

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 *hog1Δ* 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 osmotic stress 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,

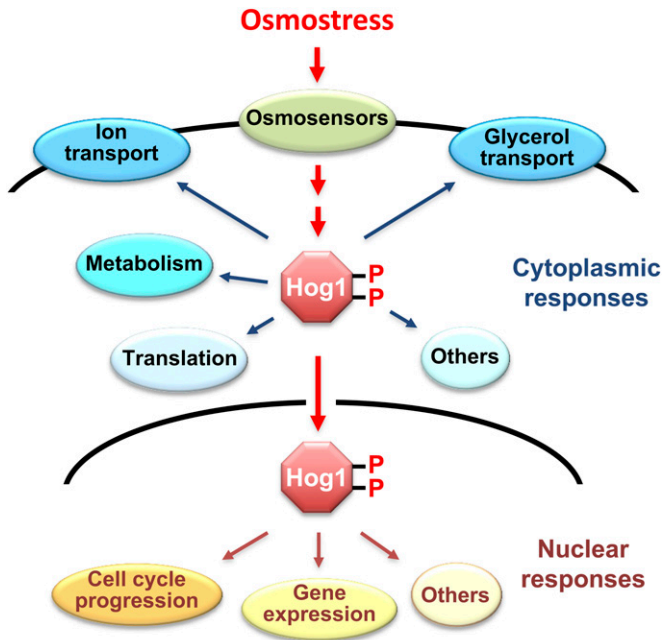


Figure 1 Osmo-adaptive responses in yeast. In response to an increase in extracellular osmolarity, the Hog1 MAPK is activated, which leads to the induction of cytoplasmic and nuclear adaptive responses. Cytoplasmic responses include the control of ionic fluxes and glycerol transport, metabolic enzymes,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

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 osmstress 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 osmstress 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

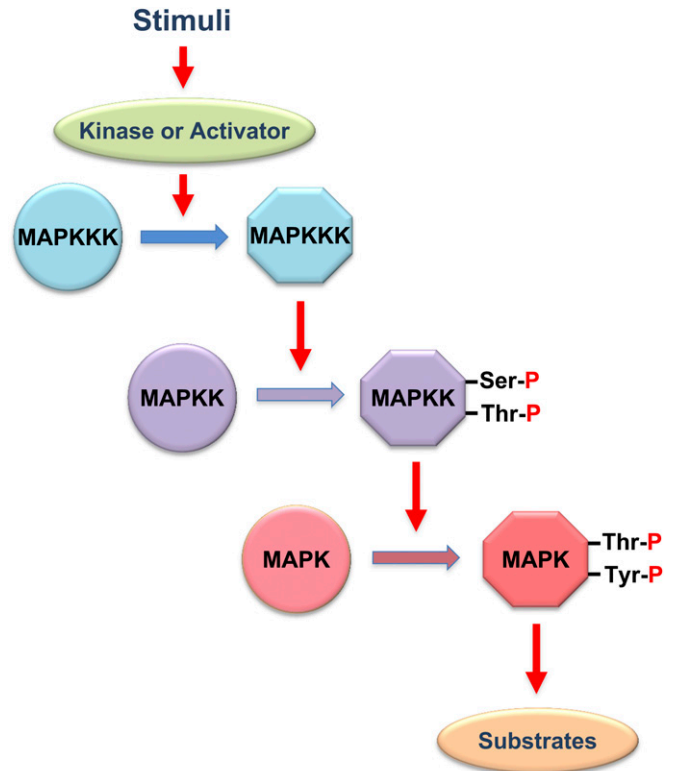


Figure 2 A schematic diagram of the MAP kinase module. Circles and hexagons represent, respectively, inactive and active forms of kinases. MAPK, MAP kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase.

