Spatial and temporal signal processing and decision making by MAPK pathways

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Mitogen-activated protein kinase (MAPK) pathways are conserved from yeast to man and regulate a variety of cellular processes, including proliferation and differentiation. Recent developments show how MAPK pathways perform exquisite spatial and temporal signal processing and underscores the importance of studying the dynamics of signaling pathways to understand their physiological response. The importance of dynamic mechanisms that process input signals into graded downstream responses has been demonstrated in the pheromone-induced and osmotic stress–induced MAPK pathways in yeast and in the mammalian extracellular signal-regulated kinase MAPK pathway. Particularly, recent studies in the yeast pheromone response have shown how positive feedback generates switches, negative feedback enables gradient detection, and coherent feedforward regulation underlies cellular memory. More generally, a new wave of quantitative single-cell studies has begun to elucidate how signaling dynamics determine cell physiology and represents a paradigm shift from descriptive to predictive biology.

Introduction

MAPK pathways sense aspects of the extracellular environment and transmit this information to regulate various cellular processes. Most proteins involved in the MAPK pathways were identified in the 1970s and 1980s and then were characterized and linked together in a signal transduction pathway in work that was largely complete more than a decade ago. Following this achievement, the field began working on understanding how dynamic extracellular pheromone signals are processed for cellular decision making. Progress has been driven by single-cell studies taking advantage of microfluidics technologies combined with fluorescence imaging and automated cell tracking and segmentation algorithms (Carpenter et al., 2006; Gordon et al., 2007; Taylor et al., 2009; Doncic et al., 2013). The mathematical nature of these new results, which allows prediction of cellular response to any arbitrary dynamic input signal or proposed inhibition of a pathway element, represents a paradigm shift from descriptive to predictive analysis in biology.

Although advances in our understanding of the dynamics of signaling have taken place in virtually all studied pathways, the pheromone response pathway in yeast is perhaps the best understood. This pathway processes extracellular pheromones to control transcription, arrest the cell cycle, and polarize growth toward the nearest mating partner. Although the MAPK signaling and the gradient tracking branches of the pheromone response pathway have mostly been examined separately, these two branches are intimately linked. In this context, new studies have demonstrated that the spatial and temporal dynamics are used for both cellular signaling and the tracking of pheromone concentration gradients.

Physiological function of the yeast pheromone-induced pathway

In each cell division cycle, haploid budding yeast decide whether or not to arrest the cell division cycle and try to find a mating partner (Chen and Thorner, 2007). The pheromone response is important because if yeast arrest but fail to mate, they lose time that could have been used to proliferate. Getting this decision right is clearly important because cells are able to track concentration gradients of only a few percent and the elimination of MAPK signaling increases the growth rate by ∼2% in rich media lacking pheromone (Segall, 1993; Moore et al., 2008; Lang et al., 2009).

To initiate signaling, a pheromone binds a G protein–coupled receptor, which leads to the G protein's α subunit (Gα) to be released from the β and γ subunits (Gβγ; Nomoto et al., 1990; Klein et al., 2000). Free Gβγ then activates the signaling branch responsible for regulating cell division and the cell polarity branch responsible for polarized growth (Fig. 1; Bardwell, 2004; Chen and Thorner, 2007; Johnson et al., 2011).

The signaling branch is responsible for cell cycle arrest and the main transcriptional response. In this branch, the Gβγ subunit binds to a Ste5–Ste11 complex and to the Ste20 kinase (Whiteway et al., 1995; Leeuw et al., 1998). Ste5 is the scaffold protein, whose recruitment to the membrane initiates a phosphorylation cascade that starts with the phosphorylation of Ste11 by Ste20 and culminates in the activation of Fus3, the main MAPK of this pathway (Chol et al., 1994; Wu et al., 1995; Pryciak and Huntress, 1998; van Drogen et al., 2000; Lamson et al., 2006). Kss1, the primary MAPK for the filamentous growth pathway, is also activated in response to pheromone, but plays only a minor role in the pheromone response of wild-type cells

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Abbreviations used: ERK, extracellular signal-regulated kinase; GAP, GTPase-activating protein; GDI, guanine dissociation inhibitor; HOG, high osmolarity glycerol; PAK, p21-activated kinase.

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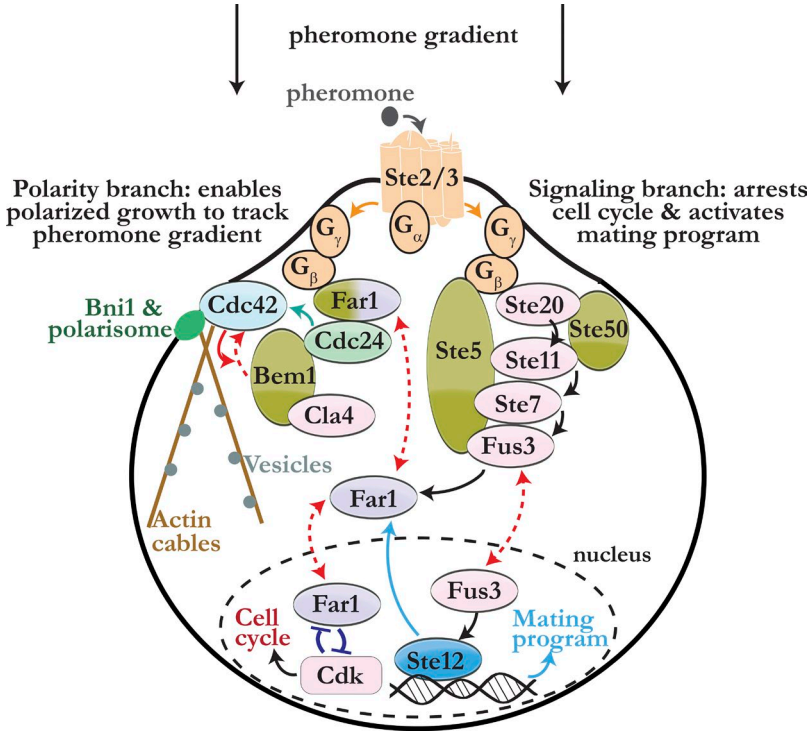

Figure 1. **Schematic illustrating the pheromone-dependent MAPK pathway in budding yeast.** The two branches of this pathway are responsible for generating cell polarity and tracking pheromone gradients and for arresting the cell division cycle and activating the expression of the mating program. The receptor is Ste2 or Ste3 for mating type a or α, respectively. Molecules and interactions of a similar type are color coded. Orange denotes receptors and G proteins, pink denotes kinases, and dark green denotes scaffold molecules. Black arrows denote phosphorylation, blue arrows denote synthesis, and red arrows denote translocation or recruitment. See text (section Physiological function of the yeast pheromone-induced pathway) for a more detailed description of protein interactions.

(Breitkreutz and Tyers, 2002; Schwartz and Madhani, 2006). After its activation, Fus3 translocates to the nucleus to activate the transcription factor Ste12 that targets ~200 genes (Errede and Ammerer, 1989; Roberts et al., 2000; Chou et al., 2006). Fus3 also phosphorylates and activates the cell cycle inhibitor Far1, which, in turn, associates with and inhibits G1 cyclin–Cdk1 complexes to arrest the cell cycle in G1 phase (Elion et al., 1993; Peter et al., 1993; Peter and Herskowitz, 1994).

