# Expression of *YAP4* in *Saccharomyces cerevisiae* under osmotic stress

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*YAP4*, a member of the yeast activator protein (*YAP*) gene family, is induced in response to osmotic shock in the yeast *Saccharomyces cerevisiae*. The null mutant displays mild and moderate growth sensitivity at 0.4 M and 0.8 M NaCl respectively, a fact that led us to analyse *YAP4* mRNA levels in the *hog1* (high osmolarity glycerol) mutant. The data obtained show a complete abolition of *YAP4* gene expression in this mutant, placing *YAP4* under the HOG response pathway. *YAP4* overexpression not only suppresses the osmosensitivity phenotype of the *yap4* mutant but also relieves that of the *hog1* mutant. Induction, under the conditions tested so far, requires the presence of the transcription factor Msn2p, but not of Msn4p, as *YAP4* mRNA levels are depleted by at least 75 % in the *msn2* mutant. This result was further substantiated by the fact that full *YAP4* induction requires the two more proximal stress response elements. Furthermore we find that *GCY1*, encoding a putative glycerol dehydrogenase, *GPP2*, encoding a NAD-dependent glycerol-3-phosphate phosphatase, and *DCS2*, a homologue to a decapping enzyme, have decreased mRNA levels in the *yap4*-deleted strain. Our data point to a possible, as yet not entirely understood, role of the *YAP4* in osmotic stress response.

Key words: HOG (high osmolarity glycerol) pathway, Msn2p, stress response element (STRE), yeast activator protein (Yap1p), *YAP4* (*CIN5*).

## INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* modulates gene expression in response to environmental cues, such as an increase in temperature and osmolarity or exposure to oxidizing agents [1,2,3]. Upon exposure to increased external osmolarity, yeast cells immediately arrest growth [4,5] and subsequent specific responses are triggered in order to repair molecular damage and to induce adaptation to the new conditions. Hyperosmotic stress leads to altered transcription of stress genes and to an increase in glycerol metabolism [6,7]. This is, to a great extent, mediated by the HOG (high osmolarity glycerol) mitogen-activated protein kinase (MAPK) pathway through the modulation of the expression of stress-responsive genes. Several transcription factors have been proposed to act under the control of the Hog1p MAPK, including Msn1p [8], Msn2/4p, Hot1p [6,8] and Sko1p [9,10] among others.

The zinc finger transcription factors, Msn2p/Msn4p bind one or more copies of the stress response element (STRE) motif, CCCCT [11], and are required for the stimulation of gene expression after exposure to osmotic shock, oxidative stress, nutrient starvation and other forms of environmental stimuli [12,13,14]. Induction of their target genes by environmental stimuli not only results in increased cell protection but also allows for a degree of cross-protection towards further, more severe, forms of stress [15]. DNA microarray analyses indicate also that a large number of genes induced by abrupt environmental changes show condition-specific and gene-specific regulation by a number of different signalling pathways [1]. Treger et al. [16] had demonstrated that many stress-responsive genes containing heat shock elements (HSEs) and STREs are co-regulated by HSF (heat shock factor) and Msn2/4p. For example, *HSP26* and *HSP104* show condition-specific co-operative regulation by these two transcription factors [17,18].

*YAP4* is a gene encoding a bZIP transcription factor belonging to the YAP (yeast activator protein) family of eight trans-activators in *S. cerevisiae* [19]. It encodes a protein initially characterized as a chromosome instability mutant, having therefore been designated as Cin5p [20]. Its overexpression was shown to confer salt tolerance [21] as well as resistance to antimalarial drugs [22] and cisplatin [23]. Furthermore, recent results from several DNA microarray analyses indicate an induction of the *YAP4* gene under various conditions, including those of oxidative and osmotic stress [1,6,7].

Our studies aim at determining the key components involved in the regulation of *YAP4* gene expression when yeast cells are subjected to hyperosmotic stress. The results obtained indicate that not only does the null mutant show a salt-sensitive phenotype, but also that Msn2p regulates *YAP4* gene expression through the more proximal STRE elements (at positions −430 bp and −716 bp). Furthermore, it is also shown that Yap4p is a downstream component of the HOG pathway and that its overexpression partially rescues the salt-sensitive phenotype of the *hog1* single mutant. Finally, *GCY1*, encoding a putative glycerol dehydrogenase, and *GPP2*, encoding a NAD-dependent glycerol-3-phosphate phosphatase, two genes whose products are involved in glycerol biosynthesis, as well as *DCS2*, encoding a homologue to an mRNA decapping enzyme, have been found to be partially dependent on Yap4p for full induction under hyperosmotic stress.

## EXPERIMENTAL

### Strains and growth conditions

*S. cerevisiae* strains used in this study are listed in Table 1. The complete coding regions of the *YAP1*, *YAP4* and *SKO1* (suppressor of protein kinase A overexpression) genes were deleted by the microhomology PCR method [26]. Deletion was confirmed by

Abbreviations used: CIN, chromosome instability mutant; CIP, calf intestinal phosphatase; HOG, high osmolarity glycerol; HSE, heat shock element; HSF, heat shock factor; MAPK, mitogen-activated protein kinase; ORF, open reading frame; SKO, suppressor of protein kinase A overexpression; STRE, stress response element; TCA, trichloroacetic acid; YAP, yeast activator protein; YRE, Yap1p response element.

