Phosphatase specificity and pathway insulation in signaling networks

Supporting Information

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Contents

1 Systems of Ordinary Differential Equations

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

In order to characterize the effects of phosphatase saturation and competition on phosphatase-mediated crosstalk we used the 2-Kinase/1-Phosphatase Loop with 2 Substrates model that we have previously characterized [1]. The equations described below were derived and analyzed in our previous work; we include them here for completeness. The set of enzymatic reactions for the 2-Kinase/1-Phosphatase Loop with 2 Substrates model are:

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

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

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

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

Each contain three rates: the complex formation (k_+), the rate of complex dissociation (k_-), and catalytic rate (k_{cat}). These reactions are diagrammed in Fig. $2A$ of the main text. The set of ODEs describing the free enzymes are:

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

The set of ODEs describing the free unphosphorylated substrates are:

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

The set of ODEs describing the free phosphorylated substrates are:

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

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

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

For purposes of display we used the following rate parameters:

Parameter	Value
$k_{+,K,i}$	0.001 $\text{nM}^{-1}\text{s}^{-1}$
$k_{-,K,i}$	0.1-999.1 s^{-1}
$k_{cat,K,i}$	0.9 s^{-1}
$k_{+,P,i}$	0.001 $\text{nM}^{-1}\text{s}^{-1}$
$k_{-,P,i}$	0.1-999.1 s^{-1}
$k_{cat,P,i}$	0.9 s^{-1}

where $i = 1$ or 2. The ranges listed for the dissociation rates (i.e. $k_{-,K,i}$) are used to set the K_M's of the enzymes in different simulations. Note that, while the values of these parameters are not meant to describe any specific enzyme, they are within the range of values obtained for kinases and phosphatases experimentally [2–4].

We used the following initial conditions for all of our simulations:

Molecular Species	Initial Concentration
K_1	0-20 nM
K_2	0-20 nM
P	10 nM-1mM
S_1	10 μM
S_2	0,10 μM

with the remaining molecular species having initial concentrations of 0. The ranges of concentrations of K_1 and K_2 are used to vary the values of r_1 and r_2.

This model was used to generate Fig. 1*B* and *C* of the main text. The concentration of K_2 was set to 0 for $r_2 = 0$ and to 20 nM for $r_2 = 2$. The concentration of K_1 was set between 0-20 nM to vary r_1 between 0 and 2. Both substrates are present at a concentration of 10μM. The values of $k_{-,K,1}$ and $k_{-,P,1}$ were both set to 999.1 s^{-1} so that $K_{M,K,1} = K_{M,P,1} = 100 \times [S_1]_0$, while $k_{-,K,2}$ and $k_{-,P,2}$ were set to 0.1 s^{-1} so that $K_{M,P,2} = K_{M,K,2} = 100 \times [S_2]_0$.

We also used this model to generate Fig. 2 of the main text. In Fig. 2*A*, $K_{M,P,i}$ was set by changing the values of $k_{-,P,1}$ and $k_{-,P,2}$ between 0.1-999.1 s^{-1}. The value of r_1 was set in Fig. 2*B* by setting the concentration of K_1 between 0-20 nM, and the concentration of K_2 was set to 0 for

$r_2 = 0$ and to 20 nM for $r_2 = 2$. For Fig. 2*C*, we first ran the model with $K_2 = S_2 = 0$ to steady state with the initial concentration of K_1 at 20 nM ($r_1 = 2$). We then removed all K_1 molecules from the system. The bound S_1 was added back to the concentration of unphosphorylated S_1. The simulations were then resumed to obtain the time courses visualized in Fig. 2*C*. $K_{M,P}$ was set by using values of $k_{-,P,2,1} = 0.1$ s^{-1}, 9.1 s^{-1}, and 99.1 s^{-1}. The fraction of phosphorylated S_1^* was normalized so that $\hat{S}_1^*(t) = (max(S_1^*) - S_1^*(t))/(max(S_1^*) - min(S_1^*))$. Fig. 2*D* was obtained using the same procedures as in Fig. 3*C*, setting $K_{M,P}$ by using values of $k_{-,P,2,1} = 0.1$-999.1 s^{-1}. The half-time of S_1^* phosphorylation was obtained by finding the time $t_{1/2} = t$ where $\hat{S}_1^*(t) = 0.5$. The total concentration of the phosphatase was set to either 10 nM (magenta curve), or 1 μM (purple curve).

To test the effectiveness of an increased phosphatase concentration in insulating substrates against phosphatase crosstalk while maintaining strong $K_{M,P,i}$ values, we set $K_{M,P,i} = 1\mu$M, $r_1 = 0.05$, $r_2 = 2$ and varied the concentration of the phosphatase from 10 nM to 1 mM (Fig. S1).

