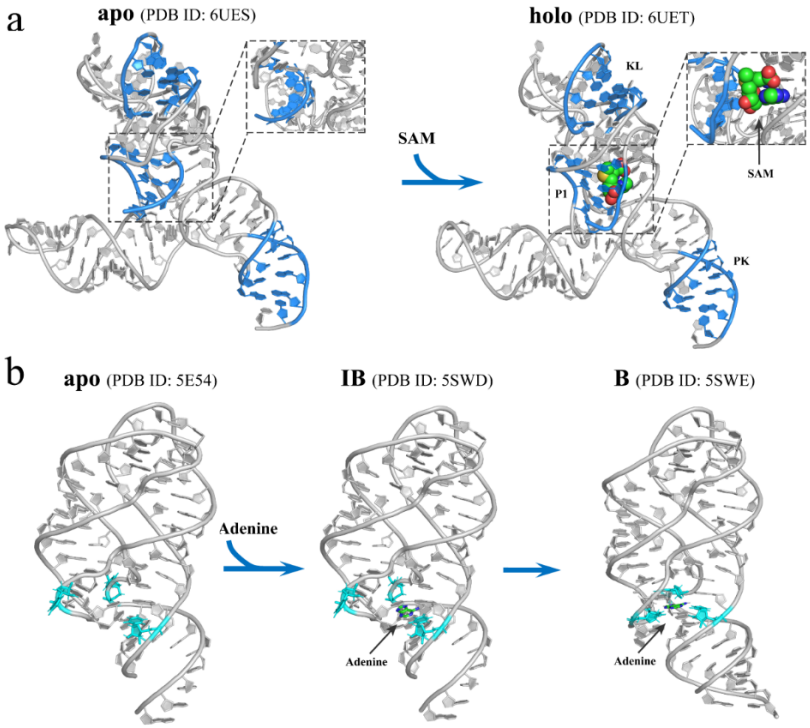

Figure 2. Dynamic folding of riboswitches. Cryo-EM and XFEL have been applied to obtain the high-resolution dynamic folding of SAM-IV (**a**) and adenine riboswitches (**b**). SAM and adenine molecules are shown with spheres and sticks, respectively. Adenine interacting bases are displayed with cyan sticks. Abbreviation: KL (Kissing loop), PK (Pseudoknot), IB (Intermediate-bound state), B (Bound state).

The *Bacillus subtilis* S-adenosyl-L-methionine-I (SAM-I) riboswitch regulates gene expression through the formation of a transcriptional terminator upon SAM binding. Single-molecule Förster resonance energy transfer

(single-molecule FRET, or smFRET) studies have mapped the conformational energy landscape of this riboswitch (Figure 1c), demonstrating its transition from a transcriptionally inactive *apoB* state to an active *apoA* state that facilitates SAM binding. Cryo-electron microscopy (cryo-EM) has emerged as a powerful tool for investigating RNA conformational dynamics. Recent structural determination of the SAM-IV riboswitch (50 kDa) by cryo-EM revealed that both *apo* and *holo* conformations, providing mechanistic insights into its regulatory function [35] (Figure 2a). Complementary to high-resolution techniques, small-angle X-ray scattering (SAXS) has been extensively employed to monitor global structural changes in various riboswitches, including TPP (Thiamine pyrophosphate), SAM-II, and c-di-GMP (Cyclic diguanosine monophosphate) riboswitches, under ligand-free and ligand-bound conditions [36–39]. These lower-resolution studies have significantly contributed to our understanding of riboswitch structure-function relationships from a dynamic perspective.

3. Applications of Riboswitches as Antibiotic Targets

Riboswitches are small molecule-sensing RNA widely distributed in both Gram-positive and Gram-negative bacteria, with rare occurrences in eukaryotes and absence in mammalian cells. The flavin mononucleotide (FMN) riboswitch, which regulates genes involved in flavin metabolism, has been identified in numerous pathogenic bacterial species [40,41]. FMN serves as a crucial coenzyme for flavoproteins and as a biosynthetic precursor for flavin adenine dinucleotide (FAD). Natural and synthetic compounds targeting FMN riboswitches have emerged as potential antibacterial agents. Roseoflavin, a naturally occurring FMN analog, exhibits antibacterial activity against Gram-positive bacteria by binding to FMN riboswitches and suppressing expression of riboflavin transporters, thereby inhibiting bacterial growth [42,43]. However, Gram-negative bacteria demonstrate intrinsic resistance to roseoflavin. Through systematic screening, ribocil was identified as an FMN riboswitch-binding compound effective against Gram-negative pathogens [44,45]. Structural studies revealed that ribocil exists as two enantiomers, *R*-ribocil and *S*-ribocil, with the *S*-form exhibiting high binding affinity and antibacterial activity (Figure 3a). Further optimization promoted the development of ribocil-C, an *S*-ribocil derivative demonstrating potent activity against *Escherichia coli* strains [44].

