Crosstalk and competition in signaling networks

Supporting Material

Michael Rowland[1], Walter Fontana[2] and Eric J. Deeds[1,3]

[1]Center for Bioinformatics, The University of Kansas, 2030 Becker Dr., Lawrence, KS 66047, USA
[2]Department of Systems Biology, Harvard Medical School, 200 Longwood Avenue, Boston MA 02115, USA
[3]Department of Molecular Biosciences, The University of Kansas, 2030 Becker Dr., Lawrence, KS 66047, USA

Email: Michael Rowland - mrowland@ku.edu; Walter Fontana - walter@hms.harvard.edu; Eric Deeds - deeds@ku.edu;

Contents

1 Systems of Ordinary Differential Equations

1.1 1–Kinase/1–Phosphatase Loop with 2 Substrates

The set of enzymatic reactions for the 1K1P loop with two substrates is as in equation **[2]** of the main text:

$$S_1 + K \underset{k_{-,K,1}}{\overset{k_{+,K,1}}{\rightleftharpoons}} KS_1 \xrightarrow{k_{cat,K,1}} S_1^* + K$$
$$S_2 + K \underset{k_{-,K,2}}{\overset{k_{+,K,2}}{\rightleftharpoons}} KS_2 \xrightarrow{k_{cat,K,2}} S_2^* + K$$
$$S_1^* + P \underset{k_{-,P,1}}{\overset{k_{+,P,1}}{\rightleftharpoons}} PS_1^* \xrightarrow{k_{cat,P,1}} S_1 + P$$
$$S_2^* + P \underset{k_{-,P,2}}{\overset{k_{+,P,2}}{\rightleftharpoons}} PS_2^* \xrightarrow{k_{cat,P,2}} S_2 + P$$

Each contain three rates: rate of complex formation, (k_+), rate of complex dissociation (k_-), and catalytic rate (k_{cat}). The set of ODEs describing the free enzymes are:

$$\frac{d[K]}{dt} = -\left([S_1]\cdot[K]\cdot k_{+,K,1} + [S_2]\cdot[K]\cdot k_{+,K,2}\right) + \left([KS_1]\cdot(k_{-,K,1}+k_{cat,K,1}) + [KS_2]\cdot(k_{-,K,2}+k_{cat,K,2})\right)$$
$$\frac{d[P]}{dt} = -\left([S_1^*]\cdot[P]\cdot k_{+,P,1} + [S_2^*]\cdot[P]\cdot k_{+,P,2}\right) + \left([PS_1^*]\cdot(k_{-,P,1}+k_{cat,P,1}) + [PS_2^*]\cdot(k_{-,P,2}+k_{cat,P,2})\right)$$

The set of ODEs describing the unmodified substrates are:

$$\frac{d[S_1]}{dt} = -\left([S_1]\cdot[K]\cdot k_{+,K,1}\right) + \left([KS_1]\cdot k_{-,K,1} + [PS_1^*]\cdot k_{cat,P,1}\right)$$
$$\frac{d[S_2]}{dt} = -\left([S_2]\cdot[K]\cdot k_{+,K,2}\right) + \left([KS_2]\cdot k_{-,K,2} + [PS_2^*]\cdot k_{cat,P,2}\right)$$

The set of ODEs describing the modified substrates are:

$$\frac{d[S_1^*]}{dt} = -\left([S_1^*]\cdot[P]\cdot k_{+,P,1}\right) + \left([PS_1^*]\cdot k_{-,P,1} + [KS_1]\cdot k_{cat,K,1}\right)$$
$$\frac{d[S_2^*]}{dt} = -\left([S_2^*]\cdot[P]\cdot k_{+,P,2}\right) + \left([PS_2^*]\cdot k_{-,P,2} + [KS_2]\cdot k_{cat,K,2}\right)$$

The set of ODEs describing the enzyme-substrate complexes are:

$$\frac{d[KS_1]}{dt} = -\left([KS_1]\cdot(k_{-,K,1}+k_{cat,K,1})\right) + \left([S_1]\cdot[K]\cdot k_{+,K,1}\right)$$
$$\frac{d[KS_2]}{dt} = -\left([KS_2]\cdot(k_{-,K,2}+k_{cat,K,2})\right) + \left([S_2]\cdot[K]\cdot k_{+,K,2}\right)$$
$$\frac{d[PS_1^*]}{dt} = -\left([PS_1^*]\cdot(k_{-,P,1}+k_{cat,P,1})\right) + \left([S_1^*]\cdot[P]\cdot k_{+,P,1}\right)$$
$$\frac{d[PS_2^*]}{dt} = -\left([PS_2^*]\cdot(k_{-,P,2}+k_{cat,P,2})\right) + \left([S_2^*]\cdot[P]\cdot k_{+,P,2}\right)$$

For purposes of display in Fig. 2*A* of the main text we used the following values for each of the rate constants:

Parameter	Value
$k_{+,K,i}$	0.001 $\text{nM}^{-1}\cdot\text{s}^{-1}$
$k_{-,K,i}$	0.001 s^{-1}
$k_{cat,K,i}$	0.999 s^{-1}
$k_{+,P,i}$	0.001 $\text{nM}^{-1}\cdot\text{s}^{-1}$
$k_{-,P,i}$	0.001 s^{-1}
$k_{cat,P,i}$	0.999 s^{-1}

Where $i = 1$ or 2.

Our simulations started with the following initial concentrations:

Molecular Species	Initial Concentration
K	0 - 2 nM
P	1 nM
S_1	100 nM
S_2	0 - 20 μM

With the remaining molecular species having initial concentrations of 0. The range of initial concentrations of K and S_2 were used to vary r_1 and $[S_2]_0/K_m$, respectively, in Fig. 2*A* in the main text.

1.2 1–Kinase/1–Phosphatase Loop with Many Substrates

The set of enzymatic reactions for the 1K1P loop with many substrates is:

$$
\begin{aligned}
S_1 + K &\underset{k_{-,K,1}}{\overset{k_{+,K,1}}{\rightleftharpoons}} KS_1 \xrightarrow{k_{cat,K,1}} S_1^* + K \\
S_2 + K &\underset{k_{-,K,2}}{\overset{k_{+,K,2}}{\rightleftharpoons}} KS_2 \xrightarrow{k_{cat,K,2}} S_2^* + K \\
&\vdots \\
S_N + K &\underset{k_{-,K,N}}{\overset{k_{+,K,N}}{\rightleftharpoons}} KS_N \xrightarrow{k_{cat,K,N}} S_N^* + K
\end{aligned}
$$

$$
\begin{aligned}
S_1^* + P &\underset{k_{-,P,1}}{\overset{k_{+,P,1}}{\rightleftharpoons}} PS_1^* \xrightarrow{k_{cat,P,1}} S_1 + P \\
S_2^* + P &\underset{k_{-,P,2}}{\overset{k_{+,P,2}}{\rightleftharpoons}} PS_2^* \xrightarrow{k_{cat,P,2}} S_2 + P \\
&\vdots \\
S_N^* + P &\underset{k_{-,P,N}}{\overset{k_{+,P,N}}{\rightleftharpoons}} PS_N^* \xrightarrow{k_{cat,P,N}} S_N + P
\end{aligned}
$$

The set of ODEs describing the free enzymes are:

$$
\begin{aligned}
\frac{d[K]}{dt} =& -\left([S_1]\cdot[K]\cdot k_{+,K,1} + [S_2]\cdot[K]\cdot k_{+,K,2} + \ldots + [S_N]\cdot[K]\cdot k_{+,K,N}\right) \\
&+ \left([KS_1]\cdot(k_{-,K,1}+k_{cat,K,1}) + [KS_2]\cdot(k_{-,K,2}+k_{cat,K,2}) + \ldots + [KS_N]\cdot(k_{-,K,N}+k_{cat,K,N})\right) \\
\frac{d[P]}{dt} =& -\left([S_1^*]\cdot[P]\cdot k_{+,P,1} + [S_2^*]\cdot[P]\cdot k_{+,P,2} + \ldots + [S_N^*]\cdot[P]\cdot k_{+,P,N}\right) \\
&+ \left([PS_1^*]\cdot(k_{-,P,1}+k_{cat,P,1}) + [PS_2^*]\cdot(k_{-,P,2}+k_{cat,P,2}) + \ldots + [PS_N^*]\cdot(k_{-,P,N}+k_{cat,P,N})\right)
\end{aligned}
$$

The set of ODEs describing the unmodified substrates are:

$$
\begin{aligned}
\frac{d[S_1]}{dt} &= -\left([S_1]\cdot[K]\cdot k_{+,K,1}\right) + \left([KS_1]\cdot k_{-,K,1} + [PS_1^*]\cdot k_{cat,P,1}\right) \\
\frac{d[S_2]}{dt} &= -\left([S_2]\cdot[K]\cdot k_{+,K,2}\right) + \left([KS_2]\cdot k_{-,K,2} + [PS_2^*]\cdot k_{cat,P,2}\right) \\
&\vdots \\
\frac{d[S_N]}{dt} &= -\left([S_N]\cdot[K]\cdot k_{+,K,N}\right) + \left([KS_N]\cdot k_{-,K,N} + [PS_N^*]\cdot k_{cat,P,N}\right)
\end{aligned}
$$

