Multilevel Regulation and Translational Switches in Synthetic Biology

Margarita B. Kopniczky, Simon J. Moore, and Paul S. Freemont

***Abstract*—In contrast to the versatility of regulatory mechanisms in natural systems, synthetic genetic circuits have been so far predominantly composed of transcriptionally regulated modules. This is about to change as the repertoire of foundational tools for post-transcriptional regulation is quickly expanding. We provide an overview of the different types of translational regulators: protein, small molecule and ribonucleic acid (RNA) responsive and we describe the new emerging circuit designs utilizing these tools. There are several advantages of achieving multilevel regulation via translational switches and it is likely that such designs will have the greatest and earliest impact in mammalian synthetic biology for regenerative medicine and gene therapy applications.**

***Index Terms*—Biomedical engineering, circuit design, logic devices, protein, ribonucleic acid (RNA), riboswitch, synthetic biology, translation.**

I. Introduction

SYNTHETIC Biology is an application-focused field that is providing a conceptual framework for engineering novel biological systems based on the engineering principles of standardization, modularity and abstraction [1]. New functions extend the natural capabilities of cells by taking elements from evolutionarily distant organisms or molecules designed entirely *de novo* with potential applications in the fields of energy, agriculture, healthcare and manufacturing. However, there are only a limited number of molecular mechanisms that can be used in predictable and controllable ways to construct synthetic devices [2], [3]. This lack of tools and modules makes it technically challenging to design higher-level logic and implement multilevel regulation There are four main biological processes in terms of circuit design that regulation can be imposed namely at the transcription level, ribonucleic acid (RNA) degradation level, the translational level and the protein degradation level. Synthetic designed gene circuits predominantly use logical operations carried out at the transcriptional level whereas natural systems are regulated at all possible levels. It is estimated that regulation at the translation level accounts for variability in gene expression by 40% [4], although in disagreement 9% variability has also been reported [5].

Despite being ubiquitous in natural regulation, synthetic circuits are predominantly controlled by transcription in preference to other levels of control [1]. The first logic gates and simple devices in synthetic biology were assembled from such components: two repressing transcriptional modules can be assembled into a toggle switch [6] and three such elements into a repressillator [7]. The advantages of transcriptional regulation are that there is remarkable flexibility in the design space for promoter sequences and DNA binding proteins and that known modules can be composed into larger systems [8]. The response of a promoter to various inputs can be changed by shuffling its repressor binding sites [9], introducing point mutations into its sequence [10] or screening randomized libraries [11]. Transcription can be controlled via various modular RNA mediated mechanism. Such examples include the CRISPR/Cas9 system, [12], [13] and a new type of RNA-based transcriptional regulators called small transcription activating RNAs [14].Moreover, orthogonal synthetic TAL effector [15], [16] and zinc finger [17] proteins have also been engineered. However, the complexity of a circuit entirely relying on transcriptional regulation is limited due to the loading effects, limited number of orthogonal regulators, and the time that it takes to process each step of gene expression [18], [19]. Some transcriptional repressors exert their effect by modifying the chromatin structure surrounding the target region, such epigenetic effects can strongly delay the reactivation of many transcriptional regulators [20], [17], [21]. In contrast, translational regulation possesses quicker dynamics due to the ‘skipping‘ of the transcription step in gene expression. In contrast, translational regulation processes have quicker dynamics due to the ‘skipping‘ of the transcription step in gene expression. A decrease of burden can be achieved if RNA regulators are used instead of protein regulators of either transcription or translation. Production of RNA is ‘cheaper` in terms of nutrient requirements and does not divert ribosomal resources away from the translation of other cellular proteins. Notwithstanding, there is a greater need to broaden the range of available tools for post-transcriptional regulation [22], especially for applications where a tightly controlled, predictable and reliable circuitry is essential. In mammalian synthetic biology, the application areas include recombinant protein production, post-translational modifications, stem cell reprogramming, gene therapy and cancer cell detection and eradication [23], [24]. For such applications, the space for error is narrow and small deviations can lead to unwanted effects like therapeutic gene circuit designed to identify and kill cancer cells could accidentally eradicate some healthy cells for example [25], [16]. Recently, there

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has been an increased effort to develop RNA based post-transcriptional tools to indirectly influence translation via mRNA stability and splicing or to directly control translation via interfering with the recruitment of ribosomes [26], [27]. New developments in foundational technology for synthetic biology will lead to fundamentally new circuit designs, with increasing sophistication and complexity. In this review, we focus on the new tools available to directly regulate translation and we describe some of the new emerging circuit designs that leverage these tools.

II. Background

Translation happens in fundamentally different ways in prokaryotes and eukaryotes. In prokaryotes, translation generally takes place simultaneously with transcription, from multiple Open Reading Frames (ORFs) on polycistronic mRNA. In contrast, this process is spatially separated in eukaryotes due to the nucleus and nuclear envelope. To guide nascent mRNAs into the cytoplasm, eukaryotes add a 7-methylguanylate cap to the 5` end of the mRNA, before translation initiation factors and ribosomes bind. The translation start site is defined by the Kozak sequence, which is equivalent – although evolutionary distinct – to the Shine-Dalgarno Sequence (SDS) found in prokaryotes. Apart from the 5' capping process, eukaryotic or viral mRNAs can use various independent mechanisms. For example the internal ribosome entry site (IRES) [28], an RNA element with stable secondary structure that is able to either directly recruit ribosomes or bind initiation/elongation factors. The most common start codon in all domains of life is AUG, with various elongation factors to assist in the process of translation.