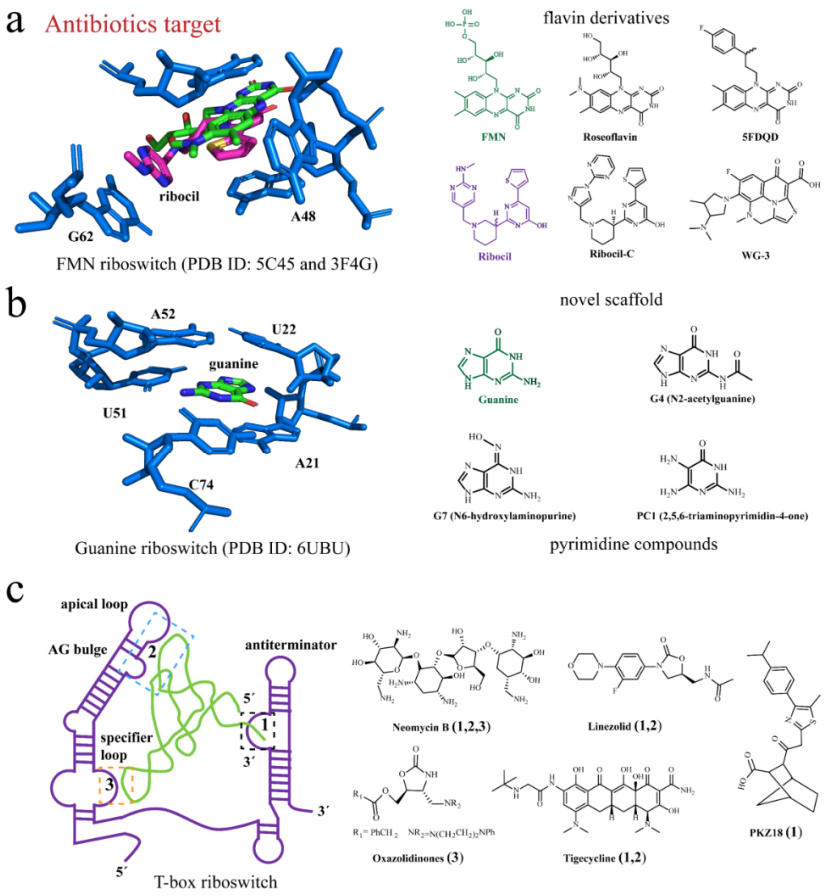

Figure 3. Riboswitch-based biotechnology contains the novel antibiotics discovery using the ligand-binding mechanisms of FMN (**a**), guanine (**b**), and T-box riboswitch (**c**). FMN and the antibiotic Ribocil are shown with green and purple sticks, respectively.

The guanine riboswitch in *Bacillus subtilis* serves as a key regulatory element for genes involved in purine transport and de novo biosynthesis [46]. Leveraging structural insights from high-resolution crystal structures, researchers have developed two pyrimidine analogs (G4, N2-acetylguanine and G7, N6-hydroxylaminopurine) that effectively target this riboswitch. These compounds demonstrate significant growth inhibition of *B. subtilis* in culture medium (Figure 3b), highlighting their potential as antibacterial drugs [47–49]. Similarly, T-box riboswitches have emerged as promising therapeutic targets due to their essential role in regulating amino acid metabolism and transport genes [50–55]. These riboswitches can be disrupted by small molecules including oxazolidinones and neomycin B, which competitively inhibit tRNA binding [56,57] (Figure 3c). Such interference with T-box riboswitch function leads to bacterial cell death, demonstrating the therapeutic potential of targeting these RNA regulatory elements.

4. Riboswitches' Applications as Biosensors

Riboswitch functionality can be characterized through reporter gene fusion assays, where riboswitches are coupled with genes encoding β-galactosidase or green fluorescent protein (GFP), enabling quantitative assessment of ligand-dependent regulatory activity via reporter protein expression levels [20,58,59]. For example, this approach has been instrumental in elucidating the roles of various transporters in maintaining vitamin B12 homeostasis, where reporter constructs were employed to monitor B12-dependent regulation (Figure 4). As an essential cofactor in numerous biochemical reactions, vitamin B12 serves as a key ligand for these regulatory studies [60]. Similarly, riboswitch-based reporter systems have proven to be valuable for monitoring intracellular signaling molecules. The second messenger c-di-GMP, which regulates critical physiological processes including motility, biofilm formation, and virulence, has been effectively quantified in vivo using engineered c-di-GMP riboswitch constructs [61] (Figure 4).

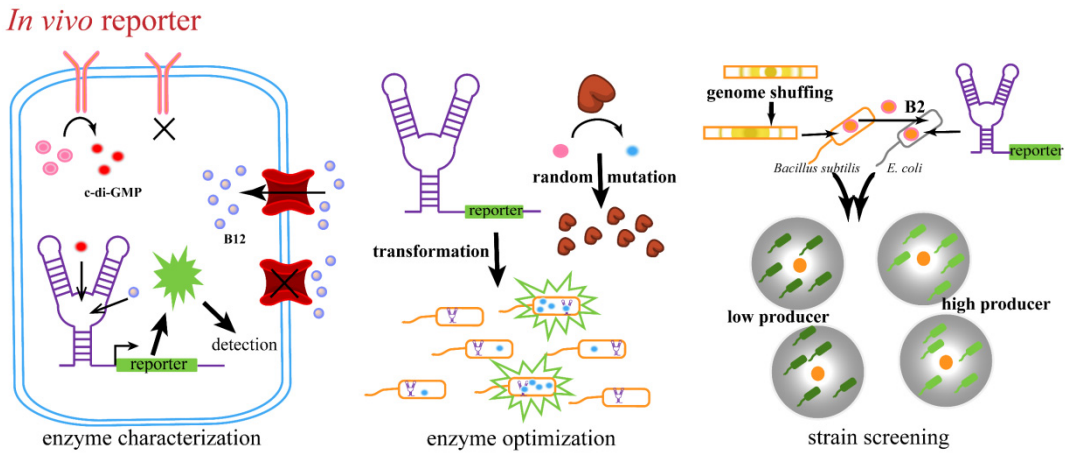

Figure 4. Riboswitch as in vivo reporter for enzyme function characterization, enzyme activity optimization, and high-yield strain screening.

Beyond their native regulatory roles, riboswitches have been developed as powerful tools for enzyme evolution and metabolic engineering. High-throughput screening platforms leveraging lysine and synthetic theophylline riboswitches have successfully identified enzyme variants with enhanced catalytic activity, including theophylline synthase from monooxygenase libraries and improved aspartate kinase mutants [62,63]. Furthermore, riboswitch-based reporters enable sensitive selection of microbial strains with optimized production of target metabolites [20,64], demonstrating their versatility in metabolic engineering applications. Traditional approaches for real-time biomolecule detection in living systems have predominantly employed protein-based biosensors, such as fluorescent proteins [65]. However, riboswitch-based biosensors offer several distinct advantages that make them an attractive alternative. First, their production through in vitro transcription bypasses the complexities of protein expression systems, significantly accelerating biosensor development. Second, riboswitches inherently possess high ligand specificity and undergo conformational changes upon binding, enabling direct signal transduction. These unique properties have been exploited to develop aptamer-based biosensors for monitoring diverse cellular metabolites, including fundamental cofactors (e.g., TPP), second messengers, and industrially relevant compounds (e.g., caprolactam) [66–69]. A notable example is the Spinach RNA aptamer system, where integration with a TPP riboswitch creates a fluorescent reporter that activates upon simultaneous binding of TPP

and the fluorogen DFHBI (3,5-Difluoro-4-hydroxybenzylidene imadazolinone) [66] (Figure 5a). This design principle has been successfully extended to monitor various signaling molecules, including adenine, SAM, c-di-GMP, c-di-AMP, and c-AMP-GMP, through their respective riboswitch-Spinach fusions [70,71] (Figure 5a). Beyond fluorescent activation, riboswitches can also employ quenching mechanisms. The cobalamin riboswitch, for instance, functions as an RNA tag that exhibits fluorescence enhancement upon cobalamin binding due to relief of intrinsic quenching [72–74] (Figure 5b). Furthermore, riboswitches responsive to toxic ligands (e.g., fluoride and guanidine) have been repurposed as biosensors in cell-free systems for environmental xenobiotic detection (Figure 5c), demonstrating their versatility across diverse applications [75–79].

