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*Designing Biological Circuits: Synthetic  
Biology Within the Operon Model and Beyond*

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*Annual Review of Biochemistry*

# Designing Biological Circuits: Synthetic Biology Within the Operon Model and Beyond

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## Keywords

synthetic biology, gene regulatory networks, synthetic epigenetics, molecular genetics, operon model

## Abstract

In 1961, Jacob and Monod proposed the operon model of gene regulation. At the model's core was the modular assembly of regulators, operators, and structural genes. To illustrate the composability of these elements, Jacob and Monod linked phenotypic diversity to the architectures of regulatory circuits. In this review, we examine how the circuit blueprints imagined by Jacob and Monod laid the foundation for the first synthetic gene networks that launched the field of synthetic biology in 2000. We discuss the influences of the operon model and its broader theoretical framework on the first generation of synthetic biological circuits, which were predominantly transcriptional and posttranscriptional circuits. We also describe how recent advances in molecular biology beyond the operon model—namely, programmable DNA- and RNA-binding molecules as well as models of epigenetic and posttranslational regulation—are expanding the synthetic biology toolkit and enabling the design of more complex biological circuits.

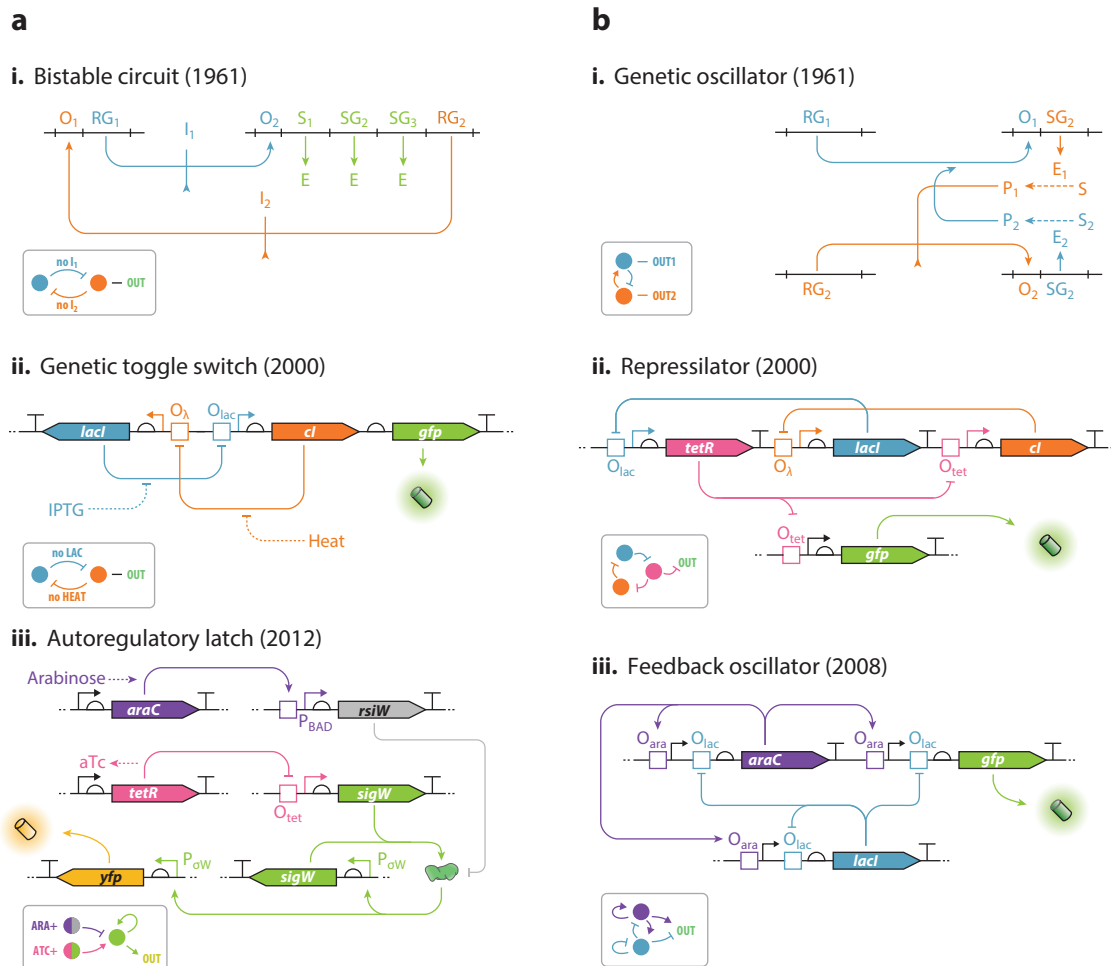
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## 1. INTRODUCTION

In a series of landmark papers published in 1961, Jacob and Monod consolidated contemporary findings on the structure of genes and their expression patterns into a comprehensive and far-reaching theory of gene regulation: the operon model (1–3). This framework drew from their own work on the induction of  $\beta$ -galactosidase activity in *Escherichia coli* grown in the presence of lactose, as well as from several other studies on adaptive enzymes and  $\lambda$  phage. To explain the phenomena of metabolic adaptation and differentiation, Jacob and Monod presented blueprints for model genetic circuits. These networks were composed from three classes of genetic elements: regulatory genes (e.g., *lacI*), their target operators (e.g., *lacZYAo*), and structural genes (e.g., *lacZ*) (**Figure 1**). The operon model set out several testable predictions, including the following: (a) Analogous mechanisms may be conserved across the domains of life; (b) the elements are modular, meaning that operators and regulators can be decoupled from downstream operons; and (c) the elements can be composed to form gene regulatory networks with complex emergent behaviors. These features of natural gene regulatory networks, refined through decades of research (4), underpinned and inspired the field of synthetic biology (5).

In this review, we examine how the circuit blueprints imagined by Jacob and Monod laid the groundwork for the construction of the first generation of synthetic gene circuits (6, 7) (**Figure 1**). Central to these efforts is the idea that complex behaviors can be programmed by composing simple functional units, all operating according to codified molecular mechanisms. We then use a series of examples from microbial synthetic biology to illustrate the striking analogies between the most widespread synthetic gene regulatory strategies and the predictions made decades earlier by Jacob and Monod. The first generation of synthetic gene circuits had important contributions to our understanding of the structure and function of gene regulatory networks, as well as to the role of noise in the regulation of gene expression (8). In the second part of this review, we highlight the role of biochemistry and molecular biology in the discovery and development of suites of new molecular tools well suited to both the detection of user-defined cues and the programmable actuation of endogenous behaviors (9). We explore how fundamental advances in our understanding of gene regulation have opened up parallel engineering strategies beyond the operon model and how synthetic gene circuits are helping to refine contemporary biological models. These examples highlight the continued, constructive interplay between fundamental biology and synthetic biology.



**Figure 1**

The operon model as the blueprint for first-generation synthetic biology. (a) (i) To account for observations of differentiated phenotypes, Jacob and Monod proposed a bistable circuit composed of transcriptional repressor genes, operators, and structural genes. Panel *i* adapted with permission from Reference 1. (ii) The genetic toggle switch represents the physical instantiation of this blueprint: Two repressors (LacI and *cl*) inhibit one another via their cognate operators (*O*<sub>lac</sub> and *O*<sub>cl</sub>). The structural gene encodes the GFP. (iii) In an alternative bistable switch architecture, the sigma factor gene *sigW* is placed under positive autoregulation (13). The system can be switched on through the aTc-mediated induction of the TetR-repressed copy of *sigW* and can be reset through arabinose-mediated activation of the antisigma factor RsiW, which sequesters SigW. (b) (i) Jacob and Monod proposed a hypothetical feedback oscillator to incorporate dynamic behaviors into their operon model. Metabolic cross talk between two operons containing structural genes (*SG*<sub>1</sub> and *SG*<sub>2</sub>) and operators (*O*<sub>1</sub> and *O*<sub>2</sub>) is mediated by their cognate repressive regulator genes (*RG*<sub>1</sub> and *RG*<sub>2</sub>). The enzymes *E*<sub>1</sub> and *E*<sub>2</sub> catalyze the formation of the metabolic products *P*<sub>1</sub> and *P*<sub>2</sub>, which allosterically inhibit *RG*<sub>2</sub> and induce *RG*<sub>1</sub>, respectively. Panel *i* adapted with permission from Reference 1. (ii) The repressilator is an alternative genetic oscillator: Three regulator genes (*tetR*, *lacI*, and *cl*) are organized in a daisy chain arrangement, with the TetR repressor also regulating the expression of the structural gene *gfp* (7). (iii) A two-component oscillator architecture uses multi-input promoters to connect the arabinose-induced activator AraC and the IPTG-inhibited LacI (14). Each transcription factor is also under autoregulatory control, and both are connected to the structural gene *gfp*. Abbreviations: aTc, anhydrotetracycline; E, enzyme; *gfp*, green fluorescent protein; I, inducer; IPTG, isopropyl thiogalactopyranoside; O, operator; RG, repressor gene; SG, structural gene; *yfp*, yellow fluorescent protein.

