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$^{D170A+F318L}$ in cells lacking the Hog1 activator Pbs2. This system allowed monitoring the effects of Hog1 by itself. Expression of Hog1$^{D170A+F318L}$ in *pbs2Δ* 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 *hot1Δ* 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; Causton *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 osmostress 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 Herskowitz, 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 (Schuller et al., 1994; Rep et al., 1999; Proft and Struhl, 2002; Proft 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 osmostress, 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 Herskowitz, 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 Herskowitz, 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 inducible expression of intrinsically active Hog1 in hog1Δpbs2Δ 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'-CATTTGGC-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 hog1Δpbs2Δ 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 Herskowitz, 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, Hog1[D170A+F318L], in hog1Δpbs2Δ cells. To avoid constitutive activity of Hog1 that might generate selective pressure throughout the cell's life and create a nonrelevant transcriptome, we used an inducible expression system. In this system, every change in RNA levels after induction of Hog1[D170A+F318L] 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-HOG1[D170A+F318L] plasmid, an "empty" plasmid, or a MET3-HOG1[WT] plasmid into hog1Δpbs2Δ 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 Hog1[D170A+F318L] 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 Hog1[WT] is not (Figure 1B, bottom).

mRNA samples were analyzed on a microarray of Agilent SurePrint G3 (Yeast), one-color, 8 × 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 Hog1[WT] or Hog1[D170A+F318L] 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 Hog1[WT] (Table 1) and a list of genes induced or suppressed in response to expression of Hog1[D170A+F318L] relative to their expression in cells harboring an "empty" vector (Tables 2A and 2B).
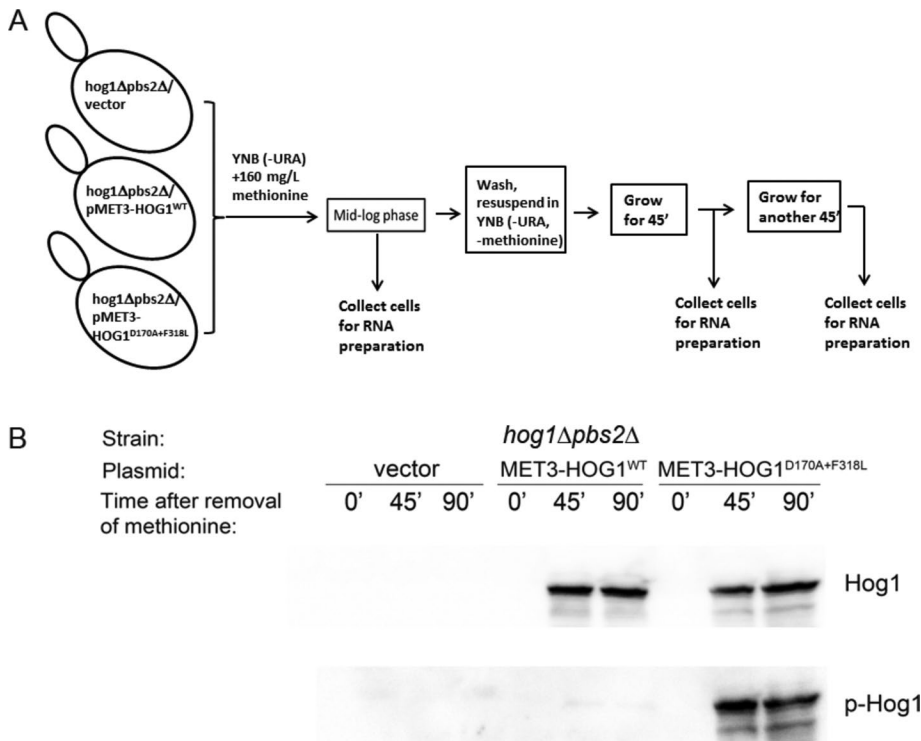
FIGURE 1: The experimental system. Induced expression of intrinsically active Hog1 in *hog1Δpbs2Δ* cells. (A) Schematic description of the experimental setup. Cells of the indicated three strains were grown to mid log phase in medium containing 160 mg/l methionine, which suppresses the expression of ectopic Hog1. The cells were then washed, resuspended in 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 Hog1$^{D170A+F318L}$ 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).

Expression of Hog1^{WT} in *hog1Δpbs2Δ* cells had a minor effect on gene expression. No genes were found to be significantly suppressed, and only 2 genes, *GPD1* and *GPP2*, were induced by >2-fold (Table 1). The effect of Hog1$^{D170A+F318L}$ 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). Of note, however, only 37 of the 105 genes were induced by ≥4-fold, and only 13 were induced by ≥10-fold (Table 2A). None of the 26 suppressed genes was suppressed by >3.4-fold (Table 2B). Thus the effect of active Hog1 by itself on gene expression was significantly milder than anticipated (induction of ~300 genes was expected according to Capaldi et al. (2008) and ~600 genes according to O'Rourke and Herskowitz (2004); Posas et al., 2000; Rep et al., 2000). To verify some of the microarray findings, we measured

Induced genes		Suppressed genes	
Gene name	Fold change	Gene name	Fold change
GPD1	4.62		
GPP2	2.08		

Fold change was calculated as the ratio between expression levels in *hog1Δpbs2Δ/*MET3-Hog1^{WT} and *hog1Δpbs2Δ/*MET3-empty vector cells 90 min after methionine removal.

TABLE 1: Genes induced or suppressed in *hog1Δpbs2Δ* cells after induction of Hog1^{WT} expression.

mRNA levels of the 12 genes most highly induced by Hog1$^{D170A+F318L}$ using real-time reverse transcription (RT)-PCR. The results (Figure 2) supported the microarray measurements by showing that the tested genes were specifically induced in cells expressing Hog1$^{D170A+F318L}$. Obviously, genes that were perhaps missed in the microarray experiment (false negatives) could not be verified, and the possibility remains that more genes are induced by Hog1$^{D170A+F318L}$ in *hog1Δpbs2Δ* cells.

We consider the genes regulated by Hog1$^{D170A+F318L}$ in *hog1Δpbs2Δ* cells (Tables 2A and 2B) to be bona fide Hog1 targets because they are exclusively activated by Hog1$^{D170A+F318L}$.

Identification of the Hog1-responsive and NaCl-responsive *cis*-element within the *STL1* promoter

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 (Alepuz et al., 2003; Capaldi et al., 2008; Gomar-Alba et al., 2012). *STL1* and *RTC3* are the most highly induced genes by Hog1$^{D170A+F318L}$, 88- and 75-fold, respectively (Table 2A). More putative Hot1 target genes—*SED1*, *FIT1*, *SPI1*, *FM48*, and *NQM1*—were also induced by Hog1$^{D170A+F318L}$ but less strongly (Table 2A). Because Hot1 seems to be a central mediator of the Hog1$^{D170A+F318L}$ 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 osmostress-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 uninducible in *hog1Δ* cells harboring an "empty" vector (Figure 3B). In *hog1Δ* cells harboring the MET3-HOG1$^{D170A+F318L}$ construct, the –704STL1-LacZ reporter was strongly induced after removal of methionine (Figure 3B), whereas in *hog1Δ* cells harboring the MET3-HOG1^{WT} plasmid, it was induced only after both removal of methionine and exposure of cells to osmostress (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 response element (STRE) sequence, 5'-CCCCT-3', located 175 base pairs upstream from 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*, *msn2Δmsn4Δ*, and *ras2Δmsn2Δmsn4Δ* 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.

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	RHO5	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-Hog1[D170A+F318L] and *hog1Δpbs2Δ*/MET3-empty vector cells 90 min after removal of methionine.

TABLE 2A: Genes induced in *hog1Δpbs2Δ* cells after induction of Hog1[D170A+F318L] expression.

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 form the *STL1*

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-Hog1$^{D170A+F318L}$ 90 min after removal of methionine.

TABLE 2B: Genes suppressed in *hog1Δpbs2Δ* cells after induced expression of Hog1$^{D170A+F318L}$.

