CHAPTER I. INTRODUCTION

Intracellular signal transduction networks propagate and integrate the information that cells sense from environmental stimuli (Asthagiri and Lauffenburger, 2000). The quantitative performance of signaling networks regulates cell decisions in complex microenvironments, and aberrations in network performance lead to pathologies, such as cancer (Hanahan and Weinberg, 2000). It has been proposed that signaling networks are composed of modular sub-networks and that a quantitative understanding of these modular building blocks would provide deeper insights into cellular decision-making (Asthagiri and Lauffenburger, 2000; Hartwell et al., 1999). One such prominent signaling module is the Mitogen-Activated Protein (MAP) kinase cascade. This signaling cascade controls diverse cellular processes, such as proliferation, differentiation, and apoptosis in eukaryotic species ranging from *S. pombe* to *H. sapiens* (Lewis et al., 1998). While the principal molecular components and mechanisms that define the MAP kinase module are well established, how these components and mechanisms work together to determine the quantitative performance of the module remains an area of intense research. A better understanding of this relationship between individual module components and the integrated behavior of the module would provide design strategies for re-engineering module performance by targeting critical components within the module.

1. The canonical MAP kinase cascade

The canonical MAP kinase cascade consists of three serially activating kinases: the MAPK kinase kinase (MAPKKK) phosphorylates and activates MAPK kinase

(MAPKK), which in turn phosphorylates and activates MAPK (Figure I-1). This structure is a widely recurring motif in intracellular signaling pathways across a range of species and has, therefore, garnered the label of a "signaling module."

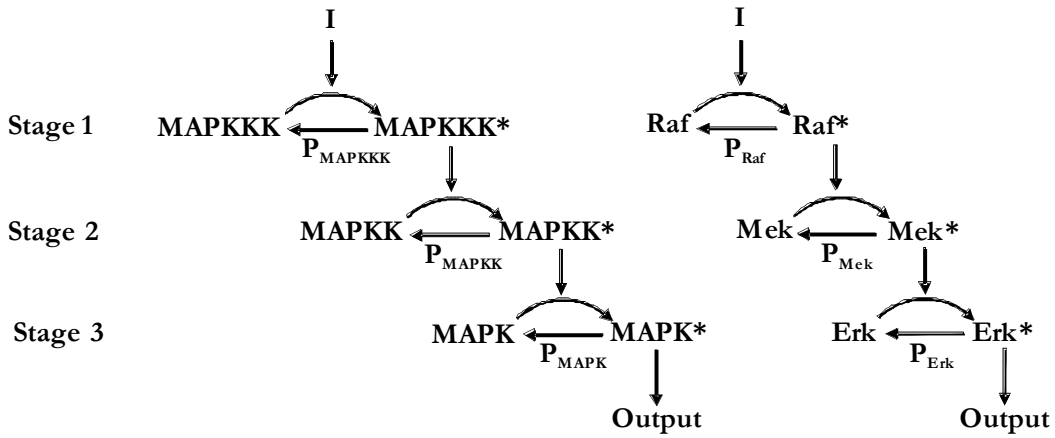

Figure I-1. MAP kinase model schematic.

The canonical MAP kinase cascade is depicted above as three serially-activated kinases (K_S), balanced at each stage of the cascade by a deactivating phosphatase (P_S). On the right, the Erk subfamily is shown.

In mammalian cells, MAP kinase cascades are categorized into one of three subfamilies: Erk, JNK, and p38. The best-characterized subfamily is the Erk module (Figure I-1), which contains the kinases Raf (MAPKKK), Mek (MAPKK), and Erk (MAPK). Commonly stimulated by growth factors, the output of the Erk module, active Erk, proceeds to activate transcription factors and other proteins ultimately influencing important cellular processes such as proliferation and differentiation (Lewis et al., 1998; Pearson et al., 2001; Widmann et al., 1999).

MAP kinase modules play a key role in many non-mammalian eukaryotic species as well. In *Saccharomyces cerevisiae*, multiple MAP kinase modules regulate diverse responses such as mating, invasive growth, and the high-osmolarity glycerol (HOG) stress response (Gustin et al., 1998). Consistent with the conserved architecture, the yeast mating pathway consists of three serially-activating kinases: Ste11 (MAPKKK), Ste7 (MAPKK), and Fus3 (MAPK). Pheromone-mediated stimulation of this pathway culminates in Fus3 activation, which precipitates cell cycle arrest and morphological changes associated with mating (Gustin et al., 1998; Widmann et al., 1999).

