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Engineering synthetic signaling proteins with ultrasensitive input/output control

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Many signaling proteins are built from simple, modular components, yet display highly complex signal-processing behavior. Here we explore how modular domains can be used to build an ultrasensitive switch—a nonlinear input/output function that is central to many complex biological behaviors. By systematically altering the number and affinity of modular autoinhibitory interactions, we show that we can predictably convert a simple linear signaling protein into an ultrasensitive switch.

Many eukaryotic signaling proteins are regulated by a modular allosteric mechanism: a catalytic 'output' domain—which in isolation is constitutively active—is repressed by modular 'input' domains that participate in steric/conformational autoinhibitory interactions[1,2]. Inputs that disrupt these interactions function as activators of the catalytic activity. This modular framework is hypothesized to facilitate the evolution of new input/output functions by simple recombination events and, similarly, may allow engineering of signaling functions. In support of this model, regulatory and catalytic domains have been swapped to yield synthetic switches that respond to novel inputs[3]. Such switches have been used to reprogram the behavior of living cells[4].

Although simple synthetic switch functions can be engineered, many natural signaling proteins display far more complex behavior. Most native proteins do not simply respond to a single input in a linear (Michaelian) fashion, but often perform more complex processing, such as integration of multiple inputs or conversion of a linear input into a nonlinear, ultrasensitive output[5–7]. Here we explore computationally and experimentally how a modular domain framework can be used to build ultrasensitive signaling switches.

Ultrasensitive switches are an important engineering target because they are critical components in higher-order regulatory systems that produce amplification, oscillation and toggling behavior[8,9]. These switches approximate digital behavior, providing an input detection threshold at which small changes in input concentration lead to large changes in output behavior.

In principle, an ultrasensitive switch could be built using multiple identical modular autoinhibitory domains that function in a cooperative manner (**Fig. 1**). Whereas a single autoinhibitory domain would give linear signaling, multiple autoinhibitory domains might yield cooperative input binding where binding of one input ligand energetically increases the favorability of binding subsequent ligands. This proposed mechanism is analogous to that used by classical cooperative switches such as hemoglobin[10,11].

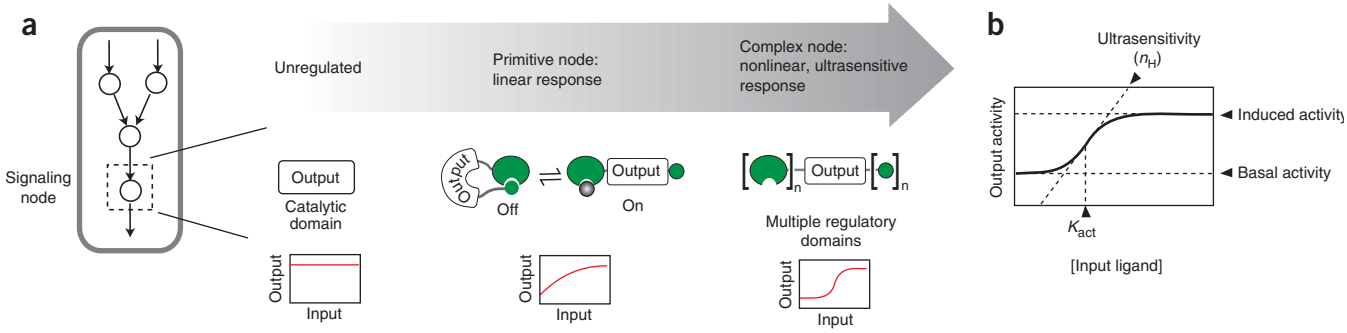

Figure 1 Potential steps in the evolution or engineering of an ultrasensitive signaling node. (**a**) Many signaling node proteins are regulated by modular allostery: they have a core catalytic domain (e.g., kinase, phosphatase) that exhibits constitutive activity, and modular autoinhibitory interaction domains that are used to gate function in an input-dependent manner. Complex behavior, such as ultrasensitivity may arise when multiple autoinhibitory domains are used to regulate one output domain. (**b**) Key behavioral parameters for synthetic signaling switches include: basal activity (with no input), maximal activity (saturating input), K_{act} (concentration of input for half-maximal activation) and ultrasensitivity (Hill coefficient, n_H).

