Nonessential Sites Improve Phosphorylation Switch

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ABSTRACT Multisite phosphorylation is a common form of posttranslational protein regulation which has been used to increase the switchlike behavior of the protein response to increasing kinase concentrations. In this letter, we show that the switchlike response of multisite phosphoproteins is strongly enhanced by nonessential phosphorylation sites, a mechanism that is robust to parameter changes and easily implemented in nature. We obtained analytic estimates for the Hill exponent (or coefficient) of the switchlike response, and we observed that a tradeoff exists between the switch and the kinase threshold for activation. This also suggests a possible evolutionary mechanism for the relatively large numbers of phosphorylation sites found in various proteins.

Received for publication 29 May 2010 and in final form 13 July 2010.

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It has been argued that a large number of phosphorylation sites could form the basis for a strong switchlike response (large Hill exponent) to an increase in kinase concentration (1,2). Such a property is desirable in situations where a highly ultrasensitive response is expected (such as cell division in response to a growth factor), or in the design of multistable systems (3). However, it was demonstrated in Gunawardena (4) through a mathematical model that the dose-response curve of the fully phosphorylated protein cannot be switchlike. Several plausible mechanisms have been suggested to enhance the switchlike response, including cooperativity among different sites (4), temporal cooperativity (5), or introducing other players such as membrane (6) and scaffold proteins (7).

Here, we propose a simple mechanism that substantially improves ultrasensitivity of a dose response. Instead of considering only the fully phosphorylated substrate as in Gunawardena (4), Qian and Cooper (5), and Serber and Ferrell (6), we include substrates with at least k phosphorylated sites, out of a total of n sites, as active (Fig. 1). We call k the minimal activation number. A possible way to achieve this threshold protein activation is through an entropic binding mechanism, as described in Lenz and Swain (8). The potential improvement of ultrasensitivity for such mechanism has been suggested in a recent study on bistability by multisite phosphorylation (9). The steady-state fraction of activated substrates is given by

$$\rho_{n,k} = \frac{s_k + \cdots + s_n}{1 + \cdots + s_k + \cdots + s_n}, \quad (1)$$

where s_i denotes the steady-state concentration of the corresponding protein. There is increasing evidence supporting this assumption. A classical example is the yeast cell cycle regulator, Sic1, which has nine phosphorylation sites, among which any combination of six is sufficient to trigger the onset of S phase (1,10,11). At first glance, the inclusion of partially phosphorylated substrates as active seems futile, because a system with minimal activation number k out of a total of n sites seems similar to a system with a total of k sites where full phosphorylation is required for activation. Rather surprisingly, we find that the remaining $n-k$ sites markedly improve the ultrasensitivity of the dose-response curve (Fig. 2 *A*).

In general, phosphorylation and dephosphorylation can follow either a sequential or a nonsequential mechanism. In the sequential mechanism, (de)phosphorylation takes place in a specific order (Fig. 1) (12). Denote by λ_i the relative efficiency of a substrate being phosphorylated versus being dephosphorylated at the i^{th} residue (detailed definitions given in Text S1 *A* in the Supporting Material). When $\lambda_i \approx \lambda$, the effective Hill exponent of the dose-response curve can be estimated as (see Text S1 *A* in the Supporting Material)

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

where $\alpha = k/(n+1)$. In particular, when $n = 2k-1$, i.e., approximately half of the phosphorylation sites are nonessential, and Eq. 1 can be rewritten as (see Text S1 *A* in the Supporting Material)

$$\rho_{2k-1,k}(u) = \frac{(\lambda u)^k}{1 + (\lambda u)^k}, \quad (3)$$

where u (dose) is the ratio of the steady-state free kinase to phosphatase. Clearly, Eq. 3 is a Hill function with Hill exponent k, consistent with the Hill exponent estimated by Eq. 2 when $n = 2k-1$. In comparison, the effective Hill exponent of the traditional k-site phosphorylation model in Gunawardena (4) is $2k/(k+1)$, a number always smaller than k for $k > 1$.

