

Engineering Modular Protein Interaction Switches by Sequence Overlap

Nathan A. Sallee, Brian J. Yeh, and Wendell A. Lim*

Contribution from the Chemistry and Chemical Biology Graduate Program and the Department of Cellular and Molecular Pharmacology, University of California, San Francisco, California 94158

Received October 10, 2006; E-mail: lim@cmp.ucsf.edu

Abstract: Many cellular signaling pathways contain proteins whose interactions change in response to upstream inputs, allowing for conditional activation or repression of the interaction based on the presence of the input molecule. The ability to engineer similar regulation into protein interaction elements would provide us with powerful tools for controlling cell signaling. Here we describe an approach for engineering diverse synthetic protein interaction switches. Specifically, by overlapping the sequences of pairs of protein interaction domains and peptides, we have been able to generate mutually exclusive regulation over their interactions. Thus, the hybrid protein (which is composed of the two overlapped interaction modules) can bind to either of the two respective ligands for those modules, but not to both simultaneously. We show that these synthetic switch proteins can be used to regulate specific protein-protein interactions in vivo. These switches allow us to disrupt an interaction with the addition or activation of a protein input that has no natural connection to the interaction in question. Therefore, they give us the ability to make novel connections between normally unrelated signaling pathways and to rewire the input/output relationships of cellular behaviors. Our experiments also suggest a possible mechanism by which complex regulatory proteins might have evolved from simpler components.

Introduction

Precise regulation of protein-protein interactions is critical for determining the wiring of cellular signaling pathways. An impressive array of elaborate mechanisms, including allostery and direct competition, have evolved to couple the binding of an input molecule to the modulation of a downstream interaction. A classic example is the protein calmodulin, which acts as a ligand-gated interaction switch—upon binding to Ca^{2+} , it undergoes a conformational change that allows it to bind to specific downstream effector proteins (Figure 1).¹ Our goal has been to find a general method for engineering new protein interaction switches like calmodulin. Coupling novel inputs with specific effector proteins would be a powerful tool for rewiring cellular regulatory behavior.²

Our strategy was inspired by another natural protein interaction switch—the GTPase-binding domain (GBD) found in proteins such as Wiskott-Aldrich syndrome proteins (WASPs)³ and p21-activated kinases (PAKs) (Figure 1).⁴ The GBD acts to autoinhibit the catalytic activities of WASPs and PAKs by interacting with autoinhibitory peptides found in these proteins. However, binding of the GBD to Cdc42 (a Rho family GTPase also known as p21) disrupts the GBD-peptide interaction,

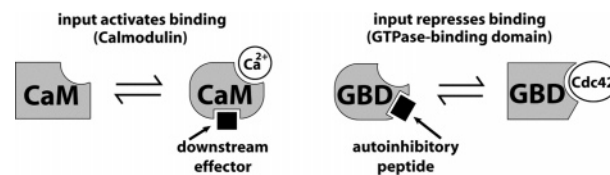


Figure 1. Naturally occurring protein interaction switches. Shown are two example proteins that respond to their input ligands (shown in white) by either activating (calmodulin, CaM) or disrupting (GTPase-binding domain, GBD) interactions with downstream effectors (shown in black).

thereby stimulating WASP and PAK activity.^{3,4} Only the GTP-bound, activated state of Cdc42 can cause this activation because the GBD has dramatically reduced affinity for Cdc42(GDP).⁵

Structural studies of the WASP GBD bound to its two alternative ligands (the autoinhibitory peptide³ and Cdc42⁶) reveal why it acts as a Cdc42-gated peptide interaction switch. Two distinct regions of the GBD are responsible for the two interactions; however, these regions overlap in sequence (Figure 2A). Specifically, amino acids 230–277 (the p21-binding domain, PBD) of WASP can independently bind Cdc42(GTP), while amino acids 250–310 can independently bind the autoinhibitory peptide ligand. The overlapped region (amino acids 250–277) adopts a different conformation in each of the two liganded states; these two conformations are incompatible and this renders the interactions mutually exclusive.⁷

