PERSPECTIVE
https://doi.org/10.1038/s41589-021-00926-z

nature chemical biology

The evolution of synthetic receptor systems

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Receptors enable cells to detect, process and respond to information about their environments. Over the past two decades, synthetic biologists have repurposed physical parts and concepts from natural receptors to engineer synthetic receptors. These technologies implement customized sense-and-respond programs that link a cell's interaction with extracellular and intracellular cues to user-defined responses. When combined with tools for information processing, these advances enable programming of sophisticated customized functions. In recent years, the library of synthetic receptors and their capabilities has substantially evolved—a term we employ here to mean systematic improvement and expansion. Here, we survey the existing mammalian synthetic biology toolkit of protein-based receptors and signal-processing components, highlighting efforts to evolve and integrate some of the foundational synthetic receptor systems. We then propose a generalized strategy for engineering and improving receptor systems to meet defined functional objectives called a 'metric-enabled approach for synthetic receptor engineering' (MEASRE).

Engineered mammalian cells are transformative for therapeutics, diagnostics, drug discovery and fundamental research. These advances are driven by technologies and understanding that enable the genetic encoding of customized functions. A particularly useful capability is linking a cell's detection of extracellular and intracellular cues with the initiation of user-defined responses using receptors. The first synthetic (non-natural) receptors were inspired by natural cellular signaling, the fundamental process by which information is transferred within and between cells. Subsequent receptor engineering has increasingly strived for evolution—a term we employ here to mean improvement and expansion—of foundational technologies. With many synthetic receptors now available, selecting and integrating receptor components and optimizing their combined performance present substantial opportunities and concomitant design challenges. Here, we survey the contemporary toolkit of synthetic receptors and strategies to systematically evolve and integrate synthetic receptor systems. We draw upon these examples to propose a conceptual framework for designing receptor systems to meet performance objectives and address unmet biomedical needs.

The toolkit for synthetic signaling in mammalian cells

In this section, we provide an overview of available modalities for controlling mammalian cell functions in response to extracellular and intracellular cues.

Composition of synthetic receptor systems. Receptor systems (both natural and synthetic) each perform two key operations: sensing and actuation (Fig. 1). Sensing is the interaction of the receptor with the target cue (or input) in a manner that causes a change in the receptor state. In this new state, the receptor actuates—effects a change in the cell (called 'output'). Actuation and output can be directly linked, or in some cases, a signal-processing module can act downstream of actuation to regulate output. Output comprises a specified change in cell state, such as regulation of gene expression, targeted protein degradation, phosphoregulation of downstream mediator proteins or induction of cellular processes such as apoptosis. Each operation may be performed by different molecular components depending on the system, and sensing and actuation domains can often be combined in a modular fashion. Synthetic receptors can be constructed using components that are natural and/or engineered in origin. This Perspective focuses on protein-based receptors, but these terms can also apply to sensors and receptors that are not protein-based and that have been reviewed elsewhere[1].

Below, we summarize currently available synthetic receptors classified based on which operations (sensing, actuation or both) have been systematically engineered. We chose this framework because these features determine how synthetic receptors can be rationally improved or integrated to implement sophisticated functions.

Synthetic receptors that combine engineered sensing with natural actuation. By engineering sensing, synthetic receptors can rewire endogenous signaling pathways to respond to new inputs (Fig. 2). Early strategies of this type involved mutating natural receptors to alter ligand-binding specificity through site-directed mutagenesis or directed evolution, for example, to enable external, selective control of G protein–coupled receptor (GPCR) signaling pathways using synthetic, small molecule ligands as inputs[2]. Input sensing can also be engineered by replacing the sensing domains of native receptors with other ligand-binding domains such as single-chain antibody variable fragments (scFvs), nanobodies (single-domain camelid antibody fragments) or small molecule-binding domains. These chimeric receptors can be engineered to sense either natural or synthetic inputs. An archetype of this approach is the chimeric antigen receptor (CAR), which binds cancer cell-surface antigens via an extracellular antibody-based domain and then activates T cell signaling pathways[3,4]. T cells engineered to express CARs targeting tumor antigen CD19 are approved by the US Food and Drug

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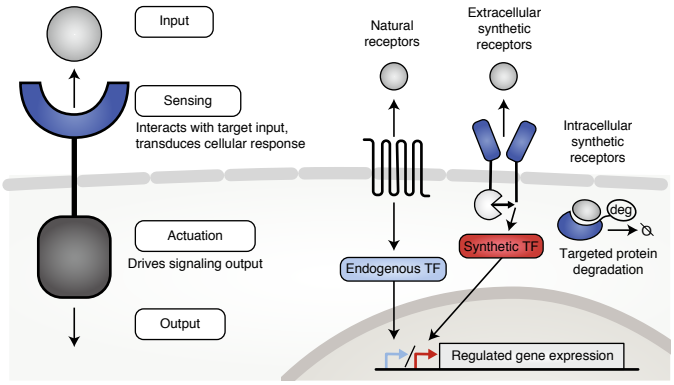


Fig. 1 | Generalized operational components of synthetic receptors. Generalized receptors involve two universal components: the sensor, which facilitates detection of the target input, and the actuator, which transduces the sensor activity into output (left). Examples of the wide range of natural and synthetic, extracellular and intracellular receptors are shown to highlight the range of types of domains that can serve as sensor and actuator components (right).

Administration for treating some B cell malignancies. Progress in engineering CARs has been reviewed elsewhere[3,4]. We expand specifically on CAR evolution below (see 'Systematic evolution of individual synthetic receptors'). Beyond CARs, this chimeric receptor engineering strategy has been used to program cells with a range of other non-native functions, including T cell receptor (TCR)-like signaling in nonimmune cells[5], antigen-specific B cell receptor signaling[6], and target cell–specific invasion and fusion[7]. Other examples of this strategy include synthetic cytokine receptors, which pair user-defined ligand-binding domains with native or modified transmembrane and intracellular domains of cytokine receptors[8–10]. Ligand-binding domains have been paired with intracellular signaling domains from the growth-factor receptor VEGFR2 to wire user-defined inputs, including small molecules and proteins, to calcium-mediated signaling to control directed migration, exocytosis and apoptosis[11]. To reconstitute and elucidate growth and patterning processes driven by morphogen gradients in *Drosophila*, a natural morphogen receptor was modified to bind green fluorescent protein using anti–green fluorescent protein nanobodies as sensor domains[12]. Each of these strategies is tailored to a specific input–output combination.

Recently, a modular system was developed to streamline chimeric receptor engineering for user-defined inputs and natural signaling outputs. The generalized extracellular molecule sensor (GEMS) is amenable to sensing various soluble ligands and signaling through multiple natural pathways[13]. GEMS receptors contain a standard transmembrane scaffold with user-defined extracellular ligand-binding domains and intracellular signaling domains derived from the cytokine receptor chain IL-6RB, the growth-factor receptor FGFR1, or VEGFR2. These examples highlight the potential to connect customized receptor sensing to many types of natural signaling pathways.

Synthetic receptors with natural sensing and engineered actuation. Synthetic receptors can redirect native sensing to engineered actuation pathways (Fig. 2) by harnessing the natural regulation of protein–protein interactions. In this approach, receptor actuation is mediated by synthetic transcription factors (TFs) and promoters. For example, Tango receptors employ natural ligand-binding-induced receptor phosphorylation to recruit an exogenously expressed protease to active receptors[14]. This function is achieved by tethering the protease to a protein that naturally binds to phosphorylated residues, such as β-arrestin and Src homology 2 domains. The actuation step is proteolytic release of a receptor-tethered synthetic TF via the recruited protease. Thus, the input remains the endogenous receptor input—for example, a GPCR ligand—but the output is user-defined gene activation. Tango receptors have been used to redirect signaling from targets of native GPCRs and receptor tyrosine kinases (RTKs) to customized transcriptional output and have been used to interrogate druggable targets for native receptors[15].

