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Publication Date
2016-08-01

DOI
10.1016/j.sbi.2016.06.012

Peer reviewed

HHS Public Access

Author manuscript

Curr Opin Struct Biol. Author manuscript; available in PMC 2017 August 01.

Published in final edited form as:

Curr Opin Struct Biol. 2016 August ; 39: 106–114. doi:10.1016/j.sbi.2016.06.012.

Modular engineering of cellular signaling proteins and networks

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Abstract

Living cells respond to their environment using networks of signaling molecules that act as sensors, information processors, and actuators. These signaling systems are highly modular at both the molecular and network scales, and much evidence suggests that evolution has harnessed this modularity to rewire and generate new physiological behaviors. Conversely, we are now finding that, following nature's example, signaling modules can be recombined to form synthetic tools for monitoring, interrogating, and controlling the behavior of cells. Here we highlight recent progress in the modular design of synthetic receptors, optogenetic switches, and phospho-regulated proteins and circuits, and discuss the expanding role of combinatorial design in the engineering of cellular signaling proteins and networks.

Introduction: why design and engineer signaling proteins?

A major goal of modern cell biology is to understand how molecular signaling circuits enable cells to sense their environment and mount an appropriate response. This goal is currently being addressed using two distinct but complementary approaches: research aimed at the dissection, mapping, and analysis of naturally occurring systems, and efforts to engineer new cell signaling pathways. As the traditional analytic approach has revealed the wide diversity of mechanisms and molecular components that underlie cellular communication, a set of common mechanistic themes in signaling have emerged [1,2]. The synthetic approach provides a complementary method for rigorously testing that conceptual framework and for elucidating the core design principles that are used to achieve fundamental classes of response behaviors. By constructing signaling systems, one can precisely alter them in a highly controlled way, and map the landscape of physiological genotype/phenotype relationships. By using orthogonal components, one can ask questions free from the pleiotropic functional entanglement of natural proteins. Thus, these forward engineering approaches may help us better predict how changes wrought by evolution, disease, or therapy will impact cellular behaviors. In addition, the ability to engineer cells with customized signaling responses could also be useful for therapeutic applications. There have been remarkable recent advances in using engineered cells for cancer immunotherapy,

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Conflict of interest statement

Wendell Lim is a Founder of Cell Design Labs and a member of its scientific advisory board.

treatment of autoimmunity, and regenerative medicine [3], and improving our ability to precisely design therapeutic cells is of growing interest.

Driven by the twin motivations of understanding natural signaling networks and building cells with useful behaviors, researchers are developing methods for engineering diverse cellular signaling molecules and systems [4,5]. Recent efforts in the synthetic biology of signaling are distinct from the transcriptional engineering that dominated early synthetic biology, which largely focused on using gene expression modules to control protein abundance. In cell signaling, protein based receptors and posttranslational protein regulation play a principal role in mediating the cell's rapid response to changes in its environment. Engineering such fast and spatially coordinated cell signaling behaviors intrinsically focuses on engineering proteins.

Signaling proteins are highly modular in structure, often comprising distinct functional domains — some that catalyze particular information processing reactions (e.g. kinases and phosphatases) and others that mediate regulation or localization. One emerging strategy for engineering posttranslational regulation thus centers on generating novel combinations of modular domains and regulatory elements, which can result in rewiring new connections in the context of a larger cellular circuit. In this review, we will consider three areas of signaling protein design in which this modular approach has been highly successful and has shown recent progress: engineered synthetic cell-surface receptors, optogenetic sensors that allow light control of signaling pathways, and the engineering of synthetic phosphorylation-regulated signaling proteins.

Hierarchical logic of signaling proteins and networks

To communicate and respond to its environment, any cell must have at least three components: sensors or receptors that receive input, a downstream layer that processes these inputs, and physiological outputs that change in response to this information (e.g. changes in transcription, cell fate, cell migration or shape, etc.). Remarkably, even if one looks at the scale of individual signaling proteins, one can find the same type of organization. Even within an individual molecule one can find domains responsible for sensing inputs, domains or interactions that mediate decision making, and domains that control output (Figure 1). With this hierarchical architecture, new cellular behaviors — novel physiological INPUT/OUTPUT relationships — do not require the evolution of new systems, but merely new linkages between existing decision-making subsystems.

