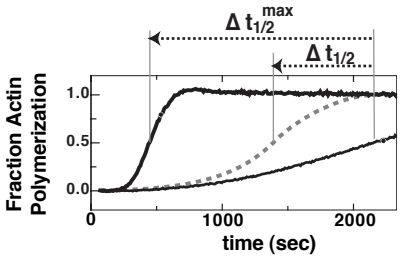


$t_{1/2}$ = time required to reach 50% maximal actin polymerization

relative activity = $\Delta t_{1/2} / \Delta t_{1/2}^{max}$

no switch: spontaneous actin polymerization (MINIMAL ACTIVTY)

experimental point: actin polymerization of switch in presence/absence of inputs (EXPERIMENTAL ACTIVITY)

OUTPUT actin polymerization in presence of constitutively active output domain (VCA) (MAXIMAL ACTIVITY)

S1. **Metric for relative activity of N-WASP switches based on half-time of actin polymerization.** Activity of switch proteins was determined using a fluorescence-based actin polymerization assay. Time required to reach 50% polymerization ($t_{1/2}$) was used as a metric for activity. Minimal activity was defined as the $t_{1/2}$ observed with spontaneous actin polymerization under these conditions in the presence of Arp2/3 but no nucleation promoting factors. Maximal activity was defined as the $t_{1/2}$ in the presence of the isolated output domain. Relative activities of individual constructs were scored by measuring the change in $t_{1/2}$ relative to the difference between maximum and minimum activities.

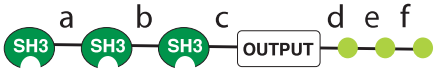
A.5.5

a	SGSGSGSGSGT	f	KL
b	SGSGSGSGSPG	g	AAAGSGSGS
c	SGSGSGSGSEF	h	SGSGSGSGS
d	SGSGSGELSGS	i	ACSGSGSGSGS
e	SGSGSGSAS	j	GSGSGSGSG

A.1.1

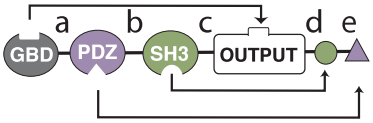
a	SGSGSGSAS
b	KL

A.3.3



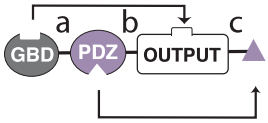
a	SGSGSGSGSEF	d	KL
b	GSGSGELSGS	e	AAASGSGS
c	SGSGSGSAS	f	SGSGSGSGS

B.3.1



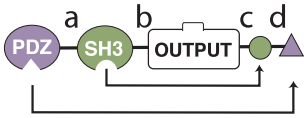
a	SGSGS	d	no linker
b	SGSGS	e	SGSGSGSGS
c	no linker		