This strategy has also been extended to alternative configurations. For example, when ChaCha receptors bind to their endogenous GPCR ligands, they recruit a cytoplasmic TF to a GPCR-tethered protease[16]. Proteolysis then induces nuclear localization and TF-mediated transcription. Other configurations recruit both the TF and protease separately after ligand binding[17] or require blue light as a second input[18]. A variation on this mechanism employs intracellular calcium-regulated protein–protein interactions to control transcriptional output[19]. These examples effectively rewire natural regulation of protein–protein interactions involved in native receptor signaling to engineered actuation in the form of transcriptional output. The synthetic TFs that perform the engineered actuation discussed in this section can also implement downstream signal processing (see 'Strategies for signal processing with engineered transcriptional programming').

Synthetic receptors with both engineered sensing and engineered actuation. Synthetic receptor systems that signal with both engineered sensing and actuation can be orthogonal to, or completely independent of, endogenous sensing and actuation. This strategy enables construction of signaling pathways with both user-defined input and output that do not rely on pre-existing natural receptors (Fig. 2).

To detect surface-bound ligands, typically via cell–cell contact, the synthetic Notch (synNotch) receptor system was developed[20,21]. SynNotch receptors contain an extracellular antibody-based ligand-binding domain, the native mouse Notch receptor transmembrane core and an intracellular tethered TF. While both sensing and actuation are engineered with synNotch receptors, the system relies on native (not engineered) processes to transduce sensing into actuation. The native Notch receptor transmembrane core is cleaved by ubiquitous proteases to release the TF after binding of a target ligand. This signaling mechanism requires a pulling force to uncover the protease cleavage site, making synNotch ideally suited for targeting surface-bound ligands. Recently, the synNotch architecture was expanded into a set of receptors called 'synthetic intramembrane proteolysis receptors' (SNIPRs), which incorporate domains from natural receptors other than mouse Notch that are similarly cleavable by endogenous membrane proteases[22]. Like synNotch, SNIPRs bind to surface-bound antigens and actuate by releasing a tethered TF. To detect extracellular soluble ligands, the modular extracellular sensor architecture (MESA) was developed[23,24]. MESA comprises two transmembrane proteins that each contain an extracellular ligand-binding domain (which determines the target input and can be antibody based or a small molecule–binding domain), a transmembrane domain and either an intracellular tethered TF and protease recognition sequence or a protease. MESA receptors dimerize after ligand binding, triggering an intracellular proteolytic *trans*-cleavage reaction that releases the TF. This system has also been modified recently to signal via a split protease reconstitution[23,25] or split TF mechanisms[26]. For both synNotch and MESA, selection of ligand-binding domains and TFs enables customization of both sensing and actuation steps when targeting extracellular inputs.

Other synthetic receptor systems achieve orthogonal sensing and actuation for intracellular ligands (Fig. 2). One example is the modular intracellular protein sensor-actuator, wherein ligand-binding domains dimerize around a soluble, cytoplasmic ligand to release a membrane-tethered TF via proteolysis (similar to the MESA

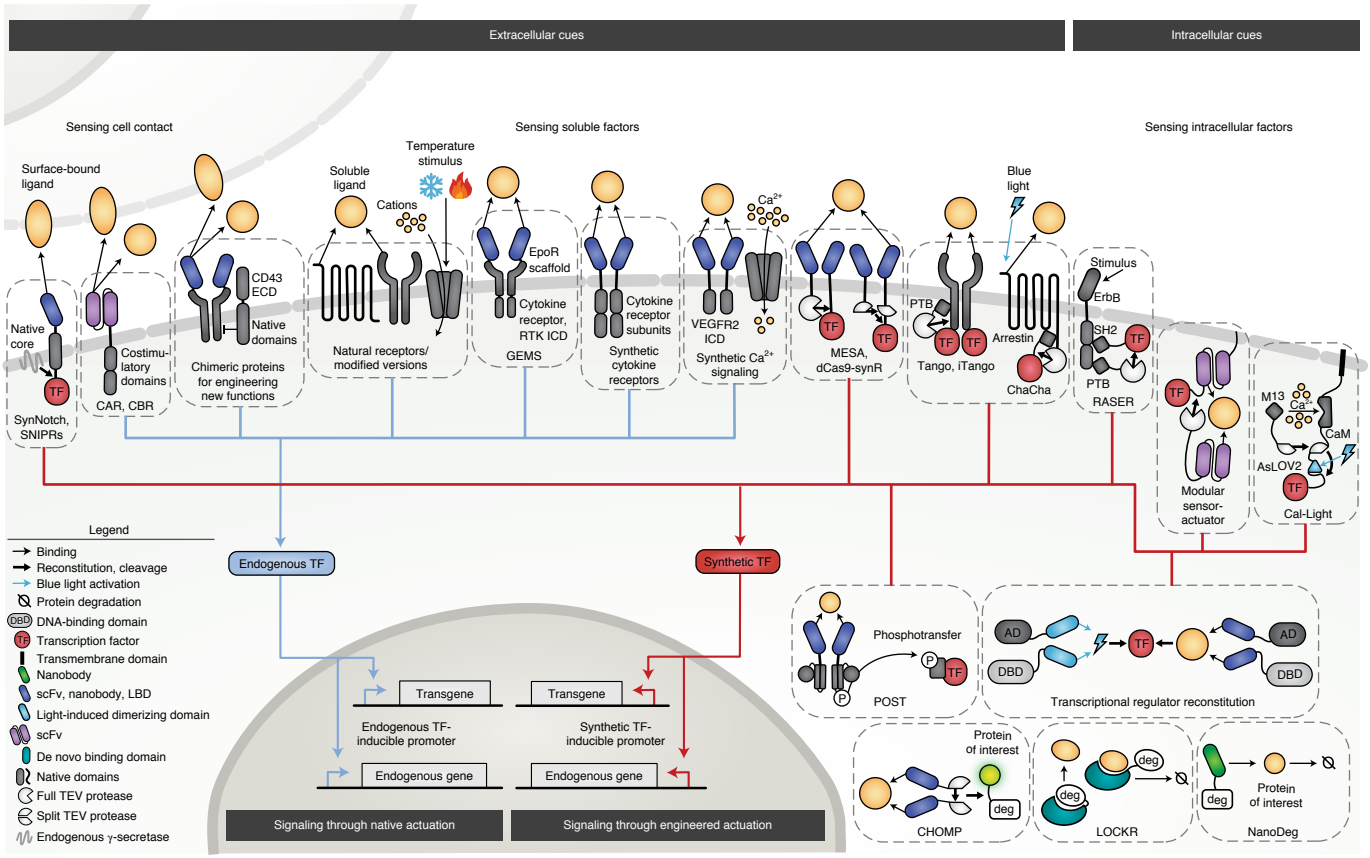


Fig. 2 | The mammalian synthetic receptor toolkit. Existing mammalian synthetic receptors can detect a range of inputs (extracellular surface-bound, extracellular soluble and intracellular factors), and signal via native or engineered actuation pathways. Synthetic receptors can have engineered sensing, actuation or both. From left to right: synNotch receptors[20,21,81,89–91], SNIPRs[22], CARs[4], chimeric B cell receptors (CBR)[6], chimeric proteins for engineering new functions[5,76], rewiring of natural receptors[36], modified natural receptors[2], GEMS[13], motif-engineered receptors[10], synthetic cytokine receptors[8], synthetic calcium signaling[11], MESA[23–25,78], dCas9 synthetic receptors (dCas9-synRs)[26], Tango[14], iTango[18], ChaCha[16], RASER[17], modular intracellular sensor-actuator[27], Cal-Light[19], phosphoregulated orthogonal signal transduction (POST)[32], transcriptional regulator reconstitution[28–31], CHOMP[34], LOCKR[35], NanoDeg[33]. AD, activation domain; AsLOV2, *Avena sativa* light, oxygen or voltage domain; BCR, B cell receptor; CaM, calmodulin; DBD, DNA-binding domain; deg, degron; ECD, extracellular domain; EGF, epidermal growth factor; EpoR, erythropoietin receptor; ErbB, epidermal growth factor receptor; ICD, intracellular domain; LBD, ligand-binding domain; P, phosphate group; PTB, phosphotyrosine-binding domain; SH2, Src homology 2 domain, TEV, tobacco etch virus.