Ultimately, reconnecting signaling subsystems requires rewiring individual signaling molecules that lie at the junctions of these higher order subsystems. Links in the cell's signaling networks are often mediated by protein domains that perform specific functions: protein–protein interaction, subcellular localization, and catalysis. These domains are often found in multi-domain proteins [6] where their combination can yield switch-like enzymes gated by upstream signals, or scaffolds that rewire and guide signaling cascades (Figure 1). In the context of evolution [7,8], development [9], differentiation [10], and disease [11,12], it is clear that new cellular behaviors often arise when existing molecular modules are recombined to generate new receptors, sensors and downstream signaling protein. In this

review, we highlight recent advances in the design of synthetic signaling systems made by following the same approach. In other words, by learning how to rewire individual signaling proteins, we are at the same time learning how to rewire whole networks.

Engineering new sensor/receptor molecules

Like a microscopic Argus,[3] each cell perceives its environment through an array of molecular sensors and receptors. Synthetic biology now affords us the ability to further expand the cell's 'field of view.' Modularly engineered receptors and sensors can be used readily to link a variety of new inputs to a critical cellular response (as in the case of chimeric antigen receptors), or use a single input (light) to selectively modulate dozens of intracellular signaling systems with precise spatial and temporal control.

CARs: extracellular receptor proteins that detect user-specified antigens

It is hard to believe that simply replacing the extracellular domain of a receptor protein with an unrelated recognition module would allow one to redirect its input sensing, but that is exactly how chimeric antigen receptors (CAR) work. A CAR is a fusion protein combining an extracellular single chain antibody (scFv) with intra-cellular regulatory domains of the T-cell receptor complex. Remarkably, when a CAR is expressed in a T cell, this can be sufficient to reprogram the cell to detect and attack tumor cells bearing the cognate antigen [13,14] (Figure 2a). Initial clinical results with CARs have been highly promising for treatment of B cell cancers (targeting the B cell specific antigen CD19) [15], although over-activation (cytokine storm) and off-target damage are severe problems [16,17]. To address these issues of safety, more precisely regulated CAR variants were recently developed by separating the sensing and intra-cellular signaling domains of the CAR into two separate molecules, and then inducibly reuniting the two components with a modular drug induced heterodimeric interaction. In this way, the split-CAR is essentially a version of the T cell receptor that has been engineered to be controlled by two novel inputs — the disease antigen and the small molecule [18•]. Likewise, CARs gated by the presence [19,20] or absence [21] of secondary antigen on the target cell have been generated using these secondary antigens as a target for co-recruitment of synthetic modulatory receptors that contain intracellular activating or inhibitory domains, respectively.

A second class of engineered receptors harnesses the regulatory mechanism observed in the native Notch receptor. Notch engagement of an extracellular ligand triggers proteolysis of the receptor, releasing a transcription factor (contained in the receptor's intracellular fragment) that enters the nucleus and drives downstream gene expression. Following this model, synthetic systems have been constructed in which TEV protease and a membrane tethered transcription factor are co-localized by activated GPCRs (G-protein coupled receptors) and RTKs (receptor tyrosine kinases) [22], or — in a more general form — by any ligand that induces receptor dimerization [23]. It was recently discovered that the proteolytic core of Notch is a modular regulatory element — enabling the generation of synthetic Notch receptors (synNotch) in which both extracellular targeting and induced gene

[3]Watchman in Greek mythology whose body was covered with 100 eyes.

expression are fully customized (Figure 2a) [24]. Importantly, synNotch and CAR receptors are highly complementary. T cells engineered so that synNotch activation drives CAR expression show high specificity for dual-antigen tumors in vivo [25].

