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Title

Using RNA as molecular code for programming cellular function

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Abstract

RNA is involved in a wide-range of important molecular processes in the cell, serving diverse functions: regulatory, enzymatic, and structural. Together with its ease and predictability of design, this lends it to become a useful handle for biological engineers with which to control the cellular machinery. By modifying the many RNA links in cellular processes, it is possible to re-program cells towards specific design goals. We propose that RNA can be viewed as a molecular programming language that, together with protein-based execution platforms, can be used to re-write wide ranging aspects of cellular function. In this review, we catalogue developments in the use of RNA parts, methods, and associated computational models that have contributed to the programmability of biology. We discuss how RNA part repertoires have been combined to build complex genetic circuits, and review recent applications of RNA-based parts and circuitry. We explore the future potential of RNA engineering and posit that RNA programmability is an important resource for firmly establishing an era of rationally designed synthetic biology.

Introduction

Many diverse roles of RNA in organisms from all kingdoms and lifestyles have been uncovered in the last few decades[1–6]. Consequently, the understanding of RNA has transformed from that of primarily an informational molecule to that of one with diverse functions: regulatory, enzymatic, and structural[7–12]. RNA's involvement in all major molecular processes of the cell– including replication, transcription, and translation– allows it to control various aspects of genetic information processing and function. This, in turn, enables synthetic biologists and biotechnologists to use RNA as a tool for reprogramming cellular machinery towards a range of metabolic, diagnostic, therapeutic and environmental applications[13–16].

Central to our ability of being able to design RNA tools for biological engineering is the fact that intra- and inter-molecular RNA interactions, as well as DNA:RNA interactions, follow simple base-pairing rules that can be used to computationally predict cis- and trans- secondary structures for a given sequence using free-energy minimization algorithms[17–19]. Conversely, it is also possible to design by inverse folding RNA sequences that meet specific structural constraints[20–22]. The available set of computational methods has successfully bridged the gap between sequence and RNA secondary structure, although its *de novo* tertiary structure prediction still remains a challenge[23,24]. A range of RNA molecules with defined biomolecular function, such as transcription termination or catalytic cleavage, are now included in libraries of standard biological parts fundamental to synthetic biology[25]. Fortunately, many RNA-based regulatory functions of these parts depend on secondary structural interactions, or can be abstracted as such, placing their manipulation firmly within our reach. We can introduce specific interactions or eliminate undesirable ones, thereby achieving modularity and orthogonality of RNA parts, or creating allosteric

regions within a given part[26–31]. RNA predictive power is in stark contrast to *de novo* prediction of protein structure, much less protein:protein interactions or protein:nucleic acid interactions, computational solutions for which remain elusive or highly resource intensive[32–35].

The programmability of RNA has led to the development of a large repertoire of genetic parts, natural and synthetic, that can control wide-ranging functions within the cell[27,31,36–38]. In addition, a number of advanced computational tools have been constructed that predict and design the functionality of these parts based on higher-order mathematical models incorporating RNA interaction energies[22,39–43]. In this review, we start with conducting an examination of the various RNA parts, methods and computational tools available for use in synthetic biology. We elucidate their design principles, and consider their input-output potential for interfacing with each other in multi-component circuitry for onward information processing and signal conversion. Next, we look at examples of successful circuit design using one or more RNA part types together. Finally, we present a range of applications facilitated by RNA parts and circuits, focusing on those that exploit the "programmable" features of RNA, and expand on the challenges and potential for the future.

RNA Parts and Tools

Large libraries of RNA parts affecting almost every step of biological control have become available in the last few years of synthetic biology. In addition to the amenability of programming, RNA parts also offer other advantages over protein effectors of biological function. Due to their higher turnover rates[44–46], RNAs offer faster regulation kinetics compared to proteins[47,48]. Although the metabolic burden of maintaining plasmids that code for synthetic parts would be similar for RNAs and proteins[49,50], protein overexpression is often accompanied by toxicity effects due to the

"protein cost" that depletes the cell of ribosomal resource[51–53]. Consequently, despite comparable output, genetic designs with higher mRNA transcription rates have better efficiency than those with higher protein translation rates since the latter divert more ribosomal resource[54]. RNA production being less costly allows the advantage of achieving higher abundance of effector RNAs relative to the target, in turn, reducing chances of retroactivity[55]. Here we discuss the key RNA parts and associated tools that have advanced RNA circuit programming to a whole new level[13,56] (Figure 1).

