

Intracellular mechanism by which arsenite activates the yeast stress MAPK Hog1

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ABSTRACT Stress-activated MAPKs (SAPKs) respond to a wide variety of stressors. In most cases, the pathways through which specific stress signals are transmitted to the SAPKs are not known. In this study, we delineate the intracellular signaling pathway by which the trivalent toxic metalloid arsenite [As(III)] activates the yeast SAPK Hog1. We demonstrate that, to activate Hog1, As(III) must enter the cell through the glycerol channel Fps1 and must be metabolized to methyl arsenite [MAs(III)] by the dimeric methyltransferase Mtq2:Trm112. We found that Mtq2:Trm1 displays SAM-dependent methyltransferase activity toward both As(III) and MAs(III). Additionally, we present genetic and biochemical evidence that MAs(III), but not As(III), is a potent inhibitor of the protein tyrosine phosphatases (Ptp2 and Ptp3) that normally maintain Hog1 in an inactive state. Inhibition of Ptp2 and Ptp3 by MAs(III) results in elevated Hog1 phosphorylation without activation of the protein kinases that act upstream of the SAPK and raises the possibility that other Hog1-activating stressors act intracellularly at different points along the canonical Hog1 activation pathway. Finally, we show that arsenate [As(V)], a pentavalent form of arsenic, also activates Hog1, but through a pathway that is distinct from that of As(III) and involves activation of the Hog1 MEK Pbs2.

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INTRODUCTION

Arsenic is among the most common poisons found in the environment (Rosen and Liu, 2009). Human exposure to arsenic is mainly through food, water, and air, and contamination of groundwater is a worldwide health problem (Smedley and Kinniburgh, 2002). Inorganic aqueous arsenic exists mainly as oxyanions of trivalent arsenite [As(III)] and pentavalent arsenate [As(V)]. Chronic exposure to inorganic arsenic is associated with cardiovascular disease and hyperten-

sion, diabetes mellitus, and various forms of cancer (Abernathy *et al.*, 2003; Beane Freeman *et al.*, 2004). Despite these health effects, As(III) is in current use as a highly effective treatment for acute promyelocytic leukemia (Rehman and Naranmandura, 2012). Therefore, it is important to understand the various mechanisms by which arsenic is transported and metabolized by eukaryotic cells, as well as the cellular responses mobilized by arsenic-induced stress.

In mammalian cells, uptake of As(V), which is an analog of inorganic phosphate, is mediated by the high-affinity phosphate transporter NaPi-IIb, whereas As(III) enters cells through the aquaglyceroporins and the glucose permeases (Maciaszczyk-Dziubinska *et al.*, 2012). Once inside the cell, As(V) is reduced to As(III) by Cdc25 protein phosphatases/arsenate reductases (Bhattacharjee *et al.*, 2010). As(III) is metabolized in mammals principally by As(III) S-adenosylmethionine (SAM) methyltransferase (AS3MT), which catalyzes the transfer of methyl groups from SAM to As(III) to produce methyl arsenite [MAs(III)] and dimethyl arsenite [DMAs(III)] (Ren *et al.*, 2011; Cullen, 2014; Dheeman *et al.*, 2014; Dong *et al.*, 2015), both of which are more toxic than inorganic As(III) (Dong *et al.*, 2015).

In the yeast *Saccharomyces cerevisiae*, As(V) uptake takes place similarly through the phosphate transporters (Wysocki and Tamas, 2010), whereas As(III) enters the cell principally through the aquaglyceroporin Fps1, a bidirectional channel that normally functions to

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Abbreviations used: As(III), arsenite; As(V), arsenate; AS3MT, SAM methyltransferase; ABC, ATP-binding cassette; ATP, adenosine triphosphate; colP, coimmunoprecipitation; DIC, differential interference contrast; DMAs(III), dimethyl arsenite; DTT, dithiothreitol; GST, glutathione-S-transferase; HOG, high osmolarity glycerol; MAs(III), methyl arsenite; MEK, MAP kinase kinase; MEKK, MAP kinase kinase; SAH, S-adenosyl homocysteine; SAM, As(III) S-adenosylmethionine; SAPK, stress-activated MAP kinase; SDM, synthetic defined medium; TAP, tandem affinity purification.

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transport glycerol (Wysocki *et al.*, 2001; Maciaszczyk-Dziubinska *et al.*, 2012). As(V) is reduced to As(III) in yeast by the arsenate reductase Acr2 (Mukhopadhyay and Rosen, 1998), and As(III) is actively transported out of the yeast cell through the plasma membrane metalloid/H⁺ antiporter Acr3 (Maciaszczyk-Dziubinska *et al.*, 2011), but also flows out of the cell through Fps1 when produced intracellularly by reduction of As(V). Alternatively, As(III) can be conjugated to glutathione and sequestered in the yeast vacuole through the ABC family transporters Ycf1 and Vmr1 (Maciaszczyk-Dziubinska *et al.*, 2012). Methylation of As(III) has not been reported in yeast, but a candidate As(III) methyltransferase, Mtq2, has been identified on the basis of increased As(III) tolerance of an *mtq2Δ* mutant (Ren *et al.*, 2011).

With regard to signaling, As(III) stimulates the mammalian stress-activated MAPK (SAPK) p38 (Elbirt *et al.*, 1998; Verma *et al.*, 2002) and both SAPKs of yeast—Hog1 of the high osmolarity glycerol (HOG) pathway (Sotelo and Rodríguez-Gabriel, 2006; Thorsen *et al.*, 2006) and Mpk1 (Sit2) of the cell wall integrity pathway (Ahmadpour *et al.*, 2016). As(V) also activates Mpk1 (Matia-Gonzalez and Rodríguez-Gabriel, 2011), but has not been shown to activate Hog1. In all cases, there is evidence that the activated SAPKs play significant roles in tolerance to these metalloids. In yeast, both As(III) and As(V) activate transcription of arsenic-specific detoxification genes by the AP-1-like transcription factor Acr1 (also known as Yap8; Wysocki *et al.*, 2004). Although the mechanism by which arsenic induces transcription is not fully understood, in the case of As(III) stress, this cysteine-reactive toxin binds directly to Acr1 to induce transcription (Kumar *et al.*, 2016). However, its ability to activate Acr1-driven transcription is partially dependent upon Hog1 (Sotelo and Rodríguez-Gabriel, 2006). Another Hog1-dependent response to As(III) is the

closure of Fps1, consistent with the glycerol channel serving as an adventitious port for As(III) entry (Thorsen *et al.*, 2006). Here again, however, the mechanism by which this regulation occurs is not known.

The HOG pathway of yeast has been well characterized with regard to its regulation by hyperosmotic stress (Saito and Posas, 2012). However, Hog1 is also stimulated by a wide array of unrelated stress signals, including heat shock (Winkler *et al.*, 2002), cold shock (Panadero *et al.*, 2006), citric and acetic acid (Lawrence *et al.*, 2004; Mollapour and Piper, 2006), oxidative stress (Bilsland *et al.*, 2004), methylglyoxal (Aguilera *et al.*, 2005), bacterial lipopolysaccharide (Marques *et al.*, 2006), glucose starvation (Piao *et al.*, 2012), curcumin (Azad *et al.*, 2014), cadmium (Jiang *et al.*, 2014), and arsenite (Sotelo and Rodríguez-Gabriel, 2006; Thorsen *et al.*, 2006). Mammalian p38 SAPK is the functional ortholog of Hog1 (Han *et al.*, 1994), and is similarly activated by most of these stress-inducing agents (Ono and Han, 2000; de Nadal *et al.*, 2002). This raises two important and related questions. First, do these various stresses activate the SAPK through a common pathway, or through alternative inputs? This is especially important because the cell surface osmosensors of the HOG pathway are the only known inputs to Hog1. Second, how does an activated SAPK mount a specific response appropriate to the particular stress experienced? We have begun to address these questions with an analysis of HOG pathway signaling in response to arsenic-induced stress.

In this study, we demonstrate that As(III) activates Hog1 indirectly through an intracellular mechanism that requires its metabolism to MAs(III) by the dimeric methyltransferase Mtq2:Trm112. MAs(III) activates Hog1 through inhibition of the protein tyrosine phosphatases (Ptp2 and Ptp3) that normally maintain Hog1 in an inactive state, rather than through activation of the canonical HOG signaling pathway stimulated by cell surface osmosensors. Intracellular activation of SAPKs through different input nodes in response to various stressors may have broad implications for consequent SAPK target specificity.

RESULTS

Hog1 is known to be activated by As(III), but not by As(V). To investigate further the activation of Hog1 by these metalloids, we used antibodies that specifically recognize dually phosphorylated (on Thr and Tyr) Hog1 (α -phospho-p38). We chose 1 mM As(III) and 3 mM As(V) for treatments because these doses induced slight growth impairment (Supplemental Figure S1). We found that, similarly to As(III), As(V) can activate Hog1 (Figure 1A), and under these conditions, we observed stronger activation by As(V) than by As(III). Activation of Hog1 by both stressors was quite rapid, peaking within 5–10 min after treatment and declining by 30 min after treatment (Figure 1A). Because As(V) is converted to As(III) by the arsenate reductase Acr2 (Mukhopadhyay and Rosen, 1998), we asked whether As(III) generated by Acr2 was responsible for As(V) activation of Hog1. An *acr2Δ* mutant displayed slightly higher basal Hog1 phosphorylation than the wild type,

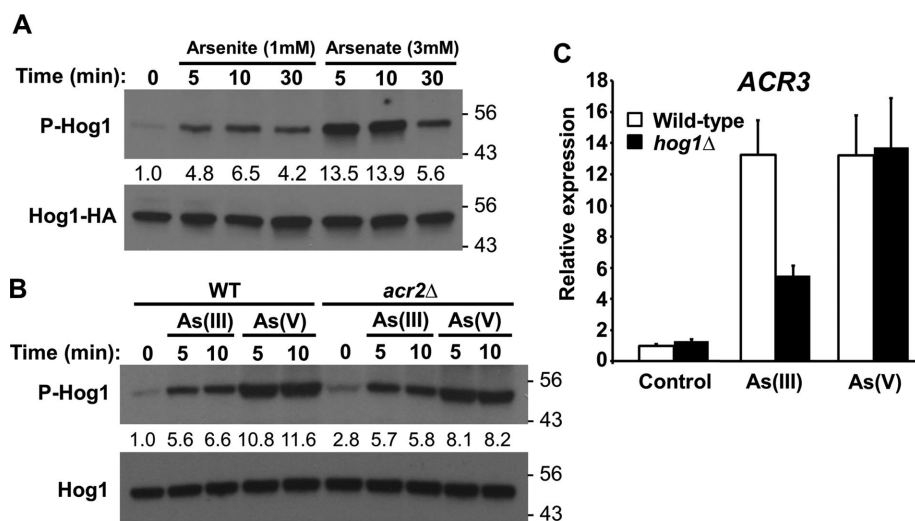


FIGURE 1: As(III) and As(V) activate Hog1. (A) Activation of Hog1 by As(III) and As(V). Wild-type yeast cells (DL3187) expressing Hog1-HA (from p3225) were treated as indicated and extracts were separated by SDS-PAGE prior to examination by immunoblot for activated (phospho)-Hog1 and total Hog1-HA. Fold activation values are relative to the untreated sample and normalized to the Hog1-HA input. Molecular mass markers (in kDa) are on the right. An α -Hog1 antibody was used for all subsequent immunoblots to detect endogenous Hog1. (B) Activation of Hog1 by As(V) does not require its conversion to As(III). Wild-type or *acr2Δ* cells (DL4341) were treated as indicated with 1 mM As(III) or 3 mM As(V) and examined for activation of endogenous Hog1. Fold activation values are relative to the untreated wild-type sample and normalized to the Hog1 input. Note that the *acr2Δ* mutant displays slightly higher basal Hog1 phosphorylation than the wild type. (C) Induction of the arsenic protection gene *ACR3* by As(III), but not by As(V), is partially dependent upon *HOG1*. *ACR3* expression was measured by quantitative real-time PCR in wild-type cells and *hog1Δ* cells (DL3158) after treatment with 1 mM As(III) or 3 mM As(V) for 5 min. Each data point represents the mean and SD of three biological replicates.