Sensors that detect bioorthogonal stimuli such as light and small molecules

Another approach to engineering novel control over cell behavior is to construct signaling proteins that are responsive to flexible user-controlled inputs, such as small molecules and light. Small molecules and light can act within the cell and eliminate the need for transmembrane receptors that are able to transmit signals across the membrane. Inducible signaling proteins help us understand and engineer signaling networks by enabling us to observe network function in response to precisely defined pathway inputs. The first generation of inducible signaling systems relied on chemically induced dimerization (CID) of modular binding domains that homodimerize or heterodimerize upon binding of a small molecule ligand. These have been previously reviewed [26•]. Below, we focus on the next generation of tools that use light as an inducer (broadly termed ‘optogenetics’), providing new strategies for protein control with exquisite spatial and temporal precision. Optogenetic proteins are engineered from natural photoreceptor domains that coordinate with light-sensing cofactors. Absorption of light by these cofactors induces photoisomerization that drives a conformational change in the associated protein domain. This conformational change is then coupled to larger allosteric changes or enhanced protein–protein interactions. A small set of photoreceptor modules that undergo light-induced dimerization, oligomerization, or steric regulation (Figure 2b) have been recurrently used to achieve optogenetic control over diverse signaling proteins and networks.

Light-induced dimerization—Optogenetic protein homo-dimerization and hetero-dimerization has been used in multiple strategies to regulate signaling nodes throughout the cell. Receptor tyrosine kinases (RTKs) EGFR, FGFR, and Ret have been endowed with blue light regulation through fusion of their intracellular tails to a small, blue-light-sensing homo-dimerization domain [27•]. Hetero-dimerization has been used to regulate signaling through protein recruitment to particular cellular compartments. Plasma membrane recruitment of activating guanine nucleotide exchange factors (GEFs) has been an effective strategy for control of small GTPases Rac, Rho, Cdc42, and Ras [28•,29], and, conversely, recruitment of an inhibitory GTPase activating protein (GAP) was reported as a method to inhibit G-protein coupled receptors [30]. Membrane recruitment was also used to regulate signaling through Raf-1 [31] and PI3K [32]. Inducible nuclear recruitment enabled optogenetic control of transcription factor activity [33], and optogenetic recruitment to other cellular compartments was reported as a general strategy for titrating away signaling proteins [34,35]. Still other dimerization-based approaches used homo-association of Dronpa mutants to sterically or allosterically regulate activity of a catalytically active GEF protein [36], and optogenetic dissociation of a constitutive dimer enabled optical control over protein trafficking and secretion [37].

Light-induced oligomerization—Multivalency and higher-order protein assembly play key regulatory roles in many cellular signaling systems [38], and recent work illustrates how protein clustering can be placed under optogenetic control. Blue light-induced

oligomerization of the Arabidopsis Cryptochrome2 protein has enabled activation of RTKs, both exogenous [39,40,41] and endogenous [41], as well as the Orai1 calcium channel [42] and the canonical Wnt pathway co-receptor LRP6 [43•]. Within the cytoplasm, clustering has been used to regulate Rho GTPase [43•] and Raf1 kinase [44] activity. Inducible clustering has also been used to regulate DNA damage signaling in the absence of DNA damage through oligomerization of TopBP1 [45].

Light-induced steric regulation—The blue light-induced conformational change of the LOV2 domain from *A. sativa* phototropin has been successfully used to sterically regulate small peptides controlling multiple cellular functions in a modular fashion. These functions include protein interaction [46,47], protein degradation [48,49], and nuclear translocation [50,51]. Steric occlusion of the appropriate peptides has also yielded optogenetic inhibition of specific kinases [52•] and activation of calcium channels [53].

Engineering phosphorylation control: intracellular posttranslational circuitry

Once a cellular sensor is activated, the signal is often relayed through a posttranslational regulatory network that processes that information and directs the cell to execute an appropriate response. Phosphorylation is the most common posttranslational modification [54], and efforts to engineer phospho-signaling proteins have shed light on how posttranslational networks function and how they can be rewired.

