Rewiring Cells: Synthetic Biology as a Tool to Interrogate the Organizational Principles of Living Systems

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modularity, network, engineering, evolvability

Abstract

The living cell is an incredibly complex entity, and the goal of predictively and quantitatively understanding its function is one of the next great challenges in biology. Much of what we know about the cell concerns its constituent parts, but to a great extent we have yet to decode how these parts are organized to yield complex physiological function. Classically, we have learned about the organization of cellular networks by disrupting them through genetic or chemical means. The emerging discipline of synthetic biology offers an additional, powerful approach to study systems. By rearranging the parts that comprise existing networks, we can gain valuable insight into the hierarchical logic of the networks and identify the modular building blocks that evolution uses to generate innovative function. In addition, by building minimal toy networks, one can systematically explore the relationship between network structure and function. Here, we outline recent work that uses synthetic biology approaches to investigate the organization and function of cellular networks, and describe a vision for a synthetic biology toolkit that could be used to interrogate the design principles of diverse systems.

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INTRODUCTION: WHY REWIRE CELLS?

The application of engineering principles toward the construction of novel biological systems—a discipline that has become known as synthetic biology—has received a great deal of attention in recent years because of its potential to deliver a wide array of technological benefits. Revolutionary applications have been envisioned that range from engineering microbes to perform industrial tasks such as biofuel production, to the reprogramming of human cells for therapeutic purposes. To a large extent, the practice of synthetic biology consists of co-opting molecular parts from natural systems and using them to construct new networks that fulfill specific design goals.

Although the promise of synthetic biology is vast, many scientists wonder whether we understand enough about cells and complex biological system to begin engineering them. After all, we are getting close to assembling a complete parts list of the molecules in a living cell, but we are far from having a predictive understanding of how these components work as a system to carry out complex biological functions. If this is the case, then how can we have the audacity to try to engineer cells? Would it not be better to first fully understand the cell and then try to engineer it?

We argue here that, in addition to its applications, synthetic biology is and will become an increasingly powerful discovery tool for understanding the organization and function of cellular networks and other complex biological systems. At the current stage of intellectual development in biology, we have extensive knowledge of molecular parts but a limited understanding of their functional organization and design principles. Thus, it can be incredibly valuable, and perhaps even necessary, to use a learning by building approach. Working in partnership with other traditional methods of cell biology research, synthetic biology can be used to evaluate hypotheses on how complex behavioral phenotypes arise from cellular network structure. Synthetic biology has already had success in engineering simple regulatory circuits that recapitulate natural circuit behavior (11, 49). In the future, it should be possible to use an engineering approach to systematically identify multiple alternative

topologies for biological circuits and to quantitatively compare their performance. This type of analysis will clarify the fundamental principles of how evolution chooses a network design to fulfill a specific functional need. Ultimately, obtaining engineering control over a broad range of cellular functions will provide an experimental toolkit that significantly augments the traditional discovery tools of cell biology.

USING SYNTHETIC BIOLOGY TO DEFINE THE EVOLUTIONARY BUILDING BLOCKS OF CELLULAR ORGANIZATION AND FUNCTION

A fundamental issue in biology is how complex systems are organized, and how that organization changes during the process of evolution. The concept of hierarchical modularity has provided a useful framework for understanding this organization (4). Systems are considered modular if the parts that compose them (modules) can be rearranged and retain their function in a context-independent fashion. It is undeniable that most biological systems exhibit features of modular organization. Viewing biological networks in terms of a hierarchy of interlinked, functional modules is a useful way to parse biological complexity into parts that are more easily understood. Thus, a systems level understanding of a complex entity such as a cell relies on our ability to identify functionally important constituent modules and to delineate their relationship to one another within the cell's organizational hierarchy.

Another reason to understand the modular organization of biological systems is to gain insight into the process of evolution. Modularity might itself be a property that improves the evolvability of biological systems by facilitating the rapid reconfiguration of network structure (35, 36, 45). In other words, the reconnection of modules may provide a system with the ability to rapidly generate functional diversity in the face of continually changing selective pressures. Therefore, the identification of functional modules, and understanding the extent to which they can be rewired, can provide insight into the evolutionary process.

Synthetic biology is a potentially useful approach for investigating the modular organization of cellular networks. By attempting to identify a part or subsystem that can be used to either rewire an existing network or construct a new network, a synthetic biologist implicitly evaluates hypotheses about the modularity of that part or subsystem. For example, if the component in question is indeed a functional module, then it should retain its native functionality when wired into a synthetic network. By the same token, a deeper understanding of modularity at the molecular level may allow us to create entirely new types of connectivities within cellular networks. Below we describe how efforts to rewire cells have helped to functionally identify the modular building blocks of several types of cellular regulatory networks and have lent general support to the idea that modularity promotes diversification of network function.

Recombining Gene Expression Modules

The organization of genetic regulatory networks offers one of the clearest examples of hierarchical modularity in a cellular system (**Figure 1*a***). Nodes in genetic networks (genes) are composed of regions containing *cis*-regulatory elements (promoters) and protein-coding regions. Network linkages are created when *cis*-acting factors that are coded for by an upstream gene interact with the promoter of a downstream gene, forming a regulatory interaction. Thus, a gene constitutes a functional module, with the input defined by the interaction between promoter and upstream *cis* factors, and the output defined by the identity of the transcribed coding region. Groups of genes are often found linked together in frequently reused, modular patterns of connectivity known as motifs (2). Motifs have defined input and output properties and specific information-processing functions (45). Higher-order behavioral complexity of genetic networks is thought

Synthetic biology: discipline that applies engineering principles toward the construction of novel biological systems that exhibit specified behaviors

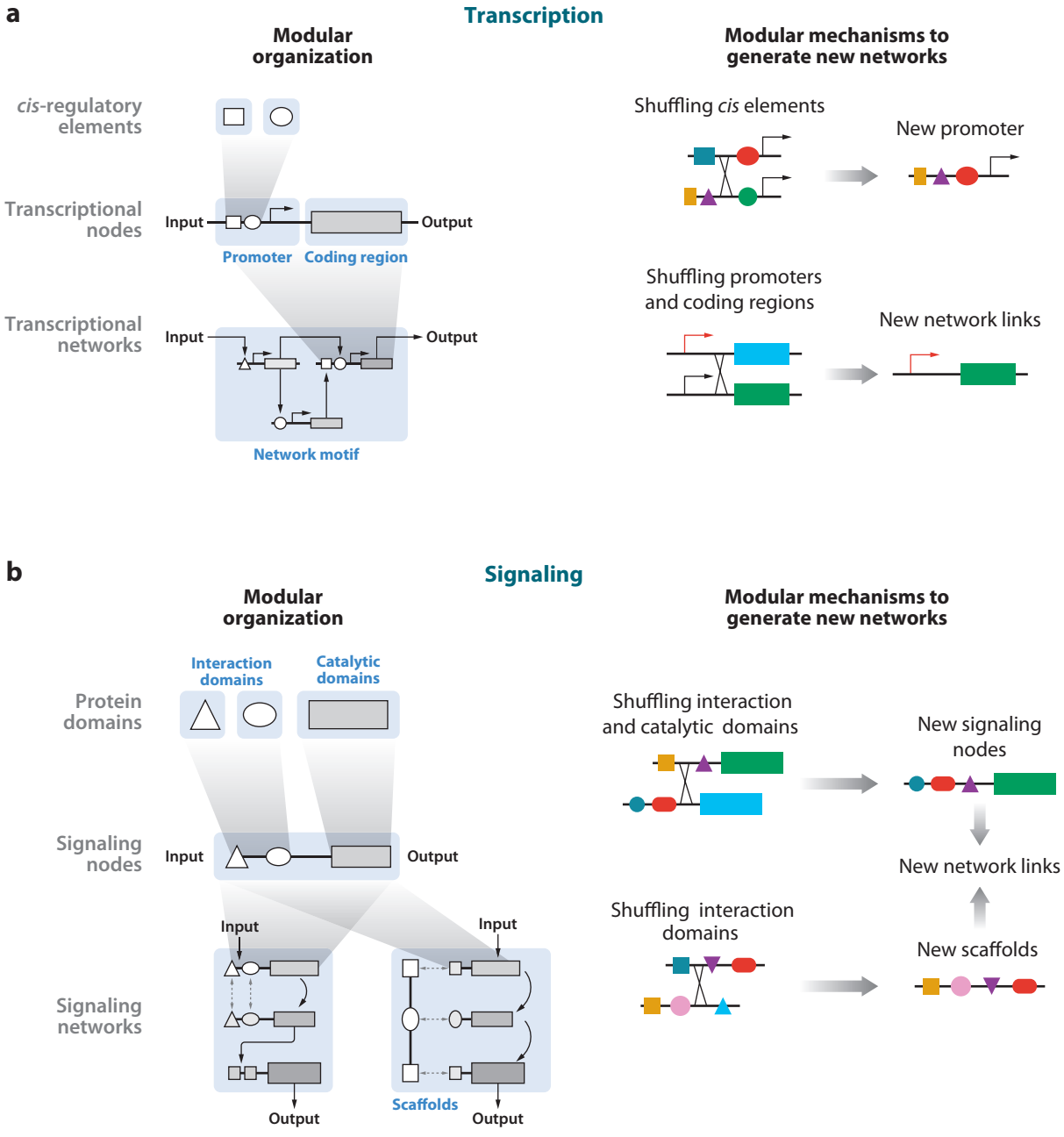
Cellular network: a collection of molecules, macromolecules, or molecular assemblies that are linked together by some mode of interaction

