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Using Synthetic Strategies to Rewire the Yeast Mating Pathway

by

Caleb Jay Bashor

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biophysics

in the

GRADUATE DIVISION

of the

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by

Caleb Jay Bashor

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**Chapter 1** of this thesis contains materials from a recently submitted review which will appear in volume 39 of the journal *Annual Review of Biophysics*: Bashor, C.J., Horwitz, A.A. Peisajovich, S.G., and Lim, W.A. (2010) Rewiring Cells: Synthetic Biology as a Tool to Interrogate the Organizational Principles of Living Systems. This chapter provides an overview of how the emerging discipline of synthetic biology can be used as a discovery tool to establish relationships between the structure and function of cellular networks. It can be viewed as an introductory framework and intellectual rationale for the experimental work described in later chapters on rewiring of the yeast mating MAP kinase pathway.

**Chapter 2** describes largely unpublished work that was foundational for the studies performed in Chapter 3. This includes the development of a single-cell fluorescent transcriptional reporter for monitoring yeast mating pathway induction. This reporter was used to not only characterize the WT mating pathway response, but also to identify pathway modulators that could be used to up- and down-regulate the mating pathway in a localization-dependent manner. These pathway modulators proved to be critical components of the synthetic circuits that were constructed in the final two chapters.

**Chapter 3** is reproduced from the following manuscript:

Bashor C.J., Helman N.C., Yan, S., and Lim, W.A. Using Engineered Scaffold Interactions to Reshape MAP Kinase Signaling (2008) *Science* 319, 1539 – 1543.

(<http://www.sciencemag.org/cgi/content/full/319/5869/1539>). The work described in this section involved the creation of scaffold-mediated, synthetic feedback circuits used to quantitatively reshape signaling of the MAP kinase pathway that regulates mating in yeast.

# Using Modular Rewiring Strategies to Reshape Kinase Signaling

Caleb Jay Bashor

## Abstract

In eukaryotic cells, networks of signaling proteins are responsible for converting environmental inputs into the appropriate regulatory outputs. The proteins that comprise signaling networks are highly modular, and are typically made up of multiple, functionally distinct domains. These domains are of two varieties: 1) catalytic domains (eg. kinases, GTPases), which leave a chemical imprint on a downstream target, and 2) interaction domains, which facilitate recognition between network members. As such, information flow in signaling networks is potentiated by catalytic domain function, while protein-protein interaction domains direct this catalytic function to specific targets, thereby defining network connectivity.

In signaling networks, groups of proteins that mediate a particular response (pathways) are often found co-localized with one another into complexes. These complexes are coordinated by scaffolds proteins, which typically are composed of multiple, modular interaction domains. By defining which catalytic components are co-localized within a pathway complex, the interaction domains in a scaffold encode the connectivity for that pathway. It has been hypothesized that scaffolding may

have facilitated the evolutionary elaboration of signaling by acting as a modular hub for the addition of new linkages in a pathway: since scaffolds create specificity by co-localization, addition of new components to a pathway may be easily accomplished by addition of a new interaction domain to the scaffold.

We reasoned that the same modularity that may have made scaffolded signaling pathways highly evolvable might also make them engineerable. In the present work we provide evidence that supports this premise: scaffolding can be exploited to generate novel, synthetic pathway linkages, and thus can be used to reshape the I/O of a signaling pathway. We reprogrammed the I/O behavior of the mating response pathway in *S. cerevisiae*, a classically studied MAP kinase signaling pathway that utilizes a scaffold protein, Ste5, to coordinate members of the pathway. We demonstrate that Ste5 can be used as an assembly platform for the synthetic recruitment of factors that positively and negatively regulate mating pathway activity. By incorporating these recruited modulators into simple transcriptional feedback loops, we demonstrate that the scaffold can be used as a junction to incorporate artificial feedback control into the pathway's response, dramatically altering its I/O. Using a limited set of molecular components, we constructed a variety of feedback circuit architectures and were able to generate diverse classes of behavior.

The results of the work described herein demonstrate the essential plasticity of a signaling network that utilizes scaffolding to define its connectivity. By exploiting

these features, we were able to easily introduce new network linkages into a signaling pathway and dramatically reshape its signaling behavior. The variability of circuit behaviors achieved from a small collection of constituent parts supports the idea that the facile rewiring of regulatory linkages using scaffolding may have played an important role in facilitating the evolution of protein signaling networks. Furthermore, the approaches we used for rewiring the mating pathway may be generalizable to a variety of other signaling pathways both in yeast and other organisms.

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## **Chapter 1**

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

## **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 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.

## **Introduction**

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<sup>11,48</sup>. 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<sup>4</sup>. 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<sup>35, 36, 37</sup>. 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 1-1A**). Nodes in genetic networks (genes) are composed of regions containing *cis*-regulatory elements (promoters) and protein-coding regions. Network linkages are created when 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<sup>2</sup>. Motifs have defined input and output properties and specific information-processing functions<sup>44</sup>. Higher-order behavioral

complexity of genetic networks is thought to derive from the interaction of different types of motifs.

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<sup>30</sup> 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<sup>12, 13</sup> served as validation of this principle and formed the basis for the yeast two-hybrid screen<sup>21</sup>. 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.<sup>16</sup> 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<sup>63</sup>. For example, modular alterations to promoter structure have been

proposed as a key mechanism for generating anatomical diversity in the animal kingdom<sup>14, 28, 47</sup>. 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.<sup>31</sup>, 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 1-1B**). Proteins found in signaling networks are made up of multiple, independently folding domains<sup>9</sup>. 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 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<sup>41</sup>. 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<sup>46</sup>. To establish the modularity of N-WASP regulation, Dueber et al.<sup>18</sup> 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<sup>32</sup>, 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<sup>45</sup>. 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 (unpublished data, Peisajovich S, Wei P, Garabino J, and Lim W) 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 wildtype 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 1-2A**). Two recent studies<sup>56,57</sup> 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 1-2B**). 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.<sup>6</sup> evaluated the potential for using synthetic feedback regulation to reprogram the input/output of the yeast mating MAPK pathway (**Figure 1-2C**). 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 dramatically changed wildtype 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.<sup>34</sup> 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 48), including circuits that produce gene expression oscillations, bistable switches that act as epigenetic memory devices<sup>1,27</sup>, circuits that perform combinatorial logic operations<sup>18, 31, 58</sup>, and circuits that count cellular events<sup>23</sup>. 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.

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<sup>26</sup>? 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 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<sup>53</sup>. 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<sup>20</sup> (**Figure 1-3A**). 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.<sup>62</sup> 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<sup>5</sup>, 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 period and amplitude of oscillations are independently tunable. A second *E. coli*--based circuit was subsequently built<sup>3</sup>. 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.<sup>55</sup> 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<sup>60</sup>, and yet another oscillator was constructed using transcriptional feedback combined with the enzymatic interconversion of a metabolite pool<sup>25</sup>.

### ***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 topologies and large areas of parameter space could enumerate circuit topologies that accomplish a certain target behavior (**Figure 1-3B**). Ma et al.<sup>42</sup> 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<sup>16, 19</sup>, and previously described studies involving the small-scale rearrangement of network modules<sup>18, 26, 31</sup> 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 1-4**). 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<sup>10</sup>. 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<sup>61</sup>.

More recently, light-modulated interaction domains from plants have been exploited to mediate synthetic linkages in mammalian cells. Levskaya et al.<sup>39b</sup> have adapted a plant phytochrome light-inducible dimerization system<sup>52</sup> as a noninvasive tool for the creation of network linkages with a high degree of temporal and spatial specificity (**Figure 1-4B**). Wu et al.<sup>64</sup> 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.

### ***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<sup>17</sup>. 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.<sup>29</sup> 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 (where information is encoded by whether a gene product is expressed or not) and protein complex assembly (where 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 1-5A**).

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 1-5B**). 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 1-5C**). In one example of an engineering such a connection, Yeh et al.<sup>65</sup> 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 mamalian 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 to which the reader/writer/eraser paradigm can be applied (**Figure 1-5B**). Ubiquitination tags are written to a protein target by ubiquitin (E3) ligases, read by a variety of ubiquitin binding domains (UBDs)<sup>33</sup>, and erased by deubiquitinating (DUB) enzymes<sup>22</sup>.

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<sup>15</sup>. 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 1-5B**). 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<sup>54</sup>. 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<sup>59</sup>. 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<sup>40, 54</sup>. Histone modifications are also reversible. This includes lysine methylation, for which no eraser was identified until the recent discovery of histone demethylases<sup>51</sup>. In addition to its great combinatorial potential, the currency of histone modification is particularly interesting because it may encode epigenetic memory.

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 1-5D**). Using a 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.

### ***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<sup>50</sup>. 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<sup>49</sup>. 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.<sup>37</sup> 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<sup>39</sup>, or to use an autoregulatory module to lower expression noise for a gene of interest<sup>7</sup>.

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<sup>66</sup>. 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 1-4C**) was used to track the number of times an event of interest takes place, for example, cell divisions before senescence<sup>23</sup>. 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<sup>38</sup>. 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 1-6**). 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

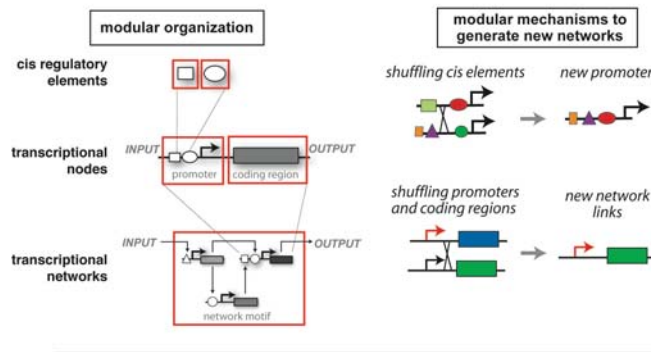
and social behavior. Principles of systems rewiring could also be applied to control cell-cell communication to help identify molecular- and systems-level determinants for tissue differentiation and developmental patterning. In each of these potential areas, a close relationship with between synthetic and discovery biology will be 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.

## **Acknowledgments**

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Figure 1

### A. TRANSCRIPTION



### B. SIGNALING

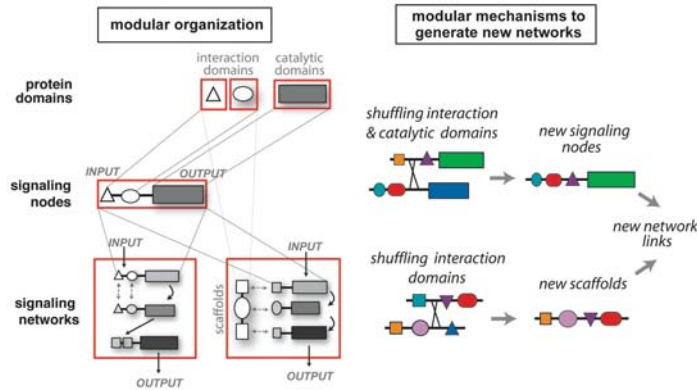


**Figure 1-1** Synthetic rewiring experiments can help to define the modular hierarchy of transcriptional and signaling networks. Cellular regulatory networks are built up from hierarchies of interlinked modules. Function is achieved from the assembly of molecular building blocks into network nodes that perform a defined input/output function. 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.

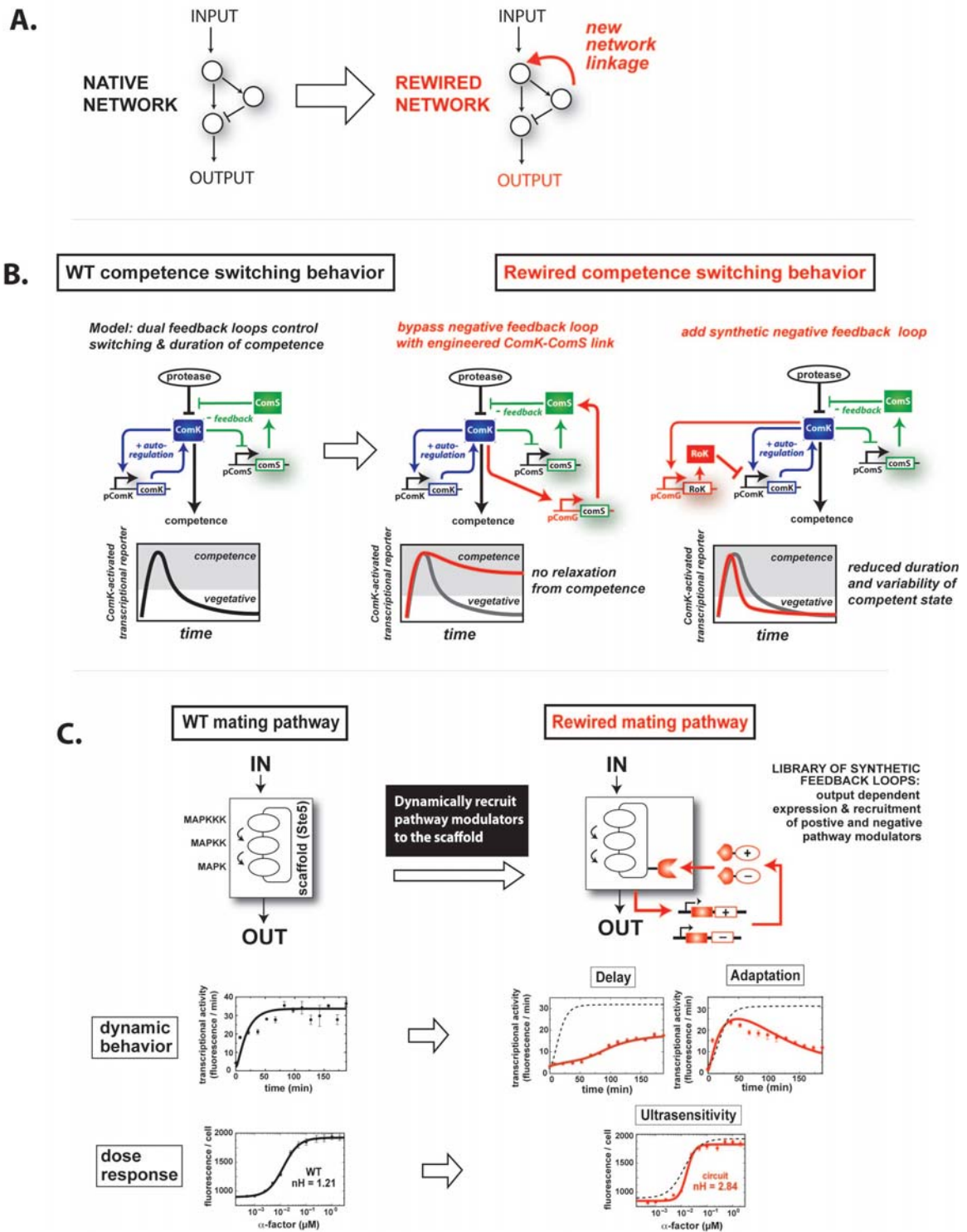
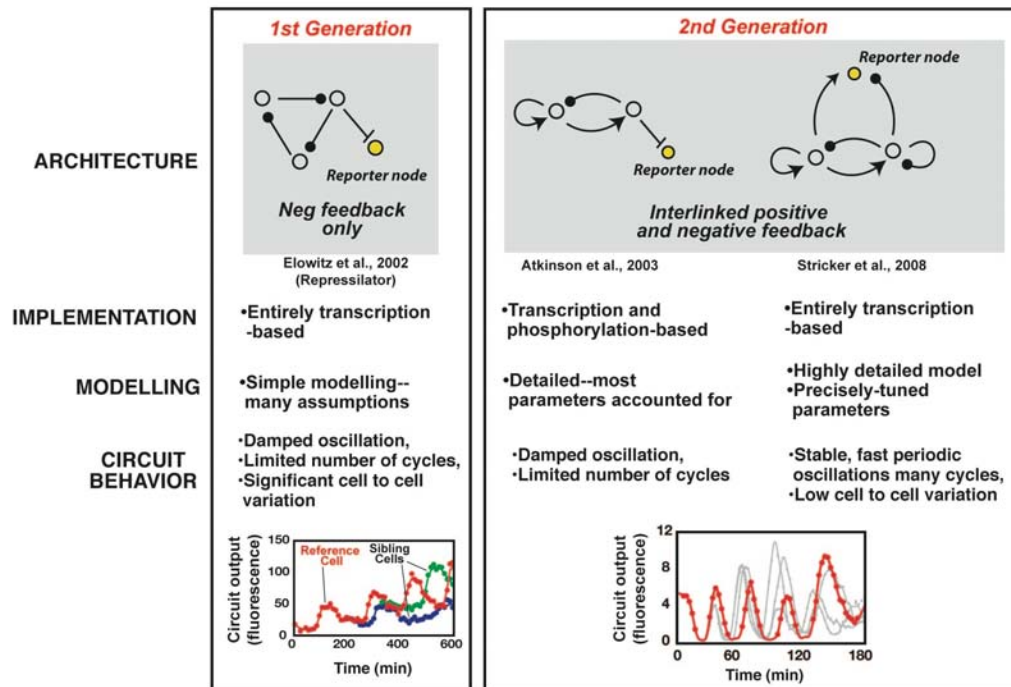


Figure 1-2. (continued on the following page)

**Figure 1-2.** 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.<sup>56, 57</sup> 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 replicas of data from figures in references 56 and 57 redrawn with permission. **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.<sup>6</sup> 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 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 redrawn from Bashor et al.<sup>6</sup>.

