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Rewiring Cells: Synthetic biology as a tool to interrogate the organizational principles of living systems

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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 perturbing them through genetic or chemical means. The emerging discipline of synthetic biology offers an additional, powerful way 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. Additionally, by building minimal "toy" networks, one can systematically explore the relationship between network space (linkages and parameters) and functional space (the system's physiological behavior). 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.

I. 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 based on 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 and biomass conversion to the reprogramming of human cells for therapeutic purposes. In practice, synthetic biology consists of co-opting molecular "parts" from natural systems and using them to construct new networks that fulfill specific design goals. These artificial networks can range from gene regulatory circuitry designed to precisely control the expression patterns of genes, to new metabolic systems that produce useful metoabolites.

While 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, although we are getting close to assembling a complete parts list of the molecules in a living cell, 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 expect to build engineered cells? Would it not be better to first understand the cell, then try to engineer it?

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We argue here that in addition to its applications, synthetic biology is and will become an increasingly powerful discovery tool for understanding the organization of cells and other complex biological systems. At the current stage of intellectual development in biology, we have extensive knowledge of molecular parts and components, but little knowledge of functional organization and principles. Thus, it can be incredibly valuable, and perhaps even necessary, to use a "learning by building" approach. Working in partnership with systems biology and other traditional methods of cell biology research, synthetic biology can be used to evaluate sophisticated hypotheses on how complex behavioral phenotypes arise from cellular network structure. For example, by functionally testing putative parts in engineered systems, we can identify modular functional units, and uncover the logic of how units can be linked together in hierarchical fashion to yield new function. Synthetic biology has already had early success in engineering simple regulatory circuits that recapitulate some of the behaviors of natural circuits (13). In the future, it should be possible to use an engineering approach to systematically identify multiple, alternative topologies for biological circuits, and quantitatively compare their performance. This type of analysis might shed light on the fundamental principles of how evolution chooses a network design to fulfill specific functional needs. Ultimately, obtaining engineering control over a broad range of cellular functions will provide an experimental toolkit that augments the traditional discovery tools of cell biology. In our estimation, using synthetic biology approaches to rewire biological networks will become a standard investigative approach.

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

One of the most fundamental issues in biology is how complexity is organized, and how that organization changes during the process of evolution. The concept of **hierarchical modularity** has provided a usefully framework for understanding this organization. Systems are considered modular if the parts that comprise 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 cellular systems in terms of a hierarchy of interlinked, functional modules is one useful way to parse complexity into parts that are more easily understood. Thus, one can argue that understanding a complex entity like a cell will rely on our ability to decompose it into its most important hierarchical modules and describe the relationship of these modules to one another.

Another reason to understand the modular organization of biological systems is to gain insight into the process of evolution. Many people have postulated that modularity might itself be an adaptive trait that improves the **evolvability** of biological systems by facilitating the rapid reconfiguration of network structure (42,43,52). In other words, modularity may be adaptive because the reconnection of modules provides a simple system with the ability to rapidly generate functional diversity in the face of continually changing selective pressures. Therefore, the identification of functional modules, and learning the extent to which they can be rewired can provide insight into evolutionary process.

Synthetic biology is a powerful approach for investigating a cell's modular organization. By attempting to identify a part or subsystem that can be used to construct new networks, 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 placed in a synthetic network. By the same token, if the modular protocol for creating connectivity within a network is well understood, then it should be possible to use that knowledge to introduce diverse new linkages. Below we describe how efforts to rewire cells have helped to functionally identify the modular building blocks of

several types of regulatory networks, and lent general support to the idea that modularity promotes phenotypic diversification.

Recombining Gene Expression Modules

The organization of genetic regulatory networks offers perhaps the clearest example of hierarchical modularity in a cellular system (figure 1a). Nodes in genetic networks (genes) are composed of regulatory regions (promoters) and protein coding regions. Network linkages are defined when *cis*-acting factors 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 linked together in frequently reused, modular patterns of connectivity known as motifs (2). Motifs also have defined input and output properties, as each class performs specific information processing functions (52). The higher order behavioral complexity of genetic networks is thought to derive from the interaction of different types of motifs (51).

A considerable body of work has experimentally demonstrated the high degree of modularity in gene transcriptional control. Much of this work predates the recognition of synthetic biology as a formal discipline, and occurred at a time when there was little formal knowledge on how gene regulatory networks worked. (56). The establishment of systems for the heterologous expression of proteins (35) was a key demonstration of the universal modularity of gene structure that exists across species boundaries. The early use of chimeric transcriptional activators to regulate chimeric gene modules consisting of eukaryotic coding regions and bacterial regulatory elements (15,16) served as demonstration of this principle, and formed the basis for the yeast-two-hybrid screen (24).

More recent studies into the modularity of gene structure--motivated by the desire to understand transcriptional regulation in a precisely quantitative way for the purpose of genetic circuit engineering--have explored the effects of modular reshuffling of promoter architecture. In one study, Cox et al. used a library of promoter elements to combinatorially vary the placement, number, and affinity of operator sites (20) in a promoter regulated by two repressors (two-input regulation). Despite focusing on very simple changes in promoter architecture, the library generated expression strengths over a broad dynamic range, and showed a variety of logic gating properties for the two inputs. This demonstrated that simple, modular variations in a promoter can be used to generate a breadth of regulatory diversity, allowing for tuning both the strength of genetic network linkages and how it acts on a node.

An understanding of the how variations in genetic network connectivity can be generated opens a path to understanding how evolution may have shaped network structure. The high degree of apparent modularity in gene network architecture, and the assumed plasticity of *cis* regulatory elements between different promoter regions have led to the hypothesis that changes in *cis* regulatory elements have played a major role in the evolution of phenotypic diversity. For example, alterations to promoter structure have been proposed as the primary mechanism for anatomical diversity in the animal kingdom (17,32,55). How an evolutionarily important recombination event might have occurred is difficult to ascertain based on phylogenetic evidence alone. However, there is experimental evidence suggesting that the shuffling of promoters and coding regions can be used to generate a surprising assortment of regulatory diversity. In a study conducted by Guet et al. (36), a combinatorial approach was used to shuffle various promoter and coding elements into a library of three-node networks. When the library constituents were analyzed, it was found that the resulting motifs exhibited a variety of gating properties, and were able to achieve simple computational functions. These results hint at the potential for regulatory and coding regions to generating behavioral diversity via the simplest rearrangements of promoter structure.

Recombining Signaling Modules

Protein signaling networks mediate the processing of external signals and, like gene regulatory networks, have evolved modular network structures (figure 1b). Proteins found in signaling networks are made up of multiple, independently-folding domains (10), at least one of which carries out a catalytic function (e.g. a kinase domain, which transfers a phosphate to a target protein). Additionally, signaling proteins typically contain one or more protein-protein interaction domains that specify the interaction of the catalytic domain with its target or play a regulatory role (e.g. through autoinhibition). Thus, for a functional module in a protein signaling network, input is defined by the interaction of the regulatory domain with an interaction partner, while output is defined by the activity of the catalytic domain. However, a notable difference between nodes for protein signaling networks compared with genetic networks is the diversity by which input can be regulated. For example, a regulatory domain can modulate a signaling protein's catalytic output in *cis*, by intramolecular auto-regulation of catalytic function. Input, which can come in the form of a binding event or a chemical modification (e.g. a phosphorylation event), can either switch on catalytic function or switches it off. Regulatory domains can also modulate 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 specific subcellular region where it can create the necessary proximity for interaction with a specific upstream or downstream target.. In that sense, modular recruitment can potentially specify both the input and the output for a signaling protein. Recruitment also plays a notable role in organizing higher-order assemblies of signaling proteins. Adaptors and scaffolds are proteins that are comprised entirely of regulatory domains that co-localize proteins into complexes.

