

# Control of Ubp3 ubiquitin protease activity by the Hog1 SAPK modulates transcription upon osmopressure

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**Protein ubiquitylation is a key process in the regulation of many cellular processes. The balance between the activity of ubiquitin ligases and that of proteases controls the level of ubiquitylation. In response to extracellular stimuli, stress-activated protein kinases (SAPK) modulate gene expression to maximize cell survival. In yeast, the Hog1 SAPK has a key role in reprogramming the gene expression pattern required for cell survival upon osmopressure. Here, we show that the Ubp3 ubiquitin protease is a target for the Hog1 SAPK to modulate gene expression. *ubp3* mutant cells are defective in expression of osmoresponsive genes. Hog1 interacts with and phosphorylates Ubp3 at serine 695, which is essential to determine the extent of transcriptional activation in response to osmopressure. Furthermore, Ubp3 is recruited to osmoresponsive genes to modulate transcriptional initiation as well as elongation. Therefore, Ubp3 activity responds to external stimuli and is required for transcriptional activation upon osmopressure.**

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## Introduction

Ubiquitylation is a reversible post-translational modification with essential roles in various signal transduction cascades and determines protein stability (Amerik and Hochstrasser, 2004; Komander *et al.*, 2009; Reyes-Turcu *et al.*, 2009). The balance of ubiquitin ligases and ubiquitin proteases determines the ubiquitylation state of a protein. To date, several ubiquitin-specific proteases (UBPs) have been identified in the budding yeast *Saccharomyces cerevisiae*. The large

number and the diversity of these enzymes suggest that each might have a specific function in regulating biological functions. Indeed, de-ubiquitylating enzymes are implicated in many processes, including free ubiquitin generation and ubiquitin removal from substrate fragments after proteasomal degradation. Also, they are responsible for the reversal of ubiquitylation to prevent degradation or to modify substrate activity (reviewed by Amerik and Hochstrasser, 2004; Soboleva and Baker, 2004; Komander *et al.*, 2009). Ubp3, the yeast homologue of human USP10, was originally isolated by Baker *et al.* (1992) on the basis of the ability to cleave a ubiquitin- $\beta$ -galactosidase fusion protein. Ubp3 requires Bre5 as a cofactor to form an active de-ubiquitylation symmetric heterotetrameric complex (Li *et al.*, 2005, 2007). Ubp3 has the ability to remove ubiquitin chains from target proteins before the ubiquitylated target binding to the proteasome and thus can negatively regulate proteasome-mediated degradation (Brew and Huffaker, 2002; Cohen *et al.*, 2003a,b; Baxter *et al.*, 2005; Kraft *et al.*, 2008; Ossareh-Nazari *et al.*, 2010). Ubp3 has also been implicated in transcription since it interacts with Sir4 (Moazed and Johnson, 1996) as well as the general transcription factor TFIID (Auty *et al.*, 2004). Recently, it was shown that Ubp3 acts as a transcriptional co-activator of Gal4 and Gcn4 by protecting protein Tbp1/Spt15 from degradation (Chew *et al.*, 2010). In addition, Ubp3 acts during transcriptional elongation by reversing RNA Pol II ubiquitylation (Kvint *et al.*, 2008).

p38-related stress-activated protein kinase (SAPK) Hog1, which is activated in response to osmopressure, has a pivotal role for reprogramming the gene expression capacity of the cell (reviewed by de Nadal and Posas, 2010; Martinez-Montanes *et al.*, 2010; Weake and Workman, 2010). Hog1 regulates different steps of the transcription process. At initiation, it modulates several unrelated transcription factors, each of them responsible for controlling the expression of a subset of osmoresponsive genes directly or in collaboration with other factors (Capaldi *et al.*, 2008; Ni *et al.*, 2009). Active Hog1 interacts with and directly phosphorylates some of these transcription factors, which alters their level of activity (Proft *et al.*, 2001; de Nadal *et al.*, 2003). Hog1 also associates with DNA at stress-responsive promoters via such specific transcription factors (Alepez *et al.*, 2001; Pascual-Ahuir *et al.*, 2006; Pokholok *et al.*, 2006; Proft *et al.*, 2006) and functions as a platform to recruit general transcription factors, chromatin modifying activities and RNA Pol II (Proft and Struhl, 2002; Alepez *et al.*, 2003; de Nadal *et al.*, 2004; Zapater *et al.*, 2007). During elongation, Hog1 SAPK serves as a selective transcriptional elongation factor for stress-responsive genes (Pascual-Ahuir *et al.*, 2006; Pokholok *et al.*, 2006; Proft *et al.*, 2006). In addition, Hog1 recruits the RSC chromatin remodelling complex at the stress-responsive genes to modify nucleosome organization (Mas *et al.*, 2009). Here, we report crosstalk between

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phosphorylation and ubiquitin signalling networks. We demonstrate that Hog1 SAPK interacts with Ubp3 upon osmotic stress and regulates its de-ubiquitylase activity to determine the extent of transcription outcome. Therefore, Ubp3 and the control of its activity by the SAPK is an important factor in the modulation of gene expression in response to osmolarity.

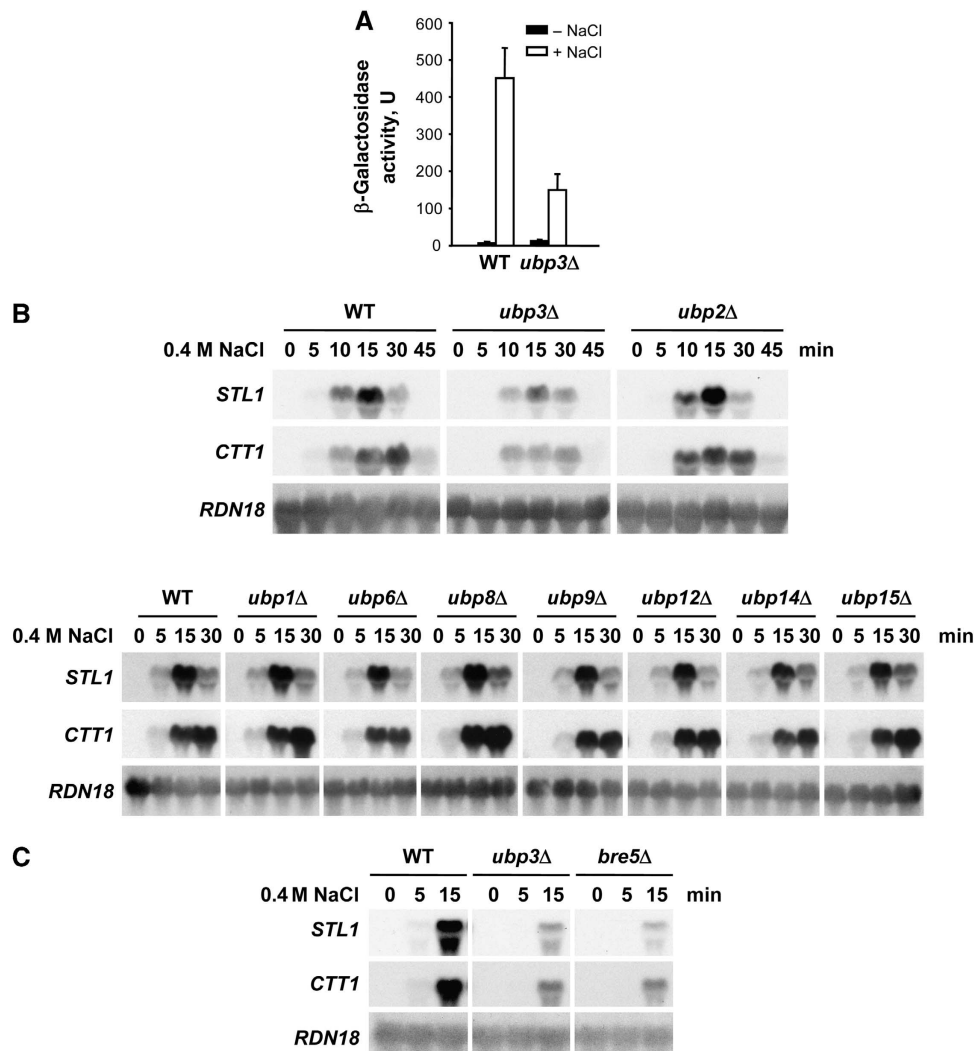
## Results

### *Ubp3 is required for full transcriptional response upon osmolarity*

Cells rapidly change their pattern of gene expression upon osmolarity. To uncover novel activities required for maximal gene expression in response to osmolarity, we undertook an exhaustive genome-wide genetic screen searching for mutations that rendered cells with defective expression of an osmolarity-responsive gene reporter at high osmolarity. A high-

throughput screen was performed by robotically pinning an ordered array of ~800 haploid yeast deletion mutants related to transcriptional processes and containing an integrated osmolarity-responsive gene reporter construct (*STL1::LacZ*) onto YPD and YPD 0.4 M NaCl X-gal plates and scoring their ability to induce gene expression in response to stress ( $\beta$ -galactosidase production at 0.4 M NaCl). One of the mutants identified corresponded to the Ubp3 ubiquitin protease and induction of the *STL1::LacZ* reporter upon stress was impaired in cells deficient in *UBP3* (Figure 1A).

