

satisfy these tests, then this model is shown to be inappropriate and the parameter values derived therefrom of dubious value. We recommend that reviewers of submitted manuscripts containing such analyses insist on self-consistency as one criterion for acceptability for publication.

Finally, we suggest that readers apply these extremely simple tests to already published results as one way (but not the only way<sup>2</sup>) of evaluating their trustworthiness.

#### Acknowledgments

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## Tripping the switch fantastic: how a protein kinase cascade can convert graded inputs into switch-like outputs

James E. Ferrell, Jr

Recent experimental work has shown that the mitogen-activated protein (MAP) kinase cascade can convert graded inputs into switch-like outputs. The cascade could therefore filter out noise (signals of insufficient magnitude or duration) and still respond decisively to supra-threshold stimuli. Here, we explore the biochemical mechanisms likely to be at the root of this behavior.

**THE MAP KINASE** cascade is a set of three protein kinases – a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK) and a MAP kinase (MAPK) – that function as a signal-relaying module<sup>1–6</sup> (Fig. 1). MAPKKKs activate MAPKKs through the phosphorylation of two residues (usually serines); the active MAPKKs activate MAPKs through phosphorylation of a threonine and a tyrosine residue. MAP kinase cascades often receive inputs from plasma membrane-associated signaling molecules in response to extracellular stimuli. The cascades might also monitor the internal status of the cell. For example, MAP kinase-like activities have been

implicated in the spindle assembly checkpoint<sup>7</sup>.

MAP kinase cascades have been found in animals, plants, fungi and protists. Many cells possess a number of MAP kinase cascades operating in parallel – budding yeasts possess at least five, and mammalian cells at least three<sup>1,5,6,8</sup>. Even a single MAP kinase cascade might bring about different changes in a cell's function in different contexts. For example, the well-studied cascade comprising Raf-1 (a MAPKKK), Meks 1 and 2 (MAPKKs), and Erks 1 and 2 (MAPKs) can trigger mitogenesis in many tissue culture cells, transdifferentiation or mitogenesis in PC12 cells, cell-fate induction in developing embryos, and activation of Cdc2–cyclin B complexes in oocytes<sup>4,9,10</sup>.

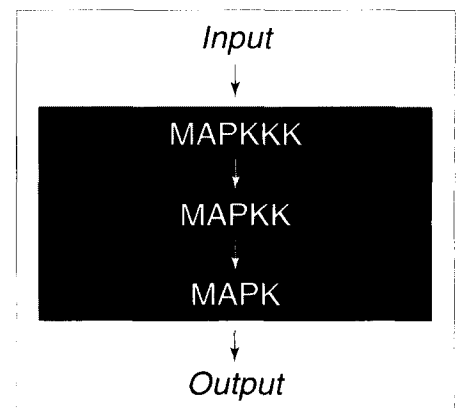
Thus, MAP kinase cascades are evolutionarily conserved and biologically versatile; evidently the three-kinase scheme has been a successful way of

transmitting information. But why are three kinases employed rather than one? The adenylate cyclase–cAMP signaling system transmits signals from the membrane to the nucleus using a single protein kinase, protein kinase A. The JAK–STAT systems also use a single kinase. Why are there so many intermediaries in the MAP kinase system?

#### The magnitude of the response: signal amplification

One attractive possibility is that a three-kinase system provides the cell with a high degree of signal amplification, in the same way that a photomultiplier tube converts a small pulse of photons into a large photocurrent. If each kinase rapidly phosphorylates, for example, 1000 target molecules, then the cascade would produce a heroic 10<sup>9</sup>-fold amplification.

This degree of amplification is probably unnecessary and, in fact,



**Figure 1**

The mitogen-activated protein kinase (MAPK) cascade. MAPKKK denotes MAP kinase kinase kinase; MAPKK denotes MAP kinase kinase.

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the amount of amplification actually achieved by the cascade is much more modest. Take, for example, the mating-pheromone response pathway in the budding yeast *Saccharomyces cerevisiae*. The  $\alpha$ -factor receptor, which initiates the response, is present at a level of about 5000 to 8000 molecules per haploid 'a' cell<sup>11,12</sup> (Table I), and some mating-pheromone responses are half-maximal when the receptor is about 10% occupied<sup>13</sup>. The relevant MAPKs (Kss1p and Fus3p) are each present at a level of about 5000 molecules per haploid cell<sup>14</sup>. Thus, approximately 500 activated receptors bring about the activation of fewer than 10000 MAPK molecules; the total amplification between the  $\alpha$ -factor receptor and the MAPKs is at most 20-fold. This degree of amplification could certainly be accomplished without the intermediacy of a three-kinase cascade.

The Raf-Mek-Erk MAP kinase cascade, which operates in many animal cells, can be activated by the Ras protein. Ras is present in mammalian tissue culture cells at about  $\sim 20000$  molecules per cell (Table I), and under conditions where 10–50% of the Ras molecules become GTP-bound, downstream effects occur<sup>15–18</sup>. The Erk1 and Erk2 MAP kinases together are present at levels of the order of 1000000 molecules per cell, and almost all of these MAP kinase molecules are activated under conditions where 2000 to 10000 Ras molecules are activated. So, the amplification between Ras and MAPKs is about 100- to 500-fold – higher than it was in the yeast case, but still not so high that it is clear that a cascade of enzymes should be required.

