Post-translational tools expand the scope of synthetic biology
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Synthetic biology is improving our understanding of and ability to control living organisms. To date, most progress has been made by engineering gene expression. However, computational and genetically encoded tools that allow protein activity and protein–protein interactions to be controlled on their natural time and length scales are emerging. These technologies provide a basis for the construction of post-translational circuits, which are capable of fast, robust and highly spatially resolved signal processing. When combined with their transcriptional and translational counterparts, synthetic post-translational circuits will allow better analysis and control of otherwise intractable biological processes such as cellular differentiation and the growth of tissues.

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Introduction
Synthetic biologists control living cells by dictating when, where and to what extent gene products, typically proteins, are active. The simplest strategy for controlling the activity of a protein is to regulate its concentration, which is commonly done by varying the rate of transcription or translation. In nature, however, post-translational regulatory processes coordinate biochemical events over smaller length and time scales, allowing more sophisticated and reliable behaviors to be encoded (Figure 1). For example, allosteric processes can change protein activity thousands of fold in milliseconds, while protein–protein interactions (PPIs) and enzyme cascades can give rise to switch-like phenotypic changes. Various modes of post-translational regulation can also buffer the impact of gene expression noise, making cellular processes more predictable [1–9].

Genetically encoded tools for post-translational control have lagged behind those for gene expression for several reasons. First, protein function is, in general, more difficult to engineer at the level of primary sequence than that of a promoter or regulatory RNA. Second, gene expression dynamics occur over experimentally tractable timescales of minutes to hours while post-translational signaling occurs in seconds or less (Figure 2). Finally, gene expression can often be treated as homogeneous across an entire cell, while many post-translational processes require spatial heterogeneity on the micron scale.

Despite these challenges, recent technological advances have allowed direct control of protein function in live cells. In particular, PPIs are now being designed computationally [10**,11**], and light-switchable proteins [12,13*] are being used to control an increasing number of post-translational events in real time. Here, we discuss new developments and their dual scientific and engineering impact using the example of programmable stem cell differentiation.

Engineered allosteric control of proteins
Post-translational circuits can be constructed using proteins that are allosterically regulated by unnatural inputs [14,15]. In classic work, the autoinhibitory domains of the cytoskeletal regulator N-WASP were functionally replaced (modularly recombined) with PDZ and SH3 peptide-binding domains and their ligands [16]. By arranging different numbers of synthetic regulatory modules in various configurations across the protein, N-WASP was controlled logically [16] and cooperatively [17] by unnatural peptide inputs in vitro. Modular recombination has since been used to rewire the input [18–21] and output [22] specificities of kinases, G-protein-coupled receptors [23], and to construct a synthetic cytoskeletal regulatory cascade [14].

Allostery can also be engineered by adding a regulatory domain N-terminal or C-terminal to a protein of interest. Since its structure was solved in 2003 [24], the blue-light-switchable light oxygen voltage (LOV) domain from A. sativa Phototropin 1 (AsLOV2) has been used to put many proteins under optical control [25]. Light absorption by a flavin mononucleotide (FMN) chromophore triggers dissociation of a C-terminal helix that can de-repress a sterically or conformationally inactivated output domain. The ‘lit’ state of AsLOV2 is stable for ~30 s, resulting in reversible switching. In 2008, Strickland et al. demonstrated that the DNA binding affinity of E. coli TrpR could be modulated by the fusion of an N-terminal AsLOV2 domain [26]. This led the way for many other
LOV-regulated proteins including the histidine kinase FixL [20], *B. subtilis* lipase A [27] apoptosis-inducing Caspase-7 [28], and Rac1 [29**].

Rac1 is a GTPase that binds various effector proteins to trigger cytoskeletal rearrangements. Hahn and coworkers fused AsLOV2 to the N-terminus, resulting in a photo-activated Rac1 (PA-Rac1) with 10-fold increased affinity for the effector PAK in the ‘lit’ state [29**]. Illumination of 20 μm spots revealed that gradients of Rac1 activity can polarize mammalian cells, allowing directional movement. Moreover, a spot of Rac1 activation triggers a wave of RhoA inhibition (observable by FRET) that travels across the cytoplasm in under 2 min [29**].