The set of ODEs describing the modified substrates are:

$$
\begin{aligned}
\frac{d[S_1^*]}{dt} &= -\left([S_1^*]\cdot[P]\cdot k_{+,P,1}\right) + \left([PS_1^*]\cdot k_{-,P,1} + [KS_1]\cdot k_{cat,K,1}\right) \\
\frac{d[S_2^*]}{dt} &= -\left([S_2^*]\cdot[P]\cdot k_{+,P,2}\right) + \left([PS_2^*]\cdot k_{-,P,2} + [KS_2]\cdot k_{cat,K,2}\right) \\
&\vdots \\
\frac{d[S_N^*]}{dt} &= -\left([S_N^*]\cdot[P]\cdot k_{+,P,N}\right) + \left([PS_N^*]\cdot k_{-,P,N} + [KS_N]\cdot k_{cat,K,N}\right)
\end{aligned}
$$

The set of ODEs describing the enzyme-substrate complexes are:

$$\begin{aligned}
\frac{d[KS_1]}{dt} &= -\left([KS_1]\cdot(k_{-,K,1}+k_{cat,K,1})\right)+\left([S_1]\cdot[K]\cdot k_{+,K,1}\right)\\
\frac{d[KS_2]}{dt} &= -\left([KS_2]\cdot(k_{-,K,2}+k_{cat,K,2})\right)+\left([S_2]\cdot[K]\cdot k_{+,K,2}\right)\\
&\vdots\\
\frac{d[KS_N]}{dt} &= -\left([KS_N]\cdot(k_{-,K,N}+k_{cat,K,N})\right)+\left([S_N]\cdot[K]\cdot k_{+,K,N}\right)\\
\frac{d[PS_1^*]}{dt} &= -\left([PS_1^*]\cdot(k_{-,P,1}+k_{cat,P,1})\right)+\left([S_1^*]\cdot[P]\cdot k_{+,P,1}\right)\\
\frac{d[PS_2^*]}{dt} &= -\left([PS_2^*]\cdot(k_{-,P,2}+k_{cat,P,2})\right)+\left([S_2^*]\cdot[P]\cdot k_{+,P,2}\right)\\
&\vdots\\
\frac{d[PS_N^*]}{dt} &= -\left([PS_N^*]\cdot(k_{-,P,N}+k_{cat,P,N})\right)+\left([S_N^*]\cdot[P]\cdot k_{+,P,N}\right)
\end{aligned}$$

The following values for rate constants were used in the simulations presented in Fig. $2B$ of the main text:

Parameter	Value
$k_{+,K,i}$	0.001 $\text{nM}^{-1}\cdot\text{s}^{-1}$
$k_{-,K,i}$	0.001 s^{-1}
$k_{cat,K,i}$	0.999 s^{-1}
$k_{+,P,i}$	0.001 $\text{nM}^{-1}\cdot\text{s}^{-1}$
$k_{-,P,i}$	0.001 s^{-1}
$k_{cat,P,i}$	0.999 s^{-1}

The different molecular species were initialized with concentrations:

Molecular Species	Initial Concentration
K	0 - 2 nM
P	1 nM
S_i	500 nM

$i = 1, 2, \ldots, N$

The remaining molecular species had initial concentrations of 0. The range of initial concentrations of K was used to vary the value of r_1, and N was varied to obtain the surface in Fig. $2B$ in the main text.

1.3 1–Kinase/2–Phosphatase Loop

The set of enzymatic reactions for the 1K2P loop is:

$$S_1 + K \underset{k_{-,K,1}}{\overset{k_{+,K,1}}{\rightleftharpoons}} KS_1 \xrightarrow{k_{cat,K,1}} S_1^* + K$$

$$S_2 + K \underset{k_{-,K,2}}{\overset{k_{+,K,2}}{\rightleftharpoons}} KS_2 \xrightarrow{k_{cat,K,2}} S_2^* + K$$

$$S_1^* + P_1 \underset{k_{-,P,1}}{\overset{k_{+,P,1}}{\rightleftharpoons}} P_1S_1^* \xrightarrow{k_{cat,P,1}} S_1 + P_1$$

$$S_2^* + P_2 \underset{k_{-,P,2}}{\overset{k_{+,P,2}}{\rightleftharpoons}} P_2S_2^* \xrightarrow{k_{cat,P,2}} S_2 + P_2$$

The set of ODEs describing the free enzymes are:

$$\frac{d[K]}{dt} = -\left([S_1]\cdot[K]\cdot k_{+,K,1} + [S_2]\cdot[K]\cdot k_{+,K,2}\right) + \left([KS_1]\cdot(k_{-,K,1}+k_{cat,K,1}) + [KS_2]\cdot(k_{-,K,2}+k_{cat,K,2})\right)$$
$$\frac{d[P_1]}{dt} = -\left([S_1^*]\cdot[P_1]\cdot k_{+,P,1}\right) + \left([P_1S_1^*]\cdot(k_{-,P,1}+k_{cat,P,1})\right)$$
$$\frac{d[P_2]}{dt} = -\left([S_2^*]\cdot[P_2]\cdot k_{+,P,2}\right) + \left([P_2S_2^*]\cdot(k_{-,P,2}+k_{cat,P,2})\right)$$

The set of ODEs describing the unmodified substrates are:

$$\frac{d[S_1]}{dt} = -\left([S_1]\cdot[K]\cdot k_{+,K,1}\right) + \left([KS_1]\cdot k_{-,K,1} + [P_1S_1^*]\cdot k_{cat,P,1}\right)$$
$$\frac{d[S_2]}{dt} = -\left([S_2]\cdot[K]\cdot k_{+,K,2}\right) + \left([KS_2]\cdot k_{-,K,2} + [P_2S_2^*]\cdot k_{cat,P,2}\right)$$

The set of ODEs describing the modified substrates are:

$$\frac{d[S_1^*]}{dt} = -\left([S_1^*]\cdot[P_1]\cdot k_{+,P,1}\right) + \left([P_1S_1^*]\cdot k_{-,P,1} + [KS_1]\cdot k_{cat,K,1}\right)$$
$$\frac{d[S_2^*]}{dt} = -\left([S_2^*]\cdot[P_2]\cdot k_{+,P,2}\right) + \left([P_2S_2^*]\cdot k_{-,P,2} + [KS_2]\cdot k_{cat,K,2}\right)$$

The set of ODEs describing the enzyme-substrate complexes are:

$$\frac{d[KS_1]}{dt} = -\left([KS_1]\cdot(k_{-,K,1}+k_{cat,K,1})\right) + \left([S_1]\cdot[K]\cdot k_{+,K,1}\right)$$
$$\frac{d[KS_2]}{dt} = -\left([KS_2]\cdot(k_{-,K,2}+k_{cat,K,2})\right) + \left([S_2]\cdot[K]\cdot k_{+,K,2}\right)$$
$$\frac{d[P_1S_1^*]}{dt} = -\left([P_1S_1^*]\cdot(k_{-,P,1}+k_{cat,P,1})\right) + \left([S_1^*]\cdot[P_1]\cdot k_{+,P,1}\right)$$
$$\frac{d[P_2S_2^*]}{dt} = -\left([P_2S_2^*]\cdot(k_{-,P,2}+k_{cat,P,2})\right) + \left([S_2^*]\cdot[P_2]\cdot k_{+,P,2}\right)$$

For purposes of display in Figs. 3A and B in the main text, we used the following parameters in the model:

Parameter	Value
$k_{+,K,i}$	0.001 nM^{-1}·s^{-1}
$k_{-,K,i}$	0.001 s^{-1}
$k_{cat,K,i}$	0.999 s^{-1}
$k_{+,P,i}$	0.001 nM^{-1}·s^{-1}
$k_{-,P,i}$	0.001 s^{-1}
$k_{cat,P,i}$	0.999 s^{-1}

$i = 1$ or 2

Each of the molecular species in the model started with the following initial concentrations:

Molecular Species	Initial Concentration
K	1 nM
P_1	0.5-100 nM
P_2	0.5-100 nM
S_1	100 nM
S_2	0, 20 μM

The remaining molecular species had initial concentrations of 0. The range of initial concentrations for P_1 and P_2 were used to independently set r_1 and r_2, respectively, in Figs. 3A and B in the main text. In Fig. 3A $[S_2]_0 = 0$ and in Fig 3B $[S_2]_0 = 20nM$.