## A. DESIGNED BIOLOGICAL OSCILLATORS



## B.

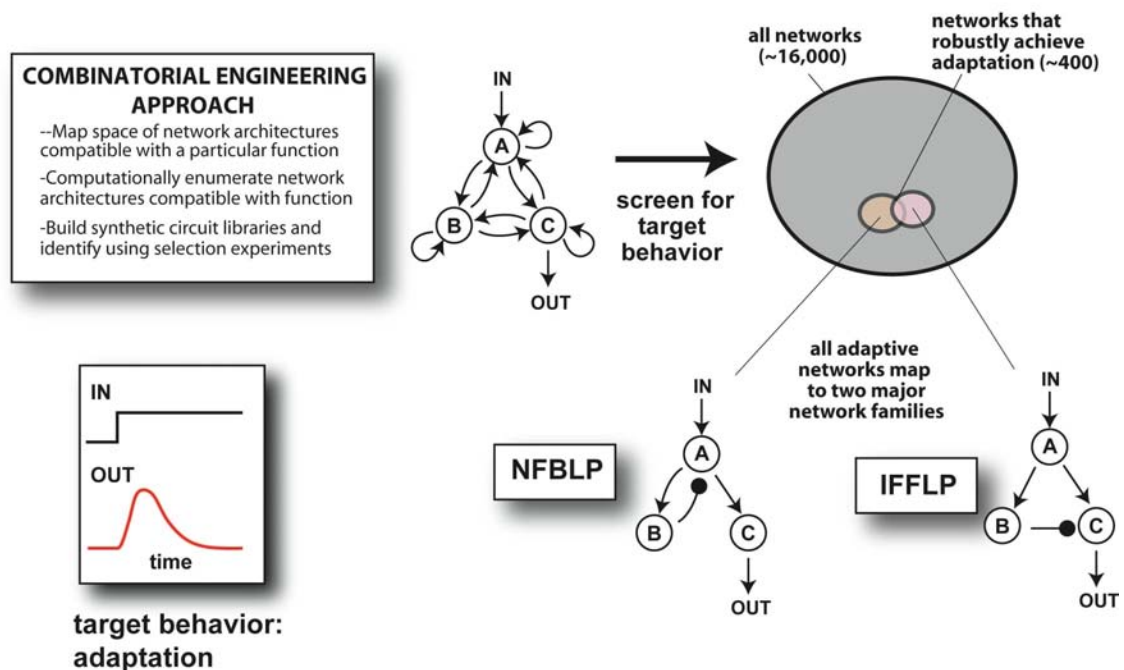


Figure 1-3. (continued on the following page)

**Figure 1-3.** 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 node in the circuit. The first oscillator design (repressilator) was a three-member ring network based on repressor-operator interactions<sup>20</sup>. Subsequent circuits<sup>3, 55, 60</sup> utilized an interlinked positive and negative feedback design that was shown computationally to be more robust to parameter variation<sup>62</sup>. The circuit constructed by Atkinson et al.<sup>6</sup> was transcription based, but it also utilized phosphorylation to mediate one branch of the feedback. The robust, stable oscillator constructed by Stricker et al.<sup>55</sup> 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. Oscillator time course data are redrawn, with permission, from references 20 and 62. **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.<sup>42</sup> 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 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.

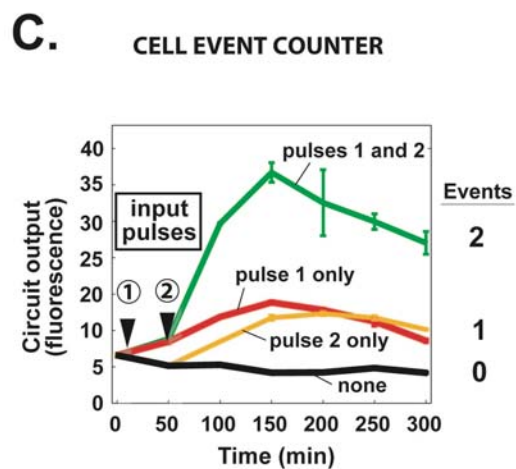
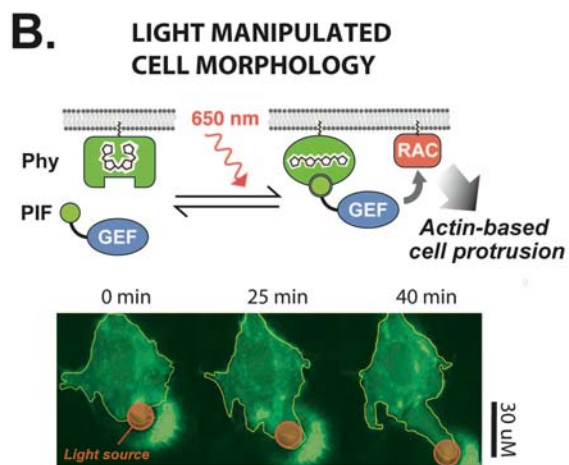
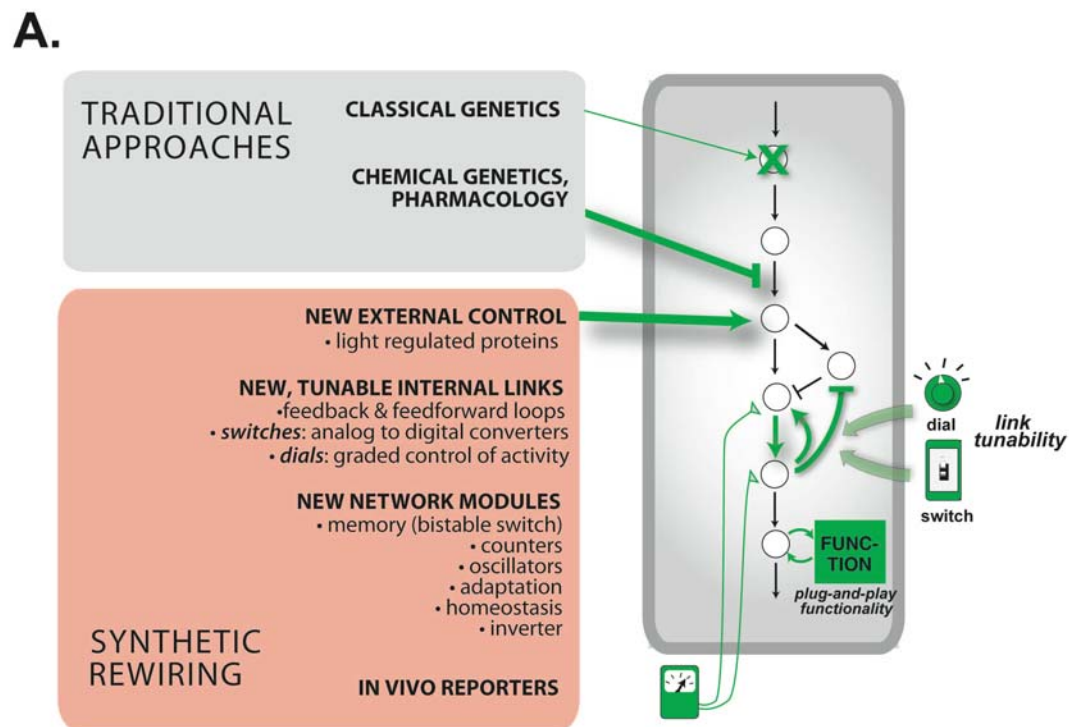


**Figure 1-4.** (continued on the following page)

**Figure 1-4.** 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 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 non-invasively direct highly localized protein-protein association events within a cell. In a recent example in Levskaya et al.<sup>39b</sup>, actin polymerization was induced 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. In work by Friedland et al.<sup>23</sup>, a circuit was implemented which activates only after the occurrence of two consecutive stimulation events (data redrawn from reference 23 with the authors' permission).

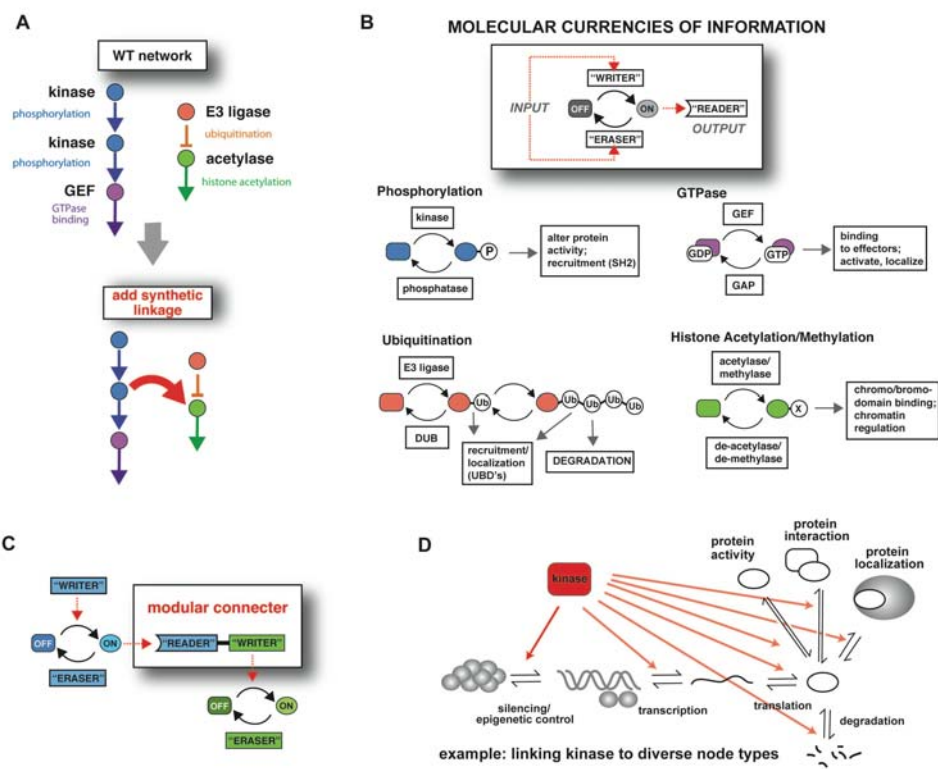


**Figure 1-5.** (continued on the following page)

**Figure 1-5.** 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 connector 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 connector 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.

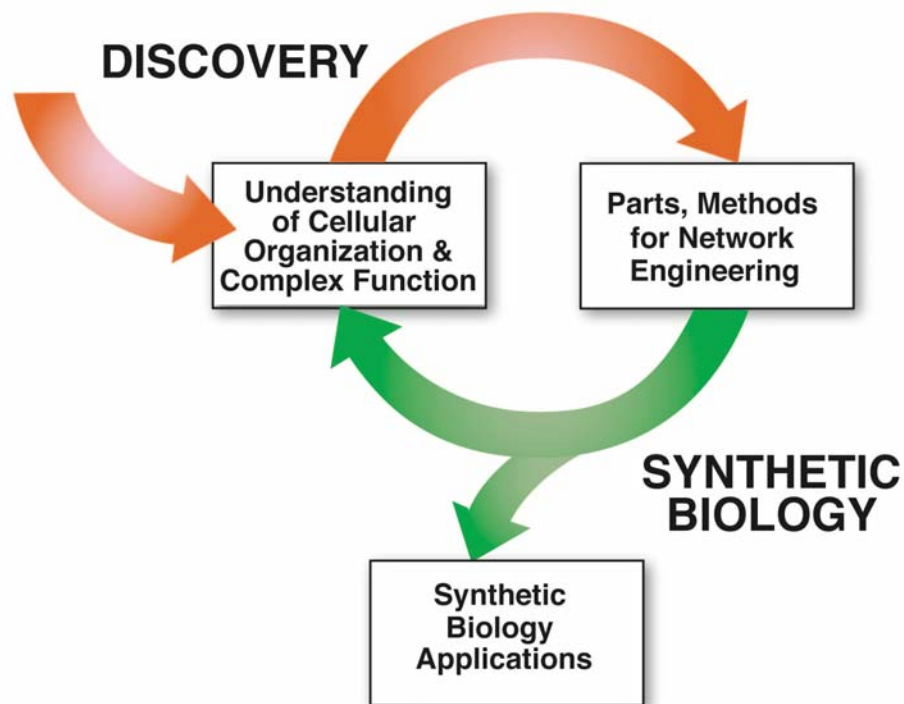


**Figure 1-6.** Complementarity of discovery and engineering approaches in reaching a deeper understanding of complex biological systems. Discovery biology generates the medium that synthetic biology appropriates for engineering purposes. In the process of creating useful applications and tools, synthetic biology uncovers principles of design and organization that improve our understanding of biological systems.

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## **Chapter 2**

# **Development of a Fluorescent Transcriptional Reporter for Single-cell Analysis of the Yeast Mating Pathway and Application Toward the Identification of Reversible, Localization-dependent Pathway Modulators**

# **Development and Characterization of a Fluorescent Transcriptional Reporter Strain for Monitoring Yeast Mating Pathway Induction by Flow Cytometry**

## ***Introduction***

The goal of re-engineering the yeast mating MAP kinase pathway demands a reliable, quantitative reporter for measuring pathway output. Such a reporter has several requirements: **1)** The reporter must serve as a reliable surrogate for mating pathway output. Since the target of our engineering efforts is the mating MAP kinase cascade module, the reporter should provide a metric for the activity state of Fus3, which is the output of this module. Dual phosphorylation of Fus3 activates the kinase, allowing it to phosphorylate its downstream target<sup>1</sup>. **2)** Since we are interested in testing many circuit configurations, the reporter must be amenable to high throughput analysis. **3)** Finally, in order to properly assess the output of our engineered circuits, it is critical that the reporter allow us to measure pathway induction in single cells. MAP kinase pathways have demonstrated non-linear, bi-modal induction profiles in other species and cell types. There are several examples of studies in which bulk measurements of MAP kinase pathway activation were unable to detect bi-modality, and single cell measurements were needed<sup>3</sup>.

In order to meet these requirements, we constructed a fluorescent transcriptional reporter strain (CB009) in which GFP is transcribed upon pathway induction (**Figure 2-1A**). The strain harbors an artificial cassette containing the mating

pathway-activated promoter, which drives transcription of a gene coding for enhanced, yeast-optimized GFP<sup>4</sup>. During mating pathway induction, Fus3 phosphorylates transcriptional regulatory factors that induce the transcription of a variety of genes involved in the mating response. The promoter from one of the more strongly expressed of these genes, *FUS1* was chosen to drive transcription of the reporter<sup>5</sup>. Here, we describe the analysis of the reporter strain and its validation as a mating pathway readout that meets our listed requirements.

### ***Results and Discussion***

When the *pFUS1*-GFP reporter strain, CB009, was induced with saturating (>10  $\mu$ M) concentrations of mating pheromone ( $\alpha$ -factor), cellular fluorescence increased dramatically over time as measured by GFP-FACS (**Figure 2-1**). When cellular fluorescence was plotted against forward light scatter (an approximate index of cell size), cells demonstrated only marginal changes in cell size between induced (high fluorescence) and uninduced (low fluorescence) populations (**Figure 2-1A**).

Histograms plotted from cells gated for a standard size range (corresponding to single cells) showed approximately symmetric, gaussian distributions of cells for both the induced and uninduced populations (**Figure 2-1B**). Values for mean population intensity used in experiments throughout this study were calculated from these distributions.

Early efforts to create a reporter strain that permitted accurate measurement of pathway induction over extended time courses (60 - 180 min) were confounded by

two factors: **1)** Cell cycle arrest, a normal consequence of mating pathway induction<sup>6</sup>, caused cells to swell over time (**Figure 2-1C**). Larger cells exhibit higher levels of fluorescence, and the relationship between cell size and measured fluorescence is non-linear (data not shown). Additionally, cell cycle arrest alters the steady state that arises from basal rates of GFP synthesis and decay during exponential growth, and GFP levels are highly dependent on cytoplasmic dilution during cell division. Taken together, these factors make quantitation of GFP accumulation resulting from mating pathway-induced increases in GFP transcription difficult in arrested cells. In order to avoid these complications, we knocked out the gene that codes for Far1, the protein that mediates mating pathway-induced cell cycle arrest.  $\Delta far1$  cells do not arrest, and continue dividing at approximately the same rate as uninduced cells, changing substantially in size only after 3-4 hours of treatment with saturating pheromone. **2)** Bar1, a protease that degrades  $\alpha$ -factor, is secreted upon induction of the pathway. The accumulation of Bar1 in the culture media during a mating pathway induction time course experiment made extended time course experiments impossible, even at supersaturating concentrations of  $\alpha$ -factor this was indicated by sharp drops in GFP fluorescence intensity after two hours (**Figure 2-1B**). To address this issue we generated a strain in which *BAR1* was knocked out. This eliminates  $\alpha$ -factor degradation, and resulted in the linear accumulation of GFP for up to and beyond 180 min. CB009 harbors both  $\Delta far1$  and  $\Delta bar1$  knockouts, as do all strains subsequently generated from it (**Table 2-1**).

Dose- and time-dependent analysis of the CB009 reporter strain revealed that mating pathway induction is both linear and graded with respect to  $\alpha$ -factor concentration. In **Figure 2-2A**, dose-response analysis performed at 90 minutes after pheromone treatment shows that the pathway is linear with respect to receptor occupancy ( $nH = 1.08$ ). Induced cells demonstrate a unimodal, approximately gaussian fluorescence distribution at all doses of  $\alpha$ -factor, indicating that the pathway is graded in its response to input (**Figure 2-2B**). Induced CB009 cells displayed monotonic increases in cell fluorescence at all concentrations of  $\alpha$ -factor for the entirety of a 180 min time course (**Figure 2-2C**). Slope for the increase in GFP was relatively constant between 30 and 180 min, indicating a steady state in pathway activation. Unimodal, gaussian fluorescence intensity distributions were maintained throughout the time courses. At all concentrations of  $\alpha$ -factor stimulus, when stimulus was removed, signal decayed rapidly, demonstrating that pathway activation was reversible (**Figure 2-2D**).

Taken together, these results indicate that the *pFus1*-GFP reporter strain fulfills requirements for a quantitative, single-cell reporter of mating pathway output. Furthermore, the reporter demonstrated that the mating pathway exhibits uncomplicated input/output behavior: pathway activation is linear and graded, increases monotonically to a maintained steady state, and is reversibly inactivated with the removal of stimulus. These features predispose the mating pathway to engineering work in which this simple input/output behavior alter higher order, non-linear behaviors are

## ***Materials and Methods***

The background strain used for experiments done in this section, CB008, (see **Table 2-1**) contained *BAR1* and *FAR1* knockouts generated from the strain SΦ992. The *Δbar1* mutation was generated using primer amplification of a G418 resistance cassette and transformation<sup>7</sup>. The *Δfar1* mutation was created using a pop-in/pop-out cassette derived from the plasmid CB098 (**Table 2-2**).

The Fus1-GFP reporter plasmid pCB095 was constructed from the vector pRS306<sup>8</sup> using standard cloning techniques (see **Table 2-2**). The plasmid contained 1.5 kb region of the *MFA2* locus , followed by 750 bp of the *FUS1* 5' UTR, including the ATG start codon of *FUS1*. Yeast optimized eGFP was cloned downstream of the stop codon, followed by a 250 bp region of the *MFA2* 3' UTR. A HindIII site located within the 3' region was used to linearize and x-form the vector for transformation onto synthetic dropout (SD) –uracil media. Colonies were screened for GFP fluorescent signal, and then replated onto SD complete + 5-fluoroorotic acid in order to loop out the *URA* gene cassette. *Aura* colonies were screened for  $\alpha$ -factor dependant increase in fluorescent signal, and the strain with the greatest difference between induced and uninduced fluorescent signal. The reporter strain CB009 was constructed from strain CB008 using this approach, and strain CB010, 011 and 012 were constructed by making the appropriate knockouts in CB009 (see **Table 2-1** for strain genotypes and lineages).

For quantitative experiments performed in this section using either strains CB009 or CB011 (See **Table 2-2**) cells were grown overnight in 1 mL of the appropriate SD media at 30 °C under constant shaking. This was followed by 4-5 hours of outgrowth to log phase in 3 mL cultures. Cells were treated with indicated concentrations of  $\alpha$ -factor and sampled at indicated times using an LSR-II flow cytometer (BD Biosciences). Just prior to reading, cells were exposed to brief sonication at low power to disrupt cell clumps. Cellular fluorescence was measured by exciting at 488 nm with a 20 mW Sapphire argon ion laser (Coherent) detecting emission on the FITC channel using 515-545 nm filters. For each reading, 10,000 events were recorded.

