

# The Hog1 SAPK controls the Rtg1/Rtg3 transcriptional complex activity by multiple regulatory mechanisms

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**ABSTRACT** Cells modulate expression of nuclear genes in response to alterations in mitochondrial function, a response termed retrograde (RTG) regulation. In budding yeast, the RTG pathway relies on Rtg1 and Rtg3 basic helix-loop-helix leucine Zipper transcription factors. Exposure of yeast to external hyperosmolarity activates the Hog1 stress-activated protein kinase (SAPK), which is a key player in the regulation of gene expression upon stress. Several transcription factors, including Sko1, Hot1, the redundant Msn2 and Msn4, and Smp1, have been shown to be directly controlled by the Hog1 SAPK. The mechanisms by which Hog1 regulates their activity differ from one to another. In this paper, we show that Rtg1 and Rtg3 transcription factors are new targets of the Hog1 SAPK. In response to osmotic stress, RTG-dependent genes are induced in a Hog1-dependent manner, and Hog1 is required for Rtg1/3 complex nuclear accumulation. In addition, Hog1 activity regulates Rtg1/3 binding to chromatin and transcriptional activity. Therefore Hog1 modulates Rtg1/3 complex activity by multiple mechanisms in response to stress. Overall our data suggest that Hog1, through activation of the RTG pathway, contributes to ensure mitochondrial function as part of the Hog1-mediated osmoadaptive response.

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## INTRODUCTION

Cells can monitor and respond to changes in the state of mitochondria by inducing changes in the expression of nuclear genes. This response is mediated by the retrograde (RTG) signaling pathway (Parikh *et al.*, 1987; Liao and Butow, 1993; Butow and Avadhani, 2004; Srinivasan *et al.*, 2010). In yeast, the RTG signaling pathway functions as a homeostatic or stress response mechanism to adjust

various biosynthetic and metabolic activities when mitochondrial dysfunction occurs (Liao *et al.*, 1991; Small *et al.*, 1995; Liu and Butow, 1999). The key transcriptional activators of the RTG pathway are the Rtg1 and Rtg3 basic helix-loop-helix leucine Zipper transcription factors, which activate transcription by binding as a heterodimer to the consensus sequence GTCAC, which is referred to as the R-box (Liao and Butow, 1993; Jia *et al.*, 1997). Rtg1 and Rtg3 function as a heterodimer, because neither protein alone is able to bind to R-box sites (Jia *et al.*, 1997). Although transcription activation requires both Rtg1 and Rtg3, only Rtg3 has been shown to contain transcriptional activation domains (Rothermel *et al.*, 1997). In respiratory-competent cells, Rtg1 and Rtg3 exist as a complex largely in the cytoplasm, in contrast with cells with mitochondrial dysfunction (e.g., cells without mitochondrial DNA), in which they exist as a complex predominantly localized in the nucleus (Sekito *et al.*, 2000; Ferreira Júnior *et al.*, 2005). Another member of the RTG pathway is Rtg2, a cytoplasmic protein that may act as a proximal sensor of mitochondrial dysfunction, being required for Rtg1/3 complex nuclear accumulation and subsequent activation of gene expression (Rothermel *et al.*, 1997; Liu and Butow, 2006). Remarkably, the RTG pathway is known to be negatively regulated by the

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Abbreviations used: AP, alkaline phosphatase; ChIP, chromatin immunoprecipitation; DTT, dithiothreitol; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin; MAPKK, mitogen-activated protein kinase kinase; MD, minimal dextrose; NLS, nuclear localization site; ORF, open reading frame; Pol II, polymerase II; RTG, retrograde; SAPK, stress-activated protein kinase; TCA, trichloroacetic acid; TOR, target of rapamycin; YPD, yeast-peptone-dextrose.

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target of rapamycin (TOR) pathway (Wullschleger *et al.*, 2006). Nuclear accumulation of Rtg1/3, as well as expression of RTG target genes, is induced by addition of rapamycin (Komeili *et al.*, 2000).

Exposure of yeast cells to increases in external osmolarity activates the Hog1 stress-activated protein kinase (SAPK), which is essential for the induction of diverse osmoadaptive responses (Hohmann, 2002; Hohmann *et al.*, 2007; O'Rourke *et al.*, 2002; Westfall *et al.*, 2004). One of the main functions of activated Hog1 is the regulation of gene expression (Martinez-Montanes *et al.*, 2010; Weake and Workman, 2010; de Nadal and Posas, 2010; de Nadal *et al.*, 2011). Genome-wide transcriptional analyses showed that a large number of genes are regulated by osmstress in a Hog1-dependent manner, including genes that encode proteins implicated in carbohydrate metabolism, general stress protection, protein production, and signal transduction (Posas *et al.*, 2000; Rep *et al.*, 2000; Causton *et al.*, 2001; Capaldi *et al.*, 2008; Miller *et al.*, 2011). One mechanism by which Hog1 modulates gene expression is the direct regulation of transcription factors. For instance, it has been proposed that Sko1, Hot1, the redundant Msn2 and Msn4, and Smp1 are controlled by the Hog1 SAPK (Rep *et al.*, 1999, 2000; Proft *et al.*, 2001; de Nadal *et al.*, 2003). These factors are unrelated, and the mechanisms by which Hog1 regulates their function differ from one to another. Sko1 is an ATF/CREB factor that represses gene expression under nonstress conditions by the recruitment of the general corepressor complex Cyc8-Tup1. In response to osmstress, Hog1 phosphorylates Sko1, switching its activity from a repressing to an activating state, which involves the recruitment of the SWI/SNF and SAGA complexes (Proft *et al.*, 2001; Proft and Struhl, 2002; Guha *et al.*, 2007; Kobayashi *et al.*, 2008). Hot1 physically interacts with Hog1, and its binding to DNA is regulated by Hog1 kinase activity. Moreover, Hog1 mediates RNA polymerase II (Pol II) recruitment at Hot1 target promoters (Rep *et al.*, 1999; Alepuz *et al.*, 2001, 2003). Msn2 and Msn4 are generic stress factors controlled by PKA and Hog1 (Rep *et al.*, 2000; Alepuz *et al.*, 2001). Finally, Smp1 is phosphorylated by Hog1 in response to osmstress, and its transactivation activity is dependent on phosphorylation by the SAPK (de Nadal *et al.*, 2003). However, the transcription factors reported to be under the control of Hog1 cannot account for the regulation of all Hog1-dependent genes (Capaldi *et al.*, 2008; Miller *et al.*, 2011), which suggests that additional transcription factors under the control of the SAPK are required for gene expression in osmstress.

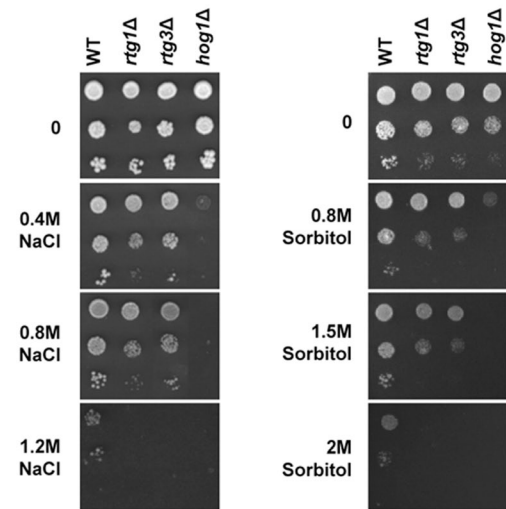
In this paper, we show that Hog1 controls Rtg1/3 transcription factor complex to induce RTG-dependent genes in response to osmstress. Hog1 interacts with the Rtg1/3 transcription complex in vivo. Remarkably, the SAPK is required for the nuclear accumulation of the complex, the recruitment of the complex at Rtg1/3-responsive promoters, and the regulation of Rtg3 transcriptional activity. Therefore the SAPK acts on the RTG complex at multiple levels to mediate gene expression. In addition, our data point out a close communication between mitochondria and nucleus via the RTG pathway to ensure mitochondrial function during adaptation to stress.