1.4 2–Kinase/1–Phosphatase Loop

The set of enzymatic reactions for the 2K1P loop is:

$$S_1 + K_1 \underset{k_{-,K,1}}{\overset{k_{+,K,1}}{\rightleftharpoons}} K_1S_1 \xrightarrow{k_{cat,K,1}} S_1^* + K_1$$

$$S_2 + K_2 \underset{k_{-,K,2}}{\overset{k_{+,K,2}}{\rightleftharpoons}} K_2S_2 \xrightarrow{k_{cat,K,2}} S_2^* + K_2$$

$$S_1^* + P \underset{k_{-,P,1}}{\overset{k_{+,P,1}}{\rightleftharpoons}} PS_1^* \xrightarrow{k_{cat,P,1}} S_1 + P$$

$$S_2^* + P \underset{k_{-,P,2}}{\overset{k_{+,P,2}}{\rightleftharpoons}} PS_2^* \xrightarrow{k_{cat,P,2}} S_2 + P$$

The set of ODEs describing the free enzymes are:

$$\frac{d[K_1]}{dt} = -\left([S_1]\cdot[K_1]\cdot k_{+,K,1}\right) + \left([K_1S_1]\cdot(k_{-,K,1}+k_{cat,K,1})\right)$$
$$\frac{d[K_2]}{dt} = -\left([S_2]\cdot[K_2]\cdot k_{+,K,2}\right) + \left([K_2S_2]\cdot(k_{-,K,2}+k_{cat,K,2})\right)$$
$$\frac{d[P]}{dt} = -\left([S_1^*]\cdot[P]\cdot k_{+,P,1} + [S_2^*]\cdot[P]\cdot k_{+,P,2}\right) + \left([PS_1^*]\cdot(k_{-,P,1}+k_{cat,P,1}) + [PS_2^*]\cdot(k_{-,P,2}+k_{cat,P,2})\right)$$

The set of ODEs describing the unmodified substrates are:

$$\frac{d[S_1]}{dt} = -\left([S_1]\cdot[K_1]\cdot k_{+,K,1}\right) + \left([K_1S_1]\cdot k_{-,K,1} + [PS_1^*]\cdot k_{cat,P,1}\right)$$
$$\frac{d[S_2]}{dt} = -\left([S_2]\cdot[K_2]\cdot k_{+,K,2}\right) + \left([K_2S_2]\cdot k_{-,K,2} + [PS_2^*]\cdot k_{cat,P,2}\right)$$

The set of ODEs describing the modified substrates are:

$$\frac{d[S_1^*]}{dt} = -\left([S_1^*]\cdot[P]\cdot k_{+,P,1}\right) + \left([PS_1^*]\cdot k_{-,P,1} + [K_1S_1]\cdot k_{cat,K,1}\right)$$
$$\frac{d[S_2^*]}{dt} = -\left([S_2^*]\cdot[P]\cdot k_{+,P,2}\right) + \left([PS_2^*]\cdot k_{-,P,2} + [K_2S_2]\cdot k_{cat,K,2}\right)$$

The set of ODEs describing the enzyme-substrate complexes are:

$$\frac{d[K_1S_1]}{dt} = -\left([K_1S_1]\cdot(k_{-,K,1}+k_{cat,K,1})\right) + \left([S_1]\cdot[K_1]\cdot k_{+,K,1}\right)$$
$$\frac{d[K_2S_2]}{dt} = -\left([K_2S_2]\cdot(k_{-,K,2}+k_{cat,K,2})\right) + \left([S_2]\cdot[K_2]\cdot k_{+,K,2}\right)$$
$$\frac{d[PS_1^*]}{dt} = -\left([PS_1^*]\cdot(k_{-,P,1}+k_{cat,P,1})\right) + \left([S_1^*]\cdot[P]\cdot k_{+,P,1}\right)$$
$$\frac{d[PS_2^*]}{dt} = -\left([PS_2^*]\cdot(k_{-,P,2}+k_{cat,P,2})\right) + \left([S_2^*]\cdot[P]\cdot k_{+,P,2}\right)$$

For purposes of display in Figs. 3*A* and *C* in the main text we used the following parameters:

Parameter	Value
$k_{+,K,i}$	0.001 $\text{nM}^{-1}\cdot\text{s}^{-1}$
$k_{-,K,i}$	0.001 s^{-1}
$k_{cat,K,i}$	0.999 s^{-1}
$k_{+,P,i}$	0.001 $\text{nM}^{-1}\cdot\text{s}^{-1}$
$k_{-,P,i}$	0.001 s^{-1}
$k_{cat,P,i}$	0.999 s^{-1}

$i = 1$ or 2

Each molecular species were initialized at the following concentrations:

Molecular Species	Initial Concentration
K_1	0 - 2 nM
K_2	0 - 2 nM
P	1 nM
S_1	100 nM
S_2	0, 20 μM

The remaining molecular species had initial concentrations of 0. The range of initial concentrations of K_1 and K_2 were used to set the values of r_1 and r_2, respectively, in Figs. $3A$ and C in the main text. In Fig. $3A$, $[S_2]_0 = 0$ and in Fig. $3C$, $[S_2]_0 = 20nM$.

1.5 Cascade with Multiple Phosphatases

The set of kinase enzymatic reactions for the cascade with multiple phosphatases is:

$$
\begin{aligned}
S_1 + K &\underset{k_{-,K,1}}{\overset{k_{+,K,1}}{\rightleftharpoons}} KS_1 \xrightarrow{k_{cat,K,1}} S_1^* + K \\
S_2 + S_1^* &\underset{k_{-,K,2}}{\overset{k_{+,K,2}}{\rightleftharpoons}} S_1^* S_2 \xrightarrow{k_{cat,K,2}} S_2^* + S_1^* \\
&\vdots \\
S_N + S_{N-1}^* &\underset{k_{-,K,N}}{\overset{k_{+,K,N}}{\rightleftharpoons}} S_{N-1}^* S_N \xrightarrow{k_{cat,K,N}} S_N^* + S_{N-1}^*
\end{aligned}
$$

Note that K is the input kinase and S_i^* serves as the kinase for S_{i+1}. The set of phosphatase enzymatic reactions is:

$$
\begin{aligned}
S_1^* + P_1 &\underset{k_{-,P,1}}{\overset{k_{+,P,1}}{\rightleftharpoons}} P_1 S_1^* \xrightarrow{k_{cat,P,1}} S_1 + P_1 \\
S_2^* + P_2 &\underset{k_{-,P,2}}{\overset{k_{+,P,2}}{\rightleftharpoons}} P_2 S_2^* \xrightarrow{k_{cat,P,2}} S_2 + P_2 \\
&\vdots \\
S_N^* + P_N &\underset{k_{-,P,N}}{\overset{k_{+,P,N}}{\rightleftharpoons}} P_N S_N^* \xrightarrow{k_{cat,P,N}} S_N + P_N
\end{aligned}
$$

The set of ODEs describing the free enzymes are:

$$\begin{aligned}
\frac{d[K]}{dt} &= -\left([S_1]\cdot[K]\cdot k_{+,K,1}\right) + \left([KS_1]\cdot(k_{-,K,1}+k_{cat,K,1})\right)\\
\frac{d[P_1]}{dt} &= -\left([S_1^*]\cdot[P_1]\cdot k_{+,P,1}\right) + \left([P_1S_1^*]\cdot(k_{-,P,1}+k_{cat,P,1})\right)\\
\frac{d[P_i]}{dt} &= -\left([S_i^*]\cdot[P_i]\cdot k_{+,P,i}\right) + \left([P_iS_i^*]\cdot(k_{-,P,i}+k_{cat,P,i})\right)\\
\frac{d[P_N]}{dt} &= -\left([S_N^*]\cdot[P_N]\cdot k_{+,P,N}\right) + \left([P_NS_N^*]\cdot(k_{-,P,N}+k_{cat,P,N})\right)
\end{aligned}$$

The set of ODEs describing the unmodified substrates are:

$$\begin{aligned}
\frac{d[S_1]}{dt} &= -\left([S_1]\cdot[K]\cdot k_{+,K,1}\right) + \left([KS_1]\cdot k_{-,K,1} + [P_1S_1^*]\cdot k_{cat,P,1}\right)\\
\frac{d[S_i]}{dt} &= -\left([S_i]\cdot[S_{i-1}^*]\cdot k_{+,K,i}\right) + \left([S_{i-1}^*S_i]\cdot k_{-,K,i} + [P_iS_i^*]\cdot k_{cat,P,i}\right)\\
\frac{d[S_N]}{dt} &= -\left([S_N]\cdot[S_{N-1}^*]\cdot k_{+,K,N}\right) + \left([S_{N-1}^*S_N]\cdot k_{-,K,N} + [P_NS_N^*]\cdot k_{cat,P,N}\right)
\end{aligned}$$

The set of ODEs describing the modified substrates are:

$$\begin{aligned}
\frac{d[S_1^*]}{dt} &= -\left([S_1^*]\cdot[P]\cdot k_{+,K,1}\right) + [S_2]\cdot[S_1^*]\cdot k_{+,K,2})\\
&\quad + \left([P_1S_1^*]\cdot k_{-,P,1} + [KS_1]\cdot k_{cat,K,1} + [S_1^*S_2]\cdot(k_{-,K,2}+k_{cat,K,2})\right)\\
\frac{d[S_i^*]}{dt} &= -\left([S_i^*]\cdot[P_i]\cdot k_{+,K,i}\right) + [S_{i+1}]\cdot[S_i^*] * k_{+,K,i+1})\\
&\quad + \left([P_iS_i^*]\cdot k_{-,P,i} + [S_{i-1}^*S_i]\cdot k_{cat,K,i} + [S_i^*S_{i+1}]\cdot(k_{-,K,i+1}+k_{cat,K,i+1})\right)\\
\frac{d[S_N^*]}{dt} &= -\left([S_N^*]\cdot[P_N]\cdot k_{+,K,N}\right) + \left([P_NS_N^*]\cdot k_{-,P,N} + [S_{N-1}^*S_N]\cdot k_{cat,K,N}\right)
\end{aligned}$$

