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Regulation of the Osmoregulatory HOG MAPK Cascade in Yeast

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The budding yeast *Saccharomyces cerevisiae* has at least five signal pathways containing a MAP kinase (MAPK) cascade. The high osmolarity glycerol (HOG) MAPK pathway is essential for yeast survival in high osmolarity environment. This mini-review surveys recent developments in regulation of the HOG pathway with specific emphasis on the roles of protein phosphatases and protein subcellular localization. The Hog1 MAPK in the HOG pathway is negatively regulated jointly by the protein tyrosine phosphatases Ptp2/Ptp3 and the type 2 protein phosphatases Ptc1/Ptc2/Ptc3. Specificities of these phosphatases are determined by docking interactions as well as their cellular localizations. The subcellular localizations of the osmosensors (Sln1 and Sho1), kinases (Pbs2, Hog1), and phosphatases in the HOG pathway are intricately regulated to achieve their specific functions.

Key words: HOG pathway, localization, MAP kinase, phosphatase, yeast.

Through evolution, living organisms have developed elaborate and sensitive adaptation mechanisms that enable them to detect changes in their environment, to signal intracellularly, and to adapt properly. MAP kinase (MAPK) cascades are important components in such cellular adaptation programs. Each MAPK cascade is composed of three sequentially activating kinases; a MAPK kinase kinase (MAPKKK) phosphorylates and activates a MAPK kinase (MAPKK), which then activates a MAPK. The budding yeast *Saccharomyces cerevisiae* has at least five signal pathways containing a MAPK cascade: the cell-wall integrity pathway, the spore wall assembly pathway, the filamentous/invasive growth pathway, the pheromone response pathway, and the high osmolarity glycerol (HOG) pathway (Fig. 1) (*1*). The HOG MAPK pathway is essential for yeast survival in a high osmolarity environment, and is the main subject of this mini-review article. The osmotic environment of the budding yeast growing in the wild is especially capricious, as typified by ripening fruits with very high glucose contents. Because the yeast cells are immotile and cannot escape from a hostile environment, they have to make internal adjustments to adapt to the increased external osmolarity. They do so by synthesizing and retaining the compatible osmolites such as glycerol to increase the internal osmolarity (*2*), by modifying water efflux, and by adjusting cell cycle progression.

The Hog1 MAPK, the key element in the HOG pathway, is activated upon hyperosmotic stress by phosphorylation of conserved threonine and tyrosine residues, via two independent upstream mechanisms that converge on the Pbs2 MAPKK. Activated Hog1 rapidly, but transiently, accumulates in the nuclear compartment, where Hog1 participates in a modification of the transcriptional pattern in response to osmotic stress. Two transmembrane osmosensors, Sln1 and Sho1, have been identified that can independently mediate activation of the HOG pathway in response to increased external osmolarity. The two upstream branches of the HOG pathway are thus referred to as the SLN1 branch and the SHO1 branch. The Sln1 transmembrane protein is a homolog of bacterial two-component signal transducers, and contains a cytoplasmic histidine kinase (HK) domain as well as a receiver (Rec) domain. Under normal (relatively low) osmotic conditions, the Sln1 HK domain is kept in the active conformation and phosphorylates a histidine residue in the HK domain. The phosphate is then transferred from the histidine to an aspartate residue in the Rec domain near the C-terminus of Sln1. The phosphate is further transferred via a phospho-relay mechanism to an intermediary phospho-carrier protein Ypd1, and eventually to an aspartate residue in another Rec domain protein, Ssk1 (*3–6*). Under hyper-osmotic conditions, Sln1 responds to changes in cellular turgor pressure (*7*), and the Sln1 HK domain is altered to the inactive conformation, resulting in a cessation of the Sln1-Ypd1-Ssk1 phospho-relay reaction. Ssk1 is rapidly dephosphorylated by an unknown mechanism; the resulting dephosphorylated Ssk1 binds and activates the redundant Ssk2 and Ssk22 MAPKKKs, which then activate the Pbs2 MAPKK (*8*). The phospho-aspartate residue in Ssk1 is intrinsically unstable and prone to spontaneous hydrolysis, but it is stabilized when Ssk1 is bound to Ypd1 (*9*, *10*). Thus, the dissociation of the relatively stable Ssk1-Ypd1 complex may be the first step in the Ssk1-dependent activation of the downstream elements. Dephosphorylated Ssk1 is eventually degraded by the Ubc7-dependent ubiquitin-proteasome system, down-regulating the HOG pathway after the completion of the osmotic adaptation (*11*).