The polarity branch of the pheromone response pathway controls polarization of cell growth to form a mating projection called a shmoo that tracks the pheromone gradient (Mackay and Manney, 1974; Butty et al., 1998). The shmoo tip typically contains a polarity patch of proteins driving polarized growth. This is initiated by Gβγ binding to a Far1–Cdc24 complex in the cytoplasm (Butty et al., 1998; Nern and Arkowitz, 1999; Shimada et al., 2000). Cdc24 is a guanine exchange factor that activates Cdc42, a small Ras-like GTPase. Cdc42 then recruits the scaffold protein Bem1 that is bound to Cdc24 and Cla4, an effector protein in the p21-activated kinase (PAK) family (Kozubowski et al., 2008). The polarity patch also recruits other factors, including Bni1, a formin that nucleates and tethers actin cables to the polarization site (Evangelista et al., 1997). Once these cables are tethered to the polarization site, the myosin motors on actin cables deliver secretory vesicles to the site to result in polarized growth (Qi and Elion, 2005).

Principles of signal transduction in the MAPK pathway

Spatial localization through scaffold proteins drives signaling. Spatial organization of pathway components is critical to the function of both branches of the pheromone response pathway. In particular, the Ste5 scaffold protein interacts with and brings together many components of the MAPK pathway to the shmoo tip to direct signaling (Pryciak and Huntress, 1998; Lamson et al., 2006; Moore et al., 2008). Accordingly, Ste5 abundance directly affects MAPK signal propagation (Chapman and Asthagiri, 2009; Thomson et al., 2011), and exogenously targeting Ste5 to the plasma membrane further stabilizes the protein and activates downstream signaling (Pryciak and Huntress, 1998; Garrenton et al., 2009). Consistent with the idea that the increased concentration of pathway components in the shmoo tip promotes signaling, the tethering of partner kinases to a scaffold increases signaling (Page and Jencks, 1971; Chapman and Asthagiri, 2009; Good et al., 2011).

In addition to recruiting MAPK components to a restricted space, scaffolds can also ensure insulation of different MAPK pathways that share components so that input to one MAPK pathway does not activate another. For instance, Ste7 functions both in the pheromone and filamentous growth pathways in yeast. Thus, it was unclear how Fus3 activation was prevented under conditions activating the filamentous growth pathway without the presence of pheromone. This question was resolved when it was discovered that whereas Fus3 is intrinsically a poor substrate for the upstream kinase Ste7, the binding of Ste7 and Fus3 to Ste5 increases the k_{cat} of Fus3 phosphorylation by Ste7 by ~5,000-fold (Good et al., 2009). Moreover, in cytosolic Ste5, two distinct domains of Ste5 interact with each other to block the ability of Ste7 to activate Fus3. Ste5 recruitment to the membrane by pheromone relieves this autoinhibition (Zalatan et al., 2012). Thus, the membrane recruitment of Ste5 functions to allow signaling only in the presence of pheromone.

MAPK pathway output reflects a graded response to extracellular pheromone input. One way to describe signal processing by a pathway is to measure its input–output relationship. This is typically done by measuring the steady-state downstream response at different input concentrations. Although such input–output relationships do not show how the pathway processes dynamic input signals, they do reveal whether or not the response is switch-like, where the output increases rapidly near a threshold input, or if the response is graded, so that it gradually increases over a wide range of input concentrations. Both graded and switch-like responses can be useful

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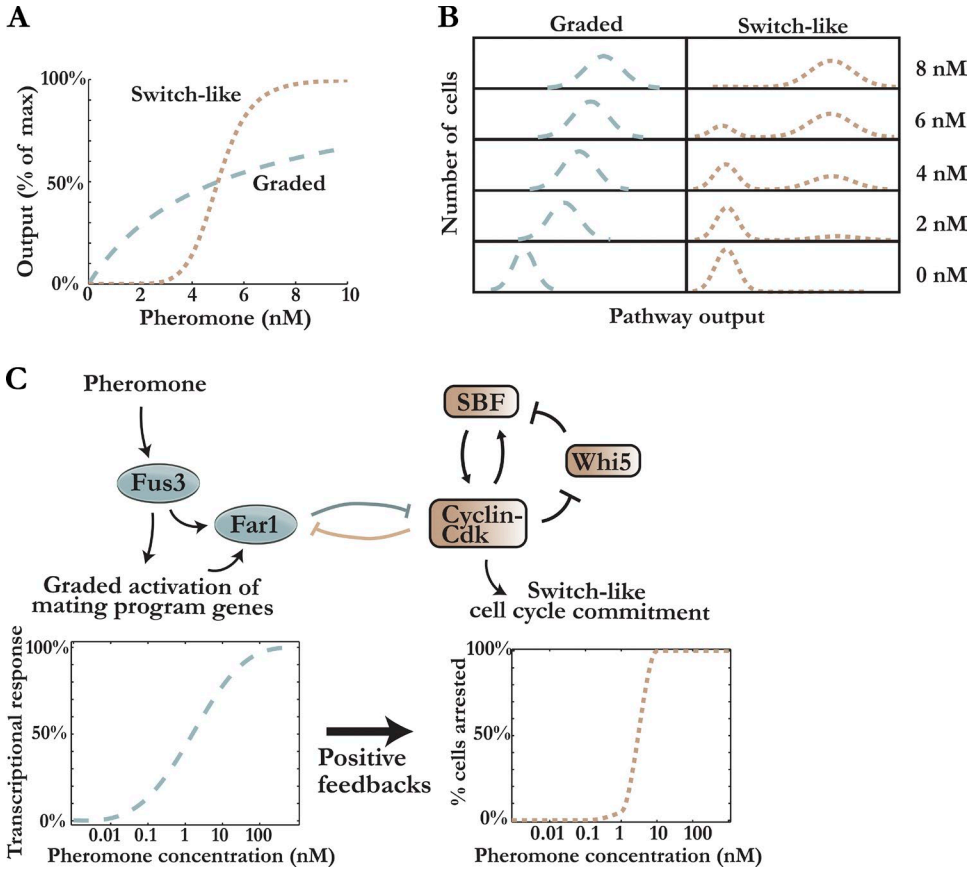

Figure 2. **Graded and switch-like features of the pheromone response.** (A) Schematic of a switch-like and graded response to pheromone. (B) Schematic of how noise results in a distribution of outputs for both graded and switch-like responses. Note the presence of bimodal distributions in the switch-like response. (C, top) Simplified network schematic of the interaction of the G1/S cell cycle control network and the pheromone-dependent MAPK pathway. (Bottom left) Illustration of the graded response of MAPK pathway outputs, such as the fraction of Fus3 that is active or the Ste12-dependent transcription rate. (Bottom right) Illustration of how the graded pheromone pathway output is converted to a switch-like cell cycle response by multiple positive feedback loops in the cell cycle network.

depending on the physiological context. Graded relationships can provide a variety of responses, whereas switch-like relationships produce binary, all-or-none responses (Fig. 2, A and B).

Graded responses are common in mammalian signaling pathways that require a proportional response in the entire range of input signals, such as those where a varied response functions to maintain homeostasis. Examples include the insulin, acetylcholine and thyroid-stimulating hormone, and angiotensin II pathways (Lin and Goodfriend, 1970; Cuatrecasas, 1971; Kasai and Changeux, 1971; Amir et al., 1973). In contrast, switch-like responses frequently characterize pathways responsible for the control of cell fate decisions, where the output is one of two cellular states. From cell cycle progression and *Xenopus laevis* oocyte maturation to apoptosis and Sonic Hedgehog signaling in fly development, switch-like signaling responses enable cells to either be in one or the other state and thereby to avoid mixed states with large fitness costs (Cross and McKinney, 1992; Xiong and Ferrell, 2003; Nair et al., 2004; Skotheim et al., 2008; Doncic et al., 2011; Balaskas et al., 2012).