B.2.1



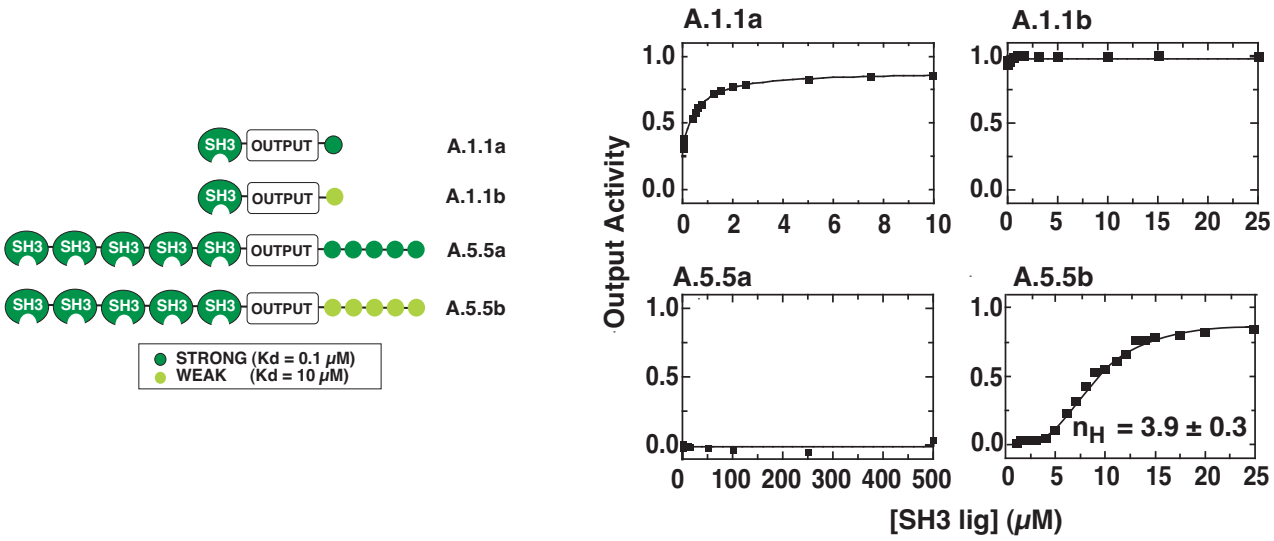
a	SGSGS
b	SGSGSGSGS
c	no linker

B.2.2

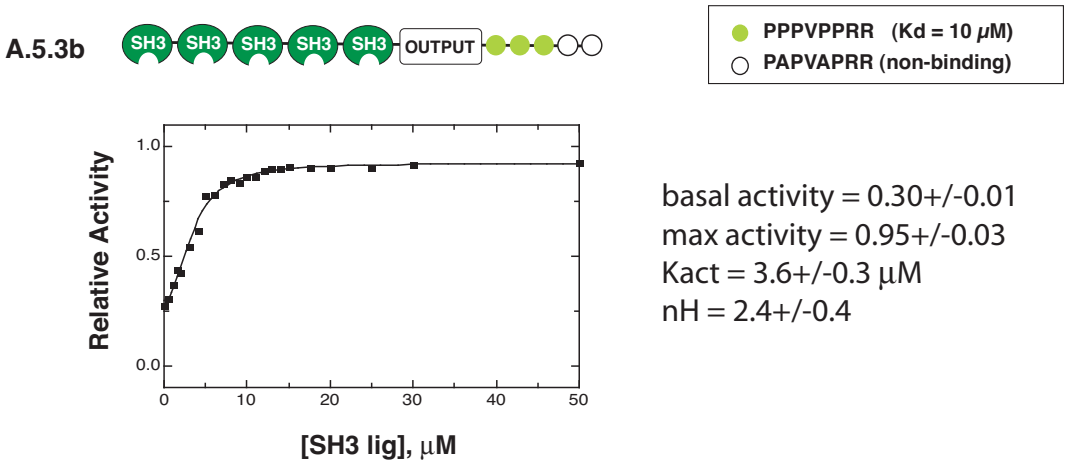


a	SGSGS	c	no linker
b	no linker	d	SGSGSGSGS

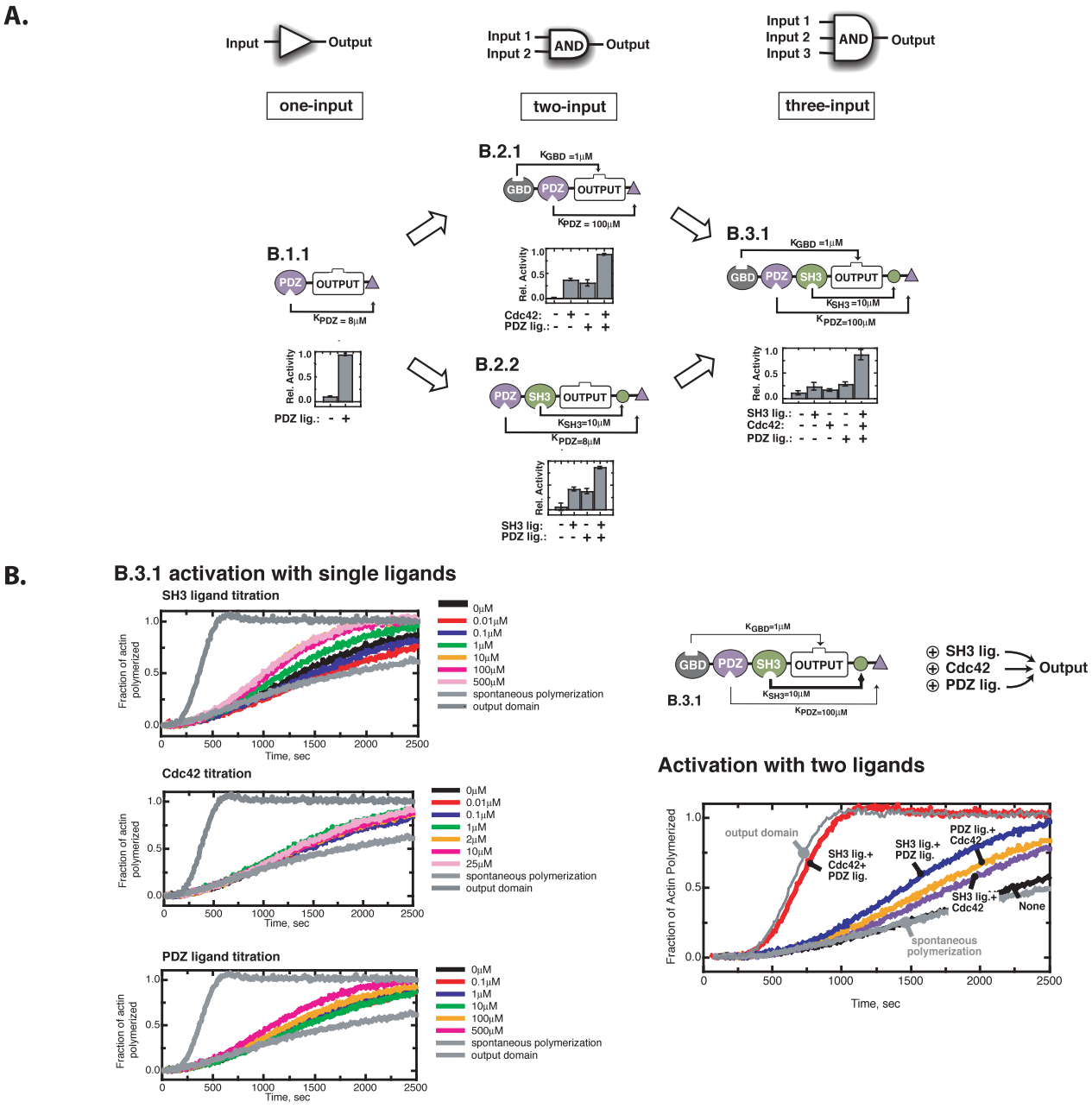
S2. **Composition of switch linkers.** Poly-Ser-Gly linkers were used to connect interaction domains and motifs. In the SH3-ligand responsive switches, amino acids other than Ser and Gly are present as an artifact of unique restriction enzyme sites engineered in the gene sequence to aid sequential addition of domains and motifs. All linkers were designed to be long enough to allow all possible intramolecular interactions to form.