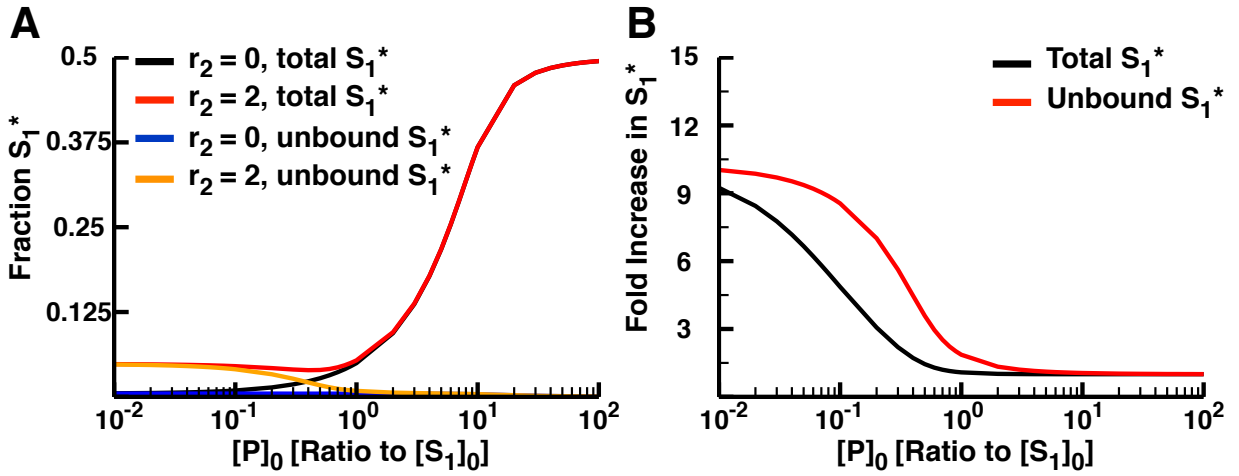

Figure S1: The effects of increased phosphatase concentration on substrate crosstalk with strong $K_{M,P,i}$s (*A*) The fraction S_1^* as a function of the concentration of the phosphatase. The concentration of the kinase is increased in order to maintain the values of r_1 and r_2 with constant catalytic rates for different concentrations of the phosphatase. At low concentrations of P, S_1 phosphorylation is increased by activation of the S_2 pathway, moving from $r_2 = 0$ (black) to $r_2 = 2$ (red). As P is expressed in concentrations greater than the substrates, the difference between the curves is removed. However, the fraction S_1^* is greatly increased. Additionally, the fraction of unbound S_1^* decreases with $[P]_0$, indicating that the increase in total fraction S_1^* is likely due to it being bound to the phosphatase. (*B*) The fold increase in S_1^* as a function of the concentration of the phosphatase. As stated above, the crosstalk between S_1 and S_2 is removed when the phosphatase is present in concentrations larger than those of the substrates.

1.2 2-Kinase/1-Phosphatase Loop with 2 Substrates and 2 Reservoir Proteins

In order to characterize the effects of reservoir proteins that bind to and shield phosphorylated substrates from dephosphorylated on phosphatase-mediated crosstalk we expanded the 2-Kinase/1-Phosphatase Loop with 2 Substrates model to include two substrate-specific reservoir

proteins, R_1 and R_2. The set of enzymatic reactions for the model are:

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

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

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

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

$$R_1 + S_1^* \underset{k_{-,R,1}}{\overset{k_{+,R,1}}{\rightleftharpoons}} R_1S_1^*$$

$$R_2 + S_2^* \underset{k_{-,R,2}}{\overset{k_{+,R,2}}{\rightleftharpoons}} R_2S_2^*$$

The set of ODEs describing the free enzymes are:

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

The set of ODEs describing the free unphosphorylated substrates are:

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

The set of ODEs describing the free phosphorylated substrates are:

$$\frac{d[S_1^*]}{dt} = [PS_1^*]k_{-,P,1} + [K_1S_1]k_{cat,K,1} + [R_1S_1^*]k_{-,R,1} - [P][S_1^*]k_{+,P,1} - [R_1][S_1^*]k_{+,R,1}$$
$$\frac{d[S_2^*]}{dt} = [PS_2^*]k_{-,P,2} + [K_2S_2]k_{cat,K,2} + [R_2S_2^*]k_{-,R,2} - [P][S_2^*]k_{+,P,2} - [R_2][S_2^*]k_{+,R,2}$$

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

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

The set of ODEs describing the free reservoir proteins are:

$$\frac{d[R_1]}{dt} = [R_1S_1^*]k_{-,R,1} - [R_1][S_1^*]k_{+,R,1}$$
$$\frac{d[R_2]}{dt} = [R_2S_2^*]k_{-,R,2} - [R_2][S_2^*]k_{+,R,2}$$

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

$$\frac{d[R_1S_1^*]}{dt} = [R_1][S_1^*]k_{+,R,1} - [R_1S_1^*]k_{-,R,1}$$
$$\frac{d[R_2S_2^*]}{dt} = [R_2][S_2^*]k_{+,R,2} - [R_2S_2^*]k_{-,R,2}$$

For purposes of display we used the following rate parameters:

Parameter	Value
$k_{+,K,i}$	0.001 $\text{nM}^{-1}\text{s}^{-1}$
$k_{-,K,i}$	0.1-999.1 s^{-1}
$k_{cat,K,i}$	0.9 s^{-1}
$k_{+,P,i}$	0.001 $\text{nM}^{-1}\text{s}^{-1}$
$k_{-,P,i}$	0.1-999.1 s^{-1}
$k_{cat,P,i}$	0.9 s^{-1}
$k_{+,R,i}$	0.001 $\text{nM}^{-1}\text{s}^{-1}$
$k_{-,R,i}$	0.01 s^{-1}

where $i = 1$ or 2. The ranges listed for the dissociation rates (i.e. $k_{-,K,i}$) are used to set the K_M's of the enzymes in different simulations. Note that, while the values of these parameters are not meant to describe any specific enzyme, they are within the range of values obtained for kinases and phosphatases experimentally [2–4].

We used the following initial conditions for all of our simulations:

Molecular Species	Initial Concentration
K_1	0-20 nM
K_2	0-20 nM
P	10 nM, 1μM
S_1	10 μM
S_2	0,10 μM
R_1	0-100 μM
R_2	0-100 μM

with the remaining molecular species having initial concentrations of 0. The ranges of concentrations of K_1 and K_2 are used to vary the values of r_1 and r_2.