The activation and deactivation of MAP kinases is driven by the opposing actions of upstream kinases and protein phosphatases, respectively. Generally, signaling pathways require the constitutive action of protein phosphatases in order to dampen kinase activity when stimulus is absent or has been removed, restoring kinases to their inactive state (Keyse, 2000). Protein phosphatases also contribute to the quantitative performance of MAP kinase modules. By altering their expression level, protein phosphatases modulate MAP kinase signal properties, ultimately influencing cell behavior (Bhalla et al., 2002; Keyse, 2000).

In addition to the core kinases and phosphatases, protein scaffolds play a prominent role in MAP kinase signal propagation. By definition, scaffold proteins bind multiple members of the MAP kinase module, bringing together various kinases onto a single, physical backbone. Protein scaffolds have been associated with virtually all MAP kinase cascades ranging from yeast to humans (Dard and Peter, 2006). Despite their

widespread prevalence (and in contrast to the kinases of the MAP kinase cascade), MAP kinase scaffolds share little structural homology between orthologous pathways, leaving only functional similarities to define and guide our understanding of signaling scaffolds.

The role that scaffolds play in signal transduction is a current area of intense research. Scaffolding proteins confer signal specificity by assembling the appropriate group of upstream activators in order to selectively direct activation of a target MAP kinase. For example, the yeast scaffold Ste5 recruits Ste11 (MAPKKK), Ste7 (MAPKK) and Fus3 (MAPK) (Bardwell, 2006). Meanwhile, another yeast scaffold, Pbs2, recruits Ste11 and Hog1 (MAPK); Pbs2 itself possesses the MAPKK functionality. Thus, Ste5 provides a route for Ste11 → Fus3 signal transfer, while Pbs2 provides an alternative route: Ste11 → Hog1 (Figure I-2). By providing specific routes to activate Fus3 and Hog1, the scaffolds encode the appropriate cellular response, mating or osmolarity response, respectively. Moreover, the stimulus-response identity can be reassigned by simple concatenation of scaffold binding domains (Park et al., 2003), highlighting the potential for re-directing signal flow through molecular engineering of scaffolds.

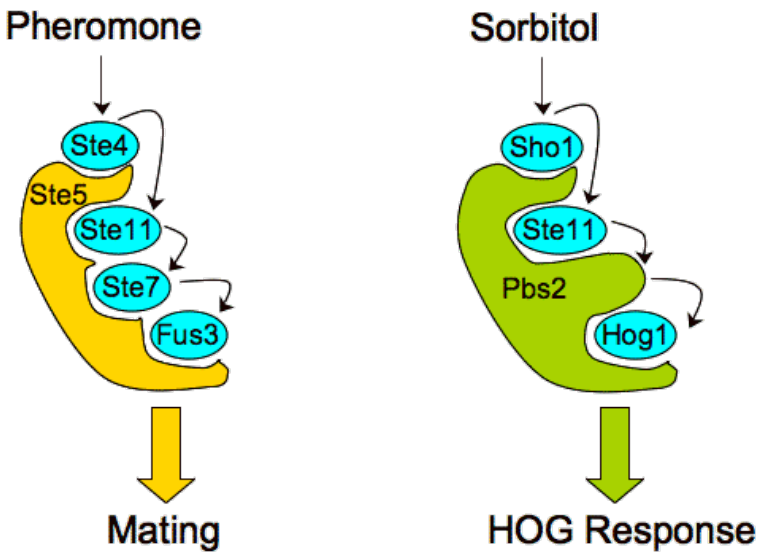

Figure I-2. MAP kinase scaffolds direct signal flow.

2. Quantitative attributes of MAP kinase pathways

2.1 Ultrasensitivity: The MAP kinase module as a biochemical switch

The conserved architecture of the MAP kinase cascade has raised questions regarding what signaling attributes such a structure might confer. One phenomenon that has been investigated extensively is the role of cascade structure in generating a steep steady-state stimulus/response curve, a property termed ultrasensitivity (Figure I-3). As originally defined, ultrasensitive responses are those that achieve a steeper output response with respect to stimulus than a hyperbolic reference curve, described by the Michaelis-Menten equation (Goldbeter and Koshland, 1981). Typically, the Hill

coefficient (n_H)[1] is used to quantify the sharpness of the curve, and has been generalized to describe the degree of the ultrasensitive response (Huang and Ferrell, 1996).

