Chapter 1

Introduction

Cellular differentiation, organism development, and tissue homeostasis require precise temporal and spatial regulation of cellular responses to external inputs. Improper regulation and coordination of these processes can lead to deformities, disease, and death. Controlling cell fate offers the potential to coordinate and redirect cellular trajectories. Efforts to control cell fate have primarily focused on regulating the chemical, biochemical, and mechanical environments in the extracellular space. Yet for a wide range of applications, environmental cues alone are insufficient to alter cellular behaviors. Further, many *in vivo* applications preclude precise control of the extracellular environment. In these instances, effective control strategies must often be performed in antagonistic environments. Synthetic gene regulatory networks that control the internal decision-making process offer an alternative approach to directing cellular fate.

Synthetic gene regulatory networks to control biological systems

The examples of synthetic gene regulatory networks encoding sophisticated functions have steadily increased over the last decade, aided by improved fabrication and sequencing methods that have reduced the cost of cloning [1-5]. Circuits capable of exhibiting dynamic behaviors, processing logical functions, and communicating cellular information have been demonstrated in synthetic gene regulatory networks [6-11]. Complex, multicellular behaviors such as synchronized oscillation and edge detection have been achieved by rational coupling of these various networks [12, 13]. Additionally, building genetic networks from the bottom-up has provided well-defined systems that can be used to study fundamental biological mechanisms [14]. Comparing the performance of

modeled and experimentally realized networks has informed our fundamental understanding of the importance of various process in biological systems, including degradation, cooperativity, and noise [15-17]. Despite remarkable advances in the realization of synthetic circuits, translation of these systems to real-world applications has been limited by the availability of methods to connect them to the requisite information in living cells [18]. There exists a need for modular interfaces that can extract specific information from biological systems and route this information to synthetic gene networks capable of programming rational responses via their interaction with native regulatory networks (Figure 1.1).

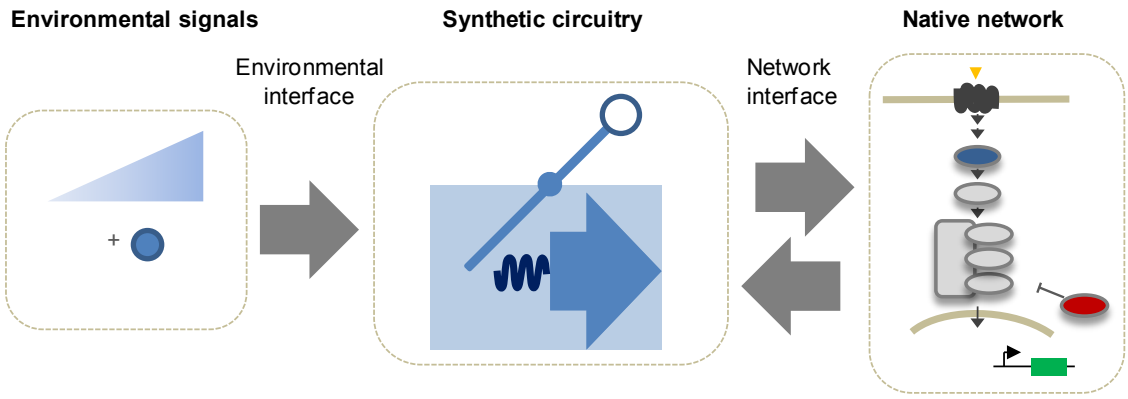

Figure 1.1. Building synthetic circuitry that interfaces with the environment and native regulatory networks to control cellular behavior. Environmental signals are transduced into changes in the native regulatory network via synthetic circuitry. The composition of the synthetic circuitry dictates the environmental interface, how environmental information is input into the synthetic circuit, and the network interface, how the circuitry implements regulation and extracts information from the native network.

Building a modular interface requires that circuitry be composed of modular parts such sensors, actuators, and other components that may be swapped in and out to connect to different environmental cues and various native networks. A synthetic circuit's ability to actuate changes in a native network depends on the properties of the circuit's parts, as well as, their interaction with each other (Figure 1.2A, B). An optimally placed circuit

runs quiescently until activated by the proper environmental cue to trigger a change in the native network (Figure 1.2C). The development of modular parts with tunable properties will enhance design flexibility, facilitating the optimal positioning of synthetic circuits for a broad range of applications.

