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Designing New Cellular Signaling Pathways

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All cells respond to signals from the environment. Extracellular stimuli activate intracellular signal transduction pathways that make decisions about cell identity, behavior, and survival. A nascent field aims to design and construct new signaling pathways beyond those found in nature. Current strategies exploit the structural modularity of many signaling proteins, which makes them inherently amenable to domain-swapping tactics that exchange their input and output connections. The results reveal a remarkable degree of functional plasticity in signaling proteins and pathways, as well as regulatory logic that can be transported to new proteins. Modified adaptor and scaffold proteins can reroute signal traffic and adjust the response behavior of the pathway circuit. These synthetic biology approaches promise to deepen our understanding of existing signaling pathways and spur the development of new cellular tools for research, industry, and medicine.

Rewiring Cellular-Signaling Circuits for New Purposes

Decades of research have provided biologists with an impressive understanding of how cell fate and behavior are controlled by external signals. Many intracellular signaling pathways have been dissected to an extent that the complete ''parts list'' is known and the activity of each individual protein component is understood (Hunter, 2000). Consequently, researchers have begun to harness this knowledge to engineer new signaling pathways (Pawson and Linding, 2005; Andrianantoandro et al., 2006; Bhattacharyya et al., 2006; Drubin et al., 2007). Although this emerging field is still in its early stages, some initial efforts have been stunningly successful, in ways suggesting that conceptually simple regulatory strategies may be transportable between different signaling proteins. As a result, repeated iteration of pretested modules, motifs, and tactics may allow signaling proteins to be controlled in increasingly predictable ways. Ultimately, cellular engineers may be able to use relatively straightforward principles to design new signaling circuits with predetermined properties. This commentary will explore some of the underlying concepts, recent progress, and future directions.

Motivations and Promise

Why engineer new signaling pathways? First, it provides a way of testing whether we really understand how signaling pathways work or whether critical aspects remain undiscovered (Benner and Sismour, 2005). Second, it could foster the development of new research tools that allow cellular events to be probed with new or more precise control. Third, synthetic signaling pathways could have industrial or therapeutic applications, such as biosensors that detect and report on the presence of toxins, modified industrial microbes that execute desired metabolic activities only when conditions meet predetermined set points, or cell-based delivery systems that seek out a target niche for localized drug delivery. Fourth, it is conceivable that engineered cells could be developed as computational devices that rival electronic microprocessors. Finally, by attempting to mimic how sophisticated signaling pathways emerged in nature, synthetic approaches may help test ideas about the mechanisms of evolution.

This commentary will focus on synthetic signaling pathways, as opposed to synthetic gene expression circuits (constructed using transcriptional activators, repressors, and promoters) that have already yielded many interesting circuit behaviors, such as switches, oscillators, and memory (Hasty et al., 2002; Sprinzak and Elowitz, 2005; Stricker et al., 2008; Swinburne et al., 2008). What advantages or differences can be offered by engineering signaling pathways? One important difference is speed, because some signal transduction responses (both activation and inactivation) can occur within seconds, which is too dynamic to wait for transcriptional and translational synthesis. Posttranslational responses may also pose a lower energetic burden on the cell. Another difference is that signaling pathways can mediate spatially restricted responses that are confined to a localized region of the cell. Finally, direct regulation of protein activity, rather than just protein levels, offers extra variety and precision of control over cellular events. However, the complexity of signal transduction pathways may make computational prediction of circuit behavior considerably more difficult than it has been for gene expression circuits.

Advances in ''rational design'' (Sterner et al., 2008) may eventually allow new signaling proteins to be created de novo, but in the near future it will be considerably less laborious and more promising to modify existing signal transduction components in ways that co-opt their functions for new purposes. Importantly, it will be advantageous if cellular engineers do not have to start from scratch for each new protein or pathway. Rather, it will be preferable to develop methods that are generalizable and portable, so that common principles and reagents can be used repeatedly and predictably. This strategy would lend itself toward standardization of parts and practices that is a foundation of other engineering disciplines (Endy, 2005; Andrianantoandro et al., 2006; Drubin et al., 2007), where designers can build systems and devices with minimal concern for the inner

workings of the component parts. Below we will consider how some fundamental properties of cell signaling could be harnessed toward these goals.

