CHAPTER IV. FUTURE WORK

In this study, we elucidated how the expression level of network constituents—kinases, phosphatases, and scaffolds—affects the quantitative performance of the MAP kinase module. In Chapter II, we presented a model that focuses on the three stages of the cascade and established a simple metric—the resistance to activation—to quantitatively gauge module performance. In Chapter III, our experimental results of the effect of scaffold abundance on the mating MAPK response revealed that many module properties quantitatively depend on scaffold abundance, including pathway ultrasensitivity, maximum signal throughput, and baseline leakage. These findings provide new quantitative insights and lay a foundation for intriguing future directions that are outlined below.

1. Experimental sensitivity analysis of scaffold perturbation in the HOG pathway

Through the systematic variation of Ste5 expression, we demonstrated that various signaling properties of the mating MAP kinase pathway, such as pathway ultrasensitivity, maximum signal throughput, and baseline leakage, all varied biphasically with respect to scaffold abundance. However, the quantitative role of Ste5 in the mating pathway may not be representative of scaffolds in general. It would be intriguing to use the experimental platform that we have developed to quantify the role of other scaffolds in signal propagation.

In yeast, scaffold-mediated MAP kinase signaling plays a critical role in both the mating and the HOG pathways (Elion, 2001; Gustin et al., 1998; Hohmann, 2002). These two pathways control very different phenotypic responses: the mating pathway induces the cell to grow chemotropically in the direction of the pheromone gradient and ultimately facilitates cell fusion, while the HOG pathway monitors and responds to pressure changes in the cell's osmolar environment. Given that these MAP kinase pathways govern such diverse responses, these pathways offer a compelling context to compare and contrast the quantitative role of scaffolds in signal propagation. Such studies would begin to address how general the quantitative relationship between scaffold abundance and MAP kinase signal response may be.

This new study centered on the osmolarity pathway would involve systematically varying the HOG scaffold, Pbs2, using the same vector-based expression system that was described in Chapter III. Wild-type yeast cells have two signaling pathways that regulate the response to high osmolarity (Hohmann, 2002). To properly study the HOG MAP kinase pathway in isolation, an *ssk2Δ ssk22Δ* strain that ablates the non-MAP kinase signaling must first be obtained (Park et al., 2003). Then, the osmolarity pathway output can be assessed by quantifying the level of phospho-Hog1 via Western blot. However, this method is time consuming and low-throughput. Therefore, as a supplement to phospho-Hog1 detection, it may be effective to use a Hog1-reporter level readout such as pSTL1-RFP (McClean et al., 2007).

2. The effect of scaffold abundance on signal dynamics in the MAP kinase mating pathway

Scaffold abundance may not only affect steady-state throughput of signal activation, but may also be a key factor in determining its duration. Recently, Monte Carlo simulations of scaffold-based MAP kinase signaling predicted that scaffolds may influence signal dynamics by broadening the distribution of time scales for kinase activation (Locasale and Chakraborty, 2008). The model predicted that an optimal scaffold concentration would maximize signal duration.

The dependence of signal duration on scaffold abundance can be tested using our panel of yeast strains that express different levels of Ste5. The pheromone-induced response can be monitored by Western blot of the phospho-MAPKs. Once the most appropriate time points have been selected, the duration of the MAP kinase signal as a function of Ste5 abundance can be quantified. The physiological time scale for mating is approximately four hours and is consistent with the duration in phospho-MAPK signals that has been reported (Sabbagh et al., 2001). In our yeast strains that express Ste5 at wild-type levels, preliminary results indicate that the activation of the mating MAPKs, Fus3 and Kss1, persists for two hours with peak activation at 15 minutes (data not shown).

Recently, an experimental protocol was reported that uses the pFUS1-GFP transcriptional response to measure the signaling dynamics of the mating pathway (Bashor et al., 2008). While Western blot detection of phospho-MAP kinase signals is

straightforward, monitoring pathway output by FACS analysis of the pFUS1-GFP transcriptional response would permit more rapid, higher throughput quantitation of the MAP kinase pathway. Ultimately, this procedure would provide the easiest platform by which to generate high resolution time-course data of MAP kinase signal activation.

3. Extension of resistance metric to a scaffold-based MAP kinase cascade

The MAP kinase model presented in Chapter II defines a metric, the resistance to activation, which predicts salient features of module performance. The resistance captures the opposing contributions of pathway activators (kinases) and deactivators (phosphatases) that represent the players in this network. A meaningful extension of this model would be to incorporate the effect of scaffolding and to develop a new resistance metric that can predict module performance in the context of a scaffold.

To construct the model, we would generate a system of ODE's similar to our previous model. Restriction of the new model to 2-stages would reduce the number of additional scaffold species; a 2-stage cascade would introduce 9 new molecular species, while a 3-stage cascade would introduce 27. By non-dimensionalization of model variables, groups containing the total expression of the kinases, phosphatases, and scaffold can be scrutinized.

