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Msb2 is a Ste11 membrane concentrator required for full activation of the HOG pathway

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ABSTRACT

The high osmolarity glycerol (HOG) pathway, composed of membrane-associated osmosensors, adaptor proteins and core signaling kinases, is essential for the survival of yeast cells under hyper-osmotic stress. Here, we studied how the MAPKKK Ste11 might change its protein interaction profile during acute stress exposure, with an emphasis on the sensory system of the so-called Sho1/Msb2 signaling branch. To characterize the transience of protein–protein interactions we utilized a recently described enzymatic *in vivo* protein proximity assay (M-track). Accordingly, interaction signals between Ste11 and many of its signaling partners can already be detected even under basal conditions. In most cases these signals increase after stress induction. All the interactions are completely dependent on the function of the Ste11-adaptor protein Ste50. Moreover, the presence of either Msb2 or Hkr1 is necessary for observing the interaction between Ste11 and scaffolding factors such as Sho1 and Pbs2. Additional assays suggest that Msb2 is not only in close proximity to Ste11 but might function as an individual Ste11 concentrator at the plasma membrane. Our results confirm the existence of negative feedback systems targeting the protein levels of Ste11 and Msb2 and also hint at changes in the dissociation rates of intermediate signaling complexes.

1. Introduction

Constant changes of environmental conditions raise the need for a cell to be able to transiently alter signal transduction and protein–protein interactions (PPIs) to adapt to new conditions. The High Osmolarity Glycerol (HOG) pathway of *Saccharomyces cerevisiae* is a well-studied MAP kinase module that has served as a model for various signaling features, including cross-talk between pathways and the use of scaffolding proteins to insulate components of the different MAP kinase cascades [1,2]. The HOG pathway is essential for yeast survival in high osmolarity environments, where the activation of the pathway initiates a number of downstream events that ultimately lead to adaptation through the production of the osmolyte glycerol, reorganization of ion transporters and water efflux, control of cell cycle progression, and regulation of gene expression. The central component of the pathway is the MAPK Hog1, which is directly activated by the MAPKK Pbs2. Two independent and functionally distinct upstream branches activated by hyperosmotic stress converge at Pbs2: the Sln1 branch mediated by the redundant MAPKKKs Ssk2/Ssk22 and the Sho1 branch mediated by the MAPKKK Ste11. Interestingly, Ste11 is the common MAPKKK of two other distinct MAPK cascades: the mating and the filamentous growth pathways. However, activation of Ste11 upon a specific stimulus only induces, in each case, a specific and appropriate adaptive response (reviewed in [1,3–6]).

Activation of the Sho1 branch of the HOG pathway depends on the membrane proteins Msb2 and Hrk1 which seem to act redundantly via slightly different mechanisms [7–10]. Both proteins are characterized by a single-path transmembrane (TM) domain with highly glycosylated extracellular domains that have been shown to have inhibitory functions in HOG signaling [9,11]. Msb2 and Hrk1 have been proposed to activate the PAK-like redundant kinases Ste20 and Cla4 in response to increases in osmolarity [12]. A recent study has shown that Msb2, but not Hkr1, interacts through its cytoplasmic domain with the actin-binding protein Bem1, which in turn recruits the Ste20/Cla4 either of which can activate Ste11 by direct phosphorylation [10]. Together, these observations have led to the conclusion that Msb2 and Hkr1 are genuine osmo-sensors. The Sho1 protein, which has been proposed to function both as a co-osmosensor as well as a Pbs2-scaffolding factor, is characterized by an N-terminal region with four transmembrane segments, a C-terminal cytoplasmic tail containing a linker region involved in Ste11 recruitment, and an SH3 domain. Sho1 localizes Pbs2 to the plasma membrane through a highly specific interaction between its SH3 domain and an N-terminal proline-rich sequence in Pbs2 [13,14]. After stress stimulation, the recruitment of Pbs2 to the membrane

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decreases, which indicates that once activated, Pbs2 dissociates from Sho1 [15].

Several studies have documented a complex functional relationship between Msb2 and Sho1. Msb2 was initially proposed as a third osmo-sensor of the HOG pathway, as an additive deletion of Msb2 in a strain containing mutations in the SLN1 branch and Sho1 decreased dramatically its tolerance to salt stress [7]. Moreover, deletion of Msb2 or Sho1 reduced cross-talk through Ste11, and a double mutation in both genes shows additional reduction in cross-talk, indicating that Msb2 and Sho1 have partially redundant roles in the activation of Ste11 [7]. Msb2 was found to interact with Sho1 [9,11] and genetic studies using hyperactive mutants suggested that Msb2 acts as an upstream element of Sho1 [9]. Deeper analyses of Msb2–Hkr1–Sho1 inter-relationships suggested that they may activate the HOG pathway by two separate modes, involving different Msb2 and Sho1 protein domains [9].

Two other proteins, Ste50 and Opy2, are also necessary for proper signaling through the Sho1 branch of the HOG pathway. Ste50 acts as an adaptor protein that interacts with Ste11 through the SAM domains present in both proteins [16–18]. Opy2 is a single-path transmembrane protein with the suggested essential function in the Sho1 branch of recruiting the Ste50/Ste11 complex to the plasma membrane [19,20]. These upstream elements allow successful Ste11 activation by two major events: the binding of Ste50 to Ste11 N-terminal SAM domain, which dissociates the N-terminal inhibitory domain from the C-terminal kinase catalytic domain [17], and the phosphorylation of Ste11 by Ste20/Cla4 [21]. The Ste50–Ste11 interaction is constitutive, whereas the phosphorylation of Ste11 occurs only upon hyperosmotic shock. However, neither of these two events is sufficient for full activation of the HOG pathway, as a phospho-mimetic Ste11 or a truncated version of Ste11 lacking the auto-inhibitory domain can constitutively activate the HOG pathway only when overexpressed [18,21,22]. Moreover, the hyperactivation of the pathway mediated by Sho1 mutants with single point mutations in the SH3 domain requires an additional hyperactive Ste11 mutation [18]. All these results suggest that full activation of the HOG pathway requires a high concentration of activated Ste11 at the membrane [21]. Ste11 membrane localization is mediated by its association with Ste50, and this is achieved by the interaction of Ste50 with three membrane proteins: the anchor protein Opy2, the small GTPase Cdc42 and Sho1 [16,18,19]. Moreover, the architecture of the HOG membrane-associated complex is established by direct interactions between Ste11 and the cytoplasmic tail of Sho1, as well as its substrate Pbs2, which is also localized at the membrane by interacting with Sho1 [13,18]. All these interactions seem to enable an efficient binding of the Ste11 kinase with its substrate Pbs2. Additionally, the insulation of Ste11 at the sites where components of the HOG cascade assemble, by the use of docking interactions and scaffold proteins, is of special relevance to avoid undesirable cross-talk with the pheromone response and filamentous growth pathways [1,6,14,23].