Modularity and Functional Plasticity

Many signal transduction proteins have a modular architecture, such that their ultimate function derives from the combined properties of multiple independent domains (Figure 1A). Often, one domain (the "activity" or "output" domain) harbors a catalytic activity (e.g., kinase, phosphatase, or nucleotide exchange factor), and this is linked with other motifs, such as protein-protein interaction domains that dictate the connections to upstream regulators and downstream targets (Figure 1Bi–iii). Because these domains and motifs are often structurally autonomous and independently folding, they can confer their individual functional properties in a context-independent fashion. This arrangement is thought to make signaling pathways inherently evolvable through domain shuffling mediated by genetic recombination (Pawson and Nash, 2003; Bhattacharyya et al., 2006; Moore et al., 2008). Thus, if evolution has exploited the modular nature of signaling proteins to increase the diversity of natural pathways, then perhaps cellular engineers can use similar shuffling approaches to create new signaling proteins and pathways (Figure 1A, right).

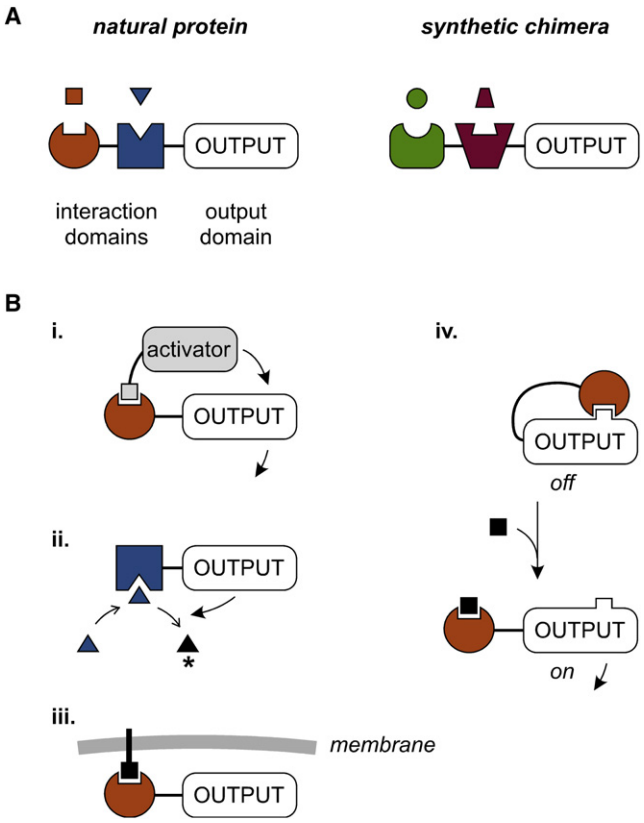

Figure 1. Modular Architecture of Signaling Proteins

(A) Natural signaling proteins often combine a catalytic ("output") domain with interaction domains that determine its connections. Exchanging interaction domains can create synthetic chimeras that connect to new stimuli or targets. (B) Interaction domains can link output domains to activators (i), substrates (ii), or subcellular locations (iii). They can also regulate protein activity by autoinhibitory binding (iv).

Indeed, existing evidence suggests that these structurally independent domains can be functionally independent and interchangeable. For instance, proteins in the MAP kinase family interact with their activators and targets by recognizing specific "docking sites" (Biondi and Nebreda, 2003; Bhattacharyya et al., 2006; Ubersax and Ferrell, 2007). These sites remain functional when moved to different positions in the partner protein and can be replaced with unrelated docking sequences from other partners (Grewal et al., 2006). In another example, signaling in the yeast pheromone pathway requires two components (Ste5 and Ste20) to localize to the plasma membrane via both protein-membrane and protein-protein interactions (Winters et al., 2005; Garrenton et al., 2006; Takahashi and Pryciak, 2007). Yet each component remains functional when its polybasic membrane-binding motif is replaced with a structurally unrelated phospholipid-binding domain, and in one case even the protein-protein interaction can be replaced with a surrogate interaction (Winters et al., 2005; Takahashi and Pryciak, 2007). Finally, activation of Notch-family receptors triggers proteolytic release of the cytoplasmic tail; when this tail is replaced with heterologous domains (e.g., a transcription factor), new responses can be activated by Notch ligands (Struhl and Adachi, 1998). These and other examples illustrate how the modular architecture of signaling proteins confers an intrinsic degree of functional plasticity. Therefore, further elaboration of these domain swap strategies could readily alter the inputs, outputs, and/or subcellular locale of signaling events.