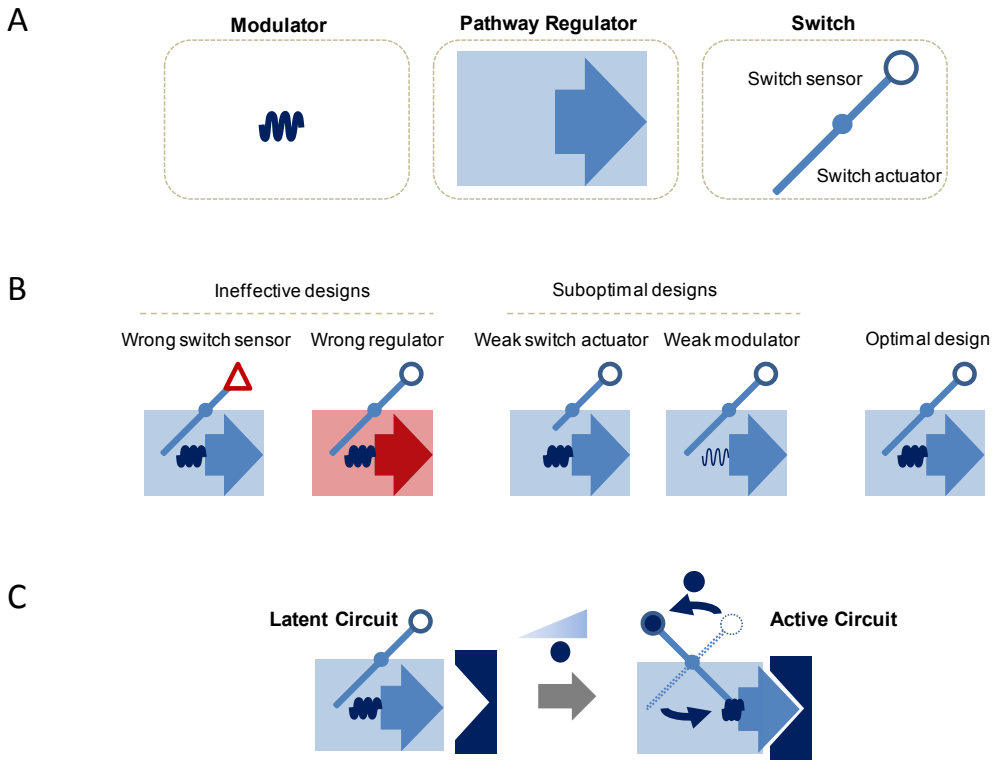

Figure 1.2. Components for interfacing with the environment and native regulatory networks. A. Parts used to interface with the environment and network. Function of the circuit relies on the properties of the elements and how they are integrated into the larger device. **B.** Composing circuits for optimal performance requires selection of the proper parts to efficiently transduce the environmental input into changes in the regulatory machinery. **C.** The latent circuit is activated by increasing environmental signal transduced by a sensor to imbedded synthetic circuitry. The synthetic circuit processes increasing levels of input by raising the profile of regulatory machinery that interfaces with the native network, mediating changes in the native network behavior.

Building a modular interface from ncRNAs

The modular, tunable, and programmable nature of RNA makes it an ideal candidate to perform sensing, actuation, and regulatory functions within a synthetic circuit [19]. Over the last twenty years the scientific community has become increasingly

aware of the role of non-coding RNAs (ncRNAs) in cellular control over gene expression. ncRNAs were thought to provide rather generic functions as ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) in translation and as small nuclear RNAs (snRNAs) in splicing. Studies of prokaryote genomes and their regulation forged the central dogma of biology, dictating that RNA had a relatively passive role in the transfer of genetic information from genes to proteins. In addition to specifying cellular state, proteins were thought to direct the trajectory of cellular fate by controlling gene expression networks. The vast tracks of ncRNA found in eukaryotes were hypothesized to be the evolutionary accumulation of inert sequences. However, large sets of these sequences are transcribed [20]. In fact, the majority of transcribed sequences in higher eukaryotes are never translated [21]. Additionally, the percentage of non-coding transcripts scales with organism complexity, while the number of coding genes does not [22]. With increasing discoveries of small ncRNAs that modulate gene expression [23-25] as well as with the discovery of the RNA interference (RNAi) pathway [26-28], ncRNAs appear to be the defining layer of sophisticated biological control that differentiates species with remarkably similar genomes [29].

While prokaryotes are known to use ncRNA to regulate gene expression, their dominant layer of control relies on protein-based regulation of transcription. Therefore, it is not surprising that prokaryotes have a relatively diverse genome and proteome from which to select elements to serve as regulators of gene expression. In contrast, eukaryotic organisms rely on a diversity of ncRNA to compensate for a largely stable and relatively small genome. As suggested by eukaryotic gene regulation strategies, control schemes for modulating gene expression are likely to require additional layers of control including the

implementation of RNA based-control systems that provide post-transcriptional control. To date, synthetic network engineering has largely focused on protein-based regulatory elements to build control systems [30]. However, there is a fundamental limitation to the complexity and specificity of protein-based regulatory schemes, which have a higher energetic cost. The burden of carrying additional genes encoding single protein regulators increases exponentially as complexity is introduced. Ultimately, in these accelerating networks, the cost of producing another protein regulator exceeds the benefit to the organism [31]. Regulation through alternative mechanisms such as ncRNA offers organisms an alternative that allows for specificity and diversity at a lower energetic cost [32].

The recurrence of ncRNA as a dominate regulatory motif in higher eukaryotes suggests that ncRNA-based regulators may improve the construction of complex regulatory architectures in synthetic circuits and facilitate connections between synthetic and endogenous circuitry. Additionally, the tunable properties of RNA and the potential to predict structure stability through existing algorithms make RNA a powerful substrate on which to build synthetic gene expression control systems. While post-transcriptional control of gene expression generally exhibits modest regulatory activities compared to transcriptional control, placement of post-transcriptional regulators at sensitive control points can mediate significant effects [33, 34]. Further, moderate activities endow these regulators with additive effects that provide a robust mechanism for tuning gene expression [35] and buffering the noise of gene expression in natural systems [36]. Finally, the additive nature of ncRNAs with modest activities provides a mechanism for synthetic circuits to logically process multiple inputs to orchestrate rational responses

[37]. In composing synthetic circuits, ncRNAs provide an additional degree of freedom for tuning circuit performance within the desired application.