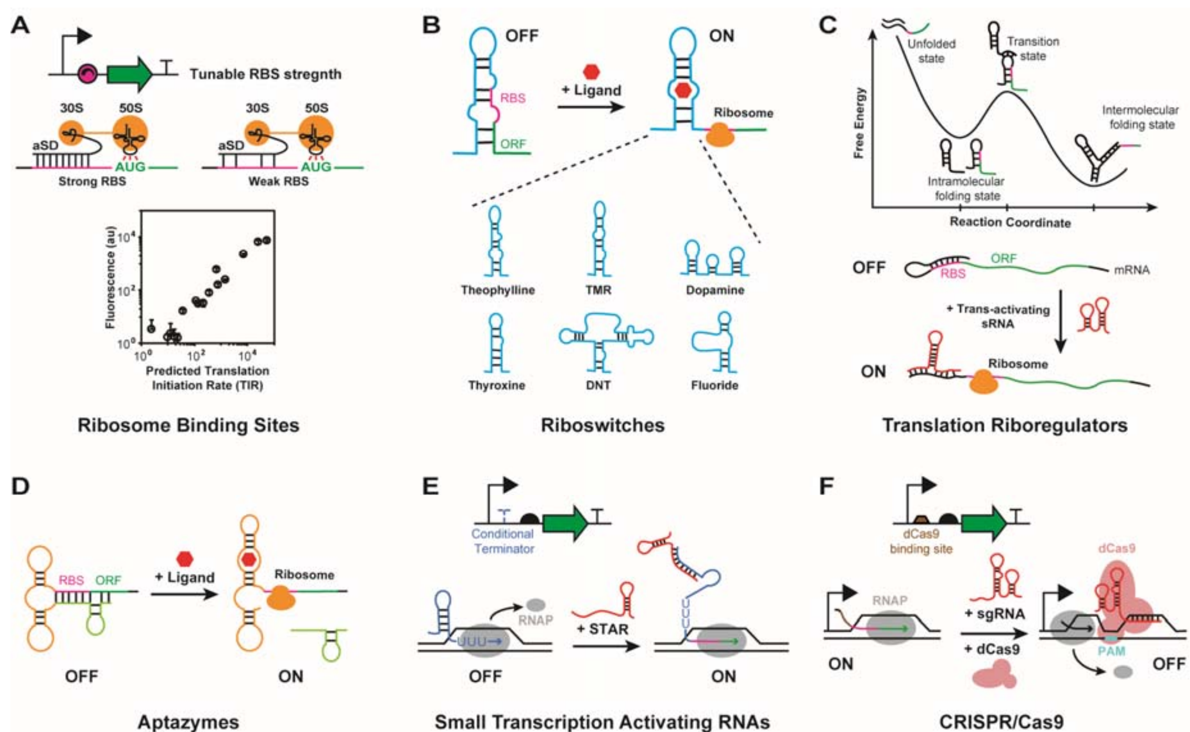

Figure 1. RNA parts can be used to regulate a wide range of genetic control functions. **(A)** Strength of interaction between the anti-Shine Dalgarno (aSD) sequence and the ribosome binding site (RBS) is an important determinant of translation initiation. By modelling these interactions, the RBS Calculator model can predict the TIR of mRNA ORFs across multiple species[40]. **(B)** Ligand-binding aptamers can be placed upstream of a gene of interest to create riboswitches, such that binding-induced conformational change alters access of the translation machinery to the gene's RBS. Shown below are the ligand-bound secondary structures of six aptamers[41]. **(C)** By modelling the energetics of RNA:RNA interaction, trans-activating small RNA regulators of translation can be designed computationally[22]. **(D)** Aptamers can be fused with ribozymes to create aptazymes, which can regulate translation of a downstream gene upon ligand binding. **(E)** Small transcription activating RNAs prevent transcription termination by disrupting the terminator hairpin loop that would otherwise cause RNAP dislocation[38]. **(F)** Binding of the dCas9 protein can also block transcription by preventing RNAP translocation along the DNA template.

Ribosome Binding Sites

Ribosomes are large multi-subunit ribonucleoproteins (RNPs) composed of ribosomal proteins and RNAs (rRNAs) that translate protein coding mRNAs. Although the tertiary structure of rRNAs forms the bulk of the ribosome[57], to the biological engineer interested in regulating protein expression, bacterial ribosomes can be abstracted to be represented by their anti-Shine Dalgarno (aSD) sequence, the 16S rRNA 3'end that interacts with the ribosome binding site (RBS) on the 5'UTR of the mRNA to form the translation initiation complex. By manipulating the interaction between the aSD and the RBS, the rate of binding of the ribosome to the mRNA, and hence the translation initiation rate (TIR) can be altered. This was previously achieved by mutating the RBS sequence towards a desired protein expression level[58].

Of late, *de novo* design of RBS sequences has been made possible by various computational tools that can predict the TIR of a given mRNA ORF as well as reverse engineer mRNA sequences for ORFs with a specified TIR. Two such tools, the RBS Calculator and the UTR Designer (Table 1)[42,43,59], have been used successfully in multiple bacterial species for tuning protein expression levels across several orders of magnitude[40,60–63] (Figure 1A). They use similar models to predict the TIR of a given mRNA ORF by calculating the energy of interaction between the ribosome and the mRNA, incorporating contributions from energies of: (a) rRNA:mRNA binding, (b) $tRNA^{fMet}$:start-codon binding, (c) compensation for non-optimal spacing between the SD and the start-codon, and (d) penalty due to 5'UTR structure that affects ribosome-mRNA interaction. However, the two use different methods to calculate energy penalty due to the structured 5'UTR[42,43]. The RBS Designer tool takes a different approach by calculating "translation efficiency" as the probability of binding of a free ribosome to the SD sequence on an mRNA[64], while EMOPEC uses a position specific weight matrix to determine translation rate from a specific SD sequence in *E. coli*[65].

The above tools also provided evidence that the same RBS sequence can have vastly different translation rates (530-fold in one example[59]) for two different ORF sequences, underlining the importance of building good mathematical models and the limits of standardized parts libraries as used in synthetic biology[25,59]. Since these tools report TIR on a relative scale specific to a given ORF, the effects of independent variables like DNA copy number, transcription rate, codon usage, protein size, protein solubility or other factors affecting translation elongation or termination can be ignored. However, this also implies that comparing TIR values across different ORFs is unlikely to correlate with protein abundance, as was recently found by ribosome profiling[66]. Although much progress has been made in the predictable tuning of protein expression, further improvements could include the kinetics of mRNA folding and ribosome:mRNA interaction in the initiation model, account for differences in mRNA and protein half-lives, and incorporate elongation and termination into a comprehensive translation model[67,68].

Riboswitches

Riboswitches are structured RNA elements usually found in the untranslated regions of the mRNA, such as 5'UTRs or the transcriptional terminators, of their regulated genes. They consist of a ligand-binding domain, the aptamer, which is coupled to an expression platform such that a ligand-induced conformational switch in their structure alters access to a translationally relevant part of the mRNA, like a prokaryotic RBS or a eukaryotic 5'cap, or the production of an intrinsic transcriptional terminator or anti-terminator stem[69–71] (Figure 1B). Riboswitches are found in all domains of life and can bind to a wide range of ligands including purines, antibiotics, vitamins and secondary metabolites[72]. Natural riboswitches have been engineered to recognize alternative natural and unnatural ligands[73], while some synthetic

riboswitches have been designed from ligand-binding aptamers in parts by rational design and functional screening[74–77]. However, despite SELEX-driven identification of a number of aptamer molecules *de novo* design of riboswitches remains considerably challenging[78,79]. A recent computational biophysical model was used to design 62 translation-regulating riboswitches from six ligand-binding aptamers, achieving up to 383-fold activation[41]. Like the RBS Calculator model, the Riboswitch Calculator (Table 1) considers the free energy change from a folded mRNA state unbound to the ligand and the ribosome to a ligand-bound mRNA in complex with the ribosome, and uses it to calculate the activation fold of the riboswitch. The work leading up to the thermodynamic model confirms previous observations that riboswitch activation requires co-transcriptional kinetic trapping of the mRNA in the ligand-bound state[75], and uncovers the role of molecular crowding in riboswitch activation. It also calculates the theoretical limits of using a hypothetical ideal riboswitch as a biosensor, predicting that based on thermodynamic considerations the best translation riboswitch will only activate ~20-fold at nanomolar ligand concentrations. However, these theoretical thermodynamic limits could be circumvented by the kinetics of the various steps involved in riboswitch activation: (1) mRNA folding, including transcription rate, (2) ligand binding, including aptamer preorganization and kinetic proofreading, and (3) ribosome binding to the mRNA[80–84].