Adaptation to osmostress entails a transient activation of the Hog1 MAPK, where the down-regulation of Hog1 activation requires its own kinase activity [24]. This negative feedback has multiple branches such as the induction of dedicated protein phosphatase activities. However, within the Sho1 branch the inhibition of upstream elements in the HOG pathway also seems to make major contributions to the attenuation of the system. To date, the direct phosphorylation of Sho1 and Ste50 by Hog1 has been described. For example, Hog1 modifies Ste50 at multiple sites to mediate the dissociation of Ste50 from the Opy2 membrane anchor, thereby reducing Ste11 membrane localization [20]. Phosphorylation of Ste50 by Hog1 also prevents activation of invasive growth under high osmolarity conditions [25]. Moreover, activated Hog1 also phosphorylates Sho1 at an amino acid residue located between the four TM domains and the C-terminal SH3 domain [26]. However, the role of this phosphorylation is still not completely clear.

Although the Sho1 branch of the HOG pathway has been extensively studied, it still lacks a unifying model explaining the role of the individual components in turning on the signal upon hyperosmotic shock due to technical limitations of techniques to study protein–protein interactions *in vivo*, under physiological conditions and using a direct read-out. In this study, we have utilized an enzymatic approach [27] to directly measure transient protein–protein interactions in the Sho1-branch. This method has already been successfully applied to study interactions in the Hog1 [27,28], PP2A [27] and autophagy [29,30] signaling pathways. We determined the interdependencies between pathway components and showed the essential function of the Ste50 adaptor for all Ste11 interactions in the Sho1 branch. Moreover, our results revealed a novel osmostress-regulated interaction between Msb2 and Ste11, as well as identifying several negative feedback systems.

2. Materials and methods

2.1. Yeast strains

Yeast strains used in this work are summarized in Table S1. Gene disruption was carried out by a polymerase chain reaction (PCR)-based strategy.

2.2. Plasmids and plasmid construction

Plasmids used in this study are summarized in Table S2. DNA constructs were generated using conventional PCR, restriction and ligation methods. Detailed cloning strategies and information on the individual constructs can be obtained upon request. Otherwise indicated, fusion proteins were expressed from centromeric plasmids and under native promoters.

2.3. Growth conditions and stress

Yeast transformants were grown overnight in synthetic complete selective media at 30 °C and were then diluted and grown until exponential phase. For osmotic stress experiments, cultures were either treated with mock or with 0.4 M NaCl for 30 min or for the indicated time.

2.4. Western blot analysis

To prepare protein extracts, about 10 OD_{600} units of cells were harvested by centrifugation and protein extracts were obtained by a post-alkaline extraction method [31]. Western blot were performed as described in [32]. For the detection of trimethylation of the histone tag, membranes were blocked with 1% milk for 1 h at room temperature, and were then incubated with specific anti-me3K9H3 antibody (mouse monoclonal, clone 6F12H4, Millipore) or with anti-me3K9H3p antibody (rabbit polyclonal, no. 2236, kindly provided by T. Jenuwein) [27]. Anti-me3K9H3 monoclonal antibody was diluted 1/2000 in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) containing 1% yeast extract and with protease inhibitor cocktail (Roche). Anti-me3K9H3p polyclonal antibody was diluted 1/3000 in 3% BSA-PBS. HA and myc tags were detected using anti-HA clone 16B12 (Covance Research Products) and anti-myc clone 4A6 (Millipore), respectively. Anti-mouse HRP-conjugated secondary antibody used was provided by Promega. Anti-rabbit HRP-conjugated secondary antibody used was provided by GE Healthcare. Immunoblots were detected using ECL Prime kit (GE Healthcare).

2.5. Western blot quantifications

Same amounts of total protein were loaded for each sample and this was checked by Pounceau S staining. Band intensities were quantified with the luminescent image analyzer ImageQuant LAS 4000 mini (GE Healthcare) using the program ImageQuant TL (GE Healthcare), or

analyzed from films using the program ImageGauge 4.0 (Fuji Software). Histone methylation signal was normalized against HA expression for each sample, and this was then compared to the signal of the control strain without stress. To quantify Msb2 protein expression, myc signals were normalized against the myc signal of the control strain without stress. Figures show representative westerns blots and the averages and standard deviations (SD) are calculated from 2 to 6 independent experiments.

3. Results

3.1. Interaction between Ste11 and Pbs2 requires Ste50, Opy2, Msb2/Hkr1 and Sho1

Short-lived protein interactions are difficult to trace biochemically. We followed the interaction of several components of the Sho1-branch of the HOG pathway (Fig. 1A) by the M-track method [27]. This novel method used to detect transient and stable protein–protein interactions involves a bait protein expressed as a fusion with a hyperactive mutant version of the mouse histone lysine methyltransferase (HKMT), and a prey protein fused with tandem array copies of the N-terminal part of histone H3 (H3) followed by an HA epitope to allow the detection of both the protein and its methylation modification by western blotting. All fusion proteins used are competent in osmotic stress signaling ([27] and Supplementary Fig. 1). Using this assay, the short-lived Ste11–Pbs2 interaction was recently studied and shown to increase during osmotic stress [27]. Here, we further tested to what extent HOG signaling components (Fig. 1A) are necessary for obtaining this Ste11–Pbs2 interaction signal (Fig. 1B). As expected, the Ste11–Pbs2 signal was completely dependent on the Pbs2-scaffold Sho1. Also, as shown in Fig. 1B and C, the Ste11-adaptor protein Ste50, the transmembrane anchoring protein Opy2, and the osmo-sensors Msb2 and Hkr1 were all required for this interaction. An interaction signal, albeit at low level, was detected in a strain carrying a deletion in the PAK kinase Ste20 (Fig. 1B and C). Surprisingly, we observed a higher level of methylation in *hog1* under non-stress conditions, which increased significantly after NaCl treatment (Fig. 1B). Without stress, this increase in Ste11–Pbs2 interaction signal in the absence of Hog1 is due to an increase in the Ste11 protein level (1.5 ± 0.4 fold in *hog1* respect to wild-type), suggesting a negative feedback on Ste11 protein stability by Hog1. However, under osmotic stress, the observed increase in the ratio of the methylation signal versus Ste11 protein amount (3.6 ± 1.2 fold in *hog1* in respect to wild-type, Fig. 1C) suggests that Hog1 activity might not only act on Ste11 protein stability but also affect the dissociation rate of the Ste11–Pbs2 complex either directly or indirectly via other signaling components.