In addition, it is not clear that every step of the cascade contributes to the amplification. The MAPKKKs Raf and Mos are scarce enzymes (see Table I) and Mek is a relatively abundant enzyme, so there is a potential for substantial amplification at the MAPKKK-to-MAPKK step. For example, during *Xenopus* oocyte maturation, the oocyte synthesizes and apparently activates about  $1 \times 10^9$  Mos molecules<sup>19</sup>, and this brings about activation of most or all of the cell's  $350 \times 10^9$  Mek-1 molecules<sup>20</sup> (C-Y. F. Huang and J. E. Ferrell, Jr, unpublished). The activation of Mek-1 brings about activation of about  $100 \times 10^9$  Erk2 molecules.

Table I. Approximate abundances of MAP kinase cascade components

Protein		Molecules per cell <sup>a</sup>	Concentration (nM)	Refs
<b>Mating pheromone response pathway in budding yeast</b>				
(upstream)	$\alpha$ -factor receptor	$8 \times 10^3$	160	11, 12
MAPKKK	Ste11p	?	?	
MAPKK	Ste7p	$< 2 \times 10^3$	$< 35$	14
MAPKs	Kss1p	$5 \times 10^3$	100	14
	Fus3p	$5 \times 10^3$	100	14
<b>Mitogenesis in tissue culture cells</b>				
(upstream)	Ras	NIH 3T3 cells	$2 \times 10^4$	33
MAPKKK	Raf-1	Cos cells	$1 \times 10^4$	17
MAPKK	Mek-1	NIH 3T3 cells	$36 \times 10^4$	600
		CHO cells	$80 \times 10^4$	1300
MAPKs	Erk1/2	PC12 cells	$75 \times 10^4$	1250
		CHO cells	$170 \times 10^4$	2800
<b>Xenopus oocyte maturation</b>				
MAPKKK	Mos	$1 \times 10^9$	3	19
MAPKK	Mek-1	$350 \times 10^9$	1200	c
MAPK	Erk2	$100 \times 10^9$	330	c

<sup>a</sup>To convert from concentrations to molecules per cell, the volume of a haploid yeast was assumed to be 0.1 pL; the volume of a tissue culture cell was assumed to be 1 pL; and the cytoplasmic volume of a *Xenopus* oocyte was assumed to be 0.5  $\mu$ L.  
<sup>b</sup>The estimate of Raf abundance in Cos cells was provided by D. Stokoe (Onyx Pharmaceuticals).  
<sup>c</sup>The Mek-1 abundance data, the Erk1/Erk2 data from Chinese hamster ovary (CHO) cells and the *Xenopus* oocyte Mek-1 and Erk2 abundance data are from our laboratory.  
<sup>d</sup>The PC12 Erk1/Erk2 data were provided by M. Cobb (University of Texas Southwestern).

