The yeast Hot1 transcription factor is critical for activating a single target gene, STL1

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ABSTRACT Transcription factors are commonly activated by signal transduction cascades and induce expression of many genes. They therefore play critical roles in determining the cell's fate. The yeast Hog1 MAP kinase pathway is believed to control the transcription of hundreds of genes via several transcription factors. To identify the bona fide target genes of Hog1, we inducibly expressed the spontaneously active variant Hog1\textsuperscript{D170A+F318L} in cells lacking the Hog1 activator Pbs2. This system allowed monitoring the effects of Hog1 by itself. Expression of Hog1\textsuperscript{D170A+F318L} in pbs2\textsuperscript{Δ} cells imposed induction of just 105 and suppression of only 26 transcripts by at least twofold. We looked for the Hog1-responsive element within the promoter of the most highly induced gene, STL1 (88-fold). A novel Hog1 responsive element (HoRE) was identified and shown to be the direct target of the transcription factor Hot1. Unexpectedly, we could not find this HoRE in any other yeast promoter. In addition, the only gene whose expression was abolished in hot1\textsuperscript{Δ} cells was STL1. Thus Hot1 is essential for transcription of just one gene, STL1. Hot1 may represent a class of transcription factors that are essential for transcription of a very few genes or even just one.

INTRODUCTION

Transcriptional activators and suppressors, known as transcription factors, are major components in determining the spectra and levels of gene expression (Struhl, 1989; Treisman, 1996; Lemon and Tjian, 2000; Levine et al., 2014). These molecules exert their effects on transcription when they associate with specific binding sites (cis-elements), which commonly reside adjacent to the promoters of their target genes (Lee et al., 2002; Babu et al., 2004; Harbison et al., 2004; Meireles-Filho and Stark, 2009; Aerts, 2012; Levine et al., 2014). Current understanding is that an individual transcription factor governs the expression of multiple target genes, which harbor its preferred binding site in their promoters. Prominent examples are the mammalian transcription factors c-Jun, CREB, MyoD, and NFκB (Rothwarf and Karin, 1999; Florin et al., 2004; Bailey and Europe-Finner, 2005; Bailey et al., 2005; Cao et al., 2006). An exceptional example is c-Myc, believed to control transcription of thousands of genes (Dang et al., 2006; van Riggelen et al., 2010). A similar phenomenon is observed in the yeast Saccharomyces cerevisiae. The yeast transcriptional activator Gcn4, for example, controls ∼539 genes (Natarajan et al., 2001), the yeast heat shock factor 1 controls at least 165 genes (Hahn et al., 2004; Yamamoto et al., 2005), and the Msn2/4 activators regulate 80–140 genes (Boy-Marcotte et al., 1998; Gasch et al., 2000; Cusson et al., 2001). Because such factors modify transcription of many genes and thereby determine the cell’s fate, they are regarded as “master genes” or “primary factors.”

By contrast, in this article, we describe the case of the yeast transcription factor Hot1, which is involved in controlling just a handful of genes and is essential for transcriptional induction of just one gene, STL1. Hot1 is activated in response to osmotic pressure by the Pbs2/Hog1 mitogen-activated protein kinase (MAPK) pathway (Rep et al., 1999; Alepuz et al., 2003). This pathway allows adaptation to osmостress and consequently cell division under these conditions, primarily by enhancing the synthesis of glycerol (Sprague, 1998; Hohmann, 2002; O’Rourke et al., 2002; Saito and Tatebayashi, 2004;
Saito and Posas, 2012; Westfall et al., 2004; Maayan et al., 2012). The Pbs2/Hog1 pathway also controls all phases of the cell cycle and modulates the transcription of hundreds of genes (O’Rourke and Herskovitz, 2002; de Nadal et al., 2011; Saito and Posas, 2012; Duch et al., 2013). Cells deficient for the genes encoding the MAPK Hog1 or the MAPK kinase (MAPKK) Pbs2 do not carry out these activities and cannot proliferate under osmotic pressure (Brewster et al., 1993; Maayan and Engelberg, 2009; Saito and Posas, 2012). Hog1 affects gene expression mostly via the intermediary transcriptional activators Msn2/4, Sko1, and Hot1 (Schuler et al., 1994; Rep et al., 1999; Profit and Struhl, 2002; Profit et al., 2005; Alepuz et al., 2003). Large-scale gene expression analysis suggests that Msn2/4, Sko1, and Hot1 combined are responsible for 88% of Hog1-dependent gene activation (Capaldi et al., 2008). The mechanism proposed for Hog1-mediated Hot1 activation is unusual. Although Hog1 phosphorylates Hot1, this phosphorylation seems not to be essential for Hot1 transcriptional activity (Alepuz et al., 2003). Instead, Hot1 associates physically with its target promoters, and in response to osmotic stress, it binds active Hog1, thereby recruiting Hog1 to the promoter. Once bound to the promoter, Hog1 functions as a transcription factor and increases transcription initiation rate by recruiting the chromatin-remodeling component Rpd3, as well as by directly associating with RNA PolII and components of the mediator complex (Alepuz et al., 2003; de Nadal et al., 2004). Several critical aspects of this proposed mechanism are still unknown. For example, the cis-element(s) recognized by Hot1 have not yet been defined. In addition, the mechanism involving interaction of Hog1 + Hot1 + RNA PolII was proposed on the basis of observations made on the promoter of the STL1 gene, and it is not yet known how many other promoters are targeted in a similar Hot1 + Hog1-dependent mechanism.

Besides our lack of knowledge of the target genes of the Hog1 + Hot1 system, the identity of the specific bona fide target genes of the Hog1 cascade is not clear. Up to now, target genes of the Hog1 pathway have been defined as genes whose expression level changes in response to osmotic pressure in wild-type cells but not in hog1Δ cells (Posas et al., 2000; Rep et al., 2000; O’Rourke and Herskovitz, 2004; Capaldi et al., 2008). The experiments on which this definition is based showed that changes in expression (increase or decrease) of ~300 genes (Capaldi et al., 2008) or even 580 genes (O’Rourke and Herskovitz, 2004) are Hog1 dependent. However, genes identified this way represent those for which Hog1 is essential as a modulator of expression but does not necessarily suffice to initiate it. Defining the genes controlled by Hog1 per se would necessitate exclusive activation of Hog1, that is, without exposure of the cell to any stimulus that concomitantly activates other pathways.

To generate such a situation, we expressed a Hog1 molecule that is intrinsically active, meaning that its biochemical and biological activities are independent of any upstream signal and of Pbs2/MAPKK activation (Bell et al., 2001; Bell and Engelberg, 2003; Yaakov et al., 2003; Maayan et al., 2012). Because this Hog1 molecule is spontaneously active in yeast cells not exposed to any stress, they should be capable of precisely disclosing the bona fide downstream targets of Hog1. We found that inductive expression of intrinsically active Hog1 in hog1Δ pbs2Δ cells leads to induction of mRNA levels of 105 genes (by twofold or more), only 13 of which were induced by 10-fold or more. Five of the 13 most highly induced genes, including the top 2, STL1 (88-fold) and RTC3 (75-fold), were reported as targets of the transcriptional activator Hot1 (Rep et al., 2000; Capaldi et al., 2008; Gomar-Alba et al., 2012). Because the cis-element through which Hot1 activates transcription had not been identified, we focused on the STL1 promoter, dissected it thoroughly, and identified a novel osmostress- and Hog1-regulated cis-element (which we termed the Hog1 responsive element [HoRE]). The HoRE contains two short identical repeats of the sequence 5’-CTTGGCC-3’ and a similar third repeat. We showed that this element binds a recombinant Hot1 protein in vitro. Its activation in vivo requires both Hot1 and Hog1, and for full induction requires Sko1 as well. Intriguingly, we could not find identical or similar HoREs in other yeast promoters, including promoters of proposed Hot1 targets. In addition, comparing of mRNA molecules expressed in hot1Δ and wild-type cells exposed to various types of stress revealed that the only gene whose mRNA was barely detected in hot1Δ cells was STL1. These observations combined suggest that Hot1 is likely to be essential for transcription of only STL1.