3.2. SH3 domain of Sho1 is not required for the Sho1–Ste11 interaction, but influences the dissociation of the complex

The SH3 domain of Sho1 is essential for stress signaling through the Sho1-branch by recruiting Pbs2 to the plasma membrane. We observed that Sho1–Pbs2 interaction was constitutive and independent of Hog1, Opy2 and the osmosensors Msb2/Hkr1 (Supplementary Fig. 2). In contrast, the interaction between Ste11 and Sho1 seems to occur independently of the Sho1-SH3 domain [14,18]. We therefore re-investigated the Ste11–Sho1 interaction with the M-track assay to reassess the role of the Sho1-SH3 domain. Overall, we detected an increase in Ste11–Sho1 interaction signal during osmotic stress (Fig. 2A and B). Supporting previous data [14], Ste11 was found to be able to interact with a Sho1 truncated version lacking the SH3 domain (Sho1SH3Δ) (Fig. 2C and D). Furthermore, the Ste11–Sho1SH3Δ interaction was found to be

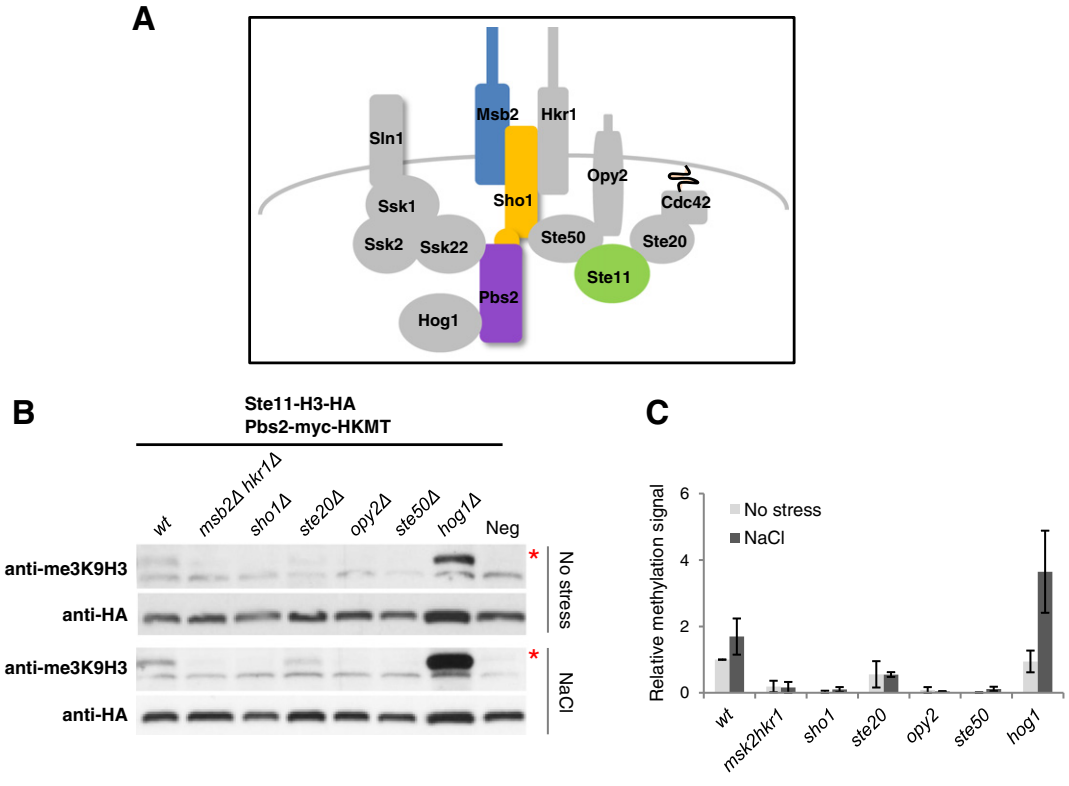

Fig. 1. Stress-regulated Ste11–Pbs2 interaction requires osmosensors and scaffold proteins and is negatively regulated by Hog1. (A) Scheme showing HOG pathway signaling components. (B) Interaction of Ste11-H3-HA (PAZ119) and Pbs2-HKMT (PAZ138) was detected with anti-meK9H3 antibodies in the indicated mutants (YCF189, YAZ120, YCF209, YCF163, YCF7 and PAY185) and wild-type (K699) strains in the absence (no stress) or presence (NaCl) of Na-induced osmotic stress. Asterisks indicate the specific methylation band. Negative control corresponds to the wild type (K699) strain transformed with the plasmids Ste11-H3-HA (PAZ119) and Pbs2 fused to a catalytically inactive methylase (PAZ139). Immunoblots were also developed with anti-HA antibody to detect Ste11-H3-HA expression. (C) Quantification of the methylation signal normalized against HA signal and compared to the wild type in the absence of stress. The average and standard deviations (SD) of 2 to 6 independent experiments for each strain are shown. This figure shows one representative experiment.