mechanism for extracellular sensing)[27]. Another example is the reconstitution of synthetic TFs after intracellular ligand binding[28–30] or in response to blue light[31]. The phosphoregulated orthogonal signal transduction system employs a bacterial two-component system to transduce intracellular ligand binding into transcriptional output through a phosphorylation relay[32]. Intracellular ligand binding can also initiate output protein degradation[33] or, conversely, reconstitute a protease that removes degron tags from an output protein to prevent degradation[34]. Lastly, de novo control of protein degradation was designed with the latching orthogonal cage-key proteins (LOCKR) system[35]. In LOCKR, a de novo designed protein preferentially binds to a target protein, causing the exposure of a degron tag that marks the bound protein for degradation. These examples illustrate the diverse signaling options afforded by engineering both the sensing and actuation operations of synthetic receptors.

Strategies for signal processing with engineered transcriptional programming. As many synthetic receptor systems actuate output through transcriptional regulation, we devote a subsection to the available tools for engineering transcriptional signal processing—the downstream steps that connect actuation to a transcriptional output. For synthetic receptor systems employing either native or engineered actuation, there now exist many options for signal processing via transcriptional regulation, some of which have already been implemented in synthetic receptor systems (Fig. 3).

For applications for which a natural receptor already exists for a ligand of interest, one can simply rewire specific components of the downstream signaling from that receptor (Fig. 3a). For example, promoters can be engineered to be responsive to the native TFs involved in endogenous signaling pathways. Typically, this includes response elements (binding sites) for key native transcriptional regulators upstream of a minimal promoter controlling transgene expression[36]. Alternatively, signaling from native receptors and synthetic receptors that perform native actuation can be redirected to user-defined endogenous or transgenic targets using proteins called 'generalized engineered activation regulators'[37] (Fig. 3b).

To implement functions that are not achievable with fully native receptors or native actuation, synthetic TFs can be useful. The toolkit of synthetic TFs has grown considerably in the past decade to enable regulation of endogenous genes and transgenes. Most prominently, dead Cas 9 (dCas9)-based TFs include tethered transcriptional regulation domains and bind to DNA sequences complementary to a provided guide RNA (gRNA)[38]. Programming these TFs by gRNA choice can be used to target either endogenous genes or transgenes and provides handles to tune expression[39,40]. Transcription activator–like effectors also contain both a

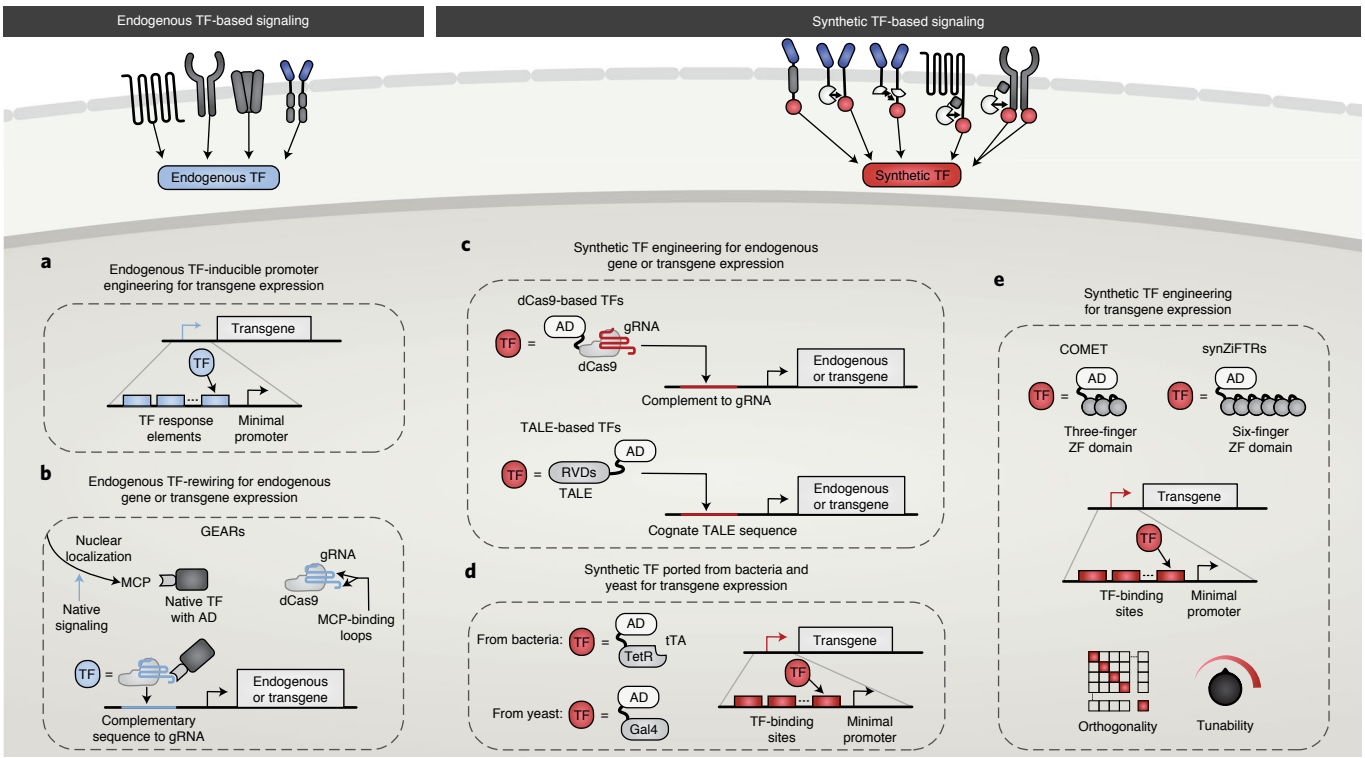


Fig. 3 | The mammalian transcriptional programming toolkit. Strategies for signal processing with transcriptional programming from synthetic receptors through endogenous (left) or synthetic (right) pathways. For receptors that signal via endogenous pathways, signaling can be rewired to customized outputs in the recently reported system generalized engineered activation regulators (GEARs)[37] or through other native pathways by engineering promoters responsive to endogenous TFs. For receptors that signal via synthetic TF release or activation, TFs can involve a dCas9 protein[38,39], transcription activator-like effectors (TALEs)[41], regulatory proteins derived from bacteria[42] or yeast[43], or zinc finger-based TFs from the recently reported COMET[28,45] or synthetic zinc finger transcription regulators (SynZiFTRs)[47]. MCP, MS2 coat protein; RVD, repeat variable di-residue; TetR, tetracycline repressor; ZF, zinc finger.

programmable DNA-binding domain and a transcriptional activation domain and can thus interface with either endogenous or engineered promoters[41]. For transgene regulation, workhorse TFs include the tetracycline-responsive transcriptional activator[42] (tTA) and Gal4-based activator[43], which each contain a transcriptional activation domain and DNA-binding domains derived from bacteria or yeast, respectively (Fig. 3d). Because tTA and Gal4 TFs are orthogonal to one another, they can be used in the same cell[20,44]. To enable genetic programs requiring more than these two canonical regulars, a library of synthetic three-finger zinc finger-based TFs (originally characterized in yeast[45]) and compatible promoters, called the 'composable mammalian elements of transcription' (COMET), was developed[28] (Fig. 3e). COMET TFs can be rationally tuned and are orthogonal to one another, enabling the use of multiple TFs in the same cell. A recent study employed and expanded the COMET system to enable model-guided predictive design of genetic programs and to process signals from multiple receptors using sophisticated logic[46]. Similarly, a panel of six-finger zinc finger–based TFs called 'synthetic zinc finger transcription regulators' was developed, with design focused on achieving specific binding to target DNA (avoiding binding to genomic sites) and exploring the use of human transcriptional regulation domains[47]. There exists substantial potential to apply this growing toolkit for transcription signal processing to enable new applications and functions.

