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Ultrasensitivity Part II: Multisite phosphorylation, stoichiometric inhibitors, and positive feedback

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Abstract

In this series of reviews we are examining ultrasensitive responses, the switch-like input-output relationships that contribute to signal processing in a wide variety of signaling contexts. In the first part of this series we examined one mechanism for generating ultrasensitivity, zero-order ultrasensitivity, where the saturation of two converting enzymes allows the output to switch from low to high over a tight range of input levels. In this second installment, we focus on three conceptually distinct mechanisms for ultrasensitivity: multisite phosphorylation, stoichiometric inhibitors, and positive feedback. We also examine several related mechanisms and concepts, including cooperativity, reciprocal regulation, coherent feedforward regulation, and substrate competition, and provide several examples of signaling processes where these mechanisms are known or are suspected to be applicable.

Michaelian responses and zero-order ultrasensitivity

Complex networks of signal transduction proteins function as rheostats, switches, amplifiers, pulse generators, timers, memory devices, and so on [1]. Understanding how these systems-level behaviors are achieved requires an understanding of how the elementary signaling monocyclus, out of which the networks are built, respond to their immediate upstream regulators. In the first part of this series of reviews, we discussed hyperbolic, Michaelian responses, which have a law-of-diminishing-returns character, discussed the concepts of sensitivity and ultrasensitivity, and then showed that sigmoidal, ultrasensitive responses can be generated if the enzymes generating a signaling output are running close to saturation [2]. This phenomenon is termed “zero-order ultrasensitivity,” and it was discovered by Goldbeter and Koshland in the early 1980’s in the course of theoretical studies of signal transduction [3–5]. However, zero-order ultrasensitivity is not the only mechanism for generating ultrasensitive responses. Here we examine three other ways for amplifying the sensitivity of a response: multistep processes like multisite phosphorylation, competitive

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inhibitors or substrates, and positive feedback loops. We begin by examining the post-translational regulation of the cell cycle regulator Cdc25C.

Ultrasensitivity in the response of Cdc25C to Cdk1

The protein phosphatase Cdc25C (cell division cycle protein 25C, a highly specific phosphoprotein phosphatase) is a critical activator of Cdk1 (cyclin-dependent kinase 1), which is the master regulator of mitotic entry for eukaryotic cells. Cdc25C is also activated by Cdk1 by phosphorylating multiple specific Ser and Thr residues in what is believed to be an intrinsically disordered regulatory region of the protein. Although mitosis in general and Cdc25C regulation in particular is a highly dynamical process, Cdc25C quickly attains maximal levels of phosphorylation (hyperphosphorylation) during mitotic entry, which means that the steady-state response of Cdc25C to Cdk1 is relevant to the behavior of the system.

In *Xenopus* egg extracts the steady-state hyperphosphorylation of Cdc25C is a very highly ultrasensitive function of the Cdk1 activity, with an effective Hill coefficient of about 11 (Fig 1A,B). Even in vitro, where cell cycle-regulated phosphatases [6–8] cannot contribute to the ultrasensitivity, the effective Hill coefficient for the phosphorylation of the Cdc25C N-terminus is about 4.5, a large number as Hill coefficients go (Fig 1C). Based on dilution studies, zero-order ultrasensitivity appears to not contribute much to the observed response [9], which raises the question of what might generate the ultrasensitive response of Cdc25C.

The answer lies in the fact that Cdc25C is regulated through multisite phosphorylation rather than through phosphorylation of a single site. This is shown in Fig 1C: if three of the conserved phosphorylation sites in the Cdc25C N-terminus are mutated to Glu residues, the resulting N-terminus can still be phosphorylated by Cdk1, but now the phosphorylation is Michaelian (with an effective Hill coefficient of ~0.9) rather than ultrasensitive [9].

Here we discuss how multisite phosphorylation can generate an ultrasensitive response; how this ultrasensitivity is (probably) enhanced by extra inessential phosphorylation sites and by cooperativity, which can be generated by priming; and how other types of coherent feed-forward regulation can also yield ultrasensitive responses.

Multisite phosphorylation and multi-step ultrasensitivity

Like Cdc25C, most phosphoproteins are multiply phosphorylated, either by multiple kinases or by one kinase phosphorylating multiple sites. This means that the protein may require n phosphorylation events to become activated (or inactivated), and such a multistep process can generate an ultrasensitive response. Different sorts of mechanisms can describe such a multisite phosphorylation process. It could be processive (multiple phosphorylations and/or dephosphorylations occurring after a single collision of the kinase or phosphatase with the substrate) or distributive (one phosphorylation/dephosphorylation per collision); ordered or random; cooperative or non-cooperative; and saturated or unsaturated. In addition, there could be AND gate or OR gate logic, or something in between, in how the phosphorylations combine to regulate the substrate. All of these variations have an impact on the steady-state input-output relationship of the system, and probably all of these variations are relevant

somewhere in signal transduction. Here we will begin with an ordered, processive dual phosphorylation mechanism, with mass action kinetics, AND gate logic, and no significant sequestration of the enzymes by the substrate. It sounds complicated, but actually this is a pretty simple way to start.

As shown in Box 1, the input-output relationship for this model is given by:

$$\frac{XPP_{ss}}{X_{tot}} = \frac{kinase^2}{K_1 K_2 + K_2 kinase + kinase^2} \quad \text{Eq 1}$$

Box 1

Ultrasensitivity from multisite phosphorylation

Suppose we have a dual phosphorylation mechanism that leads to the activation of substrate X (Fig 2A), as is the case with MAP kinases. Also, for simplicity, suppose there is only one mono-phosphoform (i.e., the phosphorylations and dephosphorylations occur in a strictly ordered fashion), and suppose that both the phosphorylation and dephosphorylation reactions are distributive rather than processive. That is, one or both of the enzymes dissociates from the substrate between the two phosphorylations or dephosphorylations. Finally, assume that the phosphorylation and dephosphorylation reactions can be described by mass action kinetics; that is, the enzymes are operating far from saturation, so that zero-order ultrasensitivity is not possible.

This scheme is an example of coherent feedforward regulation, with the input (the kinase) feeding into the output (XPP) both directly, through the phosphorylation of XP , and indirectly, from the production of the species (XP) from which XPP is produced. Systems with coherent feedforward regulation have several characteristic dynamical features [52, 53]. For present purposes, though, we will focus on the fact that they can yield ultrasensitive steady-state responses, even in the absence of zero-order effects.

The dual phosphorylation reaction yields three rate equations for the three species:

$$\frac{dX}{dt} = -k_1 kinase \times X + k_{-1} p'ase \times XP \quad \text{Eq 1.1}$$

$$\frac{dXP}{dt} = k_1 kinase \times X - k_{-1} p'ase \times XP - k_2 kinase \times XP + k_{-2} p'ase \times XPP \quad \text{Eq 1.2}$$

$$\frac{dXPP}{dt} = k_2 kinase \times XP - k_{-2} p'ase \times XPP \quad \text{Eq 1.3}$$

Here $kinase$ and $p'ase$ denote the concentrations of kinase and phosphatase, respectively; k_1 and k_2 are the rate constants for the first and second phosphorylation reactions; and k_{-1} and k_{-2} are the rate constants for the dephosphorylation reactions. At steady-state all three derivatives are equal to zero, yielding three algebraic equations plus the conservation relationship $X_{tot} = X + XP + XPP$. This system of equations can be solved

simultaneously, yielding expressions for the steady-state concentrations of X , XP , and XPP . The output of the system is XPP ; the steady-state concentration of XPP (denoted XPP_{ss}), as a fraction of X_{tot} is given by:

$$\frac{XPP_{ss}}{X_{tot}} = \frac{kinase^2}{\frac{k_{-1}k_{-2}p'ase^2}{k_1k_2} + \frac{k_{-2}p'ase}{k_2}kinase + kinase^2} \quad \text{Eq 1.4}$$

