Nbp2 targets the Ptc1-type 2C Ser/Thr phosphatase to the HOG MAPK pathway

James Mapes and Irene M Ota*

Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO, USA

The yeast high osmolarity glycerol (HOG) pathway signals via the Pbs2 MEK and the Hog1 MAPK, whose activity requires phosphorylation of Thr and Tyr in the activation loop. The Ptc1-type 2C Ser/Thr phosphatase (PP2C) inactivates Hog1 by dephosphorylating phospho-Thr, while the Ptp2 and Ptp3 protein tyrosine phosphatases dephosphorylate phospho-Tyr. In this work, we show that the SH3 domain-containing protein Nbp2 negatively regulates Hog1 by recruiting Ptc1 to the Pbs2-Hog1 complex. Consistent with this role, *NBP2* acted as a negative regulator similar to *PTC1* in phenotypic assays. Biochemical analysis showed that Nbp2, like Ptc1, was required to inactivate Hog1 during adaptation. As predicted for an adapter, deletion of *NBP2* disrupted Ptc1-Pbs2 complex formation. Furthermore, Nbp2 contained separate binding sites for Ptc1 and Pbs2: the novel N-terminal domain bound Ptc1, while the SH3 domain bound Pbs2. In addition, the Pbs2 scaffold bound the Nbp2 SH3 via a Pro-rich motif distinct from that which binds the SH3 domain of the positive regulator Sho1. Thus, Nbp2 recruits Ptc1 to Pbs2, a scaffold for both negative and positive regulators.

Introduction

Mitogen-activated protein kinase (MAPK) pathways regulate growth, development and the response to stress in all eucaryotes (Gustin *et al*, 1998; Pearson *et al*, 2001; Hohmann, 2002; Johnson and Lapadat, 2002). The three-kinase cascade common to these pathways contains a MAP/ERK kinase kinase (MEKK) or Raf, which, when activated, phosphorylates and activates MAP/ERK kinase (MEK), which in turn activates MAPK via dual phosphorylation of a Thr and Tyr residue in the activation loop. The *Saccharomyces cerevisiae* high-osmolarity glycerol (HOG) MAPK pathway is activated primarily by osmotic stress (reviewed in Gustin *et al*, 1998; Hohmann, 2002; de Nadal *et al*, 2002) and also by heat stress (Winkler *et al*, 2002). Upstream of the three-kinase cascade are two putative membrane-bound stress sensors, Sln1, a two-component signaling protein, and Sho1, a novel protein containing an SH3 domain (Figure 1A). The Sln1 branch

*Corresponding author. E-mail: IreneMOta@aol.com

contains Ypd1 and Ssk1, which forms a three-component signaling system orthologous to bacterial signaling proteins. Ssk1 negatively regulates the downstream MEKKs, Ssk2 and Ssk22. The Sho1 branch signals via the Cdc42 GTPase, Ste50 a novel protein, the Ste20 p21-activated protein kinase (PAK) and the Ste11 MEKK. Both the Sln1 and Sho1 branches converge on the Pbs2 MEK-Hog1 MAPK module. In addition to activating Hog1, Pbs2 has also been shown to act as a scaffold in this pathway, binding Sho1, Ste11, Ssk2/22 and Hog1 (Posas and Saito, 1997; Tatebayashi *et al*, 2003).

As important as the kinases that activate these pathways are the protein phosphatases that inactivate them. Mutation of phosphatases leads to aberrant signaling in yeast and *Drosophila melanogaster* MAPK pathways (Maeda *et al*, 1993; Jacoby *et al*, 1997; Martin-Blanco *et al*, 1998; Mattison *et al*, 1999; Zeitlinger and Bohmann, 1999; Winkler *et al*, 2002). Two groups of protein phosphatases inactivate the HOG pathway (Figure 1A). One group is the protein tyrosine phosphatases (PTPs) Ptp2 and Ptp3, which inactivate Hog1 by dephosphorylating the phosphotyrosine residue in the activation loop (Jacoby *et al*, 1997; Wurgler-Murphy *et al*, 1997). Ptp2 has been shown to reside in the nucleus, and is the stronger negative regulator in this pathway (Jacoby *et al*, 1997; Mattison and Ota, 2000).

The second group of phosphatases that inactivate the HOG pathway is the type 2C Ser/Thr phosphatases (PP2C), which are called PTCs in yeast. Three PTCs, Ptc1, Ptc2 and Ptc3, inactivate this pathway (Maeda *et al*, 1994; Warmka *et al*, 2001; Young *et al*, 2002). Ptc1 maintains low basal Hog1 activity and inactivates Hog1 during adaptation to osmotic stress (Warmka *et al*, 2001), while Ptc2 and Ptc3 play a complementary role by setting the maximal limit to which stress can activate Hog1 (Young *et al*, 2002). None of these three PTCs has an obvious effect on the upstream Pbs2 (Warmka *et al*, 2001; Young *et al*, 2002). Similarly, the *Schizosaccharomyces pombe* PP2C phosphatases inactivate the Spc1/Sty1 stress-activated MAPK, but do not appear to act on the upstream MEK (Nguyen and Shiozaki, 1999). However, PP2C in vertebrate signaling pathways can inactivate MEKK, MEK and MAPK in stress-activated pathways. PP2Cβ-1 inactivates the TAK1 MEKK (Hanada *et al*, 2001), while PP2Cα inactivates the MKK4 and MKK6 MEKs, and the p38 MAPK (Hanada *et al*, 1998; Takekawa *et al*, 1998).

Specificity is crucial to MAPK signaling pathways. In yeast and in vertebrates, multiple MAPK cascades coexist, regulating responses to a variety of signals. In each pathway are MEKKs, MEKs and MAPKs, which share significant sequence similarity within each group. In the absence of specificity conferring mechanisms, inappropriate crosstalk could occur between these pathways. One means to insulate these pathways is via scaffold proteins such as Pbs2 and Ste5 in the yeast HOG and pheromone response pathways (Posas and Saito, 1997; Elion, 2001), respectively, and JIP and MP-1 in vertebrate JNK and ERK1 pathways (Schaeffer *et al*, 1998; Whitmarsh *et al*, 1998), respectively. Another mechanism
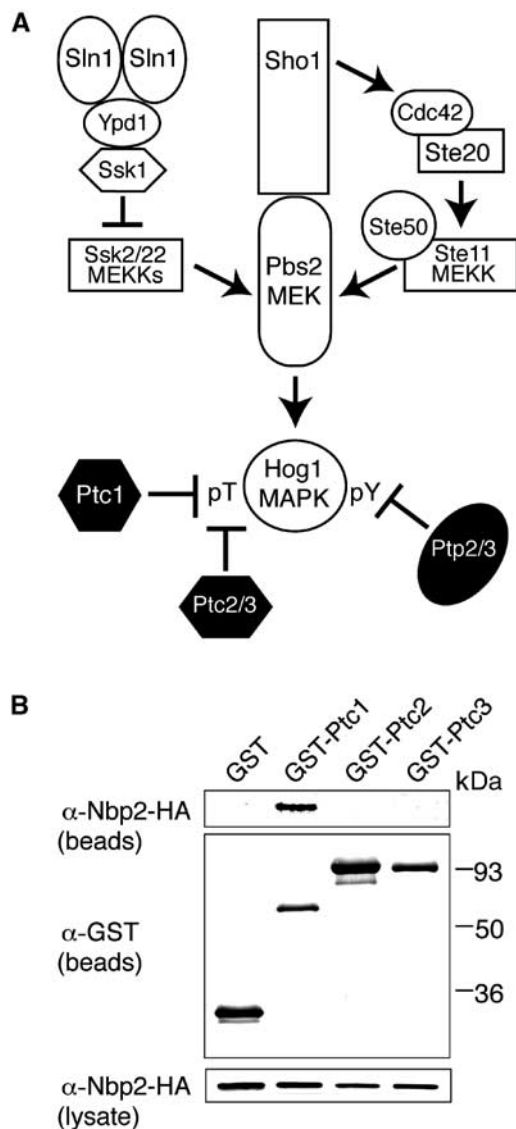
A

B

Figure 1 Activation of the HOG MAPK pathway and its negative regulation by protein phosphatases. (**A**) The yeast stress-activated HOG MAPK pathway contains a three-kinase cascade, which comprises the Ssk2, Ssk22 and Ste11 MEKKs, the Pbs2 MEK and the Hog1 MAPK. The Sln1-Ypd1-Ssk1 branch negatively regulates the Ssk2/22 MEKKs, while Sho1 positively regulates the Ste11 MEKK. Two classes of phosphatases regulate Hog1, which requires phosphorylation of Thr and Tyr in the sequence TGY for activation. Ptp2 and Ptp3 dephosphorylate pY, while Ptc1, Ptc2 and Ptc3 dephosphorylate pT. (**B**) Ptc1 but neither Ptc2 nor Ptc3 co-precipitates with Nbp2. The ability of Ptc1, Ptc2 and Ptc3 to associate with Nbp2 was examined in the $nbp2\Delta$ strain JHM17 expressing GST-PTCs and Nbp2-HA. Yeast were cultured, lysed, and co-precipitation assays performed as described in Materials and methods. The bound material was examined by SDS–PAGE and immunoblotting with anti-GST and anti-HA antibodies. The amount of Nbp2-HA in the lysate is also shown.

is via binding motifs contained within MEKs, MAPKs, its regulators and its substrates (Bardwell *et al*, 2001; Tanoue and Nishida, 2002).

