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Protein-based parts and devices that respond to intracellular and extracellular signals in mammalian cells

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Synthetic biology aims to rewire cellular activities and functionality by implementing genetic circuits with high biocomputing capabilities. Recent efforts led to the development of smart sensing interfaces which integrate multiple inputs to activate desired outputs in a highly specific and sensitive manner. In this review, we highlight protein-based interfaces that sense intracellular or extracellular cues providing information about dynamic environmental changes and cellular state. We will also discuss different mechanisms of regulation of gene expression connected to the sensors to develop diagnostic and therapeutic devices. We conclude discussing challenges and opportunities for biomedical applications of synthetic mammalian protein-based devices.

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Introduction

Engineered synthetic networks that actuate genetic programs with predictable behavior are demonstrating a promising way for the rational design of sensitive diagnostics and safer therapeutics [1–4]. To achieve sophisticated control of timing, localization, and strength of transgene expression, genetic devices have evolved modular *smart interfaces* composed by sensing and actuating modules with high biocomputing capabilities that respond to intracellular or extracellular stimuli and rewire cellular activities and fate.

A large number of sensors leverage on native or engineered proteins, as they constitute structural components of cells and tissues, are involved in the catalysis of several biochemical reactions, and are responsible of signal transmission. Actuators are often based on transcriptional regulation, using synthetic [5–7] or natural transcription factors coupled to promoters designed to ensure minimal off-target effects and leakiness [8,9]. Recently, also actuators that operate post-transcriptionally and post-translationally are being explored, in line with increasing efforts toward RNA-encoded circuits [10,11••,12•].

Here, we review the most recent protein-based interfaces to engineer mammalian cells that respond to extracellular and intracellular signals in a customized fashion. We will discuss the applications of these devices for real-time monitoring of biological and medical relevant markers, and to implement control mechanisms that prevent non-specific, undesired activation of transgene expression.

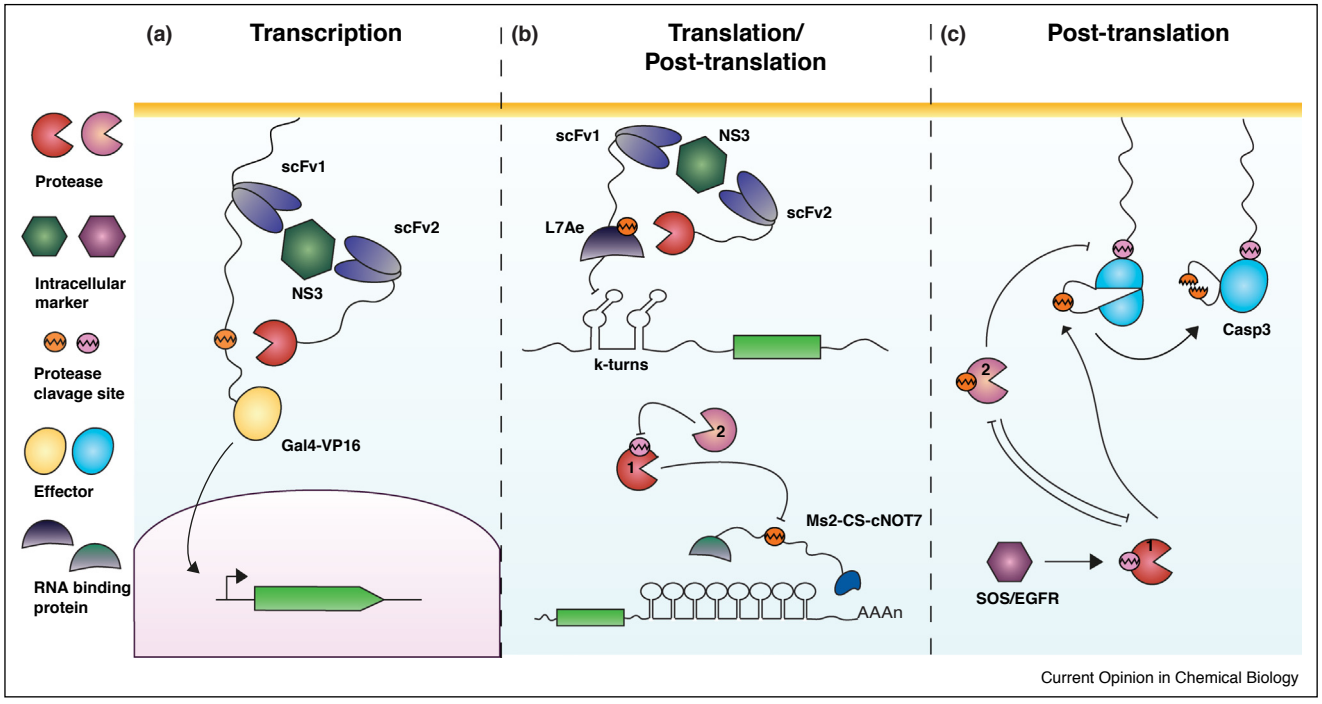
Protein devices that respond to intracellular signals