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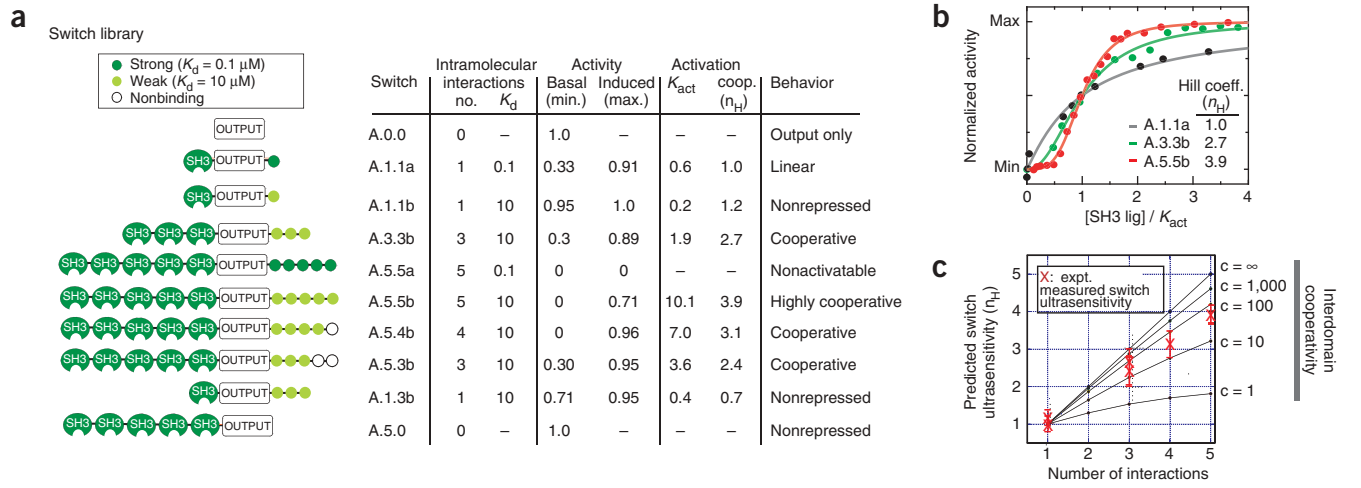

Switch	Intramolecular interactions no.	Intramolecular interactions K_d	Activity Basal (min.)	Activity Induced (max.)	Activation K_{act}	Activation coop. (n_H)	Behavior
A.0.0	0	–	1.0	–	–	–	Output only
A.1.1a	1	0.1	0.33	0.91	0.6	1.0	Linear
A.1.1b	1	10	0.95	1.0	0.2	1.2	Nonrepressed
A.3.3b	3	10	0.3	0.89	1.9	2.7	Cooperative
A.5.5a	5	0.1	0	0	–	–	Nonactivatable
A.5.5b	5	10	0	0.71	10.1	3.9	Highly cooperative
A.5.4b	4	10	0	0.96	7.0	3.1	Cooperative
A.5.3b	3	10	0.30	0.95	3.6	2.4	Cooperative
A.1.3b	1	10	0.71	0.95	0.4	0.7	Nonrepressed
A.5.0	0	–	1.0	–	–	–	Nonrepressed

Figure 2 Design and synthesis of a modular ultrasensitive switch built from the N-WASP output domain and multiple SH3 modules. (**a**) Synthetic switches designed in this study and summary of their behavioral parameters with respect to activation by external SH3 ligand (SH3 ligand = peptide YEVPPPALPPKRRR). (See **Supplementary Fig. 1** online for more details about actin polymerization activity assays and **Supplementary Fig. 2** and **Supplementary Table 1** online about switch construction.). Intramolecular SH3 ligands are color-coded for by affinity (for isolated domain-peptide interaction). (**b**) Comparisons of input/output functions for switches A.1.1a, A.3.3b and A.5.5b. To compare ultrasensitivity, each switch's relative activity was plotted as a function of the concentration of input ligand normalized by K_{act}. Observed ultrasensitivity scales with the number of autoinhibitory interactions. (**c**) Computationally predicted switch ultrasensitivity (see **Supplementary Methods** online) as a function of number of interaction domains and interdomain cooperativity 'c'. The plotted lines were derived assuming each interaction within a given switch has the same 'c' value (that is, c1 = c2 = c3, and so forth). Apparent ultrasensitivities (Hill coefficient, n_H) experimentally measured for actual designed switches (from **Fig. 2a**) are shown in red. Error bars show 1 s.d. from an average of at least three separate experiments.