Although protein phosphatases are often considered to be nonspecific, there are several examples of MAPK phosphatases that counter this notion. For example, the vertebrate dual-specificity phosphatase (DSP) MKP-3 is highly specific for ERK. Within its N-terminal noncatalytic domains lies

the MAPK docking site known as the D domain or kinase interaction motif (KIM), which interacts with the common docking (CD) motif found in ERK (Tanoue and Nishida, 2002). In yeast, the PTPs also attain specificity for MAPK family members via their N-terminal noncatalytic domains. Ptp3 contains a D domain/KIM and a Cdc25 homology (CH) domain that bind to the Fus3 CD motif (Zhan and Guan, 1999), and Ptp2 binds Hog1 via the CD motif (Mattison and Ota, 2000) (D Heidysch, C Mattison and IM Ota, unpublished).

The means by which PP2Cs target the three-kinase MAPK cascade are not understood. Previous studies showed that PP2C proteins co-precipitate with MAPK cascade members, suggesting that they specifically bind them. For example, the *S. pombe* Ptc1 co-precipitates with Spc1/Sty1 MAPK (Nguyen and Shiozaki, 1999), and vertebrate PP2Cβ-1 binds TAK1 specifically, as it did not bind MEKK2, MKK4, MKK6, JNK or p38 (Hanada *et al*, 2001). Other studies suggested that PP2C can discriminate between phosphorylated and unphosphorylated substrates. PP2Cα bound phosphorylated, but not unphosphorylated p38 (Takekawa *et al*, 1998). However, PP2Cβ-1 binding to TAK1 did not require phosphorylation (Hanada *et al*, 2001). For each of these PP2C-MAPK pathway substrate interactions, it is not clear whether they are direct or occur via a third protein, since co-precipitations were performed using cell lysates. However, an *Arabidopsis* PP2C, MPC2, can bind the stress-activated SIMK MAPK directly (Meskiene *et al*, 2003).

We sought to identify how the yeast Ptc1 might target Hog1. A proteome-wide analysis of protein–protein interactions in *S. cerevisiae* by two groups using the yeast two-hybrid method found that Ptc1 interacted with Nbp2 (Ito *et al*, 2000; Uetz *et al*, 2000). Not much is known about Nbp2, a 237-residue protein containing an SH3 domain. Nbp2 was initially identified as a Nap1-binding protein (Shimizu *et al*, 2000), where Nap1 plays a role in bud morphogenesis and cell cycle progression (Kellogg *et al*, 1995; Altman and Kellogg, 1997). Beyond Nbp2 interaction with Ptc1, additional evidence suggested a possible role for Nbp2 in the HOG pathway. An analysis of protein complexes in yeast showed that Pbs2 co-precipitated with Nbp2 and Ptc1 (Ho *et al*, 2002). Since previous studies showed that Pbs2 acted as a scaffold that binds Hog1, Sho1, Ste11 Ssk2/22 (Posas and Saito, 1997; Tatebayashi *et al*, 2003), and Ptc1 was shown to inactivate Hog1 (Warmka *et al*, 2001), we predicted that Nbp2 might act as an adapter, recruiting Ptc1 to the MAPK cascade via the Pbs2 scaffold. Indeed, we find that Nbp2 mediates Ptc1 binding to the Pbs2-Hog1 MEK-MAPK module. In this way, Nbp2 enables Ptc1 inactivation of Hog1.

Results

Nbp2 binds Ptc1 but not other PTCs in the HOG pathway

In the HOG pathway, three PTCs have been shown to inactivate Hog1. Ptc1 maintains low Hog1 activity in the absence of stress, and inactivates Hog1 during adaptation to stress (Warmka *et al*, 2001), while Ptc2 and Ptc3 set maximal Hog1 activity (Young *et al*, 2002). Ptc2 and Ptc3 are closely related to each other, but differ structurally from Ptc1 in containing a noncatalytic C-terminal domain (Young

et al, 2002). Since Nbp2 and Ptc1 were shown to interact by the yeast two-hybrid method (Ito *et al*, 2000; Uetz *et al*, 2000; Ho *et al*, 2002), but no evidence was found for Ptc2 and Ptc3, we tested whether Nbp2 was specific for Ptc1. GST-PTCs and Nbp2-HA were coexpressed in yeast from multicopy plasmids, and their ability to associate was examined in lysates. GST-Ptc1 co-precipitated with Nbp2-HA, but GST-Ptc2 and GST-Ptc3 could not bind Nbp2-HA (Figure 1B). GST-Ptc1 also bound Nbp2-HA when they were each expressed at lower levels from their endogenous promoters in low-copy *CEN*-based plasmids (data not shown). Thus, Nbp2 is specific for Ptc1.

NBP2 negatively regulates the HOG pathway

Since Ptc1 inactivates the HOG pathway by dephosphorylating Hog1 (Warmka *et al*, 2001), and Nbp2 binds Ptc1, we tested whether *NBP2* might also act as a negative regulator of the HOG pathway similar to *PTC1*. Previously, it was shown

that deletion of the phosphatases that regulate Hog1 dual phosphorylation on the activation loop Thr and Tyr exhibited a severe growth defect (Maeda *et al*, 1993), and that this was due to Hog1 hyperactivation (Jacoby *et al*, 1997). That is, *ptc1Δ ptp2Δ* had a severe growth defect while *ptc1Δ ptp2Δ hog1Δ* grew nearly as well as wild type (Jacoby *et al*, 1997). We tested whether deletion of *NBP2* together with *PTP2* might also exhibit growth defects. Indeed, the *nbp2Δ ptp2Δ* spore clones from a cross between *nbp2Δ* and *ptp2Δ* grew significantly worse than either strain alone (Figure 2A). To test whether this defect was due to *HOG1*, *ptp2Δ hog1Δ* was crossed to the *nbp2Δ* strain. The resulting *nbp2Δ ptp2Δ hog1Δ* spore clones grew significantly better than the *nbp2Δ ptp2Δ* double mutant (Figure 2B), demonstrating that *HOG1* was responsible for these defects. It was further tested whether the *nbp2Δ ptp2Δ* growth defect was simply due to delayed germination, or whether defects could be seen in vegetatively growing cells. When grown in liquid culture
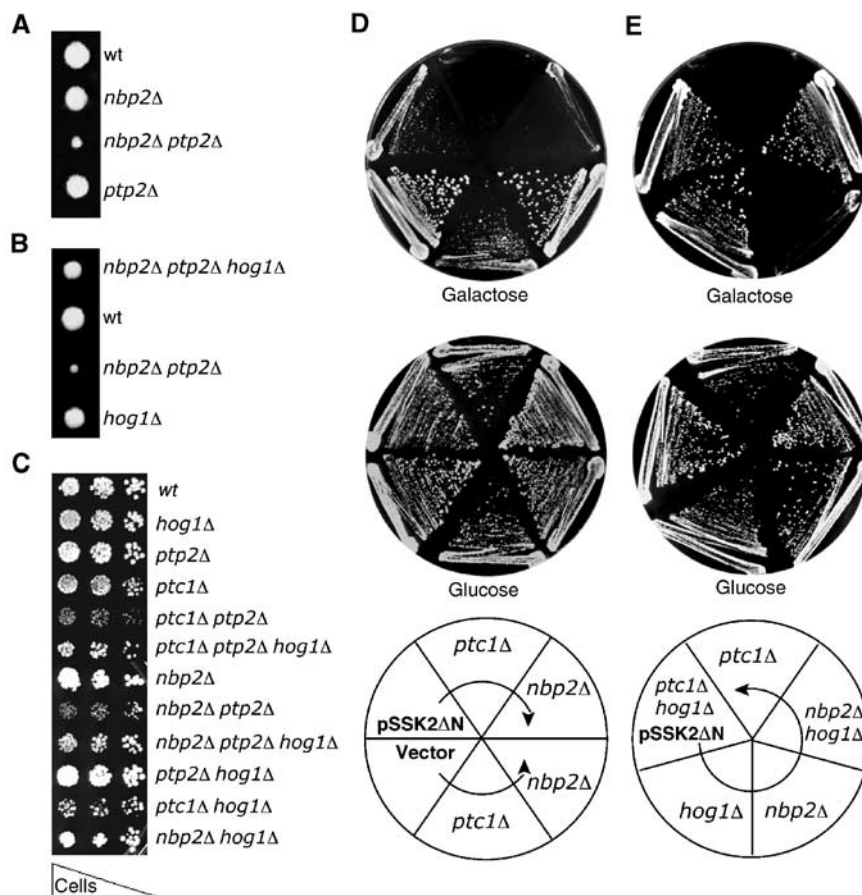