The intracellular environment provides information about the state of the cell. Thus, protein-based networks have been engineered to respond to a variety of inputs including transcription factors, microRNAs and translational modulators, to record intracellular events [13], trigger immunomodulation of the microenvironment niches [14,15••], and identify specific cell types [15••,16,17].

Proteins that are biomarkers of onset and progression of diseases may serve as inputs to synthetic circuits activation [18–20] that exert downstream transcriptional, translational and post-translational regulation (Figure 1). In order to establish a customizable framework that links intracellular protein sensing to programmed cellular responses, we recently developed a modular cellular intrabody-based sensing-actuating platform [15••]. The devices detect cytosolic proteins in a highly specific manner through two intracellular antibodies, one membrane-tethered and fused to a transcription factor (TF) via a TEV protease cleavage site (TCS), and the other fused to the viral TEV protease (TEVp) (Figure 1a). The presence of the target protein and subsequent binding of the two intrabodies results in TEVp cleavage of TCS and release of TF, converting protein detection into transcriptional output. The system successfully detects Huntington disease and HCV and HIV infection protein biomarkers, and demonstrated cytokine immunomodulation in HIV infected T cells [15••]. Notably, this was the first modular platform that can be tailored to target potentially any cytosolic protein with customized intrabodies [21,22].

Post-transcriptional, translational and post-translational regulation, may be a valid alternative to transcriptional circuits, since a general advantage of RNA-encoded

Figure 1



Protein-based devices that respond to intracellular signals. **(a)** System from Siciliano *et al.* [15••]. A modular cellular intrabody-based sensing-actuating platform detects proteins connected to the onset and development of a disease. In the example shown the target protein is the serine protease NS3, a marker of HCV infection. The two intrabodies scFvs bind different epitopes of NS3, one is linked to Gal4-VP16 and the other one to a viral protease. The viral protease recognizes a cognate cleavage site in the linker of the other intrabody and releases Gal4-VP16, that translocates to the nucleus driving a transcriptional response. **(b)** Control of translation by newly engineered RBP-proteases signaling [11••]. Top: NS3 detected by intrabodies (scFv1 and scFv2) fused to L7Ae-CS and protease respectively. L7Ae-CS binds to k-turn motifs in the 5′UTR of target mRNA inhibiting its translation. When NS3 is detected the protease disrupts L7Ae-CS and translation is ON. Bottom: a three-stage cascade is designed to control translation of selected transcripts. The transcript is bound at the 3′UTR by the fusion protein Ms2-cNOT7 with a protease cleavage site in the linker. CNOT7 chops the poly(A) of the transcript inhibiting its translation. When protease 1 is present, the output level is restored. Protease 1 is engineered to contain a cleavage site for the second protease (protease 2). When protease 2 is present, protease 1 is cleaved which restores Ms2-cNOT7, resulting in translation repression. **(c)** System from Gao *et al.* [12•]. Post-translational control is obtained by engineering a membrane bound Casp3. Ras-induced SOS is used as input of the system to reconstitute a viral protease (protease 1) that in turn activates Casp3. A second viral protease (protease 2) attenuates the activation of the system by disrupting the other viral protease and unleashing Casp3 from the membrane. This additional control reduces system activation in the OFF-state.

devices is the reduced probability of harmful integration in the host genome and immunogenicity, fostering broad biomedical, industrial, and pharmaceutical applications.

Differentially expressed microRNAs provide a useful marker of lineage [17], cancer development [16] and gene expression robustness [23,24]. In recent works from Wroblewska *et al.* [10] and Matsuura *et al.* [25] the RNA-binding protein (RBP) L7Ae that regulates mRNA translation by binding a responsive motif in the 5′UTR of the target mRNA, was engineered to respond to cell-specific microRNAs. This approach enabled the implementation of RNA-encoded circuits including cancer classifiers [10] and complex Boolean logic gates [25], that tune the expression of reporter genes for diagnostic (e.g. fluorescent proteins) or therapeutic (e.g. pro-apoptotic genes) purposes.

Transcriptional and post-transcriptional regulatory devices are conveniently modular and orthogonal, but a disadvantage is the slow response upon activation. Protein–protein regulation may provide faster responses over transcription and post-transcription-based devices.

