Chapter 1

RNP-Based Control Systems for Genetic Circuits in Synthetic Biology Beyond CRISPR

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Abstract

Ribonucleoproteins (RNPs) are RNA-protein complexes utilized natively in both prokaryotes and eukaryotes to regulate essential processes within the cell. Over the past few years, many of these native systems have been adapted to provide control over custom genetic targets. Engineered RNP-based control systems allow for fine-tune regulation of desired targets, by providing customizable nucleotide-nucleotide interactions. However, as there have been several engineered RNP systems developed recently, identifying an optimal system for various bioprocesses is challenging. Here, we review the most successful engineered RNP systems and their applications to survey the current state of the field. Additionally, we provide selection criteria to provide users a streamlined method for identifying an RNP control system most useful to their own work. Lastly, we discuss future applications of RNP control systems and how they can be utilized to address the current grand challenges of the synthetic biology community.

Key words Ribonucleoproteins, Genetic circuits, Posttranscriptional regulation, CRISPR-Cas, Engineered RNP control

1 Introduction

In recent years, synthetic biology has seen the developments of standardized genetic tools that allow regulation across a diverse set of systems, from *Escherichia coli* or *Saccharomyces cerevisiae* to mouse or human cell lines. These tools typically rely on transcriptional, translational, and other common signaling pathways and have been utilized to control various bioprocesses including engineering microbial metabolism to produce value-added chemicals [1–3], editing genomes directly [4], enabling cells to track and respond to environmental signals or stimuli [5], and allowing organisms to dynamically regulate their own metabolisms in response to stress [6].

In particular, ribonucleoprotein (RNP)-based control systems are proving increasingly valuable for the design and construction of orthogonal regulatory systems in organisms because they can be

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tuned via nucleotide-level interactions to direct metabolic pathways and cellular responses. As their name implies, RNP-based systems consist of an RNA-binding protein (RBP) complexed with an RNA transcript. Such complexes are frequently used by nature to control key aspects of gene regulation [7], providing a useful starting point for engineering the regulation of cellular responses. This methods chapter reviews key RNP-based control systems in both prokaryotic and eukaryotic systems that have been adapted as genetic circuitry for orthogonal control. We detail useful selection criteria for choosing RNP-based control systems for bioprocessing applications and point out future directions for expanding their use and versatility.

2 Current Systems

There are a wide variety of native RNPs [8, 9], but only a few that have been adapted to genetic circuitry and orthogonal regulation. Because of differences between the basic molecular biology of prokaryotes and eukaryotes, we consider RNP-based circuitry that involves these two domains separately, although the field is beginning to move toward more general and universal methods for engineering RNP control.

2.1 Engineered RNPs in Prokaryotes

There are a variety of native RNPs that have key regulatory functions in prokaryotes, such as the SRP [10, 11], RNase P [12], RNA-binding proteins (RBPs) complexed with RNAs and PNPase [13]; some of these have been adapted for use within engineered circuits. In addition, more general control by widely used CRISPR RNPs has been developed (*see* Fig. 1).

2.1.1 RNA-Binding Protein (RBP)-RNA Regulated Systems

Some of the earliest engineered RNP-controlled systems come from reprogramming bacterial RNase P ribozymes, particularly the RNase P from *E. coli*. Natively, RNase P cleaves tRNA precursors to generate the 5′ termini of mature tRNA molecules [14]. The RNP utilizes a guide sequence, which hybridizes to its target RNA, and directs cleavage of the target. Li et al. [15] first demonstrated that by engineering specific "external guide sequences" (EGS), one could specifically target RNase P cleavage of desired mRNA transcripts. The authors utilized RNase P from *E. coli* and engineered EGS to cleave mRNA sequences of B-galactosidase from *E. coli* and nuclease A in vitro. Additionally, they demonstrated almost 90% reduction in B-galactosidase activity using the engineered EGS-RNase P complex. After this breakthrough, work began to expand the capability of RNase P targeting of mRNA targets. Guerrier-Takada et al. [16] engineered external guide sequences with RNase P from *E. coli* to target chloramphenicol resistance genes in cultures of *E. coli*. The authors eliminated nearly all growth of chloramphenicol-resistant cultures that

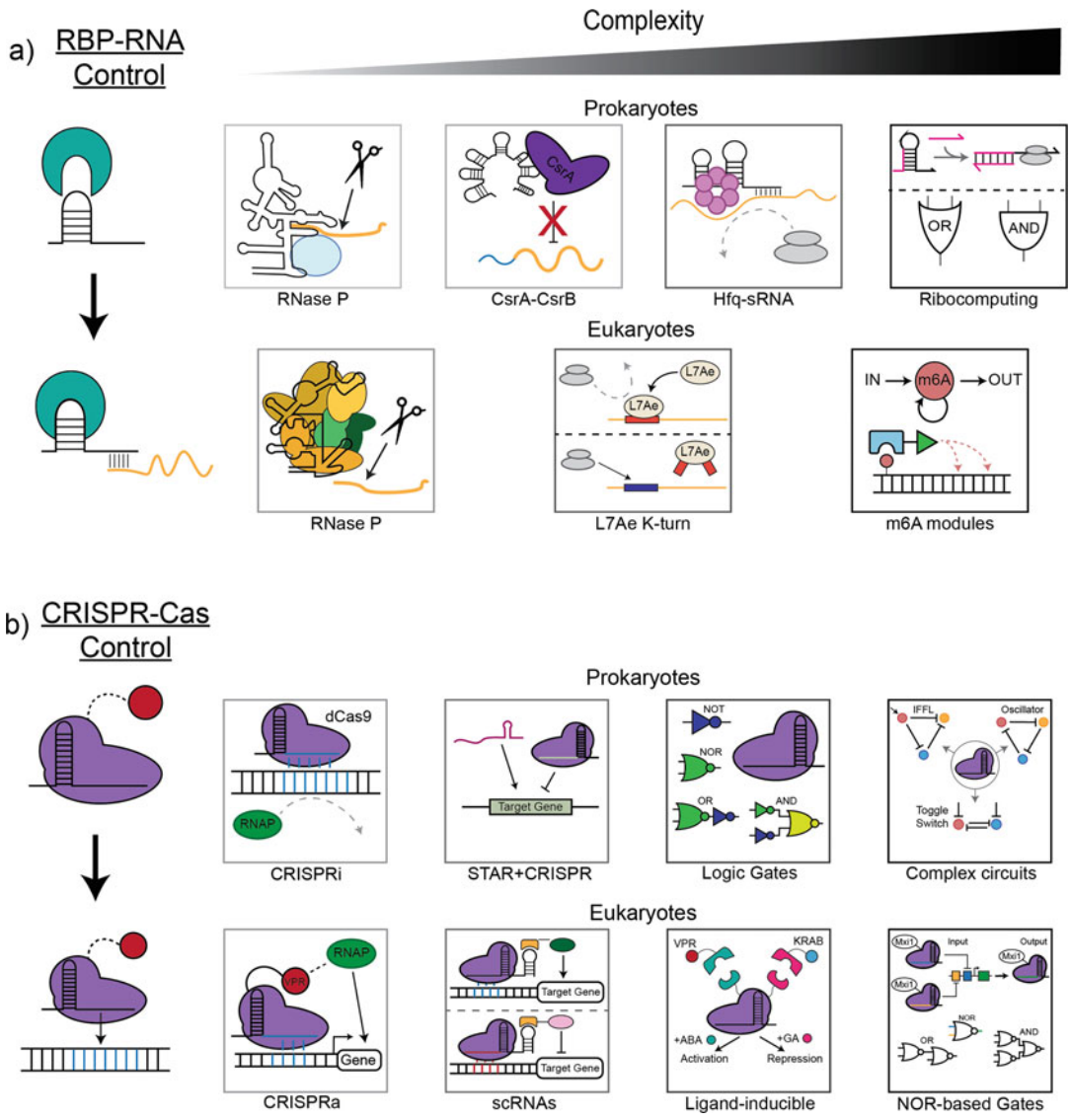

Fig. 1 Ribonucleoprotein-derived genetic control schemes developed in prokaryotic and eukaryotic systems. (**a**) Examples of RNP control schemes that utilize RNA-binding proteins (RBPs) and a guide RNA to achieve desired regulation arranged with increasing genetic circuit complexity. (**b**) Examples of RNP control schemes that utilize a CRISPR-Cas scaffold as a basis for developing engineered RNP-based control arranged with increasing genetic circuit complexity. Abbreviations are as follows: *m6A* N6-methyladenosine, *RNAP* RNA polymerase, *STARs* small transcription activating RNAs, *IFFL* incoherent feedforward loop, *VPR* VP64-p65-Rta domain, *KRAB* Kruppel-associated box, *ABA* abscisic acid, and *GA* gibberellin

contained plasmid-based chloramphenicol acetyltransferase (CAT) by expressing two CAT external guide sequences to drive cleavage via RNase P. The two EGS were put under control of an IPTG-inducible promoter, thus creating one of the earliest "NOT" gates, which targeted a drug-resistant phenotype *in E. coli*. Specifically, in

this simple single input logic gate, if IPTG is present as an input, chloramphenicol resistance of the *E. coli* cultures is effectively eliminated (i.e., in the presence of the inducer, the output was "negated"). Additionally, this genetic control scheme was adapted for human health applications. Cobaleda et al. [17] engineered two EGS to target oncogene products expressed in leukemia cells that inhibit apoptosis. By expressing the EGS and RNase P from *E. coli* in a mammalian cell model containing the two oncogene products, they demonstrated at least a 96% reduction in cell viability for cells expressing the two oncogenes, while minimizing off-target effects to only a 5% reduction in cell viability. This study was the first to engineer an EGS-RNase P complex that directly targeted and reduced oncogene expression in a mammalian cancer cell line. Subsequently, Trang et al. [18] used the RNase P RNP complex from *E. coli* to develop an engineered external guide sequence (EGS) to target the ICP4 mRNA transcript, a key protein involved in human herpes simplex virus 1 (HSV-1). Using the engineered EGS-RNase P complex, they achieved an 87% reduction in ICP4 mRNA and an 80% reduction in ICP4 protein in $^{\varphi}$CRE fibroblast cells. Additionally, cells expressing the engineered EGS-RNase P complex demonstrated a 1000-fold reduction in viral growth 18 h after exposure, while an inactive version of the complex demonstrated no reduction in viral growth. Recently, Yang et al. [19] developed a mutant RNase P ribozyme from *E. coli* by screening a library of rationally designed mutants. The identified mutant demonstrated 50-fold improved cleavage of viral mRNA from human cytomegalovirus (HCMV) relative to the wild-type version. In vivo, the mutant RNase P ribozyme achieved a 98% reduction in HCMV mRNA, which was 13% greater than the wild-type RNase P. This engineered RNase P was then introduced to the human cell line U373MG that were infected with HCMV. Five days after introducing the RNase P ribozymes to the infected cell lines, the cells with the mutant RNase P demonstrated 3500-fold reduction in HCMV mRNA, while cells containing the WT RNase P only showed a 100-fold reduction in HCMV mRNA.

