# Response to Hyperosmotic Stress

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**ABSTRACT** An appropriate response and adaptation to hyperosmolarity, *i.e.*, an external osmolarity that is higher than the physiological range, can be a matter of life or death for all cells. It is especially important for free-living organisms such as the yeast *Saccharomyces cerevisiae*. When exposed to hyperosmotic stress, the yeast initiates a complex adaptive program that includes temporary arrest of cell-cycle progression, adjustment of transcription and translation patterns, and the synthesis and retention of the compatible osmolyte glycerol. These adaptive responses are mostly governed by the high osmolarity glycerol (HOG) pathway, which is composed of membrane-associated osmosensors, an intracellular signaling pathway whose core is the Hog1 MAP kinase (MAPK) cascade, and cytoplasmic and nuclear effector functions. The entire pathway is conserved in diverse fungal species, while the Hog1 MAPK cascade is conserved even in higher eukaryotes including humans. This conservation is illustrated by the fact that the mammalian stress-responsive p38 MAPK can rescue the osmosensitivity of *hog1*Δ mutations in response to hyperosmotic challenge. As the HOG pathway is one of the best-understood eukaryotic signal transduction pathways, it is useful not only as a model for analysis of osmostress responses, but also as a model for mathematical analysis of signal transduction pathways. In this review, we have summarized the current understanding of both the upstream signaling mechanism and the downstream adaptive responses to hyperosmotic stress in yeast.

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*SACCHAROMYCES* (literally, sugar yeast) thrive, in their natural habitat, on decomposing fruits, including grape, where sugar (such as glucose, fructose, and sucrose) is abundant. As the fruits dry, the sugar concentration may approach its saturation point. This high sugar concentration poses a dilemma to the yeast, as the abundant food also brings unfavorable osmotic conditions that are a potential threat to their survival. Increased external osmolarity induces water efflux, an increased concentration of cytosolic ions (especially $Na^+$), and cell shrinkage, which are all detrimental to cell growth [for general biological effects of osmostress, see Wood (1999, 2011)]. Amazingly, yeast can grow and vigorously ferment in media containing as much as 40% (2.2 M) glucose (Watanabe *et al.* 2010), which is obviously a highly dangerous osmotic condition.

Therefore, to cope with such an increased external osmolarity, yeast initiates a complex adaptive program that includes temporary arrest of cell-cycle progression, adjustment of transcription and translation patterns, and the synthesis and retention of the compatible osmolyte glycerol (Figure 1). These adaptive responses are mostly governed by the high osmolarity glycerol (HOG) signaling pathway,

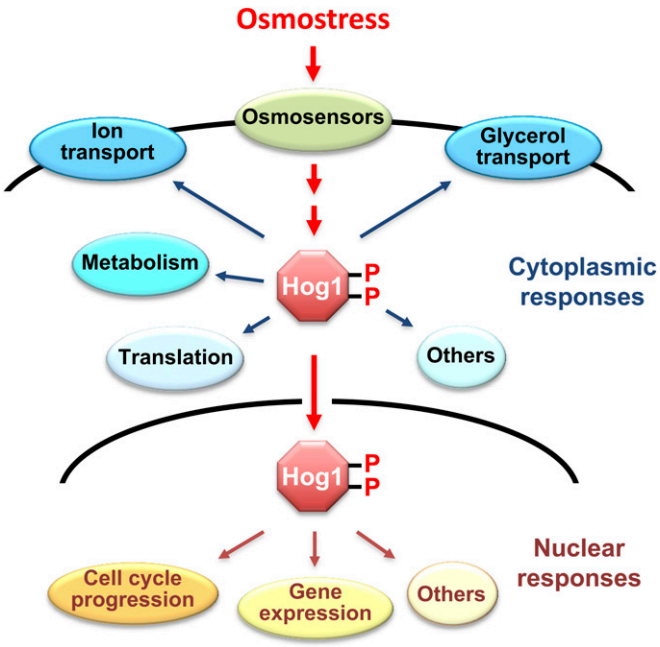


**Figure 1** Osmo-adaptive responses in yeast. In response to an increase in extracellular osmolarity, the Hog1 MAPK is activated, which leads to the induction of cytoplasmic and nuclear adaptive responses. Cytoplasmic responses include the control of ionic fluxes and glycerol transport, metabolic enzymes, and protein translation. Nuclear responses include the modulation of cell-cycle progression and the control of gene expression.

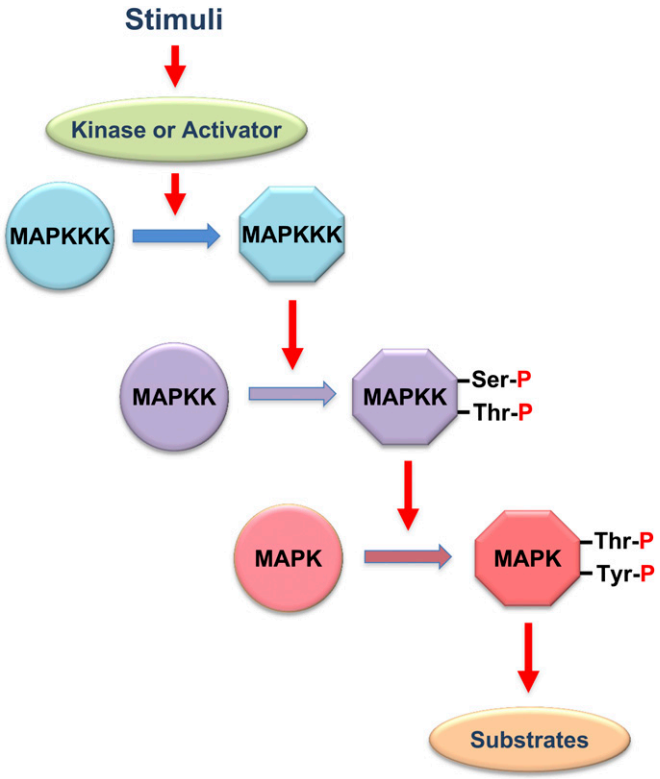


**Figure 2** A schematic diagram of the MAP kinase module. Circles and hexagons represent, respectively, inactive and active forms of kinases. MAPK, MAP kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase.

whose core is the Hog1 MAP kinase (MAPK) cascade. In this review, we have summarized the current, often fragmentary, understanding of both the upstream signaling mechanism of osmostress and the downstream adaptive responses. Because the HOG pathway is highly conserved across fungal species, elucidation of the signaling and effector mechanisms in *Saccharomyces cerevisiae* will be highly relevant to the studies of other yeasts and fungi (Krantz *et al.* 2006a,b). We endeavored to be as comprehensive as possible, but due to space limitations, many interesting subjects had to be left out. Readers who are interested in various aspects of yeast osmostress responses are encouraged to consult a number of excellent review articles (Gustin *et al.* 1998; Sprague 1998; Chellappan 2001; Hohmann 2002a,b, 2009; O'Rourke *et al.* 2002; Saito and Tatebayashi 2004; Schwartz and Madhani 2004; Sheikh-Hamad and Gustin 2004; Chen and Thorner 2007; Hohmann *et al.* 2007; de Nadal and Posas 2010).

## Upstream Signaling Mechanisms

### *Overview of the HOG pathway*

The central core of the HOG pathway is the Hog1 MAPK cascade. MAPK cascades are evolutionarily conserved signaling units that are utilized in many intracellular signal transduction pathways in diverse eukaryotic organisms, including fungi and yeast (Chen *et al.* 2001). Each MAPK cascade is composed of three sequentially activating kinases (Figure 2). A MAPK is activated by a MAPK kinase (MAPKK) by dual phosphorylation of the conserved Thr and Tyr residues in the TXY motif within the activation loop. A MAPKK is similarly activated by a MAPKK kinase (MAPKKK) by phosphorylation of the Ser/Thr residues in its activation loop. The first kinase of the cascade, MAPKKK, is activated either by phosphorylation by an upstream kinase, sometimes called MAPKKKK, or by binding of an activator protein, depending on the pathway. Each MAPK module is activated by specific types of stimuli and induces specific adaptive responses.

The upstream part of the HOG pathway comprises the functionally redundant, but mechanistically distinct, Sln1 and Sho1 branches (Figure 3). A signal emanating from either branch converges on a common MAPKK, Pbs2, which is the specific activator of the Hog1 MAPK (Brewster *et al.* 1993; Maeda *et al.* 1994). The Sln1 branch activates the redundant Ssk2 and Ssk22 MAPKKKs, which then activate Pbs2 (Maeda *et al.* 1995). The Sho1 branch activates the Ste11 MAPKKK, which also activates Pbs2 (Posas and Saito 1997). Thus, a mutant that lacks both the *SSK2* and *SSK22* genes (an *ssk2*Δ *ssk22*Δ mutant) is totally dependent on the Sho1 branch for activation of the Hog1 MAPK, whereas a mutant that lacks *STE11* is dependent on the Sln1 branch. Once activated, a substantial fraction of the Hog1 MAPK is transported into the nucleus where it regulates transcription and the cell cycle, although there are also Hog1 targets in the cytoplasm. As adaptation proceeds, and osmotic balance is re-established, Hog1 activity goes down to near basal

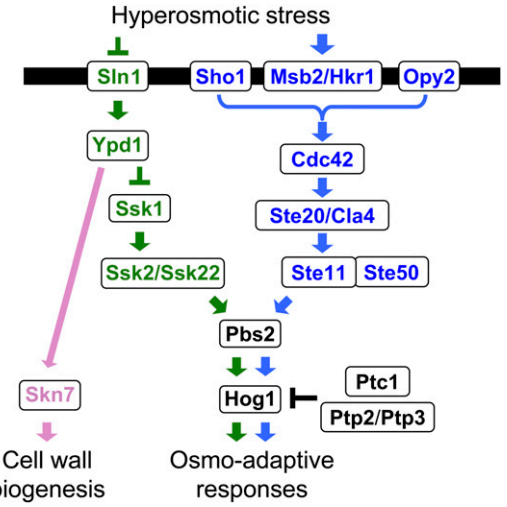


**Figure 3** A schematic diagram of the yeast HOG pathway. The protein names separated by a thrash (/) are functionally redundant. Proteins that are specific to the Sln1 branch are colored green, those that are specific to the Sho1 branch are colored blue, and those that are common are colored black. The black horizontal bar represents the plasma membrane. Arrows indicate activation, whereas the T-shaped bars represent inhibition.

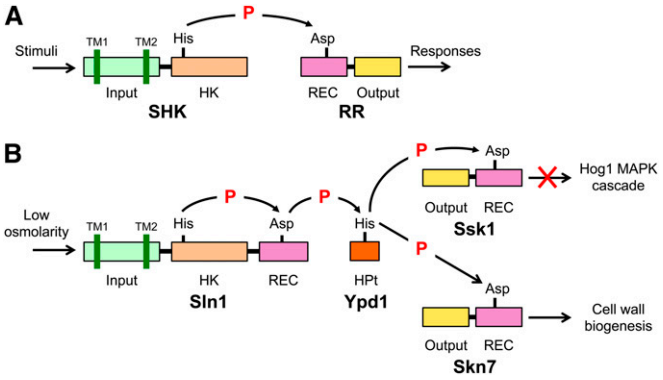


**Figure 4** Schematic diagram of two-component signaling systems. (A) The prototypical two-component system that is characterized by the conserved phosphotransfer reaction between a histidine residue and an aspartate residue. (B) The Sln1-Ypd1-Ssk1 multistep phosphorelay. SHK, sensor histidine kinase; RR, response regulator; HK histidine kinase domain; REC, receiver domain; HPt, histidine-containing phospho-transfer protein; TM, transmembrane segment; P, phosphoryl group.

levels, and Hog1 is exported back to the cytoplasm. Thus, there are mechanisms that control Hog1 nuclear import/export, as well as downregulation of Hog1 activity.

There are several other signal pathways that utilize a MAPK cascade in yeast, which are involved in the mating response, filamentous and invasive growth (FIG), and regulation of cell-wall biogenesis. Surprisingly, three of these pathways (HOG, mating, and FIG) share many of the same signaling elements, including the Ste11 MAPKKK. Thus, it is important to prevent signal leakage from one pathway into another pathway. This aim seems to be attained by insulation and exquisite network of reciprocal cross-regulation among the signaling pathways.

### *Sln1 branch of the HOG pathway*

***Two-component signal transduction system:*** The Sln1 branch of the HOG pathway is a variation of the so-called two-component system. Two-component systems are ubiquitous in prokaryotes, plants, and fungi (for comprehensive reviews, see Stock *et al.* 2000; Gao and Stock 2009; Casino *et al.* 2010; Schaller *et al.* 2011). As the name implies, the prototypical two-component system is composed of two proteins (Figure 4A): the first is a sensor histidine kinase (SHK) that contains an input (or sensor) domain, a HK catalytic domain, and a histidine auto-phosphorylation site, and the second is a response regulator (RR) that contains an output (or effector) domain and a receiver (REC) domain. When the input domain senses a relevant stimulus, the HK is activated (or inactivated), and a histidine residue located near the HK domain is phosphorylated (or dephosphorylated). This phosphoryl group is then transferred to the acceptor aspartate residue in the REC domain of a cognate RR. This phosphotransfer reaction is termed the His-Asp phosphorelay. Because both histidine phosphate and aspartate phosphate are energetically activated, they are often symbolized as His∼P and Asp∼P. In bacteria, numerous simple two-component systems exist that are composed of an SHK and a cognate RR. However, there are also more complex variations of this theme, where the basic His-Asp phosphorelay reaction is repeated twice so that a phosphoryl group is transferred sequentially through a His-Asp-His-Asp multistep phosphorelay (Figure 4B). In a complex two-component system, a phosphoryl group is initially transferred from a HK domain to a cognate REC domain as in the simple systems. This phosphoryl group, however, is then transferred to an intermediate phospho-carrier termed histidine-containing phospho-transfer (HPt) protein, which catalyzes specific phospho-transfer reactions between two REC domains. The phosphoryl group is then transferred from HPt to a second REC domain. The Sln1 branch of the yeast HOG pathway is an example of complex two-component systems (Posas *et al.* 1996; Saito 2001). In the budding yeast, there are three REC proteins (Sln1, Ssk1, and Skn7), but only one SHK (Sln1) and one HPt (Ypd1). In fact, Sln1 governs two distinct signaling pathways: the Sln1-Ypd1-Ssk1 multistep phosphorelay, which regulates hyper-osmolarity responses, and the Sln1-Ypd1-Skn7 multistep phosphorelay, which makes a contribution to hypo-osmolarity responses.

***Sln1-Ypd1-Ssk1 multistep phosphorelay:*** The N-terminal half of Sln1 is the sensor domain that is composed of an extracellular domain (ECD) flanked by two transmembrane segments, TM1 and TM2 (Ota and Varshavsky 1993; Maeda *et al.* 1994). The C-terminal half is composed of a HK domain and a REC domain; hence Sln1 is termed a "hybrid histidine kinase." When activated, the Sln1 HK auto-phosphorylates His-576 near the HK domain, using ATP as a phospho-donor (Posas *et al.* 1996). This phosphoryl group is then transferred to Asp-1144 in the Sln1 REC domain. It is likely that the HK

catalytic site of one molecule phosphorylates the His phosphorylation site in another molecule in an Sln1 dimer. The phosphate is then transferred to His-64 of Ypd1, an HPt protein. The phosphoryl group on Ypd1 is finally transferred to Asp-554 in the REC domain of Ssk1.