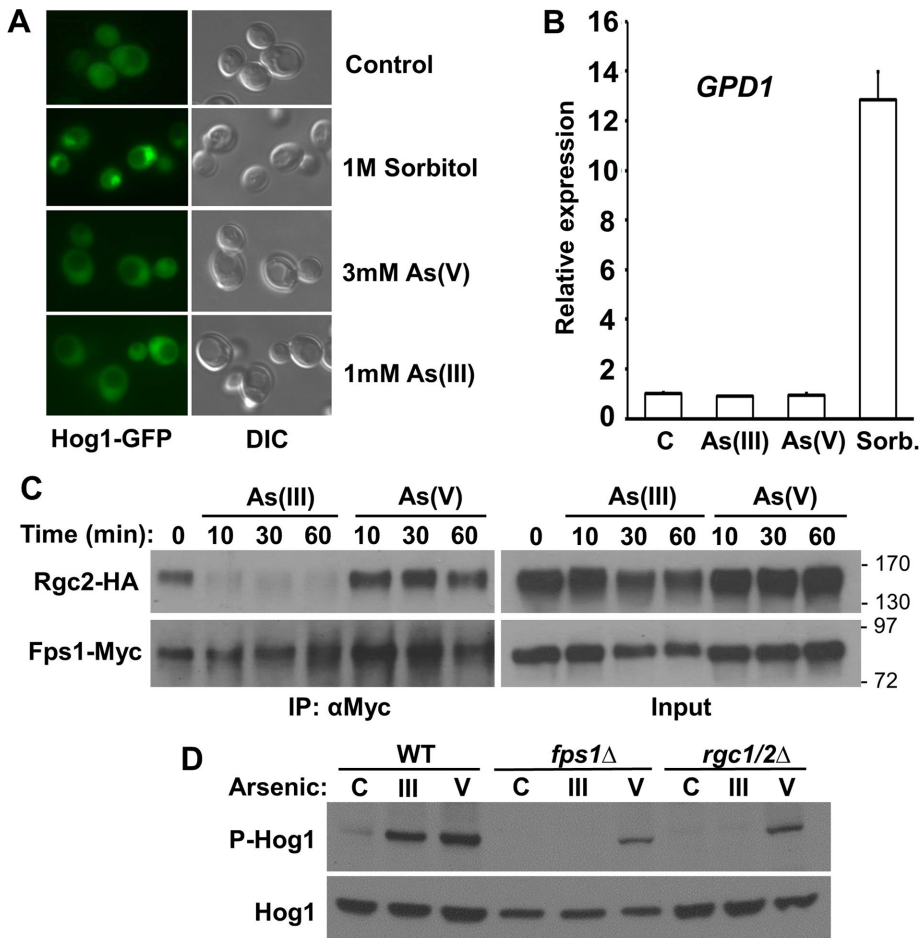


FIGURE 2: Hog1 output in response to As(III) or As(V) treatment and requirement for As(III) to enter cell for Hog1 activation. (A) Neither As(III) nor As(V) induces nuclear accumulation of Hog1. Wild-type cells (DL3187), expressing Hog1-GFP from a centromeric plasmid (p3177), were treated as indicated and visualized by fluorescence microscopy and differential interference contrast (DIC) within 5 min to detect nuclear accumulation of Hog1. (B) The osmoreponsive gene *GPD1* is induced by treatment with sorbitol, but not with As(III) or As(V). *GPD1* expression was measured by quantitative real-time PCR in wild-type cells (DL3187) after treatment with 1 M sorbitol (to induce hyperosmotic shock), 1 mM As(III), 3 mM As(V), or no treatment "C" for 5 min. Each data point represents the mean and SD of three biological replicates. (C) As(III), but not As(V), treatment induces eviction of Rgc2 from Fps1. CoIP of Rgc2-HA and Fps1-Myc in wild-type cells (DL3187) treated with 1 mM As(III) or 3 mM As(V) for the indicated times. Anti-Myc immunoprecipitates (IPs) were separated by SDS-PAGE and subjected to immunoblot analysis. Molecular mass markers (in kDa) are shown on the right. (D) As(III) fails to activate Hog1 in *fps1Δ* or *rgc1Δrgc2Δ* mutants, which are blocked for As(III) entry through the glycerol channel, Fps1. Extracts from wild-type (DL3187), *fps1Δ* (DL3226), and *rgc1Δrgc2Δ* cells (DL3207) were examined by immunoblot for activation of Hog1 after treatment with 1 mM As(III), 3 mM As(V), or no treatment "C" for 5 min.

but was not appreciably impaired for Hog1 activation by As(V) (Figure 1B), suggesting that activation by As(V) does not require its conversion to As(III). Both As(III) and As(V) stimulate arsenic-protective gene expression, including the *ACR2* and *ACR3* genes, through the AP-1-like transcription factor Acr1 (Wysocki et al., 2004). In the case of As(III), this transcriptional induction is partially dependent on Hog1 despite the fact that As(III) does not induce nuclear accumulation of Hog1 (Sotelo and Rodríguez-Gabriel, 2006; Thorsen et al., 2006). Therefore, we investigated the requirement for Hog1 in As(V)-induced transcription of *ACR3*. Unlike As(III), As(V) induction of *ACR3* was completely independent of Hog1 (Figure 1C). However, similarly to As(III), As(V) treatment failed to induce nuclear translocation of Hog1 (Figure 2A) and failed to induce transcription of the osmopro-

protective gene *GPD1*, which is induced by Hog1 in response to hyperosmotic shock (Figure 2B).

Because As(III) enters yeast cells through the glycerol channel Fps1 and this channel is closed in a Hog1-dependent manner in response to As(III) treatment (Thorsen et al., 2006), we examined the effect of both As(III) and As(V) treatment on Fps1 closure. Hog1-catalyzed eviction of the glycerol channel regulator, Rgc2, from Fps1 occurs in response to hyperosmotic shock and is responsible for the closure of Fps1 (Lee et al., 2013). We found through coimmunoprecipitation (coIP) experiments that As(III) treatment similarly induced rapid eviction of Rgc2 from Fps1, whereas As(V) did not (Figure 2C). This makes sense from a physiological perspective, because As(V) does not enter the cell through Fps1. However, we do not currently have a mechanistic explanation for this differential behavior.

Intracellular activation of Hog1 by As(III)

Previous reports indicated that upstream signaling components of the HOG pathway are required for activation of Hog1 by As(III) and for tolerance to As(III) (Sotelo and Rodríguez-Gabriel, 2006; Thorsen et al., 2006), the implication being that Hog1 is activated through signal propagation from the cell surface. However, we made an observation that suggested that As(III) must enter the cell to activate Hog1. Because, as noted above, As(III) entry to yeast cells is mainly through Fps1, we assessed the ability of As(III) to activate Hog1 in either an *fps1Δ* mutant or an *rgc1Δrgc2Δ* mutant, which is impaired for Fps1 channel activity and displays tolerance to As(III) similar to that of an *fps1Δ* mutant (Beese et al., 2009). In these settings, As(III) failed to activate Hog1, whereas As(V), which enters through the phosphate transporters, was able to activate Hog1 (Figure 2D).

If As(III) activates Hog1 through an intracellular mechanism, rather than through activation of the canonical HOG pathway, it

must do so in one of two ways. First, As(III) might activate another signaling pathway that stimulates the HOG pathway at a point below the cell surface osmosensors. However, this explanation seems unlikely because the HOG pathway is composed of two independent branches (from the Sln1 and Sho1 osmosensors) that feed into Pbs2, the MEK of the HOG pathway (Figure 3A), and both branches must be blocked to prevent activation of Hog1 by As(III) (Sotelo and Rodríguez-Gabriel, 2006), thus suggesting that As(III) stress would have separate inputs to both branches. The second possibility is that As(III) treatment might amplify basal signal that flows through the pathway to Hog1. In this case, upstream pathway components would be required to provide that basal signal, but would not be activated by As(III) treatment.