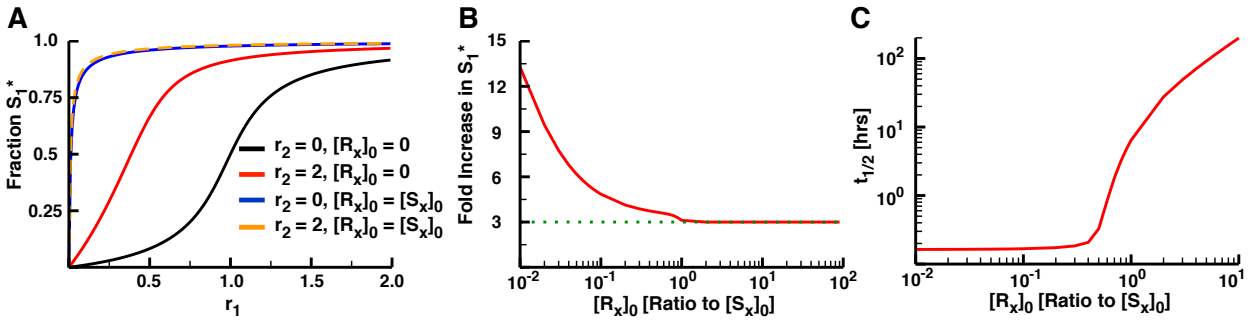

Figure S2: The effects of reservoir proteins on substrate crosstalk (A) The fraction S_1^* as a function of r_1. Without the reservoir proteins, S_1 responds to signals from S_2 (red versus black curves). The crosstalk is removed with the addition of the reservoir proteins (orange versus blue curves). Note, however, that the response becomes hyperbolic in r_1. (B) The fold increase in S_1^* as a function of reservoir protein concentration. As the concentrations of the reservoir proteins increases, the crosstalk between the substrates is gradually removed. (C) The half-life of S_1 phosphorylation as a function of the concentration of reservoir proteins. Note that when the reservoir proteins are at stoichiometric or greater concentrations, the time required to completely dephosphorylate the substrates greatly increases.

1.3 2-Kinase/1-Phosphatase Loop with Many Substrates

The expression for the fraction of phosphorylation S_1^* in a 2K1P loop in which kinase K_2 and P act upon N substrates is similar to the expression for a 2K1P loop with 2 substrates we have previously derived (equation 1 in the main text) [1]. The only difference in this case is the value of $\alpha_{P,1}$, the inhibitory term that captures the effects of the competing substrates on the phosphatase. In a model with N substrates, this term becomes:

$$\alpha_{P,1} = 1 + \sum_{i \neq 1} \frac{[S_i^*]}{K_{M,P,i}} \tag{1.1}$$

Note that the value of $\alpha_{P,1}$, and thus the inhibition of the phosphatase, depends on the total saturation of the phosphatase across all its substrates. A set of substrates that all respond to the same signal can thus cause phosphatase crosstalk with other proteins in the network, even if none of those substrates is at high enough concentration to saturate the phosphatase individually.

1.4 1-Kinase/1-Substrate Model with Synthesis and Degradation

In order to characterize the effectiveness of synthesis and degradation of a substrate as a replacement for the phosphatase, we created the 1-Kinase/1-Substrate model with Synthesis and Degradation. The set of enzymatic reactions for this system are:

$$K + S \underset{k_-}{\overset{k_+}{\rightleftharpoons}} KS \xrightarrow{k_{cat}} K + S^*$$

$$S \xrightarrow{k_{deg,U}} \emptyset$$
$$KS \xrightarrow{k_{deg,U}} K$$
$$S^* \xrightarrow{k_{deg,P}} \emptyset$$
$$\emptyset \xrightarrow{k_{synth}} S$$

where $k_{deg,U}$ and $k_{deg,P}$ are the degradation rates of unphosphorylated and phosphorylated S. This model includes separate degradation rates for the unphosphorylated and phosphorylated substrate ($k_{deg,U}$ and $k_{deg,P}$), since phosphorylation of the substrate might either increase or decrease the stability of the protein. The ODE describing the free kinase is:

$$\frac{d[K]}{dt} = [KS]k_- + [KS]k_{cat} + [KS]k_{deg,U} - [K][S]k_+$$

The ODE describing the free unphosphorylated substrate is:

$$\frac{d[S]}{dt} = [KS]k_- + k_{synth} - [K][S]k_+ - [S]k_{deg,U} \tag{1.2}$$

The ODE describing the free phosphorylated substrate is:

$$\frac{d[S^*]}{dt} = [KS]k_{cat} - [S^*]k_{deg,P}$$

The ODE describing the concentration of kinase-substrate complex is:

$$\frac{d[KS]}{dt} = [K][S]k_+ - [KS]k_- - [KS]k_{cat} - [KS]k_{deg,U}$$

In order to understand the effects of degradation at steady state, note that the total substrate concentration is defined as:

$$[S]_T = [S] + [S^*] + [KS]$$

and the change in total substrate concentration can thus be written:

$$\frac{d[S]_T}{dt} = \frac{d[S]}{dt} + \frac{d[S^*]}{dt} + \frac{d[KS]}{dt}$$

At steady state, $d[S]_T/dt = 0$. By substituting the above ODEs and simplifying, we get:

$$\frac{d[S]_T}{dt} = k_{synth} - ([S] + [KS])k_{deg,U} - [S^*]k_{deg,P} = 0$$

We can then solve for k_{synth}:

$$k_{synth} = ([S] + [KS])k_{deg,U} + [S^*]k_{deg,P}$$

We can substitute this equation into the original differential equation for $[S]$ (equation 1.2):

$$\frac{d[S]}{dt} = [KS]k_- - [K][S]k_+ + [KS]k_{deg,U} + [S^*]k_{deg,P} \tag{1.3}$$

Equation 1.3 is useful for two reasons. For one, there is a positive term in the equation corresponding to the degradation of phosphorylated substrate ($[S^*]k_{deg,P}$). This term reflects the fact that, in order for $[S]_T$ to remain constant at steady state, new, unphosphorylated substrate molecules must be synthesized to replace S^* molecules that are degraded. There is thus an "effective" dephosphorylation rate in this system where S^* molecules are converted to S, which corresponds mathematically to an unsaturateable first-order phosphatase. Secondly, we used equation 1.3 instead of 1.2 in our numerical integration, so $[S]_T = [S]_0$ for all time; in other words, while the concentration of unphosphorylated and phosphorylated substrate might change in our simulations, the total concentration of substrate remains constant. This allows us to control total substrate levels by setting the initial substrate concentration, as we do in our other models. One could of course simulate equation 1.2 with a constant k_{synth} that allows total substrate concentration to vary with time; while such transients might have interesting effects on the system, we leave consideration of those effects to future work.

