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A renaissance in RNA synthetic biology: new mechanisms, applications and tools for the future

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Since our ability to engineer biological systems is directly related to our ability to control gene expression, a central focus of synthetic biology has been to develop programmable genetic regulatory systems. Researchers are increasingly turning to RNA regulators for this task because of their versatility, and the emergence of new powerful RNA design principles. Here we review advances that are transforming the way we use RNAs to engineer biological systems. First, we examine new designable RNA mechanisms that are enabling large libraries of regulators with protein-like dynamic ranges. Next, we review emerging applications, from RNA genetic circuits to molecular diagnostics. Finally, we describe new experimental and computational tools that promise to accelerate our understanding of RNA folding, function and design.

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Introduction

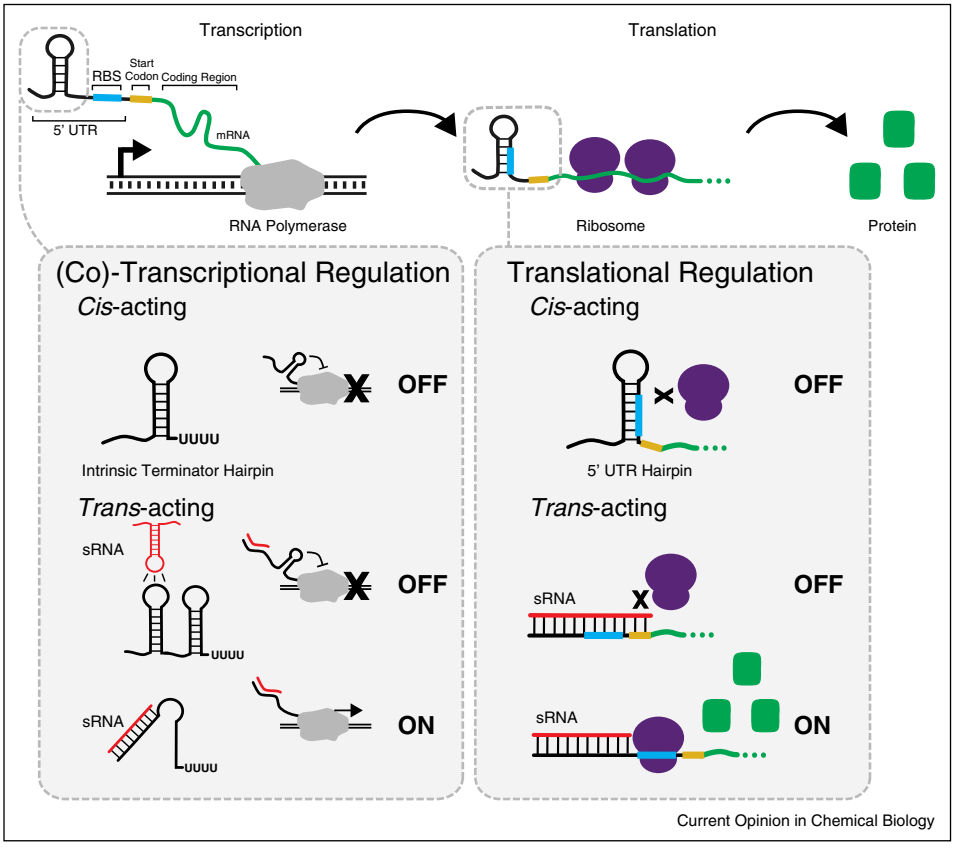
Many biotechnologies seek to harness the natural abilities of cells to produce compounds from renewable feedstocks and to sense and respond to changing environments. These natural cellular abilities are the result of networks of regulatory molecules that dynamically control the expression of specific genes in response to environmental cues. Therefore, our ability to engineer biological systems is directly related to our ability to control gene expression. Consequently, there has been a great deal of work within synthetic biology to develop versatile and programmable genetic regulators that enable the precise control of gene expression [1].