Since the initial development of RNase P-regulated genetic control, development of novel prokaryotic RNP control systems has expanded to global RNA-binding proteins (RBPs) to posttranscriptionally regulate translation of mRNA targets. Two prevalent examples include the complexing of the Hfq or CsrA bacterial proteins and a small noncoding RNA (sRNA) [20]; we refer to these as RBP-sRNA regulated systems. The most common example of this RNP control scheme is Hfq-sRNA regulation. In this system, the Hfq protein binds to an sRNA and facilitates binding to a specific mRNA target, allowing for the sRNAs to hybridize and directly regulate the posttranscriptional fate of the mRNA itself [21]. This mechanism has been adapted to create many

Hfq-sRNA control systems in bacteria. One key example is from Na et al. in which the authors employed the Hfq-sRNA-mRNA interaction to downregulate expression of desired genes within *E. coli* [22]. The authors achieved this by engineering the MicC sRNA (an sRNA involved in regulation of the EnvZ-OmpR two-component signaling pathway [23]) with a custom seeding region to trans-actively downregulate a desired mRNA target sequence. They demonstrated the utility of the Hfq-MicC control system in a bioprocessing application by improving de novo production of tyrosine and cadaverine in *E. coli*. The authors improved overall product yield by downregulating several genes involved in competing metabolic pathways for tyrosine and cadaverine production. Noh et al. expanded upon this system by developing a MicC sRNA with tunable transcription levels [24]. This provided a system with a greater dynamic range of MicC sRNA transcriptions, which allowed for tunable repression of target genes. They utilized these modified constructs to improve overall titers of proline and putrescine in *E. coli* by knocking down genes involved in competing metabolic pathways of the two compounds. Importantly, systems like these, which have been previously reviewed [1], allow the user to fine-tune control over expression of desired genes by providing a customizable nucleotide seed sequence in the modified MicC sRNA. Moreover, users do not have to spend time engineering the genome to knockout or knockdown these genetic targets. An additional example of utilizing Hfq-sRNA posttranscriptional regulation comes from Lahiry et al. [25], in which the authors rationally redesigned the DsrA sRNA of *E. coli* to target mRNA transcripts involved in the n-butanol synthesis pathway of *Clostridium acetobutylicum*. The authors utilized the regulatory mechanism of the Hfq-sRNA-mRNA RNP complex to downregulate two key mRNA sequences shown to reduce n-butanol production. The authors iteratively redesigned the native stem loops of the DsrA sRNA to ultimately achieve over fourfold reduction in expression of both target genes. This work provides another framework for exploiting nucleotide-level interactions to drive RNP-based control within the Hfq-sRNA system.

In addition to Hfq, the bacterial global regulator protein CsrA has been utilized in an engineered RNP-based control system for posttranscriptional regulation of mRNA targets. CsrA is best known to prevent translation of mRNA targets via binding in the 5′ untranslated region (UTR) and occlude ribosome binding, although native, alternative mechanisms of activation and binding to other mRNA regions have also been reported for CsrA [26]. Omics studies of the Csr system such as Sowa et al. [27] and Leistra et al. [28] have identified multiple candidate genes that could be posttranscriptionally upregulated by CsrA. Moreover, recent work from Renda et al. [29] confirmed first the first

mRNA target posttranscriptionally activated due to binding of CsrA in the 5′ UTR. Thus, CsrA sits as a potential control node for simultaneous activation and repression of genetic targets. The RBP is primarily regulated by two small RNAs, CsrB and CsrC, which can each sequester multiple copies of CsrA and block formation of the RNP complex between CsrA and its target mRNAs [26]. In Leistra et al., the authors rationally engineered mutants of the CsrB sRNA of *E. coli* to have a gradient of stronger or weaker affinity for CsrA [30]. The authors created 20 total mutants, 10 with weaker affinity for CsrA than WT CsrB and 10 with stronger affinity. The strongest CsrB mutant demonstrated almost 3.5-fold stronger affinity with CsrA. Lastly, they demonstrated that the CsrB mutants could be utilized to control CsrA-regulated metabolic processes, such as glutamate production. Overexpressing the strongest CsrB mutant led to a 1.75-fold increase in glutamate production relative to overexpression of WT CsrB. This work is one of the first systems to engineer the Csr system and offer an approach to control the formation of CsrA-based RNP complexes by engineering competing RNA-CsrA interactions. This system offers a potential future opportunity to engineer tunable RNP-based control systems utilizing CsrA.

While not an RNA-binding protein, the ribosome can also be considered a RNP at the system level and, as such, affords broad opportunities for regulatory control. Genetic circuits have been developed to control translation by blocking or promoting formation of the ribosomal RNP complex. One key example comes from Green et al. [31], in which the authors developed "ribocomputing" devices utilizing sRNAs to unwind toehold switch hairpins that contained the ribosome binding site for a downstream gene of interest. In this scheme, when the toehold switch was unwound, the ribosome could bind and begin translation. The authors developed several devices of increasing complexity. They began with an individual "OR," as well as an "AND" logic gate. The "OR" gate consisted of two consecutive hairpins such that when provided with an sRNA complementary to *either* hairpin, the RBS was freed and allowed the ribosome to bind. The "AND" logic gate utilized a single hairpin that required two sRNAs to hybridize and rearrange the hairpin; only upon binding of both sRNAs, the RBS was liberated for ribosome binding. Next, they constructed multi-input gates such as a 4-sRNA "AND" gate, as well as a 6-sRNA "OR" gate. Ultimately, they constructed a 12-sRNA input circuit that utilized five consecutive "AND" gates. Each of these constructed gates achieved a range between 25-fold and 41-fold activations of exogenous GFP. The type of flexibility of this ribocomputing system is an enormous asset given the potential distinct advantages of "OR" and "AND" logic gates for gene regulation in different context. For example, work from Amalfitano

et al. [32] utilized these ribocomputing gates to control a glucose reporter that can be measured by store bought glucose meters. These circuits were utilized to specifically detect the presence of bacterial RNA from *Salmonella typhi*, the bacterium responsible for typhoid fever, at clinically relevant levels. Moreover, these circuits were then modified to specifically detect viral RNA from SARS-CoV-2 within an hour, while not being activated by any other control viral RNAs present. These ribocomputing circuits may provide the opportunity for portable diagnostics for use in clinical and affordable human health applications.

2.1.2 CRISPR-Cas-Regulated Systems

The second and potentially most well-known category of RNP-based control in bacteria are engineered CRISPR-Cas systems. Natively, CRISPR-Cas systems utilize a Cas protein complexed with a crRNA (CRISPR RNA, complementary to the DNA target) and tracrRNA (transactivating crRNA, a scaffold for Cas9 protein binding) for targeted genome editing [4]. While native CRISPR-Cas systems were known for at least two decades [33, 34], their engineering utility was first presented by Jinek et al., where they demonstrated targeted genome editing in *E. coli* by utilizing a Cas9 nuclease protein from *S. pyogenes* and a single guide RNA (gRNA) chimera adapted from a fusion of the tracrRNA and crRNA [35]. Since then, many iterations of CRISPR-Cas systems have been developed for targeted gene editing as well as precise control of gene expression. One of the biggest breakthroughs to CRISPR-Cas systems was the introduction of a catalytically inactive Cas9 protein (dCas9) from Qi et al. [36]; here, the authors introduced two point mutations to remove Cas9 endonuclease activity, effectively creating a customizable DNA-binding protein (DBP). Using this construct, the authors developed the CRISPR interference (CRISPRi) system. The dCas9 protein and gRNA formed an RNP that operated as a DNA-binding complex that could repress various targeted genes based on the seeding sequence of the gRNA. They were able to demonstrate almost 1000-fold repression of specific target genes in *E. coli* and demonstrated the ability to target multiple genes simultaneously by using multiple gRNAs [36]. The development of dCas9 has allowed for novel CRISPR-Cas9 regulatory tools. As an example, Nielsen et al. expanded the versatility of CRISPR-Cas regulation in genetic control schemes [37] by utilizing dCas9 to construct multiple genetic circuits within *E. coli*. The authors first constructed an arabinose-responsive "NOT" gate, such that in the presence of arabinose, the gRNA sequence was expressed and complexed with dCas9 to transcriptionally repress a desired target gene by binding within the promoter sequence of the target gene. The authors then built more complex gates such as two consecutive "NOT" gates, a "NOR" gate (where the lack of two imposed conditions leads to a desired

output), an "OR" gate, as well as an "AND" gate. The two consecutive "NOT" gates utilized anhydrotetracycline (aTc), and 2,4-diacetylphloroglucinol (DAPG)-inducible promoters to achieve a double inverter circuit. In the presence of aTc, the gRNA sequence was transcribed to direct dCas9 repression of the downstream target. Upon addition of DAPG, a gRNA sequence was expressed that blocked the aTc promoter sequence via dCas9, alleviating repression of the downstream target. The "NOR" gate utilized a DAPG and arabinose-responsive promoter to control expression of a gRNA to target a downstream gene with dCas9. In the presence of either or both compounds, the downstream target was repressed. The "OR" gate combined the "NOR" and "NOT" gates consecutively to activate the downstream target in the presence of DAPG or arabinose. Lastly, the "AND" gate combined the arabinose-responsive and DAPG-responsive "NOT" gates into the "NOR" gate to generate an "AND" gate, such that only in the presence of both arabinose and DAPG would the downstream target be activated. These engineered circuits measured at least a 100-fold change in target gene expression for each gate, using plasmid-based red fluorescent protein (RFP) as a reporter. Finally, the authors applied the "OR" gate to control the *malT* gene and demonstrated control of the lambda phage susceptibility phenotype under the control of Ara and/or DAPG inducers. Along with interference, methods to activate genes using CRISPR-Cas components (CRISPRa) in bacteria were developed. Bikard et al. first demonstrated activation of endogenous target genes by fusing dCas9 to the omega subunit of RNAP in *E. coli* [38]. While novel, activation using CRISPR proved to be more difficult, as the authors could only achieve a maximum of 23-fold activation of endogenously expressed GFP, which was comparable to other transcriptional activation systems at the current time [39, 40]. Importantly, Dong et al. [41] expanded upon the CRISPR activation regulatory scheme originally developed by Bikard et al. To achieve enhanced CRISPR-based activation in bacterial systems, the authors utilized modified gRNAs fused to an RNA hairpin that binds an RNA-binding protein (RBP), which itself is fused to a transcriptional activator protein domain. A transcriptional activator protein can be defined as a protein or protein domain, which natively recruits RNA polymerase machinery [42]. After screening several bacterial transcriptional activators, they found the SoxS activator fusion could achieve almost a three-fold activation in expression of targeted endogenous genes relative to the original CRISPR. The complex was modified to improve CRISPR activation activity, while minimizing native SoxS DNA binding affinity. Lastly, the authors demonstrated both aTc and arabinose-inducible genetic regulation of multiple genetic targets using the CRISPRa constructs and the CRISPRi system from Qi

et al. in tandem. This work was one of the first demonstrations of simultaneous activation and repression of endogenous genes in bacterial systems. These developments provided novel opportunities to engineer complex genetic circuits from CRISPR-based regulation.