***Regulation of Sln1 HK activity:*** Genetic analyses of various mutants in the Sln1 pathway suggest that the Sln1 HK domain is catalytically active under normal osmotic conditions, whereas it is inactivated when the environmental osmolarity is increased (Maeda *et al.* 1994; Fassler and West 2010). *In vitro* reconstitution of the Sln1-Ypd1-Ssk1 multistep phosphorelay reactions supports the same conclusion (Posas *et al.* 1996). As expected, the ECD and its flanking transmembrane (TM) domains are important for regulation of the HK activity. For example, deletion of TM1 constitutively activates, whereas removal of both TM1 and ECD inactivates, Sln1 HK (Ostrander and Gorman 1999). *In vivo*, Sln1 seems to respond to changes in turgor pressure (the pressure exerted by water inside the cell against the cell wall). When yeast is exposed to high external osmolarity, turgor pressure decreases as the cytoplasm shrinks. An earlier study suggested that turgor change rather than water loss activates the HOG pathway (Tamás *et al.* 2000), which was later supported by biophysical analyses (Schaber *et al.* 2010). Consistent with these findings, Sln1 HK activity is inhibited when turgor is reduced by the antifungal antibiotic nystatin or by enzymatic removal of the cell wall (Reiser *et al.* 2003). Conversely, Sln1 HK activity is enhanced by increased turgor pressure caused by raised intracellular glycerol concentration (Tao *et al.* 1999). In a more recent study, it was found that the presence of the abundant GPI-anchored cell-wall mannoprotein Ccw12 has a role in Sln1 HK activation (Shankarnarayan *et al.* 2008). These results suggest that Sln1 responds to osmolarity-induced changes in the cell wall. On the other hand, it was also found that the Sln1 branch of the HOG pathway is activated when membrane fluidity is reduced by a rapid downshift in temperature to $<10°$ or by dimethyl sulfoxide treatment (Hayashi and Maeda 2006; Panadero *et al.* 2006). Hypoxia also activates the Sln1 branch, perhaps by an altered membrane fluidity caused by depletion of heme and ergosterol (Hickman *et al.* 2011). These results suggest that Sln1 might respond to changes in the plasma membrane. Cold activation of the HOG pathway might be physiologically important because Hog1-dependent accumulation of glycerol would protect yeast from freezing. In any case, it is clear that further studies are needed to establish the biophysical nature of the stimuli that control Sln1 activity.

***HPt protein Ypd1:*** Ypd1 is a small protein of 167 aa and is composed of a four-helix bundle with the phospho-accepting histidine (His-64) in the middle of the third helix (Song *et al.* 1999; Xu and West 1999). Ypd1 interacts with three different REC domains, one each in Sln1, Ssk1, and Skn7. A systematic Ala-scanning mutagenesis of Ypd1 coupled to two-hybrid interaction analyses indicated that the REC domains of Sln1, Ssk1, and Skn7 interact with Ypd1 at overlapping binding sites (Porter *et al.* 2003; Porter and West 2005). The $\alpha$1 helix of the Ssk1 REC domain was identified as the interaction site with Ypd1 by isolation of Ssk1 mutants that cannot interact with Ypd1 (Horie *et al.* 2008). The structure of a complex between Ypd1 and the REC domain of Sln1 is consistent with these mutational studies (Xu *et al.* 2003; Zhao *et al.* 2008).

Phosphotransfer reactions involving wild-type Ypd1 are very rapid, reaching steady-state levels in $<5$ sec *in vitro* (Janiak-Spens and West 2000). Thus, detailed kinetic analyses are possible only by using a rapid quench flow apparatus (Kaserer *et al.* 2010). Perhaps the most important finding is that phosphotransfer from Ypd1∼P to Ssk1 is both very rapid (160 $sec^{-1}$) and irreversible, whereas that from Ypd1∼P to Skn7 is slower (1.4 $sec^{-1}$) and readily reversible (Janiak-Spens *et al.* 2005). These and other kinetic properties of Ypd1 are consistent with the notion that Ssk1 is constitutively phosphorylated under normal osmotic conditions.

***Activation of the Ssk2/Ssk22 MAPKKKs by Ssk1:*** Ssk1 activates a pair of homologous, and functionally redundant, MAPKKKs termed Ssk2 and Ssk22 (Maeda *et al.* 1995). Like many other members of the MAPKKK family, the kinase catalytic domain of Ssk2/Ssk22 is near the C-terminal end, and there is an auto-inhibitory domain (AID) in the N-terminal region. Ssk1 binds to the N-terminal region of Ssk2/Ssk22, and, perhaps by conformational change, relieves the catalytic domain from inhibition by the AID (Posas and Saito 1998). Since the Sln1 HK is active under normal osmotic conditions, Ssk1 is constitutively phosphorylated by Ypd1∼P. However, under hyperosmotic conditions, unphosphorylated Ssk1-OH will accumulate, and it binds and activates Ssk2/Ssk22. Consistent with this notion, expression of unphosphorylatable Ssk1 mutants such as Ssk1-D544S or Ssk1 mutants that cannot interact with Ypd1 (and thus cannot accept phosphate from Ypd1∼P), such as Ssk1-I514T, hyperactivate the Hog1 MAPK cascade (Horie *et al.* 2008).

Asp∼P is chemically unstable and is spontaneously hydrolyzed. Indeed, the half-life of purified Ssk1∼P is only ∼13 min *in vitro* (Janiak-Spens *et al.* 2000). If it is similarly unstable in cells, then it is unlikely that all of the Ssk1 is stably converted to Ssk1∼P, and therefore there is a possibility that persistent Ssk1-OH would activate the Hog1 MAPK cascade in the absence of any osmotic stimulation. However, several mechanisms exist that prevent erroneous activation of the Hog1 MAPK cascade. First, the half-life of Ssk1∼P dramatically increases to 40 hr when Ypd1 is included in the incubation reaction *in vitro* (Janiak-Spens *et al.* 1999). It was proposed that Ypd1 forms a stable complex with Ssk1∼P and sterically shields the phosphorylated Asp residue from hydrolysis (Janiak-Spens *et al.* 2000). Such enhanced stability of Ssk1∼P would maintain the levels of Ssk1-OH in unstimulated cells at a level low enough that inadvertent activation of the Hog1 MAPK cascade would be

prevented. Second, any residual Ssk1-OH that may still exist would not contribute significantly to Ssk2/Ssk22 activation because only a doubly dephosphorylated Ssk1 dimer, $(Ssk1\text{-}OH)_2$, can activate Ssk2 and Ssk22 (Horie *et al.* 2008). For example, when 1% of Ssk1 is dephosphorylated, only 0.01% of Ssk1 dimer is doubly dephosphorylated. Third, Ssk1-OH is degraded by a ubiquitin-proteasome-dependent mechanism, which may serve as an additional safeguard against spontaneous activation of Ssk2/Ssk22 in the absence of osmostress (Sato *et al.* 2003). Finally, it should be noted that there is in fact a low basal signaling in the Sln1 pathway in the absence of any external stimulation, which may allow more rapid response upon osmostress (Macia *et al.* 2009).

Although stable Ssk1∼P is required to prevent spontaneous Hog1 activation under nonstimulated conditions, it causes another difficulty under high-osmolarity conditions. When yeast is exposed to hyper-osmolarity, activation of the Hog1 MAP kinase cascade occurs within minutes, which requires a much faster dephosphorylation of Ssk1∼P than the observed half-life of 40 hr *in vitro* in the presence of Ypd1. Higher osmolyte concentrations decrease the Ssk1∼P half-life by two-fold in *in vitro* reactions, but this modest effect alone would not be sufficient to account for the rapid *in vivo* activation of the MAPK cascade (Kaserer *et al.* 2009). Therefore, the dephosphorylation of Ssk1∼P might be accelerated under stress conditions *in vivo*, perhaps by an as-yet-unidentified phosphatase.

The actin cytoskeleton is important for the survival of yeast under osmostress, as many mutations in actin cause osmosensitivity (Wertman *et al.* 1992). Hyperosmotic stress causes a rapid disassembly of actin cables, followed by depolarization of actin patches leading to a cell-cycle delay (Chowdhury *et al.* 1992). The reassembly of the actin cytoskeleton occurs only after osmotic balance is re-established (Brewster and Gustin 1994). During osmostress, Ssk2 concentrates in the neck of budding cells and forms a complex with actin, and following reestablishment of osmotic balance, Ssk2 promotes actin cytoskeleton recovery (Yuzyuk *et al.* 2002). This recovery mechanism requires a polarized distribution of Ssk2, its actin-interacting activity and its kinase catalytic activity, but, interestingly, does not require Ssk1 (Yuzyuk and Amberg 2003; Bettinger *et al.* 2007). Although Ssk1 is the only known activator of Ssk2/Ssk22, osmostress does cause slight activation of the Hog1 MAPK in *ssk1*Δ *sho1*Δ mutants, whereas no activation is observed in *ssk2*Δ *ssk22*Δ *sho1*Δ mutants (Maeda *et al.* 1994; Reiser *et al.* 2000). These findings suggest that there may be an as-yet-unknown mechanism that can activate Ssk2/Ssk22 without Ssk1.

***Ssk2/Ssk22-Pbs2-Hog1 kinase cascade:*** Once activated, the Ssk2/Ssk22 MAPKKK initiates a kinase cascade reaction that involves the Pbs2 MAPKK and the Hog1 MAPK (Boguslawski 1992; Brewster *et al.* 1993). Although there are several other MAPKKs and MAPKs in yeast with similar sequences, activated Ssk2/Ssk22 exclusively phosphorylates, and thereby activates, Pbs2, and activated Pbs2 phosphorylates only Hog1. These specific interactions are due to the presence of specific docking sites in Pbs2. An Ssk2/Ssk22-specific docking site is located in the Pbs2 N-terminal regulatory region (Tatebayashi *et al.* 2003). Fusion of this Pbs2 docking site to the Ste7 MAPKK, which is not a substrate of Ssk2/Ssk22, allows phosphorylation of Ste7 by Ssk2/Ssk22. Pbs2 has two specific binding sites for Hog1: one is in the N-terminal regulatory region, and another is near the C terminus (Murakami *et al.* 2008).

The activity of wild-type Hog1 is absolutely dependent on double phosphorylation of its TGY motif by Pbs2. However, several Hog1 mutants that are partially active without any phosphorylation by Pbs2 have been isolated (Bell *et al.* 2001; Bell and Engelberg 2003). By using these mutants, Hog1-dependent effects can be studied without exposing cells to osmostress, which would induce both Hog1-dependent and -nondependent effects (Yaakov *et al.* 2003).

Stress-responsive MAPK cascades that are homologous to the Hog1 MAPK cascade are found in both lower and higher eukaryotes (Sheikh-Hamad and Gustin 2004). For example, the mammalian stress-responsive p38 MAPK is structurally highly similar to Hog1, and p38 can complement mutant strains of yeast that lack the Hog1 MAPK (Han *et al.* 1994). Also, the kinase domain of the mammalian stress-responsive MAPKKK termed MTK1 (also known as MEKK4) is highly similar to the kinase domains of Ssk2 and Ssk22, and expression of constitutively active MTK1-ΔN can complement the *ssk2*Δ *ssk22*Δ double mutation (Takekawa *et al.* 1997). MTK1 is activated by binding of its specific activator, Gadd45, in a manner similar to activation of Ssk2 and Ssk22 by Ssk1, although these activators are unrelated and not functionally exchangeable (Takekawa and Saito 1998; Mita *et al.* 2002; Miyake *et al.* 2007).

***Sln1-Ypd1-Skn7 multistep phosphorelay:*** Ypd1 donates its phosphoryl group not only to Ssk1 but also to Skn7 (Figure 4B). Skn7 is composed of an N-terminal DNA-binding domain and a C-terminal REC domain and is highly conserved among fungi (Brown *et al.* 1994). A phosphotransfer reaction from Sln1 to Skn7 via the intermediary Ypd1 was demonstrated *in vitro* (Li *et al.* 1998; Ault *et al.* 2002). Although Skn7 is exclusively localized in the nucleus and Ssk1 is mostly in the cytoplasm, Ypd1 is found in both the nucleus and the cytoplasm, which is consistent with its ability to transfer phosphate to both Skn7 and Ssk1 (Lu *et al.* 2003). The Sln1-Ypd1-Skn7 phosphorelay regulates a response that is complementary to that of the Sln1-Ypd1-Ssk1 phosphorelay: whereas Ssk1 is activated under hyperosmotic conditions, Skn7 is activated under hypo-osmotic conditions. Skn7 regulates oxidative stress-responsive genes, and *skn7*Δ mutants are hypersensitive to oxidative stresses such as exposure to hydrogen peroxide (Krems *et al.* 1996; Raitt *et al.* 2000a). However, the role of Skn7 in oxidative responses is not dependent on Sln1, and the phospho-accepting Asp-427 of Skn7 is not required (Morgan *et al.* 1997; He *et al.* 2009).

In contrast, induction of hypo-osmostress responsive genes, such as *OCH1*, is dependent on Sln1 and requires the Asp-427 of Skn7 (Ketela *et al.* 1998; Li *et al.* 2002; Shankarnarayan *et al.* 2008). *OCH1* encodes the mannosyltransferase in the *cis*-Golgi apparatus that initiates N-linked glycosylation of secreted/membrane proteins and thus is a key enzyme in cell-wall maintenance. Although the *skn7*Δ mutants are not osmosensitive, the suppression of the hypo-osmotic stress sensitivity of a *pkc1*Δ mutant by *SKN7* overexpression suggests that Skn7 and the PKC pathway coordinately regulate cell-wall integrity that is critical for growth under hypo-osmotic conditions (Brown *et al.* 1994). For more details on Skn7, see a recent comprehensive review by Fassler and West (2011).

### *Sho1 branch of the HOG pathway*

Unlike the Sln1 branch, which is a variation of the well-understood two-component paradigm, the activation mechanism of the Sho1 branch is still only vaguely defined. Although many important observations have been made, there is still a lack of a unifying mechanism that incorporates all of the separate facts. Thus, we will first present an overview of the current hypothesis of how the Sho1 branch might be activated and will then discuss the details of individual steps in the following sections.

***Overview:*** A signaling response in the Sho1 branch is initiated by the putative osmosensors Msb2 and Hkr1, which are highly glycosylated single-pass TM proteins (Tatebayashi *et al.* 2007). Through an as-yet-undefined mechanism that seems to involve an interaction between the Msb2/Hkr1 osmosensors and the Sho1 co-osmosensor, this response leads to activation of the PAK-like kinases Ste20 and Cla4 by inducing their association with the membrane-bound small G-protein Cdc42 (Lamson *et al.* 2002). Activated Ste20/Cla4 then phosphorylates and activates the Ste11 MAPKKK (Raitt *et al.* 2000b; van Drogen *et al.* 2000), which in turn phosphorylates and activates the Pbs2 MAPKK that is associated with the Sho1 membrane anchor (Maeda *et al.* 1995; Tatebayashi *et al.* 2006). Because both the Cdc42-Ste20 and the Sho1-Pbs2 complexes are localized on the membrane, Ste11 must also be localized to the membrane so that efficient activator/substrate interactions between Ste20 and Ste11, as well as between Ste11 and Pbs2, can take place. Membrane localization of Ste11 is mediated by the Ste50 adaptor protein, which forms a stable complex with Ste11 (Posas *et al.* 1998; Wu *et al.* 1999), primarily via association of Ste50 with the membrane anchor protein Opy2 (Ekiel *et al.* 2009; Yamamoto *et al.* 2010), and secondarily by Ste50–Cdc42 and Ste50–Sho1 interactions (Tatebayashi *et al.* 2006; Truckses *et al.* 2006). Activation of the Hog1 MAPK by Pbs2 seems to proceed as in the Sln1 branch.