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**Table 1 *S. cerevisiae* strains**

MAT, mating type; KAN, kanamycin resistance.

| Strain | Genotype | Source |
|---|---|---|
| FY1679 | MAT$\alpha$ his3-200 ura3-52 GAL2 | [24] |
| *yap1* | MAT$\alpha$ yap1::KAN | This study |
| *yap4* | MAT$\alpha$ yap4::KAN | This study |
| *yap1/yap4* | MAT$\alpha$ yap1, yap4::KAN | This study |
| W303-1A | MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC mal0 | [25] |
| W303-1B | MAT$\alpha$ leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC mal0 | [25] |
| *hog1* | MATa hog1::TRP1 | Dr S. Hohmann |
| *hog1/yap4* | MATa hog1::TRP1, yap4::KAN | This study |
| *sko1* | MATa sko1::KAN | This study |
| *yap1* | MATa yap1::KAN | This study |
| *yap4* | MATa yap4::KAN | This study |
| *yap1/yap4* | MATa yap1, yap4::KAN | This study |
| *msn2* | MAT$\alpha$ msn2::HIS3 | Dr F. Estruch |
| *msn4* | MATa msn4::TRP1 | Dr F. Estruch |
| *msn2/msn4* | MATa msn2::HIS3, msn4::URA3 | Dr F. Estruch |
| *msn2/yap4* | MAT$\alpha$ msn2::HIS3, yap4::KAN | This study |
| *hot1* | MATa hot1::KAN | [36] |
| *msn1* | MAT$\alpha$ msn1::URA3 | [36] |
| *skn7* | MATa skn7::KAN | Dr S. Hohmann |
| *yap4YAP4* | MATa yap4::KAN, YIpYAP4 | This study |
| *yap4YAP4*$_{\Delta STRE430}$ | MATa yap4::KAN, YIpYAP4$_{\Delta STRE430}$ | This study |
| *yap4YAP4*$_{\Delta STRE716}$ | MATa yap4::KAN, YIpYAP4$_{\Delta STRE716}$ | This study |
| *yap4YAP4*$_{\Delta YRE}$ | MATa yap4::KAN, YIpYAP4$_{\Delta YRE}$ | This study |
| *yap4YAP4*$_{\Delta STRE430/716}$ | MATa yap4::KAN, YIpYAP4$_{\Delta STRE430/716}$ | This study |
| *yap4YAP4*$_{\Delta STRE430/YRE}$ | MATa yap4::KAN, YIpYAP4$_{\Delta STRE430/YRE}$ | This study |
| *yap4YAP4*$_{\Delta 430/716/YRE}$ | MATa yap4::KAN, YIpYAP4$_{\Delta STRE430/716/YRE}$ | This study |

PCR analysis of genomic DNA using upstream and downstream primers. The *hog1*, *hot1*, *msn1* and *skn7* mutants in the W303-1A strain were a gift from Dr S. Hohmann (University of Göteborg, Sweden) whilst the *msn2* and *msn4* single and double mutants were a gift from Dr F. Estruch (University of Valencia, Spain). Yeast strains were grown in YPD medium [1 % (w/v) yeast extract/2 % (w/v) peptone/2 % (w/v) glucose] or SC medium [0.67 % yeast nitrogen base without amino acids, 0.6 % casamino acids (Difco, West Molesey, Surrey, U.K.)] supplemented with 2 % (w/v) glucose and the appropriate selective amino acids. Standard liquid cultures were incubated with orbital shaking (200 r.p.m.) at 30 °C.

Osmotic-shock induction in liquid cultures was carried out on growing cultures at early log phase ($D_{600} = 0.4$–$0.5$) by addition of 5 M NaCl to a final concentration of 0.4 M or 0.8 M, and samples were collected at the indicated time points by centrifugation at 2700 ***g*** for 5 min. Samples for RNA and protein extraction were washed and stored at − 80 °C.

Phenotypic growth assays were carried out on solid media by spotting 5 $\mu$l of a serially diluted culture (1:10 starting from $D_{600} = 0.1$). Growth was recorded after 1–2 d at 30 °C. Standard methods were used for genetic analysis [27], cloning [28] and transformation [29].

### *YAP4* overexpression

*YAP4*-overexpression studies used the entire *YAP4* ORF (open reading frame) plus 1043 bp upstream and 190 bp downstream cloned into the multicopy vector YEp356R [30]. The region of interest was amplified by PCR using the following primer sequences: 5′-GGTTAAGCTTAGTGCCATTCGGTGAG-3′ and 5′-GCTCCATGACAACATTCG-3′ and cloned by *Hin*dIII digestion to generate construct YEpYAP4.

### Site-directed mutation of the YAP4 promoter *cis*-elements

Site-directed mutagenesis of *YAP4 cis*-elements were generated by first cloning, as before, the same entire *YAP4* ORF, with its 1043 bp upstream region, into the YIplac211 [31] *Hin*dIII site. Mutations were subsequently generated by PCR amplification of the entire construct using complementary primers containing the desired mutation as follows: $\Delta STRE_{430}$, 5′-GACGCAATGCGTGAGTTGATTTCCCGAG-3′ (that alter the STRE sequence from GGAGGGG to TGAGTTG); $\Delta STRE_{716}$, 5′-GTAAAACTTGTTGTACGGAGAGTTGTCGAGAAAAG-3′ (that alter the STRE sequence from GAGGGG to GAGTTG); $\Delta$YRE, 5′-CGTTAATTTGTAAGTTATGCAACCAAGTGC-3′ (that alter the recognition site TTAGTAA to TAAGTTA). These amplifications were treated with *Dpn*I prior to *E. coli* transformation. The purified plasmids were linearized by digestion with *Nco*I and transformed into the *yap4* mutant strain. Double and triple mutants were generated by subsequent amplification of mutant constructs.

### Construct sequencing

All constructs generated in this study were sequenced using the ABI Prism DyeDeoxy Terminator Cycle Sequencing Kit (PerkinElmer Applied Biosystems Inc., Warrington, Cheshire, U.K.) and ABI Prism 373A Automatic Sequencer (PerkinElmer).