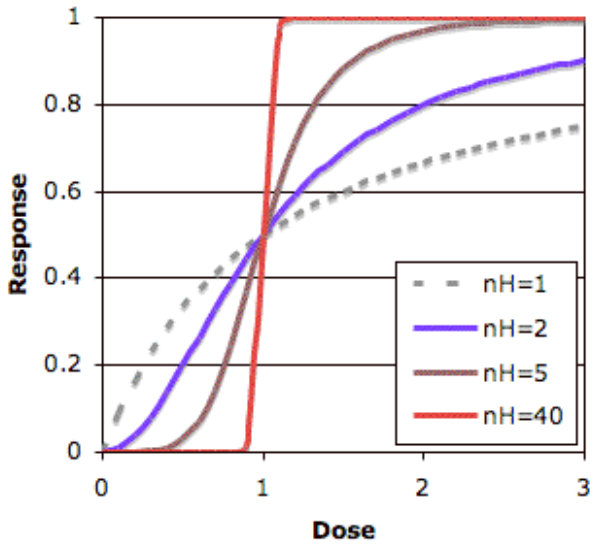

Figure I-3. Hill equation characterizes MAP kinase ultrasensitivity.

As the Hill coefficient increases, the dose-response curve becomes more switch-like. The non-sigmoidal Michaelis-Menten curve (n_H=1) serves as a reference for comparison.

In Huang and Ferrell's model of the MAP kinase cascade, a particular form of multi-step activation that results in an ultrasensitive response is identified. In the model, the activation of MAPKK and MAPK by MAPKKK and MAPKK, respectively, proceeds through a distributive, two-collision mechanism rather than through a processive, one-collision mechanism. Experimental studies in *Xenopus* oocytes confirmed that the

[1] The Hill coefficient compares the 'steepness' of a stimulus/response curve to that of the Michaelis-Menten reference curve – the greater the Hill coefficient, the steeper the response. Responses characterized by Hill coefficients < 1 are not ultrasensitive.

activation of p42 MAP kinase by Mek indeed occurs by a distributive, two-collision mechanism (Ferrell and Bhatt, 1997; Huang and Ferrell, 1996). Furthermore, the multi-level cascade structure itself contributes to the overall ultrasensitive response through multiplicative amplification of the individual responses at each stage (Brown et al., 1997; Ferrell, 1997; Kholodenko et al., 1997). The model predicts that the distributive activation mechanism, in concert with the combinatorial effect of the cascade structure, can result in an ultrasensitive response characterized by a Hill coefficient of 4.9. These predictions are in excellent agreement with experiments performed in *Xenopus* oocyte extracts (Huang and Ferrell, 1996).

However, experimental analysis of MAP kinase activation in intact *Xenopus* oocytes revealed that the ultrasensitive response in single cells was characterized by a Hill coefficient of 42, far exceeding the Hill coefficient of 4.9 observed in protein extracts and predicted by the model (Ferrell and Machleder, 1998). This discrepancy was attributed to the fact that the MAP kinase cascade is embedded in a positive feedback loop *in vivo*, where p42 MAP kinase activation promotes the accumulation of Mos (MAPKKK), thereby strengthening signal throughput (Matten et al., 1996). Indeed, positive feedback is yet another mechanism by which to generate ultrasensitivity. The intrinsic ultrasensitivity of the cascade together with the positive feedback loop results in a biochemical switch, where a graded stimulus is converted into a binary output (Ferrell, 1999).

2.2 Bistability: Discrete transitions and biochemical 'memory'

Bistability in signaling networks has generated particular interest due to its ability to explain discrete, often irreversible, transitions that are commonly observed in biology (Sible, 2003). The maturation of *Xenopus* oocytes is an excellent example of a cellular event that progresses through distinct, stable phases. The ultrasensitive MAP kinasse response has been placed at the center of the biochemical network that controls the irreversible transition inherent in *Xenopus* oocyte maturation (Ferrell, 2002). By adjusting the gains of the positive and negative feedback loops, a monostable, switch-like response can bifurcate into a bistable, hysteretic response (Ferrell and Xiong, 2001). Experimental analysis has provided abundant evidence confirming that the Mos/Mek/p42 MAP kinase cascade indeed controls and stably maintains the irreversible oocyte maturation decision (Ferrell and Machleder, 1998; Xiong and Ferrell, 2003).