Engineered scaffolds for phospho-signaling

Proteins with multiple interaction domains can serve as molecular scaffolds, organizing multiple proteins in a signaling pathway into a complex (Figure 1). In yeast, the scaffold protein Ste5 orchestrates the mating pheromone MAP kinase (MAPK) pathway. Ste5 co-localizes a kinase cascade (MAPKKK → MAPKK → MAPK) and serves as a platform for the spatial and temporal control of these enzymes. To investigate how modular interactions mediate kinase cascades, Ryu and Park designed synthetic scaffolds using strings of repeated peptide binding domains (PDZ) and fused complementary PDZ ligands to each of the Ste5-associated kinases: Ste11 (MAPKKK), Ste7 (MAPKK), and Fus3 (MAPK). Synthetic scaffolds that co-localized two or more kinases (MAPKKK AND [MAPKK OR MAPK]) at the cell membrane were sufficient to functionally replace Ste5 [55] (Figure 3a). Moreover, these minimal scaffolds demonstrated logic gate properties and could be tuned by the co-recruitment of negative regulatory phosphatases. This result matches findings with endogenous MAPK pathways, where recruitment of regulators to engineered scaffolds reshapes the amplitude and timing of pathway behavior [56]. This approach was recently extended by the use of bacterial effectors [57]. The utility of this approach is exemplified by OspF, a toxic protein that irreversibly inactivates MAPKs by catalyzing a beta-elimination reaction that removes the hydroxyl group of the key phosphothreonine side chain, thereby preventing MAPK phosphorylation and consequent activation. This mechanism enables *Shigella flexneri* to disable human epithelial and dendritic cells. Repurposed as a tool for synthetic biology, this pathogenic inhibitor has been used to engineer a negative feedback

loop that reshapes the dynamic response of the yeast osmolarity pathway (Figure 3a) and a T-cell ‘pause switch’ for adoptive immunotherapy [57].

Phospho-regulated linear motifs

Signaling proteins are enriched in unstructured regions (linear motifs) where the ‘writers’, ‘readers’, and ‘erasers’ of phosphorylation (kinases, phospho-binding domains, and phosphatases, respectively) collectively regulate protein binding, concentration, and localization [58,59] (Figure 1). Phospho-regulated linear motifs have been used to create dynamic reporters of intracellular signaling. FRET reporters for specific kinases link a phospho-regulated intramolecular binding event (phosphorylated substrate peptide + phospho-binding domain) to fluorescent protein co-localization [60]. More recently, a synthetic, phospho-stabilized, version of the destabilizing PEST domain was fused to a fluorescent protein to construct a live cell reporter of Erk activity [61]. In a third example of phospho-engineering, Regot *et al.* built a reporter that translocates in response to c-Jun N-terminal kinase (JNK) activation by combining a nuclear export signal (NES) activated by JNK phosphorylation, a phospho-inhibited nuclear import signal (NLS), and a fluorescent protein [62••] (Figure 3b). Importantly, this design can be readily adapted to multiple types of kinases by either substituting the docking site for JNK with that of another MAP kinase (MAPK), or mutating a kinase’s naturally occurring substrate to introduce the NLS and NES modules. This work illustrates the potential for modular engineering with linear motifs, and complements recent advances in the computational design of posttranslational regulation [63].

Scaling up phospho-circuit design

In proteins that contain multiple phosphorylation sites, phospho-regulated linear motifs can collectively form information processors that integrate inputs, set response thresholds [64], tune binding affinities [65], amplify weak signals, and serve as ‘conduits’ for sequential signal transduction [66]. Analogous synthetic ‘devices’, built from combinations of phospho-regulatory modules, may ultimately make it possible to endow engineered signaling proteins with complex information processing behaviors.