RESULTS

Expression of intrinsically active Hog1 in hog1Δ pbs2Δ cells affects only 131 genes

Previous studies identified genes whose induction or suppression in response to osmotic pressure are critically dependent on Hog1 (Posas et al., 2000; Rep et al., 2000; O’Rourke and Herskovitz, 2004; Capaldi et al., 2008). To identify genes for which Hog1 is not merely essential but is actually sufficient for modifying their expression, we expressed an intrinsically active variant of Hog1, Hog1D170A+F318I, in hog1Δ pbs2Δ cells. To avoid constitutive activity of Hog1 that might generate selective pressure throughout the cell’s life and create a nonreceptive transcriptome, we used an inducible expression system. In this system, every change in RNA levels after induction of Hog1D170A+F318I expression could be specifically attributed to Hog1 activity. For inducible expression, we used the MET3 promoter, which can be efficiently shut off in medium supplemented with methionine and is rapidly activated upon methionine removal (Mumberg et al., 1994; Yaakov et al., 2003). We introduced the MET3-HOG1D170A+F318I plasmid, an “empty” plasmid, or a MET3-HOG1WT plasmid into hog1Δ pbs2Δ cells. Cells of the resulting three strains were grown to mid log phase on medium containing methionine, washed, and resuspended in medium lacking methionine. Samples for mRNA isolation were collected before removal of methionine (time point 0), as well as at 45 and 90 min after removal of methionine. The experimental setup is schematically presented in Figure 1A. Western blot analysis verified that removal of methionine resulted in induction of the Hog1 molecules (Figure 1B, top). Because Hog1D170A+F318I protein is autoactivated via spontaneous autophosphorylation (Bell et al., 2001; Bell and Engelberg, 2003; Yaakov et al., 2003), we verified that it is phosphorylated after induction of expression, whereas Hog1WT is not (Figure 1B, bottom).

mRNA samples were analyzed on a microarray of Agilent SurePrint G3 (Yeast), one-color, 8 x 60K-format slides. Data were analyzed with the aid of the specialized microarray analysis software Genespring GX. We first calculated the ratio of expression levels of every gene at each time point in cells expressing Hog1WT or Hog1D170A+F318I relative to those in cells harboring the “empty” vector. mRNA molecules with a ratio of less than two were excluded. This calculation enabled us to eliminate genes that were induced or suppressed after methionine removal in all three strains (mostly genes involved in methionine synthesis), as well as genes that were not affected at all by methionine removal in the three strains. From the remaining genes, we selected those whose expression levels were changed at least twofold at the 90-min time point, and thus we obtained a list of genes specifically induced in response to expression of Hog1WT (Table 1) and a list of genes induced or suppressed in response to expression of Hog1D170A+F318I relative to their expression in cells harboring an “empty” vector (Tables 2A and 2B).
Expression of Hog1WT in hog1Δpbs2Δ cells had a minor effect on gene expression. No genes were found to be significantly suppressed, and only 2 genes, GPD1 and GP22, were induced by >2-fold (Table 1). The effect of Hog1D170A+F318L expression in hog1Δpbs2Δ cells was more dramatic, leading to induction of 105 genes (Table 2A) and suppression of 26 genes by >2-fold (Table 2B).

Among the 13 genes that are most strongly induced by active Hog1 (≥10-fold; Table 2A), five genes, STL1, RTC3/HGI1, THI4, GPD1, and GPP2, were previously reported to be targets of the transcription factor Hot1 (Alepez et al., 2003; Capaldi et al., 2008; Gomar-Alba et al., 2012). STL1 and RTC3 are the most highly induced genes by Hog1D170A+F318L, 88- and 75-fold, respectively (Table 2A). More putative Hot1 target genes—SED1, FIT1, SPI1, FM48, and NQM1—were also induced by Hog1D170A+F318L, but less strongly (Table 2A).

Because Hot1 seems to be a central mediator of the Hog1D170A+F318L effect on transcription, we sought to find which element it recognizes on its target promoters. We focused for this purpose on the most highly induced gene, STL1, assuming that identification of the Hog1- and/or osmmostress-responsive regions within its promoter would disclose the Hot1 target element. To identify the Hog1-responsive element within the STL1 promoter, we cloned from the yeast genome the 704 base pairs located upstream of the first codon of STL1 and inserted them upstream to the β-galactosidase gene. In wild-type cells (the YPH102 strain), the –704STL1-LacZ gene was strongly activated after exposure of cells to 0.7 M NaCl (top bar in Figure 3A). It was inactive and could not be induced in hog1Δ cells harboring the “empty” vector (Figure 3B). In hog1Δ cells harboring the MET3-HOG1D170A+F318L plasmid, the –704STL1-LacZ reporter was strongly induced after removal of methionine (Figure 3B), whereas in hog1Δ cells harboring the MET3-HOG1WT plasmid, it was induced only after both removal of methionine and exposure of cells to osmmostress (Figure 3B). This experiment verifies that the 704–base pair promoter region is activated by osmotic pressure as expected and also by activation of Hog1 by itself. Promoter sequence analysis revealed a single stress responsive element (STRE) sequence, 5′-CCCCT-3′, located 175 base pairs upstream of the start codon, raising the possibility that the Ras/cAMP pathway via Msn2/4 is involved in regulating STL1. However, mutating the STRE (Figure 3C) or testing the –704STL1-LacZ in ras2Δ, msn2Δ/msn4Δ, and ras2Δ/msn2Δ/msn4Δ cells (Figure 3D) showed that the Ras/STRE system is not involved in STL1 promoter activation by active Hog1 or in response to osmotic pressure.

**FIGURE 1:** The experimental system. Induced expression of intrinsically active Hog1 in hog1Δpbs2Δ cells. (A) Schematic description of the experimental setup. Cells of the indicated three strains were grown to mid log phase in medium containing 160 mg/l methionine, which suppresses the expression of ectopic Hog1. The cells were then washed, resuspended in methionine-deficient medium lacking methionine, and allowed to continue proliferating. mRNA samples were collected before removal of methionine (time point 0) and at 45 and 90 min after removal of methionine. (B) Hog1 molecules were monitored 45 and 90 min after removal of methionine, and Hog1D170A+F318L was spontaneously phosphorylated. Protein lysates were prepared from cells collected at the time points at which RNA was isolated (A) and analyzed by Western blot using anti-Hog1 antibodies (top) and anti–phospho-p38 antibodies (bottom).

| Gene name | Fold change | Gene name | Fold change |
|-----------|-------------|-----------|-------------|
| GPD1      | 4.62        |           |             |
| GP22      | 2.08        |           |             |

Fold change was calculated as the ratio between expression levels in hog1Δpbs2Δ/MET3-Hog1WT and hog1Δpbs2Δ/MET3-empty vector cells 90 min after methionine removal.

