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Yeast go the whole HOG for the hyperosmotic response

Sean M. O'Rourke, Ira Herskowitz and Erin K. O'Shea

An evolutionarily conserved mitogen-activated protein kinase pathway – the high osmolarity glycerol (HOG) pathway – mediates the hyperosmotic response in *Saccharomyces cerevisiae*. A variety of powerful approaches has generated a comprehensive picture of how cells respond to this stress condition. Several presumptive osmosensors on the cell surface recruit and activate downstream signaling components, which regulate the activity of transcription factors to control gene expression.

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Cells respond to diverse stresses by adapting their physiology to meet the imposed conditions. Most cells adapt to increased osmolarity by accumulating compatible solutes to balance the cellular osmotic pressure with the external environment. The compatible solute differs in various organisms but is generally a small molecule that does not harm cellular components; for example, glycine betaine, sorbitol, sucrose, glycerol and amino acids [1]. *Saccharomyces cerevisiae* uses glycerol as a compatible solute. How this yeast responds to high osmolarity to produce glycerol and make other physiological adjustments is now being elegantly elucidated.

Yeast cells challenged with hyperosmotic shock rapidly shrink and begin synthesizing glycerol [2]. A breakthrough in our understanding of the control of the yeast osmoresponse came when Gustin and colleagues performed a genetic screen for osmosensitive mutants and identified two of the founding members of the high-osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway: Pbs2 and Hog1 [3]. Mutations in *PBS2* or *HOG1* [encoding a MAPK kinase (MAPKK) and a MAPK, respectively], cause osmosensitivity and accumulation of reduced levels of glycerol [3]. Furthermore, Hog1 is rapidly phosphorylated in a manner that is dependent on Pbs2 and hyperosmotic stress. Thus, the yeast response to elevated osmotic pressure uses a MAPK pathway to respond to an extracellular stimulus, analogous to the much studied yeast pheromone-response MAPK pathway [4,5].

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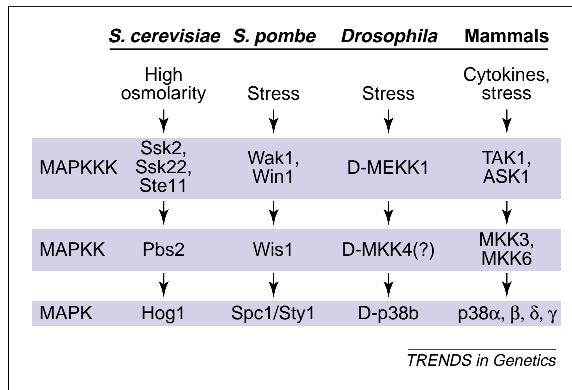

	S. cerevisiae	*S. pombe*	*Drosophila*	Mammals
	High osmolarity	Stress	Stress	Cytokines, stress
MAPKKK	Ssk2, Ssk22, Ste11	Wak1, Win1	D-MEKK1	TAK1, ASK1
MAPKK	Pbs2	Wis1	D-MKK4(?)	MKK3, MKK6
MAPK	Hog1	Spc1/Sty1	D-p38b	p38α, β, δ, γ

TRENDS in Genetics

Fig. 1. Examples of MAPK cascades containing Hog1/p38 homologs. The *S. cerevisiae* HOG pathway responds to high osmolarity stress. The *S. pombe* Spc1/Sty1 pathway responds to multiple stimuli such as heat, osmotic, oxidative and nutrient deprivation stress. *Drosophila* p38b is activated by heat and osmotic stress; the relevant MAPKK for stimulating D-p38b in response to stress is unknown but might be D-MKK4. A second *Drosophila* MAPKK, encoded by *licorne*, is thought to activate p38 to set up developmental patterning (not shown). In mammalian cells, p38 isoforms are activated by stress and cytokines, effecting a variety of biological consequences. Abbreviations: Hog, high osmolarity glycerol; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; *S. cerevisiae*, *Saccharomyces cerevisiae*; *S. pombe*, *Schizosaccharomyces pombe*.