Portable Control via Autoinhibition

Signaling proteins can be activated by allosteric conformational changes that propagate from a regulatory site to the active site (Bhattacharyya et al., 2006), but designing this form of regulation de novo is inherently difficult and protein specific. In contrast, many signaling proteins are controlled by fundamentally simpler "relief of inhibition" mechanisms that may be readily adapted for synthetic purposes. Here, binding interactions block the protein's function, and activating signals turn it on by disrupting the inhibitory interactions (Figure 1Biv); the negative domains commonly occur *in cis* and hence are autoinhibitory. This class of regulatory mechanism includes both "intrasteric regulation," in which inhibitory domains directly bind and occlude the catalytic site, as well as "modular allostery," in which the active state is either sterically or conformationally prevented by interactions away from the catalytic site (Kobe and Kemp, 1999; Bhattacharyya et al., 2006). In principle, any other modification or binding interaction that is mutually exclusive with the autoinhibited state (due to steric, electrostatic, or conformational incompatibility) could be appended to the protein and used to trigger its activation artificially.

Indeed, this strategy already has been spectacularly successful. The mammalian protein N-WASP regulates actin assembly via an output domain that is controlled by autoinhibition (Figure 2A); it is then turned on when activating factors disrupt the inhibitory conformation (Prehoda et al., 2000). Lim and colleagues (Dueber et al.,

2003) mimicked this mode of regulation with heterologous sequences, by attaching a common peptide-binding motif known as a PDZ domain at one end and its cognate target peptide at the other (Figure 2B). The resulting intramolecular PDZ-peptide interaction inhibited the intervening N-WASP output domain, and the hybrid protein could be turned back on by the addition of soluble target peptide. In effect, this converted native N-WASP into a form that can be activated by a foreign signal, and in a way that is remarkably straightforward at both conceptual and technical levels. Similar regulation was achieved using another peptide-binding motif (an SH3 domain), and incorporation of both the PDZ-peptide and SH3-peptide pairs into the same molecule generated more sophisticated circuit behaviors, such as an "AND gate" in which protein activation required the addition of both peptide ligands. Separately, if the output domain was flanked with multiple tandem copies of the SH3 domain and its target peptide, the resulting cooperative binding changed the dose-response behavior from linear to sigmoidal ("ultrasensitive") (Dueber et al., 2007).

In another remarkable example, the same core idea was applied to unrelated proteins (Yeh et al., 2007). Starting with two guanine nucleotide exchange factors (GEFs) that activate distinct Rho-family GTPases, the authors flanked the isolated catalytic domains with a PDZ domain and its cognate peptide (Figure 2C), as in the N-WASP experiments. As a new twist, they modified the peptide sequence so that it could be phosphoryated by protein kinase A (PKA), and this disrupts PDZ binding. Strikingly, each GEF was not only turned off by the intramolecular PDZ-peptide interaction, but was now activated by PKA. These reagents functioned both in vitro and in vivo, generating new controls over mammalian cell morphology. In essence, the authors transported the regulatory logic from one protein (N-WASP) to new proteins (GEFs) using artificial protein-peptide interaction pairs that need not engage the output domain per se but rather "snap shut" its activity via flanking interactions. The significance of this strategy lies in its conceptual simplicity and potential broad applicability. That is, artificial autoinhibition may constitute a regulatory strategy that is highly portable and generalizable.

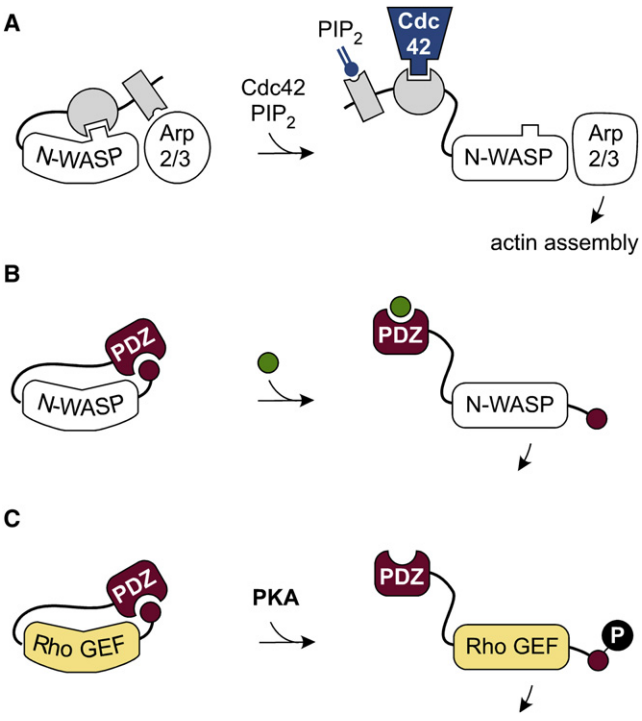

Figure 2. Regulating Protein Activity with Foreign Autoinhibitory Interactions

(A) N-WASP stimulates the actin nucleation complex Arp2/3. The GTPase Cdc42 and the phospholipid PIP_2 activate N-WASP by relief of autoinhibition.