Recently, Santos-Moreno et al. [43] greatly expanded upon the complexity of CRISPRi-regulated genetic circuits. The authors developed the classic toggle switch, feedforward loop, and oscillator in *E. coli*. The toggle switch expressed two gRNA sequences that responded to either arabinose or AHL. In the absence of either inducer, two gRNAs were expressed to repress each other, which was measured by low fluorescence of super-folder green fluorescent protein (sfGFP). This was achieved by constitutively expressing two gRNA sequences. The first gRNA sequence (sgRNA-1) repressed the second gRNA sequence (sgRNA-4) and the *sfGFP* gene, while the second gRNA (sgRNA-4) repressed sgRNA-1. Thus, the two cancelled each other out and kept sfGFP expression low. Concurrently, a second copy of sgRNA-1 was controlled by an arabinose-inducible promoter, and a second copy of sgRNA-4 was under an AHL-inducible promoter. In the presence of AHL, the switch was toggled to high activity by induction of the second copy of sgRNA-4; this was indicated by high sfGFP fluorescence. Conversely, in the presence of arabinose, the second copy of sgRNA-1 was expressed to maintain repression of sfGFP, measured by low fluorescence. The authors developed an arabinose-inducible feedforward loop, in which a single gRNA repressed two targets: an sfGFP sequence and a second sgRNA. The second sgRNA also repressed the sfGFP sequence. Thus, across an arabinose induction gradient, there is a small concentration that allows for a spike in sfGFP fluorescence, while all other arabinose concentrations lead to basal levels of sfGFP fluorescence. Lastly, the authors constructed the "Repressilator" circuit, termed the "CRISPRlator," in which three sgRNAs consecutively repressed each other. This work was the first demonstration of using CRISPRi to construct the toggle switch, incoherent feedforward loop, and oscillator, three classic genetic circuits originally published by Gardner et al. [44] (toggle switch), Magnan et al. [45] (incoherent feedforward loop), and Elowitz et al. [46] (oscillator), respectively.

Along with developing CRISPR-based genetic control schemes in model organisms, such as *E. coli*, there has been development of regulatory systems in more industrially relevant prokaryotes. One of the best examples comes from Gordon et al. [47]. The authors developed a CRISPRi toolbox for the cyanobacteria *Synechococcus sp*. Strain PCC 7002 to repress endogenous gene targets by adapting the CRISPRi system previously developed by Qi et al. [36]. They placed the sgRNA and dCas9 constructs under an anhydrotetracycline (aTc)-inducible promoter, effectively creating

an aTc-responsive "NOT" gate. The authors first demonstrated sevenfold repression in endogenously expressed EYFP using the "NOT" gate. Next, they applied the regulatory system to redirect carbon flux for improved lactate production in cyanobacteria. By repressing glutamine synthesis I (*glnA*), they were able to achieve over 8 mM in lactate synthesis, which was the highest titer of lactate photosynthetically produced. Additionally, the rate of lactate production was twofold greater than that of the nonengineered, wild-type strain. This work demonstrates the versatility of CRISPR-based regulatory circuits and the ability to utilize RNP control in industrially relevant microbes. Concurrently, Cleto et al. [48] developed a CRISPRi system for the industrial microbe *Corynebacterium glutamicum*, which is heavily used for large-scale batch production of many amino acids. The authors developed an IPTG-responsive "NOT" gate to control repression of endogenous target genes. Next, they utilized the CRISPRi system to knockdown genes in glycolysis to redirect carbon flux to improve production of L-lysine and L-glutamine. By using the CRISPRi system to downregulate the *pgi* or *pyk* genes, the authors achieve a 1.31-fold and 3.04-fold increase of L-lysine and L-glutamine, respectively, relative to a wild-type strain. The utilization of the CRISPRi-based gate demonstrated the versatility and ease for gene regulation without genomic editing in a key industrial microbe, which could help speed up time required for industrial strain engineering.

Additionally, CRISPR-based control schemes have been utilized in tandem with additional methods of genetic control to produce novel circuities. One paramount system that exemplifies this comes from works by Chappell et al. 2015 [49] and Chappell et al. 2017 [50], in which they developed a scheme to control transcription termination of RNA polymerase (RNAP) on its target DNA sequences. The authors developed and improved upon the small transcription activating RNAs (STARs) control system, respectively. The authors developed sRNA sequences to disrupt a transcription terminator that was placed upstream of a gene of interest. Addition of the STAR allowed for 94-fold activation of the downstream gene. The RNAP complex was not disrupted by the termination sequence, allowing for transcription elongation of the downstream target genes. These STARs were utilized to create RNA-based logic gates for transcription and regulate multiple genes in metabolic pathways, as well as used in tandem with CRISPRi to create a feedforward control circuit to drive accelerated CRISPRi response.

2.2 Engineered RNPs in Eukaryotes

Eukaryotic systems contain both homologous RNPs found in prokaryotes, such as RNase P, as well as additional native eukaryote RNPs including the spliceosome [51], telomerases [52], and snRNPs [53]. Some of these systems have been adapted for targeted genetic control in eukaryotes. Additionally, as was the

case with prokaryotes, engineered RNP schemes based on CRISPR-Cas control are now abundant.

2.2.1 Engineered RNA-Binding Protein-RNA-Regulated Systems

Just as in prokaryotic systems, some of the first engineered RNP-based control schemes in eukaryotes involved the RNase P complex. Primarily, RNase P from humans utilized in complex with engineered external guide sequences (EGS) to cleave specific mRNA targets. Moreover, with the foundation of utilizing RNase P from *E. coli* to target viral mRNA transcripts, most systems adapted the human RNase P complex to achieve similar results. Jiang et al. [54] were one of the first to utilize engineered external guide sequences (EGS) to direct human RNase P to target and cleave the protease mRNA sequence from human cytomegalovirus (HCMV). The engineered target sequence was 35-fold more active directing RNase P cleavage of the target mRNA sequence in vitro than when using a wild-type-derived EGS. Additionally, the engineered EGS-RNase P complex achieved a 95% reduction in expression of the protease and a 4000-fold reduction in viral growth in U373MG cells, compared to the wild-type-derived EGS, which only achieved an 85% reduction of HCMV protease and 150-fold reduction in viral growth. This work opened the door for utilizing RNase P RNP complexes as a means to treat human health issues, such as HCMV infections.

Concurrently, implementing native RNA-binding proteins (RBPs) for custom genetic regulation began emerging as a popular field of interest for developing RNP-based control schemes. Most often, specific RBPs are fused to another protein editor that is typically utilized to modify desired RNA transcripts. The review by Shotwell et al. [55] provides an in-depth review of the selection and design of RBPs in these regulatory systems. One of the first examples of this approach comes from Saito et al. [56]. The authors developed a modular synthetic translational repressor and activator to regulate translation of exogenous mRNA transcripts, utilizing the archaea-derived L7Ae-box C/D kink-turn motif RNP to control translation. This RNP consists of the archaeal RNA-binding protein, L7Aa, which binds a short guide RNA sequence that contains two conserved motifs: box C (RUGAUGA) and box D (CUGA). The constructed repressor design consists of the C/D guide RNA motif sequence fused directly upstream of the start codon of a desired transcript. When expressed, the ribosomal L7Ae protein binds the guide RNA C/D box motif in mRNA fusion transcript and blocks translation of the downstream gene. The authors demonstrated a 90% reduction in translation of a box C/D-enhanced green fluorescent protein (EGFP) target fusion, measured by a 90% reduction in EGFP fluorescence. To design the activator, the authors replaced the original C/D box motif with an anti-C/D box motif, the complementary sequence, directly upstream of the EGFP target gene. Concurrently, the original C/D

box sequence was constitutively expressed, such that the two sequences could hybridize and the C/D turn motif would block translation through RNA interference. However, when the L7Ae protein is expressed, the protein binds the C/D RNA sequence and alleviates the RNA interference and translational repression of the transcript. Using this scheme, they achieved over 70% activation of the anti-C/D motif EGFP transcript, measured by EGFP fluorescence, relative to fluorescence of only expressing the unmodified EGFP transcript. Additionally, they demonstrated this system could simultaneously repress and activate two different targets, showing nearly 100% repression of a model DsRed transcript and almost 100% activation of another model EGFP transcript. Lastly, L7Ae was put under an anhydrotetracycline (aTc)-inducible promoter to create a tetracycline-responsive "NOT" gate using the repressor construct in HeLa cells, showing titratable repression of an EGFP transcript. This study was one of the first to utilize a specific RNA-binding protein and an RNA sequence in eukaryotic cells to regulate expression of a desired mRNA transcript. Utilizing RBPs in the aforementioned manner to directly engineer epitranscriptome control in eukaryotes has been of particular interest over the last few years. One of the most exciting examples comes from Liu et al. (2019) [57], in which the authors focus on an engineered N^6-methyladenosine (m6A) writer that allows for directed m6A edits on desired mRNA transcripts [45]. The authors developed the novel m6A writer by fusing the human METTL3/METTL14 heterodimer, a native m6A writer, to the dCas9 enzyme and were able to selectively target mRNA sequences with gRNAs. Additionally, the authors developed a novel m6A eraser by fusing either human ALKBH5 or FTO to dCas9 to exploit the native eraser capabilities of these two proteins to selectively remove m6A modifications. Using this system, they demonstrated selective addition and removal of m6A modifications within the human *Malat1* mRNA sequences, which drove structural remodeling of the sequence and downstream interactions with the human heterogeneous nuclear ribonucleoprotein C (HNRNPC). Removal of an m6A modification within a hairpin of the *Malat1* transcript restructures the hairpin, such that HNRNPC cannot bind. Removal of the m6A modification by the engineered m6A eraser reduced binding by 50%. These circuits could be used in the future to potentially control downstream regulatory effects of transcripts containing m6A modifications in humans. In addition to utilizing human m6A writer/eraser dCas9 fusions, others have imported components from prokaryotes to achieve similar novel RBP fusion regulatory systems, such as in Park et al. (2019) [58]. The authors developed a synthetic "read-write module" to detect or add m6A modifications to mRNA transcripts utilizing multiple fusion proteins. The module first contained an "initiator" complex,

which consisted of DNA adenine methyltransferase (Dam) *from E. coli* fused to an engineered zinc finger protein domain that binds specific DNA sequence in the AAV1 locus in the human genome. The zinc finger coordinated the Dam protein adjacent to the AAV1 locus, such that Dam protein could add an m6A modification to a GATC motif in the locus. Next, they built the "reader" portion by fusing the DNA binding domain of Dpn1 from *S. pneumoniae* to DNA effector domains (VP64 for activation, KRAB for repression). Dpn1 would bind to the specific m6A modifications, while the DNA effector domain would "read" out transcription of AAV1 locus, which contained a copy of EGFP integrated into the genome. To create the "write" portion, the authors fused Dam to DpnI, such that the write module could "write" additional m6A modifications to the AAV1 locus. The full "read-write" module demonstrated inducible transcriptional activation of EGFP in the AAV1 locus, measured by EGFP fluorescence. Upon induction, the "initiator" added a single m6A modification, the "writer" bound to the single modification, and added additional m6A modifications to downstream GATC motifs. The multiple m6A modifications enhanced recruitment of the "reader" to activation expression of EGFP from the AAV1 locus. The full module demonstrated a 30-fold activation or repression of EGFP depending if the VP64 or KRAB domain was utilized in the "reader." The authors lastly utilized this three-component system to confer "epigenetic memory" to the genome via m6A modifications that were conserved across daughter cells, meaning new cells contained the parent cell m6A modifications, which activated EGFP expression from the AAV1 locus. The cells containing the three-component system retained at least 50% of the population continued to upregulate EGFP transcription for ~14.3 days, relative to cells utilizing inducible plasmid-based transcription factor activator proteins, which was only 2.8 days.