The set of ODEs describing the enzyme-substrate complexes are:

$$\begin{aligned}
\frac{d[KS_1]}{dt} &= -\left([KS_1]\cdot(k_{-,K,1}+k_{cat,K,1})\right) + \left([S_1]\cdot[K]\cdot k_{+,K,1}\right)\\
\frac{d[S_{i-1}^*S_i]}{dt} &= -\left([S_{i-1}^*S_i]\cdot(k_{-,K,i}+k_{cat,K,i})\right) + \left([S_i]\cdot[S_{i-1}^*]\cdot k_{+,K,i}\right)\\
\frac{d[S_{N-1}^*S_N]}{dt} &= -\left([S_{N-1}^*S_N]\cdot(k_{-,K,N}+k_{cat,K,N})\right) + \left([S_N]\cdot[S_{N-1}^*]\cdot k_{+,K,N}\right)\\
\frac{d[P_1S_1^*]}{dt} &= -\left([P_1S_1^*]\cdot(k_{-,P,1}+k_{cat,P,1})\right) + \left([S_1^*]\cdot[P_1]\cdot k_{+,P,1}\right)\\
\frac{d[P_iS_i^*]}{dt} &= -\left([P_iS_i^*]\cdot(k_{-,P,i}+k_{cat,P,i})\right) + \left([S_i^*]\cdot[P_i]\cdot k_{+,P,i}\right)\\
\frac{d[P_NS_N^*]}{dt} &= -\left([P_NS_N^*]\cdot(k_{-,P,N}+k_{cat,P,N})\right) + \left([S_N^*]\cdot[P_N]\cdot k_{+,P,N}\right)
\end{aligned}$$

Where $i = 2, \ldots, N-1$.

For purposes of display in Fig. $3D$ in the main text, we used the following parameters:

Parameter	Value
$k_{+,K,i}$	0.001 $\text{nM}^{-1}\cdot\text{s}^{-1}$
$k_{-,K,i}$	10^{i-8} s^{-1}
$k_{cat,K,i}$	$0.999 \cdot 10^{i-5}$ s^{-1}
$k_{+,P,i}$	0.001 $\text{nM}^{-1}\cdot\text{s}^{-1}$
$k_{-,P,i}$	10^{i-8} s^{-1}
$k_{cat,P,i}$	$0.999 \cdot 10^{i-5}$ s^{-1}

$i = 1, 2, \ldots, N$

The k_{cat}'s and k_{-}'s were calculated as $0.999 \cdot 10^{i-5}$ s^{-1} and 10^{i-8} s^{-1}, respectively; the kinetic parameters of reaction i in the cascade were thus varied so that each substrate concentration was $10 \cdot K_m$ in respect to its kinase and phosphatase.

The molecular species in the system started with the following initial concentrations:

Molecular Species	Initial Concentration
K	10^{-18} - 0.1 nM
P_i	0.01 nM
S_1	1 nM
S_2	10 nM
$\vdots$	$\vdots$
S_i	$10 \cdot S_{i-1}$
$\vdots$	$\vdots$
S_{N}	$10 \cdot S_{\text{N-1}}$

$i = 1, 2, \ldots, N$

The remaining molecular species had initial concentrations of 0. We systematically increased the initial concentration of the S_i's ($[S_i]_0 = 10 \cdot [S_{i-1}]_0$); since S^*_{i-1} is the kinase for S_i, this ensured that all substrates were at higher concentrations than their enzymes. The range of initial concentrations of K were used to vary the value of r in Fig. $3D$.

1.6 Cascade with a Single Phosphatase

The set of kinase enzymatic reactions for the cascade with a single phosphatase is:

$$
\begin{aligned}
S_1 + K \quad &\underset{k_{-,K,1}}{\overset{k_{+,K,1}}{\rightleftharpoons}} \quad KS_1 \quad \xrightarrow{k_{cat,K,1}} \quad S_1^* + K \\
S_2 + S_1^* \quad &\underset{k_{-,K,2}}{\overset{k_{+,K,2}}{\rightleftharpoons}} \quad S_1^* S_2 \quad \xrightarrow{k_{cat,K,2}} \quad S_2^* + S_1^* \\
&\vdots \\
S_N + S_{N-1}^* \quad &\underset{k_{-,K,N}}{\overset{k_{+,K,N}}{\rightleftharpoons}} \quad S_{N-1}^* S_N \quad \xrightarrow{k_{cat,K,N}} \quad S_N^* + S_{N-1}^*
\end{aligned}
$$

The set of phosphatase enzymatic reactions is:

$$
\begin{aligned}
S_1^* + P \quad &\underset{k_{-,P,1}}{\overset{k_{+,P,1}}{\rightleftharpoons}} \quad PS_1^* \quad \xrightarrow{k_{cat,P,1}} \quad S_1 + P \\
S_2^* + P \quad &\underset{k_{-,P,2}}{\overset{k_{+,P,2}}{\rightleftharpoons}} \quad PS_2^* \quad \xrightarrow{k_{cat,P,2}} \quad S_2 + P \\
&\vdots \\
S_N^* + P \quad &\underset{k_{-,P,N}}{\overset{k_{+,P,N}}{\rightleftharpoons}} \quad PS_N^* \quad \xrightarrow{k_{cat,P,N}} \quad S_N + P
\end{aligned}
$$

The set of ODEs describing free enzymes are:

$$
\begin{aligned}
\frac{d[K]}{dt} &= -\left([S_1]\cdot[K]\cdot k_{+,K,1}\right) + \left([KS_1]\cdot(k_{-,K,1}+k_{cat,K,1})\right) \\
\frac{d[P]}{dt} &= -\left([S_1^*]\cdot[P]\cdot k_{+,P,1} + [S_2^*]\cdot k_{+,P,2} + \ldots + [S_N^*]\cdot k_{+,P,N}\right) \\
&\quad + \left([PS_1^*]\cdot(k_{-,P,1}+k_{cat,P,1}) + [PS_2^*]\cdot(k_{-,P,2}+k_{cat,P,2}) + \ldots + [PS_N^*]\cdot(k_{-,P,N}+k_{cat,P,N})\right)
\end{aligned}
$$

The set of ODEs describing unmodified substrates are:

$$
\begin{aligned}
\frac{d[S_1]}{dt} &= -\left([S_1]\cdot[K]\cdot k_{+,K,1}\right) + \left([KS_1]\cdot k_{-,K,1} + [PS_1^*]\cdot k_{cat,P,1}\right) \\
\frac{d[S_i]}{dt} &= -\left([S_i]\cdot[S_{i-1}^*]\cdot k_{+,K,i}\right) + \left([S_{i-1}^* S_i]\cdot k_{-,K,i} + [PS_i^*]\cdot k_{cat,P,i}\right) \\
\frac{d[S_N]}{dt} &= -\left([S_N]\cdot[S_{N-1}^*]\cdot k_{+,K,N}\right) + \left([S_{N-1}^* S_N]\cdot k_{-,K,N} + [PS_N^*]\cdot k_{cat,P,N}\right)
\end{aligned}
$$

The set of ODEs describing modified substrates are:

$$\begin{aligned}
\frac{d[S_1^*]}{dt} &= -\left([S_1^*]\cdot[P]\cdot k_{+,K,1}\right) + [S_2]\cdot[S_1^*] * k_{+,K,2}) \\
&\quad + \left([PS_1^*]\cdot k_{-,P,1} + [KS_1]\cdot k_{cat,K,1} + [S_1^* S_2]\cdot(k_{-,K,2} + k_{cat,K,2})\right) \\
\frac{d[S_i^*]}{dt} &= -\left([S_i^*]\cdot[P]\cdot k_{+,K,i}\right) + [S_{i+1}]\cdot[S_i^*] * k_{+,K,i+1}) \\
&\quad + \left([PS_i^*]\cdot k_{-,P,i} + [S_{i-1}^* S_i]\cdot k_{cat,K,i} + [S_i^* S_{i+1}]\cdot(k_{-,K,i+1} + k_{cat,K,i+1})\right) \\
\frac{d[S_N^*]}{dt} &= -\left([S_N^*]\cdot[P]\cdot k_{+,K,N})\right) + \left([PS_N^*]\cdot k_{-,P,N} + [S_{N-1}^* S_N]\cdot k_{cat,K,N}\right)
\end{aligned}$$

The set of ODEs describing enzyme-substrate complexes are:

$$\begin{aligned}
\frac{d[KS_1]}{dt} &= -\left([KS_1]\cdot(k_{-,K,1} + k_{cat,K,1})\right) + \left([S_1]\cdot[K]\cdot k_{+,K,1}\right) \\
\frac{d[PS_1^*]}{dt} &= -\left([PS_1^*]\cdot(k_{-,P,1} + k_{cat,P,1})\right) + \left([S_1^*]\cdot[P]\cdot k_{+,P,1}\right) \\
\frac{d[S_{i-1}^* S_i]}{dt} &= -\left([S_{i-1}^* S_i]\cdot(k_{-,K,i} + k_{cat,K,i})\right) + \left([S_i]\cdot[S_{i-1}^*]\cdot k_{+,K,i}\right) \\
\frac{d[PS_i^*]}{dt} &= -\left([PS_i^*]\cdot(k_{-,P,i} + k_{cat,P,i})\right) + \left([S_i^*]\cdot[P]\cdot k_{+,P,i}\right) \\
\frac{d[S_{N-1}^* S_N]}{dt} &= -\left([S_{N-1}^* S_N]\cdot(k_{-,K,N} + k_{cat,K,N})\right) + \left([S_N]\cdot[S_{N-1}^*]\cdot k_{+,K,N}\right) \\
\frac{d[P_N S_N^*]}{dt} &= -\left([PS_N^*]\cdot(k_{-,P,N} + k_{cat,P,N})\right) + \left([S_N^*]\cdot[P]\cdot k_{+,P,N}\right)
\end{aligned}$$

Where $i = 2, \ldots, N-1$.