For purposes of display we used the following rate parameters:

Parameter	Value
k_+	0.001 $\text{nM}^{-1}\text{s}^{-1}$
k_-	0.1 s^{-1}
k_{cat}	0.9 s^{-1}
$k_{deg,U}$	1x10^{-7} - 1x10^{-4} s^{-1}
$k_{deg,P}$	1x10^{-7} - 1x10^{-4} s^{-1}

The ranges of $k_{deg,U}$ and $k_{deg,P}$ were used to vary the degradation rate of the substrate across simulations. The simulations started with the following initial concentrations:

Molecular Species	Initial Concentration
K	0 - 1 nM
S	10 μM
KS	0

The range of K was used to set the value of r_{deg} across simulations.

This model was used to generate Fig. 3*B* and *C* of the main text. In Fig. 3*B* we used $k_{deg,U} = k_{deg,P} = \log 2/t_{1/2}$ for $t_{1/2} = 10$, 31, and 187 hrs. The value of r_{deg} was set between 0 and 2 by changing the initial concentration of K so that $[K] = (r_{deg}[S]_0 k_{deg,P})/k_{cat}$ (where r_{deg} was set to the desired value, see Section 3 for the derivation of r_{deg}), which ends up giving a value between 0-0.186 nM. In Fig. 3*C* we first ran the model to steady state with an initial concentration of K at 0.186 nM ($r_{deg} = 2$). All kinase molecules were then removed from the system, and the simulation continued in order to obtain the time courses shown in Fig. 3*C*.

1.5 2-Kinase/1-Phosphatase Loop with "Ordered" Phosphatase Adaptors

In order to characterize the effectiveness of phosphatase adaptors in insulating pathways from phosphatase crosstalk, we first developed the 2-Kinase/1-Phosphatase Loop with Ordered Phosphatase Adaptors. The set of enzymatic reactions for this model are:

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

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

$$PA_1 + S_1^* \underset{k_{-,P,1}}{\overset{k_{+,P,1}}{\rightleftharpoons}} PA_1S_1^* \xrightarrow{k_{cat,P,1}} PA_1 + S_1$$

$$PA_2 + S_2^* \underset{k_{-,P,2}}{\overset{k_{+,P,2}}{\rightleftharpoons}} PA_2S_2^* \xrightarrow{k_{cat,P,2}} PA_2 + S_2$$

$$P + A_1 \underset{k_{-,A,1}}{\overset{k_{+,A,1}}{\rightleftharpoons}} PA_1$$

$$P + A_2 \underset{k_{-,A,2}}{\overset{k_{+,A,2}}{\rightleftharpoons}} PA_2$$

where $k_{-,A,i}$ represents the dissociation rate of the phosphatase-adaptor complex and $k_{+,A,i}$ represents the association rate. The reactions that include a phosphatase molecule are diagrammed in the inset of Fig. $4A$ of the main text. The set of ODEs describing the concentration of free enzymes are:

$$\frac{d[K_1]}{dt} = [K_1S_1]k_{-,K,1} + [K_1S_1]k_{cat,K,1} - [K_1][S_1]k_{+,K,1}$$
$$\frac{d[K_2]}{dt} = [K_2S_2]k_{-,K,2} + [K_2S_2]k_{cat,K,2} - [K_2][S_2]k_{+,K,2}$$
$$\frac{d[P]}{dt} = [PA_1]k_{-,A,1} + [PA_2]k_{-,A,2} - [P][A_1]k_{+,A,1} - [P][A_2]k_{+,A,2}$$

The set of ODEs describing the concentration of free unphosphorylated substrates are:

$$\frac{d[S_1]}{dt} = [K_1S_1]k_{-,K,1} + [PA_1S_1^*]k_{cat,P,1} - [K_1][S_1]k_{+,K,1}$$
$$\frac{d[S_2]}{dt} = [K_2S_2]k_{-,K,2} + [PA_2S_2^*]k_{cat,P,2} - [K_2][S_2]k_{+,K,2}$$

The set of ODEs describing the concentration of free phosphorylated substrates are:

$$\frac{d[S_1^*]}{dt} = [PA_1S_1^*]k_{-,P,1} + [K_1S_1]k_{cat,K,1} - [PA_1][S_1^*]k_{+,P,1}$$
$$\frac{d[S_2^*]}{dt} = [PA_2S_2^*]k_{-,P,2} + [K_2S_2]k_{cat,K,2} - [PA_2][S_2^*]k_{+,P,2}$$

The set of ODEs describing the concentration of adaptor-bound phosphatase are:

$$\frac{d[PA_1]}{dt} = [P][A_1]k_{+,A,1} + [PA_1S_1^*]k_{-,P,1} + [PA_1S_1^*]k_{cat,P,1} - [PA_1]k_{-,A,1} - [PA_1][S_1^*]k_{+,P,1}$$
$$\frac{d[PA_2]}{dt} = [P][A_2]k_{+,A,2} + [PA_2S_2^*]k_{-,P,2} + [PA_2S_2^*]k_{cat,P,2} - [PA_2]k_{-,A,2} - [PA_2][S_2^*]k_{+,P,2}$$

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

$$\frac{d[K_1S_1]}{dt} = [K_1][S_1]k_{+,K,1} - [K_1S_1]k_{-,K,1} - [K_1S_1]k_{cat,K,1}$$
$$\frac{d[K_2S_2]}{dt} = [K_2][S_2]k_{+,K,2} - [K_2S_2]k_{-,K,2} - [K_2S_2]k_{cat,K,2}$$
$$\frac{d[PA_1S_1^*]}{dt} = [PA_1][S_1^*]k_{+,P,1} - [PA_1S_1^*]k_{-,P,1} - [PA_1S_1^*]k_{cat,P,1}$$
$$\frac{d[PA_2S_2^*]}{dt} = [PA_2][S_2^*]k_{+,P,2} - [PA_2S_2^*]k_{-,P,2} - [PA_2S_2^*]k_{cat,P,2}$$

