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Reprogramming Control of an Allosteric Signaling Switch Through Modular Recombination

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Many eukaryotic signaling proteins are composed of simple modular binding domains, yet they can display sophisticated behaviors such as allosteric gating and multi-input signal integration, properties essential for complex cellular circuits. To understand how such behavior can emerge from combinations of simple domains, we engineered variants of the actin regulatory protein N-WASP (neuronal Wiskott-Aldrich syndrome protein) in which the "output" domain of N-WASP was recombined with heterologous autoinhibitory "input" domains. Synthetic switch proteins were created with diverse gating behaviors in response to nonphysiological inputs. Thus, this type of modular framework can facilitate the evolution or engineering of cellular signaling circuits.

Cellular behavior is mediated by circuits of interconnected signal transduction proteins. Many of these proteins are allosteric—their catalytic output activity is gated by specific upstream inputs such as ligand binding or covalent modification. Most eukaryotic signaling proteins are composed of modular domains with binding or catalytic functions (1, 2). It has been proposed that domain recombination could facilitate the evolution of proteins with novel signaling functions (1–4).

Consistent with such a model, complex allosteric gating in some signaling switches is mediated by modular, autoinhibitory interactions (4, 5). For example, the actin regulatory switch N-WASP (6, 7) (Fig. 1A), which displays sophisticated signal integration, con-

tains an output region ("VCA" domain) that in isolation is constitutively active—it stimulates actin polymerization by binding and activating the actin-related protein (Arp) 2/3 complex. However, two modular domains, a highly basic (B) motif and a guanosine 5'-triphosphatase (GTPase)-binding domain (GBD) repress activity through autoinhibitory interactions (8, 9). Two activating stimuli, the phosphoinositide PIP₂ and the activated GTPase Cdc42, bind the B and GBD motifs, respectively, and disrupt autoinhibition (9, 10). Because the two inputs act cooperatively, N-WASP approximates an AND gate in which strong activation is only observed in the presence of both inputs (9, 11). Such multi-input regulation is thought to yield precise spatial and temporal control over actin polymerization.

We explored the flexibility of such modular regulation by attempting to use domain recombination to reprogram input control of N-WASP. As a simple test of whether mod-

ular autoinhibition is interchangeable, we engineered a synthetic signaling switch gated by a single heterologous ligand (Fig. 1B). The design involved tethering an unrelated modular domain–ligand pair—in this case a PDZ domain and its cognate C-terminal peptide ligand—to the termini of the N-WASP output domain. This design would create a potential autoinhibitory interaction that could be relieved by competitive binding of an external PDZ ligand.

Under basal conditions, this synthetic switch was repressed in an *in vitro* actin polymerization assay (12) (Methods, fig. S1). Repression required an intact, intramolecular autoinhibitory interaction: constructs containing only one interaction partner were not repressed, and addition of saturating free PDZ domain (~10-fold > K_d) *in trans* to a construct bearing only the PDZ ligand did not yield repression (fig. S2). The intramolecular PDZ interaction likely locks the output domain in an inactive conformation or restricts dynamic properties required for activity.

The switch was activated by increasing concentrations of free PDZ ligand (Fig. 1C), with maximal activity close to that of the isolated output domain. Half-maximal activation (K_{act}) required 50 μ M input. Precise gating behavior was dependent on the affinity of the autoinhibitory interaction (fig. S3); reducing affinity of the internal ligand resulted in lower basal repression but increased input sensitivity (reduced K_{act}), as would be expected if the intramolecular PDZ interaction was required for repression.