2.2.2 CRISPR-Cas-Regulated Systems

The most well-studied category of engineered RNP genetic circuits in eukaryotes involves CRISPR-Cas machinery. After Jinek et al. [35] demonstrated the use of CRISPR-Cas9 for gene editing in bacteria, work from Cong et al. [59] and Mali et al. [60] quickly followed to establish functional CRISPR-Cas9 in eukaryotic cells for precise gene editing. In Cong et al., the authors demonstrated Cas9 from *S. pyogenes* can be programmed to target multiple genomic locations in human 293FT cells, using variable gRNA sequences. Additionally, they demonstrated this system could utilize multiple gRNA sequences simultaneously for multiplexed genome modifications. Work from Mali et al. [60] demonstrated that a similar CRISPR-Cas9 system could achieve homologous recombination in 293T, K562, and induced pluripotent stem cells using Cas9 from *S. pyogenes* and engineered gRNAs. Additionally,

they constructed a library of 190,000 predicted gRNA sequences which covers 40.5% of the human genome.

After demonstrating functional CRISPR-based systems in eukaryotes, the first major development in CRISPR-regulated genetic circuits in eukaryotes came from work by Qi et al. [36], which was detailed in the previous section. Along with developing a CRISPRi system in bacteria, they demonstrated the CRISPRi system could also work in HEK293 cells with at least twofold repression of an endogenous EGFP-coding region. With Qi et al. demonstrating that dCas9 could regulate desired exogenous and endogenous transcripts in eukaryotes, the first CRISPR-driven genetic control schemes appeared. Kiani et al. [61] developed some of the first CRISPR-based repressors in HEK293T cells. They achieved over 100-fold repression of exogenous EYFP by using the engineered gRNA-dCas9 RNP to target the promoter of the EYFP gene. They next layered two repressor constructs consecutively, creating one of the first double inverter circuits in eukaryotes. The inner layer expressed "gRNA-a" to complex with dCas9 and repress EYFP transcription, while the outer layer expressed "gRNA-b" to repress transcription of the inner gRNA. This double inverter achieved a 27-fold change in activity of EYFP. Lastly, the authors created a doxycycline-dependent "NOT" gate by regulating gRNA transcription via a doxycycline-responsive promoter. The doxycycline-dependent "NOT" gate demonstrated 2–30-fold repression of plasmid-based EYFP-based doxycycline induction concentration in HEK293T cells.

To improve repression strength by dCas9 in eukaryotes, work was done to fuse dCas9 to various effector domains. Gilbert et al. [62] demonstrated that fusing the chromatin modifier Kruppel-associated box (KRAB) domain could achieve at least 15-fold repression of endogenous EGFP in HEK293 cells; the rationale for the dCas9-KRAB fusion confers localization of the KRAB domain via dCas9 to allow for chromatin remodeling and silence transcription through histone deacetylation, meaning the target sequence would become inaccessible to transcriptional machinery at the chromatin packs more tightly around the histones. Using the dCas9-KRAB fusion protein, the authors developed a "NOT" gate, which achieved 15-fold repression of plasmid-based EGFP, measured by fold reduction of EGFP fluorescence. This demonstrated to the field that dCas9-DNA effector domain fusions could improve the overall efficacy of CRISPR-Cas-based repression and also be applied to create user-controlled genetic circuits. Gander et al. [63] greatly expanded dCas9-based genetic circuits by synthesizing a library of "NOR" gates in *S. cerevisiae*. The authors enhanced target repression fusing dCas9 to the Mxi1 domain from human, a protein that was shown to effectively recruit histone deacetylase enzymes for chromatin remodeling. The

rationale was similar to that of Gilbert et al. [62], where they repress genes of interest by driving chromatin remodeling to make target sequences sterically inaccessible. The dCas9-Mxi1 fusion achieved a maximum of 98% reduction in plasmid-based GFP expression, while standard dCas9 had a maximum of only 42% reduction in GFP expression. The "NOR" gate used two gRNA sequences simultaneously to repress one target; 20 gRNAs and target sequences were designed, allowing for a possible 400 unique gate combinations. To demonstrate the utility of this system, the authors constructed increasingly complex gates using different combinations of "NOR" gates, including "OR," "AND," "NAND," "XNOR," and "XOR" gates. Lastly, by using two of the same targeting gRNAs for the "NOR" gate, the authors converted the "NOR" gate to a simple "NOT" gate. They demonstrated seven consecutive "NOT" gates could still achieve a twofold repression. Achieving a twofold repression through a seven-tier regulatory cascade is remarkable, as this was the first instance of a CRISPR-based regulatory cascade in a eukaryotic system achieving significant target repression that consisted of more than two tiers of regulation within the circuit. Moreover, this library provided the first highly customizable system to build complex genetic circuits in eukaryotes. Yeo et al. [64] further enhanced dCas9-based repression in eukaryotes by fusing the human MeCP2 effector domain to the dCas9-KRAB fusion protein developed by Gilbert et al. [62]. The MeCP2 protein further improves chromatin remodeling, as it natively recruits deacetylase enzymes [65]. This configuration now maximizes recruitment of deacetylase enzymes to remodel chromatin around target sequences and repress transcription. The dCas9-KRAB-MeCP2 fusion showed almost twofold increased repression of endogenous genes than the previous dCas9-KRAB fusion. Next, they integrated the dCas9-KRAB-MeCP2 fusion into exogenous and endogenous gene circuits to show improved control. The authors constructed a doxycycline-inducible "NOT" gate, which achieved almost 40-fold repression of exogenous EYFP, which was four times greater than the dCas9-KRAB containing "NOT" gate. They also built a doxycycline-inducible double inverter circuit, similar to the double inverter in Kiani et al., which led to a threefold activation of exogenous EYFP. Lastly, they constructed a double inverter circuit to regulate the endogenous CXCR4 gene. The dCas9-KRAB-MeCP2 achieved an eightfold change in CXCR4 regulation, which was at least twofold greater than other dCas9 constructs used in the inverter.

In parallel to developing CRISPRi-based genetic circuits, there has been significant effort to develop CRISPR-activated (CRISPRa) control schemes in eukaryotes. CRISPR-Cas-based gene activation was first demonstrated in works from Gilbert et al.

(ref#), Cheng et al. [66], and Perez-Pinera et al. [67]. All three works utilized dCas9 fused to multiple copies of the herpes simplex virion protein (VP16) transcriptional activation domain, which natively complexes with transcription factors to drive transcriptional activation, and were able to achieve targeted gene activation of both exogenous and endogenous targets in human cell lines. The work of Nissim et al. [68] was one of the first works to develop CRISPRa-based genetic circuits. The authors utilized a dCas9-VP64 (taCas9) fusion protein to build a transcriptional genetic cascade in HEK293T cells. The transcriptional cascade utilized "gRNA1" complexed with taCas9 to activate expression of "gRNA2," which complexed with taCas9 to drive transcription of the ECFP protein.

To further improve CRISPR-based gene activation, Chavez et al. [69] rationally designed a VP64-p65-Rta (VPR) tripartite activator to fuse to dCas9. The VP64 domain, the technical term for four consecutive copies of the VP16 domain, the p65 domain, and the Rta domain are all well-established transcriptional activator domains. VP16 and Rta domains are activators from herpes simplex and Epstein-Barr viruses, while p65 is the most potent transcriptional activator found in humans [70]. Thus, the expected additive functionality of fusing the three domains is potent transcriptional activation. The dCas9-VPR fusion demonstrated a 22-fold to 320-fold increase in endogenous gene expression relative to activation by the dCas9-VP64 fusion from Gilbert et al. [62] in HEK293T cells. Of significance to the field, development of the dCas9-KRAB CRISPRi and dCas9-VPR CRISPRa platforms vastly expanded the potential for CRISPR-based RNP control, such that users could now develop powerful genetic circuits to either significantly activate or repress target genes in eukaryotic systems. Gao et al. [71] developed chemically inducible control of CRISPRa and CRISPRi genetic circuits from modified dCas9 fusion proteins. The authors modified the dCas9-VPR fusion protein from Chavez et al. [69], such that the VPR activation domain was fused via chemically inducible heterodimerization linker domains. Meaning, a proper functional fusion between the dCas9 protein and the VPR domain can only form in the presence of a specific chemical inducer, as the chemical allows the two parts of the heterodimer to successfully link to one another. After screening multiple domains, they found that an abscisic acid (ABA)-inducible and a gibberellin (GA)-inducible heterodimerization domain, previously discovered by Liang et al. [72] and Miyamoto et al. [73], could achieve a 165-fold and 94-fold activation of plasmid-based EGFP in HEK293T cells, respectively. Next, they replaced the VPR activation domain with the KRAB repression domain and observed 5.6-fold and 3.2-fold repression of EGFP only in the presence of ABA and GA, meaning the dCas9 and KRAB domains could only

fuse together and achieve desired regulation via successful heterodimerization, which required the presence of either ABA or GA. The authors next created an "OR" as well as an "AND" gate by fusing both ABA-inducible and GA-inducible heterodimerization domains to a single dCas9 protein. The "OR" gate was operated by fusing the GA-inducible domain to the N-terminus of dCas9, while fusing the ABA-inducible domain to the C-terminus of dCas9. Therefore, in the presence of either or both inducers, dCas9 could fuse to the VPR domain, as the VPR domain was fused to the other half of either the ABA-inducible or GA-inducible domain. Thus, the VPR domain could properly fuse to the dCas9 protein and activate a plasmid-based EGFP target. The authors demonstrated at least 76-fold EGFP activation for any of the inducer combinations used. The "AND" gate was constructed by fusing one half of the GA-inducible domain to the N-terminus of dCas9. The other half of the GA-inducible domain was fused to one half of the ABA-inducible domains. Lastly, the remaining half of the ABA-inducible domain was fused to the VPR domain. Thus, the full dCas9-VPR fusion protein required the presence of both ABA and GA to be present for the complete fusion to be successfully constructed, as GA allowed for dCas9 to fuse to the intermediate domain and ABA allowed for VPR to fuse to the other half of the intermediate domain. The "AND" gate ultimately achieved 49-fold activation of exogenous EGFP. Lastly, the authors constructed a dCas9 fusion that could either activate or repress a desired target. To do this, they modified the "OR" gate by replacing the VPR domain that was fused to one half of the ABA-inducible domain with the KRAB repression domain. Thus, in the presence of GA, the dCas9 construct could fuse to VPR and activate its desired target, while in the presence of ABA, dCas9 would fuse to KRAB and repress its target. This is diagrammed in Fig. 1b, within the "ligand-inducible" box. Using this system, they were able to achieve 2.7-fold repression and 2.5-fold activation.