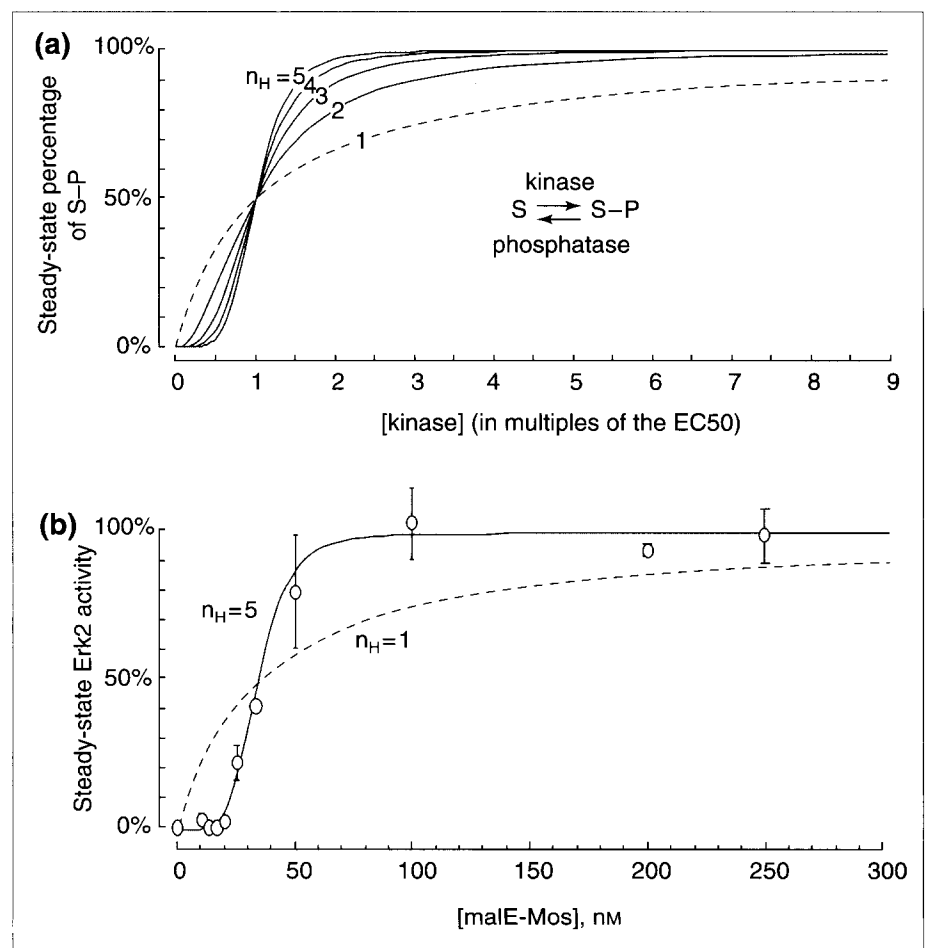


Figure 2

Steady-state responses in various systems. (a) Comparison between a hyperbolic, Michaelian response (dashed red line) with ultrasensitive responses for systems with Hill coefficients of 2, 3, 4 and 5 (solid blue lines). (b) The experimentally determined steady-state response of the Erk2 mitogen-activated protein (MAP) kinase to various concentrations of added recombinant malE-Mos in a *Xenopus* oocyte extract system. A Michaelian stimulus-response curve (dashed red line) and an ultrasensitive stimulus-response curve (with a Hill coefficient of 5, solid blue line) are shown for comparison. Redrawn from Ref. 22, with permission.

The net amplification is about 100-fold, but the signal is attenuated rather than amplified when the signal is passed down the cascade from Mek-1 to Erk2. Why does the cell not do away with

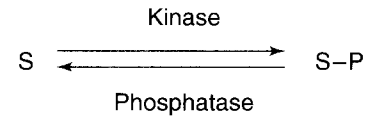
Mek-1, or with both Mek-1 and Mos, and let Erk2 be regulated more directly and efficiently? Is something other than amplification accomplished by interposing Mek-1?

**The sensitivity of the response: the shape of the stimulus-response curve**

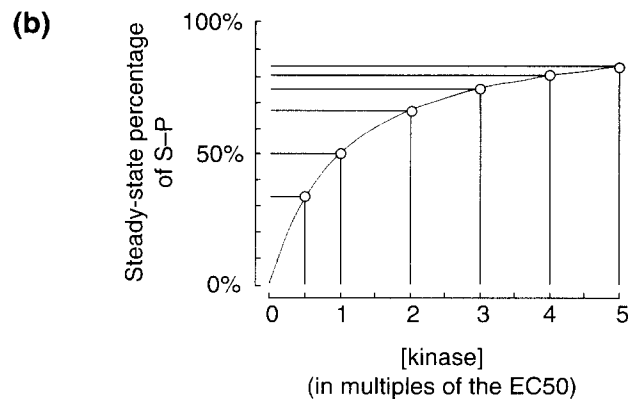
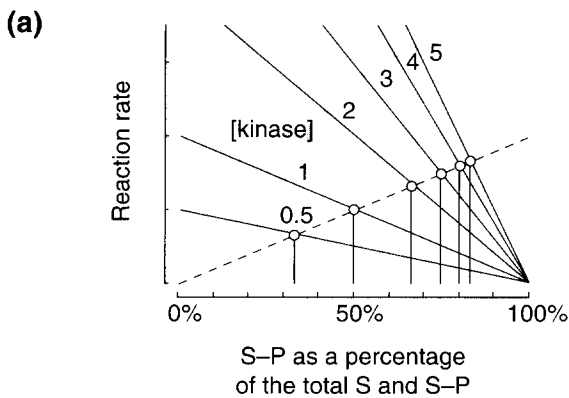
Magnitude amplification is only one of several important aspects of the steady-state behavior of a signaling system.

**Box 1. Graphical derivation of stimulus-response curves**

Here, we shall use simple geometrical arguments to derive the Michaelian hyperbolic stimulus-response curve, and to show how multistep processes and enzyme saturation can produce sigmoidal stimulus-response curves indistinguishable from those of cooperative enzymes. In each case, we shall consider a simple phosphorylation-dephosphorylation system: a substrate S is phosphorylated by a kinase to yield S-P, which can be dephosphorylated by a phosphatase to yield S again (right).



**Hyperbolic (Michaelian) sensitivity.** Assume that the converting enzymes – the kinase and phosphatase – are far from saturation. (a) The rates of the forward and reverse reactions will then linearly increase (the reverse reaction; dashed red line) or decrease (the forward reaction; solid blue lines) as the percentage of phosphorylated substrate present in the reaction increases from 0% (no S-P present) to 100% (no S present). Where the dashed red and solid blue lines intersect, the forward and reverse reactions are running at the same rate; the system is at steady-state. Initially, assume that equal amounts of kinase and phosphatase activity are present, which means that the steady-state level of S-P is 50%.