Hierarchical modularity: type of organizational structure in which a collection of units with their own independently identifiable functions are grouped together into larger units with an indentifiable function

Node: the most basic unit of a network that can convert an input into an output

to derive from the interaction of different types of motifs (44).

A considerable body of experimental work has demonstrated the high degree of modularity in gene transcriptional control. The establishment of systems for the heterologous expression of proteins (30) was an important affirmation of the universal modularity of gene structure that exists across species boundaries. The early use of chimeric transcriptional activators and the use of chimeric gene modules consisting of eukaryotic coding regions and bacterial regulatory



elements (12, 13) served as validation of this principle and formed the basis for the yeast two-hybrid screen (21). More recent studies of the modularity of gene structure have explored the effects of modular reshuffling on promoter architecture. In one study, Cox et al. (16) used a library of promoter elements to combinatorially vary the placement, number, and affinity of operator sites in a promoter regulated by two repressors (two-input regulation). They demonstrated that simple variations in the modular structure of a promoter can be used to generate a breadth of regulatory diversity.

An understanding of how variations in genetic network connectivity can be generated leads to an understanding of how evolution may have shaped network structure. The high degree of modularity in gene network architecture and the apparent plasticity of *cis*-regulatory elements have led to the hypothesis that changes in *cis* regulation played a major role in the evolution of phenotypic diversity (64). For example, modular alterations to promoter structure have been proposed as a key mechanism for generating anatomical diversity in the animal kingdom (14, 28, 48). How evolutionarily important rewiring events might have occurred is difficult to ascertain on the basis of phylogenetic evidence alone. However, experimental evidence suggests that regulatory diversity can be generated by shuffling promoters and coding regions in synthetic gene networks. In a study conducted by Guet et al. (31), combinatorial rearrangement of promoters and coding elements into a library of three-node networks resulted in motifs that exhibit a variety of complex gating properties. These results hint at the potential for regulatory and coding regions to generate diversity in signal-processing behavior via simple modular rearrangements.

Recombining Signaling Modules

Protein signaling networks mediate the processing of external signals and, like gene regulatory networks, have evolved modular network structures (**Figure 1*b***). Proteins found in signaling networks are made up of multiple, independently folding domains (9). These domains fall primarily into two classes: catalytic domains (which execute chemical reactions, e.g., a kinase domain, which transfers a phosphate to a target protein) and regulatory domains (which target, localize, or regulate the catalytic domain). Thus, for a functional module in a protein signaling network, input is defined by the interaction of the regulatory domain with a partner, and output is defined by the activity of the catalytic domain. However, a notable difference between nodes in protein signaling networks and nodes in genetic networks is the diverse means by which signaling input can be regulated. For example, a regulatory domain can modulate a signaling protein's output in *cis* by intramolecular autoregulation of catalytic function. Input, which arrives in the form of a binding event or a chemical modification (e.g., addition of a phosphate group), can switch catalytic function either on or off. Regulatory domains can also modulate

Figure 1

Synthetic rewiring experiments can help to define the modular hierarchy of transcriptional and signaling networks. Cellular regulatory networks are built up from hierarchies of interlinked modules. Function is achieved from the assembly of molecular building blocks into network nodes that perform defined input/output functions. Nodes, in turn, are assembled into network motifs—patterns of connectivity that execute specific information-processing tasks. By attempting to rewire the components of cellular networks, we can impose upon those components a test for functional modularity. (*a*) In transcriptional networks, nodes are composed of *cis*-regulatory elements, which define input, and coding regions, which specify output. Groups of nodes are often found interlinked in specific connectivity patterns known as motifs, which often perform specific information-processing functions. (*b*) In protein signaling networks, interactions between signaling proteins (nodes) are mediated by interaction domains (input modules) that recruit catalytic domains (output modules) to specific targets. Interaction domains can also allosterically regulate catalytic domains, creating protein switches. Scaffold proteins are assemblies of regulatory domains that bind multiple catalytic components and thereby organize the connectivity of entire pathways.

N-WASP: neuronal Wiskott-Aldrich syndrome protein

PIP2: phosphatidylinositol 4,5-bisphosphate

SH2: Src homology 2

DED: death effector domain

MAPK: mitogen-activated protein kinase

signaling protein connectivity by acting in *trans*, through recruitment interactions with other regulatory motifs in other signaling proteins. In this capacity, a regulatory domain can localize a signaling protein to a particular subcellular region where it can create the necessary proximity for interaction with a specific upstream or downstream target. Recruitment also plays a notable role in organizing higher-order assemblies of signaling proteins. Adaptors and scaffolds are proteins that colocalize multiple proteins into complexes.

Synthetic biology studies have demonstrated the flexibility with which modular signaling switches with diverse behaviors can be built using protein modules. N-WASP (neuronal Wiskott-Aldrich syndrome protein), a switch protein with actin-polymerizing activity, is one notably well-studied example of a protein that displays modular autoregulation (42). N-WASP activation occurs when Cdc42 and the phospholipid PIP2 (phosphatidylinositol 4,5-bisphosphate) bind to N-WASP and abrogate autoinhibition. As both inputs are required for N-WASP activation, the protein effectively acts as an AND-gate (47). To establish the modularity of N-WASP regulation, Dueber et al. (18) replaced the native N-WASP regulatory domains with heterologous regulatory domains and showed that activity could then be gated by those inputs. By varying the architecture of synthetic switch construction, a small library of synthetic switches was created that displayed surprisingly complex signal integration, recapitulating the native AND-gate behavior but also displaying other types of gating. Their findings show that autoregulation of catalytic activity can be entirely modular—gating function can be fully decoupled from catalytic activity.

As is the case with genes, groups of signaling proteins are often organized into characteristic motifs that are key to network-information-processing function. Linear cascades are a common motif for kinase signaling pathways; feedback regulation is also common. Regulatory domains often mediate the connectivity of signaling pathways, linking upstream motifs (e.g., a GTPase switch) with downstream ones (e.g., a kinase cascade).

A number of important studies have demonstrated the modularity of pathway connectivity by testing the ease with which manipulation of regulatory domain–mediated recruitment can be used to alter the input/output relationship of a signaling response. In one study (32), a chimeric adaptor protein assembled from an SH2 (Src homology 2) domain and a DED (death effector domain) domain was used to couple an upstream proliferative signal to the downstream activation of an apoptotic pathway. Other studies have focused on scaffold modularity by exploring their ability to specify information flow in a pathway. For example, yeast mitogen-activated protein kinase (MAPK) signaling cascade connectivity was altered with synthetic scaffold chimeras (46). By constructing scaffolds that bound components of both the mating and osmolarity response MAPK pathways, the authors demonstrated the ability to reroute signaling input from one MAPK pathway into the output of the other. This result suggests that it is possible to reprogram pathway connectivity in a modular fashion by altering scaffold-binding properties, and that scaffold proteins may allow for the creation of new pathways during evolution.