In the case of the pheromone-induced MAPK pathway, arguments could be made for the physiological function of both switch-like and graded responses. On the one hand, arresting the cell cycle reflects a binary decision indicative of a switch-like system. On the other hand, certain morphological aspects of the pheromone response, such as cell polarization and gradient sensing, clearly indicate a graded response. Phenotypically, the evidence seemed to be in favor of switch-like pathway dynamics because the majority of cells switched from dividing to being arrested over a narrow range of pheromone concentration (Paliwal et al., 2007). It was later suggested that the competition between the kinase Fus3 and the phosphatase Ptc1 for phosphorylation sites on Ste5 was the mechanism behind this switch-like output (Malleshaiah et al., 2010). However, other work called into question the switch-like nature of the pheromone pathway. Both MAPK activity and transcription was remarkably graded in a 100-fold pheromone concentration range, consistent with earlier work (Poritz et al., 2001; Colman-Lerner et al., 2005; Takahashi and Pryciak, 2008; Conlon et al., 2016). But, if the pheromone pathway is graded, then why did the phenotypic response appear to be switch-like?

There are two answers as to why the phenotypic response to pheromone appeared to be switch-like. The first is that pheromone-induced phenotypes are often quantified by digitizing data so that each cell is classified into a single bin. Then, even though the underlying phenotype is gradually varying, a population of cells transition from one data bin to the next with a very small increase in pheromone. Second, the interaction between the pheromone pathway and the switch-like cell cycle G1/S network results in switch-like outputs for some phenotypes at the population level. As Far1 represses G1 cyclin–Cdk complexes

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that are required for G1/S progression, G1/S Cdk activity represses the pheromone pathway by targeting Far1 for degradation and by inhibiting Ste5 membrane localization, which effectively dismantles the pheromone pathway (Henchoz et al., 1997; Gartner et al., 1998; Lamson et al., 2006; Strickfaden et al., 2007). Importantly, this dismantling of the pheromone pathway only happens after a sharp increase in cyclin–Cdk activity that commits cells to divide. Because this commitment point is driven by multiple positive feedbacks in the G1/S network, the degradation of the nuclear Far1 and subsequent dismantling of the pheromone pathway is also switch-like (Fig. 2 C). Indeed, when the effect of the cell cycle on the pheromone pathway is removed, the pheromone response is clearly graded (Colman-Lerner et al., 2005; Yu et al., 2008). In addition, it is important to avoid difficult-to-control cell-density effects by conducting experiments in strains lacking the secreted pheromone protease Bar1. In summary, whereas the MAPK pathway has a graded input–output relationship over a large extracellular pheromone concentration range, its interactions with the switch-like positive feedback–driven G1/S cell cycle network can partially mask this graded response.

Cause and consequences of a graded pathway response. The graded response of the pheromone pathway over a large range of pheromone concentrations may have been selected for its ability to transmit more information about the extracellular environment that can be used to generate multiple cellular responses (Jackson and Hartwell, 1990; Segall, 1993). When a cell is surrounded by multiple mating partners, it strongly prefers to mate with partners that produce the highest pheromone concentration (Jackson and Hartwell, 1990), suggesting that the cell can distinguish different pheromone concentrations. Indeed, gene expression was shown to change over a 100-fold range of pheromone concentration (Poritz et al., 2001; Colman-Lerner et al., 2005; Yu et al., 2008). One possible reason for this graded response in the genes expressed is that gene products required for steps later in the mating program would only be activated at later times when cells sense higher pheromone concentrations. That is, different genes required for different physiological responses such as arrest and projection formation reach their half-maximum level of transcription at different pheromone concentrations (Moore, 1983; Lahav et al., 2007). Although it is not clear how this is accomplished in all cases, in one specific case, Ste12 first activates the expression of the transcription factor Kar4, and then, later, both Kar4 and Ste12 together activate the expression of Kar3 and Prm2, two proteins involved in nuclear fusion (Lahav et al., 2007). Because the Kar4-dependent subset of Ste12 targets requires a higher pheromone concentration to reach half-maximum expression, this coherent feedforward regulation coordinates the differential expression of Ste12 targets.

The ability to have a graded response to pheromones depends not only on the receptor occupancy but also on the dose–response alignment between the upstream receptor and downstream effectors. Indeed, a recent study suggests that alignment between the upstream signal and downstream effectors in the pheromone pathway allows the optimal transmission of information and that this alignment is achieved by a Fus3-mediated negative feedback on upstream components of the pathway (Yu et al., 2008). It is likely that multiple other negative feedbacks contribute to the graded response, including secretion of the pheromone protease Bar1, up-regulation of the Gα subunit GTPase activator Sst2, inhibitory phosphorylation of Ste5 by Fus3, and activation and increased transcription of the phosphatase Msg5 targeting Fus3 (Doi et al., 1994; Dohlman et al., 1996; Ballensiefen and Schmitt, 1997; Apanovitch et al., 1998; Barkai et al., 1998; Garrison et al., 1999; Bhattacharyya et al., 2006; Maeder et al., 2007; Jin et al., 2011; Bush and Colman-Lerner, 2013). Deletion of individual negative feedback elements modestly reduces the dynamic range of the graded response (Takahashi and Pryciak, 2008). This implies that multiple negative feedbacks act together to produce a graded response.

Cell-to-cell variability and molecular noise. Graded signaling responses are ideal for producing differential cellular responses to different extracellular input concentrations. However, the ability of cells to accurately distinguish different pheromone concentrations is in conflict with the frequently observed significant cell-to-cell variation. When genetically identical yeast cells are exposed to the same pheromone concentration, they exhibit significant cell-to-cell variation in gene expression, cell division, and morphology. The sources of such cell-to-cell variation are frequently decomposed into intrinsic and extrinsic noise, where intrinsic noise stems from random births and deaths of individual mRNA and protein molecules involved in the pathway. In contrast, extrinsic noise reflects variation in global gene expression machinery, such as RNA polymerases and ribosomes, or cellular state, such as the phase of the cell cycle (Elowitz et al., 2002; Swain et al., 2002; Kærn et al., 2005).

In the pheromone pathway, extrinsic noise is particularly dominant and has two major sources: mother–daughter cell type differences and the cell cycle positions at the time of pheromone addition. Yeast asymmetrically divide into a larger mother and a smaller daughter cell, which respond differently to pheromone. The larger mother cell typically requires higher pheromone concentrations to arrest the cell cycle. This is in part caused by the larger size of the mother, which is known to drive G1/S progression, and some proteins that are asymmetrically partitioned at division (Johnston et al., 1977; Colman-Lerner et al., 2001; Di Talia et al., 2007; Caudron and Barral, 2013). Asymmetric inheritance of the daughter cell–specific transcription factors Ace2 and Ash1 down-regulate the G1 cyclin *CLN3* to make cells easier to arrest (Laabs et al., 2003; Di Talia et al., 2009). In addition, cell cycle position is a large source of extrinsic noise because cells in G1 are much more responsive to pheromone. In fact, half of total cell-to-cell variation in the system output is caused by preexisting differences in cell cycle positions at the time of the pheromone addition (Colman-Lerner et al., 2005; Conlon et al., 2016). After controlling for these sources of extrinsic noise, there is still some intrinsic and extrinsic noise present in the system, but this residual variation is small enough so that it does not have a big impact on the graded response (Colman-Lerner et al., 2005).

Signal integration, cellular memory, and decision making. In response to intermediate pheromone concentrations, cells temporarily arrest in G1. Fluctuations in MAPK activity, caused by either fluctuations in the extracellular pheromone concentration or by intrinsic noise, could then lead cells to prematurely decide to divide. As the MAPK pathway response is both fast and graded, there is no obvious mechanism to prevent signaling fluctuations from dominating decision making. This raises the question of whether and how cells distinguish genuine mating opportunities from noise.

