

# Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin $\beta$ homologs NMD5 and XPO1

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**MAP kinase signaling modules serve to transduce extracellular signals to the nucleus of eukaryotic cells, but little is known about how signals cross the nuclear envelope. Exposure of yeast cells to increases in extracellular osmolarity activates the HOG1 MAP kinase cascade, which is composed of three tiers of protein kinases, namely the SSK2, SSK22 and STE11 MAPKKs, the PBS2 MAPKK, and the HOG1 MAPK. Using green fluorescent protein (GFP) fusions of these kinases, we found that HOG1, PBS2 and STE11 localize to the cytoplasm of unstressed cells. Following osmotic stress, HOG1, but neither PBS2 nor STE11, translocates into the nucleus. HOG1 translocation occurs very rapidly, is transient, and correlates with the phosphorylation and activation of the MAP kinase by its MAPKK. HOG1 phosphorylation is necessary and sufficient for nuclear translocation, because a catalytically inactive kinase when phosphorylated is translocated to the nucleus as efficiently as the wild-type. Nuclear import of the MAPK under stress conditions requires the activity of the small GTP binding protein Ran-GSP1, but not the NLS-binding importin  $\alpha/\beta$  heterodimer. Rather, HOG1 import requires the activity of a gene, *NMD5*, that encodes a novel importin  $\beta$  homolog. Similarly, export of dephosphorylated HOG1 from the nucleus requires the activity of the NES receptor XPO1/CRM1. Our findings define the requirements for the regulated nuclear transport of a stress-activated MAP kinase.**

**Keywords:** MAP kinase/nuclear transport/osmotic stress/protein phosphorylation

## Introduction

Eukaryotic cells have evolved a variety of mechanisms to relay information from the extracellular environment to the nucleus. One such mechanism consists of MAP (mitogen-activated protein, or microtubule associated protein) kinase cascades that typically transduce signals from plasma membrane-associated receptors to nuclear transcription factors, culminating in altered expression of target genes. One such pathway is the budding yeast high osmolarity

glycerol response (HOG) pathway (Brewster *et al.*, 1993). In this pathway, the MAP kinase HOG1 is activated following the phosphorylation of two key residues (Thr174 and Tyr176) by the dual-specificity MAP kinase kinase PBS2 (Brewster *et al.*, 1993). Activation of PBS2 in turn requires its phosphorylation by a MAP kinase kinase; this can be either STE11, or one of two partially redundant MAPKKs, SSK2 and SSK22 (Posas *et al.*, 1998). STE11 acts in conjunction with the transmembrane SH3 domain-containing protein SHO1, while SSK2 and SSK22 are regulated by a prokaryotic-like two component phospho-relay system consisting of SLN1, YPD1 and SSK1 (Maeda *et al.*, 1994; Posas *et al.*, 1996). The essential features of stress-activated MAP kinase pathways are conserved from yeast to humans (Ip and Davis, 1998), but how the activation of a protein kinase cascade postulated to reside in the cytoplasm may lead to changes in gene transcription remains unclear. Presumably, some component must cross the nuclear envelope. Although it has been shown that a mammalian MAP kinase homolog can translocate into the nucleus of PC12 cells under certain conditions (Traverse *et al.*, 1992, 1994), it remains to be shown whether this is a requirement for the transduction of the signal, and exactly how translocation is achieved.

Recent work has elucidated the basic mechanisms for the import of proteins into the nucleus. Many newly synthesized proteins that are destined for the nucleus contain a 'classical' nuclear localization signal (NLS). The NLS is recognized in the cytoplasm by the importin  $\alpha$  subunit of a heterodimeric NLS receptor, and targeted to the nuclear pore complex (NPC) by the importin  $\beta$  subunit. Translocation across the NPC requires the activity of the small Ras-related GTP binding protein Ran. It has been suggested that the nucleotide state of Ran may function either to determine the direction of movement (into or out of the nucleus) (Corbett *et al.*, 1995; Koepp *et al.*, 1996) or to regulate the loading of cargo onto nuclear transport factors (Melchior and Gerace, 1998). Recently, new homologs of importin  $\beta$  have been identified (Gorlich *et al.*, 1997), and have been found to play roles in such diverse pathways as the export of tRNA (Arts *et al.*, 1998; Kutay *et al.*, 1998) or proteins (Stade *et al.*, 1997; Kehlenbach *et al.*, 1998; Toone *et al.*, 1998) from the nucleus, or the recycling of hnRNPs back into the nucleus (Pollard *et al.*, 1996; Bonifaci *et al.*, 1997; Fridell *et al.*, 1997; Pemberton *et al.*, 1997; Siomi *et al.*, 1997; Senger *et al.*, 1998; Truant *et al.*, 1998). Thus, nuclear transport is now thought to involve a multiplicity of importin  $\beta$  homologs that serve to ferry specific cargoes in both directions across the nuclear envelope, in a manner that is regulated by Ran-GSP1.

Here, we localize the components of the HOG1 signaling module, identify the regulatory mechanisms that determine the subcellular localization of HOG1 MAPK, and charac-

terize the mechanism by which HOG1 is imported into, and subsequently exported from, the nucleus. Our data suggest a new function for the regulatory phosphorylation sites of protein kinases of the MAP kinase superfamily, and by revealing a new nuclear import pathway, shed further light on the complex mechanisms that determine the compartmentalization of eukaryotic cells.

## Results

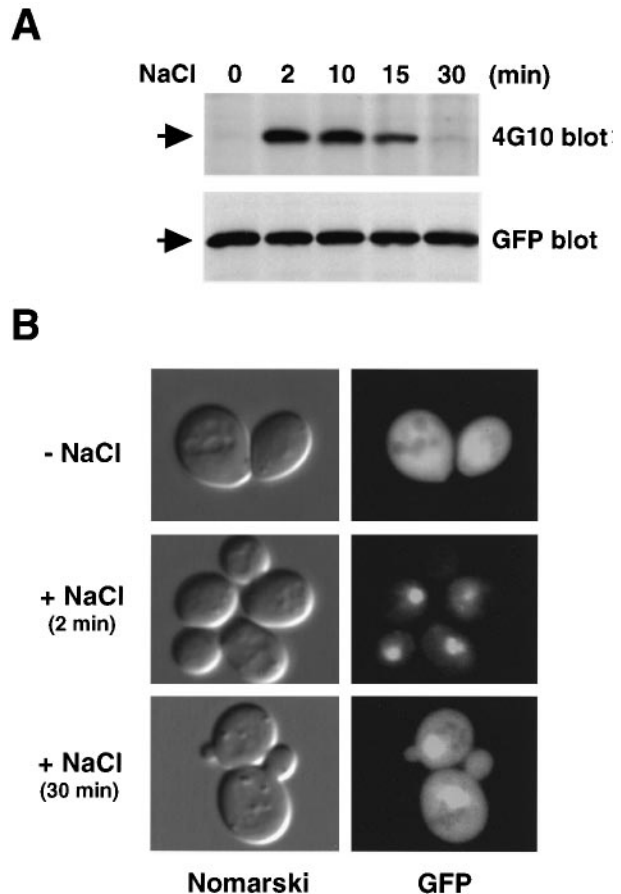
### **The HOG1 MAP kinase translocates transiently to the nucleus after osmotic stress whereas the MAPKK PBS2 and the MAPKKK STE11 remain cytoplasmic**