For purposes of display we used the following rate parameters:

Parameter	Value
$k_{+,K,i}$	0.001 $\text{nM}^{-1}\text{s}^{-1}$
$k_{-,K,i}$	0.1 s^{-1}
$k_{cat,K,i}$	0.9 s^{-1}
$k_{+,P,i}$	0.001 $\text{nM}^{-1}\text{s}^{-1}$
$k_{-,P,i}$	0.1 s^{-1}
$k_{cat,P,i}$	0.9 s^{-1}
$k_{+,A,i}$	0.001 $\text{nM}^{-1}\text{s}^{-1}$
$k_{-,A,i}$	0.1 s^{-1}

where $i = 1$ or 2. Our simulations started with the following initial concentrations:

Molecular Species	Initial Concentration
K_1	0-20 nM
K_2	0-20 nM
P	10 nM
S_1	10 μM
S_2	10 μM
A_1	10^{-1}-10^4 nM
A_2	10^{-1}-10^4 nM

This model is used to generate Fig. 4 of the main text. In Fig. $4A$, r_1 is set to 0.1 by having an initial concentration of K_1 of 1 nM and r_2 is set to 2 by having an initial concentration of K_2 of 20 nM. The initial concentration of the adaptors A_1 and A_2 were then concurrently varied between 10^{-1} to 10^4 nM. In Fig. $4B$, A_1 and A_2 were initialized with total concentration of 10 nM each. The values of r_1 and r_2 were set between 0 and 2 by setting the initial concentrations of K_1 and K_2 between 0 and 20 nM.

1.6 2-Kinase/1-Phosphatase Loop with "Unordered" Phosphatase Adaptors

We also developed a model of a 2-Kinase/1-Phosphatase Loop with Unordered Phosphatase Adaptors. The set of enzymatic reactions for this model are:

$$
\begin{aligned}
K_1 + S_1 &\underset{k_{-,K,1}}{\overset{k_{+,K,1}}{\rightleftharpoons}} K_1S_1 \xrightarrow{k_{cat,K,1}} K_1 + S_1^* \\
K_2 + S_2 &\underset{k_{-,K,2}}{\overset{k_{+,K,2}}{\rightleftharpoons}} K_2S_2 \xrightarrow{k_{cat,K,2}} K_2 + S_2^* \\
PA_1 + S_1^* &\underset{k_{-,P,1}}{\overset{k_{+,P,1}}{\rightleftharpoons}} PA_1S_1^* \\
PA_2 + S_2^* &\underset{k_{-,P,2}}{\overset{k_{+,P,2}}{\rightleftharpoons}} PA_2S_2^* \\
P + A_1S_1^* &\underset{k_{-,A,1}}{\overset{k_{+,A,1}}{\rightleftharpoons}} PA_1S_1^* \\
P + A_2S_2^* &\underset{k_{-,A,2}}{\overset{k_{+,A,2}}{\rightleftharpoons}} PA_2S_2^* \\
PA_1S_1^* &\underset{}{\overset{k_{cat,P,1}}{\rightleftharpoons}} PA_1 + S_1^* \\
PA_2S_2^* &\underset{}{\overset{k_{cat,P,2}}{\rightleftharpoons}} PA_2 + S_2^* \\
P + A_1 &\underset{k_{-,A,1}}{\overset{k_{+,A,1}}{\rightleftharpoons}} PA_1 \\
P + A_2 &\underset{k_{-,A,2}}{\overset{k_{+,A,2}}{\rightleftharpoons}} PA_2 \\
A_1 + S_1^* &\underset{k_{-,P,1}}{\overset{k_{+,P,1}}{\rightleftharpoons}} A_1S_1^* \\
A_2 + S_2^* &\underset{k_{-,P,2}}{\overset{k_{+,P,2}}{\rightleftharpoons}} A_2S_2^*
\end{aligned}
\tag{1.4}
$$

This model differs from the "ordered" model in that the adaptors can first bind the phosphorylated substrate without needing to be bound to a phosphatase catalytic core. These reactions are diagrammed in the inset of Fig. $4A$ of the main text. The set of ODEs describing the concentration of free enzymes are:

$$
\begin{aligned}
\frac{d[K_1]}{dt} &= [K_1S_1]k_{-,K,1} + [K_1S_1]k_{cat,K,1} - [K_1][S_1]k_{+,K,1} \\
\frac{d[K_2]}{dt} &= [K_2S_2]k_{-,K,2} + [K_2S_2]k_{cat,K,2} - [K_2][S_2]k_{+,K,2}
\end{aligned}
$$

$$\frac{d[P]}{dt} = [PA_1]k_{-,A,1} + [PA_2]k_{-,A,2} + [PA_1S_1^*]k_{-,A,1} + [PA_2S_2^*]k_{-,A,2} \\ - [P][A_1]k_{+,A,1} - [P][A_2]k_{+,A,2} - [P][A_1S_1^*]k_{+,A,1} - [P][A_2S_2^*]k_{+,A,2}$$

The set of ODEs describing the concentration of free unphosphorylated substrates are:

$$\frac{d[S_1]}{dt} = [K_1S_1]k_{-,K,1} + [PA_1S_1^*]k_{cat,P,1} - [K_1][S_1]k_{+,K,1}$$
$$\frac{d[S_2]}{dt} = [K_2S_2]k_{-,K,2} + [PA_2S_2^*]k_{cat,P,2} - [K_2][S_2]k_{+,K,2}$$

The set of ODEs describing the concentration of free phosphorylated substrates are:

$$\frac{d[S_1^*]}{dt} = [PA_1S_1^*]k_{-,P,1} + [A_1S_1^*]k_{-,P,1} + [K_1S_1]k_{cat,K,1} - [PA_1][S_1^*]k_{+,P,1} - [A_1][S_1^*]k_{+,P,1}$$
$$\frac{d[S_2^*]}{dt} = [PA_2S_2^*]k_{-,P,2} + [A_2S_2^*]k_{-,P,2} + [K_2S_2]k_{cat,K,2} - [PA_2][S_2^*]k_{+,P,2} - [A_2][S_2^*]k_{+,P,2}$$

