REVIEWS

Designing customized cell signalling circuits

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Abstract | Living cells have evolved a broad array of complex signalling responses, which enables them to survive diverse environmental challenges and execute specific physiological functions. Our increasingly sophisticated understanding of the molecular mechanisms of cell signalling networks in eukaryotes has revealed a remarkably modular organization and synthetic biologists are exploring how this can be exploited to engineer cells with novel signalling behaviours. This approach is beginning to reveal the logic of how cells might evolve innovative new functions and moves us towards the exciting possibility of engineering custom cells with precise sensing–response functions that could be useful in medicine and biotechnology.

Living cells are dynamic systems that use complex molecular signalling circuits to monitor external and internal states, and to execute the appropriate physiological responses. Like any sensory machine, evolved or man-made, these cellular signalling circuits contain decision-making subsystems that act as sensors and processors (such as receptors and their effectors) that ultimately control various response subsystems (such as gene transcription and cytoskeletal dynamics) (FIG. 1a). A major goal of modern cell biology is to understand how these molecular signalling systems achieve their complex responses, which are optimally tuned for their physiological role. Although most research is aimed at dissecting, mapping and analysing cell signalling networks, our increasing understanding of how these systems work has led to the emergence of a radical new approach — efforts to design and build custom synthetic signalling circuits[1,2].

Here, I focus on the synthetic biology of signalling, looking at how the signalling circuitry of eukaryotic cells can be engineered to construct cells with designed behaviours. Eukaryotic cells use protein signalling networks to sense their environment and mediate rapid responses. As signal-processing networks in cells function in a three-dimensional setting, they also control complex spatial or morphological cellular responses. I discuss how signalling circuits with precise response behaviours can be generated by considering how the specificity of a response is determined (that is, what sets of outputs are linked to a specific input), how the precisely tuned dose response or temporal dynamic profiles of responses are optimized for particular physiological functions and how complex spatial and morphological control can be achieved (FIG. 1b). I also consider why efforts to design and build custom synthetic signalling circuits have emerged, how they might provide a deeper perspective on the design principles and mechanisms of molecular signalling systems, and how customized response behaviours could be applied in medicine and biotechnology. Finally, I discuss how future tools and methods could be developed to make the engineering of cellular behaviours easier.

Why engineer cell signalling?

Before considering specific examples of engineered signalling pathways, it is useful to discuss the motivations for engineering cell signalling. Attempting to create new signalling behaviours in cells may seem like an audacious goal, given that we do not yet have a complete or reliably predictive understanding of the natural signalling circuits of cells. However, the engineering of cell signalling is not simply a process for applying an already well-developed understanding — it offers an approach for 'understanding by building'. Whereas biology has traditionally been a science of analysis and deconstruction to identify genes and molecules that are important for a particular process, synthetic biology offers an inverse approach, focusing on how individual molecular parts can be assembled into systems that carry out complex behaviours[3–7]. As we have access to fully sequenced genomes and a vast amount of proteomic data we do not lack a complete list of molecular parts, but rather an understanding of how these parts fit together in a functionally coherent way. Engineering new cell signalling networks offers an approach for us to test and expand our understanding of the organizational principles of complex molecular systems.

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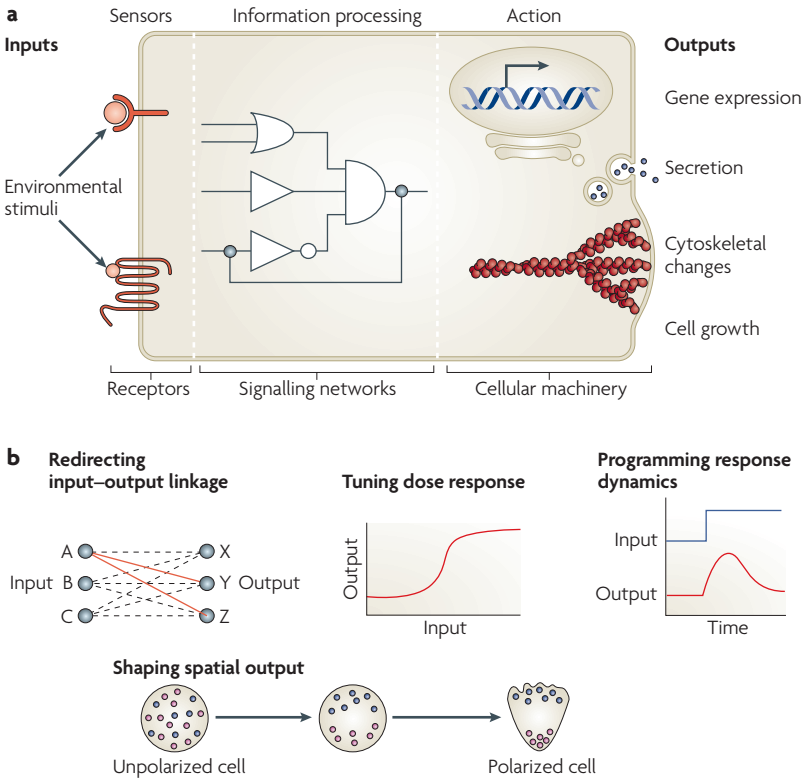

Figure 1 | **The general organization and behaviour of cell signalling circuits.** **a** | Cells generally sense environmental stimuli through sensors, such as receptors. This information is then processed by intracellular signalling networks, which engage various cellular outputs, including gene expression, secretion, cytoskeletal changes and cell growth. **b** | Some of the major challenges in the evolution or engineering of novel signalling circuits are achieving the correct linkage between specific inputs (A, B and C) and outputs (X, Y and Z), tuning the quantitative behaviours (dose response and dynamics) of the signalling response so that they are optimal for their physiological function, and generating robust spatially self-organizing processes, such as those associated with cell polarization, directed motility, division and compartmentalization.

In this sense, the synthetic biology of signalling is not simply oriented towards achieving an application goal, such as building a cell with a target function, but it is also an exploratory science in which it is important to understand what designs 'work' and how they relate to designs that 'don't work'. For example, for a natural signalling network that carries out a complex behaviour of interest, traditional genetic deconstruction can be used to identify molecules and linkages that are necessary and important for function (FIG. 2a). However, synthetic approaches can then be used to systematically explore many types of changes, such as alternative network linkages, the tuning of linkage strength and the addition of new linkages, to test which networks are compatible with the behaviour of interest. By dissecting the natural network, or engineering a single successful circuit, it is unlikely that the deeper understanding of the functional landscape will be gained that a more complete and systematic synthetic circuit exploration can yield[5–7] (FIG. 2b). In this sense, attempting to engineer cellular behaviours is akin to the early history of synthetic organic chemistry, whereby synthesis of new or modified molecules provided a complimentary approach to chemical analysis in the development of the fundamental theories of chemical bonding, structure and reactivity[8].

Exploring the plasticity of signalling pathways, and how their functions can be tuned, is also relevant to the pathology and treatment of disease. Many cancers harbour oncogenic mutations that effectively 'rewire' the cell signalling networks that control the balance between cell growth, differentiation and death[9]. Similarly, many intracellular pathogens, including bacteria and viruses, produce specific proteins that rewire endogenous signalling pathways[10–12]. Many bacterial pathogen proteins interface with host signalling pathways, often to suppress the host immune response or to enhance infection (see Supplementary information S1 (box)). Thus, by using synthetic biology to understand the plasticity of pathways and how their behaviour is changed by network perturbations, we can gain a better framework for understanding the strategies adopted by pathogens to exploit the inherent fragilities of signalling networks. Moreover, we can develop strategies for shifting a diseased network back to a stable, non-pathological behaviour. The most stable network-based therapies may not involve simply blocking the primary oncogenic protein with a drug, but rewiring the network so that it shows a new but stable behaviour.