## RESULTS

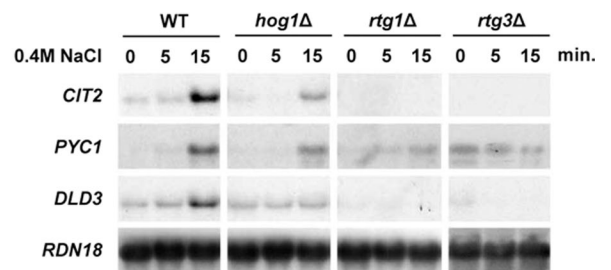
### Rtg1 and Rtg3 transcription factors are required for cell survival upon osmstress

In an exhaustive genome-wide genetic screen searching for deletions that render cells osmosensitive, we identified several protein complexes involved in transcription, such as SAGA, Mediator RSC, and Ubp3 (Zapater *et al.*, 2007; Mas *et al.*, 2009; Sole *et al.*, 2011). In addition, we found that mutations in *RTG1* and *RTG3* genes rendered cells unable to grow at high osmolarity. To characterize in more detail the phenotype of osmosensitivity of the *rtg1* and *rtg3* mutants, we

## A



## B



**FIGURE 1:** The Rtg1 and Rtg3 transcription factors are essential for adaptation to osmstress. (A) Mutations in *RTG1* and *RTG3* genes render cells osmosensitive. Wild-type and the indicated mutant strains were grown to logarithmic phase and diluted to 0.05 OD<sub>660</sub>. Tenfold serial dilution series were spotted on plates with or without NaCl or sorbitol at the indicated concentrations. Growth was scored after 2–5 d. (B) Rtg1, Rtg3, and the Hog1 SAPK are required for the induction of RTG target genes in response to osmstress. Indicated yeast strains were grown on MD-Gln and treated with 0.4 M NaCl at the indicated times. Total RNA was analyzed by Northern blot analysis for transcript levels of *CIT2*, *PYC1*, and *DLD3*, with *RDN18* as a loading control.

individually spotted these mutant strains onto yeast–peptone–dextrose (YPD) plates with different concentrations of salt and sorbitol. Deletion of *RTG1* and *RTG3* genes affected cell growth at high osmolarity both in salt and sorbitol media, indicating that these transcription factors are important for adaptation to osmstress (Figure 1A).

### RTG-dependent genes are induced in response to osmstress in a Hog1-dependent manner

The requirement of *RTG1* and *RTG3* for cell survival at high osmolarity prompted us to analyze the expression of genes known to be under the control of Rtg1 and Rtg3 transcription factors in response to osmstress. Activation of the RTG pathway is dependent on the source of assimilable nitrogen. In the presence of glutamine, Rtg1/3 transcription factors are retained in the cytoplasm and RTG-dependent genes are repressed (Liao and Butow, 1993; Liu and Butow, 1999; Komeili *et al.*, 2000). Growth media for all the experiments

was supplemented with glutamine to ensure the RTG pathway remained inactive and Rtg1/3 target genes were repressed (see *Materials and Methods*). Among the typical Rtg1/3 target genes are *CIT2* (encoding a peroxisomal isoform of citrate synthase), *PYC1* (encoding pyruvate carboxylase), and *DLD3* (encoding a cytoplasmic isoform of D-lactate dehydrogenase). All of them show a strong increase in expression upon respiratory dysfunction (Liao *et al.*, 1991; Chelstowska *et al.*, 1999; Epstein *et al.*, 2001). Notably, these genes were also strongly induced in response to osmostress, and their transcriptional activation was completely dependent on *RTG1* and *RTG3* transcription factors (Figure 1B). It is worth noting that the expression of *CIT2* in YPD medium, in which the RTG pathway is already activated in the absence of stress, still showed an increase upon osmostress (Supplemental Figure S1). When cells suffer mitochondrial dysfunction, the cytoplasmic Rtg2 protein is required for activation of the retrograde response (Liao and Butow, 1993; Ferreira Júnior *et al.*, 2005). Remarkably, under osmostress conditions, cells lacking *RTG2* also displayed impaired transcription of the RTG-dependent genes (Figure S2A). These data indicate that the integrity of the RTG pathway is required for gene activation upon osmostress.

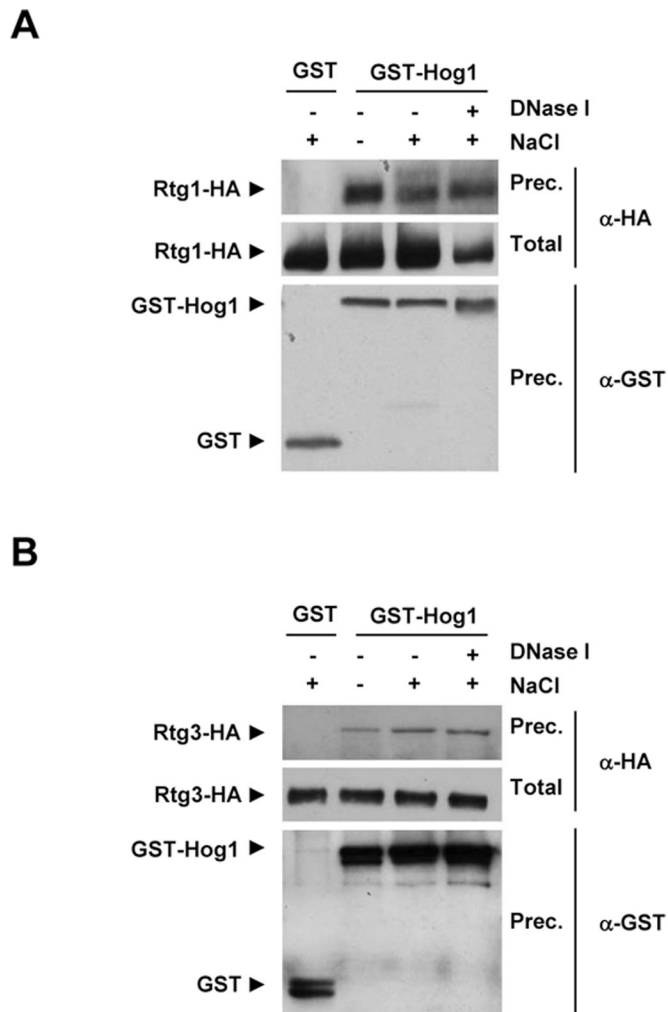
We then tested whether induction of the Rtg1/3 target genes in response to osmostress was dependent on Hog1. Expression of *CIT2*, *PYC1*, and *DLD3* was strongly reduced upon stress in a *hog1Δ* strain when compared with wild-type (Figure 1B). Therefore Hog1 is required for activation of RTG-dependent genes upon osmostress. To test whether the role of Hog1 on RTG-dependent genes was restricted to osmostress, we analyzed RTG-dependent gene expression in response to rapamycin. Activation of RTG-responsive genes upon addition of rapamycin was not altered in a *hog1Δ* strain (Figure S2B). Thus the role of Hog1 in RTG-mediated gene expression is restricted to osmostress. Of note, induction of osmoreponsive genes regulated by transcription factors such as *CTT1*, *STL1*, and *GRE2* was not affected in cells lacking *RTG1* or *RTG3* (Figure S2C). Therefore Rtg1 and Rtg3 transcription factors are required specifically for activation of a specific subset of osmoreponsive genes.

### The Hog1 SAPK interacts with the Rtg1/3 complex in vivo

Hog1 is able to interact with some of the transcription factors regulated by the SAPK (de Nadal and Posas, 2010). To investigate the relationship between Hog1 and the Rtg1/3 transcription complex, we tested whether Hog1 was able to interact with Rtg1 and Rtg3. We performed glutathione S-transferase (GST) pull-down experiments in extracts of cells expressing GST-Hog1 and endogenously hemagglutinin (HA)-tagged *RTG1* and *RTG3*. Hog1 was able to co-precipitate with both Rtg1 and Rtg3 proteins upon osmostress and under nonstressed conditions (Figure 2). Binding of Rtg1/3 to Hog1 was similar when protein extracts were treated with DNase I, suggesting that binding occurs in solution rather than through chromatin (Figure 2). Thus the Hog1 SAPK physically interacts with the Rtg1/3 complex, which provides biochemical evidence for the relationship between the SAPK and the RTG transcription complex.