### Northern-blot analysis

Total RNA was extracted by the hot acid/phenol method [28] from early log-phase cultures that were either untreated (control) or exposed to 0.4 M or 0.8 M NaCl. Approx. 30 $\mu$g of RNA was loaded per lane, separated in formaldehyde gels [28] and transferred onto nylon membranes (MagnaCharge; Schleicher and Schuell, Dassel, Germany; Hybond XL, Amersham Biosciences, Frieburg, Germany). Probes (a 0.74 kb intragenic YAP4 PCR fragment, a 0.6 kb intragenic HSP26 PCR fragment, a 0.2 kb intragenic U3 PCR fragment, a 1.8 kb intragenic YAP1 PCR fragment, a 0.86 kb intragenic GCY1 PCR fragment, a 0.48 kb intragenic GPP2 PCR fragment, and a 0.62 kb intragenic DCS2 PCR fragment) were radiolabelled by random priming with [$\alpha$-$^{32}$P]ATP (MegaPrime; Amersham Biosciences), G50-purified and hybridized overnight in Church hybridization buffer [0.25 M sodium phosphate (pH 7.5), 7 % (w/v) SDS, 1 mM EDTA, 1 % (w/v) BSA] at 65 °C. Radioactive blots were washed [20 mM sodium phosphate (pH 7.5), 0.1 % SDS, 1 mM EDTA] and the signal was detected either by exposure to radio-sensitive film (Biomax MR; Kodak, Rochester, NY, U.S.A.) or to a Molecular Dynamics (Little Chalfont, Bucks., U.K) Phosphor Screen, and the signal detected by a Storm 860 Scanner. mRNA levels were quantified (ImageQuant, Molecular Dynamics) and normalized against those of the internal loading control, U3, a small nuclear RNA (SNR17A).

### Generation of YAP4–TAP tag, protein extraction and Western analysis

#### TAP-tag cloning

*Afl*III restriction sites were introduced into the desired *YAP4* fragment by PCR amplification of pRS416*YAP4*, a centromeric vector [32] containing the entire *YAP4* ORF cloned in this study (as described above) using primers 5′-CCCACATGTTGGCTCACTTACAGATC -3′ and 5′-CCCACATGTATTCTTTTAATTTCGACTTTA-3′. The amplified *YAP4* fragment was

then digested with *Afl*III and cloned into the pBS1479 [33] *Nco*I site, placing it in frame with the TAP tag plasmid sequence. The *YAP4*–TAP tag fragment was then digested with *Mls*I and *Xba*I and cloned back into pRS416*YAP4*.

### Protein extracts

Samples collected at the indicated time points were harvested by centrifugation at 21000 ***g*** for 5 min at 4 °C, washed with 20 % (w/v) TCA (trichloroacetic acid) and lysed by vortexing. The supernatant was centrifuged for 1 min at 2700 ***g*** and the pellet resuspended in Laemli buffer [62.5 mM Tris/HCl (pH 8.7), 2 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol, 10 % (v/v) glycerol and 0.01 % Bromophenol Blue], neutralized with 1.0 M Tris (pH 8.0) and heated for 5 min at 95 °C. Finally, the solution was centrifuged again for 1 min at 2700 ***g*** and the supernatant proteins were quantified using the Bradford method [34] with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.). All equipment and reagents were pre-cooled before use.

### Western-blot analysis

Samples were separated by SDS/PAGE (10 % gel), transferred onto a PROTRAN® (Schleicher and Schuell) nitrocellulose transfer membrane and incubated with anti-TAP tag antibody [PAP rabbit antibody (Sigma); 1:1.000 dilutions in PBS, 0.1 % Tween, 1 % (w/v) dry milk]. Chemiluminescent detection was performed using the ECL® Western Blotting Detection System (Amersham Biosciences). The TCA-precipitated pellet of protein extracts of *yap4* strains, expressing TAP-tagged Yap4p, were acetone-washed, dried in a speed vac, dissolved in 75 mM iodoacetamide, 1 % (w/v) SDS, 100 mM Tris/HCl, pH 8.0, 1 mM EDTA and complete protease inhibitors (Boehringer Mannheim) and subjected to CIP (calf intestinal phosphatase; New England Biolabs) incubation for 45 minutes at 37 °C prior to SDS/PAGE and immunoblot [35].

### Intracellular glycerol content

To measure the internal glycerol levels, the assay was performed as described previously [36] with minor modifications. Cells were harvested by centrifugation for 1 min at 2700 ***g***, washed and resuspended in cold water. After measuring the $D_{600}$, samples were heated for 15 min. at 95 °C. The glycerol released into the supernatant was quantified with a glycerol determination kit (Roche, Mannheim, Germany). The results presented are the average of six independent experiments.