Translation Riboregulators

Trans-acting riboregulators are small RNA (sRNA) molecules that, like riboswitches, regulate mRNA translation by controlling access of the cell's ribosomal machinery to the mRNA[85,86]. The most common point of control is translation initiation, when the 16S rRNA aSD interacts with the RBS to form the translation initiation complex. sRNA-mediated activation is possible in mRNAs where a structured 5'UTR

occludes the RBS in the OFF state, and only allows ribosomal access upon conformational change induced by sRNA binding[22,87].

In addition to the advantage of RNA programmability, sRNA-based regulators have faster regulation kinetics than proteins due to their shorter half-lives[48]. Additionally, translation regulators have quicker response times than transcriptional ones since all the interacting molecules have already been transcribed[88]. The sRNA:mRNA interaction is initiated at a seed site of sequence complementarity between the two molecules and proceeds along the thermodynamic energy gradient. The energetics of these interactions and the RNA structural constraints were used to build a thermodynamic model of sRNA-mediated translation activation and design many synthetic riboregulators of up to 10-fold activation[22,89] (Figure 1C). The model was also published as an online tool for riboregulator design, the Ribomaker (Table 1)[89]. While the original strategy employed structural constraints to position the RBS in a hairpin stem as a way of maintaining the OFF state, later work has employed a different strategy in the design of "toehold switches" where the RBS is maintained in the unpaired loop of the folded hairpin but translation initiation is prevented by burying the translation start site in the paired stem. The latter design has the advantage that the folding energies of the hairpin-loop structure and the RBS strength can be tuned relatively independently.

sRNA-mediated translation repression also follows similar rules of sRNA:mRNA interaction, except in this case the binding of the sRNA results in the occlusion of the RBS. This process of translation repression can either be aided by the Hfq RNA chaperone, or operate independently of it[90]. Unlike sRNA-mediated translation activation that requires a structured 5'UTR in the OFF state, sRNA-mediated repression is possible to design for almost any mRNA as long as the designed

repressing sRNA can bind it with sufficient strength to prevent translation initiation or elongation[15,91].

Table 1: Computational tools for RNA synthetic biology

Tool	Modes	Modes	Refs.	Model details
RBS Calculator (v1, v1.1, v2) (Online)	Forward Engineering (v1.1)	Designs ribosome binding site with specified translation initiation rate (TIR)	40,42,59	• Calculates TIR as a function of the strength of binding between the 16S rRNA 3'end and the mRNA. • Version 2 accounts for structured standby sites upstream of the Shine Dalgarno sequence based on biophysical constraints. • Most frequently updated model.
	Forward Engineering Library (v1.1)	Designs a degenerate library of ribosome binding sites with a specified range and resolution of TIRs	40	
	Reverse Engineering (v1, v1.1, v2)	Predicts TIRs of start codons in the input mRNA sequence	40,42,59	
UTR Designer (Online)	Forward Engineering	Designs 5'UTR with specified expression level	43	• Calculates protein expression level by calculating strength of binding between the 16S rRNA 3'end and the mRNA. • Accounts for local structure near the Shine Dalgarno sequence based on ensemble average. • Allows codon optimization of the ORF to achieve desired expression level.
	Forward Engineering Library	Designs a degenerate 5'UTR with a specified range and resolution of expression levels	92	
	Reverse Engineering	Predicts expression level of the input gene	43	
Riboswitch Calculator (Online)	Reverse Engineering	Predicts TIR of the gene in the riboswitch mRNA, in the presence and the absence of the ligand	41	• Calculates TIR as a function of the strength of binding between the 16S rRNA 3'end and the mRNA, accounting for different aptamer structures in the presence and the absence of the ligand. • Also predicts the fold activation at varying concentrations of the ligand and the mRNA
Ribomaker (Online)	Forward Engineering	Designs sRNA and/ or mRNA sequences for specified structural constraints in the resulting heterodimer	89	• Designs one or more RNA sequences based on the biophysics of intermolecular interaction
CasOT (Downloadable)	Search single-gRNA, paired-gRNA, and target-and-off-target	Search for target sites of a gRNA sequence through specified genomes	93	• Uses pattern matching for the sgRNA-PAM combination to determine target and off-target hits

Ribozymes

Ribozymes are a class of enzymatic RNA molecules that adopt a specific tertiary structure allowing for cleavage of the RNA molecule at a defined locus[94]. The self-cleaving property of these ribozymes has been used in synthetic biology for insulation of RNA parts from their surrounding context, or for self-processing of large RNAs[95,96]. With the knowledge of the ribozyme structure, its cleavage may be regulated

by the insertion of an aptamer whereby the necessary structural confirmation required for catalytic activity is met only when the aptamer binds to a small molecule ligand. Such ligand-induced ribozymes, called aptazymes, can be designed to regulate gene expression and therefore act as molecular sensors of small molecules[97–99] (Figure 1D). Using similar principles of conformational switching, computational methods have been used to calculate secondary structure of the ribozyme molecules to engineer ribozymes for ligand-induced release of riboregulators[37]. These sRNA-releasing ribozymes, called "regazymes", can also be re-programmed to sense specific sRNAs, converting them into signal relay molecules. As with riboswitches, induced conformational changes in aptazymes are often co-transcriptional, making kinetic RNA folding method like Kinefold more useful for their design[100,101].

Transcriptional Regulators

Rho-independent or intrinsic transcriptional terminators represent one of the most well-known example of functional structured RNAs, and are responsible for ~80% of all transcription termination events in *E. coli*[102]. They consist of a conserved RNA hairpin-loop motif, with a ~5-9 nt long stem and 3-5 nt loop, and are followed by a U-tract of 7-9 nt. While the U-tract causes the RNA polymerase to pause during transcription, the formation of the hairpin destabilizes the transcription elongation complex resulting in the eventual dislocation of the RNA polymerase from the DNA[103,104]. With the knowledge of the molecular mechanism in action, many synthetic terminators have been designed with a wide range of termination efficiencies, often surpassing those of natural terminators, or with engineered bidirectional functionality[105–107]. Based on the measured termination efficiencies and an analysis of sequence and secondary structural features, linear regression and biophysical models have been built to predict termination function of a given terminator sequence[106,107].