To analyze the spatial organization of the HOG1 signaling pathway in yeast, we genetically fused the open reading frames of the MAPK *HOG1*, the MAPKK *PBS2* and the MAPKKK *STE11* to the N-terminus of the green fluorescent protein (GFP). When expressed from their own promoters from a low-copy number (*CEN*) plasmid, each GFP fusion was able to rescue the osmosensitivity of cells lacking the corresponding wild-type gene (data not shown), demonstrating that each fusion protein is functional, and reflects the behavior of the corresponding wild-type protein. Moreover, immunoblotting of extracts from cells expressing these GFP-tagged proteins, confirmed that the proteins were expressed, and no free GFP was observed (data not shown).

Initially, we focused on the HOG1 MAPK. Microscopic examination of cells expressing HOG1-GFP revealed that HOG1 localizes throughout the cytoplasm and nucleus of unstressed cells (Figure 1B, -NaCl). When cells carrying HOG1-GFP were exposed to a brief osmotic shock (0.4 M NaCl for 2 min), HOG1 changed its distribution from being mainly cytoplasmic to accumulating completely within the nucleus (Figure 1B, +NaCl). After 30 min in the presence of NaCl, the levels of HOG1-GFP in the cytoplasm were partially restored, indicating that nuclear localization of HOG1 is a transient event. Interestingly, nuclear localization correlates with the transient HOG1 phosphorylation that is observed after osmotic stress (Figure 1A, 4G10 blot). The appearance of HOG1-GFP in the cytoplasm after 30 min of stress is the result of nuclear export, rather than new protein synthesis, since addition of the protein inhibitor cyclohexamide does not affect the observed distribution (data not shown). In addition, the levels of HOG1-GFP were found not to change during the osmotic stress response (Figure 1A, GFP blot).

We asked whether the translocation of HOG1-GFP into the nucleus was specific for osmotic stress (the only form of stress known to activate HOG1). Indeed, only osmotic stress (0.4 M NaCl or 1 M sorbitol) could induce HOG1-GFP translocation (Figure 2); 7.5% ethanol, 1 mM *t*-butyl hydrogen peroxide and 20 min temperature shift (37°C) all failed to drive the nuclear translocation of HOG1 (Figure 2). These data show that translocation of HOG1 into the nucleus is an integral feature of the osmotic stress response.

We next asked whether the kinases upstream of the HOG1 MAPK would co-localize with HOG1. A functional fusion of the STE11 MAPKKK and PBS2 MAPKK to GFP localized exclusively to the cytoplasm of unstressed



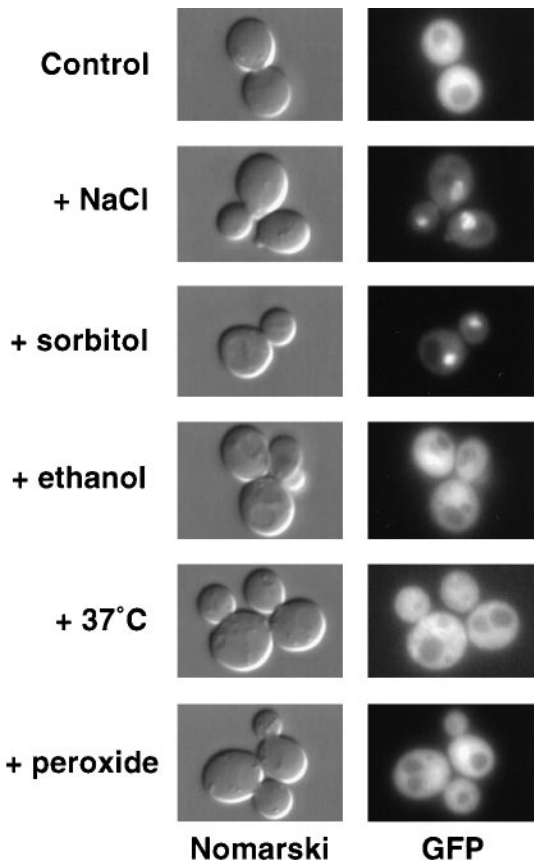
**Fig. 1.** HOG1 MAPK is transiently phosphorylated and translocated to the nucleus after osmotic stress. **(A)** Transient phosphorylation of HOG1 after osmotic stress. A *hog1*Δ strain (TM233) was transformed with GFP-tagged HOG1. Cells were exposed to 400 mM NaCl for the time indicated, and tyrosine-phosphorylated HOG1-GFP (Hog1p) was detected by immunoblotting with monoclonal antibody 4G10 to phosphotyrosine. The substitution of KCl for NaCl had no effect on the outcome, in this or any of the following experiments. **(B)** Nucleo-cytoplasmic redistribution of HOG1 after osmotic stress. The *hog1*Δ strain (TM233) was transformed with the HOG1-GFP plasmid. Cells were grown to mid-log phase and aliquots were taken before (-NaCl) or after the addition of 0.4 M NaCl (+NaCl) at the indicated times. HOG1-GFP was detected by fluorescence microscopy as described in Materials and methods.

cells, and did not relocate to the nucleus of stressed cells (Figure 3). These data indicate that the MAPK is the only element of the pathway that is relocated to the nucleus after stress.