N-WASP, a switch protein with actin polymerizing activity, is one notably well-studied example of a protein that displays modular autoregulation (48). N-WASP activation occurs when Cdc42 and the phospholipid PIP2 bind to N-WASP and abrogate autoinhibition. As both inputs are required for N-WASP activation, the protein effectively acts as an AND-gate (54). In order to demonstrate the modularity of N-WASP regulation, Dueber et al. (21) replaced the native N-WASP regulatory domains with heterologous regulatory domains and demonstrated that activity could then be gated by heterologous inputs. By varying the architecture of synthetic switch construction, a small library of synthetic switches was created which displayed surprisingly complex signal integration, recapitulating the native AND-gate behavior, but also demonstrating other types of gating. This work shows that domain-mediated autoregulation of catalytic activity can be entirely modular—gating function can be decoupled entirely from catalytic activity. The ease with which N-WASP could be synthetically gated suggests an easily generalizable mechanisms for imposing control on unregulated proteins. As is the case with genes, groups of signaling proteins are often found organized into characteristic motifs that are key to signaling network information processing function. Linear cascades are a common motif for kinase signaling pathways, while feedback regulation is also common. Regulatory domains often mediate the connectivity of signaling pathways, linking upstream motifs (eg. a G-protein switch) with downstream ones (eg. a kinase cascade). A number of important studies have tested the modularity of pathway connectivity by demonstrating the ease with which manipulation of regulatory domain-mediated recruitment can be used to dramatically change the input/output relationship of a signaling response. In one study (38), a single domain rearrangement in an adaptor protein 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 pathway connectivity. For example, yeast MAPK signaling cascade connectivity was altered using synthetic scaffold chimeras. By constructing the scaffolds to bind components of both the mating and osmolarity response MAPK pathways, the authors demonstrated the ability to re-route signaling input from one MAPK pathway into the output of the other (37). 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 (53).

As is the case for transcriptional networks, the modular architecture of post-translational signaling components may have contributed to the evolutionary diversification of signaling network architecture. However, the question remains as to whether domain rearrangements play the same role in evolutionary diversification in protein networks as do the shuffling of cis-regulatory elements in genetic networks. Peisajovich et al. (REF) recently addressed this question using a library of chimeric N- and C-terminal domain fusions created from various components of the yeast mating pathway. When the library of chimeras was expressed, significant alterations to signaling occurred, while pathway response was essentially robust to over-expression of the constituent proteins, as well as to expression of individual domains. These results suggest the potency of domain recombination as a mechanism to alter phenotype in protein signaling networks.

III. Using synthetic biology to perturb and probe network mechanism

In modern biology, it is increasingly common to apply the approaches of systems and computational biology to generate a model that can explain the behavior of a complex molecular regulatory 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 biology approaches to change the links and parameters of the network, thus allowing one to identify the key properties of a network that are critical for function, as well as its inherent robustness and fragilities. Instead of simply analyzing one network architecture and parameter set, this approach offers the possibility of fuller understanding of the relationship by which network properties map to functional behavior. This approach should not only enhance our understanding of the principles governing network function, but it is likely to also prove critical for network medicine – understanding how pathogenic perturbations rewire and disrupt function and how therapeutic intervention can be use to guide the network back to a functional state. Thus, overall, synthetic biology can be seen as the partner of systems biology in dissecting network mechanism.

Tinkering to probe mechanism and explore plasticity & robustness

For genetic networks, rewiring experiments have proven to be a useful way to quantitatively test predictions about the relationship between network structure and behavior (figure 2a). A recent pair of studies (64, 65) examining a genetic circuit that regulates the stochastic switching between states of competence and vegetative growth in *B. subtilis* serves as prime example of this type of approach (figure 2b). In the study, the authors measured circuit dynamics using fluorescent transcriptional reporters, and developed a quantitative model in which cells transiently pass through competence 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, while decay of the excited state (exit from competence) is regulated by an opposing negative feedback pathway. 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 author's model: adding in an additional positive feedback loop that bypassed the negative feedback loop (postulated to drive exit from competence) caused cells to be permanently locked into competence, while adding an additional negative feedback loop, by contrast, 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 a scaffolded yeast MAP kinase pathway (figure 2c). 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 were able to dramatically reshape the otherwise graded, linear mating pathway response into various non-linear behaviors like acceleration, pulse generation, delayed regulation, and ultrasensitive dose response. In essence, using the yeast mating MAP kinase pathway as a core element, the authors were able to generate the range of behaviors that MAP kinase pathways in diverse cells and organisms can show. These results demonstrate the intrinsic flexibility of mating MAP kinase pathway signaling and offer a potentially generalizable approach for synthetically tuning 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 *E. coli* transcriptional network, Isalan et al. (40) 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 very little negative effect on fitness, and caused only marginal changes in genome-wide transcription. Several library members actually enhanced fitness under certain selective conditions. These results suggest that the native *E. coli* transcriptional network has a high evolutionary capacity to tolerate random 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 on the creation of simple genetic networks that recapitulate fundamental information processing tasks. Several classes of these so-called “toy” circuits have been constructed (reviewed in (13,57)), including circuits that produce gene expression oscillations, bistable switches that act as epigenetic memory devices (1,31), circuits that perform combinatorial logic operations (21,36,66), and circuits that count cellular events (26). 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.

Questions have been raised as to whether toy systems tell us anything meaningful about natural systems. 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 (30)? 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 perhaps instructive to consider the engineering of man-powered flight (see **box 1**. Understanding principles of flight through engineering). Historical attempts to construct aircraft based on imitations of avian flight were failures. Flight was only achieved once underlying mechanical forces were decomposed and understood through successive engineering attempts; Sir George Cayley, Father of Aeronautics was the first to separate the forces of lift and drag, and was able to build a functioning glider based on this insight (18). The successful engineering platform of fixed-wing aircraft required the decoupling of lift (provided by wings), propulsion (provided by propellers) and control (provided by rudder and ailerons), in a way that is extremely distinct from the integrated way in which birds solve these problems. It can be argued that this artificial “synthetic” system facilitated a deeper quantitative understanding of the principles of flight, and became useful for understanding the more complex implementation of flight in animals (in addition to the direct utility of the airplane).