We assessed whether expression of osmolarity-responsive genes was affected in *ubp3* cells upon stress to determine the importance of Ubp3 in the regulation of gene expression. Expression of osmolarity-responsive genes such as *STL1*, *CTT1* and *ALD3* was reduced in a *ubp3* strain as compared with the wild type (WT) (Figure 1B and data not shown). *S. cerevisiae* contains several UBPs and we followed gene expression in yeast strains lacking individual ubiquitin proteases



**Figure 1** The Ubp3–Bre5 de-ubiquitylase complex is essential for the transcriptional response upon osmolarity. (A) Activation of the *STL1::LacZ* reporter upon stress is impaired in cells deficient in *UBP3*. WT and *ubp3* mutant strains carrying a multicopy *STL1::LacZ* reporter construct were assayed for  $\beta$ -galactosidase activity (expressed in nmol/min per mg) in cells grown to mid-log phase before (filled bars) or after (open bars) a brief osmotic stress (0.4 M NaCl for 30 min). The results are presented as the mean  $\pm$  s.d. of three independent experiments. (B) Impaired gene expression in a *ubp3* mutant strain upon osmolarity. WT and the indicated mutant strains were grown to mid-log phase in rich medium and then subjected to osmolarity (0.4 M NaCl) for the indicated length of time. Total RNA was assayed by northern blot for transcript levels of *STL1*, *CTT1* and *RDN18* (as a loading control). (C) Osmolarity-impaired gene expression of cells lacking *BRE5*. Northern blot of WT, *ubp3* and *bre5* strains was done as described for (B).

to determine their role in gene activation in response to osmolarity. Specific deletion of other ubiquitin proteases did not have any particular effect on gene expression upon stress (Figure 1B; Supplementary Figure S1A). These results define a specific role for Ubp3 de-ubiquitylase in modulating gene expression upon osmolarity. Albeit having altered gene expression, *ubp3* mutant cells were not osmosensitive (Supplementary Figure S1B). Ubp3 forms a complex with the accessory factor Bre5 (see Introduction). Expression of *STL1* and *CTT1* was also strongly reduced by deletion of *BRE5* (Figure 1C), suggesting that Ubp3 and Bre5 function together in transcriptional activation in response to osmolarity.

### Ubp3 interacts with Hog1 in an osmotic stress-dependent manner

Hog1 interacts with a number of substrates to control gene expression. The possibility that Hog1 and Ubp3 physically interact was addressed by testing whether Hog1 was immunoprecipitated with Ubp3 from cell extracts. Yeast cells expressing Myc-tagged Ubp3 and HA-tagged Hog1 expressed from their genomic locus were subjected to osmolarity and Ubp3 was immunoprecipitated using specific monoclonal antibodies against the Myc epitope. As shown in Figure 2A, Ubp3 was able to coprecipitate Hog1 in a stress-dependent manner. Similarly, Hog1-HA was precipitated with Ubp3 in response to osmolarity (Figure 2B). Moreover, *in vitro* experiments using purified proteins from *Escherichia coli* showed that Ubp3 binds directly to Hog1 (Supplementary Figure S7A). Thus, the data show that these proteins interact upon osmolarity, which provides biochemical evidence

for the relationship between Ubp3 ubiquitin protease and the SAPK.

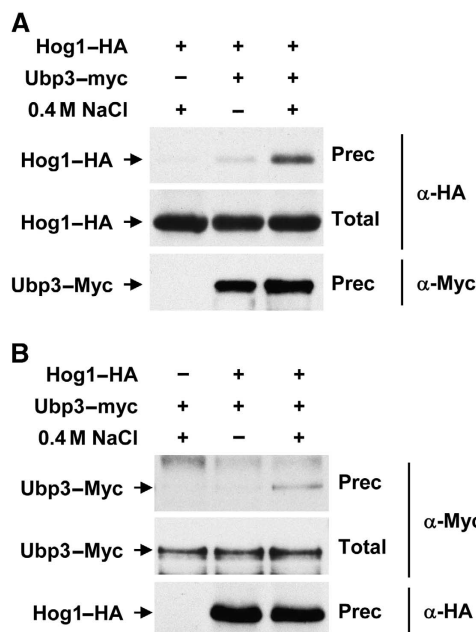
### Ubp3 associates with osmolarity-responsive genes upon stress

Hog1 associates with stress-responsive gene promoters and throughout the entire transcribed region of target genes in response to stress (Alepuz *et al*, 2001; Pokholok *et al*, 2006; Proft *et al*, 2006). We asked whether Ubp3 was recruited to osmolarity-responsive genes upon stress. We used chromatin immunoprecipitation (ChIP) to follow the binding of Ubp3 to promoters and ORFs of osmolarity-responsive genes (such as *STL1* and *CTT1*) before and after the addition of NaCl. Chromatin from WT and from *hog1* cells, both expressing integrated tagged Ubp3, was immunoprecipitated and quantified by real-time PCR. As shown in Figure 3A, Ubp3 associated specifically with osmolarity-responsive promoters and ORFs only in response to osmolarity. However, the binding of Ubp3 to stress-responsive promoters was weaker than that observed at ORFs. Furthermore, no binding of Ubp3 was observed in a *hog1* strain upon stress, indicating that recruitment of Ubp3 to chromatin in response to osmolarity is dependent on Hog1 (Figure 3A). It is noteworthy that binding of Hog1 to stress-responsive promoters was not affected in a *ubp3* strain (see Supplementary Figure S2). Therefore, Hog1 is required for Ubp3 recruitment at stress-responsive genes upon osmolarity.