***Putative osmosensors Msb2 and Hkr1:*** Both Msb2 and Hkr1 are highly glycosylated single-path transmembrane proteins (Figure 5). The extracellular domains of these

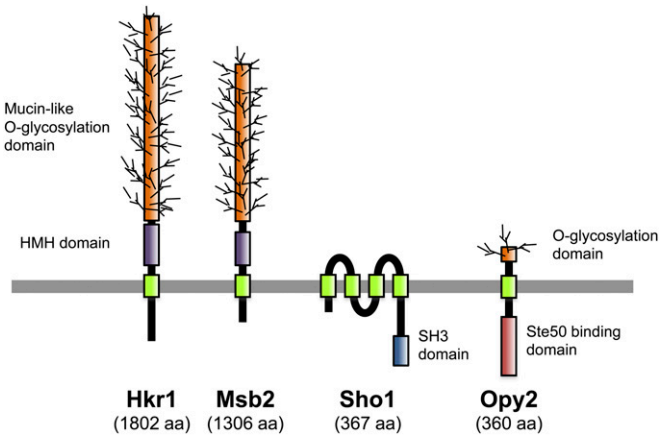


**Figure 5** Schematic representations of the four transmembrane proteins involved in the Sho1 branch of the HOG pathway. HMH, Hkr1-Msb2 homology domain. Not drawn to scale.

proteins are highly Ser/Thr rich and contain numerous *O*-glycosylation sites that are glycosylated by the protein *O*-mannnosyl transferase Pmt4 (Yang *et al.* 2009). The *MSB2* gene was originally identified as a multicopy suppressor of a *cdc24* mutant (Bender and Pringle 1989). Since Cdc24 is a guanine exchange factor for Cdc42, it is believed that Msb2 somehow regulates the activity of Cdc24 or Cdc42. Indeed, a weak binding between Msb2 and Cdc42 has been observed (Cullen *et al.* 2004). However, how Msb2 controls Cdc42 activity is unclear.

The possible involvement of Msb2 in the HOG pathway was initially suggested by the observation that the weak osmo-tolerance of the *ssk1*Δ *sho1*Δ mutant was abolished in the *ssk1*Δ *sho1*Δ *msb2*Δ triple mutant (O'Rourke and Herskowitz 2002). This observation was interpreted at that time as indicating that Msb2 is a third osmosensor in the HOG pathway (Sln1 and Sho1 being the other two). A later study, however, revealed that Msb2 and another transmembrane glycoprotein, Hkr1, are the more likely osmosensors in the Sho1 branch, but that Sho1 itself has a downstream function as a co-osmosensor (Tatebayashi *et al.* 2007). This conclusion is partly based on genetic epistasis tests that indicated that *MSB2/HKR1* functions upstream of *SHO1*: a constitutively active *SHO1* mutant can activate Hog1 MAPK even in the *msb2*Δ *hkr1*Δ double-mutant cells, but a constitutively active *MSB2* or *HKR1* mutant cannot activate Hog1 in a *sho1*Δ mutant.

The Ser/Thr-rich glycosylation domains of Msb2 and Hkr1 have a negative regulatory function, as their deletion converts Msb2 and Hkr1 into constitutively active forms (Cullen *et al.* 2004; Tatebayashi *et al.* 2007). Furthermore, inhibition of *O*-glycosylation by *pmt4*Δ mutation, together with inhibition of *N*-glycosylation by tunicamycin, activates the Hog1 MAPK cascade in an Msb2-dependent manner (Yang *et al.* 2009). Based on these observations, two possible mechanisms of activating these osmosensors have been proposed. One is by proteolytic cleavage in the extracellular domain by the aspartyl protease Yps1, which eliminates the

Ser/Thr-rich glycosylation domain (Vadaie *et al.* 2008). Another is by an osmostress-induced conformational change in the oligosaccharide structure (Tatebayashi *et al.* 2007). However, the actual mechanism remains unclear.

***Co-osmosensor Sho1:*** The *SHO1* gene was initially identified by isolation of mutants that are **s**ynthetically **h**igh **o**smolarity sensitive in the presence of mutations that inactivate the Sln1 branch of the HOG pathway (Maeda *et al.* 1995). Sho1 is a relatively small protein (367 aa) that is composed of an N-terminal bundle of four transmembrane segments (TM1–TM4) and a C-terminal, cytoplasmic SH3 domain (Figure 5). The Sho1 SH3 domain binds to a Pro-rich motif (KPLPPLPV) in the N-terminal regulatory region of Pbs2 and serves to localize Pbs2 to the membrane (Maeda *et al.* 1995). Of the 27 SH3 domains found in the yeast proteome, only the Sho1-SH3 binds Pbs2, indicating a very high level of selectivity (Zarrinpar *et al.* 2003). The Sho1–Pbs2 interaction is required for activation of Pbs2 by the Ste11 MAPKKK. The Sho1 SH3 domain can also bind to Pro-rich motifs in Fus1 (KPLPLTPN) (Nelson *et al.* 2004) and in Ste20 (QPLPPIPP) (K. Tanaka, K. Tatebayashi, H.-Y. Yang, and H. Saito, unpublished results). Thus, during a mating response, induced Fus1 might downregulate the Sho1 branch by competitively inhibiting the Sho1–Pbs2 interaction. The role of the Sho1–Ste20 interaction seems to be redundant with that of other signaling elements in the Sho1 branch because this Pro-rich motif in Ste20 is required for activation of the Sho1 branch only in some mutants, but not in wild-type cells.

A few lines of evidence suggest that Sho1 might serve additional roles in signaling other than membrane targeting of Pbs2 and Ste20. First, Pbs2 appears to dissociate from Sho1 upon activation of the Pbs2 MAPKK, as suggested by decreased membrane localization of Pbs2 following osmostress stimulation, and this dissociation is hindered in a *ste20*Δ or a *ste11*Δ mutant or in a *pbs2*Δ mutant in which a catalytically inactive Pbs2-K389M is expressed, but interestingly not in a *hog1*Δ mutant (Reiser *et al.* 2000). These observations suggest that the Sho1–Pbs2 interaction might be dynamically regulated by a feedback phosphorylation by activated Pbs2. Second, an experimental replacement of the Sho1 SH3 domain with another SH3 domain derived (and modified) from the Fyn kinase resulted in a hybrid Sho1 that bound to Pbs2 just as well as the wild-type Sho1. Nonetheless, such a hybrid Sho1 is functionally defective, implying that the Sho1 SH3 domain has other functions in addition to Pbs2 binding (Marles *et al.* 2004). Third, and possibly related to the previous point, several proteins, notably Ste11 and Ste50, have been shown to interact with Sho1, but this binding is independent of the Pro-rich-motif-binding ability of the Sho1 SH3 domain (Zarrinpar *et al.* 2004; Tatebayashi *et al.* 2006). These interactions might enable Ste11 to efficiently interact with Pbs2 that is associated with Sho1. Finally, there are a number of Sho1 mutants that are constitutively activated in the sense that their expression will activate the Hog1 MAPK in the absence of any osmostress (Tatebayashi *et al.* 2006, 2007; Vadaie *et al.* 2008). These mutations are found both in the TM region and in the cytoplasmic region, suggesting that Sho1 might engage in dynamic interaction with other molecules through both its TM and cytoplasmic regions. Thus, the potentially dynamic functions of Sho1 are still far from being understood.

***Adaptor protein Ste50:*** *STE50* was originally identified as a gene that is required for an efficient mating response, as its deletion mutants are moderately sterile (Ramezani Rad *et al.* 1992; Xu *et al.* 1996). Ste50 is essential for the Sho1 branch of the HOG pathway (Posas *et al.* 1998; Wu *et al.* 1999) and is also necessary for the filamentous and invasive growth pathway that activates the Kss1 MAPK (Ramezani Rad *et al.* 1998; Jansen *et al.* 2001). Thus, all three signal pathways that involve Ste11 are dependent on Ste50. Structurally, Ste50 is composed of an N-terminal sterile-α motif (SAM) domain and a C-terminal Ras association (RA) domain (Ramezani-Rad 2003) (Figure 6). A SAM domain is a protein interaction module of ~70 amino acids that can homo-dimerize and hetero-oligomerize with other SAM domains (Qiao and Bowie 2005). *In vivo* binding studies have shown that the Ste50 SAM domain binds to the SAM domain in Ste11 (Posas *et al.* 1998; Wu *et al.* 1999; Jansen *et al.* 2001), while *in vitro* studies demonstrated that the Ste50 SAM domain can homo-dimerize as well as hetero-dimerize with Ste11 SAM (Bhattacharjya *et al.* 2004; Grimshaw *et al.* 2004; Kwan *et al.* 2004, 2006). The SAM-mediated Ste50–Ste11 interaction is essential for all the known activities of Ste50 (Ramezani-Rad 2003).

In spite of its name, the Ste50 RA domain does not seem to interact with Ras proteins. Genetic evidence suggests that the RA domain might interact with the Cdc42 GTPase, which is supported by a coprecipitation assay that showed that the Ste50 RA domain interacted equivalently with either GTP- or GDP-bound Cdc42 (Tatebayashi *et al.* 2006; Truckses *et al.* 2006). A Ste50 mutant that lacks the RA domain (Ste50-ΔRA) is functionally defective and cannot activate the Hog1 MAPK in response to osmostress. However, forced localization of Ste50-ΔRA to the plasma membrane, by attachment of a membrane-targeting signal, results in efficient activation of the Hog1 MAPK, indicating that an essential function of the RA domain is to aid Ste50 membrane localization (Tatebayashi *et al.* 2006; Truckses *et al.* 2006; Wu *et al.* 2006). In wild-type cells, Ste50 membrane localization could be attained, in principle, by an interaction of the Ste50 RA domain with the membrane-associated Cdc42 GTPase. However, the major factor that recruits Ste50 to the membrane appears to be the membrane anchor protein Opy2 (Wu *et al.* 2006; Yamamoto *et al.* 2010). Importantly, membrane-targeting of Ste50-ΔRA, using the Ras C-terminal prenylation signal, can rescue the osmostress-induced Hog1 activation in the absence of Opy2, implying that the Ste50–Opy2 interaction and resulting Ste50 membrane localization is the main function of the

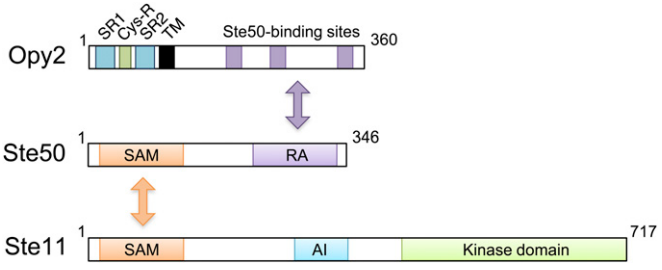


**Figure 6** Schematic diagram of the Ste11/Ste50/Opy2 complex. Ste11 and Ste50 bind together through their SAM domains, whereas the RA domain of Ste50 binds to any of three binding sites in Opy2. AI, auto-inhibitory domain; Cys-R, cysteine-rich domain; SR, Serine rich domain; TM, transmembrane domain.

Ste50 RA domain (Tatebayashi *et al.* 2007). Ste50 has also been shown to interact with the membrane protein Sho1 (Tatebayashi *et al.* 2006), but the roles of Ste50–Sho1 interaction in signaling remain to be determined. In summary, the main function of Ste50 seems to be to serve as an adaptor between the Ste11 MAPKKK and the membrane anchor Opy2, so that Ste11 is efficiently recruited to the membrane.

***Membrane anchor Opy2:*** The *OPY2* gene was initially identified as a multicopy suppressor that downregulates the mating MAPK signal pathway (Edwards *et al.* 1997). However, disruption of *OPY2* does not have any significant impact on the mating pathway. It was later found that the *opy2*Δ mutation, together with a defect in the Sln1 branch, causes synthetic osmosensitivity, indicating that Opy2 has an essential function in the Sho1 branch of the HOG pathway (Wu *et al.* 2006).

Opy2 is a single-path transmembrane protein of 360 aa. Its short extracellular domain is composed of, from the N terminus, a highly Ser-rich (SR1) domain, a Cys-rich (Cys-R) domain, and another Ser-rich (SR2) domain followed by the TM segment (Figure 6). The SR1 domain, but not SR2, is highly *O*-glycosylated by the protein *O*-mannnosyl transferase Pmt4, but deletion of SR1 does not have any observable effect on Opy2 functions (Hutzler *et al.* 2007; Yang *et al.* 2009). The Cys-R domain is characterized by an arrangement of eight cysteine residues, and genes that encode a similar Cys-rich motif are found in a wide range of fungal species. The cytoplasmic region of Opy2 is intrinsically disordered as revealed by NMR spectroscopy (Ekiel *et al.* 2009) and comprises four short well-conserved regions (CR-A to CR-D) interspersed among nonconserved sequences (Yamamoto *et al.* 2010).

The essential function of Opy2 in the Sho1 branch is to recruit the Ste50/Ste11 complex to the plasma membrane. Earlier studies suggested that there is more than one Ste50-binding site in Opy2 (Wu *et al.* 2006; Ekiel *et al.* 2009). A more recent study extended this hypothesis and showed that there are actually three independent Ste50-binding sites in Opy2, which correspond to the conserved regions CR-A, CR-B, and CR-D. CR-A and CR-D seem to constitutively bind Ste50, whereas CR-B (DIRSHITLGSSIL) binds Ste50 only when the Ser and Thr residues are phosphorylated by the casein kinase I isoforms, Yck1 and Yck2 (Yamamoto *et al.* 2010). Yck1/Yck2 are activated when glucose availability is high (Zaman *et al.* 2008). In fact, Opy2 CR-B is phosphorylated only when there is abundant glucose in the media. Opy2 is required not only for the Sho1 branch, but also for the FIG pathway, which is activated under limited nutrition and activates the Kss1 MAPK. Interestingly, CR-B seems to function only in the Hog1 pathway, but not in the FIG pathway. Thus, it is possible that under glucose-rich environments the phosphorylation of CR-B shifts Opy2 activity away from Kss1 and toward Hog1.

In summary, the main function of Opy2 is to serve as a membrane anchor for the Ste11 MAPKKK through its binding to the adaptor protein Ste50. Opy2 also integrates signals from the osmosensors and the glucose sensors.

***Activation of Ste20/Cla4:*** Ste20 is a member of the p21-activated kinase (PAK) family of protein kinases that are activated by the small GTPase Cdc42 (Bokoch 2003). In the absence of stimuli, PAK family kinases are inhibited by their N-terminal auto-inhibitory domain that binds to their C-terminal kinase domain (Lei *et al.* 2000). This auto-inhibition is relieved when GTP-bound (activated) Cdc42 binds to the p21-binding domain termed "CRIB" that is close to the auto-inhibitory domain (Peter *et al.* 1996; Leberer *et al.* 1997; Lamson *et al.* 2002; Ash *et al.* 2003). Ste20 was initially identified as a kinase that is required to activate the Ste11 MAPKKK in the mating signal pathway (Leberer *et al.* 1992). Later, Ste20 was shown to participate in two other signal pathways, the FIG and the Sho1 branch of the HOG pathway (Mösch *et al.* 1996; O'Rourke and Herskowitz 1998; Raitt *et al.* 2000b). Cla4 is another PAK family kinase and is involved mainly in cell-cycle regulation, such as septin formation and polarized growth (Tjandra *et al.* 1998). Although both *ste20*Δ and *cla4*Δ mutants are viable, the *ste20*Δ *cla4*Δ double mutation is lethal (Cvrcková *et al.* 1995). Thus, it is believed that Ste20 and Cla4 share at least one essential function, although the nature of that essential function is not known.