Remarkable progress has been made in creating libraries of genetic regulators that can be configured to control the expression of individual genes, or to implement advanced programs via genetic circuits [1]. Although synthetic RNA regulators were one of the early successes in this endeavor [2], historically there has been a greater emphasis placed on engineering protein regulators [3]. This is in part due to the large dynamic ranges of protein repressors and transcription factors, and the large repertoire of natural regulators to draw from [1]. Recently however, there has been a resurgence of interest in engineering and applying synthetic RNA regulators to control gene expression [4,5]. This has been motivated by our increasing appreciation of the widespread use of RNA regulation in natural systems [4] and our deepening knowledge of RNA folding and function.

RNA-mediated control of gene expression often involves the formation of specific structures within mRNAs, particularly in bacteria. For example, certain structures can regulate gene expression in *cis* by preventing transcription elongation, in the case of intrinsic terminator hairpins, or by preventing translation initiation by occluding ribosome binding sites (RBS) (Figure 1). Moreover, the formation of these *cis*-acting structures can also be regulated by interactions with *trans*-acting RNAs, creating genetic switches that are flipped at the RNA level. Although RNA secondary structures are potentially highly designable, being largely determined by Watson–Crick base-pairing of the four letter nucleotide code, the design of high functioning synthetic RNA regulators has historically been challenging.

In this review, we focus on recent advances in RNA synthetic biology that are transforming the way we think about using RNAs to engineer biological systems. We start by discussing new synthetic regulatory mechanisms and design strategies that have uncovered new classes of modular RNAs with protein-like performances. Next, we describe how RNA regulators are being applied to improve and deliver on new applications, ranging from metabolic engineering to new molecular diagnostics. Finally, we discuss how new high-throughput RNA structure characterization experiments, and computational RNA design tools, are helping unlock the RNA sequence–structure–function relationship for further engineering of this powerful molecule.

Figure 1



RNA structures regulate gene expression. Dashed boxes highlight examples of how RNAs can regulate transcription and translation in *cis* and *trans* in bacteria. Formation of intrinsic transcription terminator hairpins (co-transcriptionally) in 5′ untranslated regions (5′ UTRs) of mRNAs result in premature termination of transcription elongation (gene expression OFF). Hairpins in 5′ UTRs can also sequester ribosome binding sites (RBS), preventing ribosome binding and translation initiation (gene expression OFF). Both of these *cis*-acting RNA structures can be regulated by interaction with *trans*-acting sRNAs that allow or prevent the *cis*-acting RNA structures to form. These sRNAs thus flip RNA genetic switches and can be used to turn gene expression OFF or ON.