## 2. SYNTHETIC BIOLOGY WITHIN THE OPERON MODEL

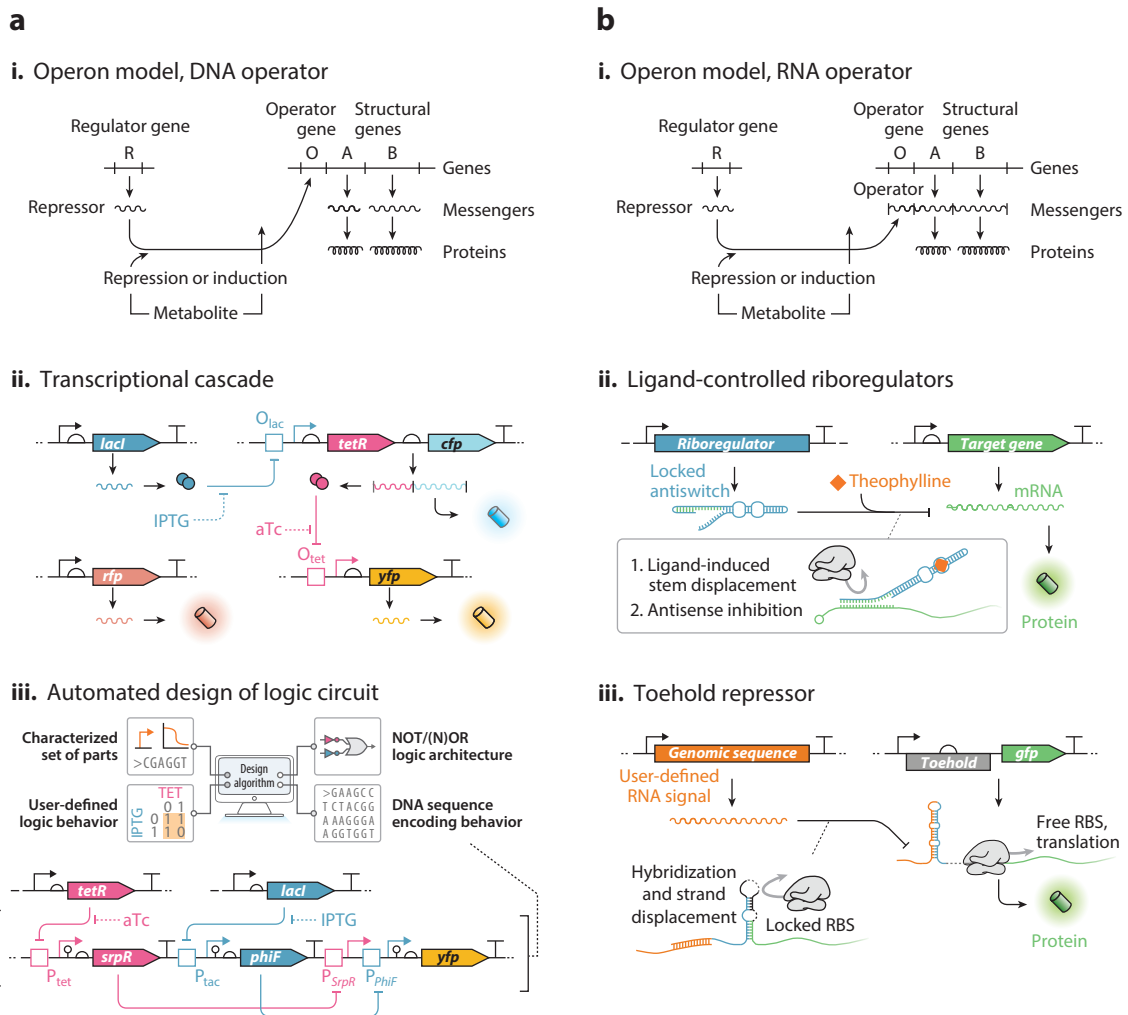
In this section, we discuss the extensive influences of the operon model and its broader theoretical framework on early efforts in synthetic biology (3). To provide the necessary scientific context, we can summarize the pertinent conclusions and predictions made by this model as follows. (a) The expression of structural genes, encoding the structure of proteins such as metabolic enzymes (e.g., LacZ), is mediated by transient intermediaries—now identified as mRNAs. (b) The expression of structural genes is regulated by the functional interplay between regulator genes and operators. (c) The products of regulator genes are cytosolic repressors (e.g., LacI); Jacob and Monod initially favored the hypothesis that these regulators were RNAs. (d) Regulators control information flow from structural genes to proteins without influencing the chemical nature of the proteins themselves. (e) The operator sequence is physically adjacent to the structural genes at the DNA level (or is possibly on the RNA molecule) and can control several cistrons (e.g., *lacZYA* regulates the expression of three coding sequences in the *lac* operon). (f) Specific small molecules, such as the products of metabolic enzymes (e.g., allolactose), bind to regulators and structural enzymes and allosterically tune their biochemical activities.

We begin this section by highlighting the striking analogies between early synthetic gene circuits and the diagrams proposed by Jacob and Monod (**Figure 1**). We discuss the expansion of transcriptional regulation strategies to encompass the full breadth of behaviors permitted within the operon model and note that transcriptional circuits remain a predominant mode of cellular reprogramming across synthetic biology (10) (**Figure 2a**). Moving along the central dogma, we then underscore the parallels between the alternative cytoplasmic operator model suggested by Jacob and Monod for posttranscriptional regulation and efforts to develop and implement RNA-based tools to build synthetic circuits (11) (**Figure 2b**).

### 2.1. Transcriptional Circuits

Jacob and Monod used a series of hypothetical gene circuit architectures to illustrate the generalizability of their operon model (1). These blueprints demonstrated how different arrangements of regulators, operators, and structural genes could explain complex behaviors such as cellular differentiation. As their model was published decades before the emergence of recombinant DNA technologies (12), Jacob and Monod lacked the tools necessary to construct these hypothetical circuits. However, the methods for DNA assembly and cloning that were developed at the end of the twentieth century allowed biological engineers to realize these hypothetical designs in living cells. Below, we show that the first synthetic gene circuits represent a proof by construction of the operon model and its extensions (**Figure 1**). We then focus on efforts to expand this framework through the incorporation of feedback regulation (**Figure 1**). Finally, we explore the emergence and continued development of layered transcriptional cascades and logic circuits (10).

**2.1.1. Blueprints for bistable switches and oscillators.** To provide an explanation for cellular differentiation within the framework of the operon model, Jacob and Monod proposed a circuit that would allow a population of genetically identical cells to maintain two stable phenotypes (**Figure 1a**). In this circuit, two hypothetical operons encode opposing repressors that are transcriptionally regulated by each other's corresponding operator. Jacob and Monod postulated that this architecture would lead to the establishment of two distinct, mutually exclusive cell states, depending on which of the two repressors is dominant. In this model, enzyme-encoding structural genes are expressed only if they are on the same operon as the dominant regulator. Furthermore, they predicted that if the repressors could be allosterically inhibited by the binding



**Figure 2**

DNA- and RNA-level operators and their implementations in synthetic biology. (a) (i) The first of the two general operon models considers the case for DNA-level operators. Panel *i* adapted with permission from Reference 3. (ii) A two-step cascade was used to study noise propagation in regulatory networks (21). Both regulation layers operate following the principles outlined in the operon model: Regulators (LacI or TetR) controlled by small molecules (IPTG or aTc, respectively) modulate the expression of the proteins encoded on their target operons (TetR and CFP, or YFP, respectively). The constitutive synthesis of RFP is used to measure extrinsic noise in the system. (iii) Cello is a computational platform that automates the design of logic circuits using a NOT/NOR-based architecture (22). Cello combines well-characterized parts (e.g., repressors) into layered circuits to implement the user-specified logic behavior, with small-molecule inducers (e.g., aTc, IPTG) as inputs. In the provided example, a NAND logic is implemented by combining two operons, each one controlled by allosterically regulated repressors (TetR, LacI). The products of these operons are also repressors (SrpR, PhiF); they jointly regulate a tandem promoter that drives the production of the output reporter (YFP). (b) (i) The second general operon model considers the scenario for RNA-level regulation. Panel *i* adapted with permission from Reference 3. (ii) The antiswitch is an RNA-based, ligand-controlled regulator (23), conceptually similar to that envisioned by Jacob and Monod. Upon binding of the small-molecule effector by the aptamer structure, the activated regulator binds on the mRNA to control translation. (iii) The synthetic toehold repressor is an operator sequence and sequesters the RBS upon hybridization of a matching RNA molecule, which acts as the regulator (24). Abbreviations: aTc, anhydrotetracycline; CFP/*cfp*, cyan fluorescent protein; *gfp*, green fluorescent protein; IPTG, isopropyl thiogalactopyranoside; RBS, ribosome binding site; RFP, red fluorescent protein; YFP/*yfp*, yellow fluorescent protein.

of their respective ligands, the system could transition between its two stable transcriptional states. Gardner et al. (6) constructed a physical instantiation of this blueprint—the genetic toggle switch—as one of the first synthetic gene circuits. On a single bacterial plasmid, the authors assembled two mutually inhibitory operons encoding the bacterial repressor LacI and the phage repressor cI (or TetR)—controlled externally by the lactose analog IPTG and temperature (or aTc), respectively. In lieu of the enzymes envisaged by Jacob and Monod, the structural gene encoded green fluorescent protein (GFP) as a reporter of the circuit's behavior. As predicted, this system exhibited two stable, mutually exclusive transcriptional states. Importantly, the two external cues, IPTG and heat (or aTc), allowed the switch to be toggled between its two stable states (GFP on and GFP off) across a population of *E. coli*.