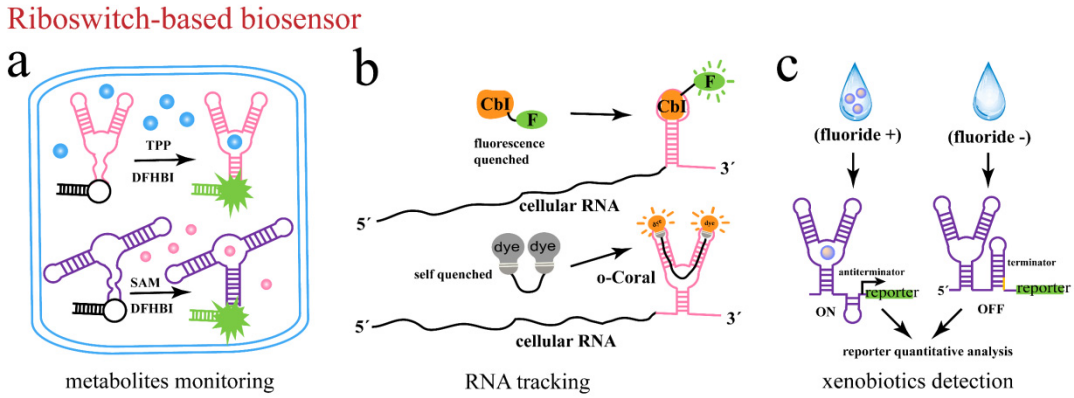

Figure 5. Riboswitches as biosensors for monitoring cellular metabolites, RNA imaging, and detection of environmental xenobiotics. (**a**) Riboswitches detect small molecules and then activate the DFHBI fluorescence by binding to a fused fluorescent RNA aptamer. (**b**) Self-quenching fluorescent molecule specifically binds to cellular RNA containing riboswitch aptamer, thereby endowing non-covalent RNA labeling for the assistance of RNA tracking. (**c**) Fluoride and other xenobiotic riboswitches regulate reporter gene expression to detect target molecule concentrations.

5. Conclusions

Riboswitches play a pivotal role in metabolic regulation by modulating gene expression in response to cellular metabolites. These structured RNA elements undergo ligand-induced conformational changes that precisely control downstream gene expression at transcriptional, translational, and post-transcriptional levels. Emerging evidence suggests that riboswitch dynamics are far more complex than the traditional two-state model, involving intricate conformational transitions that remain poorly understood. Current limitations in characterizing the structural dynamics of both aptamer domains and expression platforms upon ligand binding have significantly impeded progress in several key areas: (1) mechanistic understanding of riboswitch structure-function, (2) rational design of synthetic riboswitches, (3) component engineering for improved performance, and (4) de novo construction of ligand-specific variants. A comprehensive elucidation of riboswitch structural dynamics is essential for developing next-generation RNA-based regulators that offer precise gene expression control and expanded application potential. Such fundamental insights will provide critical guidelines for the rational design of functional RNA elements tailored to specific biotechnological and therapeutic applications.

Riboswitches serve as sophisticated molecular regulators that orchestrate metabolic processes through precise structural transitions. These naturally evolved RNA elements and their synthetic counterparts have emerged as versatile platforms for biotechnological innovation. Their unique gene-regulatory capabilities enable diverse applications spanning both in vivo and in vitro systems, particularly as promising antibiotic targets and highly specific biosensors (Figure 6). The functional repertoire of riboswitches can be substantially expanded through engineering approaches. Natural tandem riboswitch architectures provide blueprints for developing, including modular RNA systems integrating multiple regulatory functions, complex genetic circuits employing tandem riboswitch logic gates, and chimeric constructs combining distinct functional motifs. Notable advances include the successful implementation of riboswitch-riboswitch and riboswitch-ribozyme arrays in living systems. Furthermore, the strategic fusion of riboswitches with CRISPR guide RNAs has significantly enhanced the precision and tunability of CRISPR technologies [80,81]. In future, the integration of protein-binding RNA aptamers with riboswitches functionality promises to yield next-generation tools for real-time metabolic monitoring, precision genome engineering, and advanced diagnostic platforms [82].