New RNA regulatory mechanisms solve key challenges and create new capabilities

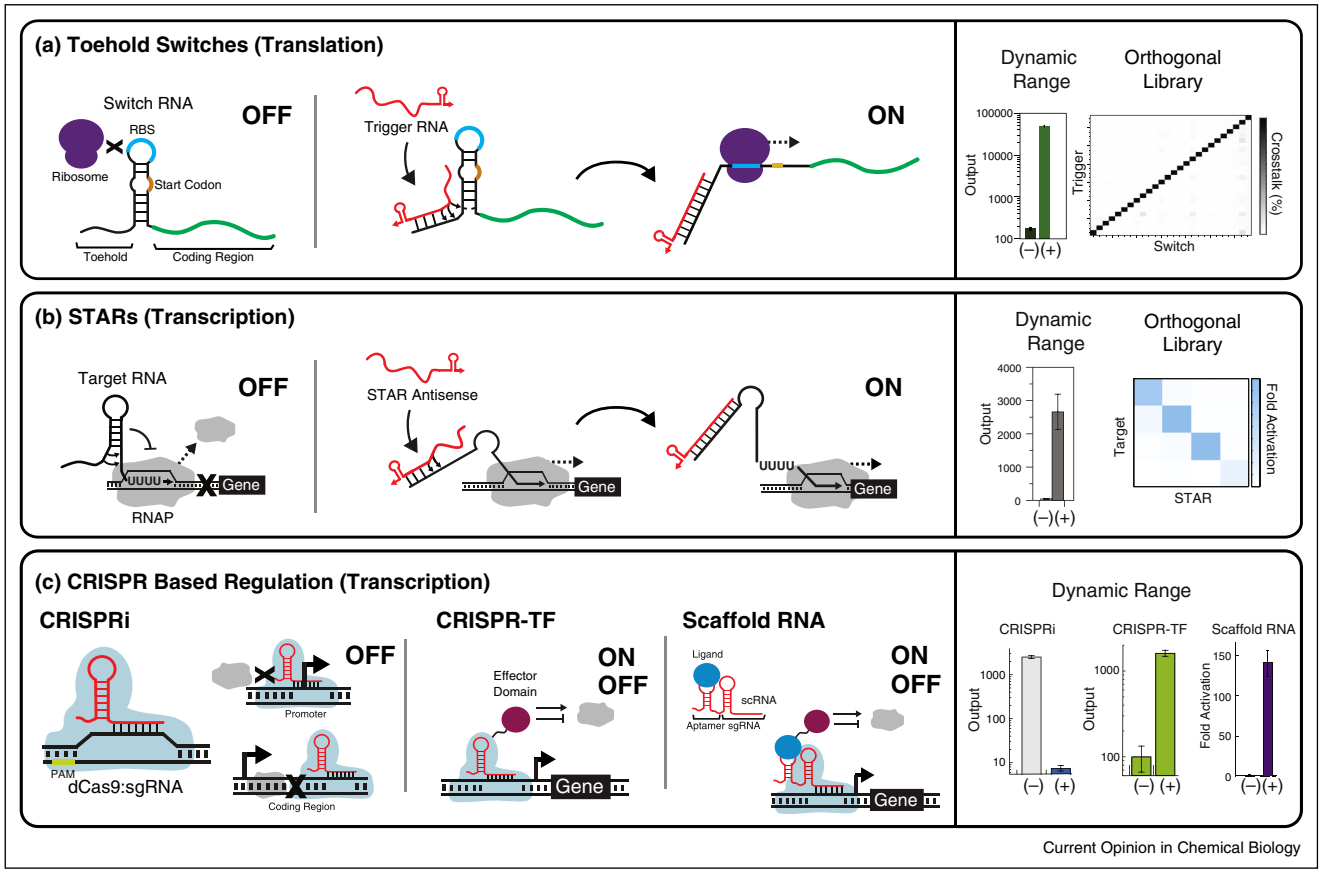
The resurgence of RNA synthetic biology has brought about a host of new RNA regulators that control transcription [6], translation [7,8], and mRNA degradation and processing [9,10]. RNA regulatory switches that respond to a range of small molecules, RNAs, and proteins have also been engineered [9–11]. While these examples highlight the versatility and designability of RNAs, RNA regulators have typically lagged behind protein regulators in terms of dynamic range and the number of independently acting, or orthogonal, regulators available for genetic circuit construction [1]. Recently, exciting new RNA-based regulatory mechanisms have been engineered that overcome these limitations.

Toehold translational switches — rethinking riboregulator design principles to enable protein-like dynamic ranges

Synthetic RNA translational activators called 'riboregulators' were one of the first synthetic regulatory RNAs described [2] and have been used in applications ranging from biocontainment [12] to probing cellular RNA folds [13]. Inspired by natural bacterial small RNA (sRNA) regulators, riboregulators use designed RNA hairpin structures to block translation in *cis* by sequestering the RBS of a gene. Translation is activated through the binding of a *trans*-activating RNA, which removes the structural blockade of the hairpin on the RBS. Although an early success, riboregulators displayed modest dynamic ranges (19-fold), and only two orthogonal variants were constructed [2].