In another example, Jacob and Monod turned their interests toward temporally varying behaviors. They outlined a hypothetical oscillator circuit that creates cyclic patterns of gene expression (**Figure 1b**). Again, their design centered around the regulatory cross talk between two distinct operons. In this scenario, each operon is repressed by an independent regulator and encodes an enzyme that catalyzes the production of a small molecule. In turn, these small molecules allosterically control the activity of the opposite regulator such that the metabolic product of operon 1 relieves repression on operon 2, while the product of operon 2 drives the corepression of operon 1. Jacob and Monod predicted that, under the appropriate biochemical parameters, the transcriptional activities of the two operons would display temporal oscillations. As one of the founding works in synthetic biology, Elowitz & Leibler (7) developed a related temporal oscillator; rather than relying on mixed regulation and enzymatic conversion, they based the so-called repressilator entirely on transcriptional repression and replaced the positive regulation invoked by Jacob and Monod with a logically equivalent double repression in *E. coli* (**Figure 1b**). In this circuit, genes encoding three well-characterized repressors—LacI, cI, and TetR—were connected in a daisy chain network. The resulting cyclic expression of a TetR-regulated fluorescent reporter confirmed the oscillatory behavior of this circuit. Importantly, the implementation of this circuit demonstrated that autonomous dynamic behaviors could be programmed in synthetic gene networks and established a minimal model for the theoretical study of circadian clocks. Together, the toggle switch and repressilator represented a proof by construction for the hypothetical extensions to the operon model, validating the prediction that regulators and operators could be composed to generate complex biological behaviors.

**2.1.2. Positive and negative feedback.** The first observations of allosteric end-product inhibition in metabolic pathways (e.g., tryptophan biosynthesis) inspired Jacob and Monod to extend the concept of feedback control to genetic networks (1). In this context, feedback can emerge from the direct activity of a regulator toward itself (autoregulation) or indirectly via the downstream products of its target operon. Extensive theoretical analyses, reviewed in detail by Wall et al. (15), have sought to establish design rules for feedback and autoregulation at the transcriptional level. These studies suggested, for example, that negative autoregulation could improve the robustness and stability of genetic circuits. In 2000, following the publication of the toggle switch and repressilator, Becskei & Serrano (16) developed a minimal synthetic gene circuit to study the case of negative autoregulation experimentally. Using a fluorescently labeled version of the TetR repressor, they measured the population-wide distribution of the protein levels in two cases: (a) an autoregulatory system in which the *tet* operators were placed upstream of the *tetR-gfp* fusion gene and (b) experimental controls in which the expression of *tetR-gfp* was unregulated. Their findings were consistent with the conclusions drawn from natural biological systems, namely that autoregulation improves the stability of the circuit output in the face of biological noise and reduces gene expression heterogeneity across an *E. coli* population.

Jacob and Monod had discussed the possibility that an operon producing its own inducer would exhibit an all-or-nothing transcriptional behavior (1). Indeed, an early synthetic biology study in yeast using a self-activating TetR transcription factor (TF) implicated positive autoregulation in the switch-like conversion of continuous inputs to binary phenotypic outputs (17). Isaacs et al. (18) developed a similar model system in *E. coli*, in which an operon encoding a temperature-sensitive mutant of the  $\lambda$  phage cI protein and a GFP reporter was placed under the control of a cI-activated target promoter. This process established a simple positive feedback loop, regulating both cI and GFP. By destabilizing the mutant cI protein at higher temperatures, the experimenters could artificially tune the level of transcriptional self-activation. Within an intermediate cI activation regime, they observed a bimodal distribution of cell fluorescence, confirming that positive transcriptional autoregulation can amplify internal molecular noise and drive the emergence of two discrete subpopulations. In a concrete demonstration of the ability of positive autoregulation to support bistability, Chen & Arkin (13) later implemented an alternative toggle switch architecture that replaced the two mutually opposed transcriptional repressors with a single, sequesterable autoactivator (**Figure 1a**). These works underscore the role of positive feedback in the establishment and genetic tuning of population heterogeneity and the long-term maintenance of cell states in response to transient cues.

The simultaneous implementation of both positive and negative autoregulation in synthetic gene circuits further expanded the genetic design space accessible to synthetic biologists, as illustrated by the development of robust two-component genetic feedback oscillators. In the original circuit proposed by Jacob and Monod, two operons are connected by a positive regulatory interaction and a negative regulatory interaction, mediated by their downstream metabolic products (**Figure 1**). Barkai & Leibler (19) later developed a theoretical framework for the construction of this class of two-component oscillators using solely transcriptional regulation; this work further emphasized the importance of interfacing positive autoregulation with negative feedback and highlighted the robustness of this architecture against internal cellular noise. In 2008, Stricker et al. (14) used this model to construct a tunable genetic oscillator with both a shorter period length and greater amplitude than previous circuits (**Figure 1b**). To interface the two branches of the feedback loop (activation and repression) and implement simultaneous positive and negative autoregulation at each node in the network, these researchers' circuit relied on dual-input, hybrid promoters responsive to the activator AraC and the repressor LacI (14, 20). The use of these two orthogonal sensors allowed Stricker et al. to fine-tune the oscillatory period and experimentally traverse their model parameters by using two chemical channels: arabinose and IPTG. As predicted, this physical instantiation of the extended Barkai & Leibler model was particularly robust to internal noise. Interestingly, Stricker et al. further explored the parameter space of their model, revealing that a simple negative autoregulation circuit consisting of a self-repressing LacI element would be sufficient to generate oscillations—an architecture known as a Goodwin oscillator. After validating this prediction experimentally, the authors attributed the module's behavior to the inherent delay between *lacI* gene expression and the formation of the active LacI TF complex. This result underscores the central role played by quantitative models in guiding synthetic gene circuit design strategies.

**2.1.3. Transcriptional cascades and logic gates.** In their hypothetical design of a bistable genetic switch, Jacob and Monod introduced the notion that transcriptional regulators could be composed to directly control other regulators (**Figure 1a**). While their model latch network had a cyclic architecture, a natural extension of this concept is the creation of layered regulatory cascades (**Figure 2a**); natural gene regulatory networks are often organized into hierarchies, which can coordinate complex phenotypic changes and define sequential transcriptional programs (25).



Building on successful implementations of the canonical circuit architectures discussed above, synthetic biologists set out to use the same circuit toolbox (e.g., LacI, cI, TetR) as a test bed for the study of natural gene regulatory networks. To characterize the sensing behaviors of layered cascades, Hooshangi et al. (26) compared the responses of linear circuits of various lengths (1–3 stages) to different steady-state input levels. To extend the cascade, the authors inserted LacI and cI repression modules between the aTc-controlled TetR input layer and the reporter output layer. They found that an increase in cascade length improved overall sensitivity to the inducer molecule while at the same time amplifying both transcriptional noise and the overall response times.

At the same time, Rosenfeld et al. (27) used a synthetic cascade to quantify the effects of cellular noise on an individual repression module. Their two-stage circuit consisted of a TetR-regulated cI–yellow fluorescent protein (YFP) fusion, which in turn controlled a cyan fluorescent protein (CFP) reporter. By measuring the levels of both fluorescent reporters at the single-cell level, the authors derived a correspondence between repressor levels (i.e., YFP levels) and CFP production rates. Their work demonstrated that the quantitative relationship between repressor level and gene expression varies significantly between individual cells and within a given cell lineage over time. This observation helps to explain the amplification of noise in multistage cascades described by Hooshangi et al. (26), as transcriptional variability is less apparent in population-averaged measurements. In a complementary approach, Pedraza & van Oudenaarden (21) combined a similar two-stage transcriptional cascade with a third, independent reporter that controlled for the contribution of extrinsic sources of noise (e.g., environmental fluctuations or the abundance of ribosomes). By simultaneously tracking all three reporters across a population of single cells, the authors decomposed the effects of transmitted and extrinsic noise on the propagation of information through their circuit (**Figure 2a**). This work highlighted that the transmission of information through a genetic cascade can amplify the relatively low level of noise attributed to individual transcriptional units.

