Annu. Rev. Genet. 2004. 38:725–48
doi: 10.1146/annurev.genet.39.073003.112634

PRINCIPLES OF MAP KINASE SIGNALING SPECIFICITY IN *SACCHAROMYCES CEREVISIAE*

Monica A. Schwartz and Hiten D. Madhani
Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143–2200; email: mschwar@itsa.ucsf.edu, hiten@biochem.ucsf.edu

Key Words MAPK, signaling, specificity, yeast

■ **Abstract** Cells respond to a plethora of signals using a limited set of intracellular signal transduction components. Surprisingly, pathways that transduce distinct signals can share protein components, yet avoid erroneous cross-talk. A highly tractable model system in which to study this paradox is the yeast *Saccharomyces cerevisiae*, which harbors three mitogen-activated protein kinase (MAPK) signal transduction cascades that share multiple signaling components. In this review we first describe potential mechanisms by which specificity could be achieved by signaling pathways that share components. Second, we summarize key features and components of the yeast MAPK pathways that control the mating pheromone response, filamentous growth, and the response to high osmolarity. Finally, we review biochemical analyses in yeast of mutations that cause cross-talk between these three MAPK pathways and their implications for the mechanistic bases for signaling specificity. Although much remains to be learned, current data indicate that scaffolding and cross pathway inhibition play key roles in the maintenance of fidelity.

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INTRODUCTION[1]

A key question in cell and developmental biology is how cells perceive and respond properly to their environment. Cells must not only sense and distinguish between stimuli, but also transduce the signal accurately to activate the appropriate responses. Many of the core eukaryotic signal transduction pathways have been elucidated, some in great detail. Questions about how these pathways carry out their function of biological information processing can be posed now that many of the components and their protein-protein interactions are known. Paradoxically, signaling pathways often share components with each other, an arrangement that would seem at odds with the goal of accurate signaling transduction (Figure 1). Thus, a central question is how such pathways maintain specificity and avoid erroneous cross-talk so that distinct signals produce the appropriate changes in cellular physiology. Cells must have evolved mechanisms to maintain specificity in order to avoid unwanted responses to stimuli, while at the same time allowing the proper response to take place. Although several theoretical proposals have been put forth for how specificity is achieved, the actual strategies used by cells to maintain fidelity, in general, remain unclear. The goal of this review is to describe the potential and actual mechanisms by which pathways with shared components maintain specificity. We focus entirely on MAP kinase signaling pathways in the model eukaryote, *Saccharomyces cerevisiae*.

S. cerevisiae lends itself to the study of signaling specificity because of the powerful genetic and biochemical tools available in this organism. Several MAP kinase signaling pathways that share multiple components, yet transduce distinct signals, have been elucidated in substantial detail. Moreover, mutations that produce a loss of specificity have been identified, providing a foundation on which models of specificity mechanisms can be built. Because MAPK pathways are well conserved from yeast to humans, fundamental insights gained from the study of yeast should be applicable to MAP kinase signaling in more complex organisms.

For this review, the term cross-talk is used to refer to unwanted communication between signaling pathways that share components. We qualify this type of cross-talk as erroneous or unwanted to distinguish it from the numerous cases where distinct signaling pathways communicate with each other positively and negatively as a normal course of action to program the final cellular response. Of course, an

[1]Abbreviations: MAPK: mitogen-activated protein kinase, MEK: MAP/ERK kinase, MEKK: MEK kinase, PAK: p21 activated kinase, HOG: high osmolarity glycerol

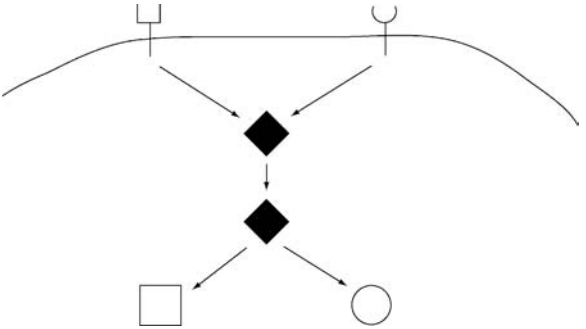

Figure 1 A depiction of the problem of signaling specificity—two separate pathways share several components yet produce distinct outcomes in response to distinct inputs. Shared components are indicated by filled diamonds. Pathway-specific components of one pathway are indicated by open circles. Pathway-specific components of a second pathway are indicated by open squares.

unwanted and erroneous event in one cellular context could be desired and accurate in another. However, for the three yeast MAPK pathways considered here, there is compelling evidence that an input of one pathway does not activate the output of another and that the pathways act as conduits for distinct types of information.

This review consists of three parts. First, to generate a basic conceptual framework for understanding signaling specificity, we discuss general mechanisms that could, in principle, explain specificity, and we briefly allude to examples from yeast or metazoans where possible. Next, we introduce the yeast mating, filamentation, and high osmolarity glycerol (HOG) MAP kinase pathways. The extensive number of shared components in these pathways highlights the crucial requirement for cells to have robust mechanisms that maintain specificity. We emphasize the key players known or thought to be required for preventing unwanted cross-talk between the pathways. Finally, we review current experimental evidence in support of various models for MAPK signaling specificity in yeast.

MODELS

This section describes theoretical mechanisms by which specificity could be achieved in cases where different pathways share components. We consider four broad categories of mechanisms that we term sequestration, combinatorial signaling, cross pathway inhibition, and kinetics.

Sequestration

One general class of mechanisms by which specificity could be maintained in cases where two pathways share components, would be to sequester pathway-specific components of one pathway away from components of another pathway.

By allowing only the components specific to one pathway to come into proximity with shared components, specificity could, in principle, be maintained. There are several different ways by which components of pathways could be physically segregated.