Recently, Green *et al.* re-designed the basic riboregulator mechanism to include an innovative 'toehold' strategy to more efficiently propagate the RNA–RNA interactions needed for translational activation [14••]. The toehold strategy [15] works by sequestering most of the riboregulator region in a designed hairpin, leaving only a small linear 'toehold' region exposed (Figure 2). This toehold region is designed to seed the interaction with the invading *trans*-activating RNA called a trigger RNA. Once

Figure 2



Advances in synthetic RNA regulators of gene expression. Mechanisms of three new classes of synthetic RNA regulators, with representative functional data showing dynamic ranges and orthogonal libraries. **(a)** Toehold switches re-design riboregulators to activate translation in response to a *trans*-acting sRNA called the trigger RNA. Switch RNAs are designed to form hairpins to repress ribosome binding and translation initiation. Trigger RNAs open this structure by first binding to a linear toehold region and then proceeding to unzip the hairpin, releasing the ribosome binding site (RBS). Dynamic range and orthogonality data adapted from [14••]. **(b)** Small transcription activating RNAs (STARs) are a new synthetic transcription activation mechanism. Target RNAs are designed to form an intrinsic terminator hairpin upstream of a coding region to be regulated. The terminator hairpin co-transcriptionally folds and prevents transcription elongation into the gene. STAR antisenses are designed to bind to the 5′ half of the intrinsic terminator hairpins, preventing terminator formation and allowing transcription elongation of the downstream gene. Dynamic range and orthogonality data adapted from [17••]. **(c)** CRISPR interference (CRISPRi) gene regulation uses a catalytically dead version of the Cas9 (dCas9) endonuclease and a chimeric small guide RNA (sgRNA) to bind DNA at either promoter or coding regions to repress transcription initiation or elongation. CRISPR transcription factors (CRISPR-TFs) and scaffold RNAs (scRNA) use fusions to the dCas9 or the sgRNA, respectively, to localize functional protein domains, such as effector proteins that can additionally regulate gene expression. Dynamic range data is adapted from [22••,24,28••].

seeded, the trigger invades the hairpin, opening it and triggering translation activation. The elegance of the toehold motif [15] allowed Green *et al.* to use the Nucleic Acids Package (NUPACK) [16] to design a library of 168 toehold switches. NUPACK is a suite of computational tools that can be used to design RNA sequences that fold into user specified structures and complexes [16]. Green *et al.* further used NUPACK to identify a set of 26 highly orthogonal switches that were later confirmed experimentally. Further design refinements resulted in 13 variants that showed up to 400-fold dynamic range. Finally, the high level of orthogonality permitted independent regulation of 12 genes simultaneously in the same cell, as well as the construction of genetic logic gates.

STARs — addressing a natural gap in regulation

Bacterial sRNAs are naturally diverse regulators of gene expression. However, to date no naturally occurring sRNA transcriptional activators have been identified, leaving a crucial gap in sRNA regulatory function. Recently, this gap was addressed with the creation of small transcription activating RNAs, or STARs [17••]. The STAR mechanism is based on modulating intrinsic terminator hairpin structures which, when formed, prevent downstream transcription (Figure 1). *Trans*-acting STAR

antisense RNAs are able to activate transcription by preventing the formation of terminator hairpins placed in the 5′ untranslated region (UTR) of a coding region [17••] (Figure 2). This STAR mechanism enabled the construction of numerous variants, with one showing 94-fold activation. Moreover, these STARs were shown to be highly orthogonal to each other as well as a library of RNA-mediated transcriptional repressors, or attenuators [6,17••,18]. STARs were also shown to be directly composable, meaning that they can be fused to each other, or to transcriptional repressors, to create higher-order functions such as new types of RNA-only transcriptional logic gates [17••,18]. Design rules were established as well, opening up the possibility for *de novo* design of STAR regulators that can leverage the large libraries of natural and synthetic transcriptional terminators that have recently been developed [19,20].

Repurposed CRISPR systems — the best of RNAs and proteins

For many years, synthetic biologists interested in designing regulators were faced with a tradeoff between ease of design and regulatory performance. While RNA regulators tended to be easier to design, they often had weaker binding, leading to smaller dynamic ranges than their protein regulator counterparts. Recent breakthroughs in the creation of the Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) interference (CRISPRi) mechanism combine the best of both worlds in a hybrid system that marries the simplicity of RNA design with the binding properties of proteins.