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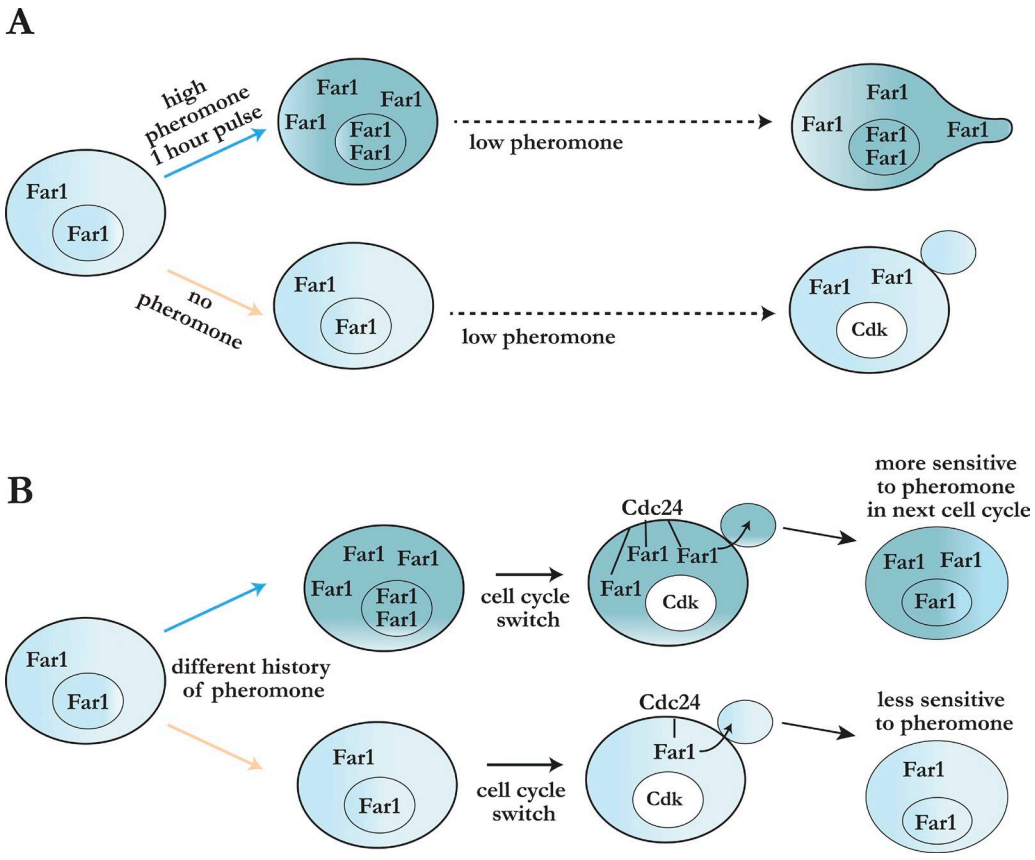

Figure 3. **Yeast use memory of past exposure to pheromone to decide to reenter the cell cycle.** (A) Yeast temporarily exposed to a high pheromone concentration (top) will remain arrested at a lower concentration than yeast only exposed to the lower concentration (bottom). Cells base the decision to reenter the cell cycle on Far1 levels, which reflect an integration of MAPK pathway activity. (B) Yeast are able to transmit memory of past pheromone exposure across generations despite the mutual inhibition of cell cycle kinases and Far1. This is because Cdc24 anchors some Far1 in the cytoplasm, which is not targeted for degradation by the predominantly nuclear cell cycle kinases. Release of the anchored Far1 in subsequent cell cycles promotes cell cycle arrest in daughter cells. Yeast may have pseudohyphae morphology during arrest under the conditions shown here. Far1 concentration is illustrated in proportion to the darkness of the blue color.

Cells are able to make accurate decisions regarding cell cycle reentry by integrating the MAPK signal over time. This occurs through the cell cycle inhibitor Far1, which inhibits cyclin–Cdk complexes. Far1 reflects an integration of the pheromone pathway activity because it is a target of the Ste12 transcription factor, whose activity, as previously discussed, is a graded function of pheromone concentration (Yu et al., 2008). Thus, total Far1 reflects an integral of the duration and the concentration of past pheromone exposure. Because cells are sensitive to Far1 levels, cells that have previously experienced higher pheromone concentrations have more Far1 and therefore stay arrested much longer at equivalent pheromone concentrations when compared with cells not previously exposed to pheromone (Fig. 3 A). In addition to this transcriptional control, Fus3, whose activity quickly changes to reflect extracellular pheromone concentration, directly activates Far1 (Yu et al., 2008). Thus, graded Fus3 activity determines the proportion of active Far1 (Doncic and Skotheim, 2013).

The feedforward regulation of Far1, which is activated by Fus3 both directly and through the Ste12 transcription factor, is the core information-processing unit of the pheromone pathway. The fast branch, direct activating phosphorylation of Far1, allows cells to rapidly respond to dramatic changes in pheromone when needed. For example, there is an initial bolus of Far1 at the end of every cell cycle regardless of pheromone concentration (McKinney and Cross, 1995; Oehlen et al., 1996). If cells are exposed to a rapid increase in pheromone, phosphoactivation of the initial Far1 is sufficient to arrest cells. Conversely, when pheromone is completely removed, Fus3 activity quickly drops to zero, Far1 is dephosphorylated, and cells rapidly reenter the cell cycle. Thus, arrest and reentry decisions are quickly made by phosphorylating and dephosphorylating an existing pool of Far1. In addition, the concentration of Far1 reflects the memory of past pheromone exposure (Doncic and Skotheim, 2013). When Far1 is mutated to have a shorter half-life, cells can no longer store extracellular pheromone information in Far1 concentration, and mating efficiency is reduced (Doncic et al., 2015).

Spatial organization of memory. The temporal integration of MAPK signaling damps out stochastic fluctuations to provide a robust memory of extracellular pheromone. Just as temporal dynamics are used to enhance signaling memory, so too is spatial organization. In particular, the nuclear and cytoplasmic pools of Far1 each enhance cellular memory through distinct molecular mechanisms. The mechanism of cellular memory of nuclear Far1 is discussed in the previous section and reflects an integration of the pheromone signal over the arrest duration. The cytoplasmic Far1, however, contributes to arrest dynamics as a result of a different molecular mechanism requiring spatial organization (Doncic et al., 2015). At the point of

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cell cycle reentry from pheromone arrest, downstream nuclear cyclin activity rapidly and irreversibly increases to a high level that quickly destroys the nuclear Far1 (Blondel et al., 2000; Atay et al., 2016). However, the cytoplasmic pool of Far1, whose exchange with the nuclear pool is slowed sufficiently because of an anchoring effect by Cdc24, remains relatively stable and is partitioned into newborn cells after cell division. In the daughter cell, this cytoplasmic pool of Far1 significantly affects the arrest duration in the next division cycle, presumably by slowly contributing to the nuclear Far1 pool (Doncic et al., 2015). When the Far1–Cdc24 interaction is disrupted, Far1 is no longer anchored in the cytoplasm, and daughter cells no longer inherit the memory of their mother's past pheromone exposure. Thus, the spatial organization of Far1 is essential for an intergenerational memory of past pheromone exposure (Fig. 3 B).

Typically, positive feedback–driven transitions lose information regarding the previous cellular state. However, in this case, cells are able to retain information from the previous state across a switch-like transition through the spatial organization of the signaling pathways described in the previous paragraph. This may be one of many examples where the exquisite spatial organization of the eukaryotic cell bestows new and unexpected signaling properties to previously characterized network motifs.