CELL TYPE A simple mechanism by which specificity could be ensured would be to restrict the expression of proteins that could potentially cause cross-talk to different cell types (Figure 2*A*). In this case, the shared components would be expressed broadly, but pathway-specific proteins whose activation would cause cross-talk would be expressed in a cell type–specific manner. Examples of such pathway-specific factors are specific effector transcription factors that are activated by a shared upstream signaling mechanism. This strategy can insulate two pathways from each other by physically sequestering pathway-specific components away from each other in different cells. One example (among many) of this straightforward mechanism comes from work on vulval signaling in *Caenorhabditis elegans*,

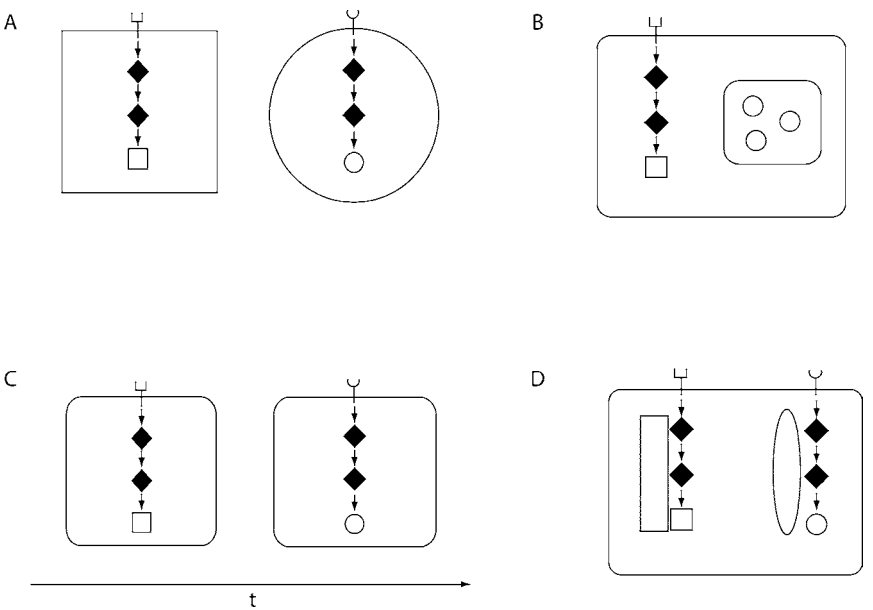

Figure 2 Potential mechanisms of sequestering shared components of two pathways from each other. (*A*) Cell type. In this model, pathway-specific factors are expressed in different cell types. (*B*) Subcellular compartmentalization. In this scenario, two pathways that share components are in distinct subcellular regions, preventing cross-talk. (*C*) Temporal separation. For this mechanism, expression of specific components at discrete times maintains specificity. (*D*) Scaffolding. Here, signaling occurs only in multiprotein complexes nucleated by a pathway-specific scaffolding factor. Symbol shapes are denoted as in Figure 1.

where two cell types express and utilize the same MAP kinase signaling components, yet express distinct MAPK-responsive transcription factors that program distinct developmental fates (71).

SUBCELLULAR COMPARTMENTALIZATION If two pathways that share components are present in the same cell, subcellular compartmentalization of pathway-specific components offers a potential mechanism for cells to avoid cross-talk (Figure 2*B*). Proteins sequestered in different cellular compartments (e.g., nucleus versus cytoplasm) would not come into direct contact with each other. As with having different cell types, keeping the two sets of specific components separate within the cell would allow shared components to activate only the appropriate response. One example is the restricted subcellular distribution of MAPK signaling components in neurons, which appears to account for the observation that Nerve Growth Factor (NGF) causes distinct responses when applied to distal axon terminals versus cell bodies (77).

TEMPORAL SEPARATION If the signals must be read in the same cellular compartment, an alternative mechanism that could promote specificity would be to restrict the expression of specific proteins temporally (Figure 2*C*). Differential expression during the cell cycle or during development could maintain specificity. One example of such temporal separation is the timed expression and activity of Cyclin-dependent kinase (Cdk) complexes in many eukaryotes (49).

SCAFFOLDING Signaling scaffolds can be defined as proteins that tether two or more signaling components of a pathway to each other. They are ubiquitous in eukaryotic signaling, and mammalian MAP kinase scaffolds have been recently reviewed (45). At least 18 scaffolds that bind one or more components of a MAPK pathway have been described. Scaffolds have been proposed to accelerate signaling reactions by binding multiple components of the same pathway. Tethering the correct components in close proximity with each other could allow the scaffolds to raise the local concentration of the bound signaling components, thereby promoting signaling reactions. We discuss evidence that supports this view of scaffold function below. Because of their ability to tether proteins, scaffolds have also been widely proposed to be involved in the maintenance of signaling specificity (Figure 2*D*) (27, 56). The conventional view is that by assembling only the correct pathway components into an active complex, a scaffold can insulate one pathway from another.

We emphasize here that this view of how scaffolds promote specificity is incomplete. In particular, current models do not account for the possibility that active signaling species could dissociate from scaffolds and thus erroneously activate components of other pathways. Thus, we propose that scaffolds may also prevent such events from happening. For example, a scaffold may have a higher affinity for an active species that could cause cross-talk than for the more innocuous inactive species. Another mechanism by which scaffolds would prevent the dissociation of

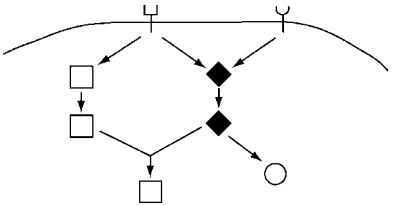

Figure 3 Combinatorial signaling. In this model, the combination of signaling pathways activated by an extracellular signal determines the ultimate cellular response. Symbol shapes are denoted as in Figure 1.

active signaling components would be to target them for inactivation or destruction specifically while bound to the scaffold. We discuss evidence for such scenarios in yeast MAPK signaling below.

Combinatorial Signaling

Some signaling mechanisms require two separate inputs to activate one distinct pathway in order to elicit a response (Figure 3). The two branches of the pathway are not redundant since in the absence of input from one of the branches, the output does not occur. Such behavior can be described in terms of Boolean logic as an AND gate. This type of combinatorial signaling has been implicated in mitigating the unwanted effects of spontaneous activity within a signaling pathway that is unrelated to the presence of the normal activating stimulus. To ensure that the signal is truly present and that the output is required, this type of "coincidence detector" circuit requires two separate pathways to be simultaneously activated in order to produce a response. One example of such a detector is provided by N-WASP, a protein that regulates the nucleation of actin polymerization by the Arp2/3 complex. The activity of N-WASP requires both Cdc42 and PIP_2, the two well-characterized upstream inputs, restricting actin polymerization to sites where both inputs are available (63, 68).