Thus, we argue that building toy systems is a highly complementary and useful way to systematically explore underlying biological principles. While the situational details of a

specific biological network may be best analyzed by a careful reverse engineering study (absolutely necessary for purposes such as understanding how a perturbation leads to disease), toy circuit construction offers a way to identify the underlying design principles that allow different varieties of circuits to be constructed from any type of cellular network (61). Additionally, a toy circuit's bottom-up construction ensures full control over circuit design--this enables a systematic, comprehensive exploration of parameter space, as well as the ability to impose tests of the functional sufficiency of alternative circuit topologies (3). While it is also useful to apply comparative genomics approaches to enumerate different types of orthologous network topologies and to use network conservation to extract what elements of a network are functionally important, this approach involves intrinsic speculation regarding similar fitness pressures on these different organisms. Moreover, network comparisons across species can be challenging because of the many simultaneous genetic changes occurring. Building toy systems and systematically changing them offers a more concrete way to test the merits of alternative designs by observing their performance upon exposure to various types of experimentally imposed challenges.

Tracing the progress in engineering genetic ***oscillatory circuits*** offers a cogent example of how iterative engineering attempts can reveal important circuit principles. The first synthetic genetic oscillator was constructed in *E. coli*, and was based on a triple-negative feedback ring design (figure 3). The behavior of this circuit, dubbed the repressilator, was damped, with oscillations persisting for no more than three periods. The circuit was also very noisy—oscillatory behavior was observable in only a fraction of cells harboring the circuit, and tremendous variability was apparent in cells that did oscillate. While this study represented a major milestone for synthetic biology, the noisy, unstable nature of the circuit's behavior should have been unsurprising, as Tsai et al. (70) computationally analyzed a number of different oscillator designs and demonstrated that designs consisting of only negative feedback linkages (like the repressilator) exhibit periodic oscillations over a very narrow region of parameter space. Designs that feature opposing positive and negative feedback loops, as pointed out earlier by Barkai and Leibler (5), appear more robust to parameter perturbation. The authors conclude that a dual positive-negative design is probably a better choice for biological systems. It is more likely to be robust to noise, and the period and amplitude of oscillations are more easily tunable by independent parameter alteration. A second *E. coli*-based circuit construction based on this design was subsequently built (4). It was more stable, but still only persisted for up to five periods. In the most recent example of an *E. coli*-based oscillator, Stricker et al. (63) 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 the previous studies--was the use of a fully descriptive quantitative model to guide their design. This helped them to realize importance of the timescale of 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. While the oscillator designed by Sticker et al. was built from a purely transcriptional network, a more recent construction in mammalian cells utilized an antisense RNA to mediate feedback (68), while yet another oscillator was constructed using transcriptional feedback combined with the enzymatic interconversion of a metabolite pool (29).

Searching function space by combinatorial network design

While 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 more non-biased fashion using a combinatorial selection approach. Such an approach could be used to sample many network toplogies and large areas of parameter space to identify sets of networks that support target behaviors. Such an analysis could be used to enumerate and

compare families of circuit architectures, as well as identify core design requirements for a given class of behavior (figure 4a). Ma and colleagues recently adopted such an approach *in silico* (50) by querying all possible three-node networks (~16,000) for perfect adaptation behavior (figure 4b). Of the topologies that were identified, all shared one of two core topologies. While these minimal core topologies were sufficient to achieve adaptation, it was determined that 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 the experimental implementation of a selection-based, forward engineering approach realistic? Previously mentioned examples hint at the promise of modular recombination strategies as a useful way to learn about design. The promoter shuffling approaches described earlier suggest the possibility of using a combinatorial approach to learn heuristic rules for promoter design (20,22), and the shuffling of network modules to generate new behaviors seems plausible (21,30,36). However, the molecular biology required to actually construct network libraries remains daunting. To even consider an experimental analog to Ma and colleagues' computational effort, several technical details will need to be addressed. The first challenge is library construction. Combinatorial cloning of modular parts (promoters elements, protein fusions etc.) could be used to generate a diverse range of constructs from an initial set of modules. A second technical challenge, especially for larger libraries, will be devising a selection or screen to efficiently assay the library. High throughput screening by flow cytometry or microscopy hold promise, but a selection would be ideal, allowing for the assaying of mixed clones. The ability to perform engineered network evolution, however, would clearly be powerful in reaching a deeper understanding of network design principles, as well as in providing a way to build synthetic systems optimized for specific functional applications.

IV. EXPANDING THE TOOLKIT OF GENETIC PERTURBATIONS WITH SYNTHETIC BIOLOGY

Moving from an inventory-level understanding of cellular biology to a systems-level understanding can be greatly assisted by a set of tools that directly manipulate cellular networks using the modules from which they are constructed. Though traditional modes of inquiry have proven useful for connecting gene and/or protein function to a particular cellular phenotype (and vice versa), they are limited in their ability to decompose systems-level function (figure 5). For example, classical reverse genetics is limited to conditional mutants and gene knockouts (deleting nodes), and chemical biology, to modulating the activity of proteins (breaking links). Synthetic biology, on the other hand, offers the investigator the ability to augment the network under investigation by rewiring old network linkages, or creating entirely new ones. As our ability to rewire cellular systems progresses, we can begin to view synthetic biology as a comprehensive 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 non-invasive 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 in mind of being able to perform rewiring experiments on the totality of cellular systems—not only to link nodes within diverse networks, but also to make linkages 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 approaches 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 time scale (12). Since 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 (69). More recently, light-modulated interaction domains from plants have been exploited in several ways to mediate synthetic linkages in mammalian cells. Levskaya et al. (2009) have adapted a plant phytochrome light-inducible dimerization system (60) as a non-invasive tool for the creation of network linkages with a high degree of temporal and spatial specificity. Wu et al. (71) have similarly adapted the plant LOV domain, which undergoes a light-induced allosteric change, to switch protein activities on and off. This type of control is absolutely crucial when studying signaling processes that proceed at the rate of diffusion, and should be especially useful for interrogating membrane localized events such as cellular polarization.

Synthetic perturbations II: creating new, tunable linkages in networks

Rewiring any type of cellular network is predicated on our ability to understand the modular basis of its connectivity. For gene regulatory networks, we have a firm understanding of how to build linkages and tune them in a precise way. Presently, other types of cellular networks offer more limited means of synthetic control. Modular protein-protein interaction domains have been used in a number of synthetic biology studies to rewire signaling proteins of various types, but a comprehensive set of generic protein recruitment elements is not yet available. Building toolkits of interaction modules that encompass different classes of binding (as well as tunable affinities) might be a useful future goal. One of the primary challenges for creating new linkage modules in cells is being able to make them in a way that is orthogonal to native networks (i.e. so that they do not inadvertently cross react with native proteins). Historically, this has been overcome by using interaction modules that are essentially heterologous to the networks being engineered, with the implicit assumption that such heterologous interactions are more likely to be orthogonal to native interactions. Another strategy is to computationally design interaction pairs that are orthogonal to native pairs. Recently Grigoryan et al. developed a computational approach to the engineering of synthetic leucine zipper interaction networks. Using a combination of experimental and computational approaches, the authors were able to demonstrate that the native network of human leucine zippers is undersampled relative to the possible interaction space available to them, and that specific binding partners could be engineered that showed minimal cross reactivity (34).

One of the challenges of trying to engineer and link together diverse elements in a cell's regulatory network is the diversity of molecular "currencies" that exist inside the cell. In addition to the currency of gene expression (information is encoded by whether a gene product is expressed or not expressed) or of protein complex assembly (information is encoded by whether a complex is formed or not), there are currencies such as post-translational covalent modifications (phosphorylation, ubiquitinylation, acetylation, etc.) and conformational currencies (e.g. GTPases). Evolution has managed to create useful links between these different currencies. A challenge for the synthetic biologist when attempting to make designed network linkages is to devise simple ways to similarly "convert" one currency into another, (figure 6a).