Therefore, the approaches to control and regulate translation in prokaryotes and eukaryotes are fundamentally different. In prokaryotes, occluding the SDS from being recognised by the ribosome is one approach and can be achieved by incorporating a complementary region on the mRNA. In eukaryotes, more sophisticated mechanisms can be designed, for example an aptamer motif with stable secondary structure or a protein binding site upstream of the start site. Eukaryotic mRNAs are normally monocistronic (contain a single ORF) but it has been observed that a second ORF can be translated if an IRES element is present or if an elF4 initiation factor is recruited upstream of the start site. As discussed later, some regulatory elements or parts of regulatory elements can be used to interfere with translation in prokaryotes and eukaryotes, but they have to be implemented according to different design rules.

III. Predicting Translation Initiation Rate

A key aspect of translational regulation is in defining the efficiency of translation which helps to fine-tune protein levels. In a recent study, the efficiency of 65,536 human translation initiation sequences (TIS), the sites in close proximity to the start codon, were compared and TIS de-regulation was found to be associated with cancer genesis [29]. Analogous to the set of constitutive promoters of predictable strengths [10], libraries of elements with increasing translation efficiencies have been constructed. In prokaryotes, it is well established that the translation rate depends greatly on the sequence complementarity

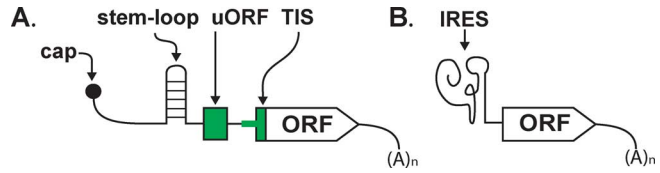

Fig. 1. Various features of mRNA that modulate translation efficiency on a continuous scale. The features are shown in the 5' region, upstream of the open reading frame (ORF) which is labeled as is the poly-A tail at the 3' end of the mRNA. (a) Cap-dependent mechanisms include stem-loop RNA structures which interfere with ribosomal scanning; upstream ORFs (uORFs) cause ribosomes to dissociate from the mRNA at the associated stop codon and therefore reduces translation of the second ORF; TIS (Translation Initiation Sequence) variants involved in the direct recognition of the translation start site by the ribosome. (b) Variants of cap-independent IRES sequences influence the affinity of ribosomes or initiation factors to the mRNA, thus affecting translation efficiency (number of protein molecules produced from an mRNA over time).

of the SDS to the ribosome and on the secondary structure of the mRNA [30]. Various algorithms have been developed to design an RBS of desired strength for a given ORF to minimize complex secondary structure formation and optimize ribosomal binding, which are easily accessed through various web-based resources [31]–[35]. The RBS calculator for example has a 47% probability of predicting RBS strength within two-fold of the target [34]. Therefore it is proposed to generate multiple elements and screen for the desired activity since the probability increases to 72%, 85% and 92% if two, three or four RBSs are generated. Recently, Mutalik *et al.* proposed a novel and modular bicistronic RBS design and achieved an improved, 93% chance of predicting RBS strength within two-fold of target [11].

Designing fine-tuned translation responses can allow the control of relative gene expression from the same mRNA transcript, or transcripts from similar promoters. The strategy to modify RBS strengths of ORFs encoding genes involved in a biosynthetic pathway was successful to optimize naringenin production for example [36]. Similar control over translation efficiency is highly desirable in mammalian synthetic biology for optimizing therapeutic protein production, programming reliable differentiation for tissue engineering or for achieving optimal gene expression dosage in gene therapy. Variations in TISs can be used to fine tune translation efficiency in mammalian cells, but only within a limited dynamic range and thus is restrictive for translational control (Table I.). To address this, Ferreira *et al.* assembled a library of upstream ORFs (uORF), which made it possible to span a range of three orders of magnitude in translation efficiency when used together with TIS prediction [37]. An uORF is a sequence in 5' from the main ORF that encodes a short peptide that may be functional in certain cases [38]. According to the proposed probabilistic leaky scanning model, ribosomes that initiate and exit translation at the uORF are more likely to dissociate from the mRNA than the fraction of ribosomes that `leaks through' and only initiates at the correct start codon [37]. Alternatively, RNA structures can also be used to decrease translation efficiency in a systematic and predictable manner via the mechanism of blocking elongation and thus slowing overall translation rate [39], [40].

Not only cap-dependent but also cap-independent translation efficiency can be modified. One of the most commonly used

TABLE I
TOOLS FOR TRANSLATION INITIATION RATE PREDICTION

	Name	Range	Expression Levels	Accuracy	Reference and link
Prokaryotic	RBS Calculator	100 000 fold	1-100 000 a.u.	$R^2 = 0.84$	[30] [34] https://www.denovodna.com/software/
	UTR Designer	10 000 fold	100-1000 000 a.u.	$R^2 = 0.81$	[32] http://sbi.postech.ac.kr/utr_designer
	RBS Designer	100 000 fold	10^{-5} - 1 a.u.	$R^2 = 0.87$	[33] http://ssbio.cau.ac.kr/web/?page_id=195
	Bicistronic Design	600 fold	1.17 - 700 a.u.	$R^2 = 0.9$	[11]
	Artificial Neural Network	-	0 - 3.6 relative strength*	$R^2 = 0.98^*$	[35]
Eukaryotic	Stem-loop	125 fold	1.6 – 200 mg/l protein expression	no quantitative model established	[40]
	uORF	1 000 fold	0.001 - 1.4 efficiency†	$R^2 = 0.92$	[37]
	TIS	70 fold	20 – 140 % efficiency†	$R^2 = 0.82$	[29]
	IRES mutant library	300 fold	0.5 – 100 % efficiency†	no quantitative model established	[41]

Range shown is calculated from the lowest and highest measurements of the experimentally characterized libraries, in various units. The measurement methods used in each instance are different and therefore the above values are only given as a guide and the referred literature should be consulted for more detailed information. Accuracy is the value given in the study as the correlation between the predictions and the experimentally observed values. The predictions can be based on thermodynamic models or on a dataset of measurements obtained from an initial library and the source literature should be consulted for further information. *The promoter and RBS strengths were both varied in this study. †Efficiencies of eukaryotic translation are reported as normalized values, and calculated based on the proportion of the fluorescence of a regulated reporter to a control reporter. Abbreviations: RBS = Ribosome Binding Site, uORF= upstream Open Reading Frame, TIS = Translation Initiation Sequence, IRES = Internal Ribosome Entry Site.