Cross Pathway Inhibition

Although insulation of pathways that share components from each other offers an elegant solution to the specificity problem, alternative mechanisms can be envisioned that do not require pathways to be fully separated from each other. For example, activation of one pathway could cause the inactivation or destruction of a specific component of the other pathway, interrupting signaling through that pathway (Figure 4). In this scenario, one input would activate all populations of shared components, but also bias activation of a pathway-specific component of one pathway by another mechanism (such as scaffolding). The ability of that component to inactivate a pathway-specific effector of the other pathway would generate only the

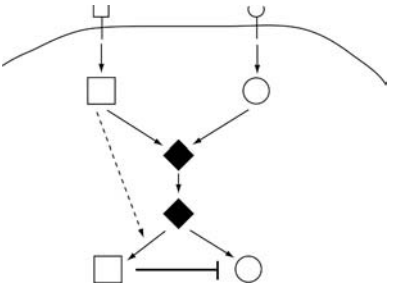

Figure 4 Cross pathway inhibition. In this model, the preferential activation of a pathway-specific component of one pathway causes the inactivation of a pathway-specific component of a second pathway. For this mechanism to promote specificity, the activation of a pathway-specific component downstream of shared components must be quantitatively biased in a way that reflects the input. Here, such a bias is indicated by the dotted line and could, for example, occur via the action of a pathway-specific scaffold. Cross pathway inhibition could then convert a modest quantitative bias into a strictly pathway-specific outcome. For simplicity, inhibition in only one direction is shown. Symbol shapes are denoted as in Figure 1.

output of the first pathway. We term such a mechanism cross pathway inhibition and distinguish it from the term negative cross-talk, which is generally used to refer to inhibitory interactions between pathways that do not share components. Such mechanisms presumably require a high degree of regulation since complete elimination of a competing pathway might be deleterious. For example, if basal, ligand-independent signaling is required or if the pathway must be rapidly induced in order to respond quickly to the appropriate signal, then its complete inactivation by cross pathway inhibition would be undesirable.

Signaling Kinetics

A popular class of models for the maintenance of specificity requires that shared components be activated with different kinetics, depending on the input. A cellular response can, in principle, be different depending on the duration or amplitude of the input signal or by two different inputs. One model for how this difference in signal amplitude or duration is decoded is through an unstable downstream factor that requires sustained signaling for stabilization. For example, accumulation of the c-Fos transcription factor requires both transcriptional induction by MAPK signaling and subsequent stabilization of the protein by direct phosphorylation by the MAPK. Thus, sustained signaling is required for the protein to accumulate (47, 48). Classic experiments in PC12 adrenal chromaffin cells demonstrated that the duration of signaling by receptor tyrosine kinases determines whether cells differentiate or proliferate (13, 74).

YEAST MAPK SIGNALING AS A MODEL SYSTEM

The *S. cerevisiae* genome encodes six MAP kinase orthologs (61). Three of these, Fus3, Kss1, and Hog1, function during the mating pheromone response, the switch to filamentous growth, and the response to high osmolarity, respectively. Remarkably, these distinct pathways share a series of components, yet cross-talk between these pathways is avoided (Figure 5). These pathways have been elucidated in considerable detail using a combination of genetics and biochemistry, setting the stage for an analysis of underlying mechanisms of specificity and other systems-level properties. As in mammalian cells, these pathways consist of a conserved module in which three kinases phosphorylate each other in sequence. The MAPK is phosphorylated by the MAPK/ERK kinase (MEK), which is itself phosphorylated by a MEK kinase (MEKK). Since MAPK pathways are evolutionarily conserved, insights from yeast should further the understanding of orthologous pathways in higher organisms.

Below we summarize each pathway briefly. In addition, for each pathway we provide more detailed background information on key players that are suspected or known to play a role in signaling specificity. However, we delay discussion of their mechanistic roles in preventing unwanted cross-talk until the penultimate section.

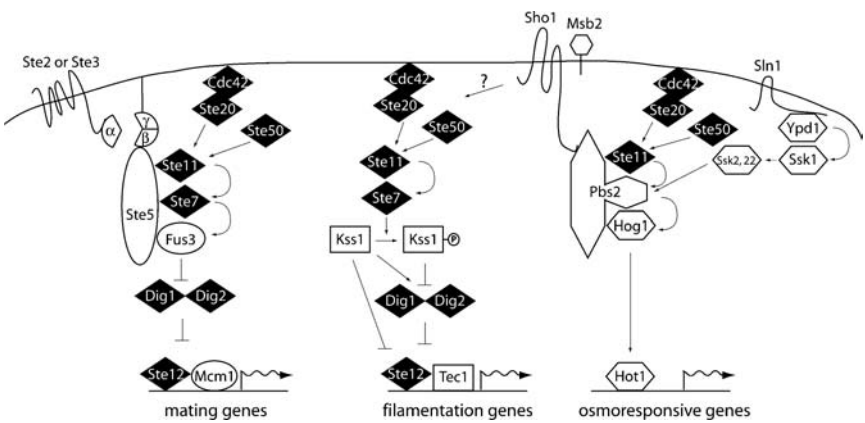

Figure 5 The *S. cerevisiae* mating, filamentation, and high osmolarity glycerol MAPK pathways. Mating pathway-specific components: open circles, only one of several possible transcription factor combinations depicted (these correspond to mating type **a**-specific genes); Filamentation pathway-specific components: open squares; HOG pathway-specific components: open hexagons, one of several known transcription factors depicted. Shared components: filled diamonds.