Polarization and gradient tracking by the pheromone response pathway

In general, cell geometry impacts signaling, and signaling controls cell geometry. For example, the primary cilium of metazoan cells is a hub of cellular signaling molecules that are both concentrated and spatially extended beyond the cell body (Goetz and Anderson, 2010). In turn, signaling controls cell geometry, including the formation of the primary cilium. Similarly, yeast polarization impacts the spatial localization and concentration of MAPK signaling molecules. In turn, these same signaling molecules help determine yeast polarity. For example, the spatial organization and regulation of the Far1–Cdc24 interaction in the pheromone pathway is not limited to providing cellular memory, but is also integral to gradient tracking (Nern and Arkowitz, 1999). This is because Far1 and its anchor Cdc24 are part of a polarity cluster required to sense and track pheromone gradients. How gradient sensing works has been the subject of recent single-cell quantitative studies, which have started to answer why only a single polarization site is established and how this site tracks pheromone gradients. The answers indicate that signaling and gradient tracking, two branches of the pheromone pathway that have long been separately studied, are intimately linked.

Symmetry breaking and polarity establishment. For yeast cells to track a pheromone gradient, they first need to form a single site of polarized growth. Even in the absence of directional cues, yeast cells choose a random direction to polarize. Because this polarity establishment requires symmetry to be broken, it suggests that there exist positive feedback mechanisms that concentrate the polarization complexes to a single site on the membrane (Drubin and Nelson, 1996). Although it is often thought that there need to be multiple positive feedbacks to reinforce a single site of polarization, a single positive feedback mechanism can be sufficient if certain considerations are met (Altschuler et al., 2008; Chau et al., 2012; Freisinger et al., 2013). Indeed, the membrane-bound GTPase Cdc42 forms clusters that recruit Cdc24, which, in turn, activates neighboring Cdc42. However, in this model, it is not Cdc42 that is limiting, but the scaffold protein Bem1 and other polarity factors that mediate the recruitment of Cdc24 to the polarity cluster (Kozubowski et al., 2008; Wu et al., 2015). Active Cdc42 in the polarity cluster recruits Bem1 from the cytoplasm, and because both Cdc24 and the PAK Cla4 bind to the same molecule of Bem1, they are also recruited to the cluster. More Bem1 leads to the recruitment of more Cdc24–Cla4 complexes, leading to the further activation of neighboring Cdc42 in a positive feedback loop (Kozubowski et al., 2008). As long as multiple loci have to compete for a polarity factor such as Bem1, and membrane-bound active Cdc42 diffuses significantly slower than cytoplasmic Cdc42 as experimentally measured, a single polarity site is likely to form (Marco et al., 2007; Goryachev and Pokhilko, 2008; Kozubowski et al., 2008; Bendezú et al., 2015; Wu et al., 2015). Consistent with this model, engineered fusions of Cdc24 and Cla4 bypass the requirement for Bem1, and when wild-type Bem1 is overexpressed, multiple transient polarity loci can be observed in a fraction of the cells (Kozubowski et al., 2008; Howell et al., 2009). Indeed, when Bem1 is exogenously tethered to the membrane in cells that lack the bud site selection protein Rsr1, a fraction of mother cells form two buds (Howell et al., 2009). Moreover, slowing down the exchange of Bem1 and Cdc24 between the membrane and cytoplasm also slows down the competition between polarity clusters, indicating that competition for the pool of polarity factors is important for single-cluster formation (Fig. 4 A; Wu et al., 2015).

Although spatial positive feedback may be sufficient for the formation of a single polarity cluster, and has been shown to be required for such polarity formation, this is not the only such feedback mechanism. It is currently debated what function additional feedbacks and other accessory proteins have for the establishment and maintenance of cell polarity. For instance, it was recently shown that the GTPase-activating protein (GAP) Bem2 and the guanine dissociation inhibitor (GDI) Rdi1 are important for actin-independent polarity establishment and that they act in combination to slow down the diffusion of Cdc42 away from the polarity patch (Slaughter et al., 2009; Smith et al., 2013; Woods et al., 2016). Interestingly, when multiple Cdc42 loci compete with each other, foci intensity oscillates, which suggests the presence of a negative feedback (Howell et al., 2012). Although the presence of negative feedback seems counterintuitive for the establishment of polarity, it is possible for negative feedback to impart robustness to the system by allowing it to be insensitive to the concentrations of Cdc42 and Bem1 over a large range (Howell et al., 2012). It was suggested that this negative feedback mechanism might be the inhibitory phosphorylation of Cdc24 by the Cdc42-activated PAK Cla4 (Kuo et al., 2014).

Sensing and tracking a pheromone gradient. The primary purpose of polarized growth is to find and fuse with a nearby mating partner. To do this, yeast track shallow pheromone gradients. However, tracking a shallow pheromone gradient is not a trivial problem because of the small size of yeast cells. Yeast are not motile, so they cannot use the bacterial strategy of sampling different locations in space tens of micrometers apart, but rather are limited in their spatial sampling to their own cell size. In practice, the gradient sensing problem is even more difficult for yeast because they concentrate receptors and other pathway components at the shmoo tip. This further reduces the length scale over which pheromone concentrations can be compared to a few hundred nanometers (Ayscough and Drubin, 1998; Moore

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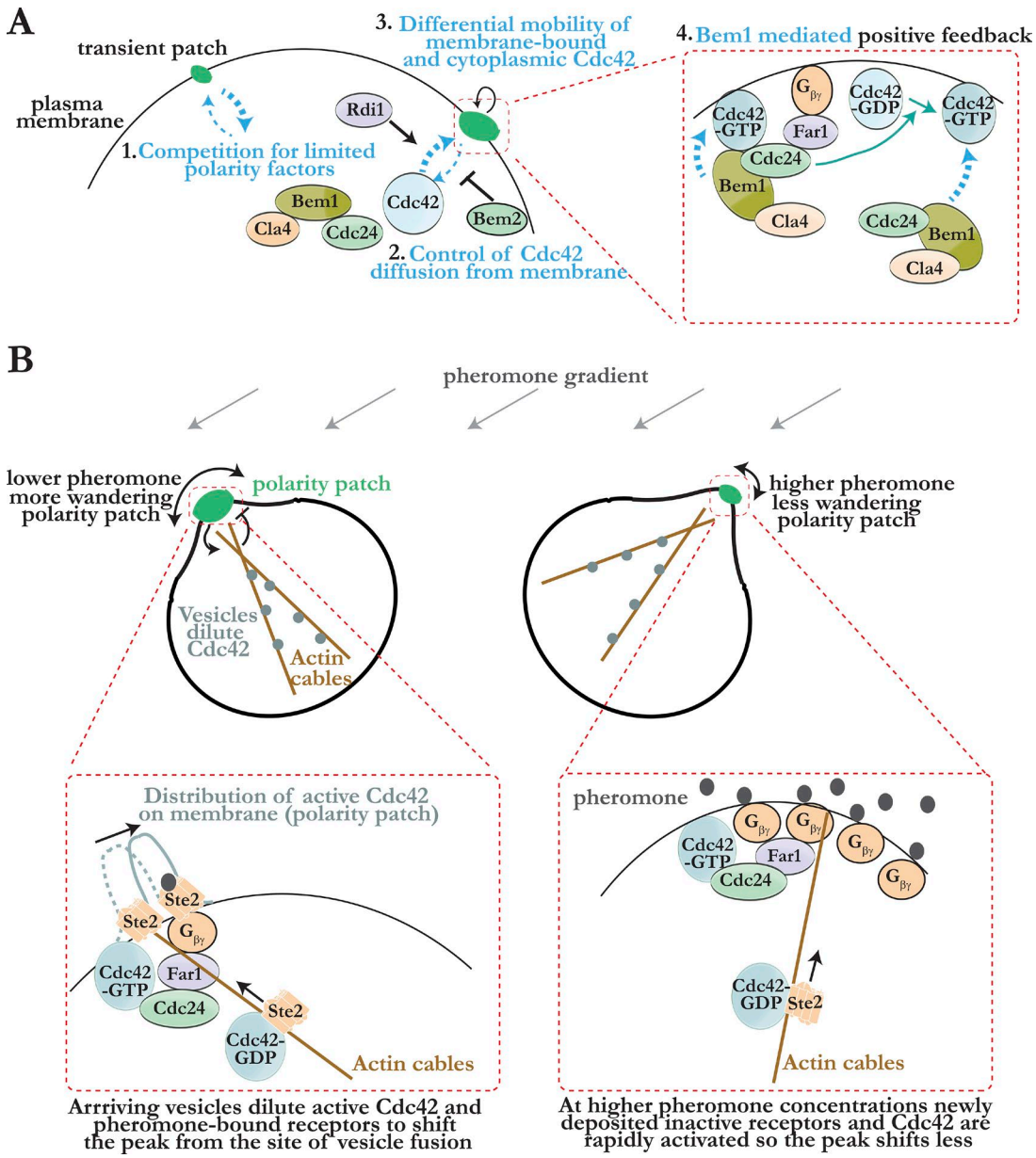