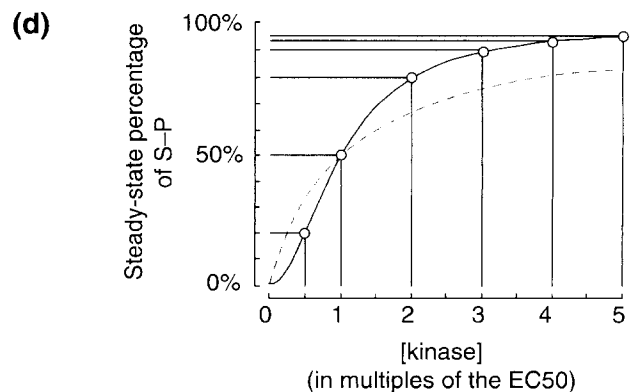
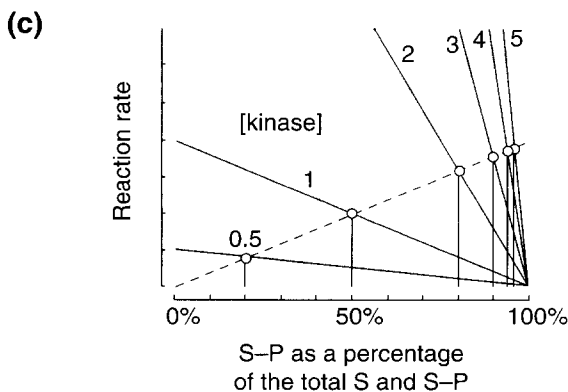


Now, double the assumed concentration of the kinase, or, equivalently, the assumed activity of the kinase. This means the slope of the forward reaction line doubles. The rate of the forward reaction will now exceed the rate of the reverse reaction, and the level of S-P will increase. As it increases, the rate of the forward reaction drops and the rate of the reverse reaction increases. Eventually, the system reaches a new steady-state, with the intersection point and the steady-state percentage of S-P shifted to the right.

(b) Plotting the predicted steady-state levels of S-P from panel (a) as a function of the assumed amount of kinase activity yields the familiar hyperbolic stimulus-response curve. The system is maximally sensitive – the stimulus-response curve is steepest – at low stimulus levels, and becomes progressively less sensitive as the stimulus increases.

**Multistep ultrasensitivity.** Next, consider how the situation changes if the rate of the forward reaction increases as the square of the amount of kinase present, as could be the case for the second step of a two-step, dual-phosphorylation reaction. (c) The rates of the forward and reverse reactions are still straight-line functions of the percentage of S-P present, but now the slope of the forward reaction line varies as the square of the amount of kinase activity present.

Assume that one unit of kinase activity is present, and that it balances the phosphatase activity to yield a steady-state with 50% S-P. Now, if the amount of kinase present is doubled, the rate of the forward reaction increases fourfold. This pushes the system further from steady-state than was the case in panel (a). The proportion of S-P present must rise to a higher level (80% vs 67%) before the forward and reverse reaction rates come back into balance and the system reaches a new steady state.



(d) The resulting stimulus-response curve, shown as the solid purple line, is sigmoidal, with a Hill coefficient of 2. The sigmoidal curve is steeper than a hyperbolic curve (the dashed green line) for stimuli around the EC50, but less steep for very small stimuli and very large stimuli. The system is more sensitive than a hyperbolic system in the middle of the response range – hence the term ultrasensitivity – but it filters out small stimuli and compresses large ones.

Another is the shape of the system's stimulus-response curve. Typical Michaelis-Menten enzymes exhibit hyperbolic stimulus-response curves (Fig. 2a, dashed curve). At very low stimulus levels, the response increases linearly with the stimulus. As the magnitude of the stimulus increases, the response to each increment of stimulus grows progressively smaller; a Michaelian enzyme obeys the law of diminishing returns. This familiar pattern of response has been referred to as hyperbolic sensitivity or Michaelian sensitivity<sup>21</sup>. An enzyme that exhibits hyperbolic sensitivity requires an 81-fold increase in input stimulus to drive it from 10% maximal activation to 90% maximal activation.

By contrast, some enzymes and systems exhibit sigmoidal stimulus-response curves, which are often well-approximated by the Hill equation (Eqn 1) (Fig. 2a, solid curves).

$$y = x^{nH} / (EC50 + x^{nH}) \quad (1)$$

The first increments of stimulus produce little response, but then once the enzyme does begin to respond, it reaches its maximal response quickly. In limiting cases, the response is switch-like; below some threshold there is no response, and then above it the response is maximal. A cell could exploit this type of response in at least two ways. The resting state of the cell could be on or near the upstroke of the

stimulus-response curve, so that a tiny change in input stimulus could lead to a large response. Or the resting state could be well to the left of the upstroke, in which case the system would filter out small stimuli and yet still allow the cell to respond decisively to stimuli of sufficient magnitude and duration.

The most familiar examples of systems that exhibit sigmoidal stimulus-response curves are cooperative enzymes. However, cooperativity is only one of several mechanisms that can generate sigmoidal stimulus-response curves and switch-like responses. Accordingly, Goldbeter and Koshland proposed a new term – 'ultrasensitivity' – to describe any system exhibiting a sigmoidal stimulus-response curve, irrespective of the underlying mechanism<sup>21</sup>. An ultrasensitive system requires less than an 81-fold increase in input stimulus to drive it from 10% maximal activation to 90% maximal activation.

