CELL BIOLOGY

Yeast Osmosensors Hkr1 and Msb2 Activate the Hog1 MAPK Cascade by Different Mechanisms

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To cope with environmental high osmolarity, the budding yeast *Saccharomyces cerevisiae* activates the mitogen-activated protein kinase (MAPK) Hog1, which controls an array of osmoadaptive responses. Two independent, but functionally redundant, osmosensing systems involving the transmembrane sensor histidine kinase Sln1 or the tetraspanning membrane protein Sho1 stimulate the Hog1 MAPK cascade. Furthermore, the Sho1 signaling branch itself also involves the two functionally redundant osmosensors Hkr1 and Msb2. However, any single osmosensor (Sln1, Hkr1, or Msb2) is sufficient for osmoadaptation. We found that the signaling mechanism by which Hkr1 or Msb2 stimulated the Hog1 cascade was specific to each osmosensor. Specifically, activation of Hog1 by Msb2 required the scaffold protein Bem1 and the actin cytoskeleton. Bem1 bound to the cytoplasmic domain of Msb2 and thus recruited the kinases Ste20 and Cla4 to the membrane where either of them can activate the kinase Ste11. The cytoplasmic domain of Hkr1 also contributed to the activation of Ste11 by Ste20, but through a mechanism that involved neither Bem1 nor the actin cytoskeleton. Furthermore, we found a PXXP motif in Ste20 that specifically bound to the Sho1 SH3 (Src homology 3) domain. This interaction between Ste20 and Sho1 contributed to the activation of Hog1 by Hkr1, but not by Msb2. These differences between Hkr1 and Msb2 may enable differential regulation of these two proteins and provide a mechanism through Msb2 to connect regulation of the cytoskeleton with the response to osmotic stress.

INTRODUCTION

In response to environmental high osmolarity, the budding yeast *Saccharomyces cerevisiae* induces an array of adaptive responses, including the synthesis and accumulation of the osmolyte glycerol, changes in the global pattern of gene expression, and a temporary arrest of cell cycle progression (*1*). These responses are all controlled by the mitogen-activated protein kinase (MAPK) Hog1, which is activated by the high-osmolarity glycerol (HOG) pathway. Upstream in the HOG pathway are two independent signaling branches, the Sln1 branch and the Sho1 branch (*2*, *3*) (Fig. 1A). The osmosensor for the Sln1 branch is the sensor histidine kinase Sln1, which transmits the signal through a two-component phosphorelay mechanism to the MAPK kinase kinases (MAPKKKs) Ssk2 and Ssk22 (collectively referred to as Ssk2/22) (*3*). In contrast, the Sho1 branch involves two functionally redundant osmosensors, Hkr1 and Msb2, which are transmembrane mucin-like glycoproteins with no known enzymatic activity (*4*–*6*). It is intriguing, therefore, that yeast has three osmosensors, when any one is sufficient to respond and adapt to the external high osmolarity. There is evidence that the stimulus recognized by the Sln1 branch is qualitatively different from that recognized by the Sho1 branch (*7*). For example, the Sln1 branch responds to changes in turgor pressure, whereas the Sho1 branch does not. However, it is unclear whether there is any functional difference between Hkr1 and Msb2.

The extracellular domains of Hkr1 and Msb2 are structurally similar to each other in that both contain a long (>700–amino acid) Ser/Thr-rich (STR) region, which is highly O-glycosylated, and a ~200–amino acid–long region termed the Hkr1-Msb2 homology (HMH) domain (Fig. 1B). For both osmosensors, an inhibitory role of the STR region and an activating role of the HMH domain are suggested by the phenotypes of mutants (*5*, *8*). In contrast, the cytoplasmic regions of Hkr1 and Msb2 share no structural similarity to each other, although they both activate the Ste11-Pbs2-Hog1 MAPK cascade. Either of the p21-activated kinase (PAK)–family kinases Ste20 and Cla4 (collectively referred to as Ste20/Cla4) activates the MAPKKK Ste11 (*9*–*11*). Ste11 localizes to the membrane through an adaptor protein (Ste50) that binds both Ste11 and the transmembrane protein Opy2, whereas Pbs2 localizes to the membrane by binding to the Src homology 3 (SH3) domain of the tetraspanning membrane protein Sho1 (*1*). Their membrane localization is essential for an efficient activation of Pbs2 by Ste11.

Here, we show that the cortical scaffold protein Bem1 and the actin cytoskeleton are essential for activation of the Ste11-Pbs2-Hog1 MAPK cascade by the Msb2 osmosensor, but not by the Hkr1 osmosensor. Bem1 is involved in cell polarity establishment and bud site selection (*12*) and interacts with many proteins, including Ste20, Cla4, the guanosine triphosphatase Cdc42, Cdc24 [a guanine nucleotide exchange factor (GEF) for Cdc42], and the actin cytoskeleton (*13*–*16*). Thus, our data suggest that the unique function of the Msb2 osmosensor is to integrate the signals from the external osmotic conditions with those from the internal cytoskeletal conditions.

RESULTS

Signaling by the redundant osmosensors Hkr1 and Msb2 is not equivalent

Here, all the yeast strains, unless otherwise noted, carry *ssk2/22*Δ double mutations so that the Sln1 branch is completely inactivated (see table S1 for all yeast strains). In the $HKR1^{+}$ $MSB2^{+}$ cells, either the kinase Ste20 or the kinase Cla4 can phosphorylate and activate the MAPKKK Ste11

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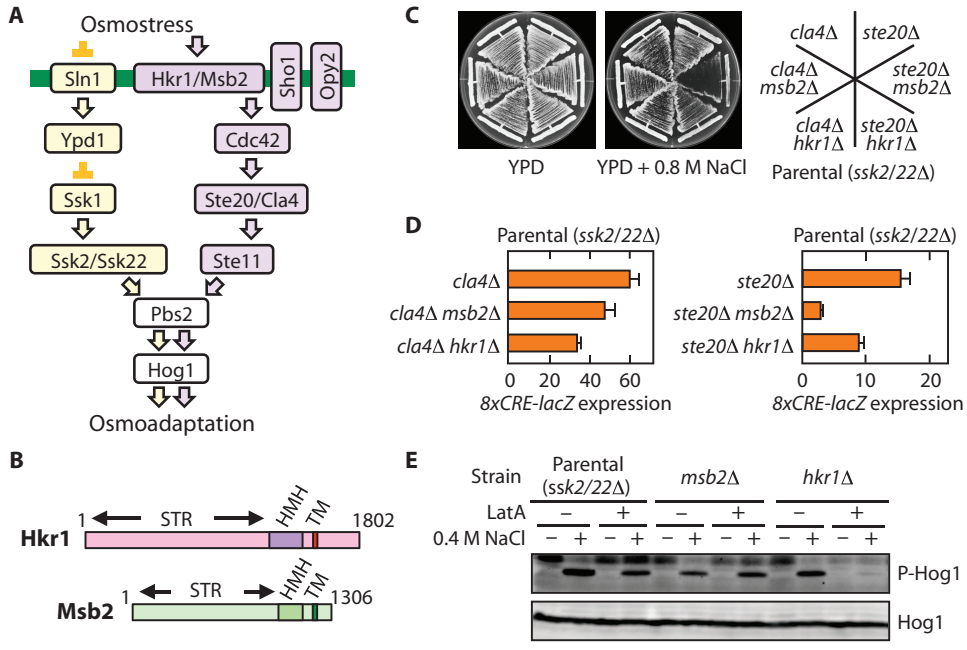

Fig. 1. Signaling by the redundant osmosensors Hkr1 and Msb2 is not equivalent. (**A**) Schematic model of the yeast HOG pathway. The green horizontal bar represents the plasma membrane. Arrows indicate positive signal flow, whereas blunt bars represent negative regulation. The Sln1 branch is indicated by yellow, and the Sho1 branch by violet. Proteins indicated with slashes represent functionally redundant components. (**B**) Schematic models of Hkr1 and Msb2. Numbers represent amino acid positions. TM, transmembrane domain. (**C**) The effects on osmosensitivity of *hkr1*Δ and *msb2*Δ mutations in the *ste20*Δ or *cla4*Δ background were tested. Data are representative of two experiments. (**D**) Osmostress-induced expression of the Hog1-specific reporter gene *8xCRE-lacZ* in the same strains as in (C) was tested. Cells were treated with 0.4 M NaCl for 30 min. Error bars represent SDs; $n \geq 3$. The value for the parental strain TM257 (*ssk2*Δ *ssk22*Δ) is 79.9 ± 23.6 ($n = 3$). (**E**) The effects of LatA on osmostress-induced Hog1 phosphorylation were examined in *hkr1*Δ and *msb2*Δ mutant strains and in the parental *ssk2*Δ *ssk22*Δ host strain. Cells were treated with 100 μM LatA (+) or with the vehicle dimethyl sulfoxide (DMSO) alone (–) for 30 min and with 0.4 M NaCl (+) or without (–) for further 5 min. Data are representative of two experiments. In (C) to (E), all strains contained the *ssk2*Δ *ssk22*Δ (*ssk2/22*Δ) double mutation to inactivate the Sln1 branch.

(*11*). However, we found that Ste20 was essential for osmoresistance of *HKR1*$^+$ *msb2*Δ host cells, in which Hkr1 is the only functional osmosensor, and that *ste20*Δ *msb2*Δ mutant cells failed to grow on high-osmolarity media (Fig. 1C). Consistent with the failure to grow under high-osmolarity conditions, the *ste20*Δ *msb2*Δ mutant could only weakly induce the Hog1-dependent reporter gene *8xCRE-lacZ* under hyperosmotic conditions (Fig. 1D). In contrast, in the *hkr1*Δ *MSB2*$^+$ host cells, where Msb2 is the only functional osmosensor, both *ste20*Δ *hkr1*Δ and *cla4*Δ *hkr1*Δ were osmoresistant and could induce the Hog1 reporter gene (Fig. 1, C and D). Thus, we found that when osmostress signaling is initiated by the Hkr1 osmosensor, only Ste20 can activate Ste11, whereas when osmostress signaling is initiated by the Msb2 osmosensor, either Ste20 or Cla4 can activate Ste11.