Figure 2 Strains lacking *NBP2* exhibit growth defects due to *HOG1*. (**A**) A strain lacking *NBP2* and *PTP2* has growth defects due to *HOG1*. A tetrad dissection of JHM20 (*MATα nbp2Δ::kanMX*) crossed to IMY21a (*MATα ptp2Δ::HIS3*) is shown. Dissectants were grown at 30°C for 72 h on standard rich media, YPD. (**B**) The *nbp2Δ ptp2Δ* growth defect is due to *HOG1*. Tetrad dissection of JHM20 (*MATα nbp2Δ::kanMX*) crossed to CMY10 (*MATα ptp2Δ::HIS3 hog1Δ::TRP1*) is shown. Dissectants were grown at 30°C for 72 h on YPD. (**C**) The growth defect of *nbp2Δ ptp2Δ*, which is suppressed in *nbp2Δ ptp2Δ hog1Δ*, can be seen in vegetatively growing cells. The growth of isogenic strains lacking *HOG1*, *PTP2*, *PTC1* and *NBP2* alone or in combination with each other in the BBY background as described in Table I was examined. The strains were grown in liquid YPD media, diluted to 1 unit ($A_{600\,nm}$), and six-fold serial dilutions were spotted onto solid YPD and incubated at 30°C for 3 days. (**D**) Deletion of *NBP2* or *PTC1* is lethal when the hyperactive MEKK allele *SSK2ΔN* is expressed. Wild-type, *ptc1Δ* and *nbp2Δ* strains in the JD52 background (Table I) were transformed with pSSK2ΔN (Warmka *et al*, 2001), a multicopy plasmid expressing *SSK2ΔN* under regulation of the *GAL1* promoter, or the empty vector pYES2 (Invitrogen). Strains were grown on selective medium containing galactose or glucose at 30°C for 3 days. (**E**) Lethality resulting from *SSK2ΔN* overexpression in the *nbp2Δ* and *ptc1Δ* strains is due to *HOG1*. The *ptc1Δ*, *nbp2Δ* and *hog1Δ* single mutants, and the *ptc1Δ hog1Δ* and *nbp2Δ hog1Δ* double mutants in the JD52 background (Table I) were transformed with pSSK2ΔN, and grown on selective medium containing galactose or glucose at 30°C for 3 days.

and plated onto rich media, both the *nbp2Δ ptp2Δ* and *ptc1Δ ptp2Δ* mutants had significant growth defects relative to the wild type and single mutants (Figure 2C). However, the triple mutants *nbp2Δ ptp2Δ hog1Δ* and *ptc1Δ ptp2Δ hog1Δ* grew better than the double mutants (Figure 2C). Thus, the synthetic growth defect of the *nbp2Δ ptp2Δ* double mutant is due to *HOG1*, similar to the *ptc1Δ ptp2Δ* mutant.

Another means to hyperactivate the HOG pathway is to overexpress the hyperactive *MEKK* allele *SSK2ΔN*, which leads to growth defects due to hyperactivation of the downstream Pbs2 MEK and Hog1 MAPK (Figure 1A) (Maeda *et al*, 1995). As predicted for a negative regulator, deletion of *PTC1* in the background of the *SSK2ΔN* overexpressor results in further activation of Hog1, which is lethal (Figure 2D and E; Warmka *et al*, 2001). That *ptc1Δ SSK2ΔN* lethality was due to *HOG1* was evident since *ptc1Δ hog1Δ SSK2ΔN* is viable (Figure 2E; Warmka *et al*, 2001). To test whether deletion of *NBP2* would have a similar effect, *SSK2ΔN* was overexpressed in the *nbp2Δ* and *nbp2Δ hog1Δ* strains. *SSK2ΔN* overexpression was lethal in the *nbp2Δ* strain (Figure 2D and E), while it was not lethal in the *nbp2Δ hog1Δ* strain (Figure 2E). These results confirm that *NBP2* is a negative regulator of the HOG pathway and suggest that Nbp2 likely acts downstream of the Ssk2 MEKK.

Nbp2 negatively regulates Hog1 kinase activity

Since genetic analysis showed that *NBP2* is a negative regulator of the HOG pathway, we tested whether Nbp2 could affect Hog1 MAPK activity as does Ptc1. Consistent with its role as a negative regulator, we showed that deletion of *PTC1* led to increased basal Hog1 kinase activity as well as an inability to inactivate Hog1 during adaptation (Warmka *et al*, 2001). Here, we find that deletion of *NBP2* has similar effects. In the *nbp2Δ* mutant, Hog1 basal activity was elevated ~3.5-fold (Figure 3) similar to *ptc1Δ*. In response to osmotic stress, Hog1 was activated, but like *ptc1Δ*, the *nbp2Δ* mutant showed an obvious defect in inactivating Hog1 during adaptation (Figure 3). As the level of Hog1 protein did not change, elevated Hog1 activity was most likely due to its increased phosphorylation. Thus, deletion of *NBP2* led to defects in Hog1 inactivation as does deletion of *PTC1*, suggesting that *NBP2* plays a role in Hog1 dephosphorylation *in vivo*.

Nbp2 mediates binding between the Ptc1 phosphatase and the Pbs2 MEK

Since Nbp2 has no sequence similarity to PP2C or other protein phosphatases, it must downregulate Hog1 by a novel mechanism. One possibility is that Nbp2 acts as an adapter, recruiting Ptc1 to Hog1 via the Pbs2-Hog1 MEK-MAPK module. This seems possible, since Nbp2 was found in a complex with the scaffold protein Pbs2 and Ptc1 (Ho *et al*, 2002). Furthermore, the Pbs2 scaffold has been shown to bind Hog1 (Posas and Saito, 1997), which would provide Ptc1 with access to Hog1. If the role of Nbp2 is to recruit Ptc1 to Pbs2, then deletion of *NBP2* should affect the ability of Ptc1 and Pbs2 to associate. Therefore, Ptc1-Pbs2 complex formation was examined in yeast lysates in the presence and absence of *NBP2*. Indeed, GST-Pbs2 co-precipitated with HA-Ptc1 in the wild type, but not in the *nbp2Δ* mutant (Figure 4). The GST-Pbs2 and HA-Ptc1 fusion proteins were expressed similarly in wild type and in *nbp2Δ*, indicating that their lack of interaction in the latter was due to the absence
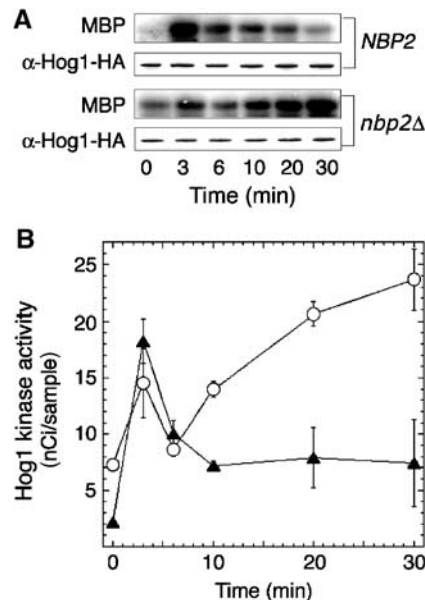

Figure 3 A strain lacking *NBP2* has increased basal Hog1 kinase activity and a defect in adaptation. (**A**) Hog1 kinase activity was examined prior to and following stress in the *NBP2* wild-type (IMY100) and in the *nbp2Δ* (JHM29) strains each carrying the Hog1-HA expression plasmid pHOG1-ha2 (Warmka *et al*, 2001). Before (time 0) and after exposure to osmotic stress (0.4 M NaCl) for various times, Hog1-HA was immunoprecipitated and incubated with MBP and [γ-^{32}P]ATP. Radiolabel incorporated into MBP was examined by SDS–PAGE and visualized using the PhosphorImager. Hog1-HA expression was the same in the two strains and unaltered by stress as indicated by the anti-HA blots of the lysates. (**B**) Hog1 kinase assays performed as in (A), for wild type (triangles) and *nbp2Δ* (circles) from three independent experiments. The plot shows the mean±s.d.

of Nbp2. Therefore, Nbp2 is required for Ptc1 to associate with Pbs2.