Fluorescence data from flow cytometry experiments were analyzed using the program FlowJo (TreeStar, Inc.). Cell populations were gated based upon forward scatter to exclude cell aggregates. After pheromone treatment, a corresponding population of cells shifted along the FITC axis to higher fluorescence at the same forward scatter. The gate used for data analysis in the absence of alpha factor was allowed to re-center on the treated population using the “magnetic gate” feature of the FlowJo software. Mean cellular fluorescence was calculated for this gated population. Data for dose-response experiments were fit using ProFit software (Quantum Soft) to a Hill equation:  $F(a) = F_{\min} + (F_{\max} - F_{\min}) * a^{n_H} / (C_m^{n_H} + a^{n_H})$ , where  $F$  = mean fluorescence,  $a$  = concentration of alpha factor,  $F_{\min}$  = mean fluorescence with no alpha factor,  $F_{\max}$  = mean fluorescence with saturating  $\alpha$ -factor,  $C_m$  = concentration of alpha factor at which fluorescence is half-maximal, and  $n_H$  = Hill coefficient.

# Identification of Recrutable Mating Pathway Modulators for Use in Mating Pathway Circuit Engineering

## *Introduction*

Scaffold proteins are thought to contribute to signaling specificity by increasing the efficiency of interaction between pathway members (see **Chapter 1**). Given the role of scaffolds in recruiting pathway components, we hypothesized that synthetic recruitment interactions could be used to harness the localization properties of a scaffold for the purpose of building new network connections into a signaling pathway. In the yeast mating MAP kinase pathway, Ste5 assembles the core MAP kinase cascade responsible for transducing mating signal. This cascade consists of Ste11 (MAPKKK), Ste7 (MAPKK), and Fus3 (MAPK). Ste5 is also responsible for localizing the cascade to the membrane in proximity to its upstream activators<sup>9,10</sup>. Because of the central role that it plays in organizing the pathway components during pathway induction, we hypothesized that Ste5 might be useful as a hub for recruiting modulating factors that could either positively or negatively impinge on pathway signaling. Once identified, the modulators could potentially be used in creating feedback circuits.

## *Results and Discussion*

We reasoned that effective localization-dependent modulators would do little to alter pathway activity when overexpressed in an unlocalized fashion, but would up- or down-regulate pathway activity dramatically when tethered to Ste5 via a flexible peptide linker. Therefore, our testing strategy involved **1)** expressing the candidates using strong, constitutively active promoter to drive expression, and **2)** expressing alleles of Ste5 in which modulator candidates were fused to the scaffold's C-terminus (see **Figure 2-3**). We tested a number of factors involved in mating pathway response. We included pathway components that are part of the mating pathway MAP kinase module, including Ste11, Ste7, Ste50, and Ste20. We also tested a weak, constitutively activating allele of Ste11, Ste11 $\Delta$ N<sup>11</sup>, as well as Fus1, a membrane anchored protein that participates in mating partner fusion and localizes to mating projections during mating response<sup>12</sup>. Various subunits that comprise the heterotrimeric G-protein that mediates receptor coupled Ste5 recruitment and subsequent pathway activation were also tested. These include, GPA1 (G $\alpha$ ), Ste4 (G $\beta$ ), and Ste18 (G $\gamma$ ), and Sst2, a GAP that regulates GPA1<sup>13</sup>. Additionally, three phosphatases (Msg5, Ptp2, and Ptp3) that are known to act on Fus3 were used in the screen<sup>14</sup>.

**Figure2-3A** shows the results of overexpression of the modulator candidates on pheromone-dependent pathway activation as assayed by FACS-GFP (see previous section for description of the reporter strains and assay). Strains harboring over expressed candidates showed only marginal effects on pathway induction when compared with the WT pathway. Several components showed weak enhancement

of activation, including Ste50, Ste11 $\Delta$ N, and Ste4. Ste11 $\Delta$ N caused a slight increase in both basal and induced pathway activation owing to its ability elevate pathway activity independently of  $\alpha$ -factor. Ste4 also demonstrate pheromone-independent activation, consistent with its role in initiating signaling via the recruitment of Ste5 to the membrane<sup>15</sup>. Overexpression of each of the three phosphatases also lowered pathway activation relative to the WT pathway.

When modulator candidates were tethered to Ste5, a number of them showed dramatic positive and negative regulation of the pathway (**Figure 2-3B**). While tethered Ste7 and Ste11 demonstrated marginal enhancement of pathway activation, the tethered Ste50 allele showed a nearly four-fold activation enhancement. Ste20-, Ste11 $\Delta$ N- and Fus1-tethered Ste5 alleles all showed full constitutive pathway activation in the absence of pheromone, and were induced only marginally by the addition of pheromone. Effects of tethering G-protein cycle-related candidates to Ste5 were varied. Tethered Ste4 showed constitutive pathway activation, while tethered GPA1, Sst2, and Ste18 had the effect of significantly lowering pathway output. The three tethered phosphatases demonstrated the most significant down-regulation of the pathway, lowering both basal and induced pathway to levels significantly below WT, and rendered the pathway insensitive to pheromone.

Based on results in **Figure 2-2**, we selected three modulators for further analysis: Ste50, Ste11 $\Delta$ N, and Msg5. Each of these modulators shows little or no regulation of

pathway induction when over expressed, but show dramatic effects when tethered. Additionally, each of these factors exhibits a different mode of pathway regulation. Ste50 is a positive, pathway-dependent modulator that, when expressed and localized, enhances pheromone activation of the pathway. Ste11 $\Delta$ N is a positive modulator that activates the pathway independently of pheromone. Msg5 is a negative modulator, the localized expression of which inhibits pheromone-dependent pathway activation. In order to fully assess the effects of the modulators on pathway signaling, we performed detailed time course and dose response experiments (**Figure 2-4**). For all modulators, increase in GFP accumulation is monotonic over a 180 min period following treatment with saturating pheromone. Only the Ste5 allele harboring tethered Msg5 showed no obvious pathway activation. Hill coefficients calculated from dose response profiles for both over expressed, and tethered modulators ( $n_H = 1.1 - 1.3$ ) were similar to the WT ( $n_H = 1.1$ ).  $EC_{50}$  values were similar to the WT as well, indicating that while the dose response curves were shifted in amplitude, the modulators caused no fundamental alteration in the linearity or sensitivity of the pathway.

We tested the ability of the pathway modulators Msg5, Ste50, and Ste11 $\Delta$ N to regulate pathway activation when reversibly bound to the scaffold. In order to mediate binding, we used a pair of synthetic, high-affinity leucine zippers<sup>16</sup> that homodimerize with a  $K_d = \sim 12$  pM, but show weak ( $>10\mu$ M) homodimerization. One of the zippers in the pair was fused to the C-terminus of each of the modulators, while the other was fused to the C-terminus of Ste5. **Figure 2-5** shows results from

these recruitment experiments. When scaffold and modulators fused to cognate zippers were expressed together, the modulators were recruited and able to regulate pathway induction. When non-cognate (homo) zippers were expressed together, the modulators had little effect on signaling. These results demonstrate that modulators can regulate mating pathway induction when reversibly recruited, and thus meet our requirements for parts that are useable for synthetic circuit construction.

We also investigated the ability of two effector proteins from the bacterial pathogen *Yersinia pestis*<sup>17</sup> to function as localization-dependent modulators. YopH and YopJ are both known to down regulate ERK signaling pathways in mammalian cells. YopH is a tyrosine phosphatase that directly dephosphorylates and inactivates ERK<sup>18</sup>, and YopJ is an acetylase which blocks MEK (MAPKK) activation<sup>19</sup>. In addition to WT versions, we also tested catalytically dead versions of each of effector in order to demonstrate that the catalytic activities associated with each of the modulators were indeed the basis of the recruited effector's regulatory effect. **Figure 2-6** shows data from overexpression, tethering, and reversible recruitment experiments, and demonstrates that both effectors can function as localization-dependent modulators, and that their effect on pathway induction is largely dependent on their respective catalytic activities.

## ***Materials and Methods***

All constructs used in this work were generated using standard molecular biology techniques (see **Table 2-1** a list of the constructs used in this study). Modulator coding sequences were obtained by PCR amplification from commercially available genomic DNA (Invitrogen). Sequences for all modulators that were tested for overexpression included both native start and stop codons. These were cloned into a pRS314 plasmid downstream of a 250 bp region immediately upstream of the ATG in *CYC1*. Tethered alleles were generated by cloning candidate ORF's (including start and stop codons) into pRS316 downstream of *STE5*, followed by sequence coding for a six amino acid linker (GSGSGS). Transcription of the fusion was driven by the promoter 500 bp 5' UTR region located immediately upstream of the Ste5 start codon. For leucine zipper recruitment experiments, the following sequences<sup>14</sup> were used for the acidic (EE12RR345L) and basic (RR12EE345L) zippers:

EE12RR345L:

```
ggatccggatctggatcgggatctgatccagatttgaaattgaagctgctttttggaaagagaaaatactgctttggaa
actagagttgctgaattgagacaaagagttcaaagattgagaaatagagtttctcaatagaactagatatggccattg
ggtggtggtaaataa
```

RR12EE345L:

```
ggatctggatctggatcgggatctgatccagatttgaaattagagctgctttttgagacaaagaaatactgctttgagaa
ctgaagttgctgaattggaacaagaagttcaaagattggaaaatgaagtttctcaatagaaactagatatggccattgg
gtggtggtaaataa
```

Zipper fusion constructs used for the reversible recruitment experiments were also pRS314- and pRS316-based with the *pCYC1* and *pSTE5* promoter sequences described above respectively driving transcription of the modulator and scaffold

fusions. Zippers were cloned downstream of the modulator and scaffold coding sequences and separated by a six amino acid linker (GSGSGS). YopJ and YopH coding sequences were generously provided by Kim Orth. All over expression experiments were conducted in CB009, while tethering and recruitment experiments were conducted using CB011 as background (**Table 2-2**). FACS analysis of mating pathway induction, including both time course and dose-response experiments, was performed as described in the materials and methods of the previous section.

**Table 2-1.** Plasmids used in this study.

| Plasmid | Parent vector | Promoter     | Gene(s)               |
|---------|---------------|--------------|-----------------------|
| CB095   | pRS306        | <i>pCYC1</i> | <i>GFP</i>            |
| CB011   | pRS314        | <i>pCYC1</i> | <i>MSG5</i>           |
| CB017   | pRS314        | <i>pCYC1</i> | <i>PTP2</i>           |
| CB018   | pRS314        | <i>pCYC1</i> | <i>PTP3</i>           |
| CB029   | pRS314        | <i>pCYC1</i> | <i>YOPH</i>           |
| CB035   | pRS314        | <i>pCYC1</i> | <i>YOPJ</i>           |
| CB041   | pRS314        | <i>pCYC1</i> | <i>SST2</i>           |
| CB042   | pRS314        | <i>pCYC1</i> | <i>GPA1</i>           |
| CB053   | pRS314        | <i>pCYC1</i> | <i>STE4</i>           |
| CB059   | pRS314        | <i>pCYC1</i> | <i>STE7</i>           |
| CB065   | pRS314        | <i>pCYC1</i> | <i>STE11</i>          |
| CB067   | pRS314        | <i>pCYC1</i> | <i>STE18</i>          |
| CB071   | pRS314        | <i>pCYC1</i> | <i>STE20</i>          |
| CB077   | pRS314        | <i>pCYC1</i> | <i>STE50</i>          |
| CB089   | pRS314        | <i>pCYC1</i> | <i>STE11DN</i>        |
| CB091   | pRS314        | <i>pCYC1</i> | <i>FUS1</i>           |
| CB101   | pRS316        | <i>pSTE5</i> | <i>STE5, MSG5</i>     |
| CB102   | pRS316        | <i>pSTE5</i> | <i>STE5, PTP2</i>     |
| CB103   | pRS316        | <i>pSTE5</i> | <i>STE5, PTP3</i>     |
| CB104   | pRS316        | <i>pSTE5</i> | <i>STE5, YOPH</i>     |
| CB105   | pRS316        | <i>pSTE5</i> | <i>STE5, YOPJ</i>     |
| CB106   | pRS316        | <i>pSTE5</i> | <i>STE5, SST2</i>     |
| CB107   | pRS316        | <i>pSTE5</i> | <i>STE5, GPA1</i>     |
| CB108   | pRS316        | <i>pSTE5</i> | <i>STE5, STE4</i>     |
| CB110   | pRS316        | <i>pSTE5</i> | <i>STE5, STE7</i>     |
| CB112   | pRS316        | <i>pSTE5</i> | <i>STE5, STE11</i>    |
| CB113   | pRS316        | <i>pSTE5</i> | <i>STE5, STE18</i>    |
| CB114   | pRS316        | <i>pSTE5</i> | <i>STE5, STE20</i>    |
| CB116   | pRS316        | <i>pSTE5</i> | <i>STE5, STE50</i>    |
| CB117   | pRS316        | <i>pSTE5</i> | <i>STE5, STE11ΔN</i>  |
| CB125   | pRS316        | <i>pSTE5</i> | <i>STE5, FUS1</i>     |
| CB321   | pRS316        | <i>pSTE5</i> | <i>STE5, ZipRR</i>    |
| CB322   | pRS316        | <i>pSTE5</i> | <i>STE5, ZipEE</i>    |
| CB334   | pRS314        | <i>pCYC1</i> | <i>MSG5, ZipRR</i>    |
| CB335   | pRS314        | <i>pCYC1</i> | <i>MSG5, ZipEE</i>    |
| CB341   | pRS314        | <i>pCYC1</i> | <i>Ste50, ZipRR</i>   |
| CB342   | pRS314        | <i>pCYC1</i> | <i>Ste50, ZipEE</i>   |
| CB354   | pRS314        | <i>pCYC1</i> | <i>Ste11ΔN, ZipRR</i> |
| CB355   | pRS314        | <i>pCYC1</i> | <i>Ste11ΔN, ZipEE</i> |
| CB356   | pRS314        | <i>pCYC1</i> | <i>YopH, ZipRR</i>    |
| CB357   | pRS314        | <i>pCYC1</i> | <i>YopH, ZipEE</i>    |
| CB358   | pRS314        | <i>pCYC1</i> | <i>YopJ, ZipRR</i>    |
| CB359   | pRS314        | <i>pCYC1</i> | <i>YopJ, ZipEE</i>    |

**Table 2-2.** Strains used in this study.

| <i>Strain</i>         | <i>Description</i>                                                                       |
|-----------------------|------------------------------------------------------------------------------------------|
| <b>SΦ992 (parent)</b> | <i>W303 MATa, STE5, BAR1, FAR1, MFA2, his3, trp1, leu2, ura3</i>                         |
| <b>CB001</b>          | <i>W303 MATa, STE5, bar1::NatR, FAR1 MFA2, his3, trp1, leu2, ura3</i>                    |
| <b>CB002</b>          | <i>W303 MATa, STE5, BAR1, FAR1 mfa2::pFUS1-GFP, his3, trp1, leu2, ura3</i>               |
| <b>CB003</b>          | <i>W303 MATa, ste5::KanR, BAR1, FAR1, mfa2::pFUS1-GFP, his3, trp1, leu2, ura3</i>        |
| <b>CB004</b>          | <i>W303 MATa, STE5, bar1::NatR, FAR1, mfa2::pFUS1-GFP, his3, trp1, leu2, ura3</i>        |
| <b>CB005</b>          | <i>W303 MATa ste5::KanR, bar1::NatR, FAR1, mfa2::pFUS1-GFP, his3, trp1, leu2, ura3</i>   |
| <b>CB006</b>          | <i>W303 MATa, STE5, bar1::NatR, FAR1, mfa2::pFUS1-GFP, his3, trp1, leu2, ura3</i>        |
| <b>CB007</b>          | <i>W303 MATa, ste5::KanR, bar1::Nat, FAR1, mfa2::pFUS1-GFP, his3, trp1, leu2, ura3</i>   |
| <b>CB008</b>          | <i>W303 MATa, STE5, , bar1::NatR, , far1Δ, MFA2, his3, trp1, leu2, ura3</i>              |
| <b>CB009</b>          | <i>W303 MATa, STE5, , bar1::NatR, , far1Δ, mfa2::pFUS1-GFP, his3, trp1, leu2, ura3</i>   |
| <b>CB010</b>          | <i>W303 MATa, ste5::KanR, , bar1::NatR, , far1Δ, MFA2, his3, trp1, leu2, ura3</i>        |
| <b>CB011</b>          | <i>W303 MATa, ste5::KanR, bar1::NatR, far1D, mfa2::pFUS1-GFP, his3, trp1, leu2, ura3</i> |

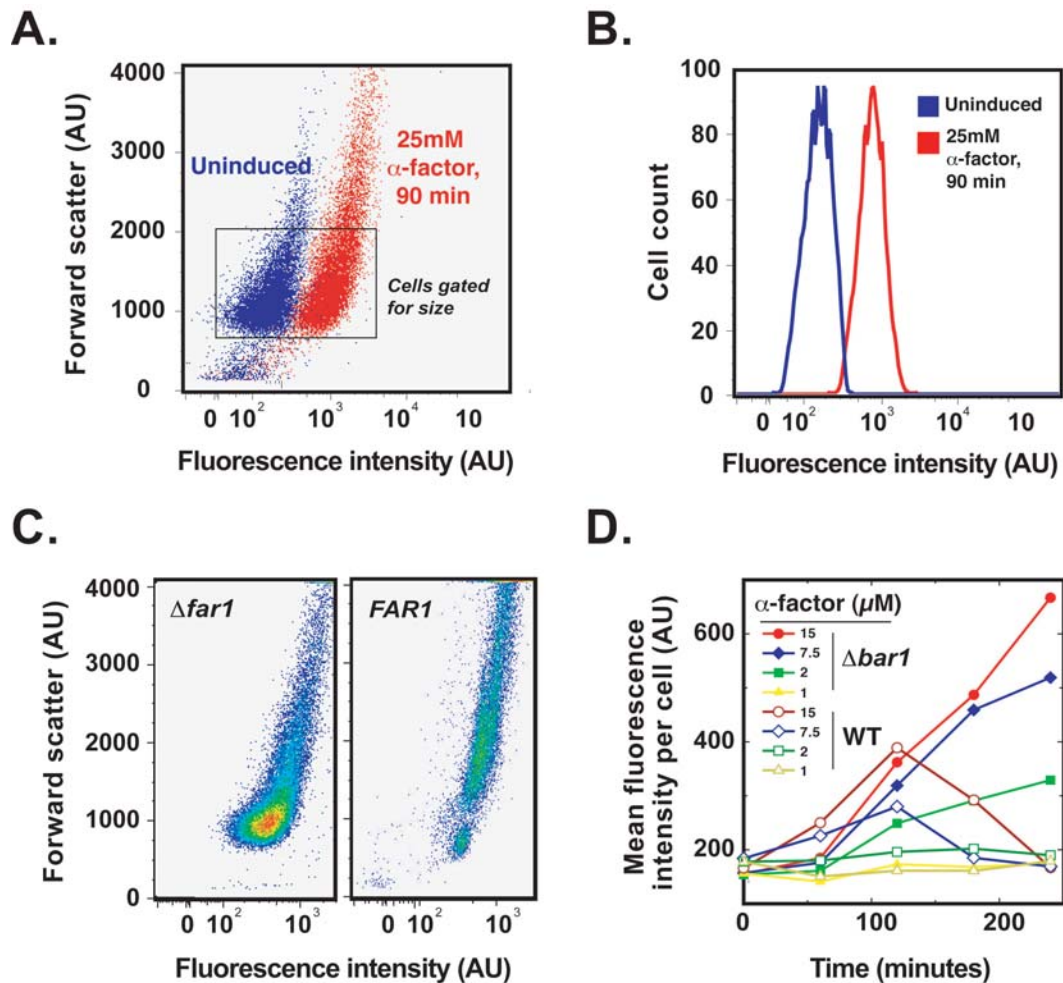


**Figure 2-1.** Construction of a fluorescent, transcription-based reporter for use in single-cell analysis of the yeast mating pathway. **A.** Upon treatment with saturating concentrations of  $\alpha$ -factor, cells harboring the *pFUS1*-GFP reporter exhibited an increase in fluorescence intensity as measured by FACS. **B.** During data analysis of pathway induction experiments, cells were gated for size based on their forward scatter. Mean fluorescence intensity per cell values used throughout this study were calculated from these populations. **C.** A knockout of the gene *FAR1* was incorporated into the reporter strain in order to avoid cell swelling and GFP retention associated with mating pathway-induced cell cycle arrest. Populations of cells depicted were treated with pheromone for 90 minutes prior to being assayed by FACS. **D.** Knocking out *BAR1* from the reporter strain eliminated pathway-dependent degradation of alpha factor, permitting the analysis of the pathway reporter. Cultures of either *BAR1* or  $\Delta bar1$  cells harboring the reporter cassette were treated with various concentrations of pheromone and sampled at various time points for analysis by FACS-GFP assay

