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RNA-based dynamic genetic controllers: development strategies and applications

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Dynamic regulation of gene expression in response to various molecules is crucial for both basic science and practical applications. RNA is considered an attractive material for creating dynamic genetic controllers because of its specific binding to ligands, structural flexibility, programmability, and small size. Here, we review recent advances in strategies for developing RNA-based dynamic controllers and applications. First, we describe studies that re-engineered natural riboswitches to generate new dynamic controllers. Next, we summarize RNA-based regulatory mechanisms that have been exploited to build novel artificial dynamic controllers. We also discuss computational methods and high-throughput selection approaches for *de novo* design of dynamic RNA controllers. Finally, we explain applications of dynamic RNA controllers for metabolic engineering and synthetic biology.

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Introduction

Dynamic regulation of gene expression in response to extracellular or intracellular signals like small molecules or proteins is crucial for both natural organisms and engineered biological systems. Natural organisms have evolved diverse regulatory mechanisms for sensing specific cues and control gene expression levels to survive in changing environments. Dynamic controllers may also serve as fundamental tools for engineering biological systems for various tasks including inducible control of gene expression, high-throughput detection of specific molecules, and dynamic regulation of metabolic pathways. Therefore, discovering new classes of natural controllers and developing artificial controllers will broaden our understanding of natural strategies for gene regulation and improve our ability to create complex biological systems.

In addition to protein-based controllers, the versatile roles of ribonucleic acid (RNA) as dynamic genetic controllers in nature have become widely recognized recently. RNA-based dynamic controllers form alternative structures depending on the binding of cognate ligands to control gene expression during transcription, post-transcription, or translation [1]. Ligands for natural RNA controllers range from inorganic ions to metabolites [2]. This competence of RNA for dynamic regulation of gene expression has motivated researchers to create artificial RNA controllers.

RNA has unique advantages as a material for constructing dynamic genetic controllers. In addition to ligands detected by natural RNA controllers, RNA can bind to various types of molecules including antibiotics, dyes, fluorescent probes, and proteins with specificity and sensitivity similar to protein-based controllers [3]. Binding events can be readily associated with regulatory activities because of the flexibility of RNA, which allows transformation between alternative structures. Moreover, the regulatory activity of RNA can be modulated or even designed by adjusting base pairings that largely determine intra- and intermolecular interactions. Finally, because of the small size of RNA controllers (generally $<$200 nt), they impose lower burdens on cells compared to protein-based controllers which require longer mRNA transcripts and translation into proteins [4]. This small size also permits construction and examination of numerous variants in a high-throughput manner to create and optimize new controllers.

Recent advances in experimental methods, biophysical modeling approaches, and computational algorithms enabled the development of new dynamic RNA controllers at an unprecedented rate. Natural RNA controllers have been re-engineered to sense new molecular signals based on information about their structures and

mechanisms. Biophysical modeling and high-throughput screening enabled *de novo* design of dynamic RNA controllers. These dynamic RNA controllers have been utilized for high-throughput screening for metabolic engineering and inducible control of cellular behavior. In this review, we summarize emerging strategies for developing RNA-based dynamic genetic controllers and applications of RNA controllers.

Re-engineering of natural riboswitches

Natural RNAs can control gene expression in response to diverse molecules and their repertoire is continually expanding. However, a reliable method for constructing RNA controllers that detect new ligands is required. For example, orthogonal expression systems that do not interfere with the host metabolism are difficult to achieve using natural controllers. Moreover, the demand for methods to detect new molecules such as important disease marker proteins and valuable metabolic products is rapidly increasing. Thus, natural RNA-based dynamic controllers were engineered to sense new signals, as the most intuitive method for developing artificial biological components is to exploit nature.

Natural riboswitches contain both sensors and expression platforms. Binding of a cognate metabolite to the sensor, an RNA aptamer, induces structural changes of the expression platform to control gene expression. Each riboswitch component can be engineered independently because of the modularity of this system. Riboswitch aptamers are primary choices for modification to broaden the range of ligands. For example, specific nucleotides in the *addA* riboswitch aptamer from *Vibrio vulnificus* were fully mutagenized based on its crystal structure [5•] (Figure 1a). Mutant riboswitches were screened for orthogonal gene activation using a library of non-natural analogues of the original ligand, adenine. Mutant riboswitches activated gene expression upon addition of ammeline or azacytosine, but showed no response to adenine. Furthermore, additional aptamer-ligand pairs were discovered in a subsequent analogue screening study [6]. This approach was applied to identify novel aptamer-ligand pairs by altering the specificity of the $PreQ_1$ class I riboswitch from *Bacillus subtilis* [7]. Aptamers of natural riboswitches were utilized as scaffolds to select aptamers for new ligands. Nucleotides in the binding pockets of natural aptamers were randomized to produce potential aptamer libraries for *in vitro* selection (SELEX) [8••] (Figure 1b). Selected aptamers for 5-hydroxytryptophan and 3,4-dihydroxyphenylalanine were successfully coupled to the readout domain, producing fluorescence upon binding of their cognate ligand *in vitro* and *in vivo*. The ability of natural aptamer scaffolds to properly fold in the cellular context ensures that aptamers evolved *in vitro* can be successfully employed in riboswitch construction for *in vivo* applications.