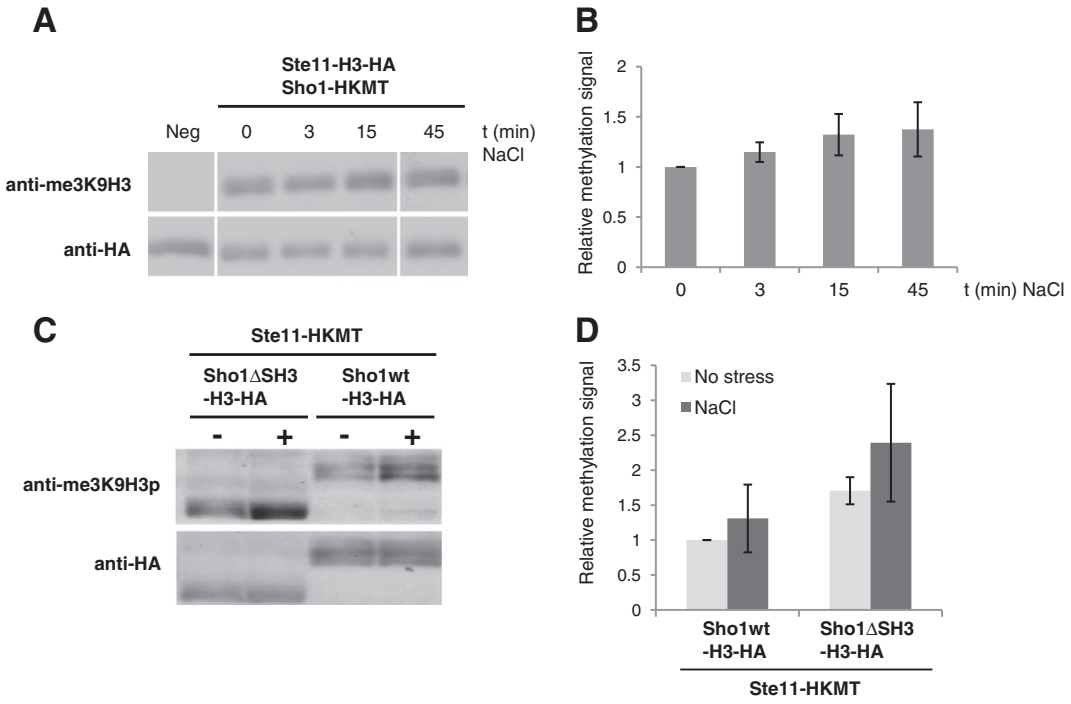

Fig. 2. Ste11 interaction with Sho1 is regulated by stress and through the SH3 domain of Sho1. (A) Ste11–Sho1 interaction increases with osmotic stress. *ste11Δ sho1Δ* mutant strain (YAZ82) containing the centromeric plasmids Ste11-H3-HA (PAZ119) and Sho1-HKMT (PCF54) was incubated with 0.4 M NaCl at the indicated times. Negative control corresponds to a *ste11Δ* mutant strain (YAZ80) containing Ste11-H3-HA (PAZ119) and an empty plasmid. All rows were cut from same western. Immunoblot was developed with the indicated antibody. (C) M-track analysis of the interaction between Ste11-HKMT (PAZ85) and two Sho1-H3-HA versions: with (Sho1wt, PID228) and without the SH3 domain (Sho1ΔSH3, PID381) in *ste11Δ sho1Δ* double mutant strain (YAZ82), in non-stress (−) or Na-induced osmotic stress (+) conditions. Fusion proteins were expressed from multicopy plasmids. Immunoblot was developed with the polyclonal anti-me3K9H3p antibody. (B and D) Quantifications of the methylation signals in (A) and (C) respectively, normalized as in Fig. 1. The average and SD of 3 (B) and 2 (D) independent experiments are shown. The western blots shown in (A) and (C) are representative experiments.

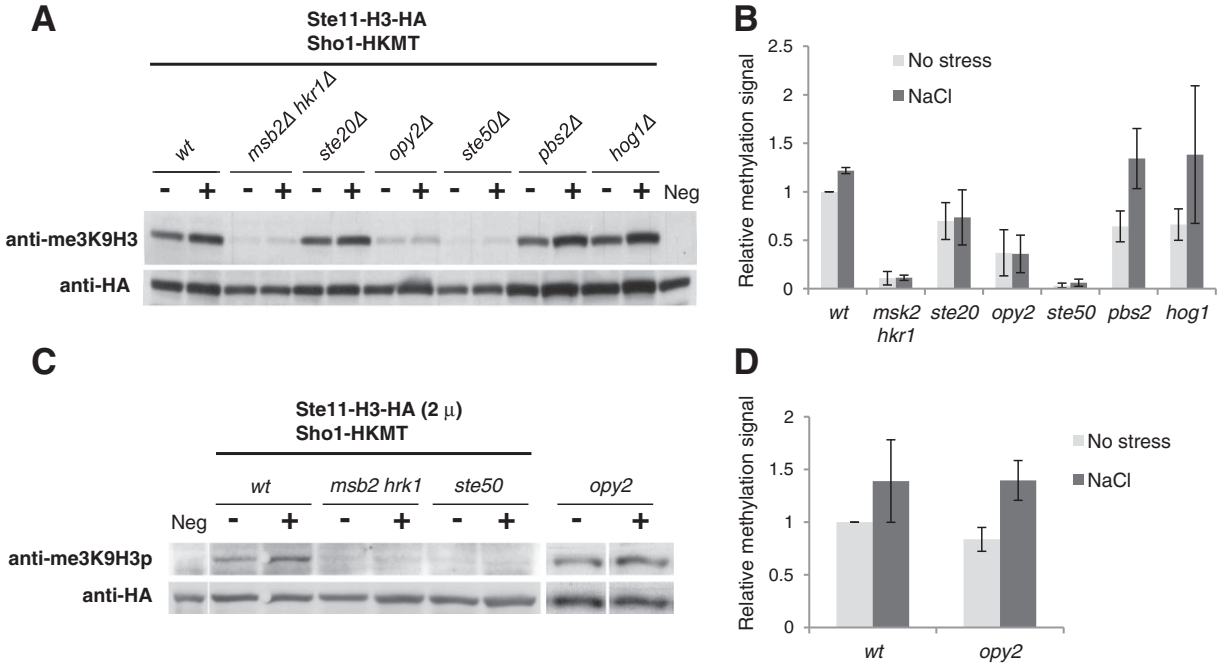

Fig. 3. Factors required for Ste11 interaction with Sho1. (A) Interaction of Ste11-H3-HA (PAZ119) with Sho1-HKMT (PCF54) in the indicated mutants (YCF189, YCF209, YCF163, YCF7, YVR10 and PAY185) and wild-type (K699) strains, with (+) or without (−) osmotic stress. Immunoblots were developed with the indicated antibodies. (C) Interaction of overexpressed Ste11-H3-HA (multicopy plasmid PAZ56) with Sho1-HKMT (PCF54) in wild-type (K699), *msb2Δ hkr1Δ* (YCF189) and *ste50Δ* (YCF7) and *opy2Δ* (YCF163) strains, under stress (+) and non-stress (−) conditions. Negative control used in (A) and (C) corresponds to a wild type strain transformed with the plasmids Ste11-H3-HA (PAZ119) and Sho1 fused to a catalytically inactive methylase (PAZ140). (B and D) Quantifications of the methylation signals in (A) and (**C**) respectively, normalized as in Fig. 1. The average and SD of 3 or 4 independent experiments for each strain in (B) and 3 independent experiments in (D) are shown. The western blots shown in (A) and (C) are representative experiments.

stronger than the interaction with full-length Sho1 (Fig. 2C and D). Similarly, Ste11–Sho1 interaction increased when using a Sho1-W338A mutant (Supplementary Fig. 3), which contains a modification in the SH3 domain and is unable to bind Pbs2 [18]. These results suggest that the presence of Pbs2 in the complex negatively regulates Ste11–Sho1 interaction. Since the assay was performed in a background that is still able to induce Hog1 activity via the Sln1 branch, we surmise that the presence of Pbs2 might enhance the dissociation rate of the Ste11–Sho1 complex.