**TABLE 1:** Genes induced or suppressed in hog1Δpbs2Δ cells after induction of Hog1WT expression.
−626, gradually reduced promoter responsiveness (Figure 3A). This suggested that the upstream promoter region, between −654 and −626, may harbor the NaCl-responsive and active-Hog1–responsive cis-element(s).

To test whether the upstream elements of the STL1 promoter are sufficient to render a heterologous promoter responsive to both osmostress and Hog1, we inserted fragments derived from the STL1 promoter via sequence analysis, we used an unbiased approach to identify the Hog1-responsive element by preparing a series of constructs carrying systematic truncations of the STL1 promoter (Figure 3A). We observed that a promoter as short as 654 base pairs was responsive to NaCl at the same efficiency as the −704STL1-LacZ construct (Figure 3A). However, further truncations, up to position 626, gradually reduced promoter responsiveness (Figure 3A). This suggested that the upstream promoter region, between −654 and −626, may harbor the NaCl-responsive and active-Hog1–responsive cis-element(s).

Because no other plausible element was identified in the promoter via sequence analysis, we used an unbiased approach to identify the Hog1-responsive element by preparing a series of constructs carrying systematic truncations of the STL1 promoter (Figure 3A). We observed that a promoter as short as 654 base pairs was responsive to NaCl at the same efficiency as the −704STL1-LacZ construct (Figure 3A). However, further truncations, up to position −626, gradually reduced promoter responsiveness (Figure 3A). This suggested that the upstream promoter region, between −654 and −626, may harbor the NaCl-responsive and active-Hog1–responsive cis-element(s).

To test whether the upstream elements of the STL1 promoter are sufficient to render a heterologous promoter responsive to both osmostress and Hog1, we inserted fragments derived from the STL1

| Gene name | Fold change | Gene name | Fold change | Gene name | Fold change |
|-----------|-------------|-----------|-------------|-----------|-------------|
| STL1      | 87.68       | BAG7      | 4.02        | YGR149W   | 2.45        |
| RTC3      | 75.61       | SPI1      | 3.97        | PRX1      | 2.45        |
| HSP12     | 47.21       | YPK2      | 3.59        | YNR066C   | 2.42        |
| KDX1      | 26.33       | ALD3      | 3.52        | FLC2      | 2.42        |
| GPD1      | 18.19       | YMR103C   | 3.50        | ERR1      | 2.39        |
| CWP1      | 16.78       | YPS3      | 3.47        | RGS2      | 2.37        |
| PNS1      | 13.88       | YIL108W   | 3.45        | SFA1      | 2.36        |
| GRE2      | 13.01       | YPR1      | 3.27        | FBP26     | 2.34        |
| PRM10     | 12.68       | YNR065C   | 3.27        | SLT2      | 2.31        |
| THI4      | 12.38       | YDL206W   | 3.25        | PTP2      | 2.3         |
| GPP2      | 11.07       | PRR2      | 3.22        | ERR2      | 2.3         |
| YLR042C   | 10.71       | CHS1      | 3.11        | SMF1      | 2.27        |
| FMP43     | 10.03       | YMR173W-A | 3.11        | WSC3      | 2.22        |
| YHR022C   | 9.78        | CRG1      | 3.04        | YIR035C   | 2.22        |
| YER053C-A | 8.09        | CSH1      | 3.02        | PFK26     | 2.19        |
| FSH1      | 7.73        | CTT1      | 2.96        | YJL132W   | 2.19        |
| YML131W   | 7.72        | PUT4      | 2.95        | GDE1      | 2.19        |
| YJL107C   | 7.65        | SOL1      | 2.93        | YPL088W   | 2.19        |
| SED1      | 7.30        | SHH3      | 2.90        | PCM1      | 2.18        |
| PIR3      | 6.77        | DDR48     | 2.85        | YCL049C   | 2.18        |
| HAL1      | 6.56        | DAK1      | 2.82        | YMR226C   | 2.18        |
| HSP32     | 6.25        | MGA1      | 2.80        | EXG1      | 2.16        |
| HBN1      | 6.21        | SRL3      | 2.76        | DFG5      | 2.12        |
| SNO4      | 6.08        | CIN5      | 2.73        | TiR2      | 2.10        |
| YKL162C-A | 5.86        | YKE4      | 2.72        | CHS6      | 2.09        |
| YDL023C   | 5.41        | CRH1      | 2.71        | PST1      | 2.08        |
| YHR033W   | 5.39        | TRS65     | 2.69        | RH05      | 2.06        |
| FIT1      | 5.20        | FMP33     | 2.67        | PAU15     | 2.05        |
| ARI1      | 5.15        | YMR122W-A | 2.63        | MSB3      | 2.05        |
| YKL102C   | 4.95        | YIL024C   | 2.61        | GPP1      | 2.04        |
| AFR1      | 4.76        | DDI3      | 2.58        | VHS3      | 2.03        |
| HSP33     | 4.51        | DDI2      | 2.57        | AVO2      | 2.02        |
| HXT1      | 4.25        | SSK22     | 2.56        | POF1      | 2.02        |
| GRE3      | 4.18        | YOL150C   | 2.52        |           |             |
| FMP48     | 4.16        | PTP3      | 2.49        |           |             |
| NQM1      | 4.03        | YPS6      | 2.46        |           |             |

Fold change is the ratio between expression levels in hog1Δpbs2Δ/MET3-Hog1D170A+F318L and hog1Δpbs2Δ/MET3-empty vector cells 90 min after removal of methionine.

TABLE 2A: Genes induced in hog1Δpbs2Δ cells after induction of Hog1D170A+F318L expression.
promoter upstream of the CYC1 minimal promoter, which is cloned upstream to the LacZ gene. The CYC1 minimal promoter alone allowed very low transcription initiation rate of the LacZ gene, reflected in just 1 U of β-galactosidase activity, which was not further induced by salt treatment. However, when a fragment containing the sequence between −704 and −533 of the STL1 promoter was inserted upstream of the minimal CYC1 promoter, the resulting chimeric promoter was strongly induced by 0.7M NaCl (Figure 4A, top). A shorter fragment, −665 to −533, was also strongly inducible by salt, but a further, shorter fragment, −626 to −533, was not (Figure 4A), suggesting that the responsive element resides within the 40 base pairs of the −665 to −626 fragment. To narrow the

| Gene name | Fold change | Gene name | Fold change | Gene name | Fold change |
|-----------|-------------|-----------|-------------|-----------|-------------|
| DIP5      | 3.40        | GPD2      | 2.28        | YLR460C   | 2.13        |
| SFG1      | 3.14        | ERG3      | 2.27        | DAL1      | 2.12        |
| CIT2      | 2.90        | PEX21     | 2.26        | DSE1      | 2.11        |
| ATO3      | 2.75        | CPA2      | 2.25        | CTP1      | 2.10        |
| YGR035C   | 2.73        | PDH1      | 2.21        | LYS12     | 2.09        |
| FRE7      | 2.63        | YLR346C   | 2.20        | NDJ1      | 2.05        |
| LYS2      | 2.39        | CAR2      | 2.20        | MIG3      | 2.01        |
| SHU2      | 2.33        | HIS4      | 2.18        | SRD1      | 2.00        |
| DUR3      | 2.29        | DSE2      | 2.14        |           |             |

Fold change was calculated as the ratio between expression levels in hog1Δpbs2Δ/MET3-empty vector cells and hog1Δpbs2Δ/MET3-Hog1D170A+F318L 90 min after removal of methionine.