We also found that another difference between Hkr1 and Msb2 signaling is the involvement of the actin cytoskeleton. Because the actin cytoskeleton is rapidly reorganized upon exposure of cells to osmostress (*17*), filamentous actin (F-actin) has been implicated in hyperosmotic stress responses. Although it is likely that the actin cytoskeleton is important for protection of cells from mechanical stress caused by hyperosmolarity, it is also possible that actin is involved in osmostress signaling. To test if the actin cytoskeleton is important for osmostress signaling, we preincubated various mutant strains with an inhibitor of actin polymerization, latrunculin A (LatA), and then assayed Hog1 phosphorylation following hyperosmotic stress (Fig. 1E). LatA pretreatment strongly suppressed Hog1 phosphorylation in *hkr1*Δ *MSB2*$^+$ cells, but not in *HKR1*$^+$ *msb2*Δ cells. This result showed that the actin cytoskeleton is indispensable for signaling by the Msb2 osmosensor, but not for signaling by the Hkr1 osmosensor.

Bem1 is necessary for signaling by the Msb2 osmosensor

We hypothesized that there might be additional factors that are required for signaling by one or other of the osmosensors. To identify such factors, we selected candidate proteins on the basis of their published functional or physical interaction(s) with Ste20, Cla4, or both (the candidates tested were Bem1, Bem2, Bem3, Boi1, Boi2, Bud6, Msb3, Msb4, Rga1, Rga2, Sla1, Sla2, Spa2, and Sph1) and deleted the genes that encode those candidate proteins in three strains: an *ssk2/22*Δ parental strain and its *hkr1*Δ and *msb2*Δ derivatives. If a gene (*XYZ*) is only necessary for signaling through Msb2, then *hkr1*Δ *xyz*Δ mutants should be osmosensitive because these mutants should have the same phenotype as *hkr1*Δ *msb2*Δ, whereas *msb2*Δ *xyz*Δ mutants should be osmoresistant because they are phenotypically similar to *msb2*Δ alone. Using these criteria, we found that only Bem1 among the 14 proteins was essential for signaling by the Msb2 osmosensor but not for Hkr1 signaling. Whereas *hkr1*Δ *bem1*Δ was highly osmosensitive, *bem1*Δ and *msb2*Δ *bem1*Δ mutants were osmoresistant (Fig. 2A). Activating phosphorylation of Hog1 following hyperosmotic stress was almost undetectable in the *hkr1*Δ *bem1*Δ mutant cells, whereas in other mutants (*bem1*Δ, *msb2*Δ, *msb2*Δ *bem1*Δ, and *hkr1*Δ), this response was similar to that of the parental (*ssk2/22*Δ) cells (Fig. 2B). Expression of the Hog1-dependent reporter gene *8xCRE-lacZ* followed the same pattern as Hog1 phosphorylation (Fig. 2C).

If Bem1 functions in a signaling pathway that involves Msb2 but not Hkr1, *bem1*Δ mutant cells should not activate Hog1 in the absence of Ste20. Double mutant *ste20*Δ *bem1*Δ cells did not induce the Hog1 reporter gene under osmostress, suggesting that signaling in the absence of Ste20 (that is, when only activation of Ste11 by Cla4 is possible) depended on Bem1, just as it depended on Msb2 (Fig. 2D). Thus, the data indicate that Bem1 and F-actin are required for the osmostress signaling initiated by Msb2, but not for that initiated by Hkr1 (Fig. 2E).

Previously, we showed that Msb2 could signal through Sho1 in two different manners, termed mode 1 and mode 2 (*8*). In mode 1, the cytosolic domain of Msb2 is dispensable, and Sho1 transmembrane domains serve an active role in Hog1 activation; in mode 2, the cytosolic domain of Msb2 is required, but the Sho1 transmembrane domains are necessary only for membrane localization of Sho1 (fig. S1). In contrast, Hkr1 only signals through Sho1 by a mode 1 mechanism. As a result, certain Sho1 transmembrane mutants that are defective in mode 1 signaling, such as Sho1-P63E, can

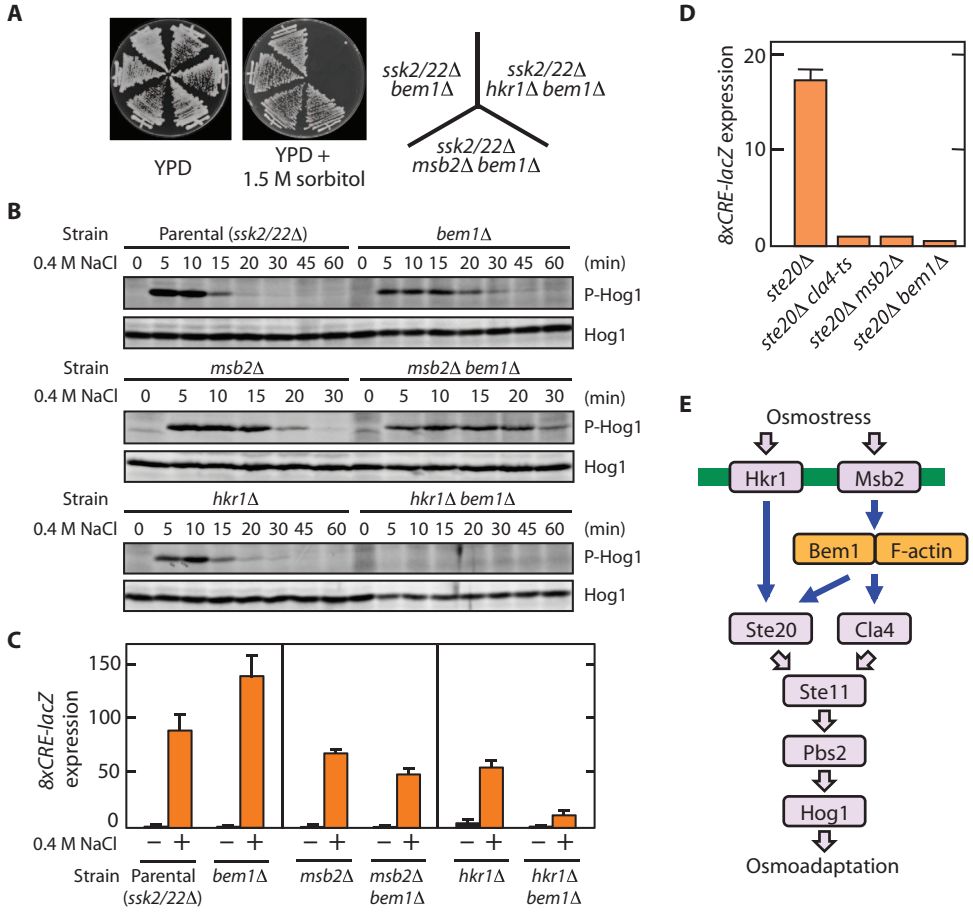

Fig. 2. Bem1 is necessary for signaling by the Msb2 osmosensor. All strains contained the *ssk2*Δ *ssk22*Δ double mutation to inactivate the Sln1 branch. (**A**) Ability of the *bem1*Δ mutant and *msb2*Δ *bem1*Δ and *hkr1*Δ *bem1*Δ double mutants to survive osmotic stress induced by 1.5 M sorbitol. Two independently chosen cells were tested for each genotype. (**B**) Ability of indicated mutant strains to stimulate Hog1 phosphorylation in response to osmotic stress (0.4 M NaCl). Note that the *msb2*Δ and *msb2*Δ *bem1*Δ mutants were analyzed over 30 min, whereas the others were analyzed over 60 min. (**C** and **D**) Ability of indicated mutant strains to stimulate *8xCRE-lacZ* reporter expression in response to osmotic stress. Error bars represent SDs; $n \geq 3$. In (C), the cells were treated with (+) or without (−) 0.4 M NaCl for 30 min. In (D), the cells were treated with 0.4 M NaCl for 30 min at 30°C, which is nonpermissive to the *cla4-ts* mutant. (**E**) Schematic summary of the signaling flow in the Sho1 branch of the HOG pathway.

support Hog1 activation only in the presence of Msb2, which can support mode 2 signaling (*8*). To further corroborate the idea that Bem1 is specifically required for Msb2 signaling, we tested whether the *bem1*Δ mutant cells could activate Hog1 in *sho1-P63E* mutant cells. As expected, *sho1-P63E bem1*Δ double mutant cells neither activated Hog1 (Fig. 3A) nor induced the Hog1-specific reporter gene (Fig. 3B).

The cytoplasmic domain of Msb2 is required for Bem1 to function in the HOG pathway

To determine what difference between Hkr1 and Msb2 required Msb2 to signal through Bem1, we used the hyperactive ΔSTR mutants of Hkr1 and Msb2 in which we deleted the inhibitory STR domain (Fig. 3C). In wild-type ($BEM1^+$) cells, expression of either Hkr1ΔSTR or Msb2ΔSTR robustly induced the Hog1-specific reporter, whereas in the *bem1*Δ mutant host, only Hkr1ΔSTR induced the reporter, further indicating that Msb2 requires Bem1 for signaling (Fig. 3D). When the cytoplasmic domains of these two hyperactive constructs were swapped, only the construct that contained the Hkr1 cytoplasmic domain induced the reporter in the absence of Bem1, indicating that the Msb2 cytoplasmic domain mediated the requirement of Bem1 for HOG pathway signaling activated by Msb2.

