Deciphering the role of Yap4 phosphorylation under stress conditions

Jorge Pereira

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Instituto de Tecnologia Química e Biológica
Universidade Nova de Lisboa

Supervisor
Professor Claudina Rodrigues-Pousada

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From left to right: Professor Isabel Sá Nogueira, Professor Margarida Casal, Professor Pedro Moradas-Ferreira, Jorge Pereira, Professor Claudina Rodrigues-Pousada, Professor Frédéric Devaux, Professor Cecília Arraiano and Professor Miguel Teixeira.

Members of the Jury

Professor Miguel Teixeira

(Metalloproteins and Bioenergetics, ITQB, President of the Juri)

Professor Frédéric Devaux

(Laboratoire de Génétique Molèculaire, CNRS, France)

Professor Margarida Casal

(Department of Biology, CBMA, Universidade do Minho, Braga)

Professor Pedro Moradas-Ferreira

(Cellular and Applied Microbiology, IBMC, Universidade do Porto)

Professor Cecília Arraiano

(Control of Gene Expression, ITQB, Universidade Nova de Lisboa)

Professor Isabel Sá Nogueira

(Microbial Genetics, ITQB, Universidade Nova de Lisboa)

Professor Claudina Rodrigues-Pousada

(Genomics and Stress, ITQB, Universidade Nova de Lisboa, Supervisor)

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(A copy of these manuscripts can be found at the end of the thesis)

This work is dedicated to:

Idalina

my parents and my sister

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Abstract

The existence of molecular mechanisms of response, repair and adaptation, many of which are greatly conserved across nature, gives to the cell with the plasticity it requires to adjust to its ever-changing environment, a homeostatic event that is termed the stress response. In the budding yeast *Saccharomyces cerevisiae* there is a particular family of transcription factors, the Yap family, which has been shown to have a relevant role in yeast adaptation to several stress conditions. In particular, Yap1 is the major regulator of the transcriptional response to oxidative stress and Yap2 and Yap8 play important roles upon cadmium and arsenic exposure, respectively.

Another Yap member, Yap4, was initially associated to chromosome instability (*CIN5*) and resistance to cisplatin and other antimalarial drugs under overexpression conditions. Later, both *YAP4* and *YAP6* were identified as genes that confer salt tolerance when overexpressed in the *ena1* mutant. Under this context, we have studied *YAP4* regulation and we showed that *YAP4* is responsive to a broad range of stress conditions, including osmotic and oxidative stress, temperature shift and arsenic exposure, among others. This is due to the existence of a promoter region that is very rich in different *cis*-elements, including several Stress Response Elements (STREs), known to be binding sites of the transcription factor Msn2, Heat Shock Elements (HSEs) recognized by Hsf1, as well as a Yap1 Recognition Element (YRE). Upon osmotic stress, Msn2 and Hog1 have been shown to be important for transcriptional regulation of *YAP4*. Under oxidative stress, *YAP4* transcription is regulated by interplay between the transcription factors Yap1 and Msn2.

The results obtained in this work showed that Yap4 is a phosphoprotein highly induced upon the different stress conditions previously shown to increase the transcription of its gene, as well as during stationary phase. This modification was confirmed after alkaline phosphatase treatment that reduces the two phosphorylated Yap4 isoforms with slower mobility shifts to the single, unphosphorylated and fast migrating isoform. The same pattern was obtained in a null Protein Kinase A (PKA) mutant, showing that Yap4 phosphorylation is PKA-dependent. Furthermore, we searched for putative intermediate kinases for this modification having observed that Yap4 phosphorylation is independent of kinases that crosstalk with the PKA pathway, namely Rim15, Sch9 and Yak1. Additionally, Hog1, the MAP kinase of the HOG pathway, which is involved in the transcription regulation of *YAP4*, does not phosphorylate Yap4. Yap4 phosphorylation is also independent of Slt2, the PKC1 MAP kinase and of the kinases Ste20, Ptk2 and Mck1, showed to be able to phosphorylate Yap4 *in vitro*. This protein has several putative phosphorylation sites and S99 and S210 were predicted to be PKA targets *in vivo*. However, our data did not confirm this prediction, showing that only the mutation of residues T192 and S196 impairs Yap4 phosphorylation. The abolishment of phosphorylation in both mutants is independent of the stress conditions applied and did not affect Yap4 nuclear localization or its ability to partially rescue the *hog1* severe osmosensitivity phenotype. Phosphorylation seems, however, to be required for Yap4 stability as the non-phosphorylated form has a shorter half-life compared to the phosphorylated one (12.1 ± 1.1 min for the wt version and 7.9 ± 0.7 min for the non-phosphorylated one). Yap4 localization was also studied and it was observed that removal of the leucine zipper abolishes its constitutively nuclear localization, suggesting that Yap4 dimerization could precede its nuclear import. This mechanism, however, does not involve the importin Pse1, previously shown to be responsible for Yap1 import into the nucleus. Ongoing studies will clarify the mechanisms involved in Yap4 nuclear localization as well as its precise role in the yeast stress response.

Resumo

A existência de mecanismos de resposta, reparação e adaptação, muitos dos quais conservados ao longo da evolução desde a bactéria ao Homem, dotou as células com a flexibilidade necessária para se ajustar ao seu meio ambiente em constante mudança. Estes mecanismos necessários à manutenção da homeostasia interna da célula constituem a resposta ao stress.

A levedura *Saccharomyces cerevisiae* contém uma família particular de factores de transcrição, designada por família Yap, que tem um papel relevante na adaptação da levedura a diferentes condições de stress. Concretamente, o Yap1 é o principal regulador da resposta transcripcional ao stress oxidativo e o Yap2 e Yap8 desempenham papéis importantes na resposta da levedura à exposição ao cádmio e arsénico, respectivamente.

Outro membro da família Yap, o Yap4, foi inicialmente associado à instabilidade cromossomática (sendo designado por *CIN5*) e à resistência à cisplatina e outras drogas contra a malária, em condições de sobre-expressão. Mais tarde, o *YAP4* e o *YAP6* foram identificados como genes que conferiam tolerância ao sal no mutante *ena1*, novamente quando sobre-expressos.

Neste contexto, estudamos a regulação transcripcional do *YAP4* e mostramos que este gene responde a um vasto leque de situações de stress, incluindo o stress osmótico e oxidativo e exposição ao calor e ao arsénico, entre outros. Tal fica a dever-se ao facto de a região promotora do *YAP4* ser rica em diferentes elementos reguladores, incluindo elementos de resposta ao stress (STRE) aos quais o Msn2 se liga, elementos de choque térmico reconhecidos pelo Hsf1 e um elemento de ligação do Yap1 (YRE). Neste sentido, mostrou-se que

após um stress osmótico, os factores Msn2 e Hog1 são necessários para a regulação transcripcional do *YAP4* e no caso do stress oxidativo, esta regulação é efectuada pelos factores Yap1 e Msn2.

Os resultados obtidos neste trabalho mostram que o Yap4 é uma proteína fosforilada, sendo induzida nas diferentes condições de stress que anteriormente mostramos aumentarem a expressão do gene que a codifica, e ainda durante a fase estacionária. Esta modificação pós-traducional foi confirmada após tratamento dos extractos proteicos com fosfatase alcalina que converteu as duas isoformas fosforiladas do Yap4 na isoforma não fosforilada, com uma maior mobilidade electroforética. O mesmo padrão de migração é obtido no mutante para a proteína cinase A (PKA), mostrando que a fosforilação do Yap4 é dependente desta cinase. Além disso, pesquisamos a existência de possíveis cinases relacionadas ou não com a PKA que pudessem ser intermediárias entre a PKA e o Yap4. Verificamos que a fosforilação do Yap4 era independente das cinases Rim15, Sch9 e Yak1. Mostramos também que a cinase Hog1, a "Mytogen-Activated Protein" cinase (MAPK) da via de sinalização HOG de resposta ao choque hiperosmótico, e que anteriormente mostramos regular a expressão do *YAP4*, não fosforila o Yap4. O mesmo sucedeu com a cinase Slt2, a "Mytogen-Activated Protein" (MAPK) cinase da via de sinalização da proteína cinase C envolvida na manutenção da integridade da parede celular. Além disso, foi mostrado *in vitro* que as cinases Ste20, Ptk2 e Mck1 fosforilam o Yap4, mas os nossos resultados *in vivo* não confirmaram aqueles dados. A proteína Yap4 possui múltiplos resíduos potencialmente fosforiláveis, tendo sendo a S99 e a S210 previstas serem fosforilados *in vivo* pelo PKA. Novamente os nossos resultados não confirmaram esta previsão, mostrando que apenas as mutações nos resíduos T192 e S196 anulam a fosforilação do factor Yap4. A ausência de fosforilação nestes dois mutantes é independente do tipo de stress aplicado e não afecta a localização nuclear do Yap4 nem a sua capacidade de, em condições de sobre-expressão, aliviar a severa osmo-sensibilidade do mutante *hog1* em stress osmótico. No entanto, a fosforilação parece ser necessária para a estabilidade do Yap4, pois a sua forma não fosforilada tem uma semi-vida mais curta que a versão nativa (7.9 ± 0.7min para a forma não fosforilada contra 12.1 ± 1.1min para a forma nativa). A localização do Yap4 também foi estudada, tendo-se observado que a remoção do "leucine zipper" impede a localização constitutivamente nuclear do Yap4, sugerindo que a sua dimerização pode ocorrer antes do seu importe nuclear. Porém, este mecanismo de internalização não envolve a importina Pse1, que está envolvida no tráfico nuclear do Yap1. Estudos em curso ajudarão a esclarecer os mecanismos envolvidos na localização nuclear do Yap4, assim como no seu papel na resposta da levedura ao stress.

Thesis outline

The work presented in this thesis is a contribution to the understanding of Yap4 role in the budding yeast *Saccharomyces cerevisiae* under stress response.

In Chapter I we review the state of the art regarding transcription factors, the specific Yap proteins and the mechanisms of stress response and signalling in yeast.

This study started with the characterization of Yap4 expression upon osmotic, oxidative and other forms of stress presented as shown as shown in Chapter II.

Yap4 phosphorylation was investigated and the results are described in Chapter III.

The data obtained on the study of Yap4 localization are presented in Chapter IV.

Finally, in Chapter V the main achievements of this work are discussed as well as the perspectives for further development.

Abbreviations and symbols

^{32}P	isotope 32 of the chemical element phosphorous
AP-1	activator protein 1
ARE	AP-1 recognition element
bp	base pairs
BR	basic region
BSA	bovine serum albumine
bZIP	basic DNA-binding domain and leucine zipper
cAMP	adenosine-3’,5’-cyclic monophosphate
cCRD	C-terminal cysteine rich domain
Ci	Curie
CIP	calf intestinal alkaline phosphatase
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI	4’,6’-diamidino-2-phenylindole, dihydrochloride
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxy-any base-5'-triphosphate
DTT	1,4-dithiothreitol
dTTP	3'-deoxythymidine 5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
ESR	environment stress response
GFP	green fluorescent protein
GSR	general stress response
GTF	general transcription factor
HOG	high osmolarity glycerol
HSE	heat shock element
HSF	heat shock factor
HSP	heat shock protein
KAN	kanamycin
kb	kilo base pairs
kDa	kiloDalton
LB	Luria-Bertani
LZ	leucine zipper
MAP	mitogen activator protein
MAPK	MAP kinase
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
n-CRD	N-terminal cysteine rich domain
NES	nuclear export signal
NLS	nuclear localization signal
nm	nanometers
nt	nucleotides
OD_{600nm}	optical density at 600 nm
ºC	degree Celsius
Oligo	oligonucleotide
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylenoglycol
pH	-log10[H+]
PKA	protein kinase A
PSA	ammonium persulfate
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rotations per minute
RT	room temperature

S. cerevisiae	*Saccharomyces cerevisiae*	STRE	stress responsive element
S. pombe	*Schizosaccharomyces pombe*	TF	transcription factor
SDS	sodium dodecyl sulphate	YRE	Yap response element

Amino acids

A	Ala	alanine	N	Asn	asparagine
B	Asx	asparagine or aspartic acid	P	Pro	proline
C	Cys	cysteine	Q	Gln	glutamine
D	Asp	aspartic acid	R	Arg	arginine
E	Glu	glutamic acid	S	Ser	serine
F	Phe	phenylalanine	T	Thr	threonine
G	Gly	glycine	V	Val	valine
H	His	histidine	W	Trp	tryptophan
I	Ile	isoleucine	Y	Tyr	tyrosine
K	Lys	lysine	Z	Glx	glutamine or glutamate
L	Leu	leucine	X	X	any aa residue listed above
M	Met	methionine			

Nucleotide bases

A	adenine
C	cytosine
G	guanine
T	thymine
N	any base listed above

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I. Introduction

I.1 Transcription factors

I.1.1 Characterization of the different families of Transcription Factors

Gene transcription and its regulation in eukaryotic cells is a highly coordinated process, involving hundreds of proteins organized in different complexes. DNA sequence-specific proteins, known as transcription factors (TFs), are able to decipher the transcriptional regulatory code by binding particular DNA sequences (binding sites known as *cis*-regulatory elements) in the promoter region of the targets genes. This differential occupancy of the promoter regions by the different TFs will favour the assembly and disassembly of the transcription machinery (Levine and Tjian, 2003) and consequently the expression levels of the targets genes.

The activity of the transcription factors can also be regulated by many other mechanisms which act under different conditions producing transcriptional changes of certain genes, and in consequence to differential physiological responses. Some TF are constitutively expressed while others are active only after exposure to a particular stimulus. Additionally, different genes contain different *cis*-regulatory elements and therefore the combination of particular binding sites in a given promoter determines the binding of the transcription factors which will in turn modulate the gene transcription. TFs are also involved in other important cell functions such as the response to environment cues, response to intercellular signals, control of cell cycle and development.

TF can be divided according to their function in three major groups: the General Transcription Factors (GTFs: TFIIA, -B, -D, -E, -F, and –H), the induced sequence-specific TFs and the chromatin remodelling and modification associated TFs, also designated transcription co-factors (Fazzio *et al*., 2001; Erkina *et al*., 2008; Steinfeld *et al.* 2007). The GTFs are basal TFs, acting at the primary level of transcription and they are necessary for the promoter recognition and correct recruitment of RNA polymerase II and other associated regulators. The sequence-specific DNA binding TFs are regulatory TFs that, unlike GTFs, bind to sequences further away from the initiation site and serve to modulate the transcription of the targets genes. TFs expression and activation is also modulated as consequence of different cell challenges. The chromatin remodelling and modification associated TFs are present in complexes that assist the transcriptional apparatus to navigate through chromatin (Levine *et al*., 2003).

From a structural point of view, TFs are modular proteins, possessing a DNA-binding domain (DBD) that will bind directly to the DNA and a trans-activating domain which contain binding sites for other proteins, such as transcription co-regulators, that will allow the recruitment of the basal transcription machinery (Kadonaga, 2004). In addition, TFs can also possess one or more activation or repression domains with a signal sensing motif responsible for the sensing of external signals through the binding of a ligand. In many cases, however, the DBD and the signal sensing domains belong to different proteins that associate within the transcription complex to regulate gene expression (Amoutzias *et al*., 2008).

The ability of TFs to dimerize increase the complexity of the gene transcription control as even a few numbers of TFs that are able to homo or heterodimerize, give rise to many new TF dimers with distinct DNA binding properties. Consequently many novel genetic regulatory networks that will allow a fine-tuning of gene expression are generated. Regarding the heterodimerization, the concentration of each monomer in the cell, the possibility of post-translational modifications, such as phosphorylation, and different binding affinities for other monomers will determine which dimer (hetero or homodimer) will be formed and, consequently, which signalling process will prevail over the others. This is the case of the Myc-Max and Mad-Max heterodimerization system that defines whether a large number of targeted genes will be expressed or silenced. The TFs Jun and ATF2 are another example as they present different binding activities from the Jun or ATF2 homodimers (Amoutzias *et al*., 2008 and references within). The case of Jun and Fos is also paradigmatic as each of them forms homodimers but only the heterodimer is functional (O'Shea *et al*., 1992).

Considering the TFs that are able to dimerize, a very heterogenic group with several motifs that characterize the DBD and define different families was described (reviewed in Amoutzias *et al*., 2008 and references therein). The general structure of the most relevant families of TF that exert their function as dimers is represented in Fig. 1.1.