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, while 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 of the target protein activity. However, in many cases there are generic "reader" modules – like SH2 domains that bind to phospho-tyrosine motifs – that control output by facilitating the formation of a new complex. Nearly all molecular information currencies are regulated by analogous modular writer/eraser/reader systems (figure 6b). For example for GTPases, guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) act as writers and erasers that activate or deactivate the GTPase, while effector modules that recognize only the GTP bound state of the GTPase act as reader modules. Connections between nodes in a regulatory network are largely made when the reader module of an upstream node regulates the writer or eraser modules of a downstream node (figure 6c). The modularity of both kinase and GTPase triads has already been exploited, in a limited way, to create new linkages in cellular networks (72). In these cases modular reader domains were used to regulate downstream kinase or GEF domains, either through recruitment or through modular autoinhibition.

If a synthetic biologist wants to connect arbitrary nodes within a natural network, it will often involve linking distinct molecular currencies. In natural networks that involve multiple molecular information currencies, conversion between currencies occurs when a reader module of one currency regulates the writer or eraser modules of another currency. Thus, in principle, a synthetic biologist could achieve novel connections by linking the output (reader) modules of one currency to the input (writer and eraser) modules of another currency. To achieve this goal, we need to understand the mechanisms used to regulate diverse molecular currencies.

What are some previously unexplored, reversible chemical currencies that could be used for generating new linkages? The reversible, enzyme catalyzed attachment of ubiquitin to various protein targets may be one such system to which the reader/writer/eraser paradigm can be applied (figure 6b). Ubiquitination tags are written to a protein target by E3 ligases, are read by a variety of ubiquitin binding domains (UBD's) (39), and can be erased by de-ubiqutination (DUB) enzymes (25). Polyubiquitination of a protein is classically considered a signal that targets cellular proteins for proteasomal degradation. However, in many systems, ubiquitin also plays a recruitment and regulatory function beyond degradation. Monoubiquitination and alternative polyubiquitin linkages specify for a variety of non-degradation outcomes, including altered cellular localization, mediation of kinase activity, and DNA damage repair (19). 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 controlling protein lifetime, but also for introducing novel recruitment interactions.

A second, yet unexploited currency is histone modification, which may be the most complex currency in the cell in terms of combinatorial potential. 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 tocomprise a "histone" code (62) read by modular binding domains that recruit transcriptional and remodeling activities. For example, histone acetylation is read by bromodomains and is associated with active transcription, while methylation, read by chromodomains, can be repressive at some residues and is required for active transcription at others (67). Indeed, individual proteins and protein complexes have been characterized that contain binding domains for multiple types of marks, hinting at the existence of a combinatorial code (47,62). Histone modifications are also reversible, including lysine methylation, for which no eraser was identified until the recent discovery of histone demethylases (59). In addition to its great combinatorial potential, the currency of histone modification is particularly interesting since it may encode epigenetic memory. Histone deacetylation and histone methylation (of specific residues) are both associated with persistent transcriptional repression, and though interactions with DNA methylation, likley contribute to an epigenetic lock on the expression of silenced genes (28) [**BOX 2: Chromatin**].

Developing a more complete understanding of the modularity underlying various types of reversible signaling currencies will allow us to diversify the types of connections we can make. Eventually, it may be possible to create linkages within and amongst the majority of cellular network types. For example, if one focuses on protein phosphorylation, one would ideally like to be able to use the output of phosphorylation by a specific kinase to regulate arbitrary target proteins using a diversity of downstream events, involving distinct molecular processes (figure 6d). For example, using a phospho-peptide recognition domain as a "reader" module, one might be able to use this interaction to build a synthetic E3 ligase (ubiquitinating enzyme) that will specifically ubiquitinate the target protein only in response to kinase activity, thereby leading to phospho-regulated proteasomal degradation of the target protein. Similarly, it may be possible to use the same phospho-recognition "reader" module to control recruitment of specific chromatin modifying enzymes to a particular DNA binding complex. In this way one could potentially engineer epigenetic changes in gene expression in response to phosphorylation. These examples are only a few of the types of novel connections that could be precisely engineered with a better understanding of regulatory input and output modules and their connectability. With such an expanded toolkit of "connection" elements, our ability to explore mechanistic questions in network biology will be dramatically enhanced.

Synthetic Perturbations III: Inserting entire modular functional blocks into networks

Although the concept of wiring entire synthetic subsystems into living organisms (figure 5) may sound somewhat like science fiction, this goal has already been achieved in an important way. The Cre-Lox system for conditional knockout of gene expression has become a crucial tool in mouse genetics, and clearly represents what can be done with a synthetic biological modular subsystem. Cre-Lox knockouts allow the deletion of a gene in a site-specific or temporal manner, permitting study of 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 inducible promoter in a Cre-transgenic line. This mouse is then bred to a mouse with a loxP (the Cre recognition site) flanked gene of interest. The resulting double transgenic mice are deleted for the gene of interest in a tissue specific or a conditional manner. This system is masterpiece of synthetic biology, incorporating a bacteriophage recombination module and a synthetic tetracycline inducible or a heterologous tissue specific promoter (58). In essence, this is an example of a modular toy subsystem built from bacterial parts that has been ported 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 (44) has already demonstrated that a genetic toggle device can be coupled various input and output modules and used to introduce programmed phenotypes in to E. coli. The authors report one engineered strain that harbors a toggle circuit controlling the conversion of a transient induction of the SOS pathway into 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 that are already being developed, as portable sub-blocks of function to control far more complex systems. Today's toy systems may have incredible potential applications in the near future that include using toggle devices to encode cellular memory in a variety of cell and tissue types, and using induced switching to control cell differentiation programs (46). An auto-regulatory module could be utilized to lower expression noise for a gene of interest (8).

It is also possible to envision submodules that play a sophisticated reporter function. The approach of using modular recruitment domains to build *in vivo* FRET sensors of various protein activities is already well established (74). But one can imagine an extension of this idea which incorporates whole genetically- encoded subsystems that play a reporter function. For example, it may be possible to use the recently reported counter to track the number of events

of interest that take place which could then be used to control triggering of some response (26). Similarly complex detectors that have filtering or logical functions could be used to report on very specific combinatorial events.

Designing novel genetic screening tools using rewired components may also be a useful discovery tool. It is worth noting that the yeast-two-hybrid screen, one of the true workhorses of modern cell biology, was made possible by application of modular rewiring—fusion of a bacterial repressor protein with a eukaryotic activator domain. Future applications could focus on using synthetic modules to screen for complementarity to certain types of mutations. This type of approach, in principle, would allow for the identification of cellular factors that carry out complex endogenous function, so long as that function could be encoded in a synthetic construct. In a recent example in yeast, a chimeric protein that created a synthetic tether between the ER and mitochondria was tested for complementation of a library of mutants that were deficient in organelle fusion (45). This synthetic screen approach allowed the researchers to evaluate whether organelle fusion was the key defect in a given mutant.