As in electronic circuits, complex cellular regulation often requires multi-input integrating gates (AND, OR, XOR, etc.) used in combinatorial control or feedback and feed-forward loops (4). We attempted the design of synthetic AND-gate switches by covalently tethering two modular domain–ligand pairs to N-WASP's output domain such that the intramolecular interactions

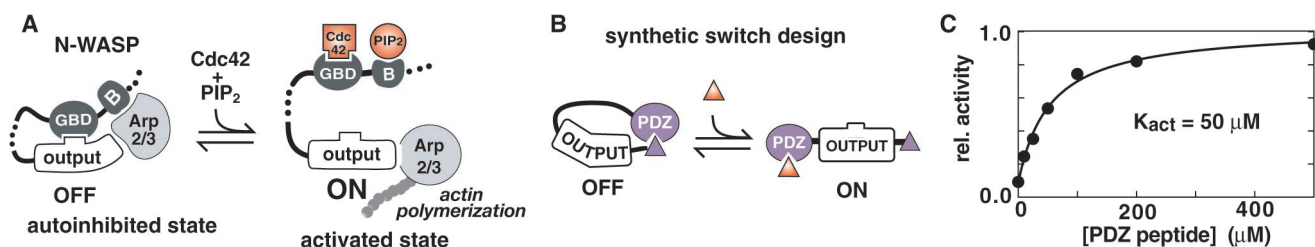


Fig. 1. Design of synthetic switch gated by heterologous ligand. (A) N-WASP is a modular allosteric switch: its output domain constitutively stimulates Arp2/3-mediated actin polymerization but is repressed by autoinhibitory interactions involving two domains, the GTPase-binding domain (GBD) and a basic (B) motif. Input ligands activate by disrupting autoinhibitory interactions: GTP-loaded Cdc42 binds GBD; PIP₂ binds B motif. These two inputs act synergistically (9, 11), thus, N-WASP resembles an AND gate. (B) Design strategy for a synthetic single-input switch using N-WASP's output domain and a PDZ domain–ligand pair as heterologous autoinhibitory module (α -syn trophin PDZ; ligand comprising NH₂-GVKESLV-COOH; $K_d = 8 \mu$ M). (C) Synthetic switch protein is

basally repressed but can be activated by addition of exogenous PDZ ligand. We tested switches with an *in vitro* pyrene-actin polymerization assay (fig. S1), using the time required to reach 50% polymerization ($t_{1/2}$) as the (relative) activity metric. Basal repression is observed only in constructs containing the intramolecular PDZ domain–ligand pair (fig. S2). Peptide concentration required for half-maximal activation (K_{act}) is 50 μ M. Studies with variant switches show that degree of repression is correlated with affinity of the intramolecular ligand (K_{PDZ}), whereas sensitivity to external PDZ ligand shows an inverse correlation (fig. S3). Assays in this and all other figures were performed with 50 nM switch protein, 5 nM Arp2/3, and 1.3 μ M actin (10% pyrene-actin).

might cooperatively repress activity. Such a switch would respond cooperatively to the combination of both competing external ligands (Fig. 2A). Because of increased complexity of two-input switches, we created a combinatorial library in which switch design parameters including domain type, domain-ligand affinity, linker length, and domain architecture were varied (Fig. 2B). To further increase variability, we used two forms of the N-WASP output domain, long and short; both display constitutive activity (13).

Two classes of switches were designed. For the first class—"chimeric" switches—the target behavior was dual regulation by PDZ ligand and Cdc42, a nonnative and a native N-WASP regulator, respectively. We con-

structed these switches using a PDZ domain and the native N-WASP GBD as regulatory modules. The GBD binds a peptide within the N-WASP output region (residues 461 to 479), an interaction that is competitively disrupted by activated Cdc42 (8). Although the intramolecular GBD interaction is required for autoinhibition in native N-WASP, it is not sufficient: the interaction does not repress N-WASP activity unless combined with the autoinhibitory interaction of the B module (the PIP₂ responsive element). (9). For the second class—"heterologous" switches—the target behavior was dual regulation by PDZ and SH3 domain ligands, two nonnative inputs. We constructed these switches using the PDZ domain from α -synthrophin and the SH3

domain from Crk. SH3 domains recognize short proline-rich motifs (14, 15).