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
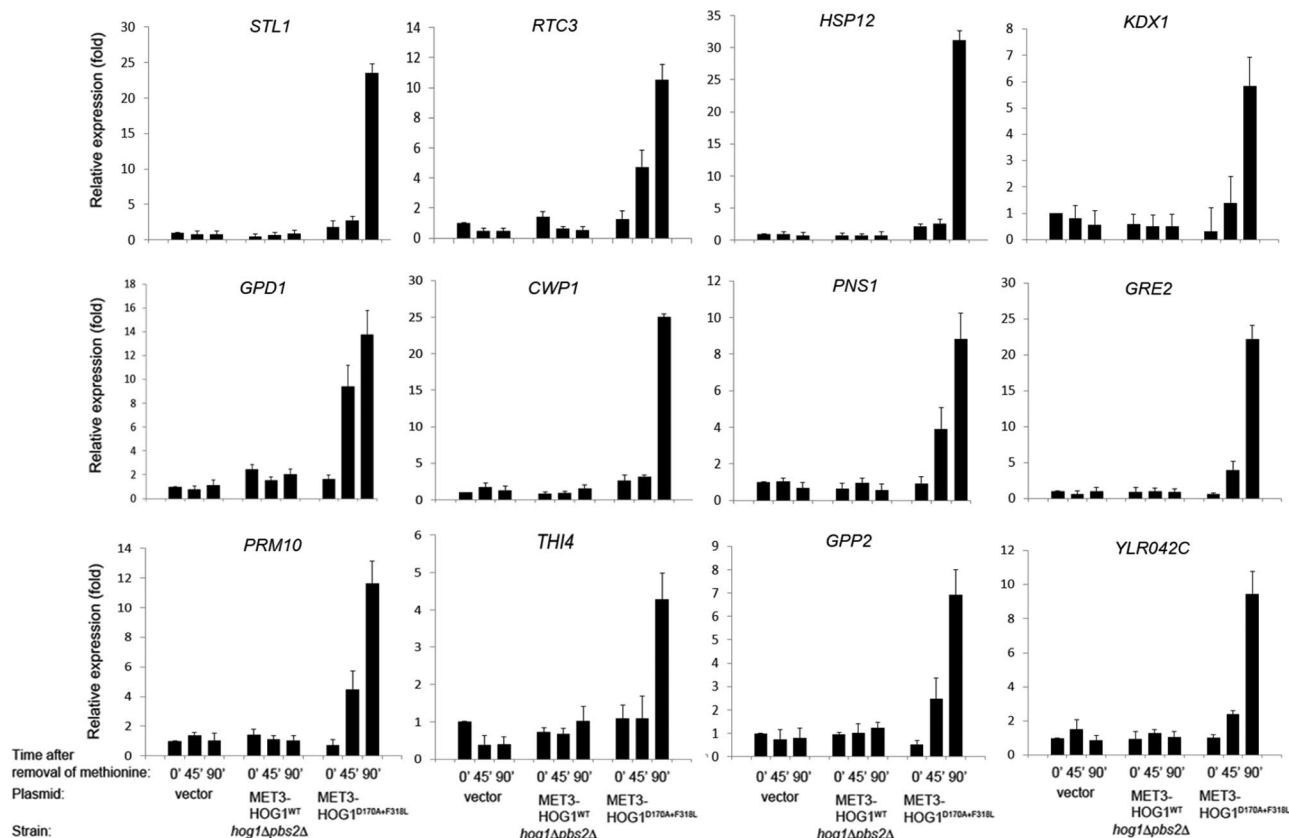

FIGURE 2: Real-time quantitative RT-PCR analysis confirmed the microarray data for the 12 most highly induced genes. mRNA levels of the indicated genes were analyzed by real-time quantitative RT-PCR. The values were normalized to the levels of *ACT1* mRNA, which served as an internal control. mRNA levels are presented as the ratio of their levels to those of the same genes at time 0 in cells harboring the empty vector. Experiments were performed in triplicate with two independent RNA preparations.
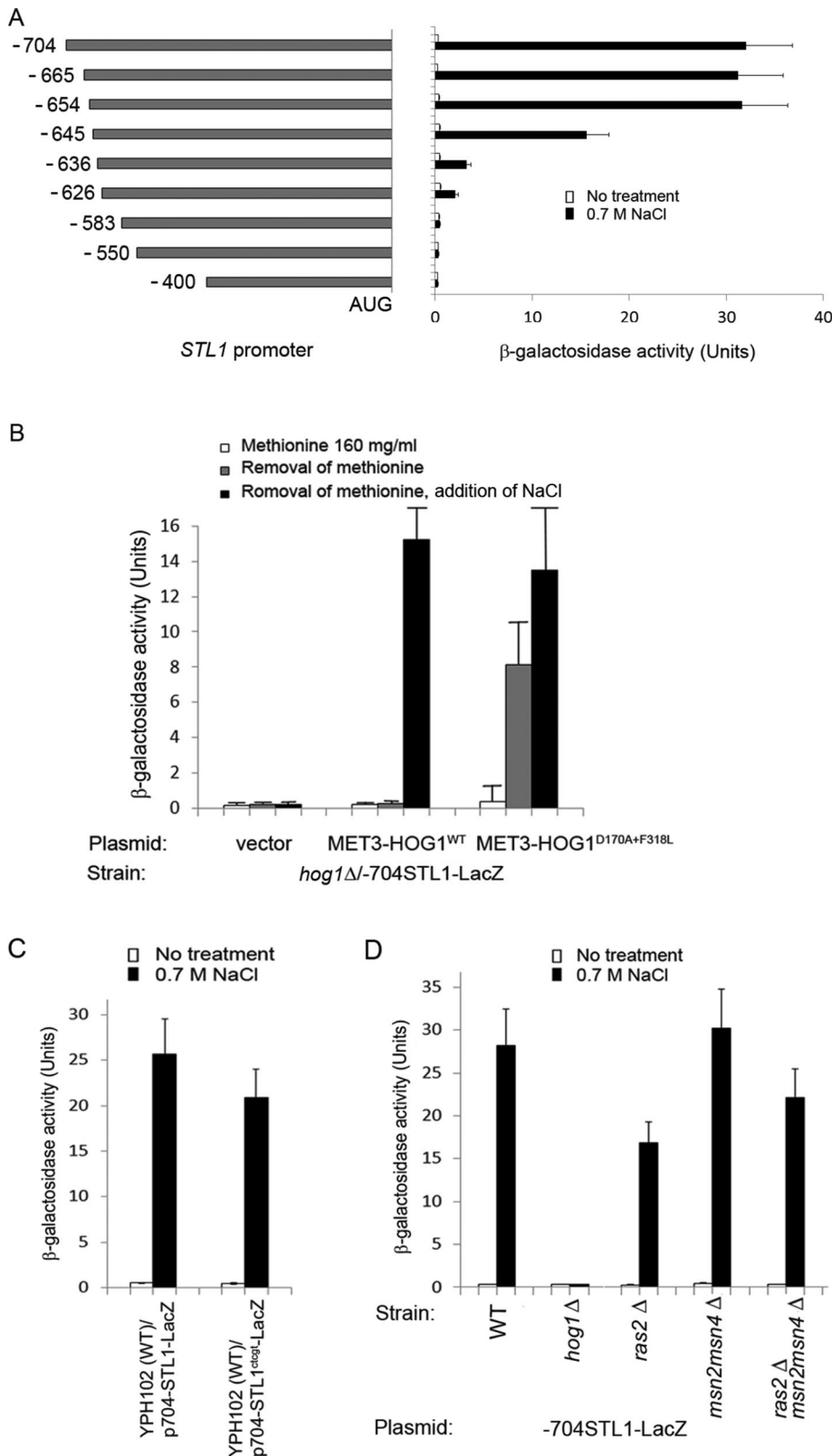
FIGURE 3: The region between −654 and −626 in the *STL1* promoter harbors an osmostress- and Hog1-responsive activity. The promoter is not induced via the Ras/Msn2/4 STRE system. (A) The indicated *STL1* promoter fragments were subcloned upstream to the β-galactosidase gene (LacZ), and the resulting vectors were tested in wild-type (YPH102) cells exposed or not exposed to 0.7 M NaCl for 1 h. Left, promoter regions; right, the corresponding β-galactosidase activities. (B) Expression of intrinsically active Hog1, but not Hog1^WT, is sufficient to strongly induce the *STL1* promoter. The −665STL1-LacZ construct was introduced into the indicated