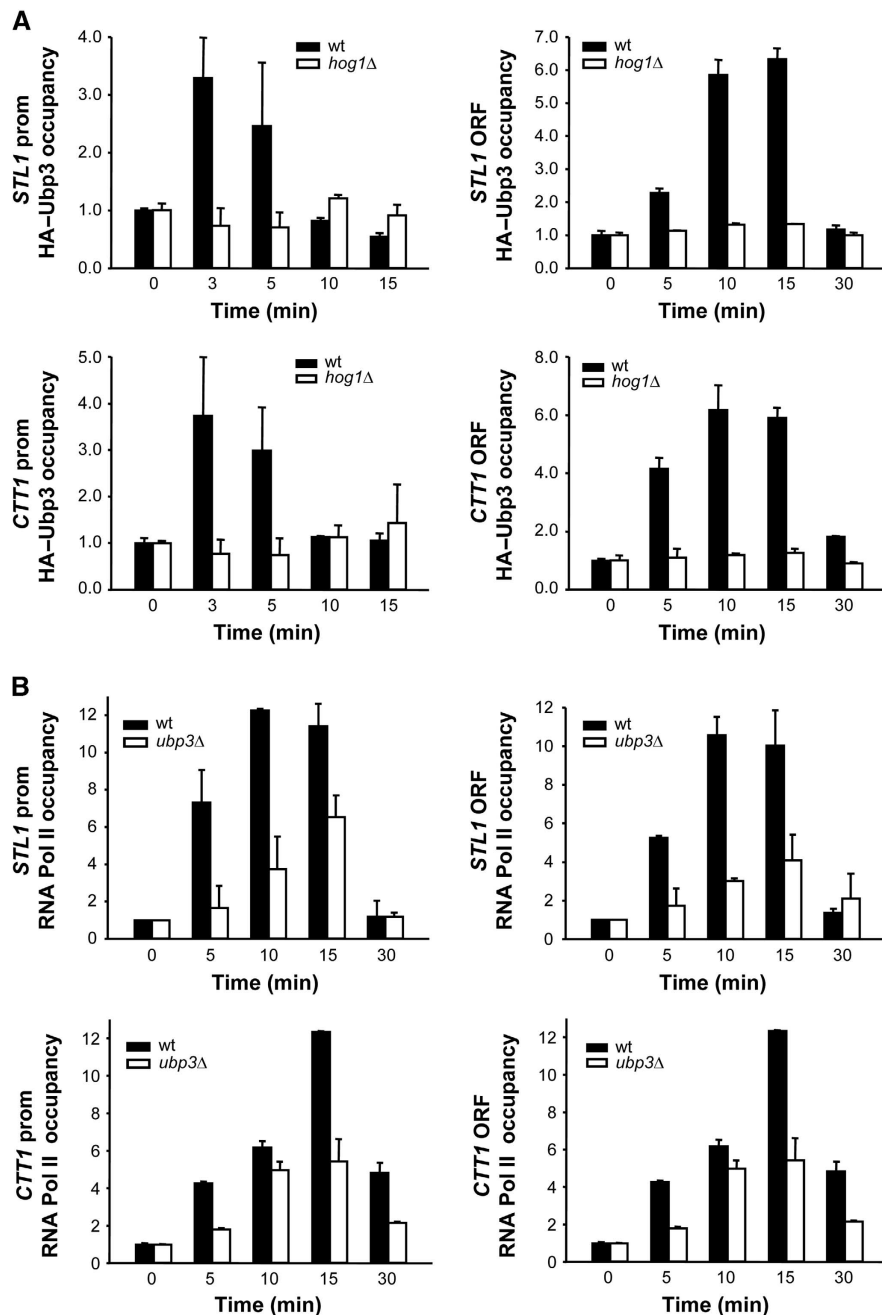
We used ChIP to assess the presence of RNA Pol II (Rpb1) at the promoter and coding region of stress-responsive genes in WT and *ubp3* cells in response to osmolarity. RNA Pol II was recruited at the promoters and ORFs of *STL1* and *CTT1* only in response to osmolarity. It is noteworthy that recruitment of RNA Pol II upon osmolarity was strongly reduced in *ubp3* cells (Figure 3B). These results are consistent with the fact that in a *ubp3* strain, *CTT1* and *STL1* gene expression is reduced in response to stress (Figure 1B). Taken together, these data provide evidence that Ubp3 is involved directly in the regulation of transcription of osmolarity-responsive genes.

### Phosphorylation of Ubp3 by the Hog1 SAPK is required for the transcriptional response upon osmolarity

To determine if Hog1 was controlling Ubp3 function, we assessed whether Ubp3 phosphorylation was crucial for transcriptional activation upon stress. There are 12 sequences corresponding to the consensus phosphorylation site for SAPKs (Ser-Pro or Thr-Pro) in the Ubp3 protein. Initially, we created a single-copy plasmid carrying a mutant version of Ubp3 replacing all 12 putative Ser and Thr sites by SAPKs to Ala (Ubp3-12m), transformed in a *ubp3* strain and assessed transcriptional activation *in vivo*. As shown in Figure 4A (upper panel), cells expressing Ubp3-12m were unable to restore *STL1*, *CTT1* or *ALD3* expression upon stress, when compared with the WT, indicating that phosphorylation of Ubp3 might be important for Ubp3 function in the regulation of gene expression. We reverted each of the 12 mutations to the WT to identify the specific Ubp3-phosphorylation site relevant to stress transcriptional activation. Strikingly, when a mutant carrying 11 mutated sites and containing only Ser695 (Ubp3-11m) was expressed, the transcriptional expression pattern was virtually indistinguishable from that of the WT (Figure 4A, upper panel), suggesting that the essential phosphorylation site in Ubp3 is Ser695. We then



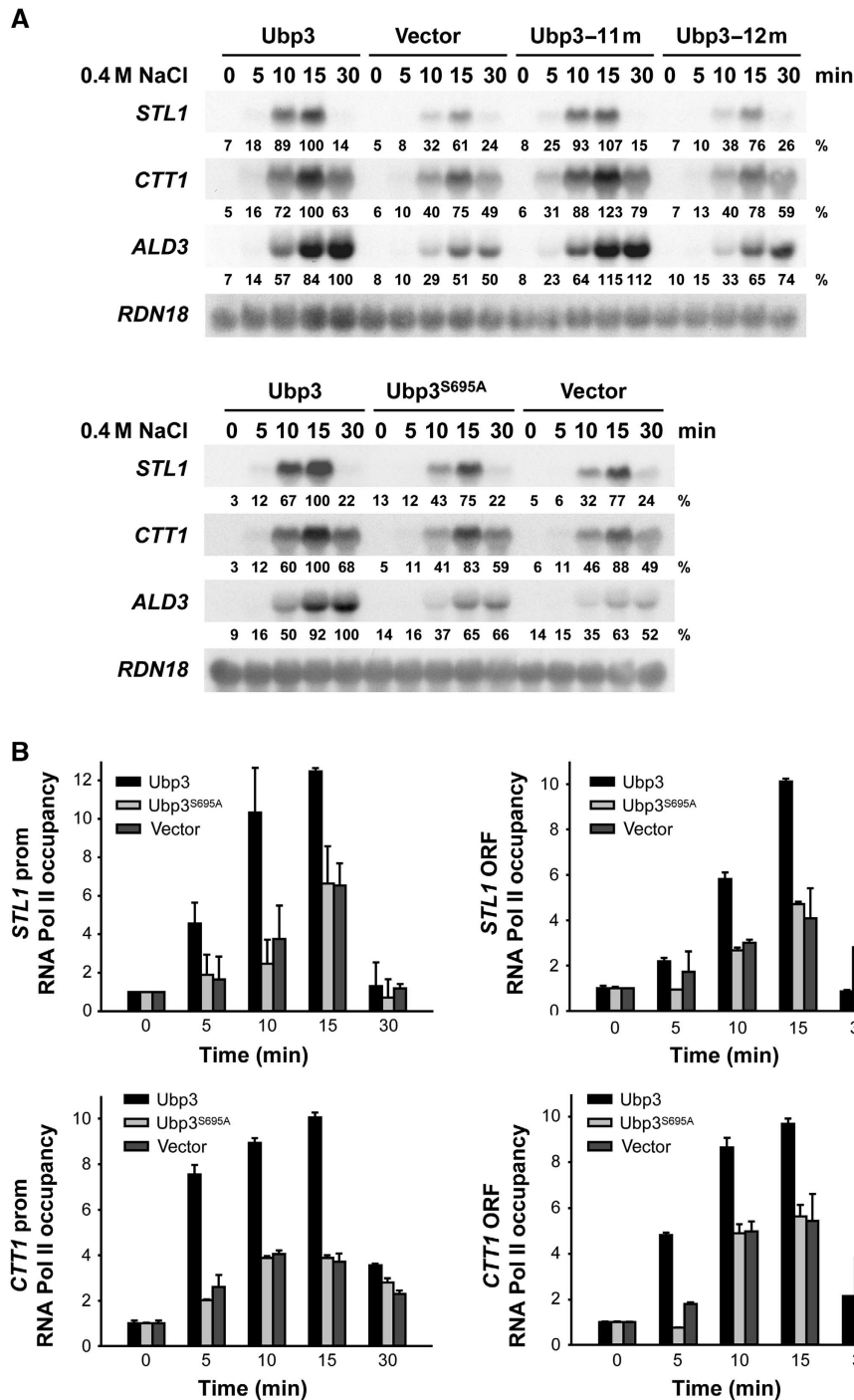
**Figure 2** *In vivo* binding of Hog1 and Ubp3. Hog1-HA and Ubp3-Myc-tagged proteins were expressed from the WT locus. Samples were taken before (-) or 10 min after (+) the addition of 0.4 M NaCl. (A) Ubp3-Myc was immunoprecipitated by monoclonal antibodies against Myc and the Ubp3-Myc (bottom) and Hog1-HA (top) proteins were detected by western blotting against Myc and HA epitopes, respectively. Total represents 2.5% of total input protein (middle). (B) Hog1-HA was immunoprecipitated by monoclonal antibodies against HA and proteins were detected as described for (A).