The Nbp2 SH3 domain binds Pbs2 and the novel N-terminal domain binds Ptc1

The above results showed that Nbp2 is needed to form a complex between Pbs2 and Ptc1. If Nbp2 recruits Ptc1 to Pbs2, then Nbp2 should be able to bind Ptc1 and Pbs2 simultaneously. If so, separate binding sites should exist on Nbp2 for Pbs2 and Ptc1. We predicted that the Nbp2 SH3 domain might bind Pbs2, as Pbs2 contains five Pro-rich or SH3 interaction motifs (*SIMs*) having the sequence PXXP, and one of these mediates binding to the upstream activator Sho1, which contains an SH3 domain (Posas and Saito, 1997). To map Nbp2 binding sites for Pbs2, GST-Nbp2 and -Nbp2 fragments containing or lacking the SH3 domain (Figure 5A) were coexpressed in yeast with Pbs2-HA. Full-length Nbp2 and Nbp2 fragments containing the SH3 domain bound Pbs2-HA, while Nbp2 fragments that lacked the SH3 domain could not bind Pbs2-HA (Figure 5B). Furthermore, the Nbp2 SH3 domain alone, residues 113–170, bound Pbs2-HA (Figure 5B). Thus the Nbp2 SH3 domain is necessary for binding Pbs2. Next, the Nbp2 binding site for Ptc1 was mapped. GST-Nbp2 associated with HA-Ptc1, as well as GST-Nbp2 fragments, which contained the N-terminal domain of this protein (Figure 5C). A fragment that contained the N-terminal domain alone, residues 1–115, also bound Ptc1, while neither
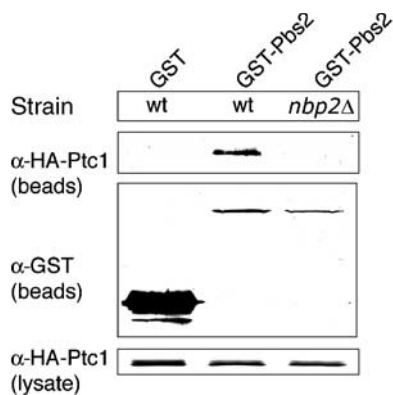
Figure 4 Ptc1 and Pbs2 associate in an Nbp2-dependent manner. Binding of HA-Ptc1 to GST-Pbs2 and GST was examined in the wild-type (JD52) and *nbp2Δ* (JHM17) strains. The strains carried pHA-PTC1 and pGST-PBS2 or the empty vector p(EG)KT, which expressed GST alone. GST-Pbs2 was isolated from yeast extracts using glutathione-sepharose, and the precipitated material was examined by SDS–PAGE and immunoblotting with anti-GST and anti-HA antibodies as described in Materials and methods. The amount of HA-Ptc1 in the lysates is also shown.

the SH3 domain alone nor the C-terminal domain alone bound Ptc1 (Figure 5C). Therefore, the N-terminal region is necessary for binding Ptc1.

The above studies do not show whether Nbp2 bound Ptc1 and Pbs2 directly, as other proteins might have mediated their interaction in yeast lysates. Therefore, Nbp2 fragments, full-length Pbs2 and Ptc1, were expressed individually in *Escherichia coli*, purified and their ability to associate was examined *in vitro*. Corroborating the results in yeast lysates, the GST-Nbp2 N-terminal domain alone bound 6xHis-Ptc1, while neither the GST-Nbp2 SH3 domain nor the GST-Nbp2 C-terminal domain could bind 6xHis-Ptc1 (Figure 5D). In addition, the Nbp2 SH3 domain bound Pbs2, while neither the Nbp2 N-terminal domain nor the Nbp2 SH3 domain could bind Pbs2 (Figure 5D). Taken together with the analysis in yeast lysates, these studies show that the Nbp2 N-terminal and SH3 domains are necessary and sufficient for binding Ptc1 and Pbs2, respectively.

We then examined how the Pbs2 scaffold might be bound to the SH3 domain of the positive regulator Sho1, and the SH3 domain of the negative regulator Nbp2. One possibility is that they might be bound via the same Pro-rich motif in Pbs2, since binding of the positive and negative regulators to Pbs2 could be mutually exclusive. Previously, the Sho1 SH3 domain was shown to bind Pbs2 via the sequence PLPPLP, residues 94–99 (SH3 interaction motif 1 (*SIM1*); Figure 6A), where the mutation, P96S, blocked signaling via this branch of the HOG pathway and inhibited binding to Sho1 (Maeda *et al*, 1995). We tested whether Nbp2 might also be bound at this site. However, Nbp2 bound the Pbs2-P96S mutant protein as well as the wild-type Pbs2 (Figure 6B). There are two other *SIMs* in the N-terminal noncatalytic domain of Pbs2 (Figure 6A). Therefore, we tested whether they might be involved in binding Nbp2 SH3. Mutation of one of these sites *SIM3*, PTRP residues 196–199 to AAAA, did not alter its ability to bind to Nbp2. However, mutation of *SIM2*, PRRP residues 187–190 to AAAA, blocked binding to Nbp2 (Figure 6B). Since Pbs2-*SIM2(AAAA)* was still able to complement the osmosensitivity of the *pbs2Δ* mutant (data
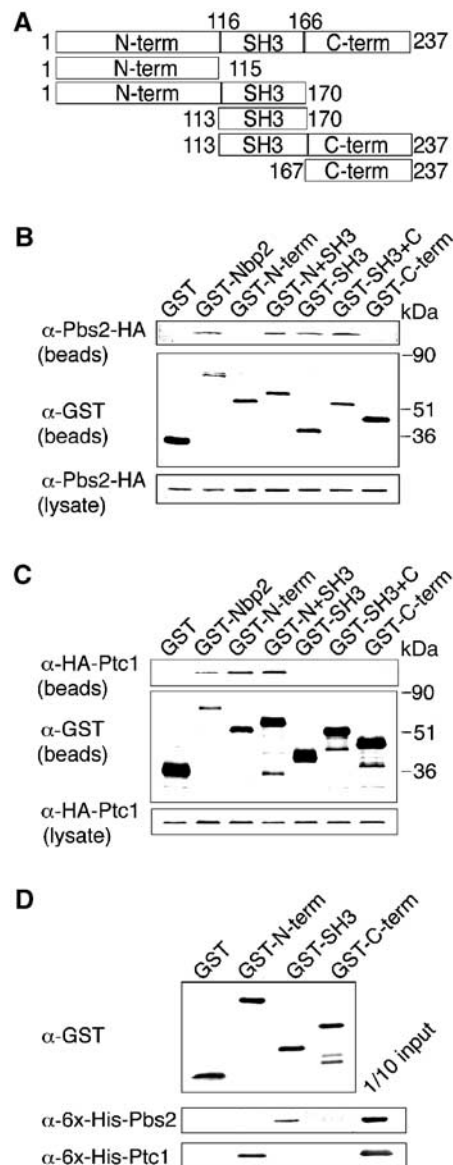

Figure 5 Identification of Nbp2 domains required for association with Ptc1 and Pbs2. (**A**) Schematic representation of Nbp2 and its fragments used in the Pbs2 and Ptc1 binding experiments. Nbp2 is a 237-residue protein with a central SH3 domain corresponding to residues 116–166. The N- and C-terminal domains are novel. (**B**) The Nbp2 SH3 domain (residues 113–170) binds Pbs2. The yeast strain AWY3 (*pbs2Δ*) expressed GST fused to full-length Nbp2, Nbp2 fragments or GST alone from the *CUP1* promoter, together with Pbs2-HA expressed from the *CUP1* promoter. GST-containing proteins were isolated using glutathione-sepharose, and the bound material was examined by SDS–PAGE and immunoblotting with anti-GST and anti-HA antibodies. The level of Pbs2-HA in the lysates is also shown. (**C**) The Nbp2 N-terminal domain (residues 1–115) binds Ptc1. The yeast strain ASY1 (*ptc1Δ*) expressed GST fusions to *NBP2* and its fragments or GST alone and HA-Ptc1 from the plasmid pHA-PTC1. Association between GST-containing proteins and HA-Ptc1 was examined as described in (B). (**D**) Recombinant Nbp2 SH3 domain and Nbp2 N-terminal domain bind Pbs2 and Ptc1, respectively. The ability of GST-Nbp2 N-terminal, GST-Nbp2 SH3 and GST-Nbp2 C-terminal domains, or GST alone to bind 6xHis-Pbs2 and 6xHis-Ptc1 was assessed as described in Materials and methods. The bound material was examined using anti-GST and anti-6xHis antibodies. The rightmost lane shows one-tenth the amount of 6xHis-Pbs2 and 6xHis-Ptc1 that was incubated with the GST-Nbp2 fusion proteins.
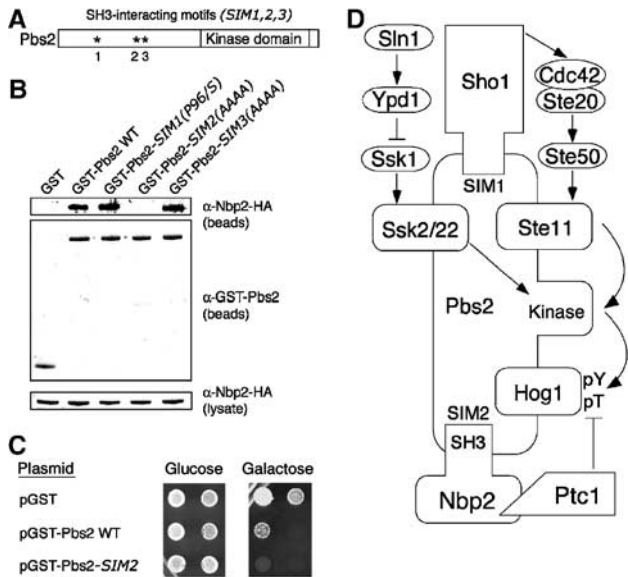
Figure 6 Nbp2 and Sho1 recognize different SH3 interaction motifs in Pbs2. (**A**) Schematic representation of the SH3 interaction motifs found in Pbs2. SH3 interaction motif 1 (*SIM1*, PLPPLP residues 94–99) is required for the Sho1-SH3 to bind. *SIM2* (PRRP residues 187–190) and *SIM3* (PTRP residues 196–199) have not been implicated previously in SH3 domain binding. (**B**) Pbs2-*SIM2* is required to bind Nbp2. GST fusions to Pbs2 and Pbs2 with mutations in *SIM1, 2* or *3* were coexpressed with Nbp2-HA in the *pbs2Δ* strain JHM61. GST fusion proteins were isolated using glutathione-sepharose, and the co-precipitated material was examined by SDS–PAGE and immunoblotting with anti-GST and anti-HA antibodies. The amount of Nbp2-HA in lysates was similar in all experiments as shown in the anti-HA blot. (**C**) Pbs2-*SIM2* mediates Nbp2-Ptc1 negative regulation of the HOG pathway. Growth of the *pbs2Δ ptp2Δ* strain IMY108 containing empty vector (pGST), GST-Pbs2 and GST-Pbs2-*SIM2(AAAA)* was compared. GST and GST-Pbs2 fusions were under control of the *GAL1* promoter and overexpressed on galactose-containing media. Cells at 30 units ($A_{600\,nm}$) were diluted 10-fold (left to right) and were plated as described in Figure 2C. (**D**) Model for Nbp2-Ptc1 regulation of the HOG pathway via the scaffold protein Pbs2. We show here that Nbp2 negatively regulates the HOG pathway by recruiting Ptc1 to the Pbs2 scaffold, facilitating Hog1 inactivation. Previously, we showed that Ptc1 inactivates this pathway by dephosphorylating Hog1-pT (Warmka *et al*, 2001). Thus, the Pbs2 scaffold binds the negative regulators in this pathway (Nbp2-Ptc1) and the positive regulators, including Sho1, the Ste11, Ssk2 and Ssk22 MEKKs and the Hog1 MAPK. Since Pbs2 bound Nbp2 and Sho1 via two different Pro-rich motifs (*SIMs*), Pbs2 could bind the negative and positive regulators simultaneously.