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^D170A+F318L-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′-CACTTT-GAC-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 ~80% of its transcription activity but could still be induced by ~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)

strains. Activity of β-galactosidase was assayed in cells after removal of methionine with or without addition of NaCl (both treatments were applied for 90 min). (C) *STL1* promoter with a mutated STRE (the −704STL1^ctcgt-LacZ construct) is as responsive as the nonmutated promoter to NaCl (0.7 M, 60 min). (D) The −704STL1-LacZ construct is fully induced in cells of the indicated mutants but not in *hog1Δ* cells.
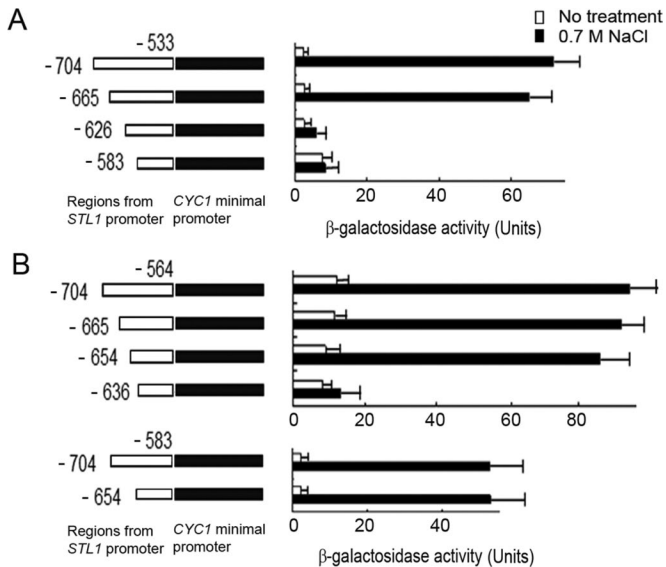
FIGURE 4: Short fragments of the *STL1* promoter, cloned upstream of the *CYC1* minimal promoter, are sufficient to render it responsive to osmotic pressure. Fragments derived from the upstream region of the *STL1* promoter were fused to the minimal elements of the *CYC1* promoter. Left, resulting constructs; 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.

Strain: YPH102 (WT)

FIGURE 5: A fragment containing two identical 5′-CATTTGGC-3′ repeats and a third, similar repeat is essential for responsiveness of the *STL1* to osmotic pressure. Top, deletions performed in the context of the full-length *STL1* promoter (704 base pairs); bottom, the β-galactosidase activities of the resulting promoters. Note that the 5′ deletion constructs −636STL1-LacZ and −626STL1-LacZ, one of which contains the third repeat and the other does not, were included in the experiment.

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, −654 to −592 fragment and the shorter, −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
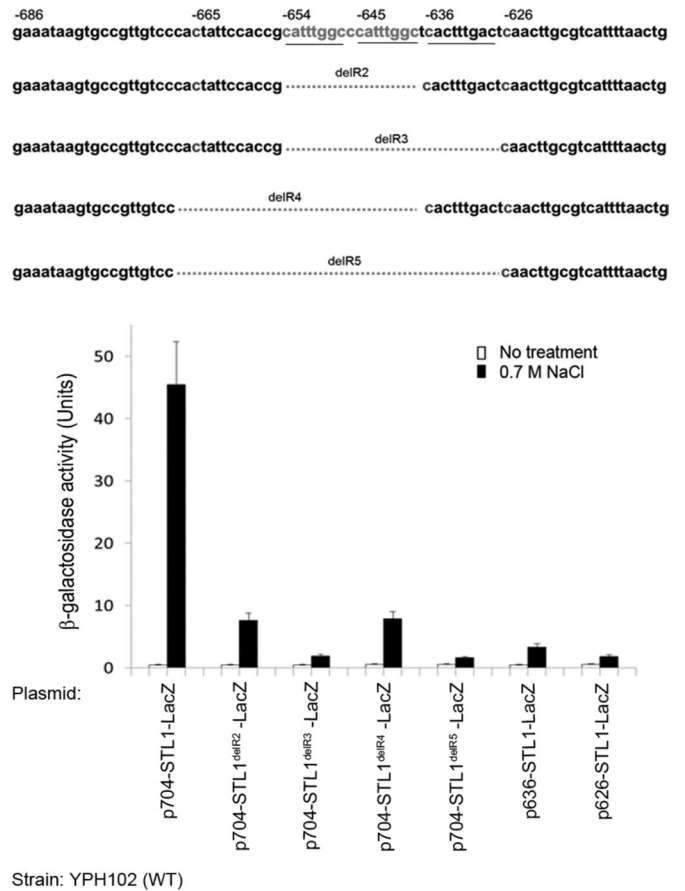
130 U, respectively; Figure 7, B and C). Shorter fragments, including the sequence that contains only the three repeats per se, were transcriptionally active but not highly inducible (E4 and E5 in Figure 7). Thus the two identical and one similar repeats are essential for the HoRE activity (Figure 6), 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). Surprisingly, 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.

HoRE activity is dependent on *HOG1* and *HOT1*

HoRE 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

| STL1 promoter | -665 | | -654 | | -645 | | -636 | | -626 |
| | ctattccaccgcatttggcccatttggctcactttgactcaacttgc |

RM1 ctattccaccgcatctggcccatttggctcactttgactcaacttgc

RM2 ctattccaccgcatttggcccatctggctcactttgactcaacttgc

RM3 ctattccaccgcatctggtccatttggctcactttgactcaacttgc

RM4 ctattccaccgcatttggtccatttggttcactttgactcaacttgc

RM5 ctattccaccgcatttggcccatctggttcactttgactcaacttgc

RM6 ctattccaccgcatctggcccatctggctcactttgactcaacttgc

RM7 ctattccaccgcatctggcccatctggctcactctgactcaacttgc

RM8 ctattccaccgcatctggtccatctggttcactttgactcaacttgc

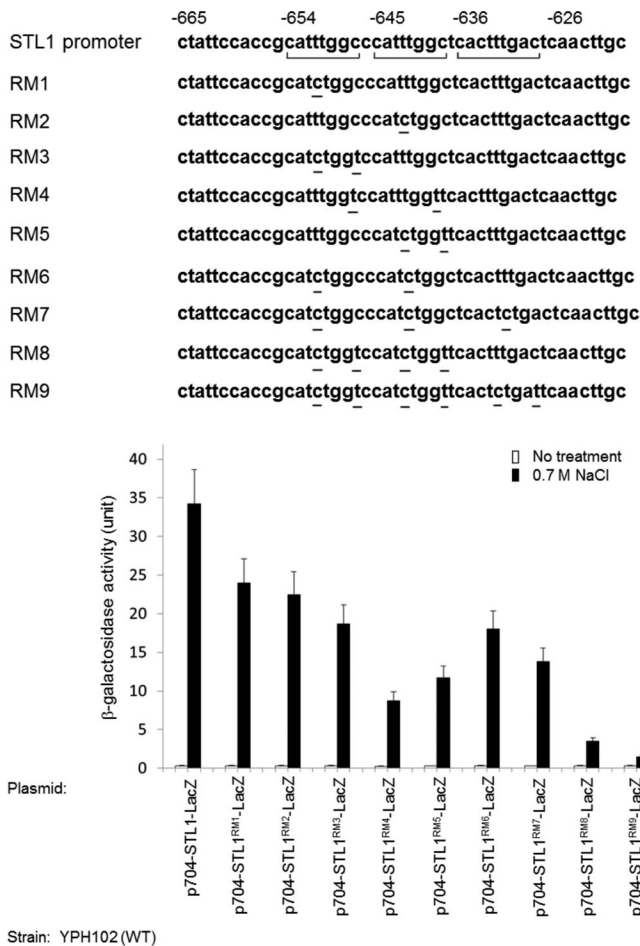RM9 ctattccaccgcatctggtccatctggttcactctgattcaacttgc

FIGURE 6: Point mutations in the two identical and one similar repeats significantly reduce promoter activity. Top, point mutations inserted in the context of the full-length 704–base pair promoter; bottom, the activities of the resulting constructs.

A

E1 -654 catttggcccatttggctcactttgactcaacttgcgtcattttaactgatatgaagggtcc -592

E2 -654 catttggcccatttggctcactttgactcaacttgcgtcattttaactgatatg -600

E3 -654 catttggcccatttggctcactttgactcaacttgcgtcattttaac -607

E4 -654 catttggcccatttggctcactttgactcaacttgc -618

E5 -654 catttggcccatttggctcactttgact -626

E6 -665 ctattccaccgcatttggcccatttggctcactttgactcaacttgcgtcattttaactgatatg -600

E7 -665 ctattccaccgcatttggcccatttggctcactttgact -626

B

C

FIGURE 7: The two identical 5′-CATTTGGC-3′ repeats plus the similar 5′-CACTTTGAC-3′ sequence are by themselves sufficient to boost the transcriptional activity of a heterologous promoter, but efficient responsiveness to Hog1 requires the presence of some additional nucleotides. (A) Sequences inserted upstream of the CYC1 minimal promoter. (B) Activities of the resulting constructs introduced into YPH102 (wild-type) cells exposed or not exposed to NaCl. (C) The short E1 fragment, when cloned upstream to the CYC1 promoter, is fully responsive to active Hog1 and not to Hog1^{WT}. Note induction of this reporter by Hog1$^{D170A+F318L}$ even in the presence of methionine, showing that residual expression of the protein dues to leakiness of the MET3 promoter is sufficient for activation of the E1 fragment.