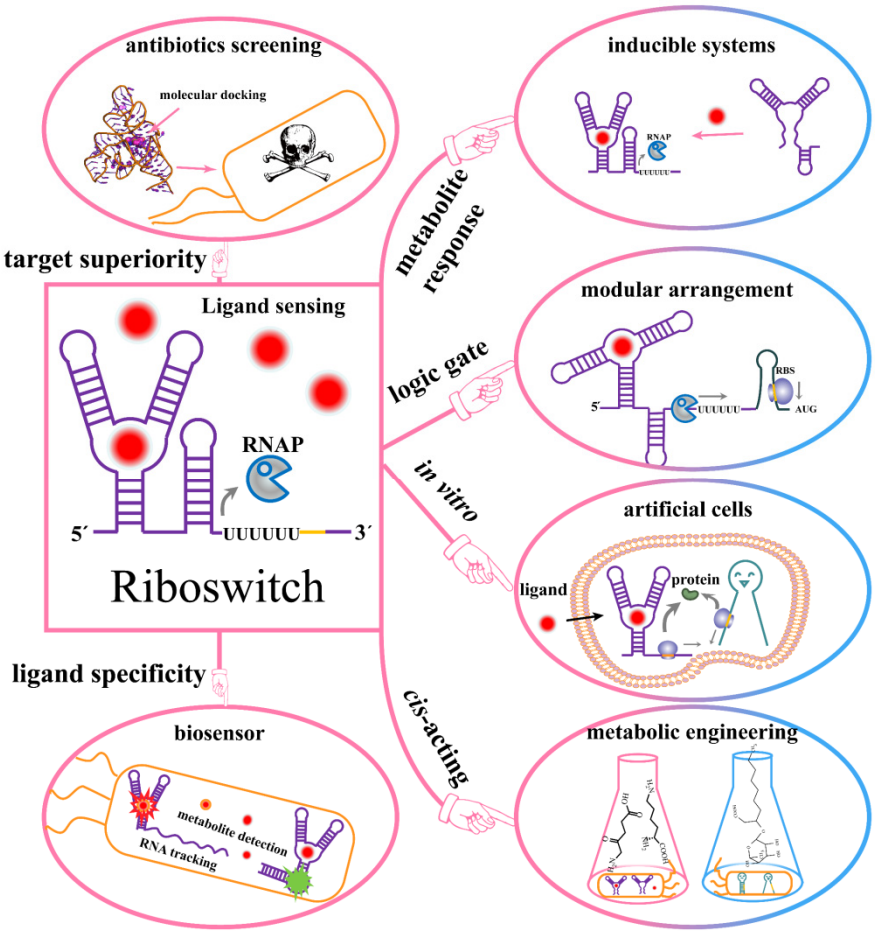

Figure 6. Schematic of riboswitches' applications in biotechnology.

Despite their potential, riboswitch applications in synthetic biology and biotechnology face several limitations. Their functionality depends on ligand-induced conformational changes, yet the underlying dynamic switching mechanisms—particularly at the single-molecule level—remain poorly characterized, constraining both natural riboswitch utilization and engineering efforts. While SELEX (Systematic Evolution of Ligands by Exponential Enrichment) has enabled expansion of RNA aptamer libraries, achieving optimal dynamic range (e.g., high ON/OFF ratios) and sensitivity (e.g., activation at physiological ligand concentrations) remains challenging. Computational approaches, including energy-based rational design and machine learning-assisted de novo design (utilizing reverse folding algorithms and RNA large language models), may offer solutions to these limitations. Additional challenges arise in biosensor development: riboswitch-based detectors currently rely on either transcriptional/translational regulation (reporter systems) or fluorescent RNA aptamers. Reporter systems lack real-time sensing capability, while fluorescent aptamers typically exhibit high background noise, low fluorescence intensity, and poor signal-to-noise ratios. Integration with smart signal output platforms, such as wireless devices or nanotechnology systems, may improve riboswitch biosensor performance. Although riboswitch-targeted antibiotics represent a novel mechanism of action, no FDA-approved drugs targeting riboswitches have been developed to date. Ribocil, currently in preclinical and early clinical studies, exhibits potential off-target effects in human cells due to structural similarities in the riboflavin pathway. However, combination therapies with conventional antibiotics may help the development of riboswitch-targeted antibiotics.