Expression platforms for natural riboswitches were engineered to construct modular components. The boundaries between aptamers and expression platforms of three *B. subtilis* riboswitches were determined based on regulatory mechanisms and secondary structures [9] (Figure 1c). The aptamers and expression platforms were reassembled in a mix-and-match fashion to create chimeric riboswitches that retained their regulatory activities to turn off transcription upon ligand binding, and even combinations using artificial RNA aptamers were successful in regulating gene expression. Furthermore, modular expression platforms that activate gene expression were developed by modifying natural expression platforms [10•]. These modular expression platforms may facilitate the development of new riboswitches simply by attaching aptamers of interest with minimal effort for optimization.

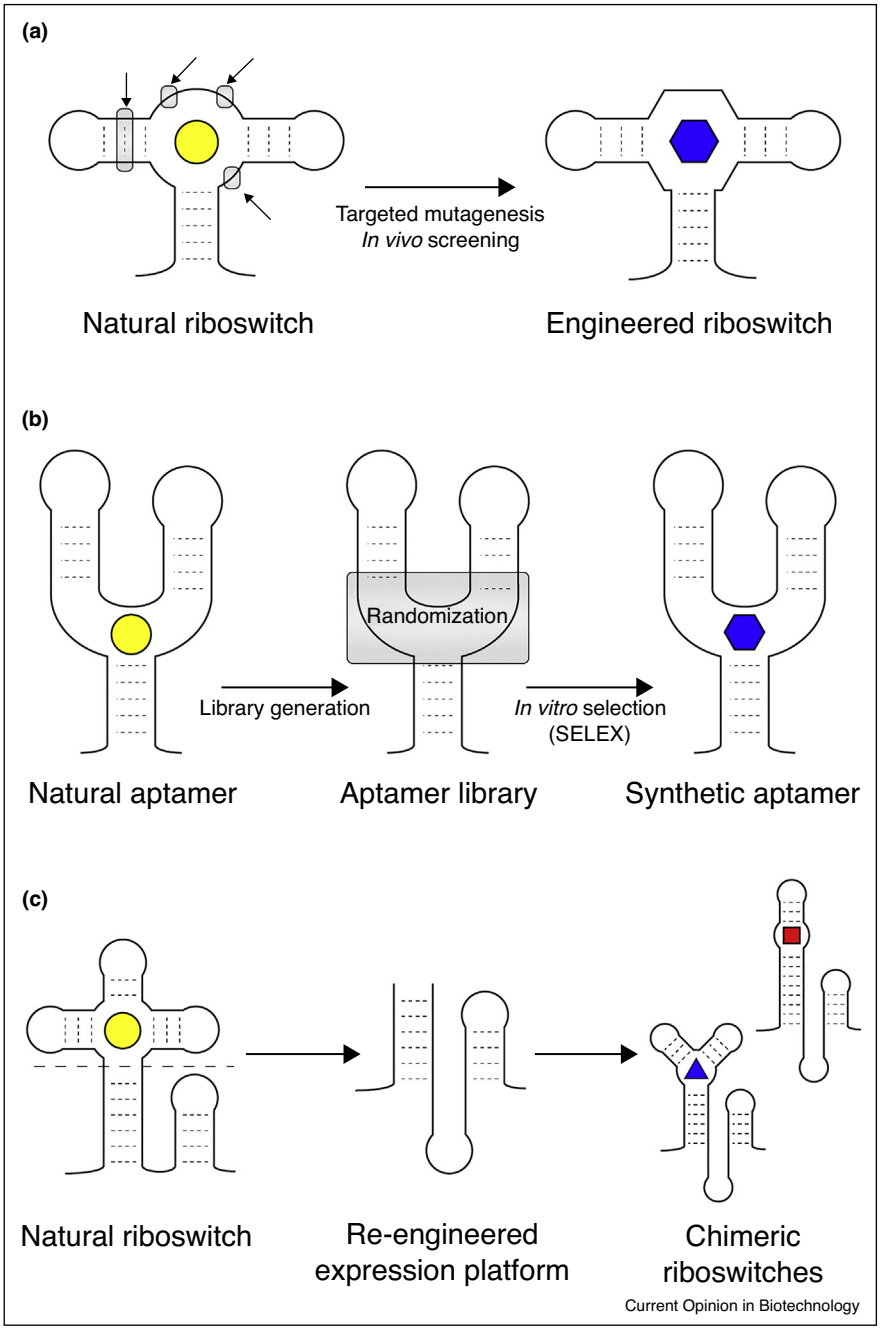
Exploiting natural RNA-based regulatory mechanisms for dynamic control

Natural RNA-based regulatory elements can be employed to dynamically regulate gene expression even if they do not contain natural ligand-binding domains. Natural aptamers or artificial aptamers created by SELEX were grafted to regulatory RNA elements to construct new dynamic RNA controllers. Engineering of natural RNA-based regulation for dynamic control of gene expression requires detailed knowledge of each mechanism.