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 smp1Δ and ras2Δmsn2Δmsn4Δ strains but was not induced in hog1Δ and hot1Δ 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 Hog1^{WT} or of Hog1$^{D170A+F318L}$ in hot1Δ cells did not activate the STL1 promoter even in cells exposed to osmotic stress (Figure 8C). Similarly, when overexpressed in hog1Δ cells, Hot1 on its own was unable to activate the STL1
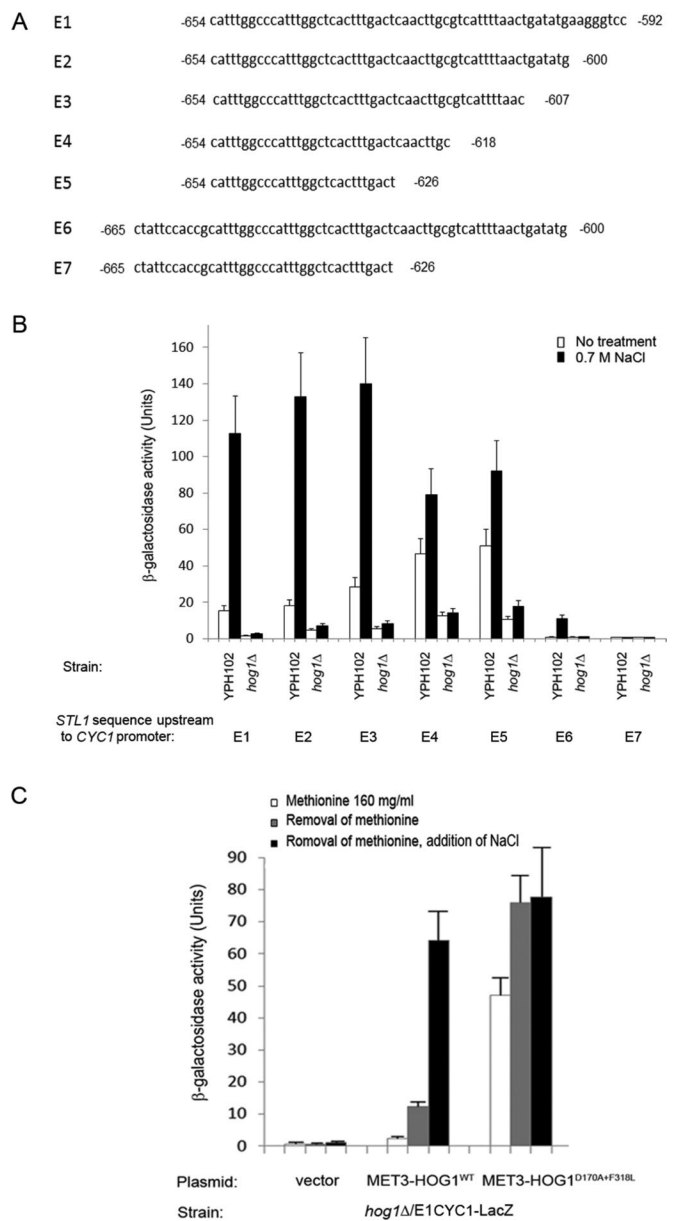
promoter (Figure 8D). These results support the notion that Hog1 and Hot1 must function together for activating the HoRE.

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
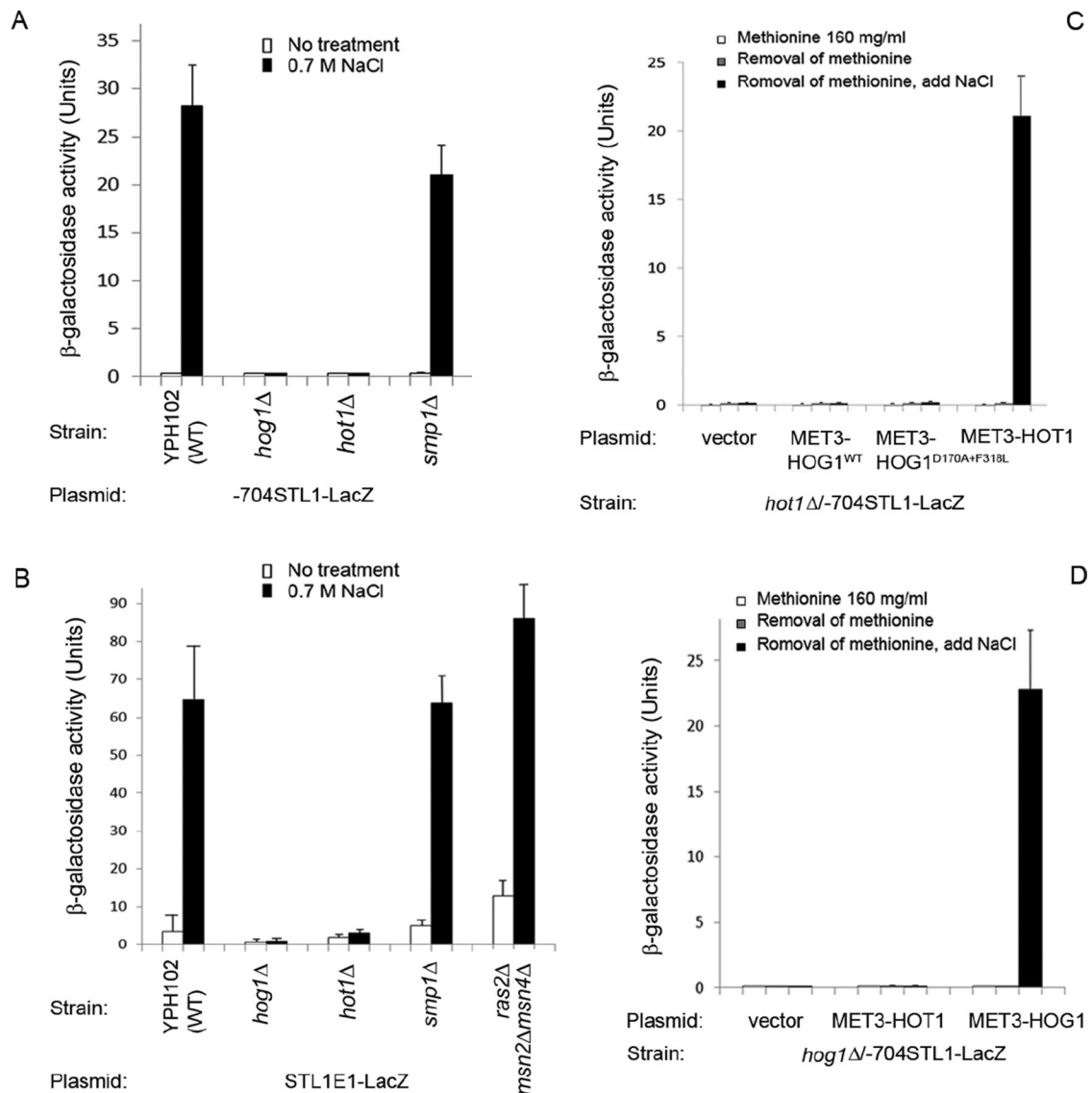
FIGURE 8: The *STL1* promoter and the HoRE are active in *smp1Δ* and *ras2Δmsn2Δmsn4Δ* cells but not in *hog1Δ* and *hot1Δ* cells. (A) The –704STL1-LacZ reporter gene was introduced into the indicated strains. Its activity was assayed in cells grown under optimal conditions and in response to 0.7 M NaCl. (B) Activity of the STL1(E1)CYC1-LacZ reporter was assayed in the indicated strains. (C) Overexpression of Hog1^WT or Hog1^D170A+F318L fails to activate the *STL1* promoter in *hot1Δ* cells. (D) Overexpression of Hot1 fails to activate the *STL1* promoter in *hog1Δ* cells.

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 STL1^delSKO1-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*.