S3. **Intramolecular affinities must be properly tuned to generate a switch with targeted behavior: basal repression and sensitivity to external input.** Interactions that are too weak can lead to high levels of background basal activity (poor repression), while interactions that are too strong lead to switches that cannot be activated within the range of input concentrations examined here. As predicted by our model, higher valency switches require weaker individual modules to remain activateable under the same concentration range as low valency switches. For example, the single SH3 peptide interaction of K_D=10 μM (A.1.1b) is too weak to produce basal repression, but five cooperative interactions of this weak affinity (A.5.5b) yields a switch that exhibits extremely strong basal repression. Similarly, a construct containing a single K_D=0.1 μM SH3 peptide interaction (A.1.1a) is activatable within the input concentration range of <100 μM ligand, whereas a construct containing five K_D=0.1 μM SH3 peptide interactions (A.5.5a) cannot be activated within this range (overrepressed). Overall, these switches show a high degree of systematic, predictable structure/function behaviors.



S4. **Mutation of two peptide ligands in the five interaction switch (A.5.3b) reduces the Hill coefficient from ~4 to 2.5.** Two of **A.5.5b's** five SH3 ligands were mutated by converting the consensus binding motif PXXP to AXXA, resulting in switch **A.5.3.b**. Titration of external SH3 ligand resulted in cooperative activation with an nH~2.5, similar to the three domain/ligand switch, **A.3.3b**.



S5. **Design and behavior of an AND-gate for three independent inputs.** A. Incremental synthesis of signaling node with 3-input AND-gate behavior. Activity of the N-WASP output domain is measured as described as described in Supplementary Fig. 1. Previously characterized switches behave as follows: B.1.1 is a single input switch responding to PDZ ligand; B.2.2 is a two input switch responding to SH3 ligand and PDZ ligand (Supplementary Fig. 2). The novel hybrid switch, B.3.1, containing all three intramolecular interaction pairs is found to behave as a strong 3-input AND-gate that displays little presence of all three. Very little activation is also observed with any pair of two inputs. B. The three input AND-gate for the three heterologous signals SH3 ligand, Cdc42, and PDZ ligand produces little activation upon addition of any single input alone or two-input combinations. Titrations of each individual ligand showed only modest activation (left plot). Co-stimulation with two ligands together also exhibited only modest activation, whereas simulataneous addition of all three inputs yielded almost full activity (in this plot [SH3 ligand] = 1 μM, [PDZ ligand] = 500 μM, and [Cdc42] = 25 μM).

Table S1. Components used in switch construction and their properties

Output	***Source***	***Residues***		***Ref.***
Output	rat N-WASP	392-501		[24]
Regulatory Domains	***Source***	***Residues***		***Ref.***
PDZ	Mouse a-syntrophin (syn)	77-171		[25]
GBD	rat N-WASP	196-274		[26]
SH3	mouse Crk	134-191		[27]
Intramol. ligands	***Sequence***	***Partner***	***Kd (μM)***	***Ref.***
[1]PDZ lig.	GVKESLV	Syn PDZ	8	[23]
[1]PDZ lig.	GVKQSLL	Syn PDZ	100	[23]
SH3 lig.	PPPALPPKRRR	Crk SH3	0.1	[16]
SH3 lig.	PPPVPPRR	Crk SH3	10	[28]
Output (C helix)	Rat N-WASP residues 461-479 (within Output A and B)	GBD	1	[14]
Input Ligands	***Sequence***	***Partner***	***Kd (uM)***	***Ref.***
[1]PDZ lig.	Ac -YVKESLV-COOH	Syn PDZ	8	[23]
SH3 lig.	Ac -PPPALPPKRRR-CONH2	Crk SH3	0.1	[16]
Cdc42-GTPγS	Residues 1-179	GBD	0.1	[14]

Intramolecular ligand affinities for partner domains are reported as measured *in trans*.