Self-cleaving ribozymes are among the most extensively engineered RNA controllers (Figure 2a). RNA aptamers were fused to self-cleaving ribozymes for ligand-dependent *cis*-regulation of mRNA stability. These aptamer-ribozyme fusions (aptazyme) generally require communication modules that transduce ligand binding into regulatory activity. Either high-throughput screening and selection [11,12,13•,14••,15] or rational design approaches [16,17] were applied to design optimal communication modules. As a result, aptazymes for small molecules, antibiotics, and proteins were developed based on hammerhead ribozymes. Additionally, newly discovered ribozymes were employed to construct new aptazymes. A twister ribozyme was utilized to build two-input logic gates by attaching two distinct aptamers at different positions on a single ribozyme scaffold [18] (Figure 2a). Moreover, hepatitis delta virus ribozyme [19] and pistol ribozyme [15] were utilized to build ligand-responsive aptazymes.

Next, RNA-based regulatory mechanisms specific to eukaryotes were engineered to design ligand-dependent control of translation. In contrast to prokaryotic translation which requires a ribosome binding site inside mRNA, eukaryotic translation generally utilizes a 5′-cap to recruit ribosomes. Therefore, a general approach for developing translation-regulating riboswitches in prokaryotes involving inserting aptamers upstream of the

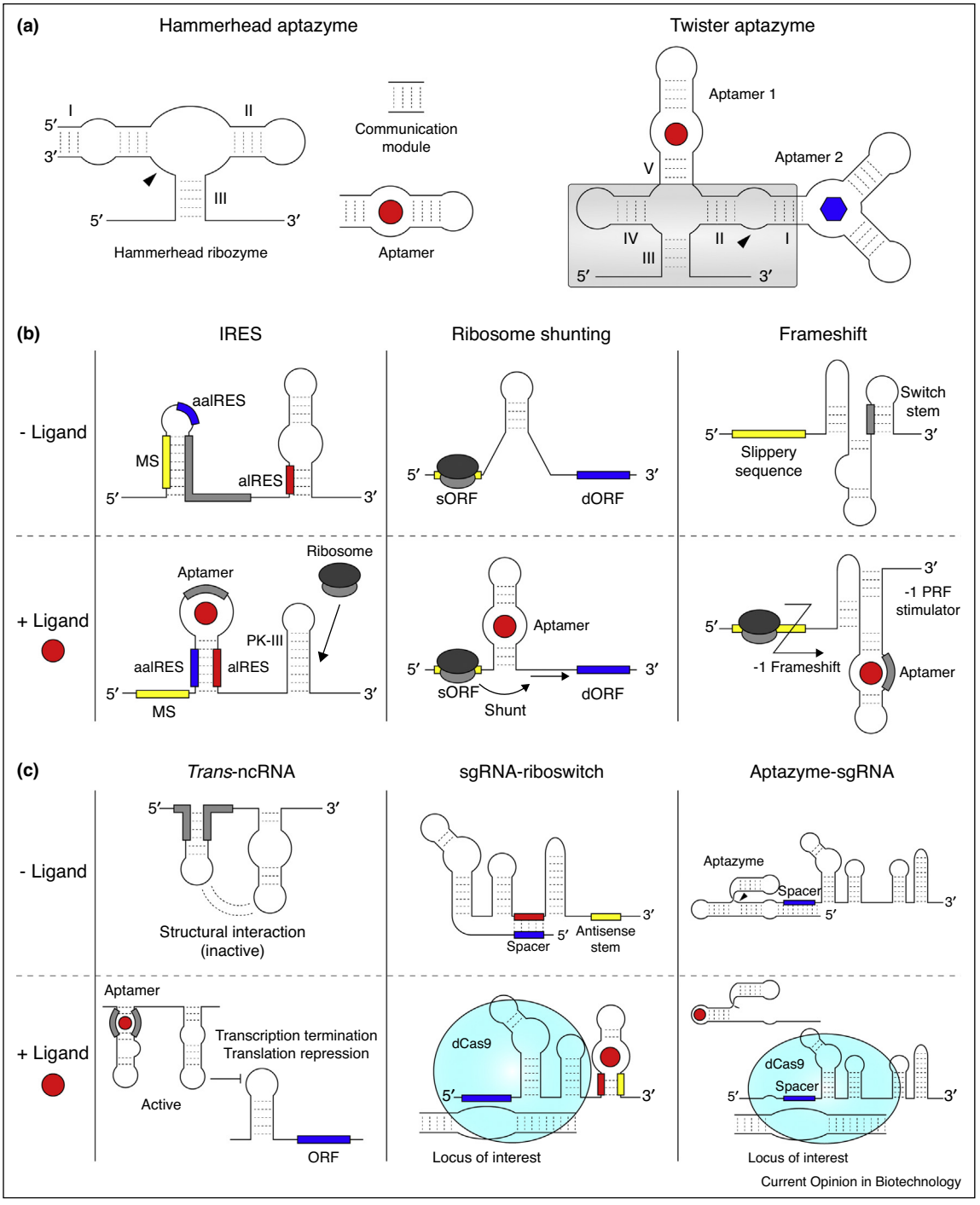
Figure 1



Re-engineering of natural riboswitches. Shaded box, randomized nucleotides; yellow circle, natural ligand; blue and red polygons, synthetic ligands. **(a)** Natural riboswitches are re-engineered to orthogonally detect synthetic ligands. Specific nucleotides are chosen based on crystal structure and fully mutagenized. Orthogonal riboswitches are identified from *in vivo* screening against synthetic analogues. **(b)** Natural aptamers are utilized as scaffolds for *in vitro* selection. Nucleotides at the three-way junction of natural aptamers are fully randomized and synthetic aptamers are evolved from *in vitro* selection. Evolved aptamers retain good *in vivo* functionality. **(c)** Expression platforms of natural riboswitches are re-engineered to construct modular platforms. A boundary between an aptamer and expression platform of a natural riboswitch is determined. Isolated expression platform is re-engineered and combined with numerous aptamers to create novel chimeric riboswitches.