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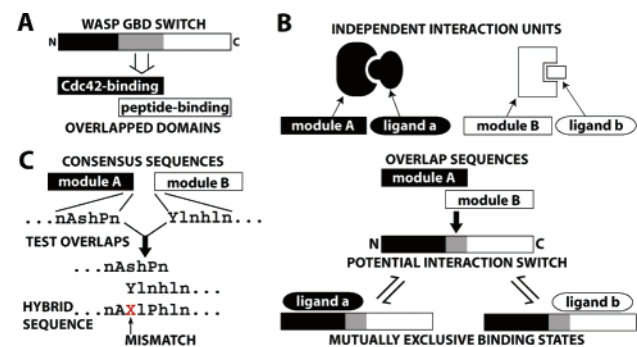


Figure 2. Strategy for design of modular protein interaction switches. (A) The WASP GBD consists of a peptide-binding autoinhibitory domain and a Cdc42-binding domain that are overlapped, rendering their interactions mutually exclusive. (B) We have used this overlapping strategy to create novel interaction switches from two unrelated binding modules. The top cartoons represent the folded structures of the protein interaction modules, while the rectangles represent their linear amino acid sequences. At the bottom, the overlapped rectangles are shown interacting with their ligands for clarity, but the modules are presumably in a folded state when bound. (C) Overview of evaluation of the overlaps performed by our PERL script. Where available, consensus sequences are used for the modules in the design process.¹⁶ Uppercase letters represent conserved amino acids that are required at that position. Lowercase letters represent groups of amino acids that are found at that position throughout the domain family—in this example, “n” is any amino acid, “s” is those with small side chains, “h” is hydrophobics, and “l” is aliphatic side chains. The script tests each overlap by evaluating the sequence compatibility of the two modules at each position in the overlap. The hybrid sequence consists of amino acids or groups of residues that can satisfy the requirements of both modules. Where the sequences are incompatible, the position is declared a mismatch (marked “X”).

Here we ask if sequence overlap of normally unrelated protein interaction elements can be used as a general method to build novel switches. Our strategy is to make chimeras of two independent protein interaction modules in which the functional regions of each module are overlapped (Figure 2B). The chimeric proteins were designed so that they should be able to interact with ligands for each of the two constituent modules, but not simultaneously. In principle, the chimeras should function as a switch in which binding of one ligand disrupts binding of the other. A conceptually related approach of domain insertion has been successful in generating allosterically regulated enzymes.⁸

Other methods exist for disrupting protein binding, but because our engineered switches are made from naturally occurring interaction proteins they provide a simple, modular way to functionally link biological interactions and processes that are normally unrelated.⁹ Most importantly, by creating novel input/output relationships between existing protein interaction modules, we assemble a set of tools for rewiring the flow of information in endogenous cellular signaling pathways.

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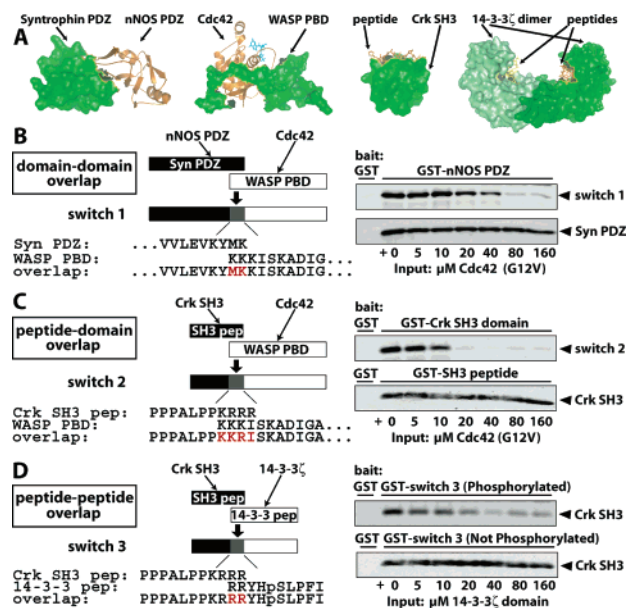


Figure 3. Constructing protein interaction switches from overlapped interaction domains and peptides. (A) Structures of the domain-ligand pairs used in our switches. The PDB accession codes are as follows: 1QAV¹⁰ (syntrophin-nNOS PDZ heterodimer), 1CEE⁶ (WASP PBD-Cdc42 complex), 1CKA¹¹ (Crk SH3-peptide complex), and 1QJA¹² (14-3-3 ζ homodimer with two peptides). (B–D) Three example synthetic switches made by overlapping protein domains and peptides. Glutathione S-transferase (GST) pull-down assays show that switch binding to one ligand is disrupted upon addition of increasing concentrations of the second ligand. In each case, the equivalent nonoverlapped interaction module is not disrupted by the competing ligand. In panels A and B, a constitutively active Cdc42 mutant (G12V) is used as the input molecule. In panel C, switch 3 is serine-phosphorylated (denoted “pS”) by PKA only in the top set of lanes.