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 *ssk2Δ ssk22Δ* 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

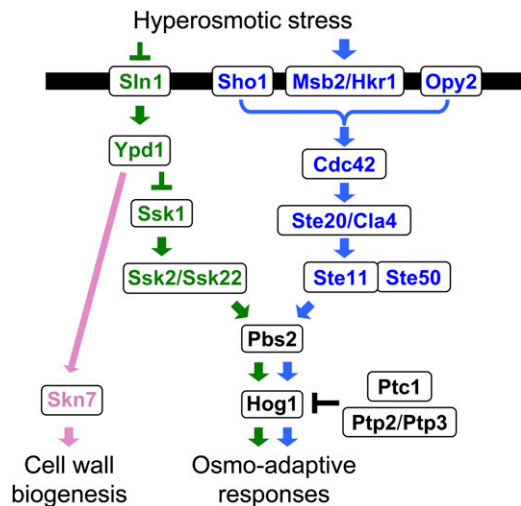


Figure 3 A schematic diagram of the yeast HOG pathway. The protein names separated by a slash (/) are functionally redundant. Proteins that are specific to the Sln1 branch are colored green, those that are specific to the Sho1 branch are colored blue, and those that are common are colored black. The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

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 phosphore-

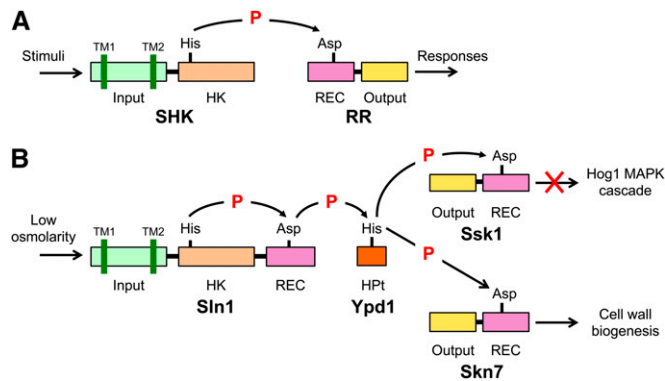


Figure 4 Schematic diagram of two-component signaling systems. (A) The prototypical two-component system that is characterized by the conserved phosphotransfer reaction between a histidine residue and an aspartate residue. (B) The *Sln1*-*Ypd1*-*Ssk1* multistep phosphorelay. SHK, sensor histidine kinase;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

lay. Because both histidine phosphate and aspartate phosphate are energetically activated, they are often symbolized as $\text{His}\sim\text{P}$ and $\text{Asp}\sim\text{P}$. 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 $<10^\circ$ 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* MAPKKs by *Ssk1*: *Ssk1* activates a pair of homologous, and functionally redundant, MAPKKs termed *Ssk2* and *Ssk22* (Maeda *et al.* 1995). Like many other members of the MAPKK 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*-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 osmotic stress (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 osmotic stress (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-unknown phosphatase.

The *actin* cytoskeleton is important for the survival of yeast under osmotic stress, 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 osmotic stress, *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*, osmotic stress does cause slight activation of the *Hog1* MAPK in *ssk1Δ sho1Δ* mutants, whereas no activation is observed in *ssk2Δ ssk22Δ sho1Δ* 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 osmotic stress, 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 *ssk2Δ ssk22Δ* 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 *skn7Δ* 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-osmotic stress 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 *skn7Δ* mutants are not osmosensitive, the suppression of the hypo-osmotic stress sensitivity of a *pkc1Δ* 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

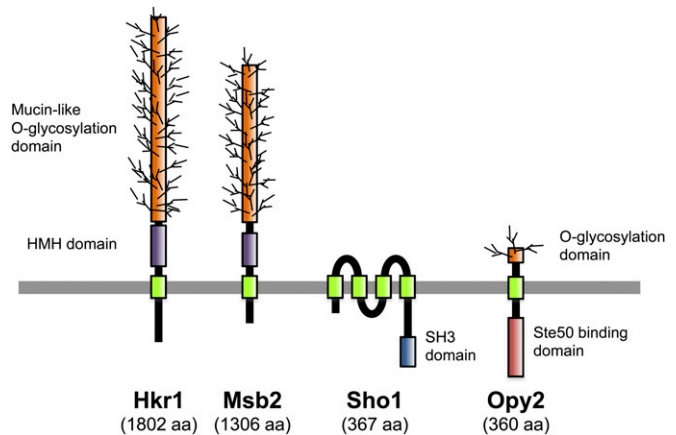


Figure 5 Schematic representations of the four transmembrane proteins involved in the *Sho1* branch of the HOG pathway. HMH, *Hkr1*-*Msb2* homology domain. Not drawn to scale.