Selecting for RNA-based sensors

To construct a modular environmental interface, requires modular sensors that can transduce the environmental input into changes in the synthetic circuit. We propose that RNA aptamers demonstrate the requisite modularity as environmental sensors that can be wired to actuation and regulatory elements. Aptamers are a class of small nucleic acids, including some ncRNAs, that bind to a wide range of ligands, such as small molecules and peptides, with sensitivity and selectivity that can rival that of proteins [38]. Aptamers are thought to bind ligands through a process called adaptive recognition, in which ligand binding occurs as the RNA molecule transitions through relatively unstructured conformations until the appropriate binding pocket is formed. Upon formation of the binding pocket, the aptamer associates with the ligand which stabilizes the ligand-bound structure. Due to evolutionary pressure during selection, the three-dimensional structure of aptamer complexes reflects highly optimized scaffolds for ligand recognition [39].

The development of new aptamer sequences to cellular molecules of interest offers the potential to connect to endogenous networks in a rational way that can direct information into exogenous control systems. Synthetic RNA aptamers have been generated *de novo* to various small-molecule and protein targets through *in vitro* selection or SELEX strategies [40, 41]. Briefly, a large library of RNA molecules ($\sim 10^{14}$–10^{15}) is incubated with the target of interest. Functional aptamers within this library space are subsequently partitioned from nonfunctional members and collected, typically using an affinity chromatography based separation strategy.

Collected sequences are then reverse transcribed and amplified to generate an enriched library that will serve as the input pool for the next round of selection. The Smolke laboratory has generated RNA aptamers that exhibit varying specificities to benzylisoquinoline alkaloids [42] and folinic acid derivates, and is developing high-throughput strategies for the direct selection and characterization of new protein- and small-molecule-responsive aptamers. Developing modular interfaces that facilitate information exchange between natural and engineered systems is critical for constructing biological control systems that program cell behavior.

Natural RNA switches as gene expression control systems

Naturally occurring RNA switches called riboswitches have been shown to regulate gene expression in response to a variety of metabolites, constructing various metabolic feedback control loops [43, 44]. Riboswitches are naturally occurring *cis*-acting RNA regulatory elements that modulate gene expression events in response to changes in intracellular metabolite concentrations [43, 44]. Riboswitches are comprised of at least two functional domains: a sensor or metabolite-binding domain and an actuator domain. Metabolite binding to the riboswitch occurs as the RNA molecule surveys equilibrium conformations. Once the appropriate binding pocket is formed, the ligand can bind the sensor domain. Ligand binding biases the equilibrium of the sensor region toward ligand-bound conformations. Through linker modules, conformational changes in the sensor domain induce a conformational change in the actuator element. In a riboswitch, the actuator is called an expression platform because it regulates gene expression [45]. Gene regulation occurs as metabolite binding events shift the equilibrium of the riboswitch toward its active or inactive conformation in which the expression platform adopts an active or inactive functional state. When the expression platform

adopts its active conformation, gene regulation occurs through an array of diverse mechanisms such as transcription termination, mRNA cleavage, or translation initiation [19].

Riboswitches are implemented by the cell as autonomous biological control systems. These RNA elements provide feedback control by sensing metabolites that are substrates and products of the riboswitch-regulated enzymes and modulating the levels of these enzymes in response to cellular metabolite concentrations. One such example is the glutamine-fructose-6-phosphate (GlcN6P) amidotransferase ribozyme-based riboswitch that is located within the 5' untranslated region (UTR) of the *glmS* gene. This enzyme metabolizes GlcN6P, which is the small-molecule effector of the riboswitch located upstream of *glmS* [46]. While some riboswitches have been discovered to promote gene expression [47], the majority repress the expression of their target gene. However, these natural RNA switches generally have evolved nonmodular architectures, in which the sequences of the sensor and actuator components interact to allow the switch to adopt different functional conformations, making the adaptation of these ncRNA controllers to new molecular inputs and regulatory mechanisms through direct component swapping unfeasible [19, 45, 47].

Synthetic RNA switches that act through ribozyme-based cleavage mechanisms

Recent studies have demonstrated the design and implementation of synthetic riboswitch counterparts by pairing RNA aptamers with various ncRNA expression platforms [34, 48, 49]. The ncRNA regulatory element encoded in the actuation domain dictates the output flexibility of the controller, in terms of the genetic targets that can be regulated, and the organisms in which the controller functions. Therefore, regulatory elements that exhibit function in diverse organisms and that can be used to flexibly

regulate diverse genetic targets are of interest for integration into these synthetic controllers. A number of design strategies have been developed to functionally couple aptamers to small molecules and proteins to diverse ncRNA regulatory elements, from miRNAs [48] to ribozymes [50], such that binding of the ligand to the aptamer domain results in a change in the activity of the ncRNA regulatory element.

