Biophysical Journal, Volume 99

Supporting Material

Non-essential Sites Improve Phosphorylation Switch

Liming Wang, Qing Nie, and German Enciso

Non-Essential Sites Improve Phosphorylation Switch

Liming Wang, Qing Nie, and German Enciso

Text S1: Supplementary Material

Contents

A Computing the Hill exponent (or coefficient) under sequential phosphorylation

In the sequential case, each phosphorylation and dephosphorylation consist of the following elementary chemical reactions respectively,

$$S_i + E \underset{k_{\text{off}_i}}{\overset{k_{\text{on}_i}}{\rightleftarrows}} ES_i \overset{k_{\text{cat}_i}}{\rightarrow} S_{i+1} + E,$$

$$S_{i+1} + F \underset{l_{\text{off}_i}}{\overset{l_{\text{on}_i}}{\rightleftarrows}} FS_{i+1} \overset{l_{\text{cat}_i}}{\rightarrow} S_i + F.$$

Here, k_{on_i} and k_{off_i} are the binding and unbinding rates of kinse E and substrate S_i, respectively; k_{cat_i} is the catalytic rate for the complex ES_i to produce S_{i+1}. Simliarly, l_{on_i} and l_{off_i} are the binding and unbinding rates of the phosphatase F and the substrate S_{i+1}, respectively; l_{cat_i} is the catalytic rate for the complex FS_{i+1} to produce S_i.

Our goal is to compute the steady state proportion of the substrates with at least k phosphorylated sites,

$$\frac{s_k + \cdots + s_n}{s_0 + \cdots + s_k + \cdots + s_n}.$$

Here, small letters denote the steady state concentrations of their corresponding proteins; the number k is referred as the *minimal activation number*. The steady state concentrations of different phosphoforms turn out to satisfy the following relations [2, 3, 4]:

$$s_{i+1} = \lambda_i u s_i, \tag{1}$$

where λ_i and u are defined as

$$u := \frac{e}{f}, \quad \lambda_i := \frac{k_{\text{cat}_i} L_{M_i}}{K_{M_i} l_{\text{cat}_i}}, \quad K_{M_i} := \frac{k_{\text{cat}_i} + k_{\text{off}_i}}{k_{\text{on}_i}}, \quad L_{M_i} := \frac{l_{\text{cat}_i} + l_{\text{off}_i}}{l_{\text{on}_i}}.$$

Note that in general the steady states are not unique for arbitrary given total concentrations of the kinase and the phosphatase [2, 3, 4], however, it is unique when considering the free kinase to phosphatase ratio as an input. Based on (1), the steady state fraction of substrates with at least k phosphorylated sites equals

$$\frac{\lambda_1\lambda_2\cdots\lambda_k u^k + \cdots + \lambda_1\lambda_2\cdots\lambda_n u^n}{1 + \lambda_1 u + \lambda_1\lambda_2 u^2 + \cdots + \lambda_1\lambda_2\cdots\lambda_k u^k + \cdots + \lambda_1\lambda_2\cdots\lambda_n u^n}. \tag{2}$$

When the relative kinase to phosphatase efficiencies are similar for each site, i.e., $\lambda_i \approx \lambda$, the above formula can be simplified to

$$r_{n,k}(x) := \frac{x^k + \cdots + x^n}{1 + x + \cdots + x^k + \cdots + x^n} = \frac{x^{n+1} - x^k}{x^{n+1} - 1}, \tag{3}$$

where $x := \lambda u$. This function $r_{n,k}(x)$ defines the input-output (also called the dose-response) curve with $\lambda e/f$ as the input and the proportion of active substrates as the output. When $n = 2k - 1$, equation (3) simplifies to

$$r_{2k-1,k}(x) = \frac{x^k}{1 + x^k},$$

which is a Hill function with Hill exponent k. The effective Hill exponent of the function $r_{n,k}(x)$, $0 < k < n$, can be estimated by the Goldbeter-Koshland Formula [1],

$$H_s(n, k) = \frac{\ln 81}{\ln(v_{n,k}/u_{n,k})}, \tag{4}$$

where

$$u_{n,k} = r_{n,k}^{-1}(0.1), \quad v_{n,k} = r_{n,k}^{-1}(0.9). \tag{5}$$

If the dose-response curve is a Hill function $\frac{x^m}{K^m + x^m}$, formula (4) recovers the Hill exponent m. For the convenience of notation, when there is no confusion, we omit the subscripts of r, u, and so on. Since $r(x)$ is an increasing function in x, both u and v in (5) are well-defined. When $k = 0$ and $n + 1$, naturally, we define

$$H_s(n, 0) = H_s(n, n + 1) = 0.$$

Let α be the ratio of k and $n+1$, termed as the *site activation ratio*. We next calculate $H_s(n,k)$ for arbitrary n and k. Define an auxiliary function

$$\bar{r}_\alpha(x) = \frac{x - x^\alpha}{x - 1}.$$

Let us denote the Hill exponent of $\bar{r}_\alpha$ by $\bar{H}$. Naturally, $\bar{H}$ equals zero when $\alpha = 0$ and $\alpha = 1$. For $0 < \alpha < 1$, we perform a change of variable with $\bar{x} = x^{n+1}$, then

$$r_{n,k}(x) = \frac{\bar{x} - \bar{x}^\alpha}{\bar{x} - 1} = \bar{r}_\alpha(\bar{x}).$$