The examples we discuss so far employed linear transcriptional cascades as minimal biological test beds to study natural systems. However, application-driven engineering contexts revealed a more direct parallel with electronic circuits that recast hierarchical architectures in the framework of digital logic (28). Building on this notion, Guet et al. (29) assembled a combinatorial library of genetic circuits by randomly assigning operators to regulator genes. This library contained several architectures that demonstrated binary logic behaviors *in vivo*. As a pertinent example, a repressive cascade consisting of a self-inhibiting *lacI*, *tetR*, *cI*, and finally *gfp* exhibited a NOR gate behavior with respect to the two input inducers, aTc and IPTG. To extend the signal processing capabilities of the operators defined in the operon model and to construct more complex logic circuits, synthetic biologists subsequently integrated multiple channels of information upstream of single transcriptional units. For instance, Guido et al. (30) combined the binding sites of a transcriptional activator and a repressor to build a hybrid promoter. By fitting a stochastic model to the responses of the two constituent modules, these authors were able to predict the behavior of a more complex mixed-input feedback circuit. Expanding the logic toolbox further, Tamsir et al. (31) created OR gates in which single operons were controlled by two tandem promoters, each harboring operators for a distinct regulator (e.g., AraC, TetR). By coupling this OR gate to the downstream expression of a repressor (e.g., cI), Tamsir et al. then created NOR gates, which are Boolean complete. Theoretically, any digital logic can be encoded in biological circuits by layering NOR gates, suggesting that the transcriptional regulation principles outlined by Jacob and Monod are sufficient to assemble complex, multi-input circuits *in vivo*.

Over the past decade, the construction of transcriptional logic circuits has continued in earnest, revealing both the potential and limitations of this approach. In an emblematic study, Moon et al. (32) constructed layered three- and four-input transcriptional AND gates; in each

gate, the products of two operons (typically a chaperone and its cognate TF) associate to form a single, functional activator. While these large synthetic gene circuits successfully performed complex logic computations, they also underscored the challenges faced by synthetic biologists operating at this scale: Differences in the timescales of parallel circuit branches can lead to logic hazards, while part defects can propagate and reduce the signal-to-noise ratio of the overall system (10). Furthermore, as transcriptional toolboxes expand, the design spaces available to synthetic biologists are becoming impossible to explore manually. To address these challenges, mathematical descriptions of TF behaviors, such as their transfer functions, can be incorporated into predictive models that facilitate the automation of synthetic gene circuit design tasks. Exploiting the Boolean completeness of digital NOR gates, Nielsen et al. (22) created a computational platform, Cello, that takes a user-defined binary logic behavior as an input and designs complex transcriptional cascades to implement that logic in *E. coli* (Figure 2a). While currently limited to steady-state digital logic, this approach can help to narrow down potential design spaces in silico. As a recent example, Cello was used to circumvent manual design iterations and to assemble 63 regulators in seven strains of *E. coli* to create a biological digital clock display (33).

## 2.2. Posttranscriptional Circuits

The role of RNA as the transient vehicle of genetic information (that is, mRNA) was still in its infancy in the early 1960s. However, Jacob and Monod, in their alternative cytoplasmic operator model, hypothesized that the regulator (either a *trans*-acting RNA or protein TF) could target an operator site on the mRNA rather than at the genetic level (3) (Figure 2b). On the basis of the evidence available at that time, Jacob and Monod concluded that the genetic operator model seemed more plausible; nonetheless, it is important to note that numerous natural RNA-level regulatory mechanisms, including antisense RNA inhibition and riboswitches, have since been discovered (34). In this section, we explore the emergence of synthetic biological circuits constructed at the posttranscriptional level (11). We show that some of the earliest RNA-level regulation strategies operate according to principles highly analogous to those presented in the operon model. We also highlight how the intrinsic ability of RNA to form structured, ligand-regulated domains has enabled the design of mRNA operator elements that respond autonomously to small-molecule cues. This expands the paradigm of metabolite sensing, described by Jacob and Monod in the context of regulatory and structural genes, to the RNA operator sequence.

**2.2.1. Controlling mRNAs with *trans*-acting regulators.** RNA-based networks have appealing properties that complement transcription-based regulation: Their responses can be faster if translation is circumvented (35), and they generally have a lower metabolic burden on the cell (36). Moreover, examples of posttranscriptional regulation are widespread in nature: Noncoding RNAs that act in *trans* to sterically block translation or destabilize the mRNA have been described in both prokaryotes (37) and eukaryotes (38). As we describe below, the earliest demonstrations of synthetic RNA regulation are reminiscent of the transcriptional control strategies outlined by Jacob and Monod: *trans*-acting molecules produced by regulatory genes were directed to specific control regions on the mRNA.

In 2004, Isaacs et al. (39) implemented a posttranscriptional control strategy in *E. coli* by using designer noncoding RNA molecules to specifically induce the translation of *cis*-repressed mRNAs. The authors utilized an engineered 5' stem-loop structure to mask the ribosome binding site (RBS), preventing translation from the synthetic mRNA unless a cognate *trans*-acting RNA was present to hybridize with the target operator and outcompete the stem-loop structure. Building on a related *trans*-acting RNA platform, Bayer & Smolke (23) developed a ligand-responsive

antiswitch system in yeast that represented a more complete analogy to the metabolite-responsive regulators envisioned by Jacob and Monod (**Figure 2b**). These RNA antiswitches were designed on the basis of metabolite-specific aptamers that act as allosterically controlled secondary structures (23). The binding of a specific small molecule (e.g., theophylline) to the aptamer domain leads to a conformational change in the antiswitch that sequesters or exposes an antisense binding domain. Through direct base pairing interactions, this antisense domain hybridizes to a region of the mRNA target around the start codon, inhibiting translation. By combining different aptamer and antisense domains, Bayer & Smolke induced or alleviated the posttranscriptional repression of multiple target genes using different chemical cues.

More recently, Green et al. (40) created toehold mRNA domains that can detect and respond to user-defined RNAs. In contrast to the strategies discussed above, in this synthetic biology technology the engineering effort is centered on the *cis*-acting operator region of the synthetic output gene, allowing the *trans*-acting regulator to be rapidly redefined with minimal sequence constraints. The original architecture was designed to sequester the RBS and start codon of the output mRNA in a stem-loop structure. Direct hybridization with the *trans* regulator is initiated at the toehold domain, leading to the rearrangement of the stem loop and the initiation of translation at the exposed RBS. Kim et al. (24) extended these designs to incorporate *trans*-regulated toehold repression as well as induction (**Figure 2b**), allowing them to design up to four-RNA input logic architectures on a single synthetic transcript. Programmable, toehold-based RNA sensors could allow synthetic biologists to better interface engineered networks with the host transcriptome and have been used as sensors in cell-free nucleic acid diagnostic technologies (41).

**2.2.2. mRNA *cis* regulators can autonomously control gene expression.** Unlike DNA-encoded operator sequences, RNA *cis*-regulatory elements can control translation rates in the absence of *trans*-acting RNAs or proteins: Natural riboswitches have evolved to regulate metabolic processes by directly coupling translation to the abundance of specific metabolites (42). The ability to condense a regulator and its operator onto a single transcript removes the need to express additional *trans*-acting factors and facilitates the modular design of synthetic RNA devices. For example, Win & Smolke (43) coupled the sensing functionality of aptamers to the activity of self-cleaving hammerhead ribozymes in yeast, thereby creating self-contained ribocomputing devices. In this synthetic biology technology, aptamer-defined ligand binding either stabilizes or disrupts a self-cleaving ribozyme actuator, thereby modulating RNA stability. The composability of the individual RNA domains allowed Win & Smolke to couple multiple aptamers to a single actuator domain or to combine multiple sensor-actuator switches on a single transcript. Acting in concert, these combinatorial devices generated complex signal processing behaviors such as Boolean logic and cooperativity.

Due to having well-defined interactions and homogeneous chemistry, RNA devices and circuits are a tractable substrate for *in silico* design, as reviewed in detail by Schmidt & Smolke (44). In an illustrative example, Carothers et al. (45) used *in silico* models of RNA folding to build synthetic transcripts controlled by ribozymes or their aptamer-gated derivatives (aptazymes) in bacteria. Having selected specific *cis*-regulator RNA domains based on the specifications of a mechanistic gene expression model, they set out to computationally design 5'UTR sequences that would meet two requirements for reliable device operation: first, that the 5'UTR sequence (including the RNA device and its variable flanking regions) not impede RBS access, and second, that the kinetics of the ribozyme/aptazyme folding process be compatible with the timescale imposed by the RNA half-life. After validating sets of 25 synthetic ribozyme and aptazyme-regulated transcripts *in vivo*, Carothers et al. found strong agreement between observed relative gene expression rates and those predicted by the initial mechanistic model. Alongside the other examples presented in

this section, this work underscores the progress made by synthetic biologists toward implementing and extending Jacob and Monod's historical cytoplasmic operator hypothesis (2).