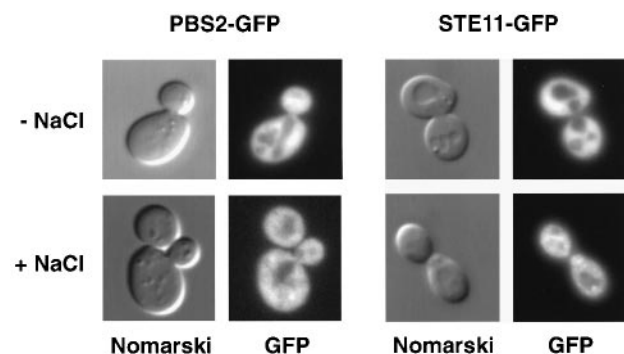
### **Phosphorylation of HOG1 is required for translocation after stress**

The stress-induced nuclear translocation of HOG1 correlates with phosphorylation by PBS2 MAPKK at residues that regulate HOG1 protein kinase activity (Figure 1A). Thus, there are three potential mechanisms that may regulate the sub-cellular localization of HOG1: (i) the phosphorylation of T174/Y176 residues; (ii) the activation of HOG1 protein kinase activity; or (iii) changes in the association of HOG1 with its MAPKK, PBS2.

The localization of HOG1-GFP was analyzed in a *pbs2*Δ strain. We observed that the protein was both nuclear and cytoplasmic, although the nuclear signal was stronger than in wild-type cells. In marked contrast to

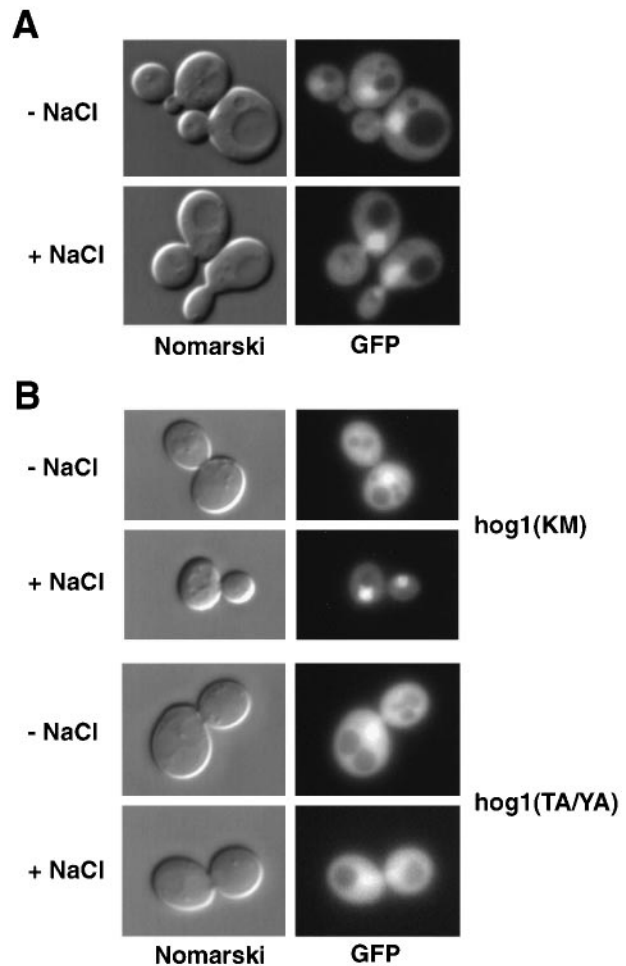


**Fig. 2.** HOG1 translocates to the nucleus only under osmotic stress conditions. The *hog1* $\Delta$  strain (TM233) was transformed with the HOG1–GFP plasmid. Cells were grown to mid-log phase and exposed to several stresses for 5 min, to a final concentration of 0.4 M NaCl (NaCl), 1 M sorbitol (sorbitol), 7.5% ethanol (ethanol), 1 mM *t*-butyl hydrogen peroxide (peroxide). Cells were incubated for 20 min at 37°C for the heat stress (37°C).



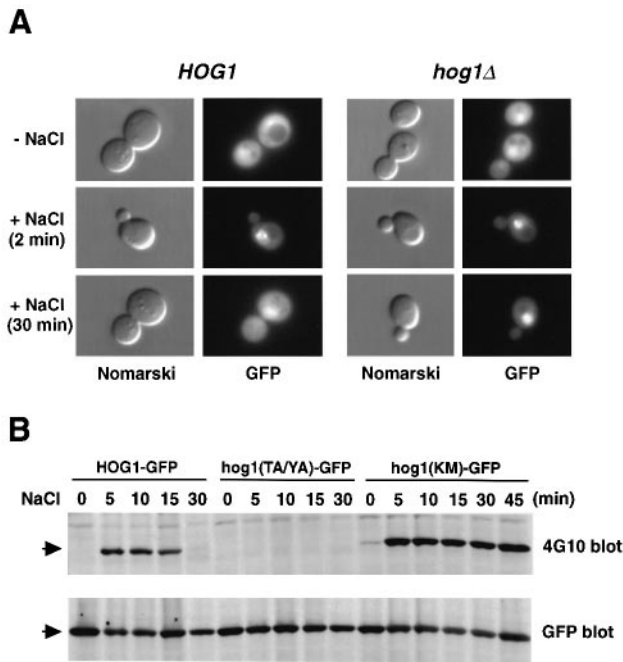
**Fig. 3.** Cytoplasmic localization of the PBS2 MAPKK and STE11 MAPKKK. PBS2 and STE11 were tagged to GFP and transformed to their respective null mutants. Cells were grown to mid-log phase and collected before (–NaCl) or 5 min after (+NaCl) the addition of 0.4 M NaCl. GFP proteins were detected by fluorescence microscopy as described in Materials and methods.

wild-type cells, nuclear translocation of HOG1–GFP did not occur after stress (Figure 4A). This indicates that the activity of the MAPKK is absolutely required for HOG1–GFP accumulation in the nucleus after stress. It is worth noting that a proportion of HOG1 always remains cytoplasmic in a *pbs2* $\Delta$  strain (Figure 4A), which indicates that PBS2 cannot be the only mechanism for tethering HOG1 in the cytoplasm.



**Fig. 4.** Phosphorylation of HOG1 is required for its nuclear translocation. (A) HOG1 does not translocate to the nucleus in a *pbs2* $\Delta$  strain. HOG1–GFP plasmid was transformed to the *pbs2* $\Delta$  strain (TM261). Cells were grown to mid-exponential phase and collected before (–NaCl) or 5 min after (+NaCl) the addition of 0.4 M NaCl. GFP proteins were detected by fluorescence microscopy as described in Materials and methods. (B) The *hog1*(TA/YA) mutant does not translocate to the nucleus after osmo-stress. A catalytically inactive HOG1, *hog1*(KM), and the non-phosphorylatable form of HOG1, *hog1*(TA/YA), were transformed into a *hog1* $\Delta$  strain (TM233). Translocation to the nucleus was tested as before by addition of NaCl 0.4 M for 5 min.