As is the case for transcriptional networks, the modular architecture of posttranslational signaling components may have contributed to the evolutionary diversification of signaling network architecture. However, the question remains whether domain rearrangements play the same role in protein networks as do the shuffling of *cis*-regulatory elements in genetic networks. A recent study (S. Peisajovich, P. Wei, J. Garabino & W. Lim, unpublished data) addressed this question by creating a library of chimeric domain fusions created from various components of the yeast mating pathway. The library of chimeras, when expressed, resulted in alterations to signaling that were far more dramatic than alterations with the simple overexpression of wild-type proteins or expression of unfused domains. These results demonstrate the

potency of domain recombination as a mechanism to alter phenotype in protein signaling networks.

USING SYNTHETIC BIOLOGY TO PERTURB AND PROBE NETWORK MECHANISM

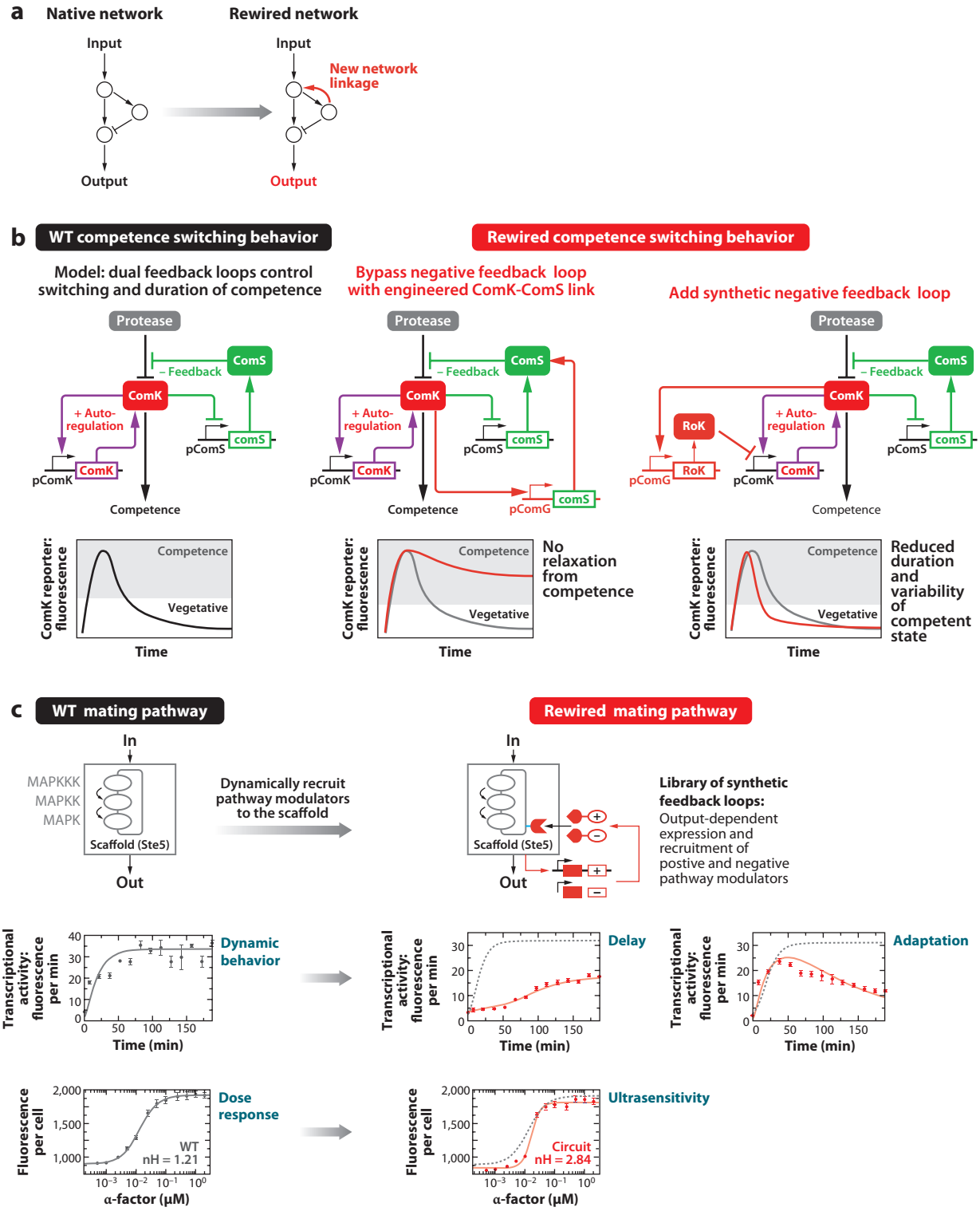
In modern biology, it is increasingly common to use the approaches of systems and computational biology to generate a model that can explain the behavior of a molecular network of interest. But how do we assess whether the model is correct or useful? Here we argue that the ultimate test for the predictive value of such models is to use synthetic rewiring to experimentally change the links and parameters of the network, thus allowing one to identify properties that are critical for function. Instead of analyzing a single network architecture and parameter set, a synthetic approach offers the chance to learn how a wide range of network properties map to functional behavior, and to understand the relationship between network performance and behavioral robustness.

Tinkering to Probe Mechanisms and Explore Plasticity and Robustness

For genetic networks, rewiring experiments are a useful way to quantitatively test predictions about the relationship between network structure and behavior (**Figure 2*a***). Two recent studies (57, 58) examining a genetic circuit that regulates the stochastic switching between states of competence and vegetative growth in *Bacillus subtilis* serve as prime examples of this type of approach (**Figure 2*b***). On the basis of experimental data, the authors developed a quantitative model to describe how cells transiently pass through the competence state in a manner similar to the excitatory state of a neuronal action potential. In this model, the transition into competence is caused by the stochastic activation of a positive feedback loop, whereas decay of the excited state (exit from competence) is regulated by an opposing negative feedback loop. To test predictions made by this model, the natural circuit was rewired with synthetic feedback loops. Results were consistent with the predictions made by the authors' model: Adding another positive feedback loop that bypassed the negative feedback loop (postulated to drive exit from competence) permanently locked cells into competence, whereas adding an additional negative feedback loop led to shorter and more precise switching times back to the vegetative state.

In a recent example of rewiring in a protein signaling network, Bashor et al. (6) evaluated the potential for using synthetic feedback regulation to reprogram the input/output of the yeast mating MAPK pathway (**Figure 2*c***). In this study, positive and negative feedback circuits were built by placing scaffold-recruited pathway modulators under the control of pathway-inducible promoters. By implementing a competitive binding sink for the modulators, and using competitive, reciprocal expression of the positive and negative modulators, the authors dramatically changed wild-type input/output behavior. The otherwise graded, linear dose response was converted to a switch-like response, and the pathway's normally monotonic temporal response was converted to various temporal behaviors such as pulse generation and delayed activation. Using the yeast mating MAPK pathway as a core element, the authors generated the range of behaviors that MAPK pathways display in diverse cells and organisms. These results demonstrate the intrinsic flexibility of MAPK pathway signaling and offer a potentially generalizable approach for synthetically tuning the behavior of scaffolded signaling cascades. More generally, this work indicates that scaffolds can serve as loci for altering signal processing in a signaling cascade, and can be used to combinatorially specify pathway connectivity as promoters do for transcription.

Synthetic rewiring approaches can also be used to evaluate general questions about the robustness and evolvability of native networks. To evaluate the robustness of the native *Escherichia coli* transcriptional network, Isalan et al. (34) shuffled sequences coding for various



transcription factors and sigma factors against their corresponding promoters, creating a library of novel network linkages. Surprisingly, when introduced into the native network, these new linkages had little negative effect on fitness and caused only marginal changes in genome-wide transcription. Several library members actually showed enhanced fitness under certain selective conditions. These results suggest that the native *E. coli* transcriptional network has a high capacity to tolerate random evolutionary rewiring events that could potentially increase fitness under changing environmental conditions.

Elucidating Design Principles by Building Toy Functional Networks

A major focus of synthetic biology in recent years has been creating simple genetic networks that recapitulate fundamental information-processing tasks. Several classes of these so-called toy circuits have been constructed (reviewed in References 11 and 49), including circuits that produce gene expression oscillations, bistable switches that act as epigenetic memory devices (1, 27), circuits that perform combinatorial logic operations (18, 31, 59), and circuits that count cellular events (23). In addition to *E. coli*, which remains the primary test bed for this work, yeast and several types of mammalian cells have been used for toy circuit construction.