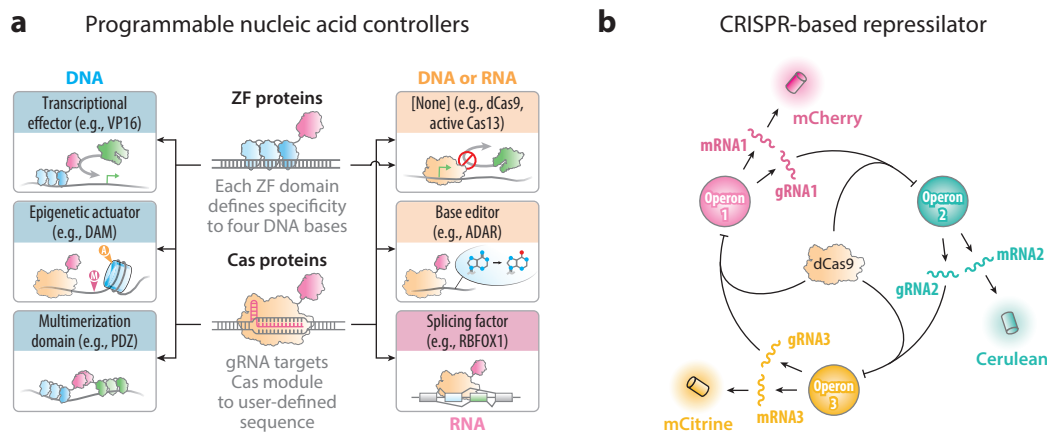
### 3. SYNTHETIC BIOLOGY BEYOND THE OPERON MODEL

In Section 2, we frame the first generation of synthetic biological circuits in terms of the operon model and the blueprints described by Jacob and Monod (1). Transcriptional regulation remains a predominant engineering format in microbial synthetic biology, underpinning the largest synthetic gene circuits to date (33) and positioning itself to deliver transformative technologies in health care and biomanufacturing (5, 9, 10). However, with the aim of approaching the diversity of regulatory strategies exhibited by living cells, a concerted effort has also been made to expand the levels at which artificial behaviors can be programmed beyond the operon model. In this section, we explore how recent, fundamental advances across the life sciences are presenting opportunities for synthetic biologists to adopt new tools for programming complex phenotypes. We begin by underscoring how classes of programmable DNA- and RNA-binding molecules characterized in the past two decades have mediated a fundamental shift in the way we design transcriptional and posttranscriptional circuits and regulators. We then focus on synthetic actuation strategies rooted in contemporary models of genetic regulation, including epigenetics and long-range DNA interactions. Finally, we discuss the growing emphasis on posttranslational regulation strategies in the assembly of synthetic biological circuits.

#### 3.1. Next-Generation Transcriptional and Posttranscriptional Regulators

The first generation of synthetic biological circuits aimed to validate biological hypotheses by reconstructing minimal circuit architectures in isolation. This focus on proof-of-concept experiments justified the reliance on a small set of specialized genetic parts performing relatively immutable functions. In this section, we describe how classes of well-characterized nucleic acid binding domains have been coopted as platform technologies for the programmable regulation of transcriptional and posttranscriptional processes (**Figure 3a**). We highlight how both the target specificity and functional activity of these tools can be rapidly repurposed, providing synthetic biologists the unprecedented ability to actuate a wide array of heterologous and native host processes.

**3.1.1. Zinc finger proteins and complex transcriptional processing.** Research in fundamental biology drives the identification, characterization, and adaptation of new classes of biomolecules that expand the synthetic biology toolbox. Building on the ubiquity of DNA-binding proteins containing zinc finger (ZF) motifs, the tandem assembly of individual ZF motifs into polydactyl ZF proteins emerged at the turn of this century as a powerful approach to design highly sequence-specific DNA-targeting domains (46). The ability to generate small DNA-binding proteins with tunable sequence specificity and affinity, along with the possibility of genetically fusing these proteins to effector protein domains (**Figure 3a**), presented a significant extension to the restricted repertoire of TF-promoter pairs that predominate synthetic gene circuits (Section 2.1). In particular, cooperative and combinatorial interactions between TFs binding on the same promoter support complex signal processing and fine-tuned gene expression regulation. In yeast, for example, Khalil et al. (47) demonstrated that synthetic ZF transcription factors (synTFs) could be functionalized with both *trans* activation domains (VP16) and protein-protein interaction domains (PDZ and peptide ligands). These PDZ domains modulated the interactions between synTFs binding on the same promoter, creating a platform for tunable cooperative behaviors (47). In an



**Figure 3**

Next-generation regulators built from programmable protein modules. (a) Both synthetic ZF domains and Cas/dCas ribonucleoprotein complexes (e.g., Cas9, Cas12) support the modular assembly of effectors that can mediate transcriptional activation and repression, epigenetic modification, and cooperativity. RNA-targeting Cas/dCas systems (e.g., Cas13) can be used to degrade mRNAs or as a scaffold to localize base editors and splicing factors to transcripts of interest. (b) A constitutive dCas9 module can be dynamically repurposed in vivo to create a single-protein, multi-gRNA repressilator (49). In this cyclical circuit architecture, the gRNAs target the dCas9 repressor to the operator of the next operon in the cycle, which encodes the subsequent gRNA and one of three orthogonal reporter genes. Abbreviations: *gfp*, green fluorescent protein; gRNA, guide RNA; ZF, zinc finger.

extension of this work, Bashor et al. (48) used a scaffold protein harboring a variable number of synTF-binding domains to program the assembly of multimeric activators. By combining these clamped activators into synthetic network motifs and fine-tuning their properties (e.g., number of binding sites, affinity), Bashor et al. successfully encoded complex circuit behaviors such as persistence filtering and frequency-dependent responses. Taken together, these examples highlight how the modular assembly of sequence-targeting elements (e.g., ZF arrays) with other protein domains facilitates the rapid redesign of transcriptional controllers to create advanced synthetic biological circuits.

**3.1.2. CRISPR-Cas-based DNA effectors.** The extensive characterization of bacterial clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) enzymes—reviewed in detail by Hille et al. (50)—has offered a powerful alternative to ZF proteins (Figure 3a). The value of these tools for biological engineers lies in their inherent programmability: A simple change in the sequence of a short guide RNA (gRNA) is sufficient to modify the target of Cas nucleases or their catalytically inactive variants (dCas) (51). Over the past decade, CRISPR-Cas systems have been adapted into a versatile toolbox with a broad range of applications, including genome engineering (52), diagnostics (53, 54), and smart materials (55).

Transcriptional repression using dCas proteins (CRISPRi) typically relies on steric hindrance and is therefore relatively species agnostic; this strategy has been used to build synthetic gene networks in both bacteria and eukaryotes. A recent report exemplifies how the facile reprogramming of dCas effectors can be used to assemble biological circuits: Santos-Moreno et al. (49) used CRISPRi to recreate classical synthetic gene circuits in *E. coli*, including a bistable toggle switch and a repressilator. These circuits center on a single dCas9 regulator (instead of two and three different TFs for the bistable toggle switch and the repressilator, respectively), with the transcriptional units regulating each other through the ad hoc production of gRNAs (Figure 3b). These

architectures demonstrate how a single dCas module can be dynamically repurposed in vivo to functionally replace several transcriptional regulators. Interestingly, dCas-binding modules can theoretically target any sequence harboring a protospacer-adjacent motif, and engineered Cas effectors with reduced targeting constraints continue to expand the addressable sequence space (56). This means that Cas-based circuits can be easily interfaced with host genomic sequences, thereby facilitating the seamless integration of synthetic constructs with native regulation mechanisms. Nielsen & Voigt (57), for example, created a layered CRISPRi-based synthetic gene circuit that converged on the regulation of an output gRNA targeting the native TF *malT*. As a result, circuit activation caused the downregulation of the endogenous maltose utilization operons and the  $\lambda$  phage receptor gene *lamB* (57).

Like ZF proteins, dCas derivatives can act as platforms for the targeting of genetic regulators to user-defined loci, expanding their activities beyond transcriptional repression (**Figure 3a**). For instance, Nakamura et al. (58) built a synthetic pulse generator circuit in metazoan cells by targeting dCas9-VPR transcriptional activators to both a reporter gene and an anti-CRISPR gene—the latter mediating a negative feedback mechanism by inhibiting the dCas9-VPR activator. While portable transcriptional activation modules are readily available in eukaryotes, analogous approaches have proved more challenging to interface with endogenous transcriptional machineries in prokaryotes. For example, Fontana et al. (59) report that the efficiency of CRISPR-based activation (CRISPRa) is highly dependent on target sequence context as well as on the geometric constraints related to DNA helix periodicity. This hurdle has so far limited the applications of CRISPRa in the assembly of synthetic bacterial gene regulatory networks, despite computational predictions that this strategy would scale particularly well in bacterial synthetic networks (60). Moreover, even recent bacterial CRISPRa technologies, based on fusions between dCas9 and viral AsiA effectors, are so far restricted to Enterobacteriaceae (61). This limitation represents a bottleneck in the actuation of endogenous genes using synthetic circuits, which would be of great interest for the activation and discovery of gene clusters producing valuable chemicals in other organisms (62).