In addition to its role in *Xenopus* oocytes, the MAP kinase cascade is prevalent in other signaling networks that have been found to exhibit bistability, such as the Epidermal Growth Factor (EGF) Receptor signaling pathway (Bhalla and Iyengar, 1999; Bhalla and Iyengar, 2001; Bhalla et al., 2002). The most notable of these studies identifies MAP Kinase Phosphatase-1 (MKP) as the "locus of flexibility" that can toggle the network between bistable and monostable regimes (Bhalla et al., 2002). Initial activation of the network by a transient stimulus elicits a sustained, supra-basal response that is maintained by positive feedback. The sustained activation of the cascade is eventually attenuated through slow-acting, negative feedback actuated by the up-regulation of MKP expression. The phosphatase serves two functions: (1) increased MKP

expression leads to signal adaptation[2], and (2) the continued presence of MKP after adaptation serves to attenuate any subsequent stimulation of the network. Thus, MKP not only precipitates signal adaptation, but also confers a 'memory' function by temporally preventing re-activation of the network.

2.3 Signal dynamics: Transient versus sustained MAP Kinase responses lead to distinct cellular fates

In addition to ultrasensitivity and bistability, the MAP kinase cascade is known to communicate biochemical information via its dynamic response. The MAP kinase response in PC12 cells serves as a paradigm that demonstrates the relevance of signal duration (Figure I-4): stimulation with EGF leads to a transient response that promotes proliferation, while stimulation with Nerve Growth Factor (NGF) leads to a sustained response that induces neuronal differentiation (Marshall, 1995). A similar transient versus sustained response occurs in *S. cerevisiae*, where a common MAP kinase cascade mediates two developmental options—invasive growth and mating. Yeast grows invasively when Kss1 activation is sustained, while when activated in a transient manner, Kss1 helps support the mating response (Sabbagh et al., 2001).

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[2] Adaptation refers to the re-setting of signal output to pre-stimulus levels.

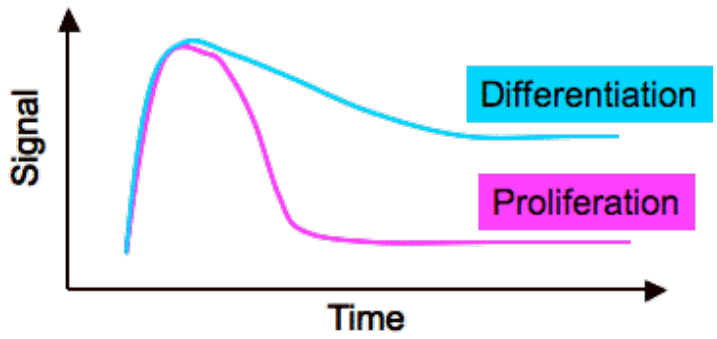

Figure I-4. MAP kinase signal duration controls cell behavior.

To examine more deeply the mechanisms that determine transient versus sustained signaling, a model of the MAP kinase signaling pathway that focused on signal adaptation as a function of multiple forms of negative feedback was developed (Asthagiri and Lauffenburger, 2001). An important conclusion reached by this model analysis was that two forms of negative feedback—decoupling deactivation and constitutive deactivation—were required to achieve full signal adaptation. Although negative feedback could mitigate continued stimulation of the cascade, constitutive deactivators were required for the specific deactivation of the accumulated output.

An ancillary effect of the decoupling negative feedback was that of ultra-desensitization of the network. Ultra-desensitization describes a situation in which the signal output decays, and continues to decay, even in the presence of an *increasing* stimulus. Thus, for sufficiently strong decoupling deactivation, it is possible to sever signal throughput completely, effectively ignoring any further change in stimulus. As a result of signal decoupling, then, the output was free to decay via constitutive deactivation (Asthagiri and Lauffenburger, 2001).