The set of ODEs describing the concentration of adaptor-bound phosphatase are:

$$\frac{d[PA_1]}{dt} = [P][A_1]k_{+,A,1} + [PA_1S_1^*]k_{-,P,1} + [PA_1S_1^*]k_{cat,P,1} - [PA_1]k_{-,A,1} - [PA_1][S_1^*]k_{+,P,1}$$
$$\frac{d[PA_2]}{dt} = [P][A_2]k_{+,A,2} + [PA_2S_2^*]k_{-,P,2} + [PA_2S_2^*]k_{cat,P,2} - [PA_2]k_{-,A,2} - [PA_2][S_2^*]k_{+,P,2}$$

The set of ODEs describing the concentration of adaptor-bound phosphorylated substrate are:

$$\frac{d[A_1S_1^*]}{dt} = [A_1][S_1^*]k_{+,P,1} + [PA_1S_1^*]k_{-,A,1} - [A_1S_1^*]k_{-,P,1} - [P][A_1S_1^*]k_{+,A,1}$$
$$\frac{d[A_2S_2^*]}{dt} = [A_2][S_2^*]k_{+,P,2} + [PA_2S_2^*]k_{-,A,2} - [A_2S_2^*]k_{-,P,2} - [P][A_2S_2^*]k_{+,A,2}$$

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

$$\frac{d[K_1S_1]}{dt} = [K_1][S_1]k_{+,K,1} - [K_1S_1]k_{-,K,1} - [K_1S_1]k_{cat,K,1}$$
$$\frac{d[K_2S_2]}{dt} = [K_2][S_2]k_{+,K,2} - [K_2S_2]k_{-,K,2} - [K_2S_2]k_{cat,K,2}$$
$$\frac{d[PA_1S_1^*]}{dt} = [PA_1][S_1^*]k_{+,P,1} + [P][A_1S_1^*]k_{+,P,1} - [PA_1S_1^*]k_{-,P,1} - [PA_1S_1^*]k_{-,A,1} - [PA_1S_1^*]k_{cat,P,1}$$
$$\frac{d[PA_2S_2^*]}{dt} = [PA_2][S_2^*]k_{+,P,2} + [P][A_2S_1^*]k_{+,P,2} - [PA_2S_2^*]k_{-,P,2} - [PA_2S_2^*]k_{-,A,2} - [PA_2S_2^*]k_{cat,P,2}$$

For purposes of display we used the following rate parameters:

Parameter	Value
$k_{+,K,i}$	0.001 $\text{nM}^{-1}\text{s}^{-1}$
$k_{-,K,i}$	0.1 s^{-1}
$k_{cat,K,i}$	0.9 s^{-1}
$k_{+,P,i}$	0.001 $\text{nM}^{-1}\text{s}^{-1}$
$k_{-,P,i}$	0.1 s^{-1}
$k_{cat,P,i}$	0.9 s^{-1}
$k_{+,A,i}$	0.001 $\text{nM}^{-1}\text{s}^{-1}$
$k_{-,A,i}$	0.1 s^{-1}

where $i = 1$ or 2. We set the $K_{D,A,i} = k_{-,A,i}/k_{+,A,i} = 100$ nM to represent a reasonably high affinity of the phosphatase catalytic core P with the adaptor domains A_i. We used the same affinity for the binding of the adaptor domain to the substrate. Note that, in this model, the affinity of the phosphatase for the adaptor domain, and the affinity of the adaptor domain for the substrate, does not depend on wether the adaptor is bound to its other partner. Our simulations started with the following initial concentrations:

Molecular Species	Initial Concentration
K_1	0-20 nM
K_2	0-20 nM
P	10 nM
S_1	10 μM
S_2	0-10 μM
A_1	10^{-1}-10^4 nM
A_2	0-10^4 nM

This model is used to generate Fig. 5 of the main text. In Fig. $5A$, r_1 is set to 0.1 by having an initial concentration of K_1 of 1 nM and r_2 is set to 2 by having an initial concentration of K_2 of 20 nM. The initial concentration of the adaptors A_1 and A_2 were then concurrently varied between 10^{-1} to 10^4 nM. In Fig. $5B$, A_1 and A_2 were initialized with total concentration of 10 nM each. The values of r_1 and r_2 were set between 0 and 2 by setting the initial concentration of K_1 and K_2 to between 0 and 20 nM.

2 The responses of the substrates of an unsaturated phosphatase are strictly hyperbolic in r

We have previously shown [1], following the derivation of Goldbeter and Koshland [5], that the change in product concentration S_1^* for an enzyme E with multiple substrates can be defined as:

$$\frac{d[S_1^*]}{dt} = \frac{V_{max,E,1}[S_1]}{\alpha_{E,1}K_{M,E,1} + [S_1]} \tag{2.1}$$

where $\alpha_{E,1} \equiv 1 + \sum_{i \neq 1} \frac{[S_i]}{K_{M,E,i}}$ is an inhibitory constant for substrate competition with S_1 for E. For a futile cycle at steady state we will have $d[S_1^*]/dt = d[S_1]/dt$. For an unsaturated 2K1P loop, $\alpha_{P,i} = 1$ since $K_{M,P,i} \gg [S_i]_0$. Given 2.1, at steady state we have:

$$\frac{V_{max,K,1}[S_1]}{K_{m,K,1} + [S_1]} = \frac{V_{max,P,1}[S_1^*]}{K_{M,P,1} + [S_1^*]} \tag{2.2}$$