Toy system: model synthetic circuit built to recapitulate a particular type of natural network behavior

Whether toy systems tell us anything meaningful about natural systems is debatable. For example, what can a ring oscillator built from a daisy chain of repressor/operator interactions really teach us mechanistically about the highly regulated oscillating networks that mediate cellular circadian clocks (26)? If we are interested in understanding biology, should we not be studying real biological systems rather than engineered toy systems?

To answer this question, it is instructive to consider the engineering history of human-powered flight. Historical attempts to construct aircraft based on imitations of avian flight were failures. Flight was achieved once underlying mechanical forces were decomposed and understood through successive engineering attempts. The successful engineering platform of fixed-wing aircraft involved the decoupling of lift (provided by wings), thrust (provided by propellers), and control (provided by rudder and ailerons) in a way that is distinct from the integrated solution employed by birds flapping wings. This synthetic system facilitated a deeper

Figure 2

Synthetic rewiring experiments can be used to test predictions about the function and plasticity of cellular networks. (*a*) Understanding the basis for modular connectivity in cellular networks allows us to design hypothesis-driven rewiring experiments. (*b*) A circuit diagram representation of the model that Suel et al. (57, 58) used to describe the transition between sporulation and competence states in *Bacillus subtilis*. Two feedback loops control levels of the master transcriptional regulator, ComK: a positive autoregulatory loop (*purple*) and a ComS-mediated triple-negative (net negative) feedback loop (*green*). This architecture defines an excitatory circuit: Stochastic fluctuation in ComK levels can cause the basal state of the circuit (which specifies sporulation) to transition to an unstable, excitatory state (which specifies competence). The transition is controlled by the positive feedback loop, whereas return to the basal state is mediated by the negative loop. Rewiring the circuit to bypass the negative feedback loop resulted in cells that switched irreversibly to competence. The addition of negative feedback regulation to the positive autoregulatory loop resulted in faster recovery from competence back to the basal state as well as lower cell-to-cell variability in switching times. Plots are redrawn from figures in References 57 and 58. (*c*) The mitogen-activated protein kinase (MAPK) pathway that mediates mating in yeast displays a graded, linear response with respect to dose and simple, monotonic activation over time. The scaffold protein Ste5 specifies mating pathway connectivity by coordinating the kinase cascade. Bashor et al. (6) recruited positive and negative pathway modulators to the scaffold using synthetic protein-protein interaction domains. These modulators up- and downregulated pathway activity in a recruitment-dependent fashion. When placed under the control of pathway-responsive promoters, positive and negative feedback loops were created. When competitive interactions were used to create a sink for modulator binding, or to create competitive, reciprocal recruitment of a modulator to the scaffold, a number of complex input/output behaviors were achieved, including adaptive and activation-delayed temporal profiles, as well as a conversion of the dose-response profile for the circuit from graded to switch like. Data panels are taken from Bashor et al. (6). WT, wild type.

quantitative understanding of the principles of flight (resulting in modern airplanes) and also proved useful for understanding the more complex implementation of flight that has evolved in animals.

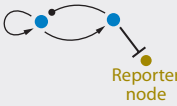
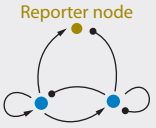
Thus, we argue that building toy systems is a highly complementary and useful way to systematically deconstruct underlying biological principles. Although the situational details of a specific circuit found within a biological network may be analyzed best by a careful reverse engineering study, toy circuit construction offers a way to identify the underlying design principles that allow different classes of circuits to be constructed from any type of cellular network (54). In addition, a toy circuit's bottom-up construction ensures full control over circuit design, enabling a systematic, comprehensive exploration of parameter space, as well as the ability to impose tests of the functional sufficiency of alternative circuit topologies.

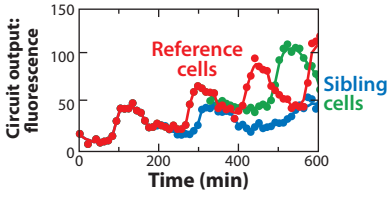
Tracing the progress in engineering genetic oscillatory circuits offers a clear example of how iterative engineering attempts can reveal important principles of circuit design. The first synthetic genetic oscillator was constructed in *E. coli* and was based on a triple-negative feedback ring design (20) (**Figure 3*a***). The behavior of this circuit, dubbed the repressilator, was damped, with oscillations persisting for no more than three periods. The circuit was also noisy—oscillatory behavior was observable in only a fraction of cells harboring the circuit, and tremendous variability was apparent in cells that did oscillate. Although this study represented a major milestone for synthetic biology, the noisy, unstable nature of the circuit's behavior should not have been surprising. Tsai et al. (63) computationally analyzed a number of different oscillator designs and demonstrated that designs consisting of only negative feedback linkages (like the repressilator) exhibit periodic oscillations within a narrow region of parameter space. Designs that feature opposing positive and negative feedback loops, as noted earlier by Barkai & Leibler (5), appear more robust to parameter perturbation. The authors conclude that a dual positive-negative feedback design is probably a better choice for biological systems. Such a design is more robust to noise, and the

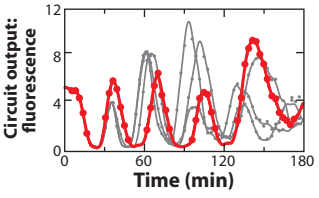
Figure 3

Using forward engineering to understand circuit design principles. Traditional reverse engineering of biological networks involves determining the structure-function relationship between one type of observed behavior and a single circuit architecture. Alternatively, a forward engineering approach may be employed in which a number of solutions that fulfill a given behavior can be evaluated either experimentally or computationally. Such an approach might illuminate basic design requirements and provide clues to achieve optimal behavior. (*a*) Progressive iteration of synthetic oscillator designs have varied in terms of architecture and implementation (type of molecules used to construct the circuit), but most designs consist of a set of transcriptional nodes that regulate each other. A reporter node that allows for the observation of circuit behavior is linked to one of the nodes in the circuit. The first oscillator design (repressilator) was a three-member ring network based on repressor-operator interactions (20). Subsequent circuits (3, 56, 61) utilized an interlinked positive and negative feedback design that was shown computationally to be more robust to parameter variation (63). The circuit constructed by Atkinson et al. (3) was transcription based, but it also utilized phosphorylation to mediate one branch of the feedback. The robust, stable oscillator constructed by Stricker et al. (56) was entirely transcription based. Quantitative modeling approaches that accompanied the designs were also variable. Repressilator modeling was simple, with numerous implicit assumptions. Whereas Atkinson et al. used a more rigorous approach for modeling, Stricker et al. used a fully parameterized model and recognized the importance of several key parameters. Time course data for single cell traces are redrawn from figures in References 20 and 63. The repressilator showed a high degree of cell-to-cell variability, as evidenced by the gradual decorrelation of sibling cells (*green and blue traces*) following septation from a reference cell (*red trace*). The oscillator developed by Stricker et al. showed rapid oscillations over multiple cycles (*red trace*) and strong synchronization among nearby cells descended from the same parent (*grey traces*).
(*b*) Combinatorially searching network space to define families of circuits that can achieve target functions is an alternative approach to learning about circuit design. Ma et al. (43) searched all possible three-node networks for topologies that exhibited perfect adaptation behavior (which was defined as a return to baseline after stimulus). The search identified ~400 robustly adapting circuits. All these networks mapped to two simple topology families that were sufficient to confer adaptive behavior: negative feedback loop with a buffering node (NFBLP) and an incoherent feed forward loop (IFFLP). These core topologies can be used for identifying possible perfect adaptation networks in natural systems and can serve as blueprints for building synthetic circuits.

a Designed biological oscillators

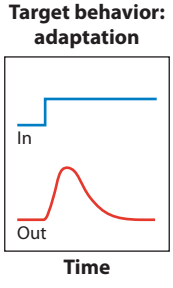
	First generation	Second generation	
	Neg feedback only	Interlinked positive and negative feedback	
Architecture	 Elowitz et al. 2002 (Reprissilator)	 Atkinson et al. 2003	 Stricker et al. 2008
Implementation	• Entirely transcription based	• Transcription and phosphorylation based	• Entirely transcription based
Modeling	• Simple modeling: many assumptions	• Detailed: most parameters taken into account	• Highly detailed model • Precisely tuned parameters
Circuit behavior	• Damped oscillation • Limited number of cycles • Significant cell-to-cell variation	• Damped oscillation • Limited number of cycles	• Stable, periodic oscillations; many cycles • Low cell-to-cell variation