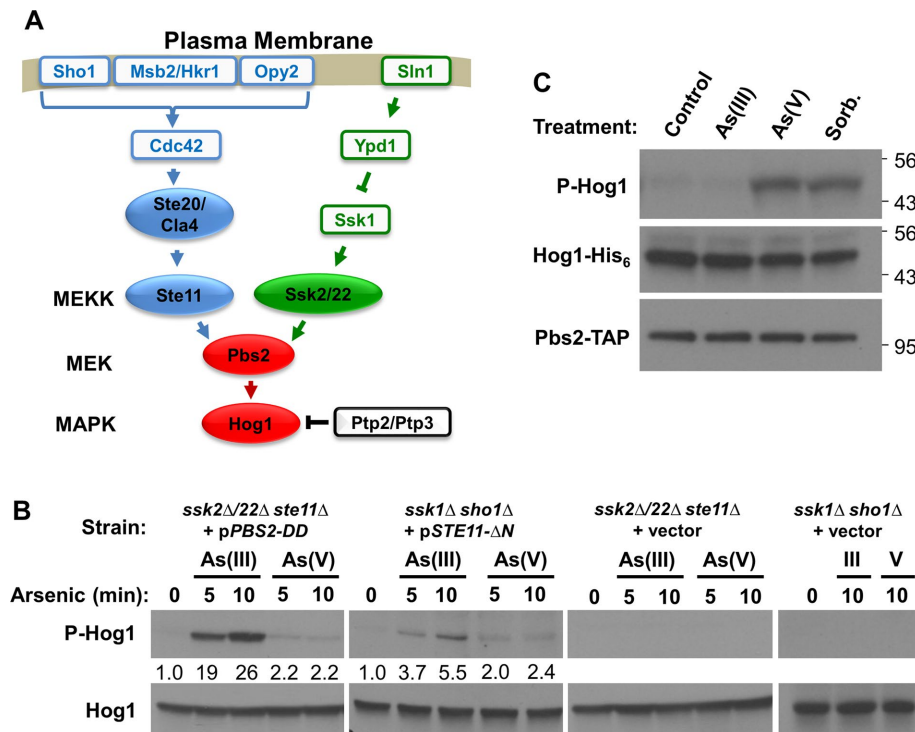


FIGURE 3: As(III) stimulates Hog1 through an intracellular mechanism that acts below Pbs2. (A) The HOG pathway showing known inputs from the cell surface osmosensors. The protein tyrosine phosphatases Ptp2 and Ptp3 maintain Hog1 in an inactive state under nonstress conditions. (B) As(III) activates Hog1 in strains in which the MAPK is mutationally severed from its upstream activators, but in which the basal signal is restored below the blockage. Strains bearing deletions in all three MEKK genes of the HOG pathway (*ssk2Δ ssk22Δ ste11Δ*; DL2344) or deletions in each of the upstream inputs (*ssk1Δ sho1Δ*; DL4306) and plasmids expressing constitutively active forms of Pbs2 (p*PBS2-DD*), Ste11 (p*STE11-ΔN*) at low level under repressing control of the *GAL1* promoter, or empty vector, were examined for activation of Hog1 after treatment with 1 mM As(III) or 3 mM As(V) for the indicated times. Strains were grown in standard SD medium to minimize expression levels of the constitutive protein kinases. Fold activation values measured from immunoblots are relative to the untreated samples and normalized to the Hog1 input. (C) As(III) treatment does not activate Pbs2. In vitro protein kinase assay for Pbs2-TAP using Hog1-His₆ as substrate. Pbs2 was isolated from untreated wild-type (DL3187) cells (Control) or cells treated with 1 mM As(III), 3 mM As(V), or 1 M sorbitol for 5 min and tested by immunoblot assay for its ability to phosphorylate Hog1. Molecular mass markers (in kDa) are on the right.

We tested this possibility in two ways. First, we examined As(III) activation of Hog1 in a pair of HOG pathway mutants in which Hog1 had been mutationally severed from its upstream activators, but with basal signal restored by a constitutive pathway mutation. In one case, both pathway branches were blocked by deletion mutations in upper-pathway components (*ssk1Δ sho1Δ*; Figure 3A). Basal signal to Hog1 was restored in this strain by low-level expression of a constitutive allele of *STE11* (*STE11-ΔN*; Cairns *et al.*, 1992), which encodes one of three HOG pathway MEKKs. The other mutant strain carries deletion mutations in all three MEKK-encoding genes of the HOG pathway (*ssk2Δssk22Δste11Δ*). In this strain, basal signal was restored below the MEKKs by low-level expression of a constitutive phosphomimetic form of *PBS2* (*PBS2-DD*) (Wurgler-Murphy *et al.*, 1997). Although high-level expression of constitutive *STE11* or *PBS2* alleles results in strong Hog1 activity and cell cycle arrest (Cairns *et al.*, 1992; Wurgler-Murphy *et al.*, 1997), low-level expression from the *GAL1* promoter under repressing conditions (glucose) did not activate Hog1 and did not interfere with cell growth. We found that As(III) activated Hog1 in both mutant strains, but only when basal signal was restored to the deletion

mutants (Figure 3B). This is in contrast to As(V), which failed to activate Hog1 appreciably in either of these strains, suggesting that As(V) activates Hog1 through a different pathway than As(III). These results demonstrate that activation of Hog1 in response to As(III) treatment is through an intracellular mechanism that impacts the HOG pathway below the level of Pbs2, yet requires a basal signal from upstream components.

The above results suggested a second test for intracellular activation of Hog1 by As(III) treatment. If As(III) activates Hog1 by amplification of the basal signal from Pbs2, rather than by active signaling of the HOG pathway through Pbs2, we should not detect Pbs2 activation by As(III) treatment. To test this, we used an in vitro protein kinase assay to detect activation of Pbs2 (Pbs2-TAP) immunoprecipitated (IP) from cells that were treated for 5 min with 1 mM As(III), 3 mM As(V), or 1 M sorbitol as a positive control for HOG pathway activation by hyperosmotic shock. Inactive Hog1 (Hog1-His₆), purified from yeast cells lacking the MEKKs of the HOG pathway (*ssk2Δ, ssk22Δ, ste11Δ*), was used as a substrate. In contrast to the sorbitol treatment, which activated Pbs2, we detected no Pbs2 activation by the As(III) treatment (Figure 3C), supporting the conclusion that As(III) activates Hog1 by amplification of basal signal, rather than by HOG pathway kinase cascade activation. Similar to hyperosmotic shock, treatment with As(V) activated Pbs2, confirming the conclusion that the pathways to Hog1 activation by As(III) and As(V) are distinct.

These results suggested the possibility that As(III) activates Hog1 by inhibition of the protein phosphatases that maintain Hog1 in an inactive state in the absence of stress. Three phosphatases have been implicated in the regulation of Hog1. These are the Tyr-specific phosphatases Ptp2 and Ptp3 and the Ser/Thr phosphatase Ptc1 (Saito and Posas, 2012). Deletion of *PTP2* and *PTP3* together, or all three of these protein phosphatase-encoding genes, greatly elevated basal Hog1 phosphorylation, with no detectable difference between the double mutant and the triple mutant (Figure 4A). However, activation of Hog1 by As(III) was completely blocked in these mutants (Figure 4B). In contrast to this, hyperosmotic shock by treatment with 0.4 M NaCl activated Hog1 further in both the double and triple phosphatase deletion mutants, supporting the hypothesis that As(III) activates Hog1 through inhibition of its protein phosphatases rather than by signal activation through the protein kinase cascade. Moreover, the similar behavior of the *ptp2Δptp3Δptc1Δ* mutant to the *ptp2Δptp3Δ* mutant suggests that Ptc1 does not play a significant role in the activation of Hog1 in response to As(III) treatment.

If As(III) activates Hog1 by inhibiting the tyrosine-specific phosphatases that normally maintain it in a silent state, mutants in these genes might display increased tolerance to As(III) by preadaptation to As(III) stress. Therefore, we examined the effect of deletion mutations of the three phosphatase genes, individually and in

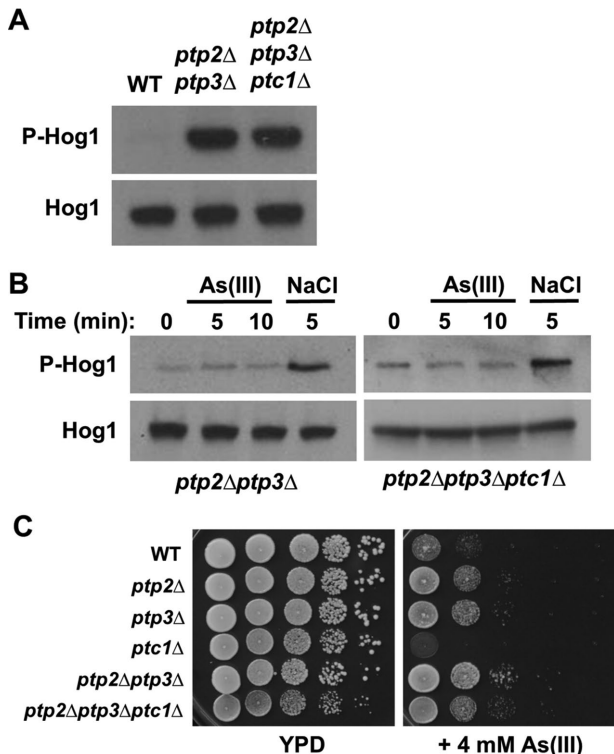


FIGURE 4: As(III) acts at the level of the Ptp2 and Ptp3 tyrosine phosphatases. (A) The basal phosphorylation of Hog1 is elevated strongly in the absence of the protein phosphatases that down-regulate it. Comparison of wild-type (DL2772), *ptp2Δ ptp3Δ* (DL4299), and a *ptp2Δ ptp3Δ ptc1Δ* mutant (DL4309) for Hog1 phosphorylation status. (B) As(III) fails to activate Hog1 in protein phosphatase mutants. The same mutant strains as above were tested for activation of Hog1 by 1 mM As(III) or 400 mM NaCl to induce hyperosmotic shock for the indicated times. (C) Mutations in *PTP2* and *PTP3* confer tolerance of As(III). Wild-type cells (DL2772), *ptp2Δ* (DL4295), *ptp3Δ* (DL4296), *ptc1Δ* (DL4297), *ptp2Δ ptp3Δ* (DL4299), and *ptp2Δ ptp3Δ ptc1Δ* (DL4309) were spotted onto YPD plates with or without 4 mM As(III) at serial 10-fold dilutions (from left to right). Plates were incubated at 30°C for 2 d.

combination, on As(III) tolerance. We found that both the *ptp2Δ* and *ptp3Δ* mutations conferred modest tolerance to As(III) and that a double *ptp2Δ ptp3Δ* mutant displayed further tolerance (Figure 4C). However, the *ptc1Δ* mutation caused As(III) sensitivity, which may reflect a multitude of other functions attributed to this phosphatase, particularly on Mkk1, a MEK of the cell wall integrity signaling pathway (Tatjer et al., 2016).

A methylated metabolite of As(III), MAs(III), inhibits the Hog1 tyrosine phosphatases

As(III) is methylated in mammals to the more toxic metabolites methyl arsenite [MAs(III)] and dimethyl arsenite [DMAs(III); Cullen, 2014; Dong et al., 2015]. Additionally, it has been reported that, in contrast to As(III), these methylated metabolites are capable of inhibiting the mammalian tyrosine-specific phosphatases PTPB1 and CD45 by reaction with active-site cysteine residues (Rehman et al., 2012). The results presented to this point suggest that As(III) activates Hog1, directly or indirectly, through inhibition of the tyrosine phosphatases that normally maintain the SAPK in a low-activity state. Therefore, we tested the ability of As(III) and MAs(III) to inhibit the yeast tyrosine phosphatase Ptp3. GST-Ptp3 was isolated from

yeast cells and used in an in vitro phosphatase assay with activated Hog1-His₆ isolated from hyperosmotically shocked yeast cells. In the absence of inhibitor, dephosphorylation of Hog1 was nearly complete after treatment with Ptp3 for 1 h (Figure 5A). In contrast to this, a catalytically inactive form of Ptp3 (GST-Ptp3-C804A; Jacoby et al., 1997), isolated in parallel with GST-Ptp3, displayed very little phosphatase activity against phospho-Hog1 after treatment for 1 h (Figure 5B), indicating that the observed activity is intrinsic to GST-Ptp3. Under these conditions, MAs(III) completely inhibited Ptp3 activity at 10 μM, whereas As(III) failed to inhibit Ptp3 even at 100 μM (Figure 5C), supporting the conclusion that As(III) methylation is essential for its inhibition of the Ptps and consequent activation of Hog1. Additional inhibition experiments revealed that MAs(III) inhibits Ptp3 in the low micromolar range (Figure 5D).