Note that if we took the two steps individually, the $EC50$ values for the first and second reactions would be given by $\frac{k_{-1}p'ase}{k_1}$ and $\frac{k_{-2}p'ase}{k_2}$, respectively. We can therefore write Eq 1.4 as:

$$\frac{XPP_{ss}}{X_{tot}} = \frac{kinase^2}{K_1K_2 + K_2kinase + kinase^2} \quad \text{Eq 1.5}$$

where the K values are the same as $EC50$ values for the two steps. Similarly, for multisite phosphorylation with n phosphorylation sites, the steady-state level of the fully n -phosphorylated substrate is given by:

$$\frac{XP_n}{X_{tot}} = \frac{kinase^n}{(K_1 \cdots K_n) + (K_2 \cdots K_n) kinase + (K_3 \cdots K_n) kinase^2 + \dots + kinase^n} \quad \text{Eq 1.6}$$

Eq 1 describes a sigmoidal curve whose exact shape depends upon the K values, which are the same as the $EC50$ values for the two individual phosphorylation steps. If the second reaction is much more favorable than the first ($K_2 \ll K_1$), then Eq 1 approaches a Hill curve with a Hill exponent of two (Fig 2B, red curves). If the first reaction is much more favorable than the second ($K_1 \ll K_2$), then the curve approaches a Michaelian response (i.e. the Hill exponent approaches one) (Fig 2B, blue curves). And if the two reactions are equally favorable, the curve is intermediate (Fig 2B, green curve). For two-site phosphorylation the three curves all look fairly similar, but for higher numbers of sites, having one of the last phosphorylations be more favorable than the previous ones can make the response curve substantially more ultrasensitive (Fig 2C).

The relationship between the relative values of K_1 and K_2 and the overall level of ultrasensitivity generated connects multisite phosphorylation to the phenomenon of cooperativity. The condition for high ultrasensitivity is that K_2 is substantially smaller than K_1 , which means that the second phosphorylation is more favorable than the first. Since the less-favorable phosphorylation nevertheless happens first, one can regard the first phosphorylation as causing the subsequent phosphorylation to become more favorable. This is analogous to the situation with a cooperative receptor, where the binding of a first ligand makes the binding of the second ligand more energetically favorable. In fact, if we write out a mechanism for the Koshland-Némethy-Filmer-style (sequential) activation of a dimeric receptor by the binding of two ligands (Fig 2D) and solve for the equilibrium concentration of active receptor as a function of ligand, the result is identical in form to Eq 1:

$$\frac{R_2 L_2}{R_{tot}} = \frac{L^2}{K_1 K_2 + K_2 L + L^2} \quad \text{Eq 2}$$

Here $R_2 L_2$ is the concentration of the active receptor-ligand complex, R_{tot} is the total concentration of receptor, K_1 (which equals k_{-1}/k_1) is the dissociation constant for the binding of first ligand, and K_2 (which equals k_{-2}/k_2) is the dissociation constant for the binding of the second. The relative values of the two dissociation constants define the cooperativity in the binding of L to the receptor, just as the ratio of the two K values determined the extent of the cooperativity in the dual phosphorylation system.

Note that with both mass action multisite phosphorylation and KNF cooperativity, the output is a sigmoidal function of the input even in the absence of cooperative phosphorylation or cooperative ligand binding (Fig 2B,C, green curves), but it is more steeply sigmoidal if these processes are cooperative (Fig 2B,C, red and purple curves). In both of these cases, the maximal effective Hill exponent one can achieve with high levels of cooperativity is 2 for a two-step process, 3 for three steps, and n for an n -step process. In the absence of cooperativity, both multisite phosphorylation and KNF-style sequential binding are better at generating a threshold than producing a complete Hill-like response [10], but with reasonable levels of cooperativity and large numbers of steps, very high Hill exponents can be achieved (Fig 2C).

Priming and cooperativity in multisite phosphorylation

Given that multisite phosphorylation generates a better switch when there is cooperativity in the phosphorylation mechanism, is there experimental evidence for cooperativity in multisite phosphorylation? The answer is yes, and one particularly striking example comes from recent studies of multisite phosphorylation by the cell cycle kinase Cdk1. Active Cdk1 complexes are composed of three subunits: the catalytic Cdk1 subunit; a mitotic cyclin, which allosterically regulates Cdk1 and also confers localization and substrate specificity upon the complex; and a Cks protein, which acts as a phosphoepitope binding subunit. Cks proteins can bind particular Thr-Pro Cdk1 phosphorylation sites [11–13]. This means that phosphorylation of one Thr-Pro sequence in a substrate could greatly facilitate the phosphorylation of a second Ser-Pro or Thr-Pro site (Fig 3). The result is cooperative phosphorylation of the second site—the priming can be thought of as a positive allosteric interaction—and an increase in the overall ultrasensitivity of the second site's response. A wide variety of Cdk1 substrates probably employ this priming. For example, the response of Cdc25C to Cdk1 is more highly ultrasensitive than one would obtain from a non-cooperative multisite phosphorylation mechanism (Fig 1C). Moreover, Cdc25C possesses phosphorylation sites that conform well to the Cks binding consensus (T48, T67, and T138), and elimination of these sites changes the response of Cdc25C from ultrasensitive to Michaelian (Fig 1C) [9, 12]. These results suggest that priming generates cooperativity and cooperativity contributes to the highly ultrasensitive response seen in this system.

Note that the cooperativity that arises from priming does not necessarily require precise spacing or even a defined structure in the substrate protein, even though in one well-studied

case, the phosphorylation of Sic1, the spacing of the priming site from the subsequent site does appear to be important [11]. In principle, priming could be a relatively simple type of cooperativity to evolve or engineer.

Extra phosphorylation sites and ultrasensitivity

Suppose we have a substrate X with 6 phosphorylation sites, all phosphorylated by the same kinase, and phosphorylation brings about activation of X . Such large numbers of sites are not unheard of, and in fact are common in substrates of Cdk1 [14]. If we wish to generate the maximum amount of ultrasensitivity, how many sites should we require be phosphorylated for activation of X ?

Many of us would probably guess 6; the greater the number of sites required, the greater the ultrasensitivity. Actually though, the answer is 3 or 4. It is best to require that only about half of the sites be phosphorylated for activation.

This result was first shown through theoretical studies by Wang and co-workers [15], and it is illustrated in Fig. 4 for the special case where all of the steps in the six-site phosphorylation are equally favorable (i.e., there is no cooperativity). As the number of sites required for activation (here called m) increases, the ultrasensitivity at the low end of the response curve improves—the threshold becomes more absolute—but the ultrasensitivity at the high end of the curve worsens. The optimal tradeoff between these two trends occurs when, for even n , the value of m is $n/2$ or $1 + n/2$; and, for odd n , when m is $(n + 1)/2$.

This phenomenon may be particularly important in cases where the function of a protein depends on the amount of phosphorylation rather than the exact identities of the sites phosphorylated. For example, the yeast protein Ste5 functions as a scaffold for mating pheromone signaling, and it must be present at the plasma membrane to serve this function. The phosphorylation of eight clustered Cdk1 sites near the N-terminus of the protein is sufficient to cause Ste5 to dissociate from the plasma membrane, and it appears that (to a first approximation) phosphorylation of any four of the eight sites is sufficient to half-maximally knock Ste5 off the membrane [16]. Wang et al.'s model of extra-site ultrasensitivity means that the inactivation of Ste5 as a function of Cdk1 would be expected to be more highly ultrasensitive than it would have been if there were only four sites, or if there were eight sites but all eight needed to be phosphorylated for inactivation. Extra-site ultrasensitivity may also contribute to the regulation of Sic1 destruction by Cdk1 [17], although recent work suggests that the various Sic1 Cdk1 sites are less equivalent than previously thought [18].