The bHLH (basic-region helix–loop–helix) family of TFs is the largest family of dimerizing TFs in humans. Many bHLH TFs are important regulators of development, differentiation and cell cycle. These TFs are characterized by an alpha-helical basic region (BR) that binds E-box DNA elements (CANNTG) as a dimer. The helix–loop–helix (HLH) forms a four-helix bundle and is responsible for the dimerization (Amoutzias *et al*., 2004). In other cases, there is an additional dimerization domain (e.g. the PAS, Orange, or leucine zipper (LZ) domains) C-terminal to the

HLH. This second dimerization domain confers higher specificity in the dimerization process.

The bZIP (basic region leucine zipper) is the second-largest family of dimerizing TFs in humans, many of them being the well-studied oncogenes. They can be generically considered as environmental biosensors and controllers of development, regulating development, metabolism, circadian rhythm, learning, memory, and response to stress and radiation (Amoutzias *et al*., 2008 and references within). bZIPs TFs structure is very similar to bHLH TFs, being the alpha-helical basic region responsible for the binding to the DNA. Again, dimerization occurs via the C-terminal coiled-coil LZ (Vinson *et al*., 2006). Fig. 1.2 highlights the structure of a bZIP dimer, focusing the residues that are important for dimerization.

The HD-ZIP (Homeodomain leucine zipper) is a three alpha helices helix–turn–helix DNA-binding domain specific to plants. In this family of TFs, DNA binding is mediated by the homeodomain and the third helix confers DNA-binding specificity. Again, the LZ domain directs dimerization (Tron *et al*., 2004).

In the MADS box family of TFs, the MADS domain directs DNA binding and the domains that mediate dimerization are located C-terminal to the MADS domain. This family is highly represented in plants, being associated to the control of organ development (de Folter *et al*., 2005).

The NF-kB (Nuclear factor-kappa B) family of TFs is involved in the regulation of the immune system, among other processes. These TFs possess a Rel homology domain (RHD), composed by two b-sheet immunoglobulin folds that are connected by a ten amino acid flexible linker. This flexible loop mediates DNA-binding, whereas the C-terminal immunoglobulin region is mainly involved in dimerization (Chen and Ghosh, 1999). NFAT (Nuclear factor of activated T cells) family of TFs is structural very similar to the NF-kB family.

NR (Nuclear receptor) family of TFs are of increasing interest since they are specific for humans and are being studied by the pharmaceutical industry as drug targets for many human diseases. In the NRs, zinc-finger (Zf) domains mediate DNA binding, whereas dimerization is mediated by both the Zf domain and a ligand-binding domain (Germain *et al*., 2006).

STAT (Signal transducers and activators of transcription) family of TFs is involved in cancer and therefore these TFs are being studied as potential targets for different therapeutics. STATs dimerization occurs between Src homology 2 (SH2) domain of one monomer and a region of a phosphorylated tyrosine within the other monomer. In these TFs, DNA binding is mediated by an immunoglobulin fold, as it happens with the NF-kB family (Chen *et al*., 1998).

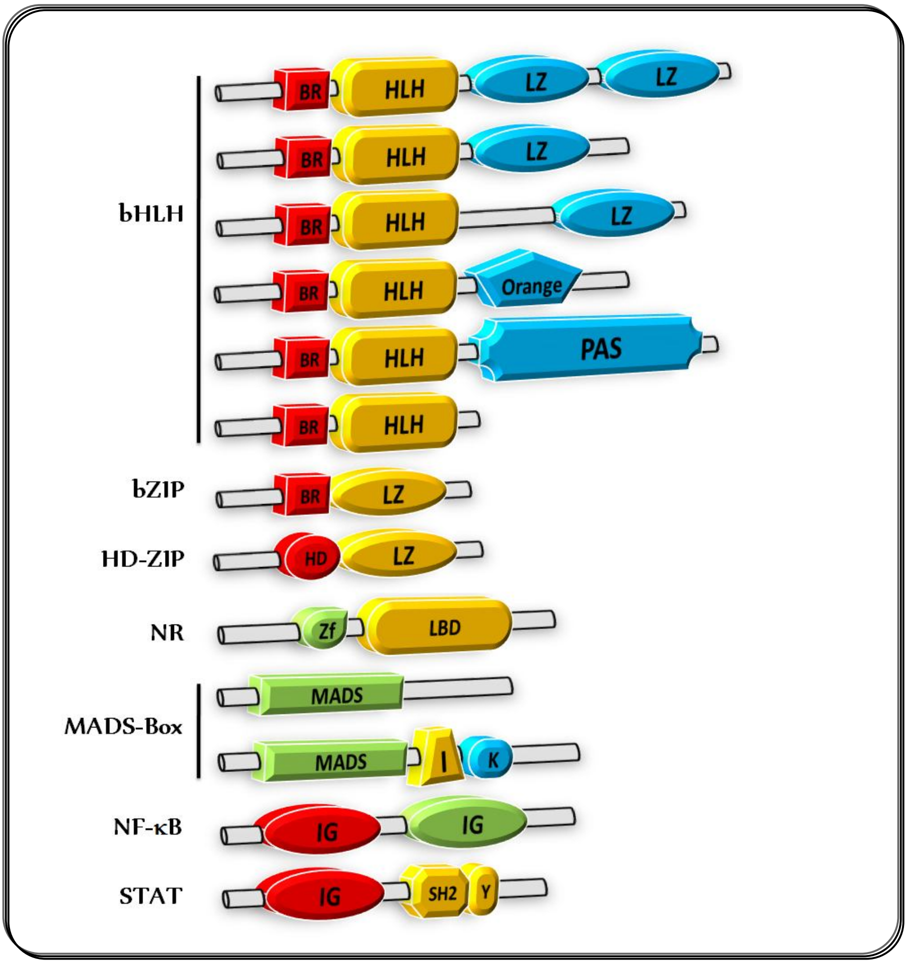


Figure 1.1: DNA-binding and dimerization domains of the major eukaryotic TF families.

For simplicity, only the DNA-binding domains (red boxes), dimerization domains (orange boxes) and additional dimerization domains that confer specificity (blue boxes) are indicated. The elements indicated in the green boxes are domains that have both DNA-binding and dimerization activity. bZIP - basic region leucine zipper TF family, bHLH - basic region helix-loop-helix TF family; BR – basic region; HLH – helix-loop-helix domain; HD – homeodomain, HD-ZIP – homeodomain leucine zipper TF family; I - specific domain of a subfamily of MADS-Box proteins; IG - immunoglobulin; K - specific domain of a subfamily of MADS-Box proteins; LBD - ligand-binding domain; LZ – leucine zipper; MADS - named after the four originally identified members: **M**CM1, **A**GAMOUS, **D**EFICIENS and **S**RF1; NR – nuclear receptor TF family; NF-κB – nuclear factor-κ B; Orange - additional dimerization domain f the bHLH domain family ; PAS - named after three proteins in which it occurs: **P**er, **A**rnt and **S**im, SH2 - Src homology 2 , STAT - Signal transducers and activators of transcription TF family, Y – dimerization domain of the STAT family of TF; Zf – zinc finger domain (adapted from Amoutzias *et al.*, 2008).

I.1.2 AP-1 transcription factors and the Yap family

bZIP-TFs are particular TFs as their main structure contains a DBD bZIP that was deduced from its primary amino acid sequence and comprises a basic region (BR) and a leucine zipper (LZ). These TF dimerize through their leucine repeats and form a coiled coil. This consists of two or more α-helices that wind around one another with a slight left-handed superhelical twist. A characteristic heptad repeat $(abcdefg)_n$ defines the placement of residues in each helix relative to the interaction interface (reviewed in Fong *et al.,* 2004). Usually, residues **a** and **d** are hydrophobic amino acids, while the exposed residues **g** and **e** are charged and polar amino acids (Fig. 2). Adjacent to the LZ dimerization domain is the highly conserved BR that contacts specific hexamers of DNA bases. bZIP were named according to the fact that the core d positions are usually leucine (or leucine-like) residues (as can be seen in Fig. 1.3).

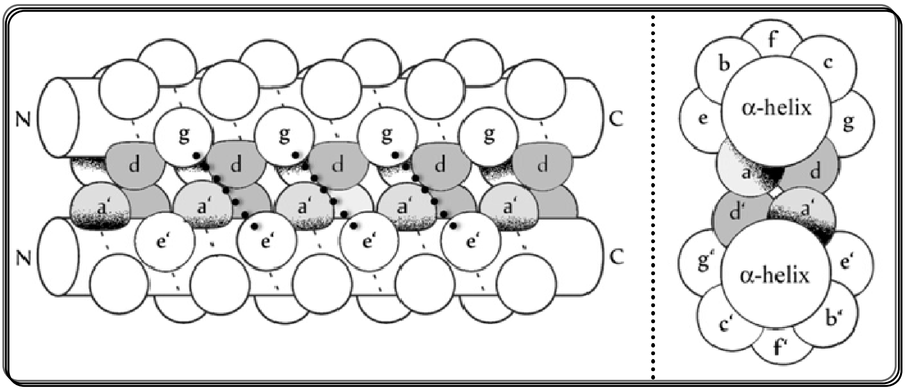


Figure 1.2: Scheme of a leucine zipper (LZ) parallel two-stranded coiled coil and side view.

In the side view (right) are indicated the relative positions of the seven residues that constitute each heptat of a LZ. At the left are highlighted the residues that constitute the interface between the two α-helices (**a, d, e** and **g).** The potential electrostatic interactions between the **g** position of one helix and the following **e′** position of the opposite helix (g····e′) are indicated by the solid dots. The prime notation was used to distinguish the residues in the two α-helices. N – Amino terminus; C – Carboxyl terminus (adapted from de Fong *et al.*, 2004; Vinson *et al.*, 2006).

Whilst this apparent simplification of bZIP sequence that allows their easy identification and prediction of binding affinities, structure and energetics of their hydrophobic interfaces through molecular modelling, bZIPs TFs exhibit a high degree of partnering selectivity. This could explain why they are able to participate in the regulation of different pathways (Fong *et al.,* 2004). This selectivity is reflected in the DNA binding sites that bZIPS are able to recognize, usually short palindromic or pseudo-palindromic target sequences. The metazoan bZIPs can recognize 6 different consensus DNA-binding sites (the TPA responsive element (TRE), AMP responsive element (CRE), CAAT box, AF recognition element (MARE), CRE-like, and PAR binding sites), while yeast bZIPs are able to bind only three consensus DNA-binding sites (the TRE-, CRE-, and YAP-binding sites) (Deppmann *et al*., 2006).

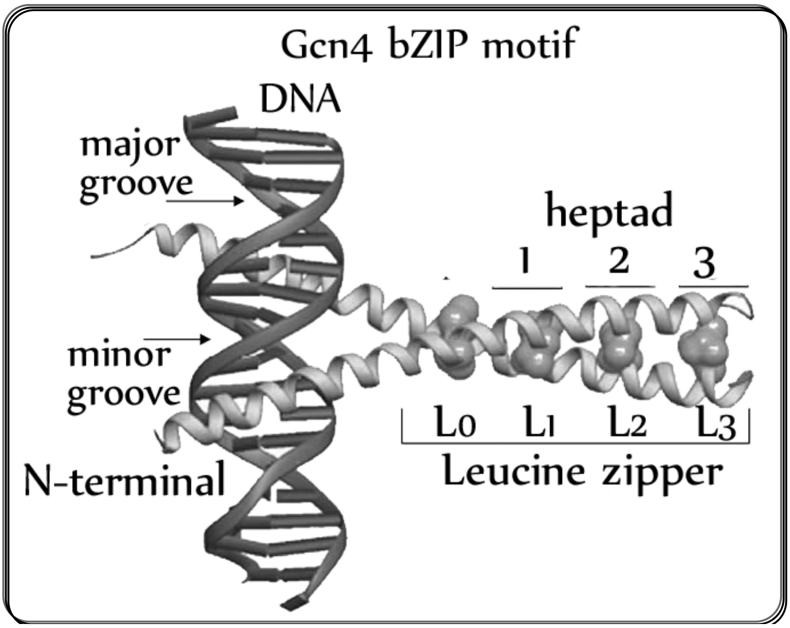


Figure 1.3: Yeast transcription factor Gcn4-bZIP binding to DNA.

The characteristic leucine residues defining the leucine zipper (LZ) as well as the heptads are indicated as L0, L1, L2 and L3 and heptad 1, 2 and 3, respectively. The N-terminal coiled-coil sequences binds to the DNA through the major (one α-helix) and minor grove (the other α-helix). Example adapted from Vinson *et al*., 2005.

There is a particular group of bZIP TFs, known as AP-1 TFs, that are able to recognize the ARE (AP-1 Responsive Element, also known as TPA-responsive element- TRE) TGACTCA nucleotide sequence, later designated as AP-1 site. AP-1 TFs are involved in several important cellular processes in mammalians, like cell proliferation, apoptosis, development and stress response, among others (Toone and Jones, 1999). The prototype AP-1 TF in the yeast *Saccharomyces cerevisiae* is Gcn4 (Fig. 3). This TF primarily regulates yeast response to amino acid starvation (Natarajan *et al.*, 2001; Hinnebusch, 2005) and is very similar to mammalian AP-1 factors Jun and Fos oncoproteins. The three TFs share the same DNA-binding specificity (Struhl, 1987) and they are able to perform the same functions in yeast and mammalian cells (Struhl, 1988).

Yap1, the first member of the family of Yaps to be described in yeast, was initially identified by its ability to bind and activate the SV-40 AP-1 recognition element (ARE: TGACTAA) (Rodrigues-Pousada *et al.*, 2004). Yap2 was isolated in a screening conferring resistance to 1,10-phenanthroline in transformed cells overexpressing a yeast library and also binds ARE *cis*-acting element. The sequencing of the *YAP1* and *YAP2* genes revealed the presence of a b-ZIP-family domain in the N-terminus homologous to the true budding yeast AP-1 factor Gcn4p and to c-Jun, its mammalian counterpart (Bossier *et al.*, 1993). Later, with the conclusion of the yeast genome sequencing project, it was possible to identify the remaining 6 Yap members (from Yap3 to Yap8). The Yap family members differ from Gcn4 because they possess different amino acid residues in the BR of the bZIP domain. They contain a glutamine at the position corresponding to Ala239 in Gcn4 and a phenylalanine at the position corresponding to Ser242 in Gcn4 (except two cases, in which the substitution is for a tyrosine residue, Fig. 4). Moreover, all

Yap members also contain a glutamine at position 234 and an alanine at position 241 and these two modifications are not found in any other bZIP proteins (reviewed in Rodrigues-Pousada *et al*., 2004).

This extended family of specific AP-1 factors is implicated in various forms of stress response.

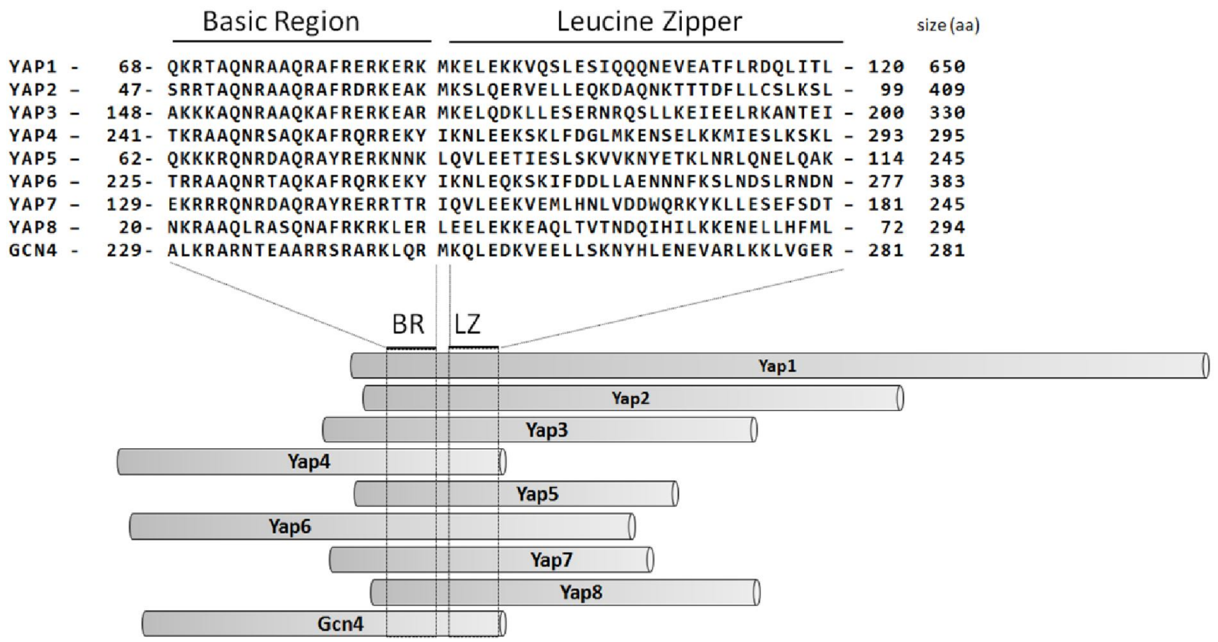


Figure 1.4: Alignment and comparison of GCN4 basic region and Leucine Zipper with the YAP family members. Conserved residues are highlighted in the text. BR – Basic Region, LZ – Leucine Zipper.