For purposes of display in Fig. 3*D*, we used the following parameters:

Parameter	Value
$k_{+,K,i}$	0.001 $\text{nM}^{-1}\cdot\text{s}^{-1}$
$k_{-,K,i}$	(10^{i-8}) s^{-1}
$k_{cat,K,i}$	$(0.999\cdot 10^{i-5})$ s^{-1}
$k_{+,P,i}$	0.001 $\text{nM}^{-1}\cdot\text{s}^{-1}$
$k_{-,P,i}$	(10^{i-8}) s^{-1}
$k_{cat,P,i}$	$(0.999\cdot 10^{i-5})$ s^{-1}

The k_{cat}'s and k_-'s were calculated as in section 1.5.

The molecular species were initialized at the following concentrations:

Molecular Species	Initial Concentration
K	10^{-18} - 0.1 nM
P	0.01 nM
S_1	1 nM
S_2	10 nM
$\vdots$	$\vdots$
S_i	$10 \cdot S_{i-1}$
$\vdots$	$\vdots$
S_{N}	$10 \cdot S_{\mathrm{N\text{-}1}}$

$i = 1, 2, \ldots, N$

Remaining molecular species were set with initial concentrations of 0. Increasing the initial concentrations of S_i ensured that $[S_i]_0 = 10 \cdot [S_{i-1}]_0$ since S^*_{i-1} is the kinase for S_i to ensure that the concentration of substrates were larger than the concentrations of their respective kinases. The range of initial concentrations of K were used to vary the value of r in Fig. 3*D*.

2 Analytical Results for the 1–Kinase/1–Phosphatase Loop

2.1 Mutual inhibition for competitive substrates

Here we will show that the 1K1P loop displays behavior dependent on r without regard for other parameters. The enzymatic reactions for an enzyme with two substrates can be written as:

$$E + S_1 \underset{k_{-,E,1}}{\overset{k_{+,E,1}}{\rightleftharpoons}} ES_1 \xrightarrow{k_{cat,E,1}} E + S_1^*$$
$$E + S_2 \underset{k_{-,E,2}}{\overset{k_{+,E,2}}{\rightleftharpoons}} ES_2 \xrightarrow{k_{cat,K,2}} E + S_2^*$$

with $E = K$ or P. The Michaelis-Menten constant and maximum velocity of the enzyme for either substrate are defined as:

$$K_{m,E,x} \equiv \frac{k_{-,E,x} + k_{cat,E,x}}{k_{+,E,x}}$$
$$V_{max,E,x} \equiv k_{cat,E,x}[E]_0$$

We can obtain the following kinetic equations:

$$\frac{d[ES_1]}{dt} = [E][S_1]k_{+,E,1} - [ES_1](k_{-,E,1} + k_{cat,E,1}) \tag{2.1.1}$$
$$\frac{d[ES_2]}{dt} = [E][S_2]k_{+,E,2} - [ES_2](k_{-,E,2} + k_{cat,E,2}) \tag{2.1.2}$$
$$\frac{d[S_1^*]}{dt} = k_{cat,E,1}[ES_1] \tag{2.1.3}$$

We also have the conservation of mass:

$$[E]_0 = [E] + [ES_1] + [ES_2] \tag{2.1.4}$$

Assuming pseudo–steady state for the enzymatic reactions, from equations 2.1.1 and 2.1.2 we get:

$$[ES_1] = \frac{[E][S_1]}{K_{m,E,1}}$$
$$[ES_2] = \frac{[E][S_2]}{K_{m,E,2}}$$

both of which can be substituted into equation 2.1.4:

$$[E]_0 = [E]\left(1 + \frac{[S_1]}{K_{m,E,1}} + \frac{[S_2]}{K_{m,E,2}}\right)$$
$$[E] = \frac{[E]_0}{1 + \frac{[S_1]}{K_{m,E,1}} + \frac{[S_2]}{K_{mE,,2}}}$$

$$[ES_1] = \frac{[E]_0[S_1]}{[S_1] + K_{m,E,1}\left(1 + \frac{[S_2]}{K_{m,E,2}}\right)}$$

This can be substituted into 2.1.3 to arrive at:

$$\frac{d[S_1^*]}{dt} = \frac{V_{max,E,1}[S_1]}{\alpha_{E,1}K_{m,E,1} + [S_1]} \tag{2.1.5}$$
$$\alpha_{E,1} \equiv 1 + \frac{[S_2]}{K_{m,E,2}}$$

where $\alpha_{E,1}$ is the inhibitory constant for S_2 competition with S_1 for E.

2.2 Steady-state solution for $[S_1^*]$

As Goldbeter and Koshland originally noted, for a futile cycle at steady state we will have $d[S_1^*]/dt = d[S_1]/dt$ [1]. Given 2.1.5, for the 1K1P loop with two substrates this yields:

$$\frac{V_{max,K,1}[S_1]}{\alpha_{K,1}K_{m,K,1} + [S_1]} = \frac{V_{max,P,1}[S_1^*]}{\alpha_{P,1}K_{m,P,1} + [S_1^*]} \tag{2.2.1}$$

Following the standard Michaelis-Menten assumptions [1, 2], we have that $[S_i]_0 >> [K]_0, [P]_0$. This gives us $[S_1]_0 = [S_1] + [S_1^*]$, which can be substituted into 2.2.1:

$$\frac{V_{max,K,1}([S_1]_0 - [S_1^*])}{\alpha_{K,1}K_{m,K,1} + ([S_1]_0 - [S_1^*])} = \frac{V_{max,P,1}[S_1^*]}{\alpha_{P,1}K_{m,P,1} + [S_1^*]}$$

Dividing both sides by $[S_1]_0$, we get:

$$\frac{V_{max,K,1}(1 - S_1^*)}{\alpha_{K,1}K_{K,1} + (1 - S_1^*)} = \frac{V_{max,P,1}S_1^*}{\alpha_{P,1}K_{P,1} + S_1^*} \tag{2.2.2}$$

$$K_{K,1} \equiv \frac{K_{m,K,1}}{[S_1]_0}\,,\; K_{P,1} \equiv \frac{K_{m,P,1}}{[S_1]_0}$$
$$S_1 \equiv \frac{[S_1]}{[S_1]_0}\,,\; S_1^* \equiv \frac{[S_1^*]}{[S_1]_0}$$

We can expand 2.2.2:

$$\alpha_{P,1}V_{max,K,1}K_{P,1} - \alpha_{P,1}V_{max,K,1}K_{P,1}S_1^* + V_{max,K,1}S_1^* - V_{max,K,1}(S_1^*)^2$$
$$= \alpha_{K,1}V_{max,P,1}K_{K,1}S_1^* + V_{max,P,1}S_1^* - V_{max,P,1}(S_1^*)^2$$

Dividing both sides by $V_{max,P,1}$, we get:

$$r_1\alpha_{P,1}K_{P,1} - r_1\alpha_{P,1}K_{P,1}S_1^* + r_1S_1^* - r_1(S_1^*)^2 = \alpha_{K,1}K_{K,1}S_1^* + S_1^* - (S_1^*)^2$$

$$r_1 \equiv \frac{V_{max,K,1}}{V_{max,P,1}}$$

which can be simplified to:

$$(1-r_1)(S_1^*)^2 + ((r_1-1) - (\alpha_{K,1}K_{K,1} + r_1\alpha_{P,1}K_{P,1}))S_1^* + r_1\alpha_{P,1}K_{P,1} = 0 \tag{2.2.3}$$

Solving for S_1^*:

$$S_1^* = \frac{(r_1-1) - (\alpha_{K,1}K_{K,1} + r_1\alpha_{P,1}K_{P,1}) + \sqrt{((r_1-1) - (\alpha_{K,1}K_{K,1} + r_1\alpha_{P,1}K_{P,1}))^2 + 4(r_1-1)r_1\alpha_{P,1}K_{P,1}}}{2(r_1-1)} \tag{2.2.4}$$