One of the best-studied examples of multisite phosphorylation is Sic1, a critical cell cycle regulator whose phospho-induced degradation triggers S phase in budding yeast [64,67]. Cyclin dependent kinase 1 (Cdk1), in complex with a cyclin and the phospho-adaptor Cks1, phosphorylates Sic1 in an ordered sequence at multiple sites, culminating in the activation of phosphodegrons within Sic1 that recruit the SCF^{Cdc4} ubiquitin ligase complex, thereby targeting Sic1 for rapid degradation. Using synthetic Cdk1 substrates, Loog and colleagues found that the rate at which Cdk1 phosphorylation propagates is determined by how well the fixed spatial orientation of the three docking sites on the cyclin-Cdk1–Cks1 complex fits with the linear pattern of phosphorylation sites along the length of substrates like Sic1 [68•]. These results suggest that a simple set of rules might define the overall pattern and rates of phosphorylation in a multisite cluster.

Eco1, a regulator of sister chromatid cohesion, is also degraded after multisite phosphorylation. The timing of Eco1 function is usually restricted to S phase by the

collective action of three different kinases (Cdk1, Cdc7, Mck1). In healthy cells, sequential phosphorylation by these kinases forms an SCF^{Cdc4} phosphodegron, triggering Eco1 destruction. However, inhibition of one kinase (Cdc7) by the DNA damage response prevents Eco1 destruction, allowing establishment of cohesion after S phase. Lyons *et al.* characterized this system and generated a mutant version of Eco1 in which Cdk1 directly primes Mck1 phosphorylation — bypassing Cdc7's phospho-regulation. This study revealed that a single point deletion converts the naturally occurring 3-pronged AND gate into a synthetic 2-pronged gate with altered cell cycle sensitivity [69•] (Figure 3b).

Overall, these findings suggest that fairly simple rules governing linear phospho-motifs are used by nature to achieve quite sophisticated information processing. As we learn to better manipulate these motifs, we may be able to test and harness these emerging rules.

Forward evolution of signaling networks

Ultimately, to engineer cellular behavior, we want to engineer cellular networks. But as described above, to engineer cellular networks we need to learn how to engineer individual signaling proteins and their connectivity. We postulate that much of the functional innovation in cellular signaling networks has evolved through repeated duplication and recombination of modular domains [7]. Researchers interested in posttranslational engineering can now use bioinformatics tools [70,71,72] to mine these naturally occurring circuits for domains that are, in essence, 'pre-validated' for a high degree of modularity (successfully functioning in many fusion contexts) and minimal crosstalk with other cellular components. These parts complement the growing toolbox of regulatory domains that have been validated (in an analogous manner) through repeated use in synthetic circuits [5,73]. With these enriched building blocks, generating new signaling proteins could potentially be a straightforward matter of screening domain combinations (combinatorial libraries) and optimizing protein expression [18•,28•,56]. The same approach can be iterated to generate posttranslational circuits of increasing complexity. One successful example has been the construction of a synthetic circuit for inducing artificial cell polarization in yeast. Modular binding domains that recognize phospho-inositide species were combined with modular catalytic domains that modify these species, yielding a set of proteins that form spatially localized positive and negative feedback loops. Together, this system of synthetic proteins generates a self-organizing asymmetric pole of the signaling molecule PIP3 [74••] (Figure 4). It is likely that in the coming years, we will see more examples of the construction of more complex synthetic signaling systems, enabled by a better understanding of modular domains, but also by advances in computational design and experimental combinatorial screening of libraries of modular synthetic circuits. As the field of synthetic signaling systems matures, a semi-predictive approach that combines computational design with combinatorial screening offers a pragmatic strategy for learning nature's design principles while tailoring cellular responses to applications in medicine and biotechnology.

Acknowledgments

The authors sincerely thank Dr. Nicholas Frankel, Dr. Ricardo Almeida, Dr. Scott Coyle, Dr. Kole Roybal and Dr. Kyle Daniels for comments on the manuscript, as well as other members of the Lim lab for helpful discussions. We apologize to authors whose relevant work was not cited in this review due to space constraints. The authors are

supported by the National Institutes of Health (P50 GM081879, PN2 EY016546, R01 GM055040, R01 GM096164, R01 CA196277), NSF Synthetic Biology and Engineering Research Center, and the Howard Hughes Medical Institute.