ribosome binding site is not applicable to eukaryotes. Thus, alternative mechanisms were exploited. For example, internal ribosome entry site (IRES) was utilized to rationally design riboswitches that activate or repress gene expression [20,21]. Aptamers were placed upstream of IRES and several base-pairing sequences were designed to control the formation of critical structures (PK-III) depending on ligand binding (Figure 2b).

Figure 2



RNA-based regulatory mechanisms for developing new dynamic controllers. Red circle and blue hexagon, ligands; black triangle, self-cleavage site. **(a)** Self-cleaving ribozymes are fused with aptamers to form aptazymes. Hammerhead ribozyme has three stems to which aptamers can be linked through a communication module. Twister ribozyme has two stems that can be combined with two distinct aptamers simultaneously to construct two-input logic gates. **(b)** Eukaryote-specific translation regulation mechanisms are exploited to create new dynamic RNA controllers. Left: Modulator sequence, anti-anti-IRES sequence (aaIRES), and anti-IRES sequence (aIRES) are designed to sequester the pseudoknot III (PK-III) which is an essential element of IRES system in the absence of ligand. When the ligand is bound to an aptamer, aaIRES and aIRES hybridize, forming PK-III. Middle: Ribosome cannot translate a downstream ORF (dORF) after translating a short ORF (sORF) in the absence of ligand. When the ligand is bound to an aptamer, an essential stem structure is formed, allowing the ribosome to shunt and reinitiate translation of the downstream ORF. Right: −1 programmed ribosomal frameshifting stimulator is formed when the ligand binds the aptamer. The ribosome shifts the reading frame at a slippery sequence. **(c)** *Trans*-acting dynamic RNA controllers. Left: A *trans*-non-coding RNA (ncRNA) is fused with an aptamer.

Another example utilized an unusual mechanism known as ribosome shunting. On a special mRNA, a ribosome that translated an upstream short open reading frame can bypass a strong stem structure and land on a specific site downstream to reinitiate translation of a downstream ORF. This stem was replaced with aptamers to control the formation of the stem in response to ligand binding [22] (Figure 2b). Ribosomal frame-shifting was also employed for dynamic control of the reading frame [23]. A theophylline aptamer was inserted along with a switch hairpin in the extra stem-loop of SARS coronavirus −1 programmed ribosomal frameshifting pseudoknot stimulator (Figure 2b). Binding of theophylline induced formation of a pseudoknot structure essential for −1 frameshifting, and the reporter gene in the −1 frame was translated.