The SHO1 branch of the HOG pathway requires Sho1, Cdc42, Ste20, Ste11, and Ste50 for transmission of the osmotic stress signal (*8*, *12–15*). The Sho1 protein comprises four transmembrane domains near its N-terminus, and a cytoplasmic SH3 domain that binds a proline-rich

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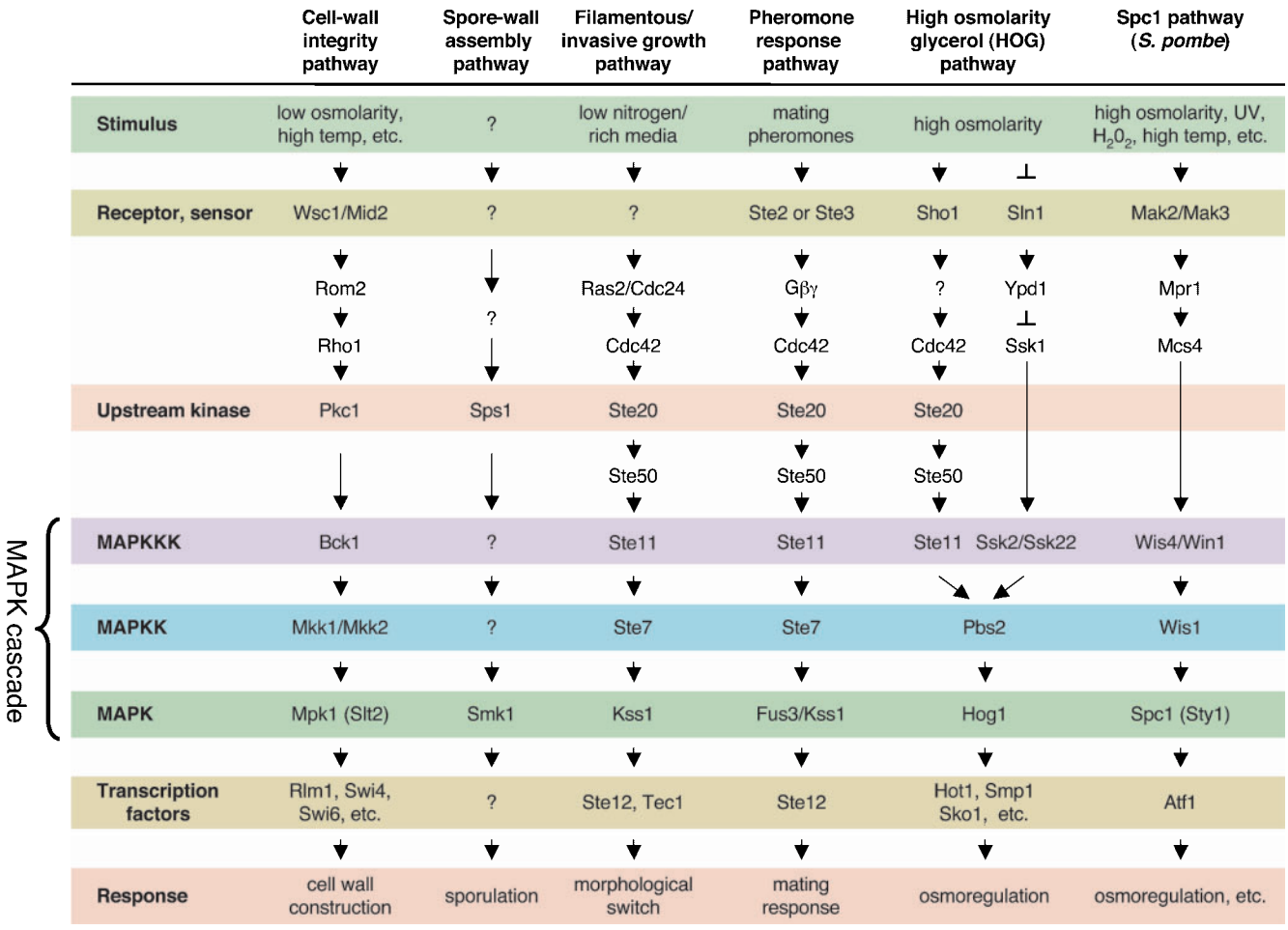