If we let $\bar{u} = u_{n,k}^{n+1}$ and $\bar{v} = v_{n,k}^{n+1}$, then $\bar{r}_\alpha(\bar{u}) = 0.1$, $\bar{r}_\alpha(\bar{v}) = 0.9$, and

$$\bar{H}(\alpha) = \frac{\ln 81}{\ln(\bar{v}/\bar{u})} = \frac{\ln 81}{(n+1)\ln(v_{n,k}/u_{n,k})} = \frac{1}{n+1} H_s(n,k).$$

Multiplying both sides by $n+1$, we obtain

$$H_s(n,k) = \bar{H}(\alpha)\,(n+1). \tag{6}$$

Next, we show that $\bar{H}(\alpha)$ is symmetric with respect to $\alpha = 1/2$. Let $\bar{u}$ be such that $\bar{r}_\alpha(\bar{u}) = 0.1$, then it is equivalent to show that $\bar{r}_{1-\alpha}(\bar{u}^{-1}) = 0.9$, which indeed holds from a straightforward computation,

$$\frac{\bar{u}^{-1} - \bar{u}^{-(1-\alpha)}}{\bar{u}^{-1} - 1} = 0.9.$$

Similiarly, let $\bar{v}$ be such that $\bar{r}_\alpha(\bar{v}) = 0.9$, then $\bar{r}_{1-\alpha}(\bar{v}^{-1}) = 0.1$, and thus the Hill exponent of $\bar{r}_{1-\alpha}$ is

$$\bar{H}(1-\alpha) = \frac{\ln 81}{\ln(\bar{u}^{-1}/\bar{v}^{-1})} = \frac{\ln 81}{\ln(\bar{v}/\bar{u})} = \bar{H}(\alpha).$$

Our numerical simulations in Figure 2B further reveal that $\bar{H}(\alpha)$ is well approximated by the quadratic function $2\alpha(1-\alpha)$, that is,

$$H_s(n,k) \approx 2\alpha(1-\alpha)(n+1) = 2k\left(1 - \frac{k}{n+1}\right). \tag{7}$$

B Intuition using biased random walks

It helps to have an intuition for why simply reducing the minimal activation number (or adding additional sites without increasing this number) increases the switch-like behavior of the system. Imagine the phosphorylation of an individual protein as a discrete stochastic event. At any given time t the protein is in a state between 0 and n phosphorylations, and it follows a random walk between these states. The propensities for phosphorylation and dephosphorylation are given by the constants e and f respectively. The probabilities $P_0(t), \ldots, P_n(t)$ for being in a specific state at time t satisfy the system of differential equations

$$\begin{aligned}
P_0' &= -eP_0 + fP_1 \\
P_1' &= eP_0 - fP_1 - eP_2 + fP_0 \\
&\vdots \\
P_n' &= eP_{n-1} - fP_n,
\end{aligned}$$

and the steady state probabilities P_i are given by the same formula (3) as the steady state concentrations for the original continuous system, in the perfect balanced case.

Now, if e is even slightly larger than f, then the bias in the random walk will intuitively make the protein spend most of its time in the top half of the states, since any biased random walk eventually moves with high probability in the direction of the bias. Similarly it will spend little time in this region if e is slightly smaller than f. This accounts for the ultrasensitive behavior. Moreover, if e is slightly larger than f, then the ball will spend slightly more time at the most phosphorylated state than if $e = f$, but not much more, since the random process itself will constantly kick it out of this location. This means that P_n as a function of e/f is much less ultrasensitive than $P_k + \cdots + P_n$.

C Unordered phosphorylation

In the unordered case, the sites are phosphorylated and dephosphorylated in a random order. Once a substrate-enzyme complex is formed, different products can be made based on their catalytic rates. The number of phosphoforms grows exponentially with n in the unordered mechanism in contrast to linearly in the sequential mechanism, and we thus expect to see pronounced difference between sequential and unordered cases as n increases.

Introduce an index vector $\vec{a}$, consisting of only zeros and ones, to represent substrates in different phosphoforms. For example, S_{001} denote the substrate with three phosphorylation sites, of which the first two sites are empty, and the last one is occupied. A general phosphorylation and dephosphorylation reaction can be decomposed into elementary reactions as

$$S_{\vec{a}} + E \underset{k^{\vec{a}}_{\rm off}}{\overset{k^{\vec{a}}_{\rm on}}{\rightleftarrows}} ES_{\vec{a}} \overset{k^{\vec{a},\vec{b}}_{\rm cat}}{\to} S_{\vec{b}} + E,$$

$$S_{\vec{b}} + F \underset{l^{\vec{b}}_{\rm off}}{\overset{l^{\vec{b}}_{\rm on}}{\rightleftarrows}} FS_{\vec{b}} \overset{l^{\vec{b},\vec{a}}_{\rm cat}}{\to} S_{\vec{a}} + F.$$

Here, the index vector $\vec{b}$ could be any vector obtained by replacing a zero in vector $\vec{a}$ by a one. For example, when $\vec{a} = (0,0,1)$, $\vec{b}$ could be $(0,1,1)$ or $(1,0,1)$, but not $(1,1,1)$. We assume that the kinase-substrate complex is determined by the reacting substrate and kinase, but not by the releasing product. That is, when $\vec{a}$ is given, different choices of $\vec{b}$ share the same kinase-substrate complex, $ES_{\vec{a}}$. This is especially suitable for the situation when the kinase has a docking site. Once the substrate binds to the docking site, any unphosphorylated residue on the substrate is a candidate to be phosphorylated.