Moreover, based on Eq. 2, we can conclude (see Text S1 *A* in the Supporting Material):

1. For fixed n, as k increases, the ultrasensitivity first increases then decreases (Fig. 2 *B*).

Editor: Herbert Levine.

doi: 10.1016/j.bpj.2010.07.030

Please cite this article in press as: Wang et al., Nonessential Sites Improve Phosphorylation Switch, Biophysical Journal (2010), doi:10.1016/j.bpj.2010.07.030

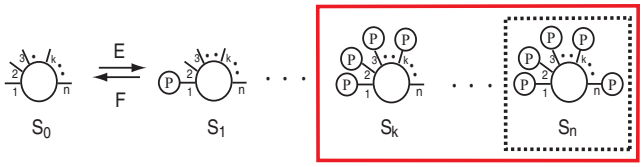

FIGURE 1 A schematic diagram of *n*-site phosphorylations and dephosphorylations under the sequential and distributive mechanism by kinase *E* and phosphatase *F*, respectively. Inside the solid box is the generalized definition of active substrates; inside the dashed box is the conventional definition.

2. When k is fixed, the ultrasensitivity is improved by including more nonessential phosphorylation sites (*black* versus *red* for the same k in Fig. 2 *B*).
3. For fixed α, the ultrasensitivity increases linearly in n (points on the same ray in Fig. 2 *B*).

Similar trends are observed through numerical simulations when the λ_i values are chosen randomly for each site (Fig. 2 *C* and Fig. S1 in the Supporting Material).

As an illustration of Eq. 2, consider the Wee1 protein in the *Xenopus* egg cell cycle. It is known that the first three sites (Ser^{38}, Thr^{53}, and Ser^{62}) tend to be phosphorylated before the other two (Thr^{104} and Thr^{150}), although the other two are essential to the activity of Wee1 (13). When the first four sites are mutated, only Thr^{150} can be phosphorylated, and this is equivalent to $k = n = 1$. The estimated Hill exponent by Eq. 2 is 1, close to the observed experimental value of 1.1 (14). When the Thr^{104} site is mutated alone (Wee1-T104E), the Hill exponent increases in experiments to 1.4 (14). Assuming for simplicity that the first three sites are phosphorylated sequentially, which corresponds to $k = n = 4$ in our model, one obtains a comparable value of 1.6.

Next, we study the effect of nonessential phosphorylation sites to the threshold of switches under the sequential mechanism. One possible definition of the threshold is EC10, i.e., the input value such that the output reaches 10% of its maximum (6). Our analytical study (see Text S1 *C* in the Supporting Material) for $\lambda_i \approx \lambda$ reveals that the threshold increases in k (Fig. 2 *D*) and decreases in n (*green* versus *red* for the same k in Fig. 2 *D*). The threshold plot of randomly chosen λ_i shows the same trend (Fig. 2 *D*, *black*).

The above analysis focuses on the sequential mechanism. In the nonsequential case, we assume that any subset of at least k phosphorylated sites is sufficient to activate the protein, regardless of the exact position of the sites. The unordered mechanism seems to be especially applicable in the context of bulk electrostatics: when a protein is sufficiently phosphorylated, it may cease to bind to negatively charged or hydrophobic regions, such as the cell membrane, regardless of exactly which sites are involved. This can alter the activity of a protein, as illustrated by the Ste5 protein in yeast (15). It has been shown that phosphorylation sites on the substrate tend to be located in poorly conserved (16) and predominantly disordered (17) regions, which suggests that the exact location

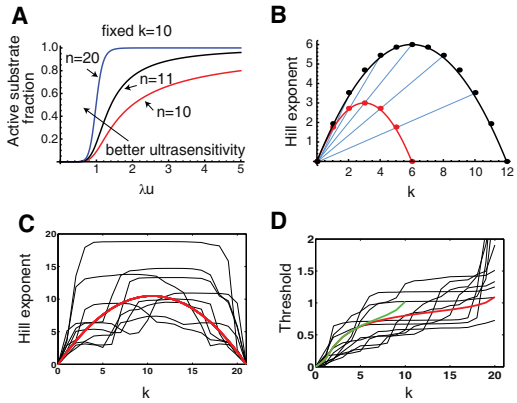

FIGURE 2 Ultrasensitivity and threshold under the sequential mechanism. (*A*) Dose-response curve. (*B*) The Hill exponents' estimation by Eq. 2 (*curve*) and computed by ln(81)/ ln(*EC*90/*EC*10) (*dots*). (*C*) Random λ_i (*black*) versus $\lambda_i \approx \lambda$ (*red*). (*D*) Threshold. (*Green*) $n = 10$, $\lambda_i = 1$. (*Red*) $n = 20$, $\lambda_i = 1$. (*Black*) $n = 20$; λ randomly chosen between 0.1 and 10.

of the sites can often be unessential for activation and may support mechanisms such as bulk electrostatics.