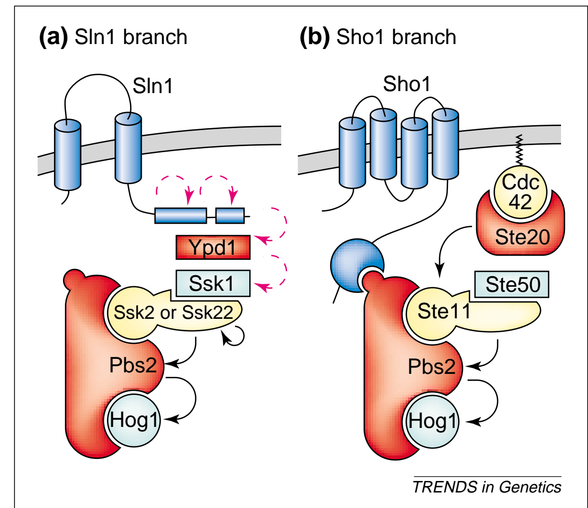

Fig. 2. Two upstream branches of the HOG pathway can each activate Pbs2 and Hog1. Solid black arrows indicate serine, threonine or tyrosine phosphorylation events during increased osmolarity. Dashed pink arrows indicate phosphotransfer events in constant osmolarity; see text for details. (a) The Sln1 branch is composed of a bacterial-like phosphorelay system that regulates the activity of the two partially redundant MAPKKKs, Ssk2 and Ssk22. Phosphotransfer in the Sln1–Ypd1–Ssk1 system occurs in constant osmotic conditions to keep Ssk1 inactive. (b) The Sho1 branch uses a novel four-membrane-spanning domain protein to recruit Pbs2 to the membrane through an SH3–polyproline interaction, after which the MAPKKK Ste11 is phosphorylated by Ste20. Abbreviations: Hog, high osmolarity glycerol; MAPKKK, mitogen-activated protein kinase (MAPK) kinase kinase.

Yeast also use MAPK-independent mechanisms to adapt to hyperosmotic stress [6]. For example, intracellular accumulation of glycerol is aided by closure of the glycerol export channel Fps1 [7].

MAPK cascades are ubiquitous in eukaryotic organisms and are composed of three sequentially acting kinases. In response to an extracellular stimulus, a MAP kinase kinase kinase (MAPKKK) phosphorylates and activates a MAPKK (e.g. Pbs2); this then phosphorylates and activates a MAPK (e.g. Hog1). The underlying logic for using a cascade of kinases is not clear, but this feature could offer multiple steps for regulation, and control commitment to signaling. Eukaryotic organisms contain multiple members of the MAPK, MAPKK and MAPKKK families, and these are organized into discrete kinase cascades [8]. There are three major subgroups of MAPKs: ERK, JNK and p38/Hog1. Hog1 orthologs are found in fungi and animals but apparently not in plants [9] (Fig. 1). Interestingly, Hog1 homologs from animals respond to cytokines, growth factors, pathogens and developmental cues in addition to diverse environmental stress conditions [10–12]. The fission yeast p38 representative, Spc1/Sty1, is activated in response to heat and oxidative stress, as well as osmotic stress [13]. By contrast, *S. cerevisiae* Hog1 seems to respond specifically to increased extracellular osmolarity [14–16]. A recent report indicates that Hog1 is also activated by heat stress [17].

The HOG pathway: two upstream branches

The HOG pathway contains two transmembrane proteins, Sho1 and Sln1, that act on downstream proteins to ultimately regulate the MAPK Hog1 (Fig. 2). Sho1 and Sln1 have been termed osmosensors, but recent studies indicate that Sho1 might, in fact, not sense osmolarity changes directly [18]. Additional osmolarity-sensing inputs have been reported [19,20], one of which requires the single-transmembrane-spanning domain protein Msb2 [21]. Importantly, the proposed high osmolarity sensors have not yet been demonstrated to respond to changes in osmolarity in an *in vitro* or heterologous system, an advance that would be extremely useful for examining the mechanism of osmosensation and for determining which proteins are the true osmosensors. Sho1 and Sln1 have very different functional domains and communicate directly to different downstream effector proteins.

The two upstream branches of the HOG pathway are often thought to be redundant. For growth on high osmolarity medium, they are, indeed, redundant. However, some differences have been noted. First, Hog1 phosphorylation in response to low solute concentrations is more dependent on the Sln1 branch [22]. Also, activity of the Sln1 branch is required to induce the expression of several reporter genes in response to very high solute levels [20]. These findings indicate that the Sln1 branch operates over a broader range of osmolarities than the Sho1 branch. In addition, we have recently found that global gene regulation in response to modest osmotic stress (e.g. 0.0625 or 0.125 M KCl) specifically requires the Sln1 branch of the HOG pathway; the Sho1 branch is dispensable (S.M. O'Rourke and

I. Herskowitz, unpublished). Mutation of the Sln pathway component *SSK1* alone impairs the induction of many genes. However, exposure of *ssk1* or *sho1* mutants to higher osmotic stress (e.g. 0.5 or 1.0 M KCl) induces gene expression similar to wild-type yeast. Together, these findings indicate that the two upstream branches of the HOG pathway are specialized for detecting different osmotic conditions.