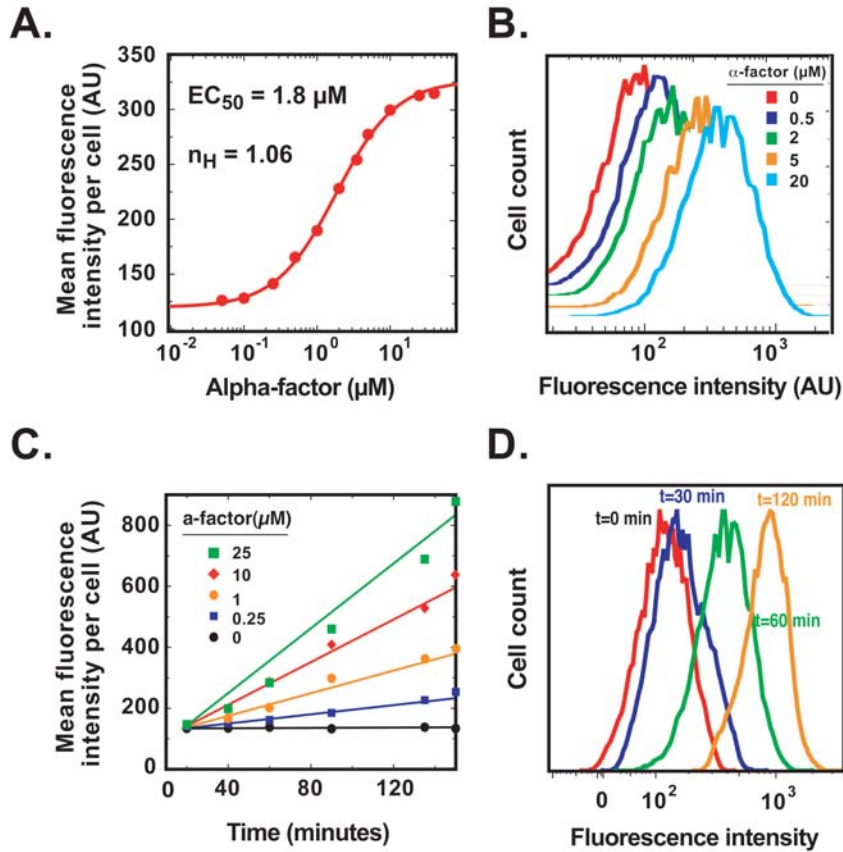
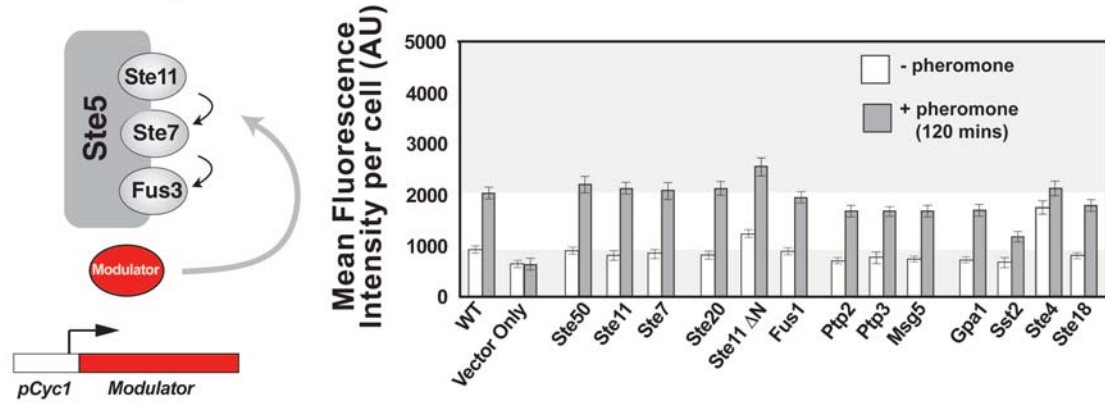


**Figure 2-2.** Dose-response and time course analysis by FACS of the *pFUS1*-GFP reporter strain reveals that the yeast mating pathway responds in a linear and graded fashion with respect to  $\alpha$ -factor dose, and exhibits simple dynamic behavior. **A.** Pathway activation in the reporter strain CB009 was measured by FACS-GFP assay following 90min of  $\alpha$ -factor treatment at various concentrations. Data were fitted to the Hill equation in order to determine  $EC_{50}$  values and Hill coefficient. **B.** Histogram representations for several of the cell populations used to calculate mean fluorescence intensity values in panel A are shown, demonstrating monomodality over the entire range of the  $\alpha$ -factor dose response curve. **C.** Over the full concentration range of the  $\alpha$ -factor dose-response curve, the pathway exhibits linear, monotonic increases in GFP fluorescence. **D.** Histogram representations of a 25  $\mu$ M trace in panel 3 demonstrates that populations of cells are monomodal at all points throughout the induction time course.

### A. Overexpression



### B. Tethering

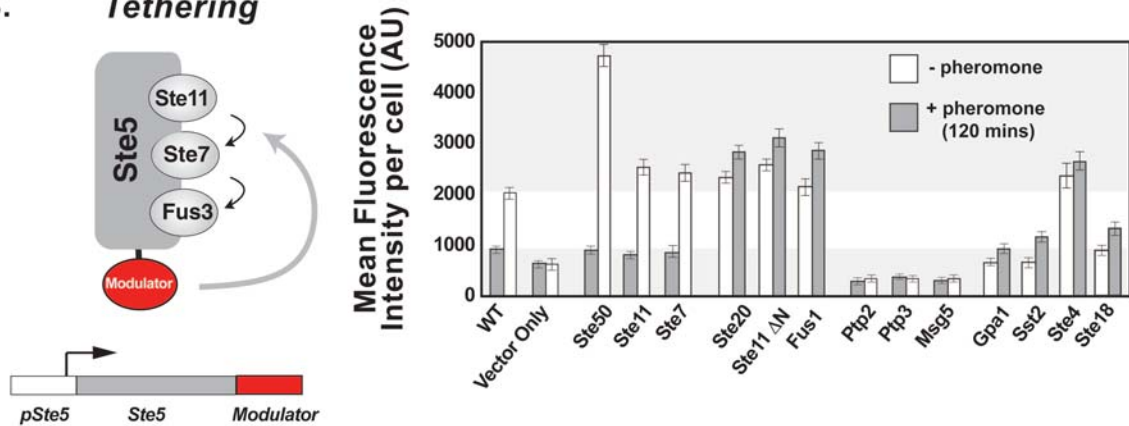


**Figure 2-3.** Identification of factors that modulate mating pathway activity in a localization-dependent manner. **A.** Screening pathway modulator candidates for effect of overexpression on pathway induction. Candidates were expressed on a single copy plasmid using *pCYC1* to drive transcription. Strains harboring overexpression constructs were sampled for pathway activation before and 120 min after treatment with a saturating concentration of  $\alpha$ -factor. **B.** Screening pathway modulator candidates for effects of covalent fusion to the scaffold on pathway induction. Candidates were expressed on a single copy plasmid in CB011.

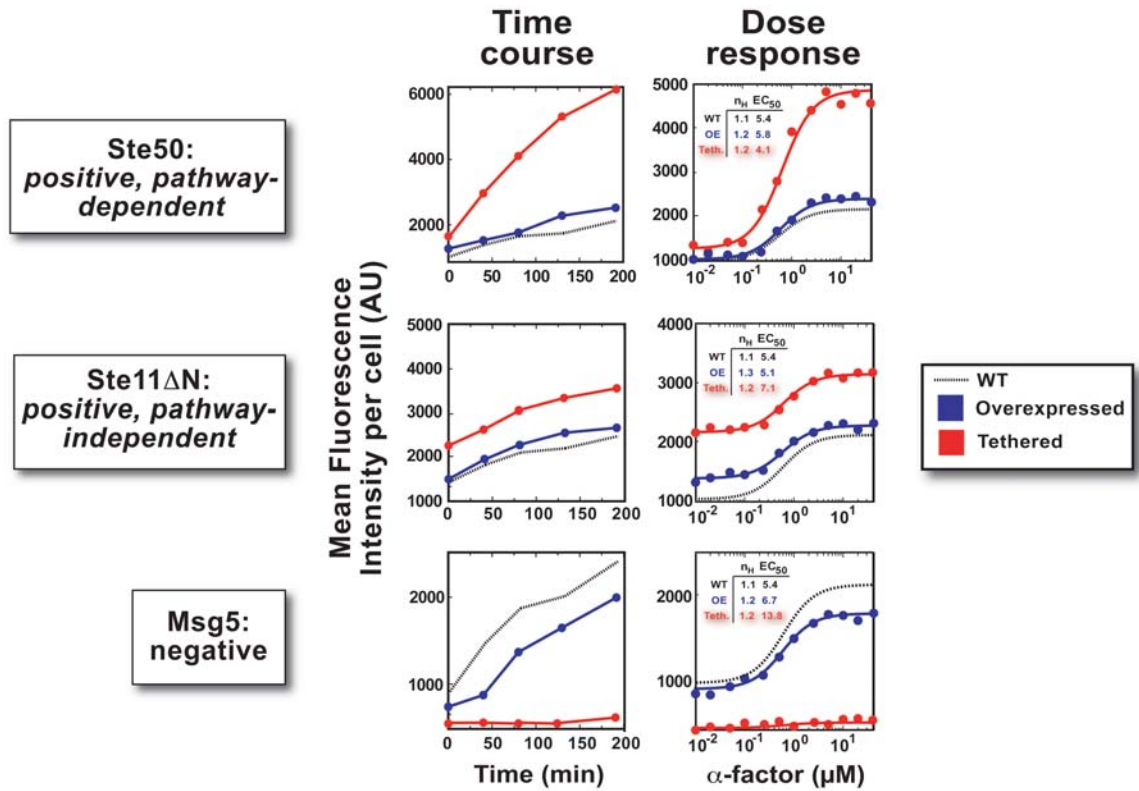


**Figure 2-4.** Time course and dose-response analysis of three modulator candidates: Ste50, Ste11ΔN, and Msg5. Strain harboring Msg5, Ste11ΔN, and Ste50 that were used in **Figure 2-3** were chosen for this analysis based on their ability to modulate the pathway when tethered but not overexpressed (see **Figure 2-3**). Experiments were conducted, and values for  $n_H$  and  $EC_{50}$  were determined, as described in previous sections.



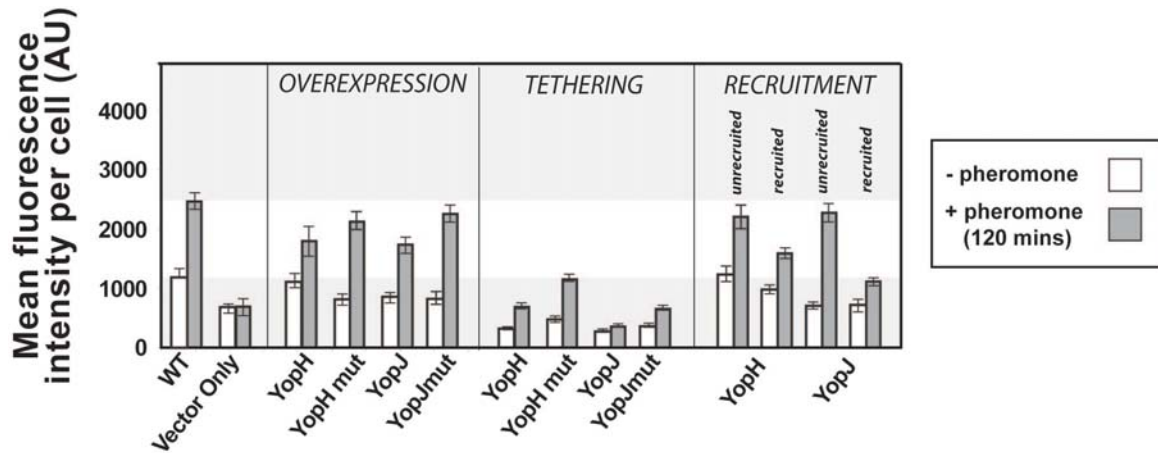


**Figure 2-6.** Bacterial pathogen effector proteins YopH and YopJ are capable of localization-dependent down regulation of the mating pathway. YopH and YopJ were evaluated for their ability to function as modulators in the mating pathway using overexpression, tethering, and leucine zipper recruitment (see materials and methods for details). Cells were assayed for mating pathway by FACS-GFP. The WT proteins were compared to catalytically inactive mutant proteins for overexpression and tethering experiments.

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## **Chapter 3**

### **Using Engineered Scaffold Interactions to Reshape MAP Kinase Pathway Signaling Dynamics**

## **Abstract**

Scaffold proteins link signaling molecules into linear pathways by physically assembling them into complexes. Scaffolds may have a higher-order role as signal processing hubs, serving as the target of feedback loops that optimize signaling amplitude and timing. We demonstrate that the Ste5 scaffold protein can be used as a platform to systematically reshape output of the yeast mating MAP kinase pathway. We constructed synthetic positive and negative feedback loops by dynamically regulating recruitment of pathway modulators to an artificial binding site on Ste5. These engineered circuits yielded diverse behaviors: ultrasensitive dose-response, accelerated or delayed response times, and tunable adaptation. Thus protein scaffolds are an extremely flexible platform for reprogramming complex cellular responses.

## **Introduction**

In cells, signaling proteins that make up a pathway are often physically organized into complexes by scaffold proteins<sup>1-3</sup>. Scaffolds direct information flow: they promote signaling between proper protein partners, and prevent improper crosstalk. Scaffolds may also play a role in shaping the quantitative response behavior of a pathway. The scaffold complex could serve as a central hub for feedback loops that modulate the recruitment or activity of pathway members on the scaffold. Such feedback loops could tune pathway dose-response and dynamics – the change in output over time. Quantitative response behavior is critical for signaling -- the behavior of a pathway must match its specific physiological function<sup>4</sup>. Scaffolds may therefore provide a platform for evolutionarily tuning response behaviors for optimal fitness<sup>5,6</sup>.

## **Results and Discussion**

We used a synthetic biology approach to explore this hypothesis: we tested whether a scaffold protein can be used as a platform for engineering synthetic feedback loops, and whether these loops can be used to systematically reshape pathway response behavior. We used the yeast mating mitogen activated protein (MAP) kinase pathway as a model system because it is highly tractable for pathway

engineering. First, connectivity of this pathway is coordinated by the scaffold protein Ste5, which binds the three core kinases – Ste11 (a MAP kinase kinase kinase or MAPKKK), Ste7 (a MAP kinase kinase, or MAPKK), and Fus3 (a MAP kinase, or MAPK) — that successively phosphorylate and activate one another (**Figure 3-1A**)<sup>7-10</sup>. Second, MAP kinase pathways appear to be functionally plastic: they are found in all eukaryotic species, but in individual cases display widely varied behaviors. For example, the yeast mating pathway shows a largely linear transcriptional response<sup>11-13</sup>, whereas the *Xenopus* oocyte maturation pathway displays an switch-like dose response<sup>14</sup>. MAPK pathways also show diverse dynamic behavior: some yield a sustained response to stimulation, whereas others show a pulse-like transient response. These different pathway dynamics are critical for determining physiological output<sup>15-20</sup>.

Our goal was overlay the endogenous pathway with synthetic feedback loops in order to systematically alter response to mating pheromone (alpha-factor) stimulation. A simple way to construct a synthetic feedback loop would be to dynamically recruit pathway modulators to the scaffold in a manner that is dependent on pathway output. We first tested whether constitutive recruitment of modulator proteins could alter pathway flux. We created a new recruitment site on Ste5 by fusing a leucine zipper heterodimerization module<sup>21</sup> to its C-terminus. Modulator proteins fused to complementary zippers were then expressed and recruited to the scaffold (**Figure 3-1B**) (this leucine zipper pair interacts with  $K_d = 6 \text{ nM}$ <sup>21</sup> - **Figure 3-S3**). Two pathway modulators were recruited: Ste50 and Msg5 (**Figure 3-1A**). Ste50 is a positive modulator – an adaptor that promotes interaction of the MAPKKK Ste11 with its upstream activator, the PAK-like kinase,

Ste20<sup>22-23</sup>. Msg5 is a negative modulator – a MAPK phosphatase that inactivates phosphorylated Fus3 MAPK<sup>24-25</sup>. When artificially recruited to the Ste5 scaffold through a leucine zipper interaction, Msg5 and Ste50 showed strong but opposite effects on pathway output, measured using a mating pathway-responsive green fluorescent protein (GFP) transcriptional reporter (See SOM or reference 26 for methods). Recruitment of the positive modulator (Ste50), increased the steady-state output of the activated pathway, while recruitment the negative modulator (Msg5), decreased output, nearly eliminating any input–stimulated response. Unrecruited Ste50 and Msg5 had much smaller effects on pathway output when expressed at the same level. Thus the impact of modulators on pathway flux is enhanced by recruitment to the scaffold.