Similarly as the sequential case, the steady state concentrations of different phosphoforms satisfy [3],

$$s_{\vec{b}} = \lambda_{\vec{a},\vec{b}}\, u\, s_{\vec{a}}, \tag{8}$$

where

$$\lambda_{\vec{a},\vec{b}} := \frac{k^{\vec{a},\vec{b}}_{\rm cat}}{K^{\vec{a},\vec{b}}_M} \frac{L^{\vec{b},\vec{a}}_M}{l^{\vec{b},\vec{a}}_{\rm cat}}, \quad K^{\vec{a},\vec{b}}_M := \frac{k^{\vec{a},\vec{b}}_{\rm off} + \sum_{\vec{a} \overset{E}{\to} \vec{b}} k^{\vec{a},\vec{b}}_{\rm cat}}{k^{\vec{a},\vec{b}}_{\rm on}}, \quad L^{\vec{b},\vec{a}}_M := \frac{l^{\vec{b},\vec{a}}_{\rm off} + \sum_{\vec{b} \overset{F}{\to} \vec{a}} l^{\vec{b},\vec{a}}_{\rm cat}}{l^{\vec{b},\vec{a}}_{\rm on}}.$$

Let us denote by s_i the total concentration of different phosphoform substrates with i sites being phosphorylated. For example, s_1 represents the sum of s_{100}, s_{010}, and s_{001}. Under the perfect balanced condition ($\lambda_{\vec{a},\vec{b}} = \lambda$), we have

$$s_i = \left(\begin{array}{c} n \\ i \end{array} \right) x^i,$$

since there are n choose k different phosphoforms with exactly k phosphorylated sites. Thus, the steady state proportion of the active substrates is,

$$g_{n,k}(x) = \frac{\left(\begin{array}{c} n \\ k \end{array} \right) x^k + \cdots + \left(\begin{array}{c} n \\ n \end{array} \right) x^n}{1 + \left(\begin{array}{c} n \\ 1 \end{array} \right) x + \cdots + \left(\begin{array}{c} n \\ k \end{array} \right) x^k + \cdots + \left(\begin{array}{c} n \\ n \end{array} \right) x^n} = \frac{\sum_{i=k}^{n} \left(\begin{array}{c} n \\ i \end{array} \right) x^i}{(1+x)^n}, \tag{9}$$

where $x = \lambda u$. Next, we prove that there exists a function σ_r that only depends on α such that $H_r(n,k)$ can be written as

$$H_r(n,k) \approx \sigma_r(\alpha)\sqrt{n+1}. \tag{10}$$

To show this, we first interpret the function $g_{n,k}(x)$ in terms of the random process of tossing coins. Define i.i.d. random variabled $Y_i \in \{0,1\}$, $i = 1, \ldots, n$, with

$$\text{Prob}(Y_i = 1) = p, \quad \text{Prob}(Y_i = 0) = q,$$

where $p + q = 1$ and $p, q > 0$. Let $Y_i = 1$ denote the ith toss being head, and $Y_i = 0$ denote the ith toss being tail. The expectation and the variance of Y_i are

$$E(Y_i) = p, \quad Var(Y_i) = E(Y_i^2) - (EY_i)^2 = p - p^2.$$

The sum of Y_i's, $W_n := \sum_{i=1}^{n} Y_i$, counts the total number of heads. That is, $\text{Prob}(W_n = k)$ represents the probability of seeing k heads in n independent experiments, which can be computed as

$$\text{Prob}(W_n \geq k) = \sum_{i=k}^{n} \left(\begin{array}{c} n \\ i \end{array} \right) p^i q^{n-i}.$$

If we let $x = \frac{p}{q}$, then

$$\text{Prob}(W_n \geq k) = \sum_{i=k}^{n} \left(\begin{array}{c} n \\ i \end{array} \right) \left(\frac{x}{1+x} \right)^i \left(\frac{1}{1+x} \right)^{n-i} = g_{n,k}(x). \tag{11}$$