I.2 Role of the Yap factors in the stress response.

I.2.1 Yap1 and Yap2

Yap1 is the major regulator of the oxidative stress response. Initial studies showed that *yap1* mutants were hypersensitive to the oxidants H_2O_2 and *t*-BOOH, and to chemicals that generate superoxide anions, such as menadione, plumbagine, methylviologen, cadmium, methylglyoxal and cycloheximide (reviewed in Rodrigues-Pousada *et al*., 2005). Using a genome-wide approach, Gasch *et al*. (2000) and Causton *et al*. (2001) demonstrated that following exposure to an oxidative insult, 24% of the genome of *S. cerevisiae* was fast and transiently modulated including several genes specifically involved in the antioxidant response as well as components of the thiol redox pathways, the heat shock proteins, drug transporters and enzymes involved in carbohydrate metabolism. *TRX2* and *GSH1* are two of these genes that were also the first two targets of Yap1 to be described as involved in the oxidative stress response to H_2O_2, diamide and t-BOOH (Kuge *et al*., 1997; Wu & Moye-Rowley 1994). Following preliminary work, more Yap1 targets related to ROS detoxification were identified. These include target genes of the thioredoxin and glutathione systems, and other antioxidants such as catalase and superoxide dismutase. Yap1 plays therefore a crucial role in regulating the adaptive response to oxidative stress induced both by H_2O_2 and chemical oxidants inducing reactive oxygen species (ROS), such as redox cycling chemicals, thiol oxidants and alkylating agents, metals, cadmium and arsenic compounds that produce oxidative stress, as side effects (Menezes *et al*., 2008).

Although little attention is given to the transcriptional regulation of Yap1, the steady-state population of their encoded mRNA levels increased upon exposure to an oxidative stimulus. However, the major regulation resides in its subcellular localization. Indeed, Kuge *et al*. (1997) showed that Yap1 nuclear retention was mediated by one of the two the cysteine-rich domains (CRD) of the protein, the one located at the C-terminus upon oxidative stress imposed by diamide and diethyl maleate. Delaunay *et al*. (2000 and 2002) and Kuge *et al*. (2001) have examined with detail the mechanism of Yap1 retention in the nucleus. Yap1 possesses a nuclear export signal (NES) that interacts with the nuclear exportin Crm1 allowing its export out of the nucleus under normal conditions. Yap1 possesses two cysteine-rich domains (one N-terminal domain (n-CRD) containing cysteines C303, C310 and C315, and one C-terminal domain (c-CRD) containing cysteines, C598, C620 and C629), and upon exposure to H_2O_2, C303 forms a disulfide bond with C598 that mask Yap1 NES preventing Yap1 interaction with the exportin Crm1 and thus Yap1 is retained in the nucleus where it can trigger the activation of its target genes. This mechanism involves two others proteins that interact with Yap1, Ybp1 (Yap1-binding protein 1) and Orp1/Gpx3 (Oxidative receptor Peroxidase 1). Orp1 acts as a sensor of oxidative stress and not as a peroxidase, reacting with H_2O_2. The oxidized Orp1 initiates a thiol-based redox relay system that culminates with Yap1 activation through its nuclear retention. This process involves the formation of an intermolecular disulfide bond between Orp1 C36 and Yap1 C598, which is then converted into the Yap1 intramolecular C303-C598 disulfide bond (Fourquet *et al*., 2008). Veal *et al*. (2003) showed that Ybp1 is crucial for the oxidation of cysteine residues of Yap1, mediating the disulfide bond between Orp1 and Yap1. Wood *et al*. (2003) have revealed that an additional intramolecular disulfide bond is formed between the n-CRD C310 and the c-CRD C629 upon exposure to H_2O_2. More recently, Okazaki *et al.*, (2007) showed that, in fact, there is a multistep disulfide bond formation in Yap1 involving the cysteines form both nCRD and cCRD domains upon exposure to oxidative stress imposed by H_2O_2.

Under oxidative stress induced by diamide and other thiol-reactive agents, Yap1 c-CRD is sufficient to mediate stress response, which suggests that Yap1 uses different redox centers to deal with different stress conditions. The mechanism of response to oxidative stress induced by diamide and other thiol-reactive agents does not required Ybp1 or the peroxidase Orp1, possibly involving a direct binding of these oxidative stress agents to the c-CRD cysteines C598, C620 and C629. (For review see Rodrigues-Pousada *et al.*, 2005). In fact, this mechanism was already shown in yeast exposed to the oxidative stress induced by N-ethylmaleimide and menadione (Azevedo *et al.*, 2003). These results suggested the presence of at least two different redox centers in Yap1: one activated upon oxidative stress imposed by H_2O_2 through intramolecular disulfide bonds (C303-C598 and C310-C629) and another upon exposure to thiol-reactive agents that bind to the cCRD cysteines (C598, C620 and C629).

Yap2, also known as Cad1, was the second member of the Yap family to be identified. As already described, Yap2 was isolated by Bossier and collaborators (1993) by its ability to bind ARE *cis*-acting element and confer resistance to 1,10-phenanthroline. Yap2 was also found to confer resistance to cerulenin and cycloheximide (Hirata *et al.*, 1994), and cadmium (Wu *et al.*, 1993), suggesting a role in the response to drug stress.

Cell exposure to cadmium stress induces the expression of *YAP2* (Fernandes *et al*., 1997) and the encoded protein is translocated to the nucleus through a Crm1-dependent mechanism, activating the transcription of its target gene *FRM2* (Azevedo *et al*., 2007). This gene encodes a protein homologous to nitroreductase, but is not clear what it is its role in metal stress response. Yap2 shares a high amino acid sequence homology with Yap1, namely in its bZIP and C-terminal CRD, conserving all three cysteine residues of Yap1 c-CRD and the hydrophobic residues that compose the NES. Taking into account this homology, Azevedo *et al*. (2007) performed the c-CRD swapping between Yap1 and Yap2, showing that the Yap2 containing Yap1 c-CRD was induced by cadmium but not by H_2O_2. Furthermore it suppresses *yap1* cadmium sensitivity but not *yap1* hydrogen peroxide sensitivity. These results suggest that the differential regulation of the oxidative stress response to H_2O_2 and cadmium is associated to the carboxyl-terminal domain of Yap1p and Yap2p, respectively. Similar to Yap1, Yap2 conserved c-CRD cysteine residues 356, 387 and 391, but not C378, are required for cadmium stress sensing (Azevedo *et al*., 2007). Unlike Yap1, Yap2 does not respond to H_2O_2. This difference could be related to the fact that Yap2 does not have conserved cysteine residues in its n-CRD, preventing the formation of the intramolecular disulfide bond necessary to mask the NES. Consequently, Yap2 is exported to the cytoplasm through the interaction with the exportin Crm1 and does not activate the transcription of any oxidative stress regulated gene.

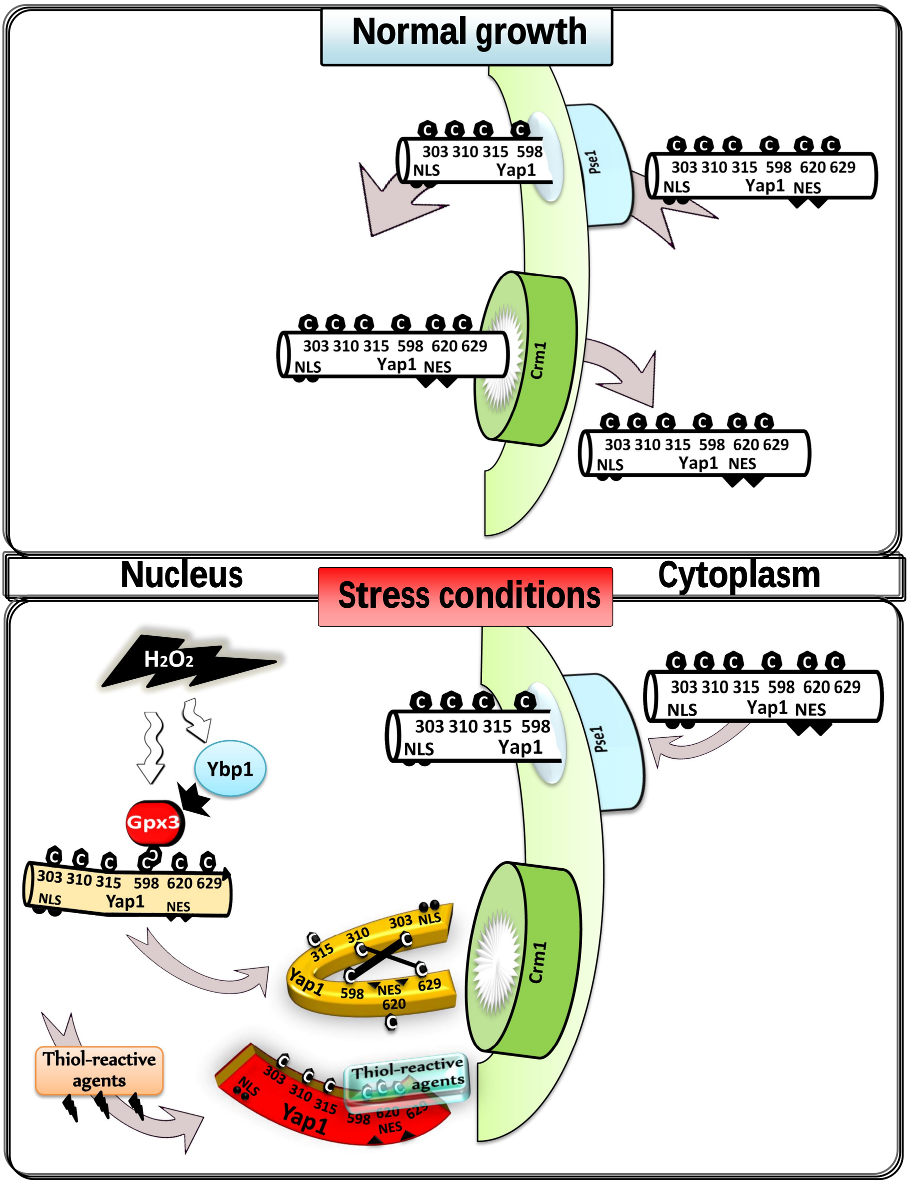


Figure 1.5: Overview of Yap1 regulation.

Under normal growth conditions, Yap1 circulates between nucleus and cytoplasm through, respectively, the interaction between its NLS and the importin Pse1 and its NES and exportin Crm1. However, exposure to oxidant conditions (H_2O_2) modifies Yap1 structure through intramolecular disulfide bond formations between its redox centers that mask the NES and blocks Yap1 interaction with Crm1, thus accumulating Yap1 in the nucleus. Additionally, Yap1 can be retained in the nucleus by thiol-reactive agents that form adducts with the C-terminal cysteines and mask the NES, again preventing its interaction with Crm1.

I.2.2 Yap8

YAP8 is the most divergent member of the Yap family. It is the main regulator of the arsenic detoxification pathway, forming a cluster (known as arsenic compounds-resistance - ACR) with the genes that encode the arsenate-reductase Acr2 and the plasma membrane arsenite efflux protein Acr3 (Wysocki *et al*., 1997). In response to arsenate exposure, Yap8 is activated and induces the expression of *ACR2* and *ACR3* (reviewed in Rodrigues-Pousada *et al*., 2004). Arsenic compounds can be unspecifically internalized by yeast cells in two forms: arsenate (As^{V}), through phosphate transporters as Pho87, and arsenite (As^{III}), through the aquaglyceporin Fps1 or the hexose permeases Hxt1, Hxt3, Hxt4, Hxt5, Hxt7 and Hxt9. The mechanism of detoxification of arsenate involves the reduction of arsenate to arsenite by the arsenate-reductase Acr2 followed by the extrusion of the arsenite compounds out of the cell mediated by the Acr3 plasma membrane arsenite efflux protein. A secondary pathway of arsenic detoxification involves GSH conjugation of arsenite [$As(GS)_3$] followed by its accumulation in the vacuole mediated by the pump Ycf1 ABC ATPase (reviewed in Bhattacharjee and Rosen, 2007).

Yap8 is constitutively expressed, being its activity mainly regulated at the level of its protein subcellular localization. Upon exposure to arsenic, Yap8 rapidly accumulates in the nucleus, and this translocation is mediated by the exportin Crm1, as it happens with the related Yap1 (Menezes *et al*., 2004). In fact, the regulation of Yap8 nuclear accumulation is triggered by the loss of interaction with Crm1. Menezes *et al.* (2004) have shown that Yap8 cysteine residues 132, 137 and 274 are essential for its subcellular localization and also for its transactivation potential.

It is not known yet if this modification involves direct binding of the arsenite to the referred cysteines as it happens with the binding of thiol-reactive agents to the c-CRD cysteines of Yap1 (Azevedo *et al*., 2003). It was thought that Yap1 and Yap8 could exert overlapping functions in the arsenic stress response as *YCF1* induction is dependent on Yap1, as well as the *ACR* genes that are also partially regulated by Yap1 (Menezes *et al*., 2004). Moreover, *yap1 yap8* double mutant is more sensitive to arsenic conditions than any of the single mutant (Menezes *et al*., 2004). However, in a recent work, Menezes *et al*. (2008) clarified Yap1 contribution to arsenic stress responses, showing that Yap1 is necessary for the prevention of oxidative damage in cells exposed to arsenic compounds, namely through the removal of ROS generated.

I.2.3 Yap3, Yap5 and Yap7

Yap3, Yap5 and Yap7 are the least characterized transcription factors of the Yap family. Yap3 shows virtually no response at the level of genomic microarray analyses to the multiple forms of environmental insults and cellular stress studied so far. However, Yap3 has a strong transactivation potential even greater than the one of Yap1 and binds the consensus YRE, TTAC/GTAA (Fernandes *et al.,* 1997). Moreover *yap3* mutant strain exhibits a high content of glycogen, which suggests that Yap3 could be related to the metabolism control (Wilson *et al*., 2002).

Yap5 has been recently shown to be strongly induced under nitrogen depletion and during stationary phase, but also amino acid starvation and diauxic phases (Ball *et al*., 2000). Using chromatin immunoprecipitation (ChIP) and genomic microarray hybridizations, Horak *et al*. (2002) found that *YAP5* is a target of the SBF (Swi4–Swi6 cell cycle box binding) transcription factor and downstream SBF, Yap5 has several putative gene targets (237 in total) involved in different mechanisms, such as DNA replication and monitoring of the fidelity of replication, DNA damage response (*RAD4*) and chromatin remodelling (*SIR4*), energy generation and amino acid metabolism (12 targets genes), as well as gamma-glutamyl kinase *PRO1* and the cyclin-dependent kinase Pho85. Moreover, Yap5 binds many promoters adjacent to genes with peak transcript levels in G1 phase and related to this, targets *CDC47*, that encodes one of a six protein complex (MCM) required for cell cycle progression and DNA replication initiation and elongation (Tye, 1999). Mollapour *et al*. (2004) also showed that *yap5* mutant is more resistant to sorbate exposure.

More importantly, Yap5 was shown to be the transcription factor that regulates iron metabolism, namely its vacuolar storage (Li *et al*., 2008). Yap5 was shown to regulate the transcription of *CCC1* (the major facilitator of iron import into the vacuole, which is the most important site of iron storage in fungi and plants). Moreover, this iron storage is stimulated by iron exposure through a Yap consensus site in the *CCC1* promoter. Yap5 is constitutively localized in the nucleus and occupies the *CCC1* promoter independent of the iron concentration, being the *yap5* mutant strain sensitive to high iron concentrations (Li *et al*., 2008). Curiously, as Yap1, Yap2 and Yap8, Yap5 also contains two cysteine-rich domains and the mutation of cysteines in each of the domains affects the transcription of *CCC1* but not Yap5 ability to bind to DNA. An additional remark that points towards the formation of disulfide bonds is the fact that iron affects the sulfhydryl status of Yap5. However, Yap5 is, as already referred, constitutively nuclear and therefore its putative disulfide bonds are not involved in the regulation of its export as in the case of Yap1, Yap2 and Yap8.