When expressed in wild-type yeast, a glutathione *S*-transferase (GST) fusion with the cytoplasmic domain of Msb2 interacted with a hemagglutinin (HA)–tagged Bem1 (Fig. 3E). This interaction was constitutive and was not affected by exposing the yeast to osmostress. To examine the role of this interaction in Msb2 signaling, we constructed an Msb2-Bem1 fusion construct (Fig. 4A). Msb2ΔSTRΔC20, in which the C-terminal 20 amino acids and the STR domain were deleted, did not induce the Hog1 reporter (Fig. 4B). This defect was completely suppressed by fusing Bem1 to the C terminus of Msb2ΔSTRΔC20, and this fusion construct, Msb2ΔSTRΔC20-Bem1, induced the Hog1 reporter in *bem1*Δ cells (Fig. 4B). Thus, a likely function of the Msb2 cytoplasmic domain is to recruit Bem1 to the site of the osmosensor action.

Bem1 recruits Ste20/Cla4 to Msb2

Bem1 is a scaffold protein involved in morphogenesis and establishment of cell polarity (*18*). By forming a complex with Ste20 (or Cla4), Cdc42, and Cdc24, Bem1 promotes activation of Ste20/Cla4 (*16*, *19*) (Fig. 4C). To define the function of Bem1 in the HOG pathway, we deleted several domains of Bem1 (Fig. 4D). By expressing these Bem1 deletion constructs in *bem1*Δ yeast also expressing the hyperactive Msb2ΔSTR, we found that both the SH3-2 domain, which binds to Ste20/Cla4 (*20*), and the CI domain, which interacts with Cdc42 (*21*), were essential for induction of the Hog1-specific reporter (Fig. 4E). To further confirm this observation, we constructed a Ste20 mutant in which the two Pro residues of the PXXP motif that binds Bem1 were mutated to Ala, which we called Ste20-PP(B)AA. Compared to Ste20 wild-type cells, Ste20-PP(B)AA mutant cells induced less Hog1 reporter expression by Msb2ΔSTR (Fig. 4F). Because this modest Msb2 signaling in the Ste20-PP(B)AA cells could be due to indirect interaction between Bem1 and Ste20 through Cdc42 (Fig. 4C), we examined whether deletion of the CRIB domain of Ste20, which binds Cdc42 (*22*), had any additional effects. Deletion of the Ste20 CRIB domain alone strongly reduced the ability of Ste20 to stimulate the Hog1 reporter by Msb2ΔSTR (Fig. 4F), which is consistent with previous reports (*22*). This residual reporter expression was completely abolished when Ste20ΔCRIB also had the PP(B)AA mutation. Similar additive effects between ΔCRIB and another mutation at the Bem1-binding PXXP site have been observed for the mating pathway, as well as the osmostress signaling pathway (*20*). In contrast

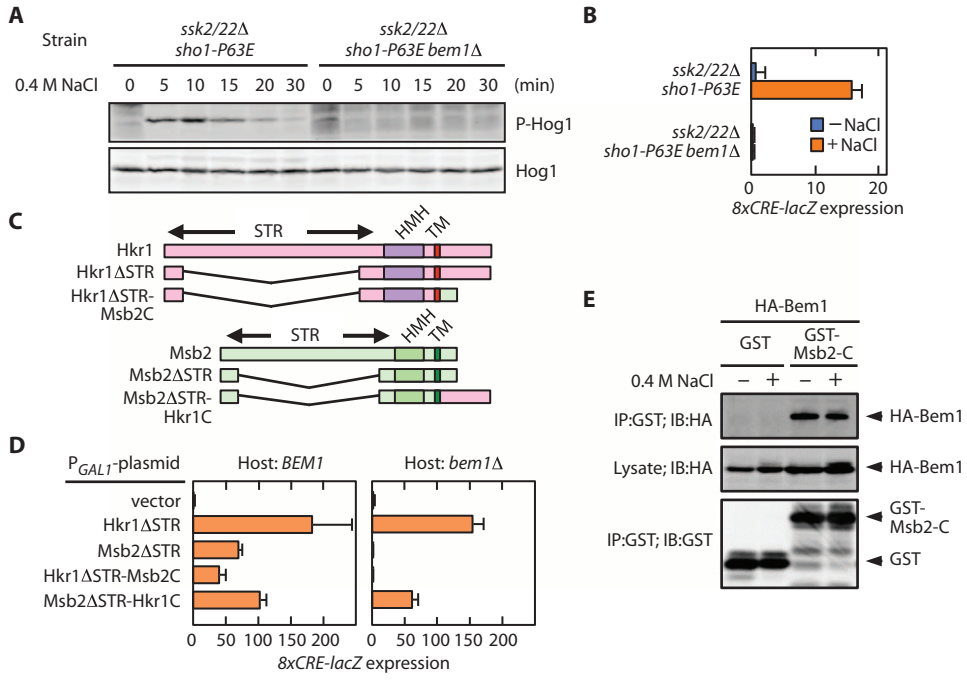

Fig. 3. The cytoplasmic domain of Msb2 is required for Bem1 to function in the HOG pathway. (**A**) Hog1 phosphorylation in response to osmotic stress in the indicated mutant strains. Sho1-P63E is a mutant that requires Msb2 for Hog1 activation (*8*). (**B**) Induction of the *8xCRE-lacZ* reporter in the indicated mutant strains in response to treatment with 0.4 M NaCl for 30 min. Error bars represent SDs; $n \geq 3$. (**C**) Schematic diagrams of the Hkr1-Msb2 chimeric constructs. The Hkr1-derived segments are colored in shades of red, whereas the Msb2-derived segments are colored in shades of green. (**D**) Induction of the *8xCRE-lacZ* reporter gene by overexpression of constitutively active Hkr1 and Msb2 mutants. Single-copy plasmids that express the indicated Hkr1 and Msb2 constructs from the inducible *GAL1* promoter (p414GAL1-Hkr1ΔSTR, p414GAL1-Msb2ΔSTR, and their derivatives) were individually transformed, together with the reporter plasmid pRS413-8xCRE-lacZ, into QG158 (*BEM1*$^+$) or into TA158 (*bem1*Δ). Expression of the Hkr1 and Msb2 constructs was induced by 2% galactose for 2 hours. Error bars represent SDs; $n \geq 3$. (**E**) Coimmunoprecipitation of Bem1 with the Msb2 cytoplasmic domain. Wild-type (WT) strain TM141 was cotransformed with the expression vectors for HA-Bem1 and GST-Msb2-C (or GST alone), and expression was induced by 2% galactose for 2 hours. Immediately before preparation of cell lysates, cells were treated with 0.4 M NaCl (+) or without (−) for 5 min. Data are representative of three experiments.

to Msb2ΔSTR, induction of the Hog1-specific reporter by Hkr1ΔSTR was not significantly affected by the Ste20 PP(B)AA mutation, consistent with our earlier observation that Hkr1 signaling did not require Bem1. However, the Ste20 mutant that could not bind to Cdc42 showed reduced activation of the Hog1 reporter by Hkr1ΔSTR, consistent with previous reports (*22*). In summary, the data indicate that binding of Bem1 to Ste20 is important for the Msb2 signaling, and we propose that Bem1 may tether Ste20/Cla4 (activated by Cdc42) to the membrane-bound Msb2.

When wild-type cells are exposed to hyperosmotic stress, only the MAPK Hog1 is robustly activated (*23*), whereas only a weak and transient activation of the MAPK Kss1 is observed (*24*). In contrast, in *hog1*Δ or *pbs2*Δ mutant cells, in which Hog1 cannot be activated, the Kss1 MAPK is strongly activated by osmostress (*25*). This crosstalk activation of Kss1 by osmostress depends on Msb2 but not on Hkr1 (*8*). Thus, we tested whether Bem1 is required for crosstalk activation of Kss1, by monitoring the specific reporter gene *FUS1-lacZ* (*25*). Indeed, consistent with the specific role of Msb2 in the crosstalk, induction of *FUS1-lacZ* expression by osmostress required Bem1 and, in particular, the SH3-2 and CI domains (fig. S2).

The Hkr1 cytoplasmic domain is involved in activation of Ste11 by Ste20

To find the functions of the cytoplasmic domain of Hkr1, we generated a series of mutants that lack parts of the Hkr1 cytoplasmic domain (Fig. 5A). An essential step in Ste11 activation is the recruitment of Ste20 to Ste11 at the plasma membrane. We postulated that a function of Hkr1 is to recruit Ste20 to the membrane and bring it into contact with Ste11, similar to how Msb2 binds Bem1 to recruit the Ste11-activating complex to the membrane. As previously shown (*8*), expression of the hyperactive Hkr1ΔSTR in wild-type cells induced the Hog1-specific reporter (Fig. 5B). This induction was abolished when either of two short segments, namely, 1592–1640 (ΔC5) and 1744–1802 (ΔC8), or the longer segment, 1534–1644 (ΔC3), was deleted in the Hkr1 cytoplasmic domain. Deletion of 1533–1591 (ΔC4) also reduced the induction moderately, perhaps due to an indirect effect on the C5 region.

Because the hyperactive Ste11-Q301P mutant mimics the effect of its phosphorylation by Ste20, cells that express Ste11-Q301P (or similar hyperactive Ste11 mutants) do not require Ste20 for Hog1 activation (*11*, *26*). However, such cells still require osmostress to activate Hog1 because osmostress is required not only for activation of Ste11 by Ste20 but also for the later step of activation of Pbs2 by Ste11 (*11*, *26*). In contrast to the results using Ste11 wild-type cells, the defects of the Hkr1 cytoplasmic deletion mutants were completely suppressed in cells that expressed Ste11-Q301P at physiological amounts (Fig. 5B), indicating that the Hkr1 cytoplasmic region is required only for activation of Ste11 by Ste20.