The CRISPRi system is derived from the type II *Streptococcus pyogenes* CRISPR system and consists of two components: a chimeric RNA named the small guide RNA (sgRNA) and a catalytically dead mutant of the Cas9 endonuclease protein (dCas9) (Figure 2) [21,22••]. Once assembled, the dCas9–sgRNA complex acts as an RNA-guided DNA binding protein, using a portion of the sgRNA to target complementary DNA sequences next to a three nucleotide protospacer adjacent motif (PAM) sequence. The sgRNA provides recognition by Watson–Crick base-pairing and can be easily designed to target promoters or open reading frame (ORF) regions to repress transcription up to 300-fold in bacteria (Figure 2) [21,22••]. The dCas9–sgRNA system is also amenable to creating fusions with additional protein and RNA modules, allowing for highly programmable genomic localization of these modules and the functions they offer. For example, fusion of protein effector domains to dCas9 has created a whole family of CRISPR/dCas9 transcription factors (CRISPR-TFs) able to activate and repress transcription in an ever increasing range of organisms [23,24]. Fusion of dCas9 to fluorescent or light-sensing proteins has also created a novel *in situ* imaging platform [25] and an optogenetic control mechanism for CRISPRi regulation [26,27]. Further functions can also be added by fusing multiple protein-binding aptamer sequences to the sgRNA for genomic localization of multi-protein complexes [28••]. In addition, the emergence of sgRNA design rules is promising to accelerate the development of CRISPRi even more [29]. Recent work is also showing that other CRISPR/Cas systems can be engineered, such as the endogenous type I-E system of *E. coli* [30•], to create a diverse range of CRIPSR/Cas-based regulators, each with unique design principles and advantages.

New applications for RNA regulators

Synthetic biologists are increasingly turning to RNA regulators to control gene expression for a range of applications. While these applications are varied, we highlight significant progress in using RNAs to construct genetic circuits, optimize metabolic pathways, and create new types of molecular diagnostics.

RNA-based genetic circuits

Genetic circuits are core elements of the cellular information processing system. They allow cells to respond to changing environments by sensing, integrating, and processing signals to make decisions that change cell state. With the emergence of large libraries of orthogonal and composable RNA regulators, a new paradigm for engineering RNA-based genetic circuits is emerging. Recently, Nielson *et al.* used CRISPRi (Section 'New RNA regulatory mechanisms solve key challenges and create new capabilities') to construct a series of NOT and NOR gates that invert transcriptional inputs with >50-fold dynamic range (Figure 3) [31•]. They also showed that CRISPRi logic gates could be configured to control the expression of additional sgRNAs, allowing layering of gates to execute more complex genetic logic. CRISPRi can also be configured into transcriptional cascades that propagate signals as sgRNAs [32,33], further demonstrating CRISPRi's use for creating synthetic genetic circuits.

The recent development of RNA-only genetic circuitry [18] has also sparked investigation into its potential

(Figure 3 Legend Continued) shows an RNA-only transcriptional cascade composed of two orthogonal transcriptional repressors (attenuators) [35]. Since the circuit signal is propagated by RNA species, dynamics are fast with response times in cell-free transcription and translation (TX–TL) reactions on the order of 5 min. **(b)** Left of panel shows how the RBS Library Calculator can be used to efficiently explore the potential expression space of three enzymes of the carotenoid biosynthetic pathway. Data adapted from [36•] shows predicted translation rates mapped onto production rates (blue low, red high) of the carotenoid pathway. Right of panel shows how naturally occurring sRNAs can be used as scaffolds to rationally design synthetic sRNAs that target endogenous genes in *E. coli* to improve yields of metabolic pathways [40•]. **(c)** Paper-based diagnostics use freeze-dried, TX–TL reactions to report on analyte presence. In the example shown, toehold switches were expressed to detect trigger RNAs designed from an Ebola virus mRNA and display a colorimetric output. Data adapted from [42••].