V. FUTURE DIRECTIONS: AREAS FOR THE APPLICATION OF SYNTHETIC BIOLOGY

In the future, will we be able to use synthetic biology-based approaches to understand more complex aspects of cellular function? Here we outline several areas that synthetic biology may have a major impact on in the future.

Building a cell: Self-assembly of complex cellular structures

Living organisms and cells have complex three dimensional structures, and the resulting compartments and shapes are critical for function. Yet we understand relatively little about how these structures and intracellular organization is achieved via the process of self-assembly (41). For example, can we understand the shapes that cells achieve or their mechanisms of dynamic shape change and movement? Can we understand how cell membranes are organized into different compartments, and how the amount of these different organelles is dynamically regulated? This class of problems seems ideally suited for the approach of understanding through attempting to build structures.

What is necessary and sufficient to build particular shapes, movements or organelles? The modules that regulate cellular organization are now quite well characterized, such as the small GTPases and phosphoinositides that regulate cell polarity, shape and trafficking, and attempts to rewire and control these master regulators may play a major role in furthering our understanding of how these systems are hierarchically organized to program specific spatial events. Many modules that directly modulate cell structure are also being discovered. For example Bar and I-Bar domains, found in a diverse set of membrane remodeling factors (27), are thought to contribute to forming membrane invaginations or protrusions, respectively. Bar and I-Bar domains are often found in proteins containing other regulatory domains, which appear to specify both localization and direct type of membrane remodeling function that Bar domains execute. A modular understanding how upstream inputs regulate cell structure assembly in these systems could provide a construction kit that allows us to test assembly principles by attempting to recapitulate particular structures, or even to build novel ones (49).

Engineering Microbial Communities

Another promising area for the application of synthetic biology is in the engineering of synthetic microbial consortia (14). It is clear that in the environment and even within our own flora, microbes function as complex communities. Microbial communities offer model systems for studying questions regarding group selection, predator-prey dynamics, and simple social

behavior. Understanding the dynamics of these systems, however, can be complicated by the heterogeneous and complex nature of native communities. As a complimentary approach, it is now possible to use synthetic biology approaches to build simple, highly controlled, genetically engineered communities to systematically explore some of these problems. Such approaches can be used to build microbial strains to execute simple behavioral programs that cause them to cooperate with one another (33) or cheat each other (33) in simple game-theory scenarios. In addition to allowing one to work out fundamental rules of community dynamics, such studies may also lay the ground work for development of engineerable modular microbial communities that carry out more complex or more diversified bioindustrial processes (7).

Engineering Multi-cellular Communication and Development

The principles of rewiring systems using optimized and orthogonal modules could easily be applied to control cell-cell communication. Such work has already been explored in bacteria (73), but could in principle also be used to explore the problems of inter-cellular communication and development in multi-cellular organisms. Such studies will require the development of modular and engineerable cell-cell communication parts, such as hormone-receptor systems, or cell-surface signaling molecules, in which the inputs that control their activity/expression, and the functional outputs that they control can be rewired. Such a toolbox of synthetic cell-cell communication parts would be invaluable in using engineering approaches to understand minimal circuits and design principles underlying multi-cellular organization and programming.

VI. Conclusions

Synthetic biology has the potential to solve a range of pressing problems that are simply not addressable with conventional approaches, and each advance in discovery biology can be viewed as grist for the applied biological engineer. However, it is equally important to note the past contributions and future promise of synthetic biology for discovery biology. Many familiar biological tools are synthetic in nature, and looking forward, there is great potential to expand this toolkit. A systems level understanding of living cells will require manipulations at the level of network linkages, and this is the core strength of the synthetic biology approach as compared to genetic or chemical biology approaches. Using synthetic biology, we stand to gain a deeper understanding of the organizational principles of cellular systems, which will have a range of important ramifications. Many disease states can be viewed as network perturbations, and synthetic biology may provide the tools and understanding to move altered networks back to acceptable stable states. Thus, the flow of information between synthetic and discovery biology is not unidirectional. Rather, we envision a state of positive feedback between these two fields (Figure 7). This vision is epitomized by a statement written by the physicist Richard Feynman, which has emerged as an informal slogan for synthetic biology: “That which I cannot create, I do not understand”.

Sidebar 1: Decomposing the forces governing of controlled flight

The earliest attempts at manned flight employed separated, flapping wings, in clear imitation of birds. Although this strategy was initially successful for Daedalus, his legend proved to be a canard, inspiring countless disasters. Even the great inventor Leonardo da Vinci produced (but luckily, never tested) designs for bird-like flying machines (Codex on the Flight of Birds, ca 1505). The first powered human flight would have to wait until 1903, when the Wright brothers, former bicycle builders, designed and flew a fixed wing craft that looked very different from a bird. The key to their success was a wing design that allowed for the functional decomposition of the forces of lift and thrust. In the bird wing, flapping provides both lift and thrust--an integrated and exquisite solution for a lightweight creature, but impractical for heavier beings and challenging to quantitatively decompose.

In the Wright brothers' plane, lift is provided by the fixed wing, while thrust is produced by propellers. By functionally decomposing these forces, it became possible to design a flying machine with enough lift to raise man, and the field of aerodynamics exploded.

Sidebar 2

In the eukaryotic cell, heterochromatin represents a problem for synthetically controlling gene expression. When integrating components of a circuit into the genome, the local chromatin context affects gene expression significantly, meaning that a given promoter cannot be considered truly modular. These effects may be reduced through the uses of insulator or boundary elements that block the spread of heterochromatin. However, chromatin is also an opportunity to harness a new mode of gene regulation with unique properties. Based on analogy to natural systems, successful adaptation of heterochromatin to synthetic circuits would provide several potential advantages. First, the possibility exists for engineering cellular memory states. Researchers have already engineered epigenetic states using synthetic transcriptional feedback control (31), (9), (46) (1). However, we have to date mostly overlooked the faithful transmission of epigenetic marks through histone and DNA modifications, which are “read” by modular proteins leading to persistent regulation of transcriptional states. Second, heterochromatin acts regionally, instead of in a promoter-specific manner, so it should be possible to control blocks of genes in a tandem manner. For example, a synthetic module comprising multiple genes in a metabolically engineered strain might be controlled coordinately. Third, heterochromatin has been shown to act 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 (a requirement for the stable differentiation of cells). Thus, heterochromatin may provide a higher level of transcriptional control for the design of synthetic systems.

Summary Points

1. In addition to delivering technological benefits, synthetic biology holds great promise as a discovery tool since it allows the biologist to manipulate the modular structure of cellular networks.
2. The complexity of biological organization can be decomposed through understanding of the modules comprise networks. Synthetic biology is dedicated to discovering and abstracting these modules, thus informing us about the fundamental organization of living systems.
3. Rewiring experiments can be used query network 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 what the minimal requirements are to achieve that behavior. The iterative development of genetic circuits has demonstrated how design relates to function.
5. Combinatorial network design, either computational or experimental, has the potential to be an efficient method of discovering network topologies that achieve a given function. 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 allows the precise interrogation of network function.