If the function of Hkr1 is to recruit Ste20 to the site of Ste11, and this event is defective for the Hkr1ΔC5 and ΔC8 mutants, then their defects might be alleviated by fusing Ste20 to Hkr1 (Fig. 5C). Indeed, both Hkr1ΔSTRΔC5-Ste20 and Hkr1ΔSTRΔC8-Ste20 induced the Hog1 reporter (Fig. 5D), suggesting that a role of the Hkr1 cytoplasmic domain is to tether Ste20 at the membrane. However, neither Hkr1ΔSTRΔC5-Ste20 nor Hkr1ΔSTRΔC8-Ste20 produced a response as strong as that of the Hkr1ΔSTR-Ste20 fusion protein, suggesting that there are additional functions of the cytoplasmic domain than binding Ste20 or that Ste20 is not optimally oriented in the context of this fusion protein. Because we could not obtain any evidence for direct binding between Hkr1 and Ste20, there is a possibility that a scaffold protein mediates an interaction between Hkr1 and Ste20.

Activated Ste20 binds to the Sho1 SH3 domain

Deletion of the entire cytoplasmic domain from Hkr1 reduces, but does not completely abrogate, the activation of Hog1 in response to hyperosmotic stimuli (*8*). Indeed, even in the absence of both Hkr1 and Msb2, overexpression

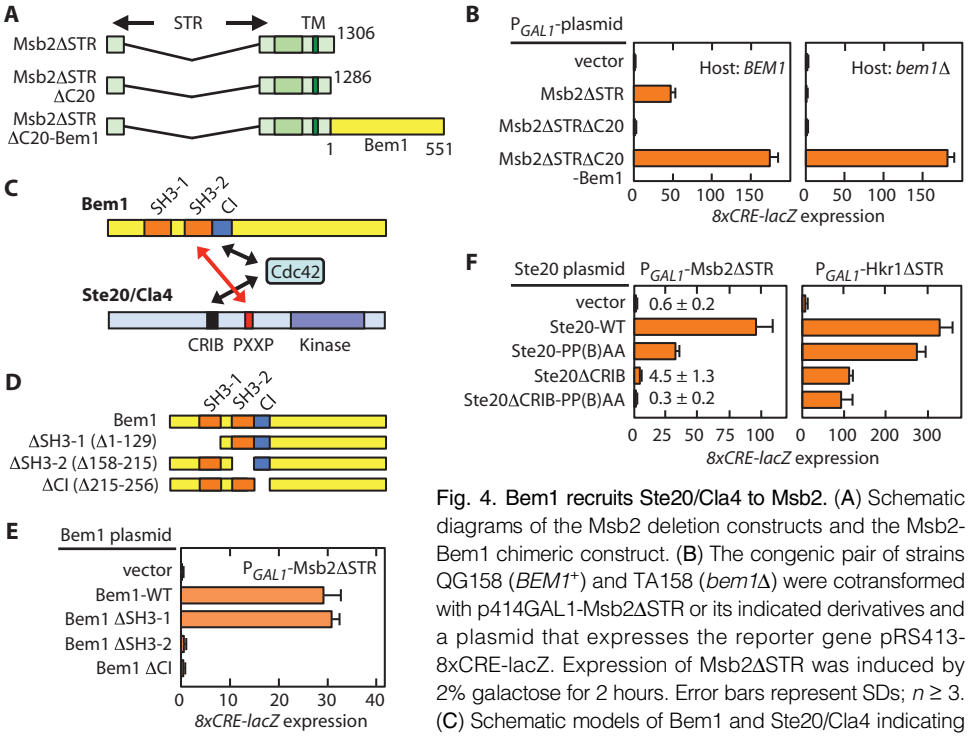

Fig. 4. Bem1 recruits Ste20/Cla4 to Msb2. (**A**) Schematic diagrams of the Msb2 deletion constructs and the Msb2-Bem1 chimeric construct. (**B**) The congenic pair of strains QG158 (*BEM1*$^+$) and TA158 (*bem1*Δ) were cotransformed with p414GAL1-Msb2ΔSTR or its indicated derivatives and a plasmid that expresses the reporter gene pRS413-8xCRE-lacZ. Expression of Msb2ΔSTR was induced by 2% galactose for 2 hours. Error bars represent SDs; $n \geq 3$. (**C**) Schematic models of Bem1 and Ste20/Cla4 indicating their mutual interaction with each other and their individual interactions with Cdc42. (**D**) Schematic diagrams of the Bem1 deletion constructs. (**E**) Activation of the Hog1 reporter gene by constitutively active Msb2ΔSTR in the presence of the indicated Bem1 mutants. The host strain TA158 (*bem1*Δ) was cotransformed with p413GAL1-Msb2ΔSTR, the reporter plasmid pRS415-8xCRE-lacZ, and the indicated derivatives of pRS414-Bem1. That these Bem1 mutants were expressed at the comparable levels as Bem1-WT was confirmed by a separate experiment (fig. S5). Expression of Msb2ΔSTR was induced by 2% galactose for 2 hours. (**F**) Activation of the Hog1 reporter gene by constitutively active Msb2ΔSTR or constitutively active Hkr1ΔSTR in the presence of the indicated Ste20 mutants. KY496 (*ste20*Δ) was cotransformed with p414GAL1-Msb2ΔSTR or p414GAL1-Hkr1ΔSTR, the HOG reporter plasmid pRS416-8xCRE-lacZ, and the indicated derivatives of pRS413-Ste20. Expression of Msb2ΔSTR or Hkr1ΔSTR was induced by 2% galactose for 2 hours. In (E) and (F), error bars represent SDs; $n \geq 3$.

of a hyperactive allele of Sho1 (P120L) activates Hog1 (*8*). Our results with the cytoplasmic deletion mutants of Hkr1ΔSTR, which activated the Hog1 reporter, although some very weakly (Fig. 5B), also suggest that there is another mechanism by which Ste20-Ste11 interaction is mediated that does not involve the Hkr1 cytoplasmic domain. We therefore analyzed the Ste20 sequence for a clue to this mechanism and discovered a previously unreported PXXP motif at amino acid positions 535 to 541 (PLPPIPP) (Fig. 6A). We refer to this site as PP(S) (that is, the PP site for Sho1), whereas the other PXXP motif at amino acid positions 472 to 480 (PSRPAPKPP) is referred to as PP(B) (that is, the PP site for Bem1). Because PP(S) is very similar to the PXXP motif (PLPPLPV) in Pbs2, which binds to the Sho1 SH3 domain (*2*), we examined if Ste20 bound Sho1. When expressed in wild-type yeast, a GST fusion with Sho1 interacted with an HA-tagged C-terminal half of Ste20 (amino acid positions 430 to 939), which contains both PP(B) and PP(S) (Fig. 6B). Ste20-Sho1 binding depended on the Ste20 PP(S) site because a PP(S)AA mutant, in which two Pro residues (underlined) in the PLPPLP sequence were mutated to Ala (Fig. 6A), did not bind to Sho1, whereas the PP(B)AA mutant did (Fig. 6C, left). Ste20-Sho1 binding also depended on the Sho1 SH3 domain because the Sho1 W338F mutant, which has a mutation at a highly conserved tryptophan that is involved in binding to Pro-rich ligands (*27*), did not bind to the Ste20 C-terminal peptide. For comparison, we also examined the Ste20-Bem1 interaction (*20*). Bem1 did not bind to Ste20-PP(B)AA, but bound to Ste20-PP(S)AA, indicating that Bem1 only binds to the Ste20 PP(B) site (Fig. 6C, right). Ste20-Bem1 binding depended on the second SH3 domain (SH3-2) in Bem1 because Bem1 W192F, which has an SH3-2 domain mutation, did not bind to Ste20.

Full-length Ste20 (Ste20-FL) did not bind to Sho1 (Fig. 6B). However, coexpression of the constitutively active Cdc42 G12V mutant (Cdc42-G/V) promoted an interaction between Sho1 and Ste20-FL that was stronger after a longer galactose induction period, suggesting that there may be a concentration-dependent effect of Cdc42 on the interaction (Fig. 6D). We propose that binding of Cdc42-G/V to the Ste20 CRIB domain induces a conformational change in Ste20 (*28*, *29*) that makes the PP(S) site accessible to the Sho1 SH3 domain (Fig. 6E).

Ste20-Sho1 binding contributes to Hog1 activation by Hkr1

We then examined the extent of the contributions of Ste20-Bem1 binding and Ste20-Sho1 binding to Hog1 activation. Ste20-Bem1 binding was important when activation of Hog1 was induced by Msb2ΔSTR because Hog1 reporter gene activation was substantially inhibited by Ste20 PP(B)AA mutation, but not by Ste20 PP(S)AA mutation (Fig. 7A, left). A double mutant (Ste20-4PA), which has both the PP(B)AA and PP(S)AA mutations, inhibited Hog1 activation to a similar extent as did the PP(B)AA mutant alone. This finding is consistent with the role of Bem1 as a scaffold that tethers both Msb2 and Ste20 (Fig. 7B). In contrast, disruption of the Ste20-Sho1 or Ste20-Bem1 binding had no apparent effect on Hog1 reporter gene activation when it was induced by expression of Hkr1ΔSTR (Fig. 7A, middle). However, activation of Hog1 by Hkr1ΔSTRΔC3 was inhibited by Ste20 PP(S)AA or by Ste20 4PA, which are defective in the interaction with Sho1, whereas Ste20 PP(B)AA mutation, which disrupts the binding between Ste20 and Bem1, had no effect (Fig. 7A, right). Thus, we concluded that Ste20-Sho1 binding contributed, although weakly, to Hog1 activation by the Hkr1 osmosensor. In the presence of the signaling mechanism that involves the Hkr1 cytoplasmic domain (Fig. 7C), this activity is not easily discerned. However, when the Hkr1 cytoplasmic domain contains a disabling deletion (such as in Hkr1ΔSTRΔC3), the signaling mechanism involving Ste20-Sho1 interaction becomes detectable (Fig. 7D). Although the Hkr1 cytoplasmic domain is dispensable for this activation mechanism, we should note that the Hkr1 extracellular domain is still required because deletion of the extracellular HMH domain rendered the constitutively active Hkr1ΔSTR inactive (fig. S3).