2.4 Protein scaffolds quantitatively affect MAP kinase output

In addition to their role in determining signal specificity, scaffolds quantitatively contribute to signal output. Scaffolds are not simply passive platforms for binding signaling components. Rather, many functionalities of scaffolds, both hypothesized and verified, suggest that they actively participate in signal transduction. For example, scaffolds may orient bound kinases for optimal interaction, thereby providing a catalytic advantage for signal activation (Dard and Peter, 2006). Some scaffolds form dimers, permitting both a *cis*- and a *trans*-phosphorylation mechanism for facilitating signal activation (Yablonski et al., 1996; Yasuda et al., 1999). Finally, recruitment of pathway effectors to scaffold complexes can quantitatively influence signaling output, a property that has been recently exploited to synthetically tune network behavior (Bashor et al., 2008).

Since scaffolds bind to multiple components of a signaling pathway, the stoichiometric relation of the scaffold to its binding partners will affect signal propagation. The effect of scaffold expression level on MAP kinase module performance was explored through computational analysis (Levchenko et al., 2000). In the model, protein scaffolds promote signaling by recruiting pathway components and localizing them onto a single complex. Thus, the scaffold provides a kinetic advantage by reducing the diffusional limitation of kinase activation; the model assumes no catalytic advantage. This model predicts a biphasic relationship between the scaffold concentration and the MAP kinase output, and is not sensitive to binding constants or cooperative binding of the kinases to the scaffold. The mechanism responsible for the decrease in signal

throughput at high scaffold concentration is combinatorial inhibition, an effect that arises when too much scaffold interferes with the optimal formation of competent signaling complexes (Figure I-5).

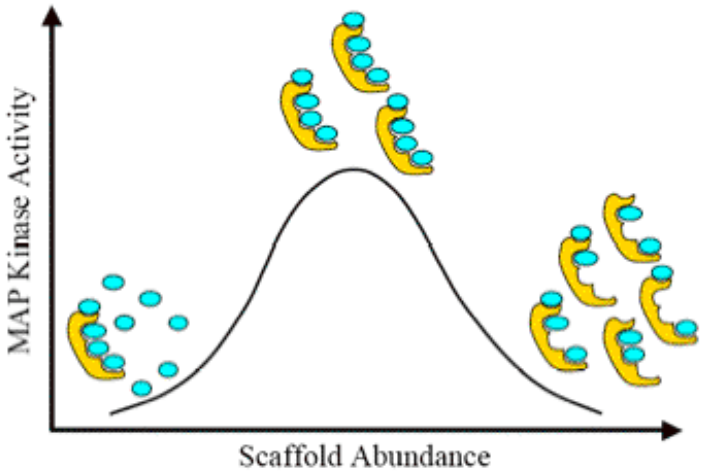

Figure I-5. MAP kinase signal propagation biphasically depends on scaffold concentration.

In addition to the biphasic effect on signal propagation, scaffolding can also influence the ultrasensitivity of MAP kinase signal propagation (Levchenko et al., 2000). Scaffold-bound kinases may be activated processively, while unbound kinases can be activated in a distributive, two-collision fashion. As a consequence, a reduction in module threshold properties may result from scaffold-mediated activation. Conversely, because they can dimerize, scaffolds may contribute to the ultrasensitivity of the MAP kinase response (Ferrell, 2000). Thus, how scaffolds quantitatively influence pathway ultrasensitivity remains to be determined.

3. Current results: The effect of varying the expression levels of module components on the quantitative performance of the MAP kinase cascade

Previous studies have identified many biologically-relevant quantitative features of MAP kinase pathways, including ultrasensitivity, bistability, and signal lifetime. Here, we probe more deeply how these quantitative properties may be affected by variations in the expression levels of the key constituents of the MAP kinase module—kinases, phosphatases and scaffolds. There are three principal reasons to focus on module sensitivity to expression level of its components. First, the expression levels of proteins vary from cell-to-cell even among genetically identical cells, i.e., cells are not quantitative clones even if they are genetic clones. Thus, understanding how sensitive the MAP kinase module is to perturbations in the expression level of its protein components will provide insights into cell population heterogeneity in MAP kinase signal propagation. Second, protein expression levels are not static. Cells dynamically alter protein expression levels to modulate cascade performance (Bhalla et al., 2002; Brondello et al., 1997; Matten et al., 1996). Understanding quantitatively how module performance is affected by these changes in protein expression would shed insight on the regulatory schemes that control MAP kinase-dependent cell processes. Finally, varying protein expression levels is a tractable design strategy that can be implemented using current molecular biology tools. Thus, it would be useful from an engineering perspective to better understand how such manipulations would affect the quantitative performance of the MAP kinase module.