### The Rtg1/3 complex and Hog1 bind to RTG-dependent promoters interdependently

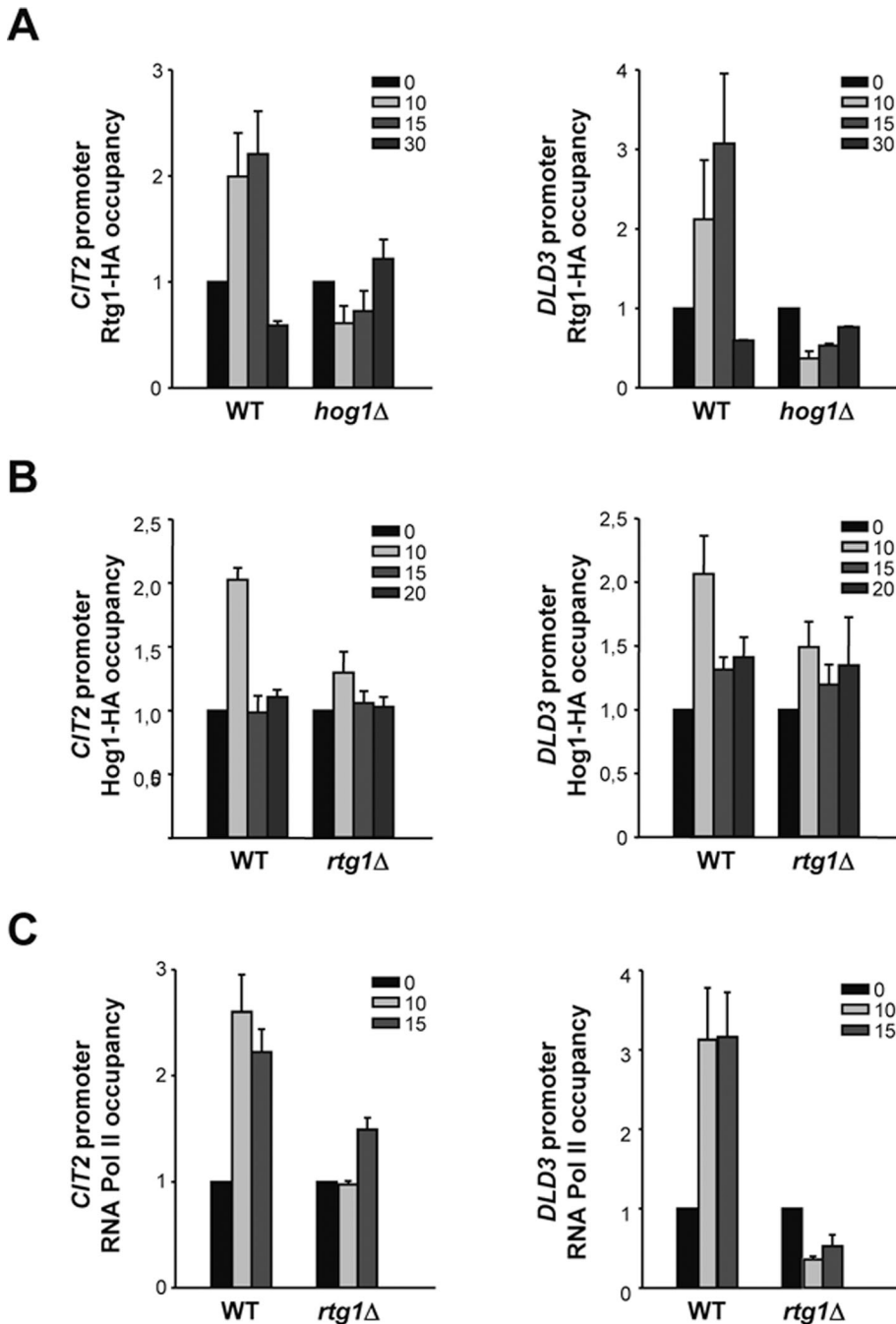
Osmoresponsive transcription factors bind to specific target promoters to regulate gene expression in response to osmostress and, in some cases, Hog1 is required to modulate their association to DNA (Alepez *et al.*, 2001, 2003). Therefore we asked whether the Rtg1/3 complex was recruited at the corresponding target promoters upon osmostress and whether this recruitment was dependent on Hog1. Rtg1 and Rtg3 form a heterodimer to bind to DNA, since



**FIGURE 2:** In vivo binding of Hog1 to Rtg1 and Rtg3. Strains expressing Rtg1-HA or Rtg3-HA from the endogenous locus were transformed with a plasmid expressing GST or GST-Hog1 under the  $P_{TEF1}$  promoter. Cells were grown in MD-Gln, and samples were taken before (–) and after (+) treatment with 0.4 M NaCl for 10 min. Protein extracts were treated or not with 200 U/ml of recombinant DNase I for 5 h at 4°C. GST-Hog1 was pulled-down by glutathione-Sepharose 4B, and the presence of Rtg1-HA (A) and Rtg3-HA (B) was probed by immunoblotting using anti-HA specific monoclonal antibody (top). Total extracts are presented in the middle panel. The amount of precipitated GST proteins was detected using anti-GST specific monoclonal antibody (bottom).

neither protein alone is able to bind to a target R-box site (Jia *et al.*, 1997). We therefore used chromatin immunoprecipitation (ChIP) of Rtg1-HA to follow the binding of the complex to *CIT2* and *DLD3* promoters upon osmostress. Rtg1 was recruited at *CIT2* and *DLD3* promoters in response to osmostress. However, binding of Rtg1 was abolished in a *hog1Δ* strain (Figure 3A). Therefore binding of the transcription complex to chromatin upon osmostress is dependent on the Hog1 SAPK.

The Hog1 SAPK is targeted to specific osmostress-responsive genes upon stress through association of specific transcription factors (Alepez *et al.*, 2001; Pascual-Ahuir *et al.*, 2006; Pokholok *et al.*, 2006; Proft *et al.*, 2006). We therefore asked whether Hog1 was recruited at the RTG-dependent promoters in response to stress and whether this recruitment was dependent on the Rtg1/3 transcription



**FIGURE 3:** Occupancy of Rtg1, Hog1, and RNA Pol II at RTG-dependent promoters. (A) Binding of Rtg1/3 complex to *CIT2* and *DLD3* promoters in response to osmstress is dependent on Hog1. Wild-type and *hog1Δ* strains carrying HA-tagged Rtg1 were grown in MD-Gln and subjected to osmstress (0.4 M NaCl) for the indicated times. Proteins were immunoprecipitated with anti-HA monoclonal antibody, and binding to the promoter regions of *CIT2* and *DLD3* loci was analyzed by PCR. Results are shown as the fold induction of treated relative to nontreated (time zero) samples normalized to *TEL2*. Data represent mean  $\pm$  SD of three independent experiments. (B) Recruitment of Hog1 to *CIT2* and *DLD3* promoters in response to osmstress is dependent on Rtg1. Wild-type and *rtg1Δ* strains were analyzed by ChIP as described for (A). (C) Rtg1 is required for recruitment of RNA Pol II at *CIT2* and *DLD3* promoters in response to osmstress. Wild-type and *rtg1Δ* strains were analyzed by ChIP as described for (A), using anti-Rpb1 antibody.

factor complex. By performing ChIP experiments, we followed binding of Hog1-HA to *CIT2* and *DLD3* promoters before and after osmstress in wild-type and *rtg1Δ* strains. Hog1 associated at these

GFP were transformed with an empty vector, a vector containing wild-type *HOG1*, or a catalytically inactive Hog1 mutant (Hog1<sup>KNN</sup>; Wurgler-Murphy *et al.*, 1997). Cells lacking *HOG1* failed to

promoters in response to osmstress, and its recruitment was dependent on the presence of Rtg1 (Figure 3B). These data indicate that the Rtg1/3 complex is anchoring the SAPK at the RTG target promoters in response to osmstress.

Moreover, we assessed the presence of RNA Pol II (the largest subunit Rpb1) at the RTG-dependent promoters in wild-type and *rtg1Δ* cells in response to osmstress. RNA Pol II was recruited at the promoters of *CIT2* and *DLD3* upon osmstress and, as expected, this recruitment was impaired in *rtg1Δ* cells (Figure 3C). These results are consistent with the fact that *CIT2* and *DLD3* genes are not expressed in response to stress in an *rtg1Δ* strain (Figure 1B).

