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Designed protease-based signaling networks

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Abstract

Regulated proteolysis is a pivotal regulatory mechanism in all living organisms from bacteria to mammalian cells and viruses. The ability to design proteases to sense, transmit, or trigger a signal opens up the possibility of construction of sophisticated proteolysis-regulated signaling networks. Cleavage of the polypeptide chain can either activate or inactivate the selected protein or process, often with a fast response. Most designs are based on sequence-selective proteases that can be implemented for transcriptional, translational, and ultimately post-translational control, aiming to engineer complex circuits that can dynamically control cellular functions and enable novel biotechnological and biomedical applications.

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Keywords

Proteolysis, Viral proteases, Endogenous proteases, Protease-based sensors, Synthetic signaling cascades.

Introduction

Proteases play important role in all aspects of cell signaling, from initiation, transmission, regulation, and termination of physiological processes. Proteases catalyze the hydrolysis of peptide bonds in essentially an irreversible reaction, and this ability to activate or inactivate proteins makes them advantageous to engineer a variety of responses. Since proteases can be adapted to function in diverse cell types, from bacteria to mammalian cells, and under diverse environmental conditions, proteases represent a powerful synthetic biology tool to interrogate and control biological processes. Proteolysis provides a potential platform for circuit design, and in recent years, important advances have been made toward designed protein networks based on proteolysis. In this review, we discuss the use of viral and endogenous proteases with a focus on proteolysis-based networks in mammalian cells.

The ectopic protease toolbox

Viral proteases cleave viral polyprotein within the host cell with high specificity to avoid massive homeostatic disorders to the host cell. Several proteases from positive-strand RNA viruses are well characterized and display stringent substrate specificity. The human hepatitis C virus proteases (HCV) and the family of plant potyvirus proteases are most widely used as tools for the development of a protease-based system (Table 1) as they recognize a substrate defined by up to seven amino acid residues, which are rarely found in the host proteome.

The HCV NS3/4A serine protease cleaves four junctions in the viral polyprotein in cis and trans [1] and is essential for viral replication. Therefore NS3/4A protease is generally considered as one of the most attractive targets for HCV drug development. Although the native form of NS3/4A is a heterodimeric multifunctional protein of 70 kDa, the catalytic subunit of the NS3/4A serine protease domain is monomeric and can be expressed in mammalian cells without significant toxicity [2]. Moreover, its substrate specificity is well characterized. Sequence analysis of individual cleavage sites defines the consensus sequence of NS3/4A as D/EXXXXC/T↓S/AXXX, with EDVVPC↓SMG as a high-affinity substrate and DEMEEC↓SQQ as an intermediate affinity substrate [3,4]. Cumulatively, all these characteristics combined, make the HCV protease an excellent tool in synthetic biology. In addition, Lin et al. have shown that NS3A lacking NS4 cofactor is active in mammalian cells with lower turnover rate [3], which could be advantageous for applications where a certain threshold should be met. Furthermore, several clinically approved inhibitors are available [5], that provide exogenous control of HCV-based systems.

The nuclear-infusion-a (NIa) protease from plant potyviruses is classified as cysteine protease that recognizes a heptapeptide sequence and displays a stringent substrate specificity. The principles of protease target site recognition and cleavage mechanism are well understood, facilitating the design of systems based on orthogonal proteases. The Tobacco etch virus (TEV)

Table 1

List of viral proteases used in synthetic circuits.