The Sln1 histidine kinase branch

Sln1 has two transmembrane regions and an intracellular histidine kinase domain that signals to two other proteins, Ypd1 and Ssk1, which together form a phosphorelay system. Phosphorelay systems comprise a histidine kinase protein (e.g. Sln1) that transfers a phosphate group to an intermediate protein (e.g. Ypd1), which then transfers the phosphate to a response regulator protein (e.g. Ssk1) [23]. The response regulator then performs some biological function; for example, triggers gene transcription. Homologs of Sln1 are found in bacterial two-component systems, where they are used to sense changes in environmental conditions [23]. Histidine kinase sensors are not as prevalent in eukaryotes as they are in prokaryotes, but some (a subset of which appear to sense increased osmolarity) have been described in *Arabidopsis*, *Dictyostelium* and various fungi [24].

Saito and colleagues have elegantly determined the phosphotransfer system used by Sln1–Ypd1–Ssk1 to regulate the HOG pathway [25] (Fig. 2a). Sln1 is constitutively active during constant osmotic conditions, leading to phosphorylation of the downstream target protein, Ypd1. Ypd1 transfers its phosphate group to the response regulator Ssk1, which is the ultimate phospho-acceptor in this phosphorelay system. Such phosphorylation of Ssk1 is thought to prevent interaction of Ssk1 with the MAPKKKs Ssk2 and Ssk22 and, consequently, the downstream components of the pathway remain inactive [26]. After exposure to increased external osmolarity, the histidine kinase activity of Sln1 is thought to be inhibited. Thus, Ypd1 and Ssk1 are dephosphorylated, and this enables binding of Ssk1 to MAPKKK Ssk2, triggering Ssk2 autophosphorylation, subsequent phosphorylation of Pbs2 and activation of Hog1 [26].

Even with this detailed view of the Sln1 branch of the HOG pathway, some questions remain. First, in a purified *in vitro* system, the half-life of Ssk1 phosphorylation is ~40 h in the presence of Ypd1 [27,28]. By contrast, Ssk1 is rapidly dephosphorylated in response to osmotic stress *in vivo* (<1 min) [25]. These observations suggest that an as-yet-unidentified phosphatase might play an important role in activation of the Sln1 branch of the pathway. Second, the method by which Sln1 actually senses changes in osmotic pressure also remains elusive. Dimerization appears to be necessary for Sln1 kinase activity, and the first transmembrane region and extracellular domains are required for osmotic stress-dependent kinase regulation [29]. Also, the levels of intracellular glycerol appear to regulate Sln1-dependent signaling, which suggests that Sln1 might sense differences in the osmotic gradient across the membrane and not just increases in external osmolarity [30,31].

The Sho1 branch

The Sho1 protein is composed of four transmembrane segments and a C-terminal SH3 domain, through which it interacts with downstream signaling elements in the HOG pathway. So far, Sho1 homologs have only been identified in fungi [32]. The physiological role of Sho1 as an osmosensor has been called into question in a recent study [18] and, thus, the activation mechanism of this branch of the pathway is unclear.

Raitt *et al.* have provided a detailed characterization of the functional domains of Sho1 [18]. They found that the function of Sho1 can be completely bypassed by overexpression of a membrane-targeted version of Pbs2. Using chimeric Sho1 proteins and truncated variants, it was shown that the transmembrane domains of Sho1 are not specifically required to stimulate the HOG pathway [18]. However, plasma membrane localization of the SH3 domain is required: a cytoplasmic Sho1 SH3 domain is inactive, whereas anchorage of the SH3 domain to the membrane using various membrane-targeting sequences is sufficient for activity *in vivo* [18]. These findings suggest that Sho1 might not sense osmolarity directly but might instead provide a docking site (via its SH3 domain) for downstream proteins. If Sho1 is not an osmolarity sensor, a different mechanism for triggering this branch of the pathway must exist.

The Sho1 branch uses proteins that are also components of the pheromone-response and filamentous MAPK pathways. This branch includes Ste20 (a p21-activated kinase), Ste50 (a SAM domain-containing protein) and the MAPKKK Ste11 [19,33,34] (Fig. 2b). Ste20 activates Ste11 by phosphorylation during pheromone signaling [35] and, presumably, also during increased osmolarity because the Ste11 phosphorylation sites are required for both responses [35]. Ste50 might be a cofactor for Ste11 because these proteins form a complex through interaction of their SAM domains [33,36]. Interestingly, Pbs2 contains an N-terminal polyproline domain that can bind the SH3 domain of Sho1 [22]. The importance of the Sho1–Pbs2 interaction is illustrated by a Pbs2 point mutant with a compromised SH3 binding site: this causes osmosensitivity when the Sln1 branch is mutated, just as does mutation of Sho1 [22]. Because Pbs2 interacts with multiple proteins (e.g. Sho1, Ste11 and Hog1), Pbs2 has been proposed to act as a scaffold, linking Sho1 to Ste11 activation and, thereby, possibly limiting cross-talk to other Ste11-dependent