Fig. 1. **Yeast signal transduction pathways that contain the MAPK module.** The budding yeast has at least five different MAPK signal pathways. Several pathways share common components. The fission yeast Spc1 pathway, which shares many similarities with the HOG pathway, is shown for comparison. Synonymous gene names are shown in parentheses.

motif in the Pbs2 MAPKK (*8*). In the SHO1 branch, activation of the Pbs2 MAPKK is effected by its phosphorylation by the Ste11 MAPKKK (*12*). The mechanism of Ste11 activation by osmotic stress is still only vaguely understood, but it is known that phosphorylation of Ste11 by Ste20 (a PAK-like kinase) is important (*16*). It is interesting to note that Ste11 is also a component of other MAP kinase modules that regulate the pheromone response and filamentation/invasive growth (*17*). There is intricate cross-regulation, both positive and negative, among these pathways. In this mini-review, we will survey recent developments in two important regulatory mechanisms in the yeast HOG MAPK pathway: the roles of protein phosphatases, and the regulation of subcellular localizations of the pathway components.

Protein phosphatases in the yeast HOG MAPK pathway

Protein phosphorylation is regulated both by protein kinases and protein phosphatases. Although more attention is usually paid to protein kinases, protein phosphatases are also critical for MAPK regulation for various purposes: *e.g.*, to reduce the basal activity, in order to prevent initiation of undesirable response in the absence of relevant stimuli; to prevent excessive MAPK activation upon stimulation; and to resume normal cell growth after adaptive responses. A MAPK is activated by dual phosphorylation at a tyrosine residue and a threonine residue; removal of either phosphate inactivates the kinase. Accordingly, at least three classes of protein phosphatases, with distinct amino acid preferences, are known to down-regulate yeast MAPK cascades. Dual-specificity phosphatases (DSPs) dephosphorylate both phospho-tyrosine (pY) and phospho-threonine (pT) and are homologous to the mammalian MAPK phosphatases (MKPs) (*18*). Yeast encodes two DSPs: Msg5 and Sdp1 (= Yil113). Protein tyrosine phosphatase (PTP) dephosphorylates only pY, and yeast encodes three PTPs: Ptp1–Ptp3. Protein phosphatase type 2C (PP2C) dephosphorylates pT and pS, although it may also dephosphorylate pY slowly (*19*). Of the six PP2C-like proteins in yeast, five (Ptc1–Ptc5) have been shown to have enzymatic activity (*20*).

Dual-specificity phosphatases. Both Msg5 and Sdp1 are involved in inactivation of the Mpk1 (= Slt2) MAPK in the cell integrity pathway (*21*, *22*). Msg5 is also important for down-regulation of the Fus3 MAPK in the pheromone response pathway (*23*, *24*). Neither DSP, how-

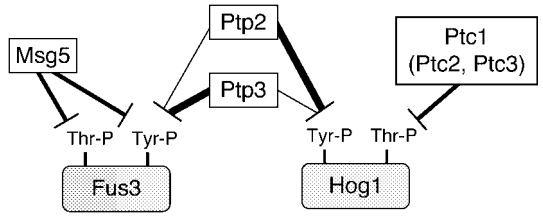

Fig. 2. **Protein phosphatases downregulating the Fus3 and Hog1 MAP kinases in yeast.**

ever, seems to have any role in the HOG pathway (*25*). The *msg5*Δ *ptp2*Δ double mutants cannot grow on media with 0.3 M $CaCl_2$, and the *msg5*Δ *ptc2*Δ double mutants cannot grow on media with either 0.3 M $CaCl_2$ or 1 M NaCl (*26*). These phenotypes suggest some roles of DSP in osmo-regulation, but details are not known.

Protein tyrosine phosphatases. Of the three PTPs in yeast, Ptp1 has no known role in MAPK regulation, even though it apparently is the most abundant and active enzyme . In contrast, Ptp2 and Ptp3 are involved in regulation of various MAPK cascades. Ptp2 and Ptp3 are, however, different in their abilities to dephosphorylate (inactivate) yeast MAP kinases. Ptp2 dephosphorylates Hog1 and Mpk1 more preferentially than Ptp3 does, whereas Ptp3 dephosphorylates Fus3 more efficiently than Ptp2 does (*24*, *25*, *27*). Ptp3 overexpression suppresses the pheromone-induced activation of the Fus3p MAPK, whereas disruption of *PTP3* gene in combination with *PTP2* results in constitutive tyrosine phosphorylation and enhanced activity of Fus3 (*24*). Fus3 activity is further increased by simultaneous disruption of *PTP3*, *PTP2*, and *MSG5*. It is believed that Ptp2 and Ptp3 are responsible for the maintenance of low basal levels of Fus3 tyrosine dephosphorylation, whereas Msg5 is important in inactivation of Fus3 following pheromone stimulation.