Results

In the switch design process, we chose to work with protein domain families that are structurally characterized and that have well-defined domain, peptide, or small molecule ligands (Figure 3A, Table 1, Table S1).¹⁵ These domains and their domain or peptide ligands were used as the modules in our design of overlapped switches (Figure 2B). Here we define domains as sequences of at least 35 amino acids that have compact folded structures. Peptide ligands are 15 amino acids or less and unfolded in the absence of binding partner. Where possible, consensus sequences for domain families (obtained from the SMART database¹⁶) were used in the design (Figure 2C).

We computationally searched for compatible sequence overlaps between domain family consensus sequences or specific sequences of domain or peptide ligands using a custom PERL script. The PERL script first separated the sequences into individual positions defined by one amino acid or a family of residues from the consensus sequence (e.g., small hydrophobics). Pairs of modules were tested for overlaps of 1–15 amino acids in both orientations (each domain or peptide was tested at the N- and C-terminus of every other domain/peptide). For each overlap tested, the PERL script sequentially analyzed each amino acid position in the potential overlap and assessed the sequence requirements for each module at that position (Figure 2C). If a particular amino acid or a subset of amino acids could fulfill the sequence requirements of both modules, they were saved

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Table 1. Domain–Ligand Pairs and Affinities

domain	origin and sequence	ligand(s)	K_d	ref
Syn PDZ	mouse α -syntrophin residues 80–164	VKESLV peptide	7 μ M	measured
Crk SH3	mouse C-Crk residues 135–189	rat nNOS PDZ	1 μ M	13
WASP PBD	human WASP residues 230–288	YPPALPKRRR peptide	20 nM	14
14-3-3 ζ	human 14-3-3 ζ residues 1–245	human Cdc42(GTP)	164 nM	measured
switch 1	[Syn 80–162]-MK-[WASP 232–288] ^b	RRYHpSLPFI peptide ^a	low nM	12
		VKESLV peptide	7 μ M	measured
		Cdc42 (GTP)	247 nM	measured

^a “pS” denotes phosphoserine. ^b Sequences of the unchanged portions of the N- and C-terminal modules are bracketed and separated by the overlap chimeric sequence.

as the hybrid sequence at that position in the overlap. When no amino acids could fulfill the requirements of both modules, the position was declared a mismatch. Potential overlap constructs were scored by the number of mismatches.

When two consensus sequences were overlapped in this fashion, we generally were able to find overlaps with no mismatches. These hybrid sequences were then searched against the sequences of individual family members.¹⁶ Specific domains were chosen based on the best fit with the overlap sequence at the relevant terminus, while also considering factors like the availability of solved structures and well-characterized ligands. In contrast, when specific sequences of domains or peptides were overlapped, there were inevitably mismatches due to the stricter sequence requirements. When available, we incorporated peptide library data¹⁷ to assess tolerance to substitution at positions with mismatches. In most cases, some mutations had to be made to one or both modules in the overlapped region. These mutations were evaluated to minimize impact on the folding and binding of either module, using all available structure and sequence information.

We constructed and tested a total of 25 switches that consisted of overlaps of two domains, one domain and one peptide, or two peptides (Table S2).¹⁵ For every switch, we tested its ability to bind its two ligands in a mutually exclusive manner. We also tested the individual constituent domains bearing any mutations that were made in the overlapped region for their ability to bind their ligand. This allowed us to separate any effects of the mutations from the effects of the overlapping of those modules.

Seven of these 25 synthetic proteins worked as interaction switches. Overlaps of two peptides showed the greatest percentage of successful switching behavior (3 of 5), and we had the least success in the domain-domain category (3 of 16). This is likely a result of the fact that domains have the complication of two competing folded structures. In most of the domain-domain overlaps we tested, one of the domains would dominate and the switch could only bind to that ligand.