Following the standard Michaelis-Menten approach [1, 5], we assume that $[S_i]_0 \gg [K]_0, [P]_0$, giving us $[S_i]_0 = [S_i] + [S_i^*]$, which can be substituted into 2.2:

$$\frac{V_{max,K,1}(1 - S_1^*)}{K_{K,1} + 1 - S_1^*} = \frac{V_{max,P,1}S_1^*}{K_{P,1} + S_1^*} \tag{2.3}$$

where $S_1 \equiv [S_1]/[S_1]_0$, $S_1^* \equiv [S_1^*]/[S_1]_0$, $K_{K,1} \equiv K_{M,K,1}/[S_1]_0$, and $K_{P,1} \equiv K_{M,P,1}/[S_1]_0$. Dividing both sides by $V_{max,P,1}$ we obtain:

$$\frac{r_1(1 - S_1^*)}{K_{K,1} + 1 - S_1^*} = \frac{S_1^*}{K_{P,1} + S_1^*} \tag{2.4}$$

where $r_1 \equiv V_{max,K,1}/V_{max,P,1}$. Since we are assuming the phosphatase to be unsaturated, $K_{P,1} \gg S_1^*$, and as such 2.4 can be simplified to:

$$\frac{r_1(1 - S_1^*)}{K_{K,1} + 1 - S_1^*} = \frac{S_1^*}{K_{P,1}} \tag{2.5}$$

Expanding 2.5, we get:

$$\begin{aligned} r_1K_{P,1} - r_1K_{P,1}S_1^* &= K_{K,1}S_1^* + S_1^* - (S_1^*)^2 \\ (S_1^*)^2 - (1 + K_{K,1} + r_1K_{P,1})S_1^* + r_1K_{P,1} &= 0 \end{aligned} \tag{2.6}$$

Solving for S_1^*, we obtain the expression:

$$S_1^* = \frac{(1 + K_{K,1} + r_1K_{P,1}) - \sqrt{(1 + K_{K,1} + r_1K_{P,1})^2 - 4r_1K_{P,1}}}{2} \tag{2.7}$$

where the negative branch of the quadratic solution is chosen to obtain physically realistic values of S_1^* (i.e. $0 \leq S_1^* \leq 1$). We can show that S_1^* for a 2K1P with an unsaturated phosphatase is strictly hyperbolic in r_1 by taking the second derivative of 2.7 with respect to r_1:

$$\frac{d^2S_1^*}{dr_1^2} = -\frac{2K_{K,1}K_{P,1}^2}{(-4r_1K_{P,1} + (1 + K_{K,1} + r_1K_{P,1})^2)^{3/2}} \tag{2.8}$$

Note that, for positive real values of both the rate constants and the concentrations, both the numerator and denominator in the above equation are positive. The second derivative is thus always negative (i.e. the curvature is concave), and the variation of S_1^* with r_1 lacks an inflection point. As a result, a system with unsaturated phosphatases cannot exhibit the sigmoidal behavior characteristic of an ultrasensitive, switch-like response.

3 Analytical solution for 1-Kinase/1-Substrate Model with Synthesis and Degradation

From the ODEs derived in 1.4, we have at steady state:

$$\frac{d[K]}{dt} = [KS]k_- + [KS]k_{cat} + [KS]k_{deg,U} - [K][S]k_+ = 0 \tag{3.1}$$

$$\frac{d[S]}{dt} = [KS]k_- + k_{synth} - [K][S]k_+ - [S]k_{deg,U} = 0 \tag{3.2}$$

$$\frac{d[S^*]}{dt} = [KS]k_{cat} - [S^*]k_{deg,P} = 0 \tag{3.3}$$

$$\frac{d[KS]}{dt} = [K][S]k_+ - [KS]k_- - [KS]k_{cat} - [KS]k_{deg,U} = 0 \tag{3.4}$$

Additionally, we can define the conservation of mass of the kinase K:

$$[K]_0 = [K] + [KS] \tag{3.5}$$

From 3.4 we can define the concentration of KS as:

$$[KS] = \frac{[K][S]k_+}{k_- + k_{cat} + k_{deg,U}} \tag{3.6}$$

Equation 3.6 can then be substituted into 3.5 to define the concentration of free kinase K:

$$\begin{aligned}
[K]_0 &= [K] + \frac{[K][S]k_+}{k_- + k_{cat} + k_{deg,U}} \\
[K]_0 &= [K]\left(1 + \frac{[S]k_+}{k_- + k_{cat} + k_{deg,U}}\right) \\
[K] &= \frac{[K]_0}{1 + \frac{[S]k_+}{k_- + k_{cat} + k_{deg,U}}}
\end{aligned} \tag{3.7}$$

We can then substitute 3.7 into 3.6 to get:

$$[KS] = \frac{[K]_0[S]}{\frac{k_- + k_{cat} + k_{deg,U}}{k_+} + [S]} \tag{3.8}$$

We can simplify 3.8 by defining:

$$K_{M,deg} \equiv \frac{k_- + k_{cat} + k_{deg,U}}{k_+}$$

where $K_{M,deg}$ is the analogue of the Michaelis-Menten constant for the kinase K, taking into account the effects of substrate degradation. Equation 3.8 then can be written:

$$[KS] = \frac{[K]_0[S]}{K_{M,deg} + [S]} \tag{3.9}$$

From 3.3 we obtain the expression:

$$[KS]k_{cat} = [S^*]k_{deg,P}$$

into which we can substitute 3.9 to get:

$$\frac{[K]_0[S]k_{cat}}{K_{M,deg} + [S]} = [S^*]k_{deg,P} \tag{3.10}$$

We can then multiply both sides of 3.10 by $[S]_0/[S]_0$:

$$\frac{[K]_0 S \cdot k_{cat}}{K_{deg} + S} = S^* k_{deg,P}[S_0]$$

where $S \equiv [S]/[S]_0$, $S^* \equiv [S^*]/[S]_0$, and $K_{deg} \equiv K_{M,deg}/[S]_0$. We can then define $r_{deg} \equiv [K]_0 k_{cat}/[S]_0 k_{deg,P}$, the ratio of the maximum velocity of the kinase to the maximum velocity of phosphorylated substrate degradation, to get:

$$\frac{r_{deg} S}{K_{deg} + S} = S^* \tag{3.11}$$

Following the standard Michaelis-Menten assumptions [1,5], we have $[S]_0 \gg [K]_0$. This gives us $[S]_0 = [S] + [S^*]$, or $1 = S + S^*$, which can be substituted into 3.11:

$$\begin{aligned} \frac{r_{deg}(1 - S^*)}{K_{deg} + 1 - S^*} &= S^* \\ r_{deg} - r_{deg}S^* &= K_{deg}S^* + S^* - (S^*)^2 \\ (S^*)^2 - (1{+}r_{deg} + K_{deg})S^* + r_{deg} &= 0 \end{aligned} \tag{3.12}$$