IRESs, the Encephalomyocarditis virus IRES was mutated to produce a library of 24 variants spanning a 300-fold expression range, which were validated across multiple cell types and genes [41]. This approach is useful for the production of antibodies by establishing an optimal light-heavy chain production ratio

IV. REGULATION WITH RNA BINDING PROTEINS

Some proteins, or domains of proteins, can bind RNA with high specificity and affinity, and are therefore ideal for crafting control of translation in a synthetic biology context. Some of these RNA Binding Proteins (RBPs) have a structural role in assisting ribosome assembly [42] or as viral coat proteins [43], whilst others are involved in RNA regulation in organelles [44] and many play a key part in translation regulation as their natural function [45]. The human genome contains 424 known and predicted RNA Binding proteins, which are involved in multiple processes [46], whilst many bind to evolutionary conserved RNA recognition motifs [47]. Novel approaches in bioinformatics and high-throughput sequencing facilitate the identification of RBPs expressed differentially in various cell types and identifying the RNA sequences that they recognize [48].

RBPs can be classified in various ways. Here, for simplicity, we distinguish Motif Binding Proteins (MotBPs), and Repeat Proteins (RPs). MotBPs recognize specific RNA sequence elements (motifs), often with distinct secondary structures. RPs in contrast, contain repeated domains, each of which is responsible for binding a single nucleotide on the target RNA. Both MotBPs and RPs hold vast potential for the regulation of translation and other RNA-related processes in synthetic biology [49].

Many MotBPs exist in eukaryotes but it has generally proven difficult to engineer them for novel uses in synthetic biology. The RNA recognition motifs can be found in hundreds of human proteins [50] and efforts have been made to create libraries from such eukaryotic MotBPs. However, they remain difficult to systematically design due to the substantial variation in the RNA binding surface and the number of bases (2–8) recognized [51]. Instead, viral and prokaryotic MotBPs are preferred for use in eukaryotic synthetic biology. They possess an added advantage of being evolutionary distant (orthogonal) and are therefore less likely to interfere with endogenous eukaryotic processes.

Viral and prokaryotic MotBPs often recognize structural motifs, usually a hairpin, where binding specificity is typically defined by the residues at the end and internal loops [52], [53]. RNA motif MotBP pairs provide an ideal tool for regulatory modules in the design of complex synthetic circuits because the RNA motif is well defined and amenable to engineering, since the binding residues can be mapped accurately using protein crystallography and homology modelling. An interesting MotBP category includes synthetically evolved RNA aptamer binding domains [54]. Nucleic acid aptamers can be generated via the technique called systematic evolution of ligands by exponential enrichment (SELEX) [55]. An increasing number of SELEX derived RNA ligands have been developed for various applications [56]. RNA aptamers binding endogenous proteins may be integrated into synthetic designs in an analogous way to viral/prokaryotic MotBP-RNA motif pairs (Fig. 2) [57].

A. MotBPs as Translational Repressors

The simplest application of an MotBP is its use as a repressor to block translation upon binding to its cognate RNA motif in the 5' untranslated region (UTR) of a gene of interest [Fig. 2(a)] [58], [59]. Multiple investigations observed that the position of the RNA motif within the 5'UTR is critical and that the maximum translation activity decreases as the