On the other hand, the central limit theorem says

$$Z_n := \frac{W_n - np}{\sqrt{p - p^2}\sqrt{n}} \sim N(0,1).$$

Therefore,

$$\begin{aligned} g_{n,k}(x) &= \text{Prob}(W_n \geq k) \\ &= \text{Prob}\left(Z_n \geq \frac{k - np}{\sqrt{p - p^2}\sqrt{n}} \right) \\ &\approx 1 - \Phi\left(\frac{k - np}{\sqrt{p - p^2}\sqrt{n}} \right) \\ &= 1 - \Phi\left(\frac{k(1+x) - nx}{\sqrt{xn}} \right), \end{aligned} \tag{12}$$

where function Φ is the cumulative distribution function of $N(0,1)$. The definition of v says

$$0.9 \approx 1 - \Phi\left(\frac{k(1+v) - nv}{\sqrt{vn}}\right). \tag{13}$$

For the simplicity of notations, we use equal sign from now on. Rearranging (13), we have

$$k(v+1) - nv = -\xi\sqrt{nv},$$

where $\xi = -\Phi^{-1}(0.1)$. In the above equation, define $\gamma := \sqrt{v}$ and replace k by $\alpha(n+1)$,

$$(\alpha - \frac{n}{n+1})\gamma^2 + \xi\sqrt{\frac{n}{(n+1)^2}}\gamma + \alpha = 0.$$

For large n, the above equation is approximately

$$(\alpha - 1)\gamma^2 + \frac{\xi}{\sqrt{n+1}}\gamma + \alpha = 0.$$

The roots are

$$\gamma_{1,2} = \frac{-\frac{\xi}{\sqrt{n+1}} \pm \sqrt{\frac{\xi^2}{n+1} - 4(\alpha-1)\alpha}}{2(\alpha-1)}.$$

Because $\alpha < 1$ and $\gamma > 0$, we have

$$\sqrt{v} = \gamma = \frac{-\frac{\xi}{\sqrt{n+1}} - \sqrt{\frac{\xi^2}{n+1} - 4(\alpha-1)\alpha}}{2(\alpha-1)}.$$

Similarly, the equation of u, where $g(u) = 0.1$, is

$$k(u+1) - nu = \xi\sqrt{nu}, \tag{14}$$

and the solution is

$$\sqrt{u} = \frac{\frac{\xi}{\sqrt{n+1}} - \sqrt{\frac{\xi^2}{n+1} - 4(\alpha-1)\alpha}}{2(\alpha-1)}. \tag{15}$$

Therefore

$$\begin{aligned}
\frac{\sqrt{v}}{\sqrt{u}} &= \frac{\frac{\xi}{\sqrt{n+1}} + \sqrt{\frac{\xi^2}{n+1} - 4(\alpha-1)\alpha}}{-\frac{\xi}{\sqrt{n+1}} + \sqrt{\frac{\xi^2}{n+1} - 4(\alpha-1)\alpha}} \\
&= \frac{\frac{\xi^2}{n+1} + 2\alpha(1-\alpha) + \frac{\xi}{\sqrt{n+1}}\sqrt{\frac{\xi^2}{n+1} - 4(\alpha-1)\alpha}}{2\alpha(1-\alpha)} \\
&= 1 + \frac{\frac{\xi}{\sqrt{n+1}}\sqrt{\frac{\xi^2}{n+1} - 4(\alpha-1)\alpha}}{2\alpha(1-\alpha)} + \frac{\frac{\xi^2}{n+1}}{2\alpha(1-\alpha)}.
\end{aligned} \tag{16}$$

Define a shorthand

$$A = \frac{\xi}{\sqrt{\alpha(1-\alpha)}}\frac{1}{\sqrt{n+1}}.$$

The third term in (16) becomes $A^2/2$, and the second term in (16) becomes

$$A\sqrt{1+\frac{\xi^2}{4\alpha(1-\alpha)(n+1)}} \approx A,$$

for fixed α and large n. Thus,

$$\frac{\sqrt{v}}{\sqrt{u}} \approx e^{\frac{\xi}{\sqrt{\alpha(1-\alpha)}}\frac{1}{\sqrt{n+1}}}, \tag{17}$$

and

$$\ln\frac{v}{u} = \frac{2\xi}{\sqrt{\alpha(1-\alpha)}}\frac{1}{\sqrt{n+1}}.$$

By the Goldbeter-Koshland Formula, we have

$$H_r(n,k) \approx \frac{\ln 81}{2\xi}\sqrt{\alpha(1-\alpha)}\sqrt{n+1} \approx 1.71\sqrt{\alpha(1-\alpha)}\sqrt{n+1} = 1.71\sqrt{k\left(1-\frac{k}{n+1}\right)}, \tag{18}$$

which is (10) with

$$\sigma_r(\alpha) = 1.71\sqrt{\alpha(1-\alpha)}.$$

Please see Figure S3A for comparisons of Hill exponents computed directly from the Goldbeter-Koshland Formula and those estimated by equation (18).

D Changes of ultrasensitivity with respect to k, n, and α

D.1 Fix n, vary k

The sequential case

$$\frac{\partial H_s}{\partial k} = 2\left(1-\frac{2k}{n+1}\right).$$

Thus, when n is fixed, as k increases, the Hill exponent first increases, then decreases, and the maximum is achieved at $2k = n+1$.

The unordered case

$$\frac{\partial H_r}{\partial k} = 1.7145\frac{1-\frac{2k}{n+1}}{\sqrt{\frac{k}{n+1}\left(1-\frac{k}{n+1}\right)}}.$$

Similarly, when n is fixed, as k increases, the Hill exponent first increases, then decreases, and the maximum is achieved at $2k = n+1$.