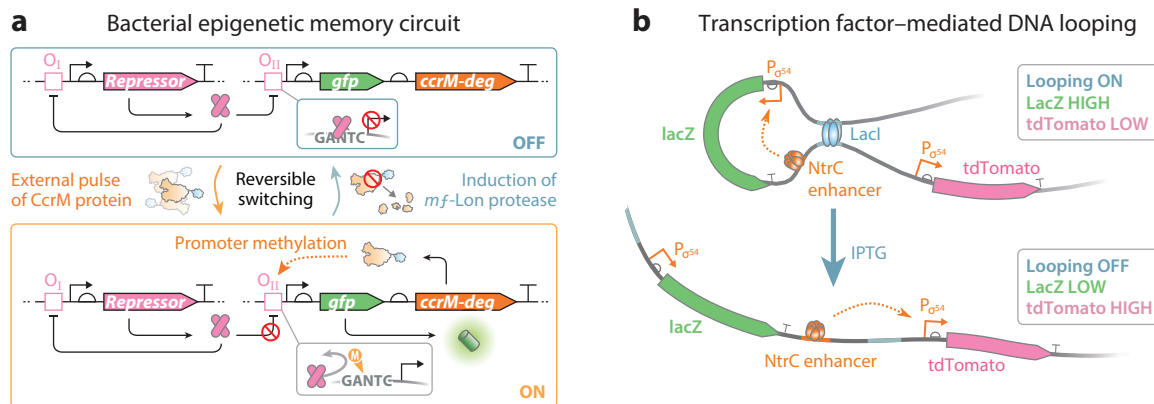
**3.1.3. Programmable RNA controllers.** We emphasize above the important additional layer of control that posttranscriptional regulation can support (Section 2.2), drawing parallels between these synthetic strategies and the alternative cytoplasmic operon model described by Jacob and Monod. Just as programmable protein architectures revolutionized the way synthetic biologists approach DNA-level control and redefined the concept of regulator genes, this new generation of modular effectors is having a similar impact, one step further along the central dogma (51) (**Figure 3a**). In these emerging technologies, the delivery of effector proteins and targeting RNA molecules is sufficient to control near-arbitrary endogenous and synthetic RNA elements. For instance, Konermann et al. (63) used the RNA-targeting nuclease Cas13 to selectively degrade mRNAs, thereby demonstrating the use of Cas effectors as a broadly applicable posttranscriptional control strategy. The inactive version of the Cas13 protein successfully mediated exon exclusion in primary transcripts, further expanding the range of RNA-level control strategies accessible with programmable Cas proteins. RNA-directed dCas effectors, like their DNA-targeting counterpart, have been fused to effector modules in metazoans to facilitate the targeting of RNA-modifying protein domains: Du et al. (64) used dCasRx to target an engineered Rbfox1 splicing factor to different sites of mRNAs, thereby mediating either the inclusion or the exclusion of user-specified exons. While some of these more recent tools have yet to be integrated into higher-level synthetic biological circuits, the plug-and-play framework for DNA- and RNA-level controllers is dramatically accelerating the way in which regulators—as defined in the operon model—are redesigned and diversified.

### 3.2. High-Level Genomic Control Strategies

Research efforts across the life sciences have established that genomes are not the static, one-dimensional entities assumed by Jacob and Monod (4). Contemporary models of genome-level structure and regulation take into account epigenetic modifications, as well as DNA topology, as major determinants of gene activity. In this section, we show how biological insight into these two modes of regulation, as well as design rules for their further engineering, can be derived from the *in vivo* construction and analysis of minimal, well-defined synthetic representations. This form of scientific inquiry based on bottom-up construction is reminiscent of the role played by the earliest synthetic gene circuits in the study of gene regulatory networks and emphasizes the ongoing interplay between fundamental biology and synthetic biology.

**3.2.1. Synthetic epigenetics.** The hypothetical circuits suggested by Jacob and Monod were in part intended to explain cellular differentiation and phenotypic heterogeneity in genetically identical populations (3) (**Figure 1**). As reviewed in detail by Allis & Jenuwein (65), it has since been established that long-term cellular memory is often mediated by epigenetic modifications. Motivated by the fundamental role of epigenetic regulation in development and human disease, research efforts have historically focused on eukaryotic systems. It is important to highlight that minimal synthetic circuits can be used to characterize the different effectors that regulate epigenetic states. Using programmable ZF DNA-targeting modules in yeast, Keung et al. (66) recruited a library of 223 chromatin regulators (e.g., histone methyl- and acetyltransferases) to different sites upstream and downstream of multiple reporter genes, both in isolation and in combinations. By varying the position of the operator sites relative to one or more reporter genes, this work revealed highly locus-dependent and long-range effects on transcription, as well as synergistic effects between TFs and epigenetic regulators. For example, they observed that the recruitment of a histone deacetylase (Sir2) to the promoter region of a reporter gene silenced not only that gene, but also two others located more than 1 kb away; furthermore, Keung et al. functionally protected user-defined genes from these long-range interactions through the ad hoc, upstream insertion of insulating DNA sequences. This work highlights the instrumental role played by programmable DNA-targeting effectors in the investigation of complex transcriptional regulation strategies that extend beyond the range of mechanisms encompassed in the classical operon model.

For synthetic biologists, the bacterial epigenetic machinery is a tractable framework for the construction of minimal epigenetic regulatory networks operating in isolation, in part due to its relative simplicity (67). Using a heterologous methyltransferase (CcrM) derived from *Caulobacter crescentus*, Maier et al. (68) converted a simple operon-like circuit into an epigenetic memory system in *E. coli*. The transient expression of CcrM in response to a preprogrammed environmental cue (e.g., UV irradiation) resulted in the methylation of the promoter of the operon, thereby preventing the binding of a constitutive ZF repressor. An additional copy of *ccrM* expressed from the activated operon ensured the maintenance of the methylation pattern in a positive feedback loop (**Figure 4a**). Within the crowded regulatory landscape of the eukaryotic nucleus, bacterial epigenetic regulators have emerged as an elegant means to create an orthogonal epigenetic code. Bacterial DNA modification systems rely primarily on adenine methylation, while animal systems typically use cytosine modification. This specificity allowed Park et al. (69) to create a stable DNA memory system based on bacterial epigenetic writers (Dam variants) and readers (DpnI derivatives) in mammalian cells. The fusion of Dam writers to programmable DNA binders (e.g., dCas9) defined the nucleation point of the epigenetic marks in the genome. A positive feedback module (Dam-DpnI fusion) could then drive the spatial propagation and long-term maintenance of the epigenetic marks. Finally, the recruitment of activators or repressors (VP64 or KRAB,



**Figure 4**

Transcriptional regulation using synthetic epigenetics and dynamic DNA looping. (a) An epigenetic switch converts transient stimulations into persistent phenotypes in *Escherichia coli* (68). The expression of the DNA methyltransferase CcrM leads to the methylation of the operon promoter, which in turn prevents the binding of a zinc finger repressor. The target operon contains a copy of *ccrM*, which creates a positive feedback loop and maintains the promoter in a methylated, derepressed state. This operon-encoded CcrM harbors a degradation tag recognized by the *mf-Lon* protease (70); the transient expression of the proteolytic enzyme can be used to reset the memory of the circuit by interrupting the epigenetic feedback loop. (b) The canonical regulator LacI can be repurposed to modulate transcription through DNA looping (71). Here, the LacI tetramer can simultaneously bind on two *lac* operators, thus forcing the close proximity of these sites. The looping state, regulated by IPTG, biases the activity of the NtrC enhancer toward one of the two  $\sigma^{54}$  promoters and favors the production of either LacZ or tdTomato. Abbreviation: *gfp*, green fluorescent protein.

respectively) to methylated sites through their fusion to the DpnI reader module allowed Park et al. to tune the expression of a target gene. In contrast to the transient responses induced by a stimulus-specific TF, this synthetic epigenetic tool established a multigenerational memory of the brief induction of the synthetic read-write platform. Together, these studies highlight how synthetic minimal representations of epigenetic memory can be employed to investigate persistent gene regulation across cell populations while also providing synthetic biologists with design rules for the bottom-up construction of insulated, multigene control strategies.

**3.2.2. Long-range genetic interactions.** In support of the conclusions drawn by Jacob and Monod, further characterization of the *lac* operon confirmed the adjacency of the *lac* operator and the genes it controls. However, updated models of gene regulation have adopted a view of genome organization that also considers spatial relationships between chromosome regions in three dimensions, including long-range interactions between distal enhancers and core promoters (72). The bottom-up assembly of synthetic enhancers in bacterial systems has become a useful test bed for researchers deciphering the biophysical mechanisms underlying DNA looping and its consequences. For instance, Brunwasser-Meirom et al. (73) built libraries of variants of a minimal *E. coli* enhancer composed of an NtrC activator binding site hundreds of bases upstream of a  $\sigma^{54}$  promoter. Using variable loop sequences harboring TF binding sites (e.g., a *tet* operator) at different positions, these researchers successfully recapitulated enhancer-quenching mechanisms observed in disparate kingdoms of life. For instance, their model demonstrated that the excluded volume of enhancer-bound TFs, which increased additively with multiple intraloop TF binding sites, mediated the quenching of NtrC-mediated DNA looping and transcriptional activation. This work shows how simple synthetic modules built from well-described transcriptional effectors can help elucidate the mechanisms underlying the spatial regulation of gene expression.