Synthetic receptor input–output configurations. The synthetic receptors discussed thus far operate by sensing a single input and yielding a single output (single input–single output) (Fig. 4a). Various strategies have been developed to achieve more complex types of input–output properties, including sensing multiple inputs and/or producing multiple outputs with a single receptor. To wire detection of multiple inputs to a single output (multiple input–single output), one strategy is to engineer the sensing component of the receptor to be bispecific, such as by selecting two tandem scFv domains. This approach has been used with CARs[48,49] (Fig. 4b) and with synNotch receptors[50]. To activate multiple outputs in response to sensing a single input (single input–multiple output), one can engineer actuation. For example, ChaCha receptors, which activate output via dCas9-based TFs[16] (Fig. 4c), can be directed to multiple transgenic or endogenous sequences using different gRNAs. Similarly, multidomain COMET TFs can drive output from multiple engineered promoters if receptor actuation involves release of a TF with multiple zinc finger DNA-binding domains[46]. Finally, to sense multiple inputs and actuate multiple outputs (multiple input–multiple output), the strategies described above could be combined. For example, synNotch receptors have been separately engineered to sense multiple inputs or to produce multiple outputs via dCas9-based TFs[26]; these approaches could be combined to perform multiple input–multiple output functions using a single receptor (Fig. 4d). The ability to encode such functions within a single receptor is a benefit of modular receptor design. Multiple receptors can also be used to construct programs with various input–output configurations, and we discuss those systems separately (see 'Integration of multiple synthetic receptors').

Systematic evolution of individual synthetic receptors

As a field, synthetic receptor engineering has matured from initial demonstrations of feasibility to extensions and improvements of these technologies. Improvement has generally been pursued with specific performance goals and characteristics in mind (Box 1).

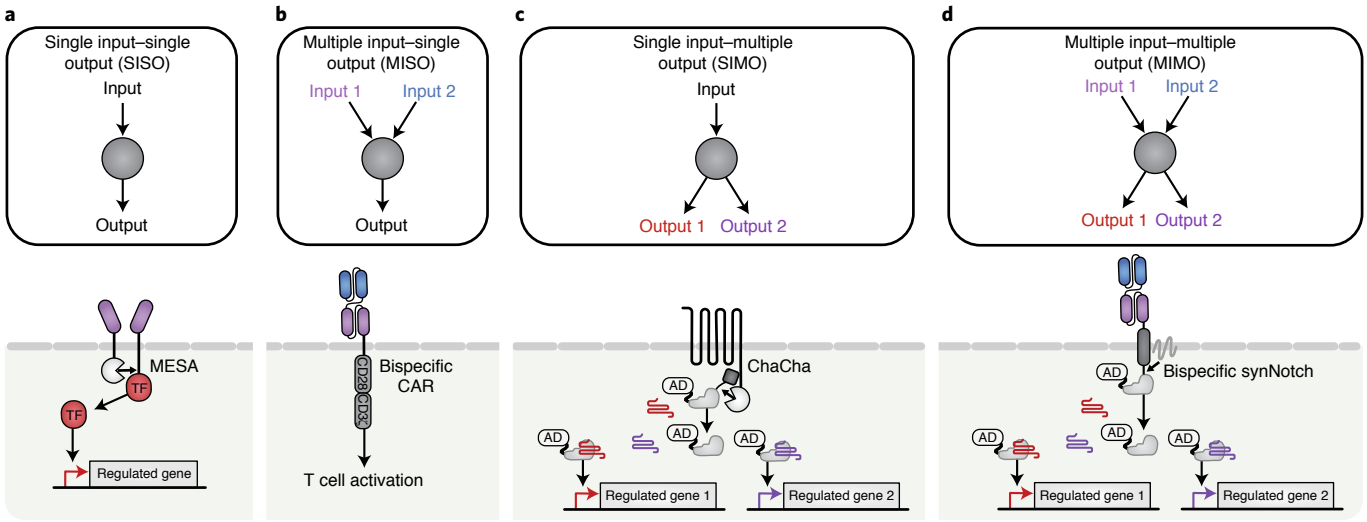


Fig. 4 | Synthetic receptor input–output configurations. Singular receptor systems can also be engineered to facilitate detection of one or more inputs and production of one or more outputs. **a**–**d**, General topologies are shown (top) alongside demonstrated examples (bottom: **a**, MESA[23,24,78]; **b**, bispecific CAR[48,49] and **c**, ChaCha[16]) and proposed examples (bottom: **d**, bispecific synNotch[50] with dCas9-based regulators).

Here, we describe performance-tuning strategies employed to improve four highlighted receptor platforms and propose general lessons for receptor engineering.

Receptors that activate T cell signaling. The most widely improved synthetic receptor is the CAR, which is used in cell-based cancer immunotherapies to link recognition of cancer antigens to T cell signaling[3] (Fig. 5a). CAR designs have evolved to improve both treatment efficacy and safety[4]. Modifications that increase signaling potency and cell persistence by adding costimulatory domains are present in US Food and Drug Administration–approved second- and third-generation CARs[4]. For treating solid tumors, clinical evidence of on-target/off-tumor toxicities motivated the need for tuning CAR signaling potency. Useful adjustments and substitutions have targeted the extracellular hinge, transmembrane and intracellular juxtamembrane domains[51,52]. Strategies to engineer transmembrane domains to control receptor oligomerization have increased cytotoxic potency without also increasing dangerous cytokine release[53]. Tonic signaling and basal T cell activation (background signaling) can be adjusted by adding torsional intracellular linkers between the transmembrane and signaling domains[52]. CAR safety can be improved by integrating multiple target inputs using molecular logic to increase specificity. For example, the iCAR employs a second receptor chain with inhibitory capacity to restrict activating functions when in contact with an off-target cell[54]. The signaling components required for T cell activation have also been split across two different CARs to create combinatorial CAR systems that require both inputs for potent signaling[55,56]. Other approaches involve designs requiring that a secondary user-provided signal (for example, a small molecule) be administered to enable CAR expression or functionality[57–60]. Similarly, CAR expression can be controlled by light, temperature and ultrasound-responsive promoters[61–64]. CAR designs in which ligand binding is mediated by a separate protein enable one to adjust sensitivity or specificity over the course of treatment. These 'switchable' CARs bind to a common (physiologically inert) adapter molecule that is fused or conjugated to the target-binding domain[65], with assembly driven by small molecule–regulated protein heterodimerization[58], noncovalent[66–72] or covalent interactions[73]. Split adapter CARs recently achieved highly specific cancer cell detection using many types of logic[59]. These examples illustrate diverse strategies for modulating CAR-based sensing and signaling.