Along with engineering the Cas protein involved in the gRNA-Cas RNP regulatory complex, significant efforts have been made to engineer the gRNA sequence to improve regulatory strength as well as to provide users with novel control methods in CRISPR-driven genetic circuits. First, work from Zalatan et al. [74] developed modified gRNAs by appending specific RNA hairpins to the 3′ end of the gRNA sequence, which recruited specific RNA-binding proteins (RBPs) fused to transcriptional activator or repression domains. These engineered gRNAs were termed scaffold RNAs (scRNAs). The authors utilized the MS2, PP7, and com RNA hairpins to recruit MCP, PCP, and Com RBPs, respectively, in the scRNA constructs. First, each RBP was fused to the VP64 transcriptional activator domain and demonstrated 50-fold, 35-fold, and 20-fold upregulation of plasmid-based mVenus in

S. cerevisiae, respectively. Additionally, the dCas9-VP64 fusion from previous works [62, 66, 67] only demonstrated a two to threefold increase in expression. This system was then transfected into HEK293T cells, in which the MCP scRNA achieved 150-fold activation of plasmid-based fluorescent protein mVenus and sixfold activation of the endogenous CXCR4 gene. Activation by the MCP scRNA was three times greater than the dCas9-VP64 fusion for both the exogenous and endogenous transcripts. The authors also replaced the VP64 activator domain with the KRAB repressor domain in each scRNA construct. The Com scRNA fusion achieved the maximum of a fivefold reduction in endogenously expressed EGFP, which was consistent with the dCas9-KRAB fusion construct. Lastly, the authors demonstrated simultaneous activation and repressor of endogenous CXCR4 and B4GALNT1, respectively, thus creating a programmable bidirectional switch in eukaryotic systems using these scRNA constructs. In this case, they achieved this by expressing two sgRNAs: one that contained MS2 RNA hairpin to recruit an MCP-VP64 RBP fusion to activate the CXCR4 target and one that contained the comb hairpin to recruit a Com-KRAB fusion to repress the B4GALNT1 target. Second, Liu et al. [75] built CRISPR-based "signal conductors" by fusing gRNA sequences to ligand-responsive riboswitches, such that the CRISPR-Cas9 complex could become active in the absence of the desired ligand, the guide sequence base pairs with the antisense stem of the riboswitch. In the presence of the desired ligand, the riboswitch undergoes a conformational change and liberates the guide sequence, such that it can interact with its target DNA target, thus activating the RNP complex. The authors coupled these riboswitch-gRNA fusions with dCas9 or dCas9-VP64 fusion proteins to either selectively repress or activate endogenous targets, respectively. They first demonstrated the circuit's ability to upregulate TP53 and CDKN1A, two antitumor genes, in the presence of the oncogenic signal nucleophosmin (NPM). The authors transfected T24 bladder cells, which have elevated concentrations of NPM, and demonstrated activation of TP53 and CDKN1A, two key genes in antitumor metabolism, measured by a twofold reduction in cell growth relative to cells with mutant sgRNA aptamers. They also demonstrated the modularity of the circuit by also upregulating tumor migration suppressor, E-cadherin, in the presence of oncogenic signal Ets-1. The circuit utilized an Ets-1-sensing aptamer fused to the sgRNA; thus in the presence of Ets-1, the gRNA could rearrange into its active state. This design achieved a fivefold reduction in tumor cell migration relative to cells containing a mutant sgRNA. Lastly, the authors constructed a circuit that could upregulate and repress genes simultaneously in response to an external signal by employing the sgRNA-aptamer fusions with dCas9-MS2 and dCas9-VP64-Rev

fusion proteins co-expressed in a single cell line. Using these constructs, the authors created an AND gate to repress BCL2 and upregulate BAX genes in response to NF-kB and B-catenin, respectively. These genes were selected, as repression of BCL2 with simultaneous activation of BAX drives apoptosis in cancer cells. The authors demonstrated they could selectively drive a sevenfold increase in apoptosis in three bladder cancer cell lines, while minimizing changes in apoptosis for three normal human cell lines. Ying et al. [76] developed a CRISPR activation construct that regulates transcription of a plasmid-based RNA aptamer that fluoresces via binding to a small molecule organic florigen. The gRNA sequence complexed with dCas9-VPR fusion contains a hairpin which prevents the guide sequence from binding to its target sequence. In the presence of specific target mRNA, the gRNA hybridizes with the mRNA transcript and undergoes structural switching such that the RNP complex can activate the transcription of the fluorescent RNA. This system was utilized in HeLA cells to detect *survivin* mRNA, also known as BIRC5, a gene upregulated in many cancers. Using this construct, fluorescent signals could be detected within an hour of induction of *survivin* mRNA, while protein-based sensing circuits took at least eight hours for signal detection.

With the development of CRISPR-based genetic circuits in eukaryotes, these systems have been recently applied to address human health issues. Liu et al. [77] developed an "AND" gate utilizing CRISPR-Cas9 in order to identify and respond to bladder cancer cells. The authors placed Cas9 and a lacI-targeting gRNA under the control of two promoters specifically activated in bladder cancer cells, hUPII and hTERT. Upon activation, the CRISPR-Cas9 complex would block transcription of LacI, which represses a downstream gene of interest. Additionally, there was no significant activation in seven other human cell lines tested. Next, they replaced the luciferase with hBax, p21, or E-cadherin gene, which drove apoptosis, reduced growth, and reduced motility, respectively. These CRISPR-based genetic circuits lay the foundation for potential gene therapies or cancer diagnostics. Recently, Fan et al. [78] developed a liposome-delivered CRISPR-Cas circuit to downregulate VEGFR2, Bcl-2, and *survivin* transcripts in human bladder cancer 5637 cell implanted into mice bladders. Liposomes specifically delivered target gRNAs and Cas13a, a homologue to the Cas9 protein that targets and cuts single stranded RNA transcripts, by using antibodies specific to VEGFR2 receptors on the tumor cells. Upon delivery, the CRISPR-Cas13a complex would form and target the desired transcripts; in this system, all target transcript expression levels were reduced to at least 40% of their levels prior to treatment. Additionally, overall tumor size was reduced to ~35% after 20 days of continuous treatment. This is one of the first instances

of using a CRISPR-based circuit in gene therapy for the treatment of human cancers. CRISPRi regulation was also utilized to program human gut bacteria. Lastly, Mimee et al. [79] developed genetic parts to program the gut bacterium *Bacteroides thetaiotaomicron*, specifically a CRISPRi-based "NOT" gate that allowed for IPTG-inducible repression of desired exogenous and endogenous targets. They colonized the gut of mice and demonstrated IPTG-inducible repression using the CRISPRi "NOT" gate by providing IPTG to the mice through drinking water. This is one of the first examples of using CRISPRi-based genetic circuits to program bacteria directly related to the human gut microbiota.

3 Selecting an Appropriate System

One of the hallmarks of synthetic biology is the fungibility of parts and circuits, and in this vein, we believe that many of the RNP-based control systems described could be cross-utilized in a variety of settings. To select an RNP-based control system that will be of the greatest utility, we review the benefits and current limitations for each of the regulatory categories reviewed above. The benefits and limitations of each category are summarized in Table 1.

In short, both prokaryotic and eukaryotic RNP control schemes broadly fall into RBP-RNA-based and CRISPR-Cas-based categories. In general, RBP-RNA-based control provides the advantage of control via engineering RNA-RNA hybridization, while also serving as a protein interface with the remainder of metabolism. For example, a global RNA-binding protein such as Hfq or CsrA can be utilized in tandem with an sRNA to achieve posttranscriptional regulation of desired mRNA transcripts, precisely because these proteins have known functional "interpretations" in a systems context. This system-wide interpretation also allows for multiplexing to target several transcripts simultaneously. These systems have been utilized for controlling the expression of multiple genes in prokaryotic systems to globally regulate cellular metabolism; for example, Na et al. [22] and Noh et al. [24] used Hfq and target sRNAs in bioprocessing applications to regulate competing metabolic pathways.

Drawbacks to RBP-RNA-based control include the fact that systemic functional interpretations are often fixed, meaning targets can often either just be repressed or activated. That said, this drawback could likely be overcome by the simple expedient of using "ribocomputing" devices to provide RNP-based regulation, for example, by using programmed strand exchange to regulate formation of an mRNA-ribosome complex. The portability of specific regulatory systems (e.g., bacterial Hfq into eukaryotes) is

Table 1
Summary of benefits and limitations of RNP-based control schemes in prokaryotes and eukaryotes

System	Mode of control	Regulatory mechanism	System benefits	Current limitations	Circuit application
Prokaryote	Ribozyme-directed mRNA regulation	mRNA transcript degraded by RNase P and external guide RNA sequence (EGS)	Target specific mRNA transcripts for degradation with minimal off-target effects	Only represses transcripts, no way to upregulate targets	[15, 16]
	RBP-RNA regulation	Hfq-sRNA driven regulation of mRNA transcripts	Multiplexed to target several genes simultaneously Reduced metabolic burden by only overexpressing sRNAs Tunable levels of endogenous gene repression without extensive genome engineering	Only represses transcripts, no way to upregulate targets Time intensive to redesign sRNA for every mRNA target Does not work in organisms lacking Hfq	[22, 24, 25]
		Engineered CsrB sRNAs with varied affinities for CsrA to titrate cellular levels	Rapidly titrate intracellular CsrA to drive changes on the cellular level Reduced metabolic burden by only requiring overexpression of CsrB sRNAs	Can only directly modify CsrA-regulated processes Rapid depletion of intracellular CsrA may have unintentional consequences on cell health Does not work in organisms lacking CsrA/homologous systems	[30]
	Ribosome-controlled regulation	"Ribocomputing" engineered hairpins and trigger sRNAs to block/allow formation of ribosome on target mRNA	Able to construct complex genetic circuits using simple composable logic gates Circuits orthogonal to native posttranscriptional regulation Rapid response by using sRNAs and eliminating the need for translating circuit triggers	Currently only able to affect expression of exogenous genes Genes can only be activated, not repressed Genetic circuits can have inconsistent fold activation	[31, 85]
	CRISPR-based regulation	Catalytically inactive Cas9 (dCas9) and custom gRNA to block transcription of target genes (CRISPRi)	Multiplexed to target several genes simultaneously Tunable levels of gene repression without extensive genome engineering	Can only repress genes, no way to activate targets Overexpression of dCas9 protein may put metabolic stress on organism	[36, 37, 43, 47, 48]