4. Investigation of MAP kinase design properties that result from scaffold dimerization

A prominent feature of scaffolds is its ability to bind concomitantly to multiple components of a signaling pathway, localizing them onto a single molecular backbone. The close proximity of scaffold-bound kinases facilitates signal transfer, although the precise mechanism of signal propagation is not well understood. Most representations of scaffolds tacitly assume a monomeric, *cis*-phosphorylation mechanism, whereby the signaling kinases bound to the same scaffold activate one another (Figure IV-1). However, an often-overlooked alternative mechanism involves dimerization. Scaffold dimerization has been demonstrated by ample experimental evidence for Ste5 and JIP (Inouye et al., 1997; Yablonski et al., 1996; Yasuda et al., 1999). In a genetics study, two mutants alleles of Ste5, one unable to bind Ste11 and the other unable to bind Ste7, display interallelic complementation (Inouye et al., 1997). While each mutant Ste5 allele alone was unable to promote mating, when co-expressed the mating phenotype was restored. Furthermore, in a pull-down assay of co-expressed myc-tagged Ste5 and GST-tagged Ste5, detection of GST-tagged Ste5 was possible after immunoprecipitation of the myc-tagged Ste5 (Yablonski et al., 1996). Thus, the differentially tagged versions of Ste5 can dimerize *in vivo*.

Dimerization may challenge basic assumptions about how scaffolds influence the quantitative properties of MAP kinase signaling networks. For example, scaffolds are thought to attenuate the ultrasensitivity of the MAP kinase response by promoting a processive rather than distributive mode of kinase activation (Levchenko et al., 2000).

However, our experimental evidence presented in Chapter III shows that shifting Ste5 to its optimal scaffold abundance *increases* the ultrasensitivity of the MAP kinase response. Interestingly, if signal activation were to require the dimerization of assembled scaffolds, then scaffolding would *generate* an ultrasensitive response (Ferrell, 2000).

An investigation of scaffold dimerization would provide an extension of the mechanistic insights that scaffolds confer to the quantitative performance of the MAP kinase cascade. To our knowledge, no scaffold model to date has reported on the effect of dimerization on pathway performance.

4.1 Robustness to perturbation in scaffold abundance

In Chapter III, we demonstrated that MAP kinase signal propagation depends biphasically on scaffold abundance. Though perturbation of scaffold abundance quantitatively altered MAP kinase signaling properties and phenotypic response, changes in signal throughput were surprisingly robust. Across a 50-fold change in Ste5 expression level the MAP kinase response varied by a maximum of 2.5-fold. This result was unexpected given that most models predict signal output to be "brittle" with respect to scaffold concentration (Burack and Shaw, 2000; Ferrell, 2000; Levchenko et al., 2000).

Scaffold models that assume a *cis*-phosphorylation mechanism do not intuitively predict robustness to variation in scaffold abundance (Burack and Shaw, 2000). Combinatorial inhibition dictates that as scaffold abundance increases, incomplete scaffold complexes will compete with one another for binding partners and inhibit

signaling. Scaffold dimerization raises the possibility that signal propagation may occur via *trans*-phosphorylation events between the kinases of two distinct scaffolds (Dard and Peter, 2006). Interestingly, a *trans*-phosphorylation scaffold model allows a mechanism by which incomplete scaffold-complexes can dimerize and permit signal propagation (Figure IV-1). A new modeling study directed at exploring the role of *trans*-phosphorylation in scaffold-mediated networks might address the discrepancy in our understanding of scaffolds.