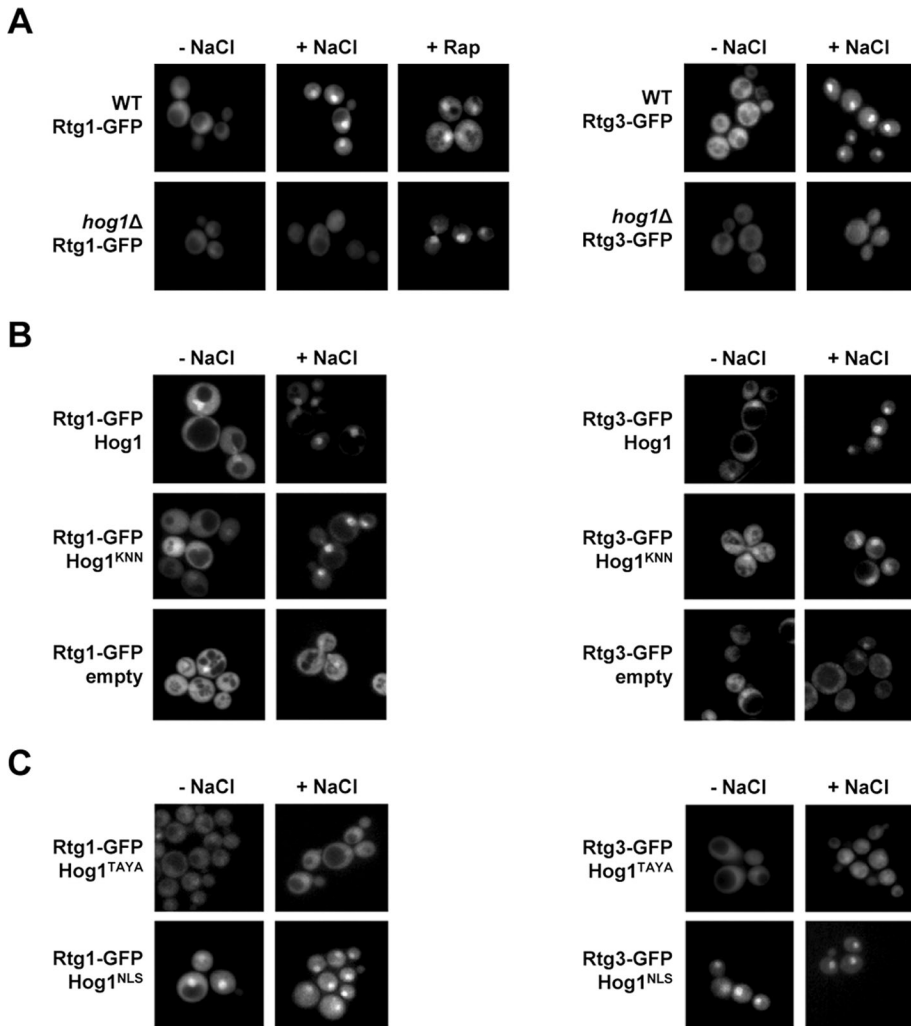
### Hog1 is required for nuclear accumulation of the Rtg1/3 complex upon osmstress

The RTG pathway is regulated by the dynamic localization of the Rtg1/3 heterodimer from the cytoplasm to the nucleus (Sekito *et al.*, 2000). Thus we assessed the subcellular localization of the Rtg1/3 transcription complex upon osmstress, using green fluorescent proteins (GFPs). Rtg1-GFP and Rtg3-GFP fusion proteins expressed under their native promoters were mainly localized in the cytoplasm in the absence of stress, whereas they showed a predominant nuclear localization when cells were subjected to a brief osmotic shock or rapamycin (Figure 4A). We next addressed whether Hog1 regulates the subcellular localization of the Rtg1/3 complex, expressing Rtg1-GFP and Rtg3-GFP in a *hog1Δ* background. As depicted in Figure 4A, the nuclear accumulation of Rtg1 and Rtg3 was impaired upon osmstress in cells lacking *HOG1*, indicating that Hog1 is required for the nuclear accumulation of the Rtg1/3 transcription complex in response to osmstress. In contrast, Rtg1 accumulated into the nucleus in *hog1Δ*-deficient cells when cells were treated with rapamycin (Figure 4A, right). Taken together, these data indicate that Hog1 regulates the subcellular localization of the Rtg1/3 complex specifically upon osmstress.

### Nuclear accumulation of the Rtg1/3 complex upon osmstress is independent on Hog1 catalytic activity

The above results prompted us to investigate whether Hog1 catalytic activity was required for Rtg1/3 accumulation into the nucleus upon osmstress. For this purpose, *hog1Δ* cells expressing Rtg1-GFP or Rtg3-





**FIGURE 4:** Role of Hog1 in regulating the subcellular localization of Rtg1/3 complex upon osmstress. (A) Rtg1/3 complex translocates into the nucleus upon osmstress in a Hog1-dependent manner. Wild-type and *hog1Δ* strains with plasmids carrying GFP-tagged Rtg1 or Rtg3 expressed under their own promoters were grown in MD-Gln to 0.5–0.7 OD<sub>660</sub> and stressed or not with 0.4 M NaCl for 5 min before being used for fluorescence microscopy analysis. Cells expressing Rtg1-GFP were treated with 1 μg/ml of rapamycin (Rap) for 15 min. (B) Rtg1/3 nuclear translocation is not dependent on Hog1 catalytic activity. *hog1Δ* strains expressing Rtg1-GFP or Rtg3-GFP with an empty monocopy vector or carrying wild-type Hog1 or Hog1<sup>KNN</sup> were treated as in (A). (C) Subcellular localization of the Rtg1/3 complex depends on the subcellular localization of Hog1. *hog1Δ* strains expressing Rtg1-GFP or Rtg3-GFP with a monocopy vector carrying wild-type Hog1, Hog1<sup>TAYA</sup>, or a vector carrying NLS-containing Hog1 (Hog1<sup>NLS</sup>) were treated as in (A).

accumulate Rtg1 and Rtg3 into the nucleus upon osmstress. Expression of wild-type Hog1 restored the ability of cells to accumulate Rtg1 and Rtg3 into the nucleus upon stress. Interestingly, cells expressing Hog1<sup>KNN</sup> concentrated Rtg1 and Rtg3 into the nucleus upon osmstress, similar to wild-type (Figure 4B). Therefore our results indicated that it is not the catalytic activity of Hog1 but the presence of Hog1 that is required for Rtg1/3 complex accumulation into the nucleus upon osmstress.

To confirm that only the presence of Hog1 is required for the localization of the Rtg1/3 complex, we followed the localization of Rtg1-GFP and Rtg3-GFP in cells expressing mutant versions of Hog1 with altered subcellular localization. We tested a Hog1 mutant containing point mutations at the Pbs2 mitogen-activated protein kinase (MAPKK) phosphorylation sites, which is unable to trans-

locate into the nucleus upon stress (Hog1<sup>TAYA</sup>; Ferrigno *et al.*, 1998), and a Hog1 fused to a nuclear localization site (NLS), which is retained in the nucleus even in the absence of stress (Ferrigno *et al.*, 1998). As shown in Figure 4C, cells expressing Hog1<sup>TAYA</sup> showed Rtg1 and Rtg3 localized throughout the cell under nonstressed conditions; this did not change upon stress. Correspondingly, cells expressing the nuclear-targeted Hog1 (Hog1<sup>NLS</sup>) showed Rtg1 and Rtg3 concentrated into the nucleus, even in absence of stress (Figure 4C). Therefore the localization of the Rtg1/3 complex depends on the localization of Hog1. Overall, these results suggest that it is not the catalytic activity of Hog1 but only the interaction of Hog1 with the Rtg1/3 transcription complex that is required for nuclear accumulation of the complex in response to osmstress.

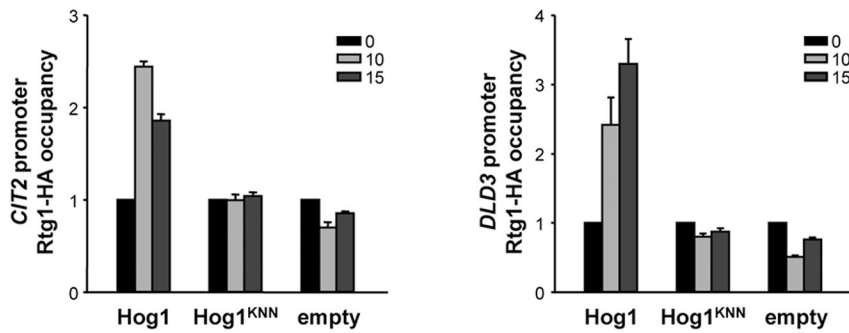
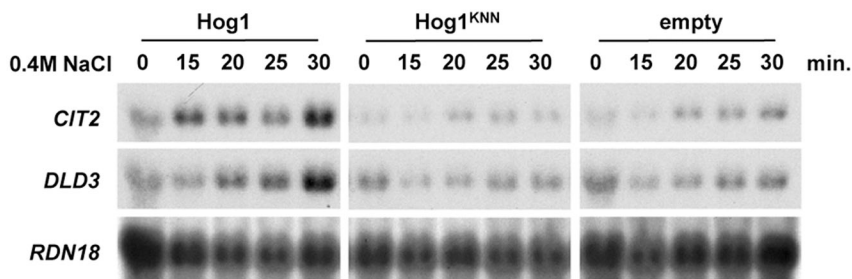
#### Hog1 catalytic activity is required for binding of Rtg1/3 at promoters and proper RTG-dependent gene expression

We then asked whether the catalytic activity of Hog1 could play a role in the regulation of the Rtg1/3 complex. We initially analyzed the role of Hog1 catalytic activity in the binding of Rtg1 to chromatin. *hog1Δ* cells with HA-tagged Rtg1 were transformed with a vector expressing wild-type Hog1, Hog1<sup>KNN</sup>, and an empty vector. As shown previously, deletion of *HOG1* resulted in impaired recruitment of Rtg1/3 complex to chromatin (i.e., *CIT2* and *DLD3* promoters) upon osmstress, which is consistent with a deficient nuclear accumulation of Rtg1 in the nucleus upon stress. However, in cells expressing Hog1<sup>KNN</sup>, Rtg1 accumulated in the nucleus upon stress, but its recruitment to chromatin upon osmstress was clearly impaired (Figure 5A). Correspondingly, cells carrying the Hog1<sup>KNN</sup> mutant were not able to induce transcription of the RTG-dependent genes upon osmstress (Figure 5B). Taken together, our data indicate that whereas the presence of the SAPK permits nuclear accu-

mulation of Rtg1 upon stress, and its activity is dispensable for this phenomenon, catalytic activity of Hog1 is absolutely required for chromatin association of the Rtg1/3 complex and proper transcriptional activation upon osmstress.