The contribution of the Sho1-Ste20 interaction to a normal hyperosmotic stimulus was not readily detectable in *HKR1*$^+$ *MSB2*$^+$ cells (fig. S4A). It

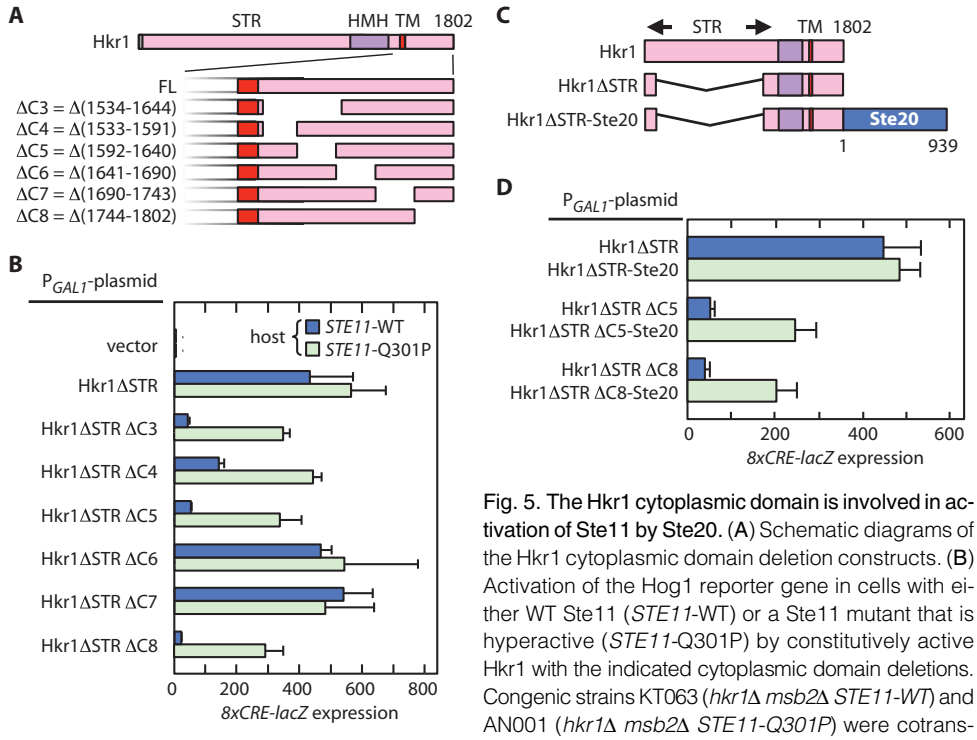

Fig. 5. The Hkr1 cytoplasmic domain is involved in activation of Ste11 by Ste20. (**A**) Schematic diagrams of the Hkr1 cytoplasmic domain deletion constructs. (**B**) Activation of the Hog1 reporter gene in cells with either WT Ste11 (*STE11*-WT) or a Ste11 mutant that is hyperactive (*STE11*-Q301P) by constitutively active Hkr1 with the indicated cytoplasmic domain deletions. Congenic strains KT063 (*hkr1*Δ *msb2*Δ *STE11-WT*) and AN001 (*hkr1*Δ *msb2*Δ *STE11-Q301P*) were cotransformed with p416GAL1-Hkr1ΔSTR (or its cytoplasmic deletion mutants as indicated) and the reporter plasmid pRS414-8xCRE-lacZ. Expression of Hkr1ΔSTR was induced by 2% galactose for 2 hours. (**C**) Schematic diagrams of the Hkr1-Ste20 chimeric. (**D**) Activation of the Hog1 reporter gene in cells expressing the indicated Hkr1 cytoplasmic deletion strains or the indicated Ste20-Hkr1 fusion proteins. KT063 (*hkr1*Δ *msb2*Δ) was cotransformed with p416GAL1-Hkr1ΔSTR or p416GAL1-Hkr1ΔSTR-Ste20 (or the indicated cytoplasmic deletion derivatives thereof) and pRS414-8xCRE-lacZ. Expression of Hkr1ΔSTR was induced by 2% galactose for 2 hours.

becomes detectable when other major mechanisms are inactivated by mutations. For example, in *hkr1*Δ *bem1*Δ *ste20*Δ mutant cells, introduction of Ste20 allows weak activation of the Hog1 MAPK by hyperosmotic stimulus, and this activation was significantly less when Ste20-PP(S)AA or Ste20-4PA was introduced (fig. S4B). In contrast, introduction of Ste20-PP(B)AA produced Hog1 reporter activity similar to that of wild-type Ste20. Thus, the Sho1-Ste20 interaction contributed, albeit moderately, to the response to normal hyperosmotic stimulus (fig. S4B).

DISCUSSION

Specific involvement of the Bem1 scaffold protein in Msb2 signaling

We found that Bem1 constitutively interacted with the Msb2 cytoplasmic domain and mediated the signaling between Msb2 and Ste20/Cla4, and that this was specific for Msb2 and not Hkr1 in the activation of the HOG pathway. That Msb2 can activate Hog1 through either Ste20 or Cla4 is consistent with the fact that Bem1 binds to either Ste20 or Cla4 (*14*, *20*, *30*).

Previously, we showed that Sho1 could signal in two different manners, termed mode 1 and mode 2 (fig. S1). The mode 2 mechanism is dependent solely on Msb2, whereas either Hkr1 or Msb2 could mediate the mode 1 mechanism (*8*). Here, we demonstrated that the mode 2 mechanism also depended on Bem1 (Fig. 3, A and B), which may explain why Hkr1 cannot signal through the mode 2 mechanism.

The Sho1 branch involves not only the osmosensors Hkr1 and Msb2 but also the transmembrane protein Opy2 (*31*, *32*). The Opy2 cytoplasmic domain is bound to the MAPKKK Ste11 through the adaptor protein Ste50 (*31*–*34*). We postulate that the interaction between the extracellular domain of Hkr1 or Msb2 and that of Opy2 brings the MAPKKK Ste11 (bound to Opy2 through Ste50) into close proximity to the MAP4Ks Ste20 or Cla4 (by Msb2 through Bem1 or by Hkr1 through an unknown mediator) and thus promotes Ste11 activation (Fig. 7, B and C).

Specific involvement of the actin cytoskeleton in Msb2 signaling

Another difference between the two osmosensors is that Msb2 signaling, but not Hkr1 signaling, is dependent on F-actin. F-actin forms actin cables in the cytoplasm, actin rings at the bud necks, and actin patches at the site of polarized growth. Although the role of F-actin in Msb2 signaling is unclear, it might be related to the ability of Bem1 to bind to actin (*13*). At the site of polarized cell growth, activated Cdc42 initiates formation of molecular assembly that incorporates Bem1 (scaffold), Ste20 (Cdc42 target), Cdc24 (GEF for Cdc42), and Cdc42 (*35*, *36*). Because Sho1 also concentrates at the site of polarized growth as well as at the bud neck (*37*), F-actin might regulate the interaction and subsequent activities of the kinases involved in Msb2 signaling. A similar role of Bem1 in the mating pheromone signaling has been suggested (*38*).

Specific interaction between the Sho1 SH3 domain and a Ste20 PXXP motif

The Sho1 SH3 domain of some yeast, including *S. cerevisiae*, binds to the MAPKK Pbs2. However, the Sho1 SH3 domain may have a function unrelated to its binding to Pbs2 because in some fungal species, for example, *Aspergillus nidulans*, Pbs2 orthologs do not contain the Sho1-binding site, although these fungi have Sho1 orthologs (*39*). We identified a previously unrecognized SH3-binding motif (PLPPIP) in Ste20 that bound Sho1. Furthermore, the Sho1 SH3 domain interacts with Ste50 in a manner that does not involve a PXXP motif (*11*), which suggests that Sho1 could potentially bind to Ste50 and Ste20 (or Pbs2) simultaneously. Because Ste50 is constitutively bound to Ste11 (*33*, *34*), we can infer that the interaction between Sho1 and Ste50 will indirectly tether Ste20 and Ste11, and the interaction of Sho1 with Pbs2 and with Ste50 will also tether Ste11 and Pbs2 (Fig. 7D). These interactions, if occurred concurrently, will activate the Hog1 by bringing the consecutive kinases in the cascade together. Although the Hkr1 cytoplasmic domain is dispensable for this minor activation mechanism, the Hkr1 extracellular domain is required. Perhaps an interaction between the extracellular domains of Hkr1 and Opy2 enhances or stabilizes the binding between Sho1 and the Opy2/Ste50/Ste11 complex.