advantages over protein-based circuitry for several reasons. First, RNA circuitry has the potential to be compact (RNA regulators are typically 10–100s nucleotides long) which permits rapid construction [31•], reduces cellular burden, and aids efficient delivery of circuits into mammalian cells [33]. Second, RNA regulators have been shown to be highly composable, which simplifies the design of RNA-only circuits [14••,17••,18] and allows easier integration with proteins to create protein–RNA hybrid circuits [34]. Finally, RNA circuits have the potential to propagate signals much faster than protein circuits, since signal propagation speed is determined by the fast degradation rates of RNAs. This was recently demonstrated by Takahashi *et al.* for a transcriptional cascade composed of two layered sRNA transcription repressors (Figure 3) [35].

RNA regulators for metabolic engineering

Metabolic engineering typically requires many rounds of optimization to maximize pathway productivity and yield. Often this process focuses on genetic optimizations to fine-tune enzyme expression levels and increase flux through the desired pathway, while minimizing flux through competing pathways. However, this is often problematic because of the vast multi-dimensional expression space that needs to be screened and the lack of regulators that can cover the necessary range of expression levels. Recently, Farasat *et al.* addressed these challenges by utilizing the RBS Library Calculator to optimize flux through the carotenoid biosynthetic pathway (Figure 3) [36•]. The RBS Library Calculator uses RNA folding algorithms to design RNA sequence/structure contexts around RBSs to give predictable protein expression levels. With this tool, Farasat *et al.* designed a set of 73 RBS variants for three enzymes of the carotenoid biosynthetic pathway. This allowed optimal expression regimes to be identified by efficient sampling of enzyme expression space [36•]. Moving forward, incorporation of dynamic regulation, as well as harnessing ligand-sensing RNAs for regulatory feedback, is likely to yield further gains as a recent theoretical study demonstrated [37].

RNA regulators have also been shown to be adept at minimizing the effects of competing pathways from the host strains. Numerous studies have highlighted the use of RNA interference (RNAi) in eukaryotes [38,39] and sRNAs in prokaryotes [40•,41] for this purpose. For example, Na *et al.* showed that natural sRNAs can be re-engineered to repress endogenous genes individually or in combination to increase flux and yield of tyrosine and cadaverine biosynthetic pathways (Figure 3) [40•].

New paper-based molecular diagnostics

Natural biological systems use molecular machinery to sense a vast range of chemical, biological, and environmental cues. This machinery can be engineered to create biosensors that report on the environment through the expression of measurable reporter genes. While promising, biosensing applications have been hindered partly because of issues related to the safety and practicality of using genetically modified organisms in the field. However, a new *in vitro* molecular diagnostic platform has the potential to open up this application space, translating biosensing from the lab to the field [42••].

At the heart of this new platform are *in vitro* cell-free transcription and translation (TX–TL) reactions that can express genetically encoded biosensors [43]. TX–TL reactions consist of a buffered cell lysate that contains gene expression machinery that can transcribe and translate genes encoded in a supplied DNA template. TX–TL reactions have been shown to robustly express a range of complex genetic circuits [43]. Recently, Pardee *et al.* showed that TX–TL systems could be made into a robust diagnostic platform. They demonstrated that freeze-dried TX–TL reaction components can be stored on filter paper for up to a year before rehydrating with an aqueous solution to activate expression [42••]. In this way, DNA-encoding biosensors and TX–TL machinery can be easily stored and later activated to report on the presence of analytes through expression of a colorimetric output (Figure 3). One powerful example of this new platform was the use of toehold riboregulators (Section 'New RNA regulatory mechanisms solve key challenges and create new capabilities') to sense trigger RNAs derived from Ebola mRNA sequences on the paper-based system (Figure 3). The flexibility of the toehold switch design, in combination with the low cost of these reactions (as low as two cents per reaction), offers a unique opportunity to rapidly develop and distribute low-cost, easy to use diagnostics, while avoiding many of the biocontainment and safety concerns surrounding traditional cell-based biosensors.