3.3. Interaction of Ste11 with Sho1 requires the scaffold Ste50 and the osmosensors Msb2/Hkr1, but not the membrane protein Opy2

A direct interaction between Ste11 and Sho1 has been documented; however, this interaction was only observed in the presence of the Ste11-adaptor protein Ste50 [14] or in the absence of Ste50 but in combination with Sho1 hyperactive mutants [18]. We observed that Ste11–Sho1 interaction was absent when Ste50 was deleted (Fig. 3A and B), suggesting that this interaction requires the formation of the Ste11–Ste50 complex, which is stable and independent of Sho1 (Supplementary Fig. 4). Ste11–Sho1 interaction was only partially lost upon Opy2 deletion and, as expected due to the presence of Cla4, the interaction in *ste20* mutant was detected at similar levels as in the wild-type strain (Fig. 3A and B). The downstream factors Pbs2 and Hog1 were not required for Ste11–Sho1 interaction, however their absence yield an increase in Ste11 protein levels under non-stress conditions (Ste11 level increases 1.7 ± 0.4 and 1.9 ± 0.3 fold in *pbs2* and *hog1* in respect to wild type, respectively), as well as a slight increase during osmotic stress (1.2 ± 0.1 and 1.4 ± 0.1 fold in *pbs2* and *hog1* in respect to wild type respectively), confirming the presence of a Hog1-regulated negative feedback over Ste11 protein level (Fig. 1). Moreover, since the Ste11 protein level increase is also seen in the absence of osmotic stress, these results suggest that basal activation of Hog1 under non-stress conditions is sufficient to mediate the negative feedback (Fig. 3A and B). Additionally, in the absence of both osmosensors Msb2 and Hrk1, only an extremely weak interaction between Ste11 and Sho1 was observed in both stress and non-stress conditions (Fig. 3A and B).

We then investigated whether the overexpression of Ste11 protein could restore its interaction with Sho1 in the absence of the signaling factors whose mutations decreased the Ste11–Sho1 interaction. Overexpression of Ste11 from a multicopy plasmid is able to fully restore the Ste11–Sho1 interaction in an *opy2* mutant, indicating that Opy2, known to be a membrane anchor of the Ste11–Ste50 complex, is not essential for this interaction (Fig. 3C and D and Supplementary Fig. 5). However, high levels of Ste11 were not sufficient to re-establish its

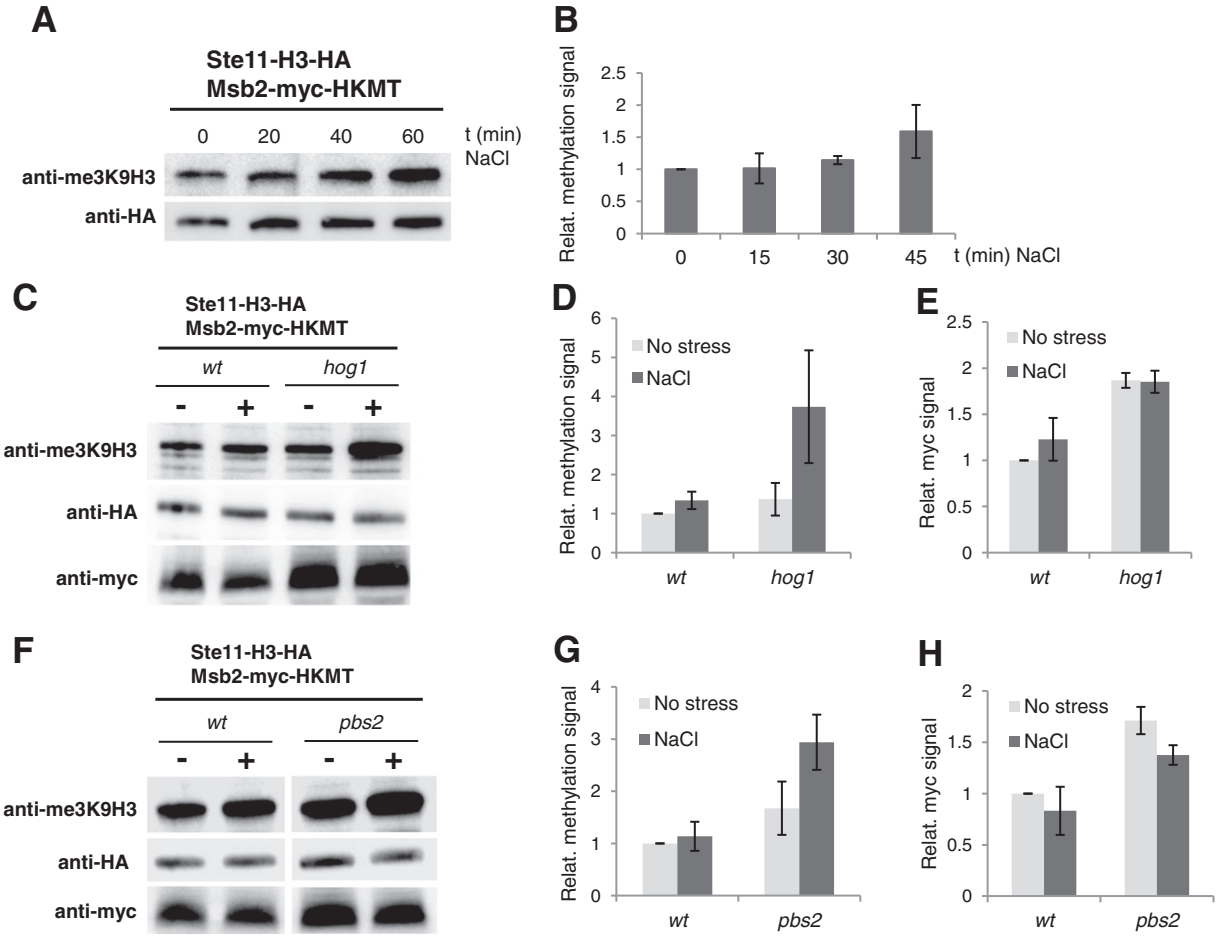

Fig. 4. Msb2 interacts with Ste11, and Msb2-protein level and interaction are negatively regulated by Hog1. (A) Ste11-H3-HA (PAZ119) interaction with Msb2-myc-HKMT (PAZ218) in *ste11Δ msb2Δ* (PAY703) strain at the indicated times of osmotic stress. Immunoblots with the indicated antibodies are shown. (C) Ste11–Msb2 M-track interaction in wild type (K699) and *hog1Δ* mutant (PAY185) strains under stress (+) and non-stress (−) conditions. Immunoblots with the indicated antibodies are shown. (F) Ste11–Msb2 M-track interaction in wild type (*ste11Δ*, YAZ80, complemented with the Ste11 plasmid) and *pbs2Δ* mutant (*ste11Δ pbs2Δ*, YID140, complemented with the Ste11 plasmid) strains under stress (+) and non-stress (−) conditions. Bands from each row are cut from the same western blot. Immunoblots were developed with the indicated antibodies. (B, D and G) Quantifications of the methylation signals in (A), (C) and (F), respectively, normalized as in Fig. 1. The average and SD of 3 (B), 2 (D) and 3 (G) independent experiments are shown. (E and H) Quantifications of the myc signals in (C) and (F), respectively, compared to wild-type signal in the absence of stress. The average and SD of 2 (E) and 3 (H) independent experiments are shown. The western blots shown in (A), (C) and (F) are representative experiments.

interaction with Sho1 in the absence of Msb2/Hkr1 or Ste50 (Fig. 3C). Altogether, these results indicate that the osmosensors Msb2/Hkr1 and the Ste11-adaptor Ste50, but not the membrane anchor Opy2, are essential for an efficient Ste11–Sho1 interaction.