The yeast SAM-dependent methyltransferase Mtq2, which is responsible for methylation of the translation release factor eRF1 (Heurgue-Hamard et al., 2005; Poleveda et al., 2006), is a candidate to catalyze the methylation of As(III) in this species. An *mtq2Δ* mutant displayed increased tolerance to As(III), but not to its more toxic metabolite MAs(III) (Ren et al., 2011; Figure 6A), suggesting that it might be responsible for conversion of As(III) to MAs(III). If this is the case, we would expect Mtq2 to be required for activation of Hog1 by As(III). We explored this possibility by first testing the ability of As(III) and MAs(III) to activate Hog1 in an *mtq2Δ* mutant. Although basal Hog1 activity was elevated slightly in this mutant, we detected no further increase in Hog1 activation by As(III) treatment (Figure 6B). In contrast to this, we detected Hog1 activation by MAs(III) treatment both in wild-type cells and in the *mtq2Δ* mutant, consistent with the dual conclusions that As(III) must be converted to MAs(III) for Hog1 activation and that Mtq2 is likely responsible for this conversion. Additionally, the *mtq2Δ* mutation did not affect the ability of As(V) to stimulate Hog1 (Figure 6C), providing further confirmation that As(V) activates Hog1 through a different pathway from that of As(III).

As a final test of the hypothesis that Mtq2 converts As(III) to MAs(III), we measured SAM-dependent methyltransferase activity of Mtq2 using As(III) as substrate. The active eRF1 methyltransferase is a heterodimer of Mtq2 with zinc-finger protein Trm112 (Heurgue-Hamard et al., 2006). Affinity-purified preparations of overexpressed Mtq2 from yeast without coexpression of Trm112 yield only weak methyltransferase activity against eRF1 (Heurgue-Hamard et al., 2005). Therefore, we coexpressed C-terminally TAP-tagged Mtq2 with Trm112 in yeast and isolated Mtq2-TAP by affinity purification. Overexpressed Mtq2-TAP in the absence of coexpressed Trm112 served as a control for this experiment. We detected a low level of As(III) methyltransferase activity from affinity-purified Mtq2-TAP alone, which was increased ~15-fold for comparable amounts of Mtq2-TAP isolated from cells that also overexpressed Trm112 (Figure 7A). The detected Mtq2:Trm112 methyltransferase activity was SAM-dependent and additionally capable of using MAs(III) as a substrate (Figure 7B). Taken together, these results demonstrate that As(III) is converted to MAs(III) by Mtq2:Trm112 and that MAs(III) is the metabolite responsible for activation of Hog1 through inhibition of its tyrosine-specific phosphatases.

DISCUSSION

There is a tendency to view SAPK signaling pathways through the same lens as growth factor signaling pathways in which signals are initiated from the cell surface. With the large and growing list of diverse stressors that activate a relatively small number of SAPKs, it seems increasingly likely that many of these stressors activate signaling through intracellular mechanisms, rather than by top-down

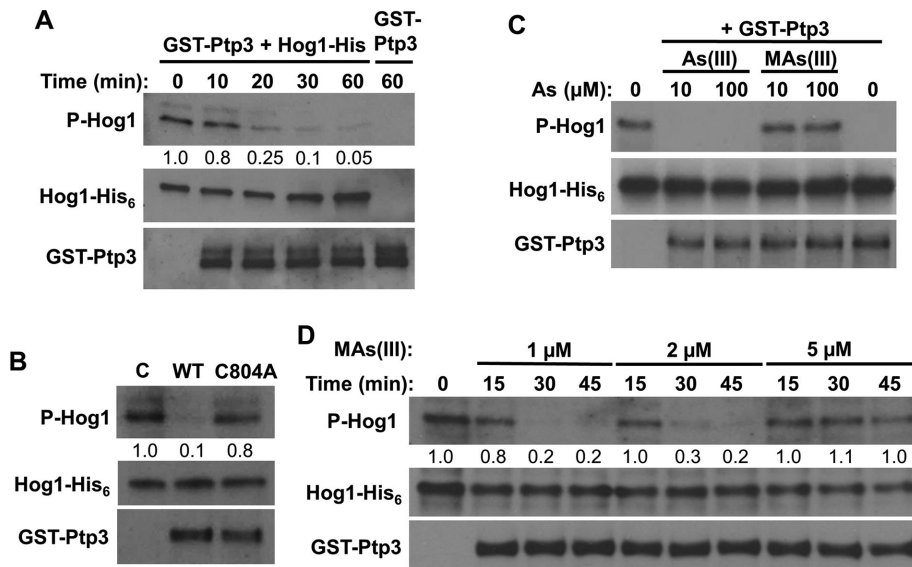


FIGURE 5: MAs(III) is the active metabolite of As(III) for inhibition of tyrosine phosphatase Ptp3 and consequent activation of Hog1. (A) *In vitro* protein phosphatase assay for GST-Ptp3 using phospho-Hog1-His₆ as substrate showing time course of phospho-Hog1 dephosphorylation by Ptp3. GST-Ptp3 was isolated from wild-type cells (DL3187) and incubated for the indicated times with purified Hog1-His₆ that had been activated by hyperosmotic shock in wild-type cells (DL3187). Values given were measured from the immunoblots and represent the fraction of phosphorylated Hog1 remaining normalized to the Hog1-His₆ input. (B) Catalytically inactive Ptp3 does not dephosphorylate phospho-Hog1. Equivalent amounts of GST-Ptp3 or GST-Ptp3-C804A isolated from a *ptp3Δ* strain (DL4296) were tested for phosphatase activity by incubation for 1 h with phospho-Hog1-His₆, as above. (C) MAs(III), but not As(III), is a potent inhibitor of tyrosine phosphatase Ptp3. The same conditions as in A were used in a protein phosphatase inhibition experiment. GST-Ptp3 was incubated for 1 h with phospho-Hog1-His₆ in the presence of the indicated concentrations of As(III) or MAs(III). (D) Inhibition of Ptp3 by MAs(III). The proteins in C were used in inhibition assays testing the indicated concentrations of MAs(III). Note the nearly complete inhibition of phosphatase activity by 5 μM MAs(III).

signaling through their canonical signaling pathways. This is particularly true for the yeast *S. cerevisiae*, which possesses only two SAPK activation pathways, both of which are well characterized from their plasma membrane sensors to their kinase cascades and transcription factors. Yet both of these SAPKs are activated by a wide range of seemingly unrelated stressors. In this study, we found that trivalent inorganic arsenic, As(III), must enter yeast cells to stimulate the SAPK Hog1, which led us to investigate the intracellular signaling pathway responsible for Hog1 activation by this toxic metalloid. We demonstrated that, in contrast to hyperosmotic stress, which signals to Hog1 by stimulation of cell surface osmosensors connected to the HOG pathway kinase cascade, As(III) treatment activates Hog1 through inhibition of the protein tyrosine phosphatases Ptp2 and Ptp3, which normally maintain Hog1 in a low-activity state. Previous reports that indicated a requirement for HOG pathway components upstream of Hog1 for its activation by As(III) (Sotelo and Rodríguez-Gabriel, 2006; Thorsen *et al.*, 2006) can be explained by our finding that basal signal through the HOG pathway, rather than activation of upstream components, is necessary for Hog1 activation by this stressor. We found that Hog1 that was mutationally severed from the signaling components known to act upstream of the SAPK could still be activated in response to As(III) treatment if a basal level of protein kinase activity was restored to its activating MEK, Pbs2, by phosphomimetic mutations. We demonstrated that this basal signal is amplified in response to As(III) treatment through the inhibition of Ptp2 and Ptp3. The further demonstration that As(III) treatment failed to activate wild-type Pbs2 supports the conclusion that Hog1

is activated in response to As(III) without stimulation of its immediately upstream protein kinase.

MAs(III) is the As(III) metabolite responsible for activation of Hog1

As(III) is metabolically activated by mammalian methyltransferases to the more toxic forms MAs(III) and DMAs(III) (Cullen 2014; Dong *et al.*, 2015). Intriguingly, these metabolites, but not As(III), are potent inhibitors of the mammalian tyrosine-specific phosphatases PTP1 and CD45 (Rehman *et al.*, 2012). On the basis of a single report by Ren *et al.* (2011), we suspected that As(III) was similarly metabolized in yeast. These authors demonstrated that deletion of yeast *MTQ2*, which encodes a SAM-dependent methyltransferase required for the methylation of translation release factor eRF1 (Heurgue-Hamard *et al.*, 2005; Poleveda *et al.*, 2006), confers resistance to As(III), but not to MAs(III), suggesting the possibility that Mtq2 converts As(III) to MAs(III). They showed additionally that overexpression of a mammalian orthologue of *MTQ2*, N-6 adenine-specific DNA methyltransferase (*N6AMT1*), in human urothelial cells catalyzed the secondary methylation of MAs(III) to DMAs(III). However, three lines of evidence support our conclusion that yeast Mtq2 is required for the conversion of As(III) to MAs(III), which is responsible for activation of Hog1 via Ptp inhibition. First, we found that Hog1 activation by As(III) was blocked in a *mtq2Δ* mutant and that the block was bypassed by MAs(III) treatment, suggesting that metabolic conversion of As(III) to MAs(III) is blocked in this mutant. Second, we found that although As(III) failed to inhibit Ptp3 *in vitro* at concentrations as high as 100 μM, MAs(III) was a potent inhibitor of this phosphatase at low micromolar concentrations, supporting the conclusion that As(III) must be converted to MAs(III) for the inhibition of Ptp2 and Ptp3 and the consequent activation of Hog1. The basis for the inhibitory activity of MAs(III) versus As(III) is not clear, but may reflect an ability of the methylated arsenical to access the active site cysteines of these enzymes. Finally, we demonstrated *in vitro* that the dimeric Mtq2:Trm112 methyltransferase was capable of SAM-dependent methyltransferase activity using As(III) as a substrate. We also detected methyltransferase activity for this enzyme using MAs(III) as substrate, consistent with the observed activity of its mammalian ortholog. However, we regard MAs(III), rather than DMAs(III), as the critical metabolite required for activation of Hog1 because *MTQ2* was not required for Hog1 activation by MAs(III) (Figure 6B). Thus, As(III) enters the cell through Fps1 and is metabolized by Mtq2:Trm112 to MAs(III), which inhibits Ptp2 and Ptp3, changing the balance of phosphorylated versus unphosphorylated Hog1 to favor the accumulation of active Hog1. This scheme is set out in Figure 8.