Activation of the Hog1 MAPK is essential for yeast viability under high osmolarity conditions, yet constitutive Hog1 activation, for example, caused by *sln1*Δ mutation or expression of hyperactive Ssk2ΔN, induces growth arrest and even lethality (*8*, *25*, *28*, *29*). Because hyperosmotic stress triggers a cell cycle delay in both G1 and G2/M phases (*30*), it is likely that the toxicity of constitutively active Hog1 is due, at least partly, to the irreversible cell cycle arrest. The lethality of Hog1 hyperactivation can be suppressed by overexpression of Ptp2 or Ptp3, suggesting that these PTPs can dephosphorylate Hog1 (*25*, *27*, *28*). The importance of phosphatases in the HOG pathway can be also seen from the observation that disruption of both *PTP2* and *PTC1* results in lethal Hog1 hyperactivation (*19*).

A negative feedback loop exists between Hog1 and Ptp2/Ptp3. Active Hog1 enhances the the Ptp2 activity (*25*), and induces Ptp3 expression (*27*). The catalytically inactive Hog1(K52M) mutant, when phosphorylated by active Pbs2, remains tyrosine-phosphorylated much longer than the wild-type Hog1, apparently because there is no induction of PTP activities (*25*).

Specificities of Ptp2 and Ptp3 are determined by their N-terminal non-catalytic domains (*31*). Thus, by exchanging their N-termini, their preferences for Fus3 and Hog1 are reversed. For Ptp3, the CH2 (Cdc25 homology 2) domain is required for Fus3 binding. The Fus3 CD domain is responsible for its Ptp3 binding, because the Fus3 D317N or D317G mutation, which is equivalent to the *Sevenmaker* (*Sem*) allele of the fruitfly Rolled (rl) MAPK, disrupts the Ptp3-Fus3 binding. An equivalent mutation in Hog1, D310N, also disrupts the stable binding between Hog1 and Ptp2 (*32*). Mutations in the Ptp3 CH2 domain do not affect its intrinsic phosphatase activity (measured using a non-specific substrate). However, the same mutants cannot inactivate Fus3 *in vivo*, demonstrating that a stable interaction between the Ptp3 CH2 domain and the Fus3 CD domain is essential for the phosphatase's physiological function.

In the fission yeast *Schizosaccharomyces pombe*, heat shock activates the Wis1 MAPKK, but only weakly compared to osmotic stress or H_2O_2. The same heat treatment, however, causes a much stronger activation of the downstream Spc1 (= Sty1) MAPK (the fission yeast homolog of Hog1) than expected from the level of Wis1 activation. This strong Spc1 response requires disruption of the interaction between Spc1 and Pyp1, the major PTP that dephosphorylates and inactivates Spc1 (*33*). Apparently, heat induces a change in Pyp1, so that it can no longer bind Spc1. In the budding yeast, heat also activates the HOG pathway, by a mechanism that involves several components in the SHO1 branch, including Sho1, Ste20, Ste50, and Ste11 (*34*). In the *ptp2*Δ *ptp3*Δ double mutant cells, heat treatment causes hyperactivation of Hog1 leading to yeast lethality, further demonstrating the importance of these PTPs in down-regulating the HOG pathway.

Protein phosphatase type 2C. Originally, the yeast PP2C enzymes Ptc1 and Ptc3 were suspected to have negative regulatory roles in the HOG pathway, because their overexpression suppressed the lethality of Hog1 hyperactivation (*28*). That *ptc1*Δ *ptp2*Δ double mutants hyperactivated Hog1 further suggested that both Ptc1 and Ptp2 were important for negative regulation of the HOG pathway (*19*, *27*). It was unclear, however, whether the yeast PP2C enzymes dephosphorylate Hog1 itself, or another upstream kinase, such as Pbs2. More recent studies demonstrated that Ptc1 dephosphorylates threonine residue of Hog1 *in vitro* (*35*). Furthermore, overexpression of Ptc1 inhibits Hog1 activation, as measured by its kinase activity, but does not affect the level of Hog1 tyrosine phosphorylation, indicating that Ptc1 inhibits the HOG pathway by dephosphorylating Hog1, rather than an upstream kinase (*35*). Ptc2 and Ptc3 may also down-regulate the HOG pathway in a similar manner (*36*). Ptc1 seems to maintain the low basal Hog1 activity as well as to inactivate Hog1 after osmoadaptation, whereas Ptc2 and Ptc3 set the maximal limit to which stress can activate Hog1 (*35*, *36*). The *S. pombe* PP2C enzymes Ptc1 and Ptc3 also dephosphorylate pT in the activating loop of the Spc1 stress MAPK (*33*).