Saturation and competition in multisite phosphorylation

So far we have considered multisite phosphorylation as though each step is described by mass action kinetics and the intermediary enzyme-substrate complexes are insignificant in concentration. What happens if we use saturable kinetics and explicitly consider the complexes, as shown in Fig 5A?

Not surprisingly, saturating the phosphorylation and dephosphorylation reactions can add additional zero-order ultrasensitivity to the overall response. What is less obvious is that by allowing the unphosphorylated and phosphorylated forms of the substrate to compete with each other for interaction with the kinase and phosphatase, the result can be a qualitative change in the response from *monostable*, where there is one value of steady-state output for each value of input, to *bistable*, where for some range of inputs there are two possible stable steady-state outputs. This surprising result was first shown by Kholodenko and co-workers in their computational studies of the distributive dual phosphorylation and dephosphorylation of the ERK2 mitogen-activated protein kinase (MAPK) kinase by the MAPK kinase MEK1 and the MAPK phosphatase MKP3 [19].

To get an intuitive idea of why bistability can occur in this situation, imagine that all X (ERK2 in this example) is initially in the non-phosphorylated state, and then add an intermediate concentration of kinase (MEK1). If X has a high affinity for the kinase, the kinase will be largely tied up in the $kinase \cdot X$ complex. This means that even when some XP is formed, there will be little free kinase to bind to it and phosphorylate it to XPP , and the system settles into a steady state with low levels of XPP .

Now suppose we perturb the system by abruptly changing some of the X to XPP . This means there is less X present as a competitor for the kinase. More kinase available to phosphorylate XP means that the rate of production of XPP will go up. That immediately depletes XP and, because X is produced from XP , eventually results in the depletion of X . The depletion of X frees up more kinase, which further increases the rate of production of XPP , which leads to further depletion of X , and on and on. Essentially we have an implicit positive feedback loop where XPP promotes its own production, and this provides the system with the potential for having two different stable steady-state levels of XPP for some concentrations of $kinase_{tot}$. One can make an analogous argument for the dephosphorylation reactions.

The situation here can be thought of as a specific example of substrate inhibition, where substrate X inhibits the production of XPP by the kinase through competition, and substrate XPP inhibits the production of X by the phosphatase through competition. And, in general, substrate inhibition can increase ultrasensitivity and generate bistability [20].

The logic described above is complicated, but it can be tested through computations. In Box 2 we show the ODEs and algebraic equations that correspond to the scheme shown in Fig 5A. Fig 5B shows the results of solving these equations for the possible steady states, under the assumption that the phosphorylation and dephosphorylation reactions are close to saturation and the substrate concentration is comparable to the concentrations of the converting enzymes. As the assumed total substrate concentration X_{tot} increases, the system goes from having a monostable (but ultrasensitive) response to having a bistable response, with bistability emerging at a substrate concentration of just above 1.5 units.

Box 2

Implicit positive feedback and bistability in multisite phosphorylation

Here we consider a more complete model of the dual phosphorylation of X . We begin by writing down the rate equations for the 7 species shown in Fig 5A (X , XP , XPP , and the four complexes denoted C_1 – C_4 ; Eqs 2.1–2.7) and three conservation equations (for X_{tot} , $kinase_{tot}$, and $p'ase_{tot}$; Eqs 2.8–2.10) [54]:

$$\frac{dX}{dt} = d_1 C_1 + k_4 C_4 - a_1 X \times kinase \quad \text{Eq 2.1}$$

$$\frac{dXP}{dt} = k_1 C_1 + k_3 C_3 + d_2 C_2 + d_4 C_4 - a_2 XP \times kinase - a_4 XP \times p'ase \quad \text{Eq 2.2}$$

$$\frac{dXPP}{dt} = k_2 C_2 + d_3 C_3 - a_3 XPP \times p'ase \quad \text{Eq 2.3}$$

$$\frac{dC_1}{dt} = a_1 X \times kinase - (d_1 + k_1) C_1 \quad \text{Eq 2.4}$$

$$\frac{dC_2}{dt} = a_2 XP \times kinase - (d_2 + k_2) C_2 \quad \text{Eq 2.5}$$

$$\frac{dC_3}{dt} = a_3 XPP \times p'ase - (d_3 + k_3) C_3 \quad \text{Eq 2.6}$$

$$\frac{dC_4}{dt} = a_4 XP \times p'ase - (d_4 + k_4) C_4 \quad \text{Eq 2.7}$$

$$X_{tot} = X + XP + XPP + C_1 + C_2 + C_3 + C_4 \quad \text{Eq 2.8}$$

$$kinase_{tot} = kinase + C_1 + C_2 \quad \text{Eq 2.9}$$

$$p'ase_{tot} = p'ase + C_3 + C_4 \quad \text{Eq 2.10}$$

At steady-state, all of the time derivatives must be zero, yielding a system of 10 algebraic equations in 7 unknowns. This can be solved numerically. For the simulations shown in Fig 6B we have assumed that all of the reactions are running close to saturation and then have selected various assumed concentrations of X_{tot} ; the higher the concentration, the more significant the competition for $kinase$ and $p'ase$ is. The parameter set was based on those used by Shvartsman and co-workers [54], with some modifications: $a_1 = 0.004$; d_1

$= 0.00016; k_1 = 0.00016; a_2 = 8; d_2 = 0.32; k_2 = 0.32; a_3 = 1; d_3 = 0.04; k_3 = 0.04; a_4 = 0.1; d_4 = 0.004; k_4 = 0.004; p'ase_{tot} = 1.$

Thus, for a dual phosphorylation mechanism, the competition between the various substrate phosphoforms for access to the kinase and phosphatase can allow the system to generate a bistable response. For multisite phosphorylation with more than two sites, multistability is possible. If the number of sites is even, there are potentially $\frac{n+2}{2}$ stable steady states, and for an odd number of sites there can be $\frac{n+1}{2}$ stable steady states [21].

Given how common multisite phosphorylation is, this is a mechanism of high potential importance for the generation of discrete cellular states. Thus far the documented instances of multistability in cellular regulation have always involved explicit positive or double negative feedback loops, rather than (or in addition to) these implicit loops [22–30]. That said, it is hard to believe that nature does not utilize Kholodenko's clever mechanism somewhere.

Note that bistability and/or increased ultrasensitivity is not an inevitable consequence of this sort of sequestration mechanism; it is parameter-dependent. Indeed, sequestration can reduce ultrasensitivity [3, 31].

Variations on the theme: reciprocal regulation

So far we have assumed that the input to the system affects only the kinase. But of course the input would have more control over the system if it both stimulated the kinase and inhibited the phosphatase, as shown in Fig 6A. This is thought to be the situation with the control of Cdk1 by the DNA damage repair checkpoint proteins Chk1 and Chk2; the Chk proteins reciprocally activate the Cdk1-inhibitory kinase Wee1 and inactivate the Cdk1-stimulatory phosphatase Cdc25C [32–35]. This type of reciprocal regulation is a recurring theme in cell cycle regulation [36]. Since the input feeds into the output twice, would the resulting input-output relationship resemble that of a dual phosphorylation mechanism?