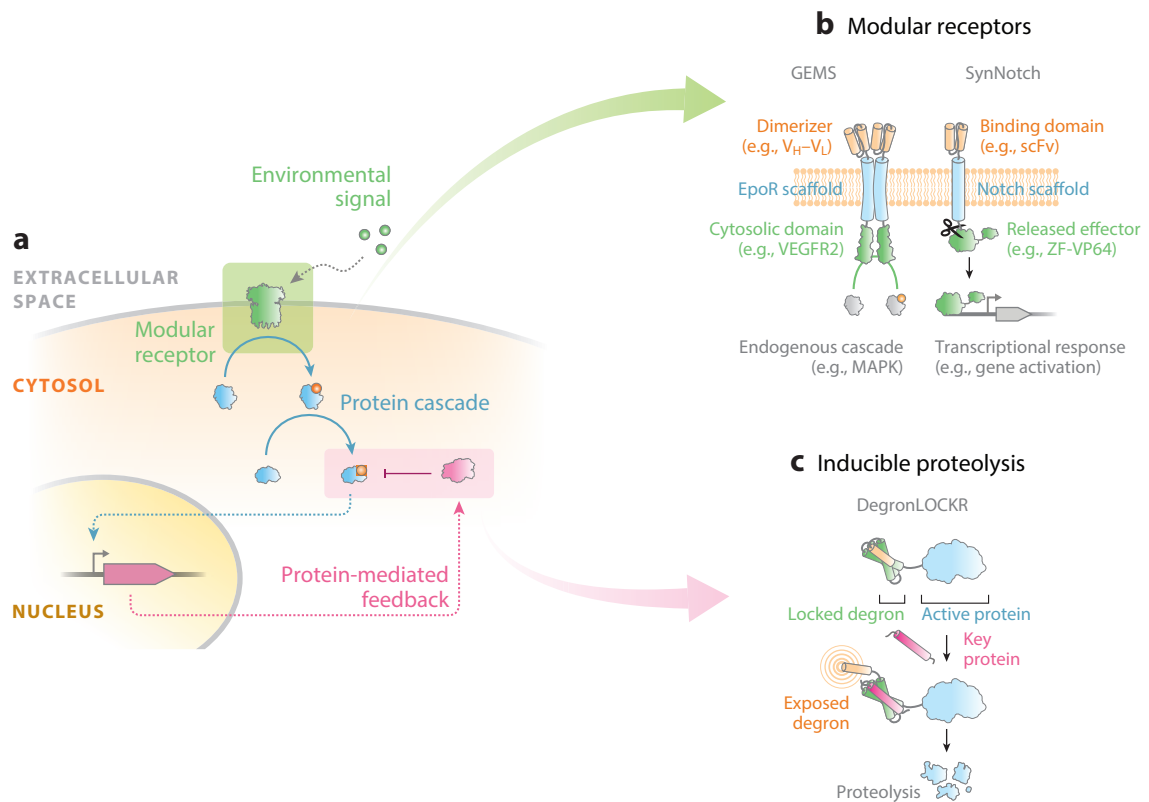


These insights are, in turn, guiding the development of methods to control synthetic gene networks by altering genome topology. Drawing from the expanding toolbox of programmable DNA-binding modules (Section 3.1), Hao et al. (74) used bivalent dCas9 complexes to force contacts between distant sites in the *E. coli* genome. By bringing a strong distal *lac* operator in close proximity to a weaker one controlling the transcription of a reporter, the authors successfully decreased transcription levels by favoring the recruitment of distant DNA-bound LacI tetramers. More recently, the same team demonstrated that forced DNA looping could be used to both positively and negatively regulate enhancer function and enforce the specific activity of an NtrC enhancer toward one of two possible  $\sigma^{54}$  promoters driving reporter gene expression (71). Hao et al. (71) placed two *lac* operators in the *E. coli* genome at positions such that the LacI-mediated bridging of these sites would form a loop containing a *lacZ* reporter and the NtrC enhancer while excluding the other reporter (*tdTomato*) (**Figure 4b**). As a result, these researchers could artificially regulate DNA topology through the reversible, allosteric modulation of the LacI tetramer. In doing so, they biased the regulatory activity of the enhancer toward either *lacZ* or *tdTomato*. These results suggest that loop engineering strategies could become a powerful way to regulate multiple genes simultaneously and to switch between genetic programs integrated at different genomic sites.

### 3.3. Protein-Level Circuits

In the operon model, Jacob and Monod postulated that the allosteric binding of small-molecule ligands to regulators (either RNA or proteins) was the predominant input format for gene regulatory circuits. They also alluded to the regulation of proteins through enzymatic modification as another example of molecular conversion (1), leaving open the possibility of protein modules acting as signal processing entities in their own right. However, Jacob and Monod's definition of regulators explicitly excluded any role of these effectors in controlling the posttranslational activity of target proteins; due to a lack of well-studied examples, protein-protein signaling was not an area of focus for the historical operon model. Subsequent advances in molecular cell biology have highlighted the ubiquitous role played by protein signaling in the flow of information through natural regulatory networks. In synthetic biology, efforts to build, perturb, and rewire protein-level circuits are now complementing established approaches at the transcriptional level (75) (**Figure 5**). Here, we follow the typical flow of information through protein signal transduction pathways, starting from receptors and moving down to the intracellular computation layer (**Figure 5a**). In doing so, we identify a common thread among many protein-based regulation strategies, namely a decomposition of proteins into modular functional domains, reminiscent of the programmable DNA and RNA actuators we describe in Section 3.1.

**3.3.1. Repurposing natural receptors as the input layer for synthetic circuits.** Transcriptional synthetic circuits, as their input, typically rely on cytosolic, small-molecule biosensors that directly couple allosteric regulation to transcriptional and translational responses (Section 2). However, in natural systems, environmental sensing and cell-cell communication are mediated by diverse receptors that couple extracellular or intracellular cues to transcriptional and post-translational responses. These receptors, and their downstream protein signaling pathways, can support complex signal integration and computational functions (76). For synthetic biologists, the emphasis on using modular protein structures to rewire signaling networks has been particularly successful in the context of membrane receptors: When the domains responsible for signal transduction can be decoupled from the signal-sensing moieties, a mix-and-match approach can then be used to couple biomolecular cues such as signaling proteins to integrated synthetic circuits or to redirect natural sensors to trigger alternative intracellular responses.



**Figure 5**

Protein-protein interactions in synthetic biological circuits. (a) Protein-level regulation is involved in environmental sensing and signal transduction. Here, a designer receptor (*green*) converts a user-defined cue into the activation of downstream protein effectors (*blue*), eventually triggering a transcriptional response that in turn mediates a posttranslational feedback loop (*pink*). (b) Modular receptor architectures such as the GEMS (80) and SynNotch (81) platforms use customizable receptor scaffolds (*blue*), on which synthetic biologists can mix and match the input (*orange*) and output (*green*) domains. The downstream response (*gray*) is either the activation of an endogenous pathway or the release of a synthetic effector. (c) The degronLOCKR is a proteolytic switch module designed in silico. The cognate key protein triggers a helix displacement that exposes a degradation tag (82).

In bacteria, environmental sensing is often mediated by arrays of orthogonal two-component systems (TCSs), in which receptor histidine kinases (HKs) control downstream response regulators (RRs) directly or via phosphotransferase cascades. The specificity of the interaction between the HKs and RRs is defined by a small, well-defined set of interfacial residues (77). McClune et al. (78) recently found that paralogous TCS pathways occupy a relatively restricted proportion of the available sequence space at these sites. To determine whether this sparsity could support the introduction of additional orthogonal pathways, they assembled a library of  $10^8$  variants of the canonical PhoQ-PhoP TCS, specifically randomizing residues at the HK-RR interface. From this library, they isolated sets of up to six insulated sensing pathways. This work highlights how protein-protein interactions can be diversified to rewire posttranslational circuits while reducing cross talk within families of sensors. Interestingly, by replacing the natural PhoQ Mg<sup>2+</sup>-sensing domain on one of these orthogonal PhoQ-PhoP pairs with a plant-derived cytokinin-sensing domain, McClune et al. created a new small-molecule biosensor. In another recent study in mammalian cells, Scheller et al. (79) reprogrammed the bacterial DcuS/DcuR TCS by using a caffeine-specific

nanobody input domain, which initiated orthogonal HK-RR phosphotransfer and downstream gene activation in response to ligand-induced receptor dimerization. These two examples of input domain swapping illustrate a general approach to create new protein-based sensors.

Similar domain recombination techniques have been applied to complex eukaryotic receptor systems, with the objective of programming intercellular signaling functions (83). In mammalian cells, for example, Scheller et al. (80) rewired the erythropoietin receptor into a versatile sense-and-respond platform. The authors replaced the receptor's extracellular ligand-binding domain with various engineered alternatives (e.g., single-chain variable antibody fragments specific to the PSA protein) and replaced the intracellular domain with the corresponding domains from other receptor families (e.g., FGFR1 or VEGFR2 cytosolic domains) (**Figure 5b**). The resulting chimeric receptors can couple user-defined binding events to downstream, endogenous signaling pathways—for instance, mitogen-activated protein kinase (MAPK) cascades or PI3K/Akt signaling. These protein-based cascades provide a built-in mechanism for signal amplification. To date, several other protein architectures, including G protein-coupled receptors (GPCRs) (84) and the Notch protein (81), have been decomposed into modular architectures in a similar way (**Figure 5b**). In the former example, GPCR signal transduction leads to the binding of a  $\beta$ -arrestin-2 adaptor to the intracellular domain of the engineered receptor, bringing together the TEVp protease and its target sequence. The subsequent proximity-induced cleavage of the target site by TEVp liberates a tethered dCas9 CRISPRa module from the arrestin adaptor, which is translocated to the nucleus to regulate downstream gene expression. As a combined synthetic biology toolbox, the modular receptor architectures discussed in this section illustrate how the design of synthetic biological circuits increasingly revolves around the isolation, characterization, and rearrangement of natural protein domains to create complex input-output relationships.