There are two important things to note about this solution. For one, the above equation is valid for $r_1 > 0$; at $r_1 = 0$ one needs to take the other branch of the solution (i.e. the branch in which the square root term is subtracted in the numerator). Also, at $r_1 = 1$, 2.2.4 has a nonessential singularity. To obtain the behavior at $r_1 = 1$, we see 2.2.3 becomes:

$$-(\alpha_{K,1}K_{K,1} + \alpha_{P,1}K_{P,1})S_1^* + \alpha_{P,1}K_{P,1} = 0 \tag{2.2.5}$$

giving us S_1^* for $r_1 = 1$:

$$S_1^* = \frac{\alpha_{P,1}K_{P,1}}{\alpha_{K,1}K_{K,1} + \alpha_{P,1}K_{P,1}} \tag{2.2.6}$$

2.3 dS_1^*/dS_2^* is always positive

We wish to show that $\frac{dS_1^*}{dS_2^*} > 0$ regardless of the values of any parameter. This would indicate that the ultrasensitivity of S_2 transfers to S_1 (*i.e.*, since S_2^* will decrease as $[S_2]_0$ increases for $r_2 < 1$, S_1^* would also decrease). To do so we notice that, by the chain rule:

$$\frac{dS_1^*}{dS_2^*} = \frac{\partial S_1^*}{\partial \alpha_{K,1}} \cdot \frac{d\alpha_{K,1}}{dS_2^*} + \frac{\partial S_1^*}{\partial \alpha_{P,1}} \cdot \frac{d\alpha_{P,1}}{dS_2^*} \tag{2.3.1}$$

This is because S_1^* is a function of $\alpha_{K,1}$, $\alpha_{P,1}$, r_1, and a vector of positive constants 2.2.4. Each of the α terms are, in turn, functions of S_2^*.

We will explore the signs of each component of 2.3.1 to show that $\frac{dS_1^*}{dS_2^*} > 0$. Using

Mathematica [3], we can obtain the partial derivative of 2.2.4 with respect to $\alpha_{K,1}$ at $r_1 \neq 1$:

$$\frac{\partial S_1^*}{\partial \alpha_{K,1}} = \frac{-K_{K,1} + \frac{K_{K,1}x}{\sqrt{x^2+y}}}{2(r_1-1)} \tag{2.3.2}$$

Where:

$$x \equiv -((r_1-1) - (\alpha_{K,1}K_{K,1} + r_1\alpha_{P,1}K_{P,1})), \qquad y \equiv 4(r_1-1)r_1\alpha_{P,1}K_{P,1} \tag{2.3.3}$$

Factoring out $\frac{-K_{K,1}}{\sqrt{x^2+y}}$ we obtain:

$$\frac{\partial S_1^*}{\partial \alpha_{K,1}} = \frac{-K_{K,1}}{\sqrt{x^2+y}} \cdot \frac{-x + \sqrt{x^2+y}}{2(r_1-1)} \tag{2.3.4}$$

Notice that the second term in 2.3.4 is the expression for S_1^*, simplifying $\frac{dS_1^*}{d\alpha_{K,1}}$ to:

$$\frac{\partial S_1^*}{\partial \alpha_{K,1}} = \frac{-K_{K,1}}{\sqrt{x^2+y}} S_1^* \tag{2.3.5}$$

Note that $K_{K,1}$, S_1^* and $\sqrt{x^2+y}$ are all positive, making $\frac{\partial S_1^*}{\partial \alpha_{K,1}} < 0$ for $r_1 \neq 1$. We can also demonstrate this for $r_1 = 1$ by taking the partial derivative of 2.2.6 with respect to $\alpha_{K,1}$:

$$\frac{\partial S_1^*}{\partial \alpha_{K,1}} = \frac{-\alpha_{P,1}K_{K,1}K_{P,1}}{(\alpha_{K,1}K_{K,1} + \alpha_{P,1}K_{P,1})^2} \tag{2.3.6}$$

Which is clearly negative, demonstrating that $\frac{\partial S_1^*}{\partial \alpha_{K,1}} < 0$ for any set of parameters.

Next it can be shown that $\frac{\partial S_1^*}{\partial \alpha_{P,1}} > 0$. We can obtain an expression the partial derivative of 2.2.4 with respect to $\alpha_{P,1}$ at $r_1 \neq 1$ with Mathematica [3] :

$$\frac{\partial S_1^*}{\partial \alpha_{P,1}} = \frac{-r_1K_{P,1} + \frac{2(r_1-1)r_1K_{P,1} + r_1K_{P,1}x}{\sqrt{x^2+y}}}{2(r_1-1)} \tag{2.3.7}$$

By factoring out $\frac{r_1K_{P,1}}{\sqrt{x^2+y}}$ we get:

$$\frac{\partial S_1^*}{\partial \alpha_{P,1}} = \frac{r_1K_{P,1}}{\sqrt{x^2+y}} \left(\frac{2(r_1-1) + x - \sqrt{x^2+y}}{2(r_1-1)} \right) \tag{2.3.8}$$

Notice that the second term in 2.3.8 is the expression for $1 - S_1^*$, simplifying $\frac{\partial S_1^*}{\partial \alpha_{P,1}}$ to:

$$\frac{\partial S_1^*}{\partial \alpha_{P,1}} = \frac{r_1K_{P,1}}{\sqrt{x^2+y}}(1 - S_1^*) \tag{2.3.9}$$

We can easily see that 2.3.9 is positive, confirming $\frac{\partial S_1^*}{\partial \alpha_{P,1}} > 0$ for $r_1 \neq 1$. We can also demonstrate this for $r_1 = 1$ by taking the partial derivative of 2.2.6 with respect to $\alpha_{P,1}$:

$$\frac{\partial S_1^*}{\partial \alpha_{P,1}} = \frac{\alpha_{K,1} K_{K,1} K_{P,1}}{(\alpha_{K,1} K_{K,1} + \alpha_{P,1} K_{P,1})^2} \tag{2.3.10}$$

Which is clearly positive, demonstrating that $\frac{\partial S_1^*}{\partial \alpha_{P,1}} > 0$ for any set of parameters.

It is easy to show that $\frac{d\alpha_{K,1}}{dS_2^*} < 0$:

$$\begin{aligned}
\alpha_{K,1} &= 1 + \frac{[S_2]}{K_{m,K,2}} \\
&= 1 + \frac{[S_2]_0 - [S_2^*]}{K_{m,K,2}} \\
&= 1 + \frac{1 - S_2^*}{K_{K,2}} \\
\frac{d\alpha_{K,1}}{dS_2^*} &= -\frac{1}{K_{K,2}} < 0
\end{aligned}$$

Similarly, we can show $\frac{d\alpha_{P,1}}{dS_2^*} > 0$:

$$\begin{aligned}
\alpha_{P,1} &= 1 + \frac{[S_2^*]}{K_{m,P,2}} \\
&= 1 + \frac{S_2^*}{K_{P,2}} \\
\frac{d\alpha_{P,1}}{dS_2^*} &= \frac{1}{K_{P,2}} > 0
\end{aligned}$$

Now we have determined the behaviors of each component of the two implementations of the chain rules presented in 2.3.1 for all values of r_1 and r_2. When we refer back to the chain rule (2.3.1) we notice that both terms are positive:

$$\begin{aligned}
\frac{dS_1^*}{dS_2^*} &= \frac{\partial S_1^*}{\partial \alpha_{K,1}} \cdot \frac{d\alpha_{K,1}}{dS_2^*} + \frac{\partial S_1^*}{\partial \alpha_{P,1}} \cdot \frac{d\alpha_{P,1}}{dS_2^*} \\
\frac{dS_1^*}{dS_2^*} &= (-)(-) + (+)(+)
\end{aligned}$$

This means that changes in S_1^* upon increases in S_2^* will always be positive. The increase in ultrasensitivity of S_2^* is thus transferred to S_1 regardless of the values of the other parameters.