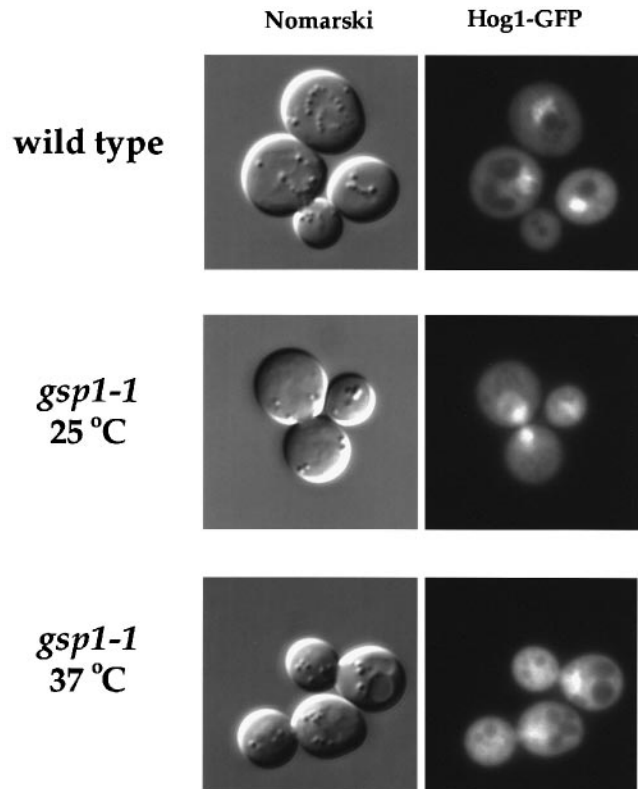
Thus, we have shown that the stress-induced nuclear translocation of HOG1–GFP correlates with its phosphorylation, and that PBS2 is required for each of these events to occur. Because phosphorylation of HOG1 mediates its activation as a protein kinase, we wished to distinguish between the possibility that the protein kinase activity of HOG1 was mediating its translocation, or that the phosphorylation of the activating residues Thr174/Tyr176 was sufficient. Two mutants were constructed; *hog1*(TA/YA) is a mutant in which the two phosphorylation sites required for MAPK activation are mutated to Ala. This mutant cannot be phosphorylated after stress (Figure 5B) and cannot complement the osmosensitivity of a *hog1* $\Delta$  strain (data not shown). The second mutant, *hog1*(KM) can be phosphorylated at Thr174/Tyr176, but this time the phosphorylated protein is devoid of protein kinase activity due to a mutation of a Lys to a Met in the active site. This mutant is also unable to complement the osmosensitivity of a *hog1* $\Delta$  strain.



**Fig. 5.** The degree of HOG1 phosphorylation determines its nuclear localization. (A) Retention of phosphorylated HOG1 into the nucleus after osmotic stress. *hog1(KM)* was transformed into a wild-type (*HOG1*) or *hog1*-deficient strain (*hog1Δ*). Cells were collected before (–NaCl) or after addition of 0.4 M NaCl (+NaCl) at the indicated times. GFP proteins were detected by fluorescence microscopy as described in Materials and methods. (B) Time course of HOG1 tyrosine phosphorylation. TM233 cells (*hog1Δ*) were transformed with the indicated alleles of *HOG1*. Cells were exposed to 0.4 M NaCl for the indicated times, and tyrosine-phosphorylated HOG1 was detected by immunoblotting with monoclonal antibody 4G10 to phosphotyrosine. GFP-fusion proteins were detected in the same blot using polyclonal anti-GFP antibody.

The *hog1(TA/YA)*-GFP allele failed to localize to the nucleus after osmotic stress (Figure 4B, bottom). This is consistent with the previous result that phosphorylation of HOG1 is required for its translocation into the nucleus. In contrast, *hog1(KM)* behaves identically to wild-type HOG1, demonstrating that the protein kinase activity of HOG1 is not required for its stress-induced translocation into the nucleus. Together, these data show that phosphorylation of HOG1, but not activation of its kinase activity, is required for HOG1 movement into the nucleus.

Dephosphorylation of HOG1 correlates with its exit from the nucleus (Figure 1). The protein phosphatases that dephosphorylate Y176 of HOG1, namely PTP2 and PTP3, have been shown to be activated by HOG1-mediated phosphorylation (Jacoby *et al.*, 1997; Wurgler-Murphy *et al.*, 1997). Thus, when a kinase-deficient allele of HOG1 is expressed in a *hog1*-deficient strain, its level of phosphorylation after stress remains higher for at least 45 min due to the lack of phosphatase activity (Figure 5B). We asked whether dephosphorylation of nuclear HOG1 is required for its return to the cytoplasm. The *hog1(KM)* mutant allele was transformed into a wild-type (*HOG1*) or a *hog1*-deficient strain (*hog1Δ*). Interestingly, return of *hog1(KM)* to the cytoplasm in the wild-type strain, where phosphatases can be activated by the wild-type allele, was faster than in the *hog1Δ* strain. This result is consistent with the idea that phosphorylation of HOG1 drives its



**Fig. 6.** HOG1 translocation requires the activity of Ran-GSP1. Wild-type cells or cells carrying a temperature-sensitive *GSP1* allele (*gsp1-1*) were transformed with the HOG1-GFP plasmid. Cells were incubated at the restrictive temperature (37°C) for 1 h and collected before (–NaCl) or 5 min after (+NaCl) the addition of 0.4 M NaCl. HOG1-GFP was localized as described above.