TABLE 2B: Genes suppressed in hog1Δpbs2Δ cells after induced expression of Hog1D170A+F318L.
responsive region, we constructed another set of chimeric STL1-CYC1 promoters (Figure 4B) and observed that a promoter containing the fragment of $-654$ to $-564$ was strongly induced by salt, whereas a promoter containing $-636$ to $-564$ was not (Figure 4B). This implies that the responsive element resides within the 19 base pairs between $-654$ and $-636$, a region that is included within the fragment mapped as NaCl- and Hog1$^{1270A+3318L}$-responsive by deletion of the STL1 promoter ($-654$ to $-626$; Figure 3A).

A cis-element composed of two 5'-CATTTGGC-3' repeats and a third, similar repeat is essential for maximal Hog1-dependent and osmostress-dependent induction of STL1 transcription

The foregoing 5' deletion analysis, combined with insertion of regions of the STL1 promoter upstream to the minimal CYC1 promoter (Figures 3 and 4), suggested that the salt-responsive and HoRE resides within the sequence between $-654$ and $-626$. This region contains two consecutive identical repeats of the sequence 5'-CATTTGGC-3' linked to a third, similar repeat, 5'-CACTTTGAC-3' (marked in Figure 5A). To determine whether these elements are essential for promoter responsiveness to osmostress and Hog1$^{D170A+F318L}$, we deleted, in the context of the full-length 704–base pair promoter, the two identical repeats from the STL1 promoter. The resulting promoter, missing the two identical repeats but still harboring the third, similar repeat (delR2; Figure 5, top), lost $\sim 80\%$ of its transcription activity but could still be induced by $\sim 20$-fold in response to salt (Figure 5, bottom). We therefore expanded the deletion toward the 5' and the 3' directions to create delR3, delR4, and delR5 (Figure 5, top). Upstream deletions (up to $-667$; delR4) did not affect activity further (the activities of delR2 and delR4, both containing the third, similar repeat, were similar). However, elimination of the third, similar repeat by expanding the deletion downstream to $-626$ (delR3 and delR5)
Hot1 induces transcription of one gene

HoRE activity is dependent on HOG1 and HOT1

Hot1 was identified in a manner that is unbiased toward any transcriptional activator. The following question remains, therefore: is HoRE the target of Hot1, reported to induce the STL1 promoter (Rep et al., 2000; Alepuz et al., 2003), or of another transcription factor? Analysis of the sequence between −601 to −655 using the YeTFaSCo (yetfasco.ccbr.utoronto.ca/) and Yeastract databases (yeastract.com/formfindregulators.php), which screen for binding motifs and transcription factors, indicated that the region between −601 to −655 is likely to be a target of HoRE. However, additional studies are required to confirm this hypothesis and to elucidate the mechanisms involved in the regulation of HoRE activity by Hot1.

To determine the importance of the repeat sequences in HoRE function, we engineered point mutations into the HoRE region of the STL1 promoter. Various mutations were introduced into the HoRE region of the STL1 promoter, and their impact on promoter activity was assessed.

FIGURE 4: Short fragments of the STL1 promoter, cloned upstream of the CYC1 minimal promoter, are sufficient to render it responsive to osmotic pressure. Fragments derived from the upstream region of the STL1 promoter were fused to the minimal elements of the CYC1 promoter. Left, resulting constructs; right, β-galactosidase activities of cells harboring these constructs. All fragments in A share the same downstream endpoint (−533). The endpoint is −564 in most of the fragments tested in B except for the two bottom constructs, in which the endpoint is −583.

Reduced promoter activity to a very low level and rendered it unresponsive to stress (Figure 5, bottom). Of note, promoter delR3, which is not active, misses only a short fragment that contains the two identical and the one similar repeats, suggesting that these sequences are essential for promoter induction.

To evaluate the importance of the accuracy of the repeats’ sequence we inserted, in the context of the full-length promoter, point mutations into the HoRE (Figure 6). A single point mutation in either the first or second repeat caused ∼30% reduction in promoter activity (constructs RM1 and RM2). Combination of mutations in repeats one and two (constructs RM3–RM6) caused a more dramatic reduction, up to 75% (RM4; Figure 6), but the mutated promoter was still efficiently induced in response to NaCl (∼16-fold; note that nonmutated promoter is induced −64-fold; Figure 6). Addition of a mutation in the third (similar) repeat (RM7) did not cause a further reduction, but more mutations in the two identical repeats (RM8) and mutation in the three repeats (RM9) reduced promoter responsiveness to salt from −64-fold to −8-fold (RM8) and 4-fold (RM9; Figure 6). Thus point mutations in the two identical and one similar repeats of the HoRE, in the context of the 704–base pair–long promoter, are sufficient to reduce responsiveness of the promoter, suggesting that accuracy of the repeats’ sequence is important for the responsiveness of the entire promoter.

Finally, to examine whether the HoRE region by itself is sufficient to render a heterologous promoter responsive to osmotic pressure, we fused a series of short oligonucleotides that include the HoRE to the CYC1 minimal promoter, cloned upstream of the LacZ gene. The 63–base pair–long fragment, −654 to −600 and −654 to −607 fragments (E1, E2, and E3 in Figure 7A) sufficed to render the CYC1 promoter transcriptionally active and fully inducible by osmotic pressure or by active Hog1 (∼8-fold and up to 130 U, respectively; Figure 7, B and C). Shorter fragments, including the sequence that contains only the three repeats per se, were transcriptionally active but not highly inducible (E4 and E5 in Figure 7). Thus the two identical and one similar repeats are essential for the HoRE activity (Figure 6), but a fully active HoRE, in the context of a heterologous promoter, is defined as the sequence that includes these repeats plus 19 base pairs downstream (E3 in Figure 7). Interestingly, fragments that include sequences upstream to the repeats (e.g., E6 in Figure 7A), although they rendered the CYC1 promoter salt responsive (10-fold), allowed just a low activity, ∼10 β-galactosidase units, indicating that the 11 base pairs between −665 and −654 might be inhibitory.

To determine whether the HoRE region by itself is sufficient to render a heterologous promoter responsive to osmotic pressure, we fused a series of short oligonucleotides that include the HoRE to the CYC1 minimal promoter, cloned upstream of the LacZ gene. The 63–base pair–long fragment, −654 to −600 and −654 to −607 fragments (E1, E2, and E3 in Figure 7A) sufficed to render the CYC1 promoter transcriptionally active and fully inducible by osmotic pressure or by active Hog1 (∼8-fold and up to 130 U, respectively; Figure 7, B and C). Shorter fragments, including the sequence that contains only the three repeats per se, were transcriptionally active but not highly inducible (E4 and E5 in Figure 7). Thus the two identical and one similar repeats are essential for the HoRE activity (Figure 6), but a fully active HoRE, in the context of a heterologous promoter, is defined as the sequence that includes these repeats plus 19 base pairs downstream (E3 in Figure 7). Interestingly, fragments that include sequences upstream to the repeats (e.g., E6 in Figure 7A), although they rendered the CYC1 promoter salt responsive (10-fold), allowed just a low activity, ∼10 β-galactosidase units, indicating that the 11 base pairs between −665 and −654 might be inhibitory.