This activation mechanism suggests the possibility that other Hog1-activating stressors may also act intracellularly at different points along the pathway. This would provide an explanation as to how so many distinct stressors activate Hog1, which is known to be connected only to the cell surface osmosensors of the HOG pathway. Detailed examination of the pathways by which other stressors

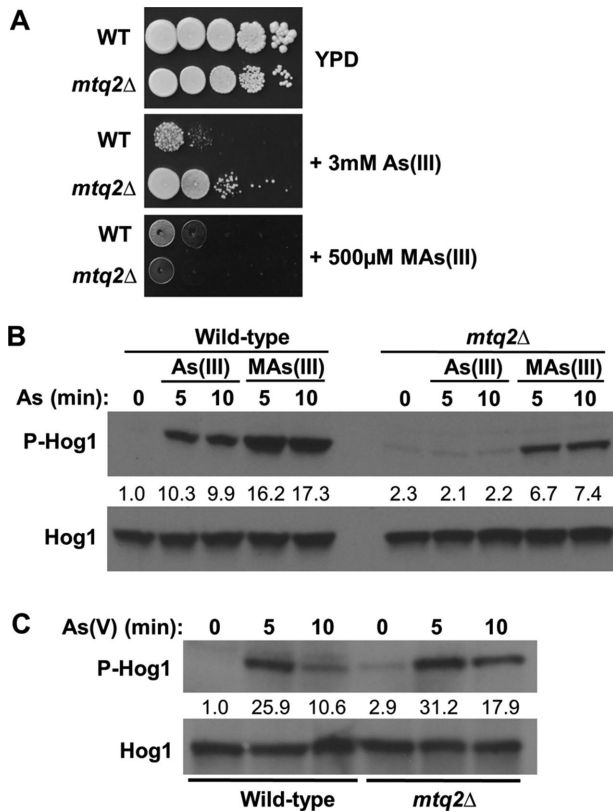


FIGURE 6: The methyltransferase encoded by *MTQ2* is required for activation of Hog1 by As(III), but not by As(V). (A) An *mtq2Δ* mutation confers tolerance of As(III), but not of MAs(III). Wild-type (DL3187) cells and an *mtq2Δ* mutant (DL4313) were spotted at serial 10-fold dilutions (left to right) onto YPD plates with 3 mM As(III) or 500 μ M MAs(III) and incubated at 30°C for 2 d. (B) An *mtq2Δ* mutation blocks Hog1 activation by As(III), but not by MAs(III). The same strains as above were treated with 1 mM As(III) or 500 μ M MAs(III) for the indicated times and tested for Hog1 phosphorylation. Fold activation values measured from the immunoblot are relative to the untreated wild-type control and normalized to Hog1 input. Note that the *mtq2Δ* mutant displays slightly higher basal Hog1 phosphorylation than the wild type. (C) An *mtq2Δ* mutation fails to block Hog1 activation in response to As(V) treatment. The same strains were tested for Hog1 phosphorylation in response to 3 mM As(V) for the indicated times. Fold activation values were determined as above.

activate Hog1 may reveal a multitude of intracellular inputs to this SAPK. Along these lines, we found that Hog1 is also activated in response to As(V) treatment, but that this activation is through a separate pathway from that described for As(III). Three lines of evidence support this conclusion. First, Hog1 stimulation by As(V) did not require its reduction to As(III) by the arsenate reductase *Acr2*. Nor did it require conversion to MAs(III) by *Mtq2*, which argues against the involvement of an unknown arsenate reductase. Second, As(V) failed to activate Hog1 in either of two engineered strains in which Hog1 responded to As(III) without a regulated signal from upstream pathway components, suggesting that the As(V) signal either emanates from the cell surface or enters the HOG pathway MEKs. Third, unlike As(III), As(V) treatment stimulated Pbs2 protein kinase activity toward Hog1 as measured *in vitro*, supporting the conclusion that As(V) treatment activates signaling of at least some components of the HOG pathway that function above Hog1.

Hog1 activated by different stresses drives divergent outputs

In this study, we considered the behavior of Hog1 activated by three different stresses: hyperosmotic shock, As(III), and As(V). When Hog1 is activated in response to hyperosmotic shock, it translocates rapidly to the nucleus and drives transcription of genes important for osmoregulation (Saito and Posas, 2012). Among these are *GPD1* and *GPP2*, which are important for glycerol biosynthesis in response to hyperosmotic stress. In contrast to this, Hog1 activated by As(III) does not translocate to the nucleus (Thorsen *et al.*, 2006) and does not drive *GPD1* expression (Sotelo and Rodríguez-Gabriel, 2006). However, Hog1 activated by As(III) contributes to arsenic-responsive transcription by *Acr1* through an unknown mechanism (Sotelo and Rodríguez-Gabriel, 2006). Our finding that As(V) also activates Hog1 provides another example of a stress-specific fate for the SAPK. Similarly to activation of Hog1 by As(III), As(V) treatment induced arsenic-responsive transcription, but did not induce nuclear translocation of Hog1 or transcriptional induction of *GPD1*. However, in contrast to As(III) treatment, Hog1 did not impact the arsenic-responsive transcription induced by As(V) treatment.

Although the bulk of Hog1 translocates to the nucleus in response to hyperosmotic shock, some is recruited to the plasma membrane glycerol channel *Fps1*, where it phosphorylates and evicts the positive regulators *Rgc1* and *Rgc2* from the *Fps1* C-terminus to induce channel closure and allow glycerol accumulation (Lee *et al.*, 2013). *Fps1* is also a key portal for entry of As(III) into yeast cells and Hog1 is important for the closure of this channel in response to As(III) treatment as an adaptation by which the cell restricts entry of As(III) (Thorsen *et al.*, 2006; Lee *et al.*, 2013). We found that As(III) treatment, similarly to hyperosmotic shock, induces the eviction of *Rgc2* from *Fps1*. The differential output of Hog1 activated by these stresses is critical to cell survival. As noted above, in response to hyperosmotic shock, Hog1 induces glycerol biosynthesis concomitant with *Fps1* closure to cause a rapid increase in intracellular osmolarity. When As(III) stress is considered, this combined action of Hog1 in the absence of high external osmolarity would cause elevated turgor and consequent cell wall stress (Beese *et al.*, 2009). Thus, As(III) stress mobilizes a Hog1 response that selectively closes *Fps1* to block As(III) entry without activating a full hyperosmotic stress response.

Another point of divergence between the responses to As(III) and As(V) is that As(V) treatment did not drive *Rgc2* eviction from *Fps1* despite its ability to activate Hog1. This differential behavior makes sense, considering that As(V) does not enter the cell through *Fps1*, but it leaves open two important questions with regard to As(V) activation of Hog1. First, what is the function of Hog1 activated by As(V) if it does not regulate either arsenic-protective gene expression or the activity of *Fps1*? One possibility is that Hog1 regulates As(V) entry through the phosphate transporters, although we are unaware of evidence to support this idea. Second, how is *Rgc2* protected from eviction by Hog1 that has been activated by As(V)? We found that Hog1 activation occurs equally rapidly in response to As(III) and As(V) treatment (within 5 min; Figure 1), implying that Hog1 activation kinetics is not responsible for its divergent behavior in response to these stressors. Nor can the relative levels of Hog1 activation by these agents explain the inability of As(V) to drive *Rgc2* eviction from *Fps1*, as it induced stronger Hog1 activation than As(III).

Perhaps additional, as yet unknown, intracellular targets of As(III) or MAs(III) contribute to the differential regulation of *Fps1* in response to trivalent versus pentavalent arsenic. For example, trivalent arsenic species are highly reactive with Cys residues in proteins (Shen *et al.*, 2013), suggesting the potential for selective regulation

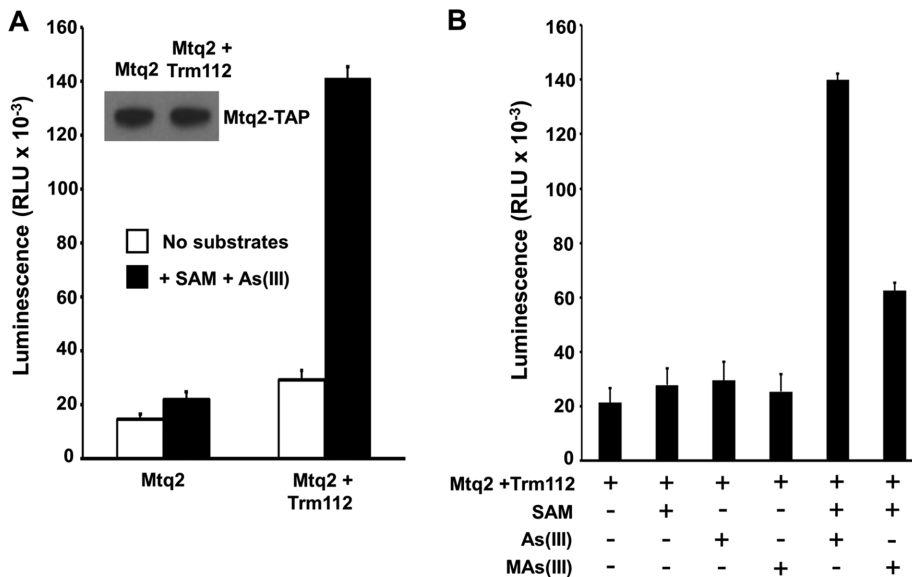


FIGURE 7: Mtq2 is a SAM-dependent As(III) and MAs(III) methyltransferase. (A) SAM-dependent methyltransferase activity of Mtq2:Trm112 using As(III) as substrate was measured in a coupled luminescence assay (see *Materials and Methods*). Mtq2 was affinity-purified from cells overexpressing Mtq2-TAP alone or coexpressing Trm112. Inset shows an immunoblot of affinity-purified Mtq2-TAP from the two samples. Output is in relative luminescence units (RLU). (B) Mtq2:Trm112 methyltransferase activity against As(III) and MAs(III) is SAM-dependent. The same preparations as in A were used to assess substrate specificity of Mtq2:Trm112. Each data point represents the mean and SD for three samples.

of Hog1 output through As(III) or MAs(III) modification of Hog1 targets, or proteins responsible for its nucleocytoplasmic shuttling. Indeed, As(III) was shown recently to bind directly to the arsenic-responsive transcription factor, Acr1, and to induce a conformational change that activates transcription (Kumar *et al.*, 2016). Therefore, the activated SAPK may cooperate with factors modified in a stress-specific manner to generate specific responses.