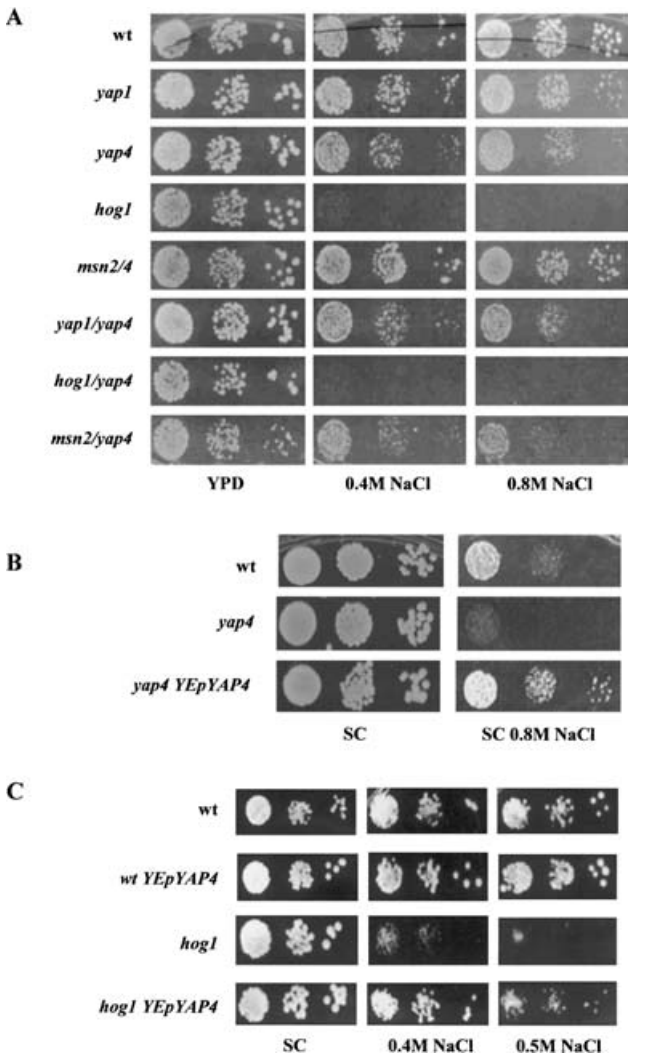


**Figure 1 Mutant strain *yap4* shows a salt-sensitive phenotype**

(**A**) Serially diluted single- and double-mutant strains (*yap1*, *yap4*, *hog1*, *msn2/4*, *yap1/yap4*, *hog1/yap4*, *msn2/yap4*) and wild-type (wt) were spotted onto YPD medium and YPD medium supplemented with 0.4 M and 0.8 M NaCl respectively. Growth was recorded after 2–3 days at 30 °C. (**B**) Recovery of the *yap4* salt-sensitive phenotype. The *yap4* mutant strain was transformed with YEp*YAP4*, a multicopy plasmid containing the complete *YAP4* ORF, and serially diluted plates were spot assayed, as described above, in SC medium supplemented with 0.8 M NaCl. (**C**) The effect of *YAP4* overexpression on the osmotic sensitivity phenotype of the *hog1* mutant strain. W303-1A and *hog1* mutant strains were transformed with YEp*YAP4*, grown as above, washed and spotted onto selective solid media with or without 0.4 M or 0.5 M NaCl. Growth was measured after 2–3 days. Wild-type strains refer to cells transformed with the empty vector.

## RESULTS

### The *yap4* mutant displays a moderate osmosensitivity phenotype

In order to assess the biological role of Yap4p, we studied the growth phenotypes of the mutant strain under both mild and moderate osmotic stress (0.4 M or 0.8 M NaCl respectively). As can be seen in Figure 1(A), whilst the *yap4* mutant is barely sensitive to 0.4 M NaCl relative to the wild-type, it shows a moderate sensitivity to 0.8 M NaCl hyperosmolarity. This osmosensitive phenotype appears strain-independent, as the same phenotypes were observed in all the tested strains (see Table 1). It was observed also under non-saline hyperosmotic conditions, using sorbitol, and is suppressed by growth at 37 °C (results not shown). Cells grown to stationary phase and used in spot assays, appear more resistant to moderate hyperosmolarity and do not display an osmosensitive phenotype (results not shown). Indeed, previous studies [21] in which phenotypic analyses had been performed using stationary phase cultures also failed to detect hyperosmotic sensitivity in the *yap4 (hal6)* mutant strain. Phenotypic analyses using *hog1*-deleted strains showed that, in agreement with previous results [37], this mutant shows severe growth impairment even at mild hyperosmolar conditions. The subsequent deletion of *YAP4* in this mutant does not aggravate this phenotype (Figure 1A) whilst analyses of the *yap1*/*yap4* and *msn2*/*yap4* mutant phenotypes do not show sensitivity beyond that observed in the single *yap4* mutant. The *msn2*/*msn4* double