A library of 34 such switches (Fig. 2B) was tested for gating by the appropriate high-affinity intermolecular ligands. Activity was tested in the presence of no inputs, each individual input, and both inputs together. Like most signaling proteins, these modular allosteric switches did not give simple binary responses; the precise response observed depended on the input concentrations used. We therefore performed activation screens under a standard set of input concentrations: 10 μ M Cdc42-GTP- γ -S [guanosine 5'-O-(3'-thiotriphosphate)], 200 μ M PDZ ligand, and 10 μ M SH3 ligand. Each of these concentrations is

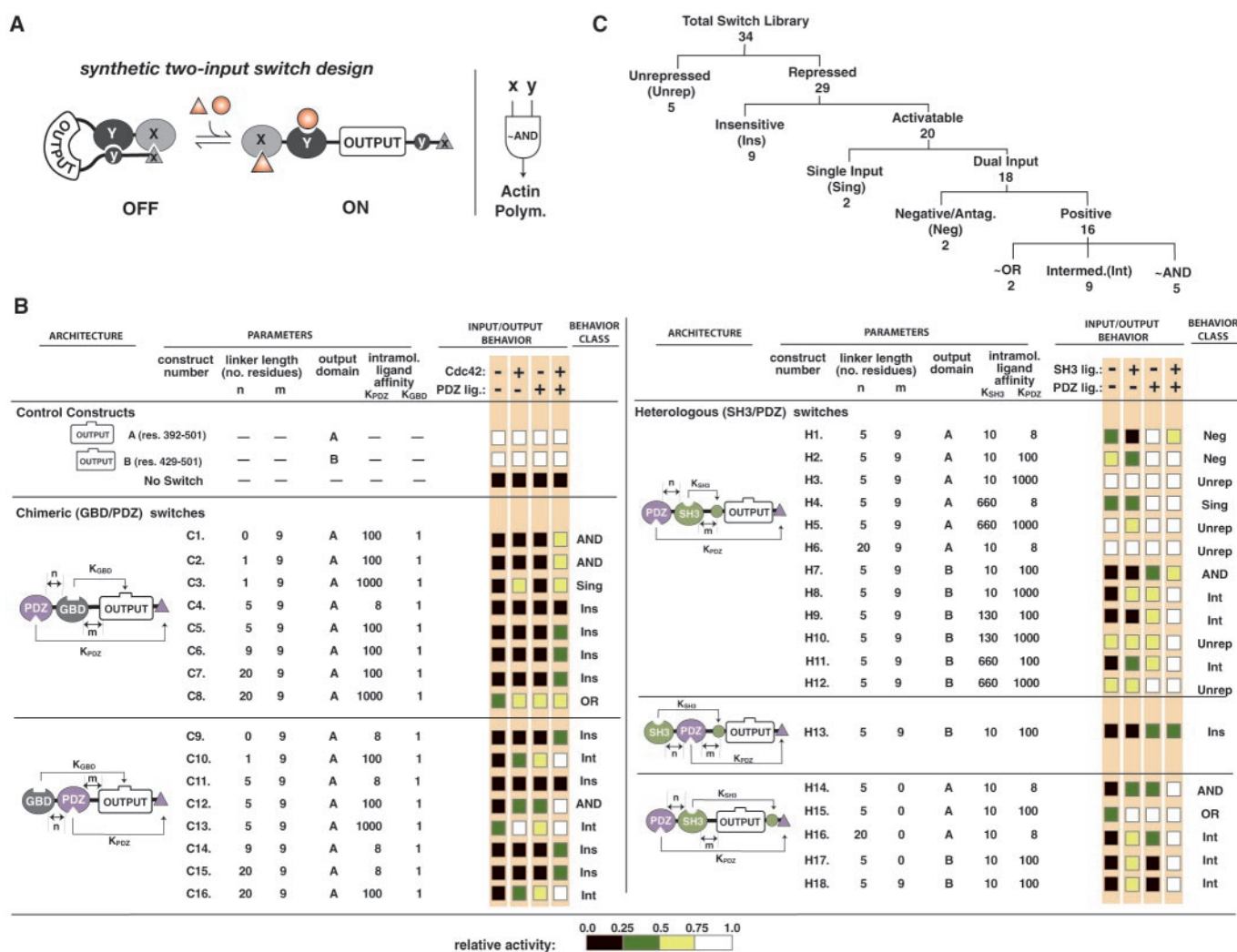


Fig. 2. Design of synthetic dual-input switch library. (A) Two-input switch design strategy. (B) Switch library constructed by domain recombination. Components used (table S1) are two output domains of N-WASP (output A and B), which differ in length; three different input domains (PDZ, SH3, GBD); from one to three intramolecular ligands of differing affinities for each of the input domains (ligand for GBD is contained within the output domain); and four different-length interdomain linkers (Gly-Ser repeats). Switch architecture and design parameters are listed at left. Component affinities are given in micromolar units. Observed gating behavior is listed at right. Activity