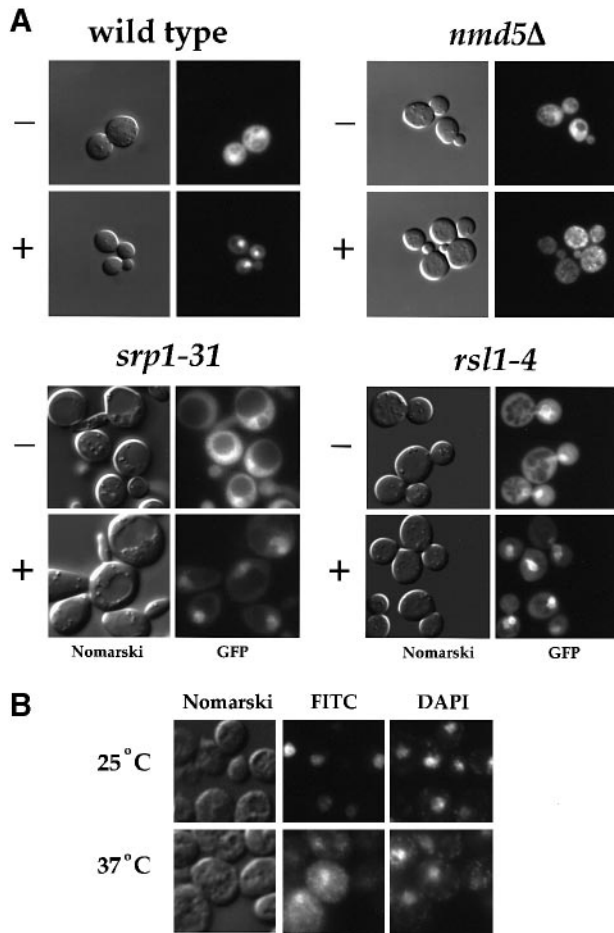
nuclear accumulation, and that its dephosphorylation mediates its return to the cytoplasm.

#### **Nuclear accumulation of HOG1 requires Ran-GSP1**

To date, all nuclear transport events require the activity of the small GTP-binding protein Ran-GSP1. We asked whether this was also the case for the nuclear translocation of HOG1-GFP. *gsp1-1* cells that carry a temperature-sensitive mutant allele of *GSP1* are unable to translocate proteins carrying prototypical nuclear import signals into the nucleus at restrictive temperatures (Wong *et al.*, 1997). Similarly, *gsp1-1* cells were unable to translocate HOG1-GFP into the nucleus in response to osmotic stress after 1 h incubation at the restrictive temperature (Figure 6). The failure to translocate HOG1 was not due to a defect in the activation of the HOG kinase cascade, as HOG1 became phosphorylated in *gsp1-1* cells even after shift to the restrictive temperature (data not shown). This finding confirms that the stress-induced translocation of HOG1 proceeds via a mechanism that, like all nuclear proteins studied thus far, requires Ran-GSP1.

#### **Nuclear accumulation of HOG1 is independent of the importin $\alpha/\beta$ heterodimer, but requires a novel importin $\beta$ homolog, NMD5**

We next addressed the mechanism whereby cytoplasmic HOG1 is targeted to the nucleus. First, we asked whether the classical importin  $\alpha/\beta$  heterodimer was responsible for this event. Cells carrying a temperature-sensitive



**Fig. 7.** Nuclear translocation of HOG1-GFP requires NMD5, but neither importin  $\alpha$  nor importin  $\beta$ . (A) Cells carrying a temperature-sensitive allele of yeast importin  $\alpha$  (*srp1-31*) or yeast importin  $\beta$  (*rsl1-4*), or cells deleted for the non-essential gene *NMD5* were transformed with the HOG1-GFP plasmid. Cells were incubated at the restrictive temperature (37°C) for 1 h and collected before (-NaCl) or 5 min after (+NaCl) the addition of 0.4 M NaCl. HOG1-GFP was localized as described above. (B) *rsl1-4* cells expressing a fusion of the SV40 NLS to invertase were incubated for 1 h at 25°C (upper row) or 37°C (lower row). The cells were processed for immunofluorescence as described (Ferrigno and Silver, 1998), and the localization of the SV40NLS-invertase detected with affinity-purified anti-invertase antibody and a FITC-conjugated secondary antibody (FITC). Nuclei were visualized by counter staining with DAPI.

mutation of the importin  $\alpha$  subunit (*srp1-31*, Figure 7A) or the importin  $\beta$  subunit (*rsl1-4*, Figure 7A) did not mislocalize HOG1-GFP to the nucleus in the absence of stress. More importantly, both *srp1-31* and *rsl1-4* cells were able to translocate HOG1-GFP into the nucleus in response to stress at the restrictive temperature (Figure 7A), even though each of these has a nuclear import defect at the higher temperature (Loeb *et al.*, 1995; Figure 7B). Together, these data suggest that the import of HOG1-GFP into the nucleus does not proceed via the importin  $\alpha/\beta$  heterodimer. Accordingly, we studied HOG1-GFP translocation through a panel of mutant strains deficient in one or more members of the importin superfamily, including *kap104Δ*, *kap123Δ*, *sxm1Δ*, *pse1-1*, *msn5Δ*, *xpo1-1*, *cse1-1*, *lph2Δ*, *los1-1*, *sok1Δ* and *mtr10Δ*. None of these showed a defect in nuclear import of HOG1-GFP after stress, and none mislocalized HOG1 to the nucleus in the absence of stress (data not shown).

In contrast, *nmd5Δ* cells completely failed to translocate HOG1 into the nucleus following stress treatment. HOG1-GFP becomes phosphorylated in *nmd5Δ* cells (data not shown), but completely fails to translocate into the nucleus even 30 min after stress (Figure 7A), by which time HOG1 has returned to the cytoplasm of wild-type cells (Figure 1). This genetic approach argues that the importin  $\beta$  homolog NMD5 is required for the complete translocation of HOG1-GFP into the nucleus of stressed cells.