Future Directions

1. Develop unbiased methods to generate and evaluate network structures (generate combinatorial libraries of networks). Address technical challenges 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. Adapt additional signaling currencies for use in synthetic biology. Ideal systems should include a writer (enzyme that catalyzes the chemical modification), a reader (binds the chemically modified substrate) and eraser (removes the modification. Promising candidates include ubiquitination and histone modifications).
3. Expand the toolkit for adding or modifying linkages to extant networks. 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. Extend synthetic biology approaches to new types of biological networks, those that regulate: cell shape, assembly of intracellular structure, cell-cell adhesion and communication, developmental regulation, behavior in microbial consortia.

Acknowledgments

We would like to thank…..

Glossary

Cellular Network	A collection of molecules, macromolecules, or molecular assemblies that are linked together by some standardized mode of interaction.
Evolvability	The capacity of a under selective pressure to generate variation that leads to adaptive behavior
Network Motif	A repeated pattern of processing interlinked nodes within a network that perform a characteristic information processing task.
Node	The most basic unit of a network that can convert an input into an output.
Synthetic Biology	Discipline that applies that engineering principles toward the construction of novel biological system that exhibit specified behaviors.
Toy Systems	Model synthetic circuits built in order to recapitulate a particular behavior (eg. oscillation, bistability). Useful for testing design principles
Hierarchical Modularity	Type of organizational structure where a collection of units with their own independently identifiable functions are grouped together into larger unit with a definable function.

Acronyms

MAPK	mitogen activated protein kinase
SH2	Src homology 2
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
PKA	protein kinase A
NFKB	nuclear factor kappa-light-chain-enhancer of activated B cells
PDZ	postsynaptic density protein-95 (PSD-95), Discs large (Dlg), Zona occludens-1
DED	death effector domain
N-WASP	neuronal Wiskott-Aldrich syndrome protein
Arp	actin-related protein
DUB	deubiquinating enzyme
UBD	ubiquitin binding domain
E1	ubiquitin-activating enzymes
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase
HAT	histone acetyl transferase
FRET	fluorescence resonance energy transfer
BAR	bin, amphiphysin (AMPH), Rvs
PTP	protein tyrosine phosphatase
LOV	light, oxygen, or voltage
ER	endoplasmic reticulum

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Annotated References:

Bashor (2008): use of dynamically controlled synthetic scaffold recruitment events to alter the temporal and dose reponse behavior in a MAP kinase pathway

Bhattacharyya (2006): extensive review outlining the modular organizational features of eukaryotic signaling proteins

Dueber (2003): demonstrated that recombining N-WASP's output domain with heterologous interaction domains can produce diverse and complex allosteric behaviors

Guet (2002): used a combinatorial shuffling approach of promoters and coding regions to generate a libarary of network motifs with varied regulatory and logic gating properties

Isalan (2008): used systematic rewiring of the *E. coli* transcriptional network to demonstrate its robustness to perturbations in its connectivity

Kormann (2009): used a synthetic biology-based genetic screen to identify a protein complex that tethers ER and mitochondria

Ma (2009): used a computational approach to enumerate circuit toplogies that supported perfect adaptation behavior

Stricker (2008): engineering of a robust, stable genetic oscillatory circuit in *E. coli*.

Suel (2006): established a quantitative model excitatory circuit in B. subtilis; used modular rewiring approach to help validate the model

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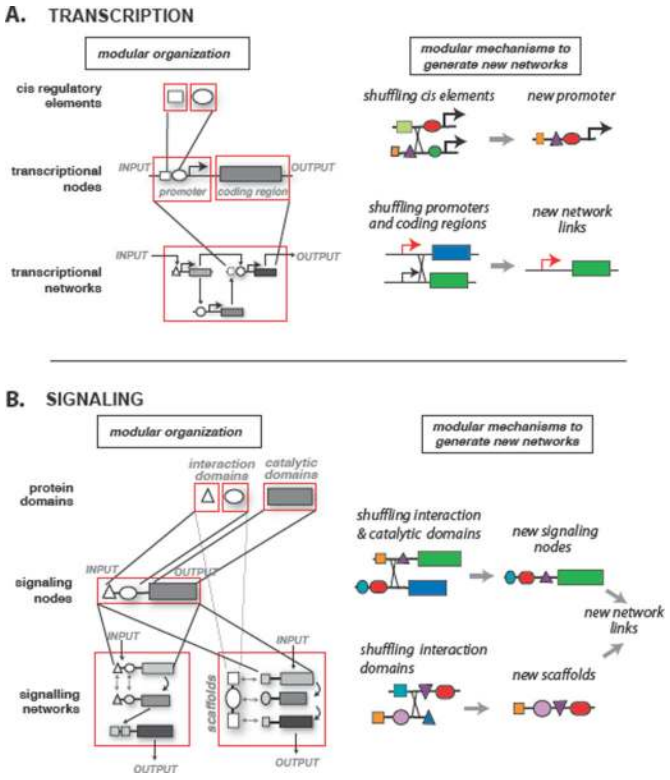

Figure 1. Synthetic rewiring experiments can help define the modular hierarchy of transcriptional and signaling networks

Different types of regulatory networks in cells are built up from hierarchies of interlinked modules. Function is achieved from the assembly of molecular building blocks into network nodes that perform a defined input/output function. Nodes, in turn, are assembled into motifs--patterns of connectivity that execute specific information processing tasks. By attempting to rewire the components of cellular networks, both at the level of nodes and motifs, we can impose upon those components a test for functional modularity. (**a**) In genetic networks, nodes are composed of *cis*-regulatory elements, which define input, and coding regions, which specify output. *Cis* elements can be shuffled experimentally to yield promoters of diverse function (20,22). Information processing functions in gene networks are performed by motifs composed of a group of interlinked genes. Genes can be shuffled to generate variation in motifs (36). It is also likely that motifs have been shuffled over the course of evolution (51). (**b**) In protein signaling networks, signaling protein (network node) interactions are mediated by regulatory domains that recruit the catalytic domain of a protein (output module) to a cellular a target. Regulatory 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. There is experimental evidence demonstrating the modularity of both switch protein function(21,72) and scaffold function (37,53)

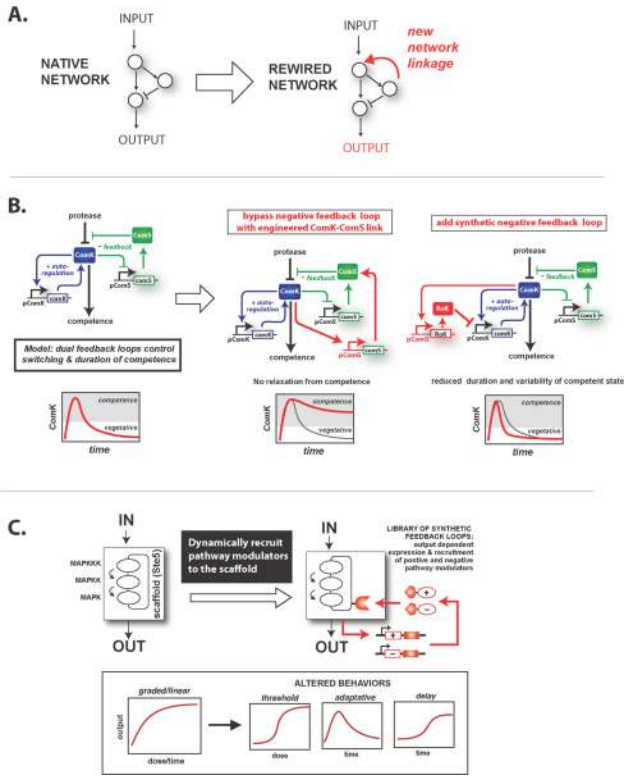

Figure 2. Rewiring experiments can be used to test predictions about the function and plasticity of cellular networks