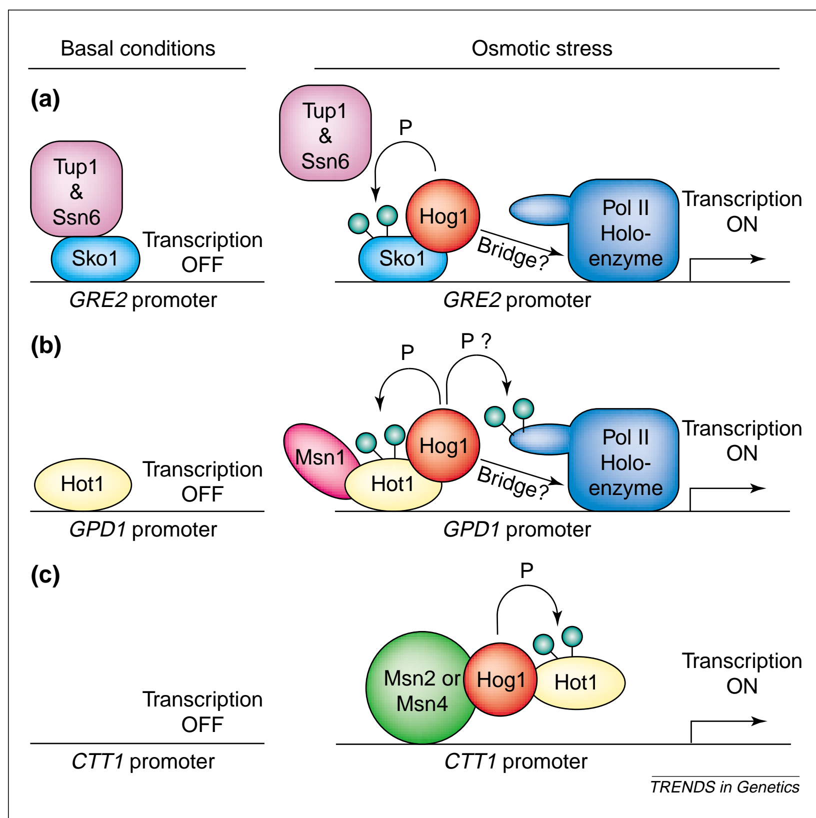

Fig. 3. Osmotic-regulated transcriptional activation of three genes. The left side shows a model of the promoters during growth in normal (basal) osmotic conditions whereas the right side depicts how Hog1 governs transcriptional induction upon osmotic stress. (a) The *GRE2* promoter is bound by the repressor Sko1, which recruits the general repressor complex Tup1–Ssn6. After osmotic shock, Hog1 phosphorylates Sko1, resulting in decreased affinity for Tup1. Sko1 then activates transcription by an unknown mechanism, perhaps interacting with the RNA polymerase II (Pol II) holoenzyme complex via Hog1. (b) Hot1 is constitutively bound to the *GPD1* promoter. After osmotic stress, Hog1 and Msn1 are recruited to the promoter via Hot1. Hog1 phosphorylates Hot1, but this phosphorylation might not be required to initiate transcription. Instead, Hog1 could again interact with, or phosphorylate, the general transcription machinery. (c) The *CTT1* promoter contains binding sites for the transcriptional activators Msn2 and Msn4. During osmotic shock, Msn2 and Msn4 enter the nucleus and bind the promoter. Hog1 is then recruited to the DNA through interaction with Msn2 and/or Msn4. Hot1 also binds to the complex if the kinase activity of Hog1 is intact.

MAPK pathways [34]. Interestingly, another signaling circuit, which also uses Sho1 to activate Ste11, does not require Pbs2 as a scaffold.

Activation of Pbs2 and Hog1

Activation of the MAPKK Pbs2 is accomplished upon phosphorylation by any of three MAPKKKs – Ste11, Ssk2 or Ssk22 [34]. Mutating the Pbs2 phosphorylation sites (Ser514 and Thr518) to alanine causes osmosensitivity [34], whereas mutation to aspartic acid constitutively activates the pathway [37]. Once activated, Pbs2 phosphorylates Hog1 on a threonine and a tyrosine residue, both of which are conserved in MAP kinases, and Hog1 is then imported into the nucleus [14,38].

The HOG pathway: inside the nucleus

Once in the nucleus, Hog1 regulates the expression of numerous genes [39,40] by controlling the activity of several transcriptional activators and repressors. DNA microarray studies indicate that Hog1 significantly regulates the expression of ~600 genes in response to increased osmolarity (Refs [39,40] and S.M. O'Rourke and I. Herskowitz, unpublished). Several recent papers are revealing the mechanisms used by Hog1 to regulate transcription factors.