Since transcriptional termination requires the slowing down of RNA polymerase and the formation of a destabilizing hairpin-loop, different anti-termination strategies have also evolved in nature to regulate termination[108–110]. These have subsequently been adapted to design multiple orthogonal parts for regulating transcription termination. Inspired by the pT181 attenuator, researchers have designed antisense sRNAs that can effectively repress transcription by stabilizing an otherwise weak terminator hairpin, resulting in premature termination of mRNA[30,111]. Conversely, small transcription activating RNAs (STARs) have been designed that activate transcription by sequestering the 5'proximal arm of the terminator hairpin, resulting in onward transcription of the mRNA[38,112] (Figure 1E). Both these sRNA-triggered methods of transcriptional regulation can be used in many combinations for genetic circuitry involving RNA-only signal propagation. Furthermore, *E. coli*'s leader-peptide regulatory element from its *tna* operon has been adapted to activate transcription by translation-mediated anti-termination[31]. Therefore, the synthetic *tna*-derived adaptor acts as a signal converter in a genetic circuit, converting a translation signal to a transcription signal by closely interfacing the ribosome and the RNA polymerase. Viewed from an electronics perspective, this is analogous to a translation current expressed as ribosomal flux (RiPS, ribosome per second) being converted to a transcription current expressed as polymerase flux (PoPS, polymerase per second) via "common signal carriers"– the ribosomes and the RNA polymerases[113,114].

CRISPR/Cas9

The Cas9 endonuclease is the enzymatic component of the RNP machinery that effects a double-strand break at a specific DNA sequence as a part of the antiviral immune system of bacteria[115]. Its specific site of action is defined by the crRNA (CRISPR RNA), which forms a complex with the tracrRNA (trans-activating CRISPR

RNA) to direct the Cas9 to its complementary DNA sequence[115,116]. The natural dual RNA complex has been engineered to a simpler single small guide RNA (sgRNA) that contains the region complementary to the target DNA sequence near a recognition motif called PAM[116,117]. The rest of the sgRNA consists of a Cas9-handle that facilitates its docking into the Cas9 protein[118]. The Cas9-sgRNA pair are self-sufficient and do not require any other host factors, as evidenced by their standalone application in diverse prokaryotic and eukaryotic hosts[119–121]. By modifying the sequence-specificity region of the sgRNA, it is possible to target it to any location in the genome or a plasmid. While the catalytically active version of Cas9 has been applied in diverse genome engineering applications[119,122], the inactive dCas9 has been used or reprogrammed, by fusion with other domains, for use as a sequence-specific activator or repressor with high specificity[123,124] (Figure 1F). The high programmability and specificity of sgRNA sequence as well as the availability of mutually orthogonal Cas9 proteins with different PAM recognition motifs has facilitated parallel application of the Cas9 technology for targeting of multiple DNA loci simultaneously using orthogonal sgRNAs[125–127]. Inducible reconstitution of split-Cas9 protein has allowed greater temporal control over its activity[128,129]. Furthermore, antisense RNA-mediated regulation of sgRNA function, and engineering additional RNA motifs onto the sgRNA has made possible the recruitment of other effector proteins with diverse functions, expanding the range of Cas9-mediated regulatory action[130–132].

RNA Circuits

With rapid expansion in the number of and types of available RNA parts, building higher-order RNA-based genetic circuits is now possible, including some RNA-only ones[88,111]. While the orthogonality of many of these parts has facilitated their simultaneous use in genetic circuits[27,73,125,133], better understanding of their design rules

has also allowed construction of many hybrid parts with composite functions[37,38,130,134]. Furthermore, RNA parts are relatively universal and can be easily ported from one species to another[135]. The improved dynamic ranges of RNA parts have also improved their ability to interface with other parts, further enhancing their composability. As a result, RNA-based circuits have now been successfully used to implement logic gates, feedback controls, feed-forward loops, and regulatory cascades[37,88,136] (Figure 2). Here we discuss some RNA circuit designs to provide better appreciation of the achievements and potential of RNA-based circuitry, putting in context the previously discussed RNA parts.