In the $\lambda_i \approx \lambda$ case, the Hill exponent of the steady-state fraction of the active substrates is estimated by (Text S1 *C* in the Supporting Material)

$$H_r(n,k) \approx 1.71\sqrt{k\left(1-\frac{k}{n+1}\right)} = 1.71\sqrt{\alpha(1-\alpha)(n+1)}, \quad (4)$$

which is approximately the square-root of H in the sequential case (Fig. S3 *A*). Therefore, the conclusions of the sequential case still hold (Fig. S3 *B*), but the ultrasensitivity increases in

$$\sqrt{n+1}$$

instead of n (Text S1 *D* in the Supporting Material). Conclusions on the threshold under the sequential mechanism also carry over to the nonsequential case (Fig. S3 *C*), except that for fixed α, the threshold increases in n, regardless of the value of α (Text S1 *E* in the Supporting Material).

In summary, under both sequential and nonsequential mechanisms, the introduction of nonessential sites appears to have opposite effects on ultrasensitivity and threshold (Table S1). Because a good switch is expected to have both high ultrasensitivity and large threshold, there seems to be an optimal range for the number of nonessential sites when the total number of sites is fixed. Thus, one possible explanation of the requirement of six phosphorylated sites in Sic1 is through the optimization of ultrasensitivity and threshold. If the phosphorylation of Sic1 follows a sequential mechanism and $\lambda_i \approx \lambda$, then the largest Hill exponent is achieved when $k = 5$ (Fig. S4 *A*). If the phosphorylation of Sic1 is random, k could be further increased to achieve a better threshold without much sacrifice in the ultrasensitivity (Fig. S4 *B*).

In this new model, by decoupling the total number of sites from the number of phosphorylations needed for activation,

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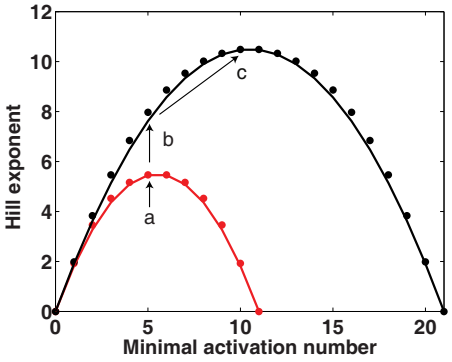

FIGURE 3 Evolutionary race. (*a*) $k = 5$, $n = 10$; (*b*) $k = 5$, $n = 20$; and (*c*) $k = 10$, $n = 20$.

we may look at the evolution of multisite phosphorylation, because a given mutation can easily change one without altering the other. For instance, a series of mutations (or a single insertion event), that lead to doubled number of sites while leaving k unchanged, may increase the ultrasensitivity dramatically (*a* versus *b* in Fig. 3) while slightly decreasing the threshold (*green* versus *red* at $k = 5$ in Fig. 2 *D*). Moreover, the evolutionary pressure may drive k to increase (*b* versus *c* in Fig. 3). This process can continue to repeat itself over time, and one can speculate that this could sometimes lead to a runaway increase in the number of sites.

Intuitively, why will the addition of the nonessential sites increase the switchlike behavior of a system?

Imagine the phosphorylation of an individual protein as a biased random walk between 0 and n phosphorylations. The propensities for phosphorylation and dephosphorylation are given by the constants E and F, respectively. When E is larger than F, the biased random walk gravitates toward states with large phosphorylated residues, and the probability of it falling in $S_k,\ldots,S_n$ is higher than that in S_n. Conversely, even if E is slightly smaller than F, the biased random walk now favors states with few phosphorylated residues, and this accounts for the ultrasensitive behavior of the switch (Text S1 *B* in the Supporting Material).