Figure 4. **Schematic of MAPK pathway–dependent cell polarization and gradient tracking.** (A) At least four mechanisms act to maintain a single polarity patch. (1) Competition of multiple sites of polarization for a limited pool of polarity factors results in the depletion of these factors by the largest site. (2) Cdc42 diffusion from the polarity site on the membrane is controlled by the GDI Rdi1 and the GAP Bem2. (3) Even in the absence of GDI-dependent control, membrane-bound active Cdc42 at the polarity patch diffuses away slowly, whereas cytoplasmic Cdc42 can rapidly reach and accumulate at the polarity patch. (4) The scaffold protein Bem1 is recruited to the polarity patch by active Cdc42-GTP. Bem1 is bound to Cdc24 so that its recruitment activates neighboring Cdc42 to complete a positive feedback loop. (B, top) Illustration showing a pheromone gradient and its effect on the polarity of cells of different orientations. Models predict that the polarity patch wanders more when the major axis of cell polarity is not aligned with the pheromone gradient. (Bottom left) Actin cables bring dilute and inactive Cdc42 and inactive pheromone receptors to the site of the polarity patch. This negative feedback pushes the center of the polarity patch away from newly arrived vesicles. (Bottom right) At higher pheromone concentrations, increased localized activation of the $G_{\beta\gamma}$ subunit results in faster local activation of arriving receptors and Cdc42 to tighten the peak and reduce the shifts caused by the negative feedback mechanism of newly arrived vesicles.

et al., 2008; Garrenton et al., 2010). Yet, yeast gradient sensing works. Thus, the question becomes how yeast are able to sense such gradients by detecting concentration differences at points located only ~100 nm apart.

The general solution to the problem of gradient detection is either spatial or temporal averaging. That is, yeast need to measure and average the pheromone concentrations across time or space to improve the signal to noise ratio. Because spatial averaging is limited to the yeast shmoo tip, yeast likely use temporal averaging. Indeed, when exposed to shallow pheromone gradients, yeast that initially polarize in the incorrect orientation will correct it over time (Segall, 1993; Moore et al., 2008). However, such reorientation conflicts with the presence of positive feedback loops that maintain the location of the polarity patch. Thus, it has remained unclear until recently how yeast cells are able to track shallow gradients in the presence of stabilizing positive feedbacks and why signaling components are spatially organized to the shmoo tip despite its apparent disadvantage in

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terms of decreasing the length scale over which concentration differences can be sensed. The main clue to answering this conundrum has been the recent observations that the system also harbors negative feedbacks that can facilitate cell reorientation (Ozbudak et al., 2005; Layton et al., 2011; Kuo et al., 2014).

To promote gradient tracking, a spatially localized negative feedback can destabilize polarization at some but not other regions of polarized growth to drive reorientation (Meinhardt, 1999). Indeed, it was found that the polarity patch wanders and that this wandering is crucial for gradient tracking (Dyer et al., 2013). Interestingly, one negative feedback that likely promotes wandering is the actin-dependent vesicle traffic that brings Cdc42 to polarity clusters. This is surprising because vesicle traffic was previously thought to be the positive feedback that drives polarity establishment (Wedlich-Soldner et al., 2003). However, recent studies showed that Cdc42 has a lower concentration in vesicles than in the polarity patch, which implies that the deposition of vesicles into the polarity patch decreases Cdc42 concentration (Layton et al., 2011; Watson et al., 2014). Vesicle fusion shifts the polarity patch peak away from the site of vesicle deposition, which would cause the patch to wander. Similarly, vesicles deposit unbound pheromone receptors and are therefore expected to decrease the concentration of pheromone-bound receptors at the location of vesicle fusion. The diffusive nature associated with wandering then arises because of the stochastic arrival of vesicles on all sides of the center of the polarity patch.

Although negative feedback can give rise to wandering, it does not immediately explain how such wandering leads to gradient tracking. Importantly, polarity patch wandering decreases at higher pheromone concentrations (Dyer et al., 2013). Thus, the patch wanders more in lower pheromone concentrations than in higher concentrations. This results in the patch spending more time on the side of the shmoo with higher pheromone concentration, thereby driving growth in this direction (McClure et al., 2015). At higher pheromone concentrations, patch wandering could be reduced by the fact that newly deposited unbound receptors are quickly bound and activated to promote continued polarization at the site of vesicle fusion (Fig. 4 B). Slower wandering at higher pheromone concentrations depends on the polarized localization of the Gβγ subunit and the Far1–Cdc24 interaction (McClure et al., 2015). In summary, while the specific molecular mechanisms underlying the concentration dependency of polarity patch wandering remain to be fully elucidated, it is now clear, at least phenomenologically, that these mechanisms enable the polarity patch to constantly wander to check the local pheromone concentration so that yeast grow up pheromone gradients.

Shared components in the signaling and gradient tracking branches of the pheromone response. Many molecules involved in the pheromone response have important roles in both MAPK signaling and gradient tracking. The secreted Bar1 pheromone protease was initially thought to be a simple negative feedback in MAPK signaling. It was later shown that Bar1 secretion also provides a mechanism for mating type a cells to create α-factor sinks and thereby modify the local pheromone gradient to improve tracking (Barkai et al., 1998; Jin et al., 2011). Similarly, the GAP Sst2 not only down-regulates signaling, but also promotes accumulation of the receptor on the shmoo tip and thus is crucial for gradient sensing and tracking (Venkatapurapu et al., 2015). In addition, it has been suggested that the slow kinetics of pheromone–receptor binding might improve the ability of yeast cells to polarize in the correct direction even when exposed to near-saturating pheromone gradients, when most of the receptors would be bound at equilibrium (Ventura et al., 2014). Finally, the MAPK Fus3 and the cell cycle inhibitor Far1 play a direct role in polarization and gradient tracking in addition to their role in cell cycle arrest. Fus3 binds to the α subunit of the G protein and activates the formin Bni1, which is required for tethering actin cables to the polarity patch (Matheos et al., 2004). Accordingly, it was shown that the binding of Fus3 to Gα is required for gradient sensing and shmoo formation (Metodiev et al., 2002; Hao et al., 2008; Errede et al., 2015). In addition, Fus3 activity also changes the availability of cytoplasmic Cdc24 by impacting the spatial localization and expression level of Far1 (Butty et al., 1998; Hegemann et al., 2015). Although nuclear Far1 concentration significantly increases with pheromone concentration, the ratio of nuclear to cytoplasmic Far1, which actually determines Cdc24 availability on the polarity patch, slightly decreases with pheromone concentration (Hegemann et al., 2015). This is because higher Fus3 activity results in an elevated level of Far1 nuclear export, leading to a relatively lower nuclear to cytoplasmic Far1 ratio and thereby an increased Cdc24 concentration on the polarity patch (Blondel et al., 1999; Hegemann et al., 2015). This higher concentration of Cdc24 on the polarity patch contributes to the decreased patch wandering at higher pheromone concentrations to promote gradient tracking (Hegemann et al., 2015). Thus, the regulation of Cdc24–Far1 spatial localization, which is crucial for memory of pheromone exposure to be transmitted to the daughter cells, also assists in gradient tracking.