Applications of engineered signalling

Engineering cell signalling behaviours might also allow us to construct cells programmed to execute precisely designed applications (FIG. 2b). Imagine if we could mimic and exceed evolution by using a tool kit of molecular parts to genetically engineer cells that carry out custom designed responses. As stem cell biology matures[13–15] and techniques such as adoptive immunotherapy develop[15,16], the possibility of using cell-based therapeutics gets closer but will require sophisticated cellular engineering to precisely control cell behaviour. For example, without novel control, how could proper stem cell migration and differentiation be directed for regenerative medicine, given the absence of normal developmental signals? Moreover, as industrial production processes, such as biofuel and materials production, engage biological organisms[17], it might be possible to engineer smarter strains that, like macroscopic production facilities, have cellular control systems that monitor external and internal states to optimize production. This may be particularly crucial as we use fermentation organisms such as yeast to produce a wide range of materials that may have toxic effects.

Although custom designed therapeutic cells lie in the future, it is useful to think about which detection and response behaviours would be valuable, as they provide useful target milestones in the development of tools and strategies for cellular rewiring.

Designed anti-cancer cells. If we could design custom therapeutic cells that sense disease signals and execute highly targeted and precisely calibrated therapeutic programmes in response, what behaviours would we want? Immune cells such as T lymphocytes or natural killer cells could be modified to identify and kill tumour cells. Such cells are already used in adoptive

Adoptive immunotherapy
A therapeutic strategy in which a patient's lymphocytes are removed, modified or manipulated *ex vivo* and retransfused to the patient. This is often used in the treatment of cancer.

T lymphocyte
A lymphocyte that expresses heterodimeric receptors associated with the CD3 complex. Effector T lymphocytes (or T cells) carry out a variety of functions, acting through interactions with other cells (for example, activating macrophages or helping B cells produce antibodies). Cytotoxic T cells kill cells infected with intracellular pathogens.

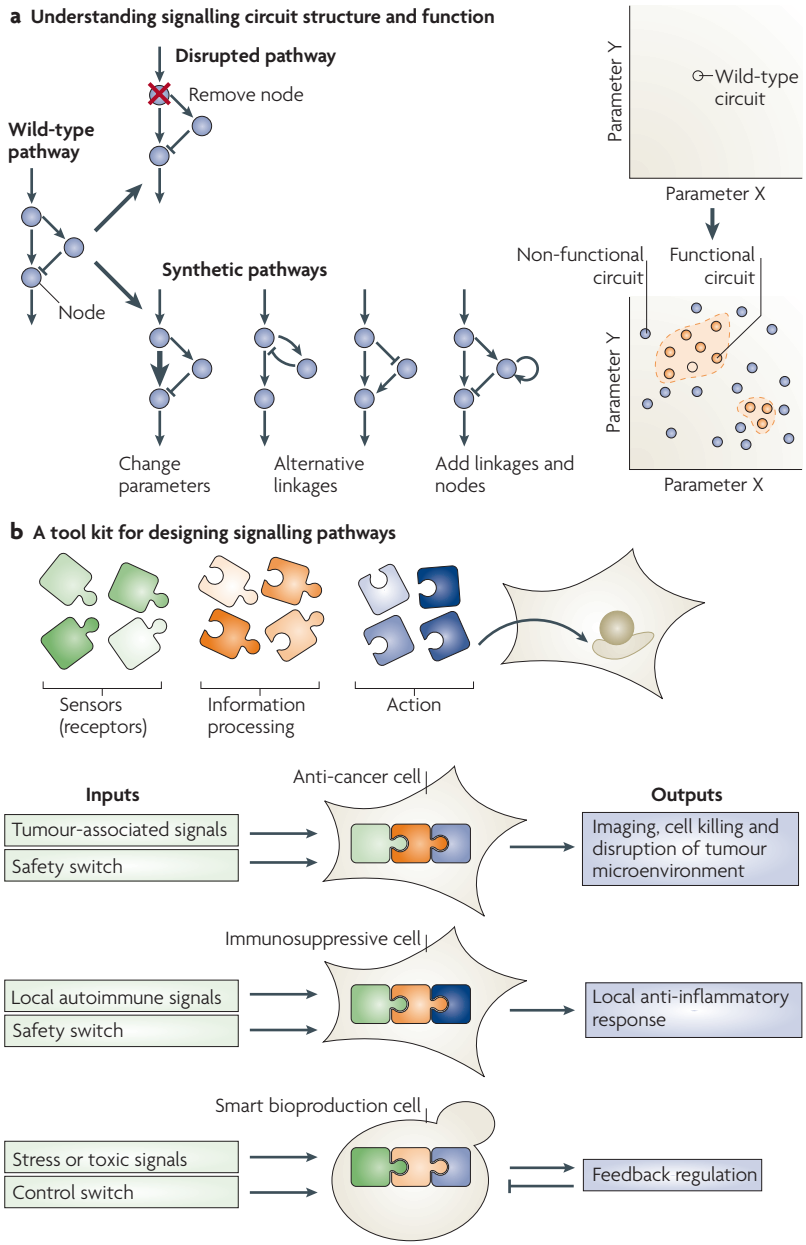

Figure 2 | **Applications of rewired cell signalling circuits. a** | Rewiring cell signalling circuits can help us to understand their design principles. Traditionally, methods such as gene disruption are used to dissect a signalling network. Synthetic approaches offer complementary information by creating alternative versions of a network that differ in connectivity or the strength of links. By mapping the space of functional (orange circles) versus non-functional (blue circles) variants, a deeper understanding of functional requirements is gained. **b** | Rewiring cell signalling circuits can also help us to construct designer signalling pathways for therapeutic or biotechnology applications. We hope to assemble a tool kit of signalling modules that can be used to create cells with designed signalling responses. An anti-cancer cell could detect a combination of tumour-associated signals (such as tumour antigens, hypoxia or tumour-promoting growth factors) and respond by producing imaging reagents, killing cells (by activating cytotoxic programmes) or secreting factors (such as pro-inflammatory cytokines or anti-angiogenesis factors) that disrupt the tumour microenvironment. It might also have safety switches that could disable the cell if needed. An immunosuppressive cell could detect a combination of autoimmune response or transplant rejection signals and trigger localized countermeasures, such as secretion of anti-inflammatory cytokines. A smart bioproduction (fermentation) cell could be engineered to precisely modulate feedback regulation, namely the flux in growth versus production pathways in response to the stress state of the cell, thus optimizing overall yield.

immunotherapy[18,19]. An anti-cancer cell could be designed to detect a combination of tumour-associated signals, including specific tumour antigens, hypoxia, organ-specific antigens and growth factors and cytokines, that are secreted by tumours to enable them to evade normal immune responses and create a tumour-promoting microenviroment[20] (FIG. 2b). Engineering cells that recognize these factors but are linked to an anti-tumour response would be ideal. It is also crucial to engineer external control (for example, by small molecules) or safety switches into these therapeutic cells, so that their behaviour can be shut off or attenuated in response to undesirable side effects.