3 Analytical Results for the 1–Kinase/1–Phosphatase Loop with Many Substrates

The 1K1P loop can be expanded to include many substrates of the kinase and phosphatase. In this case we would have a system of enzymes such that:

$$\begin{aligned} E + S_1 &\underset{k_{-,E,1}}{\overset{k_{+,E,1}}{\rightleftharpoons}} ES_1 \xrightarrow{k_{cat,E,1}} E + S_1^* \\ E + S_2 &\underset{k_{-,E,2}}{\overset{k_{+,E,2}}{\rightleftharpoons}} ES_2 \xrightarrow{k_{cat,E,2}} E + S_2^* \\ &\vdots \\ E + S_N &\underset{k_{-,E,N}}{\overset{k_{+,E,N}}{\rightleftharpoons}} ES_N \xrightarrow{k_{cat,E,N}} E + S_N^* \end{aligned}$$

where $E = K$ or P. From these equations we have:

$$[ES_1] = \frac{[E][S_1]}{K_{m,E,1}},\ [ES_2] = \frac{[E][S_2]}{K_{m,E,2}},\ \ldots\ ,\ [ES_N] = \frac{[E][S_N]}{K_{m,E,N}} \tag{3.1}$$

We also know from the conservation of mass of the enzyme:

$$[E]_0 = [E] + [ES_1] + [ES_2] + \ldots + [ES_N] \tag{3.2}$$

Substituting the system of equations from 3.1 into 3.2, we get:

$$\begin{aligned} [E]_0 &= [E]\left(1 + \frac{[S_1]}{K_{m,E,1}} + \frac{[S_2]}{K_{m,E,2}} + \ldots + \frac{[S_N]}{K_{m,E,N}}\right) \\ [E] &= \frac{[E]_0}{1 + \frac{[S_1]}{K_{m,E,1}} + \frac{[S_2]}{K_{m,E,2}} + \ldots + \frac{[S_N]}{K_{m,E,N}}} \\ [ES_1] &= \frac{[E]_0[S_1]}{[S_1] + K_{m,E,1}\left(1 + \frac{[S_2]}{K_{m,E,2}} + \ldots + \frac{[S_N]}{K_{m,E,N}}\right)} \end{aligned} \tag{3.3}$$

Substituting 3.3 into the previously defined 2.1.3, we arrive at:

$$\frac{d[S_1^*]}{dt} = \frac{V_{max,1}[S_1]}{\alpha K_{m,E,1} + [S_1]}$$

$$\alpha \equiv 1 + \sum_{i=2}^{N} \frac{[S_i]}{K_{m,E,i}}$$

From the above equation, we can proceed to solve for S_1^* as in section 2.2; as expected, one obtains equation 2.2.4, but with $\alpha_{K,1} \equiv 1 + \sum_{i=2}^{N}[S_i]/K_{m,K,i}$ and $\alpha_{P,1} \equiv 1 + \sum_{i=2}^{N}[S_i^*]/K_{m,P,i}$.

The increase in ultrasensitivity observed in Fig. $2B$ of the main text arises from the fact that, for the parameters we considered, at any $r_1 < 1$, the phosphatase has a higher maximum velocity

than the kinase. As such, the majority of any substrates present will exist in the unphosphorylated form (i.e. $S_i^* < 0.5\ \forall i$). As more substrates are added, the accumulation of these unphosphorylated substrates begins to occupy the kinase, reducing free kinase concentration and thus reducing the "effective r" of the system. In the limit where N is large, the occupation increases until the kinase is completely saturated, ultimately leading to very low phosphorylation at $r_1 < 1$. For $r_1 > 1$, a similar situation holds, but with the phosphatase occupied by the S_i^*'s.

4 Analytical Results for the 1–Kinase/2–Phosphatase Loop

In this section we will show that S_1 phosphorylation always increases in $[S_2]_0$ in the limit in which $[S_1]_0 \ll K_m$. In this system S_1^* can be derived in a similar fashion to that for the 1K1P loop, resulting in:

$$S_1^* = \frac{(r_1 - 1) - (\alpha_{K,1}K_{K,1} + r_1 K_{P,1}) + \sqrt{((r_1 - 1) - (\alpha_{K,1}K_{K,1} + r_1 K_{P,1}))^2 + 4(r_1 - 1)r_1 K_{P,1}}}{2(r_1 - 1)} \tag{4.0.1}$$

Note this is similar to 2.2.4, the difference being the absence of $\alpha_{P,1}$. This is because in this loop the substrates only share a kinase, making $\alpha_{P,1} = 1$. As such, $\frac{\partial S_1^*}{\partial \alpha_{P,1}} = 0$, by the chain rule we see:

$$\frac{dS_1^*}{d[S_2]_0} = \frac{dS_1^*}{d\alpha_{K,1}} \cdot \frac{d\alpha_{K,1}}{d[S_2]} \cdot \frac{d[S_2]}{d[S_2]_0} \tag{4.0.2}$$

Note that $\frac{dS_1^*}{d\alpha_{K,1}}$ is similar to $\frac{\partial S_1^*}{\partial \alpha_{K,1}}$ (2.3.4), the only difference being $\alpha_{P,1} = 1$ in this case. Since the value of $\alpha_{P,1}$ does not have an affect on the sign of $\frac{\partial S_1^*}{\partial \alpha_{K,1}}$, we can conclude that $\frac{dS_1^*}{d\alpha_{K,1}} < 0$ for any value of r_1 (see subsection 2.3). Additionally, we can easily show $\frac{d\alpha_{K,1}}{d[S_2]} > 0$:

$$\begin{aligned} \alpha_{K,1} &= 1 + \frac{[S_2]}{K_{m,K,2}} \\ \frac{d\alpha_{K,1}}{d[S_2]} &= \frac{1}{K_{m,K,2}} > 0 \end{aligned} \tag{4.0.3}$$

4.1 $d[S_2]/d[S_2]_0$ is always positive

Using Mathematica [3], we can obtain an expression for $\frac{d[S_2]}{d[S_2]_0}$ at $r_2 \neq 1$. To simplify the derivation, we assume $[S_1]_0 \ll K_m$ so that $\alpha_{K,2} = 1$.

$$\begin{aligned}
[S_2] &= (1 - S_2^*)[S_2]_0 \\
\frac{d[S_2]}{d[S_2]_0} &= 1 - S_2^* - \frac{dS_2^*}{d[S_2]_0}[S_2]_0 \\
&= 1 - \frac{-x' + \sqrt{(x')^2 + y'}}{2(r_2 - 1)} - \frac{z' + \frac{z'(-z') - \frac{y'}{2}}{\sqrt{(x')^2 + y'}}}{2(r_1 - 1)} \\
&= \frac{2(r_2 - 1) + x' - \sqrt{(x')^2 + y'} - z' + \frac{x'z' + \frac{y'}{2}}{\sqrt{(x')^2 + y'}}}{2(r_2 - 1)}
\end{aligned} \tag{4.1.1}$$

In which:

$$x' \equiv -((r_2 - 1) - (K_{K,2} + r_2 K_{P,2})), \qquad y' \equiv 4(r_2 - 1) r_2 K_{P,2}, \qquad z' \equiv K_{K,2} + r_2 K_{P,2} \tag{4.1.2}$$

By the definitions of x' and z' we notice that $x' = -(r_2 - 1) + z'$, which can be substituted into 4.1.1:

$$\begin{aligned}
\frac{d[S_2]}{d[S_2]_0} &= \frac{2(r_2 - 1) - (r_2 - 1) + z' - \sqrt{(x')^2 + y'} - z' + \frac{x'z' + \frac{y'}{2}}{\sqrt{(x')^2 + y'}}}{2(r_2 - 1)} \\
&= \frac{(r_2 - 1) - \sqrt{(x')^2 + y'} + \frac{x'z' + \frac{y'}{2}}{\sqrt{(x')^2 + y'}}}{2(r_2 - 1)} \\
&= \frac{(r_2 - 1)\sqrt{(x')^2 + y'} - (x')^2 - y' + x'z' + \frac{y'}{2}}{2(r_2 - 1)\sqrt{(x')^2 + y'}}
\end{aligned} \tag{4.1.3}$$

Additionally, by the definitions of x' and z', we see $(x')^2 = (r_2 - 1)^2 - 2(r_2 - 1)z' + (z')^2$ and $x'z' = -(r_2 - 1)z' + (z')^2$, which can be substituted into 4.1.3:

$$\begin{aligned}
\frac{d[S_2]}{d[S_2]_0} &= \frac{(r_2 - 1)\sqrt{(x')^2 + y'} - (r_2 - 1)^2 + 2(r_2 - 1)z' - (z')^2 - (r_2 - 1)z' + (z')^2 - \frac{y'}{2}}{2(r_2 - 1)\sqrt{(x')^2 + y'}} \\
&= \frac{(r_2 - 1)\sqrt{(x')^2 + y'} - (r_2 - 1)^2 + (r_2 - 1)z' - \frac{y'}{2}}{2(r_2 - 1)\sqrt{(x')^2 + y'}} \\
&= \frac{\sqrt{(x')^2 + y'} - (r_2 - 1) + z' - 2r_2 K_{P,2}}{2\sqrt{(x')^2 + y'}} \\
&= \frac{\sqrt{(x')^2 + y'} + x' - 2r_2 K_{P,2}}{2\sqrt{(x')^2 + y'}}
\end{aligned} \tag{4.1.4}$$

We can show that $\frac{d[S_2]}{d[S_2]_0} > 0$ for all values of r_2 by assuming the opposite:

$$\frac{d[S_2]}{d[S_2]_0} = \frac{\sqrt{(x')^2 + y'} + x' - 2r_2K_{P,2}}{2\sqrt{(x')^2 + y'}} < 0$$
$$\sqrt{(x')^2 + y'} + x' - 2r_2K_{P,2} < 0$$
$$\sqrt{(x')^2 + y'} < -x' + 2r_2K_{P,2} \tag{4.1.5}$$

If the right hand side of 4.1.5 is negative then we have already arrived at a contradiction. Otherwise we can square both sides without loss of information:

$$(x')^2 + y' < (x')^2 - 4r_2K_{P,2}x' + 4(r_2K_{P,2})^2 \tag{4.1.6}$$
$$y' < -4r_2K_{P,2}x' + 4(r_2K_{P,2})^2$$
$$4(r_2 - 1)r_2K_{P,2} < 4(r_2 - 1)r_2K_{P,2} - 4r_2K_{K,2}K_{P,2} - 4(r_2K_{P,2})^2 + 4(r_2K_{P,2})^2$$
$$0 < -4r_2K_{K,2}K_{P,2} \tag{4.1.7}$$

Which is clearly impossible, indicating $\frac{d[S_2]}{d[S_2]_0} > 0$ for $r_2 \neq 1$. Next we can obtain an expression for $\frac{d[S_2]}{d[S_2]_0}$ at $r_2 = 1$. At this point, S_2^* becomes:

$$S_2^* = \frac{K_{m,P,2}}{K_{m,K,2} + K_{m,P,2}} \tag{4.1.8}$$

As such, we can easily see that the derivative of 4.1.8 with respect to $[S_2]_0$ is equal to zero. Applying this to the previous expression for $\frac{d[S_2]}{d[S_2]_0}$ (4.1.1) we notice that at $r_2 = 1$:

$$\frac{d[S_2]}{d[S_2]_0} = 1 - S_2^* \tag{4.1.9}$$

Since S_2^* must be a value between 0 and 1, it is easy to see that $\frac{d[S_2]}{d[S_2]_0} > 0$ at $r_2 = 1$, thus showing that $\frac{d[S_2]}{d[S_2]_0} > 0$ for all values of r_2.