In summary, our results provide evidence for a role of HoRE in osmotic stress response. We have shown that HoRE is a transcriptional activator that is essential for the transcription of the STL1 promoter and other osmotic responsive genes. Further studies are required to elucidate the mechanisms involved in the regulation of HoRE activity by Hot1 and to determine the role of HoRE in other stress response pathways.
sites of transcription factors, identified several putative sites, including potential binding sites for Gcn4, Hap2, Gat3, Bas1, Skn7, and Arg80. We tested the possible involvement of these factors in regulating the STL1 promoter and observed that the −704STL1-LacZ reporter was fully functional in the corresponding knockout strains. Thus none of these factors seems to be essential for STL1 transcriptional activation. We then tested the activity of the −704STL1-LacZ construct in cells lacking transcription factors known to be activated by Hog1. Whereas deletion of SMP1 had just a small effect on promoter induction in response to 0.7 M NaCl, deletion of HOT1 totally abolished promoter activity (Figure 8A). We also tested in the mutated cells the activity HoRE by itself, that is, in the context of the CYC1 promoter. Like the full-length promoter, the STL1(E1)CYC1-LacZ reporter gene was fully responsive in cells of the smp1∆ and ras2∆msn2∆msn4∆ strains but was not induced in hog1∆ and hot1∆ cells (Figure 8B). Thus salt-induced activity of the HoRE is evidently dependent on Hog1 and Hot1 but not on Smp1 or on Msn2/4. To determine the extent to which the STL1 promoter is dependent on Hot1 and Hog1, we overexpressed each of these proteins in a strain lacking the other. Overexpression of Hog1WT or of Hog1D170A+F318L in hot1∆ cells did not activate the STL1 promoter even in cells exposed to osmotic stress (Figure 8C). Similarly, when overexpressed in hog1∆ cells, Hot1 on its own was unable to activate the STL1 promoter (Figure 8D). These results support the notion that Hog1 and Hot1 must function together for activating the HoRE.

Another transcription factor known to be activated by Hog1, Sko1, was also reported as involved in STL1 activation (Capaldi et al., 2008). A putative Sko1-binding site (marked in Figure 9C) is indeed identified within the E3 fragment, which we defined as the full-length HoRE (Figure 7). The −704STL1-LacZ reporter gene was
Volume 26 June 15, 2015 Hot1 induces transcription of one gene

With Hot1 for full activation of the promoter but is not essential for promoter activation induction of the repeats, which are the major Hog1-responsive cis-elements of the promoter. Of note, both Hot1 and Sko1 seem to bind the same E3 element (see later discussion of Figure 11D) that we defined as the fully active HoRE (Figure 7). In summary, among the mutants tested, STL1 promoter activity was totally abolished only in hog1Δ and hot1Δ and was reduced fivefold in sko1Δ. Curiously, on the basis of chromatin immunoprecipitation (ChIP) analysis, Cook and O’Shea (2012) suggested that Hot1 binds the sequence 5′-wGVRMRRKD-3′ (most preferred: 5′-T/AGGGA/GCAATG-3′) in the STL1 and RTC3 promoters. This sequence differs significantly from the HoRE identified in our study. Four different sequences that fit the 5′-wGVRMRRKD-3′ requirement reside in the STL1 promoter, all of them downstream to position −626. Given that the construct –626STL1-LacZ is not responsive to active and inducible in sko1Δ cells but showed just sixfold induction and ~25% of the activity shown in wild-type cells (Figure 9A, left bars), suggesting that Sko1 is involved in STL1 activation. Sko1 seems to affect the STL1 promoter directly and not via an effect on the steady-state levels of Hog1 or Hot1 (Figure 9B). The activity of the –636STL1-LacZ reporter, which includes the putative Sko1-binding site but lacks the two identical repeats of the HoRE, was very low in wild-type cells, ~8% of the activity of the –704STL1-LacZ reporter, and was totally abolished in sko1Δ cells (Figure 9A), suggesting that Sko1 alone, without Hot1, cannot activate the STL1 promoter. Accordingly, deleting the Sko1-binding site from E3 (see sequence in Figure 9C, top) significantly reduced but did not abolish promoter inducibility (Figure 9C, bottom). In fact, the STL1ΔSKO1-LacZ reporter was similarly induced in wild-type and sko1Δ cells, providing another indication that Hot1 can activate the promoter alone and that Sko1 is required for maximal induction. Thus Sko1 cooperates with Hot1 for full activation of the promoter but is not essential for promoter activation induction of the repeats, which are the major Hog1-responsive cis-elements of the promoter. Of note, both Hot1 and Sko1 seem to bind the same E3 element (see later discussion of Figure 11D) that we defined as the fully active HoRE (Figure 7). In summary, among the mutants tested, STL1 promoter activity was totally abolished only in hog1Δ and hot1Δ and was reduced fivefold in sko1Δ.

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osmotic pressure or to Hog1 (Figure 3A), these fragments cannot be the Hog1/Hot1 targets on the promoter. We also identified in the RTC3 promoter four sequences of 5'-wGVRMRRKD-3' (underlined in Figure 10B, top). To examine their role in regulating the RTC3 promoter in general, and by Hot1 in particular, we cloned the RTC3 promoter and prepared some deletion constructs (Figure 10B). We first tested the activity of the full-length promoter in wild-type and hot1Δ cells and found, surprisingly, that it is strongly induced in both strains (Figure 10A). Promoter activity was just slightly lower in hot1Δ cells than in wild-type cells (25% reduction; Figure 10A), suggesting that Hot1 is not essential for its activity. RTC3 promoter was more affected by knocking out the MSN2/4 genes (50% reduction; Figure 10A). Accordingly, this promoter contains four STREs (rectangles in Figure 10B, top). Deletion analysis (Figure 10B) clearly shows removal of the most downstream STRE (at position −197; Figure 10B) abolished promoter responsiveness. It seems that the proposed Hot1-binding sites play a minor role in promoter regulation. Combining the deletion analysis with the experiment in hot1Δ cells suggests that Hot1 is not a critical activator of the RTC3 promoter, explaining the lack of HoRE in this promoter.