(continued)

Table 1
(continued)

System	Mode of control	Regulatory mechanism	System benefits	Current limitations	Circuit application
		dCas9 fusion with custom gRNA to upregulate expression of target genes (CRISPRa)	Able to activate exogenous and endogenous genes using CRISPR-based regulation	Relatively low fold activation relative to other RNAP transcriptional activator systems Overexpression of dCas9-RNAP fusion may cause burden on organism	[38]
		dCas9 with modified gRNAs containing RNA-binding protein hairpins that recruit RBP-SoxS transcriptional activator fusion to upregulate targets	Titratable control of CRISPR-based activation Ability to activate and repress targets simultaneously Improved fold activation of targets relative to previous CRISPRa systems	Overexpression of dCas9 and RBP-SoxS fusion may cause a metabolic burden	[41]
	CRISPR- and RNAP-based regulation	Engineered activator sRNAs to control transcriptional elongation via RNAP of gRNAs for dCas9	Large library of composable parts for fine-tuning gene expression Operates orthogonal to the rest of the organism's metabolism Multiplexed to regulate several targets simultaneously Achieves full activation of regulated genes within an hour of induction	Only able to control expression of exogenous genes Overexpression of dCas9 can be a metabolic burden over time Only able to repress target genes, no way to activate	[49, 50]
Eukaryote	Ribozyme-directed mRNA regulation	mRNA transcript degraded by RNase P and external guide RNA sequence (EGS)	Portable method to target various mRNAs implicated in human health concerns	Limited genetic parts to provide precise user control of system	[17, 18, 54]
	RBP-RNA regulation	L7Ae-box RBP domain binding a gRNA sequence fused to the 5′ UTR of a transcript to block/allow translation	Can simultaneously activate and repress multiple targets	Can only regulate exogenous targets Must modify original transcript by fusing L7Ae binding domain to UTR of the transcript	[56]

RBP protein fusion regulation	METTL-dCas9 and ALKBH5-dCas9 fusions to selectively add or remove m6A modification to mRNA transcripts	Epitranscriptome modification of mRNA transcripts	Can only add m6A modifications to mRNA transcripts, no other modifications or regulatory effects Overexpression of dCas9 fusion proteins may cause burden on organism	[57]
	DNA adenosine methyltransferase (Dam) fused to Dnp1 to add m6A modifications, used in tandem with Dpn1 fusions to VP64 or KRAB to regulate methylated mRNA transcripts	First circuit to utilize epitranscriptome control for mRNA transcript regulatory fate Cells with "read-write" module possess regulatory "memory" of mRNA transcripts for several days	Only applied to endogenous copy of eGFP at a single locus, little studies on exogenous sequence and other loci Extensive m6A modification may cause unobserved cellular effects	[58]
CRISPR-based regulation	dCas9 or dCas9 fusions to block transcription of specific genes targeted via custom guide RNAs (CRISPRi)	Able to downregulate expression of endogenous target genes >100-fold repression of endogenous genes Large library of available complex logic gates	Overexpression of dCas9 or dCas9 fusions can be a metabolic burden on cells Circuit regulating transcription imposes lag time to achieve desired regulation Can only repress targets, no way to activate Off-target effects can occur in higher-level organisms such as mice and human cell models	[36, 62–64, 79]
	dCas9 fusions to transcriptionally activate specific genes targeted via guide RNAs (CRISPRa)	Able to activate or repress various target genes utilizing CRISPR-based circuits > 100-fold activation of endogenous targets	System is only able to activate expression of target genes Expression of dCas9 with multiple activation tags may cause metabolic burden	[62, 66–69, 71]
	Modified gRNAs for expanded regulatory control in CRISPR-Cas-based regulation	Simultaneous activation and repression of targets using CRISPR-Cas regulation Inducible control over RNP activator domain reduces leakiness of CRISPR-Cas activity CRISPR-Cas systems can respond to external metabolites or ligands	Significant time required to modify gRNA sequences to ensure desired function and maintain interaction with dCas9 Overexpression of RBP fusion proteins may cause metabolic burden	[74–76]

questionable. In this regard, there has been significantly less development of RBP-RNA-based control schemes in eukaryotes. System-level interactions have been underplayed in favor of fusing RBPs to modifying protein domains to bind and specifically modify targeted mRNA transcripts, for example, RBP protein fusions that specifically add m6A modifications to mRNA transcripts, such as in Liu et al. (2019) [57] and Park et al. [58].

CRSPR-based RNP control systems most often rely on catalytically inactive Cas9 (dCas9) fused to a transcriptional regulatory domain. These systems are highly programmable and can be used to repress (CRISPRi) or activate (CRISPRa) both endogenous and exogenous targets. Because of their modularity, CRISPRi-based systems have been integrated into genetic circuits to construct inducible "OR," "AND," as well as "NOR" logic gates. Thus, using CRISPR-based logic gates, fundamental control schemes can be constructed, such as toggle switches, feedforward loops, and oscillators, as seen in Santos-Moreno et al. [43]. Due to their programmability, CRISPR-Cas circuits can also be multiplexed to rewire cellular metabolism and improve bioprocessing product yield, although in a manner that may be significantly more complicated than relying on endogenous RBP control. Nonetheless, this feature is especially valuable in industrially relevant organisms that lack previously engineered RBP-RNA regulatory systems, such as Hfq- or CsrA-regulated systems. In this regard, dCas9 has been extensively used in eukaryotes to repress (CRISPRi) as well as activate (CRISPRa) target genes, and target genes can be regulated to greater than 100-fold wild-type levels.

A major drawback of CRISPR-based systems is the cellular burden of expressing Cas and Cas fusion proteins, which can lead in many instances to eventual circuit inactivation. In addition, there are frequently potential off-target effects due to potential mispairing of CRISPR gRNAs. Additionally, since CRISPR-based systems act through transcriptional regulation, this creates a lag time for response and can take hours to achieve the desired regulations; this is not ideal for applications that require fast-acting systems, such as those that produce toxic compounds.

Recent work suggests that it may be possible to meld the advantages of the two systems, systems integration (for RBPs) and extreme programmability (for CRISPR-Cas): guide RNA sequences have been modified to contain RBP-binding hairpins that recruit engineered RBP transcriptional regulator fusions, such as observed by Zalatan et al. [74] and Dong et al. [41]. Recruiting endogenous factors also has the advantage of allowing users to potentially simultaneously activate and repress multiple transcripts, depending on the RBP.

4 Future Perspectives

RNP-based control systems have been engineered over the last decade to provide control over major metabolic pathways and cellular responses. One potential application of RNP-based genetic control is in industrial bioprocesses, where there are already successes in producing value-added compounds such as biofuels [80] or industrial commodity chemicals [81]. Scale-up can be further improved by developing stress-responsive control schemes that quickly and continuously maximize cell viability [82]. RNP-based control systems provide unique opportunities to coordinate metabolism at the system level, providing both feedback on genetic components (transcription, replication) and on posttranscriptional regulation, ultimately reducing response lag time. Achieving complete regulation within an hour using RNP-based genetic circuits could help mitigate accumulation of toxic intermediates, which has been previously shown to improve metabolite production and cell viability [83]. Another potential avenue for RNP-based gene control is in biological therapeutics. Given that CRISPR-based methods will see increasing use in gene therapy (as a recent example, a CRISPR-Cas9 construct has been utilized to treat sickle cell disease in human patients, with edits to the exact nucleotide in 80% of the targeted alleles across multiple patients, without any observed side effects [84]), further integration of treatment with human systems biology and physiology can only further improve outcomes.

Beyond applications inside of cells, preliminary work has demonstrated potential utility of RNP-based systems in cell-free systems, such as rapid diagnostic tests for detection of antibiotics, glucose, and even specific viral strains (i.e., Ebola) [85]. In addition to diagnostic tests, RNP-based genetic circuits have the potential to be integrated into "smart materials" to detect toxic metabolites or pathogens.

Lastly, there has been a recent push to develop "minimal" or synthetic cells and organelles. These cells could serve as a chassis to create in vivo cellular systems that are entirely independent of bacterial strains and eukaryotic cell lines [86]. One key piece to improve the utility and development of these cells is finding additional ways to precisely regulate gene expression within these synthetic cells, particularly by controlling translation. Importantly, RNP control schemes (which could be easily purified and optimized for in vitro use) may serve as an excellent resource for synthetic cell systems, as they can rapidly control translation of target genes and have been utilized in vivo and in vitro systems. RNP genetic circuits have begun to be implemented into synthetic cells, such as into synthetic stress granules for spatial and temporal translational control [87]. Moving forward, combining RNP-based genetic control into synthetic cellular systems appears to be an exciting frontier in synthetic biology.

Overall, the examples of individual RNP systems that we have listed throughout highlight the opportunities for more generally engineering cells. The fact that in many instances target interactions or overall stability can be fine-tuned via nucleic acid hybridization means that well-known, quantitative thermodynamic measurements and predictions can potentially be applied to the control of gene regulation. In parallel, protein partners allow much broader systems-level integration, since proteins touch on virtually every aspect of cellular metabolism and regulation. RNPs are literally the linchpins of programmable biology, a hallmark of the ongoing revolution in synthetic biology. Our own work with CsrA shows how it is possible to allow the simplicity of nucleic acid hybridization to be parallelized and multiplexed via a single protein. Both of these features now bode well for extending interactions across the entire cellular system, where, for example, efforts to multiplex gRNAs to regulate multiple genes in parallel are mere harbingers of the possibilities available for using correlated and cross-regulated sets of nucleic acids to simultaneous regulate a wide variety of cellular processes, from intron splicing to protein translation to secretion.