#### Phosphorylation of Rtg1 does not alter RTG-mediated transcription upon stress

We next assessed whether Rtg1 was a direct substrate of Hog1 by using *Escherichia coli*-purified proteins in an in vitro kinase assay in which Hog1 was activated in the presence of a constitutive MAPKK allele (Pbs2<sup>EE</sup>; Proft *et al.*, 2001). Initially, the in vitro kinase assay revealed that Rtg1 was directly phosphorylated by Hog1 (Figure S3A). Rtg1 contains a unique putative phosphorylation site for SAPKs (Ser-Pro or Thr-Pro), namely Thr-60. We created a point

**A****B**

**FIGURE 5:** Hog1 catalytic activity is required for Rtg1/3 chromatin binding and RTG-dependent gene expression. (A) Association of Rtg1/3 complex to *CIT2* and *DLD3* promoters in response to osmstress is dependent on Hog1 catalytic activity. *hog1Δ* cells expressing HA-tagged Rtg1 with an empty monocopy vector or carrying full-length Hog1 or Hog1<sup>KNN</sup> were grown in MD-Gln and treated with 0.4 M NaCl at the indicated times. Proteins were immunoprecipitated with anti-HA monoclonal antibody, and binding to the promoter regions of *CIT2* and *DLD3* loci was analyzed by PCR. Results are shown as the fold induction of treated relative to nontreated (time zero) samples normalized to *TEL2*. Data represent mean ± SD of three independent experiments. (B) The Hog1 SAPK activity is required for the induction of RTG-dependent genes in response to osmstress. The same strains and growth conditions as in (A) were used. Total RNA was analyzed by Northern blot analysis for transcript levels of *CIT2* and *DLD3*, with *RDN18* as a loading control.

mutation version of Rtg1 in which Thr-60 was replaced with Ala (Rtg1<sup>T60A</sup>). Notably, phosphorylation of Rtg1<sup>T60A</sup> by Hog1 was completely abolished (Figure S3A), indicating that Hog1 phosphorylates Rtg1 in vitro specifically at the Thr-60 residue.

We next tested whether Rtg1 was phosphorylated in vivo in response to osmstress. Wild-type and *hog1Δ* cells expressing HA-tagged Rtg1 were subjected to a brief osmotic shock, and proteins were probed using a specific monoclonal antibody against the HA epitope. The mobility pattern of Rtg1 in SDS-PAGE was altered in wild-type cells subjected to an osmotic shock, but this change in mobility was not observed in *hog1Δ* cells (Figure S3B). When extracts from osmstressed cells were treated with alkaline phosphatase (AP), the mobility pattern of Rtg1 could be reversed, confirming that the mobility change was induced by phosphorylation (Figure S3B). Therefore Rtg1 is rapidly phosphorylated upon osmstress, and this modification depends on the Hog1 SAPK.

To assess the role of Rtg1 phosphorylation by Hog1, we studied the effect of the mutation of the Thr-60 phosphorylation site in gene expression. Vectors containing full-length *RTG1* and *RTG1<sup>T60A</sup>* allele were transformed in *rtg1Δ* cells, and the effect on transcription was

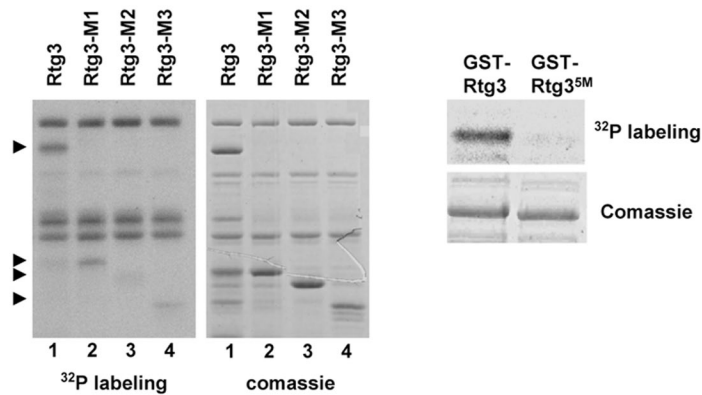
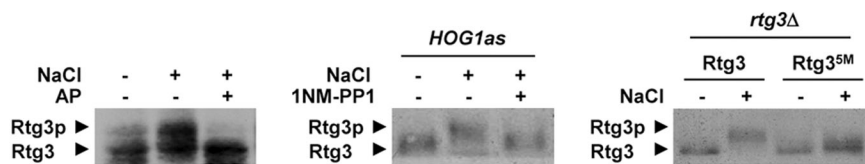
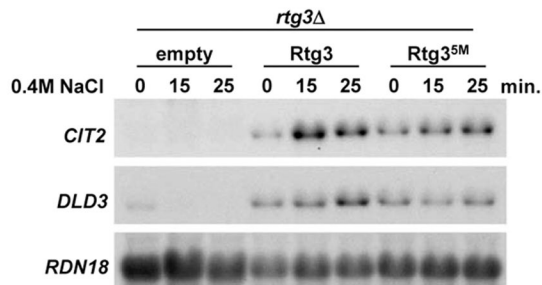
measured by Northern blot. Induction of *CIT2* and *DLD3* genes in response to osmstress was not affected in cells containing the mutant allele compared with wild-type (Figure S3C). These results indicated that Rtg1 phosphorylation by Hog1 is not a key determinant for Rtg1/3-mediated gene expression in response to osmstress.

### Rtg3 is phosphorylated by Hog1 in vitro

We then tested whether Rtg3 was directly phosphorylated by Hog1 in an in vitro kinase assay (Figure S3A). As shown in Figure 6A, full-length Rtg3 was phosphorylated by Hog1. Rtg3 contains 11 putative phosphorylation sites for SAPKs. To map the phosphorylation site(s) for Hog1 in Rtg3, we created several truncated *RTG3* alleles, expressed as GST-tagged proteins in *E. coli* and subjected to in vitro phosphorylation. Whereas a truncated form of Rtg3 containing amino acids 1–210 (Rtg3-M1) was phosphorylated by Hog1, phosphorylation of a region containing amino acids 1–183 (Rtg3-M2) was strongly reduced (Figure 6A, lanes 2 and 3). Rtg3-M1 contains a putative phosphorylation site (Thr-197) for SAPKs that is not present in the Rtg3-M2 fragment, suggesting that this residue could be a Hog1 phosphorylation site in Rtg3. Moreover, a C-terminal region of Rtg3 containing amino acids 211–486 (Rtg3-M3) was also phosphorylated by Hog1, although to a lesser extent than Rtg3-M1 (Figure 6A, lane 4). Four consensus phosphorylation sites for SAPKs are present in this region (Ser-222, Ser-227, Thr-249, and Ser-376). Notably, the combination of point mutations that replace Thr-197 and these four candidate residues (Rtg3<sup>5M</sup>) completely abolished the phosphorylation in vitro by Hog1 (Figure 6A, right).