#### Experimental studies in *Xenopus* oocyte extracts

As elaborated below, simple kinetic arguments show that the MAP kinase cascade has the potential to exhibit a markedly ultrasensitive, switch-like response. Motivated by these arguments, Huang examined the responses of Mek-1 (a MAPKK) and Erk2 (a MAPK) to the addition of recombinant Mos (a MAPKKK) in a complex *in vitro* system: undiluted crude cytoplasmic extracts from *Xenopus*

*laevis* oocytes<sup>22</sup>. He found that Erk2 exhibited a highly switch-like response, the equivalent of a cooperative enzyme with a Hill coefficient of 4 or 5 (Fig. 2b). This is a remarkably steep response; whereas a Michaelian enzyme requires an 81-fold increase in input stimulus to drive it from 10% maximal activation to 90% maximal activation, Erk2 required about a 2.5-fold increase. Mek-1 was found to respond in an intermediate fashion, with a Hill coefficient of about 1.7 (Ref. 22). Thus, there is something about the cascade arrangement that generates a switch-like response from graded stimuli.

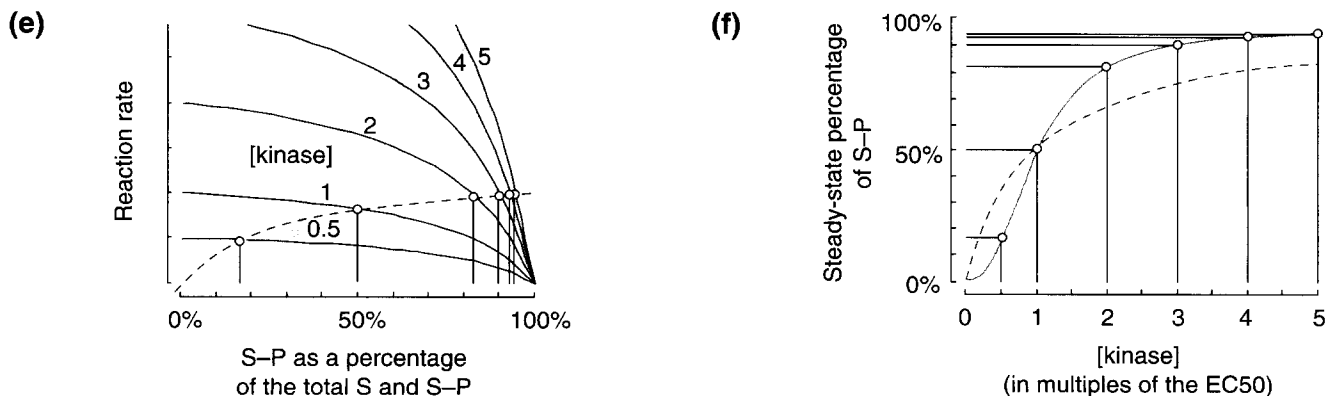
#### Dual phosphorylation as a source of multistep ultrasensitivity

As mentioned above, both MAPK and MAPKK require two phosphorylations to become fully activated. This dual phosphorylation mechanism can, under certain circumstances, give rise to an ultrasensitive response.

This can be seen by considering how one of the enzymes – for example, MAPKK – would be expected to respond to increasing amounts of stimulus (active MAPKKK). If the amount or activity of MAPKKK is small, then as MAPKKK increases, the rate of conversion of MAPKK to phosphorylated MAPKK will increase linearly. If MAPKK is phosphorylated processively – that is, MAPKKK phosphorylates both sites before dissociating from the MAPKK –

#### Box 1. Graphical derivation of stimulus-response curves (contd)

**Zero-order ultrasensitivity.** Suppose that either the kinase or the phosphatase or, as shown below, both enzymes are partially saturated. That is, their rates do not increase linearly as S-P is increased (the phosphatase) or decreased (the kinase), but instead level off at high or low percentages of S-P. The straight lines seen in panels (a) and (c) are replaced with hyperbolic curves, as shown in panel (e).



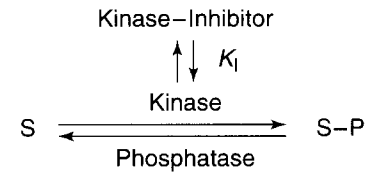
Now, imagine starting at a steady-state with 50% of the substrate phosphorylated and then doubling the amount of kinase activity. This puts the system out of balance, as was the case in panel (a), and S-P begins to accumulate. As it does, the rate of the forward reaction falls, but it does not fall as rapidly as it did in panel (a), and the rate of the reverse reaction does not rise as rapidly. As a result, it takes a larger increase in S-P to bring the system back to steady-state.

(f) Plotting the intersection points from panel (e) as a function of the amount of kinase activity yields the stimulus-response curve. The curve is sigmoidal, and, for the degree of saturation shown in panel (e), yields a Hill coefficient of 2.5. The hyperbolic curve is shown as the dashed green line.