proteins are highly Ser/Thr rich and contain numerous O-glycosylation sites that are glycosylated by the protein O-mannosyl 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 *ssk1Δ sho1Δ* mutant was abolished in the *ssk1Δ sho1Δ msb2Δ* 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 *msb2Δ hkr1Δ* double-mutant cells, but a constitutively active *MSB2* or *HKR1* mutant cannot activate *Hog1* in a *sho1Δ* 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 *pmt4Δ* 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 osmstress-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 synthetically high osmolarity 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 osmstress stimulation, and this dissociation is hindered in a *ste20Δ* or a *ste11Δ* mutant or in a *pbs2Δ* mutant in which a catalytically inactive *Pbs2*-K389M is expressed, but interestingly not in a *hog1Δ* 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 osmstress (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*- Δ RA) is functionally defective and cannot activate the *Hog1* MAPK in response to osmstress. However, forced localization of *Ste50*- Δ RA 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*- Δ RA, using the Ras C-terminal prenylation signal, can rescue the osmstress-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 *opy2Δ* 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*-mannosyl 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 *ste20Δ* and *cla4Δ* mutants are viable, the *ste20Δ cla4Δ* 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 *ste20Δ* mutants of a parental strain that is defective in the Sln1 branch, such as *ssk2Δ ssk22Δ*, is sensitive to high osmolarity, but these mutants can tolerate moderate osmotic stress (Raitt *et al.* 2000b). In contrast, *ste20Δ 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 *ste20Δ (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 path-

way, 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.*

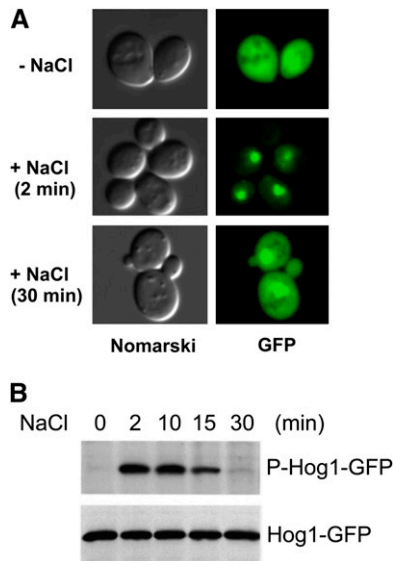


Figure 7 Transient phosphorylation and nuclear localization of the Hog1 MAPK after osmostress. GFP-tagged Hog1 (Hog1-GFP) was expressed in a *hog1Δ* host strain, and cells were exposed to 0.4 M NaCl for the time indicated. (A) Hog1-GFP was detected by fluorescence microscopy (GFP), while the cell shape 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 *ptp2Δ ptc1Δ* 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* Δ NES mutants accumulate in the nucleus, whereas *Pbs2* Δ NES Δ NLS 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 \sim 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 osmotic stress 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*), osmotic stress 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, osmotic stress 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 ($<1/200 \text{ sec}^{-1}$), 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 (McClellan *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 osmotic stress 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 osmotic stress, 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 osmotic stress (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 osmotic stress (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 osmotic stress, 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