YAP7 is up-regulated under conditions of nitrogen depletion and stationary phase (Gasch *et al.,* 2000) but its function is far from being understood. Our recent transcription profiles using the double mutant *yap1 yap8* and the respective single mutants against the wild type, all treated with arsenate, reveals that Yap7 seems to be dependent on Yap1.

I.2.4 Yap4 and Yap6

Yap4 and Yap6 are the closest related Yap family members. Yap4 is a 33kDa protein sharing almost 33% identity (Rodrigues-Pousada *et al*., 2004) with Yap6 and, unlike other Yap members, as Yap1, Yap2 and Yap8, they are constitutively located in the nucleus (Furuchi *et al*., 2001). In addition, both Yap4 and Yap6 proteins were associated to resistance to several drugs, including antimalarial drugs, chloroquine, quinine and mefloquine (Delling *et al.,* 1998), and the chemotherapeutic agent, *cis*-platinum (Furuchi *et al*., 2001), when overexpressed. Similar results were obtained in the *ena1* mutant that is more tolerant to salt when *YAP4* and *YAP6* are overexpressed. This involves a mechanism unrelated to the Na^+/Li^+ extrusion ATPase (Mendizabal *et al*., 1998). The mutant strains, however, have a divergent behaviour, at least under osmotic stress. While *yap6* mutant does not present any significant sensitivity, deletion of *YAP4* impairs growth at moderate concentrations of hyperosmolarity (Nevitt *et al*., 2004a). Furthermore, *hog1* severe osmosensitivity can be partially recovered by *YAP4* overexpression, showing that Yap4 plays a role in the yeast response to osmotic stress.

In fact, under osmotic stress, *YAP4* Msn2-mediated induction occurs in a Hog1-dependent manner through at least two STREs present in its promoter region (Nevitt *et al.*, 2004a). *YAP4* is also responsive to oxidative stress and in this case its regulation is dependent on Yap1 and Msn2 via its YRE and most proximal STRE, respectively (Nevitt *et al.*, 2004b). In both cases, the protein is transiently expressed and phosphorylated.

Genome wide approaches revealed that besides osmotic and oxidative stress, *YAP4* and *YAP6* are induced under other stress conditions, including heat and stationary phase (Gasch *et al.,* 2000; Rep *et al.,* 2000; Posas *et al.,* 2000). This result correlates well with the fact that both genes possess promoter regions rich in different *cis*-elements (Fig. 1.6), suggesting that both *YAP4* and *YAP6* respond to multiple signalling pathways.

Different computational interactome data predicts the interaction between Yap4 and Yap6, such as the one obtained by Lee *et al*. (2002). However, the possibility that this interaction occurs via heterodimerization remains to be demonstrated. Data from Deppmann *et al*. (2006) do not contemplate this mechanism but instead they propose that Yap4, Yap6, and Yap8 are neither predicted to form homodimers nor have potential partner proteins in the Yap family with which they would heterodimerize. Taking together, the data on Yap4 and Yap6 suggest that they may have a broad and general role in the yeast stress response.

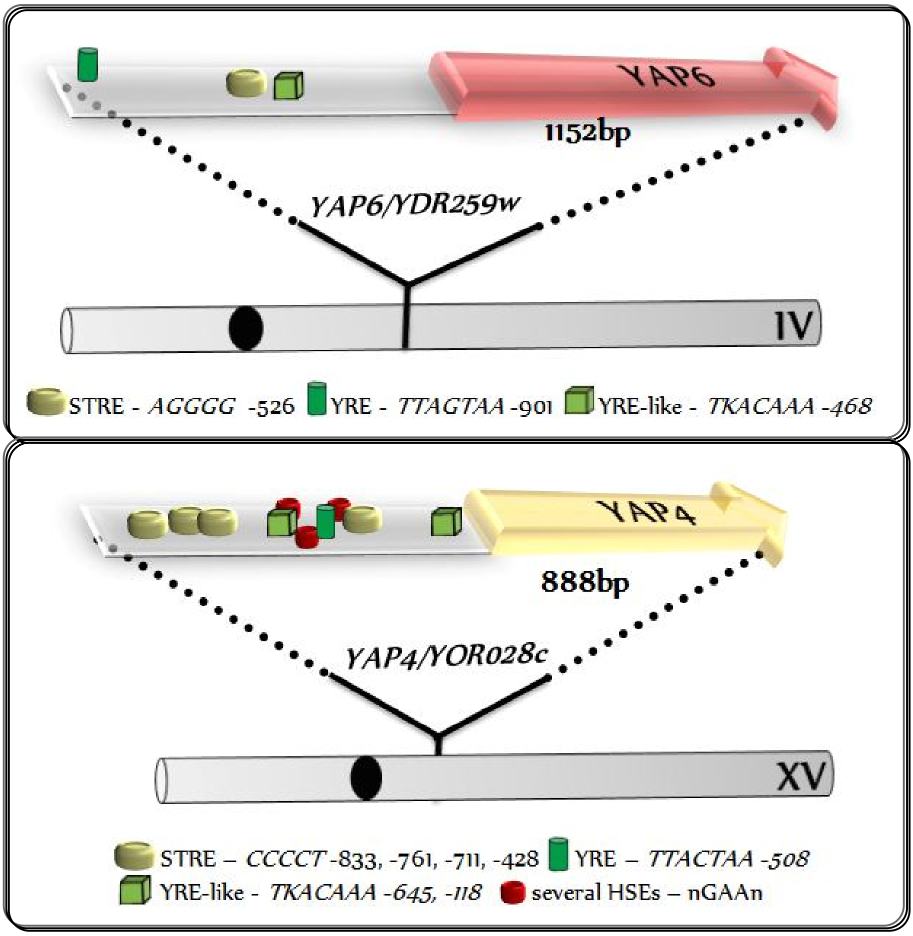


Figure 1.6: Overview of *YAP4* and *YAP6 cis*-elements.

The relative position of the *cis*-elements of *YAP4* and *YAP6* are indicated in the figure. For simplification, only three *YAP4* HSEs are indicated, although it possesses multiple nGAAn sequences in its promoter. STRE – stress-response element, YRE – Yap1 response element, HSE – heat shock element.

I.3 The Environment Stress Response

Yeast cells are continuously exposed to environmental cues that affect its viability, such as drastic changes in osmolarity, temperature, pH, nutrients and oxygen availability. In order to survive, yeasts develop several mechanisms to cope with these stress conditions. Altogether, these mechanisms can be considered as part of the yeast Environmental Stress Response (ESR). Msn2/4 and Hsf1 (General Stress Response), PKA (cAMP pathway), Hog1 (HOG pathway) and Pkc1 (protein kinase C-mediated MAP kinase pathway) are the key players of some of the regulatory pathways in *Saccharomyces cerevisiae* as represented in Fig. 1.7.

The basic mechanisms to respond to stress involves sensor proteins, usually transmembranar proteins associated with the yeast cell wall, that are able to sense the stress condition and initiate a cascade of activation of downstream effectors enabling cells to cope with the stress effects. These mechanisms are specific for the nature of the stress cells sense and many of them are conserved along eukaryotes, although some overlapping between different signalling pathways may occur. The main reason for this overlap is the fact that different stress conditions can produce the same effects in the cell (for instance, UV exposure and heat can both produce oxidative stress and will involve some common players in the yeast response).

The ESR elicits a very broad gene expression regulation program that affects almost 900 genes, two thirds of them being repressed and the remaining induced (Gasch *et al.,* 2000; Causton *et al.,* 2001). The majority of the repressed genes has already been characterized and is mainly involved in RNA metabolism and protein synthesis (Gasch *et al.,* 2002). In contrast, less than half of the induced genes have been yet functionally characterized and are associated with several cellular processes, including carbohydrate metabolism, defence from oxidative stress, cell wall modification, protein folding and degradation, cytoskeletal reorganization, DNA repair, fatty acid metabolism, metabolite transport, vacuolar and mitochondrial functions, autophagy and intracellular signalling (review in Gash, 2002).

The analysis of the gene expression profiles due to ESR reveals a very transient and graded activation of the stress response, being the magnitude of the fold change in gene expression directly proportional to the intensity of the stress encountered by the cell (Gasch *et al.,* 2000; Jelinsky *et al.,* 2000). This transient induction of ESR can probably function as an acclimation phase, allowing yeast cells to readjust their gene expression programme and be more resistant to future and otherwise lethal stress conditions. It was already widely reported that yeast cells exposure to mild stresses make them more resistant to higher doses of the same or a different stress and in most of these cases, the cross-stress protection is mediated by Msn2 and Msn4 (Berry and Gasch, 2008 and reference therein). In this way, ESR enables an acquired stress resistance to yeast cells which is very important for their survival under more aggressive conditions. This hypothesis is supported by the fact that upon an acute stress only a very small part of the ESR induced genes (less than 3%) are required for cell survival to that stress condition (Gasch, 2002). In fact, Berry and Gasch (2008) showed that mutant strains for the ESR key transcription factors Msn2 and Msn4 present defects in the acquired stress resistance but no observed sensitivity to a single dose of an acute stress. This is in fact a good example of the cross-protection elicited by ESR. Upon osmotic stress, the Sko1–Cyc8–Tup1 repressor complex is converted into an activator, inducing several different genes to protect cell from osmotic damage (Proft and Struhl, 2002). Among these genes is *MSN2*/4 encoding one of the key players of the GSR, which in turn promotes the protection to several others stress conditions, such as heat shock, oxidative stress or carbon source starvation (Proft *et al*. 2005). Berry *et al.* (2008) were able to show that Msn2 and Msn4 have much more specific roles in ESR than previously thought, being *MSN4* induced in response to a variety of stresses, while *MSN2* is preferentially induced upon osmotic challenge. Moreover, both factors display different phosphorylation profiles in response to different conditions (Garreau *et al*., 2000), although it is not known what are the consequences of such differences.

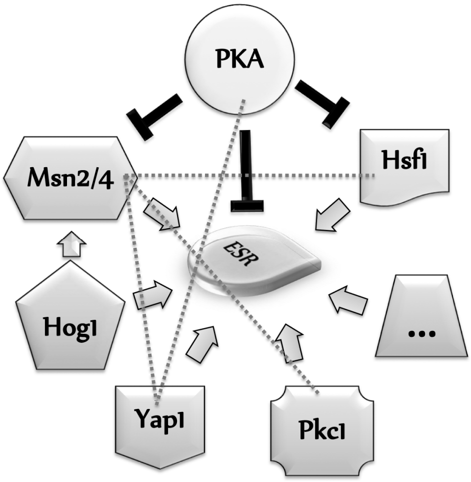


Figure 1.7: Overview of the Environment Stress Response (ESR) with the major players and pathways in which they are involved.

The yeast ESR includes several players and pathways that are induced upon different stress conditions, endowing yeast cells with a cross-protection important for their growth and survival in unfavorable environments and growth conditions. Here are represented some of the most representative players of the different pathways involved in the ESR and the regulations that are established between them. Msn2/4 is the key player of the General Stress Response and together with Hsf1 regulate the heta shock response, Hog1 is the MAPK of the HOG pathway and Pkc1 the MAPK kinase kinase of the PKC1 pathway. (...) indicate other players not considered here that can also be part of ESR.

I.3.1 The General Stress Response

The specific cross-protection elicited by Msn2 and Msn4 in the ESR can be particularly considered as the General Stress Response (GSR) and involves a subset of around 200 genes whose expression is specifically altered in under the Control of Msn2/4 (Gasch *et al*. 2000; Causton *et al*., 2001). The regulation of these genes by Msn2/4 is achieved through its binding to the *cis*-element CCCT, designated as Stress Responsive Element (STRE) (Martinez-Pastor *et al*., 1996). However, there are some genes involved in the GSR that contain this *cis*-element, as the sodium pump *ENA1*, but that are unresponsive to Msn2/4 (Alepuz *et al.,* 1997), suggesting the existence of an alternative pathway of regulation for these cases.

The Msn2/4 factors are regulated at several levels of post-translational control, since its mRNA and turnover remain stable through short periods of stress (De Wever *et al*., 2005). These forms of regulation include Mns2/4 subcellular localization and highly correlated state of phosphorylation, stability and degradation and eventually interactions with regulatory partners. Concerning the regulation of their localization, both proteins contain NES and NLS and are continuously shuttling between nucleus and cytoplasm through an efficient and oscillatory nuclear transport system that is able to discriminate the intensity of the stress insults (Jacquet *et al*., 2003) and is modulated by carbon source availability. In unstressed cells, Msn2/4 localizes predominantly in the cytoplasm. However, upon exposure to stress, Msn2/4 rapidly relocalize into the nucleus, promoting the transcription of its targets genes (Gorner *et al*., 2002 and Jacquet *et al*., 2003). This nuclear localization is prevented by a PKA-dependent hyperphosphorylation of Msn2 NLS, establishing a negative control of the PKA pathway over the GSR. As such, under normal growing cells, PKA activity is high and Msn2 is maintained in the cytoplasm (Gorner *et al*., 1998 and 2002; Garmendia-Torres *et al*., 2007). However, under stress conditions, PKA activity decreases and consequently the level of phosphorylation of Msn2/4 diminished, retaining the transcription factor in the nucleus. Msn2 possesses four PKA-consensus sequences within the NLS and one within the NES, including the serine residues S582, S620, S625, S633 and S288 respectively, that were shown to be phosphorylated under different stress conditions (Gorner *et al.,* 2002 and De Wever *et al.,* 2005). These residues are intercalated by basic arginines in both signals and most probably serine phosphorylation affects the recognition of Msn2 NLS by the karyopherins involved in its translocation, therefore regulating Msn2 distribution and activity. This model is supported by different observations. Firstly, inactivation of PKA leads to the permanent localization of Msn2 within the nucleus (Gorner *et al*., 2002; Jacquet *et al*., 2003). Secondly, Msn2 nuclear import is favoured by the dephosphorylation of the serine residues in the NLS (De Wever *et al.,* 2005). Finally, replacement of Msn2 NES (which contains the serine residue S288) by the protein kinase inhibitor (PKI) NES compromises Msn2 export from the nucleus (Garmendia-Torres *et al*., 2007). This also suggests that, unlike importins that have higher affinity for the nonphosphorylated form of the Msn2, the exportin would rather preferentially interact with the NES-phosphorylated form. Therefore one form of termination of Msn2 activity would be its nuclear phosphorylation and consequent export to the cytoplasm, where it is hyperphosphorylated in a PKA-dependent manner, preventing its cyclic internalization.

Msn2 import into the nucleus seems to involve the importins Kap121 (Pse1) and Kap123 as its internalization is prevented in the double mutant *kap121kap123* (Garmendia-Torres *et al*., 2007), while its export depends on the nuclear exportin Msn5 (Gorner *et al.,* 2002).

The NLS PKA-consensus sequences containing the phosphorylated Msn2 serine residue S582 is also consensus phosphorylation by the Snf1 kinase, the ortholog of the mammalian AMP-activated protein kinase in *Saccharomyces cerevisiae*. In fact, Snf1 directly phosphorylates this residue upon prolonged glucose depletion (De Wever *et al*., 2005). So, whilst PKA controls Msn2 activity in glucose-growing cells, phosphorylating at least the residues S582 and S620, Snf1 would be required to regulate Msn2 activity in the absence of glucose by phosphorylating the residue S582, allowing posterior PKA-dependent phosphorylation of S620 by and the consequent adaptation of the cells to glucose starvation (Mayordomo *et al*., 2002; De Wever *et al*., 2005). Snf1 is part of a complex that is essential to many different cell functions, including regulation of the transcriptional changes associated with glucose derepression; phosphorylation of histone H3; direct regulation of RNA polymerase II holoenzyme; regulation of translation, glycogen biosynthesis, and lipid biosynthesis; and regulation of general stress responses, response to salt stress and response to heat stress (Kuchin *et al*., 2000; Lo *et al*., 2001; Alepuz *et al*., 1997; Ashe *et al*., 2000; Bertram *et al*., 2002). Therefore, Msn2 downregulation by Snf1 phosphorylation would allow yeast cells to bypass growth arrest and hypothetically to coordinate the ESR with the different cells function in which Snf1 is involved.