not shown), this mutant protein was not misfolded, and argues that the *SIM2* site is essential for Nbp2 binding. Therefore, two different *SIMs* within Pbs2 provide binding sites for Sho1 and Nbp2. Since these sites are more than 90 residues apart, it is possible that the positive regulator Sho1 and the negative regulator Nbp2 are bound simultaneously to Pbs2.

Last, we tested whether Nbp2-Ptc1 exerted its negative regulatory effect via binding Pbs2-*SIM2 in vivo*. If so, a strain overexpressing the *PBS2-SIM2(AAAA)* mutant should be more susceptible to HOG pathway hyperactivation than a strain overexpressing wild-type *PBS2*, as it lacks the ability to dephosphorylate Hog1-pThr. Since strong growth defects due to Hog1 hyperactivation require increased phosphorylation on both Thr and Tyr in its activation loop, we examined the effects of *PBS2-SIM2(AAAA)* and *PBS2* overexpression in a strain that lacked *PTP2*, which is defective in Hog1-pY

dephosphorylation. Overexpression of *PBS2* was detrimental to growth, but overexpression of the mutant *PBS2-SIM2(AAAA)* was lethal (Figure 6C). The control strain expressing empty vector was viable under these conditions. Thus, by binding *SIM2* within Pbs2, Nbp2-Ptc1 exerts its negative regulatory effect on this pathway.

Discussion

The mechanisms that target PP2C Ser/Thr phosphatases to their substrates in MAPK pathways are not clear. The significance of this work is the identification and characterization of a novel PP2C targeting subunit important for inactivating the HOG pathway. We showed that Nbp2 acts as an adapter in this pathway, mediating Ptc1 interaction with the scaffold protein Pbs2 MEK, as shown in the model in Figure 6D. As predicted for an adapter, deletion of *NBP2* blocked the ability of Ptc1 to bind Pbs2 (Figure 4), and Nbp2 contained separate binding sites for Ptc1 and Pbs2 (Figure 5). Furthermore, the Pbs2 scaffold, previously shown to bind Sho1 and other positive regulators (Posas and Saito, 1997), bound Sho1 and Nbp2 via distinct Pro-rich motifs (Figure 6). Consistent with the role of Nbp2 in recruiting Ptc1, Nbp2 acted as a negative regulator of Hog1 in genetic and biochemical studies. Deletion of the negative regulators *NBP2* and *PTP2* led to growth defects due to *HOG1* (Figure 2), and a strain lacking *NBP2* showed defects in downregulating Hog1 kinase activity (Figure 3). Thus, these defects are due to an inability to target Ptc1 to the Pbs2-Hog1 complex to inactivate Hog1 normally. Since *NBP2* influenced Hog1 activity, we tested the possibility that Nbp2 bound Hog1, but we could not reproducibly detect an interaction between Nbp2 and Hog1. However, as Pbs2 is a scaffold that binds Hog1 (Posas and Saito, 1997), Nbp2-Ptc1 binding to Pbs2 would provide access to Hog1.

We also tested whether Nbp2 interaction with Ptc1, or its interaction with Pbs2, was altered by osmotic stress. However, no difference could be detected in co-precipitation assays. Therefore, the Nbp2-Ptc1 complex does not appear to require Pbs2 phosphorylation for interaction. As a result of this, it is possible that the Nbp2-Ptc1 complex has constitutive activity against this pathway. This seems possible, as even in the absence of stress, deletion of the Hog1 negative regulators *NBP2* or *PTC1* together with *PTP2* led to pathway hyperactivation as evidenced by *HOG1*-dependent growth defects (Figure 2B and C), and deletion of *NBP2* or *PTC1* was sufficient to increase basal Hog1 kinase activity (Figure 3; Warmka *et al*, 2001).

Another important consideration for the proposed role for Nbp2 as an adapter, which mediates Ptc1 inactivation of Hog1 via Pbs2, is the subcellular localization of these components. We found that Nbp2, similar to Ptc1 (Warmka *et al*, 2001), is evenly distributed between the cytoplasm and the nucleus and that its localization does not change in response to stress (J Mapes and IM Ota, unpublished). The target of Nbp2-Ptc1, Hog1, accumulates in the nucleus in response to activation similar to other MAPKs (Ferrigno *et al*, 1998; Reiser *et al*, 1999), while the Pbs2 scaffold can be cytoplasmic and nuclear. Although previous studies had shown that Pbs2 was cytoplasmic and was excluded from the nucleus (Ferrigno *et al*, 1998; Reiser *et al*, 1999), a recent study showed that it contains a nuclear exclusion signal, which,

when masked, allows Pbs2 to accumulate in the nucleus (Tatebayashi *et al*, 2003). As a result of this, it is possible for Ptc1, bound to Pbs2 via Nbp2, to inactivate nuclear-localized Hog1. However, since phosphorylated Hog1 appears to exit the nucleus (Mattison and Ota, 2000), cytoplasmic Pbs2-Nbp2-Ptc1 could also inactivate Hog1.

Since Nbp2 acts as an adapter, recruiting Ptc1 to Pbs2 to inactivate Hog1, it is reasonable to propose that Ptc1 inactivates Pbs2, and this in turn leads to Hog1 inactivation. However, in previous studies where we examined the role of Ptc1 in this pathway, we could not detect Ptc1 inactivation of Pbs2, although it clearly inactivated Hog1. For example, overexpression of *PTC1* from a multicopy plasmid blocked osmotic stress-induced Hog1 kinase activation, but did not block Hog1-Tyr phosphorylation (Warmka *et al*, 2001). The latter suggests that *PTC1* overexpression does not downregulate Pbs2 activity. If it did, a decrease in the level of both Hog1-pThr and pTyr should have been detected. We also attempted to measure Pbs2 kinase activity, but we could not detect an increase in Pbs2 kinase activity upon osmotic stress. However, overexpression of *PTC1* diminished Pbs2 kinase activity, while its deletion increased Pbs2 activity (J Mapes and IM Ota, unpublished). At this time, we cannot exclude the possibility that Nbp2-Ptc1 inactivates Pbs2; however, our current data suggest that its activity toward Hog1 may be more significant.