Principles of dynamic MAPK pathway regulation

The graded response of the pheromone pathway is instrumental for both its signal processing and gradient tracking roles. Perhaps not surprisingly, other MAPK pathways also process extracellular information into graded responses. In the pheromone-induced MAPK pathway, the graded response is seen when pheromone concentration is plotted against just about any aspect of pathway activity at any time. This is because the pathway activity rapidly adjusts to reach the new steady-state level in response to any step change in pheromone concentration. In two other well-studied MAPK pathways, the yeast high osmolarity glycerol (HOG) pathway and the mammalian extracellular signal-regulated kinase (ERK) pathway, markers of pathway activity remain dynamic for long periods of time in response to a step change in input and may in fact never reach a steady-state activity. Nevertheless, the mean transcriptional response can still be graded despite continually dynamic upstream signaling (Purvis and Lahav, 2013).

Stress-activated MAPK signaling in yeast. The yeast HOG pathway responds to hyperosmotic stress and consists of two distinct branches, each mediated by its own independent sensor for osmotic pressure: Sln1 and Sho1. These branches converge on the activation of the scaffold kinase Pbs2, which activates the downstream MAPK Hog1. In response to osmotic pressure, Hog1 regulates the accumulation of glycerol that reduces osmotic stress (Fig. 5 A; Hohmann, 2009; Brewster and Gustin, 2014). Although the pheromone and high osmolarity pathways share components such as Ste20 and Ste11, these two pathways are insulated from each other so that pheromone inputs do not inhibit the HOG response to salt, and vice versa

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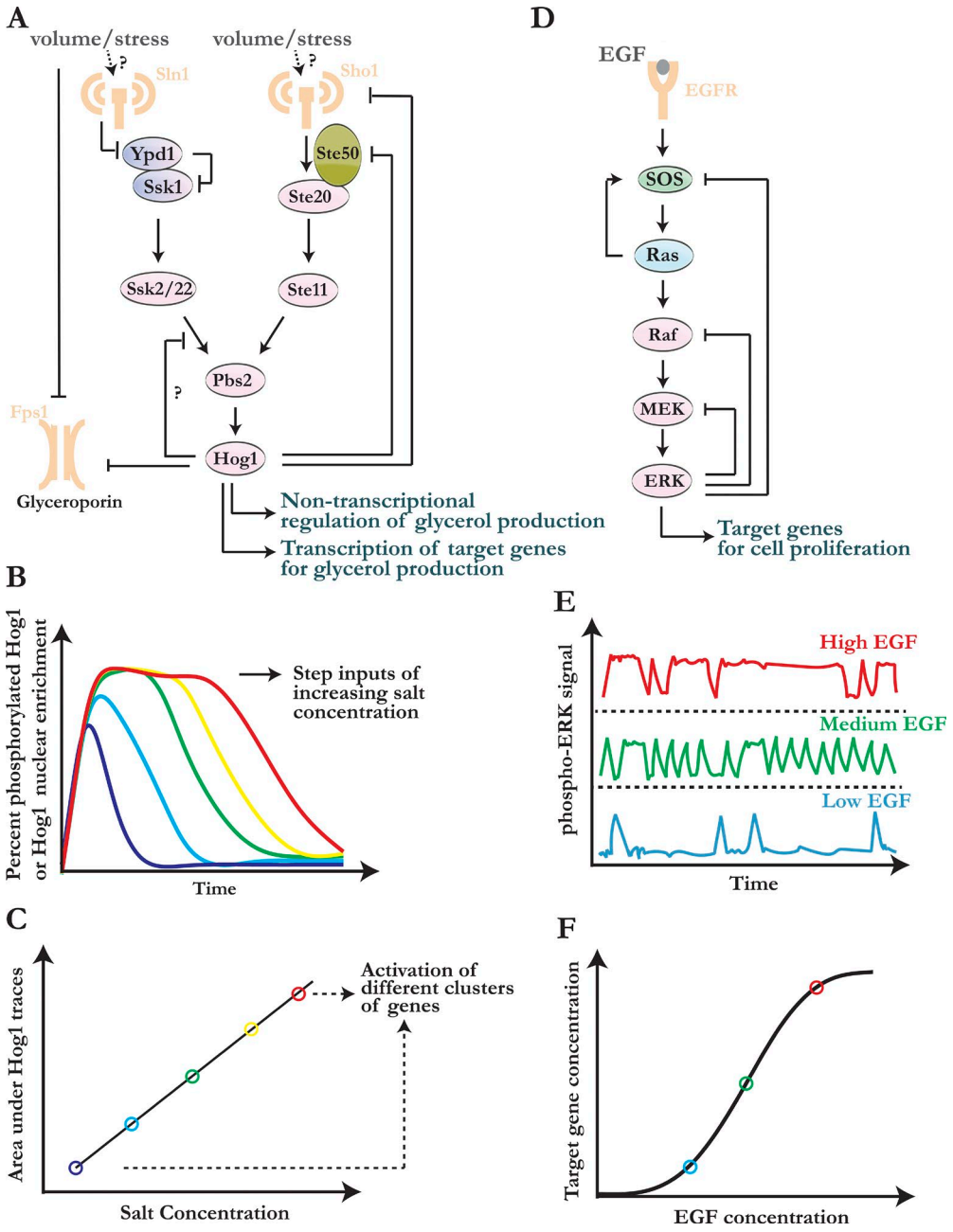

Figure 5. **Graded responses of dynamic MAPK pathways.** (A) Schematic of the HOG stress-activated pathway in budding yeast. Color coding is similar to Fig. 1, where pink denotes kinases. (B) Schematic of the activity of the MAPK Hog1 as a function of time in response to step increases of salt concentration. Note nonperfect adaptation after the decrease in Hog1 activity. (C) Schematic of the linear graded relationship between the area under the curve of Hog1 activity and the salt concentration of the step increase. Note that the downstream transcriptional response is not as linear, and that different genes are activated at different salt concentrations. (D) Schematic of the mammalian ERK pathway, which shares a common ancestral pathway with the pheromone-induced yeast MAPK pathway. Color coding is similar to Fig. 1. (E) Schematic of the Erk activity in cells exposed to different concentrations of EGF. The Erk activity is dynamic even in response to constant growth factor concentrations. Erk pulse frequency increases with EGF concentration. (F) Downstream outputs integrating Erk activity over time are a graded function of EGF input.

(Patterson et al., 2010). In fact, even though the pheromone pathway can trigger HOG response in osmolarity-adapted cells, this is not caused by molecular cross talk. Rather, the morphological output of the pheromone pathway, shmooing, generates regions of concave curvature, which triggers a stress response, activating the HOG pathway (Baltanás et al., 2013).