There are evidences that Msn2 regulation is much further fine-tuned. Srb10 kinase-induced phosphorylation of Msn2 might contribute to increase of its export (Chi *et al*., 2001) and several other groups present data that suggest a link between higher degradation rates of

Msn2 in the nucleus and cell adaptation (Durchschlag *et al.,* 2004; Lallet *et al.,* 2004; Bose *et al.,* 2005). There are also different phosphatases that are able to dephosphorylate Msn2, playing an important role in its regulation, antagonising PKA-dependent phosphorylation. De Wever and collaborators (2005) showed that upon a sudden glucose depletion in the medium, Msn2 activation was driven by a fast but transient decrease in phosphorylation of several residues in the NLS and this decrease in Msn2 phosphorylation state was mediated by the PP1 protein phosphatase (encoded by *GLC7*), making it a potential mediator of glucose starvation signals that target Msn2. Besides, Mayordomo *et al*. (2002) showed that in the *reg1* mutants (Reg1 is the regulatory subunit of the Reg1/Glc7 protein phosphatase complex), the regulation of Msn2 distribution was lost and the TF was constitutively present in the cytosol. Moreover, they showed that this behaviour was associated to the presence of an abnormal active Snf1 protein kinase that inhibits the nuclear localization of Msn2 upon carbon starvation.

Santhanam *et al*. (2004) also showed that protein phosphatase 2A (PP2A) was essential to regulate Msn2 activity under stress conditions, including heat and osmotic shock, as well as nitrogen (but not glucose) starvation, inhibiting the nuclear export of Msn2. Whi2 (negative regulator of G1 cyclin expression) and its binding partner, Psr1-phosphatase seem also to regulate Msn2 as they both are required for a full activation of the general stress response, possibly through the dephosphorylation of Msn2 as they were shown to co-precipitate with the TF (Kaida *et al*., 2002).

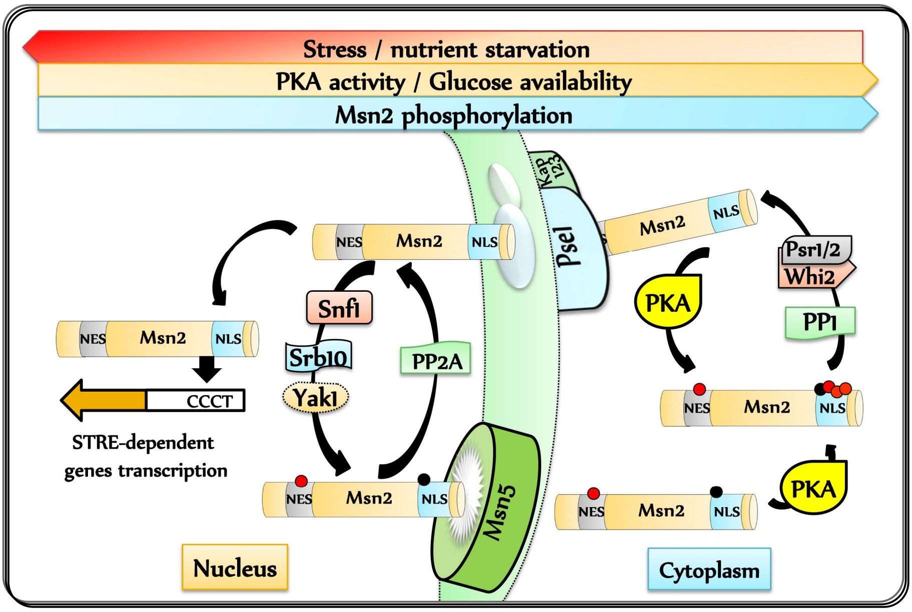


Figure 1.8: Overview of Msn2 activity via phosphorylation dependent sub-cellular localization.

The control of Msn2 activity is achieved mainly through the regulation of its nuclear localization, which is prevented by hyperphosphorylation of its NLS. This modification is driven by PKA, but in particular conditions, Msn2 phosphorylation can be dependent on other kinases, such as Snf1, Srb10 or Yak1 (see text above). Nuclear (PP2A) and cytoplasmic phosphatases (complex Whi2-Psr1/2 and PP1 (Glc7) dephosphorylate Msn2, inhibiting its export or promoting its import, respectively. There are other mechanisms of control of Msn2 activity that were not considered here, such as DNA binding affinity (Hirata *et al*., 2003), transactivation (Boy-Marcotte *et al*., 2006) and degradation of nuclear Msn2 (Lallet *et al*., 2004 and 2006).●● = Msn2 phosphorylated residues. The black residue (S582) can be differentially phosphorylated by Snf1 or PKA (see text).

I.3.2 The Heat shock response: Hsf1 and Msn2/4

The response to heat shock involves a fast induction of a conserved group of heat shock proteins (HSPs). In *S. cerevisiae*, this response is mediated by the heat shock transcription factor (Hsf1) and Msn2/4. Hsf1 is an essential and modular protein highly conserved from yeast to humans, consisting of a central core with a winged helix-turn-helix class of DNA-binding domain, a leucine zipper domain, containing two hydrophobic oligomerization repeats required for homo-trimerization, and a C-terminal trans-activation domain (CAD) (Fig. 1.9).

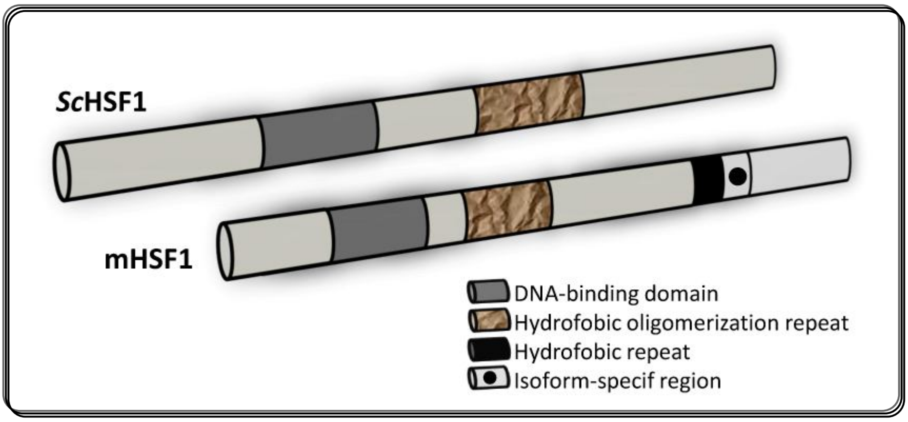


Figure 1.9: Comparison of the generic structure of Hsf1 in yeast (*Sc*) and mammalian (m) (adapted from Pirkkala *et al*., 2001).

HSFs bind a *cis*-element formed by multiple inverted repeats of nGAAn, designated as heat shock element (HSE), in the promoters of its target genes, activating their transcription. Sakurai and Takemori (2007) identified the HSEs in 59 of 62 target genes of *Saccharomyces cerevisiae* Hsf1. They verified that the Hsf1 protein recognizes continuous and discontinuous repeats of the nGAAn and the existence of some divergence in the functional HSEs. Moreover, work from Sakurai group (Hashikawa *et al*., 2007) showed that sequences of four or more HSEs are bound cooperatively by two HSF trimers. However, there are major differences in the number and regulation of HSFs from yeasts to higher eukaryotes. Concerning to the number of HSFs, while the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* have only one essential HSF, the nonvertebrate metazoans such as the fruit fly *Drosophila melanogaster* have a single HSF that is essential for several important cellular functions, such as oogenesis, early larval development and survival in response to severe stress challenges, but is dispensable for growth and viability under nonstressful conditions in adult flies (Pirkkala *et al*., 2001 and references therein). Vertebrate metazoan chickens and mammals, however, express four related HSF genes with different functions: Hsf1, Hsf2 and Hsf4 are present in mammalian cells while Hsf3 is an avian-specific factor (Voellmy, 2004). Finally, *Arabidopsis thaliana* has twenty one HSF genes tightly regulated into a network of interacting proteins (Nover *et al*., 2001; Baniwal *et al*., 2004). It is thought that plants' diversity of HSF can be related to the constant and often severe environmental aggressions, these immotile organisms have to cope with.

Regarding HSF regulation from yeasts to high metazoan, the major difference in the conserved Hsf1 regulation is related to its active trimerization form. In fact, while *S. cerevisiae* is able to discriminate different stress conditions and activate the expression of genes required to each response through a single and essential Hsf1, the three heat shock factors present in metazoan cells are activated only under specific developmental or environmental conditions. In the first case, Hsf1 forms a trimer complex and binds DNA constitutively in the HSEs (Pirkkala *et al*., 2001 and references therein), even in the absence of any heat shock, leading in some cases to the moderate expression of heat shock genes during normal growth conditions (Liu *et al.,* 1999). This implies that Hsf1 DNA-binding ability and transactivation competence are regulated independently (Voellmy, 2004) and there must be additional stimulus upon heat shock in order to activate transcription. The signal triggering the transcription of the target genes is most probably the stress-induced hyperphosphorylation of Hsf1. The evidence for this assumption became clear from a study with *Saccharomyces cerevisiae* cells expressing an oligomerization-defective

Hsf1. In this case, Hsf1 was unable to bind a specific subset of target genes containing three HSEs units and was not extensively phosphorylated in response to stress, being unable to activate genes containing this type of HSE (Hashikawa *et al*., 2007). These results also suggest that oligomerization is a prerequisite for stress-induced hyperphosphorylation of Hsf1.

There is another feature that was not evolutionary conserved and contributes for the differential regulation of the Hsf1 from yeasts to higher eukaryotes. Whereas the metazoan HSF has only one C-terminus trans-activation domain (CAD), HSFs from *Saccharomyces cerevisiae* and *Kluveromyces lactis* share an additional N-terminal trans-activation domain (NAD) (Trott and Morano, 2003 and references therein), that, together with the CAD, are thought to mediate temporal aspects of the heat shock response (Chen *et al*., 2002). The heat shock response can be divided into transient and sustained response, characterized by the increase in temperature over 35°C for less than 1h and by a prolonged time of cell growing under higher temperatures, respectively. The Hsf1 NAD (first 65 amino acids) is thought to mediate the transient response, whereas the CAD (between residues 595 and 783) is thought to be responsible for the sustained response to stress. Moreover, each trans-activation domain seems to regulate the expression of specific genes. Hsf1 CAD is critical for the heat-induced expression of *CUP1*, *HSP82*, and *HSP26*, whereas its loss has no effect on the heat-induced expression of *SSA1*, *SSA3*, and *HSP104* (Pirkkala *et al*., 2001 and references therein).

Higher eukaryotic Hsf1 is in turn, a cytoplasmic inactive monomer, being translocated to the nucleus only upon a heat shock, where it trimerizes, binds to HSEs and becomes inducibly phosphorylated and subsequently acquires transcriptional activity (reviewed in Holmberg *et al.,* 2002). It is thought that under non-stress conditions, Hsf1 from higher eukaryotic cells is maintained in its monomeric and inactive form through the binding of Hsps (heat shock proteins). Upon activation, Hsf1 trimer can be inactivated by binding of inhibitors as the heat shock factor binding protein 1 (Hsbp1). Hsbp1 interacts with the oligomerization domain of an active Hsf1, thereby negatively affecting HSF1 DNA binding activity (Satyal *et al*., 1998). The carboxyl-terminal heptad repeat (HR-C) should be involved in the suppression of Hsf1 trimerization as this domain is well conserved among the vertebrate HSFs but poorly conserved in plants and *S. cerevisiae* HSFs, what could cause the constitutive trimerization of HSF in *S. cerevisiae*. Mammalian Hsf1 regulation is further regulated at multiple levels, involving the oligomeric status of Hsf1 and its DNA-binding ability, phosphorylation in different serine residues (regulating Hsf1 transcriptional activity both positively and negatively) and sumoylation, transcriptional competence, nuclear localization or interactions with regulatory cofactors or other transcription factors (reviewed in Holmberg *et al.*, 2002; Voellmy, 2004).

Hsf1 function is not restricted to the heat shock response. It was already shown that, among other effects, Hsf1 also mediates protection to the heavy metals copper and cadmium, as well as to oxidative stress, through the activation of the *CUP1* gene (Silar *et al.,* 1991; Sewell *et al.,* 1995; Liu and Thiele, 1996). Hsf1 seems to have a role also under glucose starvation conditions, mediated by the kinase Snf1. Hahn and Thiele (2004) showed that Snf1 interacts with Hsf1 *in vivo* and was necessary for glucose starvation-induced Hsf1 phosphorylation. Moreover, Snf1 was required for a widespread activation of Hsf1 target gene expression and for the maintenance of Hsf1 binding to target gene chromatin *in vivo*. However, it is not yet known if Hsf1 is a direct target of Snf1.

The HSF is also thought to be controlled by the PKA pathway. Different evidences place Hsf1 downstream of PKA and suggest that PKA might be involved in negative regulation of Hsf1 activity (Yamamoto *et al*., 2007). However, it seems that only a subset of HSE-containing genes regulated by Hsf1 is negatively regulated by PKA, as for example the genes involved in the Hsf1-dependent response to treatment with menadione, diamide, KO_2, or 1-chloro-2,4-dinitrobenzene (Ferguson *et al*., 2005 and Yamamoto *et al*., 2007). Additionally, Hsf1 does not seem to be a direct target of PKA, as deletion of PKA causes an increase in Hsf1 phosphorylation (Ferguson *et al*., 2005).

The second regulatory system of the heat shock response, Msn2 and Msn4, is not evolutionary conserved between yeasts and humans. These transcription factors only exist in yeast and are found in the promoters of most HSPs (Estruch, 2000). There is therefore some degree of crosstalk between the two systems in order to precisely regulate the yeast response to heat and other stress response in which Hsf1 and Msn2/4 are involved. In this way, there are genes that are regulated by only one of these systems, while others, such as *HSP26* and *HSP104*, are regulated by cooperation between both Msn2/4 and Hsf1 (Amoros and Estruch, 2001; Grably *et al*., 2002). Several genome-wide analysis performed by different groups revealed that in a generic way, Msn2/4 controls the expression of chaperones and genes involved in carbon metabolism and oxidative stress, whereas Hsf1 regulates the expression of many other chaperones and genes related to cell wall maintenance and energy regeneration (Hahn *et al.,* 2004; Yamamoto *et al*., 2005; Eastmond and Nelson, 2006). In a more detailed view, Erkine *et al*. (2008) describes that Hsf1 is actively involved in chromatin remodelling events at gene promoters, while the role of the Msn2/4 system in these processes is poorly understood. Related to this the data from Yamamoto *et al*. (2008) suggest that Msn2/4 play a role before the exposure to high temperatures since, unlike Hsf1, they were not involved in the upregulation of genes necessary for the recovery period following severe heat shock and also were dispensable for cell growth during that period. Therefore Hsf1 and Msn2/4 would act differentially before and after exposure to extreme temperatures to ensure cell survival and growth.

As Hsf1 and Msn2/4 are both regulating the heat shock response, although in different aspects of yeast adaptation, we would expect the existence of common regulators able to control their activity. That is the case of the multi-functional E3 ubiquitin ligase Rsp5. In a recent report, Haitani and Takagi (2008) showed that Rsp5 is involved in the repair system of stress-induced abnormal proteins and upon environmental stresses, primarily regulates the expression of Hsf1 and Msn2/4 at the post-transcriptional level, mediating their mRNA nuclear export.

The crosstalk between different TF regulating the heat shock response is not only observed for Hsf1 and Msn2/4. A report from Raitt and collaborators (2000) shows that in some cases, transcriptional regulation of the genes involved in the heat shock response involves interplay between Hsf1 and Skn7.

I.3.3 The PKA pathway

PKA (Protein Kinase A) pathway is essential for growth and antagonizes induction of the general stress response, being also involved in many other cellular functions such as glycolysis and gluconeogenesis, aging, bud site selection, actin repolarization, sporulation and pseudohyphal differentiation (reviewed in Santagelo, 2006). This is achieved mainly through the control of the phosphorylation state of transcription activators and repressors, kinases and metabolic enzymes (Thevelein and De Winde 1999). The importance of PKA pathway for cell survival is reflected in the fact that cells deficient in PKA activity stop grow, arresting in G1 and have an increased resistance to heat stress. In contrast, cells harbouring a constitutively activated PKA fail to arrest in G1, are defective for trehalose and glycogen accumulation, rapidly lose viability, and remain highly sensitive to heat stress upon nutrient starvation (Reinders *et al.*, 1998). PKA, the effector kinase of this pathway, is thus responsible for coupling the nutrients availability with the stress conditions, allowing rapid growth of glucose-growing cells but simultaneously rendering them sensitive to environmental stresses to prevent cell damage (Griffioen and Thevelein, 2002).