Ribozymes are RNA molecules that catalyze a variety of reactions such as self-cleavage or ligation [51]. Thus, ribozyme activity is independent of cell-specific machinery, and these RNA elements may provide a regulatory strategy that can be used across diverse organisms, including bacteria and eukaryotic cells. The hammerhead ribozyme is one of the most extensively studied ribozymes [51-54]. Previous work coupled aptamers to the stem-loop regions in hammerhead ribozymes, allowing for *in vitro* allosteric ribozymes [55-57]. However, the coupling strategies used in these early allosteric ribozyme designs inactivated the ribozyme activity at physiological salt conditions, not allowing these switches to be implemented as controllers inside cells. Through elucidation of the design rules for *in vivo* catalytic activity [58, 59], the stage was set for the design of RNA switch platforms that functionally integrate hammerhead ribozymes as *in vivo* regulatory elements.

The Smolke laboratory recently demonstrated a modular and extensible framework for engineering *in vivo* ligand-regulated ribozyme switches. The described switch device contains three distinct functional domains: sensor domain, comprised of an aptamer sequence, a transmitter domain, and actuator domain, comprised of a hammerhead ribozyme sequence (Figure 1.3) [60]. When placed in the 3' untranslated region (3' UTR), the engineered ribozymes switches regulate gene expression. In the OFF

state, the ribozyme favors the active conformation, promoting cleavage of the transcript. Addition of ligand biases the ribozyme switch to the ligand-bound inactive conformation, resulting in increased gene expression. Small-molecule-dependent regulation of gene expression has been demonstrated on various heterologous genes and enabled the construction of RNA switches exhibiting up- and down-regulation of target expression levels. The design of the transmitter domain is a critical design feature that supports the insulation and modularity of the sensor and actuator components and allows the forward design and tuning of synthetic RNA switches. The resulting ability to mix-and-match sensing and actuation domains makes the modular RNA switch platforms powerful tools for developing tailored gene expression control systems.

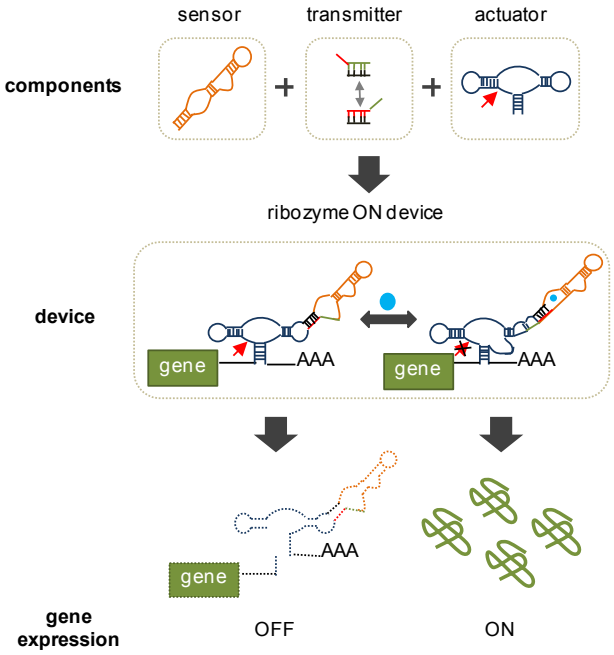

Figure 1.3. RNA-based switches regulate gene expression in response to small-molecule concentration. The switch device is constructed from three primary components, a sensor, a transmitter, and an actuator. These devices regulate the expression of a gene when placed in the 3' untranslated region (3' UTR). Shown above in the OFF state, the switch reduces gene expression in the absence of the small molecule by cleaving and destabilizing the transcript. Presence of the ligand alters the structure of the sensor which is transduced to the changes in the actuator structure via the transmitter. In the presence of ligand, the switch is ON and the actuator structure allows for increased gene expression. Adapted from Liang, J, et al. (*Submittedn*).

The construction of ligand-responsive control elements that act in *trans* will improve our ability to study natural gene networks as well as impose exogenous control in biological systems. The described ribozyme switch system allows for the control of transgenes (in cis); however, it cannot be directly used for the regulation of endogenous genes (in trans). While a number of synthetic RNA switches that allow for the regulation of endogenous genetic targets have been described that function through miRNA-[48], shRNA- [61], or antisense-based [62] mechanisms, these regulatory elements are associated with a number of drawbacks. For example, antisense-based regulatory efficiency is highly variable across targets [63]. In addition, utilizing the RNAi pathway to process synthetic substrates has raised concerns about off-target effects and competition between synthetic and native substrates [64]. Cis-acting ribozymes may be converted into to trans-acting ribozymes by splitting the stem I loop and engineering the intermolecular reaction between the ribozyme and target sequence (Figure 1.4). Facilitating the ribozyme-target binding event *in vivo* is a critical hurdle to implementing trans-acting ribozymes as regulators of gene expression [65]. Previous attempts to control target expression via trans-ribozyme have been limited by poor *in vivo* efficiency [66, 67]. Improvements in ribozyme designs and expression systems may increase ribozyme-mediated knockdown of targets *in vivo.* Elucidating the rules for the regulation of target transcripts will poise trans-acting ribozymes as unique actuators of gene expression. Finally, extension of the design principles elucidated for functional *in vivo* activity of cis-ribozyme switches to the design of trans-ribozyme switches may provide a promising alternative to target the expression of endogenous proteins without modifying the natural context of the target gene [66, 68, 69].