Here we focus on one best-characterized example from each category (Figure 3B–D). In each case, binding of the switch to its two ligands was mutually exclusive and competition between the two bound states could be driven in either direction by varying ligand concentrations. One example of a domain-domain switch is an overlap of the syntrophin (Syn) PDZ domain and the WASP PBD (switch **1**; Figure 3B). The syntrophin PDZ binds to C-terminal peptide sequences, such as VKESLV-COOH, or it can form a PDZ heterodimer with the nNOS PDZ

domain (Table 1).¹³ Switch **1** bound to nNOS PDZ and GTP-bound Cdc42 in a mutually exclusive manner.

An example of a successful peptide-domain switch is an overlap of a proline-rich SH3 peptide ligand¹¹ and the WASP PBD (switch **2**; Figure 3C). This switch could bind to the Crk SH3 domain in a manner that is inhibited by Cdc42(GTP). A successful peptide-peptide overlap is that of the same SH3 ligand with a 14-3-3 domain peptide ligand¹² (switch **3**; Figure 3D). Switch **3** incorporates phosphorylation as an additional level of regulation because 14-3-3 ζ can only bind to a form of its peptide ligand bearing a phosphoserine.¹² Therefore, the unmodified switch only interacts with the Crk SH3 domain, but when phosphorylated by protein kinase A (PKA), the switch interacts with Crk SH3 in a manner that is inhibited by the 14-3-3 ζ domain.

To ensure that switching was happening on a physiologically relevant time scale, we tested switches **1–3** in the pulldown assay for competition at short time points. Switching between the different bound states was found to occur on a time scale of seconds to minutes for all three switches.¹⁵ The affinities of switch **1** for VKESLV peptide and Cdc42(GTP) were also measured by fluorescence-based binding assays (Table 1).¹⁵ These K_d values were used in a simple equilibrium-based model of binding and along with previously published kinetic data on the constituent domains^{5,18} we were able to simulate switch **1** competition.¹⁵ The time scale of switch **1** competition in this model was consistent with the pulldown time course data.

We wanted to more carefully investigate the mechanism of switch **1** to determine whether it is a conformational switch like the GBD or a steric switch where switching occurs because of steric competition between the two ligands. We found several lines of evidence to support a steric mechanism. First, the affinities of switch **1** for its ligands were comparable to those of the individual constituent domains (Table 1). Therefore, there is likely no significant change in the ΔG of folding for either domain in the switch, which would be expected for a conformational mechanism of switching.

A second clue to a steric mechanism is that we found Cdc42 binding to be mutually exclusive with the binding of a large PDZ ligand (130-residue nNOS PDZ domain), but not with the small VKESLV peptide that binds to the same pocket on the PDZ domain. Moreover, fusing the peptide ligand to a large glutathione S-transferase (GST) domain restored some competition with Cdc42 (Figure 4A). Thus, a bulky PDZ ligand is a requirement for switching, implying that it sterically hinders Cdc42 binding.

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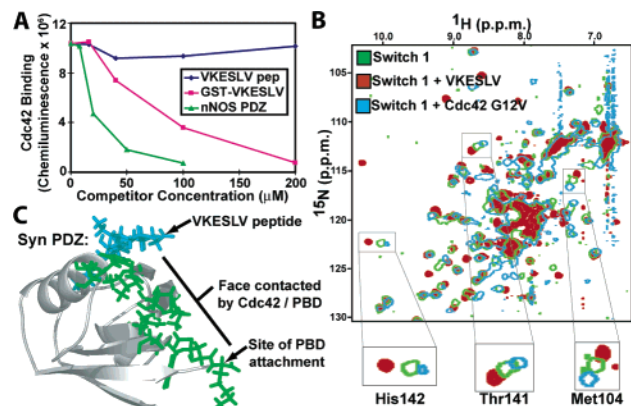


Figure 4. Mutually exclusive binding in switch 1 is likely due to steric competition between the ligands. (A) Maltose-binding protein (MBP) pulldown competitions, using MBP-Cdc42 G12V as bait and switch 1 as prey. nNOS PDZ competes with Cdc42 for switch 1 binding, but VKESLV peptide does not. VKESLV peptide tagged with GST can compete with Cdc42. (B) HSQC spectra were taken of ^{15}N -labeled switch 1 alone and with unlabeled VKESLV peptide or Cdc42 G12V ligand. Three syntrophin (Syn) PDZ side chains are highlighted whose chemical shifts show three distinct states. (C) Syn PDZ-GVKESLV NMR structure (2PDZ)¹⁹ with side chains highlighted in green where backbone amide chemical shifts were perturbed by Cdc42 binding to the neighboring PBD domain. These perturbed positions map to one surface of the PDZ domain, which is likely contacted by either Cdc42 or the folded PBD, leading to a steric clash with larger PDZ ligands.