In Chapter II, we use a computational approach to delineate how four quantitative properties of the MAP kinase module—responsiveness to input, dynamic range of output, signal amplification, and signal lifetime—depend on the relative abundances of the two core components, kinases and phosphatases. We uncover a reduced metric termed the 'resistance to activation' that predicts the quantitative properties of the MAP kinase module across a wide range of parameter values. This resistance metric successfully predicted signal lifetime, revealing two distinct regimes of signal decay: (1) stimulus limited decay and, (2) resistance limited decay. The resistance also captured other module properties such as the dynamic range and the responsiveness to input. Our analysis shows that all module attributes cannot be simultaneously optimized, revealing tradeoffs in module design. Thus, the resistance to activation captures the fundamental principles that determine cascade behavior and can be exploited to guide quantitative redesign of the MAP kinase module.

In addition to the expression levels of kinases and phosphatases, scaffold abundance will quantitatively affect MAP kinase signaling properties. In Chapter III we present an experimental sensitivity analysis that quantifies how MAP kinase module performance is affected by systematic variations in scaffold abundance. Our results show that scaffold abundance significantly affects several quantitative aspects of signal propagation, including signal throughput, pathway ultrasensitivity and baseline leakage. We demonstrate that tuning scaffold abundance comes with trade-offs in module performance: while changes in scaffold expression do not compromise signal specificity, it increases baseline leakage when no stimulus is present. These new insights into the

quantitative role of scaffolding in MAP kinase signaling suggest advantages and limitations in designing synthetic scaffold-based regulatory and metabolic circuits.

4. References

Asthagiri, A.R., and D.A. Lauffenburger. 2000. Bioengineering models of cell signaling. *Annu Rev Biomed Eng*. 2:31-53.

Asthagiri, A.R., and D.A. Lauffenburger. 2001. A computational study of feedback effects on signal dynamics in a mitogen-activated protein kinase (MAPK) pathway model. *Biotechnol Prog*. 17:227-39.

Bardwell, L. 2006. Mechanisms of MAPK signalling specificity. *Biochem Soc Trans*. 34:837-41.

Bashor, C.J., N.C. Helman, S. Yan, and W.A. Lim. 2008. Using engineered scaffold interactions to reshape MAP kinase pathway signaling dynamics. *Science*. 319:1539-43.

Bhalla, U.S., and R. Iyengar. 1999. Emergent properties of networks of biological signaling pathways. *Science*. 283:381-7.

Bhalla, U.S., and R. Iyengar. 2001. Robustness of the bistable behavior of a biological signaling feedback loop. *Chaos*. 11:221-226.

Bhalla, U.S., P.T. Ram, and R. Iyengar. 2002. MAP kinase phosphatase as a locus of flexibility in a mitogen-activated protein kinase signaling network. *Science*. 297:1018-23.

Brondello, J.M., A. Brunet, J. Pouyssegur, and F.R. McKenzie. 1997. The dual specificity mitogen-activated protein kinase phosphatase-1 and -2 are induced by the p42/p44MAPK cascade. *J Biol Chem*. 272:1368-76.

Brown, G.C., J.B. Hoek, and B.N. Kholodenko. 1997. Why do protein kinase cascades have more than one level? *Trends Biochem Sci*. 22:288.

Dard, N., and M. Peter. 2006. Scaffold proteins in MAP kinase signaling: more than simple passive activating platforms. *Bioessays*. 28:146-56.

Ferrell, J.E., Jr. 1997. How responses get more switch-like as you move down a protein kinase cascade. *Trends Biochem Sci*. 22:288-9.

Ferrell, J.E., Jr. 1999. Building a cellular switch: more lessons from a good egg. *Bioessays*. 21:866-70.

Ferrell, J.E., Jr. 2000. What do scaffold proteins really do? *Sci STKE*. 2000:PE1.