D.2 Fix k, vary n

Notice that H_s in (7) and H_r in (18) can be written either in terms of α and n or in terms of k and n. In the following computations, we use the expressions of H_s and H_r involving only k and n.

The sequential case

$$\frac{\partial H_s}{\partial n} = \frac{2k^2}{(n+1)^2} > 0.$$

Thus, when k is fixed, as n increases, the Hill exponent always increases.

The unordered case

$$\frac{\partial H_r}{\partial n} = \frac{1.7145}{2\sqrt{k\left(1-\frac{k}{n+1}\right)}}\frac{k^2}{(n+1)^2} > 0.$$

So, when k is fixed, as n increases, the Hill exponent always increases.

D.3 Fix α, vary n

In the following computations, we use the expressions of H_s and H_r in (7) and (18) invloving only α and n.

The sequential case

$$\frac{\partial H_s}{\partial n} = 2\alpha(1-\alpha) > 0.$$

Thus, when α is fixed, as n increases, the Hill exponent always increases.

The unordered case

$$\frac{\partial H_r}{\partial n} = \frac{1.7145\alpha(1-\alpha)}{2\sqrt{n+1}} > 0.$$

Therefore, when α is fixed, as n increases, the Hill exponent always increases.

E Changes of threshold with respect to k, n, and α

First, let us first prove a fact that will be repeatedly used in our analysis. Define a function

$$y(x) = \frac{a_k x^k + \cdots + a_n x^n}{1 + a_1 x + \cdots + a_{k-1}x^{k-1} + a_k x^k + \cdots + a_n x^n},$$

which is the general form of the dose-response curves used in equation (2) (the sequential case) and equation (2) (the unordered case). Here, a_is are positive real numbers. k and n are integers, and $k \leq n$. Claim that $y(x)$ is an increasing function of x, $x > 0$, i.e.,

$$\frac{dy}{dx} > 0 \text{ for } x > 0. \tag{19}$$

This is equivalent to proving that the function $\frac{1}{y(x)}$ is decreasing in x. The derivative of $\frac{1}{y(x)}$ with respect to x is,

$$\frac{\sum_{i=1}^{k-1}\sum_{j=k}^{n}(i-j)a_i a_j x^{i+j-1} - \sum_{j=k}^{n} a_j x^{j-1}}{(a_k x^k + \cdots + a_n x^n)^2}. \tag{20}$$

Notice that in the first term of the numerator, $i-j$ is always negative, and a_i and a_j are both positive, so overall (20) is negative. Therefore, $\frac{1}{y(x)}$ is decreasing in x, and $y(x)$ is increasing in x.

In the following subsections, we focus on the perfect balanced case, which corresponds to $a_i = 1$ in $y(x)$ under the sequential mechanism (the function $r_{n,k}(x)$) and $a_i = \binom{n}{k}$ in $y(x)$ under the unordered mechanism (the function $g_{n,k}(x)$).

We define the threshold of the does-response curve $y(x)$ as the value of x when $y(x)$ reaches ten percent of its maximal, i.e., $y^{-1}(0.1)$. The changes of threshold with respect to k, n, and α are analyzed in the following subsections.

E.1 Fix n, vary k

The sequential case

For fixed n, rewrite the function $r_{n,k}(x)$ as $l(k,x)$. Thus, the threshold is the solution of

$$0.1 = l(k,x). \tag{21}$$

Taking derivative of both sides of equation (21) with respect to k, we obtain

$$\frac{dx}{dk} = -\frac{\partial l/\partial k}{\partial l/\partial x}.$$

Based on (19), $\partial l/\partial x$ is positive. On the other hand,

$$\frac{\partial l}{\partial k}(k,x) = -\frac{x^k \ln x}{x^{n+1}-1} < 0$$

on both intervals $x > 1$ and $0 < x < 1$. Also, it is easy to see that $\partial l/\partial k$ is continuous at $x = 1$ with

$$\frac{\partial l}{\partial k}(k,1) = -\frac{1}{n+1}.$$

Thus, dx/dk is always positive, i.e., for fixed n, the threshold is increasing in k.

The unordered case

The threshold in the unordered case is solved from equation (14). For fixed n, taking derivatives with respect to k on both sides of equation (14), we obtain

$$\frac{du}{dk} = \frac{u+1}{n-k+\frac{\xi\sqrt{n}}{2\sqrt{u}}} > 0.$$

Thus, for fixed n, the threshold increases in k.

E.2 Fix k, vary n

The sequential case

For fixed k, rewrite the function $r_{n,k}(x)$ as $h(n,x)$. Thus, the threshold is the solution of

$$0.1 = h(n,x). \tag{22}$$

Taking derivative of both sides of equation (22) with respect to n, we obtain

$$\frac{dx}{dn} = -\frac{\partial h/\partial n}{\partial h/\partial x}.$$

Based on (19), $\partial h/\partial x$ is positive. On the other hand,

$$\frac{\partial h}{\partial n}(n,x) = \frac{x^k - 1}{(x^{n+1}-1)^2} x^{n+1} \ln x > 0$$

on both intervals $x > 1$ and $0 < x < 1$. Also, it is easy to see that $\partial h/\partial n$ is continuous at $x = 1$ with

$$\frac{\partial h}{\partial n}(n, 1) = \frac{k}{(n+1)^2}.$$

Thus, dx/dn is always negative, i.e., for fixed k, the threshold is decreasing in n.