#### **NMD5-GFP transiently translocates to the nucleus following osmotic stress**

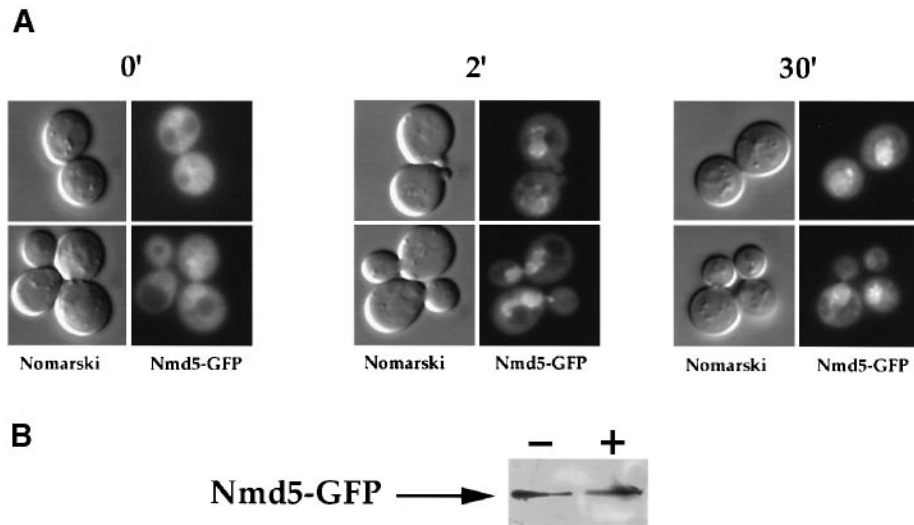
We wished to characterize further the function of NMD5. We constructed a fusion of the NMD5 ORF to GFP, or to a triple repeat of the hemagglutinin (HA) tag, and placed these fusions under the control of the *NMD5* promoter. Each of these constructs was able to rescue the phenotypes of *nmd5Δ* cells, including HOG1 translocation (data not shown), demonstrating that they were functional. In addition, immunoblotting revealed that the NMD5-GFP protein was expressed (Figure 8B). Microscopic examination of *nmd5Δ* cells expressing NMD5-GFP from *CEN* plasmids revealed that the protein localized throughout the cytoplasm of unstressed cells (Figure 8A). Within 2 min of stress, NMD5-GFP (but not other importin  $\beta$  homolog-GFP fusions, including KAP95, PSE1, SXM1 or XPO1, not shown) accumulated at the nuclear periphery and in the nucleus (Figure 8A). The NMD5-GFP fusion began to relocate to the cytoplasm within 30 min following stress. Stress was not observed to affect the levels of NMD5-GFP (Figure 8B). The correlation between NMD5 and HOG1 movement further supports the idea that HOG1 import into the nucleus may be mediated by NMD5.

#### **The export of dephosphorylated HOG1 from the nucleus requires a functional NES receptor**

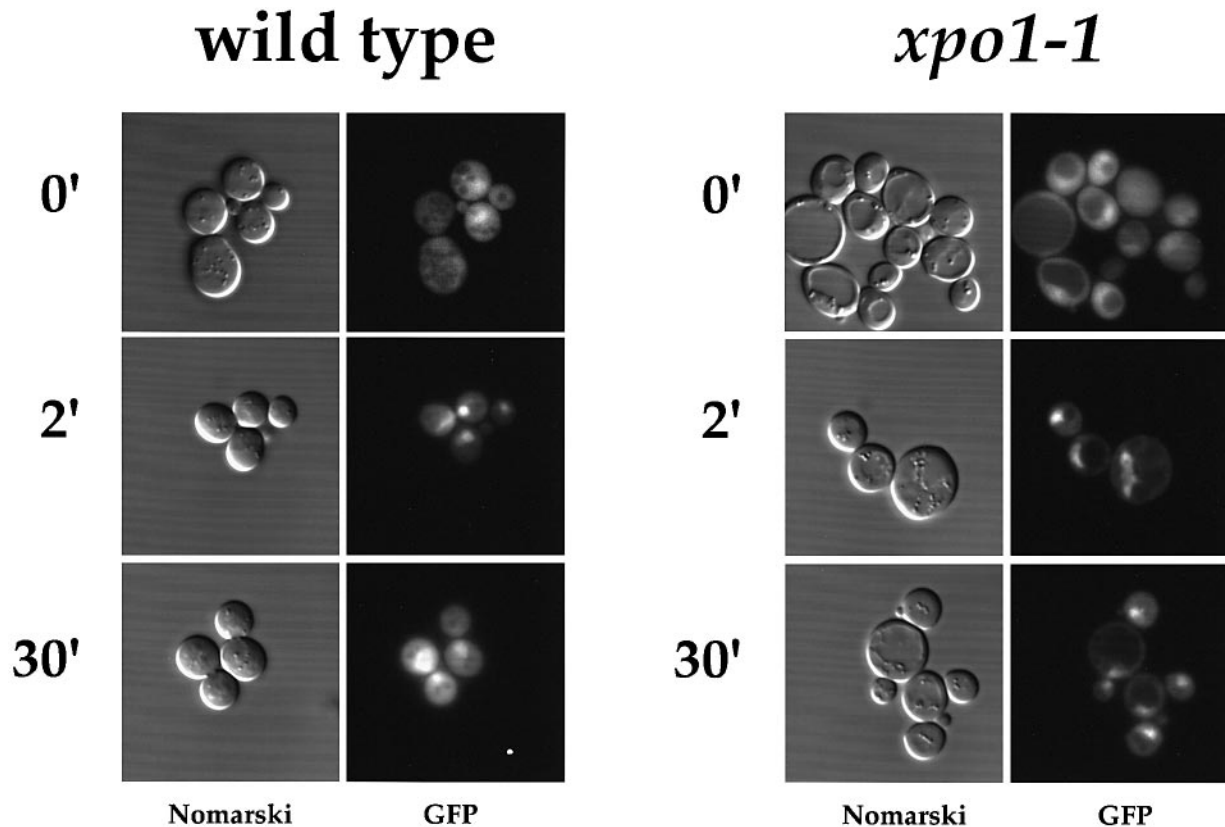
The nuclear localization of HOG1-GFP was observed to be transient (Figure 1). Even when cells remain exposed to stress, much of HOG1-GFP returns to the cytoplasm within 30 min. Nuclear export of a number of proteins has been shown to be mediated by a leucine-rich nuclear export sequence (NES) that is recognized by an importin  $\beta$  homolog, XPO1-CRM1, the so-called NES receptor (Stade *et al.*, 1997). Accordingly, we made use of a temperature-sensitive mutation of the NES receptor, *xpo1-1*, to test distribution of HOG1-GFP after osmotic stress at restrictive temperature. HOG1-GFP failed to exit the nucleus of *xpo1-1* cells (Figure 9A). No other importin  $\beta$  homolog tested (see above) was defective in HOG1 export (data not shown). Interestingly, HOG1-GFP that was trapped in the nucleus of *xpo1-1* cells was still dephosphorylated with the same kinetics as wild-type (not shown), suggesting that at least a proportion of the relevant tyrosine phosphatases PTP2 and PTP3 is in the nucleus of stressed cells.

## **Discussion**

We have localized the components of the HOG1 MAP kinase cascade in *Saccharomyces cerevisiae*. Functional fusions to GFP of the MAPKKK (STE11), the MAPKK (PBS2) and the MAPK (HOG1) allowed all three protein kinases to be localized to the cytoplasm (Figures 1 and 3).