of library members was screened in the presence of no inputs, each individual input, and both inputs simultaneously by using a standard set of input concentrations (Cdc42GTP- γ -S: 10 μ M; PDZ ligand: 200 μ M; SH3 ligand: 10 μ M; all of these concentrations are 20 to 100 times the K_d for input binding to its isolated recognition domain). Relative activity (measured as in fig. S1) under these conditions is indicated by a color code (from low to high: black, green, yellow, white). (C) Classes of gating behavior observed in the library (see fig. S4 for class definitions). For linkers n and m, see the scheme at the left; lig., ligand; polym., polymerization.

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20 to 100 times the K_d observed for input ligand binding to its isolated recognition domain.

Switches could be divided into diverse behavioral classes (Fig. 2C). At the extremes, five switches showed little or no basal repression, and nine were extremely well-repressed, but could not be activated under any of the tested conditions. Most constructs, however, showed some type of gating behavior. Of the remaining 20 switches, 16 showed positive gating (both inputs activate). Two of the proteins displayed antagonistic gating: one input activates, whereas the other represses (mechanism discussed later). The positively gated dual-input switches could be further subdivided. Two proteins showed OR gate-like behavior (roughly equivalent activation in the presence of either individual input or both together), five proteins showed clear AND gate-like behavior, whereas the remaining constructs showed intermediate behavior. Thus, this relatively small library yielded a diversity of switch behaviors, including several with the targeted AND-gate behavior.

Several design principles were revealed by examining how switch parameters alter behavior. Basal repression and input sensitivity were directly linked to the affinity of autoinhibitory interactions. For example, the chimeric switch C11, which has an intramolecular PDZ ligand with $K_d = 8 \mu\text{M}$, was well repressed under basal conditions but insensitive: It could not be activated by the standard concentrations of PDZ ligand or Cdc42, even in combination (Fig. 3A). However, if the intramolecular PDZ ligand-affinity was reduced ($K_{\text{PDZ}} = 100 \mu\text{M}$), the protein then resembled an AND gate (switch C12).

Heterologous switch behavior was also dependent on affinity of the autoinhibitory interactions. For example, switch H15, which has internal SH3 and PDZ ligands with $K_{\text{SH3}} = 10 \mu\text{M}$ and $K_{\text{PDZ}} = 100 \mu\text{M}$, resembled an OR gate (Fig. 3B). However, increasing the affinity of the internal PDZ ligand by ~ 10 -fold ($K_{\text{PDZ}} = 8 \mu\text{M}$) within the same architecture yielded a well-behaved AND gate (switch H14). Interestingly, in one architectural context, the $8 \mu\text{M}$ PDZ affinity was too high to yield AND-gate behavior (switch C11), whereas in another context this affinity was ideal (switch H14). This difference may be due to differences in the affinity of the partner domain; in C11 the partner domain is the GBD, which binds its internal ligand with $K_d = 1 \mu\text{M}$ (9, 16), whereas in H14 the partner domain is an SH3 domain with $K_{\text{SH3}} = \sim 10 \mu\text{M}$. Maintaining a balance between switch repression and sensitivity may require balancing the affinities of the highly coupled autoinhibitory interactions.

Linker length also affected switch behavior. For example, if the linker length between

the PDZ and SH3 domains in H14 was increased from 5 to 20 residues, the switch became more sensitive to the isolated inputs (switch H16), indicative of reduced domain coupling. This finding is consistent with observations that coupling between regulatory domains of Src family kinases depends strongly on conformational and energetic features of the interdomain linker (17). Within this library, however, increasing interdomain linker length did not uniformly reduce coupling, which suggests that these effects are context-dependent.