Protease	Less/more active version	Comment	Split protease	Preferred substrate	Less preferred substrate	Inhibitor	Reference
Potyviral proteases							
TEV			Yes	ENLYFQ/S	ENLYFQ/L; ENLYFQ/G	No	[13]
	TEV_E	Orthogonal protease	No[a]	ENLYFE/S		No	[7]
	TEV_H	Orthogonal protease	No[a]	ENLYFH/S		No	[7]
	TEV_EAV		No[a]	ENLYFQ/S	ENLYFQ/L; ENLYFQ/G	No	[8]
	uTEV1Δ_	Improved catalytic rate	No[a]	ENLYFQ/S	ENLYFQ/L; ENLYFQ/G	No	[9]
	Ca^{2+}-TEV	Calcium-activated TEV	No	ENLYFQ/S	ENLYFQ/L; ENLYFQ/G	No	[22]
	eTEV	Higher catalytic efficiency	No[a]	ENLYFQ/S	ENLYFQ/L; ENLYFQ/G	No	[8]
	erTEV	Active in secretory pathways	Yes	ENLYFQ/S	ENLYFQ/L; ENLYFQ/G	No	[6]
PPV			Yes	NVVVHQ/S		No	[17,40]
SbMV			Yes	ESVSLQ/S		No	[17,40]
	erSbMV	Active in secretory pathways	Yes	ESVSLQ/S		No	[40]
SuMMV			Yes	EEIHLQ/S		No	[15,17]
TVMV			Yes	ETVRFQ/S		Yes	[15,18,34–36]
TUMV			Yes	ACVYHQ/S		No	[15]
Hepatitis C virus protease							
NS3/4A			No	EDVVPC/SM	DEMEED/SQ	Yes	[45–50]
	NS3/4A 54A	Lower catalytic efficiency					[3]
NS3A			No	EDVVPC/SM	DEMEED/SQ	Yes	[3]
	NS3 54A	Lower catalytic efficiency					[3]
HIV protease							
HIV-1				SQVSQNYPIVQNLQ		Yes	
SARS-CoV-2 proteases							
Mpro			No	TTVRLQSGFRKM		Yes	
PLpro			No	IALKGGKIVNNW		Yes	

[a] Split version was not jet experimentally tested, but the same split site as used for unmodified TEV protease should work.

protease is the most characterized and widely used protease in biotechnology. The pervasive role of TEV protease in synthetic sensing and signaling is illustrated by numerous variants of TEV protease (e.g., TEV protease active in the secretory pathway [6]; TEV protease variants with orthogonal cleavage sites [7]; variants with different catalytic activities [8,9]; self-cleaving and non-self-cleaving variants [7]). These were used for target protein degradation [10], sensing protein–protein interactions [11–13], TEVp-based biosensors development [14] and finally for the development of fully synthetic signaling pathways [15–18]. In addition to the TEV protease, several potyviral homologs with orthogonal cleavage sites have been characterized, including Plum pox virus protease (PPV) [19], Soybean mosaic virus protease (SbMV) [20], Sunflower mild mosaic virus protease (SuMMV) [21], Turnip mosaic virus protease (TUMV) [15], and Tobacco vein mottling protease (TMVM) [15,16]. The development of split versions of several potyviral proteases provides an attractive method for regulating protease activity by selected signals [17]. By inserting a calcium-sensitive module into the exposed loop of the TEV protease an allosterically regulated TEV protease was developed that senses and responds to intracellular Ca influx [22]. Because potyvirus genus is one of the largest among the plant infecting RNA viruses [23], the repertoire of orthogonal homologs can potentially be further expanded by elucidating their substrate specificity.

Engagement of endogenous proteases

While the high specificity of viral proteases is perfectly suited for use in *in vitro* synthetic networks, the introduction of exogenous modules could sometimes be disadvantageous, especially with respect to therapeutic applications. More than 2% of human genome is encoded by proteases, indicating the importance of proteolysis in cell signaling. At the same time, however, the question arises as to how the endogenous proteases can be engaged or engineered with no, or minimal crosstalk to native protease-based signaling pathways. One option that introduces the desirable feature of tightly regulated activation of proteases is the development of small molecule- or light-controlled proteases, such as photoactivatable reassembly of caspase-7 [24] or caspase-3 [25]. Despite the aforementioned challenges, synthetic intramembrane proteolysis receptors, synNotch-tethering the ADAM10 protease and γ-secretase, enable the generation of fully orthogonal circuits with designed inputs and outputs. ADAMs are a family of transmembrane metalloproteases that cleave transmembrane proteins at a juxtamembrane position to