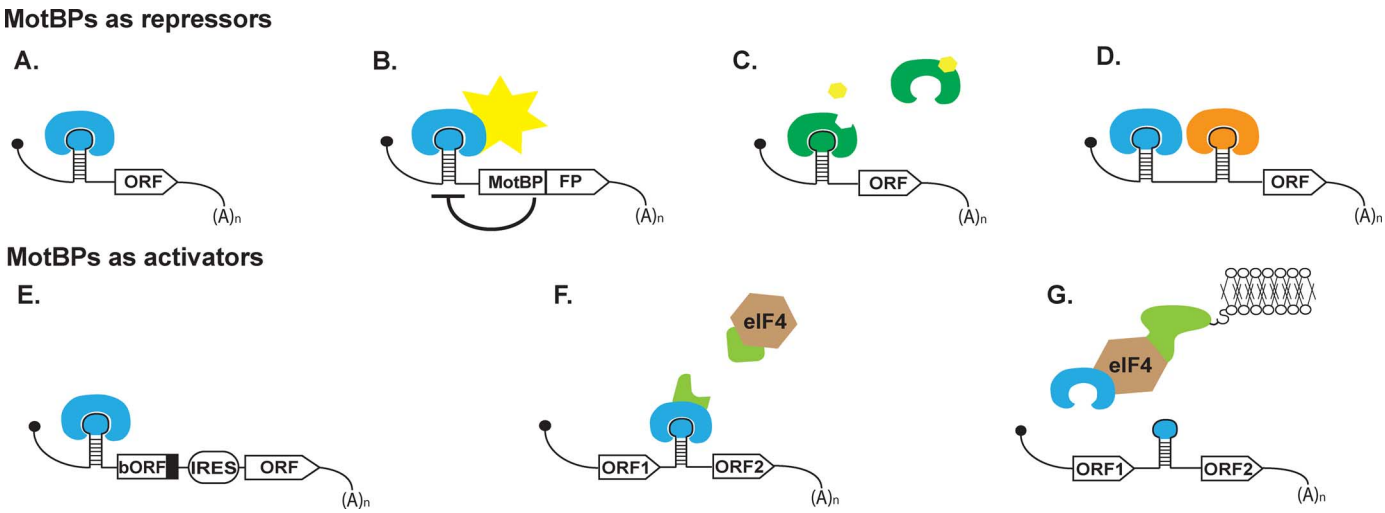

Fig. 2. RNA motif binding proteins (MotBP) can be used either as translational repressors (turn OFF translation) or as activators (turn ON translation). (a) In a simple repressor design, the MotBP (blue) binds to its cognate RNA motif (blue) positioned in the 5' untranslated region (UTR) of the mRNA in order to control expression of a downstream open reading frame (ORF) followed by the poly-A tail at the 3' end of the mRNA. (b) Negative feedback circuit designed to control the expression of an RNA motif binding protein (MotBP; blue) fused protein of interest shown as a fluorescent protein (FP; yellow) in this example [64]. (c) Reversible repression via a MotBP (green) binding a small molecule (yellow) which dissociates the MotBP from the mRNA [54]. (d) Two different MotBP binding sites (blue and orange) in the 5'UTR lead to the repression of translation in the presence of either MotBP (blue or orange). (e) Inverter module based on RNA degradation induced via the translation of a bait ORF (bORF) with premature termination codons (black box). The internal ribosome entry site (IRES) driven ORF is only expressed if the MotBP (blue) binds and represses the bORF and the mRNA is thus spared from degradation [68]. (f) Protein-protein interaction mediated translation initiation from the second ORF (ORF2) of mRNA. Ribosomes dissociate from the mRNA after the translation of the first ORF (ORF1) but can be recruited again in the presence of eIF4 (brown). This is achieved via a fusion protein of eIF4 and a light-responsive protein (green) which is recruited to the mRNA upon blue light illumination via binding of a cognate partner (green) fused to a MotBP (blue) [63]. (g) Cytoplasmic localization of an otherwise membrane bound MotBP fusion-protein induces translation from ORF2 preceded by the MotBP's cognate RNA motif (blue) [70].

distance of the structural motif to the 5' cap decreases, both in mammalian cells [60], [61] and in plants [59]. However, the same context dependency was not observed in yeast [62]. Moreover, multiple copies of the RNA motif can increase repression, with a maximum level observed with three [61] or six [63] tandem copies of RNA motifs. Interestingly, different modes of repression can often be observed with different MotBPs, some inhibiting only cap-dependent translation, and others inhibiting translation from an entire bicistronic transcript where the second ORF is translated from an IRES [45]. Such complex design rules make the systematic engineering of translational modules challenging and thus thorough characterization of the individual parts are necessary.

A general strategy was proposed to stabilize gene expression levels via incorporation of an MoBP mediated negative feedback loop at the translational level [64]. The L7Ae MotBP was fused to a fluorescent reporter and placed under the control of its cognate RNA motif. In such a configuration, the binding affinity of the MotBP to the RNA motif regulates the steady state level of the MotBP fusion protein. Furthermore, the parameters inferred from experimental measurements and the model describing this regulatory circuit could be extended to predict regulation of translation from other mRNAs in *trans*. The L7Ae protein and the RNA motif could potentially be replaced with any known MotBPs and RNA motifs with different dissociation constants, and the MotBP could be fused to or regulate any protein of interest.

Another widely used transcriptional regulator is the tetracycline repressor (TetR) protein which has recently been engineered to achieve small molecule inducible translation [54]. The repressor protein binds to an artificially selected RNA aptamer motif to block translation in the same way as discussed in the previous examples. When tetracycline analogues are added, TetR changes conformation and dissociates from the mRNA, allowing translation to happen. The method was initially developed for applications in model organisms where the tools for controlling gene expression are limited. More recently it was used to control the expression of various target proteins, independent of promoter context and mRNA localization in plasmodium to reveal anti-malarial drug targets [65].

Combination of MotBPs may also be used to carry out bio-computation. The expression of MotBPs can be under the control of different promoters that sense small molecule inputs and then transmit these input signals to regulate translation of the output signal. The MS2 and L7Ae protein-RNA motif pairs were used in the construction of such a half-adder and half-substractor circuits [66]. An important feature for the construction of the half-adder (Fig. 3) circuit was that a single ORF can be controlled by multiple MotBPs if multiple RNA motifs are inserted into the same 5'UTR [66]. The ORF will only be translated if none of the MotBPs are present in the cell [Fig. 2(d)]. MotBP mediated translation control can also be combined with transcriptional regulation in a modular fashion and also with ribozyme regulation. Ausländer *et al.* constructed a three input AND gate by composing promoter regulation, an MotBP and a protein-responsive hammerhead ribozyme [67]. These two examples demonstrate that MotBP repressors are useful tools in combination with other regulatory elements to achieve higher-order control dynamics of complex synthetic biology circuits.