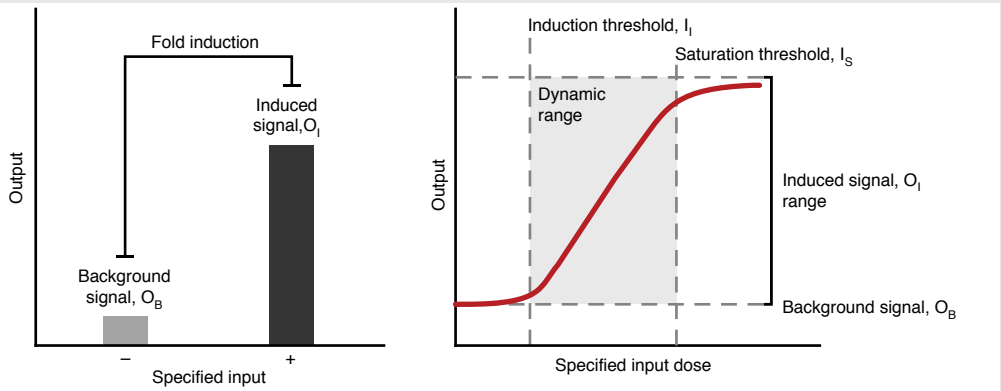
Other efforts have expanded CAR functionality. Bispecific CARs with two scFv domains in tandem help prevent treatment failure due to antigen escape[48,49]. Some CARs can sense soluble proteins[74]. Chimeric receptors that contain full TCR intracellular signaling domains (T cell receptor fusion constructs and synthetic T cell receptor and antigen receptors) tune immunomodulatory potency[75,76]. Chimeric receptors that combine components of cytokine receptors and TCRs can be employed in nonimmune cells to drive JAK-STAT signaling in response to target engagement[5]. The proliferation of CAR-family receptors evidences the rapidity with which receptors are evolving to meet clinical needs.

Receptors that signal via phosphorylation-regulated proteolysis. Engineered receptors that repurpose natural phosphorylation-regulated signaling to generate customized output have also been systematically improved (Fig. 5b). Tango tethers a synthetic TF to a truncated GPCR or RTK and separately tethers a tobacco etch virus protease (TEVp) to a phosphorylated residue-binding domain[14]. When the receptor binds the target ligand, the TEVp fusion protein is recruited to the phosphorylated receptor, mediating cleavage and TF release. A modified version of this strategy involving split TEVp was also constructed to improve sensitivity and increase fold induction[77]. To enhance the magnitude of ligand-induced signaling relative to background, the ChaCha receptor system modifies the Tango configuration by tethering the TEVp to the receptor and fusing the TF to the phosphorylated residue-binding protein such that it is excluded from the nucleus until cleavage occurs, reducing background[16]. Each ChaCha receptor can liberate more than one TF molecule, potentially contributing to the reported enhancement in fold induction compared with Tango. ChaCha development employed investigations elucidating the biophysical links between design choices and these properties. A modified version of this strategy—rewiring of aberrant signaling to effector release (RASER)—was recently developed to distinguish healthy versus oncogenic RTK signaling by integrating TF release events over time[17]. RASER was tuned to produce substantial output only when signaling is sustained over time, which is a signature of pathology in the application of interest. To enable more sophisticated

Box 1 | Performance metrics and characteristics for describing engineered receptor systems

The performance of engineered receptor systems has been historically evaluated using a variety of both quantitative and qualitative performance criteria. Here, we summarize commonly used performance metrics and characteristics to contextualize and highlight the utility of and information afforded by each. Quantification of unprocessed or primary metrics is the most standard reporting strategy. For example, signaling output can be quantified via fluorescence measurements of reporter activation using flow cytometry, or in various other ways, depending on the system. From primary metrics, lumped metrics can be derived to characterize higher order aspects of overall system performance. These metrics can vary widely in definition across studies for biosensing technologies with different constructions. Lumped metrics can be informative because they often describe ratios of signals—normalizing for effects that are not of central interest; however, interpretation requires comparison with primary metrics. For example, a system may exhibit a high fold induction even for a small ligand-induced level of output simply because the background signal is infinitesimally small. To facilitate the adoption and comparison of engineered receptor systems across labs and disciplines, reporting of lumped metrics should always be accompanied by primary metrics.

Beyond quantitative metrics, a characteristic that is less easily standardized but important for describing engineered receptor systems is robustness—the degree to which performance metrics remain constant despite variations in other system properties (that is, perturbations). Common perturbations include change of cell type (primary versus immortalized, tissue of origin, human versus other mammal derivation, and so on), variation in genetic context (transient transfection versus stable integration, random versus site-specific integration, single versus multicopy integration), variation in stoichiometry of system components, and variation in cell resource availability[97,98]. Robustness in the context of biological systems has been previously reviewed[99], and application of these concepts to receptor systems is of increasing importance as these technologies move into clinical products.



Primary metrics	
Background signal, O_B:	output signal in the absence of target input, sometimes referred to as leakiness or basal signal.
Induced signal, O_I:	output signal induced by the action of target input, such as a ligand, cell state change, light, heating or cooling.
Induction threshold, I_I:	target input threshold above which output signal is measurable above background signal, also known as sensitivity.
Saturation threshold, I_S:	target input threshold above which output signal no longer changes with increases in input.
Dynamic range, [I_I, I_S]:	range of input doses over which output changes with a change in input dose.

Lumped metrics
Fold induction, O_I/O_B: ratio of induced signal to background signal, also known as fold difference.
Specificity: percentage of output signal that is uniquely induced by the target input.
Efficiency: percentage of the target input that contributes to output signal.

information processing, the phosphorylation readout approach has been extended to sense multiple inputs and regulate output using multiple defined protein–protein interactions (that is, to implement multiple input–single output processing). In the iTango and Cal-Light systems[18,19], the two components of split TEVp are recruited separately to a GPCR containing a proteolytically cleavable TF via phosphorylation-mediated, blue light–mediated or calcium signaling–mediated interactions. Redirection of phosphorylation-based signaling can theoretically be applied to any receptor system (engineered or native) for which phosphorylation sites and protein–protein interactions are well-defined.

Receptors that signal via dimerization-based proteolysis. A third class of synthetic receptors that has been systematically improved are those that initiate engineered actuation pathways after dimerization, which causes proteolysis and release of a tethered TF. MESA was engineered to facilitate detection of soluble cues (Fig. 5c). The original MESA design comprises two chains that tether a TF at the membrane and proteolytically release this TF after extracellular ligand-binding-induced receptor dimerization[23]. Initial implementations of MESA showed ligand-inducibility, but also suffered from undesirable background signaling that resulted from transient chain collisions[24]. To reduce background signal, an alternative