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References

1. Leistra AN, Curtis NC, Contreras LM (2019) Regulatory non-coding sRNAs in bacterial metabolic pathway engineering. Metab Eng 52:190–214. https://doi.org/10.1016/j.ymben.2018.11.013
2. Yan Q, Pfleger BF (2020) Revisiting metabolic engineering strategies for microbial synthesis of oleochemicals. Metab Eng 58:35–46. https://doi.org/10.1016/j.ymben.2019.04.009
3. Jensen MK, Keasling JD (2015) Recent applications of synthetic biology tools for yeast metabolic engineering. FEMS Yeast Res 15(1):1–10. https://doi.org/10.1111/1567-1364.12185
4. Shivram H, Cress BF, Knott GJ, Doudna JA (2021) Controlling and enhancing CRISPR systems. Nat Chem Biol 17(1):10–19. https://doi.org/10.1038/s41589-020-00700-7
5. Khalil AS, Collins JJ (2010) Synthetic biology: applications come of age. Nat Rev Genet

11(5):367–379. https://doi.org/10.1038/nrg2775

6. Sowa SW, Gelderman G, Contreras LM (2015) Advances in synthetic dynamic circuits design: using novel synthetic parts to engineer new generations of gene oscillations. Curr Opin Biotechnol 36:161–167. https://doi.org/10.1016/j.copbio.2015.08.020
7. Hogan DJ, Riordan DP, Gerber AP, Herschlag D, Brown PO (2008) Diverse RNA-binding proteins interact with functionally related sets of RNAs, suggesting an extensive regulatory system. PLoS Biol 6(10):e255–e255. https://doi.org/10.1371/journal.pbio.0060255
8. Lee SR, Lykke-Andersen J (2013) Emerging roles for ribonucleoprotein modification and remodeling in controlling RNA fate. Trends Cell Biol 23(10):504–510. https://doi.org/10.1016/j.tcb.2013.05.001
9. Collins LJ, Kurland CG, Biggs P, Penny D (2009) The modern RNP world of Eukaryotes. J Hered 100(5):597–604. https://doi.org/10.1093/jhered/esp064
10. Akopian D, Shen K, Zhang X, Shan S (2013) Signal recognition particle: an essential protein-targeting machine. Ann Rev Biochem *82*(1):693–721. https://doi.org/10.1146/annurev-biochem-072711-164732
11. Han R, Fang J, Jiang J, Gaidamakova EK, Tkavc R, Daly MJ, Contreras LM (2020) Signal recognition particle RNA contributes to oxidative stress response in Deinococcus radiodurans by modulating catalase localization. Front Microbiol 11:3263. https://www.frontiersin.org/article/10.3389/fmicb.2020.613571
12. Esakova O, Krasilnikov AS (2010) Of proteins and RNA: the RNase P/MRP family. RNA. 16(9):1725–1747. https://doi.org/10.1261/rna.2214510
13. Cameron TA, Matz LM, De Lay NR (2018) Polynucleotide phosphorylase: not merely an RNase but a pivotal post-transcriptional regulator. PLoS Genet 14(10):e1007654. https://doi.org/10.1371/journal.pgen.1007654
14. Guerrier-Takada C, Gardiner K, Marsh T, Pace N, Altman S (1983) The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. Cell 35(3):849–857. https://doi.org/10.1016/0092-8674(83)90117-4
15. Li Y, Guerrier-Takada C, Altman S (1992) Targeted cleavage of mRNA in vitro by RNase P from Escherichia coli. Proc Nat Acad Sci 89(8):3185 LP–3189. https://doi.org/10.1073/pnas.89.8.3185
16. Guerrier-Takada C, Salavati R, Altman S (1997) Phenotypic conversion of drug-resistant bacteria to drug sensitivity. Proc Nat Acad Sci 94(16):8468 LP–8472. https://doi.org/10.1073/pnas.94.16.8468
17. Cobaleda C, Sánchez-García I (2000) In vivo inhibition by a site-specific catalytic RNA subunit of RNase P designed against the BCR-ABL oncogenic products: a novel approach for cancer treatment. Blood 95(3):731–737. https://doi.org/10.1182/blood.V95.3.731.003k28_731_737
18. Trang P, Kilani A, Kim J, Liu F (2000) A ribozyme derived from the catalytic subunit of RNase P from Escherichia coli is highly effective in inhibiting replication of herpes simplex virus 111Edited by J. Doudna. J Mol Biol *301*(4):817–826. https://doi.org/10.1006/jmbi.2000.4022
19. Yang Z, Vu G-P, Qian H, Chen Y-C, Wang Y, Reeves M, Zen K, Liu F (2014) Engineered RNase P Ribozymes effectively inhibit human cytomegalovirus gene expression and replication. Viruses 6(6):2376–2391. https://doi.org/10.3390/v6062376
20. Quendera AP, Seixas AF, dos Santos RF et al (2020) RNA-binding proteins driving the regulatory activity of small non-coding RNAs in Bacteria. Front Mol Biosci 7:78. https://www.frontiersin.org/article/10.3389/fmolb.2020.00078
21. Santiago-Frangos A, Woodson SA (2018) Hfq chaperone brings speed dating to bacterial sRNA. Wiley Interdiscip Rev RNA 9(4):e1475–e1475. https://doi.org/10.1002/wrna.1475
22. Na D, Yoo SM, Chung H, Park H, Park JH, Lee SY (2013) Metabolic engineering of Escherichia coli using synthetic small regulatory RNAs. Nat Biotechnol 31(2). https://doi.org/10.1038/nbt.2461
23. Shuo C, Aixia Z, Blyn LB, Gisela S (2004) MicC, a second small-RNA regulator of Omp protein expression in Escherichia coli. J Bacteriol *186*(20):6689–6697. https://doi.org/10.1128/JB.186.20.6689-6697.2004
24. Noh M, Yoo SM, Kim WJ, Lee SY (2017) Gene expression knockdown by modulating synthetic small RNA expression in *Escherichia coli*. Cell Syst 5(4):418–426.e4. https://doi.org/10.1016/j.cels.2017.08.016
25. Lahiry A, Stimple SD, Wood DW, Lease RA (2017) Retargeting a dual-acting sRNA for multiple mRNA transcript regulation. ACS Synth Biol 6(4):648–658. https://doi.org/10.1021/acssynbio.6b00261

26. Romeo T, Babitzke P (2018) Global regulation by CsrA and its RNA Antagonists. Microbiol Spectr 6(2). https://doi.org/10.1128/microbiolspec.RWR-0009-2017
27. Sowa SW, Gelderman G, Leistra AN, Buvanendiran A, Lipp S, Pitaktong A, Vakulskas CA, Romeo T, Baldea M, Contreras LM (2017) Integrative FourD omics approach profiles the target network of the carbon storage regulatory system. Nucleic Acids Res 45(4): 1673–1686. https://doi.org/10.1093/nar/gkx048
28. Andrew R, Stephanie P, Ying-Jung L, Archana P, Helen Y, Potts AH, Bevilacqua PC, Romeo T, Babitzke P, Cossart PF (2021) CsrA-mediated translational activation of ymdA expression in Escherichia coli. MBio 11(5):e00849–e00820. https://doi.org/10.1128/mBio.00849-20
29. Leistra AN, Gelderman G, Sowa SW, Moon-Walker A, Salis HM, Contreras LM (2018) A Canonical biophysical model of the CsrA global regulator suggests flexible regulator-target interactions. Sci Rep 8(1):1–19. https://doi.org/10.1038/s41598-018-27474-2
30. Leistra AN, Amador P, Buvanendiran A, Moon-Walker A, Contreras LM (2017) Rational modular RNA engineering based on in vivo profiling of structural accessibility. ACS Synth Biol 6(12):2228–2240. https://doi.org/10.1021/acssynbio.7b00185
31. Green AA, Kim J, Ma D, Silver PA, Collins JJ, Yin P (2017) Complex cellular logic computation using ribocomputing devices. Nature 548(7665):117–121. https://doi.org/10.1038/nature23271
32. Amalfitano E, Karlikow M, Norouzi M, Jaenes K, Cicek S, Masum F, Sadat Mousavi P, Guo Y, Tang L, Sydor A, Ma D, Pearson JD, Trcka D, Pinette M, Ambagala A, Babiuk S, Pickering B, Wrana J, Bremner R et al (2021) A glucose meter interface for point-of-care gene circuit-based diagnostics. Nat Commun 12(1):724. https://doi.org/10.1038/s41467-020-20639-6
33. Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A (1987) Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. J Bacteriol 169(12):5429–5433. https://doi.org/10.1128/jb.169.12.5429-5433.1987
34. Jansen R, van Embden JDA, Gaastra W, Schouls LM (2002) Identification of genes that are associated with DNA repeats in prokaryotes. Mol Microbiol *43*(6):1565–1575. https://doi.org/10.1046/j.1365-2958.2002.02839.x
35. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337(6096):816–821. https://doi.org/10.1126/science.1225829
36. Qi LS, Larson MH, Gilbert LA et al (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152(5):1173–1183. https://doi.org/10.1016/j.cell.2013.02.022
37. Nielsen AAK, Voigt CA (2014) Multi-input CRISPR/Cas genetic circuits that interface host regulatory networks. Mol Syst Biol 10(11):763. https://doi.org/10.15252/msb.20145735
38. Bikard D, Jiang W, Samai P, Hochschild A, Zhang F, Marraffini LA (2013) Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. Nucleic Acids Res 41(15): 7429–7437. https://doi.org/10.1093/nar/gkt520
39. Dove SL, Joung JK, Hochschild A (1997) Activation of prokaryotic transcription through arbitrary protein–protein contacts. Nature 386(6625):627–630. https://doi.org/10.1038/386627a0
40. Dove SL, Hochschild A (1998) Conversion of the ω subunit of Escherichia coli RNA polymerase into a transcriptional activator or an activation target. Genes Dev *12*(5):745–754. http://genesdev.cshlp.org/content/12/5/745.abstract
41. Dong C, Fontana J, Patel A, Carothers JM, Zalatan JG (2018) Synthetic CRISPR-Cas gene activators for transcriptional reprogramming in bacteria. Nat Commun 9(1):2489. https://doi.org/10.1038/s41467-018-04901-6
42. Sadowski I, Ma J, Triezenberg S, Ptashne M (1988) GAL4-VP16 is an unusually potent transcriptional activator. Nature 335(6190): 563–564. https://doi.org/10.1038/335563a0
43. Santos-Moreno J, Tasiudi E, Stelling J, Schaerli Y (2020) Multistable and dynamic CRISPRi-based synthetic circuits. Nat Commun 11(1): 2746. https://doi.org/10.1038/s41467-020-16574-1
44. Gardner TS, Cantor CR, Collins JJ (2000) Construction of a genetic toggle switch in