Recombinant Hot1 protein binds the HoRE in vitro

The foregoing findings strongly suggested that activation of the STL1 promoter in response to osmootress or to active Hog1 is mediated via the HoRE in a Hot1-dependent manner. To examine the possibility that Hot1 is capable of associating physically with HoRE, we applied an electrophoretic mobility shift assay (EMSA) and measured directly Hot1 binding. A radioactively labeled fragment containing the HoRE was incubated with a purified recombinant polyhistidine-tagged Hot1 protein and the reaction mixture was subjected to native-gel electrophoresis. When the labeled HoRE probe was incubated with the polyhistidine-tagged Hot1 protein, its migration in the gel was significantly retarded, suggesting HoRE-Hot1 association (Figure 11A). No retardation was observed when labeled HoRE was incubated with another purified polyhistidine tagged protein (JNK) or with glutathione S-transferase (GST) or bovine serum albumin (BSA; Figure 11A). Binding of Hot1 to the probe was outcompeted by the unlabeled probe but not by a probe in which HoRE was mutated (RM9; Figure 11B), suggesting that the intact sequence of the identical and similar repeats is important not only for induction of the promoter by osmootress (Figure 6), but also for association with Hot1. We also incubated the probe with yeast lysates prepared from wild-type cells and found that it is strongly induced in both strains (25% reduction; Figure 10A). Promoter activity was just slightly lower in hot1Δ cells than in wild-type cells (25% reduction; Figure 10A), suggesting that Hot1 is not essential for its activity. RTC3 promoter was more affected by knocking out the MSN2/4 genes (50% reduction; Figure 10A). Accordingly, this promoter contains four STREs (rectangles in Figure 10B, top). Deletion analysis (Figure 10B) clearly shows removal of the most downstream STRE (at position −197; Figure 10B) abolished promoter responsiveness. It seems that the proposed Hot1-binding sites play a minor role in promoter regulation. Combining the deletion analysis with the experiment in hot1Δ cells suggests that Hot1 is not a critical activator of the RTC3 promoter, explaining the lack of HoRE in this promoter.

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Knocking out HOT1 from the genome abolishes transcriptional induction in response to osmotic stress of only one gene, STL1

The HoRE that we identified in the STL1 promoter seems to be the direct target of Hot1. We therefore looked for a similar HoRE in the promoters of putative targets of Hot1. Rep et al. (2000) proposed nine genes (STL1, PHO84, YGR043C, CHA1, GPD1, GPP2, YHR087W/RTC3, YGR052W/FMP48, and PUT4; the last may be suppressed by Hot1) as potential Hot1 targets on the basis of microarray studies with hot1Δ cells in the W303 background. Gomar-Alba et al. (2012) suggested that RTC3/HGI1 is a Hot1 target, and Capaldi et al. (2008), based on large-scale microarray and ChIP analyses, proposed several more Hot1 target genes (~20; 10 of them were putative), including THI4, SED1, SP11, and FIT1, which were also identified here as targets of HOG1(D170A+F318L) (Table 2A). As shown earlier (Figure 10), Hot1 is not critical at all for induction of the RTC3/HGI1 promoter, which does not contain HoRE. Searching the promoters of all other putative Hot1 targets also did not disclose the presence of any HoRE. We therefore tested the expression of some of these genes via real-time-RT-PCR in hot1Δ cells (see Figure 12). All genes tested—GP1, GPP2, RTC3/HGI1, PHO84, and FIT1—were efficiently induced in response to NaCl, KCl, or sorbitol in hot1Δ cells (see later discussion of Figure 12), although to a somewhat lower levels than in wild-type cells. It seems that although Hot1 was found to be associated with the promoters of these genes (Capaldi et al., 2008), it is not essential for their transcriptional induction, although it does play a role in it.

Because HoREs were not found in the putative Hot1 targets, we looked for HoRE in all yeast promoters, that is, within the sequence residing 1000 base pairs upstream of the first AUG codon. A sequence containing the HoRE as is (E5, Figure 7) or even just the two identical repeats as they appear in the STL1 promoter could not be found in any of the S. cerevisiae promoters. A single 5′-CATTTGGC-3′ was found in 347 promoters. However, in those 347 promoters, the 5′-CATTTGGC-3′ sequence is not in the vicinity of any similar sequence and is most probably not affected by Hot1 (see later discussion; Tables 3 and 4). Thus a functional HoRE appears to be unique to the STL1 promoter.

Two possible explanations may account for the lack of the HoRE from any other yeast promoter. First, STL1 may be the only bona fide target of Hot1. Second, the sequence of the Hot1 binding site is not rigid, and to activate other promoters, Hot1 is not using HoRE but other cis-elements that may or not be similar to HoRE. This notion is based on many recent examples of transcription factors that were found to be associated with sequences that vary significantly from their optimal binding site (MacQuarrie et al., 2011). We opted therefore to identify the target genes of Hot1 via a functional approach in which we analyzed a whole-genome microarray to search for genes that are not induced in hot1Δ cells in response to several stress conditions. Cells of the hot1Δ and hot1Δ/HOT1 strains (a hot1Δ strain into which an intact, single-copy HOT1 gene was introduced) were grown to logarithmic phase. Then each culture was divided into four cultures that continued to grow for 1 h on yeast nitrogen base
compared with hot1Δ/HOT1 cells. Only four genes (including STL1) showed similar differences after NaCl treatment (Table 4). Some of the genes that showed changes in expression levels between the strains in the microarray analysis and were previously reported to be regulated by Hot1 were also tested directly by real-time RT-PCR (Figure 12). Of those, GPD1, GPP2, FIT1, RTC3, and YGR066C were induced to high levels in hot1Δ cells in response to osmostress, reaching levels of 70–90% of their expression levels in wild-type cells (Figure 12). ICL1, NQM1, and YNR034W-A were more severely affected by the absence of Hot1 and were induced to 30–50% of their levels in wild-type cells (Figure 12). Only STL1 showed no induction whatsoever in hot1Δ cells (Figure 12). Thus, under the conditions tested, Hot1 activity is essential for transcription of just one gene, STL1.

The absolute dependence of STL1 transcription on Hot1 and the observation that Hot1 is essential only for STL1 transcription suggest that knocking out either gene would impose the same phenotype. We tested the growth rates of hot1Δ and stl1Δ cells on several types of osmostress and could not observe any sensitivity (Figure 13).

(YNB) –URA or on YNB –URA supplemented with 0.9 M NaCl, 0.9 M KCl, or 1 M sorbitol. Total RNA was extracted from each of the eight samples and analyzed by microarray. In cells grown under optimal conditions, five genes appeared to be mildly affected by the lack of HOT1 (Table 3). Of those, PHO84 showed approximately fourfold increase in expression in hot1Δ cells, whereas the other four genes showed only twofold change in expression. Comparison of gene expression between the two strains under osmostresses showed that the most significantly affected gene was STL1. The mRNA levels of this gene in KCl- or NaCl-treated hot1Δ/HOT1 cells were 254- and 39-fold higher, respectively, than in hot1Δ/vector cells (Table 4). When hot1Δ and hot1Δ/HOT1 cells were exposed to sorbitol, STL1 mRNA levels were only 13-fold higher in hot1Δ/HOT1 cells than in hot1Δ/vector cells (Table 4C) because transcriptional induction of STL1 in response to this stress is weaker relative to its induction by KCl or NaCl. Microarray analysis disclosed that only a few more genes, in addition to STL1, were affected by knockout of HOT1, and even those were only mildly affected (Table 4). After exposure to 0.9M KCl, 21 genes showed >3-fold-reduced expression in hot1Δ/vector cells compared with hot1Δ/HOT1 cells. Only four genes (including STL1) showed similar differences after NaCl treatment (Table 4). Some of the genes that showed changes in expression levels between the strains in the microarray analysis and were previously reported to be regulated by Hot1 were also tested directly by real-time RT-PCR (Figure 12). Of those, GPD1, GPP2, FIT1, RTC3, and YGR066C were induced to high levels in hot1Δ cells in response to osmostress, reaching levels of 70–90% of their expression levels in wild-type cells (Figure 12). Only STL1 showed no induction whatsoever in hot1Δ cells (Figure 12). Thus, under the conditions tested, Hot1 activity is essential for transcription of just one gene, STL1.