**Figure 3** In response to osmotic stress, Ubp3 binds to stress-dependent genes through the Hog1 SAPK and it is required for recruitment of RNA Pol II. **(A)** Hog1 mediates the recruitment of Ubp3 to stress-responsive genes in response to osmotic shock. WT (filled bars) and *hog1* (open bars) strains carrying HA-tagged Ubp3 were grown to mid-log phase and subjected to osmotic shock (0.4 M NaCl for the indicated length of time). Proteins were immunoprecipitated with anti-HA monoclonal antibodies and binding to the promoter (left-hand panels) and ORF (right-hand panels) regions of *STL1* and *CTT1* loci was analysed. The real-time PCR results are shown as the fold induction of treated relative to non-treated (time zero) samples normalized to a telomere internal control. Data are the mean and s.d. of three independent experiments. **(B)** Ubp3 is required for proper RNA Pol II occupancy at stress-responsive genes. WT (filled bars) and *ubp3* (open bars) strains were analysed by ChIP as described for **(A)** using anti-Rpb1 antibody (8WG16, Covance).

created and expressed in *ubp3* cells a single point mutant at Ser695 (Ubp3<sup>S695A</sup>). This mutant displayed reduced expression of osmoreponsive genes upon osmotic stress similar to that of *ubp3* cells (Figure 4A, lower panel). Correspondingly, cells carrying the Ubp3<sup>S695A</sup> mutation showed reduced binding of RNA Pol II to *STL1* and *CTT1*, promoters and coding regions, compared with the WT (Figure 4B). Of note, Ubp3<sup>S695A</sup> associated with osmoreponsive promoters upon stress similar to WT. Ubp3<sup>S695A</sup> binding at coding regions was slightly reduced if compared with WT, possibly due to the

reduced amount of RNA Pol II (Supplementary Figure S3). It is worth mentioning that the protein levels of WT and mutated versions of Ubp3 were expressed equally in yeast cells (see Supplementary Figure S4). The drug 6-azauracil (6-AU) reduces the cellular pools of nucleotides and is frequently used as a tool to study the genetics of transcript elongation (Mason and Struhl, 2005). Strains lacking *UBP3* or expressing a version of Ubp3 with impaired catalytic activity (Ubp3<sup>C469A</sup>) are sensitive to 6-AU (Kvint *et al*, 2008). Expression of the unphosphorylatable allele Ubp3<sup>S695A</sup> was

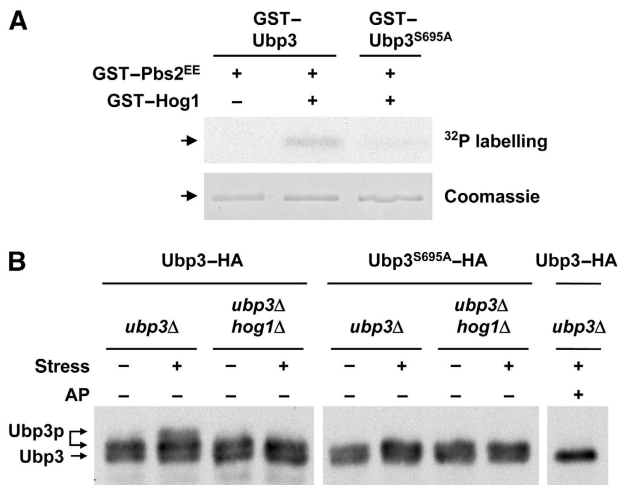


**Figure 4** Phosphorylation modulates Ubp3-mediated transcriptional activation *in vivo*. (A) Impaired gene expression in an unphosphorylatable Ubp3 version upon osmotic stress. The *ubp3* yeast strain was transformed with the indicated plasmids, grown to mid-log phase and subjected to osmotic stress (0.4 M NaCl) for the indicated length of time. Total RNA was assayed by northern blot for transcript levels of *STL1*, *CTT1*, *ALD3* and *RDN18* (as a loading control). Quantification data came from the same original blot and it was normalized to the loading control. The values of maximum gene expression of the WT strain were used as 100% reference. (B) The phosphorylation site of Ubp3 is required for proper RNA Pol II occupancy at stress-responsive genes. The *ubp3* strain was transformed with an empty vector as a control (grey bars), a WT Ubp3 (dark bars) and the unphosphorylatable Ubp3<sup>S695A</sup> proteins (light grey bars). Cells were subjected to osmotic stress (0.4 M NaCl) for the indicated length of time and binding of Rpb1 to the promoter (left-hand panels) and ORF (right-hand panels) regions of *STL1* and *CTT1* was analysed by ChIP as described for Figure 3B.

able to rescue the 6-AU-sensitive phenotype of a *ubp3* strain (Supplementary Figure S5), indicating the functionality of the Ubp3<sup>S695A</sup> allele. Together, our data suggest that the phosphorylation of Ser695 is crucial for mediation of gene expression upon osmotic stress.

#### **Ubp3 is phosphorylated upon osmotic stress in a Hog1-dependent manner**

We used *in vitro* and *in vivo* phosphorylation assays to confirm that the Hog1 SAPK directly phosphorylated Ubp3 Ser695. Initially, we asked whether Hog1 phosphorylated



**Figure 5** Ubp3 is phosphorylated by the Hog1 SAPK at Ser695 *in vitro* and *in vivo*. **(A)** *In vitro* phosphorylation of Ubp3 by Hog1. Recombinant GST-fused proteins were purified from *E. coli*. Hog1 and the constitutively activated Pbs2<sup>EE</sup> allele were incubated (when indicated) in kinase buffer containing ATP. Ubp3 or Ubp3<sup>S695A</sup> was then added in the presence of radioactive ATP. Phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography (upper panel). GST-tagged Ubp3 proteins were detected by staining with Coomassie brilliant blue (lower panel). **(B)** *In vivo* phosphorylation of Ubp3 upon osmotic stress depends on Hog1. Ubp3-HA and Ubp3<sup>S695A</sup>-HA were expressed in a low-copy vector in *ubp3* and *ubp3 hog1* cells. Samples were taken in mid-log phase and fixed in 20% TCA for SDS-PAGE and immunoblotting with monoclonal HA-specific antibodies (12CA5). Cells were subjected or not subjected to brief osmotic shock (0.4 M NaCl for 10 min) and treated or not treated with alkaline phosphatase (AP).

Ubp3 directly. *In vitro* kinase assays were done with *E. coli* purified proteins in which Hog1 was activated in the presence of a constitutive MAPKK allele (Pbs2<sup>EE</sup>) (Proft *et al*, 2001). As shown in Figure 5A, GST-fused Ubp3 expressed from *E. coli* was phosphorylated only when it was incubated with activated Hog1, indicating that Ubp3 is a direct substrate for the Hog1 SAPK. Notably, phosphorylation of Ubp3 was reduced significantly in the Ubp3<sup>S695A</sup> mutant when compared with the WT.