Mating Pheromone Response MAPK Pathway

Yeast can exist in haploid or diploid states. Haploid cells identify mating partners by responding to pheromone gradients generated by cells of the opposite sex or mating type (**a** or α). Both pheromones and the receptors are mating type-specific, ensuring that cells only respond to signals appropriate to their mating type. Cells form a mating projection (shmoo) toward the highest concentration of pheromone and arrest their cell cycle in G_1 in preparation for mating. Once mating partners are in contact, the intervening cell walls are dissolved, permitting fusion of the plasma membranes. Nuclear fusion ensues, resulting in the formation of a zygote, which resumes its cell cycle to grow as a stable **a**/α diploid cell.

The intracellular response to mating pheromone has been well-characterized (2, 17, 19) (Figure 5). Pheromone from the opposite mating type binds and activates a seven transmembrane G protein–coupled receptor (Ste2 or Ste3). Receptor activation causes a G$\beta\gamma$ complex (Ste4 and Ste18) to be released from inhibition by a Gα subunit (Gpa1), but they remain at the plasma membrane due to lipid modifications on the Gα (S-palmitoylation and N-myristoylation) and Gγ (farnesylation and S-palmitoylation) subunits. G$\beta\gamma$ interacts with a host of different proteins involved in mating and colocalizes relevant proteins to facilitate action of the pathway. In particular, it binds the scaffold protein Ste5, recruiting it to the plasma membrane where the bound kinase complex, consisting of the Ste11 MEKK, the Ste7 MEK, and the Fus3 MAPK, is activated (65). The Rho family protein Cdc42, a small GTPase localized to the plasma membrane, activates the p21-activated kinase (PAK) Ste20 upon GTP binding through an interaction with a CRIB motif in Ste20 (32, 34, 58). Ste20 also displays an interaction between its C-terminal tail and the G$\beta\gamma$ complex (35). Upon recruitment of the Ste5 complex to the plasma membrane by G$\beta\gamma$, Ste20 is thought to activate scaffold-bound Ste11 by direct phosphorylation (18). Activation likely occurs through relief of inhibition of an intramolecular interaction. Ste11 activation is assisted by the Ste50 protein, which binds to the N-terminal noncatalytic domain of Ste11 via interactions between SAM (Sterile Alpha Motif) oligomerization domains present in Ste50 and in Ste11 (66, 79). Active Ste11 is thought to phosphorylate MEK Ste7 on the Ste5 scaffold. Ste7 in turn activates the MAPK Fus3 by dual phosphorylation of the TEY motif present in its activation loop. Phosphorylated Fus3 activates mating gene transcription and promotes cell cycle arrest.

KEY PLAYERS:

Ste5 The scaffold Ste5 has no obvious homologues outside fungi, but at least 18 mammalian MAPK signaling scaffolds have been described, which themselves are unrelated to each other (45). Thus, although the primary structures of MAP kinase signaling scaffolds have not been conserved, evolution has apparently turned to scaffolding multiple times. Ste5 functions specifically in the mating pathway;

it is dispensable for filamentous growth and for the response to high osmolarity (20). Ste5 was among the first signaling scaffolds to be identified. A combination of genetic, two-hybrid, coimmunoprecipitation, and cosedimentation studies have demonstrated that Ste5 is a modular protein that can bind each of the kinases in the cascade (Ste11, Ste7, and Fus3), as well as Gβ through distinct binding domains (20). Although it is presumed that the scaffold binds all components simultaneously, this has yet to be demonstrated rigorously. A MAP kinase highly related to Fus3, Kss1, is required for mating when Fus3 is inactivated by mutation (12, 21, 38, 41). However, because Kss1 is the MAP kinase for the filamentous growth pathway (see below), we treat it as a pathway-specific component of that pathway (41). Two-hybrid and coimmunoprecipitation studies suggest that Ste5 can form dimers and/or higher-order oligomers (28, 29, 80). An intriguing study reported that in order to be competent for plasma membrane localization and binding to Ste11, Ste5 must first shuttle through the nucleus (42, 75). The current model is that shuttling enhances oligomerization, and only oligomers are competent for signaling, possibly by disrupting an intramolecular inhibitory interaction and allowing the Ste4 and Ste11 binding sites to become free. Although the details of this mechanism remain to be elucidated, it may provide for tight regulation of signaling.

Ste5 is thought to promote signaling efficiency by concentrating and perhaps even orienting and aligning the relevant components. Results of a recent study suggest that concentration of the binding partners may be sufficient for signaling (56). This study exploited previously identified point mutations that decrease the affinity of Ste5 for either Ste11 or Ste7. The defects associated with these mutations could be partially suppressed by appending artificial cognate binding domains to Ste5 and the kinase target. While signaling efficiency is reduced, these artificial interactions supported signal transduction, suggesting that the exact orientation of the kinases on the scaffold may not be essential for signaling. However, since the kinase binding domains of Ste5 were not deleted, the suppression of the missense mutations by artificial recruitment might not reflect a fundamentally different geometry of binding to the scaffold.

Fus3 Fus3 is a homolog of the mammalian ERK-type MAPKs, which are activated in response to diverse stimuli, including mitogens. Fus3 plays a central role in mating because it controls not only pheromone-dependent gene expression, but also promotes cell cycle arrest and mating projection formation (2, 17, 19). Fus3 presumably has many direct phosphorylation targets; however, few have been characterized. A key function of Fus3 is to activate the transcription factor Ste12, which is required for pheromone-dependent gene induction. Fus3 apparently activates Ste12 indirectly, by inactivating (likely by direct phosphorylation) two redundant inhibitors of Ste12, Dig1/ Rst1 and Dig2/ Rst2 (11, 72). A second key function of Fus3 is to promote cell cycle arrest by phosphorylating the Cdk inhibitor Far1 (9, 57). Fus3 also phosphorylates Ste7, but the significance of this feedback modification is not understood (3, 8, 83).

Fus3 is required for specificity between the mating and filamentation pathways. In *fus3*Δ cells or in cells harboring a kinase-dead allele of *FUS3*, the pheromone response pathway can activate the filamentation MAPK pathway (10, 24, 41, 70).