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
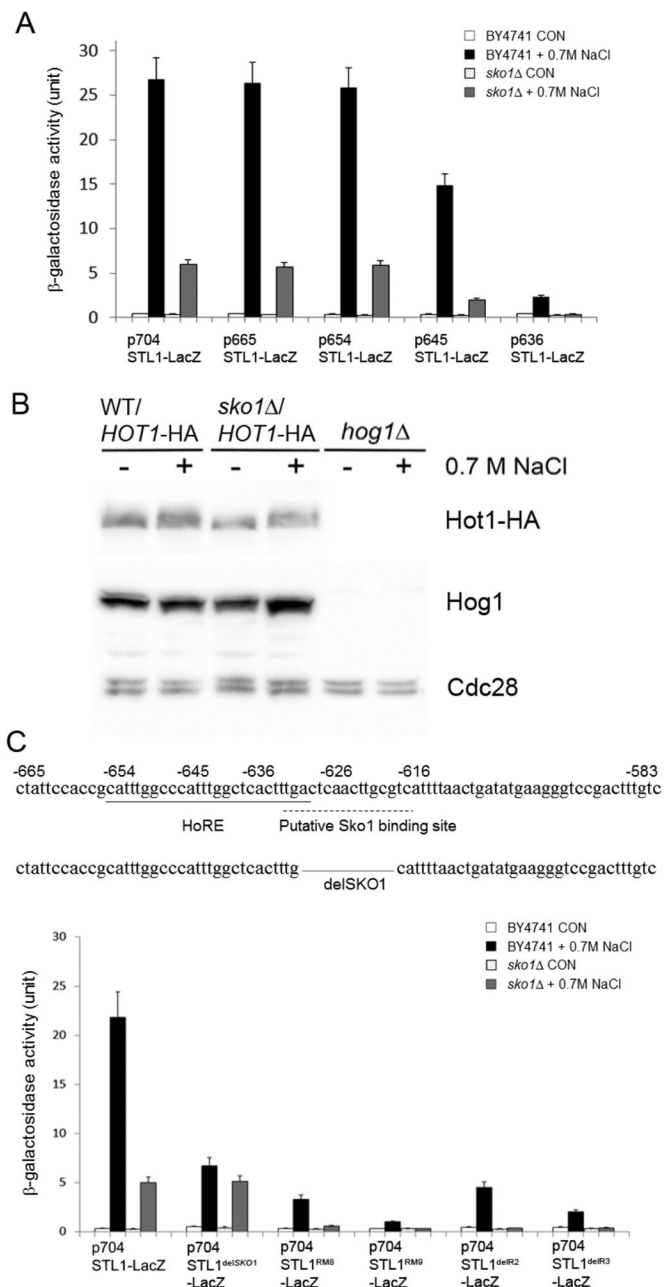
FIGURE 9: The transcription factor Sko1 cooperates with Hot1 for efficient transcription of *STL1* but is not essential for it. (A) Induction of STL1-LacZ reporter genes is reduced in *sko1Δ* cells. The indicated reporter genes were introduced into cells of the BY4741 and *sko1Δ* strains. The β-galactosidase activity in these cells was monitored before and after exposure to 0.7 M NaCl. (B) Sko1 has no effect on steady-state levels of Hog1 and Hot1 as monitored by Western blot analysis of cell lysates prepared from the indicated strains. Note that levels of Hot1 were monitored using anti-HA antibodies in strains harboring a single-copy plasmid carrying the *HOT1* gene with its native promoter. Lysate of *hog1Δ* cells was used as a control for the anti-Hog1 antibody. (C) A putative binding site for Sko1 is found (dashed line) in the *STL1* promoter within the region containing the HoRE (upper sequence). The lower sequence shows the fragment deleted in the construct 704STL1[delSKO1]-LacZ. Bottom, β-galactosidase activity of cells (BY4741 and *sko1Δ*) harboring this reporter and the other indicated reporters.

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 that 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 osmostress 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 osmostress (Figure 6), but also for association with Hot1. We also incubated the probe with yeast lysates prepared from cells of the wild-type strain BY4741 and of the *hog1Δ*, *hot1Δ*, the *hog1Δhot1Δ* strains either exposed or not exposed to 0.7M NaCl for 1 h. DNA-binding activity was observed in lysates prepared from BY4741, *hog1Δ*, and *hot1Δ* cells, but DNA–protein complex obtained with lysates prepared from *hog1Δ* and *hot1Δ* cells moved faster in the native gel (Figure 11, C and D). Binding of lysates to a mutated probe (M; Figure 11D, bottom) was significantly reduced. No HoRE binding activity was manifested by a lysate prepared from *hog1Δhot1Δ* cells (Figure 11D). Reduced binding activity was manifested by lysates prepared from *hot1Δsko1Δ* cells (lane 9, Figure 11D), suggesting that both proteins may bind E3, which contains binding sites for both. Lysates prepared from *sko1Δ* cells manifested efficient binding, suggesting that binding of Hot1 is independent of Sko1. The results with the recombinant Hot1 and cell lysates combined suggest that Hot1 associates with HoRE. Hot1 binding may be Hog1 independent (Figure 11D). It seems that Sko1 may also bind HoRE.
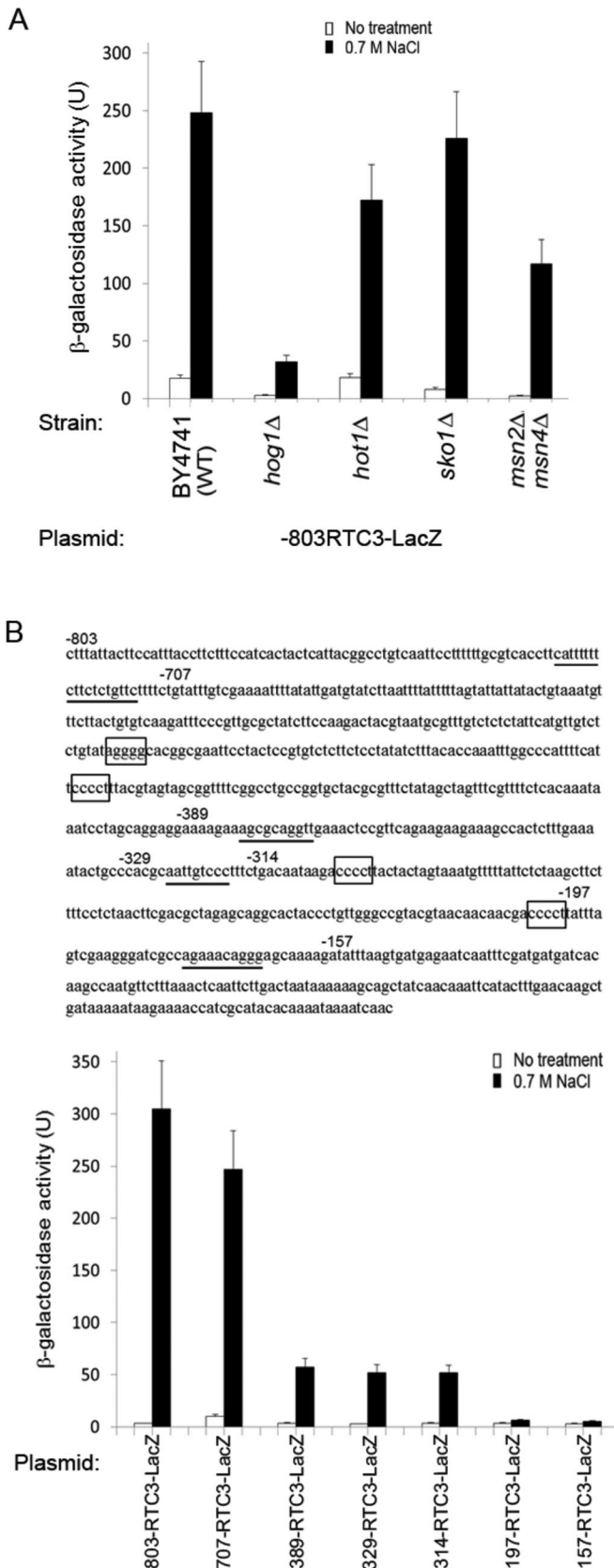
A

β-galactosidase activity (U)