Hog1 was rapidly phosphorylated and activated when the yeast cells were exposed to osmotic stress. To determine whether Ubp3 Ser695 was also phosphorylated *in vivo* by Hog1 in response to osmotic stress, fully functional Ubp3-HA and Ubp3<sup>S695A</sup>-HA-tagged proteins were transformed in *ubp3* and *ubp3 hog1* strains and probed using specific monoclonal antibodies against the HA epitope. As shown in Figure 5B, the mobility pattern of Ubp3 in SDS-polyacrylamide gel electrophoresis (PAGE) was altered in WT cells subjected to osmotic stress. Protein extracts were treated with alkaline phosphatase and the mobility shift was abolished, confirming that the shift in electrophoretic mobility was the result of phosphorylation. Strikingly, mutation of Ser695 abolished the mobility shift because of the phosphorylation of Ubp3 in response to osmotic stress. Furthermore, phosphorylation of Ubp3 in response to osmotic stress was completely dependent on Hog1 because Ubp3-HA expressed in *hog1* cells did not undergo the mobility shift observed for WT cells. It is noteworthy that Ubp3 was partially phosphorylated under basal conditions, although this phosphorylation did not depend on stress or Hog1. Taken together, these results

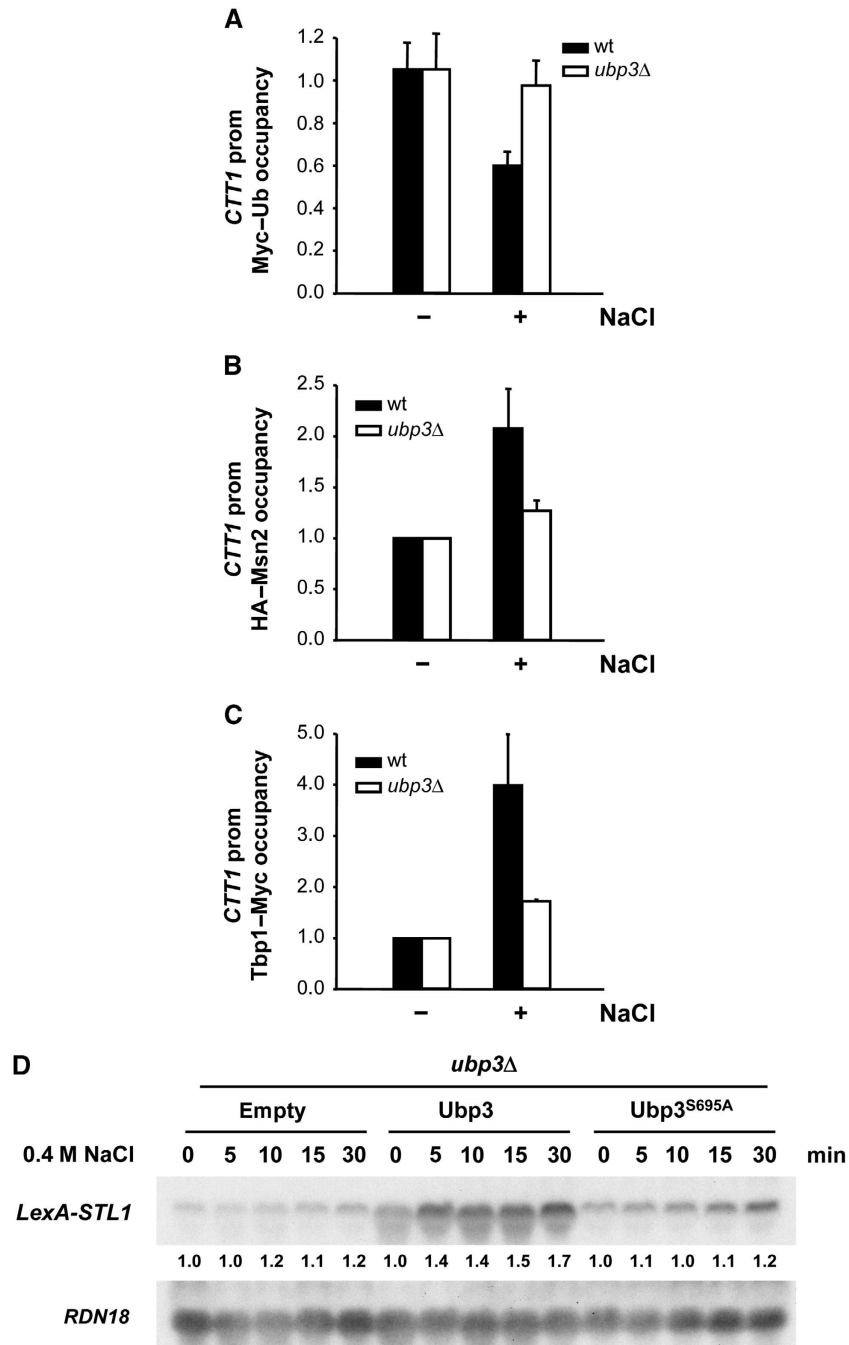
show that *in vivo*, Ubp3 Ser695 is the specific phosphorylation site for Hog1 upon osmotic shock and that this modification depends on the Hog1 SAPK.

### Ubp3 regulates transcription initiation and elongation in stress-responsive genes

Ubp3 is involved in the regulation of transcriptional initiation and elongation by controlling the ubiquitylation of different substrates (Kvint *et al*, 2008; Chew *et al*, 2010). We found that Ubp3 is recruited to promoters and ORFs of osmo-responsive genes (Figure 4A), suggesting that this ubiquitin protease might control initiation as well as elongation upon stress.

To assess the role of Ubp3 at initiation upon osmotic stress, we used ChIP from WT and *ubp3* cells expressing a single-copy Myc-tagged ubiquitin and monitored the ubiquitylation state of proteins recruited to the *CTT1* promoter upon osmotic stress. As shown in Figure 6A, the level of ubiquitylation was reduced upon osmotic stress in WT cells, whereas no change was observed in *ubp3* cells. Thus, the level of ubiquitylation of proteins at the osmo-responsive promoters was altered upon osmotic stress in a Ubp3-dependent manner. Several unrelated transcription factors that can act independently or in combination are responsible for controlling the transcriptional initiation of osmo-responsive genes (Capaldi *et al*, 2008; Ni *et al*, 2009). Among them, the activators Msn2 and Msn4 and the Tbp1/Spt15 proteins are controlled by protein ubiquitylation (Chi *et al*, 2001; Chew *et al*, 2010). We followed the occupancy of HA-tagged Msn2 and Tbp1/Spt15 to the *CTT1* promoter and found that occupancy in osmo-responsive promoters was reduced in the absence of *UBP3* (Figure 6B and C), indicating that Ubp3 modulates the presence of key elements for transcription initiation at promoters upon stress. This is consistent with reduced recruitment of RNA Pol II in *ubp3* cells (Figure 3B). Together, these results show that Ubp3 has a role in transcription initiation in response to stress.

It is known that Ubp3 has a role in transcriptional elongation by directly controlling the ubiquitylation levels of the RNA Pol II subunit 1 (Rpb1) (McCulloch *et al*, 2006; Bilsland *et al*, 2007; Kvint *et al*, 2008). To test whether Ubp3 also affected elongation upon osmotic stress, we uncoupled the processes of transcriptional initiation and elongation by driving the expression of an osmo-inducible gene with the LexA-VP16 activator (Proft *et al*, 2006). Specifically, we measured the RNA expression level of the *STL1* osmo-responsive gene, which is driven by the LexA promoter (*LexA-STL1*) and the LexA-VP16 activator in the presence or in the absence of osmotic stress. In such system, the *STL1* transcript levels in response to stress can be attributed exclusively to an effect on elongation and not to changes in initiation. As shown in Figure 6D, the expression of *STL1* mRNA was induced in response to osmotic stress. It is noteworthy that the induction of mRNA accumulation was impaired in a *ubp3* mutant strain, while the unphosphorylatable allele Ubp3<sup>S695A</sup> was unable to restore *STL1* expression in contrast to the WT. The use of a constitutive activator such as VP16 explains the observed basal transcription in the absence of stress. Expression of the non-responsive stress gene *LexA-ADH1* was not affected in *ubp3* cells (Supplementary Figure S6A). Moreover, we have assessed RNA Pol II association through the ORF of the *LexA-STL1* gene in *ubp3* cells carrying a plasmid containing the WT Ubp3 or the Ubp3<sup>S695A</sup> allele. Cells expressing Ubp3<sup>S695A</sup> showed reduced RNA Pol II at *STL1* coding region upon