Filamentous Growth MAPK Pathway

In response to nitrogen starvation and other signals, diploid **a**/α yeast cells undergo a developmental change and switch to a filamentous form of growth called pseudohyphal development (25, 40, 54). This transition includes cell elongation, a switch to a unipolar budding pattern, maintenance of attachment between mother and daughter cells, and the consequent ability to invade semisolid media. This morphological change is likely a foraging response that allows cells to scavenge for nutrients. Filamentous growth, although ubiquitous in wild yeast, has been lost from most recombinant inbred laboratory strains. Consequently, studies of filamentation have been largely restricted to filamentation-competent strains such as Σ1278b. In the most common human fungal pathogen, *Candida albicans*, mutants defective in the ability to switch between yeast and filamentous forms of growth are invariably deficient in virulence in animal models (23). In *S. cerevisiae*, haploid mating-type **a** or α cells can also switch to a filamentous growth form. Glucose starvation (14) and some alcohols such as isoamyl alcohol (a product of normal yeast metabolism) (37) promote this switch, which is termed haploid invasive growth. A close kinship between haploid invasive growth and diploid pseudohyphal growth is suggested by the observation that both are controlled by a common set of conserved signaling pathways.

The MAP kinase signaling cascade required for filamentous growth shares many components with the mating pheromone response pathway and is discussed further below. A pathway that uses cAMP as a second messenger appears to sense environmental nutrient levels and also controls filamentous growth (54). Under conditions of limited nitrogen, but a sufficient carbon source, a G protein–coupled receptor, Gpr1, appears to signal to a Gα subunit homolog (Gpa2), which in turn regulates cAMP level. The target of cAMP is Bcy1, the regulatory subunit of the cAMP-dependent kinases (PKAs). The three yeast PKA orthologs, Tpk1, 2 and 3, each play a distinct role in filamentous growth (55, 67). Both the MAPK and PKA pathways control the expression of *FLO11*, a cell surface adhesion molecule required for cell-cell attachment during filamentous growth. The *FLO11* upstream region is complex and appears to integrate information from multiple signaling pathways (69). These pathways are likely to have numerous other targets that mediate this critical developmental switch.

Cdc42 and Ste20 are components of the MAPK pathway that controls filamentous growth pathway (Figure 5). As in the pheromone response pathway, these proteins act upstream of a cascade involving the Ste11 MEKK and the Ste7 MEK. The MAP kinase for the filamentous growth pathway is Kss1 (12, 41). Dig1 and Dig2 act as inhibitors in this pathway as well, but they do not appear to be completely redundant as they are in the pheromone response pathway (4, 11, 72). Dig1

appears to play the dominant role during filamentous growth (7). Remarkably, even the transcription factor for the pheromone response pathway, Ste12, is required for filamentous growth (36). However, at filamentation promoters, Ste12 acts with the filamentation-specific factor Tec1 (6, 39, 46). These two transcription factors bind cooperatively to promoter elements, termed filamentation response elements (FREs), that have been defined in the promoters of *TEC1* and the *Ty1* retrotransposon (6, 39).

Little is known about the precise signals that activate the filamentation MAPK pathway. Diploid pseudohyphal growth is caused by nitrogen starvation in the presence of high concentrations of glucose, but it seems clear that pathways other than the MAPK pathway transduce these signals. Similarly, glucose starvation and alcohols stimulate haploid invasive growth, but it is again unknown whether they signal through the MAPK pathway. The identity of the receptor(s) for the filamentation MAPK pathway has remained elusive. Puzzlingly, Sho1, a transmembrane osmosensor that feeds into a branch of the Hog1 MAPK pathway, is required for pseudohyphal growth (52). Furthermore, overexpression of Msb2, a second putative osmosensor in the Sho1 branch of the HOG pathway, activates phosphorylation of the Kss1 MAPK and promotes haploid invasive growth (13a). It may be that Sho1 and Msb2 associate with other membrane proteins to form a receptor specific for the filamentation pathway. An association recently described between Sho1 and Fus1, a transmembrane protein involved in mating, suggests that Sho1 could act in multiple pathways via combinatorial associations (51).

KEY PLAYERS:

Kss1 Kss1, the MAPK for the filamentous growth pathway, is an ERK-type MAPK highly homologous to Fus3. Its identity as the MAPK for this pathway was difficult to elucidate because a null mutant in *KSS1* still undergoes pseudohyphal development and haploid invasive growth (41). The resolution of this apparent paradox is that Kss1can both positively and negatively regulate the filamentation transcription factor Tec1-Ste12 (12, 41). In the absence of signaling, Kss1 inhibits Tec1-Ste12 and filamentous growth in a kinase-independent manner. Once phosphorylated and activated by Ste7, Kss1 switches into an activator of filamentation gene expression (12, 41). The precise mechanisms by which Kss1 inhibits and activates filamentous growth are not understood. Current evidence suggests that unphosphorylated Kss1 acts as an inhibitor by direct binding to Ste12 as well as by promoting the inhibitory functions of Dig1 and Dig2, which also bind directly to Ste12 (4, 5). The active form of Kss1 may function by inactivating the Dig1 and Dig2 inhibitors by phosphorylation and/or by phosphorylation of Tec1-Ste12 (4, 5).

As mentioned above, Kss1 was originally assigned to the pheromone response pathway because it is required for mating in cells lacking Fus3 (70). One way of reconciling this observation with the role of Kss1 as the MAPK for the filamentous growth pathway is to propose that Kss1 functions only in mating when Fus3 is

inactive (41). However, mating pheromone treatment of cells causes phosphorylation and activation of a population of Kss1 (70). This population appears to be small because a much larger amount of phosphorylation occurs in cells lacking Fus3. Nevertheless, this phosphorylation does not lead to transcriptional activation of a filamentation reporter (*FRE-LacZ*), suggesting that the consequences of the small amount of Kss1 activation in response to mating pheromone seen in wild-type cells is suppressed before it can lead to the activation of the filamentation transcriptional program.