The growth of *ste20*Δ mutants of a parental strain that is defective in the Sln1 branch, such as *ssk2*Δ *ssk22*Δ, is sensitive to high osmolarity, but these mutants can tolerate moderate osmostress (Raitt *et al.* 2000b). In contrast, *ste20*Δ *cla4*$^{ts}$ double mutants of the same strain are highly osmosensitive and are completely unable to activate Hog1, indicating that Cla4 partially compensates for the function of Ste20 (Tatebayashi *et al.* 2006). The finding that *ste20 (ΔCRIB)* mutants are more osmosensitive than the *STE20* wild-type parental cells seems to indicate that Cdc42 binding to Ste20 is required for activation and/or membrane localization of Ste20 (Raitt *et al.* 2000b; Winters *et al.* 2005). However, overexpression of constitutively active *cdc42(G12V)* only very moderately activates Hog1, suggesting that an additional factor might be necessary for full activation of Ste20 (Raitt *et al.* 2000b). Although it is

frequently assumed that GTP association of Cdc42 is increased and that Ste20 kinase is activated in response to osmostress, there is no direct evidence for these assumptions. An alternative mechanism, in which osmostress induces the association of active Ste20 (which has been activated by an osmostress-independent manner) and Ste11, might better fit the available data. Indeed, the mating MAPK pathway is activated by an analogous mechanism, *i.e.*, by pheromone-induced association of Ste20 and Ste11 (Pryciak and Huntress 1998; Lamson *et al.* 2002).

***Activation of Ste11 by Ste20/Cla4:*** Activation of the Ste11 MAPKKK by osmostress requires at least two events. The first event is the binding of Ste50 to the Ste11 N-terminal SAM domain. This interaction helps to dissociate the N-terminal inhibitory domain from the C-terminal kinase catalytic domain, thus relieving inhibition of the kinase (Wu *et al.* 1999). However, as the Ste11–Ste50 interaction is constitutive, this effect is not likely to play an active role in regulating Ste11 activity during osmostress. The second event that is required is phosphorylation of Ste11 by Ste20/Cla4. It has been demonstrated that, in response to α-mating factor, activated Ste20 phosphorylates Ser-302, Ser-306, and Thr-307 in the N-terminal regulatory region of Ste11 (van Drogen *et al.* 2000). Based on the effects of phospho-mimetic mutations, it is believed that these Ste11 sites are also phosphorylated by Ste20/Cla4 upon osmostress stimulation (Lamson *et al.* 2006).

Ste50 binding and phosphorylation by Ste20/Cla4 are important, but not sufficient for Ste11 to transmit signals to downstream elements. Phospho-mimetic substitutions at the phosphorylation sites, or mutations in the auto-inhibitory domain, or even a deletion of the entire N-terminal regulatory region, all constitutively activate Ste11. Overexpression of one of these constitutively active Ste11 mutants activates both the Ste11-Pbs2-Hog1 and the Ste11-Ste7-Fus3/Kss1 MAPK cascades, without any stimulation (Posas and Saito 1997; Lamson *et al.* 2006; Tatebayashi *et al.* 2006). However, expression of the same constitutively active Ste11 mutants using the native *STE11* promoter does not significantly activate the Hog1 MAPK or the Fus3/Kss1 MAPK (Lamson *et al.* 2006; Tatebayashi *et al.* 2006). Constitutively active Ste11 mutants do activate the Hog1 MAPK cascade and the mating MAPK cascade in a Ste20/Cla4-independent manner upon respective stimulation (Lamson *et al.* 2006; Tatebayashi *et al.* 2006). Thus, it is clear that, in addition to activation of Ste11 by Ste20/Cla4, another stimulus-dependent signal amplification step is required to transmit sufficient signal to the downstream component (Pbs2 in the case of the HOG pathway and Ste7 in the cases of the mating and FIG pathways). The nature of this amplification step is unclear, but one possibility is a stimulus-induced membrane localization of activated Ste11 (Lamson *et al.* 2006).

***Activation of Pbs2 by Ste11:*** Ste11 can be activated by any of the three MAPK cascades: the osmoregulatory HOG pathway, the mating pathway, and the FIG pathway. When activated by osmostress, however, Ste11 activates only the Pbs2 MAPKK, while in the other pathways Ste11 activates the Ste7 MAPKK. Thus, there must be a mechanism that allows only Pbs2 to be activated by Ste11 during osmotic stimulation. As discussed earlier, Pbs2 is recruited to the plasma membrane by the membrane-associated scaffold protein Sho1 (Maeda *et al.* 1995; Reiser *et al.* 2000), and the Ste11/Ste50 complex is recruited to the membrane by the membrane anchor protein Opy2 (Wu *et al.* 2006; Ekiel *et al.* 2009; Yamamoto *et al.* 2010). However, efficient activation of Pbs2 by Ste11 seems to require, in addition to their membrane localization, direct and indirect docking interactions between Ste11 and Pbs2. It is known that Ste11 and Pbs2, Ste11 and Sho1, Ste50 and Sho1, and possibly Opy2 and Sho1 bind to each other (Posas and Saito 1997; Zarrinpar *et al.* 2004; Tatebayashi *et al.* 2006). Thus, multiple interactions between the Opy2/Ste50/Ste11 complex and the Sho1/Pbs2 complex bring Ste11 in close contact with Pbs2 for efficient activation. The relative contributions of these interactions to Pbs2 activation, as well as their regulation by osmostress, remain to be determined.

### *Activation of the HOG pathway by non-osmotic stresses*

A number of non-osmotic stresses are known to activate the HOG pathway, including cold stress (Hayashi and Maeda 2006; Panadero *et al.* 2006), heat stress (Winkler *et al.* 2002), hypoxia (Hickman *et al.* 2011), arsenite (Sotelo and Rodríguez-Gabriel 2006; Thorsen *et al.* 2006), acetic acid (Mollapour and Piper 2006, 2007), low pH (Kapteyn *et al.* 2001), inhibition of glycosylphosphatidylinositol (GPI) anchor synthesis (Toh-E and Oguchi 2001), and inhibition of sphingolipid synthesis (Tanigawa *et al.* 2012). In most cases, Hog1 is only moderately activated, and the kinetics of Hog1 phosphorylation is different from those observed upon osmostress. Although it is unclear how Hog1 is activated by these stresses, such stresses often activate either the Sln1 branch or the Sho1 branch, but not both. Adaptation to these diverse stresses, in addition to osmostress, might explain why yeast has apparently redundant osmostress-signaling branches. In this context, it is worth noting that the *Aspergillus nidulans* HogA MAPK (a homolog of Hog1) is activated only by the two-component signaling pathway homologous to the Sln1 branch, even though the mold has a Sho1 homolog (Furukawa *et al.* 2005).

### *Nuclear transport of activated Hog1*

Hog1 rapidly accumulates in the nucleus following osmotic stress (Figure 7A). Hog1 is then exported back to the cytoplasm after return to an iso-osmotic environment or after adaptation to high osmolarity (Ferrigno *et al.* 1998; Reiser *et al.* 1999). The kinetics of the transient Hog1 nuclear localization closely correlate with those found for the dual phosphorylation of Hog1 at Thr-174 and Tyr-176 (Figure 7B). Indeed, Hog1 mutations at these amino acid positions prevent Hog1 translocation into the nucleus (Ferrigno *et al.*

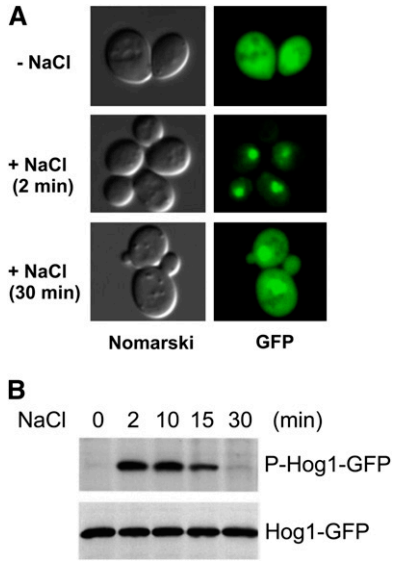


**Figure 7** Transient phosphorylation and nuclear localization of the Hog1 MAPK after osmostress. GFP-tagged Hog1 (Hog1-GFP) was expressed in a *hog1*Δ host strain, and cells were exposed to 0.4 M NaCl for the time indicated. (A) Hog1-GFP was detected by fluorescence microscopy (GFP), while the cell shape was pictured by differential interference contrast microscopy (Nomarski). (B) Total Hog1-GFP and phosphorylated Hog1-GFP were detected by immunoblotting using, respectively, anti-GFP and anti-phosphotyrosine antibody. Modified from Ferrigno *et al.*, 1998.

1998; Reiser *et al.* 1999). Hog1 phosphorylation itself, however, is not sufficient for its nuclear localization because the constitutively phosphorylated Hog1 molecules in the *ptp2*Δ *ptc1*Δ double-mutant cells do not accumulate in the nucleus (Mattison and Ota 2000). Catalytically inactive Hog1 mutants, such as D144A, cannot translocate into the nucleus after hyper-osmotic stimulation (Westfall and Thorner 2006). In contrast, other catalytic site mutants that retain partial activity, such as K52R or K52M, not only translocate into the nucleus, but also even fail to be exported out of the nucleus (Ferrigno *et al.* 1998; Mattison and Ota 2000). Thus, Hog1 catalytic activity seems to be required for its nuclear import and/or export, but its precise role remains unclear. Strains that lack the general stress activators Msn2 and Msn4, the related transcription factors Msn1 and Hot1, or the nuclear protein tyrosine phosphatase Ptp2 accumulate less Hog1 in the nucleus than wild-type cells, suggesting that these molecules bind and retain Hog1 in the nucleus (Reiser *et al.* 1999; Rep *et al.* 1999b; Mattison and Ota 2000).

Nuclear import of Hog1 is partially dependent on the activity of Gsp2 (homolog of mammalian Ran GTPase) and Nmd5 (homolog of importin β), but not on that of Srp1 and Rsl1, which encode the nuclear localization signal (NLS)-binding importin α/β heterodimer (Ferrigno *et al.* 1998). This result is consistent with the fact that Hog1 does not contain a classical NLS. Nuclear export of Hog1 requires the activity of the nuclear export signal (NES) receptor Xpo1/Crm1 (Ferrigno *et al.* 1998).

Nuclear localization is necessary for Hog1 to phosphorylate its nuclear substrates, including transcription factors and cell-cycle regulators. Indeed, cells that express plasma membrane-tethered Hog1 (Hog1-CCAAX), which cannot translocate to the nucleus, seem to have deficient expression of the Hog1-dependent genes (Westfall *et al.* 2008). Strikingly, however, membrane-tethered Hog1 permits robust growth under conditions of hyper-osmotic stress, suggesting that Hog1-mediated cytoplasmic modulation of metabolic activities, perhaps those that are necessary for glycerol synthesis and accumulation, are more important for long-term cell survival than alteration of the gene expression pattern (Bouwman *et al.* 2011).

Unlike Hog1, the Hog1-activating kinase Pbs2 is found mostly in the cytoplasm of both unstressed and osmostress-stimulated cells (Ferrigno *et al.* 1998). Nevertheless, Pbs2 has an NES at its N terminus (residues 4–18) and an NLS at its C terminus (residues 636–639). Pbs2 ΔNES mutants accumulate in the nucleus, whereas Pbs2 ΔNES ΔNLS double mutants are found in the cytoplasm (Tatebayashi *et al.* 2003). Thus, it is likely that Pbs2 shuttles between the two compartments, but the function of such shuttling is unknown.

### *Dynamics of HOG pathway signaling*

The Hog1 MAPK is only transiently activated following osmostress stimulation. Phosphorylation of the Hog1 activation sites (TGY) increases rapidly, reaches a maximal level at ~5 min, and then gradually decreases to near basal levels within 30 min (Maeda *et al.* 1995; Hao *et al.* 2007) (Figure 7B). This negative regulation is dependent on the kinase activity of Hog1 itself because phosphorylation of catalytically inactive Hog1 persists much longer than that of wild-type Hog1 (Wurgler-Murphy *et al.* 1997). Several negative-feedback mechanisms are known in the HOG pathway. Furthermore, the Hog1 MAPK pathway is part of a complex signaling network that involves at least two other MAPK pathways. The dynamic characteristics of this signal network are intensely investigated both by conventional genetic/biochemical approaches and by more recent systems biological and computational approaches.

***Negative feedback by glycerol accumulation:*** The most important negative feedback mechanism of Hog1 pathway signaling is removal of the osmostress by induced accumulation of the compatible solute glycerol (Brewster *et al.* 1993; Albertyn *et al.* 1994; Klipp *et al.* 2005; Muzzey *et al.* 2009). Although transcriptional induction of *GPD1* and other genes necessary for glycerol accumulation is important for long-term downregulation of the Hog1 pathway, such induction takes too long (at least 15 min) to account for the rapid decline of Hog1 activity (Hirayama *et al.* 1995). It has been proposed that Hog1 might more rapidly regulate glycerol accumulation by directly modulating the activities of the glycerol channel Fps1 and metabolic enzymes involved in glycerol biosynthesis (Dihazi *et al.* 2004; Klipp *et al.* 2005; Mollapour and Piper 2007; Westfall *et al.* 2008; Beese *et al.* 2009; Bouwman *et al.* 2011).

***Negative feedback by protein phosphatases:*** Although signaling from the upstream osmosensors stops when osmotic imbalance is eliminated by glycerol accumulation, it is still necessary to inactivate the kinases by dephosphorylation to bring the system to the prestimulation state. The two activating phosphorylation sites in Hog1, namely Thr-174 and Tyr-176, are dephosphorylated by different enzymes (for reviews, see Saito and Tatebayashi 2004; Martín *et al.* 2005).

Members of the type 2C Ser/Thr phosphatase family, Ptc1, Ptc2, and Ptc3, dephosphorylate Thr-174. Of these phosphatases, Ptc1 is the most important for de-activation of Hog1, as the *ptc1*Δ mutant retains high Hog1 activity even after 1 hr (Warmka *et al.* 2001). The specificity of Ptc1 toward Hog1 is indirectly conferred by the adaptor protein Nbp2 (Mapes and Ota 2004). Nbp2 binds to both Ptc1 and Pbs2, and as Pbs2 also has a high affinity for Hog1, Ptc1 is indirectly recruited to Hog1 by the Nbp2–Pbs2 complex. In contrast, Ptc2 and Ptc3 seem to have more of a subsidiary role of limiting the maximal activity of Hog1 during activation (Young *et al.* 2002).

Members of the protein tyrosine phosphatase family, Ptp2 and Ptp3, dephosphorylate Tyr-176 (Jacoby *et al.* 1997; Wurgler-Murphy *et al.* 1997). Although these tyrosine phosphatases are partially redundant, Ptp2 is primarily responsible for Hog1 dephosphorylation, whereas Ptp3 is more important for Fus3 dephosphorylation (Zhan and Guan 1999). Ptp2 is found in the nucleus, whereas Ptp3 is localized in the cytoplasm (Mattison and Ota 2000). This localization of Ptp2 seems to ensure that tyrosine dephosphorylation of Hog1 occurs only after Hog1 has entered into the nucleus. Because Hog1 is inactivated when either Thr-174 or Tyr-176 is dephosphorylated, the *ptc1*Δ *ptp2*Δ double-mutant strain is lethal because of Hog1 hyperactivation (Maeda *et al.* 1993). Phosphatases that inactivate other kinases in the Hog1 pathway have not been identified confidently.