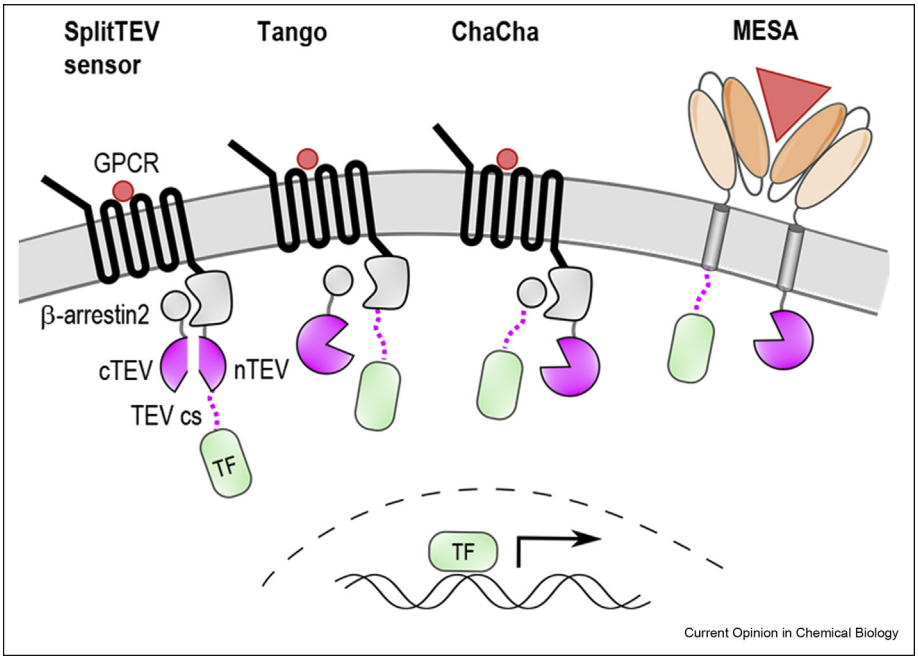
release soluble ectodomains from the cell surface. In the Notch and synNotch pathway, y-secretases mediate cleavage of the remaining part in the inner leaflet of the Notch/synNotch-expressing cell membrane, and release the intracellular portion that function as transcription factor [26]. Endogenous proteases also play an important role in degron-mediated regulation of proteins, however this involves an indirect, ubiquitination mediated engagement of protease [27].

Protease-based sensing and signaling circuits

Most frequently reported protease-based sensors use the TEV protease as a signaling mediator and transcription factor as an output. This type of sensor architecture takes advantage of the transient interaction between the ligand and receptor, resulting in stable and long-lasting transcription of a reporter gene due to the irreversibility of the protease cleavage. Potyviral proteases have been used in several designs of protease-based sensor networks (Figure 1). These include sensing of protein–protein interactions using split proteases (split TEV protease sensor connected to a GPCR sensor module) [14] or with induced proximity of a protease and its cognate cleavage site (Tango [13] and ChaCha [11] systems linked to GPCR sensor module or MESA [12,28], as a fully synthetic receptor), and chemical or light-induced protease-based sensors for targeted protein degradation (Figure 1) [29,30]. On the other hand, protease sensors for screening potential viral protease inhibitors are commonly used for antiviral drug discovery. The recent interesting work was conducted by Franko et al. in which tunable autoproteolytic gene switches (TAGS) were developed preferentially for SARS-CoV-2 antiviral drug discovery; however, the modularity of the system allows its use beyond drug discovery [31]. Virus-specific proteases have been used to trigger the designed response system, leading to the desired antiviral response or activation of cell death, where targeting an essential viral function is expected to be resistant to point mutations that may render chemical inhibitors inactive [32].