Assuming mass action kinetics (Box 3), the steady-state response is given by Eq 3.6. Under optimal conditions—the K value for the activation of the kinase is much higher than that for inactivation of the phosphatase [37]—the response curve approaches a Hill function with a Hill exponent of 2 (Fig 6B), the same limiting function we obtained for mass action dual phosphorylation. Thus, reciprocal regulation has the potential to generate ultrasensitivity.

Box 3

Reciprocal regulation

Here we derive an expression for the steady-state response of a reciprocal regulation system. As shown in Fig 6A, we will assume that an input activates the kinase and inactivates the phosphatase through simple mass action processes. At steady-state,

$$0 = k_1 (1 - kinase_{act}) input - k_{-1} kinase_{act} \quad \text{Eq 3.1}$$

$$0 = k_2 p'ase_{act} input - k_{-2} (1 - p'ase_{act}) \quad \text{Eq 3.2}$$

where $kinase_{act}$ and $p'ase_{act}$ designate the fractions of kinase and phosphatase molecules that are active. We can solve for $kinase_{act}$ and $p'ase_{act}$ as a function of $input$:

$$kinase_{act} = \frac{input}{\frac{k_{-1}}{k_1} + input} \quad \text{Eq 3.3}$$

$$p'ase_{act} = \frac{\frac{k_{-2}}{k_2}}{\frac{k_{-2}}{k_2} + input} \quad \text{Eq 3.4}$$

Now the rate equation for substrate phosphorylation is:

$$\frac{dXP}{dt} = k_3 kinase_{act} (X_{tot} - XP) - k_{-3} p'ase_{act} \times XP \quad \text{Eq 3.5}$$

Setting this expression equal to zero, for the steady-state response, and substituting Eqs 3.3 and 3.4 for $kinase_{act}$ and $p'ase_{act}$ yields:

$$\frac{XP}{X_{tot}} = \frac{K_2 input + input^2}{K_1 K_2 K_3 + (K_2 + K_2 K_3) input + input^2} \quad \text{Eq 3.6}$$

where, as usual, $K_1 = k_{-1}/k_1$, $K_2 = k_{-2}/k_2$, and $K_3 = k_{-3}/k_3$. The shape of this curve depends upon the values assumed for the kinetic parameters [37]. If all of the K values are taken equal to 1, the result is a Michaelian response, and if K_1 is large compared to K_2 , the curve approaches a Hill curve with a Hill exponent of 2 (Fig 6B).

To see why this combination of parameters yields this $n = 2$ response, note that if K_1 is

large, Equation $kinase_{tot} - \frac{input}{K_1}$ and if K_2 is small, $p'ase_{tot} - \frac{K_2}{input}$. Substituting into Eq 3.5 and setting the expression equal to zero yields:

$$\frac{XP}{X_{tot}} = \frac{input^2}{K_1 K_2 K_3 + input^2} \quad \text{Eq 3.7}$$

Eq 3.7 is a Hill equation with a Hill exponent of 2 and an $EC50$ of $\sqrt{K_1 K_2 K_3}$.

As proof of principle, Rossi and co-workers expressed a Tet-operon reporter construct together with a tetracycline-stimulated transcriptional activator, a tetracycline-inhibited transcriptional repressor, or both, in mouse myoblasts [38]. They found that expressing both the activator and repressor yielded a higher Hill coefficient ($n = 3.2$) for their reporter's response to doxycycline than either the activator ($n = 1.6$) or inhibitor ($n = 1.8$) alone did.

These findings establish that reciprocal regulation can indeed increase the sensitivity of a response.

Variations on the theme: feed-forward regulation

Note that the dual phosphorylation mechanism described above is one type of feed-forward regulation. The kinase contributes to the activation of X both directly, by catalyzing the phosphorylation of XP , and indirectly, by catalyzing the production of the XP that can then be activated. There are numerous other examples of feed-forward regulation in cell signaling. For example, $\text{PtdIns}(3,4,5)P_3$ (PIP_3) feeds into the activation of Akt directly, by binding to it and bringing it to the plasma membrane where it can be phosphorylated by membrane-bound protein kinase PDK1. PIP_3 also contributes to Akt activation indirectly, by promoting the membrane localization of PDK1 (Fig 7A). Thus PIP_3 feeds into the activation of Akt in two ways. Does this mean the steady-state response of Akt to PIP_3 should be ultrasensitive?

As shown in Box 4 and Fig 7B, the answer is yes. Assuming simple mass action binding and phosphorylation reactions, the equation for the steady-state response is similar in form to that of dual phosphorylation (Eq 4.4). When the affinity of the two proteins for PIP_3 is high (K_1 and K_2 are small) and the activation of membrane-bound Akt by PDK1 is far from saturation (K_3 is large), Eq 4.4 approaches a Hill function with a Hill exponent of 2 (Fig 7B). Thus, traditional feed-forward regulation, like dual phosphorylation, can yield an ultrasensitive response.

Box 4

Feed-forward regulation

Although dual phosphorylation can be thought of as feed-forward regulation, usually the term applies to a situation where an input affects an output through two independent pathways. An example of this is the activation of Akt by PIP_3 ($\text{PtdIns}(3,4,5)P_3$) (Fig 7A), which we model here. Assuming mass action kinetics, the fraction of the PDK1 bound to PIP_3 at the membrane is described by:

$$\frac{dPDK1_{mem}}{dt} = k_2 PIP_3 (1 - PDK1_{mem}) - k_{-2} PDK1_{mem} \quad \text{Eq 4.1}$$

The reactions of Akt are more complicated. Inactive, cytosolic Akt (Akt_{cyt}) can bind to the membrane through association with PIP_3 to yield inactive, membrane-associated Akt (Akt_{mem}). Akt_{mem} can then be activated by phosphorylation to yield Akt_{act} . So Akt_{mem} can be produced by membrane association of Akt_{cyt} or dephosphorylation of Akt_{act} , and can be taken away by membrane dissociation or PDK1_{mem}-catalyzed phosphorylation:

$$\frac{dAkt_{mem}}{dt} = k_1 PIP_3 (Akt_{tot} - Akt_{mem} - Akt_{act}) + k_{-3} Akt_{act} - k_{-1} Akt_{mem} - k_3 PDK1_{mem} Akt_{mem} \quad \text{Eq 4.2}$$

And the net production of Akt_{act} is described by:

$$\frac{dAkt_{act}}{dt} = k_3 PDK1_{mem} Akt_{mem} - k_{-3} Akt_{act} \quad \text{Eq 4.3}$$

Active *Akt* might also dissociate from the membrane, which would add one additional *Akt* species and other terms to Eq 4.3; for simplicity we are assuming this dissociation is negligible.

At steady-state, all three derivatives are equal to zero. Combining Eqs 4.1 – 4.3 then yields:

$$\frac{Akt_{act}}{Akt_{tot}} = \frac{PIP_3^2}{K_1 K_2 K_3 + (K_1 K_3 + K_2 K_3) PIP_3 + (1 + K_3) PIP_3^2} \quad \text{Eq 4.4}$$

When the affinity of the two proteins for PIP_3 is high (K_1 and K_2 are small) and the activation of membrane-bound *Akt* by PDK1 is far from saturation (K_3 is large), Eq 4.4 approaches a Hill function with a Hill exponent of 2 (Fig 7B).

Stoichiometric inhibitors and inhibitor ultrasensitivity

The p21 protein is a high-affinity stoichiometric inhibitor of several cyclin-dependent protein kinases, including the cell cycle protein kinase cyclin E-Cdk2. The p21 protein is scarce, on the order of ~1 nM, and its target Cdk2 is thought to be ~10-fold more abundant [39]. Thus, as cyclin E begins to accumulate and bind to Cdk2 during G1 phase, the resulting complexes are initially neutralized by p21. Only after the cyclin E-Cdk2 complexes exceed the concentration of p21 can they phosphorylate target substrates. Since cyclin E accumulates relatively slowly, it is likely that the activity of Cdk2 is generally close to its steady-state level. And, in principle, the steady-state response of Cdk2 to cyclin E should be ultrasensitive, with a discrete concentration threshold corresponding to the capacity of the p21 molecules for cyclin E-Cdk2.