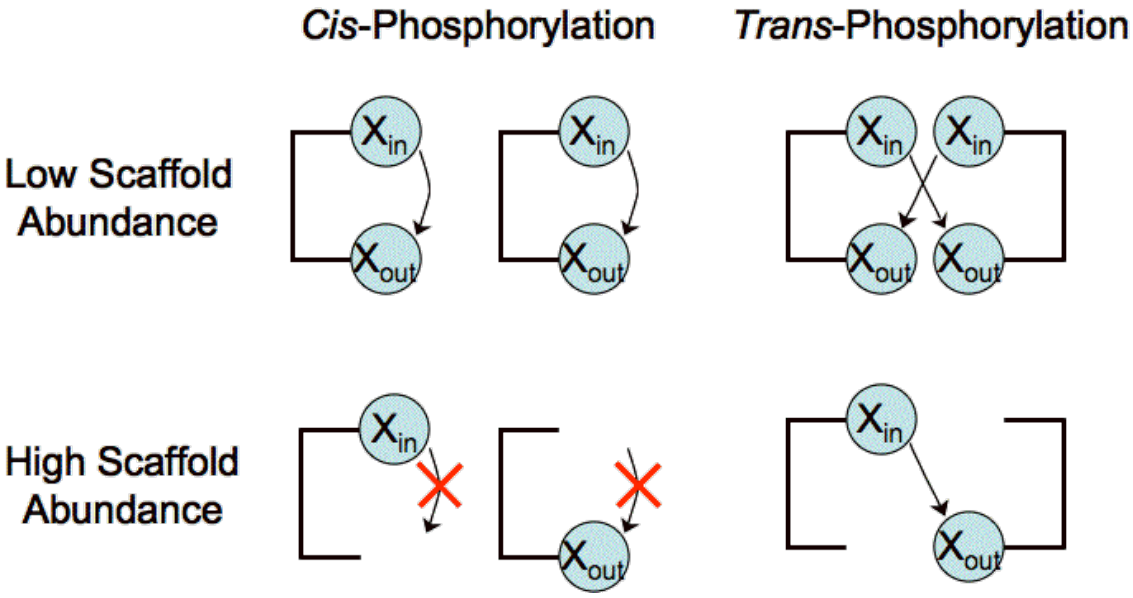

Figure IV-1. Scaffold dimerization permits signal activation for incompletely bound complexes via *trans*-phosphorylation.

When in low abundance relative to their binding partners, scaffolds are fully assembled. Signal activation can proceed for both the case of cis-acting monomer and trans-acting dimer complexes. However, when in high abundance relative to their binding partners, scaffolds are only partially assembled. While signal activation is inhibited via the *cis*-phosphorylation mechanism, the *trans*-phosphorylation mechanism provides a route for partially-assembled complexes to transmit signal.

The new model can use the framework of the Levchenko-Sternberg model, and simply add the trans-phosphorylation mechanism (Levchenko et al., 2000). Dimerization of a two-membered scaffold would introduce 27 dimeric scaffold species in addition to the nine monomeric scaffold species. The omission of off-scaffold mechanisms of signal activation would simplify model construction and analysis. To parse the effect of the *trans*-phosphorylation mechanism on the sensitivity to scaffold abundance, a reference model of *cis*-acting scaffolds (that can also dimerize) would be used for comparison.

4.2 Dimerization may augment the scaffold's contribution to signal fidelity

As discussed previously, scaffolds play an important role in signal specificity (see Chapter I-5). However, an overabundance of scaffold may compromise its ability to maintain signal fidelity. Using the yeast mating pathway as an example (Figure IV-2), an overabundance of Ste5 may promote leakage of the mating signal into the adjacent HOG pathway by activating too large a pool of Ste11, a signaling kinase shared by both pathways. Active and unbound Ste11 could then associate with Pbs2 and elicit a HOG response. Conversely, overabundant Ste5 may increase the sensitivity of the mating pathway to activation by leaky signals (Flatauer et al., 2005). Activation of the HOG pathway will lead to Ste11 activation, but an overabundant Ste5 may "soak up" any active and unbound Ste11, thereby activating the mating response.

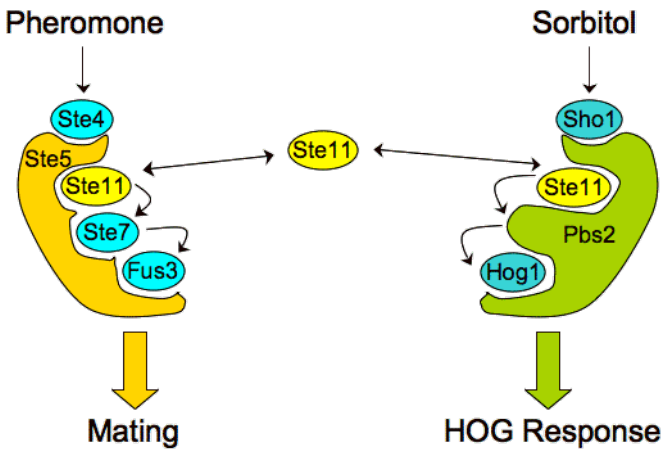

Figure IV-2. A shared signaling intermediate can facilitate signal leakage.

The mating and the HOG MAP kinase pathways in yeast have distinct inputs (pheromone and sorbitol, respectively) and distinct outputs (Fus3 and Hog1, respectively). However, they share a common signaling intermediate, Ste11. In each pathway, scaffolding helps maintain signal fidelity.

However, our experimental results show that neither of these scenarios is entirely accurate. In our sensitivity analysis of MAP kinase response to perturbation in Ste5 abundance, pheromone stimulation activated only the mating MAP kinases and did not stimulate phosphorylation of Hog1. Meanwhile, stimulation with sorbitol appropriately activated Hog1 with no cross-activation into the pheromone pathway. Thus, across nearly 50-fold change in Ste5 expression level, signal fidelity is maintained (See Chapter III.6.3).