To build synthetic feedback loops we then placed the modulators under the control of a mating-dependent promoter (*pFIG1*; **Figure 3-2A**), so that they are only expressed upon pathway activation. The positive feedback circuit, (using Ste50) increased steady-state pathway output and led to a more switch-like dose-response. The apparent Hill coefficient ( $n_H$ ) increased from  $1.12 \pm 0.08$  (wild-type) to  $2.42 \pm 0.19$ . Similar behavior was generated using positive feedback loops in which constitutive pathway alleles were expressed from a mating-responsive promoter<sup>27</sup>.

The engineered negative feedback circuit (using Msg5) displays adaptation – the cells responded initially like wild-type, but after 35 minutes, showed a decrease in output, even with continued stimulation. Adaptation is a critical for homeostatic and sensing systems. It can be important to limit the magnitude and duration of an output that is harmful or has a high metabolic cost. Adaptation is also critical for sensing systems (e.g. vision or

chemotaxis) that automatically desensitize to a continuous stimulus, allowing for detection of input changes over a large dynamic range<sup>28,29</sup>.

One advantage of engineered feedback loops is the ability to systematically explore how the alteration of specific circuit parameters affects pathway behavior. Simulations indicate that adaptation in the simple negative feedback circuit can be tuned by adjusting feedback strength (**Figure 3-3A**). We explored two methods for adjusting feedback strength. First, we changed the strength of the leucine zipper interaction used to recruit Msg5 (**Figure 3-3B**), using a set of three leucine zipper pairs that bind with the following affinities:  $K_d = 6$  nM, 40 nM, and 810 nM<sup>21</sup>. Second, we changed the strength of promoter controlling expression of recruited Msg5 (**Figure 3-3C**), using a pair of mating promoters: *pFIG1* (strong) and *pPRM2* (weak -see **Figure 3-S4**). As predicted, either method results in a decrease in the steady-state pathway output.

Because these synthetic feedback circuits rely on modulator recruitment, they could be regulated by competitive interactions that block recruitment. We tested whether competitors could be used to build more complex negative feedback circuits that displayed a pulse-like activation response with higher maximal output (**Figure 3-4A**). We constitutively expressed a decoy leucine zipper that competes with the scaffold protein (Ste5-zipper) for binding to the negative modulator (Msg5-zipper). Because the decoy has a higher affinity, it initially acts as a sink – after pathway activation, newly expressed negative modulator is bound to the decoy zipper, preventing recruitment to the scaffold. Only after the decoy zipper is saturated, is additionally expressed negative modulator recruited to the scaffold, resulting in

pathway repression. Indeed, this delayed negative feedback loop leads to a pulse-like response (higher maximal output, followed by decrease in output). Moreover, the sharpness of the response can be modulated by adjusting the level at which the decoy zipper is expressed – higher decoy expression leads to more pulse-like response.

The interplay of competing scaffold interactions and variable expression can be used to generate additional dynamic behaviors, including systems with faster or slower response time. The rise-time of a pathway– how fast a response occurs after input – can be critical for function. A pathway that detects a toxic stress may require a fast response. A delayed response may be required if the response is energetically very costly or if there is a high level of input noise (a delay circuit could prevent mis-activation by transient input fluctuations, while allowing activation by a sustained input).

We were able to alter the mating pathway to show accelerated response time, while still maintaining a wild-type level of maximal pathway output (**Figure 3-4B**). In this accelerator circuit, the positive modulator (Ste50-zipper) was constitutively expressed, but the negative modulator (Msg5-zipper, high affinity) was inducibly expressed. This result supports the paradigm that negative feedback can speed the time it takes to reach steady-state<sup>30</sup>, albeit at a reduced steady-state output. But here, the wild-type magnitude of output is achieved by the added presence of the positive modulator.

We also generated a delay circuit by constitutively expressing a negative modulator (Msg5-zipper) and inducibly expressing a high affinity decoy zipper (**Figure 3-4C**). The pathway initially showed a weak response to stimulation because the recruited negative modulator keeps the pathway shut off. After a delay of ~ 50 minutes, however, a sufficient level of pathway activation is reached, and expression of the high affinity decoy zipper displaces the negative modulator, allowing pathway activation.

Competition between positive and negative modulators can also be used to alter dose-response behavior. We built a circuit with enhanced ultrasensitive switch behavior by constitutively expressing a negative modulator (Msg5-zipper) and inducibly expressing a positive modulator (Ste50-zipper) (**Figure 3-4D**). This circuit is a double positive feedback loop – induced expression of Ste50-zipper directly increases pathway output, but also relieves the inhibitory effect of Msg5-zipper by displacing it from the scaffold. The dose response profile for the resulting circuit showed a increased in cooperativity (apparent Hill coefficient  $n_H = 2.84 \pm 0.19$  vs.  $1.21 \pm 0.06$  for no-feedback circuit and  $2.42 \pm 0.19$  for positive feedback only circuit).

We have used a simple principle – recruitment of pathway modulators to a scaffold<sup>31</sup> – to systematically alter a single MAPK pathway so that it displays a wide range of quantitative response behaviors. The evolutionary diversification afforded by scaffolds may explain their common use in diverse signaling pathways [32-35]. The success of this simple recruitment-based strategy suggests that it may be

possible to reprogram cellular responses with the precision that control engineers have in designing man-made electronic circuits. Given the central importance of MAPK cascades in mammalian and plants cells in regulating differentiation, proliferation, apoptosis, and immune and stress responses, this approach may facilitate the engineering of cells with novel therapeutic or biotechnological functions.

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## Materials and Methods

Synthetic circuits were introduced into appropriate yeast strains as two distinct plasmid-encoded components: 1) a synthetic scaffold consisting of Ste5-leucine zipper fusion 2) recruited effector/decoy constructs consisting of different promoters driving transcription of a effector/decoy-zipper fusion. General architecture of constructs containing both of these components, as well as sequence details of the promoter and terminator regions used in plasmid construction are provided in Figure 3-S1. All promoters, terminators, and yeast genes (*STE5*, *MSG5*, *STE50*) were cloned from a *Saccharomyces cerevisiae* cDNA library (Invitrogen) by PCR.

Leucine zippers used in scaffold and effector fusions were hetero-oligomerizing zippers designed by Vinson and colleagues that are derived from the human PAR family member VBP<sup>37</sup>. These zippers were chosen as generic recruitment modules because they are likely to be orthogonal to yeast proteins – exclusively interacting with each other. We experimentally confirmed that expression of zippers alone had no effect on the behavior of the mating pathway (**Figure 3-S2**, panels C and D). Furthermore, there are defined mutants of the zippers that can be used to systematically tune interaction affinity over a range of  $10^3$  (**Figure 3-S2A**). Details of leucine zipper sequences, identity of zipper binding partners, and binding affinities for different pairs are provided in **Figure 3-S2**. Specific plasmid

constructs used in this study are listed in **Table 3-S1**, and the identity of the constructs used for circuit construction in each experiment are listed in **Table 3-S2**.

Genotypes of yeast strains used in this study are listed in **Table 3-S3**. Gene knockouts were made using standard gene disruption techniques. Unless specifically indicated, all experiments were conducted in the background strain CB011, a  $\Delta far1$  strain which does not show cell cycle arrest upon  $\alpha$ -factor treatment, and shows no shmooing behavior. Use of this strain is necessary for quantitatively reproducible FACS analysis, as Far1-dependent cell cycle arrest leads to a wide variation in cell size and GFP expression per cell<sup>38</sup>. For this work, the  $\Delta far1$  strain is arbitrarily defined to be the starting “wild-type” strain, although it has slightly different behavior from a *FAR1* strain.

*STE5* synthetic scaffold constructs were derived from the parent vector HO-hisG-URA3-hisG-poly-HO (see **Figure 3-S1** for cloning details), which is designed for genomic integration into the HO locus<sup>39</sup>. Strains harboring circuits were built from CB011 by initially integrating synthetic scaffold constructs. Effector/decoy constructs were then introduced by genomic integration. These constructs were derived from either pRS305, which integrates into the *LEU2* locus, or pRS304 which integrates into the *TRP1* locus. Correct genomic integration of pRS304- and pRS305-based as well as HO-locus constructs into the appropriate marker loci was verified by performing PCR on colony lysates.

In order to realized the behaviors for circuits depicted in **Figure 3-4**, we tested multiple configurations of promoter strength and zipper affinity for each specific architecture. Generally speaking, behaviors that were depicted in **Figure 3-4** were only exhibited by a limited number of “productive” combinations of molecular components. A summary of which configurations were constructed, and which were identified as productive is summarized in **Figure 3-S3**.

Quantitative activity of the various promoters used in this study were analyzed by RT-PCR (**Figure 3-S4**). Total RNA was isolated from yeast by hot acid phenol extraction<sup>40</sup>. Extracts were treated with DNase to remove contaminating genomic DNA, and template cDNA was synthesized from 1-3 µg total RNA using T18 as a primer. To further ensure that only cDNA and not genomic DNA was amplified during the PCR, controls were run to which no reverse transcriptase was added. Real time, quantitative PCR was done using a DNA engine Opticon machine (BioRad) with SYBR green as the fluorescent probe. Relative mRNA expression level of each measured gene in a given sample was determined by normalization to the transcript level of the gene *TDH1*.

Analysis of pathway-dependent GFP expression by flow cytometry was performed largely as described<sup>41</sup> with the following specific modifications: For all FACS-GFP experiments, triplicate cultures were grown to early log phase ( $OD_{600}=0.05-0.1$ ) in complete synthetic dropout media. For time course experiments, triplicate cultures were treated with 2 µM alpha-factor (Zymo Research) to initiate the pathway. For

dose-response experiments, three separate cultures were treated with each of the indicated alpha-factor concentrations. For time course experiments, aliquots of cultures were removed at 15 min intervals. For dose response experiments, cultures were typically sampled once at indicated times. For both types of experiments, sample aliquots were treated with cycloheximide (5 $\mu$ g/mL), and dispensed into 96-well culture plates. Following incubation at room temperature for 1 hr in the dark to allow for GFP fluorophore maturation, plates containing treated cultures were analyzed with a BD LSR-II flow cytometer (BD Biosciences) using a high-throughput sampling module. 5,000 cells were counted for each reading, and GFP fluorescence was measured by exciting at 488 nm with a 100 mW Coherent Sapphire laser.

Analysis of FACS-GFP fluorescence data for alpha-factor-treated cell populations has been described previously<sup>41</sup>. For the present study, mean fluorescence intensity data in time course experiments were converted to transcriptional rate data plots in **Figures 3-2, 3-3, and 3-4**. This conversion was carried out to more accurately reflect temporal variation in pathway output – absolute GFP expression is a poor direct readout of this, since the apparent lifetime of the GFP used here is >100 minutes, and thus it continuously accumulates over the course of the experiment. Transcriptional activity was calculated according to the process outlined in **Figure 3-S5**, by taking into account GFP synthesis and degradation/dilution. Data points in figures containing timecourse data represent mean transcriptional activity values for triplicate experiments  $\pm$  std. dev. Solid line fits in these figures represent

solutions to a quantitative dynamic model consisting of coupled differential equations (see “Quantitative Modeling” section).

Data points in figures containing dose-response data represent mean fluorescence intensity values for triplicate experiments  $\pm$  std. dev. Triplicate data from dose-response profiles were fitted using ProFit software (Quantum Soft) to a Hill equation:  $F(a) = (F_{\min} + (F_{\max} - F_{\min})) * (a^{n_H} / (C_m^{n_H} + a^{n_H}))$ , where  $F$  = mean fluorescence,  $a$  = concentration of alpha-factor,  $F_{\min}$  = mean basal fluorescence (no alpha-factor),  $F_{\max}$  = mean fluorescence with maximal pathway output (saturating alpha-factor),  $C_m$  = alpha-factor concentration at which fluorescence is half-maximal, and  $n_H$  = Hill coefficient. Error for Hill coefficients are std. dev. values determined by the Profit curve fitting algorithm and reflect errors amongst experimental replicates.

Yeast cells in mid-log phase ( $OD_{600}=0.5$ ) were treated with saturating alpha-factor (2  $\mu$ M) and 5 mL of culture was harvested at indicated time points. Samples were prepared from harvested cells for western blot experiments by lysis in SDS-PAGE buffer (350  $\mu$ L) and  $\sim 20$   $\mu$ L of sample were used for immunoblot detection.

Phosphorylated Fus3 was detected using an anti-phospho p44/42 antibody (Cell Signaling Technology) as primary followed by Goat anti-Rabbit IR@800 secondary (Odyssey). Blots were visualized using a LICOR Odyssey infrared imager. For hexokinase loading controls (see **Figure 3-S6**), blots were stripped and reprobed

with a yeast hexokinase antibody (US Biological, H2305-01) followed by LICOR detection using the same Goat anti-Rabbit IR@800 secondary.

## **Quantitative Modeling**

To assess whether the behaviors of the synthetic feedback circuits could be explained by the simple model of dynamically-regulated recruitment to the scaffold, we simulated pathway behavior using a system of three coupled ordinary differential equations (see below), tracking the activation states of the scaffold complex and a transcription factor and the population of the feedback element. Fits to the experimentally observed transcriptional activity (Figures 3-4) are derived from this model.

In this highly simplified model, the scaffold complex was considered as a lumped element (i.e. black box) with a fixed total population comprised of two states: active and inactive. The total population of the transcription factor was also assumed constant, and similarly composed of active and inactive versions. Scaffolds were activated by pheromone input with Michaelis-Menten kinetics. Inactive transcription factors were activated by active scaffolds. The population of active transcription factor was the computational readout, which we compared against the experimentally measured transcriptional activity (see Figure 3-S5).

To model feedback circuits, synthesis of effector proteins from mating responsive promoters was modeled as a function of active transcription factor using a Hill

equation ( $nH = 2$ ; this closely matches experimentally observed expression from the mating responsive promoter pFig1 – see Figure 3-S5). The binding of the synthesized feedback elements to the scaffold complex was explicitly calculated using a dissociation constant corresponding to the affinity of the leucine zippers. Negative (positive) feedback elements bound to the scaffold reduced (increased) the concentration of active scaffolds at a significantly higher rate than unbound feedback elements. For circuits that consist of multiple binding partners (e.g. feedback element that can bind to either the scaffold or a decoy element), the populations of the possible binding complexes were calculated explicitly by computationally solving a cubic equation, assuming that the populations come to binding equilibrium on a time scale short with respect to the other rates in the equations. Furthermore, the simulation began 100 min before the alpha-factor stimulus was added in order to allow the system to reach an uninduced equilibrium. Most parameters were kept constant amongst all circuits: activation rates, dissociation constants, decay constants, catalytic constants, total populations of scaffold and transcription factors, etc. Parameters that were allowed to vary somewhat include background basal scaffold activity and promoter strengths (we have observed that expression from a single promoter varied over a range of  $\sim 5$ -10 fold on average from clone to clone on a single genomic integration transformation).

The equations below were used to represent the synthetic feedback circuits. The first set of equations, describing the dynamics of transcription factor activation and feedback element transcription as well as conservation rules, are common to all

simulations. The system of ordinary differential equations was solved computationally for each circuit using built-in functions in MATLAB (Mathworks, Natick, MA). Code available upon request.

## Equations used for simulating circuits:

### Common to all circuits:

$$\begin{aligned}\frac{dTF_{active}}{dt} &= (k_{TF} Scaffold_{active}) TF_{inactive} - \gamma_{TF} TF_{active} \\ \frac{dFB}{dt} &= k_{FB} \frac{TF_{active}^{n_H}}{k_{d-TF} + TF_{active}^{n_H}} + k_{FB_{basal}} - \gamma_{FB} FB \\ 1 &= f_{bound} + f_{unbound} \\ Scaffold_{TOTAL} &= Scaffold_{active} + Scaffold_{inactive} \\ TF_{TOTAL} &= TF_{active} + TF_{inactive}\end{aligned}$$

### Negative feedback:

$$\begin{aligned}\frac{d Scaffold_{active}}{dt} &= \frac{S_{\alpha}}{k_{\alpha} + S_{\alpha}} Scaffold_{inactive} f_{unbound} - \gamma_{scaffold} Scaffold_{active} \\ &\quad - (f_{bound\_neg} k_{cat\_neg}) Scaffold_{active} \\ &\quad - (f_{unbound} k_{cat\_neg} k_{cat-off-scaffold\_ratio}) FB_{unbound} Scaffold_{active}\end{aligned}$$

### Positive feedback:

$$\begin{aligned}\frac{d Scaffold_{active}}{dt} &= \frac{S_{\alpha}}{k_{\alpha} + S_{\alpha}} Scaffold_{inactive} f_{unbound} - \gamma_{scaffold} Scaffold_{active} \\ &\quad + (f_{bound\_pos} k_{cat\_pos}) Scaffold_{inactive} \\ &\quad + (f_{unbound} k_{cat\_pos} k_{cat-off-scaffold\_ratio}) FB_{unbound} Scaffold_{inactive}\end{aligned}$$

### Accelerator:

$$\begin{aligned}
\frac{d Scaffold_{active}}{dt} = & \frac{S_{\alpha}}{k_{\alpha} + S_{\alpha}} Scaffold_{inactive} f_{unbound} - \gamma_{scaffold} Scaffold_{active} \\
& + (f_{bound\_pos} k_{cat\_pos}) Scaffold_{inactive} \\
& + f_{unbound} k_{cat\_pos} k_{cat-off-scaffold\_ratio} Ste50_{unbound} Scaffold_{inactive} \\
& - (f_{bound\_neg} k_{cat\_neg}) Scaffold_{active} \\
& - (f_{unbound} k_{cat\_neg} k_{cat-off-scaffold\_ratio}) FB_{unbound} Scaffold_{active}
\end{aligned}$$

**Delay:**

$$\begin{aligned}
\frac{d Scaffold_{active}}{dt} = & \frac{S_{\alpha}}{k_{\alpha} + S_{\alpha}} Scaffold_{inactive} f_{unbound} - \gamma_{scaffold} Scaffold_{active} \\
& - (f_{bound\_neg} k_{cat\_neg}) Scaffold_{active} \\
& - (f_{unbound} k_{cat\_neg} k_{cat-off-scaffold\_ratio}) FB_{unbound} Scaffold_{active}
\end{aligned}$$

**Variables (concentrations):**

$Scaffold_{active}$  = lumped element variable that represents the activated MAPK pathway (note:  $Scaffold_{TOTAL}$  = fixed value)  
 $TF_{active}$  = transcription factor (fixed total amount, either active or inactive)  
 $FB$  = feedback effector

**Parameters:**

**Kinetic rate constants:**

$k_{TF}$  = rate constant for activation of inactive transcription factor via active Scaffold  
 $k_{FB}$  = rate constant for creation of feedback effector via active transcription factor  
 $k_{FBbasal}$  = basal rate of creation of feedback effector (in absence of pheromone)  
 $g_{TF}$  = endogenous decay rate of active TF to inactive TF  
 $g_{FB}$  = endogenous degradation rate of FB  
 $g_{scaffold}$  = endogenous decay rate of active scaffold complex to inactive scaffold complex

**Binding constants:**

$k_a$  = binding constant of alpha-factor  
 $k_{d-TF}$  = dissociation constant of TF from promoter of FB

### Catalytic constants:

$k_{cat}$  = catalytic rate of feedback effector (e.g. rate constant for phosphatase converting active scaffolds to inactive scaffolds)  
 $k_{cat-off-scaffold-ratio}$  = ratio corresponding to reduced effectiveness of unbound effectors to catalyze reactions on the scaffold.