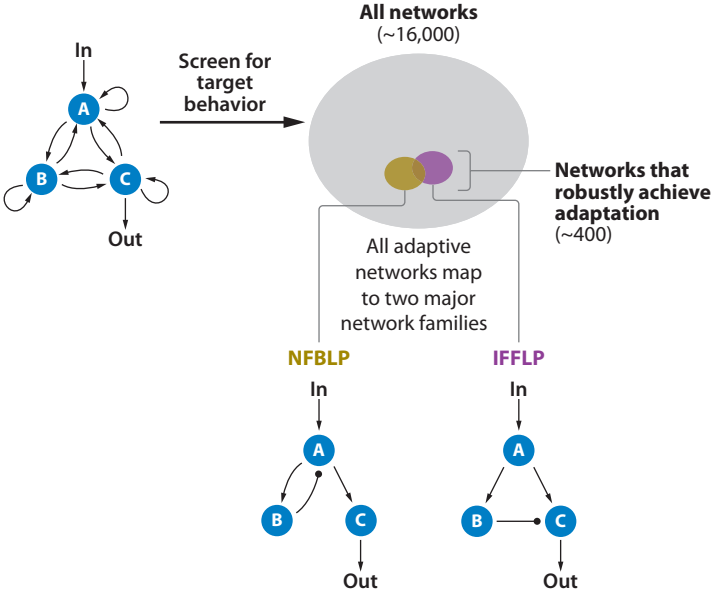




b Combinatorial engineering approach

- Map space of network architectures compatible with a particular function
- Computationally enumerate network architectures compatible with function
- Build synthetic circuit libraries and identify using selection experiments





period and amplitude of oscillations are independently tunable. A second *E. coli*–based circuit was subsequently built (3). As predicted, it was more stable but still only persisted for up to four periods. In the most recent example of an *E. coli*–based oscillator, Stricker et al. (56) used a variation of the design to realize stable, periodic oscillations that persisted over a wide range of parameter space. A key to their success—an approach that set them apart from previous studies—was the use of a fully descriptive quantitative model to guide their design. This helped them to realize the importance of the timescale of the negative feedback loop relative to that of the positive feedback loop in producing stable oscillations. It is interesting to note that several designs with the same basic architecture but with different molecular implementations have been built in recent years, demonstrating the generalizability of design principles to different systems. Although the oscillator designed by Sticker et al. was built from a purely transcriptional network, a more recent construction in mammalian cells utilized antisense RNA to mediate feedback (61), and yet another oscillator was constructed using transcriptional feedback combined with the enzymatic interconversion of a metabolite pool (25).

Searching Function Space by Combinatorial Network Design

Although toy systems have proven useful for understanding biological circuit design, the process of iterative tinkering is slow. One solution might be to explore network design space in a less biased fashion by using a combinatorial selection approach. Sampling many network toplogies and large areas of parameter space could enumerate circuit topologies that accomplish a certain target behavior (**Figure 3*b***). Ma et al. (43) recently adopted such an approach in silico by querying all possible three-node networks (~16,000) for perfect adaptation behavior. Of the networks that were identified, all shared one of two core topologies. Although these minimal core topologies were sufficient to achieve adaptation, additional linkages could widen the parameter space over which the circuits functioned, giving important insight into how the robustness of a circuit can be enhanced.

Is a selection-based, forward-engineering approach experimentally realistic? Promoter-shuffling experiments suggest that combinatorial approaches can be used to learn rules for promoter design (16, 19), and previously described studies involving the small-scale rearrangement of network modules (18, 26, 31) suggest that shuffling approaches can generate novel behaviors. However, the molecular biology required to construct large network libraries remains daunting. To even consider an experiment analogous to Ma et al.'s computational effort, two technical challenges will need to be addressed. The first is library construction. Combinatorial cloning of modular parts (e.g., promoters elements and protein fusions) could be used to generate a diverse range of constructs from an initial set of modules. The second challenge is devising a selection or screen to efficiently assay large libraries. High-throughput screening by flow cytometry or microscopy holds promise, but a selection would be ideal, allowing mixed clones to be tested. The ability to perform engineered network evolution would be powerful in reaching a deeper understanding of network design principles, and would serve as a guide for building synthetic systems optimized for specific behaviors.

EXPANDING THE TOOLKIT OF GENETIC PERTURBATIONS WITH SYNTHETIC BIOLOGY

Moving from an inventory-level understanding of cellular networks to a systems-level understanding can be assisted greatly by a set of tools that directly manipulates network structure and connectivity. Although traditional modes of inquiry are useful for connecting gene and/or protein function to a particular cellular phenotype, they are limited in their ability to decompose systems-level function (**Figure 4**). For example, classical reverse genetics is largely limited to conditional mutants and gene knockouts (deleting nodes), and chemical biology to

modulating the activity of proteins (breaking links). Synthetic biology, however, offers the investigator the ability to augment the network under investigation by rewiring native network linkages or creating entirely new ones.

As our ability to rewire cellular systems progresses, we can begin to view synthetic biology as a toolkit for biological discovery that can complement classical and chemical genetics. We could, for example, develop tools that create tunable or switchable linkages between target nodes. We could wire entire functional modules into systems—toy circuits could be used to drive custom regulatory programs. We could also use rewiring to create noninvasive reporters and novel genetic screens as tools for discovery. By continuing to decompose cellular systems into modular parts, we can systematically expand the synthetic toolkit with the eventual goal of performing rewiring experiments on the totality of cellular systems—to link nodes not only within diverse networks but also between different levels of the cellular hierarchy.

Synthetic Perturbations I: Creating New Means of External Modulation for Temporal or Spatial Control of Signaling

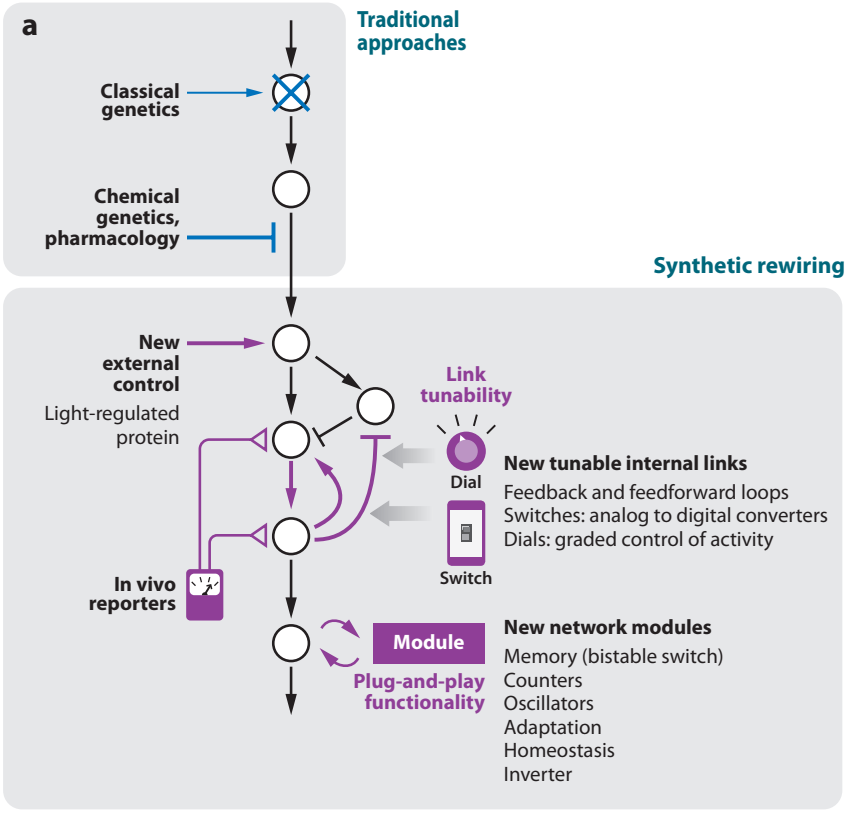
Building on the power of chemical biology and approaches such as small-molecule-induced dimerization, it may be possible to use synthetic biology to harness other classes of biological molecules to more finely control diverse target nodes. A powerful example is the engineering of light-controlled switches for molecular and cellular processes. In the field of neuroscience, light-inducible ion channels from microbes have been adapted to activate mammalian neurons on a millisecond timescale (10). Because ion channels represent the fastest signaling conduit available to biology, this burgeoning field of optogenetics has permitted researchers to manipulate patterns of neuron firing and affect behavior in living, freely moving systems (62).