In conclusion, we have proposed a mechanism through the use of nonessential sites that could account for the high ultrasensitivity observed in many multisite phosphorylation systems. For given values of the total number of sites and the minimal number of phosphorylations for activation, we have obtained estimates of the effective Hill exponent under both sequential and nonsequential mechanisms. The effect of nonessential sites on both the effective Hill exponent and the threshold of a dose-response curve are analyzed. Our results suggest that inclusion of nonessential phosphorylation sites improves ultrasensitivity, but decreases the threshold (Table S1 in the Supporting Material). Thus, to achieve a good activation switch, there is a balance between the number of nonessential sites and the total number of sites. This new mechanism could be extended to other contexts such as methylation, acetylation, or even the binding of multiple transcription factors on a promoter region.

SUPPORTING MATERIAL

Five text sections, five figures, and one table are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(10)00902-1.

ACKNOWLEDGMENTS

We thank Karen Sachs for the many stimulating conversations, and Jim Ferrell and Lee Bardwell for useful advice.

Q.N. was supported by National Institutes of Health grant Nos. R01GM75309, R01GM67247, and P50GM76516, and National Science Foundation grant No. DMS-0917492.

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Biophysical Journal, Volume 99

Supporting Material

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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.

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Figure S1

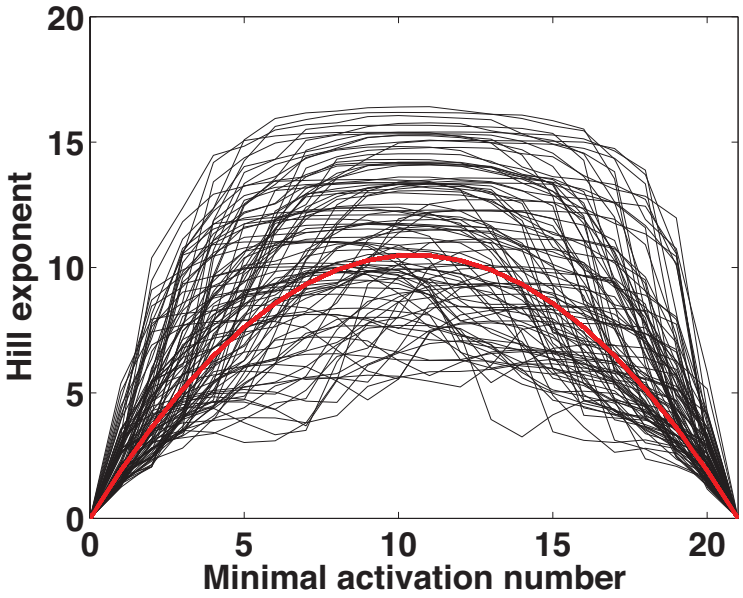

Figure S1: Plot of the Hill exponents for random λs. Each black curve corresponds to one set of λ_is. In total, 100 sets of λ_is are generated, where each $\log_{10}\lambda_i$ follows a uniform distribution on $[-1, 1]$. The red curve represents the perfect balanced case when $\lambda_i = 1$.