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References

1. Liang, X.; Chen, D.; Su, A.; Liu, Y. Divergent Molecular Assembly and Catalytic Mechanisms between Bacterial and Archaeal RNase P in Pre-tRNA Cleavage. *Proc. Natl. Acad. Sci. USA* **2024**, *121*, e2407579121. https://doi.org/10.1073/pnas.2407579121.
2. Dar, D.; Sorek, R. Regulation of Antibiotic-Resistance by Non-Coding RNAs in Bacteria. *Curr. Opin. Microbiol.* **2017**, *36*, 111–117. https://doi.org/10.1016/j.mib.2017.02.005.
3. Liu, B.; Samaniego, C.C.; Bennett, M.R.; Franco, E.; Chappell, J. A Portable Regulatory RNA Array Design Enables Tunable and Complex Regulation across Diverse Bacteria. *Nat. Commun.* **2023**, *14*, 5268. https://doi.org/10.1038/s41467-023-40785-x.
4. Schmerer, N.; Janga, H.; Aillaud, M.; Hoffmann, J.; Aznaourova, M.; Wende, S.; Steding, H.; Halder, L.D.; Uhl, M.; Boldt, F.; et al. A Searchable Atlas of Pathogen-Sensitive lncRNA Networks in Human Macrophages. *Nat. Commun.* **2025**, *16*, 4733. https://doi.org/10.1038/s41467-025-60084-x.
5. Wilson, R.C.; Doudna, J.A. Molecular Mechanisms of RNA Interference. *Annu. Rev. Biophys.* **2013**, *42*, 217–239. https://doi.org/10.1146/annurev-biophys-083012-130404.
6. Nugent, P.J.; Park, H.; Wladyka, C.L.; Yelland, J.N.; Sinha, S.; Chen, K.Y.; Bynum, C.; Quarterman, G.; Lee, S.C.; Hsieh, A.C.; et al. Decoding Post-Transcriptional Regulatory Networks by RNA-Linked CRISPR Screening in Human Cells. *Nat. Methods* **2025**, *22*, 1237–1246. https://doi.org/10.1038/s41592-025-02702-6.
7. Gao, L.; Chen, D.; Liu, Y. Ligand Response of Guanidine-IV Riboswitch at Single-Molecule Level. *eLife* **2024**, *13*, RP94706. https://doi.org/10.7554/eLife.94706.
8. Waters, L.S. Bacterial Manganese Sensing and Homeostasis. *Curr. Opin. Chem. Biol.* **2020**, *55*, 96–102. https://doi.org/10.1016/j.cbpa.2020.01.003.
9. Guanzon, D.A.; Pienkoß, S.; Brandenburg, V.B.; Röder, J.; Scheller, D.; Dietze, A.; Wimbert, A.; Twittenhoff, C.; Narberhaus, F. Two Temperature-Responsive RNAs Act in Concert: The Small RNA CyaR and the mRNA ompX. *Nucleic Acids Res.* **2025**, *53*, gkaf041. https://doi.org/10.1093/nar/gkaf041.
10. Wu, L.; Chen, D.; Ding, J.; Liu, Y. A Transient Conformation Facilitates Ligand Binding to the Adenine Riboswitch. *iScience* **2021**, *24*, 103512. https://doi.org/10.1016/j.isci.2021.103512.
11. Atilho, R.M.; Mirihana Arachchilage, G.; Greenlee, E.B.; Knecht, K.M.; Breaker, R.R. A Bacterial Riboswitch Class for the Thiamin Precursor HMP-PP Employs a Terminator-Embedded Aptamer. *eLife* **2019**, *8*, e45210. https://doi.org/10.7554/eLife.45210.
12. Sudarsan, N.; Lee, E.R.; Weinberg, Z.; Moy, R.H.; Kim, J.N.; Link, K.H.; Breaker, R.R. Riboswitches in Eubacteria Sense the Second Messenger Cyclic Di-GMP. *Science* **2008**, *321*, 411–413. https://doi.org/10.1126/science.1159519.
13. Zhao, B.; Guffy, S.L.; Williams, B.; Zhang, Q. An Excited State Underlies Gene Regulation of a Transcriptional Riboswitch. *Nat. Chem. Biol.* **2017**, *13*, 968–974. https://doi.org/10.1038/nchembio.2427.
14. Capdevila, D.A.; Rondón, J.J.; Edmonds, K.A.; Rocchio, J.S.; Dujovne, M.V.; Giedroc, D.P. Bacterial Metallostasis: Metal Sensing, Metalloproteome Remodeling, and Metal Trafficking. *Chem. Rev.* **2024**, *124*, 13574–13659. https://doi.org/10.1021/acs.chemrev.4c00264.
15. Kiliushik, D.; Goenner, C.; Law, M.; Schroeder, G.M.; Srivastava, Y.; Jenkins, J.L.; Wedekind, J.E. Knotty Is Nice: Metabolite Binding and RNA-Mediated Gene Regulation by the preQ1 Riboswitch Family. *J. Biol. Chem.* **2024**, *300*, 107951. https://doi.org/10.1016/j.jbc.2024.107951.
16. Chen, D.; Han, Z.; Liang, X.; Liu, Y. Engineering a DNA Polymerase for Modifying Large RNA at Specific Positions. *Nat. Chem.* **2025**, *17*, 382–392. https://doi.org/10.1038/s41557-024-01707-6.
17. Stagno, J.R.; Wang, Y.-X. Riboswitch Mechanisms for Regulation of P1 Helix Stability. *Int. J. Mol. Sci.* **2024**, *25*, 10682. https://doi.org/10.3390/ijms251910682.
18. Zheng, L.; Song, Q.; Xu, X.; Shen, X.; Li, C.; Li, H.; Chen, H.; Ren, A. Structure-Based Insights into Recognition and Regulation of SAM-Sensing Riboswitches. *Sci. China Life Sci.* **2023**, *66*, 31–50. https://doi.org/10.1007/s11427-022-2188-7.
19. Bushhouse, D.Z.; Fu, J.; Lucks, J.B. RNA Folding Kinetics Control Riboswitch Sensitivity in Vivo. *Nat. Commun.* **2025**, *16*, 953. https://doi.org/10.1038/s41467-024-55601-3.
20. Chen, D.; Li, J.; Wu, Y.; Hong, L.; Liu, Y. Structural Dynamics-Guided Engineering of a Riboswitch RNA for Evolving c-Di-AMP Synthases. *Sci. Adv.* **2025**, *11*, eadt8165. https://doi.org/10.1126/sciadv.adt8165.
21. Hedwig, V.; Spöring, M.; Ottlinger, J.; Köse, S.; Nar, H.; Schnapp, G.; Gottschling, D.; Klein, H.; Aspnes, G.; Klugmann, M.; et al. Engineering Oxypurinol-Responsive Riboswitches Based on Bacterial Xanthine Aptamers for Gene Expression Control in Mammalian Cell Culture. *Nucleic Acids Res.* **2025**, *53*, gkae1189. https://doi.org/10.1093/nar/gkae1189.
22. Ogawa, A.; Fujikawa, M.; Tanimoto, R.; Matsuno, K.; Uehara, R.; Inoue, H.; Takahashi, H. Cell-Free Multistep Gene Regulatory Cascades Using Eukaryotic ON-Riboswitches Responsive to in Situ Expressed Protein Ligands. *ACS Synth. Biol.* **2025**, *14*, 909–918. https://doi.org/10.1021/acssynbio.4c00840.