A survey of a fungal genome database revealed that, among the putative Ste20 orthologs, those of *Kluyveromyces* and *Zygosaccharomyces* species

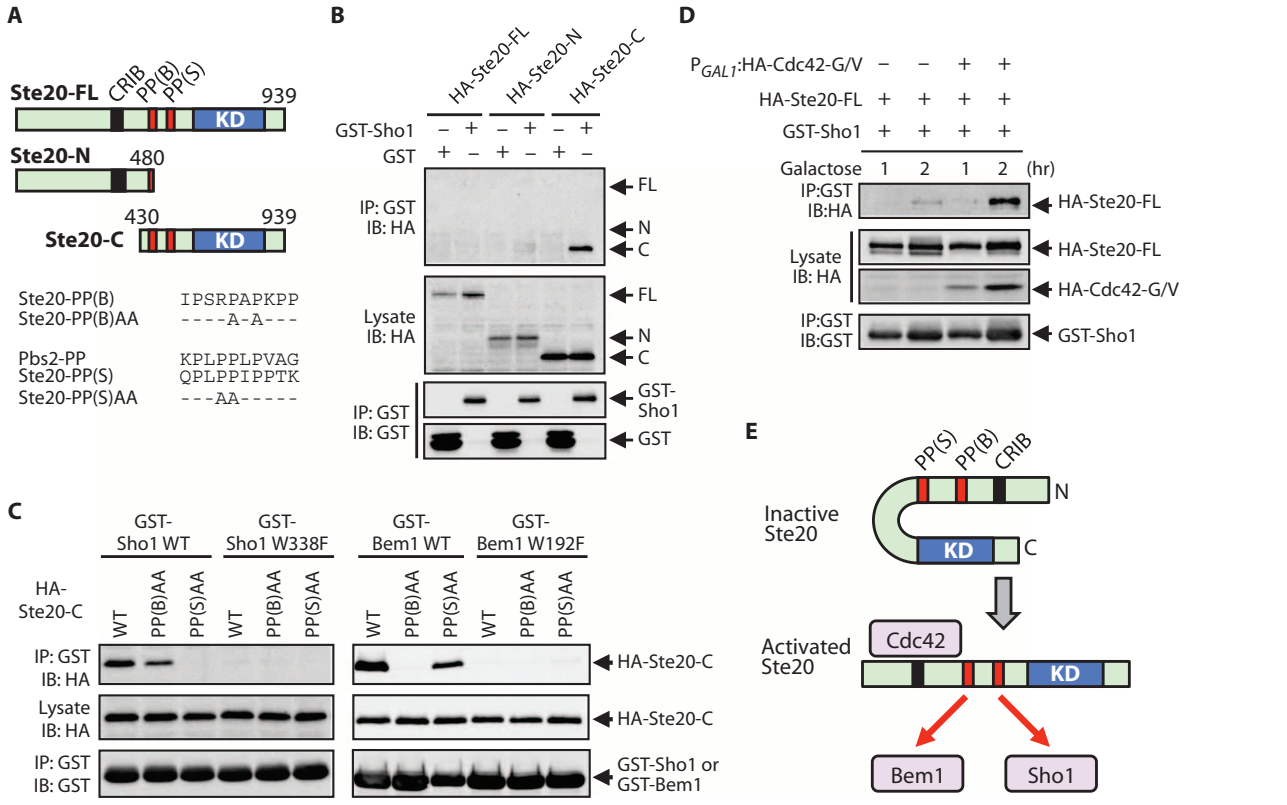

Fig. 6. Activated Ste20 binds to the Sho1 SH3 domain. (**A**) Schematic diagram of full-length Ste20 (Ste20-FL) and the N- and C-terminal truncation mutants (top), and the amino acid sequences of the Pro-rich motifs (bottom). CRIB, Cdc42/Rac interactive binding site; PP(B), a PXXP motif for Bem1; PP(S), a PXXP motif for Sho1; KD, kinase domain; FL, full length. (**B**) Coimmunoprecipitation of the indicated Ste20 proteins with Sho1. TM257 (*ssk2*Δ *ssk22*Δ) was cotransformed with pHA-Ste20 (or its indicated derivative) and pGST-Sho1 (or the control vector for GST alone). Expression of HA-Ste20 and GST-Sho1 was induced by 2% galactose for 2 hours before preparation of cell lysates for immunoprecipitation (IP). Coprecipitated protein was detected by immunoblotting (IB). (**C**) Coimmunoprecipitation of Sho1 with Ste20-PP(S) and of Bem1 with Ste20-PP(B). TM257 was cotransformed with pHA-Ste20-C and either pGST-Sho1 or pGST-Bem1 (or their derivatives as indicated). Expression of the encoded proteins was induced by 2% galactose for 2 hours before preparation of cell lysates. (**D**) Coimmunoprecipitation of full-length Ste20 with Sho1 in the presence of constitutively active Cdc42 (Cdc42-G/V). TM257 was cotransformed with pHA-Ste20-FL, pGST-Sho1, and pHA-Cdc42-G12V (G/V) as indicated. Expression of the encoded proteins was induced by 2% galactose for 1 or 2 hours as indicated. (**E**) Schematic model of the activation of Ste20 by Cdc42. Binding of Cdc42 to the CRIB domain of Ste20 induces a change in the Ste20 conformation, which allows an access of Sho1 to the PP(S) site in Ste20.

have clear Sho1-binding sequences, PLPPLP and PLPPIP, respectively. Because these species also have an Hkr1 ortholog, it is likely that Ste20-Sho1 interaction contributes to Hog1 activation in these species as well.

Functional differences between the Hkr1 and Msb2 osmosensors

In contrast to the similarity in the extracellular domains of Hkr1 and Msb2, their cytoplasmic domains are different. This difference is reflected in their functional distinctions. In addition to its involvement in the HOG pathway, Msb2 is also the most upstream signaling element in the filamentous growth (FG) MAPK signaling pathway, which activates the Ste20-Ste11-Ste7-Kss1 MAPK cascade in response to poor nutritional conditions (*5*). In contrast, Hkr1 is not involved in the FG pathway (*40*). Another, and perhaps related, difference between Hkr1 and Msb2 is their roles in the signaling crosstalk between the Hog1 MAPK pathway and the Kss1 MAPK pathway. Crosstalk occurs only when Ste11 is activated by Msb2 signaling, but not by Hkr1 signaling (*8*). Consistent with this specificity, here, we report that the crosstalk also required Bem1. Thus, crosstalk is not a leakage (or failure of insulation) of signal from osmostress-activated Ste11 to Ste7, as often interpreted, but is a more specific redirection of the signal emanated from the Msb2 osmosensor and transduced through Bem1. In spite of extensive searching, the phosphorylation target of Hog1 that is necessary to block crosstalk activation of Kss1 is unknown (*41*, *42*). In this context, our results indicate that such a target should be sought in the part of the HOG pathway that is specific to the signaling by Msb2 and Bem1 and is not shared by the signaling by Hkr1.

Concluding remarks

In this report, we uncovered the previously unsuspected complexity in the signaling machinery that mediates a seemingly simple input-output relationship, namely, activation of the MAPK Hog1 in response to hyperosmolarity. Although we have no clear explanation for the existence of multiple parallel signaling mechanisms, it is entertaining to speculate that each signaling mechanism is differentially regulated or integrates different cellular events with the osmostress signal. For example, the Msb2 branch might integrate separate inputs from the osmosensor and from the cytoskeletal components. It should be a fruitful area of investigation in the future.

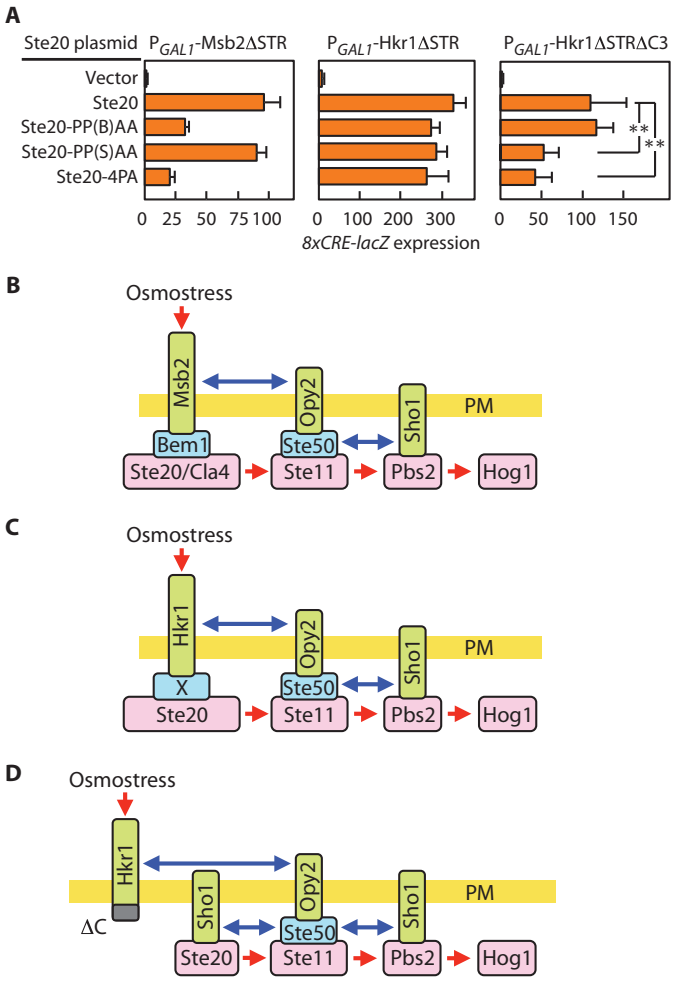

Fig. 7. Ste20-Sho1 binding contributes to Hog1 activation by Hkr1. (A) Activation of the Hog1 reporter gene by the indicated constitutively active mutants of Msb2 or Hkr1 in the presence of the indicated Ste20 mutants. KY496 (*ste20*Δ) (left and middle) and TA187 (*hkr1*Δ *ste20*Δ) (right) were cotransformed with p414GAL1-Msb2ΔSTR (left), p414GAL1-Hkr1ΔSTR (middle), or p414GAL1-Hkr1ΔSTRΔC3 (right), the HOG reporter plasmid pRS416-8xCRE-lacZ, and the indicated derivatives of pRS413-Ste20. Expression of Msb2ΔSTR or Hkr1ΔSTR was induced by 2% galactose for 2 hours. Error bars represent SDs; $n = 3$ (left and middle) and $n = 6$ (right). $^{**}P < 0.01$ (Student's *t* test, two-tailed). (**B** to **D**) Models of Hog1 activation by the Sho1 branch of the HOG pathway. PM, plasma membrane. Red arrows indicate the signal flow, and blue arrows indicate protein-protein interactions. (B) Activation of Hog1 by the Msb2 osmosensor. (C) Major mechanism of Hog1 activation by the Hkr1 osmosensor. X is a hypothetical adaptor protein that tethers Hkr1 to Ste20. (D) Alternative Hog1 activation mechanism that involves interaction between Ste20 and Sho1.