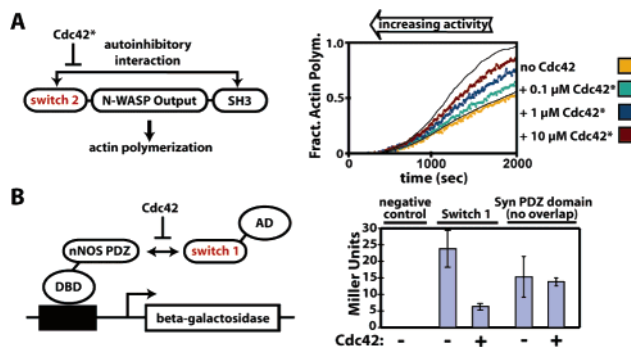


Figure 5. Using synthetic interaction switches to control biological function. (A) Switch 2 and its SH3 domain partner were fused to the output domain of N-WASP. In vitro pyrene actin polymerization assay (right) shows that this chimeric protein is autoinhibited but activatable by Cdc42 G12V (Cdc42*). The thin black lines represent uncatalyzed actin polymerization (minimal activity) and polymerization by the unregulated output domain (maximal activity). (B) Switch 1 can be used to regulate a yeast two-hybrid system. Switch 1 was fused to the activation domain (AD) and nNOS PDZ was fused to the DNA-binding domain (DBD). Strength of interaction is monitored by β -galactosidase activity (Miller units). Coexpression of Cdc42 disrupts the yeast two-hybrid interaction, but not when switch 1 is replaced by the isolated Syn PDZ domain. In the negative control, switch 1 was removed from the AD.

Finally, we collected HSQC NMR spectra of ^{15}N -labeled switch 1 on its own, bound to unlabeled PDZ peptide ligand, or bound to Cdc42(GTP). The assignments for the previously solved Syn PDZ-GVKESLV peptide structure (2PDZ)¹⁹ overlaid well with many resonances in the switch 1-VKESLV peptide spectrum, allowing us to assign most of the resonances corresponding to the PDZ domain. These data showed that the PDZ domain in switch 1 did not undergo global unfolding upon Cdc42 binding. A number of PDZ resonances shifted upon binding of switch 1 to Cdc42 (Figure 4B). These chemical shift

changes mapped to residues on the surface of the PDZ domain closest to the PBD fusion site and extending toward the PDZ binding site (Figure 4C). This surface is likely in close contact with Cdc42 or the folded PBD, thereby disrupting interactions with larger PDZ ligands.

We tested whether these switches could be integrated into larger proteins to modulate function in vitro or in vivo. First, we replaced the regulatory elements of the protein N-WASP (neuronal WASP, a close relative of WASP²⁰) with a synthetic switch. N-WASP's actin polymerization activity is normally regulated by an intramolecular interaction mediated by the native GBD. We made a synthetic protein in which N-WASP's constitutively active catalytic domain was flanked by switch 2 on the N-terminal side and its SH3 domain binding partner on the C-terminal side (Figure 5A).²¹ Like native N-WASP, the activity of this synthetic protein was basally repressed by the intramolecular SH3 interaction, but it was specifically activated by addition of Cdc42(GTP).

To determine if we could rewire cellular function, we tested the Cdc42-responsive switches (1 and 2) in a regulated yeast two-hybrid assay.²² The results for switch 1 are shown, but similar data were obtained with switch 2. Switch 1 was fused to a transcriptional activation domain, and its ligand—the nNOS PDZ domain—was attached to a DNA-binding domain (Figure 5B). In yeast cells containing these two constructs, we observed robust expression of the β -galactosidase reporter gene, showing that the two proteins interact. In cells that additionally express Cdc42, however, we observed a large decrease in β -galactosidase signal, indicating that the interaction was disrupted by Cdc42 in vivo. As a control, we tested the isolated Syn PDZ-nNOS interaction (no overlap) and observed no change in β -galactosidase signal upon expression of Cdc42.