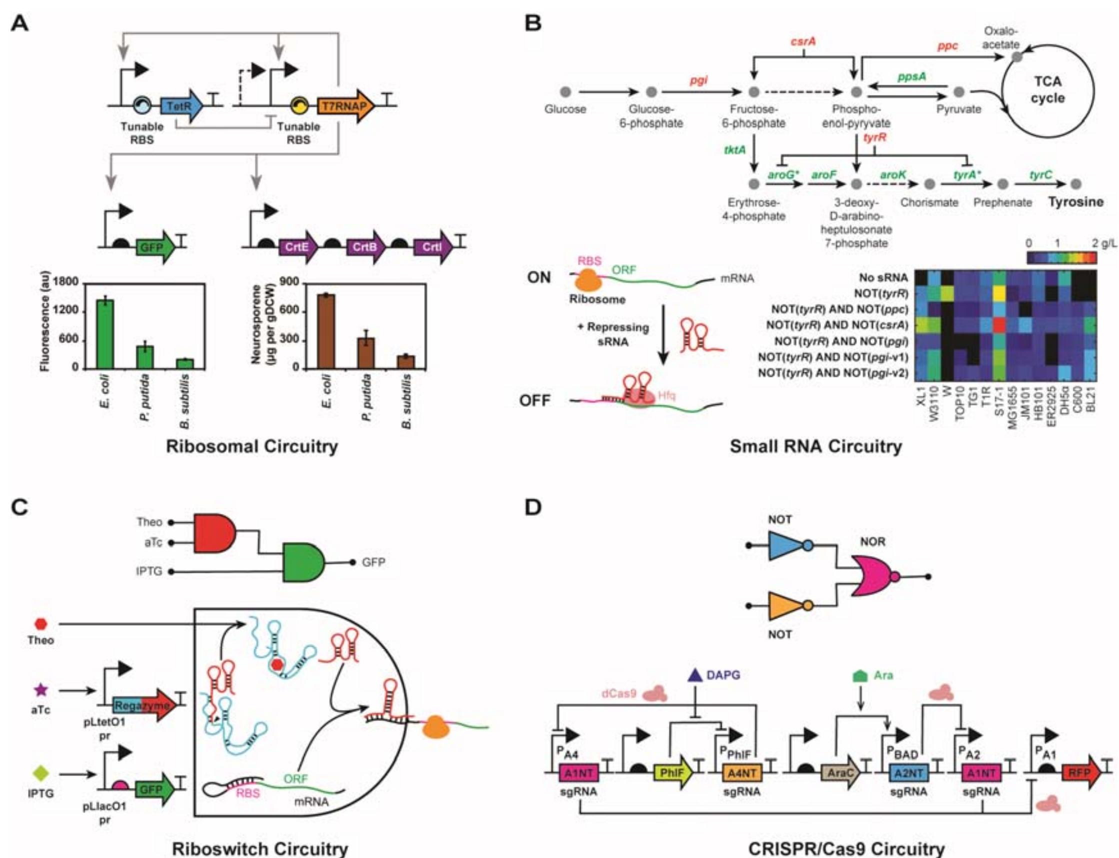

Figure 2. RNA parts can be assembled into multi-layered circuitry. (A) *Computational design of* tunable *RBS sequences allows construction of a mixed feedback loop of orthogonal polymerase (T7RNAP) and repressor (TetR) molecules, enabling gene expression in a host-promoter independent manner*[135]*.* ***(B)*** *Hfq-chaperoned binding between sRNA and mRNA results in translation repression of the latter. This has been used for metabolic engineering in E. coli to increase tyrosine production*[15]*. Genes in red were targeted for sRNA-mediated combinatorial repression while those in green were overexpressed on plasmids in fourteen strains (* indicates feedback-resistant mutant of the overexpressed gene).* ***(C)*** *Composite parts like regazymes (sRNA emitting aptazyme) can be used to build RNA-only circuits, such as the*

*3-input AND gate demonstrated here[37]. **(D)** Orthogonal sgRNAs, together with the dCas9 repressor, have been used to build higher order genetic circuits[125].*

Ribosomal circuitry

While proteins are the specialized reservoirs of the bulk of enzymatic activity in the cell, by leveraging the role of RNA in protein expression we can gain greater programmable control over their function. As discussed earlier, by engineering ribosome:mRNA interactions using translation initiation models, we can tune protein expression over a large dynamic range within the cell[42,43,59]. The RBS Library Calculator and the UTR Library Designer, add-on tools to the respective computational models, can design a degenerate RBS sequence to span a range of protein expression space, further enhances this ability[40,92]. This methodology has been used to tune the knobs of multi-dimensional enzyme expression spaces to determine the optimal stoichiometry of individual enzymes that achieve a balanced pathway for neurosporene, NADPH, lysine and hydrogen production[40,63,92]. Total enzyme expression can be subsequently increased, while maintaining relative enzyme stoichiometry, to further improve production. The ability to engineer multi-dimensional tunable protein expression has also been used to create mixed feedback loop transcriptional genetic circuits for autonomous regulation of the orthogonal T7 RNA polymerase to cap toxicity, allowing conversion from a RiBS signal to a PoPS signal, and facilitating inter-species portability of genetic parts and pathways[135] (Figure 2A).

The ability to engineer the translation process by manipulating rRNA:mRNA interaction has also been pursued from the rRNA end with exciting implications. By manipulating the aSD sequence, orthogonal ribosomes with alternative RBS specificities have been created[137,138]. Orthogonal ribosomes open up new avenues for biological engineering by helping partition the translation machinery into separate mRNA pools, one for native cellular functions and the other for the use of the biological

engineer, facilitating otherwise difficult to implement design paradigms like synthetic amino acid incorporation into peptides or quadruplet-codon decoding ribosomes[139–141]. rRNA engineering has recently led to the development of a single subunit ribosome (Ribo-T) that has the potential to further expand RNA control over protein regulation[142].

Small RNA circuitry

As seen earlier, sRNA binding to the mRNA can abolish translation initiation or elongation, thereby allowing direct control over protein expression in the cell. Recent work has expanded this approach to implement a combinatorial knockdown of multiple genes using synthetic sRNAs for metabolic engineering of biosynthetic pathways in *E. coli*. In order to redirect flux through the tyrosine production pathway, four genes (*csrA*, *pgi*, *ppc* and *tyrR*) were targeted for repression in a number of cell lines, improving tyrosine production dramatically[15] (Figure 2B). In the most productive strain, the simultaneous application of sRNAs results in a genetic NOT-AND-NOT gate for *tyrR* and *csrA* genes. In the same study, repressing six of eight target genomic loci by sRNAs led to an increase in cadaverine production by ~55%. sRNA circuits have also been built for activating translation of genes with structurally repressed 5'UTRs. A combination of these trans-activating riboregulators has been used to build RNA circuits that trigger physiological responses like λ-phage lysis, integrate simple 2-input or more complex 4-input AND gate logic, and implement transcriptional cascades *in vitro*[22,87,133,143].

In addition to manipulating expression by regulating translation, sRNAs have also been used to regulate transcription in synthetic genetic circuitry. The pT181-inspired sRNA-triggered attenuators have been used to implement RNA-only NOR gate logic and a 3-step cascade[88,111]. Similarly, the previously discussed transcription

activating STARs have been used to design many orthogonal activating RNAs reaching activation folds of up to 94-fold, and the implementation of novel RNA-only AND and NIMPLY logic gate organisations[38].

Riboswitch circuitry

As reviewed recently, simple logic gate circuit functionality already exists in a number of naturally occurring riboswitches[144]. The *glmS* riboswitch integrates glucose-6-phosphate and glucosamine-6-phosphate signals, while the *add* riboswitch integrates adenine and temperature signals in an OR gate fashion[145,146]. Similarly, the *metE* riboswitch responds to S-adenosylmethionine and vitamin B12 in a NOR gate logic[147]. Synthetic riboswitches have also been built using design and selection methodologies to process AND and NAND gate logics, or act as band pass filters[134,148]. More generalizable strategies have also been used to implement additional logic gate functions like OR and NOR, and signal filters[149].

Riboswitches can also be combined together, or joined with other RNA parts, to make composite parts with novel functionalities[134]. By fusing a ligand-binding riboswitch to a ribozyme, researchers have been able to create a larger array of logic gate circuits: AND, NOR, and NAND gates[149,150]. Similarly, researches have created an sRNA-emitting ribozyme, called regazyme, that responds to a small molecule or a specific sRNA trigger by cleaving to release another sRNA for downstream function. The regazyme can also be activated by an sRNA, acting as a signal relay inside cells[37]. The regazymes have been used to build 2- and 3-input AND gate logic circuitry (Figure 2C).