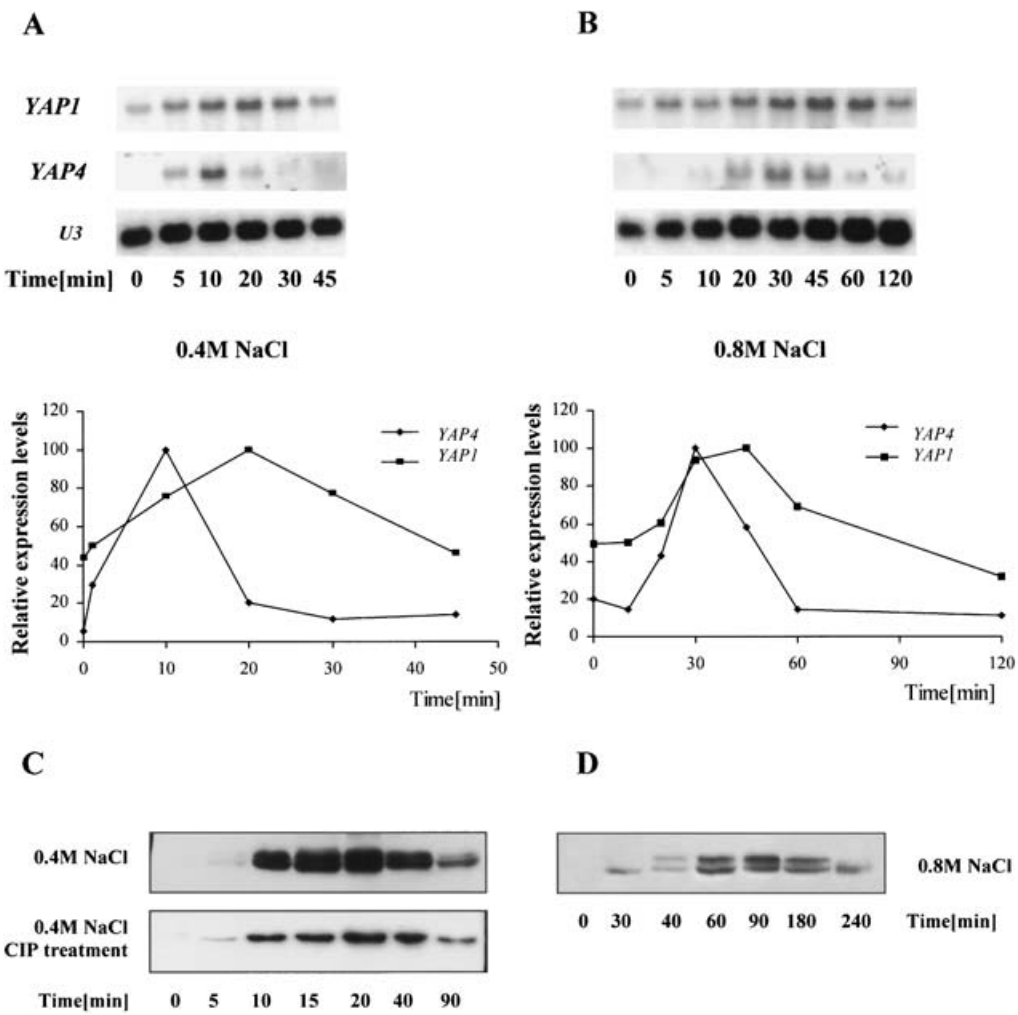


**Figure 2 Induction of *YAP4* and *YAP1* under conditions of (A) mild (0.4 M NaCl) and (B) moderate (0.8 M NaCl) osmotic stress**

Cells upshifted to these conditions were harvested at the indicated time points and the RNA was extracted and blotted (upper panels) as described in the Experimental section. The mRNA levels for *U3*, a small nuclear RNA, were used as an internal loading control against which all mRNA levels were normalized (lower panels). Gene induction reflects an increase in Yap4p protein levels at both mild (**C**) and moderate (**D**) conditions that resolve into a single band after treatment with CIP (**C**, lower panel). *yap4* mutant strains transformed with *YAP4*TAP tag were shifted to the desired concentration of stress-generating agent, and samples harvested at the indicated time points. Protein extraction, separation, transfer and immunoblotting were performed as described in the Experimental section.

mutant, as well as the *msn2* single mutant, does not display an osmosensitive phenotype (Figure 1A and results not shown) under these conditions, which is in accordance with previous studies [38]. Subsequent *YAP4* gene overexpression studies using cells transformed with a multicopy vector containing the cloned *YAP4* gene (see Experimental section) not only suppresses the *yap4* salt-sensitive phenotype (Figure 1B) but also alleviates that of the *hog1* mutant (Figure 1C).

### *YAP4* is induced under conditions of osmotic stress and requires the presence of Hog1p and Msn2p

*YAP4* gene expression analyses were performed in order to address the involvement of this transcription factor in the response to osmotic stress. Figure 2(A) illustrates the *YAP4* induction kinetics after exposure to mild osmotic stress (0.4 M NaCl), showing a rapid and transient peak after exposure for 10 min. Under moderate stress conditions (0.8 M NaCl), although the intensity is comparable with the response to mild stress, there is a delay in the induction of the *YAP4* gene with a peak at 30–60 minutes upon exposure (Figures 2B and 3B), the expression declining thereafter. These data correlate with previous observations [7,36] whereby the expression of several osmo-responsive genes is delayed at increasing osmo-shock conditions and may reflect a distinct adaptive response. In contrast with this transient expression profile is that observed for *YAP1*, whose basal expression can be observed to rise and decline upon exposure to saline stress, possibly reflecting an altered redox state of the cell imposed by an osmo-shock (Figures 2A and 2B).

Having determined the induction pattern of the *YAP4* mRNAs under hyperosmotic conditions, it became relevant to analyse whether this mRNA increase reflects an enhancement in the Yap4p protein levels. Figures 2(C) and 2(D) illustrate the results obtained using the *yap4* strain carrying an episomally expressed TAP-tagged Yap4p. The protein induction kinetics are similar to those observed for its mRNAs. Upon stress induction the appearance of two bands can be observed, which are converted into a single band after treatment with CIP (Figure 2C and results not shown) indicating that Yap4p becomes most probably phosphorylated. Indeed, Yap4p contains several putative phosphorylation sequences that may be required for protein post-activation.