(B) The normal autoinhibitory interactions in N-WASP can be replaced with foreign sequences such as a PDZ domain and its binding peptide. (It is unclear whether the foreign interactions block catalytic activity by a steric or conformational effect.)

(C) Regulation of Rho GEF activity with foreign autoinhibitory interactions. Phosphorylation of the target peptide by PKA disrupts PDZ binding and hence activates the GEF.

Exploiting Adaptors, Scaffolds, and Docking Interactions to Modify Inputs and Outputs

To stitch together customized signaling proteins into a new pathway, it is useful to consider how the direction of signaling traffic is determined in natural pathways. The choice of downstream targets can be dictated by specific sequence recognition, either at the site of a posttranslational modification (e.g., a phosphorylation site) or at a distal site such as a docking motif (Ubersax and Ferrell, 2007). In principle, transplanting docking interactions could provide a simple way to reroute signaling. Indeed, two yeast MAPKKKs (Ssk2 and Ssk22) were made to activate a new MAPKK (Ste7) by providing it with a docking site from a related MAPKK (Pbs2) that is the normal substrate (Tatebayashi et al., 2003). Although here the new substrate was in the same protein family as the usual substrate, it seems possible that entirely new substrates could be created using similar tactics.

Another way that signaling proteins choose their targets is by colocalization to discrete subcellular regions or coassembly into multiprotein complexes, which in each case is often regulated by additional proteins called adaptors and scaffolds (Figure 3A). These extremely diverse proteins are thought to serve as routers that direct signal traffic down specific paths by controlling the communication among signaling intermediates (Pawson and Scott, 1997; Pawson and Nash, 2003; Bhattacharyya et al., 2006). Indeed, in a crude progenitor to current synthetic design efforts, early experiments used scaffolds to steer the signaling output of a kinase that normally can function in multiple pathways; when the kinase was covalently attached to a pathway-specific scaffold protein, it favored substrates that bind the same scaffold, and hence activated that pathway preferentially (Harris et al., 2001). Like other signaling proteins, scaffolds and adaptors also tend to have a modular construction, making them promising targets for further derivatization.

Recently, adaptor and scaffold proteins have been used to enforce new connections and create new signaling circuits. One study switched a pathway that ordinarily promotes cell proliferation into one

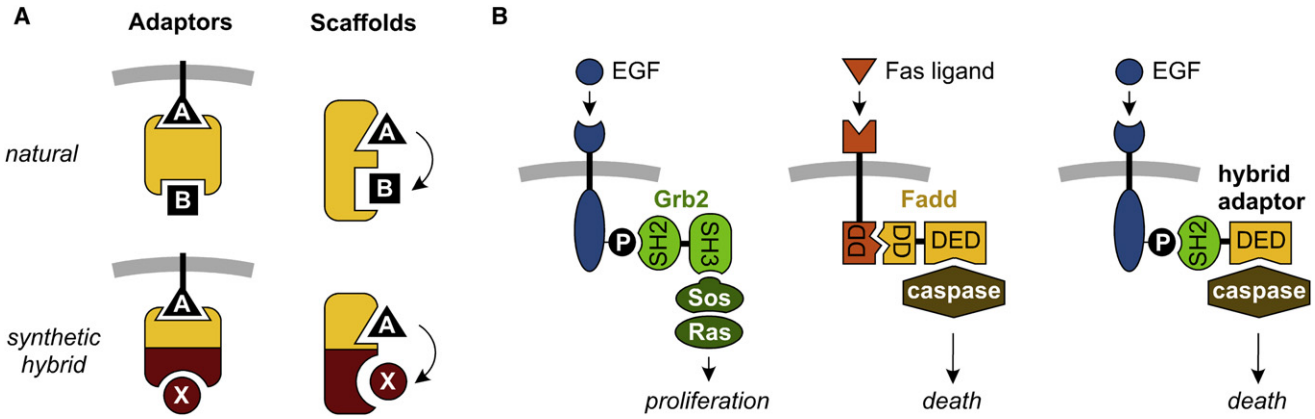

Figure 3. Adaptors, Scaffolds, and Pathway Rewiring

(A) Adaptor proteins are intermediary linkers that allow one protein to indirectly control another protein without their direct contact; this often regulates localization (e.g., to a membrane). Scaffolds serve as signal transfer platforms by binding multiple proteins and promoting their mutual interaction. Synthetic hybrids could create new connections and pathways.