### Other constants:

$S_a$  = concentration of alpha-factor (strength of alpha-factor signal)  
 $f_{bound\_neg}$  = fraction of scaffolds bound by the negative feedback effector (between 0-1)  
 $f_{bound\_pos}$  = fraction of scaffolds bound by the positive feedback effector (between 0-1)  
 $f_{unbound}$  = fraction of scaffolds not bound by any feedback effector (between 0-1)  
 $n_H$  = Hill coefficient for transcription of feedback gene  
 $Ste50_{unbound}$  = Total Ste50 (fixed value) minus Ste50 bound in complex with Scaffold.

In order to calculate the concentration of complexes with competing molecular species, the assumption that the binding reactions come to equilibrium on a fast time scale compared to other system dynamics leads to a cubic equation that can be solved numerically. Specifically, consider three molecular species A, B, and C which can form complexes AB and BC (but not AC) – namely, A and C compete for binding to B. If  $k_1$  is the dissociation constant for A binding to B and  $k_2$  is the dissociation constant for C binding to B, then the concentration of the complex AB is the solution of the following equation which is between zero and the smaller of  $A_T$  and  $B_T$ , where:

$$a \cdot [AB]^3 + b \cdot [AB]^2 + c \cdot [AB] + d = 0$$

$$k = k_1 / k_2$$

$$a = k - 1$$

$$b = 2A_T + B_T + k_1 + k \cdot (C_T - B_T - A_T - k_1)$$

$$c = -A_T \cdot (A_T + 2B_T + k_1 + k(C_T - B_T))$$

$$d = A_T^2 B_T$$

### Parameters:

#### Global (common to all simulations):

|            |       |
|------------|-------|
| $k_\alpha$ | 20    |
| $k_{TF}$   | 0.004 |

|                                 |       |
|---------------------------------|-------|
| $\gamma_{TF}$                   | 0.018 |
| $\gamma_{scaffold}$             | 0.2   |
| $k_d$ weak zipper               | 150   |
| $k_d$ medium zipper             | 15    |
| $k_d$ strong zipper             | 5     |
| $k_{d-TF}$                      | 6     |
| $TF_{TOTAL}$                    | 50    |
| $Scaffold_{TOTAL}$              | 50    |
| $k_{cat}$ negative feedback     | 0.5   |
| $k_{cat}$ positive feedback     | 1     |
| $k_{FB\_strong\_basal\_ratio}$  | 0.028 |
| $k_{FB\_weak\_basal\_ratio}$    | 0.059 |
| $k_{cat\_off\_scaffold\_ratio}$ | 0.006 |
| $n_{Hill}$                      | 2     |

### Circuit-specific parameters:

#### Parameters for Figure 2 (simple negative and positive feedback):

| circuit             | negative feedback | positive feedback |
|---------------------|-------------------|-------------------|
| feedback promoter   | strong            | strong            |
| zipper              | medium            | medium            |
| $S_{\alpha\_basal}$ | 0.003             | 0.01              |
| $k_{TF}$            | 2.1               | 0.75              |
| $\gamma_{FB}$       | 0.0025            | 0.001             |

#### Parameters for Figure 3 (variations of negative feedback):

| feedback promoter zipper | strong strong | strong medium | strong weak | weak strong | weak medium | weak weak |
|--------------------------|---------------|---------------|-------------|-------------|-------------|-----------|
| $S_{\alpha\_basal}$      | 0.018         | 0.003         | 0.002       | 0.018       | 0.001       | 0.015     |
| $k_{TF}$                 | 1.5           | 2.1           | 2.1         | 1           | 2.1         | 0.5       |
| $\gamma_{FB}$            | 0.0025        | 0.0025        | 0.0025      | 0.0025      | 0.0025      | 0.0025    |

#### Parameters for Figure 4 (advanced circuits with binding competition):

| circuit             | decoy                        | decoy                        | decoy       | accelerator                  | delay                        |
|---------------------|------------------------------|------------------------------|-------------|------------------------------|------------------------------|
| feedback promoter   | strong                       | weak                         | none        | strong                       | strong                       |
| zipper              | decoy-strong;<br>Msg5-medium | decoy-strong;<br>Msg5-medium | Msg5-medium | Ste50-medium;<br>Msg5-strong | decoy-medium;<br>Msg5-strong |
| $S_{\alpha\_basal}$ | 0.0015                       | 0.02                         | 0.005       | 0                            | 0.05                         |
| $k_{TF}$            | 2.85                         | 2.25                         | 2.55        | 4.35                         | 12                           |
| $Decoy_{TOTAL}$     | 300                          | 150                          | 0           | 0                            |                              |

|                                     |        |        |        |        |
|-------------------------------------|--------|--------|--------|--------|
| Ste50 <sub>constitutive_TOTAL</sub> |        |        | 35     |        |
| Msg5 <sub>constitutive_TOTAL</sub>  |        |        |        | 280    |
| $\gamma_{FB}$                       | 0.0025 | 0.0025 | 0.0025 | 0.0005 |

**Table 3-S1.** Plasmids used in this study

| Plasmid | Parent vector | promoter     | gene         | Leucine zipper |
|---------|---------------|--------------|--------------|----------------|
| CB500   | pRS305        | <i>pCYC1</i> | <i>MSG5</i>  | R34            |
| CB501   | pRS305        | <i>pCYC1</i> | <i>MSG5</i>  | E34(I)         |
| CB502   | pRS305        | <i>pCYC1</i> | <i>MSG5</i>  | E34(V)         |
| CB503   | pRS305        | <i>pCYC1</i> | <i>MSG5</i>  | E34(N)         |
| CB504   | pRS305        | <i>pSTE5</i> | <i>MSG5</i>  | R34            |
| CB505   | pRS305        | <i>pSTE5</i> | <i>MSG5</i>  | E34(I)         |
| CB506   | pRS305        | <i>pSTE5</i> | <i>MSG5</i>  | E34(V)         |
| CB507   | pRS305        | <i>pSTE5</i> | <i>MSG5</i>  | E34(N)         |
| CB508   | pRS305        | <i>pFIG1</i> | <i>MSG5</i>  | R34            |
| CB509   | pRS305        | <i>pFIG1</i> | <i>MSG5</i>  | E34(I)         |
| CB510   | pRS305        | <i>pFIG1</i> | <i>MSG5</i>  | E34(V)         |
| CB511   | pRS305        | <i>pFIG1</i> | <i>MSG5</i>  | E34(N)         |
| CB512   | pRS305        | <i>pPRM2</i> | <i>MSG5</i>  | R34            |
| CB513   | pRS305        | <i>pPRM2</i> | <i>MSG5</i>  | E34(I)         |
| CB514   | pRS305        | <i>pPRM2</i> | <i>MSG5</i>  | E34(V)         |
| CB515   | pRS305        | <i>pPRM2</i> | <i>MSG5</i>  | E34(N)         |
| CB517   | pRS304        | <i>pCYC1</i> | <i>GST</i>   | E34(I)         |
| CB521   | pRS304        | <i>pSTE5</i> | <i>GST</i>   | E34(I)         |
| CB524   | PRS304        | <i>pFIG1</i> | <i>GST</i>   | R34            |
| CB525   | pRS304        | <i>pFIG1</i> | <i>GST</i>   | E34(I)         |
| CB529   | PRS304        | <i>pADH1</i> | <i>GST</i>   | E34(I)         |
| CB532   | pRS304        | <i>pADH1</i> | <i>GST</i>   | E34(I)         |
| CB536   | pRS304        | <i>pCYC1</i> | <i>STE50</i> | R34            |
| CB535   | pRS304        | <i>pCYC1</i> | <i>STE50</i> | E34(I)         |
| CB538   | pRS304        | <i>pSTE5</i> | <i>STE50</i> | R34            |
| CB541   | pRS304        | <i>pFIG1</i> | <i>STE50</i> | R34            |
| CB542   | pRS304        | <i>pFIG1</i> | <i>STE50</i> | E34(V)         |
| CB543   | M4366         | <i>pSTE5</i> | <i>STE5</i>  | E34(I)         |
| CB552   | M4366         | <i>pSTE5</i> | <i>STE5</i>  | E34(I)         |
| CB553   | M4366         | <i>pSTE5</i> | <i>STE5</i>  | E34(V)         |
| CB554   | M4366         | <i>pSTE5</i> | <i>STE5</i>  | E34(N)         |

**Table 3-S2.** Plasmids used in specific experiments

| <b>Figure/<br/>Panel</b> | <b>Constructs used</b> |                                                                        |
|--------------------------|------------------------|------------------------------------------------------------------------|
|                          | <b>Scaffold</b>        | <b>Modulator</b>                                                       |
| <b>Figure 1B</b>         | CB551                  | Msg5: CB500, CB501<br>Ste50: CB535, CB536                              |
| <b>Figure 2A</b>         | CB551                  | Neg. feedback: CB510<br>Pos. feedback: CB542                           |
| <b>Figure 3B</b>         | CB551                  | Strong zipper: CB513<br>Medium zipper: CB514<br>Weak zipper: CB515     |
| <b>Figure 3C</b>         | CB551                  | Strong promoter: CB511<br>Weak promoter: CB515                         |
| <b>Figure 4A</b>         | CB553                  | No decoy: CB508<br>low decoy: CB508, CB521<br>High decoy: CB508, CB532 |
| <b>Figure 4B</b>         | CB553                  | Neg feedback only: CB508<br>Accelerator circuit: CB508, CB538          |
| <b>Figure 4C</b>         | CB553                  | Neg modulator only: CB504<br>Delay circuit: CB504, CB525               |
| <b>Figure 4D</b>         | CB551                  | Switch: CB506, CB543                                                   |

**Table 3-S3.** Strains used in this study

| <i>Strain</i> | <i>Description</i>                                                                       |
|---------------|------------------------------------------------------------------------------------------|
| <b>CB011</b>  | <i>W303 MATa, ste5::KanR, bar1::NatR, far1Δ, mfa2::pFUS1-GFP, his3, trp1, leu2, ura3</i> |
| <b>CB009</b>  | <i>W303 MATa, bar1::NatR, far1Δ, mfa2::pFUS1-GFP, his3, trp1, leu2, ura3</i>             |

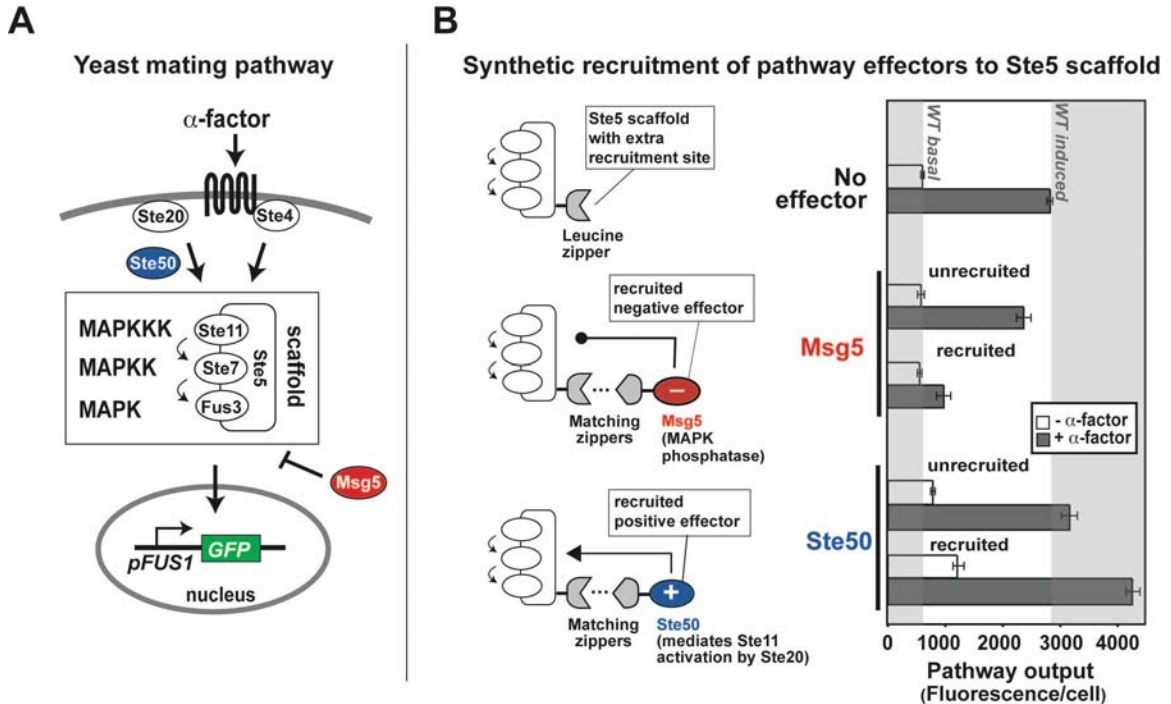


**Figure 3-1.** Output from the yeast mating MAPK pathway can be significantly increased or decreased by artificially recruiting positive or negative pathway effectors to the scaffold protein Ste5. **A.** Schematic of the mating pathway. Alpha-factor activates the G-protein coupled receptor, Ste2, and heterotrimeric G<sub>beta</sub> subunit, Ste4. Activated Ste4 recruits the Ste5 scaffold complex to the membrane, allowing PAK-kinase Ste20 (membrane-localized) to activate MAPKKK Ste11. Ste11 and its downstream kinases, Ste7 (MAPKK) and Fus3 (MAPK), are co-localized on the Ste5 scaffold protein. Activation of the MAPK cascade leads to the mating transcriptional program (pathway reporter: Fus1 promoter-GFP). In this work we have focused on pathway modulators that are not part of the core kinase cascade: Ste50 (positive effector, blue) promotes activation of Ste11 by Ste20; Msg5 (negative effector, red) is a MAPK phosphatase that dephosphorylates activated Fus3. **B.** Synthetic recruitment of pathway modulators to the Ste5 scaffold complex with a heterodimeric leucine zipper protein-protein interaction pair (see Materials and Methods). A basic zipper module was fused to Ste5 and the complementary acidic zipper module was fused to either a negative modulator, Msg5, or a positive modulator, Ste50 (this recruited leucine zipper pair has a  $K_d = 6.1$  nM). As a negative control ("unrecruited"), Ste50 and Msg5 were fused to the non-complementary zipper. Effect of recruitment on pathway output was assessed by measuring pFUS1-GFP expression by FACS. Basal output was measured prior to stimulation; induced output was measured 120 min. after stimulation with a saturating concentration of alpha-factor (2  $\mu$ M). Error bars represent std. dev. of three experiments. Unshaded area in the bar graph highlights the input-dependent output change observed for the wild-type pathway. All strains were constructed from a  $\Delta$ STE5 background using an integrated Ste5-zipper fusion expressed from the native pSTE5 promoter. Modulator-zipper fusions were

expressed from *pCyc1* promoter and integrated using pRS305 (Msg5) or pRS304 (Ste50) plasmids. At these expression levels, Ste50 and Msg5 only show significant effects on induced pathway output (Ste50 - increase; Msg5 - decrease) when recruited to Ste5, where a higher effective concentration may facilitate their actions. Control experiments in which recruitment domain orientation was reversed (basic zipper fused to modulators and acidic zipper was fused to scaffold) gave similar results (**Figure 3-S3E**).

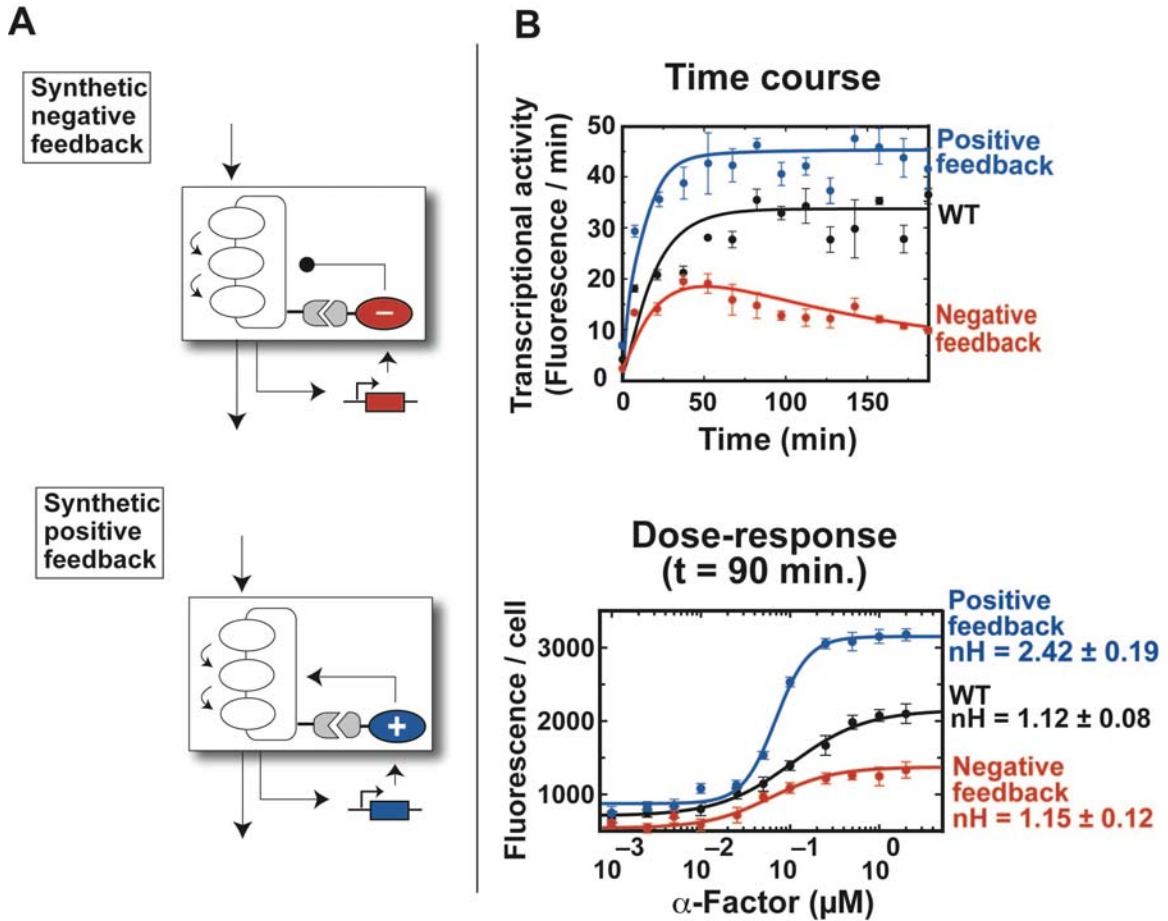


**Figure 3-2.** Dynamically regulated recruitment of pathway modulators to the Ste5 scaffold can be used to build synthetic positive and negative feedback loops. **A.** Schematic of negative and positive feedback loop design. Modulator-leucine zipper fusions (negative - Msg5; positive - Ste50) are expressed from a mating-responsive promoter (*pFig1*). Stimulation of the pathway by pheromone leads to expression of the modulator- zipper fusion, which is then recruited to the Ste5-scaffold where it can strongly exert its effect, either decreasing or increasing pathway flux. **B.** Experimental analysis of synthetic feedback loops. In order to facilitate FACS analysis, we implemented all feedback circuits in a  $\Delta FAR1$  background strain (designated as WT). This strain does not undergo mating induced cell cycle arrest, thus cells are more uniform in size for FACS analysis (see Materials and Methods for full description of strain and data collection methods). The synthetic negative feedback circuit (red), upon stimulation with 2  $\mu$ M alpha-factor, shows an initial rate of pathway activation similar to the wild-type (no synthetic feedback), but peaks at  $\sim 35$  min and shows adaptation to a steady-state that is  $\sim 3$ -fold lower than wild-type. The synthetic positive feedback circuit (blue) shows time course dynamics similar to the wild-type, but with steady-state output  $\sim 1.5$  fold greater. The dose-response curves show that the positive feedback circuit displays significantly more

switch-like activation, with an apparent Hill coefficient  $n_H = 2.42 \pm 0.19$  compared to  $n_H = 1.12 \pm 0.08$  for the wild-type. For both time course and dose response plots, data points represent mean values for three independent experiments  $\pm$  std. dev. Best fit lines shown in time course and dose-response plots were generated by fitting data points as described in Materials and Methods.