☐ No treatment
■ 0.7 M NaCl

Strain: BY4741 (WT), hog1Δ, hot1Δ, sko1Δ, msn2Δ msn4Δ

Plasmid: -803RTC3-LacZ

B

-803
ctttattacttccatttaccttcttcatcactactcattacggcctgtcaattccttttttgcgtcaccttcatttttt
-707
cttctctgttctttctgtatttgtcgaaaatttatattgatgtatcttaatttattttagtattattatactgtaaatgt
tcttactgtgtcaagatttcccgttgcgctatcttccaagactacgtaatgcgtttgtctctctattcatgttgtct
ctgtat**ggggg**cacggcgaattcctactccgtgtctcttctcctatatctttacaccaaatttggcccattttcat
tcccctttacgtagtagcggttttcggcctgccggtgctacgcgtttctatagctagtttcgtttctcacaaata
-389
aatcctagcaggaggaaaagaaagcgcaggttgaaactccgttcagaagaagaaagccactctttgaaa
-329   -314
atactgcccacgcaattgtccctttctgacaataaga**ccccт**tactactagtaaatgtttttattctctaagcttct
-197
tttcctctaacttcgacgctagagcaggcactaccctgttgggccgtacgtaacaacaacga**ccccт**attta
-157
gtcgaagggatcgccagaaacagggagcaaaagatatttaagtgatgagaatcaatttcgatgatgatcac
aagccaatgttcttaaactcaattcttgactaataaaaaaagcagctatcaacaaattcatactttgaacaagct
gataaaaataagaaaaccatcgcatacacaaaataaaatcaac

β-galactosidase activity (U)

☐ No treatment
■ 0.7 M NaCl

Plasmid: 803-RTC3-LacZ, 707-RTC3-LacZ, 389-RTC3-LacZ, 329-RTC3-LacZ, 314-RTC3-LacZ, 197-RTC3-LacZ, 157-RTC3-LacZ

FIGURE 10: The promoter of *RTC3*, a putative target of Hot1, is activated in *hot1Δ* cells. It is less efficiently activated in *msn2Δmsn4Δ* cells and possesses several STREs. (A) The –803-RTC3-LacZ reporter gene was assayed in the indicated strains. (B) Top, the sequence of the *RTC3* promoter. STREs are boxed, and the proposed Hot1-binding sites (Cook and O'Shea, 2012) are underlined. The most 5' nucleotide of each deletion construct is marked by its number. Bottom, β-galactosidase activity of the indicated deletion constructs of the *RTC3* promoter as assayed in wild-type cells.

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*, *SPI1*, 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—*GPD1*, *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
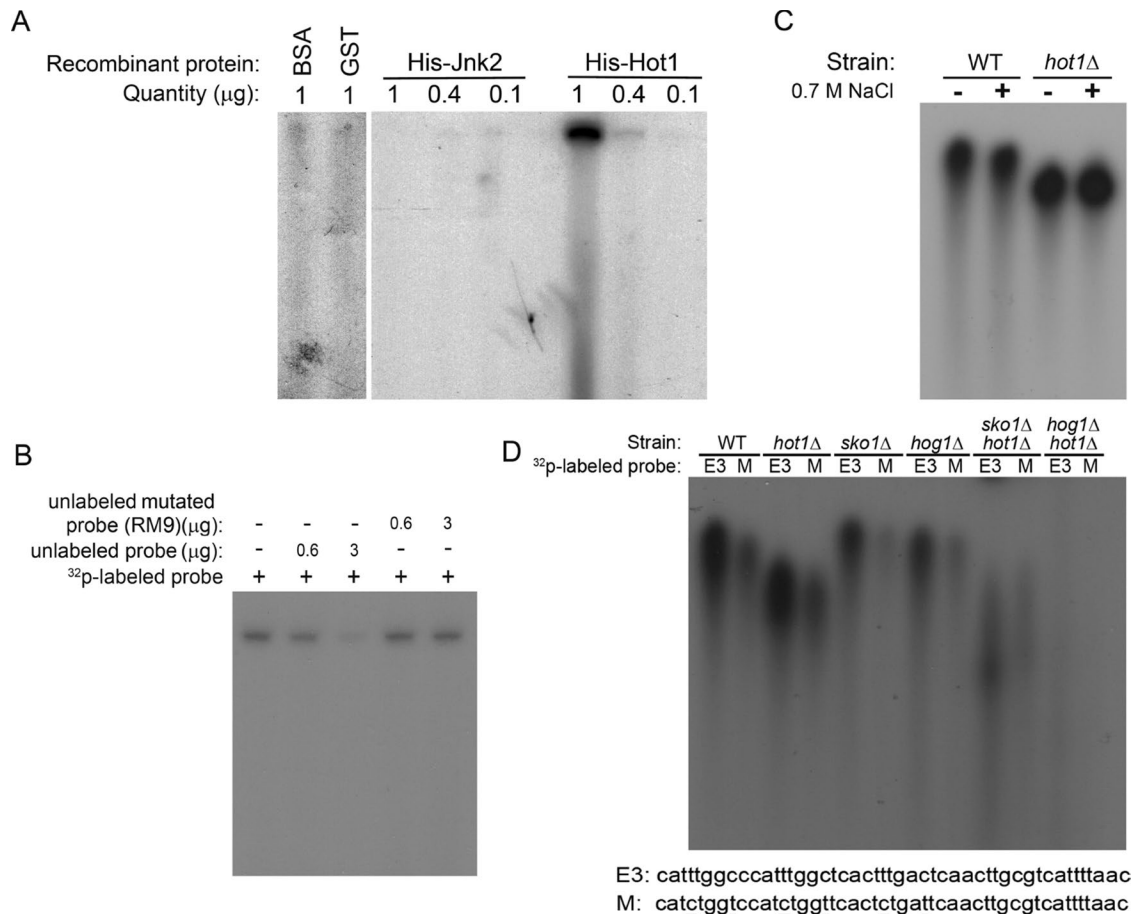
FIGURE 11: The Hot1 protein binds the HoRE of the *STL1* promoter. (A) The indicated quantities of recombinant histidine (His)-tagged Hot1 protein were mixed with ^{32}P-labeled fragment of the *STL1* promoter for 15 min and then loaded onto 5% acrylamide gel. Gels were run at 110 V for 6 h. Recombinant His-tagged JNK2, BSA, or GST protein purified from *E. coli* were tested as controls. (B) Recombinant His-tagged Hot1 (1 μg) was mixed with ^{32}P-labeled fragment together with the indicated unlabeled fragments. (C) The ^{32}P-labeled fragment was incubated with cell lysates prepared from wild-type or *hot1Δ* cells exposed or not exposed to 0.7 M NaCl. (D) Cell lysates of the indicated strains were incubated with ^{32}P-labeled E3 fragment of the *STL1* promoter (see Figure 7; see sequence at bottom) or with a mutated E3 fragment (M; see sequence at bottom).

(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 osmotresses 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 (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). *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).

Induced genes		Suppressed genes	
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.

TABLE 3: Genes induced or suppressed in *hot1Δ* cells under optimal growth conditions.

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

Gene name	Fold change	Gene name	Fold change
A. 0.9 M KCl		**B. 0.9 M NaCl**	
STL1	254.76	STL1	38.92
YML057C-A	6.24	YML057C-A	3.65
NQM1	5.19	NQM1	3.35
YGR066C	5.05	FIT1	3.34
FIT1	4.36		
YNR034W-A	4.09	**C. 1 M sorbitol**	
ICL1	4.04	Gene name	Fold change
ERR2	3.80	STL1	13.26
ERR1	3.77	NQM1	11.50
ERR3	3.72	GRE1	4.87
TMA17	3.67	TKL2	4.27
BTN2	3.64	YBR116C	4.18
SPG4	3.55	SIP18	3.63
TPS2	3.49	FIT1	3.57
RTN2	3.43	YNL067W-A	3.53
YCL046W	3.34	PAI3	3.36
TSL1	3.26	GND2	3.11
RTC3	3.23	ALD3	3.02
CUR1	3.08		
CYC7	3.04		
YNL195C	3.03		

Fold change was calculated as the ratio of expression levels in *hot1Δ/HOT1* and *hot1Δ* cells 60 min after exposure to 0.9 M KCl (A), 0.9 M NaCl (B), or 1 M sorbitol (C).

TABLE 4: Genes induced in wild-type cells, but not in *hot1Δ* cells, in response to osmotic pressure.