PKA regulation is quite complex, involving several aspects as localization of its subunits, phosphorylation, cAMP levels, among others, and is far beyond the scope of this report. Here I will give a simple overview of some PKA regulation features.

PKA consists of three functionally partly redundant catalytic subunits, Tpk1, Tpk2, and Tpk3, and, unlike cells from multicellular organisms, only one regulatory subunit, Bcy1 (Griffioen *et al.*, 2001). In the inactive form, PKA is a tetramer composed by two regulatory subunits and two catalytic subunits. PKA activation is triggered mainly by the cAMP levels, although it can be also activated independently of cAMP (Thevelein, 2008). External signals, such as a glucose shift, activate the adenyl cyclase that leads to the increase in the levels of cAMP. These molecules bind the Bcy1 regulatory subunits of PKA, causing the dissociation of the PKA inactive complex. Once released, the PKA catalytic monomers become active and phosphorylate their target proteins (Zurrita-Martinez and Cardenas, 2005) (Fig. 1.10). PKA activity is further controlled through the localization of the catalytic Tpk1 and the regulatory Bcy1. Griffioen *et al*. (2000) showed that localization of Bcy1 is very dynamic and responsive to changes in the environment, being carbon source dependent. In turn, Tpk1 subcellular distribution is dependent on cAMP levels. In this way, in exponentially glucose growing cells, PKA activity is higher in the cytoplasm because Bcy1 localizes almost exclusively in the nucleus, inactivating PKA catalytic subunits. The rise in cAMP levels leads to the activation of PKA by dissociation of the regulatory Bcy1 subunits from the catalytic Tpk1 subunits. A significant part of Tpk1 is quickly translocated to the cytoplasm, while Bcy1 subunits are maintained in the nucleus. However, in stationary cells or cells growing on a nonfermentable carbon source, both Bcy1 and Tpk1 are widespread in the cell. In addition, the same research group (Griffioen *et al*., 2001) showed that Yak1-dependent phosphorylation of Bcy1 in its N-terminal domain maintains Bcy1 in the cytoplasm. Regulation of the PKA activity through the glucose-sensing Yak1 is not restricted to the phosphorylation of Bcy1. Its activity as a growth antagonist, tuning PKA has been long time suggested by Smith *et al*. (1998), showing that Msn2/4 is necessary for the proper expression of *YAK1*. Recent data from Lee *et al*. (2008) helps to clarify this feedback mechanism. According to these authors, Yak1 may be the effector of the PKA control over the GSR, as it is able to activate both Hsf1 and Msn2 through phosphorylation when PKA activity is lowered by glucose depletion. An additional level of control of this mechanism was suggested by Moriya *et al*. (2001) that provide evidences that glucose levels may modulate the intracellular localization of Yak1.

The crosstalk between PKA pathway and GSR involving the players PKA, Msn2 and Yak1 allows to explain the viability of a strain lacking all the three catalytic subunits of PKA that is restored only with Msn2/4 deletion or in a less degree by Yak1 deletion (Smith *et al*., 1998). Fig. 1.10 provides a simplified overview of the described PKA regulation and players involved.

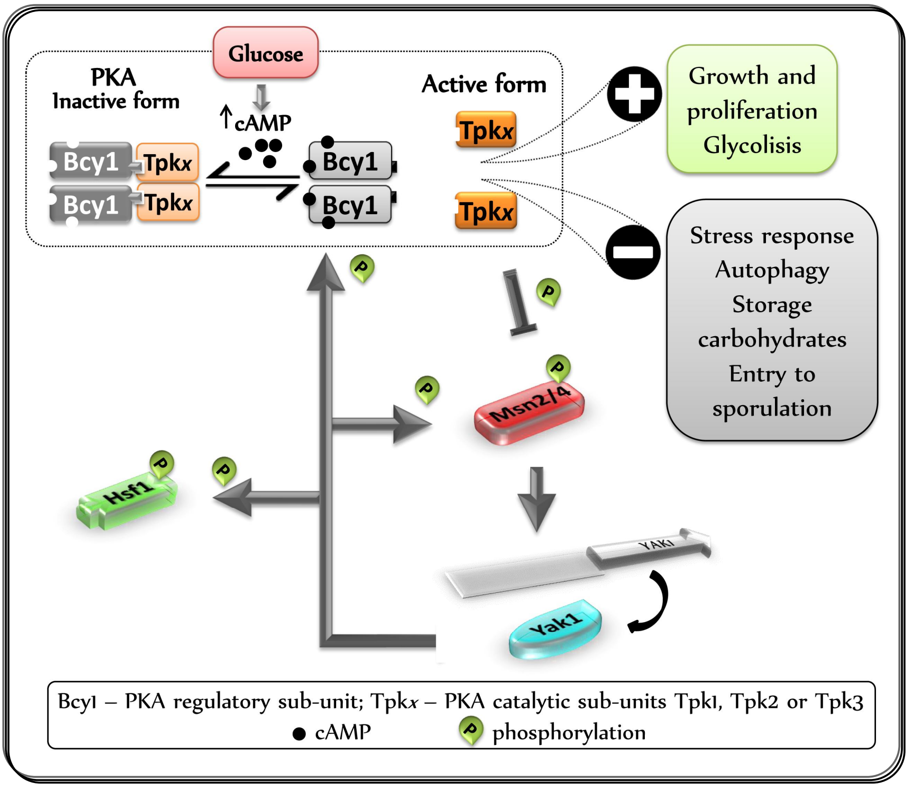


Figure 1.10: Overview of the PKA pathway crosstalk with GSR.

Schematic representation of the crosstalk between PKA and the GSR. PKA pathway is directly controlled by the cAMP levels in response to glucose availability which modulates the balance between the inactive and active forms. Under normal growing conditions, PKA favors growth and proliferation and repress the stress response through phosphorylation of its main effector, Msn2/4. In turn, Msn2/4 controls *YAK1* expression and the encoded glucose-sensing kinase Yak1 regulates Msn2/4, Hsf1 and the PKA regulatory subunit Bcy1.

I.3.4 The HOG pathway

The HOG pathway is the main pathway that yeast cells use to respond and adapt to a hyperosmotic challenge. Under such conditions, water starts to diffuse passively from the cytoplasm to the outside. As a consequence of the increased osmotic pressure, cells start to shrink and the intracellular solutes rises to dangerous levels. To counteract theses effects and avoid death, yeast cells increase the inner concentration of osmolytes, such as glycerol and trehalose (de Nadal *et al.,* 2002; Hohmann, 2002). This is achieved by an immediate growth arrest and a fast change in the gene expression program, involving transient transcriptional induction of more than 150 genes (Posas *et al*., 2000; Rep *et al*. 2000; Yale *et al*., 2001), that leads to the activation of different response mechanisms in order to repair damage and induce adaptation to the new conditions (de Nadal *et al.,* 2002; Támas *et al*, 2003). The importance of the production of the osmolytes during the yeast response to osmotic stress is reflected by the fact that most of the changes in gene expression upon an osmotic shock are involved in the shift of the metabolism and cell permeability to these compounds (O'Rourke *et al*, 2004). These changes in transcription result, among other effects, in an increase in glycerol metabolism (Rep *et al.,* 2000; Posas *et al.,* 2000) or reestablishment of ionic balance (Proft *et al*, 2004) and are mainly achieved through the activation of the HOG (High Osmolarity Growth) pathway. This is a MAPK (mitogen-activated protein kinase) pathway, whose major player is the MAP Kinase Hog1, a homologue of the p38 and c-Jun N-terminal kinase (JNK) families in mammals. These pathways used by eukaryotes cells to respond to stress challenges are also known as SAPK (Stress Activated Protein Kinases) pathways and are highly conserved. They involve a cascade of three consecutively activated kinases, from the MAP kinase kinase

kinase (MAPKKK), to the MAP kinase kinase (MAPKK) and finally the MAP kinase (MAPK). The signal that triggers this phosphorelay system is sensed by different sensor mechanisms, usually transmembranar proteins that activate the MAPKKK by phosphorylation, triggering the downstream MAPKK activation and finally the phosphorylation of the MAPK. Once phosphorylated, the MAPK accumulates in the nucleus and phosphorylates its targets. There are evidences, however, as it is the case of Hog1, that not all of the activated MAP kinase is translocated into the nucleus, regulating some cytosolic events (Ferrigno *et al*., 1998; Reiser *et al*., 1999).

The importance of Hog1 in the yeast response to osmotic stress is revealed by the severe sensitivity of the *hog1* mutant under such conditions (Siderius *et al.,* 2000), whereas the hyper-activation of the HOG pathway is lethal (Maeda *et al.,* 1993 and 1995).

In the HOG pathway two different branches, Sln1 branch and the Sho1 branch (Maeda *et al*., 1995), are able to sense the osmotic shift and phosphorylate the MAPKKKs (Ssk2, Ssk22 or Ste11). In turn, any of the MAPKKKs is able to phosphorylate the MAPKK Pbs2. Phosphorylated Pbs2 activates Hog1 through dual Thr/Tyr phosphorylation and promotes its fast translocation into the nucleus where it activates the transcription of its target genes (Támas *et al*., 2003). This includes several transcription factors, such as Msn1, Msn2/4, Hot1 and Sko1 (Rep *et al.,* 1999b; 2000; Pascual-Ahuir *et al.,* 2001).

The Sln1 branch is a phosphorelay system (Posas *et al*., 1996), having the Sln1 histidine kinase as its main key-player. This kinase has two transmembrane regions that act as osmosensors and an intracellular histidine kinase domain that signals the downstream target proteins

Ypd1 and Ssk1. Sln1 is constitutively active under normal growth conditions and continuously phosphorylates Ypd1. In turn, Ypd1 transfers its phosphate group to the response regulator Ssk1, preventing its interaction with Ssk2 and Ssk22, thus inactivating the HOG pathway. Upon an osmotic shift, the histidine kinase activity of Sln1 is inhibited, preventing Ypd1 and Ssk1 phosphorylation. Unphosphorylated Ssk1 is then able to bind to the N-terminal regulatory domain of the MAPKKK Ssk2, triggering its autophosphorylation and activating Pbs2 and in turn Hog1 (Posas *et al*., 1998). This branch acts as a negative regulator of the HOG pathway because the null mutant *sln1* is unviable, causing the permanent activation of the pathway (Maeda *et al*., 1994).

The Sho1 branch of Pbs2 activation involves the transmembrane protein Sho1, the mucin-like redundant proteins Msb2 and Hrk1 (Tatebayashi *et al*., 2007), the small GTPase Cdc42 and components of pheromone-response and filamentous MAPK pathways, namely MAPKKK Ste11 and its binding partner Ste50 and Ste20 (Posas *et al*., 1997 and 1998). Mucins Msb2 and Hrk1 are transmembrane proteins that are thought to be at the most upstream of the branch, being able to sense osmostress through its glycosilated serine/threonine rich domain (reviewed in Nadal *et al*., 2007) that is modified upon osmotic shift. This perturbation is then transmitted to the tetraspan Sho1 that will activate the downstream targets. Sho1-dependent signalling activates the small G-protein Cdc42 that binds and activates the PAK (p21-activated protein kinase) family members Ste20 and Cla4. This complex then phosphorylates Ste11 and in turn Ste11 activates Pbs2. Opy2 is another transmembrane important protein of the Sho1 branch. Its role seems to be a membrane anchor for the targeting of Ste50, the Ste11-interactor protein, for the membrane, possibly facilitating Ste11 activation by Ste20/Cla4 complex. Similar function is attributed to Cdc42 (Wu *et al*., 2006). Tatebayashi and collaborators (2006) present evidences that Cdc42, Ste50 and Sho1 act as adaptors of the Sho1 branch as they brought together the different components of the branch, allowing the flow of the osmostress signal from Ste20/Cla4 to Ste11 and finally to Pbs2. According to their work, the role of Cdc42 also involves the binding to the Ste11–Ste50 complex to bring activated Ste20/Cla4 to their substrate Ste11.

Work from Vadaie *et al*. (2008) suggests that the activity of Msb2 as osmosensor involves the processing and release of its secreted auto-inhibitory domain. In fact they identified Yps1 as the nutrient limitation-induced aspartyl protease necessary for the processing and release of the extracellular domain of Msb2 and consequent activation.

Sho1 role in the respective branch seems to be the anchoring of Pbs2 to the membrane, allowing its activation by Ste11. Sho1 is a transmembrane protein, possessing four transmembrane segments and has a C-terminal SH3 domain through which interacts with the SH3-polyproline domain of Pbs2 (Maeda *et al*., 1995). Besides, Raitt *et al*. (2000) showed that overexpression of a membrane-targeted version of Pbs2 could bypass Sho1 what may discard Sho1 as osmosensor of the branch. However, data from O'Rourke *et al.* (2002) does not corroborate this topological view of the branch. They showed that Sho1 effectively acts as an osmosensor, although it is partially redundant with Msb2. Both are necessary for maximal stimulation through this branch in response to hyperosmotic conditions (reviewed in Westfall *et al*., 2006).

Although partially redundant, these two upstream branches of the HOG pathway have some degree of specialization for detecting different osmotic conditions. It has been reported that Hog1 phosphorylation in response to low solute concentrations is more dependent on the Sln1 branch (Maeda *et al*., 1995) and that the activity of the same branch is required to induce the expression of several reporter genes in response to very high solute levels (Van Wuytswinkel, *et al*., 2000). These findings indicate that the Sln1 branch is able to respond to a broader range of osmotic shifts than the Sho1 branch.

Once activated by Pbs2-dependent phosphorylation, Hog1 is translocated into the nucleus, apparently mediated by the karyopherin Nmd5 (Ferrigno *et al*., 1998), although other mechanisms of Hog1 import could be involved as *nmd5* null mutant does not present osmosensivity (O'Rourke *et al*., 2002). Hog1 translocation is specifically dependent on the Pbs2-mediated phosphorylation of Thr174 and Tyr176 (Ferrigno *et al*., 1998; Reiser *et al*., 1999). DNA microarray studies indicate that nuclear Hog1 is responsible for the increased expression of about 600 genes (Posas *et al*., 2000; Rep *et al.,* 2000; O'Rourke *et al.*, 2004). This is achieved, for instance, by the Hog1 binding and phosphorylation of the transcription factors Sko1 and Hot1. However, several evidences show that during osmotic stress Hog1 is effectively recruited by the TF it regulates to the promoter of downstream target genes, as in the case of the Hog1 recruitment by Hot1 to the promoter of *GDP1* (Alepuz *et al*., 2001) and by Sko1 to its dependent promoters, being both Sko1 and Hog1 required for the recruitment of the SAGA histone acetylase and SWI/SNF nucleosome-remodelling complexes in response to osmostress necessary for transcription (Proft *et al*., 2002). An overview of the *S. cerevisiae* HOG pathway can be found in Fig. 1.11 (page 55).

The role of Hog1 in the yeast response to osmotic stress seems to be extended to others aspects such as the magnitude of gene induction, the duration of gene regulation and the limitation of the cross-activation of other MAPK cascades (Proft *et al.*, 2002). The results obtained by Proft and collaborators (2002) also allow to discriminate the magnitude of the stress imposed and to determine which branch of the HOG pathway is used. In this way, the Sln1-Ssk1, but not the Sho1-Ste11 branch, is used during modest osmotic stress, while at intermediate hyperosmotic stress both branches, as well as the ESR has a significant contribution to changes in gene expression. Upon extreme osmotic stress, the ESR pathways are preferentially used as Gash *et al*. (2000) have previously shown.

Nevertheless, Hog1 is also responsible for restoration of gene expression upon osmo-adaptation (O'Rourke and Herskowitz, 2004) and restart of protein translation after the pause in protein synthesis imposed by an osmotic shock (Uesono and Toh-e, 2002).

Some genetics evidences placed Msn1 and the key TFs of the ESR Msn2/4 downstream Hog1 (Martinez-Pastor *et al.*, 1996, Rep *et al.*, 1999b). This is suggested by the observation that there are many Hog1-dependent genes that are independent of Msn2/4, while the majority of the genes that are affected by Msn2/4 deletion are Hog1-dependent (Rep *et al.,* 2000). Regarding this, Alepuz *et al*. (2001) showed, for instance, that under osmotic stress the maximal induction of the Msn2/4-dependent gene *CTT1* also requires Hog1. Additionally, they showed that Msn2/4 recruit both Hog1 and Hot1 to the promoters of *CTT1* and *HSP12*. It is possible that the cross talk between the HOG pathway and the Msn2/4-dependent ESR could be mediated by the TF Sko1. In fact, upon osmotic stress, Hog1 converts Sko1 from a repressor to an activator, phosphorylating it in three sites (Proft *et al.*, 2002; Proft *et al.*, 2005), and in turn Sko1 regulates the expression of Msn2/4.