[1]Affinities measured with peptides with an N-terminal tyrosine replacing the glycine to accurately measure peptide concentration. PDZ peptides were synthesized with a N-terminal acetyl group and a C-terminal carboxyl group.

Supplementary Methods

Protein construction, expression, and purification

Switch proteins. Component domains and binding motifs used for switch protein construction are listed in Supplementary Table 1. Plasmids bearing these component DNA sequences were amplified by multi-step polymerase chain reaction (PCR), using primers that encoded for the appropriate intramolecular ligand motifs and linkers. Poly-Ser-Gly repeat linkers between individual recognition domains and motifs were chosen for their predicted flexibility and designed to be long enough to allow all possible intramolecular domain/motif interactions to form. Many of these linkers also contain one or two amino acids other than Ser and Gly as an artifact of a unique restriction enzyme site engineered at these positions to allow assembly via multiple ligations (Supplementary Fig. 2). In this manner, SH3 domains could be sequentially added. SH3 binding motifs were similarly incorporated with the exception that these sequences were directly synthesized to have the appropriate cohesive ends when annealed. Thus, the final switch constructs could be readily adapted to increase the number of SH3 domains and motifs as well as modifying the motif affinity. Plasmids were harvested using *Escherichia coli* (XL1 Blue) and proteins were expressed as fusions to a cleavable hexahistidine tag (pET19-derived vector) [13] in a BLR-DE3 strain, as previously described [14]. Desired protein was purified by chromatography on Ni-NTA resin (Qiagen). The hexahistidine affinity tag was removed by incubation with tobacco etch virus (TEV) protease at 25°C for 2 hrs, after which the reaction mixture was passed over a second Ni-NTA column. Proteins were further purified using a Source Q column (Pharmacia).

Cdc42. A soluble fragment of human Cdc42 (residues 1-179), used as an input molecule, was expressed in *Escherichia coli* (BL21-DE3) as a hexahistidine fusion, and purified as described above for switch proteins. Purified Cdc42 was activated by incubating with 10 fold excess of GTPγS for 15 min at 30 °C, followed by addition of 50-fold excess $MgCl_2$ to quench the reaction. Charged Cdc42 was dialyzed in 50 mM KCl, 1 mM $MgSO_4$, 1 mM EGTA, and 10 mM imidazole (pH 7.0).

Peptide Synthesis. Peptides were synthesized using conventional solid-phase Fmoc-amino acid chemistry. SH3 and PDZ domain ligand peptides were synthesized on Rink-Amide resin (Novabiochem) and Wang resin (Novabiochem), respectively. All peptides were N-terminally acetylated, cleaved, and purified as previously described [15]. SH3-peptide affinities were previously measured [16-18], while PDZ-binding affinities were measured by competition with dansylated-peptide as described [15].

Actin Polymerization Assays

Reagent preparation. Actin was purified from rabbit muscle [19] and was pyrene-labeled as previously described [20]. Arp2/3 was purified from bovine brain by a two-step purification scheme adapted from [21]. Briefly, brains were homogenized in an equal volume of buffer (50 mM PIPES (pH 6.8), 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 0.1 PMSF, and 0.2 mM ATP) using a blender (Waring). Insoluble materials were separated by centrifugation and the supernatant was incubated with SP-sepharose (Amersham). Fractions eluted with the above buffer (with the

addition of 100 mM KCl) were applied to an affinity column composed of GST-tagged human WASP residues 418-502. Arp2/3 was eluted with 200 mM $MgCl_2$, 150 mM NaCl, 0.2 mM ATP, 1 mM DTT, 10mM imidazole (pH 7.5). Fractions containing protein were pooled and the concentration of $MgCl_2$ reduced to 0.2 mM by dialysis.

Pyrene Actin Polymerization Assay. Actin polymerization assays were performed essentially as previously described [22] with modifications for use of a SpectraMax Gemini XS (Molecular Devices) fluorescent plate reader (excitation: 365 nm; emission: 407 nm). Briefly, a solution consisting of 10% pyrene-labeled actin was converted from a Ca-ATP to a Mg-ATP form by addition of $MgCl_2$ and EGTA to final concentrations of 50 μM and 200 μM, respectively. This solution was incubated at 25°C for 10 min in a 96 half area well plate (Corning)(10 μL per well). To initiate polymerization, a solution containing Arp2/3, switch construct, and appropriate ligand, which had been preincubated for 10 min at 25°C, was added to the actin mix. Final assay conditions were 1.3 μM actin, 5 nM Arp2/3, 50 nM switch, 50 mM KCl, 1 mM $MgSO_4$, 1 mM EGTA, 0.2 mM ATP, 1 mM DTT, 3 μM $MgCl_2$, and 11.5 mM imidazole (pH 7.0) in a volume of 150 μL.