Tec1 Tec1 was originally identified as a transcription factor required for the expression of the yeast *copia*-like retrotransposon, Ty1 (30, 31). Subsequent work showed that is was important for pseudohyphal development and haploid invasive growth (26, 46). In contrast to Ste12, Tec1 is dispensable for the pheromone response. Analysis of FRE-dependent reporters and genetic experiments demonstrate that Tec1 acts downstream of the filamentation MAPK pathway (39). It harbors a TEA/ATTS DNA binding domain that recognizes the same target sequence, CATTCY, as its homologs in *Aspergillus nidulans* (AbaA) and humans (TEF-1) (26, 39). In vitro, purified recombinant full-length derivatives of Tec1 and Ste12 bind cooperatively to FRE elements derived from the *TEC1* and Ty1 promoters (39).

Genome-wide chromatin immunoprecipitation experiments have defined the direct targets of Ste12 and Tec1 under conditions that promote mating (pheromone treatment) or filamentous growth (butanol treatment) (82). These studies demonstrate that Ste12 redistributes across the genome in a manner that depends on the environmental conditions. Ste12 tends to be bound to mating promoters under conditions of pheromone treatment, but shifts to a distinct set of genes, including many known to be involved in filamentous growth, upon treatment of cells with butanol (82). This redistribution to filamentation promoters requires Tec1, suggesting that earlier studies using isolated FREs as a model for the cooperative role of Tec1 with Ste12 apply to a significant set of endogenous genes.

HOG MAPK Pathway

S. cerevisiae adapts to high external osmolarity by inducing the synthesis of solutes to compensate for osmotic pressure. These internal osmolytes include glycerol and trehalose. A MAPK pathway, the HOG (high osmolarity glycerol) pathway, transduces the high osmolarity signal and activates the appropriate response (15, 53) (Figure 5).

This pathway has two redundant input branches that activate a common target, the MEK Pbs2 (15, 53). Active Pbs2 phosphorylates the MAPK Hog1 on two sites in a conserved TGY motif present in its activation loop. One of the two input branches is activated by the putative transmembrane osmosensors Sho1 and Msb2. A proline-rich motif present in the N-terminal noncatalytic domain of Pbs2 allows its association with the SH3 domain in the intracellular tail of Sho1. This

interaction is required for activation of Pbs2 by Ste11, the MEKK. Pbs2 also acts as the scaffold for the Sho1 branch of the HOG pathway due to its ability to interact with multiple components of this arm of the circuit (59). Pbs2 thus has two distinct functions: (*a*) the MEK for the HOG pathway and (*b*) the scaffold for a branch of the pathway that shares components with the mating and filamentation pathways. The Cdc42-Ste20 complex and Ste50, which are required for signaling by the mating and filamentation pathways, also activate Ste11 in this branch of the HOG pathway (52).

The other input branch to the HOG pathway is a prokaryotic-like "two-component" system (15, 53, 60, 62). Sln1, transmembrane histidine kinase, is the osmosensor. The intracellular histidine kinase domain transfers a phosphate to Ypd1, which in turn transfers the phosphate to Ssk1. Ypd1 and Ssk1 display homology to bacterial response regulator proteins that, in a similar manner, are activated upon receipt of a phosphate from upstream histidine kinases. Sln1 is active in the absence of osmostress, and the ensuing phosphorylation of Ssk1 inhibits its interaction with Ssk2 and Ssk22, the redundant MEKKs of this branch. Upon osmostress, Sln1 histidine kinase activity is inhibited allowing dephosphorylation of Ypd1 and Ssk1. Unphosphorylated Ssk1 can interact with the MEKKs, leading to their activation through autophosphorylation. Active Ssk2 and Ssk22 phosphorylate Pbs2, which in turn phosphorylates Hog1. Activation of the MAPK Hog1 results in its translocation to the nucleus and the activation of osmoresponsive genes.

KEY PLAYER:

Hog1 Hog1 is the yeast homolog of the mammalian p38 MAP kinase. p38 is a member of the stress activated protein kinase (SAPK) family and plays a key role in inflammatory and stress responses. In yeast, Hog1 is activated transiently, and prolonged activation has been shown to be lethal. One method of downregulation is through the action of phosphatases on Hog1. There are two families of phosphatases known to act on Hog1, the type 2C serine/threonine protein phosphatase Ptc1 and the protein tyrosine phosphatases Ptp2 and Ptp3 (44, 76). Remarkably, once in the nucleus, Hog1 has been shown to directly interact with DNA-bound transcription factors to activate gene expression (1, 16, 64). The data gathered to date have revealed intricate regulation of transcriptional activity by the Hog1 MAPK. For example, Hog1 recruits Rpd3-Sin3, a histone deacetylase complex required for osmotic induction of target genes (16). In addition to promoting the synthesis of intracellular osmolytes, Hog1 has also been implicated in suppressing protein synthesis after osmotic shock. Downregulation of protein synthesis requires phosphorylation by Hog1 of the downstream MAPK-activated kinase Rck1 (73).

Like Fus3, Hog1 is essential for the maintenance of signaling specificity. Loss of Hog1 kinase activity, by deletion of *HOG1*, *PBS2*, or expression of a kinase-deficient allele of *HOG1*, causes activation of the mating and filamentation pathways upon stimulation of the HOG pathway (52).

FACTORS REQUIRED FOR SPECIFICITY IN YEAST MAPK SIGNALING

As described in the Models section, numerous mechanisms could, in theory, prevent cross-talk between pathways that share components. The critical question becomes which mechanisms are actually used by biological systems such as the yeast MAPK signaling pathways described in the previous section. The focus of the research into these pathways (and virtually all signaling pathways) has been on determining the role of proteins, their modifications, and their interactions in signaling efficiency. Activities required for specificity may not be required for signaling per se and have therefore been overlooked unless explicitly sought.

The mating and filamentation pathways have two tiers of shared components. The first level includes the shared PAK, MEKK, and MEK, which then activates a specific MAPK (Fus3 versus Kss1). The next downstream components in the hierarchies are the inhibitors Dig1 and Dig2, and the transcription factor Ste12, which are shared between the mating and filamentation pathways. Consequently, there should be specificity mechanisms that act upstream of the MAPKs as well as those that act downstream. Depending on how "tight" these mechanisms are, it may be difficult to identify mutations that disrupt specificity upstream of the MAPKs because the downstream mechanisms may be sufficient to inhibit cross-talk. Since the HOG pathway only shares upstream components with the other pathways, there may be fewer specificity mechanisms at play.