Ferrell, J.E., Jr. 2002. Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. *Curr Opin Cell Biol*. 14:140-8.

Ferrell, J.E., Jr., and R.R. Bhatt. 1997. Mechanistic studies of the dual phosphorylation of mitogen-activated protein kinase. *J Biol Chem*. 272:19008-16.

Ferrell, J.E., Jr., and E.M. Machleder. 1998. The biochemical basis of an all-or-none cell fate switch in Xenopus oocytes. *Science*. 280:895-8.

Ferrell, J.E., and W. Xiong. 2001. Bistability in cell signaling: How to make continuous processes discontinuous, and reversible processes irreversible. *Chaos*. 11:227-236.

Goldbeter, A., and D.E. Koshland, Jr. 1981. An amplified sensitivity arising from covalent modification in biological systems. *Proc Natl Acad Sci U S A*. 78:6840-4.

Gustin, M.C., J. Albertyn, M. Alexander, and K. Davenport. 1998. MAP kinase pathways in the yeast Saccharomyces cerevisiae. *Microbiol Mol Biol Rev*. 62:1264-300.

Hanahan, D., and R.A. Weinberg. 2000. The hallmarks of cancer. *Cell*. 100:57-70.

Hartwell, L.H., J.J. Hopfield, S. Leibler, and A.W. Murray. 1999. From molecular to modular cell biology. *Nature*. 402:C47-52.

Huang, C.Y., and J.E. Ferrell, Jr. 1996. Ultrasensitivity in the mitogen-activated protein kinase cascade. *Proc Natl Acad Sci U S A*. 93:10078-83.

Keyse, S.M. 2000. Protein phosphatases and the regulation of mitogen-activated protein kinase signalling. *Curr Opin Cell Biol*. 12:186-92.

Kholodenko, B.N., J.B. Hoek, H.V. Westerhoff, and G.C. Brown. 1997. Quantification of information transfer via cellular signal transduction pathways. *FEBS Lett*. 414:430-4.

Levchenko, A., J. Bruck, and P.W. Sternberg. 2000. Scaffold proteins may biphasically affect the levels of mitogen-activated protein kinase signaling and reduce its threshold properties. *Proc Natl Acad Sci U S A*. 97:5818-23.

Lewis, T.S., P.S. Shapiro, and N.G. Ahn. 1998. Signal transduction through MAP kinase cascades. *Adv Cancer Res*. 74:49-139.

Marshall, C.J. 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell*. 80:179-85.

Matten, W.T., T.D. Copeland, N.G. Ahn, and G.F. Vande Woude. 1996. Positive feedback between MAP kinase and Mos during Xenopus oocyte maturation. *Dev Biol*. 179:485-92.

Park, S.H., A. Zarrinpar, and W.A. Lim. 2003. Rewiring MAP kinase pathways using alternative scaffold assembly mechanisms. *Science*. 299:1061-4.

Pearson, G., F. Robinson, T. Gibson, B.-E. Xu, M. Karandikar, K. Berman, and M. Cobb. 2001. Mitogen-activated protein (MAP) kinase pathways: Regulationand physiological functions. *Endocrine Reviews*. 22:153-183.

Sabbagh, W., Jr., L.J. Flatauer, A.J. Bardwell, and L. Bardwell. 2001. Specificity of MAP kinase signaling in yeast differentiation involves transient versus sustained MAPK activation. *Mol Cell*. 8:683-91.

Sible, J.C. 2003. Cell biology: thanks for the memory. *Nature*. 426:392-3.

Widmann, C., S. Gibson, M.B. Jarpe, and G.L. Johnson. 1999. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev*. 79:143-80.

Xiong, W., and J.E. Ferrell, Jr. 2003. A positive-feedback-based bistable 'memory module' that governs a cell fate decision. *Nature*. 426:460-5.

Yablonski, D., I. Marbach, and A. Levitzki. 1996. Dimerization of Ste5, a mitogen-activated protein kinase cascade scaffold protein, is required for signal transduction. *Proc Natl Acad Sci U S A*. 93:13864-9.

Yasuda, J., A.J. Whitmarsh, J. Cavanagh, M. Sharma, and R.J. Davis. 1999. The JIP group of mitogen-activated protein kinase scaffold proteins. *Mol Cell Biol*. 19:7245-54.