A second factor likely to be important in dictating the stress-specific output of Hog1 is the specific mechanism of its activation. Different activating inputs might result in different protein-protein interactions. Active Hog1 that forms different complexes depending upon its mechanism of activation may be directed to different targets. For example, Hog1 that is activated through inhibition of the Ptp phosphatases, rather than by activation of its MEK Pbs2, may engage in different interactions not only with Pbs2, which

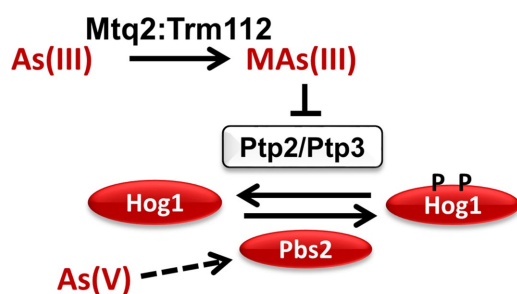


FIGURE 8: Model for the pathway by which As(III) activates Hog1. As(III) is metabolized by the methyltransferase Mtq2:Trm112 to MAs(III), which inhibits the Ptp2 and Ptp3 tyrosine phosphatases that modulate the phosphorylation state of Hog1. This results in activation of Hog1 by shifting the balance toward the phosphorylated state. As(V) activates Hog1 through a different pathway that involves the activation of Pbs2 (dashed arrow).

functions as a scaffold for the HOG pathway (Posas and Saito, 1997; Murakami *et al.*, 2008), but with other pathway components, as well. It is perhaps significant in this regard that we have found that As(III) treatment induces dissociation of Ptp3 from Hog1, a finding that contrasts with their continued association after hyperosmotic shock (unpublished data). In any case, how the variety of stress signal input mechanisms contribute to the specificity of SAPK output is an important question and may be one key to understanding the full range of SAPK signaling in yeast and in humans.

MATERIALS AND METHODS

Strains, growth conditions, transformations, and gene deletions

The *S. cerevisiae* strains used in this study were all derived from Research Genetics background S288c and are listed in Table 1. Yeast cultures were grown in YPD (1% Bacto yeast extract, 2% Bacto Peptone, 2% glucose) or minimal selective medium, SDM (0.67% yeast nitrogen base, 2% glucose), supplemented with the appropriate nutrients to select for plasmids. Yeast cultures were transformed according to Gietz *et al.* (1995).

Chromosomal deletion of the single *ACR2*, *MTQ2*, and *SSK1* genes was carried out by homologous recombination. The hygromycin-resistance gene *hphMX4* from pAG32 (Goldstein and McCusker, 1999) or the kanamycin-resistance gene *KanMX* from pFA6a-KanMX (Longtine *et al.*, 1998) was amplified by high-fidelity PCR (Phusion; ThermoFisher) using primers containing the upstream region (40 base pairs immediately before the starting ATG) and downstream region (40 base pairs immediately after the stop codon) of the target gene. The PCR products were integrated into the genome of the wild-type strain by homologous recombination. Integrates were selected on plates containing hygromycin B, or G418, yielding *acr2Δ::HPHMX4* (DL4341), *mtq2::HPHMX4* (DL4313) or *ssk1Δ::KanMX* (DL4304). Deletion of *SHO1* in the *ssk1Δ* background was generated using same method, resulting in *ssk1Δ::KanMX sho1Δ::HPHMX4* (DL4306).

Deletion of *PTP3* in the *ptp2Δ::KanMX* strain (DL4295) was generated using the *hphMX4* marker (*ptp2Δ::KanMX ptp3Δ::HPHMX4*; DL4299). Additional deletion of *PTC1* in DL4299 was generated using the *HIS3* as a selectable marker. The *HIS3* gene from pFA6a-*His3MX6* was amplified by PCR, as above. Integration into the genome of DL4299 was selected for on the plates without histidine, yielding *ptp2Δ::KanMX ptp3Δ::HPHMX4 ptc1Δ::HIS3* (DL4309). All gene replacements were validated by PCR analysis across both integration junctions.

Chemicals

Sodium arsenite (NaAsO_2) and sodium arsenate (Na_2HAsO_4) were purchased from Sigma, and methyl arsenite (CH_3AsO_2) was purchased from ChemCruz.

Plasmid construction

The *HOG1* gene was epitope-tagged on its C-terminus with the 3xHA epitope and expressed under its natural promoter for detection of

Strain	Relevant genotype	Source or reference
DL2344	<i>MATa</i> S288c <i>ssk2Δ::leu2 ssk22Δ::leu2 ste11Δ::his3</i>	Posas and Saito, 1997
DL2772	<i>MATα</i> S288c (BY4742) <i>his3Δ leu2Δ ura3Δ lys2Δ</i>	Research Genetics
DL3158	<i>MATa</i> S288c <i>hog1Δ::KanMX</i>	Research Genetics
DL3187	<i>MATa</i> S288c (BY4741) <i>his3Δ leu2Δ ura3Δ lys2Δ</i>	Research Genetics
DL3207	<i>MATa</i> S288c <i>rgc1Δ::KanMX rgc2Δ::KanMX</i>	Beese et al., 2009
DL3226	<i>MATa</i> S288c <i>fps1Δ::KanMX</i>	Research Genetics
DL4295	<i>MATα</i> S288c <i>ptp2Δ::KanMX</i>	Research Genetics
DL4296	<i>MATα</i> S288c <i>ptp3Δ::KanMX</i>	Research Genetics
DL4297	<i>MATα</i> S288c <i>ptc1Δ::KanMX</i>	Research Genetics
DL4299	<i>MATα</i> S288c <i>ptp2Δ::KanMX ptp3Δ::HPHMX4</i>	This study
DL4304	<i>MATa</i> S288c <i>ssk1Δ::KanMX</i>	This study
DL4306	<i>MATa</i> S288c <i>ssk1Δ::KanMX sho1Δ::HPHMX4</i>	This study
DL4309	<i>MATα</i> S288c <i>ptp2Δ::KanMX ptp3Δ::HPHMX4 ptc1Δ::HIS3</i>	This study
DL4313	<i>MATa</i> S288c <i>mtq2Δ::HPHMX4</i>	This study
DL4341	<i>MATa</i> S288c <i>acr2Δ::HPHMX4</i>	This study

TABLE 1: Yeast strains.

Hog1 in Figure 1. The promoter region of *HOG1* (from position -489) and the entire *HOG1* gene without the stop codon were amplified from genomic yeast DNA by high-fidelity PCR (Phusion) using the primers designed with a *XhoI* site (upstream) and with a *NotI* site (downstream) and cloned into pRS426-3HA-ADH1^T (p3150) to yield pRS426-HOG1-3HA (p3225). For subsequent experiments, endogenous Hog1 was detected using α -Hog1 antibodies.

For in vitro protein kinase assays, *HOG1* was tagged at its C-terminus with 6xHis (His₆) and expressed under the control of the *MET25* promoter. The *HOG1* coding sequence was amplified by high-fidelity PCR from genomic yeast DNA using a forward primer that contained an *XbaI* site immediately before the start codon and a reverse primer that introduced a His₆ tag followed by a stop codon and an *XhoI* site. This fragment was digested with *XbaI* and *XhoI* and inserted into pUT36 (p2415) to yield pUT36-MET25P-HOG1-HIS₆ (p3454).

For in vitro protein phosphatase assays, a catalytically inactive form of Ptp3 (Ptp3-C804A) was produced from parental plasmid GAL1^P-GST-PTP3 (p3402) using Quick Change mutagenesis (Agilent Technologies) to yield GAL1^P-GST-ptp3-C804A (p3464).

For in vitro methylation assays, *TRM112* was overexpressed under the control of the *MET25* promoter. The *TRM112* coding sequence was amplified by high-fidelity PCR from genomic yeast DNA using a forward primer that contained an *XbaI* site immediately before the start codon and a reverse primer that introduced a stop codon and a *SmaI* site. This fragment was digested with *XbaI* and *SmaI* and inserted into pYEP181-MET25P-FPS1-Myc (p3121), from which *FPS1-Myc* was removed by digestion with the same enzymes, yielding pYEP181-MET25P-TRM112 (p3460). The plasmids used in this study are listed in Table 2.

Protein extraction

Protein extraction was carried out as described previously (Kamada et al., 1995) for colP or using the rapid boiling method (Kushnirov, 2000) for direct immunoblot experiments.

Cultures for colP experiments with Rgc2-HA and Fps1-Myc were grown to mid-log phase in selective medium and starved for

methionine for 2 h to induce expression of Rgc2 and Fps1 under the control of the conditional *MET25* promoter. Cultures were treated with 1 mM As(III) or 3 mM As(V) for up to 60 min. Protein extraction and colP were carried out as described previously (Lee et al., 2013). Extracts (100 μ g of protein) were incubated with mouse monoclonal α -Myc antibody (1 μ g, 9E10; Pierce), for 1 h at 4°C and precipitated with protein A affinity beads for 1 h at 4°C. Samples were washed with IP buffer three times and boiled in SDS-PAGE buffer.

SDS-PAGE and immunoblot analysis

Proteins were separated by SDS-PAGE (7.5% gels) followed by immunoblot analysis using mouse monoclonal α -Myc antibody (9E10; Santa Cruz), α -HA (16B12; Covance), α -GST (B-14; Santa Cruz), α -tetra His (Qiagen), or goat polyclonal α -Hog1 (γ C-20; Santa Cruz) at a dilution of 1:10,000. Rabbit polyclonal α -phospho-p38 (T180/Y182, Cell Signaling) was used at a dilution of 1:2000 to detect phosphorylated Hog1. Secondary goat anti-mouse (Amersham) and donkey anti-goat (Santa Cruz) antibodies were used at a dilution of 1:10,000. Secondary donkey anti-rabbit (Amersham) was used at a dilution of 1:2000. All results involving immunoblot analyses were replicated at least once, and representative blots are shown.

Quantitative real-time PCR

Quantitative real-time PCR was conducted to quantify the levels of mRNA for *GPD1* or *ACR3* under arsenic stress or hyperosmotic shock. Wild-type cells were treated with 1 mM As(III), 3 mM As(V), or 1 M sorbitol for 5 min. Whole-cell mRNA was prepared with a NucleoSpin RNA kit (Macherey-Nagel), as directed by the manufacturer. Complementary DNA was generated using the Superscript III (Invitrogen) first-strand synthesis system for RT-PCR. Real-time PCR was performed using a Bio-Rad CFX96 Real-Time system with Bio-Rad SYBR green mix. The data from biological triplicates were plotted using Microsoft Excel.

In vitro protein kinase assay

An in vitro protein kinase assay with Pbs2 was performed using Hog1 as substrate. Pbs2 was prepared from a wild-type strain

Plasmid	Description	Source or reference
p115	pRS316	Sikorski and Hieter, 1989
p1106	pRS426	Sikorski and Hieter, 1989
p1230	pYES2-GAL1 ^P -STE11-NA	Posas and Saito, 1997
p2415	pUT36	Millson et al., 2005
p2501	pUT36-MET25 ^P -RGC2-HIS ₆	Beese et al., 2009
p2823	pAG32	Goldstein and McCusker, 1999
p3121	pYEP181-MET25 ^P -FPS1-Myc	Lee et al., 2013
p3147	pRS315-3HA-ADH1 ^T	Lee et al., 2013
p3150	pRS426-3HA-ADH1 ^T	Lee et al., 2013
p3151	pRS316-MET25 ^P -RGC2-3HA	Lee et al., 2013
p3177	pRS416-HOG1-GFP	Ferrigno et al., 1998
p3225	pRS426-HOG1-3HA	This study
p3261	pFA6a-KanMX	Longtine et al., 1998
p3263	pFA6a-His3MX	Longtine et al., 1998
p3370	pYES2-GAL1 ^P -PBS2DD	Wurgler-Murphy et al., 1997
p3396	GAL1 ^P -PBS2-TAP (6HIS-HA-ZZ)	Open Biosystems
p3402	GAL1 ^P -GST-PTP3	Open Biosystems
p3454	pUT36-MET25 ^P -HOG1-HIS ₆	This study
p3459	GAL1 ^P -MTQ2-TAP (6HIS-HA-ZZ)	Open Biosystems
p3460	pYEP181-MET25 ^P -TRM112	This study
p3464	GAL1 ^P -GST-PTP3-C804A	This study

TABLE 2: Plasmids.