(B) Phosphotyrosines in the tail of activated EGF receptor bind the SH2 domain of the adaptor protein Grb2, whose linked SH3 domains lead to activation of Ras and proliferative signaling (left). The Fas receptor recruits the DD domain of the adaptor protein Fadd, whose linked DED domain leads to activation of caspases and apoptosis (middle). When a hybrid adaptor protein was constructed in which the SH2 domain from Grb2 was linked to the DED domain from Fadd (right), stimulation with EGF now led to cell death (Howard et al., 2003).

that promotes cell death (Howard et al., 2003). This was accomplished by plucking domains from existing pathway-specific adaptor proteins and fusing them into a hybrid adaptor that now artificially linked a growth factor receptor to an activator of caspases (Figure 3B). Similarly, chimeras between two different scaffold/adaptor proteins in yeast allowed the input stimulus of one MAP kinase pathway to trigger the output response of another (Park et al., 2003). In both examples, simple domain swaps allowed existing signaling components to be rewired into a novel circuit. It is likely that further extrapolations of this same core logic could create novel pathways.

Controlling Circuit Behavior

Once new pathways are built, how can their input-output processing behaviors be controlled? That is, will the pathway circuit behave like a sharp on/off switch or a graded rheostat? Will signaling be short lived, long lived, or permanent? Some control over these response behaviors has been synthetically introduced by combining signaling regulation with gene expression. For example, one study (Ingolia and Murray, 2007) placed a constitutively active component of the yeast pheromone pathway under transcriptional control of the pathway itself; this created a self-perpetuating circuit in which the initial activation by an external stimulus trips a positive feedback loop that continues to fire the pathway even after the stimulus is withdrawn (Figure 4A). An intriguing and useful feature of such circuits is that they effectively provide a long-term memory of brief exposures to stimuli.

Another study altered the response dynamics in the same system by making derivatives of the pathway scaffold protein so that it could now recruit additional positive or negative regulators (Bashor et al., 2008). By expressing these recruited regulators from pathway-inducible promoters, feedback loops were created that changed the processing characteristics of the signaling circuit,

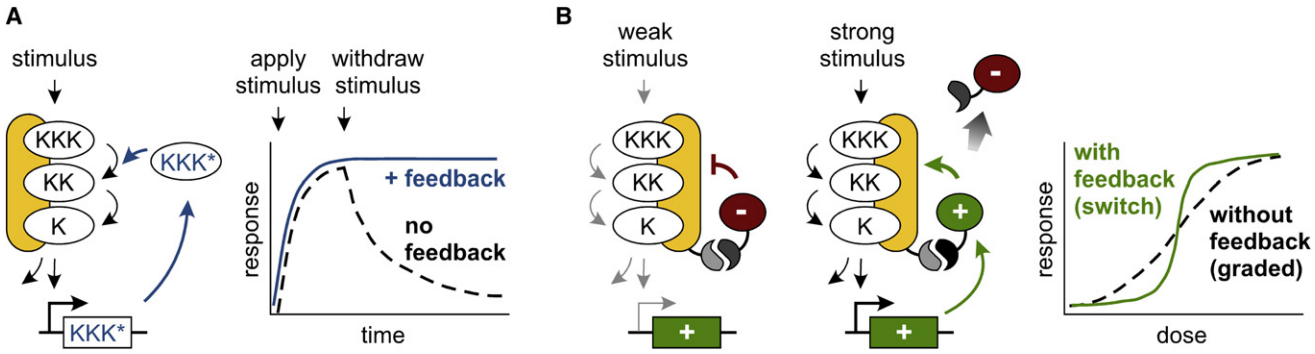

Figure 4. Controlling Circuit Behavior

(A) Creation of an irreversible, self-perpetuating signaling circuit in a MAP kinase cascade by placing a constitutively active form of one pathway component (KKK*) under transcriptional control of the pathway (Ingolia and Murray, 2007).