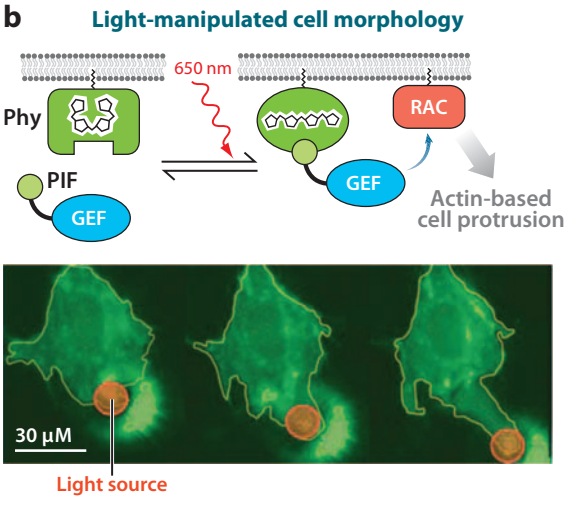
More recently, light-modulated interaction domains from plants have been exploited to mediate synthetic linkages in mammalian cells. Levskaya et al. (40) have adapted a plant phytochrome light-inducible dimerization system (53) as a noninvasive tool for the creation of network linkages with a high degree of temporal and spatial specificity (**Figure 4*b***). Wu et al. (65) have similarly adapted the plant LOV (light, oxygen, or voltage) domain, which undergoes a light-induced allosteric change, to switch protein activities on and off. This type of control is crucial when studying signaling processes that proceed at the rate of diffusion, and should be useful for interrogating membrane-localized events such as cellular polarization.

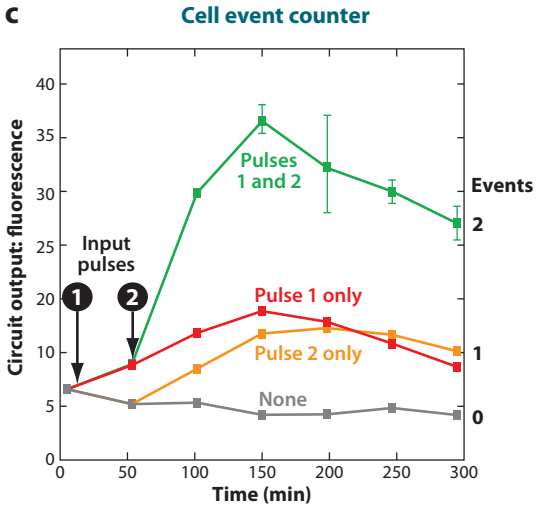
LOV: light, oxygen, or voltage

Synthetic Perturbations II: Creating New, Tunable Network Linkages

Rewiring of any cellular network is predicated on our understanding of the modular basis of its connectivity. For gene regulatory networks, we have a firm understanding of how to build and tune linkages in a precise way. However, other cellular networks offer more limited means of synthetic control. A number of synthetic biology studies have used modular protein-protein interaction domains to rewire signaling pathways and, more recently, to regulate metabolic flux in an engineered biosynthetic pathway (17). However, a comprehensive set of generic protein recruitment elements is not yet available. One of the primary challenges for creating new linkage modules in cells is designing them to be orthogonal to native networks (so they do not cross-react with native proteins). Historically, this has been overcome by using interaction modules that are heterologous to the networks being engineered and thus assumed to be orthogonal to native interactions. An alternative strategy is to computationally design interaction pairs that are orthogonal to native interaction networks. Recently, Grigoryan et al. (29) used a novel computational approach to engineering highly specific binding partners for human leucine zipper proteins. Their study demonstrated that the native network of human leucine zippers is undersampled relative to the potential interaction space available to them, suggesting that specific binding pairs that show







minimal cross-reactivity to the native network could be engineered.

One of the challenges of trying to link diverse regulatory network elements is the variety of molecular information currencies that exists inside the cell. In addition to gene expression (in which information is encoded by whether a gene product is expressed or not) and protein complex assembly (in which information is encoded by whether a complex is formed or not), currencies, posttranslational covalent modification currencies (e.g., phosphorylation and ubiquitination), and conformational currencies (e.g., GTPase switches) exist as well. Evolution has managed to create useful links between these different currencies. When attempting to engineer new linkages, the synthetic biologist is challenged to devise simple ways to similarly convert one currency into another (**Figure 5*a***).

It is worthwhile to examine the fundamental logic by which nature controls currencies such as phosphorylation. In general, the phosphorylation state of a target protein is controlled by a set of modular input enzymes: Kinases are writer enzymes that make phosphorylation marks, whereas phosphatases are eraser enzymes that remove the marks. Inputs control this event by modulating the activity of the writer and eraser. The output of phosphorylation can be controlled by direct changes to the target protein activity. However, in many cases reader modules—such as SH2 domains that bind to phosphotyrosine motifs—control output by facilitating the formation of a new complex. Most molecular information currencies are regulated by analogous modular writer/eraser/reader systems (**Figure 5*b***). For GTPases, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) act as writers and erasers that activate and deactivate the GTPase, respectively, whereas effector modules that recognize only the GTP-bound state of the GTPase act as reader modules.

Connecting arbitrary nodes within a natural network may involve linking distinct molecular currencies. In natural networks, linkages between currencies occur when a reader module of one currency regulates the writer or eraser modules of another currency (**Figure 5*c***). In one example of engineering such a connection, Yeh et al. (66) linked a GTPase triad to a kinase triad to create a novel linkage between two currency types. In their study, a synthetic, intramolecular interaction domain was used to allosterically regulate a GEF that modulates cell morphology in mammalian cells. By making the regulatory interaction responsive to protein kinase A (PKA) phosphorylation, the authors converted PKA activation into changes in cellular morphology.

What unexplored, reversible chemical currencies could be used for generating synthetic linkages? The attachment of ubiquitin to various protein targets may be one such system

GEF: guanine nucleotide exchange factor

GAP: GTPase-activating protein

PKA: protein kinase A

Figure 4

Synthetic biology offers an expanded set of research tools for making genetic perturbations in cells. (*a*) Traditional approaches for investigating cellular systems are limited in how they can investigate network structure-function relationships. Classical genetics makes mutations, which eliminate network nodes, whereas chemical biology primarily provides tools that disrupt network links by inhibiting protein function. Synthetic biology augments these approaches by providing a diverse set of research tools for the experimental perturbation of cellular networks. By co-opting the modular building blocks that are used to construct networks, synthetic biology allows an investigator to rewire a network with new linkages. These links can either be constitutive, precisely tunable (dials), or turned on and off in a controlled fashion (switches). By wiring new functional subsystems into networks, an investigator can introduce a genetically encoded functionality that can be used to alter network behavior. These functionalities include reporters that can be programmed to detect a variety of complex cellular events. (*b*) Phytochrome domains that dimerize upon light activation can be used to noninvasively direct highly localized protein-protein association events within a cell. Levskaya et al. (40) induced actin polymerization by the light-activated association of Rac and an activating GEF, resulting in localized cell protrusions. (*c*) A genetic circuit that counts events is one example of a type of network module that can be used to introduce engineered function into a cellular network. Friedland et al. (23) implemented a circuit that activates only after the occurrence of two consecutive stimulation events (data redrawn from Reference 23). Transcriptional output is given in arbitrary fluorescence units.