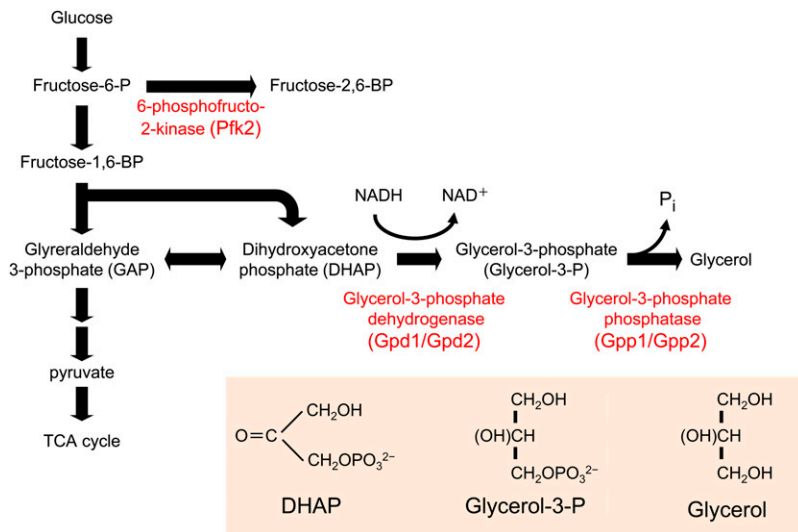


Figure 8 Glycerol biosynthetic pathway. Glycerol is synthesized from an intermediate in the glycolysis, dihydroxyacetone phosphate (DHAP), by two-step enzymatic reactions. The first enzyme is glycerol-3-phosphate dehydrogenase (Gpd1/Gpd2), which reduces DHAP using NADH as reducing agent. The second enzyme 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 osmotic stress. 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 osmotic stress, 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 osmotic stress 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 osmotic stress.

General stress responses

In addition to glycerol, a number of other organic osmolytes, including trehalose, protect yeast from osmotic stress, 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 osmotic stress have similar protective functions as these osmolytes (de Nadal and Posas 2010; Martínez-Montañés *et al.* 2010). For

example, in response to osmotic stress, 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. Osmotic stress 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°, *hog1Δ* cells can survive on moderate osmotic stress, such as 0.8 M sorbitol, better than at 30° (Siderius *et al.* 2000). This protection is not sufficient for *hog1Δ* cells to survive higher levels of osmolarity.

Regulation of gene expression by osmotic stress

Global analysis of gene expression upon osmotic stress:

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 osmotic stress 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 *hog1Δ* strain (Westfall *et al.* 2008). Therefore, cytoplasmic events caused by the rapid and transient activation of the *Hog1* MAPK in response to osmotic stress—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 suffi-

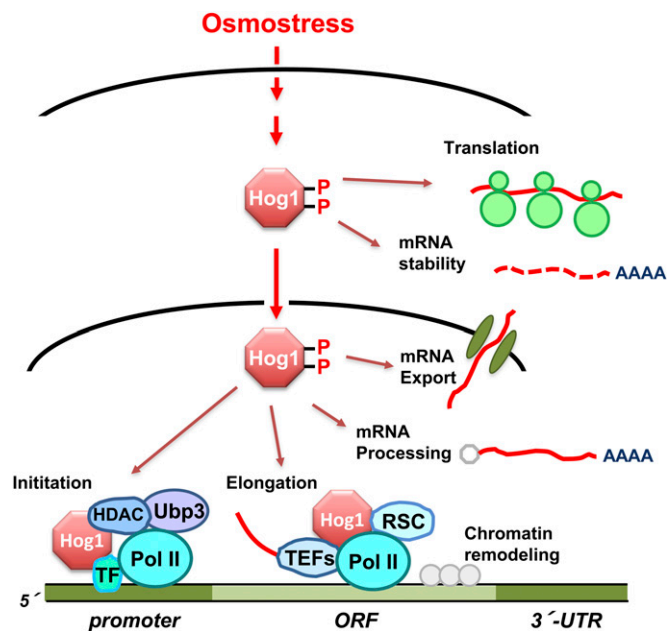


Figure 9 Control of mRNA biogenesis by the Hog1 MAPK. Once activated upon osmotic stress, Hog1 controls many aspects of mRNA biogenesis both in the nucleus and in the cytoplasm. Hog1 phosphorylates and activates transcription factors (TFs). Remarkably, Hog1 associates to loci of stress-responsive genes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

cient for the maintenance of osmotic balance under these experimental conditions without invoking induced gene expression in the nucleus.

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 osmotic stress; of these genes, there is one subset of genes that specifically responds to osmotic stress, 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, osmotic stress, 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 non-lethal 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 osmotic stress (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 osmotic stress 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 osmo-responsive 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 osmotic stress (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 osmotic stress.

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 osmotic stress-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 osmotic stress.