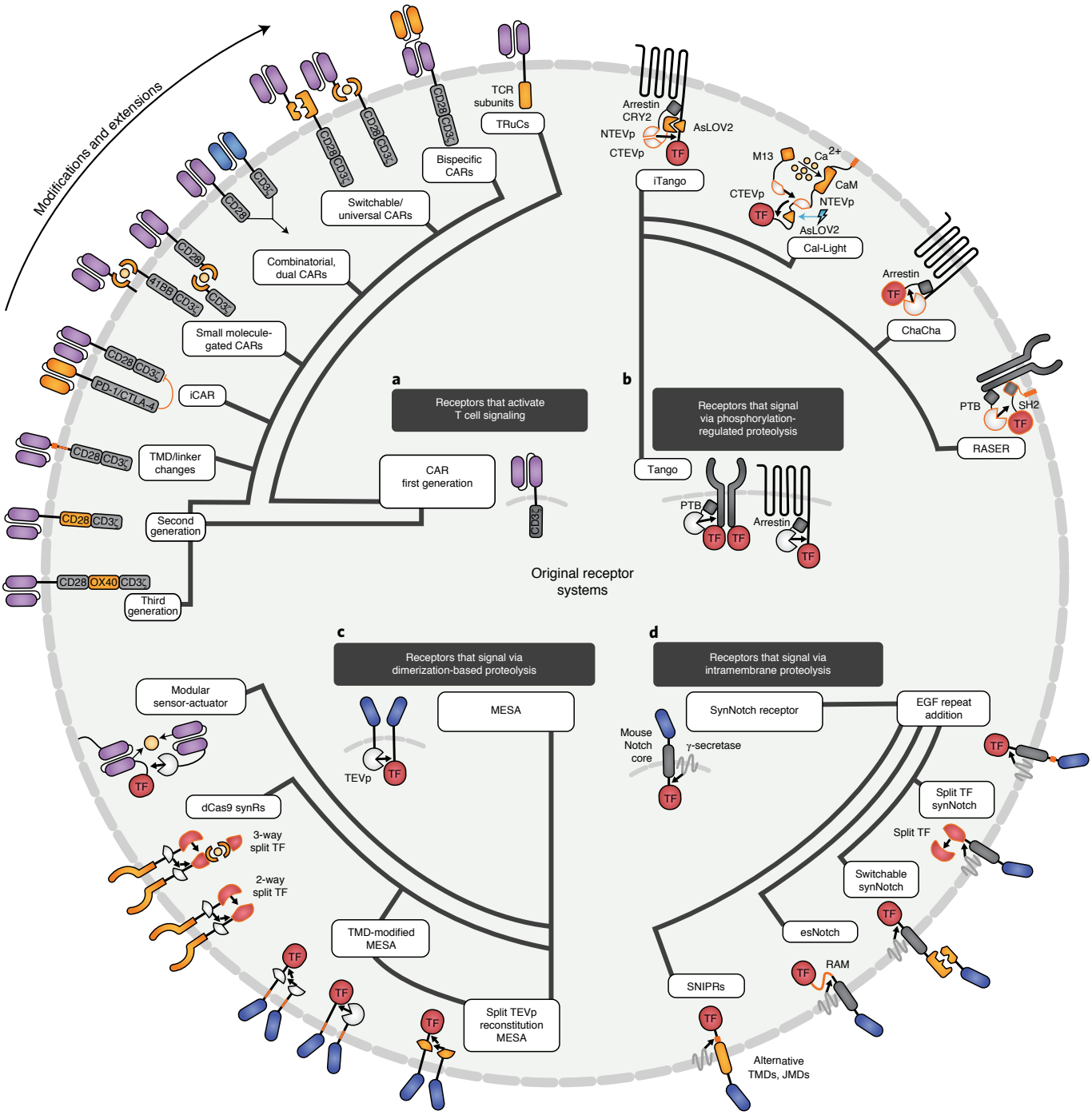


Fig. 5 | The evolutionary history of synthetic receptor systems. a–d, Improvements, modifications and extensions of four seasoned receptor systems have led to the creation of new systems with additional functionalities and altered performance metrics. **a**, Receptors that activate T cell signaling. **b**, Receptors that signal via phosphorylation-regulated proteolysis. **c**, Receptors that signal via dimerization-based proteolysis. **d**, Receptors that signal via intramembrane proteolysis. Original systems are shown in the center of the circle and descendants are shown on the outside of the circle, connected to each other via a phylogenetic tree. In some cases, singular changes were made to one system to produce a new system, whereas in other cases, simply the concept or signaling mechanism from one system was extended to a new system. Specific improvements or changes are highlighted in orange. AsLov2, *Avena sativa* light-oxygen-voltage 2 domain; CaM, calmodulin; CRY2, cryptochrome 2; CTEVp, C-terminal component of split tobacco etch virus protease; EGF, epidermal growth factor; gen, generation; esNotch, enhanced synNotch; NTEVp, N-terminal component of split tobacco etch virus protease; PTB, phosphotyrosine-binding domain; SH2, Src homology 2 domain; TMD, transmembrane domain; JMD, juxtamembrane domain; TRuCs, T cell receptor fusion constructs.

mechanism was developed in which receptor dimerization first initiates split TEVp reconstitution before cleavage, and split TEVp reconstitution propensity was tuned using a computation-guided workflow to achieve both low background and high fold induction[25]. In a separate investigation, the contribution of MESA design choices to receptor performance was systematically explored, identifying particularly important features, including transmembrane domain sequence[78]. This receptor system now

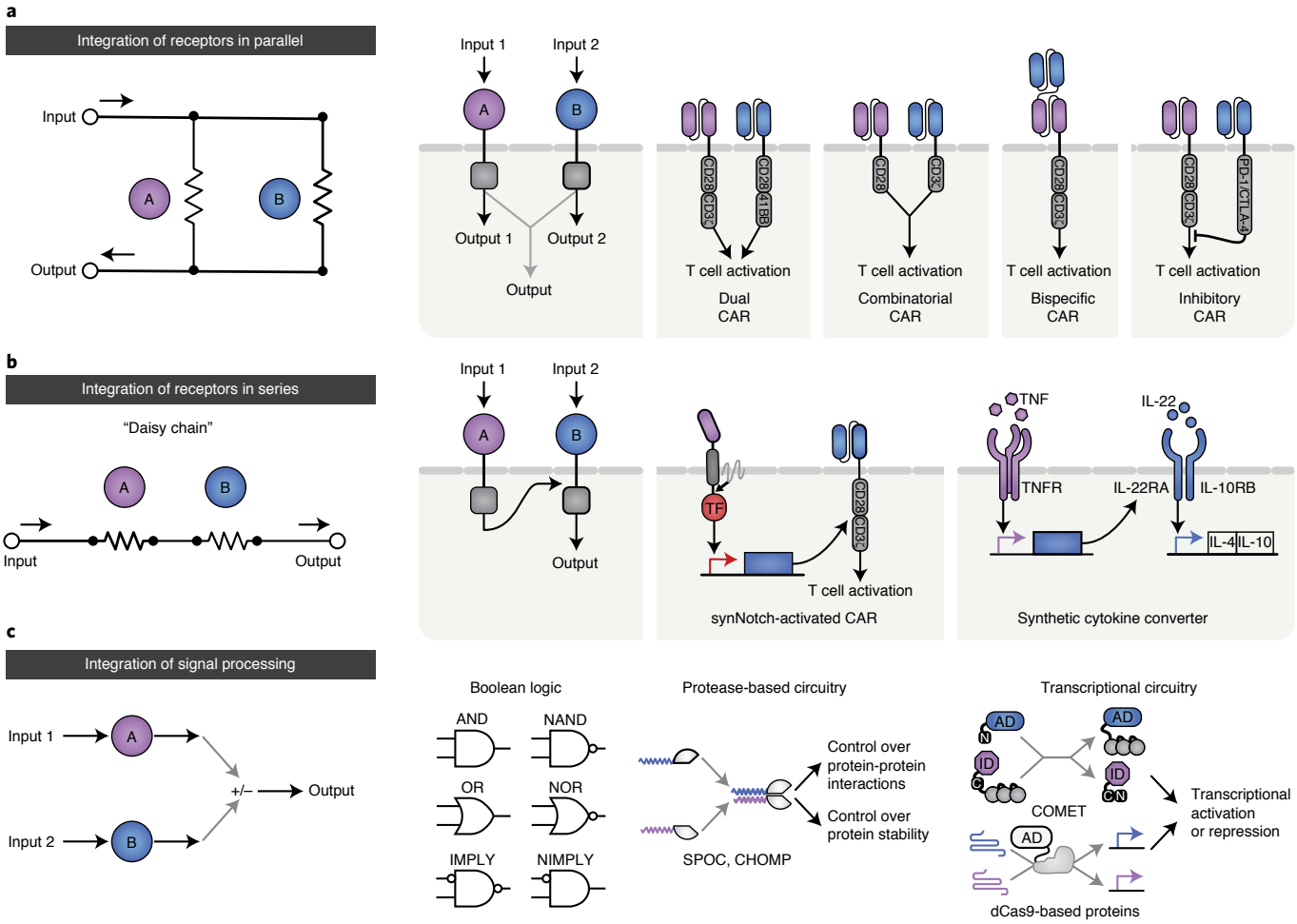


Fig. 6 | Strategies for integration of synthetic receptor systems. a,**b**, Multiple synthetic receptors can be integrated together in parallel[48,49,54–56,86] (**a**) or in series[50,79,92] (**b**) configurations to facilitate detection of multiple inputs. General topologies are shown (left) alongside demonstrated examples (right). **c**, Downstream modules consisting of synthetic biology technologies can be used to integrate signaling from multiple receptors when their expression is driven by receptor actuation (left). Protease-based circuitry[34,95] and transcriptional circuitry[28,38–40,46] represent two recently characterized approaches to perform sophisticated Boolean logic functions (right). AD, activation domain; C, gp41-1 C-terminal intein fragment; ID, inhibitory domain; IL-4, interleukin 4; IL-10, interleukin 10; IL-22, interleukin 22; IL-10RB, interleukin 10 receptor subunit beta; IL-22RA, interleukin 22 receptor subunit alpha; N, gp41-1 N-terminal intein fragment; SPOC, split-protease cleavable orthogonal CC; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor.