Escherichia coli. Nature 403(6767):339–342. https://doi.org/10.1038/35002131

45. Mangan S, Itzkovitz S, Zaslaver A, Alon U (2006) The incoherent feed-forward loop accelerates the response-time of the gal system of Escherichia coli. J Mol Biol 356(5): 1073–1081. https://doi.org/10.1016/j.jmb.2005.12.003
46. Elowitz MB, Leibler S (2000) A synthetic oscillatory network of transcriptional regulators. Nature 403(6767):335–338. https://doi.org/10.1038/35002125
47. Gordon GC, Korosh TC, Cameron JC, Markley AL, Begemann MB, Pfleger BF (2016) CRISPR interference as a titratable, trans-acting regulatory tool for metabolic engineering in the cyanobacterium Synechococcus sp. strain PCC 7002. Metab Eng 38:170–179. https://doi.org/10.1016/j.ymben.2016.07.007
48. Cleto S, Jensen JVK, Wendisch VF, Lu TK (2016) Corynebacterium glutamicum metabolic engineering with CRISPR Interference (CRISPRi). ACS Synth Biol 5(5):375–385. https://doi.org/10.1021/acssynbio.5b00216
49. Chappell J, Takahashi MK, Lucks JB (2015) Creating small transcription activating RNAs. Nat Chem Biol 11(3):214–220. https://doi.org/10.1038/nchembio.1737
50. Chappell J, Westbrook A, Verosloff M, Lucks JB (2017) Computational design of small transcription activating RNAs for versatile and dynamic gene regulation. Nat Commun 8(1): 1051. https://doi.org/10.1038/s41467-017-01082-6
51. Matera AG, Wang Z (2014) A day in the life of the spliceosome. Nat Rev Mol Cell Biol 15(2): 108–121. https://doi.org/10.1038/nrm3742
52. Shay JW, Wright WE (2019) Telomeres and telomerase: three decades of progress. Nat Rev Genet 20(5):299–309. https://doi.org/10.1038/s41576-019-0099-1
53. Vazquez-Arango P, O'Reilly D (2018) Variant snRNPs: new players within the spliceosome system. RNA Biol 15(1):17–25. https://doi.org/10.1080/15476286.2017.1373238
54. Jiang X, Bai Y, Rider P, Kim K, Zhang C-Y, Lu S, Liu F (2011) Engineered external guide sequences effectively block viral gene expression and replication in cultured cells. J Biol Chem 286(1):322–330. https://doi.org/10.1074/jbc.M110.158857
55. Shotwell CR, Cleary JD, Berglund JA (2020) The potential of engineered eukaryotic RNA-binding proteins as molecular tools and therapeutics. WIREs RNA 11(1):e1573. https://doi.org/10.1002/wrna.1573
56. Saito H, Kobayashi T, Hara T, Fujita Y, Hayashi K, Furushima R, Inoue T (2010) Synthetic translational regulation by an L7Ae–kink-turn RNP switch. Nat Chem Biol *6*(1): 71–78. https://doi.org/10.1038/nchembio.273
57. Liu X-M, Zhou J, Mao Y, Ji Q, Qian S-B (2019) Programmable RNA N6-methyladenosine editing by CRISPR-Cas9 conjugates. Nat Chem Biol 15(9): 865–871. https://doi.org/10.1038/s41589-019-0327-1
58. Park M, Patel N, Keung AJ, Khalil AS (2019) Engineering epigenetic regulation using synthetic read-write modules. Cell 176(1): 227–238.e20. https://doi.org/10.1016/j.cell.2018.11.002
59. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339(6121):819–823. https://doi.org/10.1126/science.1231143
60. Mali P, Yang L, Esvelt KM et al (2013) RNA-guided human genome engineering via Cas9. Science (80-) 339(6121):823 LP – 826. https://doi.org/10.1126/science.1232033
61. Kiani S, Beal J, Ebrahimkhani MR, Huh J, Hall RN, Xie Z, Li Y, Weiss R (2014) CRISPR transcriptional repression devices and layered circuits in mammalian cells. Nat Method 11(7):723–726. https://doi.org/10.1038/nmeth.2969
62. Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA, Lim WA, Weissman JS, Qi LS (2013) CRISPR-mediated modular RNA-guided regulation of transcription in Eukaryotes. Cell 154(2):442–451. https://doi.org/10.1016/j.cell.2013.06.044
63. Gander MW, Vrana JD, Voje WE, Carothers JM, Klavins E (2017) Digital logic circuits in yeast with CRISPR-dCas9 NOR gates. Nat Commun 8(1):15459. https://doi.org/10.1038/ncomms15459
64. Yeo NC, Chavez A, Lance-Byrne A, Chan Y, Menn D, Milanova D, Kuo C-C, Guo X, Sharma S, Tung A, Cecchi RJ, Tuttle M, Pradhan S, Lim ET, Davidsohn N, Ebrahimkhani MR, Collins JJ, Lewis NE, Kiani S, Church GM (2018) An enhanced CRISPR repressor for targeted mammalian gene regula-

tion. Nat Method 15(8):611–616. https://doi.org/10.1038/s41592-018-0048-5

65. Adkins NL, Georgel PT (2010) MeCP2: structure and functionThis paper is one of a selection of papers published in a Special Issue entitled 31st Annual International Asilomar Chromatin and Chromosomes Conference, and has undergone the Journal's usual peer review process. Biochem Cell Biol *89*(1): 1–11. https://doi.org/10.1139/O10-112
66. Cheng AW, Wang H, Yang H et al (2013) Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. Cell Res 23(10): 1163–1171. https://doi.org/10.1038/cr.2013.122
67. Perez-Pinera P, Kocak DD, Vockley CM, Adler AF, Kabadi AM, Polstein LR, Thakore PI, Glass KA, Ousterout DG, Leong KW, Guilak F, Crawford GE, Reddy TE, Gersbach CA (2013) RNA-guided gene activation by CRISPR-Cas9–based transcription factors. Nat Method *10*(10):973–976. https://doi.org/10.1038/nmeth.2600
68. Nissim L, Perli SD, Fridkin A, Perez-Pinera P, Lu TK (2014) Multiplexed and programmable regulation of gene networks with an integrated RNA and CRISPR/Cas toolkit in human cells. Mol Cell *54*(4):698–710. https://doi.org/10.1016/j.molcel.2014.04.022
69. Chavez A, Scheiman J, Vora S, Pruitt BW, Tuttle M, Iyer EPR, Lin S, Kiani S, Guzman CD, Wiegand DJ, Ter-Ovanesyan D, Braff JL, Davidsohn N, Housden BE, Perrimon N, Weiss R, Aach J, Collins JJ, Church GM (2015) Highly efficient Cas9-mediated transcriptional programming. Nat Method *12*(4): 326–328. https://doi.org/10.1038/nmeth.3312
70. Schmitz ML, Baeuerle PA (1991) The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B. EMBO J *10*(12):3805–3817. https://pubmed.ncbi.nlm.nih.gov/1935902
71. Gao Y, Xiong X, Wong S, Charles EJ, Lim WA, Qi LS (2016) Complex transcriptional modulation with orthogonal and inducible dCas9 regulators. Nat Method *13*(12):1043–1049. https://doi.org/10.1038/nmeth.4042
72. Liang F-S, Ho WQ, Crabtree GR (2011) Engineering the ABA plant stress pathway for regulation of induced proximity. Sci Signal *4*(164): rs2–rs2. https://doi.org/10.1126/scisignal.2001449
73. Miyamoto T, DeRose R, Suarez A, Ueno T, Chen M, Sun T, Wolfgang MJ, Mukherjee C, Meyers DJ, Inoue T (2012) Rapid and orthogonal logic gating with a gibberellin-induced dimerization system. Nat Chem Biol *8*(5): 465–470. https://doi.org/10.1038/nchembio.922
74. Zalatan JG, Lee ME, Almeida R, Gilbert LA, Whitehead EH, La Russa M, Tsai JC, Weissman JS, Dueber JE, Qi LS, Lim WA (2015) Engineering complex synthetic transcriptional programs with CRISPR RNA Scaffolds. Cell *160*(1):339–350. https://doi.org/10.1016/j.cell.2014.11.052
75. Liu Y, Zhan Y, Chen Z, He A, Li J, Wu H, Liu L, Zhuang C, Lin J, Guo X, Zhang Q, Huang W, Cai Z (2016) Directing cellular information flow via CRISPR signal conductors. Nat Method *13*(11):938–944. https://doi.org/10.1038/nmeth.3994
76. Ying Z-M, Wang F, Chu X, Yu R-Q, Jiang J-H (2020) Activatable CRISPR transcriptional circuits generate functional RNA for mRNA sensing and silencing. Angew Chemie Int Ed *59*(42):18599–18604. https://doi.org/10.1002/anie.202004751
77. Liu Y, Zeng Y, Liu L, Zhuang C, Fu X, Huang W, Cai Z (2014) Synthesizing AND gate genetic circuits based on CRISPR-Cas9 for identification of bladder cancer cells. Nat Commun *5*(1):5393. https://doi.org/10.1038/ncomms6393
78. Fan J, Liu Y, Liu L, Huang Y, Li X, Huang W (2020) A multifunction lipid-based CRISPR-Cas13a genetic circuit delivery system for bladder cancer gene therapy. ACS Synth Biol *9*(2): 343–355. https://doi.org/10.1021/acssynbio.9b00349
79. Mimee M, Tucker AC, Voigt CA, Lu TK (2015) Programming a human commensal bacterium *Bacteroides thetaiotaomicron* to sense and respond to stimuli in the Murine Gut Microbiota. Cell Syst *1*(1):62–71. https://doi.org/10.1016/j.cels.2015.06.001
80. Nielsen J, Keasling JD (2016) Engineering cellular metabolism. Cell 164(6):1185–1197. https://doi.org/10.1016/j.cell.2016.02.004
81. Lee JW, Na D, Park JM, Lee J, Choi S, Lee SY (2012) Systems metabolic engineering of microorganisms for natural and non-natural chemicals. Nat Chem Biol 8(6):536–546. https://doi.org/10.1038/nchembio.970
82. Wehrs M, Tanjore D, Eng T, Lievense J, Pray TR, Mukhopadhyay A (2019) Engineering

robust production microbes for large-scale cultivation. Trends Microbiol 27(6):524–537. https://doi.org/10.1016/j.tim.2019.01.006

83. Dahl RH, Zhang F, Alonso-Gutierrez J et al (2013) Engineering dynamic pathway regulation using stress-response promoters. Nat Biotechnol 31(11):1039–1046. https://doi.org/10.1038/nbt.2689
84. Frangoul H, Altshuler D, Cappellini MD et al (2020) CRISPR-Cas9 gene editing for sickle cell disease and β-Thalassemia. N Engl J Med 384(3):252–260. https://doi.org/10.1056/NEJMoa2031054
85. Pardee K, Green AA, Ferrante T et al (2014) Paper-based synthetic gene networks. Cell 159(4):940–954. https://doi.org/10.1016/j.cell.2014.10.004
86. Adamala KP, Martin-Alarcon DA, Guthrie-Honea KR, Boyden ES (2017) Engineering genetic circuit interactions within and between synthetic minimal cells. Nat Chem 9(5):431–439. https://doi.org/10.1038/nchem.2644
87. Simon JR, Eghtesadi SA, Dzuricky M, You L, Chilkoti A (2019) Engineered Ribonucleoprotein granules inhibit translation in protocells. Mol Cell 75(1):66–75.e5. https://doi.org/10.1016/j.molcel.2019.05.010