Finally, *trans*-acting non-coding RNAs were also engineered. RNA molecules that regulate translation (IS10) or transcription (pT181) were combined with aptamers to control the formation of intramolecular structures depending on ligand binding [24] (Figure 2c). Additionally, aptazymes can be connected to *trans*-acting riboregulators that expose an activating seed region after ligand-dependent cleavage [25]. These chimeric non-coding RNAs dynamically controlled gene expression *in trans*. A revolutionary technology in life science and bioengineering is clustered regularly interspaced short palindromic repeats interference (CRISPRi) and activation (CRISPRa). These methods are widely utilized for genome-wide regulation because of the ease of designing spacer sequences to target nearly any chosen genes. Recently, several groups reported extended uses of CRISPRi and CRISPRa for ligand-dependent control of gene expression [26••,27•,28]. Aptamers or aptazymes were fused to single guide RNAs (sgRNAs) to form secondary structures that shield spacer sequences in the absence of ligands (Figure 2c). When bound to cognate ligands, these fusion sgRNAs adopted alternative structures or cleaved themselves, releasing the spacer sequences to interact with target loci. Using CRISPR technologies, these allosteric sgRNAs may substantially improve our ability to dynamically control gene expression.

De novo construction of RNA-based dynamic controllers

Computational design strategies

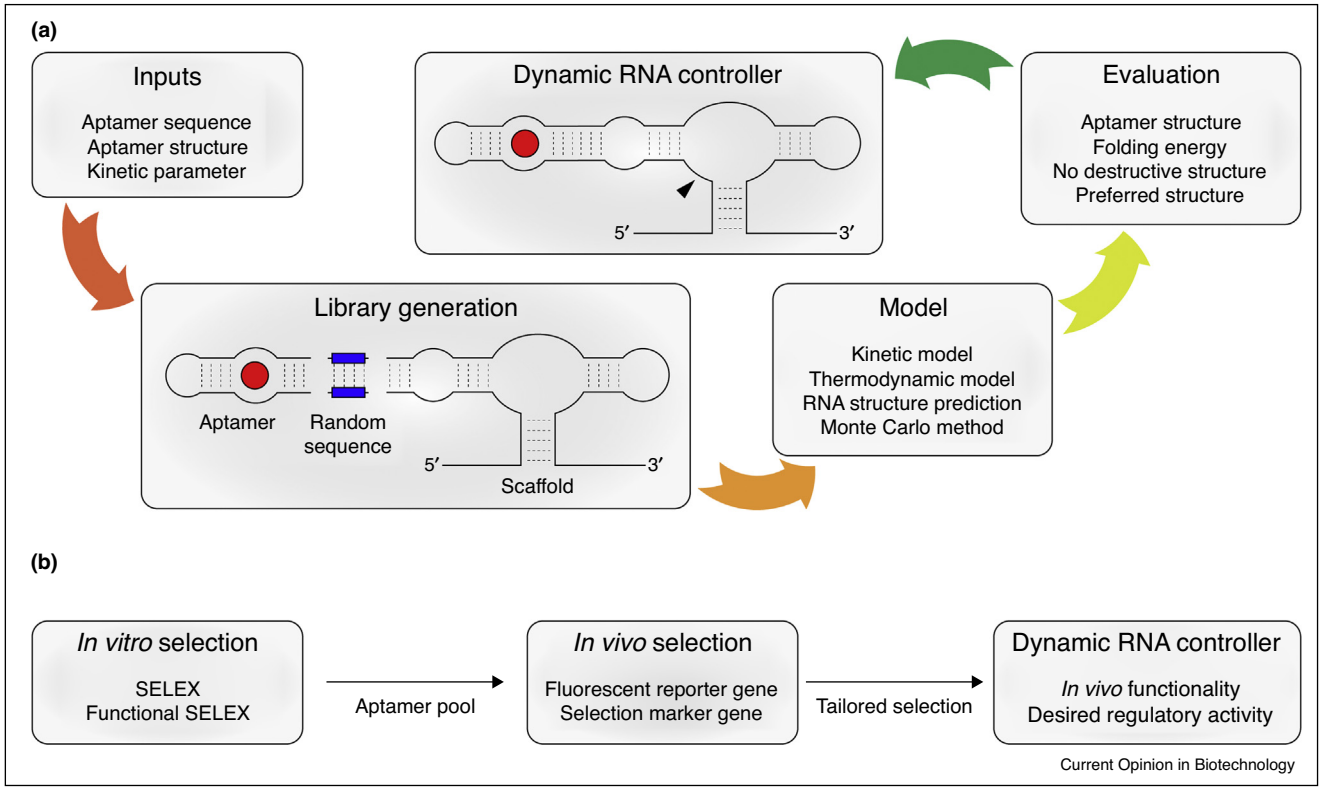
An emerging paradigm for the construction of dynamic RNA controllers involves computational design approaches based on mechanistic modeling and RNA structure prediction. Previous studies focused on developing mechanistic models for riboswitches [29] or aptazymes [30] to calculate regulatory outcomes from kinetic parameters governing discrete steps of regulation. These theoretical foundations were united with various algorithms to predict RNA structure [31–33], enabling the forward design of dynamic RNA controllers (Figure 3a). In general, specific regions in RNA controllers critical for ligand-induced regulation are randomized to generate sequence libraries. Next, computational methods predict the secondary structures of the sequence variants in the presence or absence of a ligand and screen them according to predefined criteria. The criteria include successful folding of the ligand binding pocket and rejection of any unwanted structures. Prior knowledge of the secondary structures of aptamers in their ligand-bound status and experimentally determined kinetic parameters can be reflected in the algorithm to enhance accuracy.