### Rtg3 is phosphorylated in vivo by Hog1 upon osmstress

We next attempted to determine whether Rtg3 was phosphorylated in vivo upon osmstress. It has been reported that Rtg3 is phosphorylated under nonstressing conditions and that deletion of *RTG1* abolishes this phosphorylation (Komeili *et al.*, 2000). We therefore tested whether Rtg3 was phosphorylated upon stress in *RTG1* mutant cells. HA-tagged Rtg3 was subjected to a brief osmotic shock and found to undergo a mobility shift that was reversed when samples were treated with AP (Figure 6B, left). Thus Rtg3 is phosphorylated upon osmstress. To determine whether this phosphorylation is Hog1-dependent, we analyzed the mobility of the Rtg3 protein in a yeast strain carrying a mutant allele of *HOG1* (*hog1as*) that is specifically inhibited by the small molecule inhibitor 1NM-PP1 (Macia *et al.*, 2009). We used this approach to inhibit Hog1 specifically, due to a basal change in mobility of Rtg3 in a *hog1* mutant. The addition of the inhibitor 1NM-PP1 to cells inhibited Hog1 effectively and prevented the change in mobility of Rtg3 upon stress (Figure 6B, middle). Therefore the Rtg3 phosphorylation in response to stress is Hog1-dependent. We then analyzed the phosphorylation of the non-phosphorylatable mutant Rtg3<sup>5M</sup>. As shown in Figure 6B (right), Rtg3

**A****B****C**

**FIGURE 6:** Rtg3 is phosphorylated by the Hog1 SAPK. (A) Hog1 phosphorylates Rtg3 in vitro. Left, GST-tagged full-length Rtg3 and fragments Rtg3-M1 (aa 1–210), Rtg3-M2 (aa 1–183), and Rtg3-M3 (aa 211–486) were purified from *E. coli* and assayed with in vitro activated Hog1. Phosphorylated proteins were detected by autoradiography, and proteins were detected by Coomassie Blue staining. Arrows indicate the positions of Rtg3 fragments. Right, mutations of Rtg3 Thr-197, Ser-222, Ser-227, Thr-249, and Ser-376 to Ala (Rtg3<sup>5M</sup>) abolish in vitro phosphorylation by Hog1. (B) Rtg3 is phosphorylated upon osmostress. Left, *rtg3Δ* cells expressing Rtg3-HA were grown to 0.6–1 OD<sub>660</sub>, and samples were taken before (–) or 5 min after (+) addition of NaCl to a final concentration of 0.4 M. Protein extracts were treated (+) or not (–) with 10 U of AP. Rtg3-HA mobility was assessed by immunoblotting using anti-HA monoclonal antibody. Middle, *rtg3Δhog1Δ* cells expressing Rtg3-HA and carrying a mutant allele of *HOG1* (*HOG1as*) were preincubated or not for 10 min with 5 μM of 1NM-PP1 prior to osmostress at the same conditions as in the left panel. Right, mutations of Rtg3 Thr-197, Ser-222, Thr-249, and Ser-376 to Ala (Rtg3<sup>5M</sup>) abolish phosphorylation by osmostress. *rtg1Δrtg3Δ* strains were transformed with HA-tagged Rtg3 wild-type and the Rtg3 nonphosphorylatable mutant (Rtg3<sup>5M</sup>) and processed as in the left panel. (C) Hog1 phosphorylation sites in Rtg3 are required for transcriptional activity. *rtg3Δ* strain was transformed with plasmids expressing wild-type Rtg3 or the Rtg3 nonphosphorylatable mutant (Rtg3<sup>5M</sup>), grown in MD-Gln to 0.5–0.7 OD<sub>660</sub>, and subjected to osmotic stress (0.4 M NaCl) for the indicated length of time. Total RNA was assayed by Northern blot analysis for transcript levels of *CIT2* and *DLD3*, with *RDN18* as a loading control.

phosphorylation was abolished in the *RTG3* allele that contains the mutant Hog1-phosphorylated sites (Rtg3<sup>5M</sup>). Taken together, these results show that Rtg3 is phosphorylated by Hog1 upon osmostress.

### Phosphorylation at multiple sites of Rtg3 by Hog1 affects Rtg3 function

We then aimed to determine whether phosphorylation of Rtg3 by Hog1 was important for gene expression upon osmostress. We assessed transcriptional activation in *rtg3Δ* cells carrying wild-type *RTG3* or Rtg3<sup>5M</sup>. Cells expressing the nonphosphorylatable mutant Rtg3<sup>5M</sup> showed a partial impairment of induction of transcription compared with wild-type, indicating that Hog1-dependent phosphorylation of Rtg3 is important for Rtg3-mediated gene expression (Figure 6C). Of note, the combination of Rtg1<sup>T60A</sup> with Rtg3<sup>5M</sup> does not synergize the defect in transcription of the Rtg3<sup>5M</sup> mutant upon stress (Figure S5). Because Hog1 catalytic activity is required for the recruitment of the Rtg1/3 transcription complex at the responsive promoters (Figure 5A), we asked whether Rtg3 phosphorylation by Hog1 was also required for the association of Rtg1/3 to chromatin. Recruitment of the Rtg1/3 complex to DNA in cells expressing the nonphosphorylatable Rtg3 (Rtg3<sup>5M</sup>) was almost identical to wild-type (Figure S4). It is worth noting that the reduction in gene expression observed in the Rtg3<sup>5M</sup> mutant is not as dramatic as in a *hog1*-deficient strain. This might be because Rtg3 cannot concentrate in the nucleus, associate to chromatin, nor be phosphorylated by Hog1 in a *hog1* strain, whereas the Rtg3<sup>5M</sup> is only deficient in the last regulatory step. Overall the results suggest that Rtg3 phosphorylation by Hog1 is important for regulation of its transcription factor activity.

### DISCUSSION

Yeast cells respond to increases in external osmolarity by activating the stress-activated Hog1 SAPK. A major outcome of the activation of Hog1 is the regulation of gene expression, and several transcription factors have been proposed as being controlled by the SAPK (de Nadal and Posas, 2010; de Nadal et al., 2011; Martinez-Montanes et al., 2010; Weake and Workman, 2010). However, data suggest that additional transcription factors may be required for the osmostress-induced gene expression controlled by Hog1 (Miller et al., 2011). In this paper, we show that the Rtg1/3 transcriptional complex is a new target for Hog1, linking the SAPK to mitochondrial activity during adaptation to stress. Deletion of *RTG1* or *RTG3* results in a deficient osmostress-induced expression of key genes in the trichloroacetic acid (TCA) cycle, such as *CIT2*, *DLD3*, and *PYC1*, known to be activated by the RTG pathway upon respiratory dysfunction (Liao et al., 1991; Chelstowska et al., 1999; Epstein et al., 2001). In addition deletion of *RTG1* or *RTG3* displays osmosensitivity. Of note,



the osmosensitivity observed in *rtg1* and *rtg3* mutants is stronger than that observed in deletions of other known transcription factors targeted by Hog1, but less sensitive than observed in *hog1* cells.

Osmostress alters mitochondrial function by reducing the mitochondrial electron transport in plants (Hamilton and Heckathorn, 2001). Recently it was reported that constitutive activation of Hog1 provokes a decrease in respiration levels, which leads to cell death caused by an increase in reactive oxygen species (Vendrell *et al.*, 2011). On the other hand, the Rtg proteins are essential for respiration under limiting respiratory conditions, when several TCA cycle and respiratory chain enzymes are significantly up-regulated (Fendt and Sauer, 2010). Therefore it is likely that RTG-mediated transcription increases to compensate the down-regulation of respiration that rapidly occurs upon stress. Correspondingly, mutants in different mitochondrial components showed hypersensitivity to increases in NaCl, suggesting that mitochondrial biogenesis and function are needed for efficient adaptation to osmostress (Martinez-Pastor *et al.*, 2010). Taken together, the data suggest that cells exposed to osmostress have compromised mitochondrial respiration, and Rtg1 and Rtg3 transcription factors might be important determinants for maintaining or restoring mitochondrial function.

Production of glycerol is critical for adaptation in response to osmostress. Glycerol synthesis requires the action of NADH-dependent enzymes, and mitochondria are designed to oxidize NADH back to NAD<sup>+</sup>. Thus it could seem contradictory that osmostress can up-regulate mitochondrial function. However, the pool of NADH/NAD<sup>+</sup> in the cytosol and mitochondria are different, although NADH can diffuse from the cytosol into the mitochondria in certain conditions (Rigoulet *et al.*, 2004). Therefore the function of inducing the retrograde function by osmostress might be related to the ability of the cells to reactivate respiration once cells have started to adapt rather than to maintenance of the balance between of NADH/NAD<sup>+</sup>. Alternatively, due to the delay required for the Rtg response when compared with glycerol accumulation, it is possible that an elevation in mitochondrial respiration provides more ATP to improve long-term survival under osmostress.