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 $NH_4(SO_4)_2$, 0.5% ammonium sulfate,
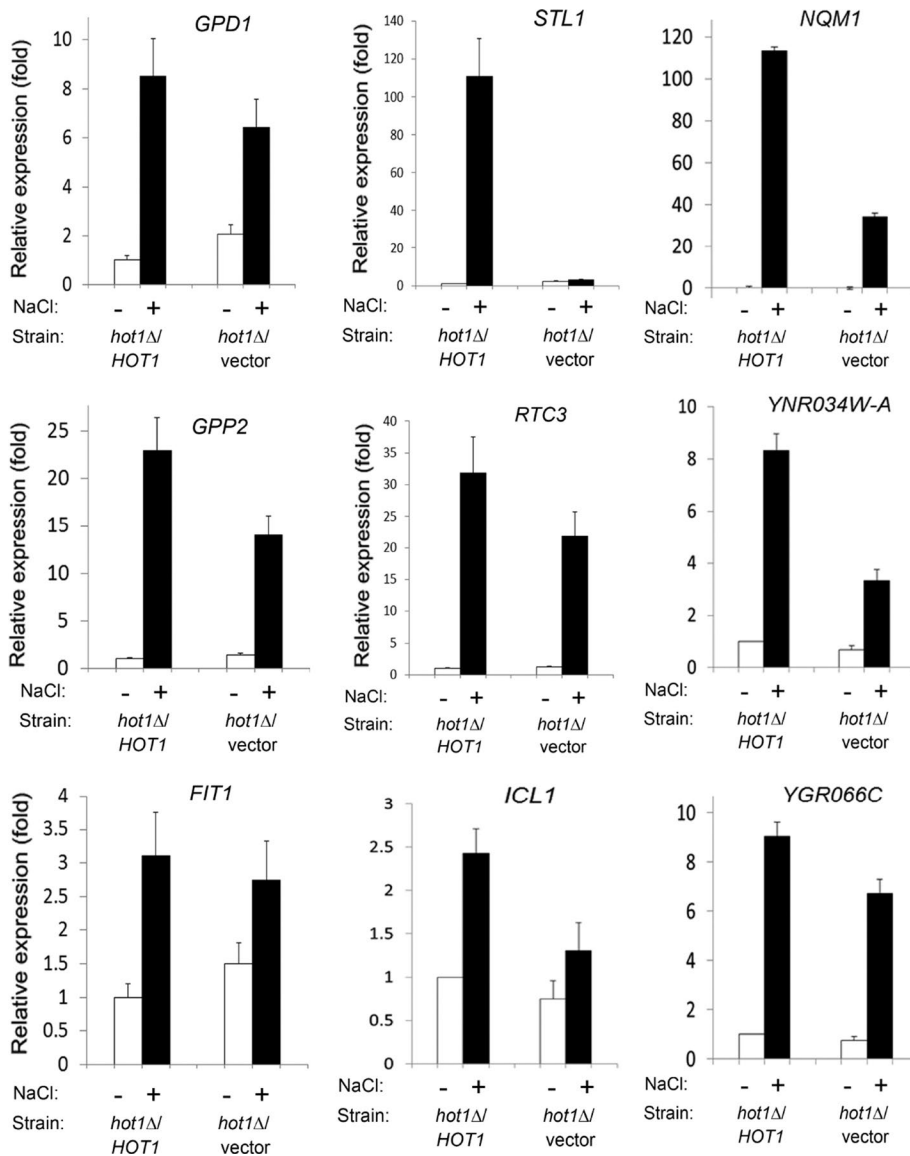
FIGURE 12: *STL1* is the only gene whose expression is abolished in *hot1Δ* cells. mRNA levels of the indicated genes were assayed by real-time quantitative RT-PCR performed on RNAs isolated from *hot1Δ* cells harboring either an empty vector or a single copy of a genomic fragment carrying the *HOT1* gene (including its native promoter). Cells were grown to mid log phase and then exposed to the indicated treatments. The value of each gene was normalized to the value of *ACT1*, which served as an internal control, and is shown as the ratio between its value in the sample to its value in untreated *hot1Δ/HOT1* cells. Values shown are averages of three independent experiments.

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 *Bam*HI and *Sal*I 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 *Xho*I, and cloned into *Xho*I-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 *Nde*I and *Not*I, 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 *Bam*HI, and cloned in a pRS316 vector digested with the same enzymes. MET3-HOG1^WT and MET3-Hog1^D170+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.

2% glucose, and 40 mg/l required nutrients), or yeast/peptone/dextrose (YPD) medium (2% glucose, 1% yeast extract, and 2% Bacto Peptone).

Plasmids

For *STL1* promoter constructs, the pLG669Z plasmid (Guarente and Ptashne, 1981) was digested with *Bam*HI and *Sal*I. Different lengths of *STL1* 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 *Bam*HI and *Sal*I and ligated to the pLG669Z vector. The resulting plasmids contained the *STL1* promoter fragments with the first ATG fused in-frame to the β-galactosidase coding sequence (Guarente and Ptashne, 1981; Grably *et al.*, 2002).

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).
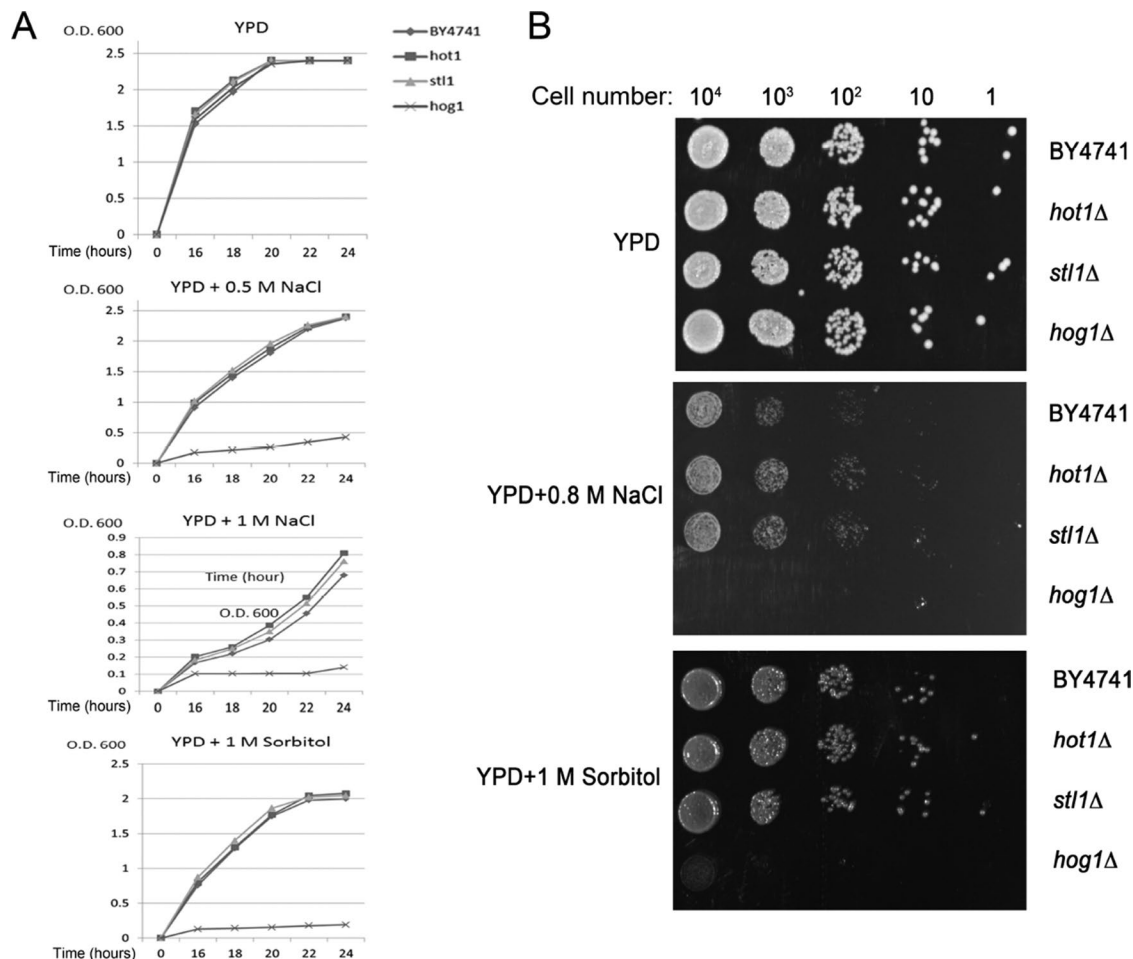
FIGURE 13: *hot1Δ* and *stl1Δ* cells are not sensitive for growth under osmostress. Cells of the indicated strains were allowed to proliferate on the indicated liquid medium (A) or on plates supplemented with agar plus the indicated medium (B).

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 C_T, 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′-ctattccaccgcatttggcccatttggctcactttgactcaacttgcgtcattttaact-gatatg-3′ and 5′-catatcagttaaaatgacgcaagttgagtcaaagtgagccaaa-tgggccaaatgcggtggaatag-3′) and labeled with T4 polynucleotide kinase (NEB, Ipswich, MA) in the presence of [γ–^{32}P]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 ^{32}P-labeled E6 probe. For competition binding by E6 fragment harboring mutations at HoRE repeats (RM9), two oligos (5′-ctattccaccgcatctggtccatctggttcactctgattcaacttgcgtcattt-taactgatatg-3′ and 5′-catatcagttaaaatgacgcaagttgaatcagagtgaac-cagatggaccagatgcggtggaatag-3′) were annealed to form double strands, and the same amount of probe was mixed with ^{32}P-labeled probe.