***Negative feedback by phosphorylation of upstream elements:*** Activated Hog1 also negatively feedback regulates the Hog1 pathway by phosphorylating upstream signaling elements. Osmostress-activated Hog1 phosphorylates Sho1 at Ser-166, which is located within the cytoplasmic linker region between the four TM domains and the C-terminal SH3 domain (Hao *et al.* 2007). Hog1 activation is slightly diminished in cells expressing the phosphomimetic Sho1-S166E. It has been shown that some mutations at Ser-166 disrupt Sho1 oligomerization. However, neither the role of Ser-166 phosphorylation in Sho1 oligomerization, nor the role of Sho1 oligomerization in Hog1 activation, is clear.

Activated Hog1 phosphorylates several amino acids in Ste50 (Ser-155, Ser-196, Ser-202, Thr-244, Ser-248, and Thr-341) (Hao *et al.* 2008). Phosphorylation of Ste50 reduces its affinity for the membrane anchor Opy2 (Yamamoto *et al.* 2010). Because the Opy2–Ste50 interaction is essential for Hog1 activation via the SHO1 branch, phosphorylation of Ste50 by Hog1 serves as a negative feedback mechanism. Indeed, the duration of Hog1 activation by osmotic stress is longer in cells that express a phosphorylation-deficient Ste50 mutant than in the control cells. Pheromone-activated Fus3 and Kss1 also phosphorylate the same Ste50 residues, suggesting that Ste50 phosphorylation may also serve as a cross-regulatory mechanism between the mating and HOG pathways (Yamamoto *et al.* 2010).

***Inhibition of crosstalk among MAPK signaling pathways:*** In general, each MAPK module is activated by specific types of stimuli and induces specific adaptive responses. To achieve this specificity would be easy if each MAPK module was composed of only unique and dedicated components. In yeast, however, three MAPK modules (the Sho1 branch of HOG pathway, the mating pathway, and the FIG pathway) share many components, including the Ste11 MAPKKK, and still maintain their individuality. Leakage of signal, or crosstalk, from one MAPK pathway to another is prevented by a number of mechanisms, in addition to the negative regulation that involves protein phosphatases (Saito 2010).

One mechanism is insulation of each MAPK pathway from the others by docking interactions and scaffold proteins (Reményi *et al.* 2005; Bardwell 2006; Dard and Peter 2006). Activation of the mating MAPK module (Ste11→Ste7→Fus3) is dependent on the presence of the Ste5 scaffold (Elion 2001; Flatauer *et al.* 2005; Winters *et al.* 2005; Garrenton *et al.* 2006; Good *et al.* 2009). In contrast, activation of the Sho1 branch of the Hog1 MAPK module (Ste11→Pbs2→Hog1) is dependent on the presence of the Sho1 scaffold (Maeda *et al.* 1995; Zarrinpar *et al.* 2004). Indeed, when a wild-type cell is costimulated with osmostress and a mating factor, dual activation of the HOG and the mating MAPK pathways occurred, indicating that these two MAPK modules are practically insulated and activated independently of each other (Patterson *et al.* 2010). The importance of docking and scaffold interactions in determining pathway specificity has also been demonstrated by artificially forcing interaction between non-native pairs of signaling elements, thus diverting the signaling flow into preselected directions (Harris *et al.* 2001; Park *et al.* 2003; Tatebayashi *et al.* 2003; Mody *et al.* 2009).

Another mechanism is cross-inhibition by one MAPK pathway of other MAPK pathways. Although the Hog1 MAPK module (Ste11→Pbs2→Hog1) shares many upstream components with the FIG Kss1 MAPK module (Ste11→Ste7→Kss1), osmostress activates the Kss1 MAPK of the FIG pathway only very weakly and transiently (Shock *et al.* 2009; Wang *et al.* 2009), and glycosylation defects that activate Kss1 do not activate Hog1 (Cullen *et al.* 2000; Yang *et al.* 2009). In the absence of Pbs2 or Hog1, however, osmostress activates Kss1 robustly and Fus3 to a lesser degree, induces Kss1/Fus3-dependent genes, and induces FIG/mating-like polarized cell growth (O'Rourke and Herskowitz 1998, 2004; Pitoniak *et al.* 2009). Using an ATP analog-sensitive Hog1 mutant, it was shown that inhibition of this crosstalk requires Hog1 kinase activity (Westfall and Thorner 2006). Although it is possible that a part of this crosstalk inhibition is achieved by modulation of FIG/mating-specific gene expression in the

nucleus (Shock *et al.* 2009), even a membrane-tethered version of Hog1, which, in principle, cannot enter the nucleus, can prevent this crosstalk, implying that a cytoplasmic substrate might be involved in this process (Westfall *et al.* 2008). However, cells expressing mutants of the known or suspected Hog1 substrate proteins (Sho1, Ste50, Opy2, Ste7, Tec1, Dig1/Dig2, and Rck1/Rck2) that lack Hog1-dependent phosphorylation sites do not display constitutive crosstalk (Hao *et al.* 2007, 2008; Shock *et al.* 2009; Yamamoto *et al.* 2010). Thus, the mechanism of cross-inhibition between the HOG and FIG/mating pathways remains obscure.

***Single-cell dynamics:*** Conventional methods used to detect MAPK activity such as immunostaining of fixed cells or immunoblotting of cell extracts using phospho-MAPK-specific antibodies can show only static snapshots and/or population averages of MAPK activation. To study the systems dynamics of a signaling pathway, it is necessary to monitor the behavior of single cells under controlled environmental conditions. The Hog1 MAPK pathway is particularly suited for this type of analysis. By using a microfluidic device to change the osmolarity of media (input), and by monitoring the nuclear translocation of fluorescent protein-tagged Hog1 (output), two groups have reported the frequency responses of HOG pathway activation (Hersen *et al.* 2008; Mettetal *et al.* 2008). At low frequency ($<1/200$ sec$^{-1}$), the HOG pathway faithfully follows the input changes, whereas at higher frequency, it responds only to the average input osmolarity. Other aspects of HOG-signaling properties have also been studied using various single-cell monitoring methods (McClean *et al.* 2007; Muzzey *et al.* 2009; Patterson *et al.* 2010; Pelet *et al.* 2011).

***In silico simulation:*** The HOG-signaling pathway is also an intense subject of *in silico* simulation, or mathematical modeling, that aims to elucidate system architecture, dynamics, and regulation based on data sets in the literature. Modeling is rapidly evolving from a simple tool that describes and summarizes the known facts into a more advanced predictive facility that can test the validity of various hypotheses (Klipp *et al.* 2005; Gat-Viks and Shamir 2007; Zou *et al.* 2007; Krantz *et al.* 2009; Rensing and Ruoff 2009; Zi *et al.* 2010; Parmar *et al.* 2011; Schaber *et al.* 2011). The popularity of the HOG pathway for such studies is undoubtedly because of its relative simplicity together with the availability of detailed mechanistic knowledge regarding this pathway and abundant quantitative and qualitative data. Thus, the HOG pathway will continue to be an excellent testing ground for algorithms that attempt to simulate and analyze more complex signal transduction networks in higher eukaryotes.

## Downstream Adaptive Responses

### *Reestablishment of osmotic balance*

***Compatible osmolytes:*** Activation of Hog1 in response to osmostress elicits a program for cell adaptation that includes short- and long-term responses. Long-term adaptation involves transcriptional and translational regulation of the genome, whereas short-term adaptation is accomplished by changes in glycerol accumulation (Albertyn *et al.* 1994) and the reestablishment of ionic balance (Proft and Struhl 2004). Exposure to increased osmolarity is known to result in loss of water, shrinkage in cell size, and a temporary arrest of growth until adaptation occurs. The major strategy for survival under high osmolarity is to produce and accumulate compatible osmolytes such as glycerol to maintain the water balance and reestablish the volume and the turgor of the cells (Blomberg and Adler 1989; Hohmann *et al.* 2007; Westfall *et al.* 2008; de Nadal *et al.* 2011). The accumulation of compatible osmolytes is a ubiquitous mechanism in cellular osmoregulation. Although there are a number of compatible osmolytes such as trehalose, amino acids, and ions that contribute differently to adaptation to osmostress, glycerol seems to be the most important compatible osmolyte for the growth of *S. cerevisiae* in the presence of high osmolarity (Hohmann *et al.* 2007).

Intracellular accumulation of glycerol is an essential response for survival under high-osmolarity conditions, and the Hog1 MAPK is responsible mainly for the accumulation of glycerol in the presence of high osmolarity (Albertyn *et al.* 1994). There are several mechanisms to control glycerol accumulation: regulation of gene expression, metabolic adjustment, and control of glycerol export and import (Hohmann 2002b).

***Glycerol accumulation:*** The expression of key metabolic enzymes that are involved in glycerol, trehalose, and glycogen metabolism is upregulated in response to Hog1 activation. The enzymes directly responsible for the synthesis of glycerol, *i.e.*, glycerol-3-phosphate dehydrogenase (Gpd1) and glycerol-3-phosphatases (Gpp1 and Gpp2), are upregulated upon osmostress (see below), and the lack of these genes severely impairs growth at high osmolarity (Figure 8) (Hohmann 2002a). Expression of sugar transporters and genes involved in sugar metabolism are also upregulated in response to osmostress (Rep *et al.* 1999a, 2000; Gasch *et al.* 2000; Tomás-Cobos *et al.* 2004; Capaldi *et al.* 2008). However, some studies indicated that regulation of gene expression by Hog1 is not absolutely required for cell survival under certain high-osmolarity conditions, especially at the initial phases of the stress and at medium osmolarity (Mettetal *et al.* 2008; Westfall *et al.* 2008). In contrast, other studies indicated that Hog1-dependent regulation of the expression of specific genes involved in glycerol metabolism is important for cell survival at high osmolarity over an extended period of time (Hohmann 2002b; de Nadal and Posas 2010; Martínez-Montañés *et al.* 2010).

Glycerol is rapidly accumulated in response to osmostress, starting within the first minute, and there is significant accumulation of glycerol after 30 min of exposure to high osmolarity (Klipp *et al.* 2005). This rapid increase in glycerol production cannot be attributed to an increase in

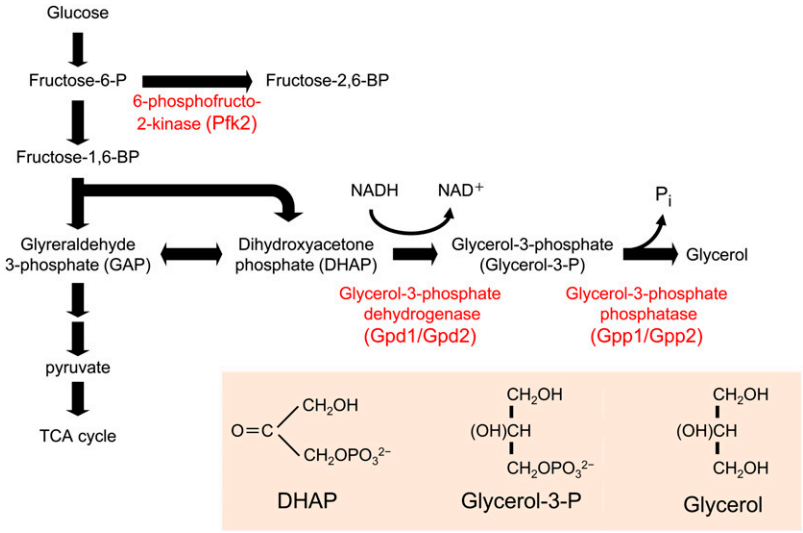


**Figure 8** Glycerol biosynthetic pathway. Glycerol is synthesized from an intermediate in the glycolysis, dihydroxyacetone phosphate (DHAP), by two-step enzymatic reactions. The first enzyme is glycrol-3-phosphate dehydrogenase (Gpd1/Gpd2), which reduces DHAP using NADH as reducing agent. The second enzyme is glycerol-3-phosphate phosphatase (Gpp1/Gpp2), which removes phosphate from glycerol-3-P to generate glycerol.

the transcription of particular genes, and therefore additional mechanisms other than transcriptional regulation must exist that permit such a rapid response. There are two main mechanisms to achieve such a rapid initial increase in glycerol concentration: changes in carbon metabolism and changes in glycerol transport.

***Metabolic adjustments:*** Adaptation to osmotic stress requires direct metabolic adjustments. Cells must redirect carbon resources toward enhanced production of glycerol, and thus there is significant modulation of central carbon metabolism during osmo-adaptation. There are indications that regulation of glycolysis is crucial for osmotic adaptation; for example, cells deficient in glycerol synthesis are highly osmosensitive. The control of glycolysis and glycerol production appears to be distributed among several enzymes through allosteric control by different metabolites (Hohmann *et al.* 2007). However, there is direct evidence indicating that the activity of the 6-phosphofructo-2-kinase, Pfk2, which is responsible for controlling the levels of fructose-2,6-bisphosphate (F2,6BP), a key activator of glycolysis, is regulated by the Hog1 MAPK (Dihazi *et al.* 2004). Therefore, Hog1 may directly control the metabolic flux in response to stress. Along the same lines, recent studies using aerobic, glucose-limited cultures suggest that metabolic regulation rather than *de novo* enzyme synthesis dominates the initial phase of the adaptive process, at least in the presence of moderately high osmolarity (1 M sorbitol) (Bouwman *et al.* 2011). Therefore, the regulation of metabolic flux is an important component in Hog1-regulated glycerol accumulation.

***Glycerol transport:*** Because the lipid bilayer has low permeability for glycerol, specific channel proteins mediate the rapid import and export of glycerol. As a consequence, the control of import and export rates is one mechanism by which the glycerol content inside of the cell can be altered. Thus, the control of the flux of glycerol through the membrane is another key factor for the initial accumulation of glycerol upon osmostress. Stl1, a sugar transporter-like protein whose expression is strongly induced by Hog1 upon stress, might contribute to glycerol accumulation by importing glycerol from the environment in response to stress. However, the fastest mechanism to alter glycerol concentration is via Fps1-mediated glycerol export (Tamás *et al.* 1999). Fps1 is a member of the aquaporin family of transmembrane channels, and cells that express Fps1 mutant proteins that are constitutively open do not accumulate glycerol and grow poorly in the presence of high osmolarity (Hohmann *et al.* 2007). In response to osmostress, the Fps1 channel closes to maintain internal glycerol, but this effect seems to be independent of Hog1 (Tamás *et al.* 1999). On the other hand, direct regulation of Fps1 transport capacity and protein stability by Hog1 has been described for arsenite transport and in response to weak acid treatment (Thorsen *et al.* 2006; Mollapour and Piper 2007; Beese *et al.* 2009). In addition, the stress-induced phosphorylation of Rgc2, a novel regulator of Fps1 channel activity, is also partially controlled by the Hog1 MAPK (Mollapour and Piper 2007; Beese *et al.* 2009). The precise mechanism by which Fps1 is controlled upon osmostress remains unclear.

The combined data indicate that the accumulation of glycerol is a key adaptive response to high osmolarity that is modulated by several mechanisms with different kinetics and different quantitative contributions to achieve proper adaptation to osmostress.