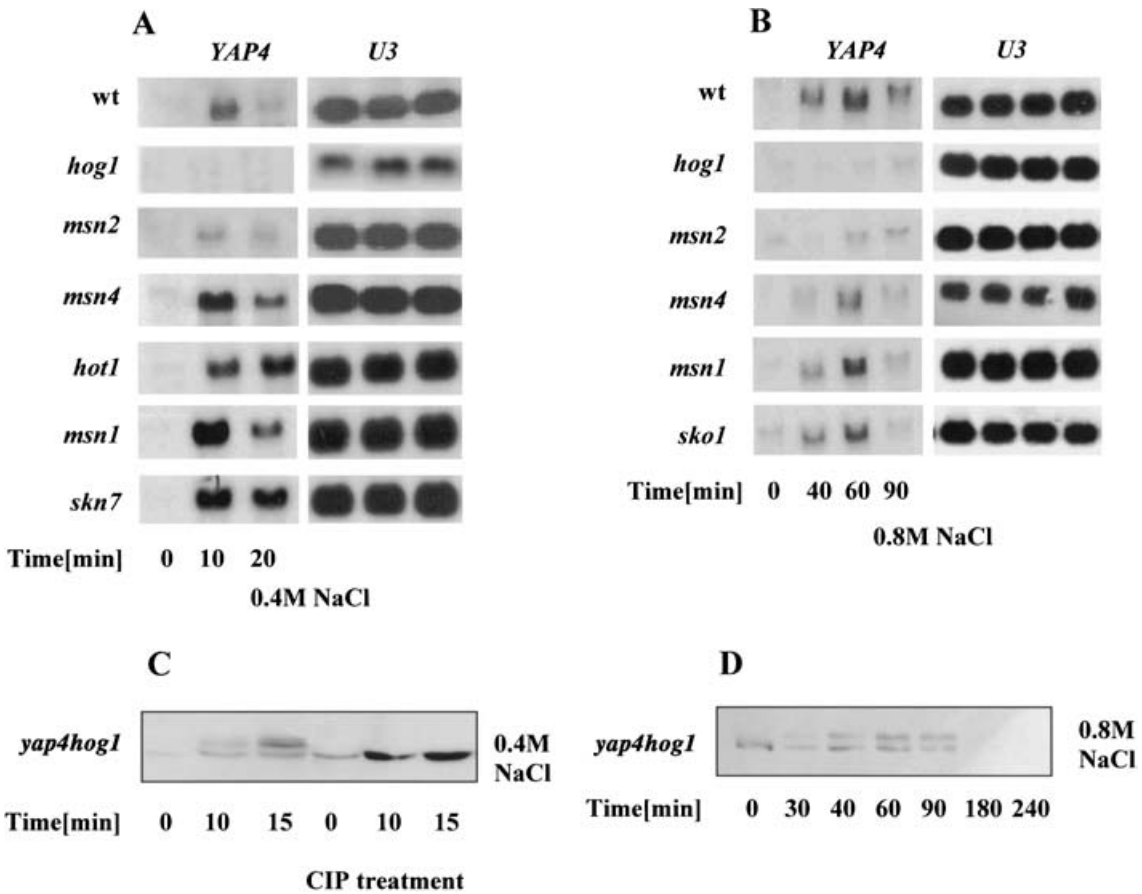


**Figure 3 Epistasis analyses using strains deleted in Hog1p and Hog1-target transcription factors**

Wild-type (wt) and mutant strains (*hog1*, *msn2*, *msn4*, *hot1*, *msn1*, *skn7*, *sko1*) were shifted to (**A**) 0.4 M NaCl or (**B**) 0.8 M NaCl and the extracted RNA was subject to Northern analysis as described above. The mRNA levels for *U3*, a small nuclear RNA, were used as an internal loading control against which all mRNA levels were normalized (**A** and **B**, right-hand panels). Yap4p induction after treatment with 0.4 M NaCl (**C**) or 0.8 M NaCl (**D**) is Hog1p-dependent, but its phosphorylation is not (**C**, CIP treatment lanes).

*S. cerevisiae* responds to osmotic shock by the immediate activation of the Hog1p MAPK cascade [39], a fact that prompted us to determine whether *YAP4* gene expression was affected in a Hog1p-deficient strain. Indeed, as indicated in Figures 3(A) and 3(B), the levels of *YAP4* mRNA are completely abolished in the *hog1* mutant strain, both in mild as well as moderate osmotic-stress conditions. This result is consistent with the observation that Yap4p protein levels are severely compromised in a *hog1* background under the same conditions (Figures 3C and 3D). However, Yap4p phosphorylation appears to be Hog1p-independent, as can be seen by the appearance of the residual double bands in these strains that, as before, resolve into a single band after treatment with CIP (Figure 3C).

Several transcription factors have been identified that act downstream of the HOG kinase pathway including, amongst others, Msn2/4p, Hot1p [8], Msn1p [8], and Sko1p [9,10]. In an attempt to place Yap4p relative to these HOG pathway downstream components, Northern analyses using several mutant strains were performed. Our results indicate that whereas the deletions of *MSN4*, *MSN1*, *SKN7* and *SKO1* genes (Figures 3A and 3B) do not appear to affect *YAP4* mRNA levels, the *hot1* mutant shows a delayed *YAP4* induction peak at 20 min. Most strikingly, however, the *msn2* mutant shows a 75% reduction of these levels under mild osmotic upshift, as determined by densitometric analysis (results not shown) and a complete loss of *YAP4* induction under moderate conditions (Figure 3B). This result suggests that *YAP4* induction requires the presence of Msn2p as well as that of Hog1p.

### *YAP4* gene regulation under osmotic stress is mediated by STRE elements

As a preliminary analysis of the *YAP4* promoter, we first searched the database for putative promoter elements present in the sequences upstream of the start site. Figure 4 is a schematic representation of some of the *YAP4* chromosomal features present upstream of the ATG. This region includes the yet uncharacterized neighbouring ORF (*YOR029w*) of unknown function. In order to evaluate the importance of these *cis*-elements, *YAP4* was cloned, and using site-directed mutagenesis, the two most proximal STREs (−430 bp and −716 bp), as well as the Yap1p response element (YRE; −517 bp) were mutated, both singly and in combination, as shown in the legend to Figure 5(A) (see Experimental section). *YAP4* gene expression was then monitored by Northern analysis. Upon osmo-shock, our results reveal that the single $STRE_{430}$ mutation leads to a reduction of approx. 50% in the abundance of *YAP4* mRNA levels, whereas the mutation in the $STRE_{716}$ mutation shows 70% of the wild-type *YAP4* gene expression. Moreover, the double mutation in these two elements shows a complete abrogation of the mRNA levels. That this is of biological significance is highlighted by our observations that the strain harbouring a mutation in all three *cis*-elements displays

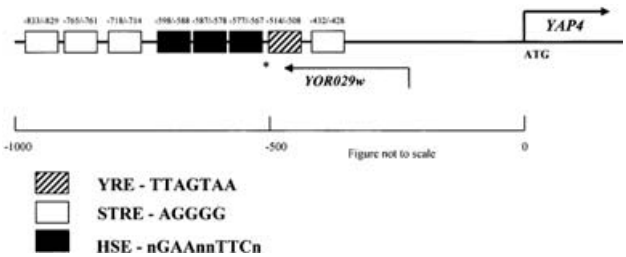


**Figure 4 Schematic representation of the YRE, HSE and STRE elements present in the *YAP4* upstream region**

The ATG start codon is shown. An upstream ORF, *YOR029w*, of as yet unknown function containing part of the *YAP4* promoter region, is also represented. The nucleotide positions of the elements (in relation to the start codon) are shown. The asterisk marks the position of the stop codon for *YOR029w* (the ORF in the complementary strand).