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### TABLE 2: Genes induced or suppressed in hot1Δ cells under optimal growth conditions.

| Gene name | Fold change | Gene name | Fold change |
|-----------|-------------|-----------|-------------|
| PHO84     | 4.14        | FIT1      | 2.17        |
| URA3      | 2.62        | BNA2      | 2.12        |
|           |             | COS12     | 2.11        |

Fold change for induced genes was calculated as the ratio of gene expression levels in hot1Δ and hot1Δ/HOT1 cells and vice versa for suppressed genes.

### DISCUSSION

This study described a new approach for identifying the bona fide target genes of Hog1, using inducible expression of intrinsically active variants. This approach is not limited to Hog1 and could be applied for the yeast MAPK SLT2/MPK1 and for all isoforms of the mammalian p38s and extracellular regulated kinases, as intrinsically active variants are available for these molecules (Askari et al., 2006, 2007, 2009; Avitzour et al., 2007; Levin-Salomon et al., 2008; Beenstock et al., 2014). Identifying the bona fide target genes of those MAPKs will show whether, similar to the case of Hog1, their activation per se results in induction of a relatively small number of target genes. In the case of Hog1 studied here, it is clear that this MAPK is essential for activation of many genes, but its individual activation in the cell is sufficient for induction of only ~100. This implies that for activation of all other genes for which Hog1 is essential, Hog1 must cooperate with other systems that are probably coactivated with it when the cell is exposed to relevant conditions. Note that transcriptional induction of Hog1 target genes is probably not required for a proper response to osmostress (Westfall et al., 2008) but may be involved in long-term adaptation to stress (Schaber et al., 2012).

Our study showed that induction of STL1 in response to osmostress or to active Hog1 is absolutely dependent on the transcription factor Hot1. Unexpectedly, STL1 seems to be the only gene for which Hot1 is essential. Other genes proposed to be activated by Hot1 do not contain the binding site HoRE identified in the STL1 promoter. In addition, the absence of Hot1 from the genome has just a partial effect on their transcription. Namely, our microarray and RT-PCR analyses showed that genes such as GPD1, NQM1, and HGI1/RTC3 are expressed and induced in hot1Δ, although to lower levels than in wild-type cells, and that the only gene whose expression is barely detectable in response to osmostress in hot1Δ cells is STL1. The possibility remains, however, that in response to particular, currently unknown, conditions, Hot1 activates transcription of more genes. It must do so, however, via a different cis-element or use the single 5′-CATTTGGC-3′ repeat found in 347 promoters.

Recruiting Hot1 to the promoters of GPD1, HXT1, HGI1/RTC3, and GPP2/HOR2 and ~15 more genes (Capaldi et al., 2008) may assist in regulating their transcription but is clearly not essential for it. It could be that Hot1 plays some accessory role, not related to transcription per se. It may be involved, for example, in DNA repair, mRNA editing, or mRNA nuclear export, activities known to accompany the transcription bubble (Fong et al., 2013; Muller-McNicoll and Neugebauer, 2013; Burns and Wente, 2014). A bigger puzzle is why STL1 transcription is absolutely dependent on a single factor, Hot1. Namely, why is STL1 left with no backup machinery? This unusual link of absolute dependence between the STL1 gene and the Hot1 protein is unexplained. STL1 encodes a sugar/glycerol transporter, and, just like HOT1, it is not essential for survival or for proliferation under osmotic pressure.

Many transcriptional activators are regarded as “master” genes because they regulate activation of many promoters and thereby determine the cell’s fate (Rothwarf and Karin, 1999; Florin et al., 2004; Bailey and Europe-Finner, 2005; Cao et al., 2006; Dang et al., 2006; van Riggelen et al., 2010). In the view of this notion, the case of Hot1, which regulates a very few genes and seems to be essential for the expression of just one gene, appears to be exceptional. However, the human genome encodes between 1400 and 2600 DNA-binding proteins (~10% of the genes in the genome), and most of them are transcriptional activators whose targets have not been revealed (Babu et al., 2004; www.biostars.org/p/53590/). Given the many similarities between yeast and higher eukaryotes (Engelberg et al., 1989, 2014), some of these human proteins may be dedicated to only a few target genes or, like Hot1, to a single one.

### MATERIALS AND METHODS

**Yeast strains and media**

Yeast strains used in this study are listed in Table 5. Commonly used media were synthetic media, YNB medium (0.17% yeast nitrogen base without amino acids and NH₄SO₄, 0.5% ammonium sulfate,
The RTC3 promoter constructs were produced in a similar way. Different lengths of RTC3 promoter were amplified by PCR using genomic DNA of the wild-type strain BY4741 as a template. Primers used are listed in Table 6. PCR products were digested with BamHI and SalI and ligated to pLG669Z, which was cut with the same restriction enzymes. For inserting elements of the STL1 promoter upstream to the CYC1 minimal promoter, regions from STL1 promoter were amplified by PCR using genomic DNA of the wild-type strain BY4741 as a template, digested with XhoI, and cloned into XhoI-digested pLG669Z-178URA (Guarente and Ptashne, 1981; Grably et al., 2002). The correct orientation was selected based on sequencing result. For producing recombinant Hot1, the coding sequence of HOT1 was amplified by PCR using genomic DNA of the wild-type strain BY4741 as a template, digested with NdeI and NotI, and cloned in the pET28 Escherichia coli expression vector digested with the same enzymes. The resulting plasmid contains the HOT1 open reading frame in-frame with and downstream to the hexahistidine tag in the vector (pET28-HOT1). To construct an integrative pRS316-HOT1 plasmid harboring the native HOT1’s promoter and terminator, the coding sequence of HOT1 plus an 800–base pair 5′ promoter sequence and a 610–base pair 3′ untranslated region was amplified by a high-fidelity PCR system (Fermentas, Vilnius, Lithuania) using genomic DNA of the wild-type strain BY4741 as a template, digested with BamHI, and cloned in a pRS316 vector digested with the same enzymes. MET3-HOG1WT and MET3-Hog1ΔD170+F318L constructs were already described (Yaakov et al., 2003).

Site-directed mutagenesis
The Stratagene QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to insert mutations in the STL1 promoter. The mutagenesis process was performed according to manufacturer’s instructions. Primers used are listed in Table 6.

β-Galactosidase assay
Cells were grown to mid log phase and divided into two cultures of 5 ml each. For salt induction, 0.81 ml of 5 M NaCl was added into 5-ml culture to make a final concentration of 0.7 M. The same volume of water was added to the other 5-ml culture. Cells were collected 60 min after addition of NaCl, disrupted, and assayed as described previously (Grably et al., 2002). Results are shown as means ± SDs of three independent experiments.

RNA extraction, real-time RT-PCR, and microarray
Total RNA was extracted from yeast cells using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized by iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA).
Gene-specific primers were used to amplify individual target genes with PCR master mix (Fermentas, Vilnius, Lithuania). Microarray analysis was performed by using Agilent SurePrint G3 (Yeast), one-color, 8 × 60K-format slide (Agilent Technologies, Santa Clara, CA). Data analyses were performed using the Genespring GX software. Real-time RT-PCR was performed with an Applied Biosystems (Foster City, CA) 7500 Fast Real-time PCR machine. cDNA was amplified by BioRad iScript Reverse Transcription Supermix following the protocol suggested by the provider. Primers used are listed in Table 6. Real-time PCR was done by the preset 7500 Fast protocol for quantitative comparative CT, SYBR Green protocol. ACT1 was used as an internal control. The value for each targeted gene was normalized to the value of ACT1.