***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* (Alepez *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 osmotic stress 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* (Alepez *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 (Alepez *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 (Alepez *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 osmotic stress, *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*, *Skp1*, 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 osmotic stress-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 osmotic stress-responsive 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 osmotic stress suggest that *Hog1* serves to recruit the basic transcriptional machinery to stress-responsive promoters (Alepez *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 Pol III 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 osmotic stress-responsive promoters. Several observations indicate that, whereas Mediator is crucial for proper gene induction under both mild and severe osmotic stress conditions, the role of SAGA is dependent on the strength of the osmotic stress. Thus, the requirement for a given transcriptional complex to regulate a promoter might depend on the severity of osmotic stress 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 osmotic stress (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 osmotic stress. *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 osmotic stress-sensitive and show compromised expression of osmotic stress-responsive genes controlled by *Hog1*. *Hog1* binds to an *Rpd3* complex (presumably *Rpd3L*) and, upon stress, recruits it to specific osmotic stress-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 osmotic stress-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 osmotic stress-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 osmotic stress, 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 osmotic stress-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 osmotic stress-responsive genes. The mechanism by which *Hog1* is recruited to the 3'-regions of osmotic stress-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, destabilization 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 *hog1Δ* 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

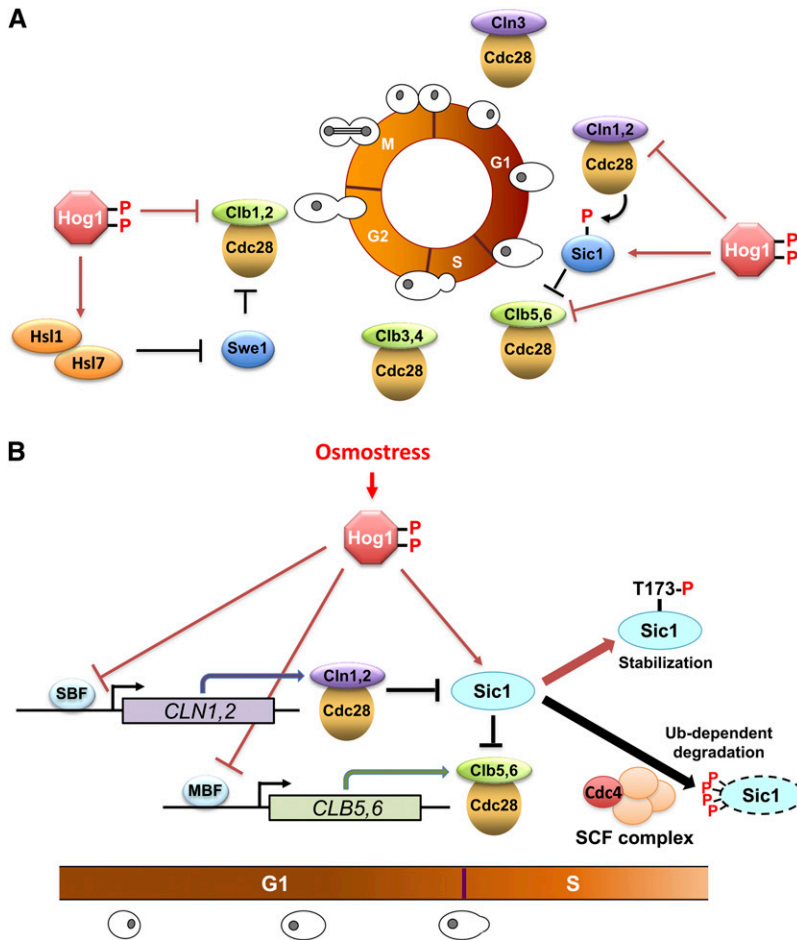


Figure 10 Control of the cell-cycle progression by the Hog1 MAPK. (A) The dominant species of the cyclin/Cdc28 complex at each cell-cycle phase are shown around the circle that represents the cell cycle ($G_1 \rightarrow S \rightarrow G_2 \rightarrow M$). Once activated by osmostress, Hog1 seems to modulate 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 cell cycle 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 osmolarity, can be demonstrated using genetic means to activate *Hog1* in the absence of osmolarity. 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 osmolarity, 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 osmolarity: 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 osmolarity conditions.

S phase: The *Hog1* MAPK is also able to modulate S-phase progression in response to osmolarity. 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 *G₁/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 osmolarity occurs in the S phase is the need to prevent replication from interfering with the necessary transcription of adaptive genes. Adaptive responses to osmolarity 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 osmolarity must be important both to provide proper adaptive gene expression and to prevent genomic instability.

G₂ 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 G₂ 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 G₂ 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 G₂. 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 adaptation 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* \sim 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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