Figure S2

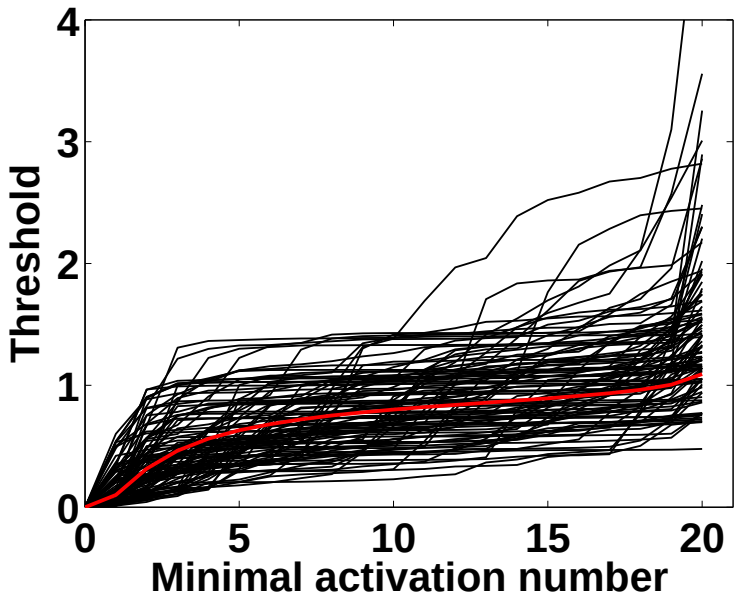

Figure S2: Plot of the thresholds for random λs. Each black curve corresponds to one set of λ_is. In total, 100 sets of λ_is are generated, where each $\log_{10}\lambda_i$ follows a uniform distribution on $[-1, 1]$. The red curve represents the perfect balanced case when $\lambda_i = 1$.

Figure S3

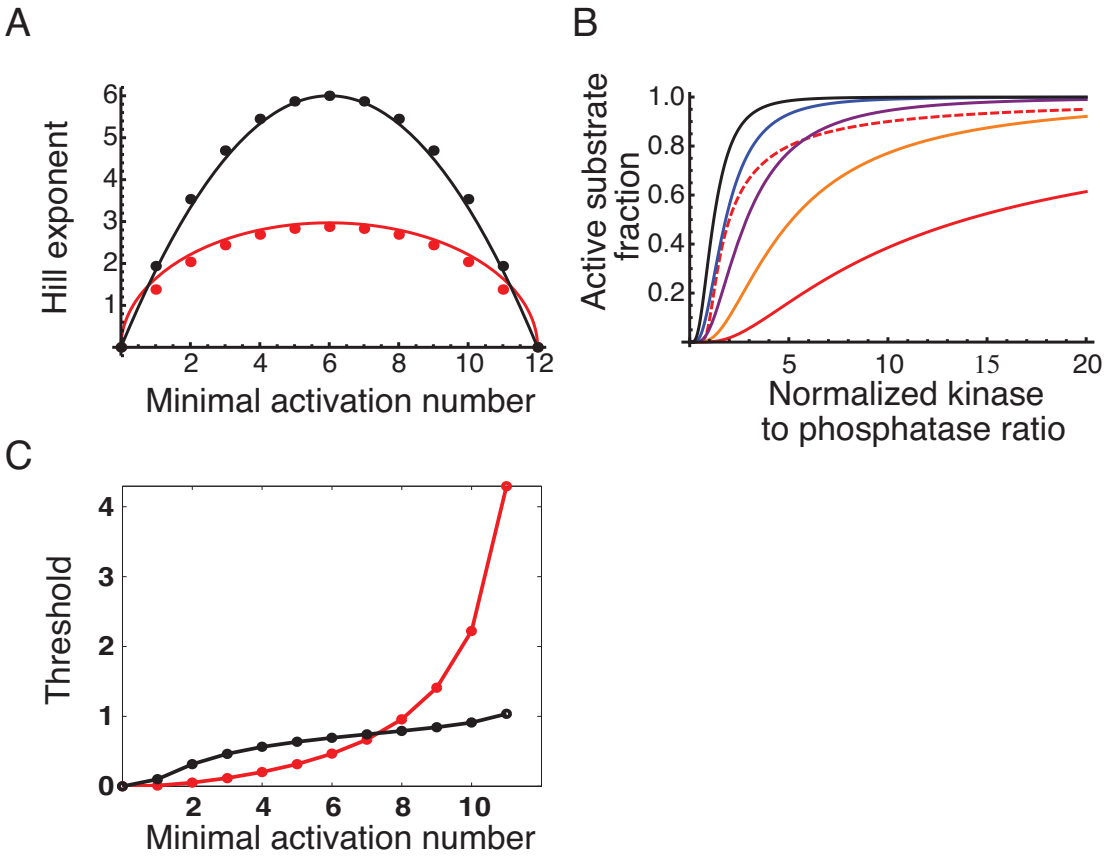

Figure S3: Comparison between the sequential and the non-sequential mechanisms. (A) The Hill exponent under the sequential (black) and non-sequential (red) mechanisms. Here, the dots are computed directly from the Goldbeter-Koshland formula, and the curves are estimations from Eq. 2 and Eq. 4 in the 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