Proteolytically-regulated signaling networks: Protease-based regulation frequently involves introduction of a specific protease substrate into the target protein, where the introduction of a peptide substrate does not interfere with the protein function, but results in activation or inactivation upon proteolytic cleavage. The usefulness of proteolysis to regulate transcription of the target gene was demonstrated by the insertion of TEV protease recognition sites into the TALE regulator. The TALE-TEVp regulator inhibits transcription initiation in the OFF state but promotes the originally repressed

Figure 1



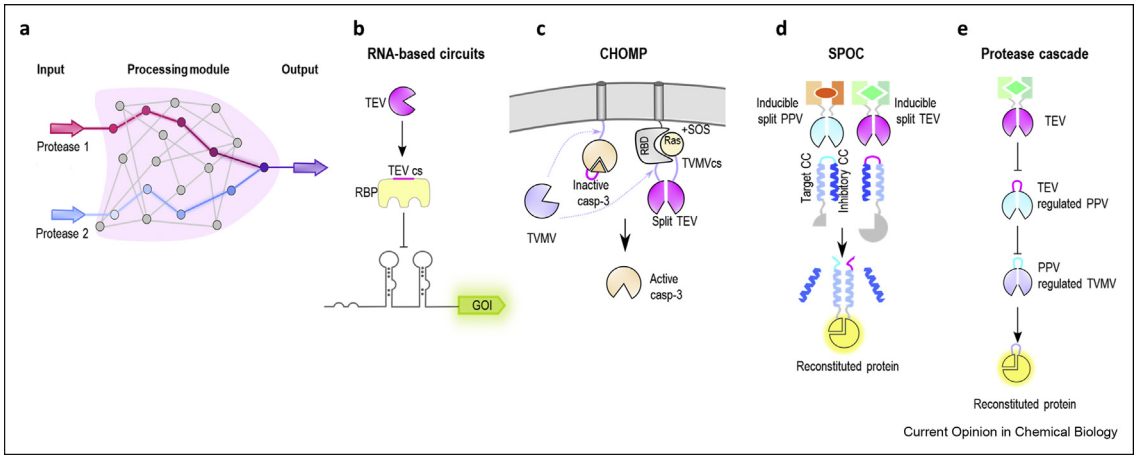
Protease-based sensors. Several protease-based sensors have been developed. Split TEV, Tango, and ChaCha are linked to a GPCR sensor module, whereas MESA engaged fully synthetic receptor based on dimerizing VEGF scFv. In the split-TEV sensor, after binding of GPCR and β-arrestin, split-TEV is reconstituted, which cleaves and releases the TF (transcription factor). In the Tango system, the protease is fused to β-arrestin and the TF is linked to the GPCR via a TEV-cleavable linker. In contrast, in the ChaCha system, the protease is fused to the GPCR and the cleavable TF to β-arrestin, reducing leakiness. MESA system consists of a synthetic sensing module based on scFV, with one scFV linked to the protease and the second to the cleavable TF. Receptor dimerization induced by ligand binding brings the protease and cleavable TF into close proximity, allowing cleavage and release of the TF.

target gene after TEVp-mediated proteolysis. Engineered TALE-TEVp system allows both repression and induction of gene expression [33]. One of the earliest examples of posttranscriptional synthetic circuits based on proteolysis relies on proteolysis-responsive RNA binding proteins (RBPs) [18]. Here, the protease cleavage site was introduced into the archaeal RBP L7Ae RBP, which binds the K-turn motif of the RNA C/D box. When bound to its target, it behaves similarly to wt L7A and acts as a repressor, by binding to the 5′UTR K-turn. However, when the protease is present, L7Ae undergoes proteolysis, preventing its binding to the 5′UTR (Figure 2b). By expanding the toolbox of proteolysis-responsive RBPs and adopting HCV and various potyviral proteases, a multilayered cascade was developed. Furthermore, a three-stage cascade was demonstrated in which the input protease controls a second protease that further transduces and amplifies the signal by cleaving the proteolysis-responsive RBP (Figure 2e). In such setting, the activation of the said protease represses translation of the reporter [18]. Circuits that combine posttranscriptional and post-translational control, benefit from the simplicity and the ability to fine-tune the RNA sequence-targeting design and also the kinetics and specificity of the proteolysis.