Evolution frequently inserts domains in unstructured protein loops, resulting in novel allosteric control and new or rewired signaling pathways [30]. Domain insertion...
is also an effective strategy for engineered allostery [31–34]. In 2003, Ranganathan and coworkers identified ‘sectors’ as coevolving networks of physically interacting amino acids that ‘wire’ together distal sites of proteins [35]. Recently, the group inserted AsLOV2 into every surface site on E. coli dihydrofolate reductase (DHFR) [36**]. Insertions at 14 of the 70 total sites generate a small degree of allosteric control, and intriguingly all 14 allosteric sites contact the DHFR sector. Although LOV is a more effective switch when fused to the N-terminus or C-terminus of a target domain [37], it appears that sectors could provide the basic road map for engineering allosteric control via domain insertion.

**Engineered control of protein–protein interactions**

PPIs underlie many cellular regulatory processes. Controlling intracellular and intercellular PPIs is therefore an important goal for synthetic biology. Along these lines, sets of orthogonal coiled coil domains have been engineered to heterodimerize with different affinities [38,39]. They have been used to rewire a MAP kinase cascade [40**], construct transcriptional logic gates [41], and engineer cooperation between kinesin motor proteins *in vitro* [42]. The peptide-binding SH3, GBD, and PDZ domains [43] and engineered self-assembling RNA structures [44] have also been used to scaffold enzymes for improved metabolic flux in bacteria.

Recently, Baker and colleagues designed a novel PPI from the ‘inside out’ [11**]. An interacting tyrosine/tryptophan pair was first encoded on the face of the Ankyrin Repeat (AR) and *P. horikoshii* coenzyme A binding proteins. The pair was then surrounded by a computationally designed hydrophobic core followed by a hydrophilic shell. The best interacting pair has a *Kₐ* of 130 nM which was improved to a remarkable 180 pm by directed evolution. Computational design and directed evolution could be combined to create a sizeable toolbox of orthogonal PPI parts that could then be linked to regulatory and catalytic domains to construct synthetic post-translational circuits. Furthermore, PPI parts with different affinities could be used to strengthen or weaken a particular interaction in the network, or even to analyze natural networks by systematically perturbing PPI strengths [45].

**Switchable protein–protein interactions**

Since it was first demonstrated in 1993 [46], the dominant approach for conditional control of intracellular PPIs has been the rapamycin-dependent heterodimerization between FKBP12 and the FRB domain of mTOR. Rapamycin has been used for inducible control of many post-translational processes [47–56], and photocaged for optical regulation of kinase signaling [57]. Nonetheless the molecule diffuses and is not rapidly reversible. To overcome these limitations, several light-switchable PPI systems have recently been engineered. These systems use blue-light induced dimerization between the *A. thaliana* cryptochrome Cry2 and its partner CIB1 [58], LOV-switchable heterodimerization domains [59], and the red/or red switchable interaction between *A. thaliana* phytochrome B (PhyB) and phytochrome interacting factor 6 (Pif6) [60**].
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The PhyB/PIF6 system (Figure 3a) can control intracellular protein signaling with seconds-scale reversibility and subcellular precision \([60**]\). This feature was recently exploited to put protein localization and phosphoinositide signaling under feedback control \([61**]\). Here, a fluorescent reporter of the PPI itself or of phosphoinositide concentration is measured at the membrane via a microscope-mounted camera and controlled with a light projector (Figure 3b). Optical feedback can reduce PPI variations between cells and produce PPI patterns that vary in time \([61**]\). This advance is particularly exciting because it could be used to reverse engineer dynamic circuit behaviors \([62]\) or precisely control the flow of signals through engineered circuits even in the face of gene expression noise and other sources of variability \([61**]\).

**Engineering orthogonal protein circuits**

Recently, Kortemme and coworkers engineered an orthogonal cytoskeletal regulatory pathway by computationally re-designing the interface between the GTPase CDC42 and its activator Intersectin (ITSN) \([10**]\). Replacement of only one ITSN and one CDC42 residue produced a functional pair with a 480 nM \(K_d\) (30 nM for wild-type) and no cross-talk with wild-type. This approach can generate new pathways while avoiding unwanted regulatory interactions that may be ported over using modular recombination. If orthogonal signaling pairs can be generated from natural versions via small numbers of mutations in general, existing pathways could be duplicated and repurposed \([15]\), allowing synthetic biologists to create many variations on a small number of themes.