**Fig. 8.** (A) NMD5-GFP relocates to the nuclear periphery following stress. Cells expressing NMD5-GFP under the control of its own promoter were observed by fluorescence microscopy before (0'), and 2 and 30 min after the addition of 0.4 M NaCl. (B) Immunoblotting analysis reveals that NMD5-GFP is expressed as a protein of ~147 kDa, whose abundance does not change following stress.



**Fig. 9.** Nuclear export of HOG1-GFP in stress-adapted cells requires the NES receptor XPO1. Wild-type cells, or cells carrying a temperature-sensitive allele of the importin  $\beta$  homolog XPO1-CRM1 (*xpo1-1*) were transformed with the HOG1-GFP plasmid. Cells were incubated at the restrictive temperature (37°C) for 1 h, and exposed to osmotic shock (0.4 M NaCl) for the indicated times.

Neither STE11 nor PBS2 were observed to undergo altered subcellular localization in stressed cells. In contrast, HOG1-GFP rapidly and reversibly translocated into the nucleus of osmotically stressed cells (Figure 1). These data indicate that activated HOG1 serves to relay the stress signal to the nucleus.

We have defined the minimum *cis*-acting requirements for the translocation of HOG1. The phosphorylation of the regulatory threonine and tyrosine residues in the TGY

motif of HOG1 is necessary and sufficient for its nuclear translocation. Because a kinase-impaired mutant of HOG1 still enters the nucleus in stressed cells, HOG1 kinase cannot be directly responsible for activating its own nuclear import. We suggest that phosphorylation of HOG1 by PBS2 either makes HOG1 available to the nuclear translocation machinery or that HOG1 continuously shuttles between the nucleus and the cytoplasm in unstressed cells, and T174/Y176 phosphorylation creates

**Table I.** The yeast strains used in this study

Strain	Genotype	Source
FY86	<i>MAT<math>\alpha</math> ura3-52 his3<math>\Delta</math>200 leu2<math>\Delta</math>1</i>	F.Winston
JTY35	<i>MAT<math>\alpha</math> ade2<math>\Delta</math>::hisG ade8<math>\Delta</math>100::KAN<sup>R</sup> ura3<math>\Delta</math> leu2<math>\Delta</math>1 his3<math>\Delta</math>200</i>	D.Amberg
TM233	<i>MAT<math>\alpha</math> ura3 leu2 trp1 his3 hog1<math>\Delta</math>::TRP1</i>	T.Maeda and H.Saito
TM261	<i>MAT<math>\alpha</math> ura3 leu2 his3 pbs2<math>\Delta</math>::LEU2</i>	T.Maeda and H.Saito
PSY962	<i>MAT<math>\alpha</math> gsp1::HIS3 gsp2::HIS3 ura3-52 leu2<math>\Delta</math>1 trp163 p CEN URA3 GSP1</i>	Wong <i>et al.</i> (1997)
PSY688	<i>MAT<math>\alpha</math> ura3 leu2 trp1 his3 ade2 srp1-31</i>	Loeb <i>et al.</i> (1995)
PSY1103	<i>MAT<math>\alpha</math> ura3-52 leu2<math>\Delta</math>1 trp1<math>\Delta</math>63 rsl1-4</i>	Koepp (1998)
<i>mtr10<math>\Delta</math></i>	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his3<math>\Delta</math>200 trp1<math>\Delta</math>1 lys2-801 mtr10<math>\Delta</math></i>	Pemberton <i>et al.</i> (1997)
<i>los1-1</i>	<i>MAT<math>\alpha</math> sup4 ade2-1 can1-100 lys1-1 trp5-48 ura3-1 his5-2 los1-1</i>	Hopper <i>et al.</i> (1980)
PSY1199	<i>MAT<math>\alpha</math> ade2<math>\Delta</math>::hisG ade8<math>\Delta</math>100::KAN<sup>R</sup> ura3<math>\Delta</math> leu2<math>\Delta</math>1 his3<math>\Delta</math>200 nmd5<math>\Delta</math>::HIS3</i>	this study
PSY967	<i>MAT<math>\alpha</math> ura3-52 leu2<math>\Delta</math>1 his3<math>\Delta</math>200 kap123<math>\Delta</math>::HIS3</i>	Seedorf and Silver (1997)
PSY1200	<i>MAT<math>\alpha</math> ura3-52 leu2<math>\Delta</math>1 his3<math>\Delta</math>200 trp1<math>\Delta</math>63 lys2 <i>shm1<math>\Delta</math></i>::HIS3</i>	Seedorf and Silver (1997)
<i>xpo1-1</i>	<i>MAT<math>\alpha</math> ade2-1 ura3-1 trp1-1 can1-1 xpo1<math>\Delta</math>::LEU2 pKW457 (HIS3, <i>xpo1-1</i>)</i>	Stade <i>et al.</i> (1997)

a binding site that would trap HOG1 in the nucleus. Our observation that a fraction of HOG1–GFP is nuclear in unstressed cells is consistent with the idea that HOG1 may continuously shuttle between the nucleus and the cytoplasm, although we have no independent evidence suggesting that this is the case. However, none of the nuclear export mutants tested showed nuclear accumulation of HOG1 in unstressed cells.

We wished to learn how phosphorylated HOG1 enters the nucleus. Macromolecules that cross the nuclear envelope do so accompanied by one or more members of the importin  $\beta$  family, and translocation of the transport complex through the nuclear pore complex requires the activity of Ran–GSP1, a Ras-related GTP-binding protein (Gorlich and Mattaj, 1996). As predicted, the translocation of HOG1 requires the activity of yeast Ran. However, nuclear import of HOG1 was independent of the function of any of the importins so far implicated in nuclear protein import, namely importin  $\alpha$  or  $\beta$  (Figure 7), KAP104 or MTR10 (not shown). Of all the importin  $\beta$  homologs that we tested, only one was required for HOG1 import, namely NMD5. In addition, we observed that NMD5 also relocates to the nucleus following stress, with the same kinetics as HOG1. Thus, we propose that NMD5 is a specific nuclear import receptor for active HOG1, although it remains possible that NMD5 has a role in the transport of other macromolecules. Moreover, a direct molecular interaction between NMD5 and HOG1 remains to be demonstrated, and it is possible that NMD5 has an alternative role, such as the release of HOG1 from a cytoplasmic anchor.