In our recent work, viral proteases were used to tune the activity of RBP and other orthogonal proteases, to expand the toolbox of genetic modules for multilayered regulation of target mRNA translation and protein–protein interaction [11••]. Differently from other systems, this was obtained by inserting cleavage sites, in the native structure of the target proteins. We first generated a TEVp-responsive L7Ae (L7Ae-CS) that is functionally disrupted in presence of the protease, recovering output expression. We then created an HCV protein sensor by fusing L7Ae-CS and TEVp to intrabodies targeting NS3

protein. NS3 expression results in TEVp-mediated cleavage of L7Ae-CS and output expression (Figure 1b).

Next, we created a switch and a cascade that combine post-transcriptional, translational and post-translational regulation where TEVp and L7Ae-CS repress each other (protein–protein and protein–RNA regulation) and microRNAs provide the input to the system (post-transcriptional control).

Similarly, we created post-transcriptional regulatory cascades by engineering TEVp-responsive, TUMVp-responsive, TVMVp-responsive and SuMMVp-responsive MS2-cNOT7 (MS2-CS-cNOT7). MS2-cNOT7 is a chimeric protein composed by MS2 which binds motifs in the 3′UTR of the target mRNA, and cNOT7 that removes the polyA signal destabilizing the transcript. The insertion of the CS between the two domains inhibits repression in presence of proteases. Mutual regulatory activity was implemented by inserting in each protease CS for orthogonal ones, and connected their functionality to MS2-CS-cNOT7 activity [11••], creating post-translational cascades (Figure 1b).

Gao *et al.* [12•] created protein–protein regulatory circuits based on viral proteases, including TEVp, TVMVp and HCVp, which were connected to reciprocally modulate their activity (e.g. by destabilizing domains) generating regulatory cascades, binary logic gates, and dynamic analog signal-processing functions. The authors linked Ras oncogene activation to the system to trigger Caspase3 (Cas3) mediated cell killing. Specifically, Ras activation leads to reconstitution of a TEVp which in turn cleaves and converts a membrane-bound Cas3 from inactive to active state. To reduce undesired activation of Cas3 in OFF-state, a TVMVp cleavage site placed between membrane-tag and Cas3 remove the latter from the membrane, attenuating its activation by TEVp (Figure 1c).

Ho *et al.* [26] used the intracellular cancer-associated sentrin-specific protease 1 (SENP1) overexpressed in several cancers to modulate the cytotoxic protein Granzyme B (GrB). Specifically, GrB is fused at the N-terminus to the small ubiquitin like modifiers (SUMO1) cleaved by SENP1. Overexpression of the latter leads to SUMO cleavage, GrB activation and killing of cancer cells. The limit of this design is that the inhibition by these peptides works only when the activity of the effector protein depends on a free N-terminus.

Protein devices that respond to extracellular signals

To monitor the extracellular milieu, mammalian cells have evolved transmembrane receptors (TRs) that sense soluble molecules and surface-bound antigens. Synthetic biology uses TRs either physiologically expressed [27,28], or newly integrated [29–33] in mammalian cell lines as the 'sensing' module to initiate a desired genetic programs.

The best-known examples of synthetic receptors that respond to surface bound molecules are the synthetic Notch (synNotch) and the chimeric antigen (CARs) receptors [34–36]. Since these receptors and their applications have been extensively reviewed [34,36–40], we will restrict our discussion to receptors that respond to soluble molecules.

The simplest networks use native ligand–receptor interactions coupled to synthetic promoters to activate gene expression. For example, diagnostic devices including sensors for pH [27] and histamine [28] levels are composed by GPCR receptors linked to a synthetic CREB1-responsive promoter via cAMP signaling pathway. This approach has enabled the engineering of mammalian cells to monitor the level of metabolites involved in tumors [29,41], infections [42], as well as metabolic [30–32], liver [33] and immune disorders [43,44,45••].