In the HOG pathway, the accumulation of glycerol results from both a fast and a slow response. The fast response shuts down the glyceroporin Fps1 to increase glycerol retention and increases glycerol production via nontranscriptional regulation (Tamás et al., 1999; Dihazi et al., 2004; Hersen et al., 2008; Schaber et al., 2012). Fast activation is possible because HOG pathway components are present even in conditions free from osmotic stress, which is similar to how pheromone pathway components are produced even in the absence of pheromone. The slow response increases transcription to gradually ramp up glycerol production (Rep et al., 1999; Mettetal et al., 2008; Schaber et al., 2012). In both fast and slow responses, the

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pathway adapts; i.e., multiple fast and slow negative feedbacks act to shut down the pathway as the higher internal glycerol concentration relieves the osmotic pressure and the cell approaches its pre-stress volume (Hao et al., 2007, 2008; Mettetal et al., 2008; Macia et al., 2009). Although this was initially thought to be perfect adaptation, i.e., that Hog1 activity would return exactly to the same pre-stress level independent of the input level (Muzzey et al., 2009), later work showed that the post-stress Hog1 activity is still higher than the pre-stress level even after adaptation (Macia et al., 2009; Baltanás et al., 2013). The higher steady-state Hog1 activity seems to be necessary to maintain the glycerol gradient between the cell and its extracellular environment at high osmolarity conditions (Baltanás et al., 2013).

The response of the HOG pathway has most often been studied in the context of the step increase in osmotic stress, e.g., by adding a salt to the medium. In response to a step change in osmotic stress, Hog1 phosphorylation and activity rapidly increases to a near-maximal level and stays there before decreasing to a much lower steady-state level (Fig. 5 B). Although the rapid Hog1 activation is similar in all levels of osmotic stress, the amount of time it remains at the near-maximal level varies. Larger stresses result in larger durations of sustained maximal Hog1 activity (English et al., 2015). Indeed, the time integral of active Hog1 before adaptation is almost perfectly linear as a function of the extracellular salt concentration (Fig. 5 C; Muzzey et al., 2009; English et al., 2015). In this way, the HOG pathway converts the amplitude of the input signal into the duration of Hog1 activity, which then leads to the approximately graded activation of Hog1 targets as a function of the input. We note that the activation of salt-responsive genes is not perfectly proportional, so there exist some genes only activated in the high salt concentrations (English et al., 2015). Thus, similar to the yeast pheromone pathway, the yeast HOG pathway is another example of a MAPK pathway that implements a graded response to varied input signals.

Although most quantitative studies investigate the HOG pathway response to step increases in input, this is only one of many different possible dynamic input signals. Examination of the HOG pathway response to simple oscillatory signals of varying frequency identified the fundamental time scales associated with pathway dynamics (Mettetal et al., 2008). Interestingly, the HOG pathway is much more active in response to an oscillatory dynamic signal than to a static input signal with the same mean level (Mitchell et al., 2015). Since the hyperactivation of HOG in response to an oscillatory signal reduces cell growth, it is likely that the signal processing properties of particular pathways have evolved to handle some, but not all, dynamic input signals.

Mammalian ERK signaling. In most mammalian cells grown in cell culture conditions, the mammalian ERK pathway detects extracellular growth factors (e.g., EGF) that trigger cell growth and division (Fig. 5 D). In brief, EGF binds the EGF tyrosine kinase receptor, which leads to the activation of the Raf–MEK–Erk MAPK pathway. This MAPK pathway and the yeast pheromone–activated MAPK pathway are descended from a common pathway present in their opisthokont ancestor, whereas the yeast HOG pathway is related to the animal stress response p38 and JNK pathways (Caffrey et al., 1999). The function of the ERK pathway depends on the cell type. Although ERK promotes proliferation in many commonly cultured cell lines, ERK appears to have the opposite effect in embryonic stem cells, where, like in yeast, this MAPK pathway promotes differentiation (Ying et al., 2008). Thus, the function of the ERK pathway, like many other signaling pathways, depends on its context in different animal cell types.

The ERK pathway in epithelial cells exhibits highly dynamic signaling in response to step changes in growth factors, like the yeast HOG pathway (Amit et al., 2007; Santos et al., 2007). In response to a step increase, the mean population response, as measured by fraction of phosphorylated active Erk, first increases to a peak before decreasing to reach a steady-state level in proportion to EGF. However, this mean response masks important dynamics in single cells (Cohen-Saidon et al., 2009). Even in steady-state conditions, the ERK pathway remains dynamic. This was revealed by the development of fluorescence resonance energy transfer (FRET) sensors for Erk kinase activity, which showed large stochastic pulses in single cells (Fig. 5 E). The rate at which these pulses appear increases with extracellular EGF concentration so that the mean population response is graded (Fig. 5 F; Albeck et al., 2013; Aoki et al., 2013). This type of cellular response, in which stochastic pulsatile dynamics transmit a graded response, was previously observed in yeast Ca^{2+} and Msn2 stress responses (Cai et al., 2008; Hao and O'Shea, 2012).

Importantly, the identity of the growth factor that triggers the ERK pathway can bias the ERK dynamics toward pulsatile or sustained ERK activation, which can then impact the proliferation-differentiation decision. EGF, which triggers pulsatile dynamics, drives proliferation, whereas NGF triggers sustained activation and differentiation. This suggests that ERK dynamics, not only mean activity, influence cell fate (Marshall, 1995). Consistent with the notion that ERK dynamics are important by themselves, the stimulation of the ERK pathway with an oscillatory NGF input can drive proliferation (Ryu et al., 2015). Although the mechanism of how EGF and NGF stimulation results in different cell fates is still not completely clear, one possibility is that ERK phosphorylates and stabilizes otherwise unstable gene products that are required for differentiation. Sustained ERK activity would then lead to sustained protein stability to promote differentiation (Murphy et al., 2002).

Finally, pulsatile Erk activity was shown to be spatially correlated in a tissue culture study of epithelial cells (Aoki et al., 2013). If one cell pulsed, neighboring cells tended to pulse at a time after the first pulse that increased with the distance between the cells. It was later revealed that these in vitro dynamics were highly relevant to in vivo dynamics when the Erk FRET sensor was introduced into mice (Hiratsuka et al., 2015). Time-lapse microscopy showed that Erk activity propagated in waves emanating from initial activation in one or a few neighboring cells. These waves were associated with cell growth and division. In places of high cell growth, such as near wounds, there were more Erk waves, suggesting a graded nature of Erk wave frequency in response to growth signals. Thus, the pulsatile nature of the dynamics, their spatial propagation, and the graded relationship between Erk activity and growth signals, which all were identified in vitro, were also present in vivo.

Concluding remarks

Although progress in understanding MAPK signaling has been rapid, it is far from complete. For many MAPK pathways, we still do not know the overall input–output relationships that govern their cellular response. For example, even in yeast, we have a poor understanding of the cell wall integrity pathway, the sporulation wall assembly pathway, and the filamentous

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growth pathway (Chen and Thorner, 2007). Nevertheless, the last decade's quantitative studies of the pheromone response pathway greatly increased our understanding of the principles of cellular signaling and decision making. We have learned that the two branches of the pheromone pathway, signaling and gradient tracking, are intricately linked and that the spatial organization of their components is integral to the functions of both branches. Cell signaling determines cell geometry, which, in turn, impacts cell signaling. In particular, spatial organization enhances signaling, bestows unexpected properties to network motifs, and provides the fundamental mechanism for polarization and gradient tracking. We anticipate that similar quantitative studies of the dynamics of MAPK pathways in yeast and animals will reveal the full complement of biological signal processing harnessing both spatial and temporal mechanisms.

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