In a system termed CHOMP (circuits of hacked orthogonal modular proteases), degrons and protease cleavage sites were fused to target proteins [16]. This enabled the removal or exposure of a degron sequence by proteolysis, resulting in degradation or stabilization of a target protein. In order to design complex circuits, protease-regulated protease was developed, which enables the construction of multilayer circuits. Boolean logic gates were engineered using leucine zipper-regulated TEV and HCV protease as binary inputs by their inclusion or exclusion in transfection. Design and functionality of a bandpass filter and adaptive pulse circuits were demonstrated using chemically regulated TEV and TVMV or HCV protease, encoded in a single DNA transcript. Finally, the utility and composability of proteolysis-based protein networks were demonstrated by a circuit that triggers cell death in response to Ras oncogene expression (Figure 2c) [16].

An important merit of proteolysis-based circuits regulated at the posttranslational level is their ability to respond rapidly, particularly in comparison to the transcriptional regulation. This has been demonstrated in a system termed split-cleavable orthogonal coiled-coil logic (SPOC logic) [17]. To develop the SPOC logic system, the toolbox of proteolysis-responsive coiled-coil (CC) elements and orthogonal split proteases was first developed to enable efficient proteolysis-based signal processing. The interaction modules consist of proteolysis-responsive auto-inhibitory CC pairs and cleavage sites for orthogonal split proteases at different positions within the CC modules. This architecture enabled the construction of all binary Boolean logic gates in mammalian cells

Figure 2



Protease-based synthetic circuits. **a)** Protease activity serves as an input to designed signaling networks whose outputs can be protein degradation, reconstitution, activation, inactivation or translocation. **b)** In RNA-based protease circuits, a protease cleaves an engineered RBP, which blocks translation when bound to the 5′UTR (RBP, RNA binding protein; GOI, gene of interest). **c)** An example of a CHOMP circuit enabling conditional activation of caspase3 in Ras-activating cells. SOS-activated Ras binds to the RBD and reconstitutes TEV protease, which in turn activates the caspase 3. TVMV protease inhibits a TEV protease and detaches caspase from the membrane thus enhancing the selectively (Casp-3, caspase3; RBP, Ras binding protein). **d)** An inducible AND gate in a SPOC logic system. Addition of rapamycin and abscisic acid reconstitutes the split TEV and PPV protease, respectively, which cleaves the inhibitory coil, allowing dimerization of the target coils and reconstitution of the split protein. **e)** Multilayer protease cascade. In all three examples of protease-based networks multilayer cascade was demonstrated in mammalian cells. Here, the input protease regulates the first protease, which serves as an input to the next layer of the protease-regulated protease, and the last protease regulates the output.