The specificity of Ptc1 to Hog1 is determined by a docking interaction. Unlike Ptp2, however, Ptc1 does not directly bind Hog1. Instead, Ptc1 binds a small adaptor protein, Nbp2, which also has a Pbs2 binding site (*37*). Because Pbs2 also binds Hog1 (*12*), a complex that contains Ptc1, Nbp2, Pbs2, and Hog1 may form. The synthetic growth defect phenotype of *nbp2*Δ *ptp2*Δ strains is

similar to that of *ptc1*Δ *ptp2*Δ strains, supporting the model in which Ptc1 can dephosphorylate Hog1 only if Ptc1 is bound to Nbp2. An interesting prediction by this model is that in the nucleus, where Hog1 is dissociated from Pbs2, Hog1 cannot be dephosphorylated by Ptc1. The physiological significance of this regulation is yet to be determined. It is also unclear how Ptc2 and Ptc3, which do not bind Nbp2, interact with Hog1 (*37*).

Subcellular localizations of the HOG pathway components

In the HOG pathway, like in other MAPK pathways, signals emanate from the osmosensors in the plasma membrane, and end mainly in the nucleus. In this dynamic process, changes in the subcellular localizations of signaling components are functionally as important as changes in their activation status. In recent years, use of the fusion products with the green fluorescent protein (GFP) has revealed interesting, sometime even counter-intuitive, aspects of the localizations and movements of the HOG pathway components.

Sln1 and Sho1 osmosensors. In unstressed yeast cells, the transmembrane osmosensor Sln1 is distributed uniformly throughout the plasma membrane. The localization of Sln1 changes in response to hyper-osmotic stress by rapidly clustering into dot-like structures (*7*). This re-localization is transient and is independent of the Hog1 kinase activity. The second osmosensor Sho1 is localized to the plasma membrane of regions of polarized cell growth, such as the emerging buds in G1-S phase cells, mother-bud necks in M phase cells, and the shmoo tips in pheromone-stimulated cells (*14*, *15*). The localization of Sho1 itself does not change upon osmotic stress (*7*), but its association with Pbs2 is induced by hyper-osmotic stress (*15*). Thus, Sho1 recruits Pbs2 to the region of the cell where other upstream activators in the SHO1 branch (Cdc42 and Ste20) are also located (*14*, *16*) . Because Pbs2 binds both Ste11 and Hog1, and Ste11 tightly binds Ste50, the transmembrane Sho1 may serve as the core of the formation of a multi-component signaling complex including Sho1, Cdc42, Ste20, Ste50, Ste11, Pbs2, and Hog1.

Ptp2 and Ptp3 tyrosine phosphatases. Ptp2 is predominantly localized in the nucleus, whereas Ptp3 is predominantly in the cytoplasm (*38*). Their distributions are not affected by any stimuli that activate yeast MAPKs (heat shock, osmotic stress, and mating pheromones). The difference in their subcellular localizations may contribute to their substrate preferences *in vivo*. Ptp2 and Ptp3 may act, respectively, as nuclear and cytoplasmic anchors for the Hog1 MAPK (*32*).

Hog1 MAPK. In unstressed cells, Hog1 localizes evenly throughout the cell (*39*, *40*). Following osmotic stress, Hog1 translocates rapidly into the nucleus (*39*, *40*). For Hog1 nuclear translocation its phosphorylation, but not its kinase activity, is essential, because a catalytically inactive kinase (such as the K52M mutant) can translocate to the nucleus as efficiently as the wild-type, whereas a phosphorylation-defective mutant (T174A T176A) cannot (*39*, *40*). The Hog1 nuclear translocation is dependent on the small G-protein Gsp1 (a Ran homolog) and Nmd5 (a homolog of importin β) (*39*). *De novo* protein synthesis is not required for the initial Hog1 nuclear import, for subsequent nuclear retention, or for nuclear export (*40*). It is unknown, however, whether Hog1 has an intrinsic nuclear localization signal (NLS) and directly interacts with the importin Nmd5, or is passively carried by another nuclear protein.