Using this approach, *de novo* design of artificial riboswitches was successfully demonstrated. One *in silico* design method was used to create bacterial riboswitches that control transcription termination in response to theophylline and tetracycline [34•,35]. This result demonstrated the power of computational methods, given that the transcriptionally controlled riboswitch was not previously achieved through high-throughput *in vivo* screening. In another study, 62 translationally controlled riboswitches were designed based on a statistical thermodynamic model using six different aptamers [36••]. In addition to the riboswitch secondary structure, this model incorporated interactions between the RNA and ribosome to predict the translation initiation rate to accurately predict the expression level.

In addition, artificial aptazymes were designed using computational methods. A pioneering work predicted the output expression levels of aptazymes through random sampling of kinetic parameters of a mechanistic model using the Monte Carlo method [37]. This model was utilized together with kinetic RNA folding simulations for designing new aptazymes. Another study showed that the model predictions improved by incorporating rate constants for a binding reaction measured *in vitro* [38]. Rather than constructing complicated mechanistic models, prediction of RNA secondary structures could be solely utilized [39]. Here, a random search algorithm based on an equilibrium partition function was applied to calculate the probabilities to form two distinct regulatory states, ON and OFF, for each aptazyme candidate sequence. Finally, aptazyme-based *trans*-acting

(Figure 2 Legend Continued) *Trans*-ncRNA is inactive when ligand is absent because of intramolecular interactions. When the ligand is bound to the aptamer, an active stem-loop structure is formed that can terminate transcription or repress translation. Middle: A single guide RNA (sgRNA) is inactive in the absence of ligand because its spacer sequence hybridizes with an antisense stem. When the ligand is bound to an aptamer, the spacer is released and sgRNA represses gene expression in combination with a catalytically inactive Cas9 (dCas9). Right: An aptazyme is attached to the 5′-end of a sgRNA to sequester a spacer sequence in the absence of ligand. When the ligand is bound to the aptamer, the aptazyme cleaves itself, leaving a free spacer sequence that can repress gene expression.

Figure 3



De novo construction strategies for dynamic RNA controllers. **(a)** General flowchart of computational methods for designing dynamic RNA controllers. **(b)** Coupled *in vitro–in vivo* selection strategy for efficient development of dynamic RNA controllers that can function *in vivo*.

riboregulators were designed using a computational technique [25]. A simple energy model was developed to calculate and minimize the energy for each regulatory step. The aptazyme-riboregulator fusion molecules cleaved themselves upon binding of the ligand and bound to the target site to activate gene expression.