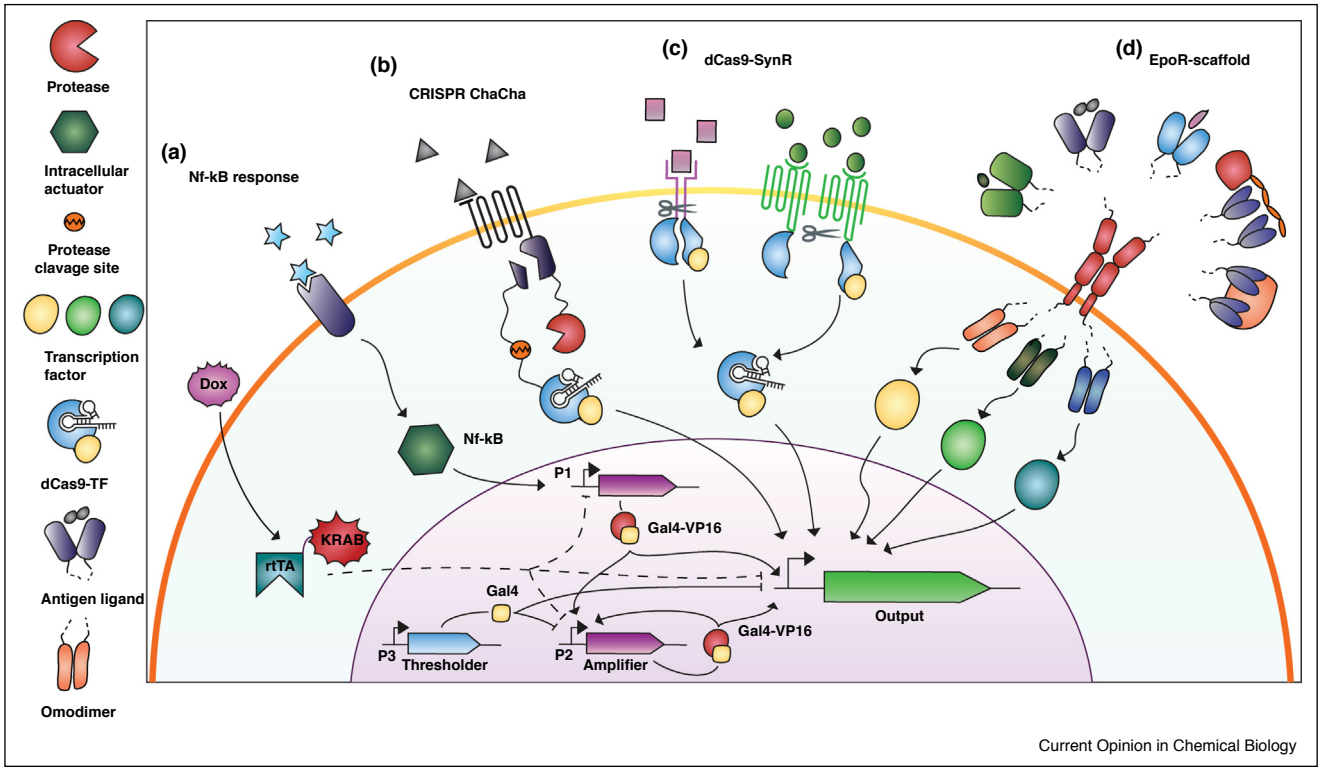
Tastanova *et al.* [29] adopted this strategy to develop a biomedical tattoo that monitor calcium levels in the blood, producing a melanin pigment when these increase in asymptomatic cancers. The tattoo consists of HEK293 cells engineered with a calcium-sensing receptor CaSR. CaSR activation triggers tyrosinase expression that converts tyrosine to melanin, producing a visible diagnostic black spot on the skin of nude mice with hypercalcemic mammary and colon adenocarcinoma [29].

Smole *et al.* [45••] designed HEK293 cells with a synthetic device that divert NF-kB-induced production by proinflammatory cytokines toward anti-inflammatory proteins such as anti-human TNF-α antibody, in combination with IL-10 or IL-1 receptor antagonist. Specifically, NF-kB activation triggers the expression of the synthetic transactivator GAL4-VP16, which amplifies its own expression while inducing anti-inflammatory proteins. Two supporting modules (a tetracycline-inducible OFF-switch and a thresholder) allow additional control of system performance. Implantation of alginate-poly-L-lysine-alginate microencapsulated engineered cells in an acute colitis mouse model show effective reduction of pathological symptoms. This device provides a means to address limitations of current anti-inflammatory treatments such as increased risks of infections and of cancer development (Figure 2a).

As compared to native receptors, synthetic ones can be tailored to sense a larger number of molecules to develop complex, orthogonal genetic programs.