Designed cells that detect these tumour-specific inputs could be engineered to yield several different responses, such as the production of imaging agents that aid in identifying tumours and metastases and the control of endogneous immune cell responses, such as chemotaxis, phagocytosis and cell killing. Perhaps most importantly, these therapeutic cells might be programmed to secrete factors that disrupt the local tumour microenvironment, such as pro-inflammatory cytokines and anti-angiogenesis factors, making it untenable for sustained tumour growth. This would be equivalent to creating a custom immune cell that disables the tumour cells and the microenviroment at multiple levels.

Targeted immunosuppression. An immune cell could also be designed to block autoimmune disease or the rejection of transplanted organs (FIG. 2b). Normal immunosuppressive drug therapy has broad and serious systemic effects. An engineered cell could be programmed to react in a local immunosuppressive manner, perhaps in response to specific autoimmune or transplant antigens in combination with the cytokine signatures of a strong autoimmune response. Such cells might be programmed to chemotax to the sites of these signals and respond by secreting anti-inflammatory cytokines that disable the inflammatory positive feedback loops that normally lead to a full-blown autoimmune or rejection response.

Can signalling networks be engineered?

There is disagreement as to whether cells can be engineered. Are cell signalling systems so finely optimized that our intervention will lead to catastrophic malfunctions or so robustly designed by evolution that the addition of new genes and network links will not markedly alter function? Clearly, evolution has been able to rewire cell signalling pathways to yield diverse responses — at some level, therefore, they are relatively plastic and evolvable. Thus, before trying to engineer new cellular behaviours, it may be instructive to consider how evolution can achieve innovative new functions.

A hallmark of signalling proteins is their modular structure, which is thought to play a big part in evolution. These modular domains can have a catalytic function or specific regulatory or interaction functions[21,22], and they are found in highly varied combinations in different signalling proteins. This has led to the model that diversity in signalling function could evolve through

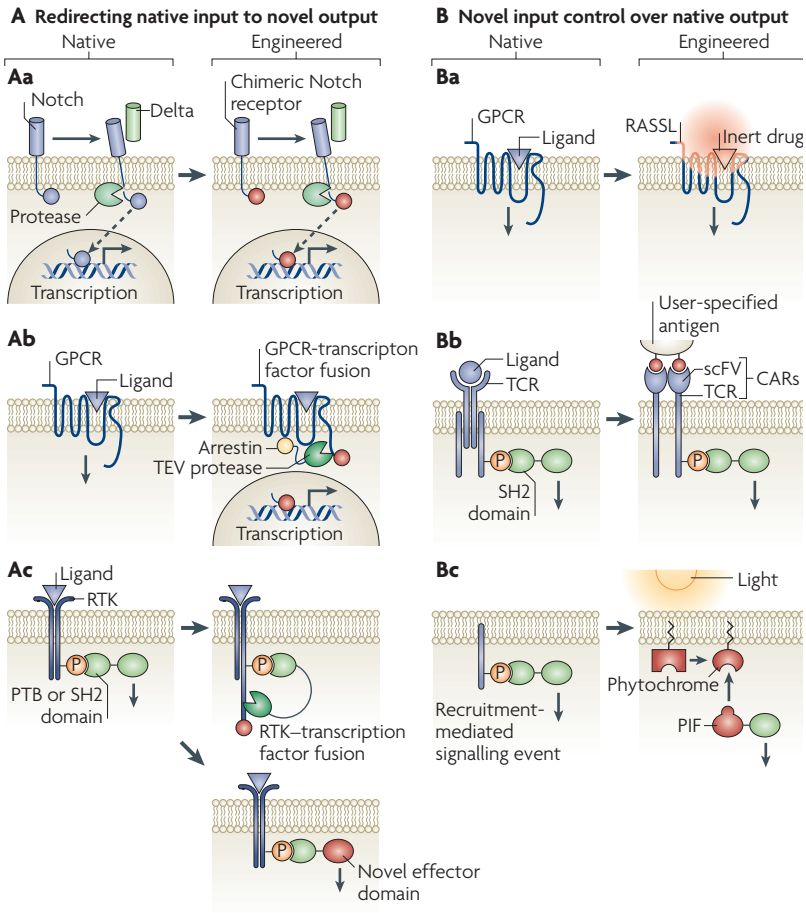

Figure 3 | **Engineering novel signalling sensors. A** | Redirecting native inputs to novel outputs. The cytoplasmic domain of Notch receptors is a transcription factor that is released by proteolysis on activation by the ligand, Delta. Replacement with an alternative transcription factor domain yields a novel transcriptional output[28] (**Aa**). G protein-coupled receptor (GPCR) output can be similarly redirected by fusing a transcription factor domain to the GPCR through a tether containing a tobacco etch virus (TEV) protease site (**Ab**). Activated GPCRs recruit the adaptor protein arrestin. If an arrestin–TEV protease fusion is expressed in the cell, GPCR activation results in the release of the transcription factor and a synthetic transcriptional output[30]. Receptor tyrosine kinase (RTK) output can be redirected through the recruitment of synthetic SH2 or PTB domain adaptors to the activated, Tyr phosphorylated receptor (**Ac**). The SH2 domain could be used to recruit a TEV protease to release an artificially tethered transcriptional domain[30], or to recruit novel effector domains such as those involved in cell death, to result in a new response, such as cell death[31]. **B** | Engineering novel input control over native responses. RASSL (receptors activated solely by synthetic ligand) GPCRs are controlled by small molecule agonists (inert drugs) because their extracellular surface is mutated so that they can't bind their endogenous ligands[34] (**Ba**). Receptors that activate T cells in response to user-specified antigens can be generated by fusing engineered single-chain antibodies (scFvs) to the intracellular region of the T cell receptor (CD3ζ chain) through its transmembrane domain (**Bb**). These receptors are called chimeric antigen receptors (CARs)[18,19]. A recruitment-mediated signalling event can be placed under light control by replacing the endogenous interaction domains with the phytochrome–PIF interaction pair from plants, which interact in response to light[46] (**Bc**).

the recombination of domains. Thus, in principle, if we could understand how evolution works with these modules, we might be able to exploit the same tool kit to find regions of behaviour that evolution has, to our knowledge, not yet explored.

Why are signalling proteins and systems so modular? Most agree that, on an evolutionary timescale, organisms are under a fitness pressure to develop innovative cellular signalling responses that might lead to advantages in changing environments and against competing organisms. Under this kind of changing fitness pressure, modular systems might spontaneously evolve as a way to facilitate the more rapid diversification of function[23]. Alon and co-workers simulated biological network evolution using evolutionary algorithms to search for simple computational networks that solve a target goal[24]. When they repeatedly switch the target goal, the resultant networks spontaneously develop more modular solutions — networks have functional subnetworks within them. These preformed subnetworks, the modules, can be rapidly reconnected in new ways to shift from one target function to another. In essence, modules seem to provide a way to rapidly move from one function space to another, while jumping over vast regions of non-functional networks. Thus, the modular organization of signalling proteins and networks may reflect the pressure on these systems to generate behaviours that fit the needs of a constantly changing environment.

The importance of modularity in facilitating the evolution of new functions fits with concepts in evolution and development, in which it is argued that much of the diversification of function and morphology of organisms evolves through the alternative regulation of existing components, rather than on the invention of radically new components[25]. Although many of these ideas have developed from focusing primarily on the regulation of genes by diverse *cis*-acting modules, they could also apply to the regulation of key catalytic signalling modules by diverse localization and regulatory modules[26,27]. Not surprisingly, many of the efforts to engineer new signalling behaviours, outlined below, exploit strategies of recombining modular functional units in novel ways; thus, in effect, harnessing an evolutionary strategy to engineer new function.