We can then solve 3.12 for S^*:

$$S^* = \frac{1 + r_{deg} + K_{deg} - \sqrt{(1 + r_{deg} + K_{deg})^2 - 4r_{deg}}}{2} \tag{3.13}$$

where we have again chosen the negative branch of the solution to ensure $0 \leq S_1^* \leq 1$. From this derivation, we can see that degradation has two effects on the fraction of phosphorylated substrate. The degradation rate of unphosphorylated substrate can change the saturation of the kinase K by the substrate through altering the Michaelis-Menten-like constant $K_{M,deg}$, in the same way as altering the dissociation or catalytic rates. Additionally, the degradation rate of phosphorylated substrate can modify the magnitude of the fraction of phosphorylated substrate by changing r_{deg}.

We can show that S^* is strictly hyperbolic in r_{deg} by taking the second derivative of 3.13 with regard to r_{deg}:

$$\frac{d^2 S^*}{dr_{deg}^2} = -\frac{2K_{deg}}{(-4r_{deg} + (1 + r_{deg} + K_{deg})^2)^{3/2}} \tag{3.14}$$

As for the response with an unsaturated phosphatase (equation 2.8), both the numerator and denominator of the above equation are strictly positive, so the second derivative is always negative. A system that relies on degradation to achieve effective dephosphorylation thus has no inflection point in r_{deg} and cannot exhibit a sigmoidal response to signals.

4 UniProt Data

We searched the UniProt database for the number of serine/threonine and tyrosine kinases and phosphatases found in all complete eukaryotic genomes [6]. For each genome, we searched for UniProt for reviewed entries that included the enzyme classification numbers for kinases and phosphatases (see Table 1). We then counted the number of entries for each genome in the search results. In order to prevent genomes with small numbers of reviewed kinases or phosphatases from unduly influencing our results, we ignored genomes with less than 5 phosphatases or 5 kinases for any given residue cass (i.e. serine/threonine or tyrosine). This resulted in 16 genomes for serine/threonine enzymes (See Table 2) and 9 genomes for tyrosine enzymes (See Table 3).

Table 1: Enzyme classification numbers used to search UniProt

Enzyme	E.C. Number
Serine/Threonine Phosphatases	3.1.3.3 / 3.1.3.16
Serine/Threonine Kinases	2.7.11.x
Tyrosine Phosphatases	3.1.3.48
Tyrosine Kinases	2.7.10.x

Table 2: The numbers and ratios of serine/threonine kinases and phosphatases from UniProt used in Figure 1 of the main text.

Species	Serine/Threonine Phosphatases	Serine/Threonine Kinases	Ratio
Arabidopsis thaliana	115	559	0.206
Bos taurus	26	81	0.321
Caenohabditis elegans	15	89	0.169
Danio rerio	14	40	0.350
Dictyostelium discoideum	21	222	0.095
Drosophila melanogaster	22	66	0.333
Gallus gallus	9	36	0.250
Homo sapiens	79	372	0.212
Mus musculus	72	374	0.193
Oryctolagus cuniculus	7	23	0.304
Oryza sativa	94	120	0.783
Pongo abelii	10	37	0.270
Rattus norvegivus	40	188	0.213
Saccharomyces cerevisiae	24	124	0.194
Schizosaccharomyces pombe	20	100	0.200
Xenopus laevis	17	72	0.236

Table 3: The numbers and ratios of tyrosine kinases and phosphatases from UniProt used in Figure 1 of the main text.

Species	Tyrosine Phosphatases	Tyrosine Kinases	Ratio
Arabidopsis thaliana	9	16	0.563
Bos taurus	13	9	1.444
Caenohabditis elegans	12	11	1.091
Drosophila melanogaster	14	21	0.667
Gallus gallus	11	32	0.344
Homo sapiens	87	95	0.916
Mus musculus	81	95	0.853
Rattus norvegivus	39	49	0.796
Xenopus laevis	11	25	0.440

Additionally, we used UniProt to determine the number of phosphoproteins in complete eukaryotic genomes. We searched UniProt for reviewed entries with keyword 'Phosphoprotein'. We then analyzed the search results and computed the number of entries for each of the 16 species from the serine/threonine enzyme results and the 9 species from the tyrosine enzyme results. The phosphatase numbers represent the total number of phosphatases from tables 2 and 3.

Table 4: The numbers and ratios of the total number of phosphatases and substrates from UniProt used in Figure 1 of the main text.

Species	Total Phosphatases	Total Substrates	Ratio
Arabidopsis thaliana	124	1295	10.444
Bos taurus	39	1759	45.103
Caenohabditis elegans	27	89	3.296
Danio rerio	14	195	13.929
Dictyostelium discoideum	21	160	7.619
Drosophila melanogaster	36	833	23.139
Gallus gallus	20	282	14.100
Homo sapiens	166	5924	35.687
Mus musculus	153	5313	34.725
Oryctolagus cuniculus	7	282	40.286
Oryza sativa	94	150	1.596
Pongo abelii	10	819	81.900
Rattus norvegivus	79	2691	34.063
Saccharomyces cerevisiae	24	2425	101.042
Schizosaccharomyces pombe	20	1067	53.350
Xenopus laevis	28	277	9.893

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