Conclusions

We have described a strategy to generate regulated protein-protein interactions through overlapping the sequences of pairs of protein interaction modules. These experiments suggest a mechanism by which complex gated proteins might have evolved from simpler modular components. In most cases, two interaction modules in a single polypeptide behave independently: one interaction does not affect the other. However, if the modules are appropriately linked, for example by sharing overlapping sequences, the interactions become interdependent: one interaction directly regulates the other.

We found that our method is particularly successful when one or both of the overlapped modules are unstructured. When we attempted to overlap two folded domains, we found that there were cases where the switch could not energetically access both of the bound states. For these switches, we found that the two isolated constituent domains bearing the overlap mutations were able to bind their ligands, but that only one domain could bind in the context of the overlapped hybrid construct. The absolute and relative affinities of the two modules for their ligands had no clear effect on likelihood of successful switching.

Of the domains we tested, the PBD showed the highest rate of success. Accordingly, the PBD is a domain that is largely

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unstructured in the absence of its ligand, Cdc42.⁶ In addition to WASPs and PAKs, a number of naturally occurring Cdc42-responsive proteins appear to have similar modes of regulation where Cdc42 binding to a PBD positively²³ or negatively²⁴ affects the interaction of a neighboring, or overlapped, domain. It seems possible that this domain is evolutionarily predisposed to regulating other protein-protein interactions.

In many cases we found that overlaps of very few amino acids were sufficient to produce the desired mutually exclusive binding behavior. This means that the requirement for sequence compatibility at the relevant termini of the interaction modules is not as prohibitive as might have been assumed. However, we also encountered a number of cases where our designed switches bound to both ligands simultaneously and showed no switching. To most effectively balance these competing considerations, we found that it was critical to have minimized protein interaction modules with no extraneous residues at either terminus that are not required for a functional interaction. We found that this greatly increased our chances of designing a functional switch because even a short overlap would likely contain residues essential for both interactions.

This method for regulating binding provides one major advantage compared to direct competitive inhibition of a protein interaction: it allows us to make novel connections between proteins that may be normally unrelated. Moreover, switches **1-3** are dependent on phosphorylation or GDP/GTP exchange for binding. This can provide another level of control: the switches require input activation through phosphorylation by a protein kinase or exchange of GDP for GTP by a guanine nucleotide exchange factor. In addition, activation can be reversed by protein phosphatases and GTPase activating proteins, respectively. Therefore, these switches have great potential to be interfaced with endogenous kinase and GTPase signaling pathways and to be coupled to different cellular responses. With these tools we can begin to rewrite the flow of information in a cell and generate new behaviors by imposing regulation such as feedback, coordinated localization, or pathway crosstalk.

Experimental Section

Protein Construction and Purification. Protein domains and ligands were expressed in *Escherichia coli* [BL21(DE3)RIL] fused to a cleavable tag: generally either hexa-histidine (6xHis) (pET19-derived vector) or GST (pGEX4T-1 vector). Fusion proteins were purified on Ni-NTA resin (Qiagen) for 6xHis tags or glutathione-agarose resin (Sigma) for GST tags. In some cases, the tag was then removed by cleavage with tobacco etch virus (TEV) protease at 25 °C for 2 h. Cleaved proteins were then purified either by a second incubation with affinity resin or by fast protein liquid chromatography (FPLC) with a Source S or Q column (Pharmacia).

1. Switch Proteins. Switches were assembled by two-step polymerase chain reaction (PCR). The first step involved PCR of each of the individual domains with the overlap sequence encoded in the primers. A second PCR step annealed the overlap regions and created the hybrid proteins, which were verified by DNA sequencing.

2. Cdc42. In each experiment, the Cdc42 used is a soluble fragment of the human protein (residues 1-179). When explicitly stated, the G12V hydrolysis-defective constitutively active mutant was used. Cdc42 was additionally expressed as a maltose-binding protein (MBP) fusion (pMAL-p2X-derived vector) and purified with amylose resin (NEB).

3. N-WASP Hybrid Switches. Switches and cognate ligands were cloned on either side of N-WASP's actin polymerizing VCA domain as described previously.²¹ Proteins were expressed in a pET19-derived vector encoding a protein with a cleavable N-terminal GST fusion and a C-terminal 6xHis tag. GST was cleaved by TEV digestion, and proteins were purified on a Source Q column.