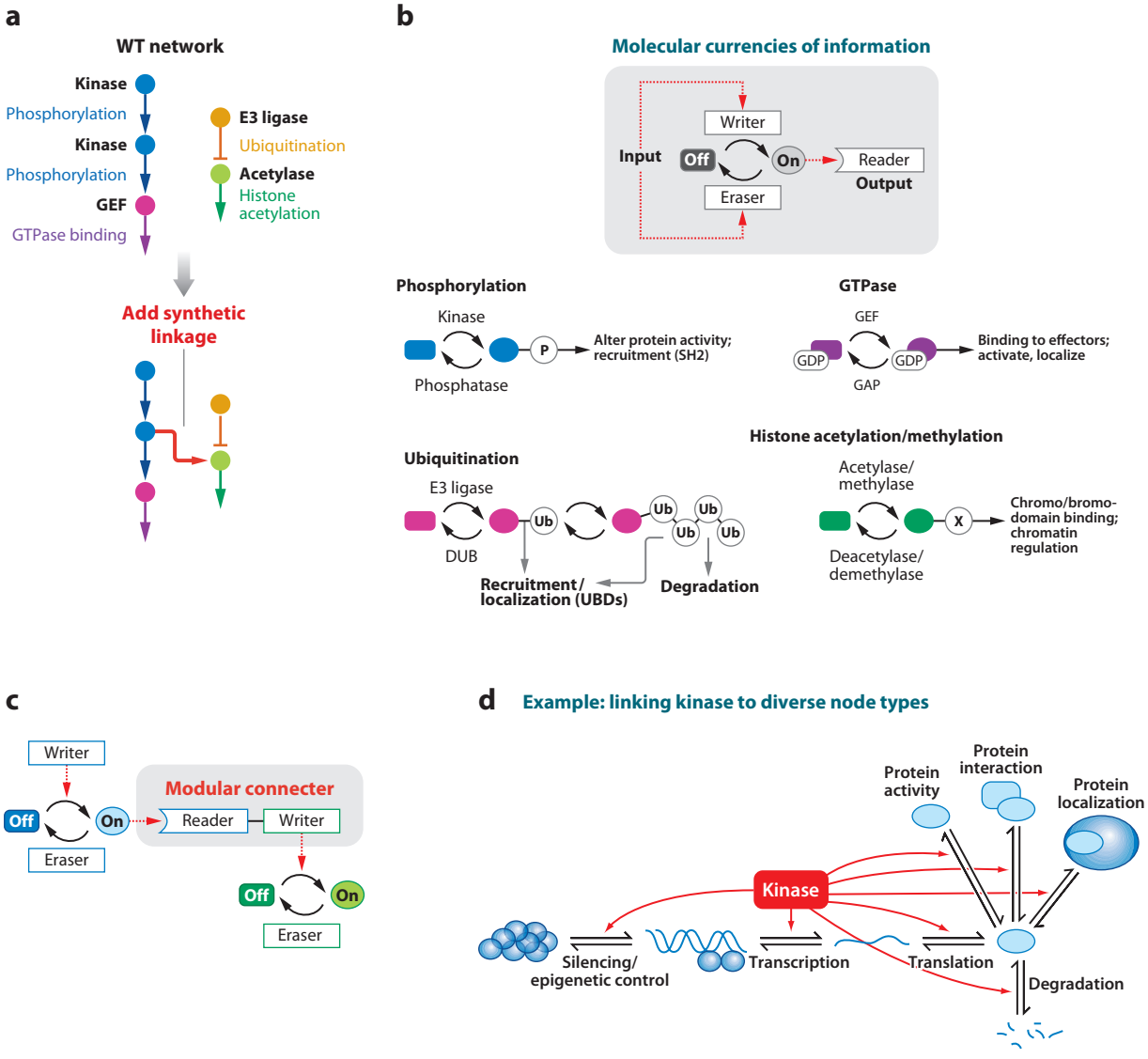
E3: ubiquitin ligase

UBD: ubiquitin binding domain

DUB: deubiquinating enzyme

to which the reader/writer/eraser paradigm can be applied (**Figure 5*b***). Ubiquitination tags are written to a protein target by ubiquitin (E3) ligases, read by a variety of ubiquitin binding domains (UBDs) (33), and erased by deubiquitinating (DUB) enzymes (22). Polyubiquitination is classically considered a signal that targets cellular proteins for proteasomal degradation. However, in many systems ubiquitin plays a recruitment and regulatory function beyond degradation. Monoubiquitination and alternative polyubiquitin linkages can specify altered cellular localization, mediation of kinase activity, and DNA damage repair (15). This diversity of outcomes and the inherent modularity of the enzymes involved suggest that control of ubiquitination would be a useful tool not only for synthetically controlling protein lifetime, but also for engineering new recruitment and regulatory interactions.

A second, yet unexploited currency is histone modification, which has remarkably



complex combinatorial potential (**Figure 5*b***). The histone tails displayed on nucleosomes act as a signaling hub for a range of chemical modifications, including methylation (lysine and arginine), acetylation (lysine), phosphorylation (serine), and ubiquitination (lysine). These marks are thought to comprise a histone code read by modular binding domains that recruit transcriptional and remodeling activities (55). For example, histone acetylation is read by bromodomains and is associated with active transcription, whereas methylation, read by chromodomains, can be repressive at some residues and is required for active transcription at others (60). Indeed, individual proteins and protein complexes that contain binding domains for multiple types of marks have been characterized, hinting at the existence of a combinatorial code (41, 55). Histone modifications are also reversible. This includes lysine methylation, for which no eraser was identified until the recent discovery of histone demethylases (52). In addition to its great combinatorial potential, the currency of histone modification is particularly interesting because it may encode epigenetic memory (see sidebar, Manipulating Chromatin to Obtain Engineering Control of Cellular Memory States).

Developing a more complete understanding of the modularity underlying various reversible cellular signaling currencies will expand the range of network manipulations available to synthetic biologists. Focusing on kinases, one can envision using phosphorylation to link an arbitrary target protein to a variety of distinct molecular processes (**Figure 5*d***). Using a

MANIPULATING CHROMATIN TO OBTAIN ENGINEERING CONTROL OF CELLULAR MEMORY STATES

Obtaining synthetic control over chromatin offers an opportunity to harness a new mode of gene regulation with unique potential advantages. First, the possibility exists for engineering cellular memory states. Although researchers have already engineered epigenetic states using synthetic transcriptional feedback control (1, 8, 27, 39), synthetic biology has largely overlooked faithful transmission of epigenetic marks through histone and DNA modifications, which leads to the persistent regulation of transcriptional states when these marks are read out by modular, chromatin-regulating enzymes (24). Second, heterochromatin acts regionally, instead of in a promoter-specific manner, so it should be possible to regulate blocks of genes in tandem. For example, a synthetic module composed of multiple genes in a metabolically engineered strain might be controlled coordinately. Third, heterochromatin acts in an all-or-none manner, with targeted genes entirely silenced or fully expressed. This binary behavior indicates a high degree of cooperativity and should make circuits robust over a wider range of parameters. Finally, heterochromatin is generally dominant over transcriptional activators. Thus, heterochromatin may provide a higher level of transcriptional control for the design of synthetic systems.

Figure 5

Harnessing the diversity of cellular information currencies to generate synthetic linkages in cellular networks. (*a*) The diversity of cellular information encoding currencies presents a challenge to the synthetic biologist who wants to engineer novel links into a network. (*b*) Many of the reversible reactions that are used as signaling currencies in posttranslational networks can be understood in terms of a reader/writer/eraser paradigm. Writers enzymatically catalyze the transfer of chemical marks onto target molecules, whereas erasers catalyze the removal of the chemical mark. Inputs to the node are used to control the writers and erasers. The presence of a mark is then read out by a reader module, which can come either in the form of an altered functionality, or as some type of binding partner that recognizes and binds to the molecule that bears the chemical mark. Reader/writer/eraser triads can be used to generate reversible linkages in a signaling network and are attractive targets for synthetic biology. Phosphorylation is the most familiar example of a chemical currency that conforms to the reader/writer/eraser paradigm. Kinases are responsible for transferring phosphates onto different types of cellular targets, whereas phosphatases act as erasers by dephosphorylating the targets. Readers for phosphate marks include phosphorylation-dependent binding partners. GTPases, ubiquitination, and histone modification follow the reader/writer/eraser paradigm as well. (*c*) Natural networks link nodes of different currencies by using modular connecter devices—devices that read in the output of the upstream currency and use it to control the input to a downstream currency. Making diverse modular connecter devices is a key goal in developing a synthetic biology toolkit. (*d*) Using phosphorylation as an example currency, we illustrate the range of downstream connections that could potentially be regulated by synthetic linkages. WT, wild type.

phosphopeptide recognition domain as a reader module, one might be able to build a synthetic E3 ligase that specifically ubiquitinates the target protein only in response to kinase activity, thereby leading to phosphoregulated proteasomal degradation of the target protein. Similarly, by altering the histone tail to create a novel phosphorylation motif, it may be possible to use the same kinase to write an orthogonal histone code.