CRISPR/Cas9 circuitry

The CRISPR/Cas9 system uses a small guide RNA (sgRNA) sequence for recognition specificity for binding to its DNA target, facilitating the use of orthogonal sgRNA sequences for targeting multiple locations in the DNA. Using the catalytically dead dCas9 protein, this has enabled the development of genetic circuitry with complex logic gate functions like NOT-NOT, NOR, NOR-NOT, NOR (NOT-NOT) where dCas9 acts as a programmable repressor in bacteria[125] (Figure 2D), repressing a gene by blocking the access of RNA polymerase (RNAP) to its promoter. Moderate success has also been achieved in turning dCas9 into a transcriptional activator by fusing it to the omega subunit of bacterial RNAP[123]. It may be possible to further improve this strategy by using stronger activation domains, or harnessing transcriptional activity from an orthogonal RNA polymerase fragment[151].

dCas9 has also been used to build higher-order layered genetic circuits for decision-making in eukaryotic cells, including yeast and mammalian[152,153]. Unlike in bacteria, dCas9 binding on its own is quite inefficient for transcriptional repression in eukaryotes[154,155], where most successful CRISPRi strategies have relied on using one or more repressor domains fused to dCas9[154–156], or a repressor protein recruited via an RNA-binding domain[130], to inactivate a promoter at the chromatin level. However, a recent report in *Drosophila* has demonstrated efficient transcriptional knockdown by using targets closely flanking the transcription start site[157]. This strategy should be attempted in more eukaryotes as it is likely to be more precise than chromatin modifying domains that have wider effect on surrounding genomic loci[158]. Similar to bacteria, dCas9-mediated activation (CRISPRa) in eukaryotes too requires an activation domain recruited to the promoter site to modify the local chromatin state[130,159–161]. As an alternative to activation domains, it may also be possible to achieve activation of a gene by dCas9-mediated disruption of +1 nucleosome

positioning that blocks the RNA polymerase from progressing downstream from the nucleosome free region[162,163].

Applications

The versatility of structure and function of RNA devices, along with their programmable nature, has permitted their use in a multitude of biological, biotechnological and medical applications (Figure 3). Many of these applications use parts and circuitry discussed earlier in this review. We consider some of them here, focusing on those applications where the programmable aspects of RNA function have been harnessed.

Riboswitch sensors for bio-production

The ability of riboswitches to control gene expression in response to small molecule binding has found them use in bio-production as sensors of desired molecular products or of metabolic intermediates. The possibility of identifying novel small-molecule binding aptamers by *in vitro* selection[78], followed by building artificial riboswitches or other aptamer-regulated RNA parts[11,37], makes them appealing as control circuits during biotechnological production of useful chemicals. Successful riboswitch-mediated enrichment of cells able to sense theophylline after mixing with non-sensing cells first hinted at the possibility of linking riboswitch based selection to directed evolution[164]. Riboswitches have subsequently been used to differentiate *E. coli* cells producing vitamin B12 from non-producers, to screen libraries of caffeine demethylase variants in yeast for higher enzyme activity, and to evolve a chimeric aspartate kinase sourced from *Bacillus subtilis* and *Thermus thermophilus* for higher lysine production[165–167]. In addition to evolving enzymes individually, riboswitch sensors have also been used to simultaneously optimize expression levels of multiple

enzymes to redirect metabolic flux towards a product of interest in both bacteria[168] and yeast[169]. Similarly, aptazyme riboswitches have been used for enhanced xanthine and vitamin B2 production in yeast and *Bacillus*, respectively[170,171] (Figure 3A).