Sko1 binds DNA motifs upstream of several osmo-inducible genes (the *GRE2* gene is shown as an example in Fig. 3a), and then recruits the general repressor complex Tup1–Ssn6 to repress transcription [41–44]. During elevated osmolarity, Hog1 phosphorylates Sko1, reducing the affinity of the protein for Tup1; this allows transcription to proceed [43,45]. Thus, in the absence of Sko1 or Tup1–Ssn6, transcription of Sko1-regulated genes is constitutive [45].

Hot1 is a transcriptional activator that exhibits a two-hybrid interaction with Hog1, and also displays Hog1- and osmotic shock-dependent phosphorylation [15,46]. Mutation of *HOT1* reduces but does not eliminate osmotic induction of the *GPD1* and *GPP2* genes, which control glycerol production. Simultaneous mutation of *HOT1* and *MSN1* (which encodes a protein related to Hot1) reduces *GPD1* expression even further [46]. In an interesting study using a chromatin immunoprecipitation PCR procedure, Alepuz *et al.* discovered that Hot1 is constitutively bound to the *GPD1* promoter and that it recruits Hog1 to the DNA during osmotic stress [15] (Fig. 3b). Because replacing the Hog1 phosphorylation sites of Hot1 with alanine does not alter the transcriptional activity of Hot1, it is proposed that Hog1 regulates alternative targets in the vicinity of the promoter; for example, by phosphorylation or protein–protein bridging interactions. Msn1 is also localized at the *GPD1* promoter, but only during osmotic stress and in *HOT1*$^{+}$ strains.

Msn2 and Msn4 are homologous, partially redundant, zinc-finger transcriptional activators that induce transcription during a variety of stress conditions, including osmotic stress [47]. The connection between these factors and Hog1 has been elusive. The transcriptional activity of Msn2 and Msn4 is regulated through nuclear localization by protein kinase A (PKA) [48]. Maximal induction of Msn2- and Msn4-dependent genes (e.g. *CTT1*) during osmotic stress also requires Hog1. Interestingly, Msn2 and/or Msn4 recruit both Hog1 and Hot1 to the *CTT1* and *HSP12* promoters, further suggesting a functional interaction between the HOG pathway, Msn2 and Msn4 [15] (Fig. 3c).

The HOG pathway: other outputs

In addition to the nuclear functions already described, Hog1 plays a role in regulating non-transcriptional processes. First, in two-hybrid screens, two groups identified Rck2 (a kinase related to mammalian MAPK-activated protein kinases) as a Hog1-interacting protein [49,50]. Moreover, Hog1 phosphorylates Rck2, increasing Rck2 kinase activity.

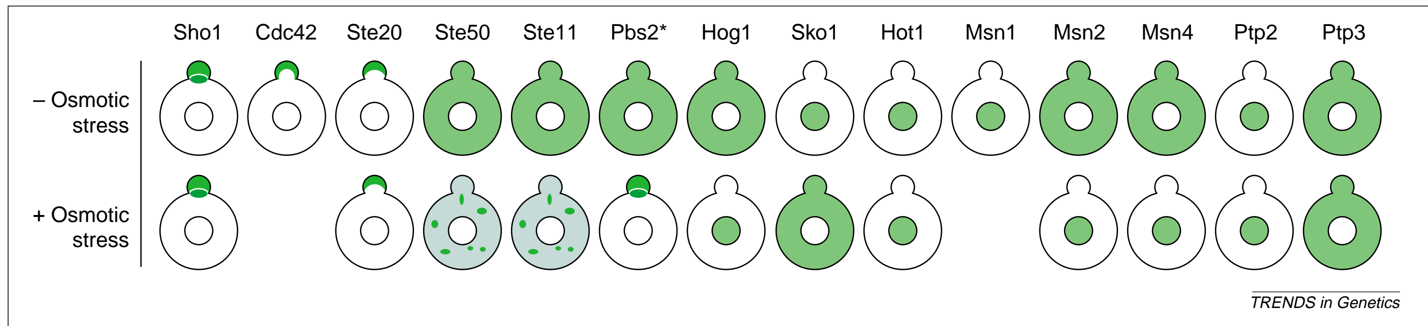

Fig. 4. Components of the high osmolarity glycerol (HOG) pathway display distinct subcellular distributions before and shortly after (generally <10 min) osmotic stress. Yeast cells are represented with small buds and a single nucleus per cell; green indicates protein. Ste50 and Ste11 display a punctate pattern after osmotic stress. Pbs2* refers to the localization of Pbs2 in *ssk1 ste11* double mutants, or of a Pbs2 protein that lacks kinase activity. See text for details and protein descriptions.

Hog1 and Rck1 are both required for the inhibition of protein biosynthesis that is observed after osmotic shock, perhaps via regulation of translation elongation factor 2 (EF-2). In fact, Rck2 is required for osmotic stress-induced phosphorylation of EF-2 [49]. The HOG pathway has also been implicated in a later stage of protein synthesis: Hog1 is responsible for resumption of protein translation after a transient pause in protein synthesis following osmotic shock [51]. Thus, Hog1 regulates the protein biosynthetic machinery during osmotic stress.