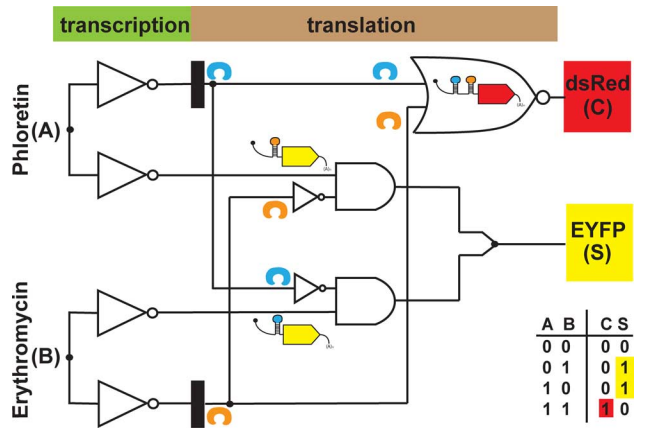

Fig. 3. The synthetic biology design features of the half-adder circuit by Ausländer *et al.* [66] can be abstracted to a logic gate circuit diagram. Logic gates can represent any type of regulation where an output is produced in response to one or multiple inputs. The types of logic gates in the above circuit include: NOT gates which only produce output if input is absent (represented by triangular symbols), AND gates which produce output if both inputs are present (half-oval symbol) and a NOR gate which produces output if neither of the inputs is present (fish-shaped symbol). The circuit design can be divided into transcriptional and translational components. The transcriptional level is regulated by two small molecules (Phloretin and Erythromycin) which repress the transcription of two reporter genes (dsRed and EYFP) and two RNA motif binding proteins (MotBP) genes, this repression is represented by NOT gates. The translational level includes unregulated steps, indicated by black boxes for the translation of two different MotBPs (orange and blue). The YFP reporter can be produced via two independent routes; there are two possible YFP-mRNAs (yellow) with two different MotBP binding motifs upstream of the YFP coding sequence (blue and orange). YFP is only produced if either variant of YFP-mRNA is present, and its cognate regulator MotBP (blue or orange) is not present. Therefore, a sequential NOT and AND gates represents this relationship. The DsRed reporter is only produced if neither of the MotBPs is present (NOR gate), the DsRed mRNA (red) is constitutively transcribed. The overall resulting function is a half-adder, a circuit where the outputs depended on the inputs according to the truth table in the bottom right.

B. MotBPs as Translational Activators

Several attempts have been made to achieve MotBP mediated induction. A set of ON switches was constructed from three different MotBPs by converting the basic OFF switch in a *cis*-acting setup where the inverter module is found on the same mRNA [68] [Fig. 2(e)]. The MotBP regulates the expression of a small peptide (152 a.a.) from a bait ORF that is terminated by two premature termination codons (OFF switches). If translated, the endogenous Nonsense Mediated Decay (NMD) response [69] is activated to degrade the entire mRNA. NMD is a general eukaryotic RNA surveillance mechanism, which targets certain mRNAs, such as those containing premature termination codons for degradation. If the translation of the bait ORF is inhibited because of binding of the MotBP to the RNA motif just upstream, the NMD is not activated, correct splicing of the mRNA can occur and IRES mediated translation can proceed from the second ORF. The second ORF is the reporter gene or any gene of interest that is inserted as part of a circuit design.

An alternative approach to switching on translation in response to MotBP binding, involves the eIF4 translation initiation factor, which has the role of recruiting ribosomes to the mRNA. It is known that a second ORF on a eukaryotic mRNA is not translated unless some sequence element is present upstream to recruit initiation factors or the ribosomes directly. Not only can an IRES element instigate this, the recruitment of eIF4 initiation factor alone, via any mechanism, is enough to initiate translation. EIF4 can be fused to a MotBP and thus the fusion protein will induce translation from a second, otherwise untranslated ORF that contains a cognate RNA binding motif upstream. In order to achieve pharmacological induction, a multi-protein fusion was constructed from a MotBP, eIF4F and an H-Ras [Fig. 2(g)] [70]. H-Ras is normally a membrane-associated signaling protein but it can dissociate in the absence of a specific protein-modification. Some therapeutically interesting small molecules such as FTI-277 can inhibit this modification and thus H-Ras is delocalized from the membrane into the cytoplasm. Thus, the fusion protein can initiate translation from the second ORF of the reporter construct. The method has been proposed for use in drug-screening but it could theoretically be adapted to sense the delocalization of any protein of interest from the membrane to the cytoplasm.

A similar approach to the translational activator module above was utilized in a design to achieve the light induced translation from the second ORF of an mRNA [63] [Fig. 2(f)]. Blue light illumination induces the CRY2PHR protein to bind CIBN protein and this protein-protein interaction provides the molecular basis for the sensor circuit. The MotBP was fused to the CIBN, the eIF4E to the CRY2PHR and therefore translation was induced from a second ORF containing the cognate RNA motif of the MotBP. Such a design may allow linking translation output to any chosen protein-protein interaction input in general.

C. Repeat Proteins

While complex synthetic circuits can be assembled using MotBPs and their corresponding RNA motifs in a modular and orthogonal fashion, their greatest limitation is that they cannot be adapted to target any desired endogenous RNA sequence. In order to interface a synthetic circuit with endogenous RNAs, repeat proteins are a plausible solution. Repeat proteins have been likened to DNA binding transcription activator-like effectors (TALEs) since they also have a one-to-one code of domain to amino acid recognition code [71]. The two known classes of repeat proteins are Pumilio and Fem–3 mRNA-binding factor (PUF) proteins and Pentatricopeptide Repeat Proteins (PPRs). Some expect that they will become just as important in manipulating RNA as TALES and Zinc Finger domain proteins are for manipulating DNA [49], [72].

PUF repeat proteins typically consist of eight tandem repeats of three-helix bundles that form a crescent-shaped structure. The amino acids at positions 12 and 16 within the second α–helix define the specificity of each repeat, according to a recognition code [73]. Natural PUFs usually recognize 8–9 nucleotides in an anti-parallel orientation and are not particularly diverse – humans only have two such proteins (PUM1 and PUM2) [74], [75]. Custom PUF domains have been used to both up and down regulate translation. In a system similar to that in Fig. 3(f), and by the same research group, light inducible translation was achieved via PUF regulation [76]. In another work, the site specific addition or removal of the polyA tail was regulated by

PUF domains fused to effectors, to influence translation efficiency and mRNA half-life, in a sequence-dependent manner [77]. Like other RBPs, repeat proteins can be utilized for various other RNA-related regulatory mechanisms when fused to other functional domains – examples that have been implemented include tracking localization and alternative splicing [78]. The recent developments in modular assembly of designer PUF domains [79] and attempts to increase the number of nucleotides recognized for increased specificity [80] are likely to facilitate their use in an increasing number of applications.