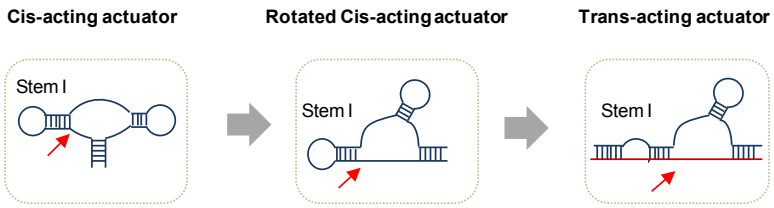

Figure 1.4. Converting cis-acting actuators to trans-acting requires engineering an intramolecular reaction into an intermolecular reaction. The cis-acting ribozyme (at left) can be converted to a trans-acting ribozyme by opening up stem I of the ribozyme to allow binding of a target transcript (shown in red).

MAPK cascades as universal signaling modules in eukaryotes

Mitogen-activated protein kinase (MAPK) cascades are highly conserved signaling pathways that control such processes as differentiation, mitosis, and apoptosis (Figure 1.5) [70]. Signaling through this pathway begins when extracellular signals are transduced across the cell membrane through receptor binding events that activate G-proteins. G-proteins relay these signals by facilitating phosphorylation of MAPKKKKs that continue phosphorylation through a three-tiered cascade until reaching the MAPK [71]. MAPKs regulate cellular behavior through interaction with repressors and transcription factors that determine entry into various cellular programs [72]. Many human cancers and other diseases are known to result from aberrant activation of cellular programs connected to MAPK signaling [73, 74]. Thus, controlling improperly activated signals has important implications in the development of therapeutics. Control systems that modulate MAPK pathways will provide an opportunity to interface with a large class of endogenous regulatory networks by which cellular fate can be programmed.

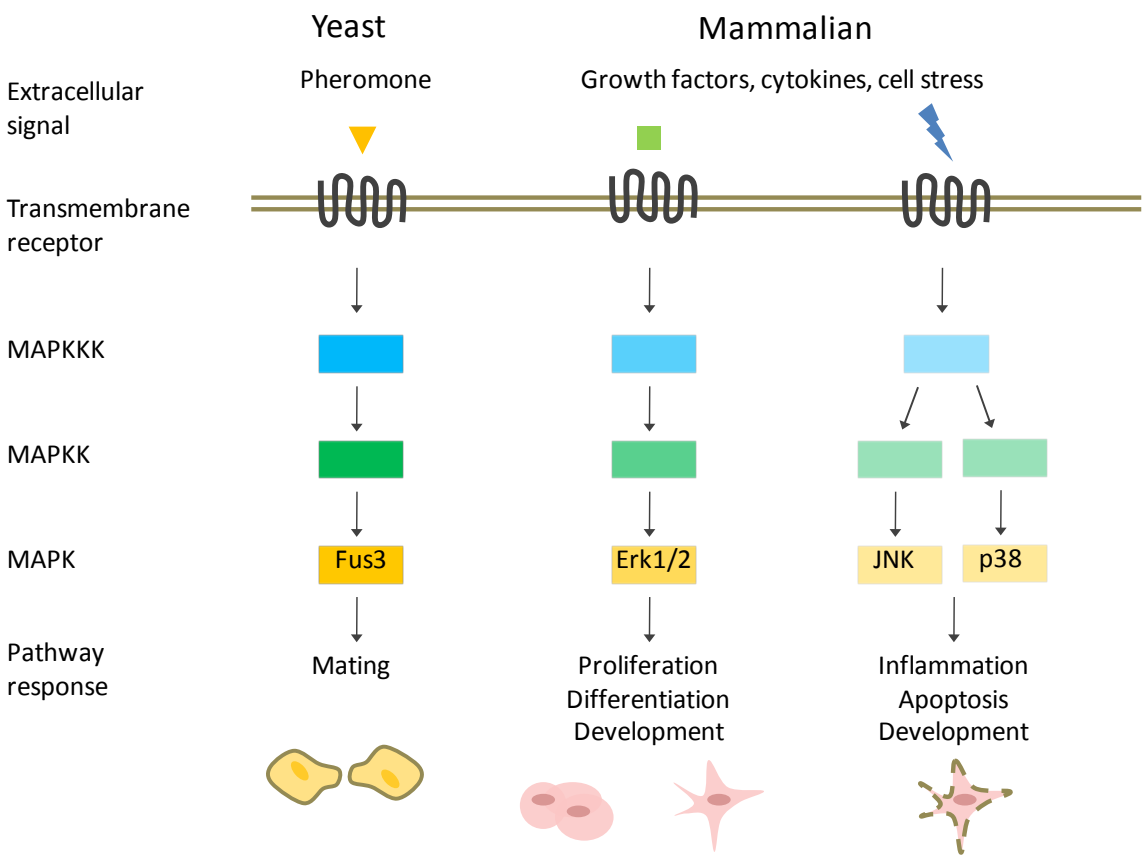

Figure 1.5. MAPK cascades as universal signaling modules in eukaryotes. From yeast to mammals MAPK pathways preserve the transmembrane receptor and downstream three-tiered MAPK cascade that ultimately generates a phenotypic response to the stimuli. Adapted from [75].