This phenomenon, termed inhibitor ultrasensitivity, is probably the simplest and easiest to understand of the various types of mechanisms for generating ultrasensitivity. It has a long history; for example, it was the mechanism Brian Goodwin invoked in his pioneering 1965 paper on biochemical oscillators to justify his use of a large Hill coefficient in one step of his oscillator model [40].

Although conceptually straightforward, inhibitor ultrasensitivity is not so easy to work through analytically. The general scheme for inhibitor ultrasensitivity is shown in Fig 8A, and in Box 5 we algebraically derive the steady-state response of a phosphorylation-dephosphorylation reaction in the presence of a stoichiometric inhibitor *I*. The result is a very complicated equation (Eq 5.5). As one would intuitively suspect, the ultrasensitivity is highest when total inhibitor concentration I_{tot} is large (which makes the threshold large), the affinity of the kinase for the inhibitor is higher than the affinity of the kinase for the substrate (which makes the threshold sharp), and even the affinity of the kinase for the

substrate is small (which makes the system switch abruptly from off to on once the threshold is exceeded) (Box 5 and Fig 8B).

Box 5

Inhibitor ultrasensitivity

To obtain an algebraic expression for inhibitor ultrasensitivity (Fig 8), we start with the rate equation for phosphorylation and dephosphorylation of X , assuming mass action kinetics (Eq 5.1). For simplicity we have left out an explicit variable for the concentration of the phosphatase—one can think of it as being incorporated into the rate constant k_{-1} .

$$\frac{dXP}{dt} = k_1 \text{kinase} (X_{tot} - XP) - k_{-1} XP \quad \text{Eq 5.1}$$

Next we write the rate equations for the binding and dissociation of the inhibitor I with the kinase (Eq 5.2).

$$\frac{d\text{kinase}}{dt} = -k_2 \text{kinase} \times I + k_{-2} \text{complex} \quad \text{Eq 5.2}$$

There are also two conservation equations:

$$I_{tot} = I + \text{complex} \quad \text{Eq 5.3}$$

$$\text{kinase}_{tot} = \text{kinase} + \text{complex} \quad \text{Eq 5.4}$$

Setting the rate equations equal to zero and solving for XP as a function of I_{tot} and the parameters yields:

$$\frac{XP}{X_{tot}} = \frac{K_1 I_{tot} + K_1 K_2 - K_1 \text{kinase}_{tot} + 2K_2 \text{kinase}_{tot} - K_1 \sqrt{I_{tot}^2 + 2(K_2 - \text{kinase}_{tot}) I_{tot} + (K_2 + \text{kinase}_{tot})^2}}{2K_1 I_{tot} - 2(K_1 - K_2)(K_1 + \text{kinase}_{tot})} \quad \text{Eq 5.5}$$

where $K_1 = k_{-1}/k_1$ and $K_2 = k_{-2}/k_2$. This equation was used to plot the curves in Fig 8B.

By defining $\rho = \frac{\text{kinase}_{tot}}{I_{tot}}$, $\chi_1 = \frac{K_1}{I_{tot}}$, $\chi_2 = \frac{K_2}{I_{tot}}$, and $\alpha = \frac{K_2}{K_1}$, Eq 5.5 can be converted to non-dimensional form:

$$\frac{XP}{X_{tot}} = \frac{1 - \rho + \alpha(\chi_1 + 2\rho) - \sqrt{1 + 2(\chi_2 - \rho) + (\chi_2 + \rho)^2}}{2 - 2(1 - \alpha)(\chi_1 + \rho)} \quad \text{Eq 5.6}$$

Note that for very tight binding of the inhibitor to the kinase, K_2 approaches zero, and so both α and χ_2 approach zero. This means that:

$$\frac{XP}{X_{tot}} = \frac{1-\rho-|1-\rho|}{2-2(\chi_1+\rho)} \quad \text{Eq 5.7}$$

If the total concentration of the kinase is less than the total concentration of the inhibitor, then $1 - \rho$ is a positive number, the numerator vanishes, and XP equals zero. Otherwise,

$$\frac{XP}{X_{tot}} = \frac{\rho-1}{\chi_1+\rho-1} \quad \text{Eq. 5.8}$$

Eq 5.8 describes a Michaelian (hyperbolic) response shifted to the right, as seen in as the high affinity limit (dashed line) in Fig 8B.

The simplicity of inhibitor ultrasensitivity makes it an attractive mechanism for engineering ultrasensitivity into synthetic regulatory circuits. Indeed, Buchler and Cross have shown that a high affinity stoichiometric inhibitor can add a threshold to the response of a synthetic C/EBP α (CCAAT-enhancer binding protein- α) reporter, resulting in ultrasensitive responses with high effective Hill coefficients (Fig 8C) [41]. In addition, Lee and Maheshri have shown that high affinity decoy transcription factor binding sites can yield a transcriptional response with a substantial threshold [42].

One can imagine a number of variations on the simple inhibitor ultrasensitivity scheme discussed here: What if the inhibitor can bind the enzyme-substrate complex as well as the free enzyme? What if the enzyme is oligomeric? For the sake of brevity these questions are left for the interested reader to explore. But here we will examine one particular variation that has cropped up in studies of protein phosphorylation, where the competitive inhibitor is itself a substrate of the kinase in question, and the competition between the two substrates contributes both ultrasensitivity and temporal ordering to a complex process.

Competing Substrates

A variation on inhibitor ultrasensitivity is to have the inhibitor actually be a high-affinity substrate of the protein kinase (or of some other signaling enzyme) (Fig 9A). A high-affinity substrate can, in principle, act as an inhibitor of lower affinity substrates by competing for access to the kinase (Fig 9A). A competing substrates mechanism like this was proposed to explain the ultrasensitive response of the Wee1 protein to cyclin B1-Cdk1 in *Xenopus* egg extracts [43].

This mechanism is shown schematically in Fig 9, and the details of the model are found in Box 6. The high-affinity substrate (X) ties up the first increments of kinase. Only after this substrate is saturated would lower affinity substrates (like Y in Fig 9A) be phosphorylated. This effect is shown in Fig 9B. When the competitor substrate is abundant enough (here we have assumed $X_{tot} = 3Y_{tot}$) and binds tightly enough (we have assumed that the K_m value for X is 10x lower than that of Y), the result is a marked threshold and an ultrasensitive response. As proof of concept, it has been shown that adding the high affinity Cdk1 substrate Sic1—which is often regarded simply as a stoichiometric inhibitor but is also a

Cdk1 substrate—to a reconstituted Cdk1/Wee1 system increases the ultrasensitivity of the Wee1 response (Fig 9C) [43].