**Box 2. Inhibitor ultrasensitivity**

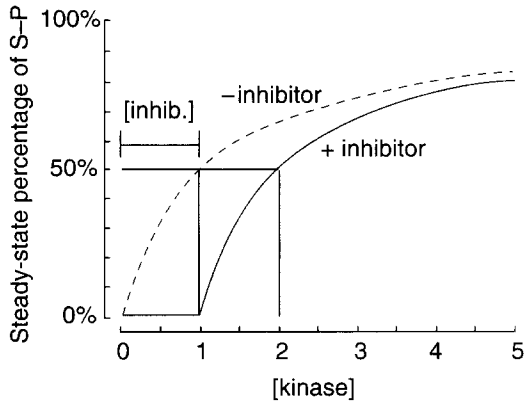
Here, we will demonstrate that a stoichiometric inhibitor can introduce ultrasensitivity into a simple system. Consider again the phosphorylation–dephosphorylation cycle examined in Box 1, but now introduce an inhibitor that can bind the kinase and prevent it from phosphorylating substrate S (right).

(a) If the affinity of the inhibitor for the kinase is very high (the  $K_i$  value is very small), the inhibitor will essentially soak up kinase until all the inhibitor is used up. Once the level of kinase exceeds the level of inhibitor, the system will respond with a hyperbolic stimulus–response curve, but shifted to the right by an amount equal to the inhibitor concentration. The original hyperbolic



curve reached 10% maximal response when the kinase concentration equaled one ninth the EC50, and 90% maximal response when the kinase concentration equaled nine times the EC50, so an 81-fold increase in stimulus was needed to drive the system from 10% to 90% maximal response. If the inhibitor soaks up the first unit of kinase, as shown in panel (a), then ten ninths of a unit of kinase activity is needed for a 10% response (five ninths the new EC50) and ten units of kinase activity is needed for a 90% response (five times the new EC50), so now only a ninefold increase in kinase activity is needed to drive the system from 10–90% activity; the system is ultrasensitive, with a steepness equivalent to that of a cooperative enzyme with a Hill coefficient of 2.

For the more realistic situation where the  $K_i$  value is not infinitesimal, an equation for the steady-state response (%S–P) as a function of the total kinase concentration, total phosphatase concentration, total inhibitor concentration and  $K_i$  value can be derived from the rate equations. At steady-state, the phosphorylation reaction rate equals the dephosphorylation reaction rate (see Eqns 1, 2), where E denotes kinase, P denotes phosphatase,  $k_f$  denotes the forward reaction rate and  $k_b$  denotes the reverse reaction rate:



$$k_f[E](100\% - \%S-P) = k_b[P]\%S-P \quad (1)$$

$$\%S-P = \frac{100\%k_f[E]}{k_f[E] + k_b[P]} \quad (2)$$

We next calculate the amount of kinase available to the phosphorylation reaction, [E], as a function of the total kinase  $[E_{tot}]$ , the total inhibitor  $[I_{tot}]$ , and the  $K_i$  value (see Eqns 3–8):

$$[E][I]/[E \cdot I] = K_i \quad (3) \qquad [E] = [E_{tot}] - [E \cdot I] \quad (4)$$

$$[I] = [I_{tot}] - [E \cdot I] \quad (5) \qquad [E \cdot I]^2 - (K_i + [I_{tot}] + [E_{tot}])[E \cdot I] + [E_{tot}][I_{tot}] = 0 \quad (6)$$

From the quadratic formula:

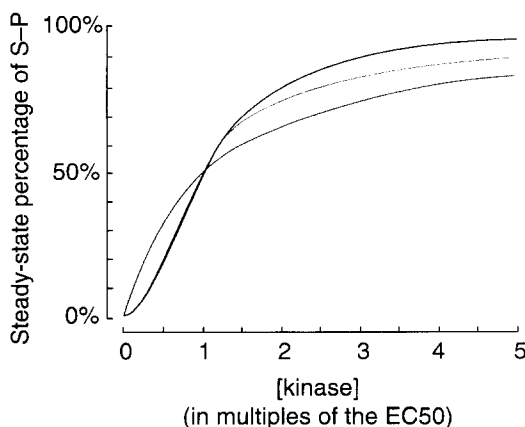
$$[E \cdot I] = 0.5(K_i + [I_{tot}] + [E_{tot}] - \sqrt{(K_i + [I_{tot}] + [E_{tot}])^2 - 4[E_{tot}][I_{tot}]}) \quad (7)$$

Substituting Eqn 7 into Eqn 4 yields:

$$[E] = 0.5([E_{tot}] - K_i - [I_{tot}] + \sqrt{(K_i + [I_{tot}] + [E_{tot}])^2 - 4[E_{tot}][I_{tot}]}) \quad (8)$$

And finally, substituting Eqn 8 into Eqn 2 yields the desired expression for the steady-state response as a function of  $[E_{tot}]$ ,  $[I_{tot}]$ ,  $[P]$ , the phosphorylation and dephosphorylation rate constants and the  $K_i$  value:

$$\%S-P = \frac{100\%k_f([E_{tot}] - K_i - [I_{tot}] + \sqrt{(K_i + [I_{tot}] + [E_{tot}])^2 - 4[E_{tot}][I_{tot}]})}{2k_b[P] + k_f([E_{tot}] - K_i - [I_{tot}] + \sqrt{(K_i + [I_{tot}] + [E_{tot}])^2 - 4[E_{tot}][I_{tot}]})} \quad (9)$$



(b) The shape of the calculated stimulus–response curve for a situation where there is one unit of inhibitor total (that is, the first unit of kinase can, in principle, be soaked up by inhibitor); the  $K_i$  value is 0.1; and the forward and reverse rate constants are 1. The inhibitor produces an ultrasensitive response – inhibitor ultrasensitivity. The stimulus–response curve predicted in the presence of the inhibitor is sigmoidal; it is similar in shape to a Hill equation curve, but it approaches the 100% S–P limit more gradually.