### *General stress responses*

In addition to glycerol, a number of other organic osmolytes, including trehalose, protect yeast from osmostress, not only by counteracting water efflux and reestablishing osmotic balance, but also by playing unique roles in antioxidation, detoxification, and the stabilization of cellular proteins and structures (Mager and Varela 1993; Yancey 2005). Notably, a number of genes that are upregulated by osmostress have similar protective functions as these osmolytes (de Nadal and Posas 2010; Martínez-Montañés *et al.* 2010). For

example, in response to osmostress, a number of genes that protect cells from oxidative damage are upregulated, including genes involved in redox metabolism, mitochondrial function, and the biosynthesis of antioxidative compounds (*e.g.*, *TRX2*, *CTT1*, *GRE3*, and *SOD2*). Genes that encode the chaperones (*e.g.*, *HSP12*, *HSP104*, and *HSP42*) that protect cells from damage by protein denaturation are also upregulated. It is worth noting that Hog1 has also been implicated in ER stress protection, which is induced in response to the accumulation of unfolded proteins (Bicknell *et al.* 2010; Torres-Quiroz *et al.* 2010; Eraso *et al.* 2011), and in the control of mitophagy, the specific autophagic elimination of mitochondria (Aoki *et al.* 2011; Mao *et al.* 2011).

One role of the transcriptional response to a specific stress is to generate a cross-protection to other types of stresses. Osmostress induces many genes that are considered to be part of general stress responses. Conversely, when cells are subjected to a mild stress (*e.g.*, oxidative stress or heat stress), stress response element (STRE)-mediated responses are induced even in the absence of Hog1 (Berry and Gasch 2008). Thus, at 37°, *hog1*Δ cells can survive on moderate osmostress, such as 0.8 M sorbitol, better than at 30° (Siderius *et al.* 2000). This protection is not sufficient for *hog1*Δ cells to survive higher levels of osmolarity.

### *Regulation of gene expression by osmostress*

***Global analysis of gene expression upon osmostress:*** Exposure of yeast to high osmolarity results in profound changes in the physiology of the cell and has a major impact on the capacity of the cell for gene expression. Analysis of the transcriptional changes mediated by Hog1 in response to osmostress may lead to a general understanding of how cells rapidly, precisely, and extremely efficiently adjust the full complement of a transcriptional program in response to extracellular stimuli. Indeed, the Hog1 MAPK plays a key role in the regulation of mRNA biogenesis by controlling several steps in the transcription process (Figure 9) (Hohmann 2002b; de Nadal and Posas 2010; Martínez-Montañés *et al.* 2010; de Nadal *et al.* 2011). Although the role of Hog1-dependent gene expression in osmo-adaptation is still incompletely understood, it is clear that long-term adaptation to high osmolarity requires regulated transcription, as a number of mutants in the transcriptional machinery render cells osmosensitive (de Nadal *et al.* 2004; Zapater *et al.* 2007; Mas *et al.* 2009). On the other hand, it has been shown that a membrane-tethered Hog1 construct abolishes short-term transcription responses at certain osmolarities (so that it cannot enter the nucleus). Nevertheless, this Hog1 construct is still able to suppress the osmosensitivity of a *hog1*Δ strain (Westfall *et al.* 2008). Therefore, cytoplasmic events caused by the rapid and transient activation of the Hog1 MAPK in response to osmostress—such as the control of glycerol production by direct modulation of metabolic enzymes (Dihazi *et al.* 2004; Bouwman *et al.* 2011) and the altered mRNA stability (Molin *et al.* 2009; Romero-Santacreu *et al.* 2009; Miller *et al.* 2011)—might be sufficient for the maintenance of osmotic balance under these experimental conditions without invoking induced gene expression in the nucleus.

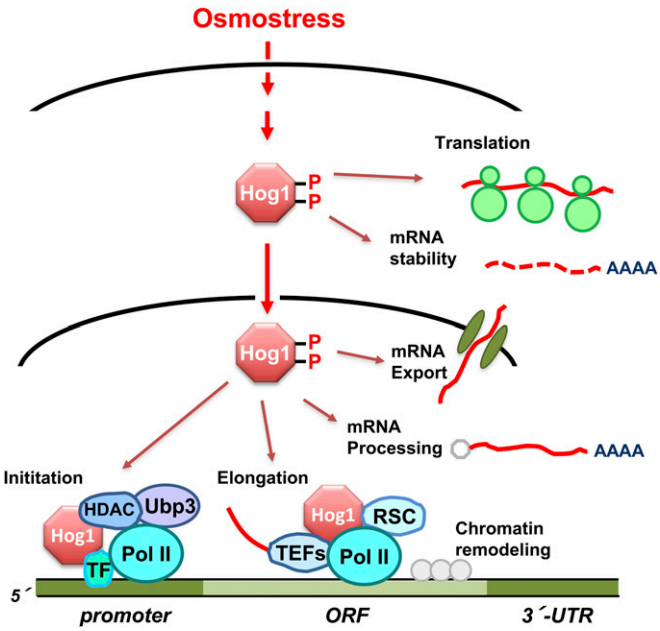


**Figure 9** Control of mRNA biogenesis by the Hog1 MAPK. Once activated upon osmostress, Hog1 controls many aspects of mRNA biogenesis both in the nucleus and in the cytoplasm. Hog1 phosphorylates and activates transcription factors (TFs). Remarkably, Hog1 associates to loci of stress-responsive genes to modulate both initiation and elongation. Hog1 also seems to control mRNA processing, nuclear export, translation and mRNA stability.

Global transcriptional responses to diverse stresses in *S. cerevisiae* have been studied in detail using gene expression profiling. There are a large number of genes whose transcription is induced in response to osmostress; of these genes, there is one subset of genes that specifically responds to osmostress, whereas another subset of genes responds indiscriminately to diverse types of stresses. Induction of the latter group of genes is known as the environmental stress response (ESR). The ESR consists of ~300 to ~600 genes whose expression is upregulated or downregulated by stresses such as DNA damage, heat shock, osmostress, or oxidative stress (Gasch *et al.* 2000; Causton *et al.* 2001; Capaldi *et al.* 2008). The extent and kinetics of the ESR appear to be dependent on the severity of the stress, since cells exposed to increasing stress often display broader changes in gene expression. This general stress response has been implicated in the phenomenon of cross-protection, whereby exposure to a nonlethal dose of one stress can protect cells against unrelated stresses (Berry and Gasch 2008). The genes upregulated by the ESR include genes involved in carbohydrate metabolism, protein metabolism, intracellular signaling, and defense against reactive oxygen species and DNA damage. On the other hand, most of the genes downregulated by the ESR are involved in protein synthesis and in growth-related processes (Gasch 2007; Martínez-Montañés *et al.* 2010).

It has been clearly established that the stress-responsive MAPKs, such as the mammalian JNK and p38 MAPKs, have a key role in the regulation of transcription upon a diverse array of stresses. In addition to the genes commonly regulated by the ESR, other genes play specific roles in adaptation to particular stresses, and these are also, in varying degrees, under the control of stress-responsive MAPKs. In *S. cerevisiae*, in which the ESR is not governed by a single regulatory system but by different signaling pathways and transcription factors depending on the type of stress, the Hog1 MAPK is critical for the regulation of ESR genes under osmostress (Posas *et al.* 2000). Indeed, studies using a *hog1*Δ mutant strain revealed that, although it depends on the severity of the stress, ~80% of the genes that are induced upon osmostress depend on the Hog1 MAPK for full induction (Posas *et al.* 2000; Rep *et al.* 2000; O'Rourke and Herskowitz 2004; Capaldi *et al.* 2008).

***Hog1 controls gene expression by regulating transcription factors:*** One of the well-characterized functions of the family of stress-responsive MAPKs including Hog1 is the regulation of gene expression at the initiation step of transcription. Indeed, Hog1 regulates several unrelated transcription factors, each of which is responsible for controlling the expression of a subset of osmoresponsive genes, either directly or in collaboration with other factors (Molin *et al.* 2009; Romero-Santacreu *et al.* 2009; Miller *et al.* 2011). These Hog1-regulated factors include the transcription activators Hot1, Smp1, Msn1, Msn2, and Msn4 and the transcription repressor Sko1 (de Nadal and Posas 2008). These factors can act independently or in combination at specific promoters to elaborate a dynamic transcriptional response to stress (Ni *et al.* 2009). A recent study that combined dynamic transcriptome analyses with an analysis of mRNA synthesis rates suggested that additional transcription factors could exist that act in association with these factors and are important for the response to osmostress (Molin *et al.* 2009; Romero-Santacreu *et al.* 2009; Miller *et al.* 2011). Therefore, these factors could also be substrates of Hog1. Overall, it is clear that a collaborative effort of many different transcription factors is needed for gene induction upon osmostress.

Direct phosphorylation of promoter-specific transcription factors is the best-understood mechanism by which the Hog1 MAPK modulates initiation of transcription. Examples of regulation by direct phosphorylation have been reported for the MEF2-like activator Smp1 and the ATF/CREB-family member Sko1 (Nehlin *et al.* 1992; Vincent and Struhl 1992). *In vivo* coprecipitation and phosphorylation studies showed that Smp1 and Sko1 interact with, and are directly phosphorylated by, Hog1 (Proft *et al.* 2001; de Nadal *et al.* 2003). Regulation of Sko1 function has an extra layer of complexity: while Sko1 acts as a transcription activator in the presence of stress, it acts as a transcriptional repressor in the absence of stress. In the absence of stress, Sko1 represses stress-inducible genes by recruiting the general transcription corepressor complex Ssn6 (Cyc8)–Tup1 to their promoters (Garcia-Gimeno and Struhl 2000; Pascual-Ahuir *et al.* 2001). Induction of Sko1-dependent genes requires the release of this repression, and this process is completely dependent on Hog1 (Pascual-Ahuir *et al.* 2001; Proft and Struhl 2002). In fact, Hog1-dependent phosphorylation of Sko1 changes Sko1 from a repressor into an activator by modifying its association with the Tup1–Ssn6 complex and allows the recruitment of the chromatin-remodeling complexes SAGA and SWI/SNF to osmostress-inducible promoters (Rep *et al.* 2001; Proft and Struhl 2002; Guha *et al.* 2007; Kobayashi *et al.* 2008). These examples illustrate that the direct phosphorylation of transcription factors by activated Hog1 is a key regulatory element for induction of gene expression in response to osmostress.

***Hog1 controls gene expression by associating with chromatin:*** Hog1 also affects the functions of transcription factors by mechanisms other than by direct phosphorylation. Indeed, phosphorylation by Hog1 may not be critical for regulation of a number of transcriptional regulators that are under Hog1 control, such as Msn2, Msn4, and Hot1 (Alepuz *et al.* 2003). Msn2 and Msn4 mostly control induction of ESR genes through the STRE (Martínez-Pastor *et al.* 1996; Schmitt and McEntee 1996), and Hot1 affects expression of a small subset of Hog1-dependent genes, including *GPD1* and *GPP2*, which are involved in glycerol biosynthesis, as well as *STL1*, which encodes a glycerol/proton symporter (Rep *et al.* 1999b, 2000; Ferreira *et al.* 2005).

The fact that the nuclear retention of Hog1 upon osmostress is dependent on the presence of the transcription factors that are downstream of Hog1 suggested that these factors could act as nuclear anchors for Hog1 by engaging in stable interactions with it (Reiser *et al.* 1999; Rep *et al.* 1999b). Indeed, Hog1 does associate with chromatin, and it does so via physical interactions with transcription factors. For example, recruitment of Hog1 to the *CTT1* promoter requires the transcription factors Msn2 and Msn4, whereas recruitment of Hog1 to the *STL1* promoter depends on the transcription activator Hot1 (Alepuz *et al.* 2001). It is worth noting that Hog1 binds only to osmo-responsive genes (Pascual-Ahuir *et al.* 2006; Pokholok *et al.* 2006; Proft *et al.* 2006). An accumulation of Hog1 in the nucleus is not sufficient for its association with chromatin, since addition of a nuclear localization signal to Hog1 does not result in its enhanced chromatin association. However, binding of Hog1 to chromatin does depend on its catalytic activity (Alepuz *et al.* 2001). Thus, in contrast to the more traditional scenario in which a MAPK controls transcription only indirectly by phosphorylating transcription factors, the persistent presence of Hog1 at target promoters clearly indicates that Hog1 itself plays an important role in the regulation of transcription initiation (Alepuz *et al.* 2001; Chellappan 2001; Proft and Struhl 2002). Systematic genome-wide analyses of the binding of transcription factors and of Hog1 to chromatin, combined with gene expression profiling, have

shown that, in response to osmostress, Hog1 can specifically regulate, and integrate, the stress responses that occur at different promoters. This effect is accomplished by Hog1 via modulation of the individual contribution of transcription factors, such as Msn2/Msn4, Sko1, and Hot1, in a promoter-specific context that results in a complex and highly specific control of transcriptional networks (Proft *et al.* 2005; Capaldi *et al.* 2008; Ni *et al.* 2009).

Other yeast MAP kinases such as Fus3, Kss1, and Mpk1 are also recruited to chromatin (Pokholok *et al.* 2006; Kim *et al.* 2008). Furthermore, structurally and functionally unrelated yeast signaling kinases, including Snf1 (Lo *et al.* 2005; Li *et al.* 2006; Pokholok *et al.* 2006), have been reported to be recruited to chromatin. Similar binding of signaling kinases to chromatin, implying their direct roles in gene regulation, has now been shown to occur in several other organisms including mammals and *Drosophila* (Chow and Davis 2006; Edmunds and Mahadevan 2006; de Nadal and Posas 2010; de Nadal *et al.* 2011).

***Transcription initiation at osmostress-responsive promoters:*** The observation that Hog1 kinase activity is needed for transcriptional activation, even though phosphorylation of transcription factors is not an absolute requirement for transcription initiation, indicates that Hog1 can induce activation of gene expression by a mechanism other than phosphorylation of transcription activators. Indeed, recruitment of the RNA Pol II machinery to osmoresponsive genes is dependent on both activated Hog1 and the presence of specific transcription factors. The facts that Hog1 tightly associates with the largest subunit of RNA Pol II and that the artificial tethering of Hog1 to chromatin is sufficient to induce gene expression upon osmostress suggest that Hog1 serves to recruit the basic transcriptional machinery to stress-responsive promoters (Alepuz *et al.* 2003).

It has been shown that the extent of transcriptional activation is regulated by the Ubp3 ubiquitin protease. Ubp3 is targeted to stress-responsive genes by Hog1, and its activity is regulated by direct phosphorylation by Hog1. Thus, the regulation of the turnover of specific transcription factors and/or RNA PolII at the promoter seems to be important for the dynamics of gene expression upon stress (Solé *et al.* 2011). Furthermore, genetic and biochemical data suggests that, in addition to binding to transcriptional activators and facilitating RNA Pol II recruitment, Hog1 is also important for the recruitment of basic transcription complexes such as SAGA, Mediator, and SWI/SNF to osmoresponsive promoters. Several observations indicate that, whereas Mediator is crucial for proper gene induction under both mild and severe osmostress conditions, the role of SAGA is dependent on the strength of the osmostress. Thus, the requirement for a given transcriptional complex to regulate a promoter might depend on the severity of osmostress and be determined through the regulation of interactions among transcriptional complexes (Zapater *et al.* 2007). The recruitment of the SWI/SNF chromatin-remodeling complex to promoters also depends on the presence of Hog1. Although elimination of components of the SWI/SNF complex does not lead to clearly observable effects on transcription, modification of chromatin might still be important for efficient transcription in response to osmostress (Proft and Struhl 2002).