Cell-cycle progression is also affected by osmotic stress. Osmotic shock causes a transient accumulation of cells in the G1 and G2 phases of the cell cycle [52]. This cell-cycle block is accompanied by transient inhibition in the cyclin-dependent kinase activity of Clb2–Cdc28. Interestingly, *hog1* mutants do not inhibit Clb2–Cdc28 activity upon osmotic shock, and also show mitotic spindle localization defects after exposure to high osmolarity [52]. The direct target of Hog1 for regulating cell-cycle functions after osmotic stress remains elusive and could represent a new cyclin-dependent kinase inhibitor analogous to Far1 and Pho81.

Compartmentalization of HOG pathway components

A striking feature of the HOG pathway is that its components are organized spatially within the cell; this organization is, in some cases, dependent on extracellular osmotic conditions (Fig. 4). Subcellular localization of components of the Sln1 branch has not been reported, but membrane-associated proteins of the Sho1 branch (Sho1, Cdc42 and Ste20) are concentrated in the bud and bud neck region of cells (Refs [18,53–56] and A. Zarrinpar, pers. commun.) Cdc42 is essential for directing growth of the bud, Ste20 is localized by Cdc42, and Sho1 is thought to recruit the Ste20 substrate Ste11 (via Pbs2). Ste11 and Ste50 display diffuse cytoplasmic staining before osmotic stress and patchy cytoplasmic localization after osmotic shock [33].

The subcellular localization of Pbs2 is particularly interesting. In wild-type cells, Pbs2 shows a diffuse cytoplasmic localization in normal or high osmolarity media [14,38]. However, in an *ssk1 ste11* double mutant strain, or in *pbs2* mutants specifically lacking kinase activity, Pbs2 is recruited to regions of polarized growth after osmotic stress [53]. It is thought that the inactive Pbs2 protein is trapped at a normally transient site. Sho1 and Cdc42 are both required for the polarized localization of Pbs2 during osmotic stress. Thus, Pbs2 localization is dependent on both osmolarity and polarized proteins.

Before osmotic stress, Hog1 is distributed throughout the cytoplasm and is excluded from the nucleus. Coincident with phosphorylation by Pbs2, Hog1 is translocated into the nucleus [14,38]. The nuclear import of Hog1 is not dependent on new protein synthesis nor on Hog1 kinase activity, but is dependent on the Pbs2-mediated phosphorylation of Thr174 and Tyr176 [14,38]. Nuclear localization of Hog1 upon osmotic stress requires the nuclear import receptor Nmd5, a member of the importin β superfamily [14]. The fact that an *NMD5* deletion mutant does not exhibit osmosensitivity (unlike *hog1* mutants) raises the possibility that another nuclear import mechanism might operate in parallel with *NMD5*, or that Hog1 has functions outside the nucleus. Because Hog1 is only transiently localized to the nucleus, an export mechanism must also be used. Indeed, nuclear export of Hog1 is dependent on a different importin β homolog, the nuclear export receptor Crm1/Xpo1 [14].

The localization of Hog1 is further modulated by protein–protein interactions. First, the transcriptional activators downstream of Hog1 mediate nuclear retention. The zinc-finger transcription factors Msn2 and Msn4 tether Hog1 within the nucleus after osmotic stress but are not required for the initial nuclear accumulation [38]. Simultaneous mutation of *MSN2*, *MSN4*, *HOT1* and *MSN1* results in a reduced duration of Hog1 nuclear accumulation, indicating that interactions of Hog1 with these transcription factors determine the length of Hog1 nuclear residence [46]. Second, the tyrosine phosphatases that dephosphorylate Hog1 – Ptp2 and Ptp3 – also regulate Hog1 localization by a mechanism that does not involve their catalytic activity [57]. Mutation of the cytoplasmic phosphatase gene *PTP3* increases nuclear localization of Hog1, whereas *PTP3* overexpression causes cytoplasmic retention of Hog1. By contrast, mutation of *PTP2* increases the amount of