N-WASP actin polymerization activity was quantified as shown in Fig. S1, as previously described [23]. Briefly, the raw fluorescence values from pyrene actin polymerization assays were normalized relative to lower and upper baselines using the equation $(F_{data}-F_{low})/(F_{high}-F_{low})$. F_{data} describes the fluorescence for each time point and F_{low} and F_{high} are average fluorescences obtained at the lower and upper baselines, respectively. Reaction half-time ($t_{1/2}$) is defined as the time required to reach 50% polymerization. A simple metric for relative activity was based on

the experimentally measured halftime: relative activity = $(t_{1/2}^{max} - t_{1/2})/(t_{1/2}^{max} - t_{1/2}^{min})$ where $t_{1/2}^{max}$ is the halftime observed with no activator (roughly equivalent to spontaneous actin polymerization; approx. 2500 sec.) and $t_{1/2}^{min}$ is the halftime observed with the constitutively active output domain (approx. 550 sec.). Relative activity was always calculated using values for $t_{1/2}$, $t_{1/2}^{min}$, and $t_{1/2}^{max}$ measured simultaneously (same 96-well plate and reagents). Data from activation experiments were fit by nonlinear least squares methods using Profit software to the following equation:

$$\text{Relative activity} = (act)_0 + ((act)_{max} - (act)_0) * (X^{n_H}/(K_{act}^{n_H} + X^{n_H}))$$

Where act_o = minimum activity, act_{max} = maximum activity, X = [SH3 ligand], n_H = Hill coefficient, and K_{act} = apparent activation constant.

Modeling of Single Input Switch Behavior

1-Interaction Switch:

Notation

Objects:

P Peptide

X Protein in open state (unbound & active)

X_R Protein in repressed state

XP Protein bound to a peptide (bound and active)

Terms:

K_{INT} Equilibrium constant for internal peptide-domain (intra-molecular) interaction. Measured in isolation.

K_{EXT} Equilibrium constant for external peptide-domain (inter-molecular) interaction. Measured in isolation.

C_{EFF} "Local Concentration Effect" – Increase in affinity of the intra-molecular interaction due to all components being in the same molecule.

Assumptions

1. [Peptide] > [Switch Protein]
2. The active states have an activity of 100%; the repressed state has an activity of 0%.

Reactions

$$XP \underset{K_{EXT}}{\Leftrightarrow} X + P \underset{\frac{K_{INT}}{C_{EFF}}}{\Leftrightarrow} X_R + P$$

States and Equilibrium Equations

$$[X]$$
$$[XP] = \frac{[X][P]}{K_{EXT}}$$
$$[X_R] = \frac{[X] \cdot C_{EFF}}{K_{INT}}$$

Activity Equation

$$\text{Relative Activity} = \frac{[X] + [XP]}{[X] + [XP] + [X_R]}$$

$$= \frac{1 + \frac{[P]}{K_{EXT}}}{1 + \frac{[P]}{K_{EXT}} + \frac{C_{EFF}}{K_{INT}}}$$

3-Interaction Switch

Notation

Objects:

P	Peptide
X	Protein in open state (unbound & active)
X_{R1}	Protein in repressed state with 1 intra-molecular binding
X_{R2}	Protein in repressed state with 2 intra-molecular bindings
X_{R3}	Protein in repressed state with 3 intra-molecular bindings
XP	Protein bound to 1 peptide (bound and active)
XPP	Protein bound to 2 peptides (bound and active)
XPPP	Protein bound to 3 peptides (bound and active)

Terms:

K_{INT} Equilibrium constant for internal peptide-domain (intra-molecular) interaction. Measured in isolation.

K_{EXT} Equilibrium constant for external peptide-domain (inter-molecular) interaction. Measured in isolation.

C_{EFF} "Local Concentration Effect" – Increase in affinity of the intra-molecular interaction due to all components being in the same molecule.

C_1 "Cooperativity Factor" – Increase in affinity of the intra-molecular interaction due to 1 existing intra-molecular binding.

C_2 "Cooperativity Factor" – Increase in affinity of the intra-molecular interaction due to 2 existing intra-molecular bindings.

Assumptions

1. [Peptide] > [Switch Protein]
2. Binding order and position does not matter.
3. The active states have an activity of 100%; the repressed states have an activity of 0%.