- multistep ultrasensitivity, from Hill eqn,  $n_H = 2$
- - - inhibitor ultrasensitivity, from Eqn 9
- ..... hyperbolic (Michaelian) sensitivity

the rate of conversion of phosphorylated MAPKK to doubly phosphorylated MAPKK will also increase linearly with MAPKKK. But, if phosphorylated MAPKK dissociates from MAPKKK, and the second phosphorylation requires a second collision, then the rate of conversion of phosphorylated MAPKK to doubly phosphorylated MAPKK will increase as the square of the stimulus concentration.

The second-order rate dependence translates into an ultrasensitive stimulus–response curve with a Hill coefficient of 2. An easy way to see this exploits a graphical representation commonly used in theoretical analyses of complicated biochemical systems<sup>23,24</sup>. In Box 1, this graphical representation is used to derive the hyperbolic stimulus–response curve of a single phosphorylation–dephosphorylation cycle (panels a, b), and the ultrasensitive stimulus–response curve of a dual phosphorylation reaction (panels c, d). Two-collision, dual-phosphorylation reactions constitute a variation on what has been termed ‘multistep ultrasensitivity’ or ‘multistep sensitivity amplification’<sup>21,25–30</sup>.

Recent studies indicate that MAPK is phosphorylated through a two-collision, distributive mechanism (J. Ferrell, unpublished), so the dual-phosphorylation process is probably an important contributor to the switch-like response observed for MAPK in oocyte extracts<sup>22</sup>. The question of whether MAPKK is activated either by a one-collision or by a two-collision mechanism has not yet been addressed.

There could be additional sources of multistep ultrasensitivity as well. For example, if a signaling molecule at one step of the cascade both activates a downstream kinase and inactivates a downstream phosphatase, this would compound the resulting ultrasensitivity. There are, in fact, some hints that this sort of reciprocal regulation does occur in the MAP kinase cascade<sup>31</sup>.

### Zero-order ultrasensitivity

Numerical solution of the rate equations of the MAP kinase cascade indicates that even if no multistep ultrasensitivity arises from the dual-phosphorylation reactions, some degree of ultrasensitivity is still expected<sup>22</sup>. Where does that come from? The answer can be found in theoretical studies from Goldbeter and Koshland in the early 1980s. They showed that ultrasensitivity can arise when the converting enzymes in a cyclic process – the

kinases and phosphatases in our case – operate near saturation<sup>21,28,30</sup>. The easiest way to see why enzyme saturation leads to an ultrasensitive response is again through graphical arguments. (This is shown in Box 1, panels e, f).

In the three systems shown in Table I, the MAP kinases are present at high enough concentration to partially saturate the relevant MAPKK. The  $K_m$  value for the phosphorylation of mammalian Erk2 by Mek-1 has been measured to be about 300 nM (N. Ahn, pers. commun.) and yeast Ste7p forms complexes with Kss1p or Fus3p with a dissociation constant of about 5 nM (Ref. 14). Given that vertebrate Mek's appear to be about as abundant as Erks, it is plausible that the Mek activation reaction might be partially saturated too. The extent to which zero-order ultrasensitivity contributes to the observed switch-like response of the MAP kinase cascade remains to be determined.

### Inhibitor ultrasensitivity

Cooperative, multistep and zero-order mechanisms are three mechanisms for generating an ultrasensitive response. Here, we can add a fourth mechanism – the presence of a stoichiometric inhibitor of the activating enzyme. The response of an ultrasensitive system to small stimuli can be thought of as being suppressed compared to the response of a hyperbolic system. The same kind of suppression could be produced by an inhibitor that soaks up some fraction of the activating enzyme. Simple geometrical arguments for how a stoichiometric inhibitor can produce an ultrasensitive response are given in Box 2, together with an algebraic derivation of the steady-state response of a system including such an inhibitor. There is no direct evidence for a stoichiometric inhibitor in the *Xenopus* oocyte MAP kinase cascade. But in processes like cyclin-dependent kinase activation, where stoichiometric inhibitors do play a role, it should be kept in mind that these inhibitors sharpen up the activation process.

### Conclusion

A protein kinase cascade like the MAP kinase cascade can do more than just amplify signals. It has the potential to convert a graded stimulus into a switch-like response, and in an oocyte extract system it appears to do just that. It will be of interest to determine to what extent this behavior is manifested in intact cells.

The switch-like response of the cascade might arise from the dual-phosphorylation mechanisms for activation of MAPK and MAPKK; from reciprocal regulation of opposing enzymes; from partial saturation of the cascade enzymes; from the presence of a stoichiometric inhibitor; or from a combination of these factors. Other signaling systems might accomplish the same ends through cooperativity. For example, the cooperative binding of calcium to calmodulin sharpens the responses of calcium-regulated enzymes. But cooperativity is not the only mechanism and indeed might not be the most common mechanism for generating switch-like responses.