Nbp2 exhibited another feature characteristic of an adapter for Ptc1. Nbp2 was not as strong a negative regulator as the Ptc1 phosphatase itself. For example, deletion of *SLN1*, which encodes a putative osmosensor and is a negative regulator of this pathway, is lethal due to Hog1 hyperactivation (Maeda *et al*, 1994). Overexpression of *PTC1* from a multicopy plasmid suppressed *sln1Δ* lethality (Warmka *et al*, 2001), while overexpression of *NBP2* from a multicopy plasmid did not (J Mapes and IM Ota, unpublished). In addition, overexpression of *NBP2* did not enhance the ability of Ptc1 to inactivate Hog1 kinase activity *in vivo* (data not shown). These outcomes seem reasonable, since overexpressing a gene encoding phosphatase catalytic activity should have a greater effect than overexpressing an adapter protein. Furthermore, these results show that Ptc1 does not become a better negative regulator of Hog1 when the level of Nbp2 protein is increased. Thus Nbp2 is unlikely to affect Ptc1 catalytic activity. Since the level of Pbs2 protein is ~10-fold lower than Ptc1 or Nbp2 (J Mapes and IM Ota, unpublished), we believe that the limiting factor in Nbp2-Ptc1 inactivation of Hog1 is the amount of Pbs2.

A study predicting interactions between yeast proteins containing SH3 domains and those proteins containing Pro rich motifs suggested that Nbp2 would bind Pbs2 in addition to other signaling proteins (Tong *et al*, 2002). One of these is the Bck1 MEKK in the cell wall integrity MAPK pathway. It is possible that Nbp2-Ptc1 could act in this pathway, since genetic analysis has linked Ptc1 to the Pkc1 protein kinase C (Huang and Symington, 1995), which acts upstream of Bck1. However, a role for Ptc1 as a negative regulator of this pathway has not been established. Another group of proposed Nbp2 interacting proteins were the p21-activated kinases (PAKs) Ste20, Cla4 and Skm1. Ste20 has been shown to act upstream of the MAPK cascades in the pheromone response and HOG pathways (Wu *et al*, 1995; Raitt *et al*, 2000). Although *CLA4* can substitute for *STE20* in the HOG

pathway, this may only occur in the *ste20Δ* strain. We tested whether Nbp2 might bind Ste20, but were not able to detect their interaction (data not shown). However, Ptc1 was found to co-precipitate with Skm1 (Ho *et al*, 2002). It will be interesting to ask whether Nbp2 mediates their interaction, and to examine the possibility that Skm1 has a role in MAPK pathways.

That PP2C and its targeting subunits are important in PAK regulation has recently been demonstrated in mammalian cells. A PP2C targeting subunit called PIX recruits PP2C phosphatases POPX1 and POPX2 to PAKs (Koh *et al*, 2002). PIX is a guanine nucleotide exchange factor, which also contains an SH3 domain that mediates the interaction with PAKs. We examined the possibility that there might be a common sequence motif that serves as a docking site for PP2C proteins on PIX and Nbp2. However, the PIX and Nbp2 PP2C binding domains share no obvious similarities. As more PP2C binding proteins are identified, it may be possible to identify a PP2C binding site consensus sequence.

A yeast synthetic lethal analysis conducted with *NBP2* and ~4700 viable yeast deletion mutants placed *NBP2* at a hub in a multinode network of interactions (Tong *et al*, 2002). Deletion of *NBP2* and genes involved in nuclear migration, spindle function, actin assembly, and actin and tubulin folding was synthetically lethal, suggesting that Nbp2 has a role in regulating the cytoskeleton. Furthermore, other nodes interacting with Nbp2 were occupied by Bni1 (which plays various roles in regulating the actin cytoskeleton), Bim1 (which binds tubulin and regulates spindle orientation), and the recently characterized Mti1/Bbc1 (which regulates the actin cytoskeleton by binding type I myosins) (Mochida *et al*, 2002). Since the HOG pathway has been implicated in cell morphogenesis in response to growth and stress signals (reviewed in Hohmann, 2002), and the cytoskeleton reorganizes in response to stress, Nbp2 might regulate these functions by acting as an adapter for Ptc1. It would be interesting to test whether deletion of *PTC1* with the same subset of genes is synthetic lethal. Alternatively, Ptc1 may have functions independent of Nbp2. If so, *ptc1Δ* may be synthetic lethal with other genes. The combination of molecular genetic, genomic and proteomic approaches such as these will shed more light on the role of this phosphatase adapter protein in yeast.

Materials and methods

Yeast strains and genetic techniques
Yeast strains (Table I) were derived from BBY45 and BBY48 and the galactose-inducible strains JD52 and JD53. The *nbp2Δ* strains were obtained by transformation with an *nbp2Δ::kanMX* allele, which was produced by PCR using oligonucleotides complementary to *NBP2* and *kanMX* contained in pRS400 (Brachmann *et al*, 1998). The *pbs2Δ ptp2Δ* strain IMY108 was produced by transforming JHM61 with the *ptp2Δ::HIS3* allele (Ota and Varshavsky, 1992). CMY1973 (*MATα hog1Δ*) was produced by transforming JD53 with *hog1Δ::hisG-URA3-hisG* (O'Rourke and Herskowitz, 1998) and growing on media containing 5-fluoroorotic acid to isolate the *hog1Δ::hisG* strain. Strains lacking *NBP2* in combination with *PTP2* and/or *HOG1* deletions were produced using standard genetic techniques.

Plasmids
Nbp2 fused to the hemagglutinin epitope (Nbp2-HA) was expressed in yeast from its endogenous promoter in the plasmid pNBP2-HA (*HIS3*, 2 μm). *NBP2* was isolated by PCR from genomic DNA

Table I Yeast strains used in this study

Strains derived from JD52 and JD53		
JD52	*MAT**a** trp1-Δ63 ura3-52 his3Δ200 leu2-3,112 lys2-801 GAL+*	Warmka *et al* (2001)
JD53	*MATα trp1-Δ63 ura3-52 his3Δ200 leu2-3,112 lys2-801 GAL+*	Warmka *et al* (2001)
JHM17	*MAT**a** nbp2Δ::kanMX trp1-Δ63 ura3-52 his3Δ200 leu2-3,112 lys2-801 GAL+*	This study
CMY1973	*MATα hog1Δ::hisG trp1-Δ63 ura3-52 his3Δ200 leu2-3,112 lys2-801 GAL+*	This study
JHM34	*MAT**a** nbp2Δ::kanMX hog1Δ::hisG trp1-Δ63 ura3-52 his3Δ200 leu2-3,112 lys2-801 GAL+*	This study
CMYX	*MAT**a** ptp2Δ::HIS3 hog1Δ::hisG trp1-Δ63 ura3-52 his3Δ200 leu2-3,112 lys2-801 GAL+*	This study
IMY107	*MATα ptc1Δ::LEU2 trp1-Δ63 ura3-52 his3Δ200 leu2-3,112 lys2-801 GAL+*	Warmka *et al* (2001)
JHM38	*MAT**a** ptc1Δ::LEU2 hog1Δ::hisG trp1-Δ63 ura3-52 his3Δ200 leu2-3,112 lys2-801 GAL+*	This study
JHM61	*MAT**a** pbs2Δ::kanMX trp1-Δ63 ura3-52 his3Δ200 leu2-3,112 lys2-801 GAL+*	This study
IMY108	*MAT**a** pbs2Δ::kanMX ptp2Δ::HIS3 trp1-Δ63 ura3-52 his3Δ200 leu2-3,112 lys2-801 GAL+*	This study
Strains derived from BBY45 and BBY48		
BBY45	*MAT**a** trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Warmka *et al* (2001)
BBY48	*MATα trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Warmka *et al* (2001)
JHM20	*MATα nbp2Δ::kanMX trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	This study
IMY21a	*MAT**a** ptp2Δ::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Ota and Varshavsky (1992)
CMY10	*MAT**a** ptp2Δ::HIS3 hog1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Mattison and Ota (2000)
JHM25	*MAT**a** nbp2Δ::kanMX ptp2Δ::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	This study
JHM29	*MAT**a** nbp2Δ::kanMX hog1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	This study
JHM21	*MAT**a** nbp2Δ::kanMX ptp2Δ::HIS3 hog1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	This study
IMY100	*MAT**a** hog1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Jacoby *et al* (1997)
ASY1	*MATα ptc1Δ::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Jacoby *et al* (1997)
ASY2	*MATα ptc1Δ::LEU2 ptp2Δ::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Jacoby *et al* (1997)
ASY3	*MATα ptc1Δ::LEU2 ptp2Δ::HIS3 hog1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Jacoby *et al* (1997)
IMY103	*MAT**a**::ptc1Δ::LEU2 hog1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Warmka *et al* (2001)
AWY3	*MAT**a** pbs2::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	Winkler *et al* (2002)
JHM29	*MAT**a** nbp2Δ::kanMX hog1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	This study

producing a fragment containing 340 bp upstream of the start codon and a *Not*I site substituted for the stop codon. The fragment was cloned into pRS423 (Sikorski and Hieter, 1989) and a *Not*I fragment containing three repeats of the HA epitope was ligated to produce pNBP2-HA. Nbp2-HA was functional as it suppressed the temperature sensitivity of the *nbp2Δ* strain.