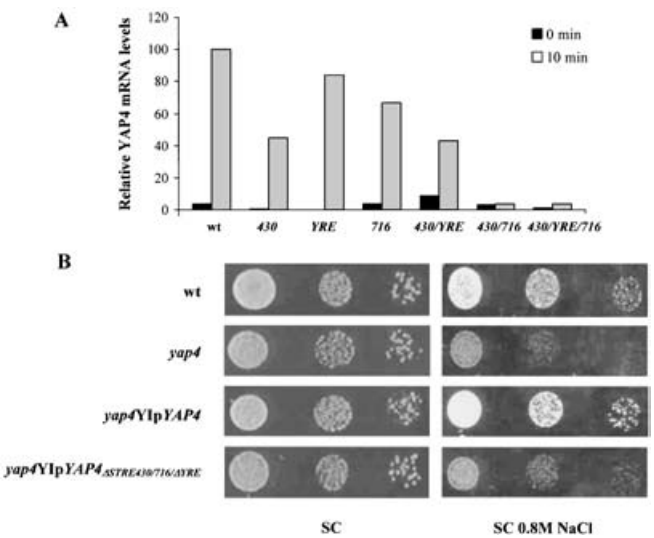


**Figure 5 Regulation of *YAP4* gene is mediated by STREs located in its promoter region**

The *yap4* mutant strain was transformed with an integrative plasmid (described in the Experimental section) containing the *YAP4* gene in which the YRE and/or two most proximal STRE sites have been mutated in the promoter. (**A**) *YAP4* was induced with 0.4 M NaCl and samples taken at 0 min and 10 min, followed by Northern analysis and mRNA quantification by densitometric analysis, as described in the legend to Figure 2. (**B**) Deletion of the two most proximal STREs as well as the YRE induces a salt-sensitive phenotype analogous to that observed in the *yap4* mutant. Cultures were grown as described in the legend to Figure 1 and spotted onto SC plates with or without 0.8 M NaCl. Growth was measured after 2–3 days.

a salt-sensitive phenotype analogous to that observed in the *yap4* mutant under moderate hyperosmolarity (Figure 5B). In contrast, the YRE is not required for the regulation of *YAP4* gene expression under osmotic stress. Indeed, although our results show an induction of *YAP1* (Figures 2A and 2B), the expression of the *YAP4* gene under hyperosmotic stress is unaffected by *YAP1* deletion (results not shown).

### Potential Yap4p involvement in glycerol biosynthesis

In order to gain a better understanding of the role of Yap4p in osmo-regulation, we have, as a parallel and ongoing study, performed DNA microarray analysis in the wild-type and *yap4* strains under mild hyperosmolar conditions for 10 min. Amongst the genes that were found to be regulated by Yap4p (T. Nevitt, J. Pereira and C. Rodrigues-Pousada, unpublished work), two

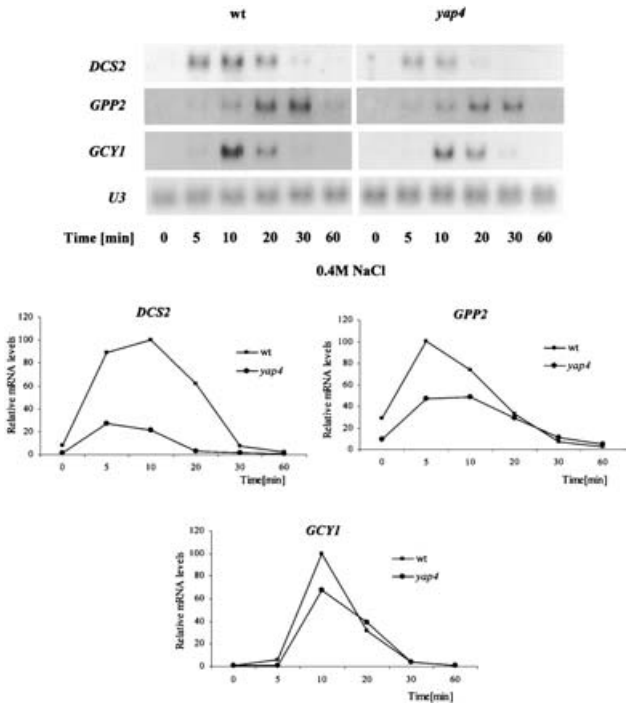


**Figure 6 Expression analysis of *DCS2*, *GPP2* and *GCY1* genes in the wild-type (wt) and *yap4* mutant strains**

mRNA samples obtained from cultures exposed to 0.4 M NaCl (as described in the legend to Figure 2) were taken at the indicated time points and were analysed by Northern analysis. The mRNA levels for *U3*, a small nuclear RNA, were used as an internal loading control against which all mRNA levels were normalized (lower panels).

involved in glycerol biosynthesis were identified, *GCY1*, encoding a putative glycerol dehydrogenase, and *GPP2*, encoding a NAD-dependent glycerol-3-phosphate phosphatase. These genes show decreased induction in the *yap4* mutant strain (Figure 6) with reduction values corresponding to 40% and 50% of the maximum levels respectively. Furthermore, *DCS2*, a gene homologous to the *DCS1*-encoded decapping enzyme, shows 80% depletion in induction levels in the *yap4* mutant upon osmo-shock (Figure 6).

In an attempt to attribute a role for Yap4p in osmotic stress we measured the intracellular glycerol levels in wild-type as well as in the *yap4* and *hog1* mutants after 1.5 h exposure to 0.7 M NaCl. As can be seen in Figure 7, the lack of *YAP4* does not appear to affect the intracellular glycerol content. As previously described [40], the *hog1* cells show a marked reduction of glycerol accumulation under these conditions.