Synthetic AND-gate switches were tested for targeted activation of actin polymerization in *Xenopus* oocyte extracts (Fig. 3C). Carboxylated polystyrene beads were coated with glutathione *S*-transferase (GST) fusions to the relevant input ligands: no ligand (GST

alone), SH3 ligand, PDZ ligand, or SH3 and PDZ ligands connected in tandem. When beads were incubated with soluble H14 switch and oocyte extract, actin filament nucleation was observed only on beads coated with the tandem SH3-PDZ ligand, consistent with multi-input targeting.

The combinatorial switch library also yielded switches with the unexpected behavior of antagonistic or negative input control (H1, H2) in which PDZ ligand acted as an activator, but SH3 ligand acted as a repressor (Fig. 4A). Detailed examination of the gating properties of switch H2 in various input concentration regimes revealed that PDZ ligand always acts as an activator; SH3 ligand, however, increased the basal level of repression (Fig. 4B). Antagonistic regulation is consistent with a model in which the intramolecular PDZ interaction is

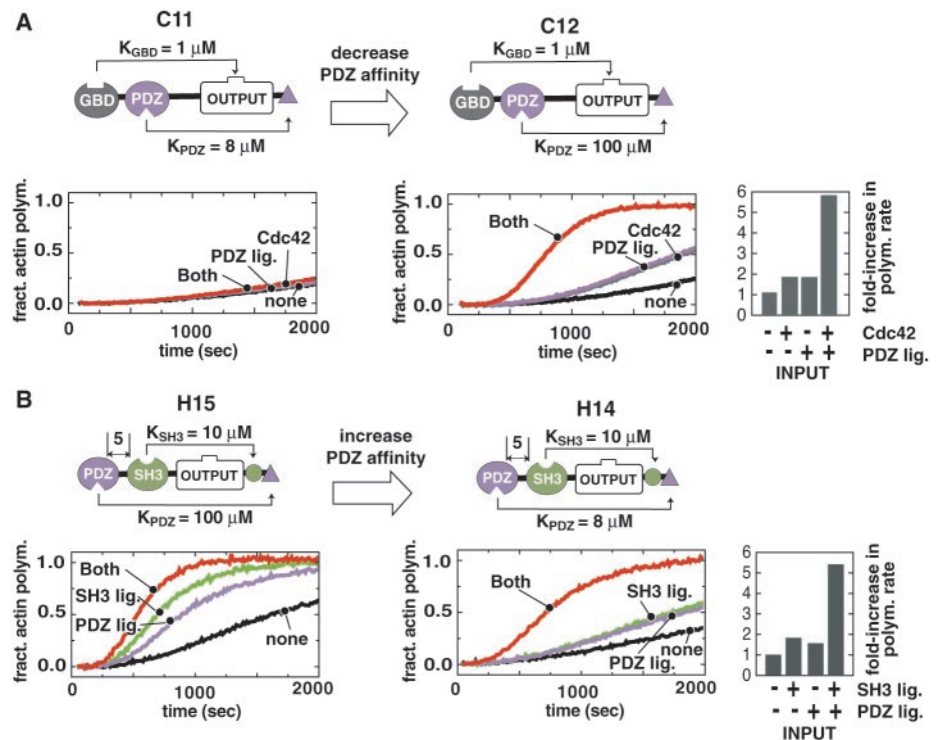


Fig. 3. Synthetic switches that resemble AND gates. (A) Chimeric switch C12 (right) resembles an AND gate; it shows strong actin polymerization only in the presence of both PDZ ligand and Cdc42. Adjacent bar graph shows maximal polymerization rates under each condition normalized to the basal rate (no input). Related switch (C11) with identical architecture but a higher affinity intramolecular PDZ ligand (left) is insensitive or overrepressed. (B) Heterologous switch H14 (right) resembles an AND gate that responds to SH3 and PDZ ligand. A related switch (H15) with identical architecture but a weaker affinity intramolecular PDZ ligand (left) resembles an OR gate; individual ligands yield relatively strong activation. (C) Switch H14, which resembles an AND gate, can spatially target actin polymerization in a *Xenopus* oocyte extract. Polystyrene beads were coated with GST fusions to no ligand, SH3 ligand, PDZ ligand, or a tandem SH3-PDZ ligand (see Methods, Supporting Online Material). The tandem ligand was used at half concentration relative to monovalent ligands. Only beads coated with the tandem ligand and incubated with switch H14 (100 μM) nucleated polymerization of rhodamine-labeled actin (red). Merge of bright-field and fluorescence images are shown. Fraction (fract.) of beads displaying actin polymerization (polym.) is given.