4.2 $dS_1^*/d[S_2]_0$ is always negative

As previously shown, we can use the chain rule to define $\frac{dS_1^*}{d[S_2]_0}$ within this motif as:

$$\frac{dS_1^*}{d[S_2]_0} = \frac{dS_1^*}{d\alpha_{K,1}} \cdot \frac{d\alpha_{K,1}}{d[S_2]} \cdot \frac{d[S_2]}{d[S_2]_0} \tag{4.2.1}$$

In which $\frac{dS_1^*}{d\alpha_{K,1}} < 0$, $\frac{d\alpha_{K,1}}{d[S_2]} > 0$ and $\frac{d[S_2]}{d[S_2]_0} > 0$. Now we can see that $\frac{dS_1^*}{d[S_2]_0} < 0$ for all values of r_1 and r_2. At $r_2 < 1$, $\alpha_{K,1} > 1$ as most S_2 will be in the unphosphorylated form. Once $r_2 > 1$, S_2 switches to its phosphorylated form, relieving the pressure on S_1 through $\alpha_{K,1}$, establishing the "gatekeeper" effect. We can see $\alpha_{K,1}$ approaches 1 as $r_2 \to \infty$, allowing S_1^* to behave as an isolated futile cycle in this limit. Since S_1^* is increasing in r_2, we can conclude that S_2 decreases

S_1^* for all values of r_2 except in the limit $r_2 \to \infty$.

5 Analytical Results for the 2–Kinase/1–Phosphatase Loop

In this section we will show that S_1 phosphorylation also always increases in $[S_2]_0$ regardless of any other parameters. In this system S_1^* can be derived in a similar fashion to that for the 1K1P loop, resulting in:

$$S_1^* = \frac{(r_1 - 1) - (K_{K,1} + r_1\alpha_{P,1}K_{P,1}) + \sqrt{((r_1 - 1) - (K_{K,1} + r_1\alpha_{P,1}K_{P,1}))^2 + 4(r_1 - 1)r_1\alpha_{P,1}K_{P,1}}}{2(r_1 - 1)} \tag{5.0.1}$$

Which is equivalent to 2.2.4, the only difference being the lack of $\alpha_{K,1}$. As such $\frac{\partial S_1^*}{\partial \alpha_{K,1}} = 0$, and we notice that by the chain rule:

$$\frac{dS_1^*}{d[S_2]_0} = \frac{dS_1^*}{d\alpha_{P,1}} \cdot \frac{d\alpha_{P,1}}{d[S_2^*]} \cdot \frac{d[S_2^*]}{d[S_2]_0} \tag{5.0.2}$$

Note that $\frac{dS_1^*}{d\alpha_{P,1}}$ is similar to $\frac{\partial S_1^*}{\partial \alpha_{P,1}}$ (2.3.7), the only difference being $\alpha_{K,1} = 1$ in this case. Since the value of $\alpha_{K,1}$ does not have an affect on the sign of $\frac{\partial S_1^*}{\partial \alpha_{P,1}}$, we can conclude that $\frac{dS_1^*}{d\alpha_{P,1}} > 0$ for any value of r_1 (see subsection 2.3). Additionally, we can easily show $\frac{d\alpha_{P,1}}{d[S_2]} > 0$:

$$\begin{aligned} \alpha_{P,1} &= 1 + \frac{[S_2^*]}{K_{m,P,2}} \\ \frac{d\alpha_{P,1}}{d[S_2]_0} &= \frac{1}{K_{m,P,2}} \end{aligned} \tag{5.0.3}$$

5.1 $d[S_2^*]/d[S_2]_0$ is always positive

We can define $[S_2^*]$ as:

$$[S_2^*] = S_2^*[S_2]_0 \tag{5.1.1}$$

And as such $\frac{d[S_2^*]}{d[S_2]_0}$ is:

$$\frac{d[S_2^*]}{d[S_2]_0} = S_2^* + \frac{dS_2^*}{d[S_2]_0}[S_2]_0 \tag{5.1.2}$$

Notice that $\frac{d[S_2^*]}{d[S_2]_0} = 1 - \frac{d[S_2]}{d[S_2]_0}$ (see 4.1.1). We can then substitute 4.1.4 in for $\frac{d[S_2]}{d[S_2]_0}$:

$$\begin{aligned}\frac{d[S_2^*]}{d[S_2]_0} &= 1 - \frac{\sqrt{(x')^2 + y'} + x' - 2r_2K_{P,2}}{2\sqrt{(x')^2 + y'}} \\ &= \frac{\sqrt{(x')^2 + y'} - x' + 2r_2K_{P,2}}{2\sqrt{(x')^2 + y'}}\end{aligned} \tag{5.1.3}$$

We can show $\frac{d[S_2^*]}{d[S_2]_0} > 0$ for any value of r_2 by assuming the opposite:

$$\begin{aligned}\frac{d[S_2^*]}{d[S_2]_0} = \frac{\sqrt{(x')^2 + y'} - x' + 2r_2K_{P,2}}{2\sqrt{(x')^2 + y'}} &< 0 \\ \sqrt{(x')^2 + y'} - x' + 2r_2K_{P,2} &< 0 \\ \sqrt{(x')^2 + y'} &< x' - 2r_2K_{P,2}\end{aligned} \tag{5.1.4}$$

If the right hand side of 5.1.4 is negative then we have already arrived at a contradiction. Otherwise we can square both sides without loss of information:

$$(x')^2 + y' < (x')^2 - 4r_2K_{P,2}x' + 4(r_2K_{P,2})^2 \tag{5.1.5}$$

Note that this expression is the same as 4.1.6, which we have already shown to be impossible, supporting the conclusion $\frac{d[S_2^*]}{d[S_2]_0} > 0$ for $r_2 \neq 0$. Next we can obtain an expression for $\frac{d[S_2]}{d[S_2]_0}$ at $r_2 = 1$. At this point, S_2^* becomes:

$$S_2^* = \frac{K_{m,P,2}}{K_{m,K,2} + K_{m,P,2}} \tag{5.1.6}$$

As such, we can easily see that the derivative of 5.1.6 with respect to $[S_2]_0$ is equal to zero. Applying this to the previous expression for $\frac{d[S_2^*]}{d[S_2]_0}$ (5.1.2) we notice that at $r_2 = 1$:

$$\frac{d[S_2^*]}{d[S_2]_0} = S_2^* \tag{5.1.7}$$

Since S_2^* must be a value between 0 and 1, it is easy to see that $\frac{d[S_2^*]}{d[S_2]_0} > 0$ at $r_2 = 1$, thus showing that $\frac{d[S_2^*]}{d[S_2]_0} > 0$ for all values of r_2.

5.2 $dS_1^*/d[S_2]_0$ is always positive

As previously shown, we can use the chain rule to define $\frac{dS_1^*}{d[S_2]_0}$ within this motif as:

$$\frac{dS_1^*}{d[S_2]_0} = \frac{dS_1^*}{d\alpha_{P,1}} \cdot \frac{d\alpha_{P,1}}{d[S_2^*]} \cdot \frac{d[S_2^*]}{d[S_2]_0} \tag{5.2.1}$$

In which $\frac{dS_1^*}{d\alpha_{P,1}} > 0$, $\frac{d\alpha_{P,1}}{d[S_2^*]} > 0$ and $\frac{d[S_2^*]}{d[S_2]_0} > 0$. Now we can see that $\frac{dS_1^*}{d[S_2]_0} > 0$ for all values of r_1 and r_2.

References

1. Goldbeter A, K. D., 1981. An amplified sensitivity arising from covalent modification in biological systems. *Proc Natl Acad Sci U S A* 78:6840–44.

2. Nelson, D. L., and M. M. Cox, 2008. Lehninger Principles of Biochemistry 5^{th} Edition.

3. Wolfram Research, I., 2010. Mathematica, Version 8.0. Champaign, Illinois.