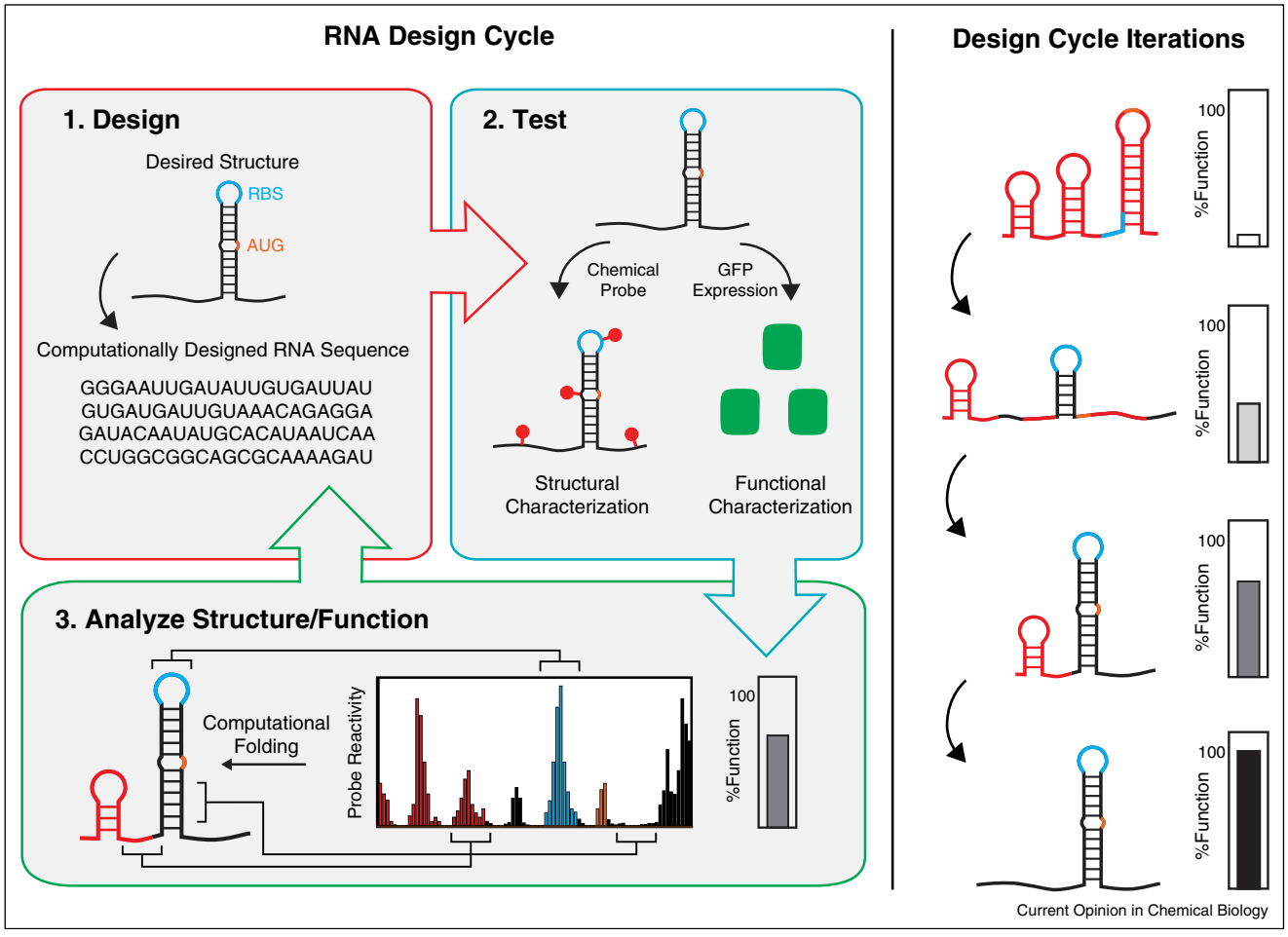
New tools and technologies to accelerate RNA engineering

The power of engineering with RNA lies in our ability to predictably alter its function by designing RNA structures. This ability is accelerating with the advent of new experimental tools that allow high-throughput characterization of RNA structures in their native cellular environment and new computational tools for modeling and designing RNA structures for precise gene regulation (Figure 4).