Beads	Switch (H14)	Beads with actin polym.
GST-SH3-PDZ lig.	-	0/12
GST-PDZ lig.	+	0/17
GST-SH3 lig.	+	0/14
GST-SH3-PDZ lig.	+	11/20

solely responsible for autoinhibition, and the intramolecular SH3 interaction destabilizes the intramolecular PDZ interaction, but, by itself, has no direct effect on output activity (Fig. 4C). We modeled this scheme by assuming that the state in which both intramolecular interactions take place is unfavorable and unpopulated (fig. S5). Such a scheme predicted an activation surface (Fig. 4C) resembling the observed behavior of switch H2 (Fig. 4B). For related switches (H1 to H3), the maximum level of repression observed (in the presence of SH3 ligand), directly correlated with PDZ affinity, a trend consistent with repression driven solely by the intramolecular PDZ interaction.

In this type of antagonistic switch, the two domains appear to act in a nested manner: The SH3 intramolecular interaction regulates the PDZ intramolecular interaction negatively, which in turn negatively regulates the

output activity (Fig. 4C). Addition of exogenous SH3 ligand, therefore, stabilizes the autoinhibitory PDZ interaction, leading to the observed inhibitory effect. In contrast, in positive integrating switches that resemble AND gates, the two domains work in concert to negatively regulate output function (Fig. 4D). Consequently, disruption of both intramolecular interactions yields activation.

This unanticipated class of switches highlights a striking feature of the library: Subtle changes in switch parameters can lead to dramatic changes in gating behavior. The architecture of antagonistic switches (H1, H2) is identical to a set of positive switches (H7 to H12) except for the size of the output domain (long output in the antagonistic switches; short in the positive switches). The geometry of the output domain must have significant impact on the coupling between

regulatory domains, presumably by altering stability of the various conformational states of the switch.

These results demonstrate that multidomain signaling switches like N-WASP are functionally modular; diverse and complex gating behaviors can be generated through relatively simple recombination events between input and output domains, even among domains with no known evolutionary relation. By allowing the establishment of novel regulatory connections between molecules with no previous physiological relation, such recombination events would be a powerful force driving evolution of novel cellular circuitry (18). This interchangeability exists because, in modular allosteric switches, regions that mediate input control are physically separable from output regions. Facile interchange of gating properties is unlikely to