3.4. Msb2 interacts dynamically with Ste11 and its protein level is regulated by a Hog1-mediated negative feedback

To establish signaling via the Sho1-branch, Ste11 interacts, directly or through Ste50, with Ste20/Cla4, Opy2, Sho1 and Pbs2 [6]. Moreover, we have shown that Msb2 and Hkr1 are required for the Ste11–Sho1 interaction. We then investigated, using M-track, whether Msb2 is able to interact with Ste11. As shown in Fig. 4A and B and Supplementary Fig. 6, the Ste11–Msb2 interaction was present in both stress and non-stress conditions, with a slight increase in response to osmotic stress *in vivo*. Provided that we had observed a negative feedback regulating Ste11 protein mediated by Hog1, we aimed to determine whether Hog1 also regulates Msb2 protein expression. We observed a 1.9 ± 0.1 fold increase in Msb2 level in *hog1*, and this increase was also seen in a *pbs2* mutant (Fig. 4C–H). Interestingly, the relative methylation signal in respect to Msb2 protein level during osmotic stress was found to be higher in both *hog1* (2.0 ± 0.6 fold) and *pbs2* (2.4 ± 0.7 fold) mutant strains compared with the wild-type strain (1.4 ± 0.3 fold), suggesting that activated Hog1 regulates not only Msb2 protein expression but also the dissociation of the Ste11–Msb2 complex (Fig. 4C and F).

3.5. Sho1 is not essential for Msb2–Ste11 interaction

Since Ste11 membrane recruitment is executed through direct or indirect interactions with Ste50, Opy2 and Sho1, we analyzed whether these factors are necessary for the binding of Msb2 to Ste11. In *opy2* and *ste50* mutant strains, the methylation signal decreased dramatically to background levels, and in the *sho1* mutant a markedly reduced signal was observed (Fig. 5). A change in Msb2 protein level was not observed in any of these mutants (Fig. 5C and F), which was expected as the SLN-branch of the HOG pathway can still activate Hog1 in these strains, and therefore, Hog1 is still able to execute the negative regulation over Msb2 protein level.

Next, we checked whether overexpression of Msb2, produced by the inactivation of the two branches of the HOG pathway, could restore Msb2–Ste11 binding in *opy2*, *ste50*, and *sho1*. With this purpose, first, we compared the methylation signal produced by Ste11–Msb2 interaction in an *opy2* mutant with an *opy2 pbs2* double mutant, where Msb2 is overexpressed (Fig. 6A to C). Under non-stress conditions, overexpression of Msb2 gave a background methylation signal, which increased after treatment with NaCl (Fig. 6A and B). Second, we studied this interaction in a *ste50* mutant that also lacks the Ssk2 and Ssk22 MAPKKKs of the SLN-branch of the pathway. In this case, the triple mutant produced a background methylation signal in the absence of stress, which was slightly increased after stress (Fig. 6D to F). However, the relative increase in Ste11–Msb2 interaction in *opy2 pbs2* with respect to *opy2* was much higher than the increase observed in *ssk2/22 ste50* with respect to *ste50* (19-fold versus 1.6-fold), suggesting that, even though both factors are essential for a physiological Ste11–Msb2 interaction, Ste50 is required in a higher extent than Opy2. Additionally, we analyzed the increase in Msb2 protein levels produced in different HOG signaling mutants. Since overexpression of Msb2 was achieved in strains harboring a Hog1 deletion (Fig. 4C) as well as in strains that inhibit Hog1 activation but contain the Hog1 protein (*pbs2* or *ssk2 ssk22 ste50*) (Fig. 6C and F), we can conclude that the Hog1-mediated negative feedback over Msb2 protein expression is not only structural but depends on Hog1 activity, either basal or induced by stress.

Third, we analyzed the contribution of Sho1 to Msb2–Ste11 binding. Msb2 has been shown to interact with Sho1 [11] and Sho1 is found to also interact with Ste11 and Ste50 [14,18]. Thus, Msb2 binding to Ste11 could occur indirectly through Sho1, and in this case, we would expect no restoration of Msb2–Ste11 interaction by increasing Msb2 levels in a *sho1* mutant. However, the overexpression of Msb2 protein produced in the triple mutant strain *ssk2 ssk22 sho1* yield high level of Msb2–Ste11 interaction, indicating that Sho1 is not essential for this interaction (Fig. 6G to I).

Taken together, these results suggest that the scaffold protein Ste50 is essential for the interaction between the osmosensor Msb2 and the