A phylogeneticaly distinct class of repeat proteins, PPRs are one of the largest nucleic acid binding protein families in land plants and bind to a great diversity of RNA molecules [44]. They are nuclear encoded but typically targeted to organelles and are known to be involved in translational stability and splicing control. PPRs bind RNA in a parallel orientation and each domain consists of about 35 amino acids and folds into a pair of antiparallel α-helices. The recognition code is defined by two amino acids, one at the beginning and one at the end of each domain [81]. In contrast with PUFs, the number of bases that natural PPRs can recognize is not fixed and can be between two and thirty [49]. This flexibility and versatility makes PPRs especially favorable for engineering. In a recent work, Coquille *et al.* created a modular consensus PPR scaffold with optimized properties for synthetic biology applications [82]. This opens up the possibility of exploring the potential of PPRs in a great variety of ways for RNA manipulation [83].

V. Regulation With Small Molecules Via Riboswitches

Riboswitches provide a fascinating ancestral link to the most primitive forms of life [84]. They represent an elegant but simple mechanism for controlling genetic regulation, using a direct interaction of a small molecule ligand to an RNA aptamer. This allows an organism to sense intracellular levels of a small molecule and respond accordingly. For example, in *Bacillus subtillis* approximately 4% of the genome is regulated by riboswitches [85], whilst it is estimated that between 10–15% of bacterial genomes encode non-coding RNA that play diverse roles in regulation [86]. From an engineering perspective, riboswitches offer a powerful tool for the development of diverse sensory circuits, logic gates and bi-phasic switches. Since the initial discovery of the thiamine pyrophosphate (TPP) riboswitch just over a decade ago [87], [88] a plethora of ligand-RNA regulatory systems have been identified in prokaryotes [89]–[91] and a finite list have been unveiled in plants [92], algae [93] and fungi [94]. Predominantly located in the 5'UTR of mRNA, riboswitches form a complex tertiary structure upon transcription and folding, forming a pocket whereby a small-molecule can bind. This ligand-aptamer interaction induces a conformational rearrangement to control gene expression. The range of ligands is restricted to biochemical intermediates of central metabolism [84], [89], [95], and ions [96]–[98].

A. Riboswitch Control Mechanisms, Discovery and Diversity

The binding of a ligand to the core of a riboswitch initiates a conformational rearrangement in the RNA tertiary structure to affect gene expression [89]. In general riboswitches possess an aptamer (ligand binding or sensor domain) and an expression platform (anti-terminator and terminator stems). Although in terms of nucleotide sequence context, these two domains are effectively interconnected through complementary stem-loops. The aptamer (sensor) is the only element that is conserved between different species [99], whereas the expression platform (or transducer) is variable and linked to the mechanism of regulation. For a comprehensive review of riboswitch regulatory mechanisms, see [84]. In brief, binding of a ligand to an RNA aptamer initiates a structural rearrangement to control formation of a terminator complex that controls gene expression [100]. For control of translational initiation, the terminator overlaps the RBS, thereby masking access for ribosome docking (Fig. 4). Binding of the ligand to the aptamer domain can either switch gene expression ON or OFF, depending on the requirements of the cell. Alternatively, transcription termination is controlled by a terminator, which in bacteria is normally followed by a poly-U sequence, which destabilises the RNA polymerase during transcription [101]. This method of regulation offers several advantages over translational control. The inherent turnover of mRNA (especially in prokaryotic systems) during transcription allows riboswitches to provide a dynamic online response through various stages of cellular growth. Firstly, less resources are required if gene expression is aborted before complete mRNA synthesis, whilst complete control can be offered for polycistronic operons, therefore preventing the buildup of intermediates and redundancy in a complex pathway. A third category of regulation has recently been discovered, showing a coenzyme B_{12} riboswitch that controls expression of a non-coding anti-sense sRNA [102]–[104]. Finally, conserved eukaryotic TPP riboswitches utilize a unique RNA splicing mechanism to control expression of thiamine biosynthetic genes [105], [106] [Fig. 4(d)]. It is likely that these latter two examples of riboswitch regulation are widespread in nature. However, developments in riboswitch discovery in eukaryotes are recent and are likely to be expanded.

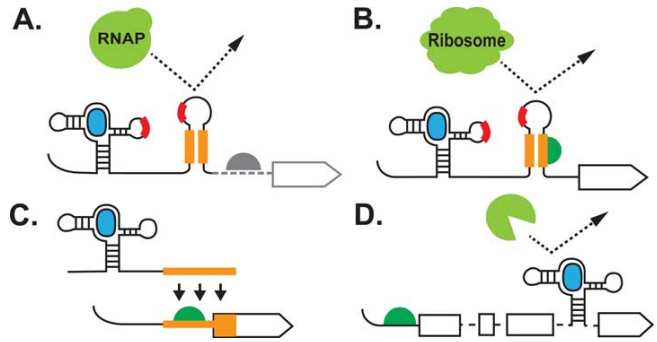

Fig. 4. Riboswitch mechanisms. Prokaryotic mechanisms include (a) transcription control provided by a terminator (orange) that stalls RNA polymerase extension in the presence of the small molecule ligand (blue). In the absence of the ligand complementary regions indicated with red form an antiterminator structure. (b) Translation initiation is prevented by masking of the RBS (green) inside the terminator stem-loop (orange) in the presence of the repressor ligand (blue). (c) Anti-sense sRNA (upper molecule) control of orthogonal genes in *trans*. A complementary region to the 5' sequence of the mRNA (orange) is revealed and inhibits translation upon ligand (blue) binding to the aptamer on the sRNA. (d) RNA splicing control is unique to eukaryotes, with a riboswitch positioned within an intron (dashed line) to hinder splicing in response to ligand binding.