4. Peptide Synthesis and Labeling. VKESLV peptide was synthesized using standard solid-phase Fmoc-amino acid chemistry. Peptide was synthesized on valine-loaded Wang resin (Novabiochem). The peptide was either N-terminally acetylated or N-terminally dansylated with dansyl chloride (Molecular Probes), then cleaved and purified as previously described.¹³

Determination of Affinity by Fluorescence Perturbation. Fluorescence measurements were performed in a Photon Technology International fluorometer at 20 °C in 20 mM Tris pH 8.0, 100 mM NaCl, 2 mM MgCl₂, and 1 mM dithiothreitol (DTT). PDZ-ligand interactions were measured as previously described.¹³ Briefly, increasing amounts of PDZ or switch were added to 400 nM dansylated VKESLV peptide with constant stirring. The sample was excited at 338 nm, and binding was monitored as an increase in fluorescence at 540 nm. PBD-Cdc42 interactions were measured as previously described.⁵ Briefly, purified Cdc42 G12V was loaded with the fluorescent nonhydrolyzable nucleotide analogue Mant-GMPPNP (Molecular Probes). Increasing PBD or switch was added to 100 nM Cdc42 G12V (Mant-GMPPNP) with constant stirring. The sample was excited at 365 nm, and binding was monitored as a decrease in fluorescence at 440 nm. All data were fit using the program ProFit (Quantum Soft).

Pulldown Binding Assays. GST and MBP-pulldown assays were performed by first binding the GST or MBP-fused bait to glutathione agarose or amylose resin, respectively. PKA phosphorylation of GST-switch **3** was done on resin-bound protein in 50 mM Tris pH 7.5, 10 mM MgCl₂, 2 mM DTT, and 1 mM ATP for 2 h at 30 °C. Each pulldown was done in 20 mM Tris pH 8.0, 100 mM NaCl, 2 mM MgCl₂, and 1 mM DTT in 200 μL total volume. Sufficient resin was added to bring the bait protein concentration to 1 μM. The 6xHis-tagged prey protein was added at 10 μM. For competitions, a series of pulldowns were set up with increasing concentrations of the ligand that competes with the bait-prey interaction. The binding incubation was done for 30 min on ice with regular vortexing. The resin was washed with 0.5 mL of phosphate-buffered saline (PBS) and 0.1% Triton X-100, then with PBS alone. The resin was suspended in SDS loading buffer, and samples were run on SDS-PAGE and transferred to nitrocellulose membrane. From this point, the signal from the pulled-down prey protein was detected two different ways: an Alpha Innotech chemiluminescence scanner or a Li-Cor Odyssey infrared fluorescence scanner. For Alpha Innotech detection, membranes were blocked with 5% milk in tris-buffered saline and 0.1% Tween-20 (TBST) and then incubated with 1:2000 diluted mouse His-probe antibody conjugated to horseradish peroxidase (HRP) (Santa Cruz Biotech) in 5% milk/TBST. Membranes were washed with TBST and incubated with chemiluminescent substrate (Pierce), and band sizes were quantitated on the Alpha Innotech instrument. For Li-Cor detection, membranes were blocked with Odyssey blocking buffer and incubated with 1:2000 diluted mouse His-probe antibody (not HRP-conjugated) in 5% milk/TBST. Membranes were then washed with TBST, incubated with fluorescent secondary antibody (goat anti-mouse IRDye800 (Rockland) diluted 1:10000 in 5% milk/TBST), washed again with TBST, and then scanned and quantitated on the Li-Cor instrument.

HSQC Protein NMR. ¹⁵N-labeled proteins were made by transferring *E. coli* cultures to M9 minimal media containing ¹⁵NH₄Cl before induction. ¹⁵N heteronuclear single quantum correlation (HSQC) spectra²⁵ were taken on a Bruker Avance800 spectrometer equipped with cryogenic ¹H[¹³C/¹⁵N] probes and with an actively shielded Z

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gradient. All spectra were measured at 25 °C in 90% H₂O/10% D₂O with 20 mM Tris pH 8.0, 10 mM NaCl, 2 mM MgCl₂, and 1 mM DTT. Experiments were run with the XWIN-NMR program (Bruker BioSpin). The resultant data were processed with NMRPipe²⁶ and analyzed using Sparky.²⁷ Assigned ¹⁵N-HSQC spectra were obtained for the previously published Syntrophin PDZ-GVKESLV peptide complex¹⁹ (2PDZ) and the WASP PBD-Cdc42 complex⁶ (1CEE). HSQC spectra were taken of a 300 μM solution of ¹⁵N-Switch **1** alone or with unlabeled VKESLV peptide or Cdc42 ligand.