Previous models have assumed that scaffolds may insulate kinases from deactivation by sterically hindering the access of phosphatases (Levchenko, 2000). In a similar fashion, if dissociation from the dimer-complex is precluded, dimerization may effectively sequester a shared signaling intermediate, thereby preventing leakage (Figure

IV-3). Thus, in addition to binding multiple signaling components, dimerization may be another mechanism by which scaffolds promote signal fidelity.

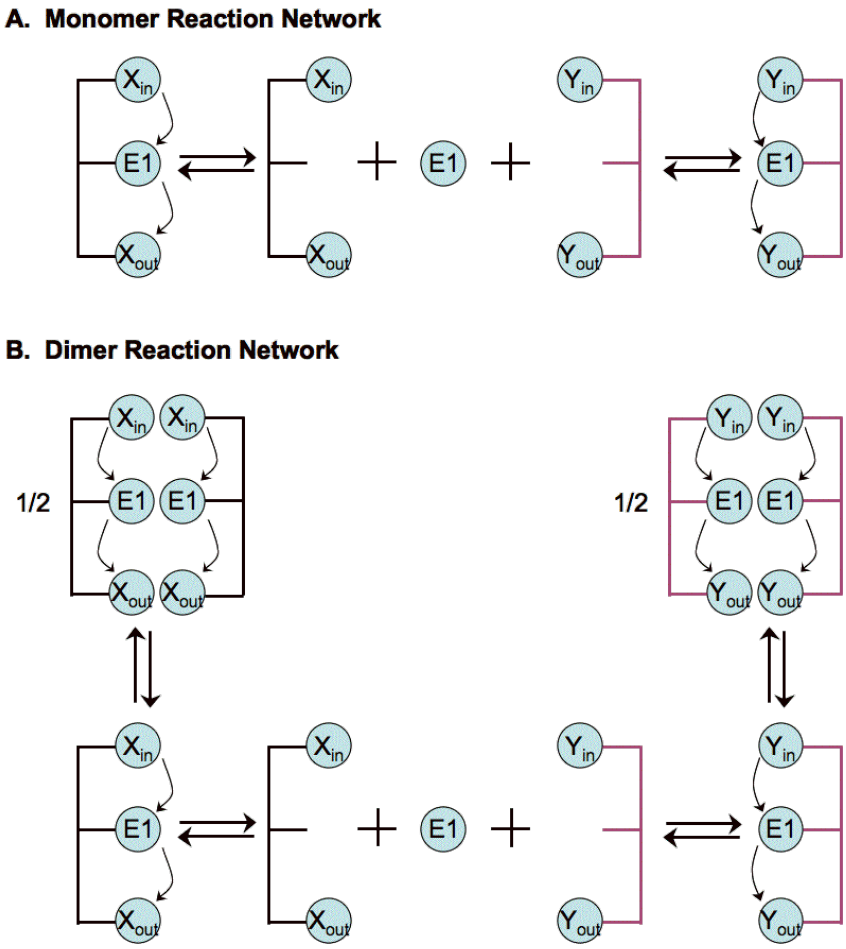

Figure IV-3. Model schematic of scaffold dimerization with signal crosstalk.

The mating and the HOG MAP kinase pathways in yeast have distinct inputs (pheromone and sorbitol, respectively) and distinct outputs (Fus3 and Hog1, respectively). However, they share a common signaling intermediate, Ste11. In each pathway, scaffolding helps maintain signal fidelity. **(A)** No Scaffold Dimerization. The E1 kinase can bind to either scaffold. When active E1 dissociates from one scaffold, it can re-associate with another, possibly of an adjacent signaling pathway, thereby facilitating signal leakage. **(B)** Scaffold Dimerization Protects E1 from Dissociation. When scaffolds dimerize, they form a complex that remains signaling competent. However, when dimerized, scaffold-bound components, including E1, cannot dissociate. Thus, scaffold dimerization may serve as a mechanism to sequester a common signaling intermediate and prevent undesirable signal crosstalk.

The effect of dimerization on signal fidelity can be investigated with an ODE model that represents the mass action kinetics of two scaffolds that homodimerize. To distinguish pathway inputs and outputs while permitting a shared signaling intermediate, a three-membered scaffold will be required. In order to monitor the effect of dimerization, a metric to assess the signal fidelity of the two distinct pathways will be required. The following definitions for signal fidelity may suffice (Komarova et al., 2005):

$$S_x = \frac{X_{out}/X_{in}}{Y_{out}/Y_{in}} \text{ and } S_y = \frac{Y_{out}/Y_{in}}{X_{out}/Y_{in}}$$

The variables X_{in} and Y_{in} represent pre-assigned inputs, while X_{out} and Y_{out} represent the terminal outputs for the two pathways. Note that X and Y are versatile representations of the signals, and can manifest as steady-state values or time-integral values of the signals (for dynamic responses).

5. References

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