A gold standard for a specificity factor is that a mutation in this factor allows signaling between pathways that do not normally interact with each other. We discuss below such occurrences as well as other data relevant to the issue of specificity.

Pathway-Specific MAP Kinases

Fus3 is required for specificity between the mating and filamentation pathways. In *fus3*Δ cells or in cells harboring a kinase-dead allele of *FUS3*, the pheromone response pathway can activate filamentation pathway-specific gene expression and produce hyperphosphorylation of the filamentation pathway MAPK Kss1 (41, 70). This phenotype can be observed even in the absence of pheromone treatment of haploid cells due to basal, ligand-independent signaling through the mating pathway. In *fus3*Δ cells, this basal signaling results in a hyperinvasive phenotype and an activation of FRE-dependent transcriptional reporters. These increases can be further enhanced by pheromone treatment. Deletion of *STE4*, which encodes the mating-specific Gβ subunit, abolished these effects of *fus3*Δ, demonstrating that this hyperactivation of the filamentous growth pathway depends upon the pheromone response pathway.

In cells lacking Fus3, the modest amount of pheromone-induced phosphorylation of Kss1 that occurs in wild-type cells is increased several-fold (70). This result suggests that Fus3 phosphorylates and activates a substrate that normally

prevents this hyperactivation of Kss1 in response to pheromone treatment. In addition, it seems likely that Fus3 has a second function in suppressing cross-talk, based on the arguments above that it seems necessary that there exist a mechanism that maintains specificity below the level of the MAPKs. Such a mechanism would also explain why a small amount of Kss1 is activated during the normal pheromone response without causing the activation of *FRE-lacZ* reporter genes. We discuss new evidence for this model below in the context of Tec1 below.

As mentioned above, Hog1 is essential for the maintenance of signaling specificity. Cross-talk in *hog1*Δ cells requires the Sho1 branch of the pathway, as cross-talk is suppressed in *hog1*Δ *sho1*Δ double mutants (52). One explanation for this result is that activation of Ste11, a shared component downstream of Sho1 in the HOG pathway, is necessary for the generation of cross-talk in a *hog1*Δ mutant. In cells lacking Hog1, activation of Ste11 in response to high osmolarity could result in erroneous participation of Ste11 in the mating and filamentation pathways. Hog1 may phosphorylate a substrate that prevents this from occurring during the normal osmoresponse.

Scaffolding

Based on the observations that scaffolds bind signaling proteins that act on each other and the expectation that increasing the local concentration of reactants should dramatically accelerate signaling reactions, it seem obvious that one function of scaffolds is to promote the efficiency of signaling. Indeed, mutational analysis of Ste5 supports this view (28). A straightforward corollary is that scaffolds would promote specificity by binding and enforcing interactions only between components of a given pathway. It does not seem coincidental that both Pbs2 and Ste5 act as scaffolds and that both bind components that are shared with other pathways.

Artificial fusions of shared components such as Ste11 to scaffolds (either Ste5 or Pbs2) can force the shared component to act in one pathway and not in another. For example, tethering an activated form of Ste11 to Ste5 restricts the activity of Ste11 to mating outputs and renders it incompetent to act in the filamentation or HOG pathways (27). This inability of the fusion to function in other pathways could be due to steric hindrance. For example, the scaffold may interact with the same face of the tethered component as proteins from the other pathway. Alternatively, specificity may stem from simply bringing into proximity one set of proteins but not the other, inappropriate set. Consistent with the latter model, an artificial hybrid scaffold consisting of a fusion of Ste5 and Pbs2 with point mutations in the Ste7 and Sho1 binding domains, respectively, can activate the HOG pathway in response to pheromone (56). This result also offers a scenario by which signaling pathways could evolve to generate novel connectivities.

As with other scaffold proteins, it has been widely proposed that Ste5 promotes signaling specificity and prevents cross-talk between pathways that also utilize the kinases involved in mating by sequestration (27, 56). While an appealing hypothesis, direct evidence for this view has been lacking. A hint comes from

experiments demonstrating that a gain-of-function mutation of Ste7 that permits its recognition by noncognate upstream kinases was most effective in functioning in a noncognate pathway in cells lacking Ste5 (81). This experiment suggests that Ste5 can sequester a mutant Ste7 from other pathways; however, the relevance of this experiment for wild-type Ste7 is unclear.

Although artificial fusion experiments have been interpreted to mean that scaffolds function entirely by enforcing the proximity of bound components, they do not offer a complete mechanism by which cross-talk is suppressed. As discussed above, current models for scaffold function do not explain how components bound to scaffolds do not dissociate to activate other pathways. This possibility cannot be assessed in artificial fusion experiments where components are permanently bound to a scaffold by genetic fusion. We have recently determined that the ability of the Ste5 scaffold to retain a bound activated kinase is, in fact, important for the maintenance of specificity (M.A. Schwartz & H.D. Madhani, manuscript in preparation). Through a genetic screen, we have identified a single amino acid, E756, in the Ste5 scaffold that is critical for suppressing cross-talk between the mating and filamentation pathways. This residue lies in the Ste7 binding domain of Ste5, and its mutation causes a shift from activation of Fus3 to Kss1 in response to mating pheromone. Biochemical analysis suggests that mutation of this residue results in the inappropriate dissociation of phosphorylated Ste7 from the scaffold, leading to pheromone-dependent hyperactivation of Kss1 and a severe defect in activation of Fus3. These data demonstrate a role for Ste5 in the maintenance of signaling specificity and elucidate a novel mechanism by which specificity is maintained. The ability of a naturally occurring scaffold to distinguish between inactive and active bound components (e.g., kinases or GTPases) offers a general mechanism by which scaffolds impart specificity to signal transduction pathways.