Engineering new sensor systems

One of the crucial tools for rewiring cellular behaviour will be the ability to engineer novel sensors and receptors for targeted inputs. However, this is perhaps the least characterized element in engineering cell signalling because the possible inputs are vast and it often involves the challenge of working with relatively complex membrane proteins. Below, recent progress in modifying or constructing diverse receptor molecules is described (FIG. 3).

Redirecting the output of natural receptors. There are several examples of a native receptor being redirected to elicit a new output. One approach exploits the modular structure of the Notch transmembrane receptors, which detect the Delta proteins presented on neighbouring cells (FIG. 3Aa). This is a crucial cell–cell communication channel in development and differentiation. When Delta binds Notch, the Notch transmembrane region is cleaved by a membrane protease, releasing the Notch carboxy-terminal domain into the cytoplasm, from where it enters the nucleus and activates gene transcription. Struhl *et al.*

showed that the Notch transcription factor module can be replaced by a synthetic transcription factor so that, when activated *in vivo*, chimeric Notch can activate genes targeted by the new transcription factor[28,29]. Although this construct was used as a reporter for Notch activation, it could easily be used to link the detection of native Delta to a completely new set of non-native target genes.

Barnea *et al.* expanded on this Notch-inspired modular strategy by engineering new transcriptional outputs for receptors that normally do not use this type of protease activation mechanism[30] (FIG. 3Ab,Ac). When G protein-coupled receptors (GPCRs) are activated by their specific ligands, they often recruit β-arrestin, which is involved in downregulating GPCR signalling. Barnea *et al.* fused β-arrestin to a highly specific protease from the tobacco etch virus (TEV), so that it was co-recruited to activated GPCRs. A synthetic transcription factor was fused to the GPCR cytoplasmic tail, linked by a TEV cleavage site. Thus, when the engineered GPCR fusion protein is activated by its endogenous ligand it recruits the β-arrestin–TEV protease partner, which cleaves and releases the transcription factor domain from the GPCR so that it can enter the nucleus and activate target genes (FIG. 3Ab). This system has been used successfully to link new transcriptional reporters to the activation of a wide range of GPCRs. The response is highly specific, owing to the specificity of TEV cleavage. In principle, this strategy could be used to link any endogenous GPCR-mediated signal to the expression of desired target genes.

Barnea *et al.* also used this strategy to link endogenous receptor Tyr kinase (RTK) signalling to novel transcriptional outputs[30] (FIG. 3Ac). Most RTKs, when stimulated, activate their kinase domains, which mediate autophosphorylation on cytoplasmic Tyr residues to recruit SH2 domain-containing proteins. In this study, the TEV protease was fused to recruited SH2 domains and a synthetic transcription factor was fused to the cytoplasmic tail of the RTK through the TEV protease cleavage site. So, RTK activation leads to the recruitment of the SH2 domain–TEV fusion, the release of the RTK-associated transcription factor and engineered gene transcription. It is remarkable that this simple modular strategy can be applied to several receptor classes, as long as they recruit a specific partner protein on activation.

Howard *et al.* harnessed the modularity of RTK signalling to redirect an oncogenic growth signal to an apoptotic response[31] (FIG. 3Ac). They engineered an SH2 adaptor protein in which an SH2 domain that recognizes an activated RTK was fused to a death effector domain from FAS-associated death domain protein (FADD). Thus, activation of the RTK led to membrane recruitment of the death domain, which induced a cell death response. The possibility of linking other novel outputs to these key recruitment events has not been well explored.

Receptors that detect novel small-molecule inputs. The above strategies take receptors that detect endogenous signalling molecules and engineer them to elicit new responses. However, in many cases, cellular engineering may require receptors that detect new, orthogonal signals for which there are no endogenous receptors, such as small molecules that could provide external control of an engineered system.

Relatively good success has been achieved in using GPCRs as a platform for engineering receptors controlled by small molecules. Certain GPCRs, such as opioid receptors, can be activated by specific small molecular agonists in addition to their endogenous ligands. Conklin, Roth and co-workers have engineered molecules known as RASSLs (receptors activated solely by synthetic ligands)[32–34] (FIG. 3Ba). These receptors are mutated so that they cannot bind their endogenous ligand but are activated by small molecular agonists.

GPCRs differ in their outputs, in part because individual receptors communicate with specific heterotrimeric G proteins. Further engineering has yielded versions of RASSLs that are specifically coupled to each of these distinct downstream pathways, thus allowing small molecule control of a highly diverse set of outputs. These RASSLs have been successfully deployed in transgenic mice — essentially rewiring signalling in a full living organism — mostly as a diagnostic and analytical tool. The applications have been diverse, given the broad usage of GPCRs throughout different tissues. For example, mice bearing taste neurons expressing RASSLs showed specific sweet (attractive) or bitter (aversive) responses to water mixed with the agonist (spiradoline), depending on which type of neuron they were expressed in[35]. In addition, expression of RASSLs in heart cells allowed heart rate to be controlled by the administration of spiradoline[36]. That these receptors work so robustly *in vivo*, hints at their potential use in more complex cellular engineering.

Chemical dimerizers form another strategy for achieving small molecule control over signalling. Such strategies have been reviewed elsewhere[37,38] and will not be discussed here.

Receptors that detect user-specified antigens. It would be ideal to engineer receptors that can sense disease-associated antigens, such as a protein expressed strongly in a tumour or infectious agent. If receptors could achieve the same diversity and selectivity of recognition as antibodies, a wide range of inputs could be detected and linked to specific responses. Chimeric antigen receptors (CARs) — receptors designed with single-chain antibodies as part of their detection mechanism — have been developed as this type of multipurpose framework (FIG. 3Bb). This strategy stems from the modularity of immune cell receptors, such as T cell receptors. Although a T cell receptor is a complex multiprotein complex, cross-linking of the cytoplasmic region of the CD3ζ chain subunit is sufficient to induce T cell signalling[39]. The CD3ζ chain contains motifs that are phosphorylated on activation by Tyr kinases such as LCK, to induce recruitment of SH2 domain-containing proteins such as the ZAP70 kinase. Fusion of the cytoplasmic region of the CD3ζ chain to an extracellular single-chain antibody yields a receptor often referred to as a 'T body', which, when expressed in T cells, causes the targeted killing of cells expressing the recognized

Natural killer cell
A class of lymphocytes that is crucial in the innate immune response. Natural killer cells exert a cytotoxic activity on target cells (such as virus-infected cells) that is enhanced by cytokines such as interferons.

SH2 domain
(SRC homology 2 domain). A protein motif that recognizes and binds Tyr-phosphorylated sequences and thereby has a key role in relaying cascades of signal transduction.

Orthogonal signal
A signal that only reacts with its cognate ligand and does not cross react with the host proteome.

Heterotrimeric G protein
A protein complex of three proteins (Gα, Gβ and Gγ). Gβ and Gγ form a tight complex, which Gα is a part of when the complex is in its inactive GDP-bound form, but dissociates from when the complex is in its active GTP-bound form. Both Gα and Gβγ can transmit downstream signals after activation.

antigen (presumably the surface antigens cross link and activate the chimeric receptors)[40,41]. Fusion of single-chain antibodies to the intracellular region of the Fc receptor (γ chain) can yield a similar type of chimeric antigen-responsive receptor. These studies highlight the modularity of these receptors: linkage of a novel extracellular recognition element to downstream intracellular signalling elements leads to a new input–output sensor.