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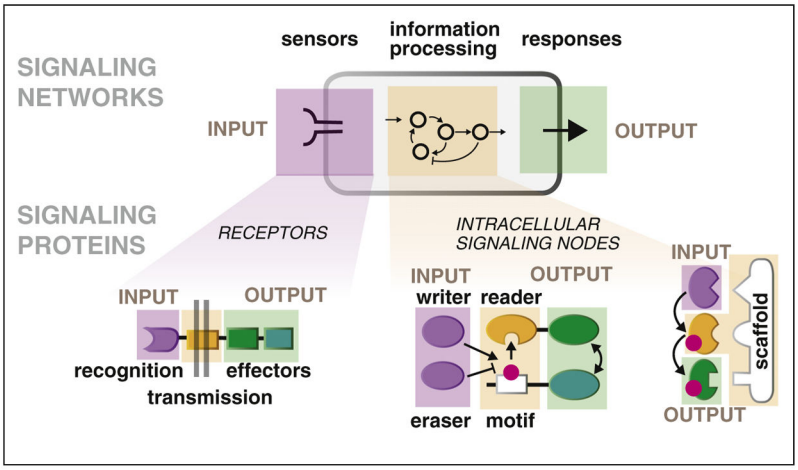

Figure 1.

Hierarchical organization of signaling systems: cells and individual proteins as input/output nodes. At any scale, a signaling system must have three components — it must have sensors to receive INPUT, an information-processing layer that decides what to make of this information, and an OUTPUT function. These components are found in individual signaling molecules, which detect and effect particular upstream and downstream molecular partners. In receptors that span the cell's membrane, ligand binding to extracellular domains (INPUT) rapidly regulates the activity of intracellular effector domains (OUTPUT). Similarly, posttranslationally-regulated binding motifs link the activities of upstream enzymes that 'write' and 'erase' the posttranslational mark (INPUT; e.g. kinases and phosphatases) to recruitment of dedicated 'reader' domains (OUTPUT). The same classes of components are found in signaling networks and whole cells, but in this case receptor molecules function as INPUT sensors, networks of intracellular proteins function as the information processing layer, and various cellular response modules control OUTPUT.