(B) A modified scaffold alters response dynamics in a MAP kinase cascade. Using leucine zippers to recruit new pathway regulators to the scaffold, signaling could be enhanced or dampened. Then, by placing expression of the recruited regulator under transcriptional control of the pathway itself, positive or negative feedback loops were established (Bashor et al., 2008). Several types of feedback circuit were constructed. Here, a pathway-induced positive regulator displaces a preexisting negative regulator, creating a response that is switchlike rather than graded.

such as whether it behaved as a rheostat versus a switch (Figure 4B) and whether the response was immediate, delayed, sustained, or transient. The magnitude of these effects could be fine-tuned (e.g., by varying promoter strength or the affinity of recruitment domains), but the results are particularly remarkable for the degree to which the altered signaling behaviors largely follow intuitive expectations. In theory, it should be possible to design nontranscriptional feedback loops if, for example, a docking interaction that controls activation of a kinase could be regulated by that same kinase (e.g., by adding phosphorylation sites within or near the docking site).

Future Approaches and Issues

To capitalize on these initial advances, new efforts must continue to focus on three primary tasks: forcing new interactions between signaling proteins, creating new mechanisms of regulation, and controlling the input-output processing behavior of the assembled circuit. Additional layers of regulation could be achieved by using targeting signals to control subcellular localization, oligomerization, and proteolysis (Devit et al., 2005; Grilly et al., 2007; Corson et al., 2008; Haruki et al., 2008). Synthetic derivatives of interaction motifs such as leucine zippers could help minimize unwanted interactions with endogenous proteins and could also promote standardization by building collections of interacting parts with predetermined specificity and affinity (Acharya et al., 2002; Bashor et al., 2008; Bromley et al., 2008). Further control over signal detection and processing can be achieved via multicellular networks that propagate responses from cell to cell (Basu et al., 2004; Andrianantoandro et al., 2006).

One avenue warranting further exploration is whether binding interactions may be generally amenable to synthetically imposed regulation through phosphorylation, by analogy to the disruptive effects of PKA phosphorylation on PDZ-peptide binding (Figure 2C) (Yeh et al., 2007). To increase the variety of usable kinases, it may suffice to place the phosphorylated sites adjacent to (rather than within) the target peptide; then, electrostatic effects of phosphorylation could either inhibit or promote binding, depending on whether the peptide-binding partner motif is flanked by electronegative or electropositive surfaces, respectively. Conceivably, varying the number of phosphorylation sites and their distance from the target peptide could adjust the strength of the effect and the sensitivity to kinase concentration (Serber and Ferrell, 2007). In principle, these strategies could be applied to many protein-protein interaction pairs.

Eventually, the ability to predict pathway behavior will benefit from computational modeling and the use of precharacterized circuit motifs (Papin et al., 2005; Brandman and Meyer, 2008). Nevertheless, substantial advances have already been accomplished through adventurous experimentation, and these studies also highlight how systematic, trial-and-error strategies can identify parameters that are critical yet unpredictable. For instance, only 5 of 34 artificial N-WASP chimeras showed the desired "AND gate" behavior in which activation required two simultaneous inputs (Dueber et al., 2003); although the majority showed some form of regulation (and some interesting surprises), the desired behavior required rather subtle variations (e.g., in module geometry, domain affinity, and linker lengths) that seem unlikely to be predictable by computational approaches anytime soon. A related issue emerges from recent studies in which unexpected features such as the subcellular location where signaling is initiated (i.e., cytoplasm, plasma membrane, or internal membranes) were found to have a strong influence on whether the input-output response behavior is graded or switchlike (Inder et al., 2008; Takahashi and Pryciak, 2008). Thus, despite our deep understanding of some pathways, unanticipated subtleties can have dramatic effects on the overall system behavior. Ideally, theoretical and empirical approaches will work together to help eliminate these lingering blind spots, some of which may actually become exposed as a by-product of synthetic research.

It seems a given that the next decade will witness increasingly sophisticated examples of custom-designed signaling pathways. As the successful strategies are refined and their applications are expanded, it will be fascinating to watch whether these efforts coalesce into a unified discipline. Will cellular engineers be able to develop new devices as routinely and rigorously as their mechanical or electronic counterparts, or is biology inherently too messy and unpredictable? Time will tell.

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