Reactions

$$
\begin{array}{ccccccc}
X + P & \underset{K_{EXT}}{\Leftrightarrow} & XP + P & \underset{K_{EXT}}{\Leftrightarrow} & XPP + P & \underset{K_{EXT}}{\Leftrightarrow} & XPPP \\
\Updownarrow \frac{K_{INT}}{C_{EFF}} & & \Updownarrow \frac{K_{INT}}{C_{EFF}} & & \Updownarrow \frac{K_{INT}}{C_{EFF}} & & \\
X_{R1} + P & \underset{K_{EXT}}{\Leftrightarrow} & X_{R1}P + P & \underset{K_{EXT}}{\Leftrightarrow} & X_{R1}PP & & \\
\Updownarrow \frac{K_{INT}}{C_{EFF} \cdot C_1} & & \Updownarrow \frac{K_{INT}}{C_{EFF} \cdot C_1} & & & & \\
X_{R2} + P & \underset{K_{EXT}}{\Leftrightarrow} & X_{R2}P & & & & \\
\Updownarrow \frac{K_{INT}}{C_{EFF} \cdot C_2} & & & & & & \\
X_{R3} & & & & & &
\end{array}
$$

States and Equilibrium Equations

$$[X]$$

$$[XP] = \frac{[X][P]}{K_{EXT}}$$

$$[XPP] = \frac{[X][P]^2}{{K_{EXT}}^2}$$

$$[XPPP] = \frac{[X][P]^3}{{K_{EXT}}^3}$$

$$[X_{R1}] = \frac{[X] \cdot C_{EFF}}{K_{INT}}$$

$$[X_{R1}P] = \frac{[X][P] \cdot C_{EFF}}{K_{INT} \cdot K_{EXT}}$$

$$[X_{R1}PP] = \frac{[X][P]^2 \cdot C_{EFF}}{K_{INT} \cdot {K_{EXT}}^2}$$

$$[X_{R2}] = \frac{[X] \cdot C_{EFF} \cdot C_1}{{K_{INT}}^2}$$

$$[X_{R2}P] = \frac{[X][P] \cdot C_{EFF} \cdot C_1}{{K_{INT}}^2 \cdot K_{EXT}}$$

$$[X_{R3}] = \frac{[X] \cdot C_{EFF} \cdot C_1 \cdot C_2}{{K_{INT}}^3}$$

Activity Equation

$$
\begin{aligned}
\text{Relative Activity} = \frac{[X] + [XP] + [XPP] + [XPPP]}{} & \\
[X] + [XP] + [XPP] + [XPPP] & \\
+ [X_{R1}] + [X_{R1}P] + [X_{R1}PP] & \\
+ [X_{R2}] + [X_{R2}P] & \\
+ [X_{R3}] &
\end{aligned}
$$

$$= \frac{1 + \frac{[P]}{K_{EXT}} + \frac{[P]^2}{K_{EXT}{}^2} + \frac{[P]^3}{K_{EXT}{}^3}}{1 + \frac{[P]}{K_{EXT}} + \frac{[P]^2}{K_{EXT}{}^2} + \frac{[P]^3}{K_{EXT}{}^3} + \frac{C_{EFF}}{K_{INT}} + \frac{[P] \cdot C_{EFF}}{K_{INT} \cdot K_{EXT}} + \frac{[P]^2 \cdot C_{EFF}}{K_{INT} \cdot K_{EXT}{}^2} + \frac{C_{EFF} \cdot C_1}{K_{INT}{}^2} + \frac{[P] \cdot C_{EFF} \cdot C_1}{K_{INT}{}^2 \cdot K_{EXT}} + \frac{C_{EFF} \cdot C_1 \cdot C_2}{K_{INT}{}^3}}$$

5-Interaction Switch

Notation

Objects:

P	Peptide
X	Protein in open state (unbound & active)
X_{R1}	Protein in repressed state with 1 intra-molecular binding
X_{R2}	Protein in repressed state with 2 intra-molecular bindings
X_{R3}	Protein in repressed state with 3 intra-molecular bindings
X_{R4}	Protein in repressed state with 4 intra-molecular bindings
X_{R5}	Protein in repressed state with 5 intra-molecular bindings
XP	Protein bound to 1 peptide (bound and active)
XPP	Protein bound to 2 peptides (bound and active)
XPPP	Protein bound to 3 peptides (bound and active)
XPPPP	Protein bound to 4 peptides (bound and active)
XPPPPP	Protein bound to 5 peptides (bound and active)

Terms:

K_{INT} Equilibrium constant for internal peptide-domain (intra-molecular) interaction. Measured in isolation.

K_{EXT} Equilibrium constant for external peptide-domain (inter-molecular) interaction. Measured in isolation.

C_{EFF} "Local Concentration Effect" – Increase in affinity of the intra-molecular interaction due to all components being in the same molecule.

C_1 "Cooperativity Factor" – Increase in affinity of the intra-molecular interaction due to 1 existing intra-molecular binding.

C_2 "Cooperativity Factor" – Increase in affinity of the intra-molecular interaction due to 2 existing intra-molecular bindings.

C_3 "Cooperativity Factor" – Increase in affinity of the intra-molecular interaction due to 3 existing intra-molecular bindings.