These first-generation CARs are relatively primitive and have met with mixed results. T cells expressing CARs directed towards tumour antigens have moderate signalling capability compared with endogenous T cell receptor responses, proliferate moderately *ex vivo* and *in vivo* and have poor survival on repeated antigen exposure[18,19]. Improvements in these behaviours have been made by incorporating additional modular domains in the intracellular regions of the CARs, including domains from co-receptor molecules that are part of normal T cell receptor activation, thus perhaps mimicking a more complete activated intracellular assembly[42,43]. Cells containing these next-generation CARs more effectively control xenograft tumours in mice and are now being used in clinical trials[18]. More sophisticated engineering of CARs may lead to further improvement in their therapeutic function.

Sensors that detect physical signals such as light. Another fascinating area of exploration is the development of genetically encoded sensors that can detect light and transduce this to a specific biological response, an area referred to as optogenetics. Naturally occurring photosensitive proteins from plants, algae and bacteria can be modified for use in higher organisms, including mammals. These tools are extremely useful as spatiotemporal dials to control and analyse complex cellular and organismal behaviour, especially when they are expressed from cell-type specific promoters. In the long term, optogenetic tools could be used to remotely control cells in therapeutic applications — light could be used to precisely activate selective cells in a living patient. Nonetheless, major technical challenges remain, such as how light can be delivered in an organism. The most commonly used optogenetic tools today are the microbial channelrhodopsin and halorhodopsin proteins, which have been used extensively to control neuronal function. These are reviewed elsewhere[44] and will not be discussed in detail here.

More recently, optogenetic tools have emerged that extend beyond ion channels to allow the control of a broader range of cell signalling systems. Airan *et al.* constructed a set of light-activated GPCRs that can communicate with both downstream Gs and Gq heterotrimeric G proteins[45]. Chimaeras of the light-sensitive visual system GPCR, rhodopsin (bovine), were made that contain intracellular loops from both Gq- and Gs-coupled adrenergic receptors. The endogenous retinal molecule is the light-sensitive chromophore. These new tools greatly expand the signalling 'vocabulary' that can be controlled by light, given the importance of Gq and Gs signalling pathways in diverse cell types.

An even more generalized strategy for light control involves the use of light-controlled protein interactions. The transient interaction of specific partner proteins is the basis of many intracellular signalling events (see below) and receptors can be bypassed so that light directly controls such intracellular interactions. Levskaya *et al.* used the plant derived phytochrome interaction system — the binding of this photoreceptor to its partner PIF domain can be toggled on and off by specific wavelengths of light — to recruit specific proteins to the membrane in a precise spatiotemporal manner[46] (FIG. 3Bc). In the case of guanine nucleotide exchange factors (GEFs), which control Rho-family GTPases, this can be used to trigger GTPase activation and downstream cytoskeletal changes, leading to light-guided cell protrusion. Although this technique is powerful and potentially applicable to many signalling interactions, the phytochrome–PIF system requires the addition of a cell-permeable chromophore that is not endogenous to mammalian cells. Wu *et al.* used a photosensitive LOV (light-oxygen-voltage) domain (found in plants, algae and bacteria) to conformationally occlude the Rac GTPase in a light-controlled manner[47]. This flavin-binding domain provides another potentially generic conformational light-controlled element, which could be used to control diverse signalling proteins.

Engineering signal processing systems

Ultimately, cells decide what response programmes to execute based on intracellular signalling networks that receive and process signals from sensor molecules (see above). Recent work in cellular engineering has focused on understanding how these networks function to make decisions and how they can be rewired.

Modular logic of signal processing. Intracellular signalling proteins are highly modular (see above). Most modules fall into one of two classes (FIG. 4a). The first class are enzymatic domains, such as kinases and phosphatases, which catalyse the post-translational modifications or conformational changes by which information is stored. In most cases, these catalytic domains come in pairs: 'writer' enzymes (such as kinases) make a modification and 'eraser' enzymes (such as phosphatases) remove the modification. The second class contains regulatory or interaction domains that modulate the activity of catalytic domains, or target them to specific partners or sites in the cell. These modules can mediate specific protein–protein interactions (either constitutive interactions or those dependent on post-translational modifications such as phosphorylation) or protein–membrane interactions. Thus, it is predominantly the regulatory and interaction domains that determine when and where the catalytic domains are activated and to what partners they transmit information[5].

These different classes of modules are found in diverse combinations and arrangements in signalling proteins (FIG. 4b). Catalytic domains fused to targeting domains can be recruited to specific complexes or membrane locations, where they will modify specific

PIF domain
(Phytochrome-interacting bHLH factor domain). A plant protein interaction domain that selectively binds to the light-activated state of the phytochrome domain. The phytochrome–PIF interaction is normally involved in transcriptional regulation of plants, but can be introduced into diverse organisms and cell types to control the recruitment and activity of fused protein activities.

Guanine nucleotide exchange factor
(GEF). A protein that activates a specific small GTPase by catalysing the exchange of bound GDP for GTP.

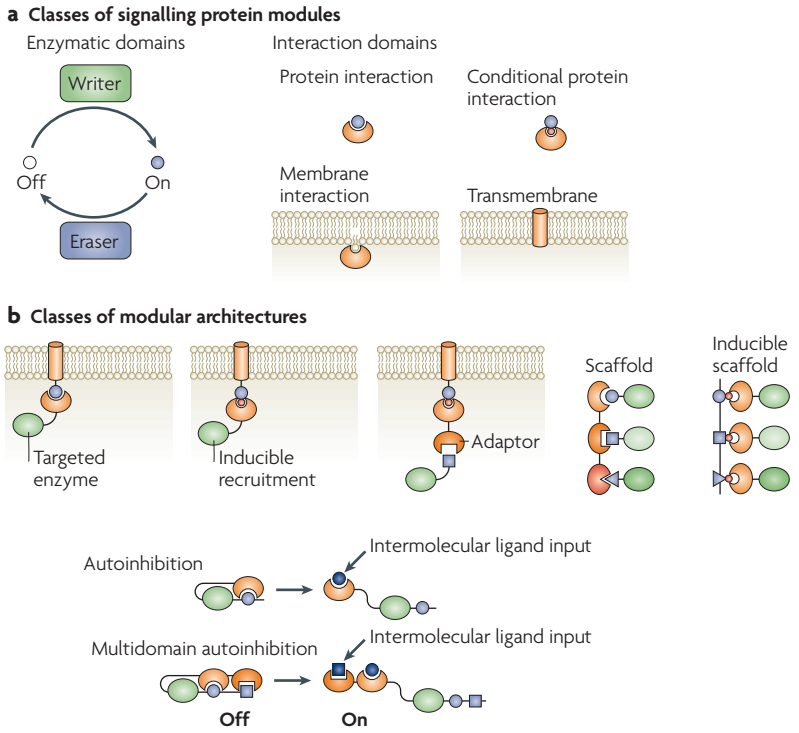

Figure 4 | **The modular logic of intracellular signalling components. a** | Modular eukaryotic signalling proteins are generally composed of enzymatic and interaction domains. Enzymatic domains, such as kinases and phosphatases, and guanine nucleotide exchange factors (GEFs) or GTPase-activating proteins (GAPs), which catalyse regulatory modifications such as phosphorylation and GTPase activation, respectively, often come in 'writer' and 'eraser' pairs with opposing activities. These enzymatic domains are regulated and targeted by interaction domains, including protein–protein interaction domains, membrane interaction domains and transmembrane domains. **b** | Different classes of modular architectures. Enzymatic domains can be directly targeted or recruited by induction to specific substrates, partners or subcellular locations by interaction domains. Alternatively, they can be indirectly targeted by adaptors or scaffold proteins, which contain multiple interaction domains. Interaction domains can also allosterically regulate catalytic domains by engaging in intramolecular autoinhibitory interactions. Such switch proteins can be activated by competing ligands that relieve autoinhibition.