**Combining transcriptional and post-translational control**

Transcriptional and post-translational devices are being combined to construct synthetic circuits with expanded functionality and improved performance \([15,40**, 63–66,67*]\). One application of combined transcriptional/post-translational circuits is to engineer ultrasensitive responses, characterized by an effective Hill coefficient \(n_{11}\) greater than one \([68]\). Ultrasensitivity is important in many switch-like behaviors such as the cell cycle and differentiation \([69]\), and is also desirable for synthetic biology \([70]\). Promoter \(n_{11}\) values typically do not exceed 4 or 5, but can be increased by incorporating post-translational regulation \([68]\). For example, when a transcriptional activator is stoichiometrically bound by an inhibitor, a small increase in its concentration can trigger a large increase in transcription rate \([71,72]\). Recently, the mammalian basic leucine zipper (bZIP) transcriptional

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activator CEBPα was expressed with its promoter in S. cerevisiae [67]. Coexpression of the inhibitor 3HF increases the activation threshold and n11 of CEBPα activation from 1 to 12, in a manner proportional to its concentration [72]. Activation threshold, ultrasensitivity, and magnitude of response can be controlled independently by varying the concentrations of kinases and inhibitors in a MAPK cascade as well [73].

Applications in cellular differentiation
In the formation of natural tissues, cells integrate chemical, mechanical, and cell–cell contact cues to regulate growth, senescence, death, locomotion, and differentiation [74]. Much of this signal-processing and information storage is carried out by post-translational circuits by way of chemical and structural protein modifications [75,76]. Current efforts in stem cell biology and tissue engineering aim to control signal transduction networks with hydrogel microenvironments that mimic those in vivo [77,78]. Synthetic biology can contribute to stem cell biology and tissue engineering on two fronts. First, natural signaling circuits can be rewired to respond to tractable inputs such as light as well as, or instead of, native inputs (Figure 4). Optical signals can then be sent into the circuits and the flow of biochemical information can be monitored with FRET/BRET or split-protein reporters. Then, with an improved understanding of circuit behaviors, light, and microenvironments can be used in combination to pattern tissue growth from the top down. Finally, cellular circuits can eventually be modified or replaced by streamlined synthetic versions constructed by engineering, rather than evolutionary processes (Figure 4). These engineered circuits can encode artificial developmental programs which, when activated, will drive cells to grow into a desired tissue with minimal external intervention. In general, the construction of integrated transcriptional and post-translational networks will allow synthetic biologists to engineer synthetic cellular behaviors that begin to approach the complexity of those seen in nature.

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:
• of special interest
**• of outstanding interest

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An orthogonal cellular signaling pathway is engineered by computational redesign of a key PPI.


This paper reports a computational strategy that produced the strongest PPI yet designed, followed by enhancement via directed evolution.


This commentary discusses the widespread impact that light-switchable proteins will have on biological research.


This paper describes the engineering of photoactivatable Rac1 and demonstrates the utility of post-translational control in space and time.


Though the motivations for the study are scientific, this paper presents a general method for identifying sites where allosteric regulation can be engineered via domain insertion.


An outstanding example of how post-translational interactions can be engineered to reshape the steady state and dynamic responses of a signal transduction cascade.


Post-translational tools expand the scope of synthetic biology

Olson and Tabor


This seminal paper reports the use of PhyB and Pho6 for light switchable control of protein-protein interactions, protein localization and signal transduction with seconds-scale reversibility and micron-scale precision in live cells.


The PhyB/Pho6 system and fluorescent proteins are used for feedback control of PPI and phosphoinositide levels at the cell membrane. Optical feedback control is used to linearly and exponentially vary protein levels at the membrane over time.


A synthetic circuit is engineered where PPI-mediated inhibition of a transcriptional activator results in an ultrasensitive transcriptional response.


Here, a mammalian MAPK cascade is refactored in yeast. Independent control of kinase and inhibitor concentrations allows the ultrasensitivity and activation threshold to be tuned.


This excellent review summarizes the various signals that stem cells use to develop into tissues and methods to engineer hydrogel microenvironments to mimic those signals.