The unordered case

The threshold in the unordered case is solved from equation (14). For fixed k, taking derivatives with respect to n on both sides of equation (14), we obtain

$$\frac{du}{dn} = -\frac{\left(\frac{\xi}{2\sqrt{nu}} + 1\right) u}{n - k + \frac{\xi\sqrt{n}}{2\sqrt{u}}} < 0.$$

Thus, for fixed k, the threshold decreases in n.

E.3 Fix α, vary n

The sequential case

For fixed α, $\bar{u} = \bar{r}_\alpha^{-1}(0.1)$ is fixed, and the threshold $u = \bar{u}^{1/(n+1)}$. So, the monotonicity of u with respect to n depends on whether $\bar{u}$ is greater than one. On the other hand, $\bar{u} > 1$ if and only if $\alpha > 0.9$. To see this, notice that the function $r_{n,k}(x)$ is increasing in x (the claim proved at the beginning of Section E). When $x = 1$, $\bar{r}_\alpha(1) = r_{n,k}(1) = 1 - \alpha$. Thus, $\bar{u} = \bar{r}_\alpha^{-1}(0.1) > 1$ if $\alpha > 0.9$; $\bar{u} = \bar{r}_\alpha^{-1}(0.1) < 1$ if $\alpha < 0.9$. Therefore, the threshold increases in n when $\alpha < 0.9$ and decreases when $\alpha > 0.9$.

The unordered case

In the unordered case, the threshold is given in (15). Rewrite $\sqrt{u}$ as

$$\sqrt{u} = \frac{2\alpha}{\frac{\xi}{\sqrt{n+1}} + \sqrt{\frac{\xi^2}{n+1} - 4(\alpha - 1)\alpha}}.$$

It is easy to see that for fixed α, u is increasing n, i.e., the threshold is increasing in n.

References

[1] A. Goldbeter and D.E. Koshland. An amplified sensitivity arising from covalent modification in biological systems. *Proceedings of the National Academy of Sciences*, 78(11):6840–6844, 1981.

[2] J. Gunawardena. Multisite protein phosphorylation makes a good threshold but can be a poor switch. *Proc. Natl. Acad. Sci.*, 102:14617–14622, 2005.

[3] M. Thomson and J. Gunawardena. Unlimited multistability in multisite phosphorylation systems. *Nature*, 460(7252):274–277, 2009.

[4] L. Wang and E.D. Sontag. On the number of steady states in a multiple futile cycle. *Journal of Mathematical Biology*, 57:29–52, 2008.

Figure S1

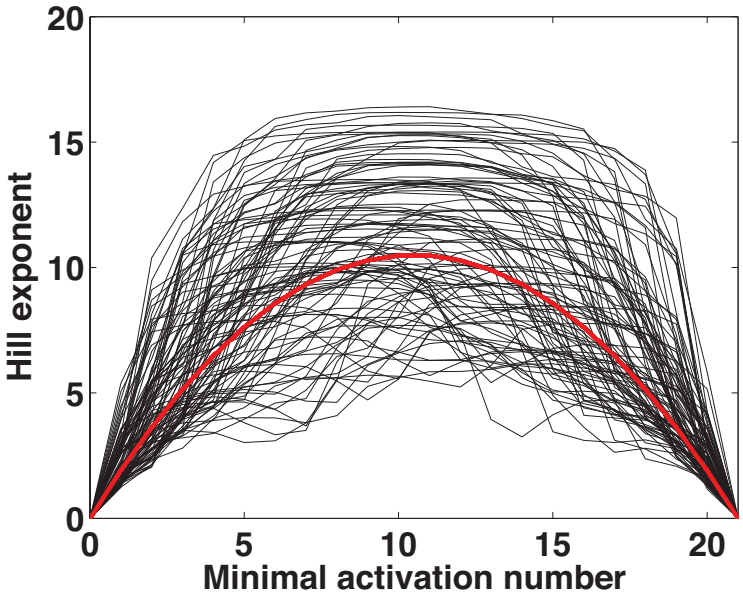

Figure S1: Plot of the Hill exponents for random λs. Each black curve corresponds to one set of λ_is. In total, 100 sets of λ_is are generated, where each $\log_{10}\lambda_i$ follows a uniform distribution on $[-1, 1]$. The red curve represents the perfect balanced case when $\lambda_i = 1$.