GST-Nbp2 and its fragments were expressed from the *CUP1* promoter in pCUP1-GST (*URA3*, 2 μm). The vector was constructed by subcloning a fragment containing the *CUP1* promoter–GST–thrombin cleavage site from the modified pYEX4T-1 plasmid (Exclone, Invitrogen) into pRS426 (Sikorski and Hieter, 1989). *NBP2* was isolated from genomic DNA using PCR and cloned into pCUP1-GST to produce pGST-NBP2. GST-Nbp2 fragments produced by PCR were expressed in yeast from pCUP1-GST. The fragments contained *Eco*RI and *Sma*I sites at their 5′ and 3′ ends, respectively, which were used to clone into pCUP1-GST.

GST-PTC fusion proteins were expressed in yeast from pKT-PTC1 (Warmka *et al*, 2001), pKT-PTC2 (Young *et al*, 2002) and pKT-PTC3. *PTC3* was isolated from genomic DNA by PCR and cloned into p(EG)KT (Mitchell *et al*, 1993). HA-Ptc1 was expressed from the multicopy plasmid pHA-PTC1. pGFP-PTC1 (Warmka *et al*, 2001) was digested with *Not*I and *Bam*HI to remove GFP, and a fragment containing three repeats of the HA epitope was substituted. The HA-Ptc1 protein was functional as it complemented the *ptc1Δ* temperature-sensitive phenotype.

Plasmids expressing *PBS2* in yeast were constructed as follows. GST-Pbs2 was expressed from the *GAL* promoter in pGST-PBS2, which contains a 2.4 kb *Bam*HI–*Sac*I fragment of *PBS2* obtained using PCR. Pbs2-HA was expressed from the *CUP1* promoter in YEplac181 (*LEU2*, 2 μm) (Gietz and Sugino, 1988). The *PBS2* stop codon was replaced with a *Not*I site, and three repeats of the HA epitope were ligated to produce pCUP1-PBS2-HA. GST fused to Pbs2, or Pbs2 where *SIMs* were mutated, were expressed in pEG(KT). Pbs2-P96S was constructed by producing two PCR products that overlapped at the mutated Pro96, using the oligonucleotides 5′-GGGAAGAGGCGACAATGGCTTATTAACAATCTG-3′ (mutation underlined) and 5′-GCCATTGTCGCCTCTTCCCGTAGC-3′ (mutation underlined). Two other *SIMs* in Pbs2 were mutated using similar methods.

Plasmids for expression in *E. coli* were constructed as follows. GST fusions to Nbp2 fragments were expressed from pGEX-6P-1 (Novagen). *Eco*RI–*Sma*I fragments from the pCUP1-Nbp2 yeast plasmids were subcloned into pGEX-6P-1. The plasmid expressing 6xHis-Ptc1 was described previously (Warmka *et al*, 2001). 6xHis-Pbs2 was expressed from pRSETa-Pbs2, which was constructed by subcloning the 2.4 kb *Bam*HI–*Sac*I fragment from pGST-Pbs2 into pRSETa (Invitrogen).

Expression of Nbp2, Ptc1 and Pbs2 in *E. coli* and purification by affinity chromatography

NBP2 fragments were expressed from the pGEX-6P-1 plasmids in *E. coli* BL21(DE3) pLysS. The strains were grown in 2xYT medium at 37°C to 0.6 units at $A_{600\,nm}$, cooled to 23°C, and induced with 300 mM isopropyl-β-D-thiogalactoside at 23°C for 2 h. Cells were lysed in sonication buffer (Tris–HCl (pH 8.0), 100 mM NaCl, 0.05% Triton X-100 and 1 mM phenyl methylsulfonyl fluoride (PMSF)), then mixed with glutathione-sepharose (Pharmacia) for 1 h at 4°C. The resin was washed with the same buffer and eluted with buffer containing 20 mM glutathione. Fractions containing the GST fusion proteins were dialyzed against phosphate-buffered saline, and the GST-tagged protein was quantified using the Bradford assay. The GST, GST N-terminal and GST SH3 domains were greater than 99% pure and the GST C-terminal fragment was ∼95% pure as judged by SDS–PAGE and Coomassie staining. Expression and purification of 6xHis-Ptc1 and 6xHis-Pbs2 was performed as described previously (Warmka *et al*, 2001).

Co-precipitation assays

Binding between Nbp2-HA and GST-PTCs or GST alone was examined in yeast strain JHM17 (*nbp2Δ*) carrying pNBP2-HA and pKT-PTC1, pKT-PTC2, pKT-PTC3, or empty vector p(EG)KT. Yeast were grown in synthetic media lacking uracil and histidine and containing 2% galactose. Cells from 150 ml of culture grown to ∼1 unit ($A_{600\,nm}$) were harvested by centrifugation and disrupted using glass beads in 600 μl of lysis buffer (50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.1% Triton X-100, 5 mM MgCl$_2$, 5 mM MnCl$_2$ and 0.1% 2-mercaptoethanol) with protease inhibitors leupeptin, pepstatin A, antipain, aprotinin and chymostatin, each at 20 μg/ml, and 1 mM PMSF. The lysates were incubated with 30 μl of glutathione-sepharose and incubated for 1.5 h at 4°C. The resin was washed extensively, boiled in sample buffer, and the bound material was analyzed by SDS–PAGE and immunoblotting with anti-GST (Pharmacia) and anti-HA (HA.11, Covance) antibodies. All other binding experiments in yeast lysates were performed similarly.

Binding between GST-tagged Nbp2 fragments or GST, and 6xHis-Ptc1 or 6xHis-Pbs2 purified from *E. coli* was performed as follows. A measure of 1 μl of 100 μM GST-tagged protein was incubated with 5 μl of 20 μM 6xHis-Ptc1 for 1 h at 4°C with 50 μl of glutathione-sepharose in 100 μl of buffer (50 mM Tris–HCl (pH 7.5), 50 mM NaCl and 0.05% Triton X-100). The resin was washed four times with the same buffer, and then four times with buffer containing 150 mM NaCl. Binding between GST-Nbp2 fragments or GST and 6xHis-Pbs2 was carried out as above, except that the wash buffer contained 250 mM NaCl. The bound material was examined by SDS–PAGE and immunoblotting with anti-GST and anti-His$_6$ antibodies (Covance).

Hog1 kinase assay
Hog1 kinase activity was examined in wild-type and *nbp2Δ* strains as described previously (Warmka *et al*, 2001). Strains were grown in synthetic media lacking histidine to exponential phase, and then left untreated or exposed to 0.4 M NaCl for various times. Cells were harvested by centrifugation, lysed using glass beads in buffer A, and Hog1-ha was immunoprecipitated with anti-HA antibody (Covance). The immunoprecipitates were washed extensively, and incubated with [γ-^{32}P]ATP (0.1 mM, 8000 cpm/pmol) and myelin basic protein (MBP) (1 μM; Sigma) for 30 min at 30°C. Incorporation of ^{32}P into MBP was assessed by SDS–PAGE and PhosphorImager analysis (Molecular Dynamics).

References

Altman R, Kellogg D (1997) Control of mitotic events by Nap1 and the Gin4 kinase. *J Cell Biol* **138:** 119–130

Bardwell AJ, Flatauer LJ, Matsukama K, Thorner J, Bardwell L (2001) A conserved docking site in MEKs mediates high-affinity binding to MAP kinases and cooperates with a scaffold protein to enhance signal transmission. *J Biol Chem* **276:** 10374–10386

Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD (1998) Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14:** 115–132

de Nadal E, Alepuz PM, Posas F (2002) Dealing with osmostress through MAP kinase activation. *EMBO Rep* **3:** 735–740

Elion EA (2001) The Ste5p scaffold. *J Cell Sci* **114:** 3967–3978

Ferrigno P, Posas F, Koepp D, Saito H, Silver PA (1998) Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin beta homologs NMD5 and XPO1. *EMBO J* **17:** 5606–5614

Gietz RD, Sugino A (1988) New yeast–Escherichia coli shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74:** 527–534

Gustin MC, Albertyn J, Alexander M, Davenport K (1998) MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* **62:** 1264–1300

Hanada M, Kobayashi T, Ohnishi M, Ikeda S, Wang H, Katsura K, Yanagawa Y, Hiraga A, Kanamaru R, Tamura S (1998) Selective suppression of stress-activated protein kinase pathway by protein phosphatase 2C in mammalian cells. *FEBS Lett* **437:** 172–176

Hanada M, Ninomiya-Tsuji J, Komaki K, Ohnishi M, Katsura K, Kanamaru R, Matsumoto K, Tamura S (2001) Regulation of the TAK1 signaling pathway by protein phosphatase 2C. *J Biol Chem* **276:** 5753–5759

Ho Y, Gruhler A, Hellbut A, Bader GD, Moore L, Adams S-L, Millar A, Taylor P, Bennett K, Boutilier K, Yang L, Wolting C, Donaldson I, Schandorff S, Shewnarane J, Vo M, Taggart J, Goudreault M, Muskat B, Alfarano C, Desar D, Lin Z, Michallckova K, Willems AR, Sassi H, Nielsen PA, Rasmussen KJ, Andersen JR, Johansen LE, Hansen LH, Jespersen H, Podtelejnikov A, Nielsen E, Crawford JVP, Sorensen BD, Matthiesen J, Hendrickson RC, Gleeson F, Pawson T, Moran MF, Durocher D, Mann M, Hogue CWV, Figeys D, Tyers M (2002) Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. *Nature* **415:** 180–183