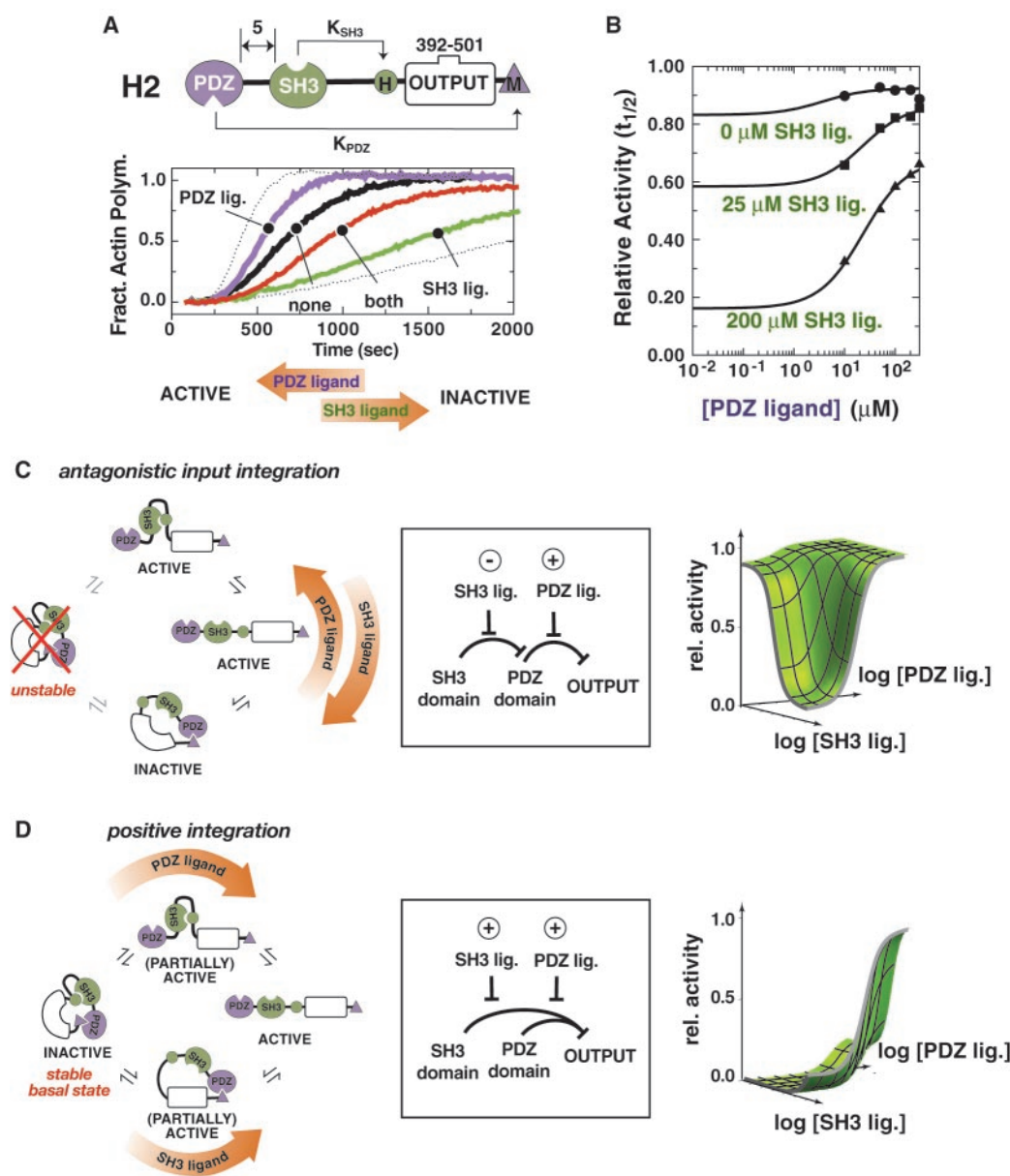


Fig. 4. Mechanism of antagonistic switch. (A) PDZ and SH3 ligands have opposing effects on the activity of switch H2; PDZ ligand is an activator, SH3 ligand is a repressor. Fraction (fract.) of beads displaying actin polymerization (polym.) is shown; lig. ligand; rel., relative. (B) Effect of PDZ ligand on switch H2 activity in the presence of different, constant concentrations of SH3 ligand. (C) Antagonistic behavior of switch H2 can be explained by a model in which the SH3 and PDZ intramolecular interactions are anti-cooperative (i.e., the state with both intramolecular interactions is unfavorable and unpopulated), and the intramolecular PDZ interaction mediates autoinhibition. Although the intramolecular SH3 interaction is neutral, it indirectly relieves repression by opposing the intramolecular PDZ interaction. A simple circuit diagram shows how this nested series of regulatory interactions yields antagonistic input control. Positive and negative net effects of inputs are indicated. Modeling of such a switch predicts an activation surface (green) consistent with experimental behavior (fig. S5). (D) Model of switches that resemble AND gates. Both intramolecular domain interactions contribute to autoinhibition. Thus, both ligands are positive regulators. Modeling yields an activation surface (green) consistent with more potent activation in the presence of both ligands simultaneously (fig. S5).

occur in conventional allosteric proteins in which input and output activities are centralized in a single folded structure, and gating is mediated by subtle conformational shifts.

Domain recombination space sampled in these experiments proved functionally rich: Although constructs showed a range of different gating behaviors (negative-positive, integrating-nonintegrating, etc.), nearly all of them show some form of gating. Gating as an emergent property, therefore, does not appear to be extremely rare, as might be expected if only very precise domain arrangements yielded regulation. This modular framework, in addition to promoting switch protein evolution, could be used to engineer proteins with novel regulatory control and, in principle, novel cellular circuits.