The mechanisms discussed here – multistep phosphorylation reactions, partial saturation of enzymes, stoichiometric inhibitors – are widespread in cellular regulation. They are encountered in receptor down-regulation, transcriptional activation, cell-cycle progression and in a variety of other contexts. These mechanisms might help ensure the sensitivity, fidelity and decisiveness of those processes, just as they have been proposed to do for the MAP kinase cascade.

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## A likely histone H2A.F/Z variant in *Saccharomyces cerevisiae*

Although core histones are extremely conserved in eukaryotic evolution, non-allelic variants have been identified for every class of histone. Perhaps the best studied non-allelic histone variants are the highly conserved F/Z variants of histone H2A. These variants are found in a variety of eukaryotes, including protozoa, fungi, invertebrates and vertebrates, where they usually account for less than 20% of the total H2A. The genes encoding the H2A.F/Z variants are thought to have diverged very early in eukaryotic evolution from those encoding more-abundant major H2As<sup>1</sup>. Disruption/deletion experiments with *Drosophila melanogaster*<sup>2</sup>, with the fission yeast *Schizosaccharomyces pombe*<sup>3</sup> and with the ciliated protozoan *Tetrahymena thermophila*<sup>4</sup> indicate that the genes encoding the H2A.F/Z variants are either essential (*D. melanogaster*, *T. thermophila*) or are required for normal growth rates and chromosome segregation (*S. pombe*), even when deletion of a gene encoding a more-abundant major H2A is without phenotypic effect<sup>4</sup>. These results argue that the H2A.F/Z variants perform important functions that differ from those of the major H2As. While the precise function of these variants is unknown, a number of studies suggest that H2A.F/Z variants are preferentially associated with transcriptionally active or potentially active chromatin (for review, see Ref. 5 and references therein).

The budding yeast *Saccharomyces cerevisiae* is an important model in which to study histones and chromatin function. Because most of its genome is transcriptionally active<sup>6</sup> and organisms that diverged earlier than (*T. thermophila*), at about the same time as (*S. pombe*), and later (*D. melanogaster*) than *S. cerevisiae* have functionally important genes encoding H2A.F/Z, it is quite surprising that a similar gene or protein has not been reported in this organism. In fact, our laboratory recently failed to find such

a gene using molecular methods<sup>7</sup>. However, the recent complete sequence of the *S. cerevisiae* genome has enabled another approach to finding an H2A.F/Z gene in this organism.

We did a TBLASTN search<sup>8</sup> of the *Saccharomyces* Genome Database (<http://genome-www.stanford.edu/Saccharomyces>) for homology to the protein sequence of hvl1 (SWISS-PROT: P08992), the H2A.F/Z variant of *T. thermophila*. This search revealed an open reading frame (SCYOL012c, accession number Z74754) on chromosome XV that was more closely related (High Score = 387,  $P = 7.9 \times 10^{-47}$ ) to the query sequence than the major H2As (P04911, P04912; High Score = 174,  $P = 1.9 \times 10^{-25}$ ). This sequence encodes an open reading frame of 134 amino acids (~14 kDa), which compares well with the sizes of known H2A.F/Z proteins (125–146 amino acids for those listed in Fig. 1). When we did a TBLASTN search of the non-redundant database for homology to this open reading frame, sequences for all of the known H2A.F/Z variants had higher scores and lower probabilities than did the major yeast H2As (Fig. 1a). Thus, this putative protein is more closely related to the H2A.F/Z proteins of distantly related eukaryotes than to the major H2As in *S. cerevisiae* itself, providing compelling evidence that the gene encodes an H2A.F/Z variant. It is likely that this gene is functional, as it is hard to imagine how such strong selection could be applied to a pseudogene. We suggest that this gene be called *HTA3*.

A consensus sequence for all of the known H2A.F/Z variants compared to the individual variants and the consensus sequence of the major H2As is shown in Fig. 1b. Most of the variants begin with the sequence M–A/S–G and have at least one GGK sequence near the amino terminus. The amino-terminal tails contain a number of common sequence elements that align reasonably well in the metazoan H2A.F/Zs and less well in the protozoan and fungal variants. Beginning at the sequence SRS, there is a conserved core of 103 residues that aligns completely in all the H2A.F/Zs. This

region is also highly homologous and largely co-linear with the consensus sequence for major H2A histones. However, consistent differences occur between the variants and the major H2A consensus sequence in this region, as noted in Fig. 1b. The carboxyl termini of the variants are highly divergent, as are those of the major H2As, although the vertebrate and sea urchin (deuterostome) sequences consist entirely of a small common motif not found in the other variants.

In summary, *S. cerevisiae* probably contains a gene encoding an H2A.F/Z variant. Coupled with the recent finding of a yeast gene encoding an H1-like protein<sup>9</sup>, the complement of yeast histones now appears to resemble that of other eukaryotes, greatly strengthening the likelihood that results from functional analyses of yeast chromatin will be generally applicable. Also, the full power of yeast genetics and molecular biology can now be applied to study linker histones and the evolutionarily conserved H2A.F/Z variants.

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