**3.3.2. Intracellular computation at the protein level.** While the ubiquity of intracellular protein signaling was not apparent from the microbial systems studied by Jacob and Monod, these pathways exemplify how posttranslational regulation can rapidly propagate and amplify cellular information. Layered phosphorylation cascades are a widespread mechanism in both prokaryotes and eukaryotes and typically mediate membrane-to-genome information propagation. Detailed studies of MAPK cascades, for example, showed that these conserved pathway architectures can provide an exceptional level of sensitivity (85), which is an attractive feature for cellular switches. Bashor et al. (86) used the yeast mating MAPK cascade as a tractable test bed for phosphorelay engineering by artificially recruiting inhibitors and activators to the MAPK scaffolding protein Ste5. By linking the production of these regulatory proteins to the activation of the MAPK cascade, they established simple feedback loops that fine-tuned the response of the overall pathway. For example, the displacement of an inhibitor protein by a heterologous activator established a positive feedback system that behaved as an ultrasensitive cellular switch. In an illustration of the interplay between fundamental and synthetic biology, Mitchell et al. (87) later used analogous feedback mechanisms to investigate the mechanisms of oscillatory hypersensitivity in the yeast osmolarity-sensing MAPK network. In this system, medium-frequency osmotic oscillations result in a sensory misperception and a corresponding fitness defect caused by the recurrent triggering of the cascade. By producing a heterologous bacterial MAPK inactivator, OspF, in response to MAPK activation, Mitchell et al. created a synthetic negative feedback loop that increases the refractory period of the system; in doing so, they demonstrated a trade-off between circuit robustness under oscillatory stress and fitness at noncritical signal frequencies. In an extension of the transcriptional feedback circuits discussed in Section 2.1.2, the two synthetic systems described above interface genetic regulation with protein-protein communication, underscoring the complementarity between transcriptional and posttranslational engineering approaches (**Figure 5a**).

Phosphotransfer-based regulation strategies can be challenging to implement from the bottom up, as they rely on interactions between proteins with strictly defined activities (e.g., phosphate donors). In contrast, proteolytic degradation is a ubiquitous regulation mechanism theoretically applicable to any protein, provided that the molecule can be conditionally targeted to cellular catabolic pathways. Such targeting is typically mediated by small degradation tags, or degrons, that can easily be appended to protein coding sequences. In one study, Cameron & Collins (70) established an inducible proteolysis platform in *E. coli* by importing an orthogonal degradation pathway from *Mesoplasma florum*. The on-demand, titratable induction of the *mf*-Lon protease allowed these authors to fine-tune the degradation rate of the tagged proteins. This system was later incorporated into the bacterial epigenetic memory circuit built by Maier et al. (68) (Section 3.2.1, **Figure 4a**): By inducing the production of *mf*-Lon, they could trigger the degradation of the tagged DNA methylase and interrupt the epigenetic feedback loop, thereby resetting the memory of the circuit.

In the examples described above, the targeted regulation of a single protein is mediated by the transcriptional activation of a protease effector gene. However, parallel proteolysis pathways can support more complex synthetic logic circuits that operate exclusively at the posttranslational level. In an extensive proof of concept, Gao et al. (88) assembled functional networks in human cells based on a set of three orthogonal viral proteases (e.g., TEVp). In these circuits, the viral proteases can positively regulate a target through the cleavage of an associated degradation tag or negatively regulate a target through the removal of a degron–masking peptide. To create layered signal processing cascades and Boolean logic gates, the authors used protease-sensitive dimerization domains to define the conditional, intracellular assembly of downstream split-protease effectors. Interestingly, several of the designer receptor architectures discussed in Section 3.3.1 rely on the viral protease–mediated release of diffusible regulators such as dCas activators following ligand recognition; this common feature may also allow for the coupling of such regulators to the downstream protease-mediated computation described by Gao et al. to create an orthogonal receptor–signaling cascade circuit.

Today, protein domains and interactions designed de novo are contributing new bio-orthogonal modules to the posttranslational toolbox. In a recent study, Ng et al. (82) created an entirely synthetic protein device harboring a degradation tag locked in a helical bundle, which is conditionally exposed upon the binding of a cognate helical key protein (**Figure 5c**). The authors fused this synthetic sense-and-respond protein module to two kinases in the yeast MAPK mating pathway. They then created artificial feedback loops by coupling MAPK signal transduction to the production of the key, thereby triggering the degradation of the predefined kinase in the cascade. Because the protein module was rationally designed in silico using biophysical models, the authors could easily tune the strength of the feedback by altering the length of the key protein. This feature exemplifies how designer protein modules can accelerate the design-build-test cycle in synthetic biology, as they facilitate the rational fine-tuning of circuit behavior. Interestingly, in this example, the protein degradation module performs its computation independently of the domains to which it is attached, and the module can be appended to a wide range of targets. In another recent example of the application of designer proteins to synthetic biology, Chen et al. (89) built a family of helical dimerization domains in silico. As the affinity between a given pair of helical modules is encoded by a defined set of hydrogen-bonding residues, the authors could program strand-displacement reactions at the protein level. For instance, a self-hybridizing molecule consisting of two interacting domains *X* and *Y* could be uncaged by a protein *X'*, provided that *X* bound more tightly to *X'* than to *Y*—thus exposing *Y* to bind to other molecules (e.g., a cognate *Y'* module). The team used these designer molecules to program and implement protein-level circuits, including complex logic behaviors in bacterial cell-free extracts, as well as yeast and human

cells. To create a binary AND gate in a bacterial cell extract, Chen et al. tested the reconstitution of a split luciferase (fragments  $L_1$  and  $L_2$ ) fused to nonmatching helical domains (yielding  $L_1$ - $X$  and  $Z$ - $L_2$ ). Upon expression of the binary input proteins ( $X$ - $Y$  and  $Y$ - $Z$ ), the four molecules formed a complex that reconstituted the functional reporter. As complementary demonstrations of the application of de novo protein design to synthetic biology, the reports by Ng et al. (82) and Chen et al. (89) are emblematic of the progressive transition away from the traditional molecular tools of the operon model and toward the adoption of customizable scaffolds with predictable biochemical activities.

#### 4. CONCLUSION

The first generation of synthetic biology began within the theoretical bounds of the operon model outlined by Jacob and Monod (1–3) and played an important role in the study of biological noise and natural gene regulatory networks. However, our fundamental understanding of the many layers of the regulation of gene expression has expanded dramatically since the early days of molecular genetics (4), informing new regulatory strategies and the design of new parts. From these engineering efforts across the modern central dogma, a general theme emerges: Historical sets of molecular tools are increasingly being complemented by modular and programmable biomolecules that can interface diverse synthetic and natural systems. In parallel, our ability to explore biological diversity for new activities and rapidly repurpose existing tools is expanding the range of behaviors that can be controlled through synthetic biology. As the vital interplay between fundamental research and biological engineering continues (8), we foresee exciting new frontiers for synthetic biology across prokaryotes and eukaryotes: Equipped with new tools and more comprehensive biological models, synthetic biologists can integrate synthetic systems more seamlessly into natural ones and minimize adverse impacts on the host. This is particularly important for applications that require the long-term stability and autonomous behavior of engineered cells outside of the laboratory. Emerging examples include living therapeutics (90, 91), as well as living diagnostics for environmental and medical monitoring (92). We are optimistic that this next generation of tools and methods will help deliver on the promised potential of synthetic biology in human health, manufacturing, and ecology. Just as important is the perspective that the lessons we learn as synthetic biologists will inform and refine contemporary models of cellular behavior.

#### SUMMARY POINTS

1. The operon model of transcription proposed by Jacob and Monod served as the blueprint for the first generation of synthetic gene circuits.
2. The interplay between fundamental biology and synthetic biology has opened up new frontiers for the application of biological circuits as tools to perturb and study natural systems.
3. Synthetic biologists are adopting and refining new gene regulatory strategies, including epigenetic control and DNA structural rearrangement.
4. Biomolecular discovery and de novo biological design are furnishing synthetic biologists with new, modular protein tools that facilitate their rapid repurposing and reprogramming.

## FUTURE ISSUES

1. Current *in silico* biological circuit design tools are well suited to the automated assembly of steady-state transcriptional logic but will need to be extended for new modes of regulation and for more complex dynamic behaviors.
2. Molecular tools based on CRISPR-Cas enzymes are a powerful platform for synthetic biologists but present challenges such as host metabolic burden and toxicity, particularly in bacteria.
3. The systematic characterization of prospective functional parts from metagenomes is a major bottleneck for the continued discovery of new classes of biomolecular tools and components.

## DISCLOSURE STATEMENT

J.J.C. is a cofounder of Synlogic and Senti Biosciences and a cofounder and director of Sherlock Biosciences. M.A.E., R.V.G., and J.J.C. are inventors on patents related to CRISPR technologies.

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