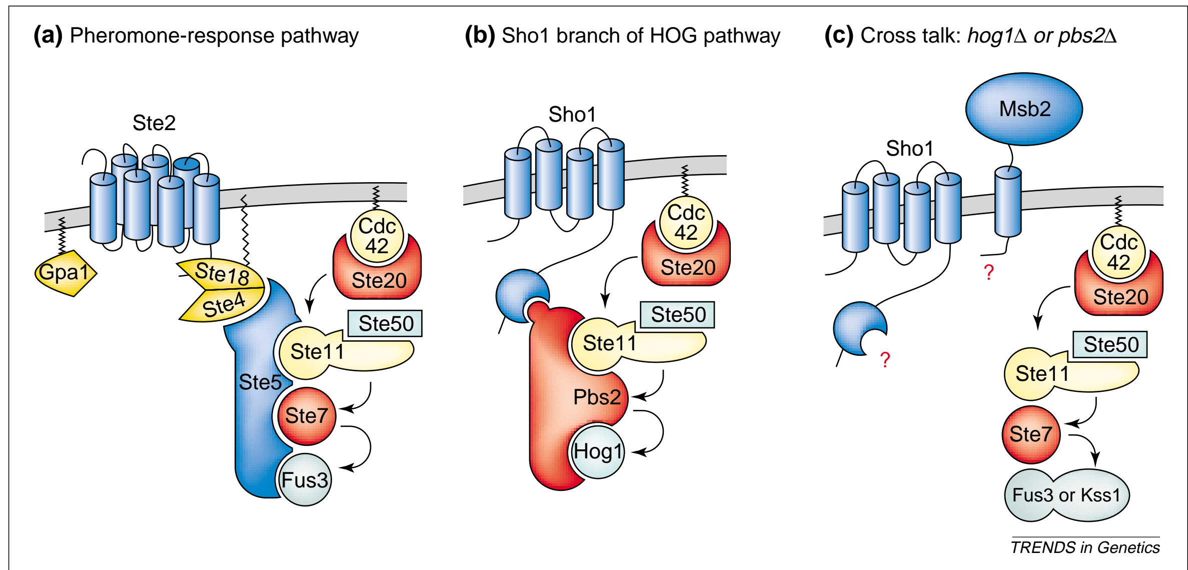

Fig. 5. Components of the Sho1 branch of the HOG pathway participate in different MAPK pathways. (a) In the pheromone-response MAPK pathway, a peptide mating pheromone stimulates a G-protein-coupled receptor (Ste2) and a heterotrimeric G protein (Gpa1–Ste18–Ste4). The Ste5 protein is recruited to the membrane through interaction with the G protein. Ste5 also binds to the MAPK cascade kinases (Ste11, Ste7 and Fus3). (b) The Sho1 branch of the HOG pathway resembles the pheromone-response pathway in that Pbs2 is recruited to the membrane and that Pbs2 interacts with other kinases of the MAPK cascade. (c) The Sho1–Ste12 cross-talk pathway operates in *hog1* and *pbs2* mutants, and perhaps in wild-type cells during various other conditions (e.g. to promote pseudohyphal development or to monitor glycosylation). The transmembrane proteins Sho1 and Msb2 can each stimulate the Ste11–Ste7–Fus3 (or Kss1) module to induce transcription of pheromone-response and filamentous genes through the transcription factor Ste12 (not shown). How Ste11 is activated in the absence of Pbs2 by Sho1 and Msb2 remains unknown (red question marks). Abbreviations: Hog, high osmolarity glycerol; MAPK, mitogen-activated protein kinase.

cytoplasmic Hog1, and *PTP2* overexpression retains Hog1 in the nucleus (even without osmotic shock). Retention of Hog1 in different cellular compartments could, therefore, specify substrate accessibility.

The transcriptional regulatory proteins that control osmotic-responsive gene expression have also been localized (Fig. 4). The repressor Sko1 is localized in the nucleus in cells cultured in media of normal osmolarity or media supplemented with 0.4 M NaCl, but is rapidly translocated to the cytoplasm during severe salt stress (1 M NaCl) [43]. Interestingly, cytoplasmic transfer is mediated not by Hog1 phosphorylation but by lack of PKA phosphorylation [48]. Msn2 and Msn4 are also localized via PKA-dependent phosphorylation. In optimal growth conditions, Msn2 and Msn4 are cytoplasmic; they translocate to the nucleus to regulate gene expression under osmotic, or other, stress conditions [48]. The transcription factor Hot1 is a nuclear protein whose distribution is not modulated by osmotic stress [46], and the related Msn1 protein is known to be nuclear in nonstressed cells [58].

Inactivation of Hog1 by phosphatases

Once Hog1 has performed its function after osmotic shock, it is important that signaling through this pathway be brought back to a basal level so that the cell is prepared for further osmotic challenge. In addition, constitutive activation of the HOG pathway causes inviability [22,34,37,59,60]. Similar to other MAPK cascades, phosphatases decrease signaling in the HOG pathway by dephosphorylating Hog1 [37,60,61]. This type of inhibition occurs during basal conditions and during adaptation to osmotic stress. Ptp2 and Ptp3 are protein-tyrosine phosphatases that remove phosphate groups from Tyr176 of Hog1 [37,60]. Mutation of both *PTP2* and *PTP3* results in constitutively phosphorylated Hog1 (i.e. phosphorylation in the absence of osmotic stress). Ptc1 is a serine/threonine-specific phosphatase that acts on Thr174 of Hog1 [61]. Mutation of *PTC1* constitutively activates Hog1. Thus, both protein-tyrosine and protein-threonine phosphatases are important for limiting the activity of Hog1, even under basal conditions.