(**a**) Understanding the basis for modular connectivity in cellular networks allows us to design hypothesis-driven rewiring experiments. The addition of new linkages to existing networks can be used can test basic assumptions about how the networks function, and how flexible their behaviors are to changes in their network structure. (**b**) Circuit diagram representation of the model that Suel, et al. (64,65) used to describe the transition between sporulation and competence states in *B. Subtilis*. Two critical feedback loops control levels of the master transcriptional regulator ComK: a positive auto-regulatory loop (purple) and a ComS-mediated triple-negative (net negative) feedback loop (11). 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) by triggering positive feedback loop activation. Competence switching is controlled by the positive feedback loop, while return to the basal state is mediated by the negative loop. Results from rewiring experiments support this model. In one case, bypassing the negative feedback loop resulted in cells that switched to irreversibly to competence. The addition of negative feedback regulationresulted in faster recovery from competence back to the basal state as well as lower cell-to-cell variability in switching times. (**c**) The MAP kinase pathway that mediates mating in yeast displays a graded, linear response with respect to input in both dose and time regimes, while other MAP kinase pathways in other organisms or cells show distinct dynamical behaviors. The scaffold protein Ste5 specifies mating pathway connectivity by coordinating the kinase cascade. In Bashor et al. (6), positive and negative pathway modulators were recruited to the scaffold using synthetic protein-protein interaction domains in order to up- and down-regulate pathway activity. When placed under the control of pathway responsive promoters, positive and negative feedback loops can be engineered. By using competitive interactions to create a sink for modulator binding, or to create competitive, reciprocal recruitment of modulator to the scaffold, a number of different types of complex input/output behaviors were achieved, including adaptive and activation-delayed temporal profiles, as well

converting the dose-response profile for the circuit from a graded to switch-like. Thus this single platform can be used to generate many of the diverse behaviors observed within the greater MAPK cascade family.

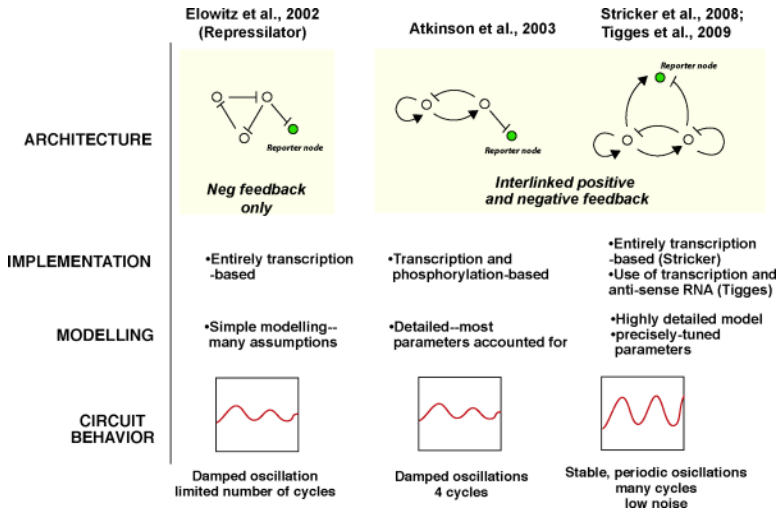

Figure 3. Empirically understanding design principles: iterative improvement of synthetic oscillator circuit behavior

Designing and building biological circuits that exhibit stable, robust oscillatory behavior has been one of the early achievements of synthetic biology. Oscillator designs vary in terms of both their architecture and implementation (type of molecules used to construct the circuit). The first oscillator design (repressilator) was as a three member ring network based on repressor-operator interactions (23). Subsequent circuits (4,63,68) utilized an interlinked positive and negative feedback design that was shown computationally to be more robust to parameter variation (70). A circuit constructed by Atkinson et al. was largely transcription-based, but utilized a phosphorylation even to mediate one branch of the feedback. The robust, stable oscillator constructed by Stricker et al. was entirely transcriptional-based, while the mamalian-based circuit (constructed in CHO cells) was implemented using a combination of transcription and antisense RNA. The nature of the quantitative modeling approaches that accompanied the designs was also variable. Modeling of the repressilator was simple, and has numerous implicit assumptions. While Atkinson et al., used a more rigorous approach to describe their circuit, Stricker et al. fully parameterized their model, and were able to use their model to recognize the importance of several key parameters in realizing a circuit that exhibited sustained oscillations.

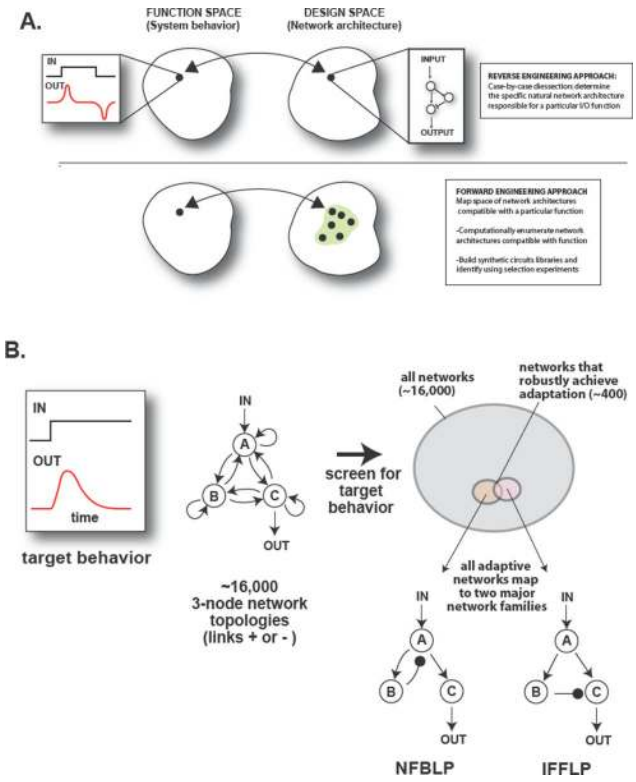

Figure 4. Combinatorially searching network space to define families of circuits that can achieve target functions

(**a**). Traditional reverse engineering of biological networks involves determining the structure/function relationship between one type of observed behavior and a single circuit architecture. As an alternative, a forward engineering approach may be employed, where a range of solutions that fulfill a given behavior are enumerated either experimentally or computationally. This approach might illuminate basic design requirements, and provide clues on how to achieve optimal behavior. (**b**) Ma et al (50) searched all possible three-node networks for topologies for those that exhibited perfect adaptation behavior (which was defined as a return to baseline after stimulus). The search identified ~300 robustly adapting circuits. All of these networks mapped to two simple topology families that were sufficient to confer adaptive behavior: negative feedback loop with a buffering node (NFBLB), and a 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.