23. Pham, H.L.; Wong, A.; Chua, N.; Teo, W.S.; Yew, W.S.; Chang, M.W. Engineering a Riboswitch-Based Genetic Platform for the Self-Directed Evolution of Acid-Tolerant Phenotypes. *Nat. Commun.* **2017**, *8*, 411. https://doi.org/10.1038/s41467-017-00511-w.
24. Serganov, A.; Huang, L.; Patel, D.J. Coenzyme Recognition and Gene Regulation by a Flavin Mononucleotide Riboswitch. *Nature* **2009**, *458*, 233–237. https://doi.org/10.1038/nature07642.
25. Degenhardt, M.F.S.; Degenhardt, H.F.; Bhandari, Y.R.; Lee, Y.-T.; Ding, J.; Yu, P.; Heinz, W.F.; Stagno, J.R.; Schwieters, C.D.; Watts, N.R.; et al. Determining Structures of RNA Conformers Using AFM and Deep Neural Networks. *Nature* **2025**, *637*, 1234–1243. https://doi.org/10.1038/s41586-024-07559-x.
26. Haack, D.B.; Rudolfs, B.; Jin, S.; Khitun, A.; Weeks, K.M.; Toor, N. Scaffold-Enabled High-Resolution Cryo-EM Structure Determination of RNA. *Nat. Commun.* **2025**, *16*, 880. https://doi.org/10.1038/s41467-024-55699-5.
27. Wang, S.; Chen, D.; Gao, L.; Liu, Y. Short Oligonucleotides Facilitate Co-Transcriptional Labeling of RNA at Specific Positions. *J. Am. Chem. Soc.* **2022**, *144*, 5494–5502. https://doi.org/10.1021/jacs.2c00020.
28. Wickiser, J.K.; Winkler, W.C.; Breaker, R.R.; Crothers, D.M. The Speed of RNA Transcription and Metabolite Binding Kinetics Operate an FMN Riboswitch. *Mol. Cell* **2005**, *18*, 49–60. https://doi.org/10.1016/j.molcel.2005.02.032.
29. Winkler, W.; Nahvi, A.; Breaker, R.R. Thiamine Derivatives Bind Messenger RNAs Directly to Regulate Bacterial Gene Expression. *Nature* **2002**, *419*, 952–956. https://doi.org/10.1038/nature01145.
30. Winkler, W.C.; Nahvi, A.; Roth, A.; Collins, J.A.; Breaker, R.R. Control of Gene Expression by a Natural Metabolite-Responsive Ribozyme. *Nature* **2004**, *428*, 281–286. https://doi.org/10.1038/nature02362.
31. Reining, A.; Nozinovic, S.; Schlepckow, K.; Buhr, F.; Fürtig, B.; Schwalbe, H. Three-State Mechanism Couples Ligand and Temperature Sensing in Riboswitches. *Nature* **2013**, *499*, 355–359. https://doi.org/10.1038/nature12378.
32. Manz, C.; Kobitski, A.Y.; Samanta, A.; Keller, B.G.; Jäschke, A.; Nienhaus, G.U. Single-Molecule FRET Reveals the Energy Landscape of the Full-Length SAM-I Riboswitch. *Nat. Chem. Biol.* **2017**, *13*, 1172–1178. https://doi.org/10.1038/nchembio.2476.
33. Neupane, K.; Yu, H.; Foster, D.A.N.; Wang, F.; Woodside, M.T. Single-Molecule Force Spectroscopy of the Add Adenine Riboswitch Relates Folding to Regulatory Mechanism. *Nucleic Acids Res.* **2011**, *39*, 7677–7687. https://doi.org/10.1093/nar/gkr305.
34. Stagno, J.R.; Liu, Y.; Bhandari, Y.R.; Conrad, C.E.; Panja, S.; Swain, M.; Fan, L.; Nelson, G.; Li, C.; Wendel, D.R.; et al. Structures of Riboswitch RNA Reaction States by Mix-and-Inject XFEL Serial Crystallography. *Nature* **2017**, *541*, 242–246. https://doi.org/10.1038/nature20599.
35. Zhang, K.; Li, S.; Kappel, K.; Pintilie, G.; Su, Z.; Mou, T.-C.; Schmid, M.F.; Das, R.; Chiu, W. Cryo-EM Structure of a 40 kDa SAM-IV Riboswitch RNA at 3.7 Å Resolution. *Nat. Commun.* **2019**, *10*, 5511. https://doi.org/10.1038/s41467-019-13494-7.
36. Reyes, F.E.; Schwartz, C.R.; Tainer, J.A.; Rambo, R.P. Methods for Using New Conceptual Tools and Parameters to Assess RNA Structure by Small-Angle X-Ray Scattering. *Methods Enzymol.* **2014**, *549*, 235–263. https://doi.org/10.1016/B978-0-12-801122-5.00011-8.
37. Baird, N.J.; Ferré-D'Amaré, A.R. Idiosyncratically Tuned Switching Behavior of Riboswitch Aptamer Domains Revealed by Comparative Small-Angle X-Ray Scattering Analysis. *RNA* **2010**, *16*, 598–609. https://doi.org/10.1261/rna.1852310.
38. Roy, S.; Lammert, H.; Hayes, R.L.; Chen, B.; LeBlanc, R.; Dayie, T.K.; Onuchic, J.N.; Sanbonmatsu, K.Y. A Magnesium-Induced Triplex Pre-Organizes the SAM-II Riboswitch. *PLoS Comput. Biol.* **2017**, *13*, e1005406. https://doi.org/10.1371/journal.pcbi.1005406.
39. Kulshina, N.; Baird, N.J.; Ferré-D'Amaré, A.R. Recognition of the Bacterial Second Messenger Cyclic Diguanylate by Its Cognate Riboswitch. *Nat. Struct. Mol. Biol.* **2009**, *16*, 1212–1217. https://doi.org/10.1038/nsmb.1701.
40. Crielaard, S.; Peters, C.F.M.; Slivkov, A.; van den Homberg, D.A.L.; Velema, W.A. Chemotranscriptomic Profiling with a Thiamine Monophosphate Photoaffinity Probe. *Chem. Sci.* **2025**, *16*, 4725–4731. https://doi.org/10.1039/d4sc06189f.
41. Eschbach, S.H.; Hien, E.D.M.; Ghosh, T.; Lamontagne, A.-M.; Lafontaine, D.A. The Escherichia Coli ribB Riboswitch Senses Flavin Mononucleotide within a Defined Transcriptional Window. *RNA* **2024**, *30*, 1660–1673. https://doi.org/10.1261/rna.080074.124.
42. Ott, E.; Stolz, J.; Lehmann, M.; Mack, M. The RFN Riboswitch of Bacillus Subtilis Is a Target for the Antibiotic Roseoflavin Produced by Streptomyces Davawensis. *RNA Biol.* **2009**, *6*, 276–280. https://doi.org/10.4161/rna.6.3.8342.
43. Mansjö, M.; Johansson, J. The Riboflavin Analog Roseoflavin Targets an FMN-Riboswitch and Blocks Listeria Monocytogenes Growth, but Also Stimulates Virulence Gene-Expression and Infection. *RNA Biol.* **2011**, *8*, 674–680. https://doi.org/10.4161/rna.8.4.15586.
44. Howe, J.A.; Wang, H.; Fischmann, T.O.; Balibar, C.J.; Xiao, L.; Galgoci, A.M.; Malinverni, J.C.; Mayhood, T.; Villafania, A.; Nahvi, A.; et al. Selective Small-Molecule Inhibition of an RNA Structural Element. *Nature* **2015**, *526*, 672–677. https://doi.org/10.1038/nature15542.