Box 6

Competing substrates

Substrate competition is conceptually similar to the competition between a stoichiometric inhibitor and a substrate that we modeled in Box 5. However, in this case we cannot obtain an analytical expression for the steady state response. Based on the scheme shown in Fig 9A, we can write down 7 rate equations for the 7 time-dependent species:

$$\begin{aligned}
 \frac{dX}{dt} &= -a_1 \text{kinase} \cdot X + d_1 \text{complex}_1 + k_{-1}XP \\
 \frac{dY}{dt} &= -a_2 \text{kinase} \cdot Y + d_2 \text{complex}_2 + k_{-2}YP \\
 \frac{d\text{complex}_1}{dt} &= a_1 \text{kinase} \cdot X - d_1 \text{complex}_1 - k_1 \text{complex}_1 \\
 \frac{d\text{complex}_2}{dt} &= a_2 \text{kinase} \cdot Y - d_2 \text{complex}_2 - k_2 \text{complex}_2 \\
 \frac{dXP}{dt} &= k_1 \text{complex}_1 - k_{-1}XP \\
 \frac{dYP}{dt} &= k_2 \text{complex}_2 - k_{-2}YP \\
 \frac{d\text{kinase}}{dt} &= -a_1 \text{kinase} \cdot X + (d_1 + k_1) \text{complex}_1 - a_2 \text{kinase} \cdot Y + (d_2 + k_2) \text{complex}_2
 \end{aligned}
 \tag{Eqs 6.1–6.7}$$

We can then either solve the differential equations numerically (for time courses) or set all of the derivatives equal to zero and numerically solve the algebraic equations for the steady-state levels of the output (YP) and the other species.

An additional interesting aspect of a system with competing substrates can be seen by examining its dynamical, rather than steady-state, behavior. Suppose that the system shown in Fig 9A is treated with sufficient kinase (1 unit) to bring about near maximal steady-state levels of Y phosphorylation (Fig 9B). Initially the concentration of X will be sufficient to tie up most of the kinase (Fig 9D, green curve). However, as X approaches its steady-state level of phosphorylation (Fig 9D, light blue curve), the amount of free X available to bind to the kinase drops, allowing the amount of kinase available to phosphorylate Y to rise (Fig 9D, dark blue). This builds a time lag into the formation of complexes between the kinase and Y (Fig 9D, orange curve), and in the phosphorylation of Y (Fig 9D, red curve).

Temporal thresholds like this might allow master regulatory proteins that control the activity of hundreds of target proteins (e.g., Cdk1) to generate distinct, temporally-ordered waves of substrate phosphorylation.

Ultrasensitivity and time lags

We have now seen that one mechanism for generating an ultrasensitive response, substrate competition, builds a time lag into the response. Is this true of other mechanisms that can generate ultrasensitivity? As shown in Fig 9E, for a simple stoichiometric inhibitor mechanism the answer is no (yellow curve); the system exponentially approaches its steady-state response, just as a simple mass action signaling system does [2]. For a system with zero-order ultrasensitivity, the time course is more linear than an exponential approach curve is (Fig 9E, blue curve). The reason for this can be seen from the rate-balance plots shown in

Fig 9F. In a rate balance plot, one plots both the phosphorylation rate (Fig 9F, green curves) and the dephosphorylation rate (Fig 9F, red curves) as functions of YP/Y_{tot} . Wherever the two curves intersect, the rates are balanced and the system is in steady state. See Part 1 of this series for more discussion of rate-balance analysis [2].

With a system that is not saturated, the rate at which the system returns toward the steady state decreases linearly as you approach the steady state (Fig 9F, left). The result is a gradual exponential approach to the steady state. However, with a saturated system, the nearly-flat plateaus of the rate curves mean that the rate at which the system approaches the steady state does not fall so fast; the rate remains closer to constant (Fig 9F, right), resulting in the more linear time course seen in Fig 9E. The result is a curve that is shaped differently from the exponential approach to steady state curve, but there is still no threshold, no inflection point in the time course. In contrast, for multisite phosphorylation (Fig 9E, green curve) and of course substrate competition (Fig 9E, red curve), a time lag is generated. In these cases the system picks up speed as it gets going.

Ultrasensitivity from positive feedback

Positive feedback loops, or double-negative feedback loops, are probably best known for their ability to generate bistable, toggle-switch responses under certain specific conditions, and we will examine such a bistable system in the next installment in this series. However, positive feedback loops can also generate monostable but ultrasensitive responses. In a sense, positive feedback allows an input to factor into an output more than once, so this ultrasensitivity is related to the multisite ultrasensitivity described above. Generally the range of parameters over which the response of a loop is ultrasensitive is larger than the range over which it is bistable (e.g. [26]), and, as shown below, in some positive feedback systems where a bistable response is impossible, ultrasensitivity still occurs.

An example of such a system is shown schematically in Fig 10A and is modeled in Box 7. The system is inspired by the positive feedback-based mitotic trigger [27, 44–48]. In the mitotic trigger, the input is the total concentration of cyclin B or, equivalently, the total concentration of cyclin B-Cdk1 complexes. When these complexes are in the correct phosphorylation state, they are active. The phosphorylation of Cdk1 is regulated by interlinked positive and double-negative feedback loops that make it so that Cdk1 activates its activator (Cdc25C) and inhibits its inhibitor (Wee1). In Fig 10A, one can think of X and X^* as the inactive and active forms of the cyclin B-Cdk1 complex, and for simplicity, we have only included a single positive feedback loop. In actuality the enzymes in the feedback loops (Wee1A and Cdc25C) exhibit highly ultrasensitive responses to Cdk1 and this allows the system as a whole to be bistable, but here we will assume that the positive feedback is a simpler process, with the rate of X activation being directly proportional to how much X there is to activate and how much X^* there is to carry out the activation (Box 7). Note that this makes the model not so relevant to how the mitotic trigger really behaves, but it instead how it would have behaved if the enzymes in the feedback loops were regulated by mass action processes.

Box 7**Ultrasensitivity from positive feedback**

As shown in Fig 10A, we assume that protein X is converted to active X^* in a bimolecular mass action process that depends upon both X^* (the activating enzyme) and X (the substrate). For example, X^* could autophosphorylate X *in trans*. We assume that X^* is converted back to inactive X by a process like dephosphorylation, and assume that this is also described by simple mass action kinetics. If the concentration of the $X \cdot X^*$ complex is small compared to X and X^* , then the system can be described by a single rate equation:

$$\frac{dX^*}{dt} = k_1 (X_{tot} - X^*) X^* - k_{-1} X^* \quad \text{Eq 7.1}$$

Steady state occurs when the derivative equals zero, which happens when:

$$X^* = 0 \text{ or } X^* = X_{tot} - \frac{k_{-1}}{k_1} \quad \text{Eq 7.2}$$

Thus the system has two steady states; however, only one of the two is stable. The first

steady state ($X^* = 0$) is stable when $X_{tot} \leq \frac{k_{-1}}{k_1}$ and unstable when $X_{tot} > \frac{k_{-1}}{k_1}$. The second steady state is non-physical (it corresponds to a negative concentration of X^*) and

unstable when $X_{tot} < \frac{k_{-1}}{k_1}$, and is both physical and stable when $X_{tot} \geq \frac{k_{-1}}{k_1}$. When

$X_{tot} = \frac{k_{-1}}{k_1}$ the system goes through a transcritical bifurcation [55].

Note that if we had assumed that the feedback was described by a Hill function with a high Hill exponent rather than by a mass action process, we could have obtained three steady states, two of which are stable and one of which represents a saddle point. More details on ultrasensitivity in a one variable non-linear positive feedback system can be found in [25].

The steady-state output of this system is shown in Fig 10C, and can be understood through the rate-balance analysis shown in Fig 10B. For low levels of the input (X_{tot}), the rate curves intersect at one point (Fig 10B) and there is one physically-meaningful steady state for the system, with $X^* = 0$ (Fig 10C). The rate curves actually intersect at a second point, not shown, but it corresponds to a non-physical negative concentration of X^* . Once the input exceeds a threshold (for the values of the kinetic parameters chosen here it is 1 unit of X_{tot} ; as can be appreciated from Fig 10B, the threshold value depends upon the rate constant for the dephosphorylation reaction, which determines the slope of the red line), the $X^* = 0$ steady state becomes unstable, and a second stable steady state appears. The value of X^* at this steady state then increases linearly with the input stimulus (Fig 10C). Thus the system has a discrete threshold below which there is no output, and then above the threshold the output increases sharply with the input. The positive feedback has not generated bistability

—there is no combination of parameters that will allow the rate curves to intersect three times, producing two stable steady states and an intermediate unstable one—but it has produced an ultrasensitive response with a sharp threshold. Sprinzak and co-workers have proposed that a similar mechanism provides a threshold and an ultrasensitive response in Notch-Delta signaling [49].