Cross Pathway Inhibition

As discussed above, it seems likely that a mechanism acts below the level of the MAPKs to promote specificity. In particular, it has been proposed that one way in which specificity might be attained would be for Fus3 to inhibit the activity of the Tec1-Ste12 transcription factor (82). We have recently determined that Tec1 is indeed inactivated by Fus3 in a manner dependent on pheromone signaling (M.Z. Bao, M.A. Schwartz & H.D. Madhani, manuscript in preparation). We have identified a consensus MAP kinase phosphorylation site in Tec1 that, when mutated, results in pheromone pathway-dependent activation of *FRE-lacZ* expression. In response to pheromone, Tec1 is rapidly degraded, and this degradation is dependent on Fus3 and the phosphorylation site. Finally, we found that the degradation event is mediated by an SCF ubiquitin ligase. These results explain why the small amount of Kss1 phosphorylation that occurs in wild-type cells in response to pheromone does not result in activation of filamentation gene expression. Moreover, they explain how specificity is maintained below the level of Fus3 and Kss1 in the face

of the downstream shared components Ste12, Dig1, and Dig2. This type of cross pathway inhibition required for signaling specificity might apply to mammalian cells where the target of the JNK MAPK, c-jun, pathway has been shown recently to be regulated by SCF-mediated destruction (50, 78).

One proposed mechanism for how the HOG MAPK pathway maintains specificity is that Hog1 activates protein phosphatases that specifically dephosphorylate Fus3 and Kss1. One candidate that could mediate such cross pathway inhibition is Msg5, a dual specificity phosphatase that can dephosphorylate those MAPKs (3).

Signaling Kinetics

It has been proposed that specificity is determined by the kinetics of MAPK signaling in yeast. This hypothesis is reminiscent of the kinetic model proposed to explain the differential effects of NGF and EGF on the cellular responses of PC12 cells (43). In one study, unstimulated cells were found to have no appreciable phosphorylated Fus3, whereas low levels of Kss1 phosphorylation were observed (70). After treatment with pheromone, both Fus3 and Kss1 were rapidly phosphorylated. After two hours, the phosphorylation of both MAPKs was reduced to preactivation levels. However, if *FUS3* was deleted, Kss1 phosphorylation was further increased and appeared to persist longer, signifying an increase in the magnitude and duration of the signal. Based on these observations, it was proposed that Kss1 is not specific to the filamentation pathway, but also plays a role in the mating pathway. In this model, transient activation of Kss1 by pheromone activates transcription of mating genes, whereas the sustained activation of Kss1 by filamentation signals activates filamentation genes (70). Although the correlation between the kinetics of Kss1 phosphorylation and cross-talk is notable, it is not clear whether there is a causal connection between the two events. Since the deletion of *FUS3* is likely to affect many processes, the change in Kss1 phosphorylation kinetics could reflect an activity of Fus3 that is unrelated to cross-talk.

A second model for the specificity of the HOG response is that the pathway is normally downregulated rapidly by Hog1-dependent feedback phosphorylation of Sho1 (52). This might result in only a small pool of Ste11 being activated, sufficient for Hog1 activation, but perhaps not enough to activate other Ste11-dependent pathways. In a *hog1*Δ mutant, Sho1 would no longer be inactivated and signaling would become persistent and result in activation of the mating and filamentation pathways. Of course, numerous other possibilities exist, and the mechanism by which the Hog1 MAPK promotes specificity awaits further investigation.

Cell Type

The mating pathway-specific components of the pheromone response pathway are haploid-specific genes (2, 17, 19). Therefore, in the diploid cell, the filamentous growth pathway cannot be activated by pheromone or by basal signaling through the pathway, since pheromone receptors, G protein, Fus3, and Ste5 are not expressed. Thus, even in the absence of other specificity-promoting mechanisms,

cell type prevents cross-talk between the mating and filamentation pathways in the **a**/α cell type.

EVOLUTION OF PATHWAYS THAT SHARE COMPONENTS

Why have *S. cerevisiae* and higher organisms evolved pathways that share components? Maintaining completely separate pathways would be simpler, since no additional mechanisms for specificity would need to evolve. The simplest hypothesis is that cross-talk between pathways may sometimes be beneficial or even necessary. That is, under certain conditions the activation of multiple pathways may be required in order for the cell to respond optimally to a signal. Such flexibility in the connectivity of signaling pathways may be highly advantageous for survival in a complex and varying environment.

One hypothesis for the preservation of the shared components between the mating and filamentation pathways is that at low levels of pheromone, it might be necessary to filament toward a mating partner in order to generate cell-cell contact. Yeast are immobile so it is not possible for a single cell to move toward a mating partner beyond the distance afforded by mating projection formation. In the wild, the two partners might also be separated by a substrate containing plant extracellular matrix. In this context, filamentous growth toward the source of the low level of pheromone may be critical for successful mating. Indeed, low levels of pheromone produce chains of cells characterized by the polar budding pattern of filamentous cells (22). That this phenomenon reflects a transient rewiring of MAP kinase signaling specificity is an appealing possibility.

PERSPECTIVE: SYSTEMS BIOLOGY OF SIGNALING

Understanding the system-level properties of signaling networks is a major challenge for the future. Mechanisms that restrict the flow of information during intracellular signal transduction are critical for accurate cellular and developmental responses. However, even in the highly tractable model system of yeast MAP kinase signaling described here, we are just beginning to understand the mechanistic underpinnings by which fidelity is maintained in situations where components are shared. The application of new methods for assaying properties of interest (specificity, noise, robustness, multistability), together with the powerful array of genetic reagents available in yeast, promises to yield many novel insights into emergent properties of the information-processing machineries of eukaryotic cells.

ACKNOWLEDGMENTS

We thank L. Bardwell, E. Elion, M. Peter, P. Pryciak, G. Sprague, and J. Thorner for sharing unpublished observations and for discussions, and we apologize to colleagues whose work could not be cited due to space limitations. We are grateful to

S. Johnson, G. Davis, M. Bao, T. Shock, and R. Tompa for their comments on the manuscript. Work on signaling specificity in our laboratory is supported by a grant from the NIH (R01-GM63670-01) to H.D.M.

The *Annual Review of Genetics* is online at http://genet.annualreviews.org

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