Despite an increase in our understanding of MAPK cascades, the development of therapeutics to intervene and redirect cellular fate through targeting components of this pathway has been primarily limited to kinase inhibitors [76-78]. These inhibitors act competitively to limit signal transduction; however, in pathways where control loops provide redundant verification of signaling, these inhibitors may be overwhelmed. Additionally, the delivery of these inhibitors is not restricted to diseased cells, which can result in unintended toxic side effects in healthy cells [79]. Effective therapeutics that redirect aberrant signaling through these pathways may need to compete with transcriptional feedback and discriminate between healthy and diseased cells. Implementation of control systems that regulate protein

levels offers the potential to rationally mediate MAPK signaling. Control systems that dictate MAPK signaling will provide a tool to elucidate our understanding of these pathways and potentially serve as a therapeutic strategy to counteract aberrant activation of cellular programs.

The homology between MAPK cascades in single-celled organisms such as yeast and higher eukaryotes allows for comparison of parallel pathway responses [71]. In particular, the study of the *Saccharomyces cerevisiae* MAPK pathways has illuminated paradigms in human MAPK signaling such as signal insulation through scaffold proteins [80, 81]. In the model eukaryotic organism *S. cerevisiae*, multiple MAPK cascades direct cellular fate via divergent regulatory programs. Decisions to halt cell cycle, upregulate excretion of a metabolite, or change cell morphology are programmed as the rational response to environmental signals. *S. cerevisiae* responds to pheromone by activating a receptor-coupled-G-protein three-tiered MAPK cascade (Figure 1.6A) [82]. The signal is transmitted from the G-proteins to the MAPK cascade of Ste11, Ste7, and Fus3. Fus3 translocates to the nucleus and phosphorylates the transcription factor Ste12. Fus3 is deactivated by the phosphatase Msg5. Upon pheromone stimulation, cells undergo cell cycle arrest, as can be seen in a halo assay, perform polarized growth to adopt the shmoo morphology (Figure 1.6B), and increase expression from the Fus1 promoter (pFUS1).

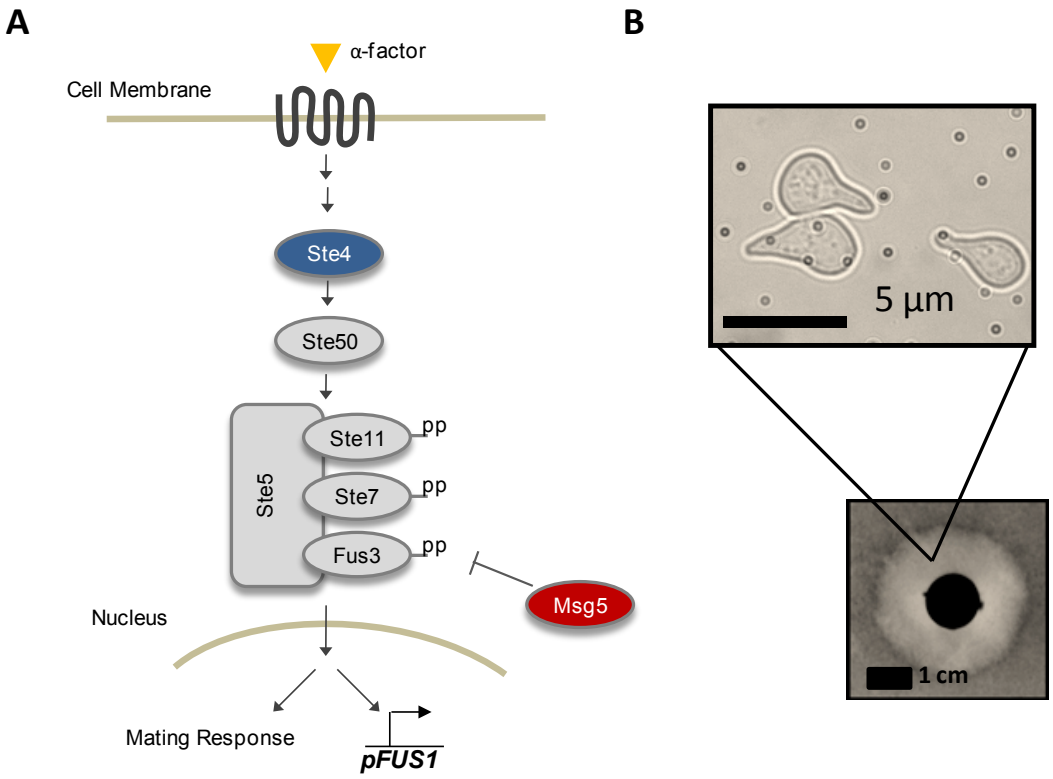

Figure 1.6. Yeast mating pathway and phenotypic response. A. Pheromone (α-factor) binding the transmembrane receptor initiates signaling in the internal G-proteins which is relayed to the canonical three-tiered MAPK cascade (Ste11, Ste7, Fus3). Phosphorylation is relayed down the cascade and culminates with phosphorylated Fus3 translocating to the nucleus to activate a range of transcription factors, transcription at mating genes, and ultimately the canonical mating response. Signaling is antagonized by Msg5, a phosphatase specific to Fus3. **B.** Phenotypic evaluation of the mating pathway can be performed via halo assay and by observing cell morphology. At bottom, a typical halo assay with a filter paper (center dark circle) saturated with pheromone establishing a gradient of pheromone. Cells within a particular radius corresponding to a particular concentration undergo pheromone-induced cell cycle arrest generating a halo in which cell density is significantly reduced relative to the plate outside of this radius. Above, cells stimulated with pheromone form "shmoos" by undergoing polarized cell growth.