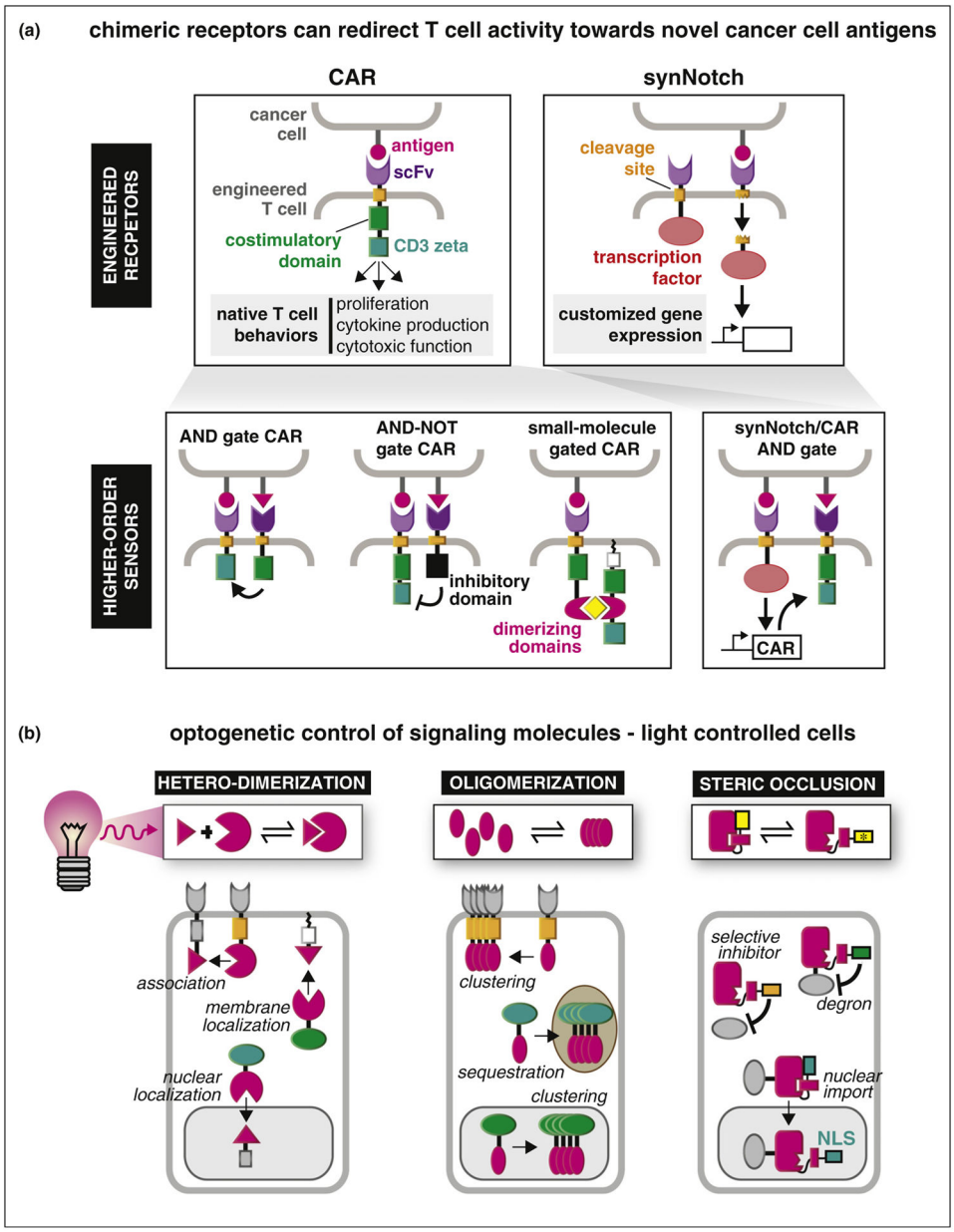

Figure 2.

Engineering new sensor/receptor systems. **(a)** The chimeric antigen receptor (CAR) was engineered to sense a tumor antigen and induce an immunogenic response against tumor cells expressing that antigen. Modular recombination of the CAR domains with new sensor modules has enhanced specificity of the CAR-T response either through logic gates requiring combinations of specific antigens or licensed by small molecule dimerization of critical signaling domains [18•,19–21]. A second type of engineered receptor based on Notch (synNotch) allows both input (target antigen) and output (gene expression) to be fully customized [24]. CAR and synNotch receptors can be combined synergistically, refining the specificity and scope of the T cell response [25]. **(b)** Modular optogenetic tools for controlling receptors and signaling proteins. Protein domains from plants that undergo light-

induced dimerization, oligomerization, or steric regulation have been used to regulate signaling activities throughout the cell in a modular fashion.