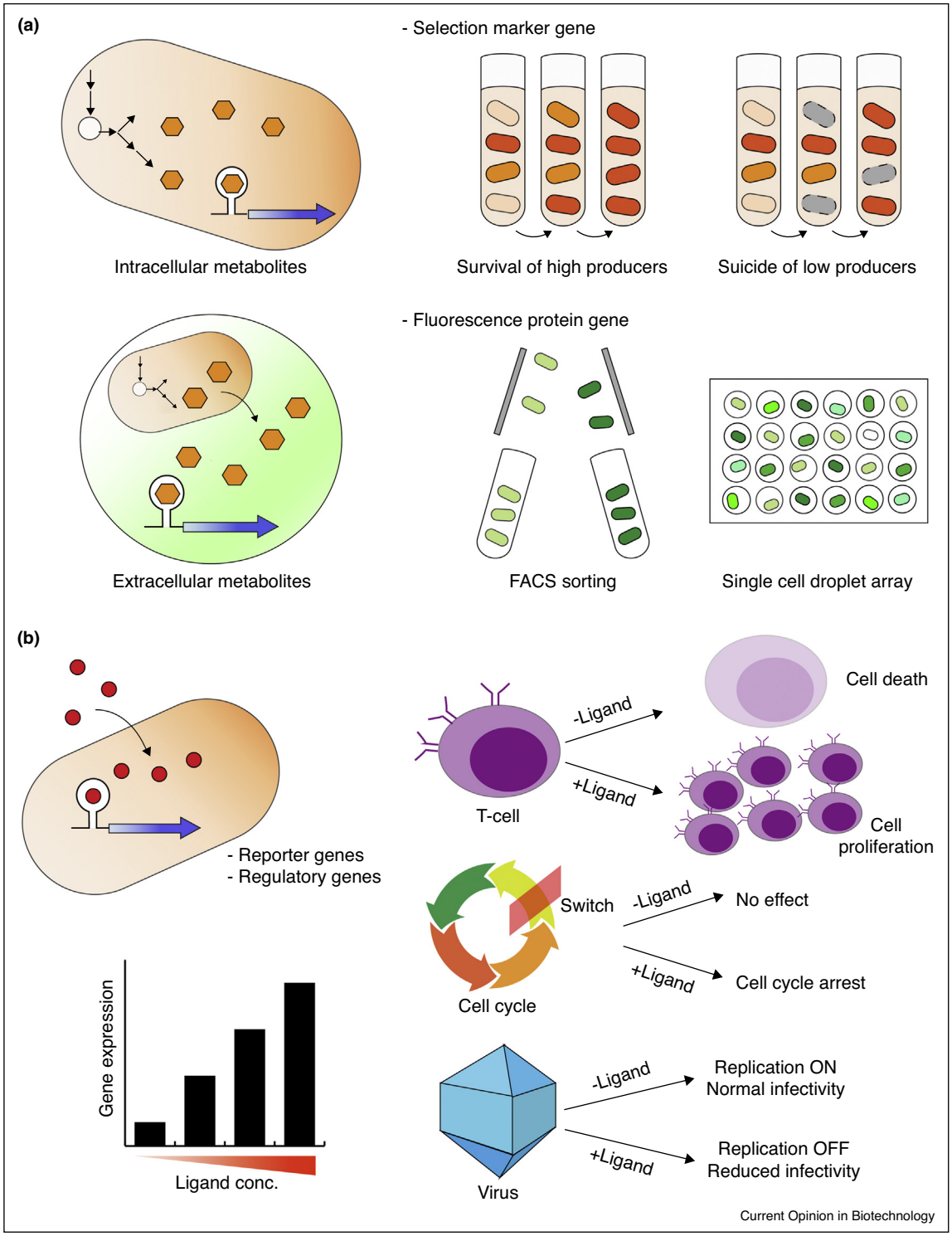
Computational methods can significantly accelerate the development of novel RNA-based dynamic controllers. These methods can screen extremely large sequence spaces compared with *in vitro* and *in vivo* selection methods. Additionally, riboswitches for ligands not readily transported across the cell membrane or that show toxicity to cells can be designed by computational methods. Finally, new design principles can be deduced from *in silico* screening.

However, there are some limitations to computational methods. These methods rely on structure-prediction algorithms to identify structural requirements and calculate free energies. However, current algorithms are limited in accurately calculating the contributions of pseudoknots and non-Watson-Crick base pairings to the overall structure [40]. Additionally, binding of a ligand to an RNA molecule can induce substantial structural rearrangements. Furthermore, newly developed aptamers may lack detailed data such as binding kinetics or secondary structures, which are important inputs for computational methods. Therefore, empirical screening methods can complement the limitations of computational methods.

In vitro and *in vivo* selection strategies

One of the long-standing difficulties in developing ligand-responsive RNA controllers is how to select RNA aptamers with switching activities *in vivo*. It is widely accepted that tight binding of an RNA aptamer to its target ligand does not necessarily guarantee switching activity *in vivo*. To address this problem, ligand-dependent strand displacement activity was selected *in vitro* [41]. In this work, RNA variants that exposed a binding site for a short DNA fragment tagged with biotin in a ligand-dependent manner were selected. Alternatively, libraries of RNA aptamers can be tested directly *in vivo* for their functionality as riboswitches. A pool of RNA aptamers enriched *in vitro* for binding to neomycin B was cloned upstream of a reporter gene [42]. Aptamers with riboswitch activities were successfully identified by *in vivo* screening based on reporter expression. The capability of *in vivo* selection was further extended in a

Figure 4



Applications of dynamic RNA controllers. **(a)** High-throughput screening and selection of metabolite production. Intracellular or extracellular metabolites can be detected using dynamic RNA controllers. Selection marker genes can control the growth rates of producer cells depending on metabolite production. Otherwise, fluorescence protein genes can be used with a fluorescence-activated cell sorting or single-cell droplet array to screen highly productive strains. **(b)** Dynamic RNA controllers can regulate gene expression in response to externally added ligands. Expression level of the gene is determined from the concentration of the ligand. RNA-based inducible gene expression is utilized to control T-cell proliferation, mammalian cell cycle, and viral replication and infection.

recently published work utilizing a coupled *in vitro–in vivo* selection strategy [43•]. In this study, an aptamer library that binds to a flavonoid, naringenin, was cloned upstream of a dual selectable marker gene. The riboswitch library was subjected to two evolutionary routes that differed by the sequences of positive and negative selection and concentration of the target ligand during positive selection. Operational ranges of the evolved riboswitches highly depended on the selection conditions, indicating that *in vivo* selection can be utilized to adjust quantitative properties of dynamic RNA controllers. Using *in vivo* screening and selection platforms for genetic switch development in prokaryotes [44,45] and eukaryotes [12,14••], the coupled *in vitro–in vivo* selection strategy may facilitate the development of novel RNA-based dynamic controllers with user-defined quantitative properties (Figure 3b). Moreover, simultaneous utilization of high-throughput measurement of activity and next-generation sequencing analysis can not only screen and optimize dynamic RNA controllers, but also provide new design principles from sequence-structure-function relationships [14••,15].