45. Rizvi, N.F.; Howe, J.A.; Nahvi, A.; Klein, D.J.; Fischmann, T.O.; Kim, H.-Y.; McCoy, M.A.; Walker, S.S.; Hruza, A.; Richards, M.P.; et al. Discovery of Selective RNA-Binding Small Molecules by Affinity-Selection Mass Spectrometry. *ACS Chem. Biol.* **2018**, *13*, 820–831. https://doi.org/10.1021/acschembio.7b01013.
46. Kim, J.N.; Blount, K.F.; Puskarz, I.; Lim, J.; Link, K.H.; Breaker, R.R. Design and Antimicrobial Action of Purine Analogues That Bind Guanine Riboswitches. *ACS Chem. Biol.* **2009**, *4*, 915–927. https://doi.org/10.1021/cb900146k.
47. Mulhbacher, J.; Brouillette, E.; Allard, M.; Fortier, L.-C.; Malouin, F.; Lafontaine, D.A. Novel Riboswitch Ligand Analogs as Selective Inhibitors of Guanine-Related Metabolic Pathways. *PLoS Pathog.* **2010**, *6*, e1000865. https://doi.org/10.1371/journal.ppat.1000865.
48. Gilbert, S.D.; Mediatore, S.J.; Batey, R.T. Modified Pyrimidines Specifically Bind the Purine Riboswitch. *J. Am. Chem. Soc.* **2006**, *128*, 14214–14215. https://doi.org/10.1021/ja063645t.
49. Kim, J.N.; Blount, K.F.; Puskarz, I.; Lim, J.; Link, K.H.; Breaker, R.R. Design and Antimicrobial Action of Purine Analogues That Bind Guanine Riboswitches. *ACS Chem. Biol.* **2009**, *4*, 915–927. https://doi.org/10.1021/cb900146k.
50. Childs-Disney, J.L.; Yang, X.; Gibaut, Q.M.R.; Tong, Y.; Batey, R.T.; Disney, M.D. Targeting RNA Structures with Small Molecules. *Nat. Rev. Drug Discov.* **2022**, *21*, 736–762. https://doi.org/10.1038/s41573-022-00521-4.
51. Falese, J.P.; Donlic, A.; Hargrove, A.E. Targeting RNA with Small Molecules: From Fundamental Principles towards the Clinic. *Chem. Soc. Rev.* **2021**, *50*, 2224–2243. https://doi.org/10.1039/d0cs01261k.
52. Gutierrez-Preciado, A.; Jensen, R.A.; Yanofsky, C.; Merino, E. New Insights into Regulation of the Tryptophan Biosynthetic Operon in Gram-Positive Bacteria. *Trends Genet. TIG* **2005**, *21*, 432–436. https://doi.org/10.1016/j.tig.2005.06.001.
53. Campos-Chavez, E.; Paul, S.; Zhou, Z.; Alonso, D.; Verma, A.R.; Fei, J.; Mondragón, A. Translational T-Box Riboswitches Bind tRNA by Modulating Conformational Flexibility. *Nat. Commun.* **2024**, *15*, 6592. https://doi.org/10.1038/s41467-024-50885-x.
54. Suddala, K.C.; Yoo, J.; Fan, L.; Zuo, X.; Wang, Y.-X.; Chung, H.S.; Zhang, J. Direct Observation of tRNA-Chaperoned Folding of a Dynamic mRNA Ensemble. *Nat. Commun.* **2023**, *14*, 5438. https://doi.org/10.1038/s41467-023-41155-3.
55. Niu, X.; Xu, Z.; Zhang, Y.; Zuo, X.; Chen, C.; Fang, X. Structural and Dynamic Mechanisms for Coupled Folding and tRNA Recognition of a Translational T-Box Riboswitch. *Nat. Commun.* **2023**, *14*, 7394. https://doi.org/10.1038/s41467-023-43232-z.
56. Orac, C.M.; Zhou, S.; Means, J.A.; Boehm, D.; Bergmeier, S.C.; Hines, J.V. Synthesis and Stereospecificity of 4,5-Disubstituted Oxazolidinone Ligands Binding to T-Box Riboswitch RNA. *J. Med. Chem.* **2011**, *54*, 6786–6795. https://doi.org/10.1021/jm2006904.
57. Anupam, R.; Denapoli, L.; Muchenditsi, A.; Hines, J.V. Identification of Neomycin B-Binding Site in T Box Antiterminator Model RNA. *Bioorg. Med. Chem.* **2008**, *16*, 4466–4470. https://doi.org/10.1016/j.bmc.2008.02.056.
58. Fowler, C.C.; Brown, E.D.; Li, Y. Using a Riboswitch Sensor to Examine Coenzyme B(12) Metabolism and Transport in *E. Coli*. *Chem. Biol.* **2010**, *17*, 756–765. https://doi.org/10.1016/j.chembiol.2010.05.025.
59. Xue, Y.; Li, J.; Chen, D.; Zhao, X.; Hong, L.; Liu, Y. Observation of Structural Switch in Nascent SAM-VI Riboswitch during Transcription at Single-Nucleotide and Single-Molecule Resolution. *Nat. Commun.* **2023**, *14*, 2320. https://doi.org/10.1038/s41467-023-38042-2.
60. Kipkorir, T.; Polgar, P.; Barker, D.; D'Halluin, A.; Patel, Z.; Arnvig, K.B. A Novel Regulatory Interplay between Atypical B12 Riboswitches and uORF Translation in Mycobacterium Tuberculosis. *Nucleic Acids Res.* **2024**, *52*, 7876–7892. https://doi.org/10.1093/nar/gkae338.
61. Gao, X.; Dong, X.; Subramanian, S.; Matthews, P.M.; Cooper, C.A.; Kearns, D.B.; Dann, C.E. Engineering of Bacillus Subtilis Strains to Allow Rapid Characterization of Heterologous Diguanylate Cyclases and Phosphodiesterases. *Appl. Environ. Microbiol.* **2014**, *80*, 6167–6174. https://doi.org/10.1128/AEM.01638-14.
62. Michener, J.K.; Smolke, C.D. High-Throughput Enzyme Evolution in Saccharomyces Cerevisiae Using a Synthetic RNA Switch. *Metab. Eng.* **2012**, *14*, 306–316. https://doi.org/10.1016/j.ymben.2012.04.004.
63. Wang, J.; Gao, D.; Yu, X.; Li, W.; Qi, Q. Evolution of a Chimeric Aspartate Kinase for L-Lysine Production Using a Synthetic RNA Device. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 8527–8536. https://doi.org/10.1007/s00253-015-6615-0.
64. Meyer, A.; Pellaux, R.; Potot, S.; Becker, K.; Hohmann, H.-P.; Panke, S.; Held, M. Optimization of a Whole-Cell Biocatalyst by Employing Genetically Encoded Product Sensors inside Nanolitre Reactors. *Nat. Chem.* **2015**, *7*, 673–678. https://doi.org/10.1038/nchem.2301.
65. Truong, L.; Ferré-D'Amaré, A.R. From Fluorescent Proteins to Fluorogenic RNAs: Tools for Imaging Cellular Macromolecules. *Protein Sci. Publ. Protein Soc.* **2019**, *28*, 1374–1386. https://doi.org/10.1002/pro.3632.
66. You, M.; Litke, J.L.; Jaffrey, S.R. Imaging Metabolite Dynamics in Living Cells Using a Spinach-Based Riboswitch. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, E2756–E2765. https://doi.org/10.1073/pnas.1504354112.
67. Paige, J.S.; Nguyen-Duc, T.; Song, W.; Jaffrey, S.R. Fluorescence Imaging of Cellular Metabolites with RNA. *Science* **2012**, *335*, 1194. https://doi.org/10.1126/science.1218298.