**Figure 6** Ubp3 regulates transcriptional initiation and elongation of stress-responsive genes. (A) Ubp3 affects the ubiquitylation state of the recruited proteins to osmoresponsive promoters. WT (filled bars) and *ubp3* (open bars) strains expressing Myc-tagged ubiquitin were grown to the mid-log phase and subjected (+) or not subjected (-) to osmstress (0.4 M NaCl for 10 min). Proteins were immunoprecipitated with anti-Myc monoclonal antibodies and binding to the promoter region of *CTT1* loci was analysed. For simplicity of presentation, the level of ubiquitin binding normalized to a telomere internal control in the absence of stress was set to 1, and the level of stressed cells was expressed relative to that. The data represent the mean and s.d. of three independent experiments. (B) Msn2 occupancy of osmstress promoters depends on Ubp3. WT (filled bars) and *ubp3* (open bars) strains expressing HA-tagged Msn2 under the control of the *ADH1* promoter were grown to mid-log phase and subjected (+) or not subjected (-) to osmstress (0.4 M NaCl for 5 min.). Proteins were immunoprecipitated with anti-HA monoclonal antibodies and binding to the promoter region of *CTT1* loci was analysed. The results are shown as the fold induction of treated relative to non-treated (time zero) samples normalized to a telomere internal control. Data represent the mean and s.d. of three independent experiments. (C) Tbp1/Spt15 occupancy to osmstress promoters is reduced in *ubp3* mutant cells. WT (filled bars) and *ubp3* (open bars) strains expressing Myc-tagged Tbp1/Spt15 were grown and stressed as described for (B). Proteins were immunoprecipitated with anti-HA monoclonal antibodies and binding to the promoter region of *CTT1* loci was analysed. The results are shown as in (B). (D) Ubp3 is required for *STL1* mRNA production when expressed by the LexA-VP16 activator. *ubp3* cells lacking endogenous *STL1* and expressing an empty vector, a single copy of WT Ubp3 or the unphosphorylatable mutant Ubp3<sup>S695A</sup> were transformed with a vector carrying *STL1* under the LexA promoter and with a plasmid containing the LexA-binding domain fused to the VP16 transcriptional activator. Cells were treated with 0.4 M NaCl for the indicated length of time and transcript levels of *STL1* and *RDN18* (as loading control) were measured. Quantification data came from the same original blot for each strain and referenced to that at 0 time point.

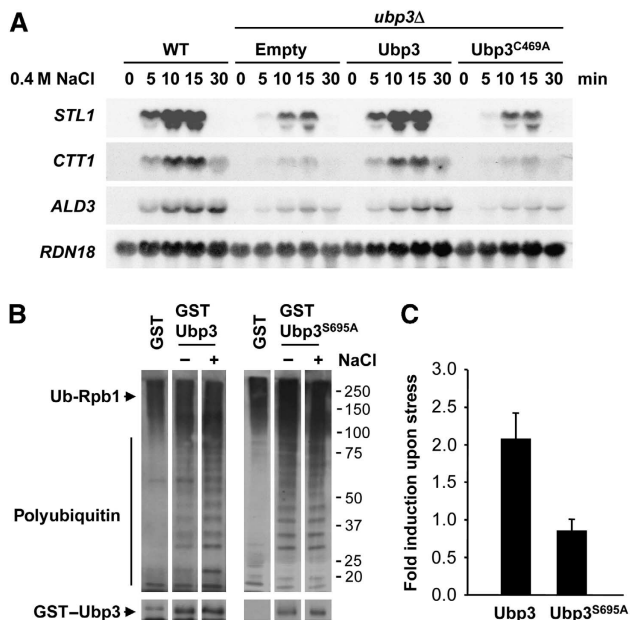
osmstress, compared with WT (Supplementary Figure S6B). Together, these results show that the phosphorylation of Ubp3 by Hog1 is crucial during transcriptional elongation and that the Ubp3 de-ubiquitylase has a role in transcription elongation in response to stress.

### Ubp3 activity is regulated via phosphorylation by Hog1 SAPK

The observation that Ser695 in Ubp3 is required to control transcriptional initiation and elongation suggested that the ubiquitin protease activity might be regulated directly by phosphorylation. First, we assessed whether the function of Ubp3 in osmstress requires its de-ubiquitin protease activity. We created a point mutant allele in Cys469 (Cys469 to Ala469), which has been shown to abolish its catalytic activity (Cohen *et al*, 2003a). Cells expressing the catalytically impaired Ubp3 mutant (Ubp3<sup>C469A</sup>) did not restore gene expression upon osmstress, whereas cells carrying a single-copy plasmid containing the WT Ubp3 rescued expression of the osmoreponsive genes in a *ubp3* strain (Figure 7A). It is noteworthy that the protein levels of WT and catalytically impaired Ubp3 proteins were expressed equally in yeast cells (see Supplementary Figure S4). These results indicate that the de-ubiquitylating activity of Ubp3 is

essential for its function in the Hog1-mediated transcription response.

We analysed Ubp3 activity on a ubiquitylated RNA Pol II substrate to assess whether the activity of Ubp3 was modulated by Hog1 phosphorylation. RNA Pol II was purified using an Rpb3-TAP-tagged strain and ubiquitylated *in vitro* by pure ubiquitylation factors Uba1, Ubc5 and Rsp5 (described by Somesh *et al*, 2005). Polyubiquitylated RNA Pol II was then incubated with purified GST-tagged Ubp3 from yeast cells treated or not treated with NaCl and samples were probed with an anti-ubiquitin antibody. RNA Pol II was de-ubiquitylated efficiently by Ubp3 from yeast as measured by the accumulation of shorter ubiquitin chains (Figure 7B). Of note, a moderate but reproducible two-fold increase of RNA Pol II de-ubiquitylation was observed when Ubp3 was purified from osmstressed cells (Figure 7C). The higher rate of Rpb1 de-ubiquitylation in response to stress suggested that Ubp3 was more active upon Hog1 phosphorylation. We used similar assays expressing the yeast Ubp3 mutant allele unable to be phosphorylated by Hog1 (GST-Ubp3<sup>S695A</sup>) to confirm this possibility. As shown in Figure 7B, the activity of Ubp3<sup>S695A</sup> did not change in response to osmstress. Of note, the activity of WT and Ubp3<sup>S695A</sup> proteins under basal conditions was very similar. Taken together, phosphorylation of Ser695 in Ubp3 by Hog1 is crucial to induce Ubp3 activity upon osmstress.



**Figure 7** Ubp3 de-ubiquitylase activity is required for adaptation to osmstress. (A) The catalytic activity of Ubp3 is essential for osmstress gene activation. *ubp3* mutant cells were transformed with a centromeric plasmid containing the WT Ubp3, a catalytically inactive allele (Ubp3<sup>C469A</sup>) or an empty vector as a control, grown to mid-log phase and subjected to osmstress (0.4 M NaCl) for the indicated length of time. Total RNA was assayed by northern blot analysis for transcript levels of *STL1*, *CTT1*, *ALD3* and *RDN18* as a loading control. (B) Ubp3 phosphorylated by Hog1 in response to osmstress is more active *in vitro*. Highly purified RNA Pol II, ubiquitylated using pure ubiquitylation factors, was de-ubiquitylated with purified WT or S695A mutant Ubp3 fractions isolated from control or osmstressed yeast. The fractions were separated by SDS-PAGE and western blotted with anti-ubiquitin and anti-GST antibodies. (C) Quantification of the results in (B), adjusting for loading of GST-Ubp3. The results are shown as the fold induction of treated relative to non-treated samples normalized to GST-Ubp3 protein levels. Error bars indicate the s.d. among experiments.

## Discussion

SAPKs are key elements for intracellular signalling networks that respond and adapt to extracellular changes. Upon osmstress, the p38-related SAPK Hog1 has a deep impact on different aspects of cellular physiology, such as cell cycle (Clotet and Posas, 2007; Yaakov *et al*, 2009), cytoskeleton reorganization, ion homeostasis and metabolic adjustments (Hohmann, 2002; Hohmann *et al*, 2007), as well as major effects on gene expression (de Nadal and Posas, 2010; Martinez-Montanes *et al*, 2010; Weake and Workman, 2010). Here, we provide evidence that the ubiquitin protease Ubp3 is a novel target of the Hog1 SAPK for modulating gene expression in response to extracellular input. First, Ubp3 copurifies with Hog1 in an osmotic stress-dependent manner. Second, Hog1 phosphorylates Ubp3 at serine 695 upon osmotic stress. Third, this phosphorylation is essential for the transcriptional response to osmstress because the non-phosphorylated mutant shows impaired osmoreponsive gene expression and reduced binding of RNA Pol II.