Figure S2

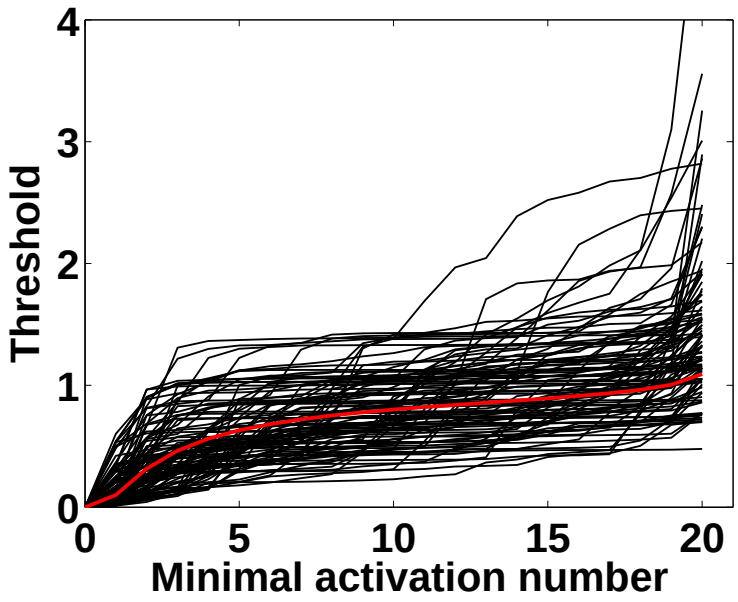

Figure S2: Plot of the thresholds for random λs. Each black curve corresponds to one set of λ_is. In total, 100 sets of λ_is are generated, where each $\log_{10}\lambda_i$ follows a uniform distribution on $[-1, 1]$. The red curve represents the perfect balanced case when $\lambda_i = 1$.

Figure S3

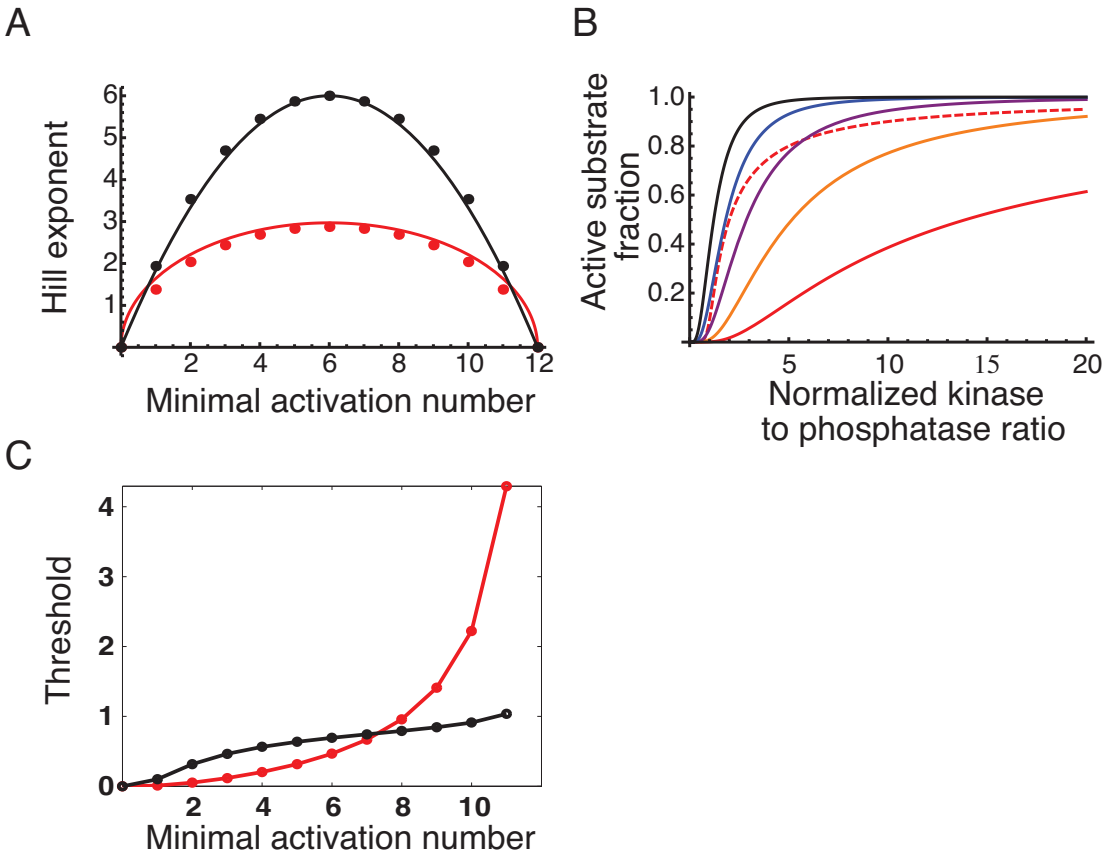

Figure S3: Comparison between the sequential and the non-sequential mechanisms. (A) The Hill exponent under the sequential (black) and non-sequential (red) mechanisms. Here, the dots are computed directly from the Goldbeter-Koshland formula, and the curves are estimations from Eq. 2 and Eq. 4 in the main text. In both plots, $n = 11$ and $\lambda_i = 1$. **(B)** Plot of the dose-response curves for the sequential case $k = 10$ (red dashed), non-sequential case $k = 10$ (red solid), non-sequential case $k = 9$ (orange solid), 8 (purple solid), 7 (blue solid), 6 (black solid). In all plots, n is fixed at 10. **(C)** The threshold under the sequential (black) and non-sequential (red) mechanisms. Here, $n = 11$ and $\lambda_i = 1$.