Gene expression analysis showed that, specifically upon osmostress, the HOG pathway is required for proper induction of Rtg1/3 target genes. The retrograde response is linked to other pathways, such as TOR signaling (Komeili *et al.*, 2000; Butow and Avadhani, 2004). However, activation of the retrograde pathway by addition of rapamycin does not require the SAPK, indicating that RTG transcription can be activated by different signaling pathways, depending on the extracellular stimuli. A key regulatory step in the regulation of the RTG pathway is the translocation of Rtg1/3 proteins from the cytoplasm to the nucleus. When the RTG pathway is inactive, Rtg1 and Rtg3 are sequestered together in the cytoplasm. On activation, Rtg3 undergoes changes in its phosphorylation state and translocates into the nucleus together with Rtg1 (Sekito *et al.*, 2000). Hog1 interacts with the Rtg1/3 complex *in vivo*, and this interaction seems to be critical for the nuclear accumulation of the complex in response to osmostress. Indeed, localization of Rtg1 and Rtg3 in cells expressing Hog1 with abolished catalytic activity and altered subcellular localization correlates with the localization of Hog1 independently upon its catalytic activity. Thus the sole interaction of the transcription factor with the SAPK is sufficient for Rtg1 and Rtg3 nuclear accumulation. There are other stress-responsive transcription factors for which translocation is the key regulatory event in stress induction of transcription. For instance, Msn2 and Msn4 activators accumulate in the nucleus under stress conditions. However, in this case, the nuclear localization seems to occur independent of Hog1 (Gorner *et al.*, 1998).

In addition to its role in the regulation of the Rtg1/3 subcellular localization, Hog1 is further required for Rtg1/3 chromatin binding and transcriptional activity. It is known that Hog1 becomes intimately linked with stress-responsive loci upon osmostress, and this binding is dependent on the presence of stress-mediating transcriptional activators (Alepez *et al.*, 2001, 2003; Pascual-Ahuir *et al.*, 2006; Pokholok *et al.*, 2006; Proft *et al.*, 2006). Similarly, Hog1 is recruited by Rtg1 to the RTG-dependent promoters in response to osmostress. Thus the Rtg1/3 transcription complex targets the SAPK to specific promoters. On the other hand, our results also showed that recruitment of the Rtg1/3 complex to RTG-dependent promoters during osmostress is strongly reduced in a *hog1Δ* strain. These results are in agreement with the subcellular localization experiments indicating that Hog1 regulates the localization of the complex upon stress. However, the association of RTG at promoters not only requires the presence of Hog1 but also its catalytic activity. For instance, in cells containing the catalytic-inactive Hog1<sup>KNN</sup>, Rtg1 and Rtg3 are nuclear upon stress but cannot associate to chromatin. Similarly, it has been described that nuclear accumulation of the MAPK or the transcription activator Hot1 are not sufficient for the association of the transcription factor to chromatin, which is also dependent on Hog1 activity (Alepez *et al.*, 2003). Overall the interdependence of binding of Hog1 and Rtg1 to promoters of target genes reveals, as in the case of other stress-mediating transcription factors, a functional connection between both proteins for making a stable complex at stress-responsive promoters.

Hog1 also directly phosphorylates Rtg1 and Rtg3 proteins. We have not been able to unravel the role of Rtg1 phosphorylation by Hog1, but Rtg3 phosphorylation seems to be important for proper gene activation. Gene induction is reduced in an Rtg3 nonphosphorylatable mutant, whereas Rtg1/3 recruitment remains unaltered. Moreover, combining Rtg1 and Rtg3 nonphosphorylatable mutants is not synergistic for proper gene activation. Thus Hog1 phosphorylation plays an important role in the regulation of Rtg3 transcriptional activity. A similar scenario has been described for other transcription factors under the control of the SAPK. This is the case of the MEF2-like activator Smp1, the transcriptional activity of which is regulated by Hog1-dependent phosphorylation upon stress (de Nadal *et al.*, 2003).

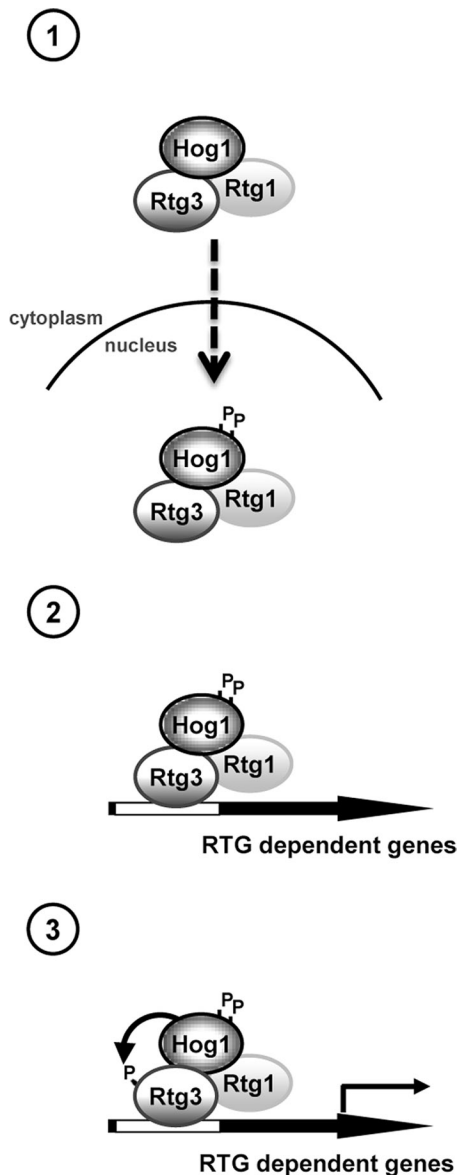
Taken together, our results show that, in contrast with what is known for the regulation of other transcription factors targeted by Hog1, the SAPK regulates the Rtg1/3 heterodimeric transcription complex by multiple mechanisms (see schematic diagram in Figure 7), which are: 1) the regulation of the nuclear accumulation of the complex, 2) the stimulation of the association of the complex at RTG-responsive promoters, and 3) the control of Rtg3 transcriptional activity. Notably, whereas the presence of Hog1 is essential for Rtg1/3 nuclear localization upon stress, the activity of the SAPK is required for Rtg1/3 chromatin binding and Rtg3 transcriptional activity regulation. Our data also provide a direct link between Hog1 and the regulation of genes important for mitochondrial activity during stress adaptation.

## MATERIALS AND METHODS

### Yeast strains

*Saccharomyces cerevisiae* strain K699 (MATa leu2-3112 ura3-1 his3-11 trp1-1 can1-100) and its derivatives YNN15 (*RTG1::kanMX4*), YNN20 (*RTG3::kanMX4*), YNN41 (*RTG2::kanMX4*), YNN17 (*HOG1::kanMX4*), and YNN51 (*RTG1::KanMX KAN::NAT RTG3::KanMX*) were used in this work. Genomic disruptions were made by long flanking-homology PCR-based gene disruption. C-terminal genomic tagging of Rtg1 in wild-type and *hog1Δ* strains yielded YNN21 (*RTG1-6HA::HIS3*)





**FIGURE 7:** The Hog1 SAPK directly modulates the Rtg1/3 complex upon osmotic stress by a triple mechanism: (1) regulating the nuclear accumulation of the heterodimeric complex (no catalytic activity of Hog1 is required); (2) stimulating the association of the complex to the responsive promoters (Hog1 catalytic activity is required, but not Rtg3 phosphorylation); and (3) controlling Rtg3 transcriptional activity (Hog1 catalytic activity and Rtg3 phosphorylation are required).

and YNN23 (*HOG1::kanMX4 RTG1-6HA::HIS3*). C-terminal genomic tagging of Rtg3 yielded YNN44 (*RTG3-6HA::HIS3*), YNN56 (*RTG1::KanMX RTG3-HA6::HIS3*), and YAD165 (*RTG1::KanMX HOG1::URA3 RTG3-HA6::HIS3*). YAD189 (*RTG1::KanMX RTG3-HA6::HIS3 HOG1::URA3::HOG1AS*) was obtained from YAD165 by introducing the *HOG1AS* mutated gene in the *HOG1* genomic locus. For Hog1 ChIP assays, YCR109 (*HOG1-6HA::HIS3*) and YCR110 (*RTG1::kanMX4 HOG1-6HA::HIS3*) were used. Tagging of genomic open reading frames (ORFs) with HA epitopes was done with a PCR-based strategy.