(DL3187) containing a plasmid expressing C-terminally TAP-tagged Pbs2 under the control of the *GAL1* promoter (p3396). Cells were grown in selective medium containing 2% glucose and transferred to selective medium containing 2% raffinose and grown to mid-log phase. Galactose was added to these cultures at a final concentration of 2% to induce expression of Pbs2-TAP for 2 h. Cultures of 10 ml were then stressed with 1 mM As(III), 3 mM As(V), or 1 M sorbitol for 5 min, collected, and lysed with 20 mM HEPES buffer (pH 7.5) containing 0.5% Triton, protease inhibitors, and phosphatase inhibitors. The lysates (500 μ l) were incubated with immunoglobulin G-sepharose 6 Fast Flow (50 μ l; GE Healthcare) for 1 h at 4°C. The beads with Pbs2-TAP were washed with IP buffer three times and used for protein kinase assays.

The unphosphorylated form of Hog1 was prepared from a strain deleted for all of the HOG pathway MEKKs, *ste11 Δ ssk2 Δ ssk22 Δ* (DL2344), which does not activate Hog1. His-tagged Hog1 was expressed in this strain from pUT36-MET25P-HOG1-HIS₆ (p3454), which was grown in 50 ml of culture to mid-log phase in selective medium, starved for methionine to induce expression of Hog1, collected and lysed using 20 mM HEPES buffer containing 0.5% triton and protease inhibitors. The protein extract (1.5 ml) was incubated with HisPur Ni-NTA Superflow Agarose (200 μ l; Thermo Scientific). The resin was washed with 20 mM HEPES binding buffer three times and the unphosphorylated form of Hog1-His₆ was eluted with 250 mM imidazole. The Hog1-His₆ eluate was dialyzed by Slide-A-Lyzer MINI Dialysis Device 10k MWCO (Thermo Scientific) against 50 mM Tris-HCl to a final volume of 1 ml.

Kinase reactions contained dialyzed Hog1-His₆ eluate (100 μ l) in protein kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 2 mM dithiothreitol [DTT]) with phosphatase inhibitors (PhosSTOP; Roche

and 10 μ l of Pbs2-TAP resin. Reactions were initiated with ATP at a final concentration of 300 μ M. Reaction mixtures were incubated at 30°C for 20 min and were stopped by adding SDS-PAGE buffer, followed by boiling for 5 min. Immunoblot analysis was performed as described above.

In vitro protein phosphatase assay

An in vitro protein phosphatase assay with Ptp3 was performed using phosphorylated Hog1 as substrate to test for inhibition of Ptp3 by As(III) and MAs(III). GST-Ptp3 was isolated from wild-type cells (DL3187) expressing GST-Ptp3 from a plasmid under the control of the *GAL1* promoter (p3402). Cells were grown in selective medium containing 2% glucose and transferred to selective medium containing 2% raffinose for further growth to mid-log phase. Galactose was added to 100 ml of culture at a final concentration of 2% to induce expression of GST-Ptp3 for 2 h. Cells were lysed in extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton) containing protease inhibitors and the extract (1.5 ml) was incubated with glutathione-superflow resin (200 μ l; Clontech) for 1 h at 4°C. The resin was washed three times with binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl). GST-Ptp3 was eluted with 20 mM reduced glutathione and dialyzed by Slide-A-Lyzer MINI Dialysis 10k MWCO against a phosphatase reaction buffer (25 mM imidazole, pH 7.5, 2.5 mM EDTA, 50 mM NaCl, 5 mM DTT) to a final volume of 1 ml.

The phosphorylated form of Hog1-His₆ was prepared from wild-type cells (DL3187) after hyperosmotic shock. A 100-ml culture of cells containing pUT36-MET25P-HOG1-HIS₆ (p3454) was grown to mid-log phase and osmotically shocked with 0.5 M NaCl. Cells were collected and lysed in extraction buffer (50 mM Tris-HCl, pH 7.5,

150 mM NaCl, 0.5% Triton) containing protease inhibitors and phosphatase inhibitors. The extract (1.5 ml) was incubated with His-Pur Ni-NTA Superflow Agarose (200 μ l; Thermo Scientific). The resin was washed with binding buffer three times and Hog1-His₆ was eluted with 250 mM imidazole buffer. Eluate was dialyzed as above against phosphatase reaction buffer and protease inhibitors were added to dialyzed eluates to a final volume of 1 ml. As(III) or MAs(III) was added to affinity purified GST-Ptp3 in reaction buffer and purified phosphorylated Hog1-His₆ was added to initiate the reaction. Phosphatase reactions were initiated by mixing 40 μ l GST-Ptp3 and 40 μ l Hog1-His₆ and were terminated by addition of SDS-PAGE buffer followed by boiling for 5 min. Immunoblot analysis was performed as described above.

In vitro arsenite methyltransferase assay

An in vitro arsenite methylation assay was performed using Mtq2 overexpressed alone in yeast or Mtq2 cooverexpressed with Trm112. Cultures were prepared from a wild-type strain (DL3187) bearing a plasmid that expresses C-terminally TAP-tagged (6HIS-HA-ZZ) Mtq2 under the control of the *GAL1* promoter (p3459), with or without a second plasmid that expresses untagged Trm112 under the control of the *MET25* promoter (p3460). Cells were grown in selective medium containing 2% glucose and transferred to selective medium containing 2% raffinose for growth to mid-log phase. Cultures (25 ml) were transferred to selective medium without methionine (to induce Trm112 expression) and with 2% galactose (to induce Mtq2-TAP expression) for 3 h. Cells were then collected and lysed with 20 mM HEPES buffer (pH 7.5) containing 0.5% Triton, protease inhibitors, and phosphatase inhibitors. The protein extract (1 ml) was precleared with agarose resin (200 μ l; Thermo Scientific) for 1 h at 4°C and incubated with HisPur Ni-NTA Superflow Agarose (200 μ l; Thermo Scientific) for 1 h at 4°C. The resin was washed three times with 20 mM HEPES (pH 7.5) buffer and Mtq2-TAP was eluted with 1 ml of 250 mM imidazole. The Mtq2-TAP eluate was dialyzed by Spin-X 5k MWCO (Corning) against 50 mM Tris-HCl and concentrated to a volume of ~100 μ l. Protein concentrations were measured by DC Protein Assay (Bio-Rad). Approximately 300–500 ng of total protein, corresponding to equivalent amounts of Mtq2-TAP based on immunoblot analysis, was used for in vitro methylation assays comparing Mtq2 alone or Mtq2 with copurified Trm112 with 100 μ M As(III) or MAs(III) as substrate. Arsenite methylation assays were performed using the MTase-Glo Methyltransferase assay kit (Promega). The basis of this assay is the conversion of methyl donor S-adenosyl methionine (SAM) to S-adenosyl homocysteine (SAH) through the action of a methyltransferase. Coupled reactions converted SAH to ATP, which was detected by a luciferase reaction. Luminescence from triplicate samples was measured by a Microplate Luminometer (Veritas).

Florescence microscopic detection of Hog1

Haploid wild-type strain (DL3187) was transformed with plasmid pRS416-*HOG1-GFP* (p3177). Transformants were grown in selective medium and visualized with a Zeiss Axio Observer Z1 fitted with a GFP filter using a 100 \times objective. For detection of Hog1 nuclear localization after stress, 1 mM As(III), 3 mM As(V), or 1 M sorbitol was added to the cells immediately before live-cell imaging.

Notes on reproducibility

All protein kinase, protein phosphatase, and arsenite methyltransferase assays were reproduced at least once in independent experiments, representatives of which are shown. Similarly, all immunoblots and colPs were reproduced at least once in independent

experiments with representative images shown. Quantitation of signals from immunoblots was done using ImageJ software.

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REFERENCES

- Abernathy CO, Thomas DJ, Calderon RL (2003). Health effects and risk assessment of arsenic. *J Nutr* 133, 1536S–1538S.
- Aguilera J, Rodríguez-Vargas S, Prieto JA (2005). The HOG MAP kinase pathway is required for the induction of methylglyoxal-responsive genes and determines methylglyoxal resistance in *Saccharomyces cerevisiae*. *Mol Microbiol* 56, 228–239.
- Ahmadpour D, Maciaszczyk-Dziubinska E, Babazadeh R, Dahal S, Migocka M, Andersson M, Wysocki R, Tamás MJ, Hohmann S (2016). The mitogen-activated protein kinase Slr2 modulates arsenite transport through the aquaglyceroporin Fps1. *FEBS Lett* 590, 3649–3659.
- Azad GK, Singh V, Thakare MJ, Barawal S, Tomar RS (2014). Mitogen-activated protein kinase Hog1 is activated in response to curcumin exposure in the budding yeast *Saccharomyces cerevisiae*. *BMC Microbiol* 14, 317–327.
- Beane Freeman LE, Dennis LK, Lynch CF, Thorne PS, Just CL (2004). Toenail arsenic content and cutaneous melanoma in Iowa. *Am J Epidemiol* 160, 679–687.
- Beese SE, Negishi T, Levin DE (2009). Identification of positive regulators of the yeast Fps1 glycerol channel. *PLoS Genetics* 5, e1000738.
- Bhattacharjee H, Sheng J, Ajees AA, Mukhopadhyay R, Rosen BP (2010). Adventitious arsenate reductase activity of the catalytic domain of the human Cdc25B and Cdc25C phosphatases. *Biochemistry* 49, 802–809.
- Bilsland E, Molin C, Swaminathan S, Ramne A, Sunnerhagen P (2004). Rck1 and Rck2 MAPKAP kinases and the HOG pathway are required for oxidative stress resistance. *Mol Micro* 53, 1743–1756.
- Cairns BR, Ramer SW, Kornberg RD (1992). Order of action of components in the yeast pheromone response pathway revealed with a dominant allele of the STE11 kinase and the multiple phosphorylation of the STE7 kinase. *Genes Dev* 6, 1305–1318.
- Cullen WR (2014). Chemical mechanism of arsenic biomethylation. *Chem Res Toxicol* 27, 457–461.
- de Nadal E, Alupez PM, Posas F (2002). Dealing with osmotic stress through MAP kinase activation. *EMBO Rep* 3, 735–740.
- Dheeman SD, Packianathan C, Pillai JK, Rosen BP (2014). Pathway of human AS3MT arsenic methylation. *Chem Res Toxicol* 27, 1979–1989.
- Dong H, Madegowda M, Nefzi A, Houghten RA, Giulianotti MA, Rosen BP (2015). Identification of small molecule inhibitors of human As(III) S-adenosylmethionine methyltransferase (AS3MT). *Chem Res Toxicol* 28, 2419–2425.
- Elbirt KK, Whitmarsh AJ, Davis RJ, Bonkovsky HL (1998). Mechanism of sodium arsenite-mediated induction of heme oxygenase-1 in hepatoma cells. Role of mitogen-activated protein kinases. *J Biol Chem* 273, 8922–8931.
- Ferrigno P, Posas F, Koepf D, Saito H, Silver PA (1998). Regulated nucleocytoplasmic exchange of HOG1 MAPK requires the importin beta homologs NMD5 and XPO1. *EMBO J* 17, 5606–5614.
- Gietz RD, Schiestl RH, Willems AR, Woods RA (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast* 11, 355–360.
- Goldstein AL, McCusker JH (1999). Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* 15, 1541–1553.
- Han J, Lee J-D, Bibbs L, Ulevitch RJ (1994). A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* 265, 808–811.
- Heurgue-Hamard V, Champ S, Mora L, Merkoulouva-Rainon T, Kisselev LL, Buckingham RH (2005). The glutamine residue of the conserved GCQ motif in *Saccharomyces cerevisiae* release factor eRF1 is methylated by the product of the *YDR140w* gene. *J Biol Chem* 280, 2439–2445.
- Heurgue-Hamard V, Graille M, Scrima N, Ulryck N, Champ S, van Tilbeurgh H, Buckingham RH (2006). The zinc finger protein Ynr046w is plurifunctional and a component of the eRF1 methyltransferase in yeast. *J Biol Chem* 281, 36140–36148.
- Jacoby T, Flanagan H, Faykin A, Seto AG, Mattison C, Ota I (1997). Two protein-tyrosine phosphatases inactivate the osmotic stress response