TABLE II
SOFTWARE TOOLS FOR RIBOSWITCH PREDICTION

Name	Short description	Reference and link
Denison Riboswitch Detector	Database tool for genome scale searching	[108] http://drd.denison.edu/
Riboswitch Explorer	Simple search program with graphical interface and database of known and predicted riboswitches, sequence length <40 kb	[109] http://132.248.32.45/cgi-bin/ribex.cgi
RNAMotif	Advanced program for searching specific RNA motifs using the command line prompt	[111] http://casegroup.rutgers.edu/casegr-sh-2.5.html
RiboSW	Basic search program, limited sequence length <10 kb	[112] http://ribosw.mbc.nctu.edu.tw/
Riboswitch finder	Basic search interface for conserved riboswitch elements	[113] http://riboswitch.bioapps.biozentrum.uni-wuerzburg.de/server.html
CMfinder	Semi-advanced RNA motif prediction tool, can analyze multiple sequences (each < 500 bp)	[114] http://wingless.cs.washington.edu/htbin-post/unrestricted/CMfinderWeb/CMfinderInput.pl

For discovering new and alternative examples of known riboswitches, a number of search algorithms are available (Table II). The earliest example is the Mfold algorithm, which uses the minimal free energy principle for predicting RNA secondary structure [107]. In addition, a number of web-based tools specific for riboswitch prediction from an input DNA sequence are available. This includes the Denison Riboswitch Detector [108], Riboswitch Explorer [109], RNA-PATTERN [110], RNAMotif [111], RiboSW [112], Riboswitch finder [113] and CMfinder [114]. These examples provide a prediction output based on conserved examples of known riboswitch classes. However, the search algorithms used are restricted on sequence length. For wider discovery, more sophisticated algorithms and rapid characterization techniques [115] are required due to the need to search larger amounts of genomic data. Experimental methods like SHAPE-Seq allow structural characterization in a high-throughput manner and may facilitate the investigation of new riboswitch classes [116]. For example, the conserved MoCo (molybdenum/tungsten) motif [98] and a number of unclassified riboswitches [91] suggest a broader role in regulation. However, some of the ligands themselves may be unknown at our current level of understanding.

An alternative strategy for creating novel riboswitch functions is to engineer a characterized riboswitch framework either through minor modifications or replacement of the aptamer or expression platform domain. The rise of SELEX [55], [56] has demonstrated a high-throughput screening method for obtaining novel RNA aptamers with affinity for a chosen ligand. However, transplanting the synthetic designs into a natural riboswitch has been difficult to achieve [117]. From a structural perspective, engineering of synthetic riboswitches may require a similar approach to *de novo* protein design [118]. At the current level of understanding, the easier approach lies with modifying single RNA residues of a starting aptamer in order to achieve binding of a new ligand similar to the original. Thus, the result is an altered aptamer with recognition highly specific to the new ligand but not binding the original ligand. A key example of constructing an orthogonal aptamer-ligand pair with this approach was the engineering of the adenine (purine) riboswitch control element [119]. This rationale provides a route to enable the synthetic engineering of metabolic pathways. In theory, with further development, this fine tuning approach can provide modular control for the transformation of a substrate into its product via a number of controlled intermediates. This could be used for flux control to prevent accumulation of toxic intermediates and decrease burden due to excess protein expression.

B. Circuit Design

An interesting circuit with great potential that relies on a prokaryotic translational riboswitch, is the Riboselector [120].

This device provides a selective advantage in response to inconspicuous metabolites and can be applied to directed evolution of high producing metabolic pathways. Another promising tool in circuit design are sets of orthogonal riboswitches that can be employed to independently tune gene expression levels from genes in the same operon [121]. Such a strategy can be utilized in optimizing the ratio of metabolic enzymes expressed in a biosynthetic pathway.

Riboswitches and related ribozymes are an attractive tool for eukaryotic and in particular mammalian synthetic biology circuit design. However, the design and application of advanced, predictable and finely tuned RNA sensing devices is still in the early stages of development. A riboswitch can provide a wide dynamic range in respect to ligand concentration [122]. Riboswitch elements can be used as key bottleneck filters to prevent unnecessary accumulation of metabolic intermediates in an engineered biosynthetic pathway. One possible area of interest is for the control of post-translational modifications in recombinant protein maturation, where homogeneity is a fundamental requirement, especially with therapeutic antibodies. Alternatively, an RNA based biosensor that could detect accumulation of undesirable metabolites or act as an active response to a depletion of essential cellular resources. A handful of synthetic examples have recently been demonstrated, which includes the use of a synthetic tetracycline riboswitch in yeast [123] and a tetracycline ribozyme in HeLa cells to induce gene expression [117]. A Biotin binding aptamer was inserted into the 5'UTRs of mammalian transcripts and resulted in a 10-fold repression of translation upon the presence of the small molecule [124]. Furthermore, a couple of studies have highlighted that natural riboswitch aptamers can be replaced with synthetic versions in a modular fashion and applied as an induction tool in eukaryotic expression systems [125].