We noted an apparent subcytoplasmic localization of HOG1–GFP in stressed, but not unstressed, *nmd5 $\Delta$*  cells. These structures co-localize with actin patches (P.Ferrigno and F.Posas, unpublished observations). The significance of this colocalization is unclear. In mammalian cells, there appears to be cross-talk between the actin cytoskeleton and stress-activated protein kinase pathways (e.g. Alberts and Treisman, 1998), and we are currently investigating a potential link between the cytoskeleton and the stress response.

We have also shown that the export of HOG1 from the nucleus requires the activity of an importin  $\beta$  homolog; screening through a panel of importin  $\beta$  mutants revealed that only *XPO1* mutants failed to export HOG1 from the nucleus after stress. Stade *et al.* (1997) have identified

*XPO1* (also known as CRM1) as a specific receptor for leucine-rich nuclear export sequences (NESs), and recent work has shown that *XPO1* homologs mediate the nuclear export of proteins in a range of species (e.g. Engel *et al.*, 1998; Toone *et al.*, 1998). Although to date we have failed to co-immunoprecipitate *XPO1* and HOG1, we suggest that *XPO1* mediates the export of HOG1 to the cytoplasm of cells adapted to stress.

Our data confirm and considerably extend several recent studies on the translocation of HOG1-homologs into the nucleus. In *Schizosaccharomyces pombe*, it has been shown that the HOG1 homolog Spc1 also accumulates transiently in the nucleus in response to osmotic stress, and in a manner that requires its activating kinase Wis1 (Gaits *et al.*, 1998). Interestingly, these investigators have shown that the nuclear accumulation of phosphorylated Spc1 is maintained at least in part by the tethering of Spc1 to its target, the transcription factor Atf1. Clearly, once the nuclear target(s) of HOG1 becomes known, it will be important to ask whether the tethering of HOG1 in the nucleus by target transcription factors also operates in *S.cerevisiae*. Recently, Khokhlatchev *et al.* (1998) have shown that the MAP kinase ERK2 also accumulates in the nucleus when phosphorylated, in a manner that does not require its enzymatic activity, and that this accumulation is driven in part by the homodimerization of ERK2.

In summary, we propose the following model for the regulation of HOG1 kinase activity. Activation of HOG1 by PBS2 in the cytoplasm is necessary but not sufficient for the stress response. Active HOG1 needs to be translocated into the nucleus, via a process that requires the nuclear transport factor NMD5, where it presumably phosphorylates specific proteins. These comprise, at the very least, transcription factors and the tyrosine phosphatases PTP2/3 that are activated by HOG1, and in turn serve to dephosphorylate and inactivate it. Because we find that HOG1 that is trapped in the nucleus in *xpo1-1* cells becomes dephosphorylated on Tyr176 (data not shown), we propose that inactivation of HOG1 occurs before it is returned to the cytoplasm. These findings may be applicable to a range of proteins, including developmentally- and cell cycle-regulated protein kinases, whose transport into the nucleus may play a key role in the regulation of their biological activity. Elucidating the molecular mechanisms whereby HOG1 interacts with the nuclear

transport machinery will likely have important implications for the control of cell growth and differentiation.

## Materials and methods

### Strains

The yeast strains used in this study are listed in Table I. To create PSY1199, the open reading frame (ORF) of *NMD5* was replaced with that of *HIS3* using a PCR-based knockout protocol (Baudin *et al.*, 1993). The haploid strains JTY32 and JTY35 (both the gift of David Amberg, SUNY Syracuse) were transformed with the PCR product to create PSY1198 and PSY1199. Replacement of the *NMD5* ORF by that of *HIS3* was confirmed by two independent PCRs using different combinations of primers. Deletion of *NMD5* had no effect on the growth of the resulting strains at any temperature. All of the experiments performed in PSY1199 (reported here) yielded identical results in PSY1198.

### Plasmids

pRS-GFP was constructed by cloning the GFP ORF and the NUF2 3' UTR from pPS934 (J.Kahana and P.Silver, unpublished) into the *XhoI* and *KpnI* sites of pRS416 (*URA3<sup>+</sup>*, *CEN*; Sikorski and Boeke, 1991). *HOG1* (from -490 to the termination codon), *PBS2* (from -738 to the termination codon) and *STE11* (from -704 to the termination codon) were amplified by PCR using primers that replaced each termination codon with a *XhoI* site. The products were cloned into the *XhoI* site of pRS-GFP to create HOG1-GFP, PBS2-GFP and STE11-GFP, respectively. Junctions were verified by DNA sequencing. Site directed mutants of *HOG1* were generated by PCR as described previously (Maeda *et al.*, 1995). The *hog1(KM)* mutant contains a single amino acid change (Lys52→Met), resulting in a catalytically inactive protein. The *hog1(TA/YA)* mutant contains a double amino acid substitution, Thr174 and Tyr176 of the kinase activation loop to Ala. These mutations abolish phosphorylation of HOG1 by the PBS2 MAPKK. Each mutation was verified by DNA sequencing, and cloned into pRS-GFP as described for the wild-type gene. The promoter and ORF of *NMD5* were amplified by PCR, and inserted into pPS934 in place of *NUF2* sequences, to create pPS1559, a *CEN* plasmid that expresses a fusion of *NMD5* to GFP. This fusion was shown to be functional by its ability to rescue the synthetic lethality of an *nmd5Δ* strain (data not shown).

### Microscopy

Yeast cells were observed using a Nikon Optiphot-2 epifluorescence microscope and a 100×Plan APO 1.4 NA DIC objective. GFP signal was elicited and detected using Chroma filter # 41018 (exciter HQ470/40; dichroic Q495LP; emitter HQ 500LP; Chroma Technology Corp., Brattleboro, VT). Images were captured using a Princeton Instruments Micromax camera equipped with a Kodak model KAF 1400 chip (1317×1035, 6.8×6.8 μm pixels; Princeton Instruments, NJ), driven by Metamorph Imaging software (Universal Imaging Corporation, West Chester, PA). Images were acquired using the Autoscale function of Metamorph, which allows images to be displayed at optimal signal-to-noise ratios. Because this function displays a range of data intensities over a uniform scale, the increased nuclear signal for HOG1-GFP in stressed cells appears the same intensity as the cytoplasmic signal in unstressed cells, which could be misinterpreted as a degradation of HOG1 following stress. The Western blot in Figure 1A demonstrates that this not the case. The final figures were produced using Adobe Photoshop without further manipulation. Further details may be found in Ferrigno and Silver (1998).

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