Cre: cyclization/recombination

Lox: locus of X-ing over

FRET: fluorescence resonance energy transfer

ER: endoplasmic reticulum

Synthetic Perturbations III: Inserting Entire Modular Functional Blocks into Networks

Although the concept of wiring entire synthetic subsystems into living organisms may sound like science fiction, this goal has already been achieved in an important way. The Cre-Lox (cyclization/recombination–locus of X-ing over) system for conditional knockout of gene expression has become a crucial tool in mouse genetics and is a clear representation of a modular, synthetic subsystem (51). Cre-Lox knockouts allow a gene to be deleted in a site-specific or temporal manner, permitting the study of gene products that are required for development. A variety of strategies have been developed, but in short, the Cre recombinase from bacteriophage P1 is expressed from a tissue-specific or tissue-inducible promoter in a Cre-transgenic line. This mouse is then bred with a mouse with a *loxP* (the Cre recognition site) flanked gene of interest, resulting in double transgenic mice harboring a conditional or tissue-specific deletion for that gene. This system is a masterpiece of synthetic biology, incorporating a bacteriophage recombination module and a synthetic tetracycline-inducible or a heterologous tissue-specific promoter (50). In essence, this is an example of a modular toy subsystem built from bacterial parts that have been imported into mammals to achieve powerful and complex genetic control. One can envision a catalogue of other similar orthogonal modules that carry out distinct functions. Work by Kobayashi et al. (37) has already demonstrated that a genetic toggle device can be coupled to various input and output modules and used to introduce programmed phenotypes into *E. coli*. The authors of this study report one engineered strain that harbors a toggle circuit controlling the conversion of a transient induction of the SOS pathway to the induction of a sustained biofilm-forming state, while another strain couples quorum sensing to the expression of a target protein. In the future, it may be possible to use oscillators, filters, logic gates, and counters as portable sub-blocks of function to, for example, control cell differentiation programs in engineered cells and tissue (39), or to use an autoregulatory module to lower expression noise for a gene of interest (7).

Submodules could be envisioned to play a sophisticated reporter function. The approach of using modular recruitment domains to build in vivo fluorescence resonance energy transfer (FRET) sensors of various protein activities is already well established (67). But one can imagine an extension of this idea that incorporates whole, genetically encoded subsystems that play a reporter function. For example, a recently reported counter submodule (**Figure 4*c***) could be used to track the number of times an event of interest takes place, for example, cell divisions before senescence (23). Similarly, complex detectors that have filtering or logical functions could be used to report on specific combinatorial events.

Designing novel genetic screens using rewired components may also be a useful discovery tool. The yeast two-hybrid screen, one of the true workhorses of modern cell biology, was made possible by application of modular rewiring—a fusion of a bacterial repressor protein with a eukaryotic activator domain. Future applications could focus on using synthetic modules to screen for functional rescue of mutations. This type of approach, in principle, would allow for the identification of cellular factors that carry out complex endogenous functions, so long as that function could be encoded in a synthetic construct. In a recent example in yeast, a synthetic, chimeric protein tether linking the endoplasmic reticulum (ER) to mitochondria was used to screen for

complementation of mutants that were deficient in organelle fusion (38). Researchers identified a protein complex that connects the ER and mitochondria by using this synthetic screen approach.

CONCLUSIONS

In the future, we foresee a deepening of the relationship between synthetic biology and discovery biology: Advances in discovery biology can be viewed as grist for the biological engineer, while the organizational and functional principles uncovered using synthetic biology will inform and advance discovery. Thus, the flow of information between the two approaches is not unidirectional, but rather a state of cyclical positive feedback (**Figure 6**). Such a relationship is epitomized by a quote from the physicist Richard Feynman that has emerged as an informal slogan for synthetic biology: "What I cannot create, I do not understand." In the future, will we be able to harness this synergism to understand more complex aspects of cellular function? Areas of future impact for synthetic biology may include systems that determine three-dimensional cellular structure. Currently, we know little about how self-assembly processes give rise to cellular structure and intracellular organization. Synthetic biology could be used to engineer minimal systems governing cell polarization and movement, assembly of cellular substructures, or organelle formation. Another area of future impact for synthetic biology is engineering of synthetic microbial consortia. Engineering of simple intercellular communication relationships between microbes could be used to explore models of game theory and social behavior. Principles of systems rewiring could also be applied to control cell-cell communication to help identify molecular- and systems-level determinants for tissue differentiation and developmental patterning. In each of these potential areas, a close relationship between synthetic and discovery biology will lead to an increased understanding of the organization and manipulability of cellular systems and should promote the realization of new, useful ways to harness them as technology.

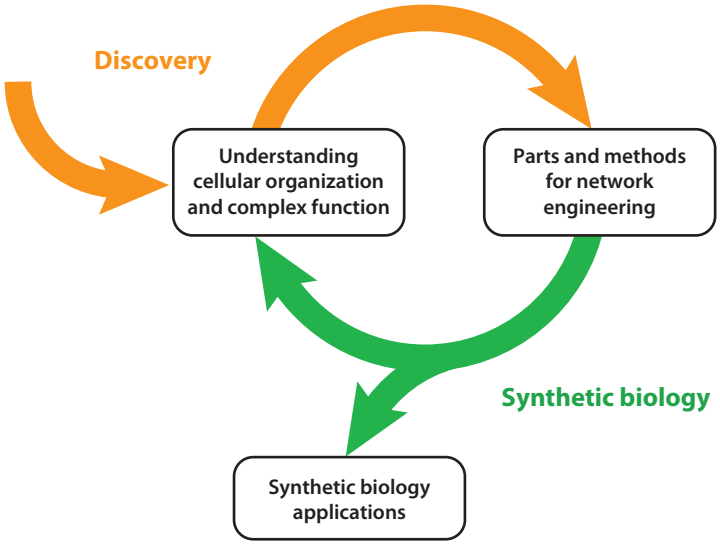


Figure 6

Complementarity of discovery and engineering approaches in reaching a deeper understanding of complex biological systems. Discovery biology generates the medium that synthetic biology appropriates for engineering purposes. In the process of creating useful applications and tools, synthetic biology uncovers principles of design and organization that improve our understanding of biological systems.

SUMMARY POINTS

1. Synthetic biology holds great promise as a discovery tool for cell biologists, allowing them to manipulate the structure of cellular networks.
2. Synthetic biology can be used to understand the complexity of biological organization by decomposing networks into the modules that comprise them.
3. Rewiring experiments can be used to query a network's function by altering its structure. The manipulation of network connectivity can be used to evaluate hypotheses.

4. Building synthetic toy networks to perform a target behavior can reveal the minimal requirements to achieve that behavior.
5. Combinatorial network design has the potential to be an efficient method of discovering functional network topologies. In contrast to a one-off design, a library of designs can provide additional insight into the relationship between circuit topology and robustness.
6. Synthetic biology can be envisioned as a toolkit that complements traditional genetic and chemical biology methods. The addition of tunable or switchable linkages as well as the use of plug-and-play functional modules permit the precise interrogation of cellular network function.

FUTURE ISSUES

1. Unbiased methods should be developed to generate and evaluate network structures (generate combinatorial libraries of networks). To realize this goal, technical challenges must be met, including the molecular biology required to generate diverse libraries and the development of high-throughput screening or selection methods to assay large numbers of constructs.
2. Synthetic biology will continue to adopt new signaling currencies. Ideal systems should include a writer (enzyme that catalyzes the chemical modification), a reader (binds the chemically modified substrate), and an eraser (removes the modification). Promising candidates include ubiquitination and histone modifications.
3. The toolkit for adding or modifying linkages to extant networks will continue to be expanded. Current work harnesses light as a signal to switch modular domains or ion channels. These devices will allow interrogation of signaling systems on a timescale that is informative with respect to diffusible signals.
4. Synthetic biology approaches will be extended to new types of biological networks including those that regulate cell shape, assembly of intracellular structure, cell-cell adhesion and communication, developmental regulation and behavior in microbial consortia.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Errata

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