enables sensing target ligands via small molecule–binding domains, scFvs and nanobodies. A mechanistically similar panel of dCas9 synthetic receptors, called 'dCas9-synRs', signal using a dimerization-induced proteolysis mechanism and employ natural receptor-derived ligand-binding domains[26]. Another related system enables detection of intracellular soluble ligands[27]. Together, these explorations identify biophysical design principles that guide both enhancement of specific performance characteristics and adaptation to novel applications.

Receptors that signal via intramembrane proteolysis. As described above, synNotch receptors were originally developed based on natural Notch receptor mechanisms[20,21], employing regulated intramembrane proteolysis to connect contact-dependent sensing to custom transcriptional output (Fig. 5d). Since its original construction, synNotch has been extended to detect clinically relevant target antigens through the substitution of scFv, nanobody, de novo–designed and natural receptor-binding domains[21,79–81]. SynNotch receptors have been designed to actuate via transcriptional output[20,21,79,82] and CRISPR–Cas-mediated genome editing[83]. Recent efforts have also focused on tuning synNotch receptor signaling potency. For example, the fold induction was altered by adding[20] or deleting[83] one or more extracellular epidermal growth factor repeats in the Notch core regulatory domain. To overcome challenges with high background signaling, the synNotch intracellular juxtamembrane domain was altered to include an additional short hydrophobic sequence present in native Notch receptors, leading to the enhanced synNotch system[84]. Interestingly, this approach reduced background at the expense of antigen-induced signaling, without much change to the fold induction. To enable sensing beyond cell- or surface-tethered ligands, a recent study developed anchor cells that bind to and display soluble ligands to initiate synNotch signaling, thereby enabling the generation of long-range, morphogenetic-like patterning[85]. Several new sensing and actuation functionalities, inspired by improvements to CARs, have been incorporated in synNotch receptors to confer advanced cancer cell recognition. New sensing functionalities include detecting intracellular antigens displayed on cancer cell surfaces via presentation by major histocompatibility complexes, the ability to detect more than one target antigen using tandem scFv domains and, like CARs, the ability to sense via 'switchable' ligand recognition domains[50,73]. New actuation programs include rapid initiation of apoptosis of the engineered cell

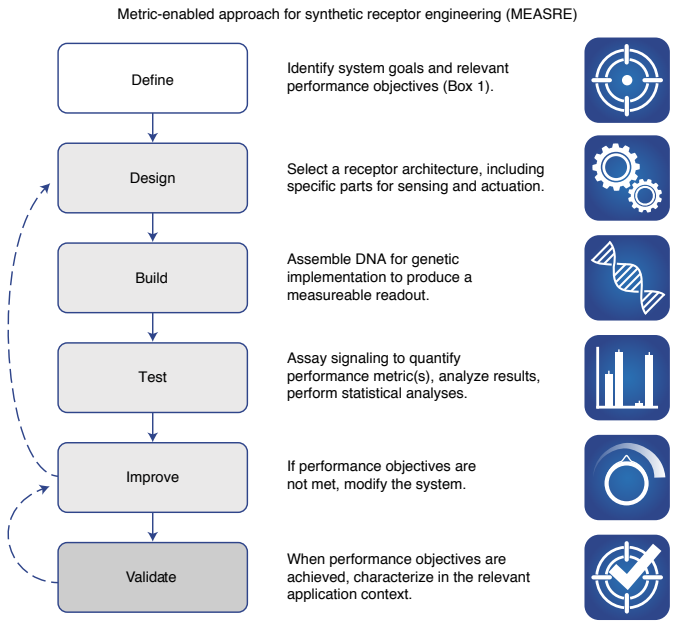


Fig. 7 | Metric-enabled approach for synthetic receptor engineering. This framework combines the design–build–test–learn cycle and Six Sigma improvement strategy to construct synthetic receptors, emphasizing measurement of performance metrics as important for optimization[96]. 'Define' involves specifying design objective(s) for the engineered cell and required ranges for performance metrics—quantifiable attributes of synthetic receptor performance (Box 1). For cell-based therapies, performance metrics can be equated to critical quality attributes, which are physical, chemical and biological properties of a therapeutic molecule that are evaluated to ensure comparability, quality and safety of the product. For example, if a synthetic receptor system is intended to drive production of a cytotoxic drug, background signal (or ligand-independent activation) should remain below a threshold to minimize off-target cytotoxicity. 'Design' focuses on the selection and construction of receptors to perform sensing and actuation. Descriptive and predictive mathematical models can help avoid infeasible implementations and identify potentially high-performing implementations before experimentation. Beyond selection of a receptor architecture, choice of DNA delivery method, sensitivity of its performance to intercellular heterogeneity, and assay development are critical components of this step. 'Build' involves assembly of a cohesive, working prototype in vitro. This includes codon optimization of protein-coding sequence(s), assembly into vectors for the chosen delivery method and selection of promoters appropriate for the cell of interest. 'Test' evaluates performance metrics and compares these against overall design objectives. Assays based on techniques with single-cell resolution (for example, flow cytometry) provide more useful information than do with bulk population-averaged measurements. 'Improve' identifies and tests design variations to overcome performance limitations. Such improvement strategies may include minor adjustments such as swapping a domain for an alternative with similar properties, or varying protein sequences to change biophysical properties. If fine-tuning is insufficient, MEASRE cycles back to 'Design' to revisit the overall receptor architecture and mechanism. Finally, 'Validate' tests whether a design that meets performance objectives when implemented in a model testbed (for example, a cell line tested in vitro) performs as required in a translationally relevant context (for example, engineered primary cells tested in an animal model). Failure at this step could inform the definition of improved performance metrics (and/or design objectives), guiding a return to the 'Improve' step.

as an inducible safety feature[50]. As described above, SNIPRs were developed by systematically investigating design choices in the synNotch receptor architecture[22]. Substitution of transmembrane and juxtamembrane sequences and the addition of extracellular regulatory elements led to performance improvements, including both background reduction and enhanced target-induced signal. Notably, many high-performing SNIPRs employ human receptor domains and humanized TFs, generating receptors that are less likely to elicit immune rejection, which could facilitate clinical translation[47]. This expanding class of receptors enables sophisticated cellular programming for diverse applications.

Integration of multiple synthetic receptors

An attractive emerging strategy is integrating distinct receptor systems into a single program that evaluates multiple environmental inputs. Here, we survey several general approaches and discuss associated capabilities, selection of receptors and methods for signal processing. We illustrate each case using the example goal of improving targeting specificity in CAR T cell therapy to overcome on-target/off-tumor adverse effects, such as B cell aplasia after treatment of B cell malignancies. Integrated receptor systems have proven useful for this purpose, with receptors programmed to function in either 'parallel' or 'series' configurations.