#### Plasmids

GFP-fused constructs were generated by amplifying *RTG1* and *RTG3* coding regions plus promoter regions (~800) from genomic

DNA and cloning them into the *XhoI* site of the pRS416-GFP construct (Raitt *et al.*, 2000). To analyze the role of Hog1 catalytic activity, we used YCpLac111-Hog1-3HA and YCpLac111-Hog1<sup>KNN</sup>-3HA vectors (Wurgler-Murphy *et al.*, 1997). pGBT9 (Gal4DBD-*HOG1*) and pRS426TEG2-Hog1<sup>TAYA</sup> were also used in this study (Ferrigno *et al.*, 1998). Full-length *RTG1* and *RTG3* coding regions were PCR-amplified from genomic DNA and cloned into the *BamHI* site of the bacterial expression vector pGEX-4T (GE Healthcare, Waukesha, WI), which allowed the expression of GST-tagged proteins in *E. coli*. pGEX-4T plasmids containing mutated alleles (described in Results) were generated by PCR amplification with the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, Agilent, Santa Clara, CA) from the pGEX-4T plasmids containing the wild-type *RTG1* and *RTG3* ORFs. Each mutation was verified by DNA sequencing. pGEX4T-Hog1 and pGEX4T-Pbs2<sup>EE</sup> (*PBS2* with Ser-514-Glu and Thr-518-Glu mutations) are described in Bilsland-Marchesan *et al.* (2000). For obtaining HA fusion proteins, the promoter (~800) and coding regions of wild-type *RTG1* and *RTG3* were PCR-amplified and cloned into the *BamHI* site of the pRS415 vector. Mutated alleles (described in Results) were generated and verified as before. The 6xHA tags were inserted before the stop codon by recombination, using a specific cassette containing the HA epitopes.

#### Buffers and media

Buffer A consisted of 50 mM Tris-HCl (pH 7.5), 15 mM EDTA, 15 mM ethylene glycol tetraacetic acid, 2 mM dithiothreitol (DTT), 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 5 µg/ml of pepstatin, and 5 µg/ml of leupeptin. AP buffer consisted of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 10 mM MgCl<sub>2</sub>. STET buffer consisted of 10 mM Tris-HCl (pH 8.0), 0.1M NaCl, 1 mM EDTA, and 5% Triton X-100. Kinase buffer consisted of 50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, and 2 mM DTT. Phosphatase inhibitor cocktail contained 10 mM NaF, 1 mM sodium pyrophosphate, and 10 mM β-glycerophosphate. For all experiments, cell cultures were grown in minimal dextrose (MD) media that contained 0.7% yeast nitrogen base and 2% dextrose (pH 5.5). Glutamine was added to MD media to a final concentration of 0.2%. To supplement the auxotrophic requirements of strains, we added required amino acids (0.006% histidine, 0.008% leucine, 0.006% adenine, 0.005% tryptophan, 20 µg/ml uracil, and 20 µg/ml methionine), lacking for the specific ones to select for plasmid maintenance. Cells were grown in YPD (1% yeast extract, 2% peptone, and 2% dextrose) as indicated in Results.

#### Cell viability assays

Yeast cultures were grown to early log phase (0.5–0.8 OD<sub>660</sub>) and diluted to a 0.05 OD<sub>660</sub>. Serial dilutions of cultures were spotted directly onto YPD plates containing glutamine and the indicated concentrations of salt and sorbitol (Figure 1A) and were incubated at 30°C. Growth was scored after 2–5 d.

#### Northern blot analysis

Yeast cultures were grown to early log phase (0.5–0.8 OD<sub>660</sub>) and either subjected to stress (0.4 M NaCl, times indicated in the figure legends) or untreated. Total RNA was extracted from 15 ml of culture by acid phenol treatment. Twenty micrograms of total RNA per sample was run in 1% agarose gels using electrophoresis. RNAs were transferred to a nylon membrane (Roche, Indianapolis, IN) by a vacuum blotter (model 785; Bio-Rad, Hercules, CA). Total RNA and expression of specific genes were probed by using radiolabeled PCR fragments containing the ORF region of *CIT2*, *DLD3*, *PYC1*, *CTT1*, *STL1*, or *GRE2*, or the noncoding exon of *RDN18* as a loading

control. Signals were quantified by using a storage phosphor screen in a Typhoon 8600 phosphorimager.

### In vivo coprecipitation assays

In vivo interaction of Rtg1-HA and Rtg3-HA fusion proteins with GST-Hog1 was determined by GST pull-down experiments. Exponentially growing cells (0.5–0.8 OD<sub>660</sub>) were subjected to a brief osmotic shock (0.4 M NaCl for 10 min). Two milligrams of yeast extract in a mixture of buffer A plus 150 mM NaCl and phosphatase inhibitors was prepared and incubated with glutathione-Sepharose beads overnight at 4°C. Beads were washed extensively with buffer A, resuspended in loading buffer, and separated on SDS–15% PAGE (Rtg1-HA) or SDS–10% PAGE (Rtg3-HA). The lysis buffer used in the coimmunoprecipitation, treated or not with DNase I, was 50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 2 mM DTT, and the entire cocktail of antiproteases and phosphatase inhibitors. Protein extracts were treated with 200 U/ml of recombinant DNase I RNase-free for 5 h at 4°C (Roche) before being incubated with glutathione-Sepharose beads as above. Immunoblotting was done by using anti-HA monoclonal antibody 12CA5 (Roche) and anti-GST monoclonal antibody (Pharmacia) together with ECL reagent (GE Healthcare, Waukesha, WI).

### ChIP

ChIP experiments were performed as described before (Zapater *et al.*, 2007). Yeast cultures were grown to early log phase (0.6–0.8 OD<sub>660</sub>) before samples of the culture were exposed to osmotic stress (0.4 M NaCl; times indicated in the figure legends). For cross-linking, yeast cells were treated with 1% formaldehyde for 20 min at room temperature. Antibodies used were mouse polyclonal anti-Rpb1 (8WG16; Covance, Princeton, NJ), and monoclonal anti-HA 12CA5. For quantitative PCR analysis of osmo-responsive genes and constitutively expressed genes, the following primers, with locations indicated by the distance from the respect ATG initiation codon, were used: *CIT2*, –460/–141; *DLD3*, –253/+46; and *TEL2* (telomeric region on the right arm of chromosome VI).

### GFP fluorescence microscopy

Exponentially growing cells (0.6–0.8 OD<sub>660</sub>) were observed without fixation using a Nikon Eclipse 901 microscope (Nikon, Tokyo, Japan) with an ORCA II charge-coupled device camera (Hamamatsu, Hamamatsu City, Japan). Images were taken at 100× magnification and converted to Photoshop 7.0.1 (Adobe Systems, San Jose, CA).

### Purification of GST proteins in *E. coli* and in vitro kinase assays

GST fusion proteins encoding Pbs2<sup>EE</sup>, Hog1, and wild-type or mutant Rtg1 and Rtg3 were constructed by using pGEX-4T (Pharmacia), expressed in *E. coli*, and purified using glutathione-Sepharose beads (Pharmacia) in STET buffer, as described previously (Posas *et al.*, 1996). Phosphorylation by Hog1 of Rtg1, Rtg3, and mutant versions were monitored as follows: 1 µg of recombinant GST-Hog1 was activated by phosphorylation by using 0.5 µg of GST-Pbs2<sup>EE</sup> in the presence of kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, and 2 mM DTT) and 50 µM ATP. After 15 min at 30°C, 5 µg of GST-Rtg1, GST-Rtg2, or GST-Rtg3 was added to the previous mixture together with [<sup>32</sup>P]ATP (0.2 µCi/µl). The mixture was then incubated for 10 min at 30°C, and the reactions were stopped by the addition of 2X SDS loading buffer. The labeled proteins were resolved by SDS–PAGE, stained with Coomassie Blue, and detected by autoradiography.

### In vivo phosphorylation assays

Cells containing the *RTG1* and *RTG3* ORF fused to HA (6xHA) epitope were grown to mid-log phase (0.6–0.8 OD<sub>660</sub>), subjected to osmotic stress treatment (0.4 M NaCl for 5 min), and harvested by centrifugation. Yeast extracts were obtained in a mixture of buffer A plus 150 mM NaCl and phosphatase inhibitors. Protein concentration was determined by Bradford analysis (Bio-Rad Protein Assay). When necessary, 1 mg of total yeast extract was treated for 2 h at 37°C with 0.5 µl phosphatase alkaline (20 U/µl; Roche). In experiments that did not require the use of AP, protein extraction was performed using the TCA protocol, as described before (Macia *et al.*, 2009). 1NM-PP1 was used at 5 µM to inhibit analogue-sensitive mutants (Macia *et al.*, 2009). Total crude extracts were loaded onto SDS–15% PAGE, and the Rtg1-HA and Rtg3-HA fusion proteins were detected by immunoblotting using an anti-HA monoclonal antibody (12CA5; Roche).

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