In Vitro Actin Polymerization Assays. Actin was purified from rabbit muscle²⁸ and pyrene-labeled,²⁹ and Arp2/3 was purified from bovine brain²¹ as previously described. In vitro pyrene-actin polymerization assays were performed as described²¹ with minor changes on a SpectraMax Gemini XS fluorescence plate reader (Molecular Devices). The excitation and emission wavelengths used were 365 and 407 nm, respectively. Exact assay conditions were 1.3 μM actin (5% pyrene-labeled), 20 nM Arp2/3, 50 nM chimeric N-WASP protein, 50 mM KCl, 1 mM MgSO₄, 1 mM EGTA, 0.2 mM ATP, 1 mM DTT, 3 μM MgCl₂, and 11.5 mM imidazole pH 7.0 in a total volume of 100 μL. To determine the minimal rate of spontaneous actin polymerization, the switch was omitted from the assay (Arp2/3 alone), and to determine the maximal rate, the switch was replaced with 50 nM unregulated N-WASP VCA domain. Raw fluorescence data were normalized to the average values of the lower and upper baselines of each curve using the equation $(F_{\text{data}} - F_{\text{lower}})/(F_{\text{upper}} - F_{\text{lower}})$.

Yeast Two-Hybrid. The Matchmaker system (Clontech) was used for yeast two-hybrid experiments. Bait ligands were cloned into the pGBT9 vector which encodes a fusion with the GAL4 DNA-binding domain. Switch proteins were cloned into the pGAD424 vector which encodes a fusion with the GAL4 activation domain. Additionally, Cdc42 was cloned into a pRS423 high-copy number 2 μm plasmid, along with an N-terminal SV-40 nuclear localization signal (NLS). Expression of Cdc42 protein was placed under the control of the Cyc1 promoter. It has been shown that human Cdc42 is activated in yeast, likely by the endogenous GTPase exchange factor Cdc24p.²² pGBT9 and pGAD424

plasmids encoding pairs of interacting proteins were transformed into the *Saccharomyces cerevisiae* strain Y187 with and without the Cdc42 plasmid. Liquid cultures of the various transformants were grown to mid-log phase, their OD₆₀₀ values were measured, and 1.5–3 mL of culture was centrifuged at 14 000 rpm for 5 min. The supernatant was discarded and the pellet resuspended in 1.5 mL of Z buffer (100 mM phosphate buffer pH 7.0, 10 mM KCl, 1 mM MgSO₄). Centrifugation was repeated, and the pellet was resuspended in 0.1 mL of Z buffer. The cells were then lysed by cycling the tubes between liquid nitrogen and a 37 °C water bath for 1 min each and repeating this cycle 5–6 times. We then added 0.7 mL of Z buffer + 0.27% β-mercaptoethanol to each tube of cells, plus one blank tube containing 0.1 mL of Z buffer alone. The reaction was then started by adding 160 μL of 4 mg/mL *o*-nitrophenyl β-D-galactopyranoside (ONPG) in Z buffer and incubating the tubes at 30 °C. The reaction was stopped 45 min to an hour later by addition of 0.4 mL of 1 M Na₂CO₃. The reaction tubes were centrifuged at 14 000 rpm for 10 min, and the supernatant was read for absorbance at 420 nm. Data are reported in Miller units, which are defined by eq 1 where *t* is time in minutes of the reaction and *V* is the volume in milliliters of culture used per reaction. All reactions were performed in triplicate from three different yeast colonies.

$$\text{Miller units} = 1000 \times \text{OD}_{420}/(t \times V \times \text{OD}_{600}) \quad (1)$$

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Supporting Information Available: Summary of design and results for all 25 synthetic switch proteins. Affinity measurements and computational modeling of competition for switch **1**, and binding competition time courses for switches **1–3**. Complete ref 17b. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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