## DISCUSSION

A diverse network of pathways is typically recruited, after various forms of environmental insult, whose co-operative action results in differential modes of regulating gene expression [4]. In this study we show that *YAP4 (CIN5)* is induced under conditions of osmotic stress (Figure 2) requiring for this the presence of Hog1p as well as of Msn2p, and possibly other transcription factors (Figure 3), and may play a potential role in glycerol metabolism through the modulation of *GCY1* and *GPP2* gene expression (Figure 6).

The HOG MAPK cascade is activated upon an increase in osmolarity by a sequential signal-relay mechanism, triggered

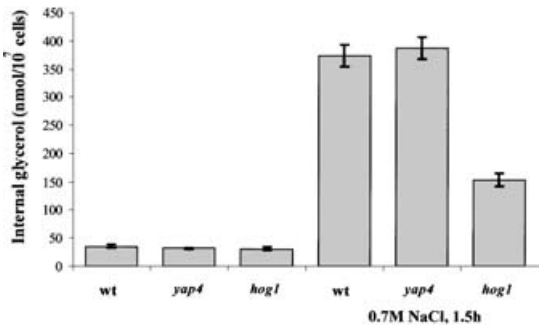


**Figure 7 Internal glycerol accumulation is unaffected by the lack of Yap4p**

Cells were grown to early-exponential phase and then either unchallenged (bars labelled *wt*, *yap4*, *hog1*; left-hand side) or exposed to 0.7 M NaCl (bars labelled *wt*, *yap4*, *hog1*; right-hand side), and samples collected after 1.5 h. Glycerol content was quantified as described in the Experimental section. In contrast with the *hog1* mutant strain, the *yap4* mutant displays intracellular glycerol content analogous to the wild-type (wt) strain.

by two putative plasma-membrane proteins [5], leading to the activation and accumulation of Hog1p in the nucleus where it ultimately modulates the activity of target transcription factors, including Hot1p [8], Sko1p [9,10], Smp1p [41] and Msn2/4p involved in downstream signal amplification. Our findings suggest that, although *YAP4* induction is completely dependent on Hog1p, transcription is activated by Msn2p (Figure 3) acting via at least two STRE elements present in the *YAP4* promoter region (Figure 5). In accordance with previous results obtained under heat shock [42], which determined that almost 85% of STRE-mediated induction is dependent on only Msn2p, we find that Msn4p is not required for *YAP4* induction. Microarray results [6] have shown previously that several genes whose expression is strongly diminished in the *msn2/4* mutant are under the control of the Hog1p MAPK. The fact that the *msn2* mutant shows a 25% *YAP4* induction under mild osmotic conditions (Figure 3A), suggests the involvement of another transcription factor. Since the *YAP4* promoter contains multiple consensus HSEs (−575 bp, −578 bp, and −587 bp from the ATG) (Figure 4), it is possible that this residual induction could be ascribed to the HSF whose co-operative action with Msn2p has been described previously [17,18,43]. Indeed, it was recently reported that multiple transcriptional regulators can be found to bind to many yeast promoters, a phenomenon only previously shown in higher eukaryotes [44]. This phenomenon may also account for the fact that strains deleted for *MSN2*, the key regulator of *YAP4* gene expression, do not reflect the *yap4* null mutant phenotype under moderate salt conditions (Figure 1A). Epistasis analyses, using several strains deleted in other Hog1p target transcription factors, reveal that none is involved in *YAP4* induction (Figure 3) suggesting that Yap4p may be located in a parallel branch governed by Msn2p. Nonetheless, although the *hot1* mutant itself does not display an osmosensitive phenotype under the tested conditions, the fact that an altered *YAP4* transcription kinetics is observed in this strain does not rule out the possibility of cross-talk between the various branches.

Our data also show that Msn2p-mediated regulation of *YAP4* expression under conditions of osmotic stress involves the synergistic contribution of two STRE elements ($STRE_{430}$ and $STRE_{716}$; Figure 5A). That these *cis*-acting elements are physiologically relevant is further supported by the observation that the mutant strain harbouring mutations in all three elements displays an osmosensitive phenotype similar to that detected in the *yap4* null mutant (Figure 5B). The existence of this moderate phenotype, along with the partial recovery of the *hog1* mutant by the overexpression of *YAP4* (Figure 1C), suggests that Yap4p may be involved in osmoprotection by regulating its targets. It has been proposed that *YAP4* overexpression was capable of conferring a salt-tolerance phenotype to *ena1* mutants through a mechanism unrelated to the $Na^+/Li^+$ extrusion ATPase [21]. Nonetheless, the regulation of *ENA1* expression is only one of the many inherent consequences of Hog1p activation and, indeed, other cation extrusion systems have been described, including *NHA1* [45] and *SNQ2* [46], as well as cation influx systems such as *TRK1* [47]. On the other hand, several other unrelated metabolic adjustments must occur upon osmotic shock; glycerol production being an immediate consequence of Hog1p target activation. However, as our results show (Figure 7), the internal glycerol content does not appear to be altered in the *yap4* null strain. Notwithstanding, our results in Figure 6 show that the expression levels of *GCY1* and *GPP2*, two genes involved in glycerol metabolism, are reduced, suggesting that the Yap4p transcription factor may contribute towards its biosynthesis, albeit not sufficiently as to be detected by the method used. *DCS2*, a homologue of the *DCS1*-encoded mRNA decapping enzyme [48], is induced under moderate osmotic stress (Figure 6) and although its role has yet to be defined, it has been shown that its homologue plays a central role in the inhibition of trehalase activity [49]. Analysis of the remaining putative Yap4p target genes may provide clues towards the determination of its functional role as well as the biological significance of the osmosensitivity phenotype associated with its deletion and the observed increased resistance resulting from its overexpression.

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