The balance of ubiquitylation is crucial to the modulation of many activities. There are several examples of control of E3 ubiquitin ligases; however, little is known about the regulation of ubiquitin proteases in response to external stimuli. Here, we show that Ubp3 phosphorylated by the Hog1 SAPK upon stress alters its activity. Ubp3p is also phosphorylated under basal conditions in alternative phosphorylation sites than Hog1, suggesting that Ubp3 can be targeted by alternative kinases in different growth conditions. Actually, analysis by mass spectrometry of purified Ubp3 from yeast cells identified several Ubp3 phosphorylation sites that were not osmo-regulated. How Ubp3 de-ubiquitylase activity is regulated is a major question. Ubp3 function is modulated by binding cofactor Bre5 (Li *et al*, 2005, 2007). Because the phosphorylatable Ubp3 serine 695 is far away



from its binding domain with Bre5 in the tertiary structure, it is unlikely that the interaction of Ubp3 with Bre5 is altered by phosphorylation. An alternative scenario is that binding between Ubp3 and its substrates is altered upon phosphorylation of Ubp3. Again, this is an unlikely scenario because the affinity between RNA Pol II, a well-known Ubp3 substrate, and the de-ubiquitylase is not affected by osmostress or by the presence of the SAPK (Supplementary Figure S7). Interestingly, our data suggest that Ubp3 activity might be up-regulated by phosphorylation that occurs on the catalytic domain of Ubp3 and the enzyme, which is able to de-ubiquitylate ubiquitylated RNA Pol II *in vitro*, does it more efficiently when phosphorylated by the Hog1 SAPK. Correspondingly, the activity of the non-phosphorylated Ubp3<sup>S695A</sup> mutant does not change in response to osmostress. In addition, the complementation of the 6-AU sensitivity of the *ubp3* mutant by Ubp3<sup>S695A</sup> shows that this allele is able to perform some of the functions of Ubp3 under normal growth conditions. Thus, these results suggest that Ubp3 activity is increased by Hog1 phosphorylation in response to stress.

Ubiquitin proteases are implicated in multiple processes, including the reversal of ubiquitylation, which functions to prevent degradation or to modify substrate activity. In this study, we found that Ubp3 is implied in regulating both initiation and elongation of transcription in response to stress. Ubp3 reverses the ubiquitylation of Tbp1/Spt15 during transcriptional activation (Chew *et al*, 2010), which opens the possibility that Ubp3 recruited by the Hog1 SAPK protects promoter-bound Tbp1/Spt15. Indeed, Tbp1/Spt15 occupancy at stress-responsive promoters is reduced in the absence of *UBP3*. Alternatively, the transcription factor Msn2, which acts downstream of Hog1, is known to be ubiquitylated (Chi *et al*, 2001). It is noteworthy that binding of Msn2 in osmo-responsive promoters is reduced in *ubp3* cells. Thus, it seems likely that the basic machinery involved in transcription initiation upon osmostress is altered in *UBP3*-deficient cells. Correspondingly, there is less expression of a reporter construct that contains only a stress-responsive promoter fused to LacZ and there is a strong reduction of the amount of RNA Pol II recruited at stress-responsive genes upon osmostress. Binding of Ubp3 occurs in both promoters and ORFs of stress-activated genes, indicating a role of this ubiquitin protease in transcriptional initiation and in elongation in response to osmostress. Ubp3 has a role in rescuing RNA Pol II from degradation by reversing the ubiquitylation of RNA Pol II (Kvint *et al*, 2008). We show that Ubp3 is important for induction of stress-activated genes at coding regions, suggesting that Ubp3 also targets elongating RNA Pol II upon osmostress. Thus, although we cannot exclude other targets of Ubp3 on osmostress-mediated transcriptional activation, Ubp3 clearly has a prominent role in this process. The Hog1 SAPK interacts and travels with elongating RNA Pol II through the coding regions of osmo-responsive genes (Proft *et al*, 2001). Because Hog1 associates specifically with ORF of osmo-responsive genes in response to stress, it seems likely that the SAPK itself dictates the specificity of targeting RNA Pol II and, presumably also Ubp3, to osmo-responsive ORFs. Together, our data suggest that the control of Ubp3 activity by the HOG pathway in response to extracellular stimuli controls the balance of ubiquitylated proteins and this is a key factor in determining the outcome of gene expression in response to external stimuli.

## Materials and methods

### Yeast strains and plasmids

*S. cerevisiae* strain BY4741 (MATA *his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*) and its derivatives YEN257 (*UBP3::NatMX*), YEN262 (*UBP3::NatMX HOG1::kanMX4*), YCS168 (*UBP5::kanMX4*) and YCS48 (*UBP2::kanMX4*) were obtained in this work. Genomic disruptions were made by long flanking homology PCR-based gene disruption. Deletion strains and strain Rpb3-TAP were obtained from the EUROSCARF collection. The tagging strains obtained in this study are YEN255 (*UBP3-6HA::HIS3*), YEN256 (*UBP3-6HA::HIS3 HOG1::kanMX4*), YCS9 (*HA-UBP3*), YCS175 (*HA-UBP3<sup>S695A</sup>*), YCS41 (*HA-UBP3 HOG1::kanMX4*), YMZ33 (*HOG1-6HA::HIS3*), YCS33 (*UBP3-18Myc::HIS3 HOG1-6HA::kanMX4*), YCS22 (*UBP3-18Myc::HIS3*), YEN254 (*HOG1-HA::HIS3 UBP3::kanMX4*), YCS98 (*TBP1-18Myc::HIS*) and YCS101 (*UBP3::kanMX4 TBP1-18Myc::HIS*). Tagging of genomic ORFs with HA or Myc epitopes was done with a PCR-based strategy.

The plasmids used in this study were pGEX4T-Hog1 and pGEX4T-Pbs2<sup>EE</sup> (*PBS2* with Ser514-Glu and Thr518-Glu mutations), which are described by Bilsland-Marchesan *et al* (2000). *UBP3* (PEN253), *UBP3-S695A* (*UBP3* with Ser695-Ala mutation) (PCS81) and *RSP5* (PCS31) were cloned into pGEX4T. *UBP3* (PEN298), *UBP3-C469A* (*UBP3* with Cys469-Ala mutation) (PEN301), *UBP3-S695A* (PCS71), *UBP3-11mut* (*UBP3* with S20A, T25A, S74A, S146A, S171A, S339A, S341A, S343A, T376A, S400A and S646A mutations) (PCS68) and *UBP3-12mut* (*UBP3* with S20A, T25A, S74A, S146A, S171A, S339A, S341A, S343A, T376A, S400A, S646A and S695A mutations) (PCS69) were cloned into pRS415. For HA tagging, *UBP3* (PCS70) and *UBP3-S695A* (PCS75) were cloned into the yeast vector pRS415-HAx6::HIS3. *UBP3* (PEN285) and *UBP3-S695A* (PCS84) were fused to glutathione S-transferase (GST) under the *TEF1* promoter (pRS426TEG1). YCpLac111 *P<sub>ADH1</sub>MSN2-3HA* was a gift from Dr C Schuller (Gorner *et al*, 1998). *P<sub>ADH1</sub>MSN2-3HA* was cloned into pRS416 (PCS95). *CEN*-based vector *P<sub>TEF1</sub>9Myc-Ubiquitin*; *LEU2<sup>+</sup>* (PGR140) was a gift from Dr M Peter. The *STL1::LacZ* fusion was constructed by insertion of the -825/+4 nucleotides of the *STL1* gene into the multicopy plasmid Yep358R containing the reporter PEN9. The LexA-binding domain fused to the VP16 transcriptional activator and the LexA-*STL1* (PGM45) and LexA-*ADH1* (PGM66) vectors are described by Proft *et al* (2006).

### Screening and β-galactosidase assays

An ordered array of 800 *MATa* viable haploid yeast gene deletion mutants obtained from EUROSCARF (*Saccharomyces* Gene Deletion Project) was crossed with *MATα STL1::LacZ* and replica pinned in duplicate onto YPD and YPD with 0.4 M NaCl, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) plates. The screen was done with an automated system in which yeast cells are transferred using a Biomek FX robot and the 384 floating-pin replicator (Biomek FX HDR 384-Pin plate) as described (Tong *et al*, 2001). The screen was done twice and the plates were incubated at 30°C for 2 days before scoring. X-gal (solid medium contains selective medium buffered with 2-(*N*-morpholino) ethanesulfonic acid (Mes)) at pH 7 plus 0.1 mg/ml X-gal. For liquid β-galactosidase assays, exponentially growing cells were subjected to osmotic stress (0.4 M NaCl for 30 min), permeabilized by treatment with ethanol/toluene and β-galactosidase measured as described (Proft *et al*, 2001).