VI. RNA-RNA RIBOREGULATION

An mRNA can not only fold onto itself to form various secondary structures and bind small molecules but it can also bind other single stranded complementary RNA molecules, according to Watson-Crick base pairing. Such trans-acting RNAs (taRNA) can be used to regulate translation in a variety of ways. The classic examples in prokaryotes rely on blocking either the SDS [126] or the Start codon [127]. In addition,

refactoring of the RNA-IN/OUT system of *E. coli* has also yielded a set of orthogonal riboregulators of translation [128]. In eukaryotes, as described above, it is less straightforward to stop or induce translation via an RNA-RNA interaction only. In the yeast antiswitch system a taRNA contains a small molecule responsive aptamer and switches conformation upon tetracycline inducer binding [129]. The sequence complementary to the Initiation site of the target mRNA is exposed and translation is repressed (turned OFF). It is known that micro RNAs can directly down-regulate translation and that miRNA binding sites are sometimes found in the 5'UTRs of mRNAs [130]. However, in synthetic constructs the miRNA binding sites are usually positioned at the 3'UTR and induce the degradation of the target via the RNA interference pathway [131], [132]. A simple MotBP OFF switch [Fig. 2(a)] can effectively be inverted if the RNA binding motif is integrated into a taRNA which induces degradation of the target mRNA only when the MotBP is absent [133], [131]. Other new methods exist to regulate RNA degradation in an RNA-regulated fashion. The Cas9/CRISPR technology has risen in popularity as it is a sequence-specific and very versatile tool for transcriptional regulation. Recent research has proven the concept that a modified Cas9, R-Cas9 can be used to selectively target RNA instead of DNA and target it for degradation [134]. This mechanism could potentially be modified to manipulate RNA in other ways than degradation.

VII. Modelling Translation

Mathematical models of translation of increasing complexity have been developed as a consequence of our increasingly detailed understanding of the underlying molecular mechanisms. A wide variety of mathematical modelling approaches have been employed to understand the interaction of a synthetic gene construct with the host organism to inform the design of optimized gene expression systems and reviewed in [135], [136]. Furthermore, along with the development of molecular tools for dynamic control of translation, there is an increasing need for computational models to aid the design of regulatory modules based on translational switches.

In Section III, we have outlined how translation initiation rates are an important rate limiting step in gene expression and can be a key point of gene regulation for synthetic biology design [31]. However, in addition to the initiation step, the elongation and termination steps are also important to include when modelling translation. The first models that incorporated these three steps of translation used non-linear Ordinary Differential Equation (ODE) based kinetics which could capture changes in elongation rate along a mRNA due to its secondary structure [137] or polysome size (ie. multiple ribosomes) [138]. Further details of endogenous regulation via initiation factors were discovered and these could be incorporated into more detailed kinetic models [139], [140]. The effect of codon composition was also incorporated into a design tool for optimizing protein expression [141].

Some aspects of translation, such as the movement of ribosomes causing collisions and 'traffic jams' are difficult to capture by deterministic models. The first stochastic model of translation by Mitrai *et al.* attempts to captures this [142]. A similar underlying framework was extended and adapted to model metabolic burden effects on the cell arising from the competition for a common pool of ribosomes [143].

A few examples exist where modelling was used to aid the design of devices assembled from various cis- and transacting control elements described in Sections IV–VI. Stapleton *et al.* constructed a negative feedback circuit from a MotBP protein (see Section IV) and an ODE based kinetic model was utilized to predict its behavior [64]. A mechanistic model was also implemented to describe the behavior of a type of ligand responsive RNA element [144] and stochastic simulations were correlated to the experimental observations from a set of designs assembled from such components.

In summary the most widely used model for translation is based around codon optimization which is used extensively in industry, for optimizing gene/protein expression. Other models are used to provide increasing insights into host – synthetic gene interactions and to help the design of complex regulatory systems.

VIII. Conclusions

It has recently become possible to predict translation efficiency on a continuous scale and to switch translation ON and OFF, similar to how transcription can be induced or repressed. With the increasingly versatile toolbox available to synthetic biologists, it is becoming possible to implement multi-level biological circuits and achieve higher-level computation. Novel translational control mechanisms will greatly facilitate such efforts and will likely rise in popularity for circuit design, especially in mammalian cells.

There are multiple features of translational regulation that make it an especially attractive approach for being used in mammalian gene circuits. Firstly, they allow completely independent regulation from those sequence elements that are responsible for regulation at the DNA-level. Once mRNA is transcribed, the promoter and genomic context surrounding the synthetic construct on the DNA level becomes irrelevant. Therefore, translational regulation can be integrated with traditional control systems in a modular fashion, as demonstrated by the complex half-adder (Fig. 3) and half-subtractor circuits implemented in mammalian cells [66].

Second, the dynamics of translational regulation are faster than that of the full gene expression process. The re-activation of a previously repressed mRNA can happen much faster than that of a transcriptionally repressed gene. Furthermore, many of the currently used transcriptional repressors often involve epigenetic modifications that take time to reverse, thus leading to slow reactivation. Not as fast as signaling cascades based on post-translational regulation, but generally faster than transcriptional regulation, translational regulation may be the ideal choice for regulating processes on the medium timescale, such as feedback regulation to balance protein levels in therapeutic or metabolic circuits [64].

Thirdly, translational regulation can be directly interfaced with various cytoplasmic components of the cell. RNA aptamers that are known to bind endogenous proteins or small molecules may directly be utilized and incorporated into protein or small molecule responsive translational switches [57].

The first examples of useful circuits with a translational switch at their core are cancer cell classifiers which can detect the level of endogenous proteins (β-catenin and NF-kB) involved in carcinogenesis [57]. Signal inputs from cytoplasmic protein-protein interactions or protein localization can be directly utilized as an input for translational regulation [63], [76], [62].

Finally, the metabolic burden imposed on the cell may be alleviated via fine tuning translation, since the competition for translational resources is generally greater than the competition for transcriptional resources [145]. Modelling and experimentally comparing burden effects on chassis cells, related to various modes of circuit regulation achieving similar function may greatly inform the design cycle of synthetic genetic circuits [146].

As synthetic genetic circuits become more sophisticated, they will employ a combination of transcriptional and various post-transcriptional regulators. There are an increasing number of new tools for translational regulation with many future applications in mammalian synthetic biology. In order to facilitate their widespread use, it is essential that such new tools are thoroughly characterized, the design rules are explored and part libraries become freely available to the synthetic biology community.

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