Strain	Relevant genotype	Source/reference
YPH102	*MATα ura3-52 lys2-801 ade2-101 his3-200 leu2-1*	Bell *et al.* (2001)
BY4741	*MATa his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0*	EUROSCARF (Bad Homburg, Germany)
JBY13	*MATa his3, leu2, ura3, trp1, ade2, lys2 hog1::TRP1*	M. Gustin (Rice University)
hog1Δpbs2Δ	*MATa his3, ura3, trp1, ade2, lys2 hog1::TRP1, pbs2::LEU2*	Bell *et al.* (2001)
hot1Δ	*MATa his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0 hot1::kanMX4*	EUROSCARF
smp1Δ	*MATa his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0 smp1::kanMX4*	EUROSCARF
ras2Δ	*MATa his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0 ras2::kanMX4*	EUROSCARF
msn2Δ	*MATa his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0 msn2::kanMX4*	EUROSCARF
sko1Δ	*MATa his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0 sko1::kanMX4*	EUROSCARF
msn2Δmsn4Δ	*MATa his3-Δ1 met15Δ0 ura3Δ0 msn2::kanMX4 msn4::LEU2*	This work
ras2Δmsn2Δmsn4Δ	*MATa met15Δ0 ura3Δ0 ras2::kanMX4 msn2::HIS3 msn4::LEU2*	This work
hog1Δhot1Δ	*MATa leu2, ura3, trp1, ade2, lys2 hog1::TRP1 hot1::HIS3*	This work
hot1Δ HOT1	*MATa his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0 hot1::kanMX4 pRS316-HOT1*	This work
hot1Δsko1Δ	*MATa his3-Δ1 leu2-Δ0 met15Δ0 sko1::kanMX4 hot1::URA3*	This work
HOT1-HA	*MATa his3-Δ1 leu2-Δ0 met15Δ0 ura3Δ0 pRS313 HOT1*	This work
sko1Δ HOT1-HA	*MATa 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.

Primers for cloning and plasmid construction		Primers for cloning and plasmid construction	
STL1-Rev	gactGGATCCggtcatggtctaaaactttctatg	STL1-E4	CAGATCCGCCAGGCGTGTA GactCTCGAGcatttggcccatttggctcactttgact
STL1-704	gactGTCGACataacggacgtacggac	STL1-E5	CAGATCCGCCAGGCGTGTA
STL1-665	gactGTCGACtattccaccgcatttgg	RTC3-803	GactGTCGACtttattacttccatttac
STL1-654	gactGTCGACatttggcccatttggctc	RTC3-707	gactGTCGACtgtatttgtcgaaaattt
STL1-645	gactGTCGACcatttggctcactttgac	RTC3-389	gactGTCGACgaaagcgcaggttgaaac
STL1-636	gactGTCGACactttgactcaacttgc	RTC3-329	gactGTCGACgcaattgtccctttctgac
STL1-626	gactGTCGACaacttgcgtcattttaac	RTC3-314	gactGTCGACtgacaataagaCCCCTta
STL1-583	gactGTCGACtttttcggccaccgcata	RTC3-197	gactGTCGACTtatttagtcgaagggat
STL1-550	gactGTCGACtccgctacctgcatttg	RTC3-157	gactGTCGACGatatttaagtgatgagaa
STL1-400	gactGTCGACtgtttctcggctatatac	RTC3-R	gactGGATCCggtcatgttgattttattttgtgtatg
STL1-704F	gactCTCGAGataacggacgtacggacg	HOT1-pt-F	gactGGATCCtcatgttttccattaatc
STL1-533R	gactCTCGAGcaaatgcaggtagcggag	HOT1-R	gactGGATCCgttattgccagaatcattg
STL1-564R	gactCTCGAGtatgcggtggccgaaaaag	HOT1-F(Nco)	gactCCATGGctTCTGGAATGGGTATTGCG
STL1-583R	gactCTCGAGacaaagtcggacccttc	HOT1-R2(N1)	gactGCGGCCGCacccttctcagaataag
CYC1-R	gactGGATCCGGTCATTATTAA gactCTC-GAGcatttggcccatttggctcactttgactcaacttgcgtcattttaactgatatgaagggtcc	**Primers for mutagenesis**	
		STL1-STRE-F	tttagcttcaattttgtcTcGttcaacgctgcttggcc
STL1-E1	AGATCCGCCAGGCGTGTA gactCTCGAG-catttggcccatttggctcactttgactcaacttgcgtcattttaactgatatg	STL1-STRE-R	ggccaagcagcgttgaaCgAgacaaaattgaagctaaa
		STL1-delR2-F	gttgtcccactattccaccgcactttgactcaacttgcg
		STL1-delR2-R	cgcaagttgagtcaaagtgcggtggaatagtgggacaac
STL1-E2	CAGATCCGCCAGGCGTGTA gactCTC-GAGctattccaccgcatttggcccatttggctcactttgactcaacttgcgtcattttaactgatatg	STL1-delR3-F	gttgtcccactattccaccgcaacttgcgtcattttaac
		STL1-delR3-R	gttaaaatgacgcaagttgcggtggaatagtgggacaac
STL1-E3	CAGATCCGCCAGGCGTGTA gactCTC-GAGctattccaccgcatttggcccatttggctcactttgact	STL1-delR4-F	gaaataagtgccgttgtcccactttgactcaacttgcg

TABLE 6: Oligonucleotides used in this study.

Primers for mutagenesis		Primers for RT-PCR	
STL1-delR4-R	cgcaagttgagtcaaagtgggacaacggcacttatttc	GRE2-RR	CTTCTTAGAACCACAGTAG
STL1-delR5-F	gaaataagtgccgttgtcccaacttgcgtcattttaac	THI4-RF	GAAGACGAAGGTGACTATG
STL1-delR5-R	gttaaaatgacgcaagttgggacaacggcacttatttc	THI4-RR	GAACAGTTTAACATTTGG
STL1-delSKO-F	cccatttggctcactttgttttaactgatatgaaggg	YLR042C-RF	GGAGGTGTAGGTTCAGTC
STL1-delSKO-R	cccttcatatcagttaaaacaaagtgagccaaatggg	YLR042C-RR	CAAATAAGCGATTGTTTC
Primers for RT-PCR		FIT1-RF	GCCGCTTTAGGCGAAAGTATT
ACT1-RF	GTGTGGGGAAGCGGGTAAGC	FIT1-RR	CTTCAGTTACTGCGGAGGTTACC
ACT1-RR	GTGGCGGGTAAAGAAGAAAATGGA	GPD1-RF	TCAATTTTTGCCCCGTATCTG
STL1-RF	GTTGCGGTATTTCATCAC	GPD1-RR	GATAGCTCTGACGTGTGAATCAACA
STL1-RR	CATAGTTGAACTGTTTACC	GPP2-RF	CGACGTCGACGGTACCATTA
CWP1-RF	CTCCACTGCTTTGTCTGTCG	GPP2-RR	CGAAATCCCTCCAGAATGCA
CWP1-RR	CAAGTATTGTAAATCCGAGC	RTC3-RF	GGGCGCTGCCTCCAA
HSP12-RF	CTGACGCAGGTAGAAAAGG	RTC3-RR	CTTCGATCTTCTTGCCCCTTACC
HSP12-RR	GAACCTTACCAGCGACCTTG	NQM1-RF	CATTACTGTTTTCCTTTAC
KDX1-RF	CACCGAGAGGTGTATTTTCC	NQM1-RR	CAGTATAGTCTTTGCCTG
KDX1-RR	GAGTTTCCTCGTTCGATTC	ICL1-RF	GACACCGTTCCAAACAAAG
PNS1-RF	CCGTTTTGGGCCTCACAC	ICL1-RR	CATCTCATCGAGTTCTTC
PNS1-RR	CAGACCAGTACCTCAAAG	YNR034W-A-RF	CCAATCACCGAAGTATTG
PRM10-RF	GCTCTATCCCTCGTTCTC	YNR034W-A-RR	GATTGGTGACTTTTCGATG
PRM10-RR	CAGCAACTAGGCTTCGAC	YGR066C-RF	GAAATGACAACATTGAAG
GRE2-RF	GTGTTCGATATGGCAAAAG	YGR066C-RR	CTCGTAAACATCACAGTC

TABLE 6: Oligonucleotides used in this study. Continued

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