### Northern blot analysis

Yeast strains were grown to mid-log phase in rich medium or in minimal medium for plasmid selection at an absorbance at 660 nm (*A<sub>660</sub>*) of 0.6–0.9 and then subjected to osmotic shock (0.4 M NaCl) for the length of time indicated. Total RNA and expression of specific genes were probed using radiolabelled PCR fragments containing the entire ORF of *STL1* (1.7 kbp), *CTT1* (1.7 kbp), *ALD3* (1.5 kbp) and the non-coding exon of *RDN18* (1.8 kbp). Signals were quantified with a Fujifilm BAS-5000 phosphorimager and autoradiographs were obtained on Kodak Biomax XAR films (Sigma-Aldrich).

### In vitro kinase assays

The GST fusion proteins encoding the WT or the Ubp3 mutant, Hog1 and Pbs2<sup>EE</sup> were expressed in *E. coli* DH5α and purified using glutathione-Sepharose beads (GE Healthcare) in STET buffer (10 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0, 5% Triton

X-100, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 2 µg/ml leupeptin, 2 µg/ml of pepstatin). A 1-µg sample of Hog1 was activated with 0.5 µg of Pbs2<sup>EE</sup> in the presence of kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 2 mM DTT) and 50 µM ATP. After 20 min at 30°C, 2 µg of WT or mutated Ubp3 protein was added to the previous mixture together with [ $\gamma$ -<sup>32</sup>P]ATP (0.1 µCi/µl) and incubated for 15 min at 30°C. The reaction was terminated by the addition of 5 × SDS loading buffer. Labelled proteins were resolved by SDS/PAGE, stained, dried and detected by autoradiography.

#### **In vitro coprecipitation assay**

GST or GST-Ubp3 was expressed in *E. coli* DH5 $\alpha$  cells and purified using Glutathione-Sepharose beads (GE Healthcare) as described above. His-tagged Hog1 was expressed in *E. coli* BL21 strain and 150 µg of total extract was incubated with TALON metal affinity resin (Clontech) in binding buffer (20 mM Tris pH 8, 150 mM NaCl plus anti-proteases inhibitors) for 1 h. A total of 0.5 µg of GST-purified proteins was then added for 3 h. Beads were extensively washed (50 mM Tris pH 8, 150 mM NaCl, 0.1% Triton X-100) and samples were boiled with 2 × SDS loading buffer. Bound proteins were analysed by western blotting using anti-GST (GE Healthcare) and anti-Hog1 ( $\gamma$ C-20, Santa Cruz Biotechnology) antibodies.

#### **In vivo Ubp3 phosphorylation assays**

*ubp3* or *ubp3 hog1* cells were transformed with HA-tagged WT Ubp3 protein or the indicated mutant form and grown in minimal medium for plasmid selection. Cells at the mid-log phase were subjected to osmotic shock (0.4 M NaCl for 5 min) and 1 ml of culture was fixed with 25% trichloroacetic acid (TCA), pelleted and washed with 100 µl of 12.5% TCA. Following the addition of glass beads, pellets were lysed by vortex mixing for 5 min, washed in 1 ml of iced acetone and dried at 55°C for 15 min. Protein precipitates were resuspended in 15 µl of 1% SDS, 100 mM Tris-HCl pH 8.0, 1 mM EDTA. Finally, 35 µl of AP buffer (100 mM Tris-HCl pH 9.5, 1 mM EDTA) was added to all samples and, when indicated, 0.2 µl of alkaline phosphatase (20 U/µl; Roche) for 30 min at 37°C. Samples were separated by SDS-PAGE and Ubp3 protein was detected by immunoblotting using monoclonal anti-HA 12CA5.

#### **In vivo coprecipitation assay**

Ubp3 and/or Hog1-tagged cells in mid-log phase (50 ml) were treated with 0.4 M NaCl for 15 min or left untreated and then collected by brief centrifugation at 4°C. Pellets were harvested with glass beads in the FastPrep-24 (Qbiogene, 30 s at speed 5) in lysis buffer A (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 15 mM EDTA, 15 mM EGTA, 2 mM DTT, 0.1% Triton X-100, 1 mM PMSF, 1 mM benzamidine, 2 µg/ml leupeptin, 2 µg/ml pepstatin, 25 mM  $\beta$ -glycerophosphate, 1 mM sodium pyrophosphate, 10 mM sodium fluoride, 100 µM sodium orthovanadate), and lysates were clarified by centrifugation and quantified by the Bradford assay (Bio-Rad Laboratories). As a control, 25 µg of whole-cell extract was blotted with anti-HA 12CA5 or anti-Myc 9E10 to check the expression levels of the tagged proteins (total, shown in Figure 2). Alternatively, 1 mg of cleared supernatant was subjected to immunoprecipitation with anti-Rpb1 (8WG16, Covance), anti-HA 12CA5 or anti-Myc 9E10 overnight at 4°C. Antibodies were recovered with protein G and were resolved by SDS-PAGE.

#### **ChIP assays**

ChIP was performed as described (Zapater *et al*, 2007). Yeast cultures were grown to early log phase ( $A_{660}$  0.6–0.9) before samples of the culture were exposed to osmotic stress (0.4 M NaCl) for the length of time specified in the legends of Figures 3 and 4. For crosslinking, yeast cells were treated with 1% formaldehyde for 20 min at room temperature. Antibodies used in this study were mouse polyclonal anti-Rpb1 (8WG16, Covance), monoclonal

anti-Myc 9E10 and anti-HA 12CA5. Quantitative PCR analysis of stress genes and constitutively expressed genes used the following primers with locations indicated by the distance from the respective ATG initiation codon: *STL1* promoter, –682/–582; *STL1* ORF, +1475/+1575; *CTT1* promoter, –432/–302 (–275/+125 for TBP1 binding); *CTT1* ORF, +736/+836; and *TEL* (telomeric region on the right arm of chromosome VI). Experiments were done on three independent chromatin preparations and quantitative PCR analysis was done in real time using an Applied Biosystems 7700 sequence detector. Immunoprecipitation efficiency was calculated in triplicate by dividing the amount of PCR product in the immunoprecipitated sample by that in the *TEL* sequence control. The binding data are presented as fold induction with respect to the non-treated condition.

#### **Recombinant protein purification**

The *ubp3* strain was transformed with the pRS426TEG1 Ubp3 or Ubp3-S695A mutant and the mid-log phase culture (2 l) was subjected or not to a brief osmotic shock (0.4 M NaCl for 15 min). GST pull-down experiments were done using glutathione-Sepharose 4B (GE Healthcare) in buffer A. Bound protein was eluted by 10 mM reduced glutathione (GSH), 50 mM Tris-HCl pH 9.5, 2 mM DTT. Eluted proteins were concentrated (Amicon Ultra centrifugal filters, 30 kDa cutoff membrane; Millipore) and resuspended in ubiquitination buffer (25 mM Tris pH 8, 125 mM NaCl, 2 mM MgCl<sub>2</sub>, 50 µM DTT). The TAP-tagged Rpb3 strain was used for the purification of RNA Pol II as described (Puig *et al*, 2001).

#### **De-ubiquitylation of Rpb1 in vitro**

For de-ubiquitylation reactions *in vitro*, 250–300 ng of RNA Pol II was incubated with 40 ng of Uba1 (Boston Biochem), 40 ng of Ubc5H5c (Boston Biochem), 90 ng of GST-Rsp5, 2 µg of ubiquitin (Boston Biochem), 2 mM ATP and 40 ng of purified Ubp3 proteins in ubiquitylation buffer for 90 min at 30°C. Reactions were stopped by the addition of SDS-PAGE loading buffer. The samples were fractionated in BisTris 4–12% polyacrylamide gel or acetate 7% polyacrylamide gel (NuPAGE Invitrogen) then blotted and probed with an anti-ubiquitin antibody (Sigma) and an anti-GST antibody (Sigma).

#### **Supplementary data**

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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*Author contributions:* CS, MN, CK, FP and EN performed the experiments and analysed the results. CS, MN, CK, MP, EN and FP participated in the experimental design. CS, FP, MP and EN wrote the paper.

## **Conflict of interest**

The authors declare that they have no conflict of interest.

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