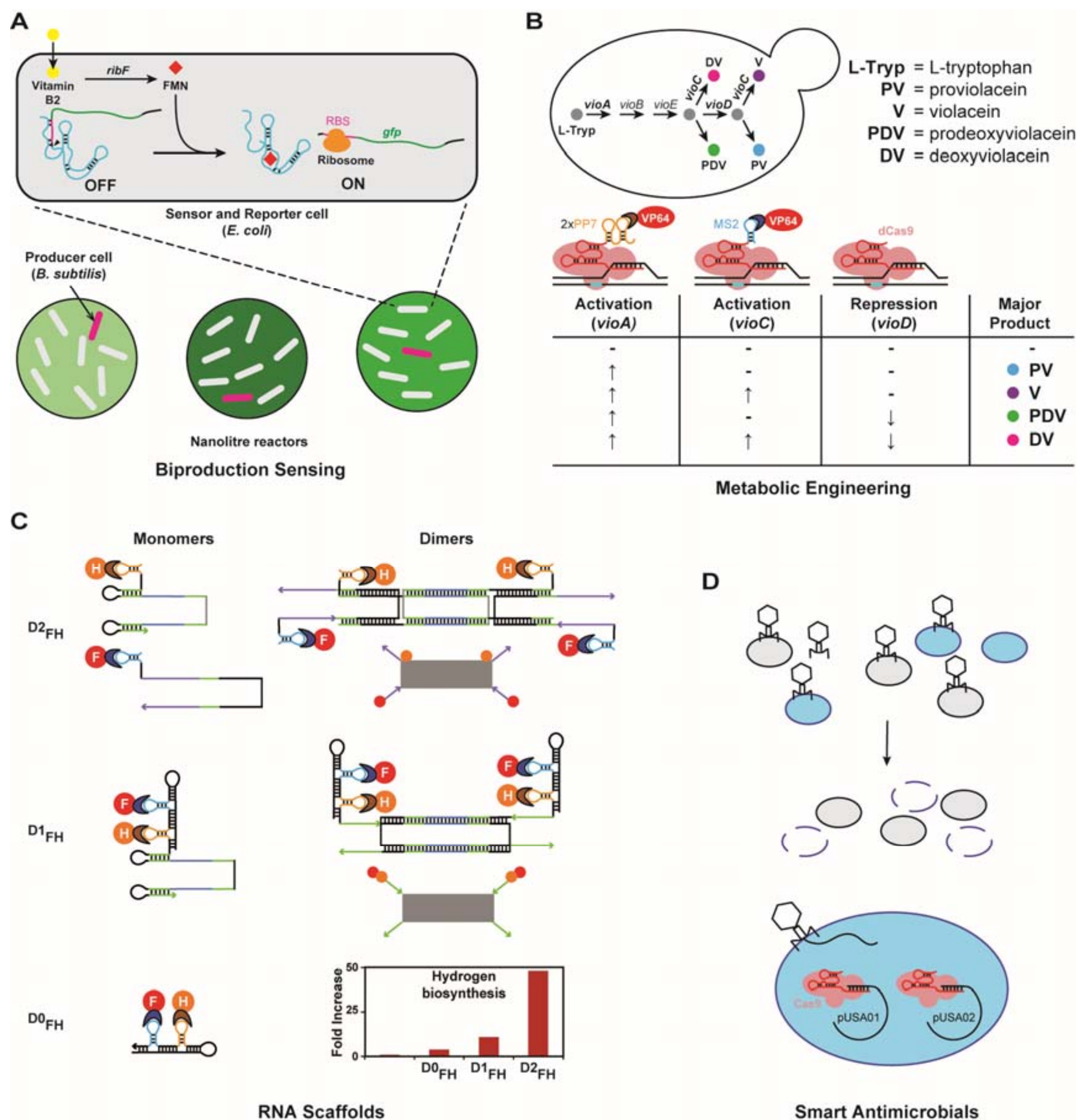

Figure 3. RNA function has been harnessed for a number of biological applications. (A) A library of vitamin B2 producing *B. subtilis* variants was screened using alginate beads as nanolitre reactors. *E. coli* cells, with an engineered riboswitch, were used for sensing and reporting B2 in the beads[171]. **(B)** Cas9-based repression and activation was used in yeast to enable programmable routing of metabolic flux from L-tryptophan to one of four different pigments produced in the violacein pathway[130]. RNA motifs MS2 and PP7 were added to the sgRNA to act as bait for the recognition domains fused to VP64 trans-activator to attract it to the relevant gene. **(C)** RNA can be used as scaffold for spatial organization of enzymes. Using RNA motifs MS2 and PP7 to recruit enzymes ferredoxin (F) and hydrogenase (H) to RNA molecules with 0D, 1D and 2D higher order structures ($D0_{FH}$, $D1_{FH}$ and $D2_{FH}$) increases hydrogen yield by up to 4-fold, 11-fold and 48-fold, respectively, compared to unscaffolded proteins (leftmost bar)[172]. The RNA-protein monomers and dimers that assemble into larger structures are also shown. The grey boxes show simplifications of the RNA-protein dimers that organize to form the higher-order structures, together with the polymerization domain tails with

RNA directionality for pairing. **(D)** Cas9 nuclease with programmable sequence-specificity has been employed with engineered phagemids to selectively kill methicillin resistant *S. aureus*[173]. The CRISPR array sequence was designed to target both resistance plasmids pUSA01 and pUSA02 for curing.

Small RNA regulators for metabolic engineering

sRNA regulators of gene expression can be easily directed against natural genes as they work through base pairing with their target sequences. As such, they can be designed with relatively high throughput, and large scale knockdown of key genes in metabolic pathways can be programmed to divert carbon fluxes and increase production. This was originally achieved using asRNAs (long antisense RNAs), and later improved by the use of PTasRNAs (paired termini asRNA)[174,175]. As discussed previously, synthetic sRNA negative riboregulators have also been used to knockdown regulators of metabolic pathways and achieve high titers of tyrosine (2 g/L) and of cadaverine (12.6 g/L)[15]. Since sRNA-mediated gene regulation can function without modifying the genome, such RNAs can be used to rapidly engineer microbial cell-factories across different species of bacteria[176]. In addition to regulating translation, CRISPRi sgRNAs can also be used in a similar way for rapid and multiplexed transcriptional repression of flux genes using the dCas9 protein, as was done for the polyhydroxyalkanoate pathway in *E. coli*[177]. Fusion of the sgRNA with other RNA motifs for accessory protein recruitment has permitted activation or repression of genes based on the sgRNA input, allowing rerouting of metabolic flux to different pigment outputs depending on the RNA program input in yeast[130] (Figure 3B).

RNA scaffolds for biotechnology

The base pairing properties of RNA allow it to be assembled into different shapes *in vitro*[178]. These properties have also been used *in vivo* to create scaffolds on which to assemble enzymes for increasing production of various molecules. A sequence-programmed RNA scaffold can be used to control the spatial organization of

hydrogen producing enzymes in *E. coli*[172]. The authors fused different RNA binding domains (MS2 and PP7) to the two enzymes required for hydrogen production in *E. coli,* to immobilize them to a specific region of the RNA scaffold. By incorporating RNA binding domains onto different RNA scaffolds that assembled into 1 or 2 dimensional structures, they were able to increase hydrogen output by up to 48-fold (Figure 3C). This method was expanded to increase pentadecane titers up to 140% and succinate up to 88%, allowing the co-localization of up to four enzymes[179].

Molecular diagnostics

The ability of RNA circuits to detect small molecules and specific RNA sequences, as well as the possibility of using the circuits in cell free extracts, has led to the exciting development of paper-based "toehold switch" riboregulators, which can be freeze-dried on a paper matrix with cell free extract and functionalized after rehydration[87,133]. These sensors can detect specific RNA sequences through binding of complementary regions and strand-displacement. Early examples include differentiating between two different *Ebola* virus strains, although the strategy can easily be expanded to detect other RNA sequences. The cell-free nature of the system makes it cheap, portable, and better suited to a regulatory environment where genetically engineered material could not be used in the field.

Smart therapeutics

The application of synthetic RNA devices and circuits for medical uses is a particularly promising route. RNA based "smart" antimicrobials have been developed to kill bacterial cells using readily programmable sequence-specific targeting. Almost 20 years ago, an sRNA acting as external guide sequence was used to target bacterial antibiotic resistance mRNAs for RNase P degradation[180]. More recently, riboregulators

and CRISPR/Cas9 nuclease have been delivered into *E. coli* to either repress antibiotic resistance genes and restore sensitivity, or to kill bacteria in a sequence specific manner[173,181,182] (Figure 3D). These proof of principle examples demonstrate the antimicrobial potential of RNA circuits for specific bacterial targeting. In addition, more complex responses based on devices that sense small molecules and other RNAs, as well as layered circuits that integrate multiple inputs, are also possible in mammalian cells. Delivered though specialized vectors[183], such circuits can read the cellular state and integrate multiple signals like viral RNAs or cancer-associated metabolites to produce conditional outputs like apoptotic or immune-stimulatory response[184]. To improve half-lives and reduce immunogenicity of the delivered RNA molecules modified bases such as pseudouridine can be substituted into them[185], while also continuing to assist RNA programmability by improving existing secondary structure prediction models to capture the altered biophysics of molecular interaction by such bases[186].