In the nucleus, Hog1 seems to be anchored by several nuclear proteins. The stress-specific transcription factors Msn2 and Msn4 may serve as nuclear anchorage proteins, because in *msn2*Δ *msn4*Δ double mutants, Hog1 exits the nucleus significantly faster than in the wild-type cells (*40*). Ptp2 may retain Hog1 in the nucleus, whereas Ptp3 retains Hog1 in the cytoplasm, because overexpression of Ptp2 segregates the majority of Hog1 to the nucleus, while Ptp3 overexpression results in predominantly cytoplasmic localization of Hog1 (*32*).

Hog1 translocation is transient: it is re-exported after return to an iso-osmotic environment or after adaptation to high osmolarity. Nuclear export of Hog1, unlike its import, requires the Hog1 kinase activity. Thus, the catalytically inactive Lys52Met and Lys52Arg mutants remain in the nucleus much longer than the wild-type Hog1 does (*39*, *40*). The time-courses of the Hog1 dephosphorylation and the Hog1 nuclear exit coincide, but dephosphorylation itself is not a requirement for Hog1 nuclear export. In *ptp2*Δ *ptp3*Δ mutants, in which Hog1 remains tyrosine-phosphorylated much longer than in the wild-type cells, Hog1 nuclear export is actually accelerated (*32*).

Pbs2 MAPKK. Pbs2 appears to be excluded from the nucleus, with or without external osmostress (*39*, *40*). This appearance, however, belies the dynamic behavior of Pbs2. Pbs2 is found predominantly in the cytoplasm, because of a nuclear export signal (NES) found near the Pbs2 N-terminus (*41*). However, when the NES is deleted, Pbs2 localizes predominantly in the nucleus. A similar NES is found in the Wis1 MAPKK, the fission yeast homolog of Pbs2 (*42*). The nuclear localization of Pbs2 NES deletion mutant is dependent on an NLS found near the Pbs2 C-terminus. These findings suggest that Pbs2 and Wis1 actually shuttle between the cytoplasm and the nucleus. Functions of these NES and NLS are not clear, however, because deletion of either or both signals does not significantly affect Hog1 or Spc1 activation (*41*, *42*).

Although Pbs2 does not seem to change its subcellular localization upon osmotic stress (*39*, *40*), it is more dynamic in this respect too. When not stimulated, the intracellular distribution of catalytically inactive Pbs2(K389M) is indistinguishable from that of the wild-type Pbs2. However, high-osmolarity stress induces transient re-localization of Pbs2(K389M) to the growing bud tip or to the mother-bud neck, which is very similar to the localization of the Sho1 osmosensor (*15*). Indeed, this re-localization of Pbs2(K389M) is dependent on its binding to the Sho1 SH3 domain, because Sho1 ΔSH3 mutation in the host cell, or P96S mutation in Pbs2(K389M), completely abrogated this re-localization effect (*15*). It is likely that high osmolarity induces Pbs2 to form a complex with Sho1, but it cannot be easily observed because the complex dissociates very rapidly by a mechanism that depends on the Pbs2 catalytic activity. The recruitment of Pbs2 to Sho1 also requires the Cdc42 small G-protein, which is an upstream element in the SHO1

branch, but not other upstream factors, Ste20, Ste50, and Ste11 (*15*).

Concluding remarks

When activated, Hog1 MAP kinase enters the nucleus, where it modulates the activities of at least four transcription activators, Hot1, Smp1, Msn2, and Msn4, and a transcription repressor Sko1 (*43–48*). Interestingly, Hot1 activation is not dependent on its phosphorylation by Hog1; instead, a complex of Hot1 and active Hog1 is formed on the osmostress-responsive genes. The complex recruits the RNA polymerase II complex to the genes (*48*). In the nucleus, active Hog1 also interacts with the Rpd3 histone deacetylase and targets the deacetylase to specific osmostress-responsive genes (*49*). Thus, Hog1 acts as integral part of transcription activation complexes (*50*). The active Hog1 also has cytoplasmic functions, such as activation of the Rck2 kinase, a calmodulin-protein kinase that regulates protein synthesis (*51*, *52*). These observations further demonstrate the importance of controlling the subcellular localization of the Hog1 protein as well as its catalytic activity. Readers are encouraged to consult recent comprehensive reviews for this and other aspects of the HOG MAPK pathway and adaptation to osmotic stress in yeast (*53–56*).

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