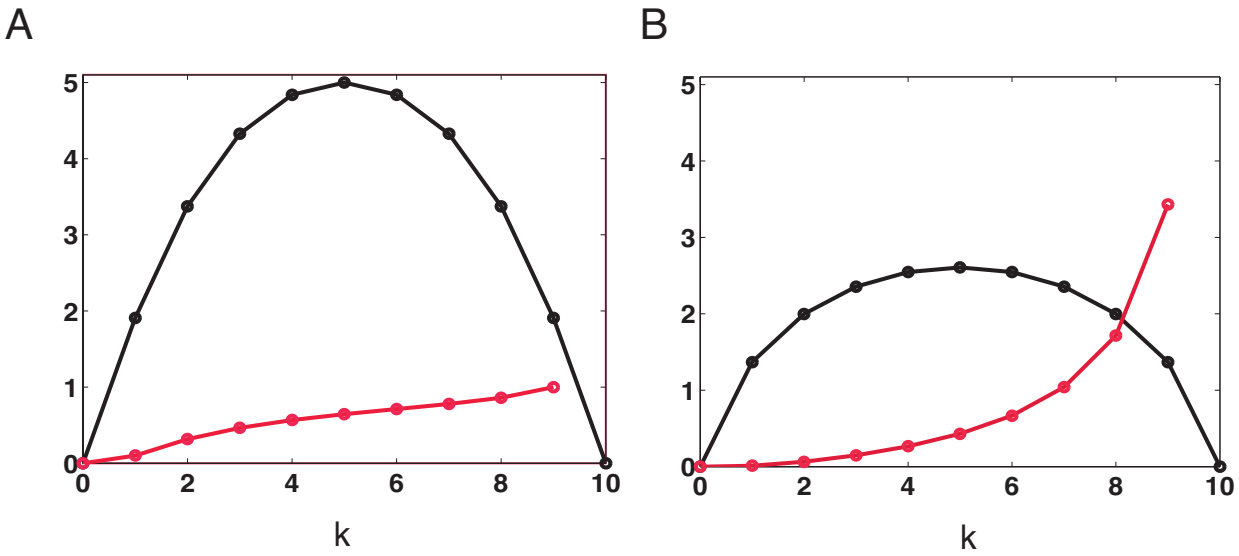

Figure S4: Ultrasensitivity and threshold under the unordered mechanism. (A) Comparing the Hill exponents computed directly from the Goldbeter-Koshland formula (dots) and from Eq. 4 (curves) for different k-values when $n = 20$ (red) and $n = 40$ (black). (B) The threshold against different k-values.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

Figure S5

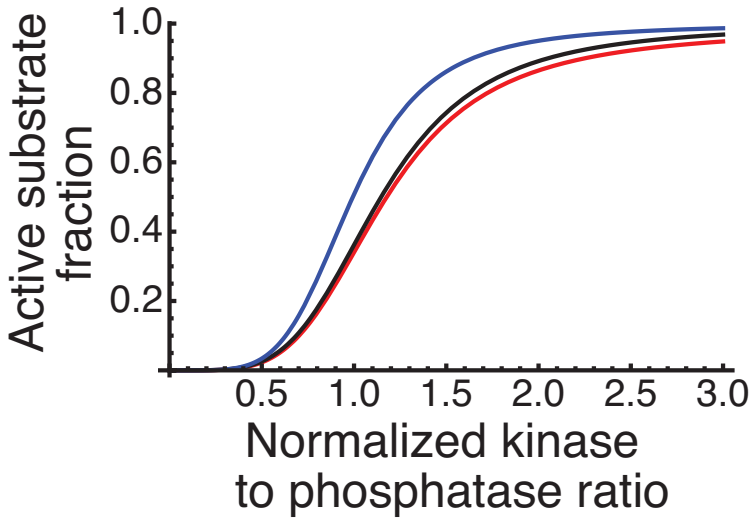

Figure S5: The combination of cooperativity and non-essential sites. The original system (red) shows high ultrasensitivity due to cooperativity with $\lambda_1, \ldots, \lambda_4 = 0.5, \lambda_5 = 16$, $n = k = 5$. The blue curve corresponds to $n = 6, k = 5$ with $\lambda_6 = 0.5$, and the black curve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