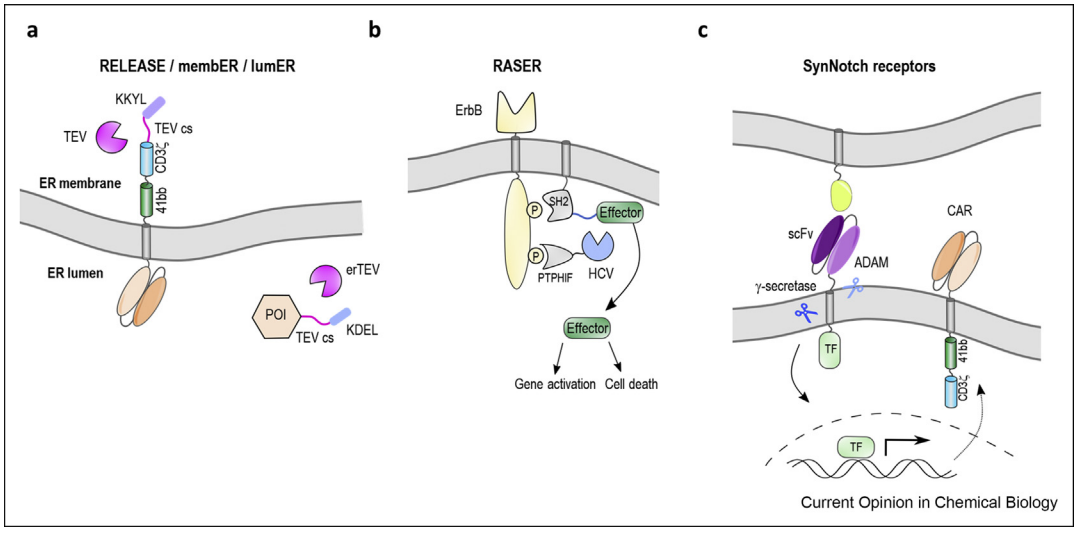
(Figure 2d). By linking the toolbox of proteolysis-dependent split potyviral proteases and proteolysis-responsive CC, multilayer cascades can be constructed. In this case, the chemically regulated split protease provides an input for the second protease-based logic layer. When the proteolysis-responsive autoinhibited CC was connected to a protease regulated by an orthogonal input protease, a proteolytic cascade was formed. Following the principle of high orthogonality, an inducible double-negation cascade with three potyviral proteases was demonstrated.

Another strategy to regulate proteolysis and design signaling networks is the release of a peptide inhibitor, genetically fused to the protease [34] [—] [36]. Peptide inhibitors have been ingeniously designed based on the peptide product of TVMV or HCV protease. Combinations of autoinhibited proteases have been combined with binding domains or protease substrate peptides to integrate them into the amplification or signaling network [34,36] or into ultrasensitive sensors [35]. By linking autoinhibited protease and a peptide-regulated allosteric switch based on calmodulin, an artificial proteolytic system was developed [37,38]. Here, the input autoinhibited protease uncage a signaling peptide, which in turn regulates the activity of target protein.

Protease networks for protein translocation: Two articles, have described a mechanism of synthetic proteolysis-driven protein secretion system. Both systems rely on the release of proteins from the endoplasmic reticulum (ER) triggered by the proteolytic cleavage of the ER retention signal. In the first system, termed RELEASE (retained endoplasmic cleavable secretion), an oncogenic RAS-sensing circuits were demonstrated [39]. A similar system was called membER by Praznik et al. [40], who went a step further and also developed a second system, termed lumER, which is based on the engineered split proteases (TEVp, SbMVp and PPVp), that can be activated within the ER. In addition to the regulated protein secretion this system can also be used for the regulated fast translocation of protein receptors to the plasma membrane, demonstrated on the regulation of the CAR T cell responsiveness (Figure 3a).

Rewiring cancer signaling with proteolysis as an underlying mechanism: A synthetic signaling pathway named RASER (rewiring of aberrant signaling to effector release) was built to specifically sense an oncogenic input signal and rewire it into a therapeutic output [3]. Here, the HCV protease was fused to the phosphotyrosine-binding (PTB) domain to sense the oncogenic activity of Erb, and a cleavable membrane-tethered cargo domain was fused to SH2. Oncogenic hyperphosphorylated Erb cells recruit the cargo domain and HCV protease via PTB and SH2 interactions. The close proximity of the cleavable cargo domain and HCV protease resulted in enhanced proteolysis and release of the therapeutic cargo from the plasma membrane. To