MATERIALS AND METHODS

Buffers and media

CAD medium consists of 0.67% yeast nitrogen base (Sigma), 2% glucose, 0.5% casamino acid (Sigma), and appropriate supplements [uracil (20 μg/ml) and tryptophan (40 μg/ml)] as needed. CAGal medium is the same as CAD, except containing 2% galactose in place of glucose. SRaf medium consists of 0.67% yeast nitrogen base and 2% raffinose with appropriate yeast synthetic drop-out medium supplement. Buffer A contains 50 mM tris-HCl (pH 7.5), 15 mM EDTA, 15 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, leupeptin (5 μg/ml), 50 mM NaF, 25 mM β-glycerophosphate, 150 mM NaCl, and 0.2% Triton X-100. Buffer Z for β-galactosidase assay contains 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, and 1 mM $MgSO_4$, adjusted to pH 7.0. SDS loading buffer (1×) contains 50 mM tris-HCl (pH 6.8), 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 700 mM 2-mercaptoethanol. Other yeast media, buffers, and standard genetic procedures are as described previously (*43*, *44*).

Reagents

LatA was obtained from Sigma and was dissolved in DMSO at 25 mg/ml to make a stock solution.

Yeast strains

All yeast mutants used in this work were derivatives of the S288C strain (table S1). Gene disruption was carried out by a polymerase chain reaction (PCR)–based strategy, and missense and intragenic deletion mutations were created by oligonucleotide-based mutagenesis (*43*).

Plasmid constructs

All mutant plasmid constructs used in this work were generated using PCR-based oligonucleotide mutagenesis and were confirmed by nucleotide sequence determination. Vector plasmids have been described (*45*, *46*). pRS414-Bem1 (= P_{BEM1}-*BEM1*, *TRP1*, *CEN6*) and pRS413-Ste20 (= P_{STE20}-*STE20*, *HIS3*, *CEN6*) are respectively full-length *BEM1* and *STE20* genomic DNA clones. p414GAL1-Hkr1ΔSTR [= P_{GAL1}-*Hkr1Δ(101–1080)*, *TRP1*, *CEN6*], p416GAL1-Hkr1ΔSTR [= P_{GAL1}-*Hkr1Δ(101–1080)*, *URA3*, *CEN6*], p413GAL1-Msb2ΔSTR [= P_{GAL1}-*Msb2Δ(100–818)*, *HIS3*, *CEN6*], and p414GAL1-Msb2ΔSTR [= P_{GAL1}-*Msb2Δ(100–818)*, *TRP1*, *CEN6*] have been described (*8*). In p416GAL1-Hkr1ΔSTR-Ste20, the Ste20 coding sequence was fused to the C terminus of Hkr1ΔSTR. In p414GAL1-Hkr1ΔSTR-Msb2C and p414GAL1-Msb2ΔSTR-Hkr1C, the cytoplasmic domain was swapped between Msb2 and Hkr1. p414GAL1-Msb2ΔSTRΔC20-Bem1 was constructed by fusing the Bem1 coding sequence to the C terminus of Msb2ΔSTRΔC20. pHA-Bem1 (= P_{GAL1}-*HA-Bem1*, *TRP1*, *CEN4*) and pHA-Ste20 (= P_{GAL1}-*HA-Ste20*, *TRP1*, *CEN4*) encode respectively N-terminally HA-tagged Bem1 and Ste20 and are based on the YCpIF16 vector. pHA-Ste20-N and pHA-Ste20-C encode respectively HA-tagged Ste20 (1–480) and Ste20 (430–939). pHA-Cdc42-G12V (= P_{GAL1}-*HA-Cdc42-G12V*, *HIS3*, *CEN6*) is based on pRS413. pGST-Sho1 (= P_{GAL1}-*GST-Sho1*, *URA3*, 2μ) and pGST-Bem1 (= P_{GAL1}-*GST-Bem1*, *URA3*, 2μ) encode respectively N-terminally GST-tagged Sho1 and Bem1 and are based on the p426GAG vector. pGST-Msb2-C (= P_{TEF2}-*GST-Msb2-C*, *URA3*, 2μ) encodes the GST protein fused to the cytoplasmic domain of Msb2 (amino acids 1216 to 1306) and is based on p426TEG.

Reporter assays

Reporter assays using the HOG reporter plasmid pRS413-8xCRE-lacZ (= *8xCRE-lacZ*, *HIS3*, *CEN6*) or its derivatives with different selective markers have been described (*11*). Throughout the figures, *8xCRE-lacZ* expression is presented as average and SDs of three or more independent samples and is expressed in Miller units (*47*).

In vivo binding assay

Cell extracts were prepared in buffer A using glass beads, essentially as described previously (*43*). To immunoprecipitate GST-tagged proteins, a 750-μg aliquot of protein extract was incubated with 50 μl of glutathione-

Sepharose beads for 2 hours at 4°C. To immunoprecipitate HA- or GST-tagged proteins, a 750-μg aliquot of protein extract was first incubated with an appropriate antibody for 2 hours at 4°C, followed by further incubation with 50 μl of protein G beads for 2 hours at 4°C. In all cases, beads were washed three times in buffer A, resuspended in SDS loading buffer, boiled for 5 min, and separated by SDS-PAGE. Immunoblotting analyses were carried out essentially as described previously (*48*). Enhanced chemiluminescence images were digitally captured using the LAS-1000 Plus (Fujifilm) equipped with a charge-coupled device camera. The following primary antibodies were used in immunoblotting: goat antibody recognizing Hog1 (yC-20, Santa Cruz Biotechnology), monoclonal antibody recognizing GST (B-14, Santa Cruz Biotechnology), monoclonal antibodies recognizing HA (12CA5 and 3F10, Roche), and antibody recognizing phosphorylated p38 (Cell Signaling), which was used to detect phosphorylated Hog1.

Other methods

Other methods including standard genetic procedures were as described previously (*43*–*46*).

SUPPLEMENTARY MATERIALS

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Fig. S1. Mode 1 and mode 2 signaling mechanisms of Sho1 and the Msb2 osmosensor.

Fig. S2. Crosstalk activation of the MAPK Kss1 by osmostress requires Bem1.

Fig. S3. The extracellular HMH domain is essential for Hog1 activation by the constitutively active Hkr1ΔSTR.

Fig. S4. Ste20-Sho1 binding moderately contributes to Hog1 activation by osmostress.

Fig. S5. Expression of Bem1 deletion constructs.

Table S1. Yeast strains used in this study.