We simulated the behavior of a series of multivalent domain switches using a simple equilibrium model, where individual states were assumed to be fully repressed in the presence of any intramolecular interactions and fully active only in the absence of all intramolecular interactions (**Supplementary Methods** online). Thus switch activity could be described as a function of how the external input ligand concentration alters the population distribution of active versus inactive states. Using this model we could explore the effect of autoinhibitory interaction number and affinity. The model also incorporates several other parameters: an effective concentration term 'C_{eff}' (the energetic effect of having both the autoregulatory domains and ligands in the same molecule) and an interdomain cooperativity term 'c' (how formation of one intramolecular autoinhibitory interaction increases the affinity of a neighboring autoinhibitory interaction). We determined how all of these microscopic energetic terms would alter the behavioral parameters of the system-input ultrasensitivity (measured by the apparent Hill coefficient, n_H), input concentration required for half-maximal activation (K_{act}) and degree of autorepression (basal activity) (**Fig. 1b**).

This model makes several simple predictions. First, ultrasensitivity is fairly independent of the affinity of the individual autoinhibitory interactions; changes in individual affinities largely affect the K_{act} but not the overall shape of the input/output transfer function. Ultrasensitivity is, however, dramatically altered by the number of autoinhibitory interactions (**Fig. 2c**). Interdomain cooperativity also contributes to ultrasensitivity. Nonetheless, even with no interdomain cooperativity (c = 1), increasing the number of interactions leads to modest increases in ultrasensitivity. Moreover, at sufficiently high interdomain cooperativity, further increases in cooperativity have little effect on ultrasensitivity—the maximal Hill coefficient is determined solely by interaction number. Nonetheless, the model also predicts key functional tradeoffs—as the number of autoinhibitory modules increases, ultrasensitivity is increased, but it also can make the switch extremely difficult to activate (nonactivatable) unless individual domain affinities are simultaneously decreased.

To experimentally test these predictions, we constructed a series of synthetic switches, using the protein N-WASP as a test bed. N-WASP has a catalytic output domain that constitutively binds and activates the Arp (actin-related protein) 2/3 complex, stimulating its actin-nucleation activity[12]. In previous studies, we showed that by combining the output domain of N-WASP with a heterologous intramolecular domain-peptide pair, we could generate a synthetic switch in which the activity of N-WASP was gated by a novel peptide input, in a simple linear fashion[3].

Here, we constructed synthetic switches in which the N-WASP output domain was combined with one to five SH3 interaction modules (**Fig. 2**, **Supplementary Figs. 1**, **2** and **Supplementary Table 1** online). The individual modules were linked by glycine-serine linkers. As predicted, a single SH3 module yielded a switch that was activated in a linear fashion by exogenous SH3-binding peptide. Increasing domain number increased ultrasensitivity (**Fig. 2b**). At the extreme, a construct with five SH3 autoinhibitory modules (switch A.5.5b) was completely repressed under basal conditions, but was activated with an apparent Hill coefficient of 3.9 (**Supplementary Fig. 3** online). Gel filtration studies are consistent with this protein adopting a monomeric state under both basal and activating conditions (E.A.M, unpublished data).

The set of constructs examined here show that it is possible to build a highly nonlinear switch using simple autoinhibitory components. As predicted, the apparent Hill coefficient scaled well with the number of autoinhibitory interactions, and fit to an apparent interdomain cooperativity 'c' approaching 100 (**Fig. 2c**). Mutational disruption of interactions led to a decrease in the Hill coefficient (**Fig. 2a** and **Supplementary Fig. 4** online). In addition, the experimental

behaviors confirmed the predicted interplay of domain number and affinity in tuning, not only ultrasensitivity, but also other key behavioral parameters, such as the threshold of activation and basal activity (**Fig. 2a**). Interactions that were too weak led to high background basal activity (poor repression; e.g., switch A.1.1b), whereas interactions that were too strong led to switches that were nonactivatable within the input concentration range examined here (e.g., switch A.5.5a).

Overall, these switches showed a high degree of systematic, predictable structure/function behaviors, indicating that it is possible to build synthetic signaling switches with targeted complex processing behaviors. We have also built switches with other complex behaviors, such as three-input signal integration (**Supplementary Fig. 5** online). The behavior of these modular synthetic switches is highly scalable: higher-order input control can be built through incremental steps that maintain the core regulatory behavior of their simpler predecessors. This scalability may explain how complex natural switches arose through a gradual evolutionary process.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

J.E.D. designed and conducted experiments depicted in this paper as well as constructing/purifying many of the synthetic switches described; prepared manuscript with W.A.L. E.A.M. constructed/purified many of the switches described, conducted experiments, and created model for switch parameter prediction; edited manuscript. W.A.L. made a major contribution to the conception of the general design of ultrasensitive switches and played a general mentorship role; prepared manuscript with J.E.D.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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