New experiments that characterize cellular RNA folding

A key component of any engineering discipline is the ability to characterize and understand why certain designs fail, leading to refinement of design principles and more efficient engineering (Figure 4). When engineering RNA, characterizing the RNA structure–function relationship is crucial. One tool available to RNA engineers is chemical probing, which provides quantitative information about RNA structure by using a chemical reaction to covalently

Figure 4



The emerging RNA design cycle. An RNA design cycle of the future that incorporates the measurement and modeling of RNA structure/function for rapid discovery of RNA design principles and efficient *de novo* design of RNA regulators. Computational design algorithms such as NUPACK [16] can be used to suggest candidate RNA sequences that fold into designed RNA structures. The cellular functions and structures of these candidates can be rapidly characterized with fluorescent gene expression assays and new high-throughput chemical probing methods. Chemical probing works by modifying RNAs in a structure-dependent manner. Modification positions can be determined with NGS to yield probe reactivity maps [45]. High levels of reactivity indicate unstructured regions of the RNAs and low levels of reactivity indicate base-paired regions or other molecular interactions with the RNA. The probe reactivity maps can also be used to refine computational RNA folding algorithms, giving more accurate models of cellular RNA folding [56•]. In this way, measurement of RNA structure and function can be directly linked, allowing RNA design principles to be discovered and incorporated into *de novo* RNA design algorithms.

modify RNA in a structure-dependent fashion. Chemical probing can be used to characterize RNA structures as they exist in the cellular environment [44], and recent efforts have brought this type of RNA structure probing into the '-omics' age by mapping the modification positions with reverse transcription and next-generation sequencing (NGS) [45] (Figure 4). The multiplexing capability of NGS has since led to the characterization of the 'structurome' of *Saccharomyces cerevisiae* [46,47], *Mus musculus* [48,49] and *Arabidopsis thaliana* [50]. These techniques are enabling the creation of large databases of information about RNA structure [51], affording an unprecedented view of cellular RNA folding that will allow the design principles of natural and synthetic RNA regulators to be uncovered.

Advances in computational RNA design

There is a rich history of computational RNA folding algorithms that can predict the properties of RNA structures from their sequence [52]. These algorithms typically serve as a powerful starting point for RNA engineering by allowing researchers to screen RNA designs *in silico* before testing *in vivo*. Recent advances in our knowledge of RNA structure–function design principles have accelerated the development of new computational tools that use these algorithms to design new RNA-based regula-

tors. For example, recent updates to the RBS Calculator now include a more rigorous treatment of structure/sequence contexts in the 5′ UTR around the RBS [53]. New tools have also been developed that design RNA regulators *de novo* to control transcription [54] or translation [8] by responding to a small molecule or RNA, respectively. Computational modeling of RNA–ligand interactions is also becoming more established [55] and will further the engineering of small molecule responsive RNA switches. Finally, improvements in using experimental RNA characterization data to constrain RNA folding algorithms [56•], and new insights gained from crowd-sourced RNA design challenges [57], promise to help usher in a new era of RNA synthetic biology driven by rational RNA design [16] (Figure 4).

Conclusion

RNA synthetic biology is transitioning into a period of rapid growth. Newly discovered design principles have led to well-characterized RNA regulators with functional dynamic ranges of proteins and the compactness and composability of RNAs. Application of new high-throughput RNA structure characterization measurements promise to vastly expand our basic knowledge of the RNA sequence–structure–function relationship, which can then be encoded into computational RNA design algorithms for rapid RNA engineering. Progress in this area has already led to RNAs being used in a variety of application spaces, ranging from metabolic engineering to new types of molecular diagnostics. The confluence of these trends promises to establish the foundations of a discipline of RNA engineering that will find increasing utility for engineering and understanding biological systems.

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