Limiting cross-talk to other MAPK pathways

Yeast contain at least five distinct MAPK pathways [62]. Of these, the pheromone-response, filamentous growth and HOG MAPK pathways use the MAPKKK Ste11 [62] (Fig. 5). In wild-type cells, there is little cross-activation of the pheromone-response or filamentous MAPK pathways by increased osmolarity [34,36,63]. Cross-talk is evident, however, when *HOG1* or *PBS2* is mutated [19,63–66]. The cross-talk circuit that operates in *hog1* and *pbs2* mutants was traced from the transmembrane proteins Sho1 and Msb2 to the MAPKs Fus3 and Kss1 (Fig. 5c). In *hog1* and *pbs2* mutants, Sho1 and Msb2 stimulate Ste50, Ste20, Ste11, Ste7, the MAPKs Fus3 and Kss1, and Ste12; this ultimately induces transcriptional targets of the pheromone-response and filamentous pathways [19,21,63].

There are two notable features of the cross-talk pathway that could reveal insights into important signaling mechanisms also used by wild-type cells:

- Sho1 is competent for signaling to Ste20–Ste50–Ste11 independently of the Sho1 SH3–Pbs2 polyproline interaction (as cross-talk occurs in *pbs2Δ* cells). This result implies that multiple mechanisms exist for Sho1 to provide input to Ste11.
- The scaffold proteins Pbs2 and Ste5 are not sufficient for insulating the MAPK pathways, as their presence does not hinder cross-activation of Ste7 by osmotic stress (as cross-talk occurs in *PBS2⁺ STE5⁺ hog1Δ* cells) [19,63,64].

Several mechanisms have been proposed to play a role in maintaining pathway specificity (which is abolished in *hog1* mutants). One proposal is that Pbs2 acts as a scaffold protein and thus tethers multiple components of the HOG pathway to limit cross-talk [34]. According to this view, formation of a complex prevents activated Ste11 from gaining access to inappropriate MAPKK targets. This idea is supported by a study from Harris *et al.*, in which fusions between Ste11 and either of its downstream MAPKK targets, Ste7 or Pbs2, were engineered and assayed for Ste11 activity *in vivo* [67]. Covalent attachment of Ste7 to Ste11 allows only mating functions, whereas fusion of Pbs2 to Ste11 allows only HOG pathway functions. Thus, robust protein–protein complexes can specify which signal transduction circuit is activated. Another model suggests that activation of the HOG pathway triggers the activity of phosphatases, which then feedback to inhibit other MAPK pathways; for example, by dephosphorylating Fus3 and Kss1 [63,64]. Such phosphatase activity has, however, not been demonstrated in wild-type cells. Yet another mechanism has been proposed involving Hog1 feedback that inhibits the Sho1 branch after osmotic stress (perhaps even acting on Sho1 itself), although such regulation has not been directly demonstrated [19]. Finally, van Drogen and Peter propose that MAPKs feedback to inhibit activated Ste11 [68]. This hypothesis would explain the inability to observe accumulation of Ste11 at signaling sites during the mating response (as is seen for the Ste5, Ste7 and Fus3 members of the pheromone-response MAPK pathway) [69], and also the observation that activated Ste11 is rapidly degraded in a MAPK-dependent manner (F. van Drogen, pers. commun.). Ste11 destruction would clearly limit the activation of spatially distant MAPKKs.

The cross-talk pathways that operate in mutant strains could reveal authentic pathways and connections used under different circumstances. Indeed, protein glycosylation defects activate transcription of *FUS1*, *FKS2* and, presumably, other Ste12 target genes, perhaps to remodel the cell wall [70,71]. Also, Sho1, Ste20, Ste50 and Ste11 are necessary for pseudohyphal development [19]. It therefore appears that yeast uses the five basic MAPK cascades, and variant cascades comprising different elements of the basic MAPK cascades, for diverse responses.

Conclusion

Studies of the *S. cerevisiae* response to elevated osmolarity have revealed both the simplicity and the complexity of signaling in eukaryotic cells. The progress in our understanding of the budding yeast p38/HOG MAPK pathway might provide insights that are useful to researchers studying even more complex metazoan signaling pathways. However, some important questions regarding osmosensing MAPK pathways remain unanswered. For example, what mechanisms are used to detect changes in osmolarity? What is the benefit of having multiple upstream branches to the HOG pathway? How is osmolarity sensed in animal cells without Sln1 and Sho1 homologs?

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