Synthetic receptors have modification in the intracellular or extracellular domains, or both. In several circuits' configurations, receptors are coupled to transcriptional activation using TEVp. This approach was pioneered

Figure 2



Protein-based devices that respond to extracellular signals. **(a)** Proinflammatory cytokines-dependent Nf-kB production to drive anti-inflammatory response [45••]. Activated Nf-kB translocates to the nucleus inducing Gal4-VP16 expression, that in turns activates an amplifier and output production. Controller 1: a thresholder module reduces OFF-state response. Controller 2: a switch shuts down the system by administering doxycycline. **(b)** ChaCha CRISPR system to detect chemokines, hormones, small molecules and so on [47]. When the receptor that includes a protease is activated by ligand binding, it induces cleavage of the transcription factor fused to a receptor-interacting module. The transcription factor translocates to the nucleus and drives output production. **(c)** dCas9-SynR uses RTKs and GPCRs receptors linked to a split dCas9 fused to a transcription factor (dCas9-TF) [48•]. The synthetic receptor allowed a dose-dependent response to a variety of ligands. **(d)** System by Scheller *et al.* They used an EpoR scaffold receptor combined with different antigen ligands in the extracellular space and with different intracellular homodimer actuators. The intracellular actuators activate downstream signaling pathways that end up with the desired output expression [49••].

by Tango system developed by Barnea *et al.* [46] that links GPCR or IGF1R receptors activation to the recruitment of TEVp, to release a membrane anchored transcription factor obtaining transgene expression. Several following works evolved the Tango architecture to respond to different stimuli, to increase the specificity of output activation [47,48•] or to enable potential activation of multiple outputs [47,48•,49••]. Kipniss *et al.* [47] developed the CRISPR ChaCha design in which, differently from Tango TEVp is membrane tethered whereas the transcription factor is fused to a cytosolic interactor (Figure 2b). ChaCha outperformed Tango system, and sensed a wide range of ligands, including chemokines, synthetic small molecules and hormones.

In the work by Baeumler *et al.* tyrosine kinases (RTKs) and GPCRs receptors were coupled to dCas9 transcription system, to gradually increase gene expression in an agonist dose-dependent manner(dCas9-synR) [48•]. To reduce OFF-state background transcriptional activity, dCas9 was split into N-terminal and C-terminal fragments fused to each of the chains of the receptor. The chimeric receptors respond to a variety of soluble ligands, including VEGF, bradykinin, LPA and glucose, demonstrating the modular applicability of the platform (Figure 2c).

Receptors that integrate different affinity modules such as single chain fragment antibodies (scFvs) may extend the range of extracellular molecules detected [49••,50].

Scheller *et al.* [49••] designed a platform with sense-and-respond capabilities by using the erythropoietin receptor (EpoR) dimers combined with different affinity domains and coupled to three different intracellular actuators to activate distinct signaling pathways. Here, the EpoR transmembrane domain was fused to the intracellular signal transduction domains of IL-6RB, FGFR1, or VEGFR2 which in turn activate downstream signaling

via JAK/STAT, MAPK, and PLCG as well as PI3K/Akt respectively (Figure 2d). Activation of the signaling pathway triggers engineered promoters to induce desired gene expression. Using this strategy, the authors demonstrated response to soluble molecules (e.g. nicotine) as well as extracellular proteins such as PSA a relevant prostate cancer biomarker, and multiplexed gene activation using orthogonal signal transmission systems. This platform provides a valuable tool for developing cell-based therapeutics that potentially target virtually any soluble marker.

Conclusion and outlook

Synthetic networks in mammalian cells are rapidly evolving toward precise control of gene expression. A critical element is to ensure tight control and timely activation of input–output processes. To this end, diagnostic and therapeutic devices incorporate smart sensors linked to circuits operation. Protein-based interfaces serve to a broad range of applications, as proteins are key regulators of all cell activities and play a major role in signal transmission. Such interfaces have been connected to a variety of intracellular and extracellular inputs including microRNAs and proteins whose altered levels or production follow dynamic changes of environmental niches [10,15••,49••].

By regulating desired outputs at transcriptional, post-transcriptional or post-translational level, synthetic devices with different fast-response characteristics can be tailored to the target application. Most devices are still implemented on DNA platforms and rely on transcriptional regulation. However, circuits that operate post-transcriptionally are gaining consideration, as the RNA-only delivery modality may address issues of immunogenicity and unwanted chromosomal integration [10,11••,14]. In addition, with protein–protein regulation the response time reduces to the order of minutes, making these devices advantageous for the treatment of metabolic disorders like diabetes where dynamic changes in metabolites level are quite fast and require precise feedback control. As the number of genetic blocks is increasing, the design of protein effectors could be of help to achieve fast response. Integrated efforts of computational approaches [51–53], along with medicinal chemistry and molecular biology [54] may provide good solutions to these unmet needs.

These tools enhance the efficacy and safety of cell-based therapies [55], and the modularity of these systems will allow to move toward precision medicine applications and sophisticated diagnostic devices.

Conflict of interest statement

Nothing declared.

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