Figure S4

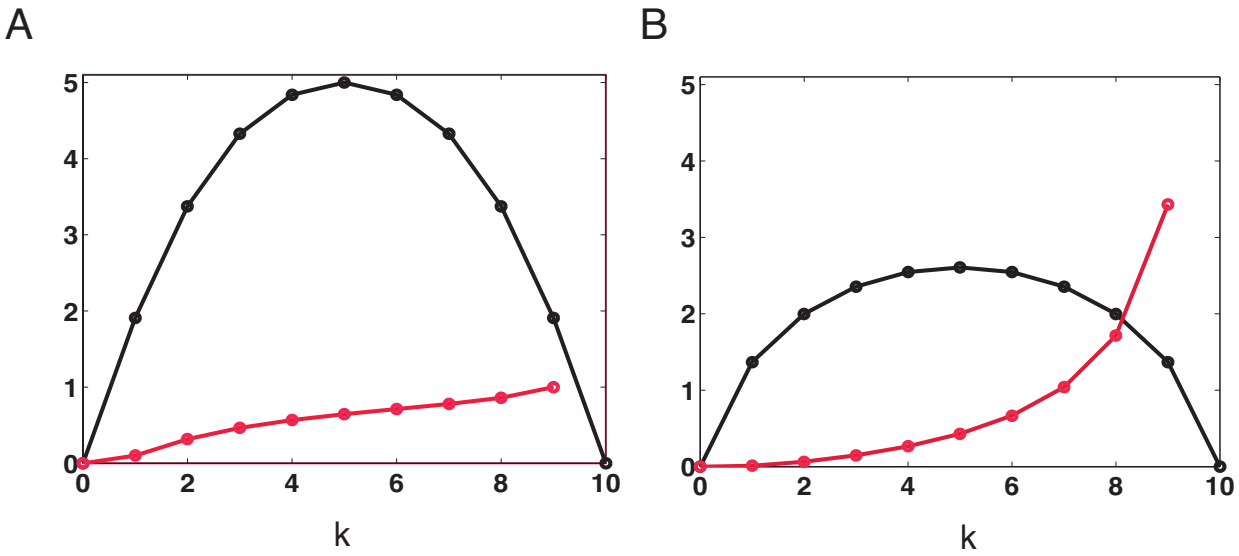

Figure S4: Ultrasensitivity and threshold under the unordered mechanism. (A) Comparing the Hill exponents computed directly from the Goldbeter-Koshland formula (dots) and from Eq. 4 (curves) for different k-values when $n = 20$ (red) and $n = 40$ (black). (B) The threshold against different k-values. Red: $n = 20$; black: $n = 40$. In both curves, $\lambda_i = 1$.

Figure S5

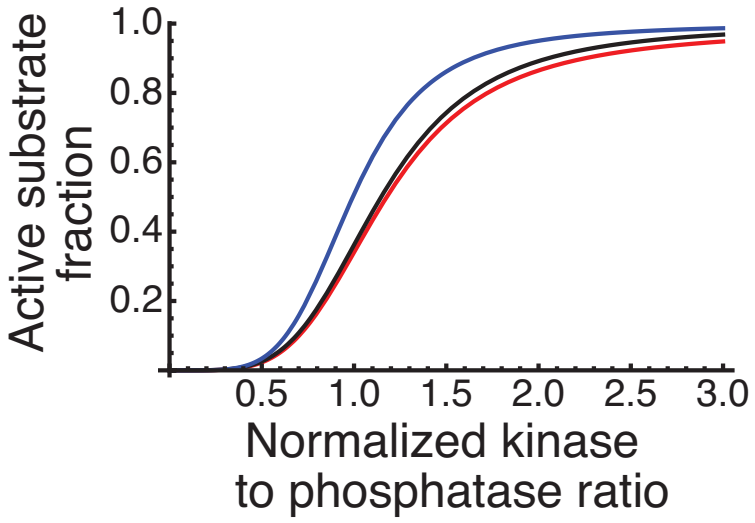

Figure S5: The combination of cooperativity and non-essential sites. The original system (red) shows high ultrasensitivity due to cooperativity with $\lambda_1, \ldots, \lambda_4 = 0.5, \lambda_5 = 16$, $n = k = 5$. The blue curve corresponds to $n = 6, k = 5$ with $\lambda_6 = 0.5$, and the black curve represents $n = 5, k = 4$.

Table S1

	Hill exponent		Threshold	
	Sequential	Non-sequential	Sequential	Non-sequential
Fix n, increase k	$\uparrow$, if $k < \frac{n+1}{2}$ $\downarrow$, if $k > \frac{n+1}{2}$	$\uparrow$, if $k < \frac{n+1}{2}$ $\downarrow$, if $k > \frac{n+1}{2}$	$\uparrow$	$\uparrow$
Fix k, increase n	$\uparrow$	$\uparrow$	$\downarrow$	$\downarrow$
Fix α, increase n	$\uparrow$	$\uparrow$	$\uparrow$, if $\alpha < 0.9$ $\downarrow$, if $\alpha > 0.9$	$\uparrow$

Table S1: Dependence of the Hill exponent and the threshold on k, n, and α in sequential and non-sequential mechanisms.