C_4 "Cooperativity Factor" – Increase in affinity of the intra-molecular interaction due to 4 existing intra-molecular bindings.

Assumptions

1. [Peptide] > [Switch Protein]
2. Binding order and position does not matter.
3. The active states have an activity of 100%; the repressed states have an activity of 0%.

Reactions

$$
\begin{array}{ccccccccccc}
X+P & \underset{K_{EXT}}{\Leftrightarrow} & XP+P & \underset{K_{EXT}}{\Leftrightarrow} & XPP+P & \underset{K_{EXT}}{\Leftrightarrow} & XPPP+P & \underset{K_{EXT}}{\Leftrightarrow} & XPPPP+P & \underset{K_{EXT}}{\Leftrightarrow} & XPPPPP \\
\Updownarrow \frac{K_{INT}}{C_{EFF}} & & \Updownarrow \frac{K_{INT}}{C_{EFF}} & & \Updownarrow \frac{K_{INT}}{C_{EFF}} & & \Updownarrow \frac{K_{INT}}{C_{EFF}} & & \Updownarrow \frac{K_{INT}}{C_{EFF}} & & \\
X_{R1}+P & \underset{K_{EXT}}{\Leftrightarrow} & X_{R1}P+P & \underset{K_{EXT}}{\Leftrightarrow} & X_{R1}PP+P & \underset{K_{EXT}}{\Leftrightarrow} & X_{R1}PPP+P & \underset{K_{EXT}}{\Leftrightarrow} & X_{R1}PPPP & & \\
\Updownarrow \frac{K_{INT}}{C_{EFF}\cdot C_1} & & \Updownarrow \frac{K_{INT}}{C_{EFF}\cdot C_1} & & \Updownarrow \frac{K_{INT}}{C_{EFF}\cdot C_1} & & \Updownarrow \frac{K_{INT}}{C_{EFF}\cdot C_1} & & & & \\
X_{R2}+P & \underset{K_{EXT}}{\Leftrightarrow} & X_{R2}P+P & \underset{K_{EXT}}{\Leftrightarrow} & X_{R2}PP+P & \underset{K_{EXT}}{\Leftrightarrow} & X_{R2}PPP & & & & \\
\Updownarrow \frac{K_{INT}}{C_{EFF}\cdot C_1\cdot C_2} & & \Updownarrow \frac{K_{INT}}{C_{EFF}\cdot C_1\cdot C_2} & & \Updownarrow \frac{K_{INT}}{C_{EFF}\cdot C_1\cdot C_2} & & & & & & \\
X_{R3}+P & \underset{K_{EXT}}{\Leftrightarrow} & X_{R3}P+P & \underset{K_{EXT}}{\Leftrightarrow} & X_{R3}PP & & & & & & \\
\Updownarrow \frac{K_{INT}}{C_{EFF}\cdot C_1\cdot C_2\cdot C_3} & & \Updownarrow \frac{K_{INT}}{C_{EFF}\cdot C_1\cdot C_2\cdot C_3} & & & & & & & & \\
X_{R4}+P & \underset{K_{EXT}}{\Leftrightarrow} & X_{R4}P & & & & & & & & \\
\Updownarrow \frac{K_{INT}}{C_{EFF}\cdot C_1\cdot C_2\cdot C_3\cdot C_4} & & & & & & & & & & \\
X_{R5} & & & & & & & & & &
\end{array}
$$

States and Equilibrium Equations

$$[X]$$

$$[XP] = \frac{[X][P]}{K_{EXT}}$$

$$[XPP] = \frac{[X][P]^2}{{K_{EXT}}^2}$$

$$[XPPP] = \frac{[X][P]^3}{{K_{EXT}}^3}$$

$$[XPPPP] = \frac{[X][P]^4}{{K_{EXT}}^4}$$

$$[XPPPPP] = \frac{[X][P]^5}{{K_{EXT}}^5}$$

$$[X_{R1}] = \frac{[X]\cdot C_{EFF}}{K_{INT}}$$

$$[X_{R1}P] = \frac{[X][P]\cdot C_{EFF}}{K_{INT}\cdot K_{EXT}}$$

$$[X_{R1}PP] = \frac{[X][P]^2\cdot C_{EFF}}{K_{INT}\cdot {K_{EXT}}^2}$$

$$[X_{R1}PPP] = \frac{[X][P]^3\cdot C_{EFF}}{K_{INT}\cdot {K_{EXT}}^3}$$

$$[X_{R1}PPPP] = \frac{[X][P]^4\cdot C_{EFF}}{K_{INT}\cdot {K_{EXT}}^4}$$

$$[X_{R2}] = \frac{[X] \cdot C_{EFF} \cdot C_1}{K_{INT}{}^2}$$

$$[X_{R2}P] = \frac{[X][P] \cdot C_{EFF} \cdot C_1}{K_{INT}{}^2 \cdot K_{EXT}}$$

$$[X_{R2}PP] = \frac{[X][P]^2 \cdot C_{EFF} \cdot C_1}{K_{INT}{}^2 \cdot K_{EXT}{}^2}$$