Applications of RNA-based dynamic controllers

Dynamic RNA controllers are employed in many applications requiring detection of specific molecules or inducible control of gene expression. The strategies described in earlier sections were utilized to create application-specific controllers. Thus, RNA-based dynamic controllers for diverse ligands were connected to a variety of outputs including genes for selection markers, genes for fluorescent proteins, and essential genes.

RNA-based dynamic controllers are widely utilized for high-throughput screening and selection of metabolite-producing microbes because of their ability to detect various metabolites (Figure 4a). Natural or artificial riboswitches were placed upstream of selection marker genes to associate cell growth with the production of amino acids in *Escherichia coli* [46••] and *Corynebacterium glutamicum* [47]. Similarly, a suicide riboswitch was constructed to select glucosamine 6-phosphate in *Saccharomyces cerevisiae* [48]. Otherwise, single-cell screenings for metabolite production were demonstrated by combining fluorescence-activated cell sorting [49] or microfluidic static droplet array [50] with artificial riboswitches. Furthermore, an artificial flavin mononucleotide ribozyme was utilized for screening of vitamin B2-producing *B. subtilis* based on a co-culture system in nanoliter reactors [51]. Finally, artificial naringenin riboswitches were utilized to monitor metabolites in co-cultures of *E. coli* strains [52].

The rapid response, small size, and no requirements for protein effectors make RNA-based dynamic controllers attractive for inducible control of gene expression in diverse biological systems (Figure 4b). For example, artificial riboswitches were employed to construct orthogonally inducible expression systems in *E. coli* [6,53] and *B. subtilis* [6] using non-natural ligands. In mammalian cell systems, aptazymes were utilized to regulate cellular processes including proliferation of mammalian T-cells [54] and control the cell cycle [55] using an externally added chemical. Furthermore, aptazymes were introduced to viral genes for ligand-dependent control of replication of viral genome or infectivity of progeny [56•].

Conclusion and future directions

The discovery of natural ligand-inducible RNA elements has led to the development of artificial RNA controllers that sense new molecular signals using different approaches. A detailed understanding of the mechanisms and structures of natural riboswitches enabled re-engineering of ligand-binding pockets or expression platforms. Diverse RNA-based regulatory mechanisms were exploited in combination with naturally occurring aptamers or artificial aptamers to create dynamic RNA controllers. Mechanistic models for RNA-based regulations and algorithms for predicting RNA structure allowed establishment of computational methods for *de novo* design of dynamic controllers. Advances in *in vitro* and *in vivo* selection made the development of dynamic RNA controllers that functions *in vivo* much more achievable. Consequently, RNA-based dynamic controllers were implemented in various applications ranging from high-throughput screening of metabolites to inducible control of cellular processes.

Although significant progress has been made in designing dynamic RNA controllers, several improvements are required. First, efficient methods of discovering novel RNA-based regulatory elements are necessary because natural RNA controllers can be used as foundations for artificial controllers and provide additional design principles. High-throughput screening of novel regulatory elements based on next-generation sequencing [57], transcriptome-wide probing of RNA structures *in vivo* [58,59], or bioinformatics-based comparative genomics [60,61] were recently demonstrated. Next, prediction algorithms for RNA structure should be refined and improved considering that the regulatory activity of RNA is largely determined by its structure. For instance, accurate calculation of the contributions of pseudoknots, non-Watson-Crick base pairings, and ligand binding would considerably improve the accuracy of structure prediction and computational design of dynamic RNA controllers. Finally, *in vitro* and *in vivo* selections should be designed deliberately to complete the development process. The computational design approaches can narrow down the number of candidates for dynamic RNA controllers after extensive *in silico* screening of vast sequence spaces. *In vitro* and *in vivo* selections can then be performed to

empirically validate each design in a high-throughput manner in an environment where the dynamic RNA controllers should work. The potential of empirical selection should be further explored because the quantitative properties of RNA controllers can be modulated using well-designed *in vivo* selection schemes [43•]. Emerging approaches in the development of dynamic RNA controllers will expand the repertoire of genetic parts for applications in metabolic engineering and synthetic biology. Future innovations in the methods for discovering natural parts, predicting RNA structure, and empirically selecting candidates will dramatically improve the design process.

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