Note that this positive feedback also introduces a time lag into the response (Fig 10D). The closer the system is to $X^* = 0$ when the input stimulus is applied, the longer the time lag (Fig 10D). And if the system starts with X^* exactly equal to zero, there will be no response to the input stimulus; the system just sits on its unstable steady state.

Concluding remarks

This concludes our survey of mechanisms that can generate ultrasensitive responses. In the last installment of this series we examined zero-order ultrasensitivity [2], and here we examined multistep ultrasensitivity (and several variations thereof), inhibitor ultrasensitivity and competing substrates, and positive feedback. Note that there really is no conceptual similarity between zero-order ultrasensitivity and multistep ultrasensitivity, or between either of those processes and inhibitor ultrasensitivity. Nevertheless, all of these mechanisms can all produce ultrasensitive responses; that is, input-output relationships that are steeper and more switch-like in character than those seen in simple mass action systems.

There is a limited amount of experimental evidence to support the occurrence of several of these mechanisms in cell regulation, beginning with classic studies of zero-order ultrasensitivity [50, 51] and continuing through more recent studies of multisite phosphorylation [9, 43] and inhibitor ultrasensitivity [41, 43]. Nevertheless, some of the most interesting theoretical predictions, such as Markevich et al.'s mechanism for bistability from dual phosphorylation plus competition [19] and Wang et al.'s analysis of the consequences of extra phosphorylation sites [15] remain to be experimentally tested.

One last question is what this ultrasensitivity is good for. In the next installment of this series we explain how and why ultrasensitive responses can contribute in critical ways to the generation of more complex systems-level behaviors, including cascade amplification, bistability, and oscillations.

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Glossary

Conservation equation

In the present context, it is an algebraic equation of the form $X_{tot} = X_1 + X_2 + \dots$, where species X is interconverted among various forms

	($X_1, X_2, \dots$) but the total concentration of X (X_{tot}) does not change with respect to time
Cooperativity	A characteristic of some multistep processes where completing some of the early steps makes a later step more favorable. Examples include the multistep binding of oxygen to hemoglobin and priming in multisite phosphorylation
EC50	Effective concentration 50; the concentration of a stimulus required for half-maximal (50%) response
Hill function	An input-output relationship of the form $Output = \frac{Input^n}{K^n + Input^n}$, where n is the Hill exponent or Hill coefficient. The larger the Hill exponent, the more ultrasensitive the response
Mass action kinetics	A simple kinetic scheme where the rate of a reaction is directly proportional to the concentration of the substrate or substrates involved in the reaction. This contrasts with Michaelis-Menten kinetics or kinetic schemes involving Hill functions
Michaelis-Menten kinetics	A model for the rate of an enzymatic reaction, premised on the assumption that the enzyme is small in concentration compared to its substrate, and that the concentration of the enzyme-substrate complex is unchanging with respect to time. In the Michaelis-Menten model the rate of an enzymatic reaction is given by $\frac{dProduct}{dt} = V_{max} \frac{Substrate}{K_m + Substrate}$
Ultrasensitivity	A property of steady-state input-output relationships that makes them switch-like in character. Goldbeter and Koshland defined input-output relationships to be ultrasensitive if it took less than an 81-fold change in input stimulus to drive the output from 10% to 90% of maximum
Zero-order	A zero-order chemical or biochemical reaction is one where the rate of the reaction is independent of the substrate concentration. Enzyme reactions approach zero-order when the enzyme is saturated with substrate

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Highlights

- To understand the responses of complex signaling networks, one must first understand the responses of the individual monocycles that comprise the network.
- Monocycles sometimes exhibit switch-like ultrasensitive responses.
- In a previous installment in this series, we showed how enzyme saturation can generate switch-like responses through zero-order ultrasensitivity.
- Here we review several other mechanisms—multisite phosphorylation, stoichiometric inhibitors, and positive feedback, and several variations thereof—that can also generate ultrasensitive responses.

Fig. 1. Multisite phosphorylation and ultrasensitivity in the response of Cdc25C to Cdk1
 (A) Schematic view of the *Xenopus laevis* Cdc25C protein, with the five putative Cdk1 phosphorylation sites highlighted. (B) Steady-state hyperphosphorylation of Cdc25C in *Xenopus* egg extracts with different levels of Cdk1 activity. The response is based on the mobility shift seen by SDS-polyacrylamide gel electrophoresis when Cdc25C is hyperphosphorylated. The Hill coefficient of the fitted Hill curve is 11. (C) Phosphorylation of the wild-type Cdc25C N terminus (red) and the N terminus with the Thr 48, Thr 67, and Thr 138 changed to Glu residues (blue). The effective Hill coefficients are 4.5 and 0.9, respectively. The response is based on ^{32}P incorporation. Adapted from [9].

Fig. 2. Ultrasensitivity from multisite phosphorylation

(A) A dual phosphorylation activation mechanism, like that of the ERK2 MAP kinase [56–58]. (B) Steady-state responses. The three solid curves represent the input-output relationship derived in Box 1 for three different assumed values of K_1 and K_2 : $K_2 = 10 \times K_1$ (blue); $K_2 = K_1$ (green); $K_1 = 10 \times K_2$ (red). In each case the two absolute K values were chosen so that the response was half-maximal at a kinase concentration of 1. The dashed curves are Hill curves with $n = 1$ (blue) or $n = 2$ (red), shown for comparison. (C) Six-site phosphorylation, assuming all 6 phosphorylations are required for activation of X . The green curve shows the response if all of the K values are equal to 1. The ultrasensitivity becomes greater and the response is overall more switch-like if the last phosphorylation is assumed to be 10-fold (red, $K_6 = 0.1$) or 100-fold (purple, $K_6 = 0.01$) more favorable than the other phosphorylation. (D) Cooperative activation of a receptor by the binding of two ligand molecules, a mechanism related to dual phosphorylation. In (A) and (D), yellow indicates inactive enzyme whereas pink indicates active enzyme.

Fig. 3. Cooperativity in multisite phosphorylation from priming

(A, B) Schematic view of how a priming phosphorylation can promote the phosphorylation of other sites through the interaction between the primed phosphoepitope and the Cks1 or Cks2 protein. The Cdk1-cyclin-Cks complex is shown in yellow. The substrate protein is shown as a disordered blue chain with two phosphorylation sites highlighted in pink. The phosphorylation of the first Thr (T) site primes the substrate for phosphorylation of the second Ser (S) residue as a result of the interaction of the pThr epitope with the Cks subunit, and can make the second phosphorylation be more favorable than the first. Adapted from [12].

Fig. 4. Extra phosphorylation sites increase ultrasensitivity

In both panels we assume non-cooperative phosphorylation, with all of the phosphorylation and dephosphorylation rate constants taken to be equal to 1. (A) For each curve, we assumed that 3 sites must be phosphorylated for activation of X ($m = 3$). The input-output relationship was then calculated assuming the total number of sites (n) to be 3 to 7. (B) For each curve, we assume that there are 6 phosphorylation sites ($n = 6$) and calculated the input-output relationship assuming that 1 to 6 of the sites must be phosphorylated for activation of X ($m = 1 - 6$). In both panels the effective Hill coefficients for the responses are shown in the inset.