targets. Often, these catalytic domains have a high intrinsic Michaelis constant (K_m) and thus require targeting by accessory interaction domains for efficient catalysis. Sometimes these targeting interactions are regulated; for example, if the interaction is dependent on a post-translational modification, such as the targeting of SH2 domain proteins to autophosphorylated Tyr sites on activated RTKs. Proteins with two interaction domains can act as adaptors that translate one interaction into a second one, leading to increased response flexibility depending on the adaptor proteins that are expressed in a particular cell type. Many interaction domain proteins can also function as scaffold proteins, which organize multiple proteins in a pathway into a complex. These interactions might be constitutive or preformed, or induced by factors such as phosphorylation or conformational changes that expose interaction sites. Thus, scaffold proteins can, in principle, determine the wiring linkages of signalling proteins and control when or where signalling happens[22,26].

Michaelis constant (K_m)
A kinetic parameter for a specific substrate in an enzyme-catalysed reaction. K_m equals the concentration of substrate that yields half-maximal velocity of the reaction. Providing certain conditions are met, the K_m for a substrate can equate to its binding constant, and the lower the value of K_m, the tighter the substrate binds.

PDZ domain
(Postsynaptic density protein of 95 kDa, Discs large and Zona occludens 1 domain). A protein-interaction domain that often occurs in scaffolding proteins and is named after the founding members of the protein family.

A second important role for interaction and regulatory domains is to directly control the activity of catalytic domains. In many cases, the interaction domains participate in intramolecular autoinhibitory interactions that sterically occlude the catalytic domain or conformationally perturb it — a type of regulation referred to as modular allostery[48]. Binding of competing intermolecular ligands to the interaction domains induces the protein's catalytic activity. Often, multiple interaction domains participate in the autoinhibition of a catalytic domain in a cooperative or hierarchical manner[49,50]. These proteins can function as complex multi-input switches that require a specific combination of inputs for proper activation. In addition, as external ligands also drive localization, such switch proteins directly couple targeting to allosteric activation.

Engineering new protein switches. Lim and co-workers explored whether the modular allosteric logic of many natural eukaryotic signalling proteins can be exploited to design new signalling switches by domain recombination[51,52] (FIG. 5a). Indeed, the catalytic domains of the actin regulatory protein neural Wiskott–Aldrich syndrome protein (N-WASP) and Rho-family GEFs can be linked to novel autoinhibitory domains to yield proteins with activities that are gated by novel ligands[51,52]. The intramolecular linkage of either of these catalytic domains to a PDZ domain and a PDZ ligand peptide can yield a switch that is activated by a competing PDZ peptide. Similarly, multiple interaction domains can be appended to yield a combinatorial switch that displays AND gate control. Depending on the exact configuration of the domains and intramolecular interactions, the types of regulation can be different in response to different competing external ligands — one ligand could activate the protein and another could repress it. These types of diverse relationships between regulatory domains are reminiscent of the diverse behaviours seen in natural signalling proteins, supporting the notion that this kind of switch architecture facilitates the evolution of diverse combinatorial regulatory switches[50]. Dueber *et al.* have also shown that synthetic autoinhibitory switches, using multivalent interactions of the same type, lead to a switch, the activation behaviour of which can be tuned cooperatively, from a linear to a digital-like response[53].

Scaffold proteins as molecular circuit boards. Intracellular signalling circuits can also be directly controlled by harnessing regulatory interactions to rewire pathway connections. For example, the catalytic domain of the Src family kinase, haemopoietic cell kinase (HCK), which is normally regulated by SH2 and SH3 domains, can be fused to a PDZ domain and directed *in vivo* to specifically phosphorylate substrates with a PDZ ligand motif[54].

Scaffold proteins can also be used to generate new pathway input–output relationships (FIG. 5b). In yeast, there are multiple functionally distinct mitogen-activated protein kinase (MAPK) pathways that regulate responses to mating pheromones and osmotic stress[55,56]. These pathways share common kinase components but remain specific because each pathway is organized by a distinct scaffold protein[57–59]. A chimeric scaffold protein that organizes select members of the mating and osmotic

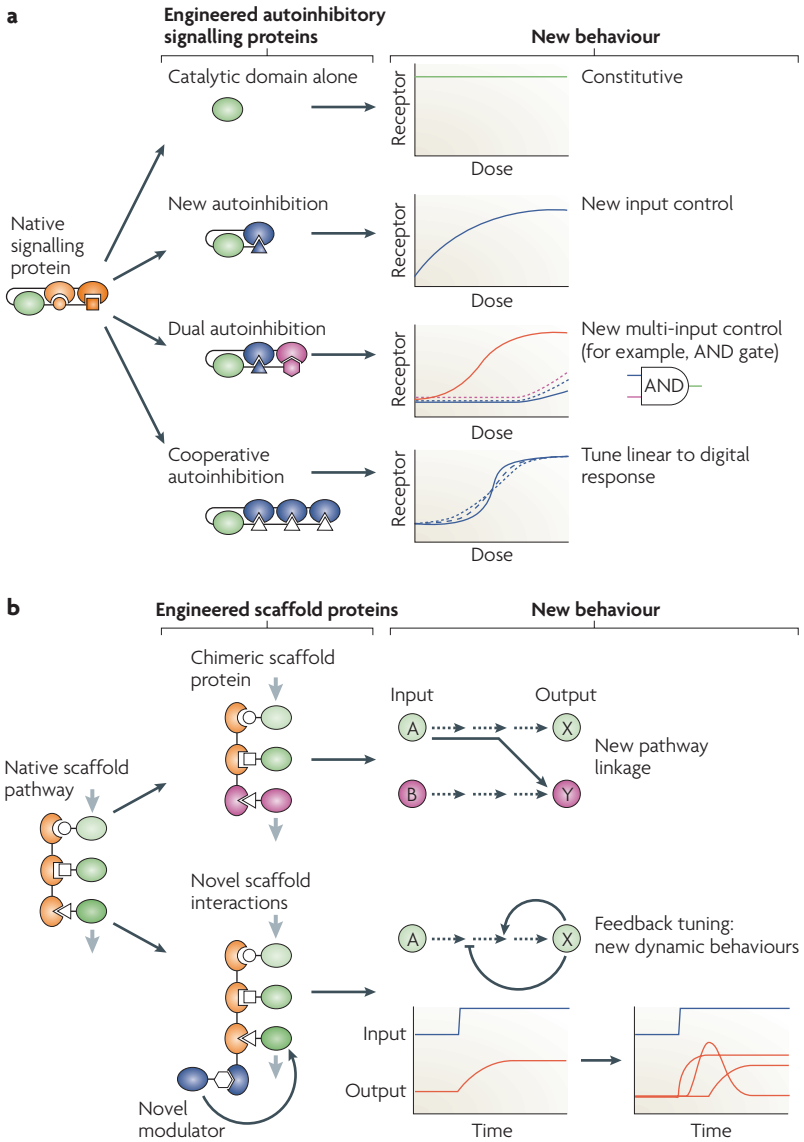

Figure 5 | **Engineering signal processing circuits. a** | Engineered allosteric protein switches. Dueber *et al.* showed that the allosteric regulation of the signalling protein neural Wiskott–Aldrich syndrome protein (N-WASP) could be reprogrammed by recombining the catalytic domain from N-WASP with different combinations of interaction domains (as depicted for a generic native signalling protein)[51,53]. Novel behaviours, as illustrated by the graphs, included multi-input (AND gate) control and highly cooperative switch-like activation (where behaviour is tuned cooperatively from a linear to a digital-like response). **b** | The use of scaffold proteins as a molecular circuit board for reshaping signalling output. The input–output linkage of a mitogen-activated protein kinase (MAPK) pathway in yeast could be redirected through an engineered chimeric scaffold that has assembled a novel combination of kinases, which leads to new pathway linkages[60]. New interaction sites can also be added to scaffolds to recruit additional modulatory factors. These additional factors can build synthetic feedback loops that can be used to generate pathways that display diverse signalling dynamics, as depicted schematically in the graphs[64].