68. Bose, D.; Su, Y.; Marcus, A.; Raulet, D.H.; Hammond, M.C. An RNA-Based Fluorescent Biosensor for High-Throughput Analysis of the cGAS-cGAMP-STING Pathway. *Cell Chem. Biol.* **2016**, *23*, 1539–1549. https://doi.org/10.1016/j.chembiol.2016.10.014.
69. Chen, Z.; Chen, W.; Reheman, Z.; Jiang, H.; Wu, J.; Li, X. Genetically Encoded RNA-Based Sensors with Pepper Fluorogenic Aptamer. *Nucleic Acids Res.* **2023**, *51*, 8322–8336. https://doi.org/10.1093/nar/gkad620.
70. Kellenberger, C.A.; Chen, C.; Whiteley, A.T.; Portnoy, D.A.; Hammond, M.C. RNA-Based Fluorescent Biosensors for Live Cell Imaging of Second Messenger Cyclic Di-AMP. *J. Am. Chem. Soc.* **2015**, *137*, 6432–6435. https://doi.org/10.1021/jacs.5b00275.
71. Wang, X.C.; Wilson, S.C.; Hammond, M.C. Next-Generation RNA-Based Fluorescent Biosensors Enable Anaerobic Detection of Cyclic Di-GMP. *Nucleic Acids Res.* **2016**, *44*, e139. https://doi.org/10.1093/nar/gkw580.
72. Braselmann, E.; Palmer, A.E. A Multicolor Riboswitch-Based Platform for Imaging of RNA in Live Mammalian Cells. *Methods Enzymol.* **2020**, *641*, 343–372. https://doi.org/10.1016/bs.mie.2020.03.004.
73. Braselmann, E.; Wierzba, A.J.; Polaski, J.T.; Chromiński, M.; Holmes, Z.E.; Hung, S.-T.; Batan, D.; Wheeler, J.R.; Parker, R.; Jimenez, R.; et al. A Multicolor Riboswitch-Based Platform for Imaging of RNA in Live Mammalian Cells. *Nat. Chem. Biol.* **2018**, *14*, 964–971. https://doi.org/10.1038/s41589-018-0103-7.
74. Bühler, B.; Schokolowski, J.; Benderoth, A.; Englert, D.; Grün, F.; Jäschke, A.; Sunbul, M. Avidity-Based Bright and Photostable Light-up Aptamers for Single-Molecule mRNA Imaging. *Nat. Chem. Biol.* **2023**, *19*, 478–487. https://doi.org/10.1038/s41589-022-01228-8.
75. Baker, J.L.; Sudarsan, N.; Weinberg, Z.; Roth, A.; Stockbridge, R.B.; Breaker, R.R. Widespread Genetic Switches and Toxicity Resistance Proteins for Fluoride. *Science* **2012**, *335*, 233–235. https://doi.org/10.1126/science.1215063.
76. Thavarajah, W.; Silverman, A.D.; Verosloff, M.S.; Kelley-Loughnane, N.; Jewett, M.C.; Lucks, J.B. Point-of-Use Detection of Environmental Fluoride via a Cell-Free Riboswitch-Based Biosensor. *ACS Synth. Biol.* **2020**, *9*, 10–18. https://doi.org/10.1021/acssynbio.9b00347.
77. Ariyarathna, M.R.; Nissanka, J.P.; Methlal, K.; Abeyrathne, K.D.; Satharasinghe, M.; Banushan, P.; Manawadu, D.; Mirihana Arachchilage, G.; Silva, G.N. Simple and Cost-Effective Fluoride Riboswitch-Based Whole-Cell Biosensor for the Determination of Fluoride in Drinking Water. *Appl. Biochem. Biotechnol.* **2025**. https://doi.org/10.1007/s12010-025-05269-2.
78. Brown, D.M.; Phillips, D.A.; Garcia, D.C.; Arce, A.; Lucci, T.; Davies, J.P.; Mangini, J.T.; Rhea, K.A.; Bernhards, C.B.; Biondo, J.R.; et al. Semiautomated Production of Cell-Free Biosensors. *ACS Synth. Biol.* **2025**, *14*, 979–986. https://doi.org/10.1021/acssynbio.4c00703.
79. Boyd, M.A.; Thavarajah, W.; Lucks, J.B.; Kamat, N.P. Robust and Tunable Performance of a Cell-Free Biosensor Encapsulated in Lipid Vesicles. *Sci. Adv.* **2023**, *9*, eadd6605. https://doi.org/10.1126/sciadv.add6605.
80. Galizi, R.; Jaramillo, A. Engineering CRISPR Guide RNA Riboswitches for in Vivo Applications. *Curr. Opin. Biotechnol.* **2019**, *55*, 103–113. https://doi.org/10.1016/j.copbio.2018.08.007.
81. Hu, L.-F.; Li, Y.-X.; Wang, J.-Z.; Zhao, Y.-T.; Wang, Y. Controlling CRISPR-Cas9 by Guide RNA Engineering. *Wiley Interdiscip. Rev. RNA* **2023**, *14*, e1731. https://doi.org/10.1002/wrna.1731.
82. Fukunaga, K.; Teramoto, T.; Nakashima, M.; Ohtani, T.; Katsuki, R.; Matsuura, T.; Yokobayashi, Y.; Kakuta, Y. Structural Insights into Lab-Coevolved RNA-RBP Pairs and Applications of Synthetic Riboswitches in Cell-Free System. *Nucleic Acids Res.* **2025**, *53*, gkaf212. https://doi.org/10.1093/nar/gkaf212.