Additionally, Sko1 controls a transcriptional feedback mechanism, being required for the induction of *PTP3*. The phosphatase encoded by this gene, together with Ptp2, function as cytoplasmic and nuclear anchors for Hog1, respectively (Mattison *et al.*, 2000), by dephosphorylating Hog1 and thus controlling its activity.

This cross talk between HOG and ESR pathways driven by Sko1 is further regulated by PKA, which controls the nuclear localization and repressor functions of Sko1 (Pascual-Ahuir *et al.*, 2001), possibly through phosphorylation. This is suggested by the ability of PKA to phosphorylate Sko1 at multiple sites *in vitro* (Proft *et al.*, 2002).

The role of the HOG pathway and its MAPK Hog1 is, as described, far beyond the osmotic stress regulation *per se*. It involves for instance the cold shock response, in which Aguilera *et al.* (2007) was able to show cooperation between the HOG pathway and Msn2/4p. It is possible that this crosstalk may also involve Sko1. Clotet and Posas (2007) reviewed Hog1 role in several aspects of the regulation of cell physiology, as the progression of cell cycle and purpose the existence of an osmotic checkpoint controlling cell cycle transitions through SAPK as Hog1.

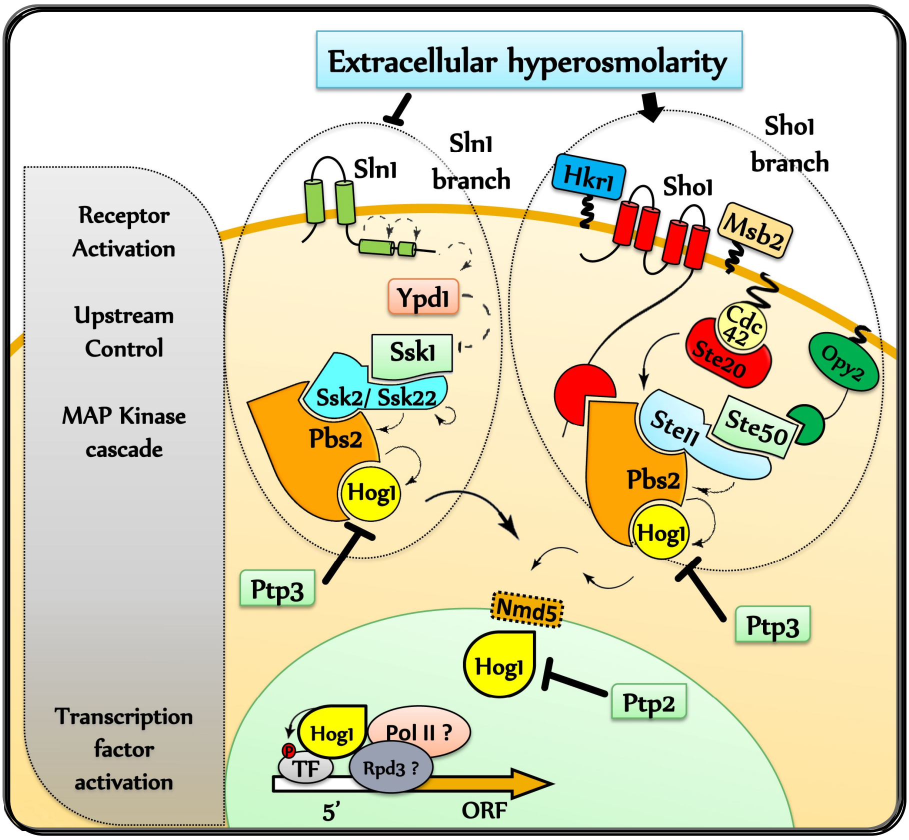


Figure 1.11: Overview of the *S. cerevisiae* HOG pathway.

The main features and players of the HOG pathway that yeast cells use to cope with osmotic stress are represented in the figure. Upon an osmotic shift, different sensor proteins present in the membrane (Sln1, Sho1 and possibly Hkr1, Msb2 and Opy2) are activated, triggering downstream controlling mechanisms that ultimately will activate the MAP kinase cascade. Sln1 and Sho1 branches of the HOG pathway converge in the phosphorylation of the MAP kinase Hog1. Once phosphorylated, Hog1 is translocated into the nucleus where it phosphorylates different target TFs such as Sko1 and Hot1. Several evidences suggest that Hog1 can modulate transcription initiation by different mechanism, such as regulation of transcription factor's activity, stimulation of the recruitment of the Pol II at osmoresponsive promoters, recruitment of the Rpd3 histone deacetylase complex, and modification of chromatin. In many cases, the TF recruits the kinase for the promoter region of the genes whose expression is being regulated by the TF. For further details about the HOG pathway regulation and specific role of its players see the text above. Dashed arrows refer to the phosphorelay system of the Sln1 branch under constant osmotic conditions. Full arrows refer to phosphorylation events under stress conditions.

Chapter II

YAP4 regulation under different stress conditions

Results and discussion

This chapter is based on the manuscripts published in:

Nevitt T, **Pereira J**, Azevedo D, Guerreiro P and Rodrigues-Pousada C (2004).
Expression of *YAP4* in Saccharomyces cerevisiae under osmotic stress.
Biochem. J. 379, 367-374;

Nevitt T, **Pereira J** and Rodrigues-Pousada C (2004). YAP4 gene expression is
induced in response to several forms of stress in Saccharomyces cerevisiae.
Yeast 21, 1365-1374;

(A copy of these manuscripts can be found at the end of the thesis)

Summary

Our previous studies on *YAP4* showed that it is responsive to a broad range of stress conditions, including osmotic, oxidative and other stress conditions. This is due to the existence of a promoter region that is very rich in different *cis*-elements, including several Stress Response Elements, known to be binding sites of the transcription factor Msn2, as well as Yap Recognition Elements. Upon osmotic stress, Msn2 and Hog1 have been shown to be important for transcriptional regulation of *YAP4*. Moreover, the null mutant *yap4* is moderately sensitive to osmotic stress and overexpression of *YAP4* bypasses this sensitivity and rescues the severe *hog1* osmosensitivity phenotype. Expression analyses in several mutant strains under pro-oxidant conditions have determined that *YAP4* is regulated by the transactivators Yap1 and Msn2. Mutation of either the Yap1-response element (YRE), located at −517bp from the ATG, or the most proximal stress response element (STRE) at −430bp, strongly compromise *YAP4* gene expression under these conditions. Furthermore, these two mutations in combination lead to a severe depletion of detectable mRNA levels, indicating an interplay between the transcription factors Yap1 and Msn2 in the regulation of *YAP4* transcription.

The results obtained in this work showed that Yap4 is a phosphoprotein highly induced upon the different stress conditions previously shown to induce the transcription of its gene, in particular osmotic stress and oxidative stress. This modification was confirmed after alkaline phosphatase treatment that reduces the phosphorylated Yap4 isoforms with slower mobility bands to the single, unphosphorylated and fast migrating isoform. Our data place Yap4 under the HOG pathway control, downstream the MAP kinase Hog1, but

Yap4 phosphorylation is independent of this kinase. We also verified if Yap4 role under osmotic stress could be related with glycerol metabolism. However, we observed that glycerol levels in a *yap4* mutant strain was the same as in the wild type strain, while in the *hog1* mutant strain those levels are half of the normal levels.

Our data also revealed that under oxidative stress Yap4 is negatively regulated by protein kinase A and its phosphorylation is completely abolished by S196A mutation, being amino acid residues S89 and T241 important for Yap4 levels.

Introduction

To survive in rapidly fluctuating environments, yeast cells developed efficient systems of sensing and adaptation, often involving simultaneous activation of several transcription factors and pathways. The general stress response (GSR) pathway is activated upon exposure to many forms of stress, including osmotic and oxidative stress, heat, low pH and nutrient starvation, among others (Estruch, 2000). The major player of this response is the two zinc finger transcription factor Msn2/4 that binds to the *cis*-element STRE (STress Response Element - CCCCT) present on target gene promoters. Previous studies have demonstrated that a significant number of genes induced by oxidative stress show Msn2/4-dependence (Gasch et al., 2000; Lee et al., 1999), pointing towards an overlap between this and the Yap1 regulon (Hasan *et al*., 2002).

Yeasts cope with hyperosmotic stress mainly through the HOG pathways, but again there is a crosstalk between this pathway and the GSR, being Msn2 activated by both pathways (Gash, 2002).

YAP4, a member of the yeast activator protein (Yap) gene family, was initially associated to resistance to cisplatin and other anti-malarial drugs under overexpression conditions (Furuchi *et al*., 2001; Delling *et al*., 1998). Later, Rep *et al.* (1999a) showed that overexpression of both *YAP4* and *YAP6* confer salt tolerance to the *ena1* mutant. This first evidence of the involvement of Yap4 in the osmotic stress response led us to further investigate its role under this stress conditions. In fact, *yap4* mutant strain displays mild and moderate growth sensitivity at 0.4M and 0.8M NaCl and *YAP4* mRNA levels in the *hog1* mutant are severely affected. This fact places Yap4 under the HOG response pathway control. Moreover, *YAP4* overexpression relieves the osmosensitivity phenotype of the *hog1* mutant. *YAP4* induction under osmotic stress also requires the presence of the transcription factor Msn2, but not of Msn4, as *YAP4* mRNA levels are depleted by at least 75% in the *msn2* mutant. This involves the two more proximal stress response elements (STRE) present in *YAP4* promoter region.

Response and adaptation to conditions of oxidative stress is mainly controlled by Yap1 that acts as sensor of different forms of oxidative stress and modulates the gene expression of a large number of genes involved in antioxidant protection and thiol redox homeostasis (Toledano et al., 2003; Delaunay *et al*., 2000; Gasch *et al*., 2000; Lee *et al*., 1999; Azevedo *et al*., 2003). Yap1 activates its targets genes through the binding to the Yap-response element (YRE-TTAC/GTAA) in the promoter region of those genes (Fernandes *et al.,* 1997). To counteract the positive regulatory effect exerted by Yap1p and Msn2p is the PKA pathway, which has been previously shown to negatively regulate both Yap1 (Fernandes *et al*., 1997) and STRE-driven induction (Gorner *et al*., 1998; Marchler, 1993).

Besides the four STREs (positioned at -428bp, -711bp, -761bp and -833bp from the *YAP4* ATG), being the two most proximal important for *YAP4* regulation by Msn2 under osmotic stress, *YAP4 cis*-elements includes one consensus YRE sequence positioned at -508bp and two YRE-like sequences located at -118bp and -645bp from the ATG. Under oxidative stress, *YAP4* expression is dependent on Yap1 and Msn2 and deletion of the YRE and most proximal STRE leads to a complete abrogation of *YAP4* mRNAs.

Taken together, these observations points out to a possible role of *YAP4* in the osmotic and oxidative stress response that are summarized in Fig. 2.1 (next page).

At the protein level, our results show that Yap4 is induced and phosphorylated under osmotic and oxidative stress following a kinetics that is in agreement with its transcriptional activation. Moreover, under osmotic stress, Yap4 is negatively regulated by Hog1, which also controls *YAP4* expression. However, Yap4 phosphorylation is independent of Hog1. Additionally, we observed that deletion of *YAP4* does not affect the accumulation of glycerol, a compound produced by yeast to balance the osmotic pressure upon an osmotic shock.

Under oxidative stress, Yap4 levels are greatly reduced in a *bcy1* strain bearing a constitutively active PKA, indicating that Yap4 is negatively regulated by PKA. S196A mutation prevents Yap4 phosphorylation and S89 and T241 residues are important in the regulation of protein levels.

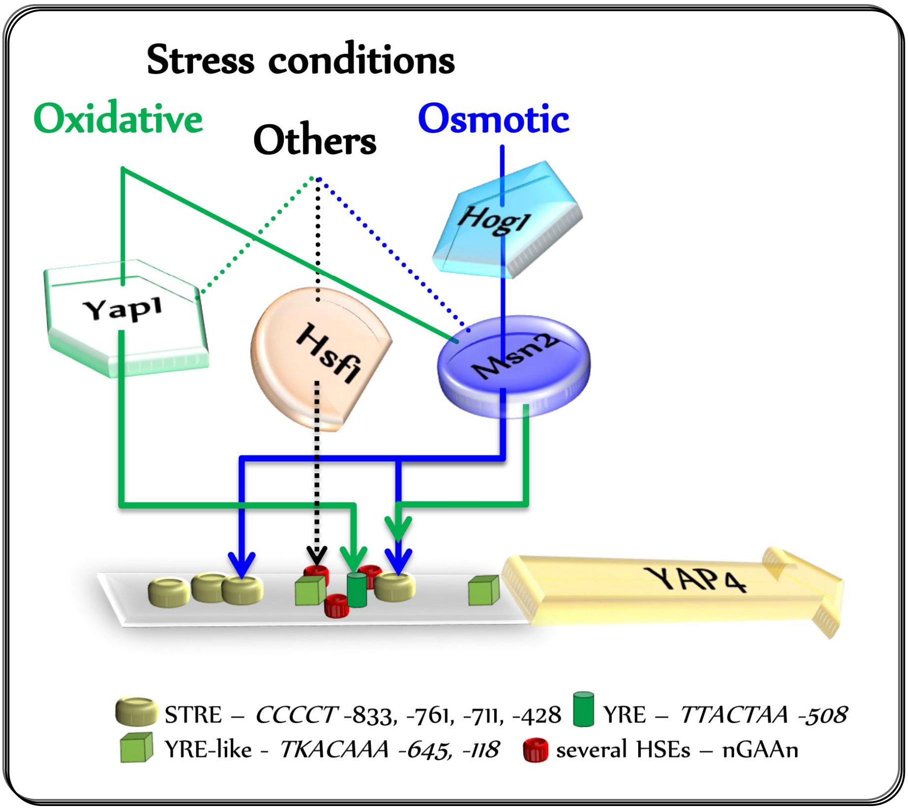


Figure 2.1: Overview of *YAP4* regulation under different stress conditions.

Upon an osmotic shift, the HOG pathway is activated and Hog1 is phosphorylated and translocated into the nucleus, thus activating its target genes. *YAP4* is downstream the HOG pathway, and its regulation involves besides the Hog1 MAP kinase, the TF Msn2, which itself can be activated both by Hog1 and independently of it. The two most proximal STREs were shown to be important for *YAP4* regulation under osmotic stress.

Under oxidative stress, the most proximal *YAP4* STRE and the YRE were shown to be the *cis*-elements more important for its regulation, which involves interplay between Msn2 and Yap1. *YAP4* also possesses multiples HSEs and therefore may also be regulated by Hsf1. As we will show, *YAP4* is also responsive to temperature shifts and the protein is induced under this stress condition. Other stress conditions, such as metals exposure and stationary phase, may also involve one or more of these two players in the regulation of *YAP4* as many stress conditions ultimately result in the production of oxidative stress through ROS (reactive oxygen species) or in the activation of the GSR (general stress response) through Msn2 activation. The *cis*-elements positions are indicated in the figure. STRE – stress response element; YRE – Yap recognition element; HSE – heat shock element; ORF - open reading frame.

Results and discussion

YAP4 is responsive to osmotic stress

To investigate the involvement of Yap4 in osmotic stress, *YAP4* gene expression analyses were performed. As Fig. 2.2 illustrates *YAP4* is induced under mild and hyperosmotic stress. Under mild osmotic stress (0.4M NaCl, Fig. 2.2A), the *YAP4* induction kinetics shows a rapid and transient peak after 10min. exposure. Upon a stronger osmotic shift (0.8M NaCl, Fig. 2.2B), there is a delay in the induction of the *YAP4* gene with a peak at 30–60min exposure, although the intensity is comparable with the response to mild stress, and the *YAP4* expression declines thereafter. These data correlate with previous observations (Posas *et al.*, 2000; Rep *et al.*, 1999) 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 observed for *YAP4*, *YAP1* exhibits a basal expression that rises and declines upon exposure to saline stress, possibly reflecting an altered redox state of the cell imposed by an osmo-shock (Figures 2.2A and 2.2B).