stress pathways yields a non-natural pathway, in which mating pheromone specifically induces the osmotic stress response programme *in vivo*[60]. Similarly, covalent fusions that, like a scaffold, force the interaction between two signalling proteins, can force signal transmission down a single pathway[61].

More recently, scaffold proteins have been shown not only to mediate the linear input–output relationship of pathways, but to coordinate the recruitment of modulatory factors that shape the dose dependence and dynamics of pathway response[62,63]. Inspired by these natural examples, Bashor *et al.* showed that the yeast mating MAPK scaffold, Ste5, can be used as a molecular circuit board to flexibly reshape the quantitative behaviour of the mating response[64] (FIG. 5b). Fusing an additional synthetic interaction site to the Ste5 scaffold (using a Leu zipper heterodimer pair) facilitates the recruitment of new modulatory factors, such as a MAPK phosphatase, which suppresses the pathway response. However, if expression and recruitment of the phosphatase is linked to pathway output, a negative feedback loop is generated that leads to adaptation (a transient response, followed by the automatic return to lower output levels), which is a key behaviour in many biological sensory systems. By linking positive and negative pathway modulators in different ways, this small tool kit of scaffold control elements could be used to generate highly diverse dose response and dynamic behaviours, including highly cooperative switching, delayed responses, accelerated responses and pulse generation. These studies show how organizing centres such as scaffolds are a rich platform for processing and shaping intracellular signalling, either through evolution or engineering.

Engineering spatial self-organization. One of the most poorly understood aspects of cell signalling is how circuits made of diffusible molecules can lead to examples of precise spatial organization in the cell, such as in directed polarization and migration. This type of self-organization is an aspect of control circuitry in which there are no good electronic or engineered counterparts, and where biology can instruct engineering.

Engineering principles are being applied to understand the mechanism of polarization in the budding yeast, *Saccharomyces cerevisiae* (FIG. 6). Polarization is controlled by the GTPase Cdc42, which ultimately localizes to one site on the mother cell, leading to the formation of a single bud that grows into the daughter cell[65]. Remarkably, this process leads to the formation of a singular bud with nearly 100% reliability. A positive feedback circuit involving Cdc42 is important in polarization: active Cdc42 at the membrane recruits the cytoplasmic GEF protein bud emergence protein 1 (Bem1), which activates and localizes additional Cdc42 (REF. 66). This feedback loop leads to the formation of Cdc42 foci, and the rapid diffusion and redistribution of Bem1 between competing foci might be important to allow one focus to become dominant, leading to singularity of budding (FIG. 6a). The effect of slowing down Bem1 diffusion and redistribution, by linking it to a transmembrane motif, has been analysed[67]. Membrane-tethered Bem1 rescued the lethality of a Bem1 knockout but could not undergo diffusion in the cytoplasm. Instead, it was delivered to the plasma membrane in vesicles by actin cables (also coordinated by Cdc42 foci) and away from membrane foci by endocytosis, and thus redistributed more slowly. Severe defects in singularity, such as

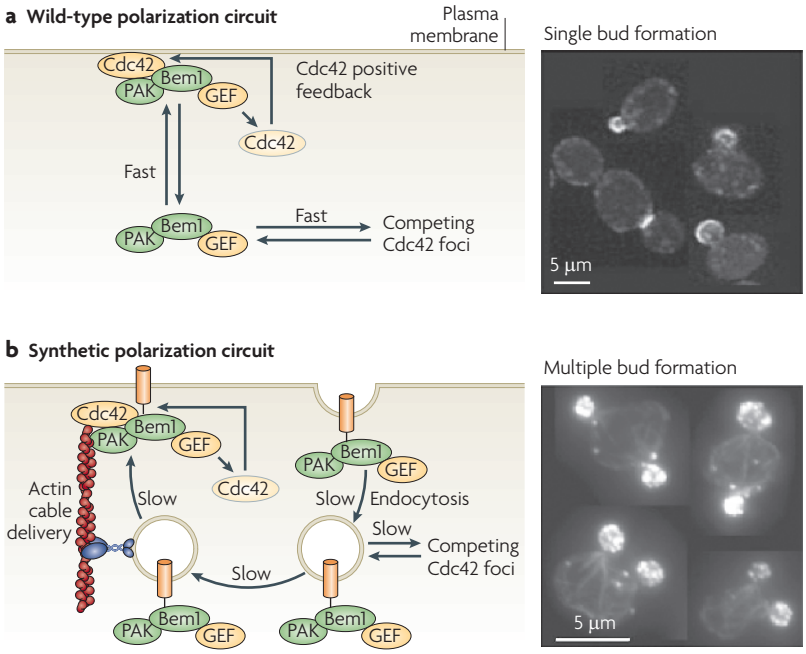

Figure 6 | **Engineering spatial regulation. a** | The wild-type yeast polarization circuit controls single bud formation. In budding yeast, localized activation of the polarity GTPase Cdc42 is amplified by a positive feedback loop: active Cdc42 recruits the cytoplasmic scaffold protein bud emergence protein 1 (Bem1), which co-assembles the p21-activated kinase (PAK) Ste20 and the Cdc42 guanine nucleotide exchange factor (GEF), Cdc24. Although a cell may have multiple Cdc42 foci, these are quickly resolved into one dominant focus, which develops into the cells only bud. A fast rate of interchange of the diffusible Bem1–PAK–GEF complex between competing Cdc42 foci is thought to be crucial for resolution into a single dominant focus. **b** | A synthetic slow polarization circuit leads to multiple bud formation. Bem1 was artificially tethered to the membrane by a fused membrane targeting motif[67]. Although membrane-tethered Bem1 can assemble the Bem1–PAK–GEF complex at sites of Cdc42 activity (that is, the positive feedback loop is intact), the exchange of the complex between competing Cdc42 foci, which is dependent on vesicular transport by actin cables and endocytosis, is slow. This synthetic polarization circuit therefore leads to poor resolution of competing Cdc42 foci and a much higher frequency (5% compared with ~ 0%) of multi-budded cells than in the wild-type circuit. The micrographs in parts **a** and **b** are reproduced, with permission, from REF. 67 © 2009, Elsevier.

AND gate
A Boolean logical operation in an output is produced only if two (or more) specific inputs are present. Many important signalling proteins and networks approximate AND gates, although none show an absolute all or none behaviour of the idealized Boolean operator.

SH3 domain
(SRC homology 3 domain). A protein module of ~50 amino acids that recognizes and binds to sequences that are typically Pro-rich.