Fig. 5. Implicit positive feedback from competition for converting enzymes

(A) A dual phosphorylation mechanism that includes intermediary complexes. This scheme is a simplified version of the distributive phosphorylation and dephosphorylation reactions that regulate the Erk2 mitogen-activated protein kinase (MAPK) [19, 58, 59]. (B) Bistable responses emerge when the enzymes are saturated and the substrate concentration is high. The model is based on Ref. [19], and this particular parameter set, adapted from Ref. [54], is as follows: $a_1 = 0.004$; $d_1 = 0.00016$; $k_1 = 0.00016$; $a_2 = 8$; $d_2 = 0.32$; $k_2 = 0.32$; $a_3 = 1$; $d_3 = 0.04$; $k_3 = 0.04$; $a_4 = 0.1$; $d_4 = 0.004$; $k_4 = 0.004$; $p'ase_{tot} = 1$.

Fig. 6. Reciprocal regulation

(A) Schematic depiction of an input that activates a kinase and inactivates an opposing phosphatase. An example of this sort of system is the checkpoint proteins Chk1 and Chk2, which activate the Cdk1-inactivating kinase Wee1 and inactivate the Cdk1-activating phosphatase Cdc25. The star shapes represent the activated forms of the kinase and phosphatase. (B) Ultrasensitivity from reciprocal regulation, based upon Eq 5.6. The blue curve assumes that all of the K values equal 1; it is a Michaelian response. The red curve assumes that $K_1 = 10$, $K_2 = 0.1$, and $K_3 = 1$. The dashed black curve is a Hill curve with a Hill exponent of 2.

Fig. 7. Feed-Forward Regulation

(A) A simplified scheme for Akt activation. PIP₃ (PtdIns(3,4,5)P₃) feeds into the activation of Akt by recruiting both Akt and its activator Pdk1 to the membrane. The pink phospholipids are PIP₃. The final activated form of Akt is denoted in pink, as is the active, membrane-associated form of Pdk1. See Ref [60] for further details. (B) Ultrasensitivity from feed-forward regulation, based on Eq 6.4. The assumed K values were $K_1 = K_2 = K_3 = 1$ (green); $K_1 = K_2 = 0.1$ and $K_3 = 100$ (blue); and $K_1 = K_2 = 0.01$ and $K_3 = 10000$ (red). The dashed black curve is a Hill equation curve with a Hill exponent of 2, for comparison.

Fig. 8. Inhibitor ultrasensitivity

(A) Schematic depiction of the inhibition of substrate (X) phosphorylation by a high affinity stoichiometric inhibitor (I) of the kinase. The rate constant for the mass action phosphorylation of X is k_1 ; the rate constant for the mass action dephosphorylation of XP is k_{-1} ; the rate constant for the association of the kinase with the inhibitor is k_2 ; and the rate constant for dissociation is k_{-2} . (B) Steady-state responses, based on Eq 1.5. The curves correspond to $X_{tot} = 1$, $I_{tot} = 2$, $K_1 = 0.1$, and $K_2 = 1$ (purple), 0.1 (blue), 0.01 (green), or 0.001 (red). The red curve has an effective Hill exponent of 9.7. The dashed black curves show responses assuming no inhibitor (left) or two units of inhibitor with a K_2 value approaching zero (right). (C) Schematic view of the synthetic inhibitor ultrasensitivity system described by Buchler and Cross [40]. Cells were engineered to express a $C/EBP\alpha$ (CCAAT-enhancer binding protein- α) reporter and various concentrations of a chimeric transcription factor ($C/EBP\alpha$ -VP16) in the presence or absence of a dominant negative $C/EBP\alpha$ protein. (D) Experimental demonstration of inhibitor ultrasensitivity in a synthetic transcriptional circuit in mammalian cells in the presence (red) or absence (blue) of the dominant-negative stoichiometric inhibitor. Adapted from [41].

Fig. 9. Ultrasensitivity from substrate competition

(A) Schematic depiction of competition between substrates X (a higher affinity substrate) and Y (a lower affinity substrate) for access to kinase. A Michaelis-Menten scheme describes the phosphorylation kinetics, and the law of mass action describes the dephosphorylation kinetics. The kinase-substrate association rate constants are a_1 (for substrate X) and a_2 (for substrate Y). Similarly, the kinase-substrate dissociation rate constants are d_1 and d_2 , and the catalytic constants are k_1 and k_2 . The rate constants for the mass action dephosphorylation reactions are k_{-1} and k_{-2} . (B) Ultrasensitive response of substrate Y to the kinase in the presence (red) or absence (blue dashed) of a high affinity competitor X. Curves are based on Eqs 6.1–6.7. The assumed parameters are: $a_1 = d_1 = k_1 = d_2 = k_2 = 1$; $a_2 = 10$; $k_{-1} = k_{-2} = 0.01$; $X_{tot} = 3$; $Y_{tot} = 1$. (C) Experimental demonstration of ultrasensitivity from substrate competition. The substrate of interest in this case is *Xenopus* Wee1; the high-affinity competitor is the budding yeast Sic1 protein. Adapted from [43]. (D) Temporal threshold from substrate competition. The rate of phosphorylation of the lower affinity substrate (Y) increases once most of the competitor substrate (X) has been phosphorylated. The assumed total concentration of the kinase was 1. In all case the concentrations are expressed as fractions of a total (e.g. XP/X_{tot} ; $kinase/kinase_{tot}$...). (E) Time courses for various systems whose steady-state responses are ultrasensitive. Temporal thresholds are seen in multisite phosphorylation and substrate competition, but not in inhibitor ultrasensitivity or zero-order ultrasensitivity. Parameters are as follows. For the stoichiometric inhibitor model, the parameters were those used for the ultrasensitive red curve in Fig 8B, and the assumed total concentration of kinase was 5. For zero-order ultrasensitivity [2], it was assumed that $X_{tot} = 1$, $K_{m1} = K_{m2} = 0.1$, and the kinase concentration was 5. For dual phosphorylation it was assumed that all of the rate constants equaled 1 and the kinase concentration was 50. For competing substrates, the parameters were the same as those used in panels B and C. In all cases, the initial level of output (XP or

YP) was taken to be 0. (F) Rate balance plots to explain the shapes of the non-saturated (left) and zero-order ultrasensitivity (right) time courses. The green curves represent the rate of phosphorylation of substrate Y as a function of how much Y is phosphorylated. The red curves represent the dephosphorylation rates. The net rate of phosphorylation is the vertical distance from the green curve to the red curve. For the non-saturated case (left), the rate of approach to the steady state decreases linearly as the distance from the steady state decreases, resulting in exponential approach to the steady state. For the saturated case (right), the rate of approach falls off more slowly, and so the system approaches steady state at a more nearly constant rate. Parameters here were chosen so that the two systems had the same steady states and the same half-times for approaching steady state.

Fig. 10. Thresholds and ultrasensitivity from positive feedback

(A) Schematic view of the system. The activation of X is assumed to be autocatalytic. (B) Rate-balance analysis. The curves show the rates of phosphorylation (green) and dephosphorylation (red) as a function of X^* for various assumed total values of the input, which is taken to be the total concentration of X present. The green curves correspond to 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 units of X_{tot} . (C) The input-output relationship for the system. The solid black dot and solid blue curve represents stable steady states. The open points and dashed blue line represents unstable steady states. Note that the system has two (non-negative) steady states when the input is greater than 1, but only one of the steady states is stable. (D) Time course for a system responding to 2 units of input, assuming various initial values of X^* . The length of the time lag is a sensitive function of $X^*[0]$.