Combining synthetic biology and systems biology to build gene regulatory networks that control cellular fate

Several modeling efforts have focused on evaluating the dynamics of MAPK cascades and posited that levels of signaling molecules are responsible for divergent cell fates [83-85]. Experimental results in the yeast pheromone-responsive MAPK pathway have demonstrated that particular profiles of signaling molecules are associated with

entry into specific fates [86-88]. Further, it has been suggested that network topology and the associated positive and negative feedback loops are ultimately responsible for these profiles and thus phenotype [87, 89]. Recent work in mammalian PC-12 cells indicates that the induced ERK MAPK network topology and resulting dynamics direct cell fate (Figure 1.7) [90]. Altering network topology routes cells to alternative fates. Questions still remain as to whether varying the induced network topology represents a conserved strategy across multiple MAPK cascades and eukaryotic organisms. Nevertheless, these results bode well for employing synthetic layers of positive and negative feedback loops as an engineering strategy to regulate cellular behavior

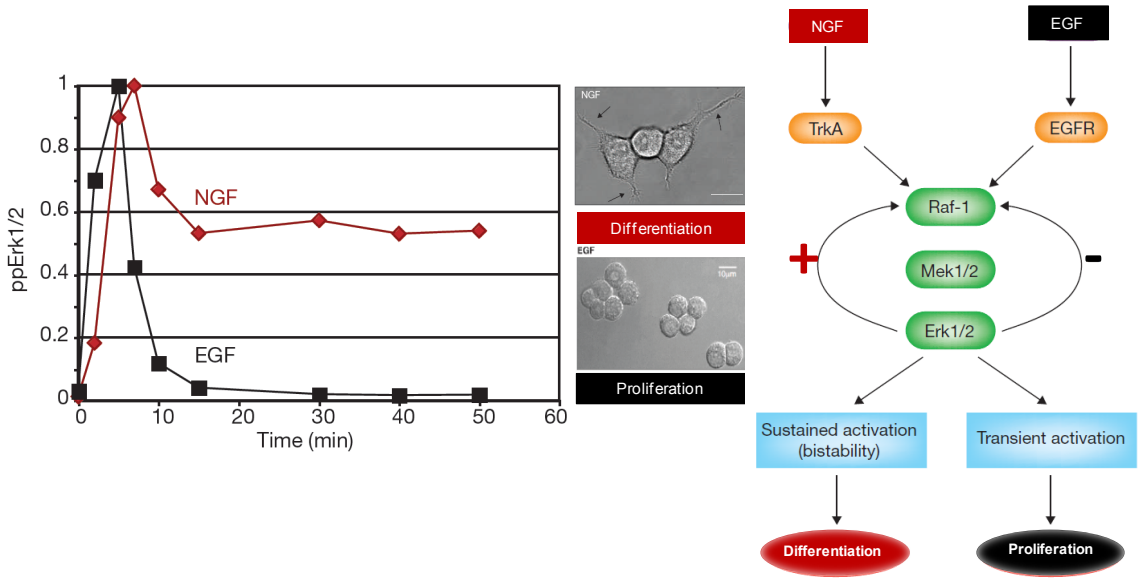

Figure 1.7. Induced network topology shapes the dynamics in a natural regulatory pathway dictating cellular fate. A. Transient stimulation with NGF and EGF leads to different Erk1/2 profiles that correspond to divergent cell fates. Adapted from [90]. **B.** Graph of network topology induced by different growth factors establishes feedback loops of different signs leading to divergent cell fates. Adapted from [91].

Construction of synthetic biological control systems that interact with natural circuits has seen success in regulating pathway activity by incorporating endogenous promoters in feedback control schemes [81]. Additionally, construction of protein

scaffold chimeras has routed cells to an alternative MAPK response [92]. While this work has successfully modulated pathway activity and/or fate, these strategies primarily rely on genetic knockouts of endogenous genes. We prefer a less invasive scheme with a paucity of genetic manipulations to the host that may minimize difficulty in transferring these control strategies to higher eukaryotes. Identification of control points within the molecular network which are sensitive to exogenous control systems will facilitate the construction of noninvasive control strategies. Modulation of the expression of pathway components at these control points can reshape the network response and redirect cellular fate. Further, constructing feedback loops at these control points can fundamentally reshape network topology, alter dynamic signaling profiles, and enhance the robustness of phenotypic selection [90, 93]. Layering these exogenous control systems with RNA-based controllers offers the potential to exogenously induce network topologies that redirect cell fate. We term these synthetic, exogenous control systems "molecular network diverters" as they conditionally divert the molecular network and consequently route cell fate. Molecular network diverters provide a means for orthogonally controlling cell fate within a genetically homogenous population via exogenously applied small-molecule input. Orthogonal control via diverters provides an additional degree of freedom in specifying cell fate preserving existing mechanical, chemical, and biochemical channels for directing cell fate. Molecular network diverters may facilitate the *ex vivo* construction of complex tissues from progenitor cells with imbedded exogenous control systems. These systems may guide progenitor cells to develop normal tissues when seeded on designer scaffolds by supplementing the missing boundary conditions normally present during *in vivo* development and serve to complement traditional tissue engineering