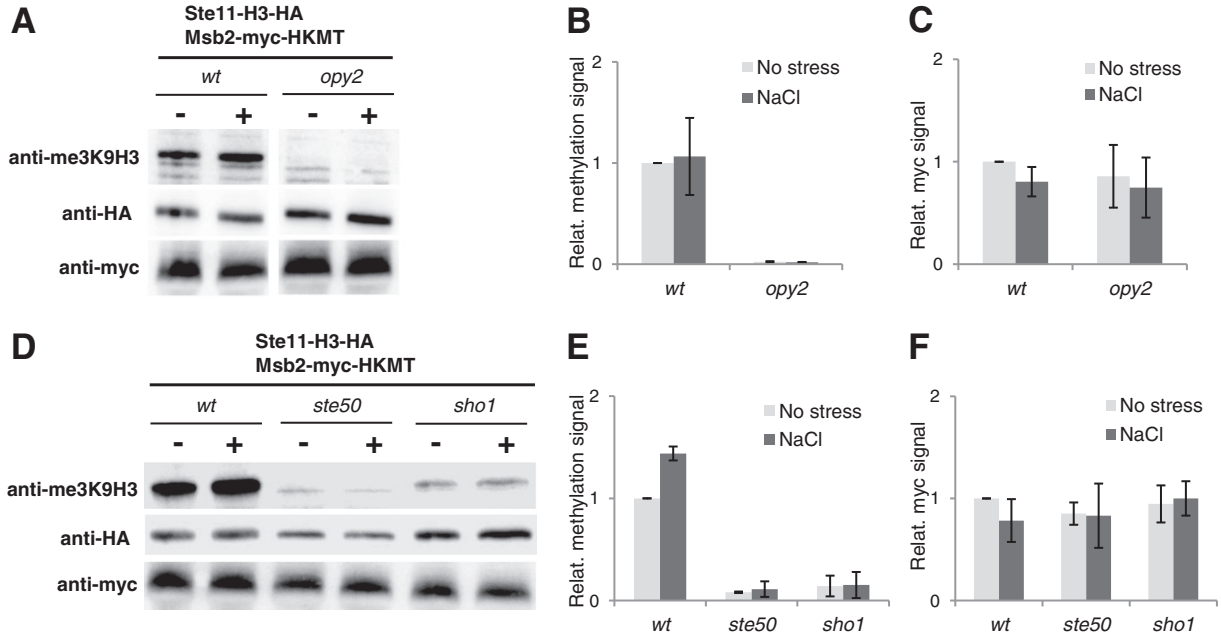

Fig. 5. Ste11–Msb2 interaction requires Ste50 and Opy2, and to a lesser extent Sho1. Ste11-H3-HA (PAZ119) interaction with Msb2-myc-HKMT (PAZ218) in the absence of Opy2 (A) and in the absence of Ste50 or Sho1 (D) in the indicated strains (K699 and YCF163 in (A); YAZ80, YAZ87 and YAZ82 in (D)) under stress (+) and non-stress (−) conditions. Immunoblots with the indicated antibodies shown in (A) and (D) were quantified as described in Fig. 1, with methylation signals represented in (B) and (E) respectively, and myc signals in (C) and (F) respectively. All quantifications show the average and SD of 3 independent experiments. The western blots shown in (A) and (D) are representative experiments. All rows were cut from same western blot.

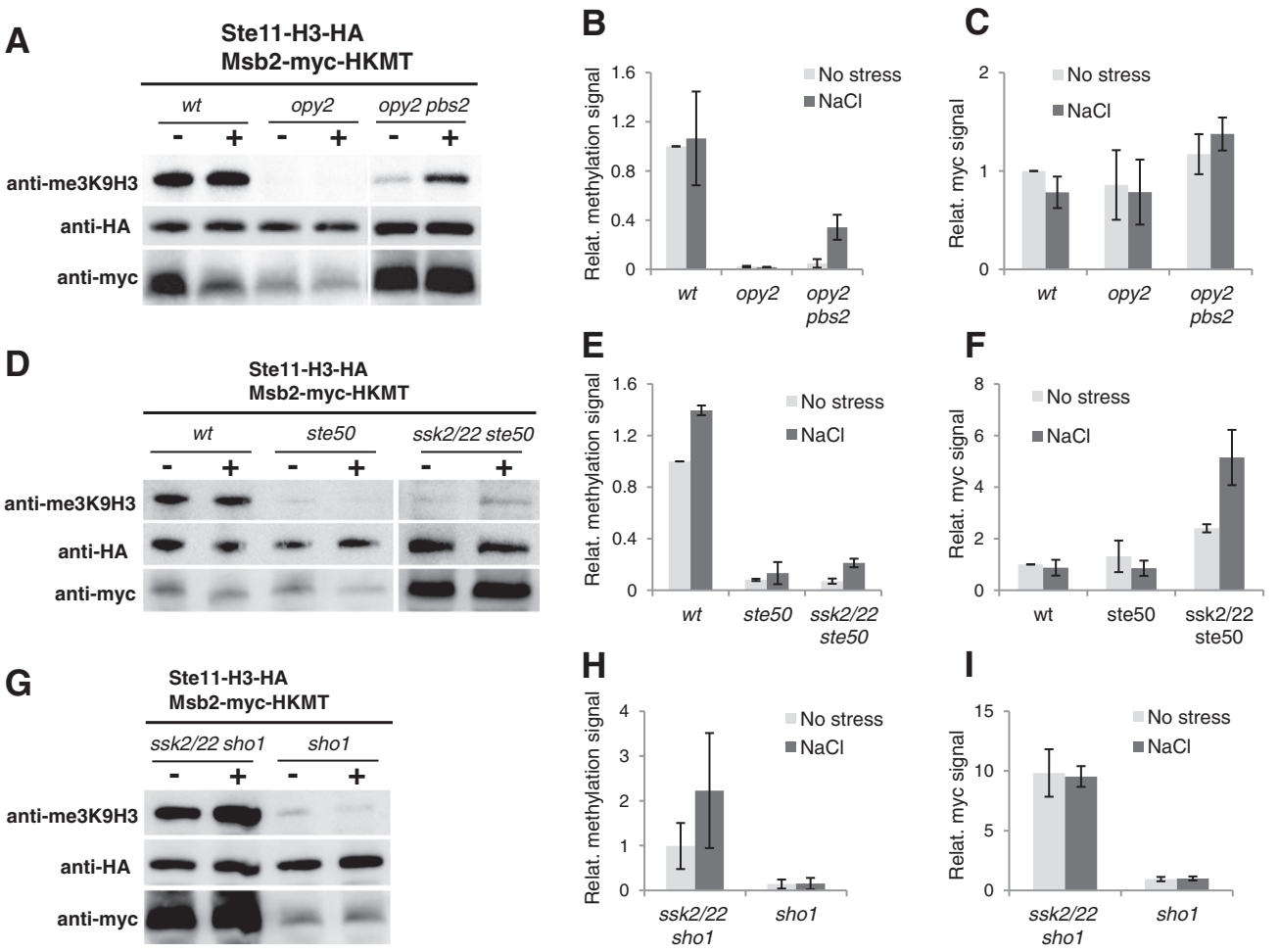

Fig. 6. Increase in Msb2 protein recovers Ste11–Msb2 interaction in *sho1*, but not in *ste50* and *opy2* mutants. (A) Ste11-H3-HA (PAZ119) interaction with Msb2-myc-HKMT (PAZ218) in wild-type (K699), *opy2Δ* (YCF163) and *opy2Δ pbs2Δ* (YCF173) strains under stress (+) and non-stress (−) conditions. (D) Ste11–Msb2 M-track interaction in wild-type (K699), *ste50Δ* (YCF7) and *ssk2/22Δ ste50Δ* (YID198) strains under stress (+) and non-stress (−) conditions. (G) Ste11–Msb2 M-track interaction in *sho1Δ* (YAZ120) and *ssk2/22Δ sho1Δ* mutant (YAZ150) strains under stress (+) and non-stress (−) conditions. Immunoblots with the indicated antibodies shown in (A), (D) and (G) were quantified as described in Fig. 1 for methylation signals represented in (B), (E) and (H), respectively, and myc signals represented in (C), (F) and (I), respectively. Quantifications show the average and SD of 3 (B and C) and 2 (E, F, H and I) independent experiments. The western blots shown in (A), (D) and (G) are representative experiments. All rows were cut from same western.