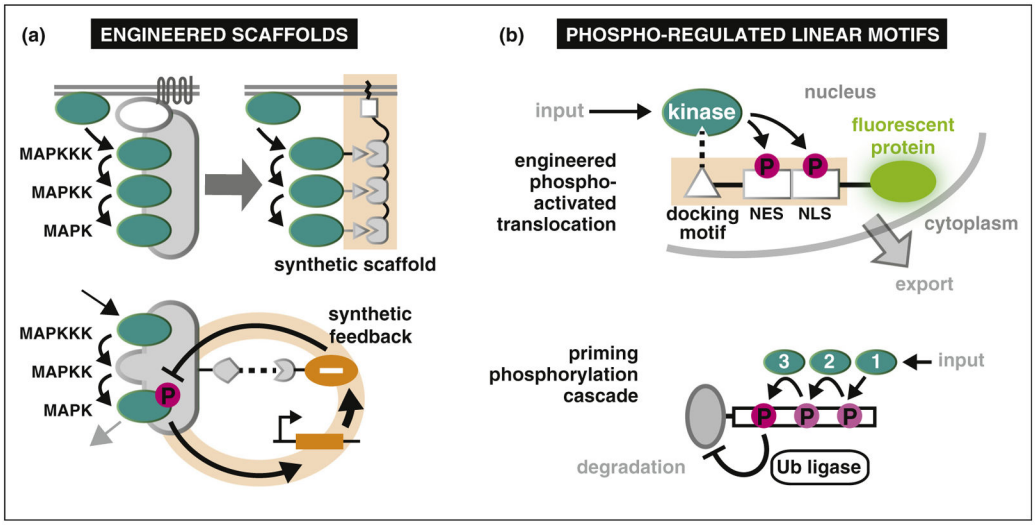

Figure 3.

Post-translational signaling: rewiring phosphorylation devices. Novel linkages between signaling modules enable new functionality in signaling proteins and networks. **(a)** Synthetic scaffolding of the MAPK pathway was sufficient to induce downstream signaling [55] (top left), while co-scaffolding with negative regulatory effectors can tune the pathway response [57]. **(b)** Combining kinase docking motifs with phospho-regulated nuclear import and export sequences is a successful strategy to create dynamic fluorescent reporters of specific kinase activity [62••] (top right). Mutation of a phospho-site in a 3-pronged AND-gate for protein degradation generated a 2-pronged degradation AND-gate [69•].

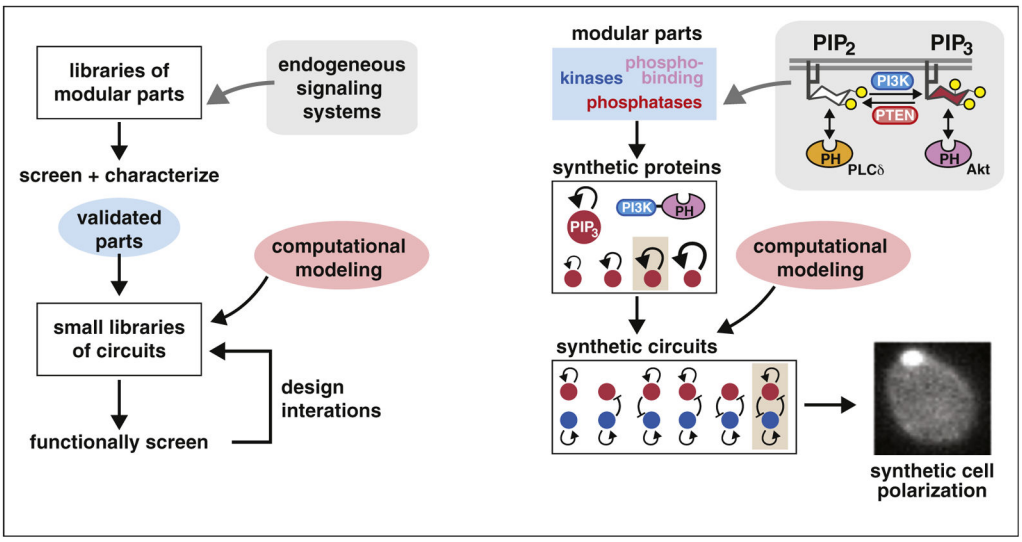

Figure 4.

Combinatorial design for engineering signaling proteins and networks. We present here a conceptual workflow for engineering signaling networks with desired properties. Small libraries of candidate circuits can be semi-rationally designed using a combination of validated signaling and regulatory components together with computational models. These circuits can then be screened and optimized for the proper function. The design of a network for cell polarization [74••] is provided as an example of this approach.