An Age-Induced Switch to a Hyper-Recombinational State

Michael A. McMurray and Daniel E. Gottschling*

There is a strong correlation between age and cancer, but the mechanism by which this phenomenon occurs is unclear. We chose *Saccharomyces cerevisiae* to examine one of the hallmarks of cancer—genomic instability—as a function of cellular age. As diploid yeast mother cells aged, an ~100-fold increase in loss of heterozygosity (LOH) occurred. Extending life-span altered neither the onset nor the frequency of age-induced LOH; the switch to hyper-LOH appears to be on its own clock. In young cells, LOH occurs by reciprocal recombination, whereas LOH in old cells was nonreciprocal, occurring predominantly in the old mother's progeny. Thus, nuclear genomes may be inherently unstable with age.

Age may be the greatest carcinogen: Cancer incidence increases exponentially near the end of human life (1). Chromosomal abnormalities are a hallmark of most tumors, and it is widely held that genomic instability is a prerequisite for tumorigenesis (2). In older individuals, there is evidence for increased genomic instability, even in noncancerous cells (3). Although numerous hypotheses exist to explain the association between aging and genomic instability (1), these have been difficult to test. To develop a mechanistic understanding of age-related genomic instability, we asked whether such a phenomenon occurs in a model biological system, the budding yeast *Saccharomyces cerevisiae*.

Heterozygosity was created in a diploid strain by the insertion of a marker gene in one

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Supporting Online Material

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Materials and Methods

Figs. S1 to S5

Table S1

References and Notes

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cell's increasing age, we isolated by micro-manipulation every daughter cell produced from a mother and allowed each daughter to form a colony (11). When the life-spans of the mother cells were complete, daughter colonies were assayed for LOH by changes in colony color. LOH was readily observed in the progeny of aging mothers by the appearance of uniformly colored colonies, or colored sectors within colonies. LOH events resulting in sectored daughter colonies were scored as half-, quarter- or eighth-sectors, which are consistent with the daughter cell or its progeny experiencing an LOH event one, two, or three generations after separation of the daughter from the original mother cell (Fig. 1, A and B).

Examination of these pedigrees revealed a marked change in LOH with the mother's age (Fig. 1C). Daughter colonies early in the life-span had no LOH events, whereas LOH was observed frequently in the colonies produced by daughters of old mothers. The first LOH events observed in the pedigrees of individual mothers did not occur until the mothers had gone through 23 cell divisions (median value); this late onset was observed at both loci analyzed (Fig. 1D, open bars). However, once an LOH event was observed in a lineage, subsequent LOH events were much more frequent, occurring in every third to fourth daughter colony (Fig. 1, B and D, solid bars). The rate of LOH per cell division in old cells was ~40 to 200 times that of young cells (Table 1). The frequency of LOH remained constant as the mother cells continued to age: After the first event, there was no significant correlation between the age of the mother and the frequency of subsequent LOH events (*MET15* $P = 0.69$ and *SAM2* $P = 0.39$ for a nonparametric Spearman correlation coefficient).

copy of a locus. Loss of heterozygosity (LOH) at the locus was detected when a genetic alteration occurred in which the marker was "lost." Although LOH in yeast can arise by multiple mechanisms, spontaneous LOH in wild-type cells occurs primarily through mitotic recombination (4). Recombination is presumed to be initiated by DNA damage along the chromosome and is typically accompanied by LOH at all centromere-distal loci (5, 6); accordingly, distal markers were more likely to undergo spontaneous LOH (table S1). Therefore, in order to maximize the chance of observing LOH events, we inserted markers distally on the two longest chromosome arms: at the *SAM2* locus on the right arm of chromosome IV and at the *MET15* locus on the right arm of XII, about 1 and 2 Mb, respectively, from their centromeres (7). Marker genes affecting colony color when lost were inserted at these loci (8, 9).

The number of daughter cells produced before death by a yeast (mother) cell defines her life-span (10). In order to determine whether genomic instability, manifested as LOH, was affected by a mother

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