MAPKKK Ste11. The transmembrane anchor protein Opy2 may be required in a lower extent. Importantly, the transmembrane protein Sho1 is not essential for Ste11–Msb2 interaction at high Msb2 levels, but required for an efficient Msb2–Ste11 binding *in vivo* (Fig. 6).

4. Discussion

4.1. Ste11 interacts with Msb2 and other signaling factors for its efficient recruitment to the membrane

In this work we have analyzed the interactions of the MAPKKK Ste11 with its substrate Pbs2, the membrane scaffold protein Sho1 and the membrane osmosensor Msb2 using a biochemically based *in vivo* protein proximity assay [27]. For Ste11–Sho1 and Ste11–Msb2 we observed a strong basal signal, and for Ste11–Pbs2 a weak signal, and for all three pairs of proteins the signal increased after osmotic stress exposure. Moreover, all three interactions were fully dependent on the presence of the Ste11-adaptor protein Ste50 (Figs. 1, 3, 5 and 6). Investigating which additional factors might be required for these interactions, we made several interesting observations that might shed more light on the staging and the dynamics of the Sho1 branch signaling system.

Most notably, we found that Ste11 and the osmosensor Msb2 may come into intimate contact during signaling (Fig. 4). However, one has to consider that the M-track assay is a proximity assay so that it is not possible to distinguish direct from spatially close indirect interactions. Moreover, one has to also take into account that the location, stoichiometry and overall structure of the effective signaling machinery is still unclear although it has established that at least Sho1 is able to assemble into multimeric structures.

We believe that our genetic analysis provides evidence that the interaction between Ste11 and Msb2 might be direct. Although there is some interdependence between Sho1 and Msb2 in how effective they produce an interaction signal with Ste11, overexpression of Ste11 does not restore Ste11–Sho1 interaction in the absence of Msb2 and Hkr1 (Fig. 3B). In contrast, overexpression of Msb2 restores Ste11–Msb2 interaction in a *sho1* mutant (Fig. 6G). Thus, Msb2 (plus Hkr1) is essential for Ste11–Sho1 interaction, but Sho1 is not absolutely required for the binding of Ste11 to Msb2 (Figs. 3B and 6G). This result suggests that the sensory system could function as an independent recruitment system for Ste11, in which Ste50 is essential. Apart from Sho1, the trans-membrane protein Opy2 also seems to be auxiliary for Ste11–Msb2 interaction. Similar to Sho1, it is not completely necessary for Ste11–Msb2 signals since the interaction can also be restored when Msb2 is overexpressed (Fig. 6). In agreement with many previous studies, Sho1 seems to provide the central hub for connecting the sensory system and the activation system of Ste11 (via Opy2) with its target the MAPKK Pbs2. While the comparatively weak Ste11–Pbs2 methylation signal decreases in the absence of Opy2, Sho1 and Msb2/Hrk1

(Fig. 1), the Sho1–Pbs2 signal is completely independent of the membrane proteins Msb2/Hkr1 and Opy2 (Supplementary Fig. 2). Accordingly, the interaction between Pbs2 and Ste11 might not contribute much, if anything, to the membrane recruitment of Ste11. Indeed, our finding that the absence of Pbs2–Sho1 interaction increases the Ste11 occupancy at Sho1 would strengthen this assessment (Fig. 2 and Supplementary Fig. 3). We therefore suggest that the sensory system as well as Opy2 might function as primary concentration and gating devices for the transfer of Ste11 to Sho1.

4.2. Hog1-dependent negative feedback regulates Msb2 and Ste11 protein levels and Ste11 interactions

A second notable observation of this work was the apparent increase of the protein interaction signals when a signaling component was deleted. Principally, there are two plausible explanations for such a result: 1) a defect in a negative feedback system or 2) the accumulation of intermediate signaling complexes, the dissociation of which depends on the function or structural presence of the deleted downstream component.

Several negative feedback systems have been proposed for the HOG pathway as essential for the transience and proper dynamics of the stress response. Here we showed that at least two of them are executed over the protein level of upstream signaling factors: Ste11 (Fig. 1) and Msb2 (Fig. 4). For both proteins we found that this regulation also occurs under non-stress conditions. Since the increase in Msb2 levels is observed not only in *pbs2* and *hog1* mutants but also in *ssk2 ssk22 sho1* mutant, we suggest that general Hog1 activity, and not only the Hog1 protein, is required for the negative feedback. It also confirms that basal Hog1 activity generated by the Sln1-branch [33] is sufficient for this negative regulation.

The signaling mucin Msb2 is at the head of the filamentous growth (FG) pathway [11] and it has been shown that HOG pathway, through its downstream components, has an inhibitory role over the FG pathway [34]. The negative regulation of Pbs2 and Hog1 over the Msb2 protein level reported here could explain this cross-regulation between these signaling pathways.

Other types of negative feedback mechanisms envisioned and proposed at protein binding levels have been detected by our work. Our data suggest that Ste11–Pbs2 and Ste11–Msb2 interactions are indeed influenced by Hog1 (Figs. 1 and 4). According to previous studies, the effects could be caused by direct Hog1-dependent phosphorylation events connected to at least two factors: Ste50 [20,25,26] and Sho1 [26]. For Ste50, it has been shown that substitutions of the phosphorylation sites clearly affect the behavior of the system. Since all interactions are dependent on the Ste50–Ste11 complex, the phosphorylation of this important scaffolding element alone could explain the changes in the protein interaction pattern.

Our results also hint at the role of downstream components in the dissociation of signaling intermediates. One obvious candidate for this observation is perhaps the increase in the interaction signal between Ste11 and Sho1 when Pbs2 is unable to bind due to the absence of the SH3 domain in Sho1. We speculate that the dissociation of Ste11 after activation of Pbs2 might be a prerequisite to keep the signaling system in a productive state, and a similar argument could be made from the observation that *hog1* shows a significant increase in the Pbs2–Ste11 interaction. The further resolution of this complex and dynamic signaling system now appears well within reach of biochemical as well as visual *in vivo* protein interaction assays.

4.3. Conclusions

In our study, we have characterized several *in vivo* interactions of the MAPKKK Ste11 with various components of the HOG pathway in order to achieve effective transduction of signal in response to hyperosmotic stress. First, the interaction between Ste11 and its substrate Pbs2 requires all upper components of the pathway. Second, the Ste11–Sho1 interaction does not require the Sho1-SH3 domain, and requires Ste50 and Msb2/Hkr1. Third, we have characterized a novel dynamic interaction between Ste11 and Msb2, where Msb2 acts as a membrane-concentrator of Ste11 requiring Ste50 but not Sho1. Finally, we have observed multiple negative feedbacks regulating protein levels and Ste11 interactions mediated by downstream components of the pathway, Pbs2 and Hog1.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagrm.2015.02.001.

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