Hohmann S (2002) Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol Biol Rev* **66:** 300–372

Huang KN, Symington LS (1995) Suppressors of a *Saccharomyces cerevisiae* pkc1 mutation identify alleles of the phosphatase gene PTC1 and of a novel gene encoding a putative basic leucine zipper protein. *Genetics* **141:** 1275–1285

Ito T, Tashiro K, Muta S, Ozawa R, Chiba T, Nishizawa M, Yamamoto K, Kuhara S, Sakaki Y (2000) Toward a protein-protein interaction map of the budding yeast: a comprehensive system to examine two-hybrid interactions in all possible combinations between yeast proteins. *Proc Natl Acad Sci USA* **97:** 1143–1147

Jacoby T, Flanagan H, Faykin A, Seto AG, Mattison C, Ota I (1997) Two protein-tyrosine phosphatases inactivate the osmotic stress response pathway in yeast by targeting the mitogen-activated protein kinase, Hog1. *J Biol Chem* **272:** 17749–17755

Johnson GL, Lapadat R (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* **298:** 1911–1912

Kellogg D, Kikuchi A, Fujii-Nakata T, Turck CW, Murray AW (1995) Members of the NAP/SET family of proteins interact specifically with B-type cyclins. *J Cell Biol* **130:** 661–673

Koh C-G, Tan E-J, Manser E, Lim L (2002) The p21-activated kinase PAK is negatively regulated by POPX1 and POPX2, a pair of serine/threonine phosphatases of the PP2C family. *Curr Biol* **12:** 317–321

Maeda T, Takekawa M, Saito H (1995) Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. *Science* **269:** 554–558

Maeda T, Tsai AY, Saito H (1993) Mutations in a protein tyrosine phosphatase gene (PTP2) and a protein serine/threonine phosphatase gene (PTC1) cause a synthetic growth defect in *Saccharomyces cerevisiae*. *Mol Cell Biol* **13:** 5408–5417

Maeda T, Wurgler-Murphy SM, Saito H (1994) A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* **369:** 242–245

Martin-Blanco E, Gampel A, Ring J, Virdee K, Kirov N, Tolkovsky AM, Martinez-Arias A (1998) puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in *Drosophila*. *Genes Dev* **12:** 557–570

Mattison CP, Ota IM (2000) Two protein tyrosine phosphatases, Ptp2 and Ptp3, modulate the subcellular localization of the Hog1 MAP kinase in yeast. *Genes Dev* **14:** 1229–1235

Mattison CP, Spencer SS, Kresge KA, Lee J, Ota IM (1999) Differential regulation of the cell wall integrity mitogen-activated protein kinase pathway in budding yeast by the protein tyrosine phosphatases Ptp2 and Ptp3. *Mol Cell Biol* **19:** 7651–7660

Meskiene I, Baudouin E, Schweighofer A, Liwosz A, Jonak C, Rodriguez PL, Jelenik H, Hirt H (2003) Stress-induced protein phosphatase 2C is a negative regulator of a mitogen-activated protein kinase. *J Biol Chem* **278:** 18945–18952

Mitchell DA, Marshall TK, Deschenes RJ (1993) Vectors for the inducible overexpression of glutathione S-transferase fusion proteins in yeast. *Yeast* **9:** 715–722

Mochida J, Yamamoto T, Fujimura-Kamada K, Tanaka K (2002) The novel adaptor protein, Mti1p, and Vrp1p, a homolog of Wiskott–Aldrich syndrome protein-interacting protein (WIP), may antagonistically regulate type I myosins in *Saccharomyces cerevisiae*. *Genetics* **160:** 923–934

Nguyen AN, Shiozaki K (1999) Heat-shock-induced activation of stress MAP kinase is regulated by threonine- and tyrosine-specific phosphatases. *Genes Dev* **13:** 1653–1663

O'Rourke SM, Herskowitz I (1998) The Hog1 MAPK prevents cross talk between the HOG and pheromone response MAPK pathways in *Saccharomyces cerevisiae*. *Genes Dev* **12:** 2874–2886

Ota IM, Varshavsky A (1992) A gene encoding a putative tyrosine phosphatase suppresses lethality of an N-end rule-dependent mutant. *Proc Natl Acad Sci USA* **89:** 2355–2359

Pearson G, Robinson F, Gibson TB, Xu B-E, Karandikar M, Berman K, Cobb MH (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* **22:** 153–183

Posas F, Saito H (1997) Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK. *Science* **276:** 1702–1705

Raitt DC, Posas F, Saito H (2000) Yeast Cdc42 GTPase and Ste20 PAK-like kinase regulate Sho1-dependent activation of the Hog1 MAPK pathway. *EMBO J* **19:** 4623–4631

Reiser V, Ruis H, Ammerer G (1999) Kinase activity-dependent nuclear export opposes stress-induced nuclear accumulation and retention of Hog1 mitogen-activated protein kinase in the budding yeast *Saccharomyces cerevisiae. Mol Biol Cell* **10:** 1147–1161

Schaeffer HJ, Catling AD, Eblen ST, Collier LS, Krauss A, Weber MJ (1998) MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. *Science* **281:** 1668–1671

Shimizu Y, Akashi T, Okuda A, Kikuchi A, Fukui K (2000) NBP1 (Nap1 binding protein 1), an essential gene for G2/M transition of *Saccharomyces cerevisiae*, encodes a protein of distinct sub-nuclear localization. *Gene* **246:** 395–404

Sikorski RS, Hieter P (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae. Genetics* **122:** 19–27

Takekawa M, Maeda T, Saito H (1998) Protein phosphatase 2Calpha inhibits the human stress-responsive p38 and JNK MAPK pathways. *EMBO J* **17:** 4744–4752

Tanoue T, Nishida E (2002) Docking interactions in the mitogen-activated protein kinase cascades. *Pharmacol Ther* **93:** 193–202

Tatebayashi K, Takekawa M, Saito H (2003) A docking site determining specificity of Pbs2 MAPKK for Ssk2/Ssk22 MAPKKKs in the yeast HOG pathway. *EMBO J* **22:** 3624–3634

Tong AHY, Drees B, Nardelli G, Bader GD, Brannetti B, Castagnoli L, Evangelista M, Ferracuti S, Nelson B, Paoluzi S, Quondam M, Zucconi A, Hogue CWV, Fields S, Boone C, Cesareni G (2002) A combined experimental and computational strategy to define

protein interaction networks for peptide recognition modules. *Science* **295:** 321–324

Uetz P, Giot L, Cagney G, Mansfield TA, Judson RS, Knight JR, Lockshon D, Narayan V, Srinivasan M, Pochart P, Qureshi-Emili A, Li Y, Godwin B, Conover D, Kalbfleisch T, Vijayadamodar G, Yang M, Johnston M, Fields S, Rothberg JM (2000) A comprehensive analysis of protein–protein interactions in *Saccharomyces cerevisiae. Nature* **403:** 623–627

Warmka J, Hanneman J, Lee J, Amin D, Ota I (2001) Ptc1, a type 2C Ser/Thr phosphatase, inactivates the HOG pathway by dephosphorylating the mitogen-activated protein kinase Hog1. *Mol Cell Biol* **21:** 51–60

Whitmarsh AJ, Cavanagh J, Tournier C, Yasuda J, Davis RJ (1998) A mammalian scaffold complex that selectively mediates MAP kinase activation. *Science* **281:** 1671–1674

Winkler A, Arkind C, Mattison CP, Burkholder A, Knoche K, Ota I (2002) Heat stress activates the yeast high osmolarity glycerol MAPK pathway; protein tyrosine phosphatases are essential under heat stress. *Eukaryot Cell* **1:** 163–173

Wu C, Whiteway M, Thomas DY, Leberer E (1995) Molecular characterization of Ste20p, a potential mitogen-activated protein or extracellular signal-regulated kinase kinase (MEK) kinase kinase from *Saccharomyces cerevisiae. J Biol Chem* **270:** 15984–15992

Wurgler-Murphy SM, Maeda T, Witten EA, Saito H (1997) Regulation of the *Saccharomyces cerevisiae* HOG1 mitogen-activated protein kinase by the PTP2 and PTP3 protein tyrosine phosphatases. *Mol Cell Biol* **17:** 1289–1297

Young C, Mapes J, Hanneman J, Al-Zarban S, Ota I (2002) Role of Ptc2 type 2C Ser/Thr phosphatase in yeast high-osmolarity glycerol pathway inactivation. *Eukaryot Cell* **1:** 1032–1040

Zeitlinger J, Bohmann D (1999) Thorax closure in *Drosophila*: involvement of Fos and the JNK pathway. *Development* **126:** 3947–3956

Zhan XL, Guan KL (1999) A specific protein–protein interaction accounts for the *in vivo* substrate selectivity of Ptp3 towards the Fus3 MAP kinase. *Genes Dev* **13:** 2811–2827