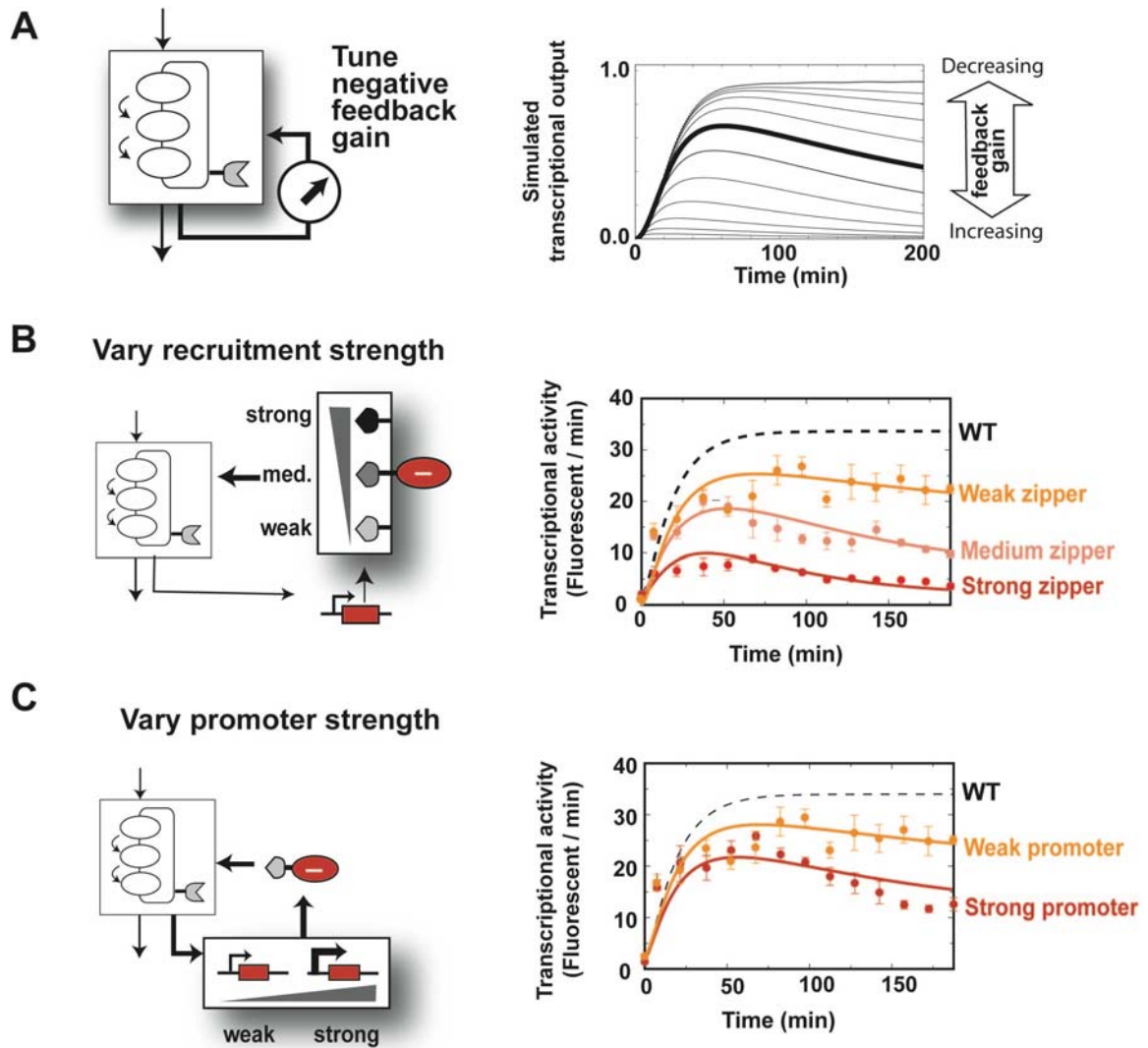


**Figure 3-3.** Strength of synthetic negative feedback circuit can be tuned by either altering recruitment affinity or inducible expression level of negative effector. **A.** Simple computational model of synthetic negative feedback loop (see Materials and Methods) predicts that adjustments in feedback gain should tune adaptation behavior. **B.** Adjusting feedback gain by varying modulator recruitment strength. Three variants of the leucine zipper were fused to Msg5. These bind the complementary, Ste5-fused zipper with different affinities (strong,  $K_d = 6.1$  nM; medium,  $K_d = 41$  nM; weak,  $K_d = 810$  nM, see Supp. Mat.). For these circuits, the promoter controlling modulator expression was held constant (*pPRM2*). **C.** Adjusting feedback gain by varying modulator promoter strength. The negative modulator, Msg5-zipper, is expressed from a mating induced promoter. Two promoters of varying inducible strength were used: strong promoter, *pFIG1*; and weak promoter, *pPRM2* (see **Figure. 3-S4** for quantitative promoter analysis). For these circuits the modulator was always fused to the weak leucine zipper. The orange plots in panels b and c represent the same data (circuit with weak zipper and weak promoter). Data points in all panels represent mean values for three independent experiments  $\pm$  std. dev.

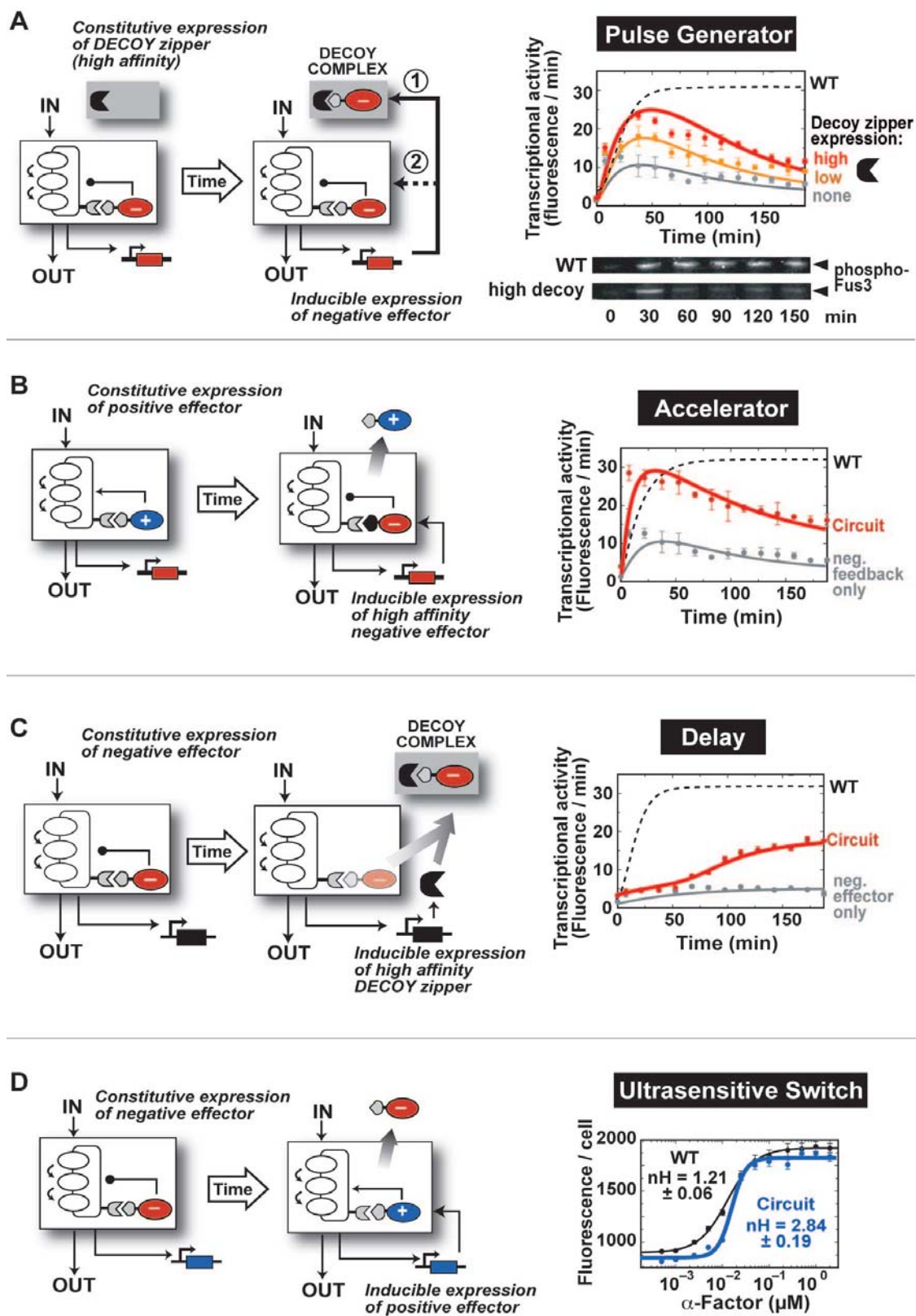


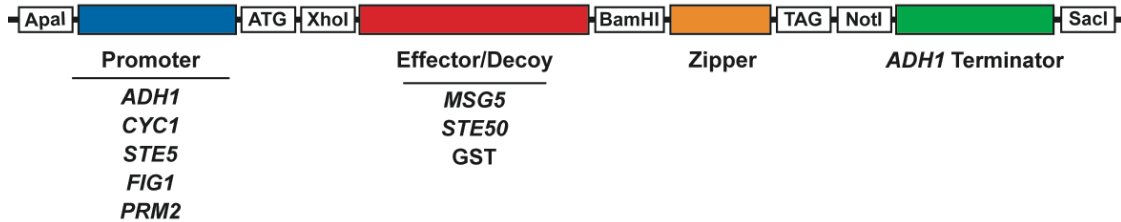
**Figure 3-4.** (continued on the following page)

**Figure 3-4.** Recruitment-based molecular toolkit can be used to engineer highly diverse pathway architectures and response behaviors. **A.** Pulse response behavior can be built from simple negative feedback loop (**Figure 3-2**) by adding a constitutively expressed decoy zipper (GST-zipper) that competes with the Ste5 scaffold zipper. The negative modulator (Msg5-zipper) is complementary to both the Ste50-zipper and the decoy zipper, but the decoy zipper binds with higher affinity ( $K_d = 6.1$  nM vs. 41 nM). Thus when the pathway is induced and the negative modulator is expressed, the decoy initially acts as a sink that prevents the modulator from binding to the scaffold and exerting any effect on pathway output. Only after the decoy is saturated will the negative modulator bind to the scaffold and repress pathway flux. Experimental circuit behavior is shown on the right, monitored by *pFUS1*-GFP transcriptional reporter (top) and anti-phospho-Fus3 western blot (bottom). Increased constitutive expression of the decoy zipper leads to increasingly sharper pulse response (constitutive promoters: low, *pSte5*; high, *pAdh1*). **B.** Accelerated circuit response is generated by constitutively expressing a recruited positive modulator (promoter: *pSTE5*; gene: Ste50-zipper) in combination with the simple negative feedback loop (**Figure 3-2**). Time course of activation (right) shows a faster rise time -- the circuit reaches the maximal output observed for the wild-type circuit, but in <20 minutes instead of ~75 minutes (Data used in grey plots in panels a and b are identical). **C.** Delayed response circuit is generated by constitutively expressing a negative modulator (promoter: *pSTE5*; gene: Msg5-zipper) and inducibly expressing a decoy zipper that is complementary to the negative effector-zipper (promoter: *pFig1*; gene: GST-zipper), but which binds with higher affinity than the Ste5-zipper ( $K_d = 6.1$  nM vs. 41 nM). The negative modulator maintains pathway in a repressed state until sufficient decoy zipper is expressed to relieve this repression. This circuit is essentially an inverted negative feedback loop, e.g. a positive feedback loop. **D.** An extreme ultrasensitive switch was built by constitutively expressing a negative modulator fused to a zipper (promoter: *pSte5*; gene: Msg5-zipper) and combining it with the simple positive feedback loop of **Figure 2-2** (inducible expression of a positive modulator Ste50 from *pFig1* promoter). Here, the positive modulator, which is fused to a zipper with equal affinity to that of the negative effector ( $K_d = 41$  nM) is displaced by inducibly expressed negative effector. This circuit is essentially a double positive loop (induction of positive modulator AND displacement of negative modulator). Dose-response analysis (right) shows significantly increased ultrasensitivity (apparent Hill coefficient  $n_H = 2.84 \pm 0.19$ , compared to the wild-type  $n_H = 1.21 \pm 0.06$  and the simple positive feedback loop  $n_H = 2.42$ ). Data points in all panels represent mean values for three independent experiments  $\pm$  std. dev. Best fit lines shown in time course and dose-response plots were generated by fitting data points as described in Materials and Methods. Errors for Hill coefficients are derived from fitting of three independent experiments. See Materials and Methods for details on construction of these and other related circuits.

## Synthetic Scaffolds



## Recruited Effectors



| Promoter regions used<br>(bases upstream of start) |        |
|----------------------------------------------------|--------|
| ADH1                                               | 1.5 kb |
| CYC1                                               | 250 bp |
| STE5                                               | 600 bp |
| FIG1                                               | 600 bp |
| PRM2                                               | 600 bp |

| Terminator regions used<br>(bases downstream of stop) |        |
|-------------------------------------------------------|--------|
| ADH1                                                  | 200 bp |
| STE5                                                  | 500 bp |

**Figure 3-S1.** Modular construction of circuit components. All vectors used in the construction of synthetic scaffold and recruited effector elements were constructed according to the architecture depicted here (See **Table 3-S1** for complete list of constructs used in this study). Sequences for effector/decoy elements were cloned as XhoI/BamHI fragments and contained the entire open reading frame for each gene except for the start ATG and stop codons. For recruited effector constructs, indicated promoter regions were cloned as ApaI and XhoI fragments included the start codon from the open reading frame of corresponding genes. For synthetic scaffold constructs, 500 bp of *pSte5* promoter was cloned with the *STE5* open reading frame as a single BglII/BamHI fragment. Zipper sequences were cloned into both scaffold and effector constructs as BamHI/NotI fragments (see **Figure 3-S2** for sequence details) and included a TAG stop codon immediately following their coding sequences. The indicated terminator regions were cloned as NotI/SacI fragments. BglII and SacI were used to clone synthetic scaffold cassettes into the BamHI and SacI sites of the HO-hisG-URA3-hisG-poly-HO multiple cloning site, while ApaI and SacI were used to clone recruited effector cassettes into pRS304 and pRS305 vectors (see **Table 3-S1** for list of constructs and their parent vectors).