REFERENCES AND NOTES

1. H. Saito, F. Posas, Response to hyperosmotic stress. *Genetics* **192**, 289–318 (2012).
2. T. Maeda, M. Takekawa, H. Saito, Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. *Science* **269**, 554–558 (1995).
3. F. Posas, S. M. Wurgler-Murphy, T. Maeda, E. A. Witten, T. C. Thai, H. Saito, Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1–YPD1–SSK1 "two-component" osmosensor. *Cell* **86**, 865–875 (1996).
4. S. Takahashi, P. M. Pryciak, Identification of novel membrane-binding domains in multiple yeast Cdc42 effectors. *Mol. Biol. Cell* **18**, 4945–4956 (2007).
5. P. J. Cullen, W. Sabbagh Jr., E. Graham, M. M. Irick, E. K. van Olden, C. Neal, J. Delrow, L. Bardwell, G. F. Sprague Jr., A signaling mucin at the head of the Cdc42- and MAPK-dependent filamentous growth pathway in yeast. *Genes Dev.* **18**, 1695–1708 (2004).
6. H.-Y. Yang, K. Tatebayashi, K. Yamamoto, H. Saito, Glycosylation defects activate filamentous growth Kss1 MAPK and inhibit osmoregulatory Hog1 MAPK. *EMBO J.* **28**, 1380–1391 (2009).
7. V. Reiser, D. C. Raitt, H. Saito, Yeast osmosensor Sln1 and plant cytokinin receptor Cre1 respond to changes in turgor pressure. *J. Cell Biol.* **161**, 1035–1040 (2003).
8. K. Tatebayashi, K. Tanaka, H.-Y. Yang, K. Yamamoto, Y. Matsushita, T. Tomida, M. Imai, H. Saito, Transmembrane mucins Hkr1 and Msb2 are putative osmosensors in the SHO1 branch of yeast HOG pathway. *EMBO J.* **26**, 3521–3533 (2007).
9. F. van Drogen, S. M. O'Rourke, V. M. Stucke, M. Jaquenoud, A. M. Neiman, M. Peter, Phosphorylation of the MEKK Ste11p by the PAK-like kinase Ste20p is required for MAP kinase signaling in vivo. *Curr. Biol.* **10**, 630–639 (2000).
10. D. C. Raitt, F. Posas, H. Saito, Yeast Cdc42 GTPase and Ste20 PAK-like kinase regulate Sho1-dependent activation of the Hog1 MAPK pathway. *EMBO J.* **19**, 4623–4631 (2000).
11. K. Tatebayashi, K. Yamamoto, K. Tanaka, T. Tomida, T. Maruoka, E. Kasukawa, H. Saito, Adaptor functions of Cdc42, Ste50, and Sho1 in the yeast osmoregulatory HOG MAPK pathway. *EMBO J.* **25**, 3033–3044 (2006).
12. H.-O. Park, E. Bi, Central roles of small GTPases in the development of cell polarity in yeast and beyond. *Microbiol. Mol. Biol. Rev.* **71**, 48–96 (2007).
13. T. Leeuw, A. Fourest-Lieuvin, C. Wu, J. Chenevert, K. Clark, M. Whiteway, D. Y. Thomas, E. Leberer, Pheromone response in yeast: Association of Bem1p with proteins of the MAP kinase cascade and actin. *Science* **270**, 1210–1213 (1995).
14. I. Bose, J. E. Irazoqui, J. J. Moskow, E. S. G. Bardes, T. R. Zyla, D. J. Lew, Assembly of scaffold-mediated complexes containing Cdc42p, the exchange factor Cdc24p, and the effector Cla4p required for cell cycle-regulated phosphorylation of Cdc24p. *J. Biol. Chem.* **276**, 7176–7186 (2001).
15. T. Takaku, K. Ogura, H. Kumeta, N. Yoshida, F. Inagaki, Solution structure of a novel Cdc42 binding module of Bem1 and its interaction with Ste20 and Cdc42. *J. Biol. Chem.* **285**, 19346–19353 (2010).
16. L. Kozubowski, K. Saito, J. M. Johnson, A. S. Howell, T. R. Zyla, D. J. Lew, Symmetry-breaking polarization driven by a Cdc42p GEF-PAK complex. *Curr. Biol.* **18**, 1719–1726 (2008).
17. J. L. Brewster, M. C. Gustin, Positioning of cell growth and division after osmotic stress requires a MAP kinase pathway. *Yeast* **10**, 425–439 (1994).
18. J. Chenevert, K. Corrado, A. Bender, J. Pringle, I. Herskowitz, A yeast gene (*BEM1*) necessary for cell polarization whose product contains two SH3 domains. *Nature* **356**, 77–79 (1992).
19. J. J. Moskow, A. S. Gladfelter, R. E. Lamson, P. M. Pryciak, D. J. Lew, Role of Cdc42p in pheromone-stimulated signal transduction in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **20**, 7559–7571 (2000).
20. M. J. Winters, P. M. Pryciak, Interaction with the SH3 domain protein Bem1 regulates signaling by the *Saccharomyces cerevisiae* p21-activated kinase Ste20. *Mol. Cell. Biol.* **25**, 2177–2190 (2005).
21. Y. Yamaguchi, K. Ota, T. Ito, A novel Cdc42-interacting domain of the yeast polarity establishment protein Bem1: Implications for modulation of mating pheromone signaling. *J. Biol. Chem.* **282**, 29–38 (2007).
22. R. E. Lamson, M. J. Winters, P. M. Pryciak, Cdc42 regulation of kinase activity and signaling by the yeast p21-activated kinase Ste20. *Mol. Cell. Biol.* **22**, 2939–2951 (2002).
23. F. Posas, H. Saito, Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: Scaffold role of Pbs2p MAPKK. *Science* **276**, 1702–1705 (1997).
24. Y. Wang, A. A. Irqeba, M. Ayalew, K. Suntay, Sumoylation of transcription factor Tec1 regulates signaling of mitogen-activated protein kinase pathways in yeast. *PLOS One* **4**, e7456 (2009).
25. S. M. O'Rourke, I. Herskowitz, The Hog1 MAPK prevents cross talk between the HOG and pheromone response MAPK pathways in *Saccharomyces cerevisiae*. *Genes Dev.* **12**, 2874–2886 (1998).
26. R. E. Lamson, S. Takahashi, M. J. Winters, P. M. Pryciak, Dual role for membrane localization in yeast MAP kinase cascade activation and its contribution to signaling fidelity. *Curr. Biol.* **16**, 618–623 (2006).
27. A. Zarrinpar, S.-H. Park, W. A. Lim, Optimization of specificity in a cellular protein interaction network by negative selection. *Nature* **426**, 676–680 (2003).
28. M. Lei, W. Lu, W. Meng, M.-C. Parrini, M. J. Eck, B. J. Mayer, S. C. Harrison, Structure of PAK1 in an autoinhibited conformation reveals a multistage activation switch. *Cell* **102**, 387–397 (2000).
29. A. Morreale, M. Venkatesan, H. R. Mott, D. Owen, D. Nietlispach, P. N. Lowe, E. D. Laue, Structure of Cdc42 bound to the GTPase binding domain of PAK. *Nat. Struct. Biol.* **7**, 384–388 (2000).
30. M. Gorelik, K. Stanger, A. R. Davidson, A conserved residue in the yeast Bem1p SH3 domain maintains the high level of binding specificity required for function. *J. Biol. Chem.* **286**, 19470–19477 (2011).
31. K. Yamamoto, K. Tatebayashi, K. Tanaka, H. Saito, Dynamic cotrol of yeast MAP kinase network by induced association and dissociation between the Ste50 scaffold and the Opy2 membrane anchor. *Mol. Cell* **40**, 87–98 (2010).
32. I. Ekiel, T. Sulea, G. Jansen, M. Kowalik, O. Minailiuc, J. Cheng, D. Harcus, M. Cygler, M. Whiteway, C. Wu, Binding the atypical RA domain of Ste50p to the unfolded Opy2p cytoplasmic tail is essential for the high-osmolarity glycerol pathway. *Mol. Biol. Cell* **20**, 5117–5126 (2009).
33. C. Wu, E. Leberer, D. Y. Thomas, M. Whiteway, Functional characterization of the interaction of Ste50p with Ste11p MAPKKK in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **10**, 2425–2440 (1999).
34. F. Posas, E. A. Witten, H. Saito, Requirement of STE50 for osmostress-induced activation of the STE11 mitogen-activated protein kinase kinase kinase in the high-osmolarity glycerol response pathway. *Mol. Cell. Biol.* **18**, 5788–5796 (1998).
35. J. B. Moseley, B. L. Goode, The yeast actin cytoskeleton: From cellular function to biochemical mechanism. *Microbiol. Mol. Biol. Rev.* **70**, 605–645 (2006).
36. A. S. Howell, N. S. Savage, S. A. Johnson, I. Bose, A. W. Wagner, T. R. Zyla, H. F. Nijhout, M. C. Reed, A. B. Goryachev, D. J. Lew, Singularity in polarization: Rewiring yeast cells to make two buds. *Cell* **139**, 731–743 (2009).
37. V. Reiser, S. M. Salah, G. Ammerer, Polarized localization of yeast Pbs2 depends on osmostress, the membrane protein Sho1 and Cdc42. *Nat. Cell Biol.* **2**, 620–627 (2000).
38. D. M. Lyons, S. K. Mahanty, K.-Y. Choi, M. Manandhar, E. A. Elion, The SH3-domain protein Bem1 coordinates mitogen-activated protein kinase cascade activation with cell cycle control in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **16**, 4095–4106 (1996).
39. K. Furukawa, Y. Hoshi, T. Maeda, T. Nakajima, K. Abe, *Aspergillus nidulans* HOG pathway is activated only by two-component signalling pathway in response to osmotic stress. *Mol. Microbiol.* **56**, 1246–1261 (2005).

40. A. Pitoniak, B. Birkaya, H. M. Dionne, N. Vadaie, P. J. Cullen, The signaling mucins Msb2 and Hkr1 differentially regulate the filamentation mitogen-activated protein kinase pathway and contribute to a multimodal response. *Mol. Biol. Cell* **20**, 3101–3114 (2009).
41. T. R. Shock, J. Thompson, J. R. Yates III, H. D. Madhani, Hog1 mitogen-activated protein kinase (MAPK) interrupts signal transduction between the Kss1 MAPK and the Tec1 transcription factor to maintain pathway specificity. *Eukaryot. Cell* **8**, 606–616 (2009).
42. P. J. Westfall, J. Thorner, Analysis of mitogen-activated protein kinase signaling specificity in response to hyperosmotic stress: Use of an analog-sensitive *HOG1* allele. *Eukaryot. Cell* **5**, 1215–1228 (2006).
43. K. Tatebayashi, M. Takekawa, H. Saito, A docking site determining specificity of Pbs2 MAPKK for Ssk2/Ssk22 MAPKKKs in the yeast HOG pathway. *EMBO J.* **22**, 3624–3634 (2003).
44. M. D. Rose, J. R. Broach, Cloning genes by complementation in yeast. *Methods Enzymol.* **194**, 195–230 (1991).
45. T. Horie, K. Tatebayashi, R. Yamada, H. Saito, Phosphorylated Ssk1 prevents unphosphorylated Ssk1 from activating the Ssk2 mitogen-activated protein kinase kinase kinase in the yeast high-osmolarity glycerol osmoregulatory pathway. *Mol. Cell. Biol.* **28**, 5172–5183 (2008).
46. Y. Murakami, K. Tatebayashi, H. Saito, Two adjacent docking sites in the yeast Hog1 Mitogen-activated protein (MAP) kinase differentially interact with the Pbs2 MAP kinase kinase and the Ptp2 protein tyrosine phosphatase. *Mol. Cell. Biol.* **28**, 2481–2494 (2008).
47. J. H. Miller, *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1972).
48. M. Takekawa, H. Saito, A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. *Cell* **95**, 521–530 (1998).

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