Although histone deacetylation has been classically associated with repression of gene expression (Robyr *et al.* 2002), there are many genes for which histone deacetylation is associated with transcription induction (Bernstein *et al.* 2000; Shahbazian and Grunstein 2007). In fact, the Rpd3 histone deacetylase (HDAC) complex plays an important role in induction of gene expression by osmostress. Rpd3 belongs to a five-member family of related histone deacetylases, and it has been reported to regulate the expression of a large number of genes (Yang and Seto 2008). There are two different Rpd3-containing HDAC complexes, the larger Rpd3L and the smaller Rpd3S, that share a common core composed of Rpd3, Sin3, and Ume1. Whereas the Rpd3L complex is recruited to promoters to enhance transcription initiation, the Rpd3S complex controls promoter fidelity by suppressing spurious intragenic transcription during elongation (Carrozza *et al.* 2005; Keogh *et al.* 2005; Li *et al.* 2007b,c; Biswas *et al.* 2008). Cells defective in Rpd3 and in other components of the Rpd3L complex are osmosensitive and show compromised expression of osmostress-responsive genes controlled by Hog1. Hog1 binds to an Rpd3 complex (presumably Rpd3L) and, upon stress, recruits it to specific osmostress-responsive genes. Binding of the Rpd3 complex to specific promoters leads to histone deacetylation, entry of RNA polymerase II, and induction of gene expression (de Nadal *et al.* 2004). It should be added that the role of the Rpd3 complex at osmostress-responsive promoters need not be restricted to alteration of chromatin structure, but it might also provide a unique binding surface or recognition motifs for the recruitment of transcription activators.

***Transcription elongation of osmostress-responsive genes:*** Elongation is also a critical phase of transcription that is highly regulated, and modification of the RNA Pol II carboxy-terminal domain is just one example of such regulation (Saunders *et al.* 2006; Egloff and Murphy 2008; Fuda *et al.* 2009). Upon osmostress, the Hog1 MAPK interacts with RNA Pol II as well as with the general components of the transcription elongation complex while these are engaged in elongation (Proft *et al.* 2006). In addition to its association with the promoter regions of osmostress-responsive genes, Hog1 is also present on the coding regions of these genes, and it travels with elongating RNA Pol II (Pascual-Ahuir *et al.* 2006; Pokholok *et al.* 2006; Proft *et al.* 2006). It should be noted that the binding of Hog1 to the coding regions is independent of promoter-bound transcription factors, but is dependent on the 3′-UTR region of osmostress-responsive genes. The mechanism by which Hog1 is recruited to the 3′-regions of osmostress-responsive genes is unclear. By fusing a Hog1-independent promoter to the coding region of

a Hog1-dependent gene, it is possible to uncouple Hog1-dependent transcription initiation from transcription elongation. Thus, it has been demonstrated that the presence of Hog1 at coding regions is essential for increased association of RNA Pol II with the coding region, suggesting that Hog1 directly affects the process of elongation (Proft *et al.* 2006). Other yeast signaling kinases, such as Fus3 or PKA, also associate with the coding regions of activated genes (Pokholok *et al.* 2006), which indicates that signaling kinases play a role in transcription beyond initiation.

***Remodeling of chromatin in response to osmostress:*** The packaging of DNA into nucleosomes affects all phases of the transcription cycle from the binding of activators and formation of a pre-initiation complex to elongation. Thus, nucleosome positioning and dynamics is another layer of transcription regulation (Cairns 2009; Jiang and Pugh 2009). As in the case of initiation, transcription elongation is also affected by chromatin structure, which is regulated by several protein factors that covalently modify histones or temporarily remove, disassemble, and reassemble nucleosomes (Li *et al.* 2007a). Chromatin-remodeling complexes utilize the energy of ATP hydrolysis to alter histone-DNA contacts by transiently unwrapping DNA, forming DNA loops, sliding nucleosomes, completely displacing the histones from DNA, or replacing histone subunits. Transcriptional responses to stress and chromatin structure alterations are tightly linked (Shivaswamy and Iyer 2008).

In response to osmostress, the nucleosome organizations of the osmostress-responsive genes undergo a dramatic change that depends on Hog1 and on the RSC chromatin-remodeling complex. The RSC complex (including Rsc1, Rsc2, Rsc3, etc.) is a distinct member of the SWI/SNF family and is known to modify nucleosome structure. Upon osmostress, the Hog1 MAPK physically interacts with RSC to direct its association with the coding region of osmostress-responsive genes, suggesting that this activity could be a major role of Hog1 during elongation. Notably, in RSC mutants, RNA Pol II still accumulates at the promoter regions of the osmostress-responsive genes, but not at their coding regions, implying that elongation is specifically suppressed. Furthermore, RSC mutants display reduced expression of osmostress-responsive genes and enhanced osmostress sensitivity (Mas *et al.* 2009). Cell adaptation under acute osmostress might thus depend on a burst of transcriptional activity that can occur only with efficient nucleosome eviction. Remarkably, the exposure of cells to mild osmostress results in bimodal expression of osmostress-responsive genes: expression levels among a population of equally stimulated cells are not continuously distributed, but display low and high peaks (Pelet *et al.* 2011). This bimodality arises at the transcriptional level: even if Hog1 is activated to a similar level in all cells, the transcriptional outcome is determined by a slow stochastic transition from a repressed transcriptional state to an activated state. This transition seems to depend on chromatin structure.

In addition to the RSC complex, two other chromatin remodelers have been found to associate with stress-responsive genes; the SWI/SNF complex, which has been discussed above, and the INO80 complex, which contains subunits such as Ino80 and Arp8. Disruption of the INO80 complex-specific gene *ARP8* results in extended expression of, and a delay in nucleosome reassembly at, stress-responsive genes during osmostress adaptation (Klopf *et al.* 2009). Therefore, chromatin-remodeling complexes and chromatin-modifying enzymes are key elements for stress-mediated gene expression, and a dynamic balance among different chromatin-remodeling complexes seems to be required for proper regulation of stress-responsive genes.

***Control of mRNA processing and stability by Hog1:*** Eukaryotic mRNAs are synthesized as precursors by RNA Pol II and are subsequently extensively modified, spliced, cleaved at the 3′-end, and polyadenylated. In addition, nuclear export and translation of mRNAs is coordinated differentially. At present, it is unclear whether osmostress-responsive genes are post-transcriptionally regulated by specific mRNA-binding proteins and what potential role the Hog1 MAPK might play in their regulation. In mammalian cells, stress-responsive p38 MAPK contributes to stabilization of cytokine/stress-inducible mRNAs, through an ARE (AU-rich elements present in 3′-UTR)-targeted mechanism. AREs regulate mRNA turnover by modulating poly(A)-shortening rates and the subsequent decay of mRNA. In *S. cerevisiae*, inhibition of the Hog1 pathway by the p38 inhibitor SB202190 leads to destabilization of ARE-bearing transcripts, suggesting that a similar mechanism exists in yeast (Vasudevan and Peltz 2001). Although this is a very interesting mechanism by which Hog1 might influence gene expression, its role in osmostress responses is unknown.

Genome-wide analyses have shown that there is a clear and specific regulation of stress-responsive mRNA in comparison with global mRNAs (Molin *et al.* 2009; Romero-Santacreu *et al.* 2009; Miller *et al.* 2011). For example, under mild osmostress, destablization of a broad range of mRNAs is induced, whereas osmostress-inducible mRNA synthesis is upregulated and the half-life of these mRNAs is extended. In a *hog1*Δ mutant, mild osmostress induces global stabilization of mRNA and P-body formation (Romero-Santacreu *et al.* 2009). Notably, stress-responsive mRNAs are selectively stabilized or degraded, depending on the phase of the response to stress, namely, initial shock, induction, or recovery (Miller *et al.* 2011). It is clear from these reports that Hog1 has an effect on mRNA stability, especially for osmostress upregulated genes. However, the mechanism by which Hog1 controls the stability of mRNAs is unclear.

### Regulation of cell-cycle progression by osmostress

In *S. cerevisiae*, a highly regulated and complex network of proteins governs cell-cycle progression, although major events are controlled by a single cyclin-dependent kinase (CDK) Cdc28. The activity of Cdc28 is regulated mainly through the synthesis and degradation of various cyclins

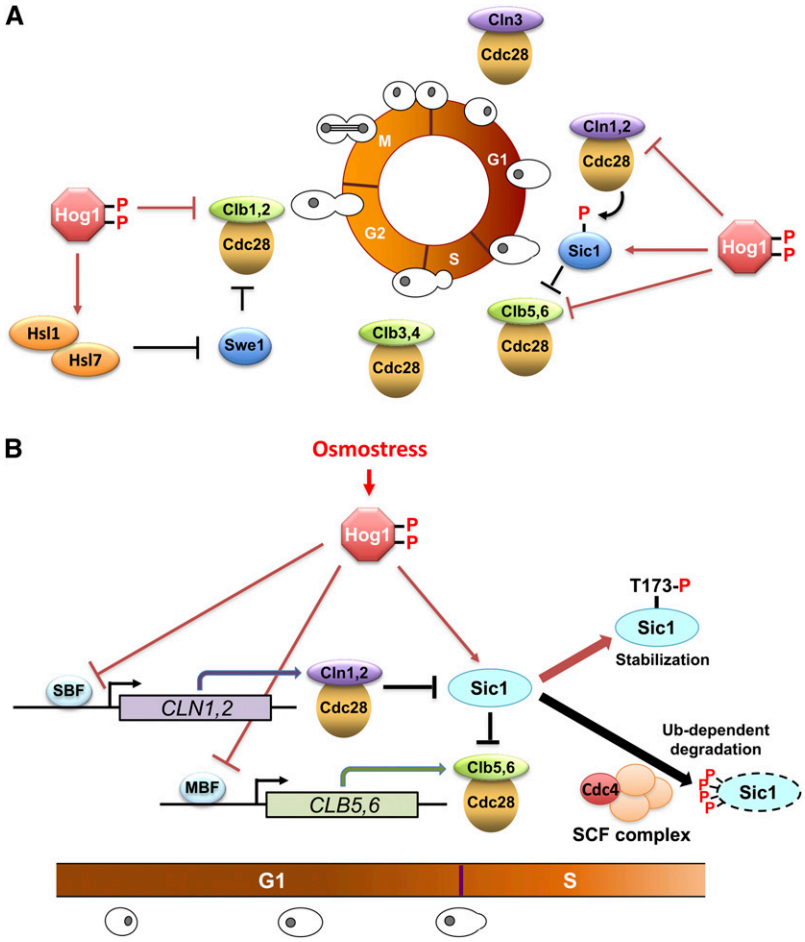


**Figure 10** Control of the cell-cycle progression by the Hog1 MAPK. (A) The dominant species of the cyclin/Cdc28 complex at each cell-cycle phase are shown around the circle that represents the cell cycle ($G_1 \rightarrow S \rightarrow G_2 \rightarrow M$). Once activated by osmostress, Hog1 seems to modulate all phases of the cell cycle. In the $G_1$ and $G_2$ phases, Hog1 controls cell-cycle regulators both directly and indirectly, and Hog1 also regulates expression of cyclins. Hog1 also modulates the S and M phases, but the mechanisms remain unclear (not shown). (B) Details of the control of the $G_1$/S transition by Hog1. The transition from $G_1$ to S phase is mediated by the expression of cyclins Cln1,2 and Clb5,6, and their binding to the Cdc28 kinase. Initially, Clb5,6/Cdc28 is inhibited by the Sic1 (CDKi). As the activity of Cln1,2/Cdc28 increases, Sic1 is phosphorylated at multiple sites, prompting ubiquitination of Sic1 by the SCF (Cdc4) complex, and its degradation by proteasome. This degradation of Sic1 releases active Clb5,6/Cdc28, which then promotes DNA replication. Osmostress-activated Hog1 delays $G_1$/S transition both by inhibiting transcription of cyclin genes (both *CLN* and *CLB*), and by directly phosphorylating Sic1 at Thr-173, which inhibits ubiquitination of Sic1 and stabilizes Sic1.

and inhibitors that bind to Cdc28 (Clotet and Posas 2007). As the presence of various stresses such as heat stress, DNA damage, and extracellular hyperosmolarity critically affects progression through the cell cycle, cells must modulate the cell cycle to allow for proper cellular adaptation (Flattery-O'Brien and Dawes 1998; Li and Cai 1999; Wang *et al.* 2000; Alexander *et al.* 2001). For cells under these stress conditions, controlled delay of cell-cycle progression is very important, since it enables cells to adapt to the new environmental conditions before moving through vulnerable cell-cycle transition periods.

As environmental stresses can occur at any cell-cycle stage, in principle, all phases of the cell cycle must be regulated by stress-activated mechanisms. In mammals, the p38 stress-responsive MAPK has been implicated in regulating $G_1$ phase, S phase, as well as a $G_2$/M checkpoint, in response to several stimuli, including osmostress (Dmitrieva *et al.* 2002; Joaquin *et al.* 2012). In yeast, the Hog1 MAPK induces a rapid and transient delay at various stages of the cell cycle to permit the full development of adaptive responses before cell-cycle progression resumes (Figure 10A) (Clotet and Posas 2007; Yaakov *et al.* 2009).

***$G_1$/S transition:*** In yeast, the $G_1$/S transition is controlled by the interplay of several cyclins. At the beginning of $G_1$, the Cln3 cyclin is sequestered in the cytoplasm by the Whi3 retention factor (Bellí *et al.* 2001; Garí *et al.* 2001). In the late $G_1$ phase, nuclear accumulation of Cln3 triggers the phosphorylation of the transcription repressor Whi5 by Cln3-bound Cdc28 (Cln3/Cdc28) (Costanzo *et al.* 2004; de Bruin *et al.* 2004). Phosphorylated Whi5 dissociates from the transcription complexes SBF and MBF, which leads to the transcription of a second wave of cyclins (Cln1, Cln2, Clb5, and Clb6). The $G_1$ cyclins Cln1 and Cln2 are functionally redundant and are abbreviated as Cln1,2; similarly, the B-type cyclins Clb5 and Clb6 are redundant and are abbreviated as Clb5,6. The activity of Cln1,2/Cdc28 stimulates bud formation and phosphorylation of the CDK inhibitor Sic1 (Figure 10B). Unphosphorylated Sic1 binds and inhibits Clb5,6/Cdc28. When several residues in Sic1 are phosphorylated by Cln1,2/Cdc28, Sic1 is poly-ubiquitinated and degraded by the proteasome (Verma *et al.* 1997). Sic1 degradation removed Sic1 inhibition of Clb5,6/Cdc28. Activated Clb5,6/Cdc28 can phosphorylate Sic1 at the same residues as those phosphorylated by Cln1,2/Cdc28. Thus, Sic1 degradation accelerates by the positive feedback loop, resulting in an abrupt rise in Clb5,6/Cdc28 activity, which drives cells into S phase. DNA replication is initiated when Clb5,6/Cdc28 phosphorylates the replication proteins Sld2 and Sld3, which are components of the pre-initiation

complex (Masumoto *et al.* 2002; Tanaka *et al.* 2007). Therefore, at the end of $G_1$, the activity of Clb5,6/Cdc28 depends on both the levels of the Clb5,6 cyclins and the levels of the inhibitor Sic1 (Schwob *et al.* 1994; Verma *et al.* 1997; Cross *et al.* 2007).