Leu zipper
A Leu-rich domain in a protein that binds to other proteins with a similar domain.

many persistent, competing Cdc42 foci, were seen, and the frequency of multi-budded cells increased to ~5% (FIG. 6b). Studies such as these help reveal the requirements for precisely controlled spatial self-organization and suggest that we can learn how to engineer signalling circuits to produce customized spatial outcomes with important therapeutic behaviours, such as regenerative medicine, that require specific cellular morphology and orientation.

Making signal engineering predictable

The studies above show that signalling systems are highly modular and plastic, and recombining modules, particularly catalytic domains with new regulatory domains, can lead to distinct response behaviours. Thus, the question is no longer whether signalling systems can be engineered to yield new behaviours, but whether they can be engineered in a way that allows us to predict what behaviours will emerge, and how successful each designed circuit will be.

Challenge of unanticipated crosstalk. One of the main issues with engineering cell signalling is that natural components — the tool kit of available domains — are being reused, which can result in unanticipated crosstalk. Will engineered interactions lead to specific phosphorylation of the desired protein, or will the domain used also cross-interact with other targets, competitively titrating out important physiological interactions and leading to unanticipated effects or failure of the designed circuit? Often natural parts do not have absolute specificity, and evolution most likely uses complex networks of cross-reactivity to yield important coordinated regulation. Although this kind of complex neural net-like system may provide advantages for a cell, it is an anathema to predictive engineering.

Envisioning the signalling tool kit of the future. One solution to this problem is to assemble a tool kit of parts that are specifically optimized for engineering. This issue is important for any type of signalling part, but the focus here is on how to assemble a useful tool kit of protein-interaction parts (FIG. 7).

Although nature has repeatedly used families of parts, such as interaction domains of a particular type, recent studies indicate that in some cases family members contain unused recognition sites in these domains. These could be exploited to engineer domain–peptide pairs that are simultaneously optimized to interact with their correct partner while avoiding cross-interaction with other members of the family[68,69]. In fact, PDZ domain–ligand pairs and heterodimerizing Leu zipper pairs have been constructed that are optimized to avoid cross reaction with natural domains of the same type[70,71]. The selectivity and predictability of existing interaction domains can also be improved by engineering composite interactions. Certainly, multidomain cooperation is a natural mechanism for increased specificity, but a new twist on this is the engineering of composite two domain clamshell interactions. Koide *et al.* have taken a PDZ domain and fused it to a fibronectin domain[72]. Using phage display they selected for variants of this tandem domain that bind a specific peptide so that it is sandwiched between the two domains. The dramatically enlarged recognition surface area leads to interactions with much higher specificity and affinity. Another solution for specificity, which is seen in nature, is differential compartmentalization. If targeting motifs could be used to localize partner proteins to specific organelles or cellular locations, then interaction motifs are likely to function in a more specific manner, especially if few or no competing interactions of this type take place at this location or organelle.

An alternative approach to achieving reliable specificity is to import domains from other organisms that do not exist in the host being engineered. For example, PDZ domains can be imported into yeast (which lack most such domains), although the possibility of fortuitous cross-reacting partners cannot be ruled out[60]. An example of an orthogonal signalling system that has been successfully imported to a new host is the bacterial Cre–Lox recombinase system, which is reliably used

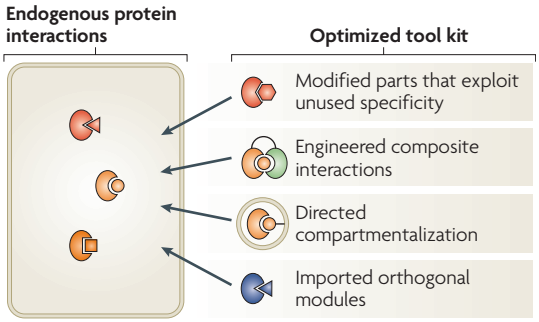

Figure 7 | **Improving the tool kit for predictable engineering of cell signalling: orthogonal interaction parts.** A native cell has its own repertoire of protein interaction modules, thus it is challenging to engineer new functions using related interaction modules that might show inadvertent crosstalk in the cell. An optimized tool kit of interaction parts could markedly increase the predictability of cellular engineering by eliminating the chances of unintended crosstalk. Several strategies for optimization include the engineering of interaction modules that exploit untapped specificity, engineering composite, multidomain interactions, combining interaction modules with subcellular targeting motifs and importing orthogonal interaction modules (either synthetically constructed or from other organisms) that are not found in the host cell.

to engineer complex chromosomal rearrangements in complex organisms, including mice[73].

Thus, imagining the tool kit of the future, one might want a set of about ten protein interaction pairs that are optimized, and orthogonal, for an organism of choice (for example, *Eschericha coli*, *S.cerevisiae* and mammals). It is also important for these interactions to be tuneable, so a series of ligands for each interaction domain that vary in affinity over several orders of magnitude would be ideal. This would allow the systematic exploration of how recruitment affinity alters system behaviour.

Combinatorial design versus prediction. Another different, but still complementary, approach to predictably engineering cell signalling is to use combinatorial variability. In natural evolution, the recombination of signalling modules to generate new function was presumably not designed or guided but rather was relatively random, and it was natural selection that identified rewiring events that led to fitness advantages. Thus, a very fruitful approach, given the lack of predictability in cellular engineering, might be to construct combinatorial libraries of synthetic circuits and to select for the desired function[27,74]. Moreover, this approach could be combined with semi-predictive design, whereby the overall architecture of engineered circuits could be designed, but combinatorial methods used to search a broader range of parameter space (using variants of each module in the library). Focusing on combinatorial selections may also provide a useful strategy in the early days of the field of synthetic biology, as it may help us learn more rapidly about core design principles.

Outlook

The goal of understanding how cells communicate and make decisions remains very attractive, especially because understanding the molecular language in a cell may allow us to communicate with cells and instruct them to carry out new programmed functions. Our ability to rewire cell signalling could provide many powerful applications, such as programming therapeutic cells to detect a selective set of disease-related signalling pathways and to locally respond in a precisely tailored way.

Although evolution has achieved this kind of innovation and precise engineering of cellular function, we are only beginning to understand how to execute this kind of goal. We have a good foundational understanding of the logic of cell signalling machinery and the sources of functional plasticity. In addition, big first steps have been made in engineering new receptor (sensor) systems, as well as new or modified intracellular signal processing circuits. Despite these tools, few efforts have been made to link these types of components in new ways to yield larger integrated circuits capable of highly refined, precision responses. Such efforts are underway. For example, the Cell Propulsion Laboratory is a National Institutes of Health nanomedicine centre that is attempting to take the relatively simple anti-tumour immune cells engineered with synthetic CARs and improve their suite of responses, such as their *ex vivo* expansion, *in vivo* survival, anti-tumour cytotoxicity and ability to disrupt a hospitable tumour microenvironment. It will be exciting to see how these efforts unfold, and how the challenges will improve the sophistication and reliability of cellular engineering.

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Competing interests statement
The authors declare no competing financial interests.

DATABASES
UniProtKB: http://www.uniprot.org
Bem1 | CD3ζ | Cdc42 | FADD | HCK | N-WASP | Ste5

FURTHER INFORMATION
Wendell A. Lim's homepage: http://limlab.ucsf.edu/
Cell Propulsion Laboratory: http://nihroadmap.nih.gov/nanomedicine/devcenters/cellularcontrol.asp
NSF Synthetic Biology Engineering Research Center: http://www.synberc.org/

SUPPLEMENTARY INFORMATION
See online article: S1 (box)

ALL LINKS ARE ACTIVE IN THE ONLINE PDF