Figure 3



Protease-based sensing-actuation. a) In the lumER systems, the protein of interest is retained in the lumen of the ER by fusion of the TEV protease-cleavable ER retention signal KDEL. After cleavage of the retention signal by the erTEV protease, the protein is secreted. In the membER and RELEASE system, the membrane protein is retained at the ER membrane via cleavable retention signal KKYL. When the cytosolic TEV protease cleaves the retention signal, the protein is translocated to the plasma membrane. **b)** The RASER system rewires the ErbB-cancer signaling pathway. In response to hyper-phosphorylated ErbB, the HCV protease and cleavable effector are recruited to the receptor, resulting in cleavage of the effector. The effector can be either a TF to activate the target gene or a proapoptotic Bid to induce cell death. **c)** Synthetic Notch receptors recognize membrane-bound antigens. Upon engagement with the antigen, the endogenous ADAM protease and γ-secretase cleave at the juxtamembrane position and release the transcription factor. In the AND gate synNotch, released TF drives transcription of CAR that sense and respond to the second antigen.

reduce the background protein release and achieve a more stringent differentiation between cancer and normal cells, the HCV module was further engineered to reduce the protease activity through protease degradation (Figure 3b). In addition, the RASER system linked to transcription factors and dCas9, enabled the activation of endogenous genes of choice leading to a variety of programmable biological outcomes [3].

Perhaps the most applicable example of signal transduction regulated by proteolysis is the development of Notch receptors [26]. In the synthetic Notch receptors both the extracellular and intracellular modules are replaced by tailored sensing and response modules, while only the proteolytically responsive transmembrane domain remains the same as in the native Notch receptor [26]. This modular design opens up a broad spectrum of user-specific cell signaling. Since their inception, synNotch receptors have proven to be a valuable tool for numerous applications, particularly in cancer immunotherapy [41]. A synNotch-based three-antigen gate AND, consisting of three synNotch receptors targeting three cancer cell-specific antigens, enables precise recognition and selectivity of cancer cells (Figure 3c) [42]. Recently, a two-step positive feedback loop was developed to distinguish cancer cells from normal cells, on basis of antigen density. Here, the high-density filter based on the low-affinity synNotch receptor triggers transcription of high-affinity CAR only when the antigen availability threshold, specific for tumor cells, is exceeded [43].

Conclusions and future perspectives

The ability to reprogram mammalian cells with a tight control provides a powerful tool to engineer cellular functions ranging from diagnostic, to therapeutic applications. Proteases implemented in mammalian cells enable the construction of sophisticated cellular networks. Since the target, recognition and cleavage mechanisms of viral proteases are well understood, and in addition, several viral proteases exhibit strong sequence specificity and orthogonality, the fulfillment of these requirements is already built in. The toolbox of available viral proteases has expanded greatly in recent years. Versions of proteases with lower and higher turnover rates, or more and less preferred substrates, allow not only tight control, but also fine-tuning of the response rate. In addition to the development of synthetic signaling cascades *in vivo* in various organisms, proteases represent an attractive tool for the development of *in vitro* proteolytic devices, such as an example of *in vitro* molecular network using protease-activatable scaffold proteins as building blocks [35,44].

Given that proteolysis is essentially an irreversible reaction, tight regulation of selectivity and specificity are the major challenges in using endogenous proteases. Several examples of light- and chemically-inducible endogenous proteases have been demonstrated for applications where spatiotemporal control is required [24,25]. Realizing the full potential of endogenous proteases in engineered synthetic circuits will require additional advances to minimize crosstalk with endogenous signals and to avoid homeostatic interference.

The modular design of many of the discussed systems enables further development and a combinatorial design to construct advanced logic gates. This is useful for incorporation of safety switches (e.g., combining two MESA systems, where one signal leads to a transcriptional activator while the second releases a transcriptional repressor) or for linking the synNotch receptor to the CAR-membER system, creating an AND gate for the increased precision of tumor recognition. Looking ahead, the integration of computational and experimental methods may facilitate the development of new types of cell regulation implemented to cell surface receptors with an aim to construct cell devices for various diagnostic and therapeutic applications.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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