$$[X_{R2}PPP] = \frac{[X][P]^3 \cdot C_{EFF} \cdot C_1}{K_{INT}{}^2 \cdot K_{EXT}{}^3}$$

$$[X_{R3}] = \frac{[X] \cdot C_{EFF} \cdot C_1 \cdot C_2}{K_{INT}{}^3}$$

$$[X_{R3}P] = \frac{[X][P] \cdot C_{EFF} \cdot C_1 \cdot C_2}{K_{INT}{}^3 \cdot K_{EXT}}$$

$$[X_{R3}PP] = \frac{[X][P]^2 \cdot C_{EFF} \cdot C_1 \cdot C_2}{K_{INT}{}^3 \cdot K_{EXT}{}^2}$$

$$[X_{R4}] = \frac{[X] \cdot C_{EFF} \cdot C_1 \cdot C_2 \cdot C_3}{K_{INT}{}^4}$$

$$[X_{R4}P] = \frac{[X][P] \cdot C_{EFF} \cdot C_1 \cdot C_2 \cdot C_3}{K_{INT}{}^4 \cdot K_{EXT}}$$

$$[X_{R5}] = \frac{[X] \cdot C_{EFF} \cdot C_1 \cdot C_2 \cdot C_3 \cdot C_4}{K_{INT}{}^5}$$

Activity Equation

$$\begin{aligned}\text{Relative Activity} = \frac{[X] + [XP] + [XPP] + [XPPP] + [XPPPP] + [XPPPPP]}{} \\ [X] + [XP] + [XPP] + [XPPP] + [XPPPP] + [XPPPPP] \\ + [X_{R1}] + [X_{R1}P] + [X_{R1}PP] + [X_{R1}PPP] + [X_{R1}PPPP] \\ + [X_{R2}] + [X_{R2}P] + [X_{R2}PP] + [X_{R2}PPP] \\ + [X_{R3}] + [X_{R3}P] + [X_{R3}PP] \\ + [X_{R4}] + [X_{R4}P] \\ + [X_{R5}]\end{aligned}$$

$$= \frac{1 + \frac{[P]}{K_{EXT}} + \frac{[P]^2}{K_{EXT}{}^2} + \frac{[P]^3}{K_{EXT}{}^3} + \frac{[P]^4}{K_{EXT}{}^4} + \frac{[P]^5}{K_{EXT}{}^5}}{1 + \frac{[P]}{K_E} + \frac{[P]^2}{K_{EXT}{}^2} + \frac{[P]^3}{K_{EXT}{}^3} + \frac{[P]^4}{K_{EXT}{}^4} + \frac{[P]^5}{K_{EXT}{}^5}}$$
$$+ \frac{C_{EFF}}{K_{INT}} + \frac{[P] \cdot C_{EFF}}{K_{INT} \cdot K_{EXT}} + \frac{[P]^2 \cdot C_{EFF}}{K_{INT} \cdot K_{EXT}{}^2} + \frac{[P]^3 \cdot C_{EFF}}{K_{INT} \cdot K_{EXT}{}^3} + \frac{[P]^4 \cdot C_{EFF}}{K_{INT} \cdot K_{EXT}{}^4}$$
$$+ \frac{C_{EFF} \cdot C_1}{K_{INT}{}^2} + \frac{[P] \cdot C_{EFF} \cdot C_1}{K_{INT}{}^2 \cdot K_{EXT}} + \frac{[P]^2 \cdot C_{EFF} \cdot C_1}{K_{INT}{}^2 \cdot K_{EXT}{}^2} + \frac{[P]^3 \cdot C_{EFF} \cdot C_1}{K_{INT}{}^2 \cdot K_{EXT}{}^3}$$
$$+ \frac{C_{EFF} \cdot C_1 \cdot C_2}{K_{INT}{}^3} + \frac{[P] \cdot C_{EFF} \cdot C_1 \cdot C_2}{K_{INT}{}^3 \cdot K_{EXT}} + \frac{[P]^2 \cdot C_{EFF} \cdot C_1 \cdot C_2}{K_{INT}{}^3 \cdot K_{EXT}{}^2}$$
$$+ \frac{C_{EFF} \cdot C_1 \cdot C_2 \cdot C_3}{K_{INT}{}^4} + \frac{[P] \cdot C_{EFF} \cdot C_1 \cdot C_2 \cdot C_3}{K_{INT}{}^4 \cdot K_{EXT}}$$
$$+ \frac{C_{EFF} \cdot C_1 \cdot C_2 \cdot C_3 \cdot C_4}{K_{INT}{}^5}$$

Switch DNA sequences

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A.1.1a
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A.1.1b
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A.3.3b
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A.5.5a
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A.5.5b
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A.5.4b
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A.5.3b

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A.1.3b
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