In the parallel configuration, two or more distinct synthetic receptors are coexpressed such that the output depends on simultaneous engagement and signaling (Fig. 6a). This type of approach often requires 'level matching' such that output from each receptor is balanced with promoter response characteristics[44]. The parallel configuration requires integration at the level of actuation, downstream signaling or final outputs. This configuration is well suited to applications in which a response to the simultaneous detection of two inputs is desired. In one example, a dual targeting mechanism was devised whereby a low-affinity, weak signaling CAR sensed one antigen, and a chimeric costimulatory receptor sensed a second antigen, such that both inputs were needed to induce T cell activation (dual CAR)[55]. Similarly, two CARs directed against different tumor antigens and coexpressed in the same cell can provide complementary costimulatory signals (combinatorial CAR)[56,86]. Additional examples are shown and described in Fig. 6a. Signaling via multiple receptors can also be distributed across separate populations of cells[59,87]. Beyond CARs, multiple synNotch receptors and multiple MESA receptors have also been integrated in the parallel configuration to implement cellular logic regulated by sensing two surface-bound ligands or two soluble ligands, respectively[20,44].

In the series configuration, one input activates a first synthetic receptor, inducing expression of a second receptor, which then transduces sensing of a second input into output (Fig. 6b). This configuration is sometimes referred to as a 'daisy chain', a term used in electrical engineering to describe devices wired in series. In a variation on this theme, the upstream receptor can regulate the activity of the downstream receptor. One example of this strategy is combinatorial antigen recognition with synNotch-regulated expression of a CAR[50,79,88–90]. This approach was systematically tuned by modulating both synNotch and CAR properties to design an ultrasensitive relationship between T cell activation and target antigen density for improved discrimination between healthy and cancerous cells[91]. More sophisticated integration topologies in series have also been implemented with synNotch receptors and CARs, including a series of two synNotch receptors that control expression of a CAR to create three-input AND and NAND gates[50]. Beyond CAR T cell therapies, the series integration configuration was also applied to natural receptors for the detection and mitigation of inflammation in a synthetic cytokine converter system[92]. The series topology can utilize synthetic TF–driven expression (for example, when synNotch is upstream) or engineered promoters that are responsive to endogenous signaling mediators (for example, in the synthetic cytokine converter).

A separate, underexplored option for creating more complex programs lies in the integration of different receptor systems at the

level of downstream outputs (Fig. 6c). This type of approach, typically involving engineered interactions between receptor outputs, will be valuable for integrating receptors that signal via different types of actuation. For example, split inteins[93] and engineered protein–protein interaction domains[20,94] can reconstitute split TFs and proteases that could be activated as outputs from different receptor systems. COMET transcriptional activators and inhibitors were recently modified to incorporate split inteins wherein splicing converts regulator function to yield high-performing logic gates[46]. The split-protease cleavable orthogonal CC (SPOC)-based and circuits of hacked orthogonal modular proteases (CHOMP) toolkits employ reconstitution of split viral proteases using protein–protein interactions and could similarly be used downstream of receptor outputs[34,95]. The integration of multiple receptor outputs is common in nature and may be useful for studying processes such as development[12]. In general, integration at the level of receptor outputs may enable new combinations of synthetic receptor types.

Challenges, opportunities and future outlook

Because mammalian cell products using synthetic receptors are applied as research tools, therapeutics and diagnostics, ensuring reliability and repeatability will become increasingly important. The development of many different types of receptors (Figs. 2 and 4) and strategies for rationally tuning receptor functions (Fig. 5) are key advances that support this overall goal. One challenge for broad application and translation of synthetic receptors has been a lack of quantitative understanding and standardization in evaluating and communicating receptor system performance. These gaps make it difficult to compare receptor systems, to transfer technologies across groups and disciplines and to select appropriate elements to combine to implement desired cell-based programs. To help address this need, we propose a set of basic quantitative performance metrics and characterizations for evaluating and describing synthetic receptors (Box 1). We posit that describing existent and next-generation receptors with these metrics will enable more efficient communication of findings, exchange of technologies and growth of the field.

The proposed metrics could form the backbone of a general approach for describing and evaluating synthetic receptor performance to help bioengineers select and build systems that meet application-specific requirements. We suggest one such generalized, systematic strategy to guide optimization of receptor design by first defining performance metrics and characteristics that may be required for a specific application and then experimentally evaluating these metrics. We call this 'methodology a metric-enabled approach for synthetic receptor engineering' (MEASRE), and it comprises six steps: define, design, build, test, improve and validate (Fig. 7). MEASRE is based on the classical synthetic biology 'design–build–test–learn' cycle with an added quality-control component adapted from the Six Sigma methodology—a data-driven, problem-solving approach guided by statistical precision that is widely used in industry, business and healthcare. Six Sigma optimization generally ensures that process development meets user-defined quality standards consistently by reducing variance outside an allowable performance range to the smallest possible frequency of occurrence[96]. Inspired by these goals, the 'define' step of MEASRE requires identification of a performance metric, also known as a 'critical quality attribute', and an allowable performance distribution that is informed by the natural biological variation of cells. The 'test' step quantitatively assesses these metrics, and the 'validate' step considers reproducibility and translatability across relevant cellular contexts and methods of genetic implementation. For example, the performance of an engineered cell-based product should consider cell-to-cell variability (within an engineered cell population) and donor-to-donor variability (for autologous or allogeneic products). Eventually, it may also be possible to evaluate recipient-specific factors that affect performance, such as the potential for undesired immune responses to engineered components, which are important but lack a framework for predictive analysis. This emphasis on quantitative description, evaluation and consistency of engineered receptor performance vis-à-vis goal-driven design may accelerate translation of engineered mammalian cell therapies that employ synthetic receptors.

An example of a specific opportunity in synthetic receptor engineering is integrating receptors and other synthetic biology technologies to design more sophisticated cell-based programs (Fig. 6). We highlight the potential for employing downstream signal processing to facilitate integration of synthetic receptors that signal through different mechanisms, and for tuning system performance (Fig. 3). As system complexity increases, structured approaches such as those proposed here will be of increasing importance and utility.

Synthetic receptor development has evolved substantially from modest modifications of natural proteins to design-driven construction, refinement and integration of modular technologies that increasingly enable true engineering of customized cellular functions. Notably, key choices in the iterative refinement of CARs were uniquely informed by evaluations in their final application context (in this case, in clinical trials[4]). Evaluating other synthetic receptor systems in application-specific contexts may prove similarly useful for guiding both the development and use of these technologies. These advances reflect a qualitative shift in the broader field of mammalian synthetic biology, wherein progress is increasingly guided by rational exploration and knowledge building, as opposed to trial and error, toward new applications of greatest clinical, scientific and societal benefit.

Received: 27 March 2021; Accepted: 18 October 2021;
Published online: 20 January 2022

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Acknowledgements

We thank M. Johnson for her critical review of this manuscript and valuable feedback. This work was supported in part by the Indo-US Science & Technology Forum (IUSSTF) and the Department of Science & Technology (DST), Government of India (J.M.); the National Science Foundation Graduate Research Fellowship Program (DGE-1842165) (H.I.E.); the National Institute of Biomedical Imaging and Bioengineering of the National Institutes of Health (NIH) under Award Number 1R01EB026510 (J.N.L.); the National Institute of Biomedical Imaging and Bioengineering of the NIH under Award Number EB021030-03, the National Institute of General Medicine of the NIH award number R35 GM138256 and the National Science Foundation award number CBET-2034495 RECODE (L.M.).

Competing interests

L.M. is an inventor of synNotch patent US9670281B2 and receives royalty payments from licensing to Gilead Inc. through UCSF; the other authors declare no competing interests.

Additional information

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Peer review information *Nature Chemical Biology* thanks the anonymous reviewers for their contribution to the peer review of this work.