When yeast cells are exposed to high osmolarity (*e.g.*, 0.4 M NaCl), the Hog1 MAPK is transiently activated for ~30 min, and a corresponding cell-cycle delay in $G_1$ is observed (Bellí *et al.* 2001). That this delay is caused by activated Hog1, and not by other effects of osmostress, can be demonstrated using genetic means to activate Hog1 in the absence of osmostress. Experimentally, this can be achieved by high-temperature inactivation of an *sln1*-ts mutant or expression of the constitutive $PBS2^{DD}$ allele, both of which are upstream of the Hog1 activation pathway. If Hog1 activity is sustained for an extended period, cells undergo a programmed cell death that requires the action of the nuclear serine proteinase Nma111 and the $SCF^{CDC4}$ ubiquitin ligase (Vendrell *et al.* 2011). However, a shorter activation of the HOG pathway results in cell-cycle delay in $G_1$. Hog1 controls the $G_1$/S transition by acting on two different components of the basic cell-cycle machinery (Escoté *et al.* 2004; Clotet and Posas 2007; Zapater *et al.* 2007 Adrover *et al.* 2011). First, Hog1-mediated $G_1$ arrest is partially mediated by downregulation of expression of the $G_1$ cyclins Cln1,2 and of the S-cyclin Clb5 (Bellí *et al.* 2001). The exact nature of the mechanism that delays the expression of these SBF/MBF-dependent genes under osmotic stress remains unknown. Second, Hog1 promotes a cell-cycle delay in $G_1$ by direct phosphorylation of Sic1 at a specific residue (Escoté *et al.* 2004). When Cln1,2/Cdc28 activities reach a threshold level, Sic1 is phosphorylated, then ubiquitinated by the Cdc4 ubiquitin ligase, and eventually degraded by the proteasome. If Hog1 is activated by osmostress, it interacts physically with Sic1 and phosphorylates another residue (Thr-173) at the carboxyl terminus of Sic1. This phosphorylation inhibits Sic1 ubiquitination (Escoté *et al.* 2004; Zapater *et al.* 2005). Thus, Sic1 degradation is inhibited, resulting in prolonged inhibition of Clb5,6/Cdc28 and a delay in the $G_1$/S transition. Incidentally, it should be noted that the same Thr-173 in Sic1 is targeted by the TOR pathway to delay cell-cycle progression in $G_1$ (Zinzalla *et al.* 2007).

Mathematical modeling and quantitative *in vivo* experiments have defined the differential roles of Hog1 in $G_1$ delay (Adrover *et al.* 2011). There are distinct effects of Hog1 at different cell-cycle stages in response to osmostress: early in $G_1$, it inhibits cyclin expression, and later in $G_1$, it inhibits Sic1 degradation. Of the three cyclin genes whose transcription is inhibited by Hog1, inhibition of *CLN1,2* expression delays bud morphogenesis, and inhibition of *CLB5* expression delays DNA replication and entry into S phase. Later in $G_1$, when these cyclins are already expressed, inhibition of cyclin expression can no longer prevent cell-cycle progression. Instead, Hog1-mediated phosphorylation and inhibition of the degradation of Sic1 prevents active Clb5/Cdc28 from initiating DNA replication. Thus, these two distinct mechanisms that operate at different time points ensure that no premature entry into S phase occurs under osmostress conditions.

***S phase:*** The Hog1 MAPK is also able to modulate S-phase progression in response to osmostress. During S phase, replication of the genome occurs, which is a highly ordered process involving many proteins. The assembly of the replication complex (RC) at origins of replication begins with the formation of the pre-replicative complex (pre-RC) during late mitosis to $G_1$. In the S phase, the pre-RC is converted into a fully assembled pre-initiation complex. Further conversion to a fully functional RC is accompanied by various molecular events, including phosphorylation of the Dpb2 subunit of the DNA polymerase by CDK. These processes are dependent on the activities of S-phase CDK (Clb5,6/Cdc28) and Dbf4-dependent kinase (the Cdc7/Dbf4 complex). When cells are stressed in early S phase, Hog1 controls the S phase by delaying the expression of the S-phase cyclins Clb5,6 (see *$G_1$/S transition*). If cells are stressed later in S phase, Hog1 interacts with components of the replication complex and delays phosphorylation of the Dpb2 subunit of the DNA polymerase (Yaakov *et al.* 2009). These effects of Hog1 are independent of the S-phase DNA checkpoint or of the known Hog1 targets Sic1 and Swe1.

One possible reason to delay the cell cycle when osmostress occurs in the S phase is the need to prevent replication from interfering with the necessary transcription of adaptive genes. Adaptive responses to osmostress require that expression of a very large number of genes is induced. It is therefore conceivable that initiating or ongoing replication might occur on the genes that are being transcribed for adaptation. It is easy to see that if the large replication complex and the transcription complex attempt to occupy the same space, they would interfere with each other's function. Furthermore, it has been shown that a collision between RNA Pol II and DNA polymerase leads to transcription-associated recombination (Aguilera 2002, 2005; Aguilera and Gómez-González 2008). Thus, delaying replication in response to osmostress must be important both to provide proper adaptive gene expression and to prevent genomic instability.

***$G_2$ phase:*** Cell-cycle progression from the S into the $G_2$ phase depends mainly on another wave of cyclin production: the mitotic cyclins Clb1 and Clb2. The Mcm1/SFF (Mcm1/Fkh2/Ndd1) complex is the transcription factor that regulates expression of *CLB1* and *CLB2* (Althoefer *et al.* 1995; Maher *et al.* 1995; Jorgensen and Tyers 2000). Further cell-cycle progression from $G_2$ into mitosis is controlled by the morphogenetic checkpoint. The $G_2$/M transition depends on the activity of CDK that is associated with either of these mitotic cyclins (Clb1,2/Cdc28). The activity of the Clb2/Cdc28 complex is inhibited by phosphorylation at a conserved tyrosine in Cdc28 by the kinase Swe1 (the ortholog of *Schizosaccharomyces pombe* and mammalian

Wee1) and is reactivated by the phosphatase Mih1 (the ortholog of *S. pombe* and mammalian Cdc25). When the formation of the septin ring is completed on the neck between mother and daughter cells, the morphogenetic checkpoint recruits a complex of Hsl1 (a septin-dependent protein kinase) and Hsl7 (a protein that binds to Swe1) to Swe1, which targets Swe1 for destruction. Swe1 does not affect cell-cycle progression under unstressed, exponentially growing conditions (Amon *et al.* 1992). However, when bud formation is impaired by various stresses, Swe1 remains active, inactivates the mitotic CDK, and delays the cell cycle (McMillan *et al.* 1999). Perturbations of the actin cytoskeleton, rather than the bud size itself, seem to stabilize Swe1 (McNulty and Lew 2005).

Activation of Hog1 upon osmostress induces a cell-cycle delay in $G_2$ by decreasing Clb2/Cdc28 activity and by downregulating *CLB2* transcription (Alexander *et al.* 2001; Clotet *et al.* 2006). The mechanisms that Hog1 uses to downregulate *CLB2* transcription are not known, but this downregulation could be a secondary effect due to the Hog1-induced decrease in Clb2/Cdc28 activity. In contrast, the mechanism by which Hog1 decreases Clb2/Cdc28 activity is clearly a result of Hog1 acting on the machinery of the morphogenetic checkpoint that controls Swe1 levels. Thus, activated Hog1 interacts with and directly phosphorylates Hsl1 at a residue within the Hsl7-docking site. This Hsl1 phosphorylation promotes the delocalization of Hsl7 from the neck, resulting in Swe1 accumulation and $G_2$ arrest (Clotet *et al.* 2006). In mutant cells that contain a nonphosphorylatable Hsl1, Hog1 activity cannot promote Hsl7 delocalization, fails to accumulate Swe1, and fails to arrest at $G_2$. This explains why the mitogenic checkpoint is sensitive to osmotic stress.

***Exit from mitosis:*** Exit from mitosis after chromosome segregation is controlled by a signaling cascade termed the Mitotic Exit Network (MEN). Activation of MEN is initiated by the activation of Tem1, a G-protein that is located in the spindle pole body (Morgan 1999). When cells undergo anaphase, the spindle pole body enters into the daughter cell where the GEF for Tem1, Lte1, is localized (Pereira *et al.* 2000). Activated (GTP-bound) Tem1 then binds to and activates the Cdc15 kinase, a critical component of MEN, which leads to activation of the phosphatase Cdc14. The Cdc14 protein phosphatase is tightly regulated by a competitive inhibitor Net1, which holds Cdc14 in an inactive state in the nucleolus during most of the cell cycle except during anaphase and telophase. Cdc14 is released by MEN or by the FEAR (Cdc Fourteen Early Anaphase Release) network, and spreads throughout the nucleus and cytoplasm to induce exit from mitosis. Cdc14 activates the Anaphase Promoting Complex (APC)/Cdh1, which promotes ubiquitination and degradation of the remaining B- type cyclins. Cdc14 also directly dephosphorylates CDK substrates; for example, Cdc14 dephosphorylates and stabilizes Sic1 (Stegmeier and Amon 2004).

Exit from mitosis could also be regulated by the Hog1 MAPK under osmotic stress. In response to osmostress, MEN mutants exit from mitosis in a manner that is dependent on Hog1. In such MEN mutants, the HOG pathway seems to drive exit from mitosis by promoting the function of FEAR network that activates Cdc14, although the exact mechanism remains unclear (Reiser *et al.* 2006).

### *Other downstream effectors of the Hog1 MAPK*

In addition to phosphorylating components of the transcriptional and cell-cycle machineries, the Hog1 MAPK also phosphorylates other cytoplasmic and nuclear proteins. Recent phospho-proteomic studies identified a number of proteins that are phosphorylated upon osmostress (Soufi *et al.* 2009). In addition, cells with mutations in kinases and phosphatases that play a role in the HOG pathway showed changes in the phospho-proteome of the cell even under normal osmotic conditions (Bodenmiller *et al.* 2010). These analyses suggest that a large number of Hog1 substrate proteins must exist that have not been previously characterized. Below, we will discuss several well-defined substrates of Hog1 that are known to have important roles in osmo-adaptation.

***Ion channels:*** Activation of Hog1 in response to osmostress induces the phosphorylation of at least two proteins located at the plasma membrane, the Nha1 $Na^+/H^+$ antiport and the Tok1 potassium channel (Proft and Struhl 2004). Immediately following the start of osmostress, passive water efflux rapidly increases intracellular $Na^+$ concentration, which causes dissociation of proteins from chromatin. Activated Hog1 phosphorylates and thus stimulates Nha1 activity, leading to rapid pumping-out of excessive $Na^+$. This activity is crucial for the rapid and selective re-association of stress-responsive transcription factors with chromatin. Phosphorylation of the Tok1 $K^+$ channel also increases its activity, although its contribution to adaption seems to be less important than that of Nha1. These initial responses to osmostress precede, and prepare for, the activation of stress-response genes that depend on Hog1.

Control of ionic fluxes during long-term adaptation occurs through regulation of the expression of the Na ATPase *ENA1*. Thus, a single MAP kinase coordinates diverse responses to stress that are temporally, spatially and mechanistically distinct, thereby providing very rapid initial relief, which facilitates subsequent changes in gene expression that permit long-term adaptation to harsh environmental conditions.

***Protein kinases regulated by Hog1:*** There is a transient decrease in protein synthesis in response to increases in external osmolarity that is caused by a decrease in amino acid uptake, repression of ribosomal protein gene expression, and a decrease in translation efficiency (Norbeck and Blomberg 1998; Uesono and Toh-E 2002). The Hog1 MAPK is not involved in the initial inhibition of translation, but rather in the reactivation of translation under stress, which functions as an adaptation mechanism (Uesono and Toh-E 2002). The cytoplasmic Rck2 kinase, which is structurally

homologous to mammalian CaM kinases, is directly phosphorylated and regulated by Hog1 (Bilsland-Marchesan *et al.* 2000; Teige *et al.* 2001). Reduction of protein synthesis upon osmostress was similar in *hog1*Δ and *rck2*Δ cells, which suggests that the effect of Hog1 on translation is mediated by the Rck2 kinase. Rck2 may affect translation by directly regulating the elongation factor EF-2, but an effect on initiation factors cannot be excluded (Teige *et al.* 2001). An analysis of polysome-associated mRNAs showed that many genes that are not transcriptionally induced are translated more efficiently under osmostress conditions. A similar analysis of *hog1*Δ cells showed that the effect of Hog1 on translation was even stronger than the effect on transcription, which highlights the importance of translational control for the fine tuning of adaptive responses (Warringer *et al.* 2010).

## Perspectives

In the second edition of "the Yeast Books" published in mid-1990s, there was only a very brief mention of budding yeast osmoregulation, which occupied no more than half a page (MacNeill and Nurse 1997). The relevant knowledge accumulated in the intervening 15 years, which we have tried to summarize in this review, is nothing less than astounding. Perhaps as a result, many important new questions have become apparent. Below, we have made a somewhat subjective list of questions that are particularly important. Considering the rapid progress made in the past 15 years, we can optimistically expect that many of these questions will be answered by the next edition of the YeastBook.

We now have a clear outline of the upstream signaling in osmostress pathway. However, under closer inspection, many unsolved questions remain. For example, the mechanism by which the Sln1 osmosensor detects changes in osmolarity is unclear. Because the Sln1 histidine kinase is structurally similar to the bacterial osmosensor EnvZ (Tokishita and Mizuno 1994; Yoshida *et al.* 2007; Wang *et al.* 2012), it is reasonable to expect that their activation mechanisms are also related. Therefore, a parallel investigation of these two osmosensors, emphasizing both their similarities and differences, might be productive. Another important question is how the Sln1-Ypd1-Ssk1 phosphorelay is regulated, and, in particular, the mechanism by which the stability of Ssk1∼P changes so drastically upon osmostress stimulation.

Although mammals do not have any homolog of the Sln1 osmosensor, they do have mucin-like transmembrane proteins that are structurally similar to the Msb2/Hkr1 osmosensors. Therefore, elucidation of the mechanism by which Msb2/Hkr1 detect osmolarity changes might shed light on the osmosensing mechanism in higher eukaryotes. Furthermore, it needs to be examined whether the Msb2/Hkr1 osmosensors actually regulate the activity of Cdc42, as currently hypothesized. The function of Sho1 in signaling should also be further delineated. Traditionally, this molecule is considered to be a passive membrane anchor for the Pbs2 MAPKK. However, genetic evidence suggests that Sho1 might have a more active role in signaling, perhaps by serving as a platform around which a signaling complex is organized.

The mechanism that controls the crosstalk among the MAPK signaling pathways is another open question. For example, how activation of the Kss1 MAPK by osmostress is prevented in wild-type cells, while this inhibition is abrogated in *hog1*Δ mutant cells, has been intensely investigated but without any clear answer. Eventually, understanding the global signaling network including the HOG signaling pathways and other intracellular signaling pathways should be an important goal in the next decade.

Regarding downstream effector functions, the number of unsolved questions is commensurate with the breadth of Hog1 functions. There is evidence to suggest that activated Hog1 elicits the production/accumulation of protective osmolytes through transcriptional induction of metabolic enzymes as well as by direct modulation of metabolic flux. Thus, establishment of how Hog1 regulates the metabolic network, both at the transcriptional and posttranscriptional levels, is important for understanding of the basic logic of the cellular response to environmental osmostress. At the metabolic level, it is essential to identify the key enzymes whose activities are directly controlled by Hog1. We must also elucidate the still unclear details of the regulation by osmostress of transcription initiation and elongation, mRNA processing, mRNA stability, nuclear export, and translation.

Osmostress induces cell-cycle delays, which permit cells to adapt to the stress before progressing into vulnerable cell-cycle transitions. Hog1 uses several molecular strategies, alone or in combination, to arrest cells at safer phases in the cell cycle until an osmotic balance is re-established. Some of these mechanisms have become clearer in recent years, but others remain obscure. Because of the advanced knowledge available regarding basic cell-cycle regulation, a model-based simulation will be particularly helpful in investigation of the modulation of the cell cycle by osmostress.

Finally, the search for Hog1 substrates is far from complete. Identification and characterization of novel Hog1 targets will serve to define new Hog1 functions as well as the regulatory mechanisms under its control.

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