Cellular RNA editing

Group I self-splicing introns can be used to engineer trans-splicing ribozymes for directed editing of RNAs using base-pairing programmable "guide sequences"[187–189]. These have been used to repair the disease form of mRNAs, in sickle cell *ß-globin* mRNA[190,191] and myotonic dystrophy *DMPK* mRNA[192] for example, or target dengue virus conserved RNA genome[193]. Other trans-splicers create specific cytotoxins[194] in response to mRNA presence or cause cell death upon viral infection[195]. They have also been used to target cancer-linked mRNAs[16], and can be delivered using viral vectors[196]. Furthermore, they can be combined with aptamers, resulting in small-molecule activated *trans*-splicers[197].

Genome engineering

CRISPR/Cas9 nuclease allows programmable genome editing by effecting RNA-guided double strand break at a precise target site that is later repaired by homologous recombination (HR) or non-homologous end joining (NHEJ) introducing recombinant DNA at the cut site[122]. However, target recognition by the small 17-20 nt gRNA often results in many off targets, particularly in large genomes[198,199]. This has promoted the development of many methodologies to deal with the undesirable off-targets. Cas9n, a nickase mutant that can only nick a single strand of the target DNA, necessitates making two separate DNA nicks for HR, eliminating NHEJ mediated repair and reducing off-target effects[200]. Systems have been developed to regulate the active time of Cas9, inducible by small molecule or light, in order to reduce off-target effects[201,202]. A recent high-fidelity Cas9 mutant has reduced strength of binding to DNA that reduces off-target effects to undetectable levels in the human genome[203]. Other methods include using bioinformatics tools, like CasOT (Table 1)[93], and computational models to predict off-target effects, and design sgRNAs to avoid them[93,204]. Due to its portability and precision programmability, the CRISPR/Cas9 genome engineering method has found widespread use for generating mouse models of disease[205], disrupting latent HIV[206], and repairing defective genes[207], among others.

Future directions

Our capacity for rational engineering of a biological system depends on our ability to predictably control the functions of its various components, and thereby its behavioral response. The idea of using RNA as the molecular programming language in which to rewrite cellular functions relies on two of its fundamental properties: versatility and structural predictability. Firstly, RNA is highly versatile and plays many roles inside the cell in its capacity as an informational as well as a catalytic molecule. In that sense, RNA can be viewed as a vital linchpin that holds many disparate processes

together, presumably as a relic from the old RNA world. As RNA engineering can only be used to affect those functions in the cell that are carried out or regulated by it, either alone or in combination with protein effectors, this functional versatility allows for wider control to the RNA coder. Secondly, RNA is a highly programmable molecule whose intra- and inter-molecular interactions rely on simple base-pairing rules and can be readily predicted. Together with the fact that most RNA functions depend on its primary sequence or its secondary structure, this predictability allows an RNA coder to rewrite these functions *de novo*.

The RNA properties of versatility and predictability are also closely related with the 'parts' and 'models' paradigms of synthetic biology. The standardized parts paradigm aims to characterize biological parts to determine their functional specifications and catalogue them. The versatility of RNA therefore results in large libraries of RNA parts that can be re-used in biological circuits where their functions are needed. While the parts paradigm improves programmability using RNA, it is effective only for modular and composable parts. When parts crosstalk or influence each other in unexpected ways, the utility of the parts paradigm gets limited. This is where the predictability of RNA interactions can be very useful. In the 'models' paradigm of synthetic biology, the functional specifications of an RNA part can be predicted from its sequence using a computational model that can determine the sequence-structure-function relationship of the part. This expands the design space for RNA circuits by allowing the coder to design novel and orthogonal parts that do not crosstalk with each other. The ability to predict RNA behavior *de novo* is closely linked to the strengths of the computational model used. For the models paradigm to be effective, models need to capture not only the core features responsible for part function, but also the effect of the surrounding

sequences on part behavior. Overall, RNA synthetic biology will benefit by a judicious use of parts and models for building biological systems.

With considerable expansion in the number and type of RNA parts and models in the past few years, as well as their improved dynamic ranges, we have reached the stage in biological engineering where RNA programmability is already being leveraged for complex biological design. The development of RNA devices capable of converting one form of signal to another[31,135], and those able to emit RNA in response to a trigger (like light, a small molecule, or another RNA) could standardize the use of sRNAs as signal mediators in gene circuits[37,47,88,133]. Such RNA-only circuits driven by broad host-range viral promoters could be transplanted across species by only replacing the interface with the host gene expression[135]. However, many further steps are necessary for achieving RNA precision control over design-to-specification for biological systems for more predictable engineering.

In order to achieve effective interfacing between different RNA parts, it is important that their input-output dynamic ranges match with each other[208]. While this is not likely to be a problem for parts with higher output dynamic range, it will severely jeopardize inter-connectivity for ribosensors of nanomolar ligand concentrations for example[41]. As connectors between incompatible RNA parts, libraries of signal amplifiers and converters need to be constructed[31,135,209]. Predictive models for RNA-based regulation of translation or riboswitches need to be developed for eukaryotes, which will further expand RNA application. Furthermore, design methodologies for RNA parts design need to be automatable and scalable such that more orthogonal parts can be generated on demand[37]. To anticipate potential bugs and avoid paths to failure, parts must be characterized at least an order of magnitude above and below their normal

range of operation, and their metabolic load and quantitative expression must be monitored *in vivo*[52,210].

As the use of the popular CRISPR/Cas9 technologies is expanded to larger circuits and genomes, it will be important to build accurate genome-wide models for predicting targeting and off-targeting efficiencies to reduce cross-talk effects of mis-targeting[211]. To expand Cas9 specificity and functionality, it will also be important to continue the search for Cas9 homologs that use longer recognition sequences and have less stringent PAM sequence requirements.

An important theoretical assumption that underlies almost all computational models describing sequence-structure-function relationships in RNA synthetic biology is that of thermodynamic equilibrium of RNA secondary structures[22,37,39,41,42,106,107]. While this may be a reasonable assumption for some applications, many RNA processes such as riboswitch activation[41,75] and ribozyme folding[212,213] are co-transcriptional and their accurate modelling requires incorporation of RNA folding kinetics[100,101]. Similarly, accurately designing switching dynamics for inducible systems will require dynamic modelling of these systems[214]. As larger RNA-only or RNA-protein genetic circuits are rationally designed[125,135], it will also be important to have an accurate characterization of their model parameters. For these purposes, *in vitro* transcription-translation systems will be a useful resource[88].

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