approaches (Figure 1.8). Such systems may be realized in the near future as researchers continue to unravel the systems biology governing cell-fate decisions [94, 95]. Advances in gene therapy delivery may allow molecular network diverters to be translated *in vivo* as cancer therapeutics targeting hyperactive MAPK pathways. Finally, the selection of new sensors responsive to pathway components may allow these diverters to perform autonomous corrective control of cell fate.

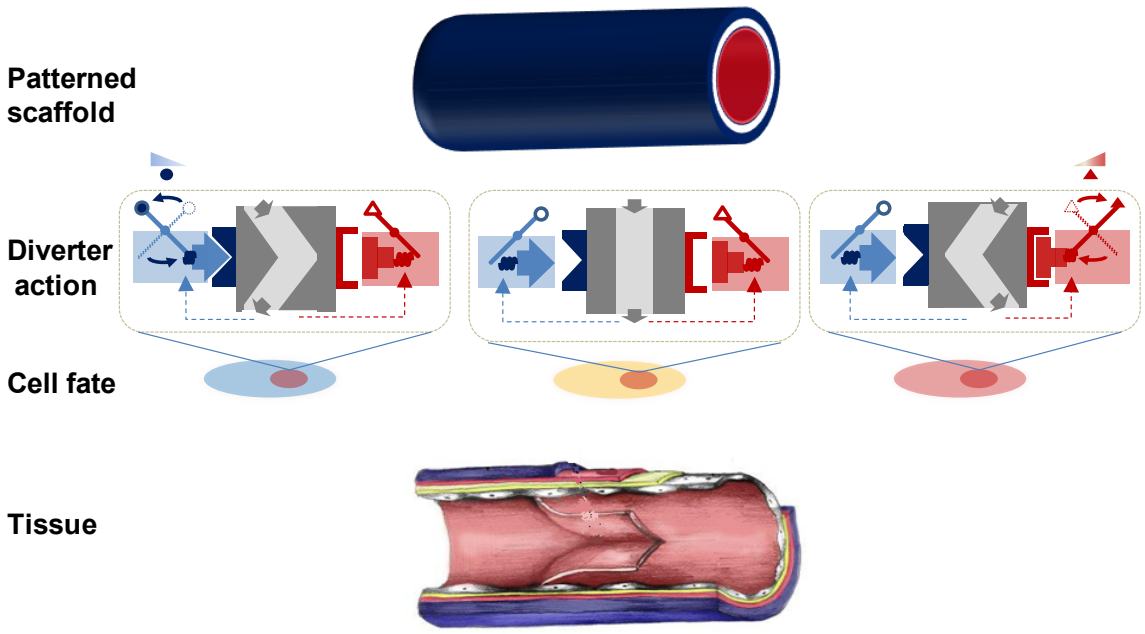

Figure 1.8. Potential application of molecular network diverters to tissue engineering via small-molecule regulated patterning of cell fate. A scaffold with the appropriate geometry is patterned with two small-molecules to trigger diverter action at particular regions within the scaffold. Activated diverters route cells to two alternative fates. All three fates are properly distributed to compose the constructed tissue.

Thesis organization

This thesis is organized into two primary sections. The first section focuses on constructing RNA-based control systems that regulate signaling in the yeast mating pathway. Chapter 2 focuses on using a synthetic titration system to identify regulators of

pathway activity and tracing the pathway response curve that routes cells to alternative fates via varying regulator expression. Using this knowledge, we construct synthetic circuits called “molecular network diverters” composed with engineered RNA controllers and feedback modules that conditionally route cellular fate. Chapter 3 examines the construction of diverters with more complex network architectures composed of multiple modules with different expression modes and RNA controllers that amplify ligand-induced phenotypic switching. We demonstrate an integrated network diverter capable of routing genetically identical cells to one of three fates dependent on environmental signals received. In the second section, chapter 4 discusses RNA-based controllers and efforts to develop a ligand-responsive trans-ribozyme platform that may be used to target both heterologous and synthetic transcript enhancing the design flexibility of synthetic control systems.

As systems biology unravels the inner workings of natural molecular networks, synthetic biology is developing the genetic regulatory tools to implement control systems that guide, tune, and override endogenous network responses. In this work, we utilize principles and tools from both systems biology and synthetic biology to compose a modular and tunable model control system by which we can conditionally direct cell fate. As the array of tools for controlling systems expands, proof-of-principle systems such as ours may be extended to applications in tissue engineering, therapeutics, and beyond.

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