- pathway in yeast by targeting the mitogen-activated protein kinase, Hog1. *J Biol Chem* 272, 17749–17755.
- Jiang L, Cao C, Zhang L, Lin W, Xia J, Xu H, Zhang Y (2014). Cadmium-induced activation of high osmolarity pathway through its Sln1 branch is dependent on the MAP kinase kinase kinase Ssk2, but not its paralog Ssk22, in budding yeast. *FEMS Yeast Res* 14, 1263–1272.
- Kamada Y, Jung US, Piotrowski J, Levin DE (1995). The protein kinase C-activated MAP kinase pathway of *Saccharomyces cerevisiae* mediates a novel aspect of the heat shock response. *Genes Dev* 9, 1559–1571.
- Kumar NV, Yang J, Pillai JK, Rawat S, Solano C, Kumar A, Grøtli M, Stemmler TL, Rosen BP, Tamás MJ (2016). Arsenic directly binds to and activates the yeast AP-1-like transcription factor Yap8. *Mol Cell Biol* 36, 913–922.
- Kushnirov VV (2000). Rapid and reliable protein extraction from yeast. *Yeast* 16, 857–860.
- Lawrence CL, Botting CH, Antrobus R, Coote PJ (2004). Evidence of a new role for the high-osmolarity glycerol mitogen-activated protein kinase pathway in yeast: regulating adaptation to citric acid stress. *Mol Cell Biol* 24, 3307–3323.
- Lee J, Reiter W, Dohnal I, Gregori C, Beese-Sims S, Kuchler K, Ammerer G, Levin DE (2013). MAPK Hog1 closes the *S. cerevisiae* glycerol channel Fps1 by phosphorylating and displacing its positive regulators. *Genes Dev* 27, 2590–2601.
- Longtine MS, McKenzie A 3rd, Demarini DJ, Shah NG, Wach A, Brachat A, Philippsen P, Pringle JR (1998). Additional modules for versatile and economic PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 14, 953–961.
- Maciaszczyk-Dziubinska E, Migocka M, Wysocki R (2011). Acr3p is a plasma membrane antiporter that catalyzes As(III)/H⁺ and Sb(III)/H⁺ exchange in *Saccharomyces cerevisiae*. *Biochim Biophys Acta* 1808, 1855–1859.
- Maciaszczyk-Dziubinska E, Wawrzycka D, Wysocki R (2012). Arsenic and antimony transporters in eukaryotes. *Int J Mol Sci* 13, 3527–3548.
- Marques JM, Rodrigues RJ, de Magalhães-Sant’ana AC, Gonçalves T (2006). *Saccharomyces cerevisiae* Hog1 protein phosphorylation upon exposure to bacterial endotoxin. *J Biol Chem* 281, 24687–24694.
- Matia-González AM, Rodríguez-Gabriel MA (2011). Sit2 MAPK pathway is essential for cell integrity in the presence of arsenate. *Yeast* 28, 9–17.
- Millson SH, Truman AW, King V, Prodromou C, Pearl LH, Piper PW (2005). A two-hybrid screen of the yeast proteome for Hsp90 interactors uncovers a novel Hsp90 chaperone requirement in the activity of a stress-activated mitogen-activated protein kinase, Sit2p (Mpk1p). *Eukaryot Cell* 5, 1914–1924.
- Mollapour M, Piper PW (2006). Hog1p mitogen-activated protein kinase determines acetic acid resistance in *Saccharomyces cerevisiae*. *FEMS Yeast Res* 6, 1274–1280.
- Mukhopadhyay R, Rosen BP (1998). *Saccharomyces cerevisiae* ACR2 gene encodes an arsenate reductase. *FEMS Microbiol Lett* 168, 127–136.
- Murakami Y, Tatebayashi K, Saito H (2008). Two adjacent docking sites in the yeast Hog1 Mitogen-Activated Protein (MAP) kinase differentially interact with the Pbs2 MAP kinase and the Ptp2 protein tyrosine phosphatase. *Mol Cell Biol* 28, 2481–2494.
- Ono K, Han J (2000). The p38 signal transduction pathway activation and function. *Cell Signaling* 12, 1–13.
- Panadero J, Pallotti C, Rodríguez-Vargas S, Rande-Gil F, Prieto JA (2006). A downshift in temperature activates the high osmolarity glycerol (HOG) pathway, which determines freeze tolerance in *Saccharomyces cerevisiae*. *J Biol Chem* 281, 638–645.
- Piao H, MacLean Freed J, Mayingier P (2012). Metabolic activation of the HOG MAP kinase pathway by Snf1/AMPK regulates lipid signaling at the Golgi. *Traffic* 13, 1522–1531.
- Poleveda B, Span L, Sherman F (2006). The yeast translation release factors Mrf1 and Sup45p (eRF1) are methylated, respectively, by the methyltransferases Mtt1p and Mtt2p. *J Biol Chem* 281, 2562–2571.
- Posas F, Saito H (1997). Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK. *Science* 276, 1702–1705.
- Rehman K, Chen Z, Wang WW, Wang YW, Sakamoto A, Zhang YF, Naranmandura H, Suzuki N (2012). Mechanisms underlying the inhibitory effects of arsenic compounds on protein tyrosine phosphatase (PTP). *Toxicol Appl Pharmacol* 263, 273–280.
- Rehman K, Naranmandura H (2012). Arsenic metabolism and thioarsenicals. *Metallomics* 4, 881–892.
- Ren X, Aleshin M, Jo WJ, Dills R, Kalman DA, Vulpe CD, Smith MT, Zhang L (2011). Involvement of N-6 adenine-specific DNA methyltransferase (N6AMT1) in arsenic biomethylation and its role in arsenic-induced toxicity. *Env Health Persp* 119, 771–777.
- Rosen BP, Liu Z (2009). Transport pathways for arsenic and selenium: a minireview. *Environ Int* 35, 512–515.
- Saito H, Posas F (2012). Response to hyperosmotic stress. *Genetics* 192, 289–318.
- Shen S, Li X-F, Cullen WR, Weinfeld M, Le XC (2013). Arsenic binding to proteins. *Chem Rev* 113, 7769–7792.
- Sikorski RS, Hieter P (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122, 19–27.
- Smedley PL, Kinniburgh DG (2002). A review of the source, behavior and distribution of arsenic in natural waters. *Applied Geochem* 17, 517–568.
- Sotelo J, Rodríguez-Gabriel MA (2006). Mitogen-activated protein kinase Hog1 is essential for the response to arsenite in *Saccharomyces cerevisiae*. *Eukaryot Cell* 5, 1826–1830.
- Tatjer L, Sacristán-Reviriego A, Casado C, González A, Rodríguez-Porrata B, Palacios L, Canadell D, Serra-Cardona A, Martín H, Molina M, Ariño J (2016). Wide-ranging effects of the yeast Ptc1 protein phosphatase acting through the MAPK kinase Mkk1. *Genetics* 202, 141–156.
- Thorsen M, Di Y, Tängemo C, Morillas M, Ahmadpour D, Van der Does C, Wagner A, Johansson E, Boman J, Posas F, et al. (2006). The MAPK Hog1p modulates Fps1p-dependent arsenite uptake and tolerance in yeast. *Mol Biol Cell* 17, 4400–4410.
- Verma A, Mohindru M, Deb DK, Sassano A, Kambhampati S, Ravandi F, Minucci S, Kalvakolanu DV, Platanius LC (2002). Cutting edge: activation of the p38 mitogen-activated protein kinase signaling pathway mediates cytokine-induced hemopoietic suppression in aplastic anemia. *J Immunol* 168, 5984–5988.
- Winkler A, Arkind C, Mattison CP, Burkholder A, Knoche K, Ota I (2002). Heat stress activates the yeast high-osmolarity glycerol mitogen-activated protein kinase pathway, and protein tyrosine phosphatases are essential under heat stress. *Eukaryot Cell* 1, 163–173.
- Wurgler-Murphy SM, Maeda T, Witten EA, Saito H (1997). Regulation of the *Saccharomyces cerevisiae* HOG1 mitogen-activated protein kinase by the PTP2 and PTP3 protein tyrosine phosphatases. *Mol Cell Biol* 17, 1289–1297.
- Wysocki R, Chéry CC, Wawrzycka D, van Hulle M, Cornelis R, Thevelein JM, Tamás MJ (2001). The glycerol channel Fps1p mediates the uptake of arsenite and antimonite in *Saccharomyces cerevisiae*. *Mol Micro* 40, 1391–1401.
- Wysocki R, Fortier PK, Maciaszczyk E, Thorsen M, Leduc A, Odhagen A, Owsianik G, Ulaszewski S, Ramotar D, Tamás MJ (2004). Transcriptional activation of metalloid tolerance genes in *Saccharomyces cerevisiae* requires the AP-1-like proteins Yap1p and Yap8p. *Mol Biol Cell* 15, 2049–2060.
- Wysocki R, Tamás MJ (2010). How *Saccharomyces cerevisiae* copes with toxic metals and metalloids. *FEMS Microbiol Rev* 34, 925–951.