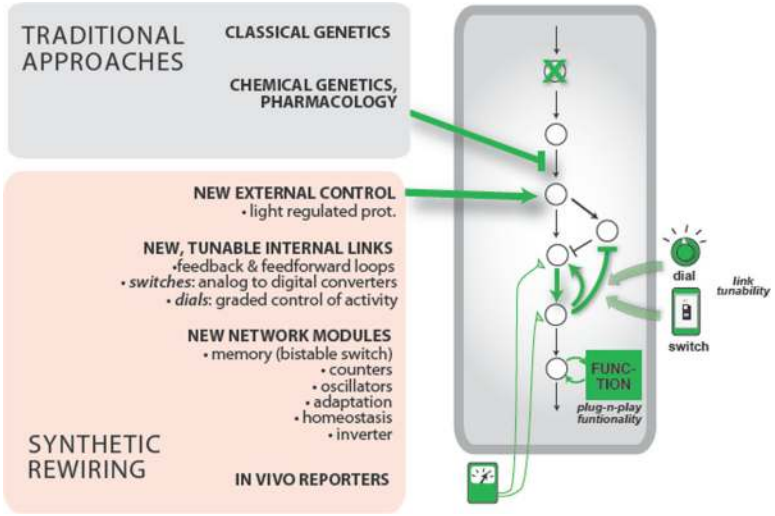

Figure 5. Synthetic biology offers an expanded set of research tools for making genetic perturbations in cells

Traditional approaches for investigating cellular systems are useful, but limited in the ways that they can test the relationship between cellular network structure and function. Classical genetics is able to make mutations, which eliminate network nodes, while 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 either be constitutive, precisely tunable (dial), or turned on and off in a controlled fashion (switches). By wiring new functional subsystems into networks, an investigator can inroduce a genetically encoded functionality that can be used to alter network behavior. These include reporters that can be programmed to detect a variety of complex cellular events

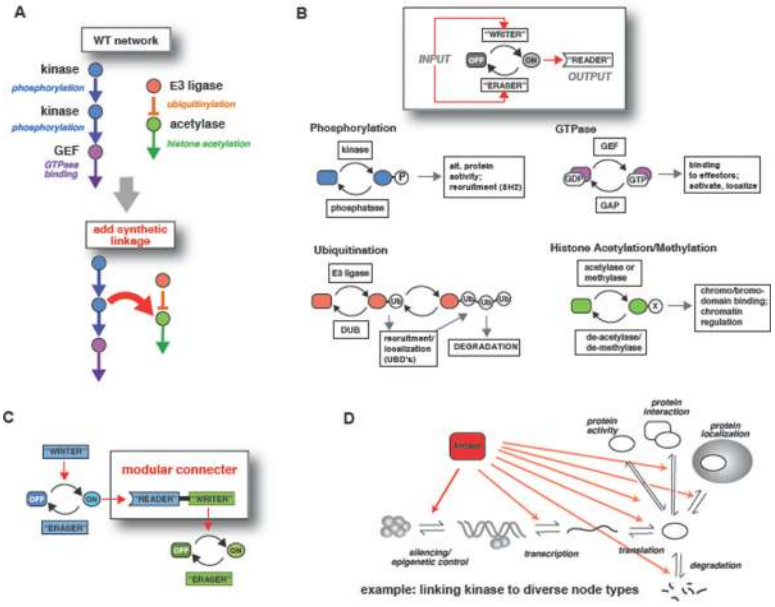

Figure 6. Harnessing the diversity of cellular information currencies to generate synthetic linkages in cellular networks

(a) Cellular networks involve many diverse information encoding currencies. This heterogeneity presents a challenge to the synthetic biologist who wants to add novel links to engineer the network. (**b**) Many of the reversible reactions that are used as signaling currencies in post-translational networks can be understood in terms of a reader/writer/eraser paradigm. Writers enzymatically catalyze the transfer of chemical marks onto target molecules; Erasers catalyze the removal of chemical mark. Inputs to the node is used to control the writers and erasers. The presence of a mark is then read out by a reader module, which can either come in the form an altered functionality, or 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 signaling a network, and are an attractive target 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 a variety of different types of cellular targets, while phosphatases act as erasers by dephosphorylating those targets. A diverse number of readers exist for phosphate marks. Phosphorylation of protein targets, for example, can result in allosteric alteration of binding surfaces such that they either bind to or disengaged from binding partners. Additionally, interaction domains that specifically recognize phosphorylated protein motifs (SH2's, WW's, FHA's) represent a common mechanisms for generating reversible interactions between nodes in a signaling pathway. GTPases follow the reader/writer/eraser paradigm as well, except that the reversible chemical mark (the structure of the guanine nucleotide) is translated into protein surface conformational changes which are read out by the interacting proteins that act as readers. The ubiquitination of protein targets and the reversible chemical modification of histones are two additional types of modular, reversible regulation currencies. In the case of ubiquitination, E3 complexes act as writers, transferring ubiquitin to protein targets. DUB's are responsible catalyzing de-ubiquitination reactions. Depending on the number of ubiquitin tags, and configuration of the tags, ubiquitination can lead to proteasome-mediated degradation, or recruitment via biding to non-proteasomal UBD's. Histone modifications constitute a diverse class of reversible chemical modifications which are used to modify the state of chromatin. Histone modifying enzymes, which acts as writers, use methylation and acetylation to write reversible marks onto resides found in histone proteins. Marks then recruit chromo domain and bromo domain-containing factors that alter chromatin state. (**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 in principle be regulated by this currency. In principle, synthetic biologists should be able to construct new connections of all of these types.

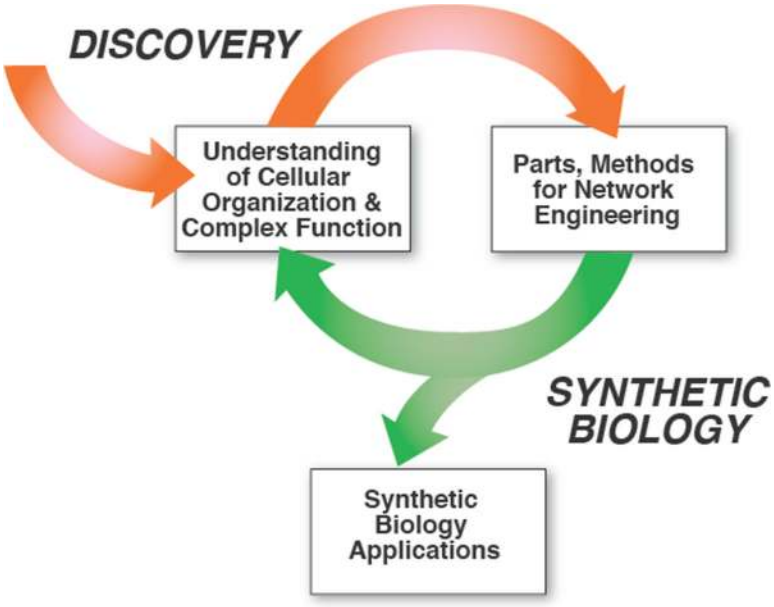

Figure 7. Complementarity of discovery and engineering approaches in reaching a deeper understanding of complex biological systems

Discovery biology supplies the medium that synthetic biology can appropriate for engineering purposes. However, in the process of creating useful applications and tools, synthetic biology uncovers principles of design and organization that improve our understanding of biological systems.