Cell lysis and Western blot analysis
Cell lysis and Western blotting were conducted as described (Yaakov et al., 2003). Anti–phospho-p38 antibody from Cell Signaling Technology (Beverly, MA) was used to detect phosphorylated Hog1. Hog1 (Y-215) antibody from Santa Cruz Biotechnology (Santa Cruz, CA) was used to detect Hog1 protein. The hemagglutinin (HA)-tagged protein was detected by HA antibody 3F10 from Roche.

Electrophoretic mobility shift assay
Whole-cell lysate binding assay was done as described (Engelberg et al., 1994). As a probe, a fragment of the STL1 promoter equivalent to E6 (Figure 7) was used. It was made by annealing two oligos (5′-ctattcaccgcatttggccatcttgaacttcgtcatatttactaatg-3′ and 5′-catatcagtttaaatgacgcaagttgagtcaagatggaccagatgcggtggaatag-3′) and labeled with T4 polynucleotide kinase (NEB, Ipswich, MA) in the presence of [γ−32P]ATP. A 10-ng amount of probe was mixed with 15 μg of total cell lysate for 15 min at 25°C before being loaded to a 5% native polyacrylamide gel. For direct binding assays, recombinant His-HOT1 and His-JNK2 proteins were purified from BL21 Rosetta strain using Ni Sepharose bead (GE Healthcare). For competition binding by wild-type STL1 E6 fragment, 3 or 0.6 μg of unlabeled double-strand E6 probe was mixed with 32P-labeled E6 probe. For competition binding by E6 fragment harboring mutations at HoRE repeats (RM9), two oligos (5′-ctattcaccgcatttggccatcttgaacttcgtcatatttactaatg-3′ and 5′-catatcagtttaaatgacgcaagttgagtcaagatggaccagatgcggtggaatag-3′) were annealed to form double strands, and the same amount of probe was mixed with 32P-labeled probe.

FIGURE 13: **hot1**∆ and stl1∆ cells are not sensitive for growth under osmostress. Cells of the indicated strains were allowed to proliferate on the indicated liquid medium (A) or on plates supplemented with agar plus the indicated medium (B).
YPH102  MATα ura3-52 lys2-801 ade2-101 his3-200 leu2-1  Bell et al. (2001)
BY4741  MATα his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0  EUROSCARF (Bad Homburg, Germany)
JBY13  MATα his3, leu2, ura3, trp1, ade2, lys2 hog1::TRP1  M. Gustin (Rice University)

hog1Δpbs2Δ  MATα his3, ura3, trp1, ade2, lys2 hog1::TRP1, pbs2::LEU2  Bell et al. (2001)
hot1Δ  MATα his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0 hot1::kanMX4  EUROSCARF

sm1Δ  MATα his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0 sm1::kanMX4  EUROSCARF

ras2Δ  MATα his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0 ras2::kanMX4  EUROSCARF

msn2Δ  MATα his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0 msn2::kanMX4  EUROSCARF

skoΔ  MATα his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0 sko1::kanMX4  EUROSCARF

msn2Δmsn4Δ  MATα his3-Δ1 met15Δ0 ura3Δ0 msn2::kanMX4 msn4::LEU2  This work

ras2Δmsn2Δmsn4Δ  MATα met15Δ0 ura3Δ0 ras2::kanMX4 msn2::HIS3 msn4::LEU2  This work

hog1Δhot1Δ  MATα leu2, ura3, trp1, ade2, lys2 hog1::TRP1 hot1::HIS3  This work

hot1ΔHOT1  MATα his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0 hot1::kanMX4 pRS316-HOT1  This work

hot1Δsko1Δ  MATα his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0 sko1::kanMX4 hot1::URA3  This work

HOT1-HA  MATα his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0 pRS313 HOT1  This work

sko1Δ HOT1-HA  MATα his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0 sko1::kanMX4 pRS313 HOT1  This work

EUROSCARF, European Saccharomyces Cerevisiae Archive for Functional Analysis, Frankfurt, Germany.

TABLE 5: Yeast strains used in this study.
Table 6: Oligonucleotides used in this study. Continued

| Primers for mutagenesis            | Primers for RT-PCR         |
|-------------------------------------|-----------------------------|
| STL1-delR5-R                        | GRE2-RR                     |
| STL1-delR5-F                        | CTTCAGAACACAGTCTAGG         |
| STL1-delR5-R                        | TH4-RR                      |
| STL1-delR5-F                        | GAGAGCAAGGTTGACTATG         |
| STL1-delSKO-F                       | TH4-RR                      |
| STL1-delSKO-F                       | GACAGTTTTAACATTGGG         |
| STL1-delSKO-F                       | YLR042C-RR                  |
| STL1-delSKO-F                       | CAAATAAGCGATTGTTT          |
| STL1-delSKO-F                       | FIT1-RR                     |
| STL1-delSKO-F                       | GCCGCTTAGCCGAAAAATTT        |
| STL1-delSKO-F                       | FIT1-RR                     |
| STL1-delSKO-F                       | CTTCAGTACCTGCGAGTAGGC       |
| STL1-delSKO-F                       | YLR042C-RR                  |
| STL1-delSKO-F                       | CAAATAAGCGATTGTTT          |
| STL1-delSKO-F                       | FIT1-RR                     |
| STL1-delSKO-F                       | GCCGCTTAGCCGAAAAATTT        |
| STL1-delSKO-F                       | FIT1-RR                     |
| STL1-delSKO-F                       | CTTCAGTACCTGCGAGTAGGC       |
| STL1-delSKO-F                       | YLR042C-RR                  |
| STL1-delSKO-F                       | CAAATAAGCGATTGTTT          |
| STL1-delSKO-F                       | FIT1-RR                     |
| STL1-delSKO-F                       | GCCGCTTAGCCGAAAAATTT        |
| STL1-delSKO-F                       | FIT1-RR                     |
| STL1-delSKO-F                       | CTTCAGTACCTGCGAGTAGGC       |
| STL1-delSKO-F                       | YLR042C-RR                  |
| STL1-delSKO-F                       | CAAATAAGCGATTGTTT          |
| STL1-delSKO-F                       | FIT1-RR                     |
| STL1-delSKO-F                       | GCCGCTTAGCCGAAAAATTT        |
| STL1-delSKO-F                       | FIT1-RR                     |
| STL1-delSKO-F                       | CTTCAGTACCTGCGAGTAGGC       |
| STL1-delSKO-F                       | YLR042C-RR                  |
| STL1-delSKO-F                       | CAAATAAGCGATTGTTT          |
| STL1-delSKO-F                       | FIT1-RR                     |
| STL1-delSKO-F                       | GCCGCTTAGCCGAAAAATTT        |
| STL1-delSKO-F                       | FIT1-RR                     |
| STL1-delSKO-F                       | CTTCAGTACCTGCGAGTAGGC       |
| STL1-delSKO-F                       | YLR042C-RR                  |
| STL1-delSKO-F                       | CAAATAAGCGATTGTTT          |
| STL1-delSKO-F                       | FIT1-RR                     |
| STL1-delSKO-F                       | GCCGCTTAGCCGAAAAATTT        |

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