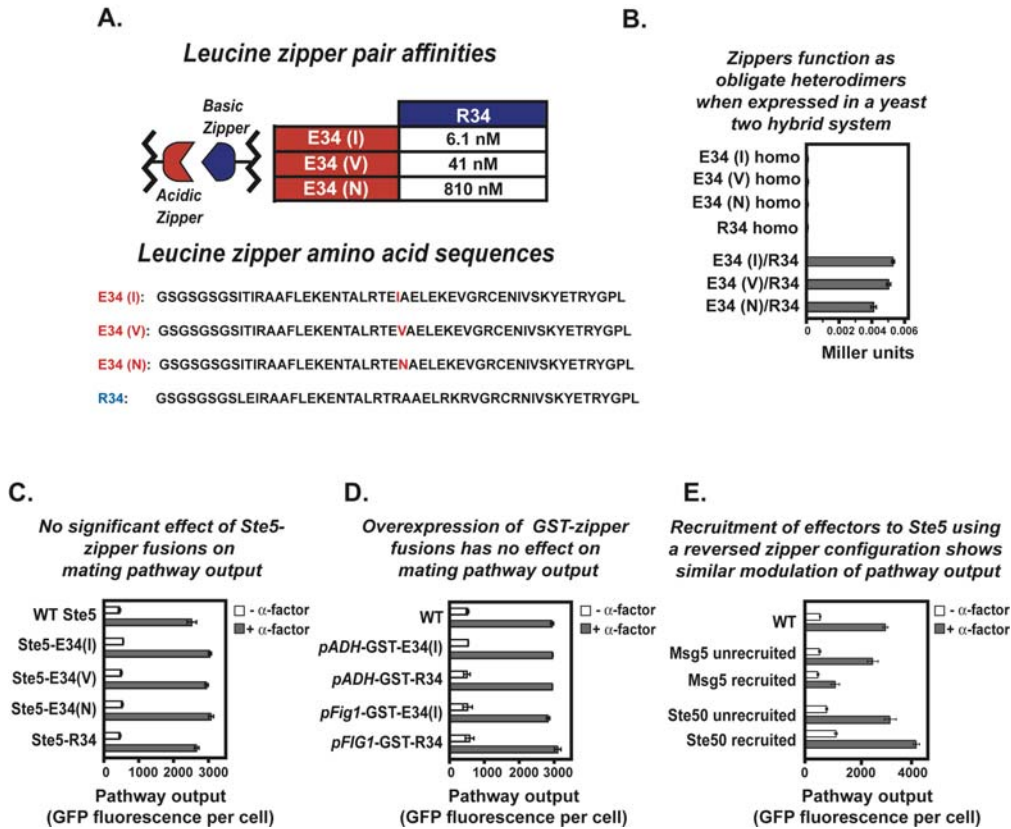


**Figure 3-S2.** Leucine zippers used in circuit construction. **A.** Specific heterooligomeric zipper interactions: zipper sequences and corresponding affinities. These leucine zippers were initially characterized by Vinson and colleagues<sup>38</sup>. **B.** Yeast two-hybrid experiments demonstrate that zippers behave as obligate heterodimers in vivo. For heterodimerizing pairs, acidic zippers were used as bait and basic zippers were used as prey. Error bars represent standard deviation for three experiments. **C.** Fusing leucine zippers to Ste5 does not significantly affect pathway output (see **Figure 3-S1** for construct details). The strain CB011 (see **Table 3-S3**) was transformed with plasmids coding for Ste5-zipper fusions (CB551, CB552, CB553, and CB554) and assessed for pathway activity in the absence of effectors by GFP FACS after treatment with 2  $\mu$ M alpha-factor for two hours. Error bars represent standard deviation for three experiments. **D.** Overexpression of leucine zippers has no significant effect on mating pathway output. The strain CB009 (see **Table 3-S3**) was transformed with plasmids coding for GST-zipper fusions (CB531, CB532, CB524, CB525) and assessed for pathway activity by GFP FACS after two hours of 2  $\mu$ M alpha-factor treatment. Error bars represent standard deviation for three experiments. **E.** Leucine zipper configuration with acidic zipper fused to Ste5 and the basic zipper fused to effectors show qualitatively similar results to the experiment in **Figure 3-1B** (where basic zipper is fused to Ste5 and acidic zipper is fused to Msg5). The experiment was conducted in the same manner as described for **Figure 3-1B**.

| Design                        | recruitment interactions<br>(zippers - FIXED)                        | promoters<br>(VARIABLE)                                                                                                                                                                                                                                                                                                                                                                           | Behavior Class                |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
|-------------------------------|----------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|--------------------------|-------------|-------------|-------------|-------------|--------------|--------------|-------------|--------------|------------------------------------------------------------------------------------|--------------|--------------|--------------|----------------------------------------------------------------------------------|
| <b>PULSE</b><br>              | scaffold: E34-V (med)<br>decoy: E34-I (strong)<br>neg. mod.: R34     | <table><tr><th>Decoy (constitutive)</th><th>Neg. Modulator (induced)</th></tr><tr><td>low (pSTES)</td><td>low (pPRM2)</td></tr><tr><td>med (pCYC1)</td><td>low (pPRM2)</td></tr><tr><td>high (pADH1)</td><td>low (pPRM2)</td></tr><tr><td>low (pSTES)</td><td>high (pFIG1)</td></tr><tr><td>med (pCYC1)</td><td>high (pFIG1)</td></tr><tr><td>high (pADH1)</td><td>high (pFIG1)</td></tr></table> | Decoy (constitutive)          | Neg. Modulator (induced) | low (pSTES) | low (pPRM2) | med (pCYC1) | low (pPRM2) | high (pADH1) | low (pPRM2)  | low (pSTES) | high (pFIG1) | med (pCYC1)                                                                        | high (pFIG1) | high (pADH1) | high (pFIG1) | adaptation<br>adaptation<br>adaptation<br><br>pulse (Fig. 4A)<br>pulse (Fig. 4A) |
| Decoy (constitutive)          | Neg. Modulator (induced)                                             |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| low (pSTES)                   | low (pPRM2)                                                          |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| med (pCYC1)                   | low (pPRM2)                                                          |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| high (pADH1)                  | low (pPRM2)                                                          |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| low (pSTES)                   | high (pFIG1)                                                         |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| med (pCYC1)                   | high (pFIG1)                                                         |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| high (pADH1)                  | high (pFIG1)                                                         |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| <b>ACCELERATOR</b><br>        | scaffold: E34-V (med)<br>pos. mod.: R34<br>neg. mod.: R34            | <table><tr><th>Pos. Modulator (constitutive)</th><th>Neg. Modulator (induced)</th></tr><tr><td>low (pSTES)</td><td>low (pPRM2)</td></tr><tr><td>med (pCYC1)</td><td>low (pPRM2)</td></tr><tr><td>low (pSTES)</td><td>high (pFIG1)</td></tr><tr><td>med (pCYC1)</td><td>high (pFIG1)</td></tr></table>                                                                                             | Pos. Modulator (constitutive) | Neg. Modulator (induced) | low (pSTES) | low (pPRM2) | med (pCYC1) | low (pPRM2) | low (pSTES)  | high (pFIG1) | med (pCYC1) | high (pFIG1) | adaptation<br>high activation<br><br>accelerator (Fig. 4B)<br>high activation      |              |              |              |                                                                                  |
| Pos. Modulator (constitutive) | Neg. Modulator (induced)                                             |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| low (pSTES)                   | low (pPRM2)                                                          |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| med (pCYC1)                   | low (pPRM2)                                                          |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| low (pSTES)                   | high (pFIG1)                                                         |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| med (pCYC1)                   | high (pFIG1)                                                         |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| <b>DELAY</b><br>              | scaffold: E34-V (med)<br>neg. mod.: R34<br>decoy: E34-I (strong)     | <table><tr><th>Neg. Modulator (constitutive)</th><th>Decoy (induced)</th></tr><tr><td>low (pSTES)</td><td>low (pPRM2)</td></tr><tr><td>med (pCYC1)</td><td>low (pPRM2)</td></tr><tr><td>low (pSTES)</td><td>high (pFIG1)</td></tr><tr><td>med (pCYC1)</td><td>high (pFIG1)</td></tr></table>                                                                                                      | Neg. Modulator (constitutive) | Decoy (induced)          | low (pSTES) | low (pPRM2) | med (pCYC1) | low (pPRM2) | low (pSTES)  | high (pFIG1) | med (pCYC1) | high (pFIG1) | delay<br>weak activation<br><br>delay (Fig. 4C)<br>weak activation                 |              |              |              |                                                                                  |
| Neg. Modulator (constitutive) | Decoy (induced)                                                      |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| low (pSTES)                   | low (pPRM2)                                                          |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| med (pCYC1)                   | low (pPRM2)                                                          |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| low (pSTES)                   | high (pFIG1)                                                         |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| med (pCYC1)                   | high (pFIG1)                                                         |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| <b>SWITCH</b><br>             | scaffold: R34<br>pos. mod.: E34-I (strong)<br>neg. mod.: E34-V (med) | <table><tr><th>Neg. Modulator (constitutive)</th><th>Pos. Modulator (induced)</th></tr><tr><td>low (pSTES)</td><td>low (pPRM2)</td></tr><tr><td>med (pCYC1)</td><td>low (pPRM2)</td></tr><tr><td>low (pSTES)</td><td>high (pFIG1)</td></tr><tr><td>med (pCYC1)</td><td>high (pFIG1)</td></tr></table>                                                                                             | Neg. Modulator (constitutive) | Pos. Modulator (induced) | low (pSTES) | low (pPRM2) | med (pCYC1) | low (pPRM2) | low (pSTES)  | high (pFIG1) | med (pCYC1) | high (pFIG1) | weak activation<br>weak activation<br><br>switch-like (Fig. 4D)<br>weak activation |              |              |              |                                                                                  |
| Neg. Modulator (constitutive) | Pos. Modulator (induced)                                             |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| low (pSTES)                   | low (pPRM2)                                                          |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| med (pCYC1)                   | low (pPRM2)                                                          |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| low (pSTES)                   | high (pFIG1)                                                         |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |
| med (pCYC1)                   | high (pFIG1)                                                         |                                                                                                                                                                                                                                                                                                                                                                                                   |                               |                          |             |             |             |             |              |              |             |              |                                                                                    |              |              |              |                                                                                  |

**Dynamic Behavior Classes**

time

**Dose-Response Behavior Classes**

[pheromone]

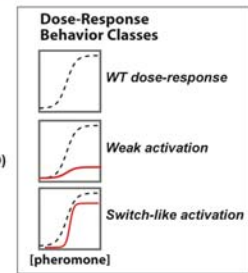
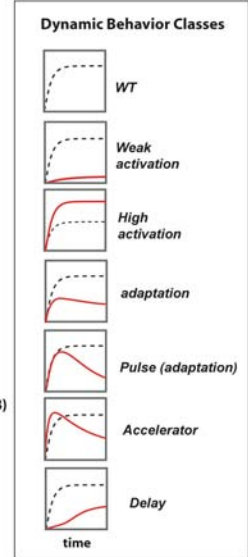


**Figure 3-S3.** Summary of circuit configurations tested during the construction of circuits in **Figure 3-4**. For each of the four circuit architectures depicted in **Figure 3-4**, multiple combinations of promoters and zippers were tested in order to determine which configurations yielded the target behaviors (pulse, acceleration, and delay time profiles, as well as switch-like dose response). For each circuit architecture, those configurations that demonstrated the richest behavior were selected for display in **Figure 3-4**. All constructs listed here were generated using the cloning strategy summarized in **Figure 3-S1**. Plots represent idealizations of characteristic temporal and dose-response behaviors.

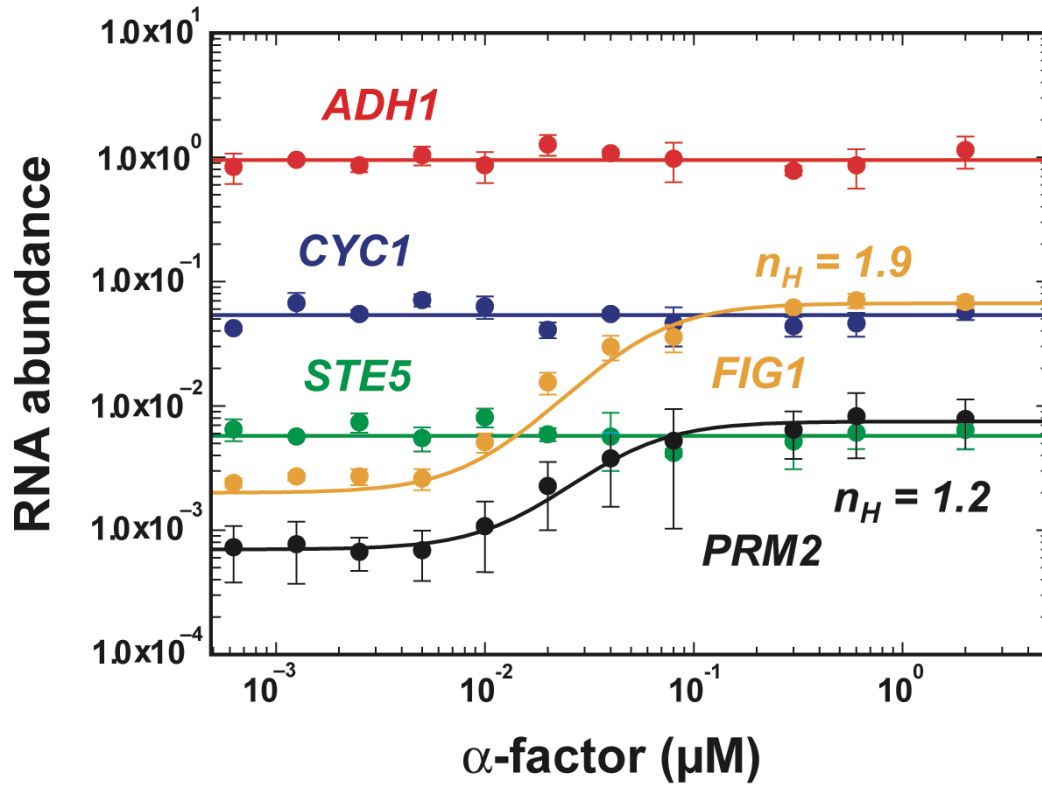


**Figure 3-S4.** RT-PCR characterization of promoters used in circuit construction. In order to identify promoters that were appropriate for circuit design, we measured mRNA transcript levels from housekeeping genes and mating pathway inducible genes by RT-PCR (see materials and methods for experimental details). Promoters identified for constitutive expression of circuit components, *pAdh1*, *pCyc1*, and *pSte5*, show no expression dependence on  $\alpha$ -factor, while promoters used for feedback of circuit components, *pFig1* and *pPrm2*, showed dose-dependent transcriptional enhancement. Error bars represent standard deviation for three separate experiments. Dose-response profiles were fitted with a Hill equation:  $R(a) = (R_{\min} + (R_{\max} - R_{\min})) * (a^{n_H}) / (C_m^{n_H} + a^{n_H})$ , where  $R$  = mean RNA abundance,  $a$  = concentration of alpha factor,  $R_{\min}$  = mean basal RNA abundance (no alpha-factor),  $R_{\max}$  = mean fluorescence with maximal pathway output (saturating alpha-factor),  $C_m$  = alpha-factor concentration at which RNA abundance is half-maximal, and  $n_H$  = Hill coefficient.

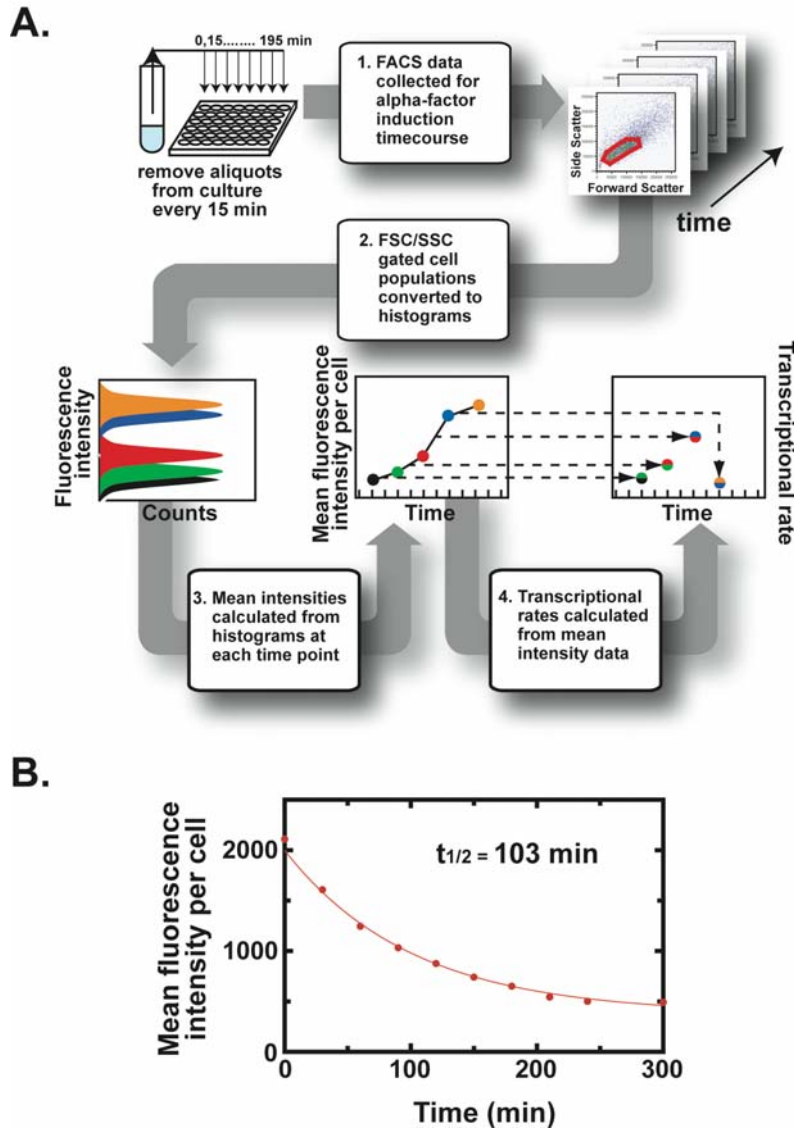


**Figure 3-S5.** Processing of data from flow cytometry experiments. **A.** Determination of transcriptional rates from GFP-FACS data. All data were analyzed for fluorescence values using the program Flowjo (Treestar). In order to determine mean fluorescence intensities at times after  $\alpha$ -factor treatment, cell populations were gated in FSC vs. SSC plots to ensure that cells of similar size and shape were compared amongst different samples and different time points. Mean fluorescence intensity data were then determined for gated populations (see reference S7 for a more detail description of mean fluorescence intensity determination). For time-dependent experiments, fluorescence intensity data were measured at 15 minute intervals. These data were converted into transcriptional rates to more accurately reflect temporal variation in pathway output – GFP expression is a poor readout of this, since the apparent lifetime of the GFP used here is >100 minutes, and thus it

continuously accumulates over the course of the experiment. Data were converted using the following equation:

$$\text{Rate}_t = ((I_{t+7.5\text{min}} - I_{t-7.5\text{min}})/15 \text{ min}) + k_{\text{decay}}(I_{t+7.5\text{min}} + I_{t-7.5\text{min}})/2$$

Where  $I$  is mean fluorescence intensity per cell at a given time point, and  $k_{\text{decay}}$  is the decay rate of GFP in the absence of  $\alpha$ -factor (a combination of GFP degradation and dilution by cell growth). We are essentially taking the time derivative of the observed intensities, after subtracting the intrinsic GFP decay rate. Transcriptional rates are therefore calculated for time points ( $t$ ) between each pair of intensity measurements ( $t+7.5$  min and  $t-7.5$  min). **B.** Experimental measurement of  $k_{\text{decay}}$  (decay rate of GFP signal in cells) using an  $\alpha$ -factor washout experiment. CB009 cells grown in liquid cultures to early log phase ( $\text{OD}_{600}=0.05$ ) were treated with alpha-factor for two hours, and then washed by centrifugation and resuspension in fresh media to remove alpha-factor. Following one hour, the data were fitted with the following equation

$$I_{\text{fluor}} = I_0 * e^{-k_{\text{decay}} * t}$$

Where  $I_{\text{fluor}}$  = fluorescence intensity,  $I_0$  = initial intensity ( $t=0$ ),  $t$  =  $t$  time.

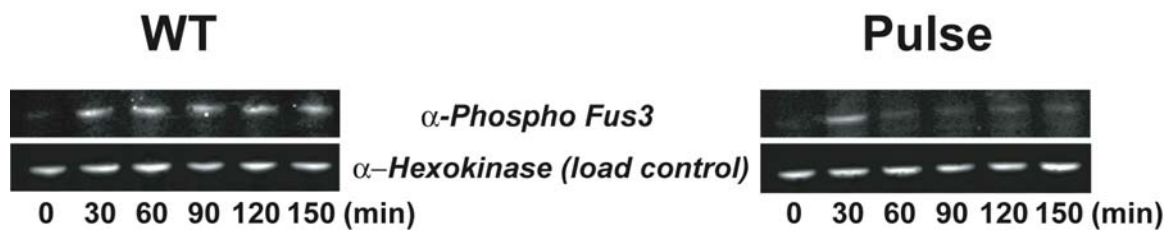


**Figure 3-S6.** Loading controls for Western blotting experiment. In order to control for sample loading, blots from the western blotting experiment shown in **Figure 2-4A** were striped and reprobed with an anti-hexokinase antibody and analyzed using fluorescence detection (see Materials and Methods).

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