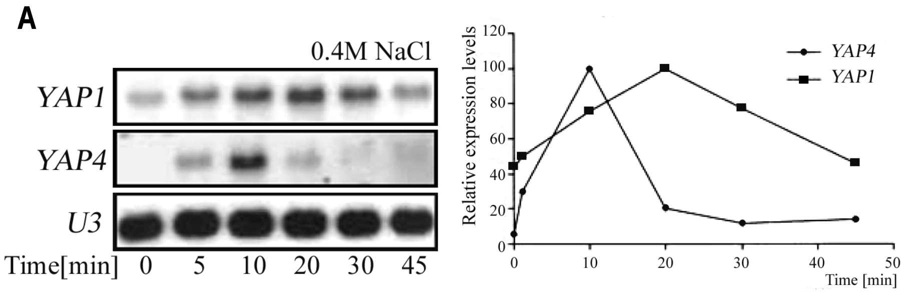


Figure 2.2: ***YAP4*** **is responsive to osmotic stress** (continue in the next page).

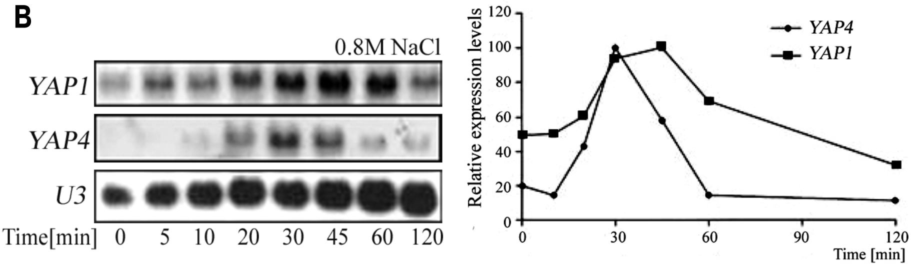


Figure 2.2: *YAP4* is responsive to osmotic and hyperosmotic stress.

Mid-log phase cells upshifted to 0.4M NaCl (A) or 0.8M NaCl (B) were harvested at the indicated time points and the mRNAs were extracted and blotted (upper panels). The mRNA levels for *U3*, a small nuclear RNA, were used as internal loading controls against which all mRNA levels were normalized (lower panels) (Nevitt *et al*., 2004a).

Yap4 levels and kinetics reflects its gene expression regulation

Having determined the induction pattern of the *YAP4* mRNAs under osmotic conditions, it became relevant to analyse whether this mRNA increase reflects an enhancement in the Yap4 protein levels. Fig. 2.3 illustrates the results obtained upon two osmotic shifts (0.4M and 0.8M NaCl), demonstrating that the protein induction kinetics is similar to those observed for its mRNAs (Fig. 2.2), showing just a small delay. Upon a mild osmotic shift, Yap4 levels increase, reaching a maximum at 20min and then decrease until at least 90min (Fig. 2.3A). Comparatively, the kinetics of Yap4 induction under a stronger osmotic challenge (0.8M NaCl) is delayed, reaching the maximum levels between 60 and 90min and only decreasing to low levels after 240min induction (Fig. 2.3C). The appearance of two bands that are converted into a single band after treatment with alkaline phosphatase (CIP - Calf intestinal alkaline phosphatase) (Fig. 2.3B), indicates that Yap4 is phosphorylated. Indeed, Yap4 contains several putative phosphorylation sequences that may be required for its activation.

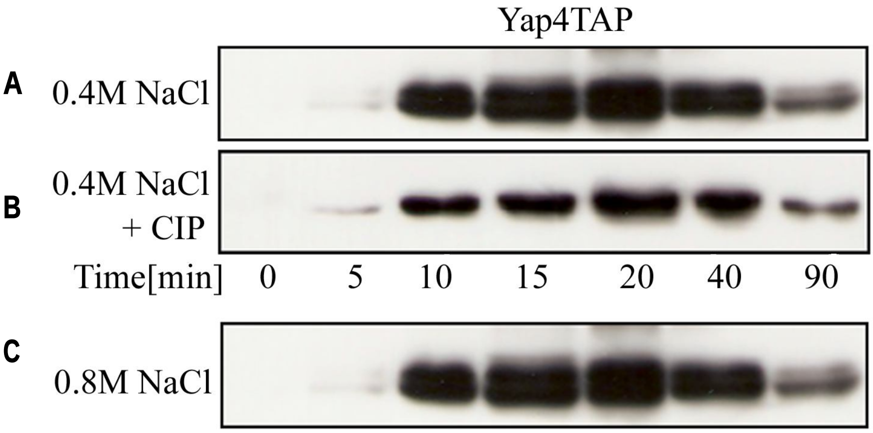


Figure 2.3: Yap4 is induced and phosphorylated upon osmotic stress.

Yap4 protein levels kinetics upon an osmotic (0.4M NaCl, panel A) and hyperosmotic shock (0.8M NaCl, panel C) is in agreement with the mRNA kinetics. The protein is phosphorylated and is resolved into a single band after treatment with CIP (panel B). *yap4* mutant strains expressing *YAP4TAP* were shifted to the desired osmolarity and samples harvested at the indicated time points. Protein extraction, separation, transfer and immunoblotting were performed as described in *Material and Methods* (Annex B, page 139).

Hog1 controls Yap4 levels but not its phosphorylation

The involvement of Yap4 in the HOG pathway downstream Hog1, being its expression severely compromised in a *hog1* mutant strain (Nevitt *et al*., 2004a) and the fact that Yap4 is phosphorylated (Fig. 2.2), led us to evaluate if Hog1 was the kinase responsible for Yap4 phosphorylation. As Fig. 2.4 shows, upon and osmotic and hyperosmotic shift, Yap4 levels in a *hog1* mutant strain are significantly affected when compared to the wild type strain, but not as much as we were expecting, since *YAP4* gene expression is severely compromised in the same mutant strain (Nevitt *et al.*, 2004b). The same result shows that Hog1 does not phosphorylate Yap4 as it conserves this modification in the *hog1* mutant strain that again is resolved into a single band after treatment with CIP (Fig. 2.3C).

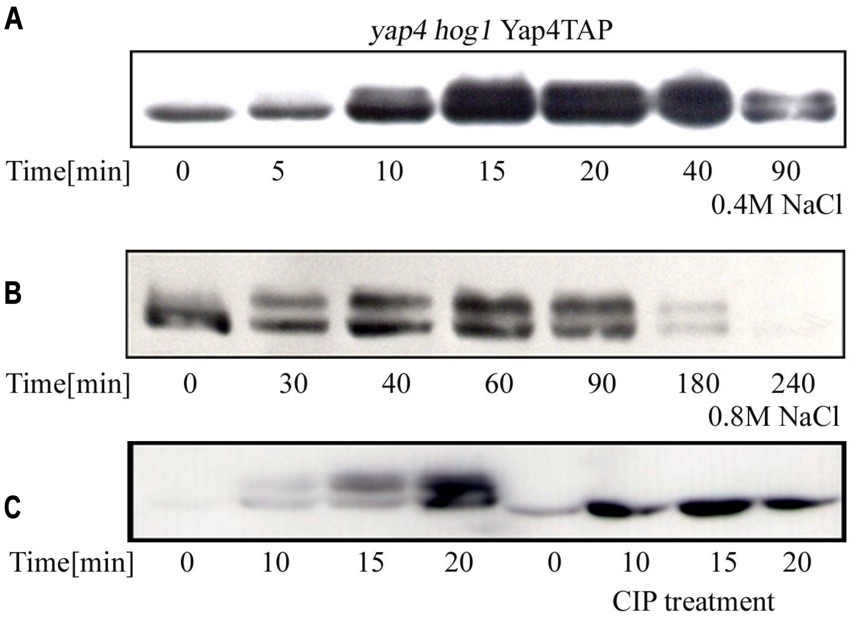


Figure 2.4: Hog1 affects Yap4 levels but not its phosphorylation.

Yap4 protein levels are significantly reduced in the *hog1 mutant* upon an osmotic (0.4M NaCl, panel A) and hyperosmotic shock (0.8M NaCl, panel B) compared to the wt strain, but phosphorylation is conserved, being only removed after CIP (alkaline phosphatase) treatment (panel C). *hog1 yap4* mutant strains transformed with *YAP4TAP* were shifted to the desired concentration of NaCl and samples harvested at the indicated time points. Protein extraction, separation, transfer and immunoblotting were performed as described in *Material and Methods* (Annex B, page 139).

Potential Yap4 involvement in glycerol biosynthesis

In order to gain a better understanding of the role of Yap4 in osmoregulation, we have, as a parallel and ongoing study, performed DNA microarray analysis in the wild-type and *yap4* strains under mild hyperosmolar conditions for 10min. Among the genes that were found to be regulated by Yap4 (T. Nevitt, J. Pereira and C. Rodrigues-Pousada, unpublished work), two involved in glycerol biosynthesis were identified: *GCY1*, encoding a putative glycerol dehydrogenase, and

GPP2, encoding a NAD-dependent glycerol-3-phosphate phosphatase, that are partially dependent on *YAP4* (Fig. 2.5) Furthermore, also *DCS2*, a gene homologous to the *DCS1*-encoded decapping enzyme (Kwasnicka *et al*., 2003) shows 80% depletion in induction levels in the *yap4* mutant upon osmoshock (Fig. 2.5). This gene is homologous to the *DCS1*-encoded decapping enzyme which was shown to inhibit trehalase activity (De Mesquita et al., 2003). This is related to osmotic response since trehalose could be used by yeast as a cellular osmolyte as glycerol does.

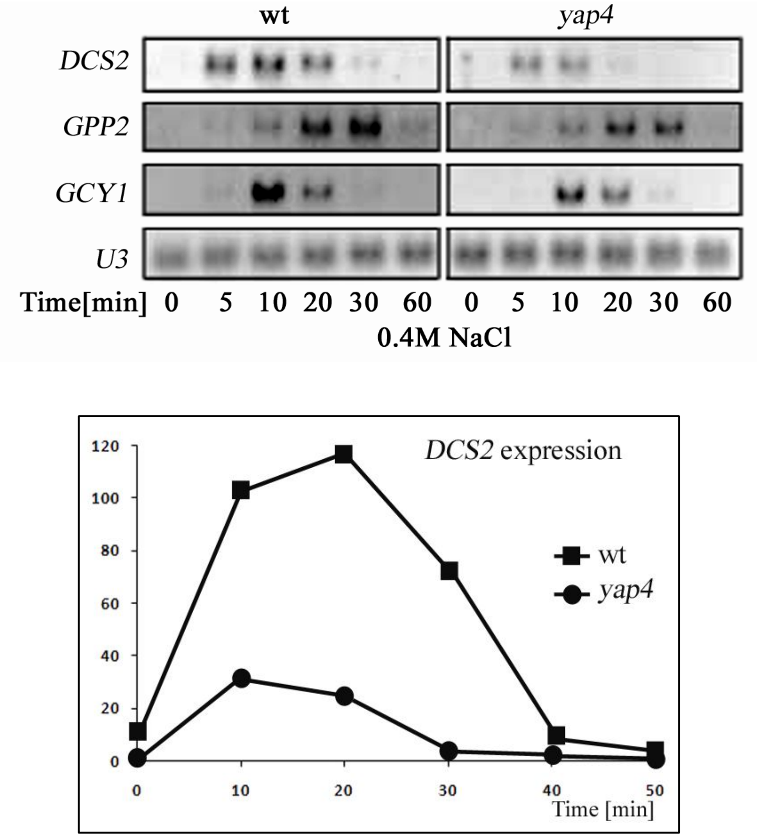


Fig. 2.5: Expression analysis of *DCS2*, *GPP2* and *GCY1* genes in the (wt) and *yap4* mutant strains (continue in the next page).

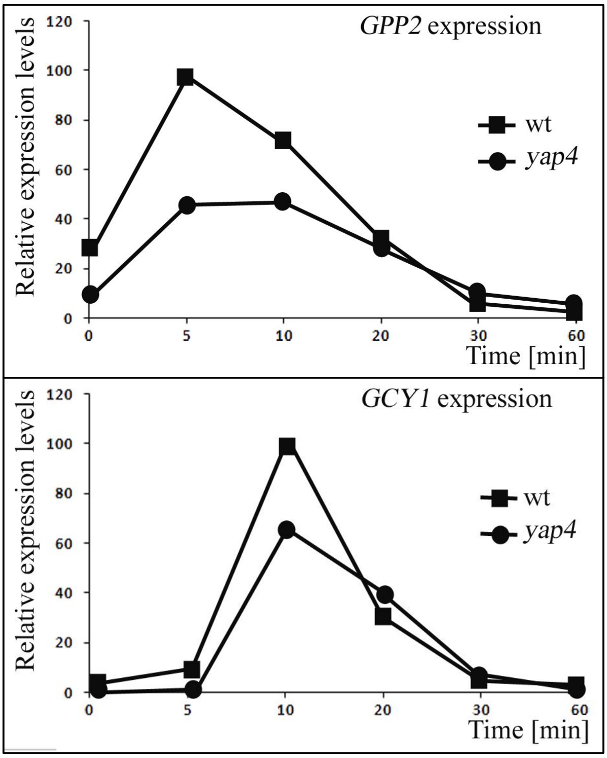


Fig. 2.5: Expression analysis of *DCS2*, *GPP2* and *GCY1* genes in the (wt) and *yap4* mutant strains (continuation of the previous page).
Wt and *yap4* mutant strain mid-log phase cells were upshifted to 0.4M NaCl and harvested at the indicated time points. The mRNAs were extracted and blotted (upper panels) as described in *Materials and Methods* (Annex B, page 139). The mRNA levels for *U3*, a small nuclear RNA, were used as internal loading controls against which all mRNA levels were normalized (lower panels).

Dcs2 protein levels were followed in wt and *yap4* mutant strains under osmotic stress. We observed that Dcs2 is induced by osmotic stress and its synthesis is partially dependent on Yap4 as its levels are significanty affected by deletion of *YAP4* (Fig. 2.6A). We also performed the same assay in a medium supplemented with glycerol as a carbon source as our unpublished results suggest that Dcs2 is related with glycerol metabolism. When cells were shifted to a medium supplemented with glycerol instead of glucose, Dcs2 levels were much increased, but again decrease when *YAP4* is not present (Fig. 2.6B). These results confirm that *DCS2* is a target of Yap4, although only partially dependent on it.

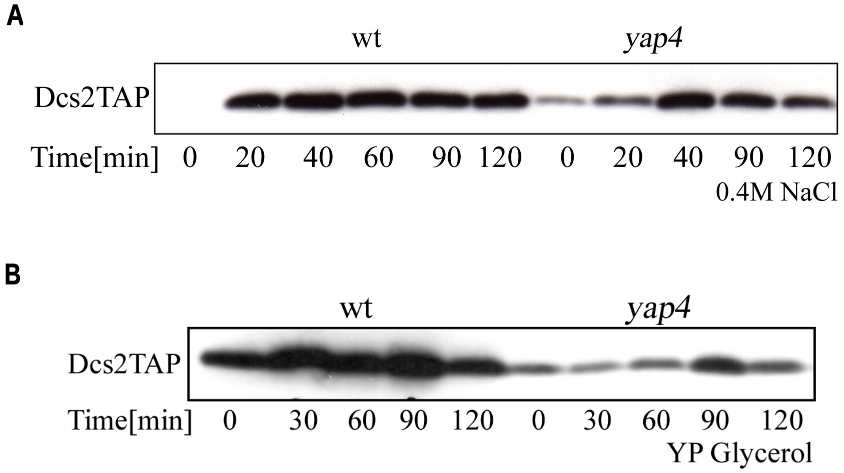


Figure 2.6: *YAP4* deletion affects the expression of Dcs2.

Dcs2TAP protein levels were monitored in the wild type and *yap4* mutant strains grown to early-exponential phase and exposed to 0.4M NaCl (A) or cells were shift to a rich medium containing glycerol as carbon source (B). Samples were harvested at the indicated time points indicated. Protein extraction, separation, transfer and immunoblotting were performed as described in *Material and Methods* (Annex B, page 139).

Internal glycerol accumulation is unaffected by the lack of Yap4

Following this putative involvement of Yap4 in glycerol metabolism, which would explain its role in the osmotic stress response, we measured the intracellular glycerol levels in wild-type as well as in the *yap4* and *hog1* mutants after 1.5 h exposure to 0.7M NaCl.

As can be seen in Fig. 2.7, deletion of *YAP4* does not appear to affect the intracellular glycerol content. As previously described (Brewster *et al.*, 1993), the *hog1* cells show a marked reduction of glycerol accumulation under these conditions.

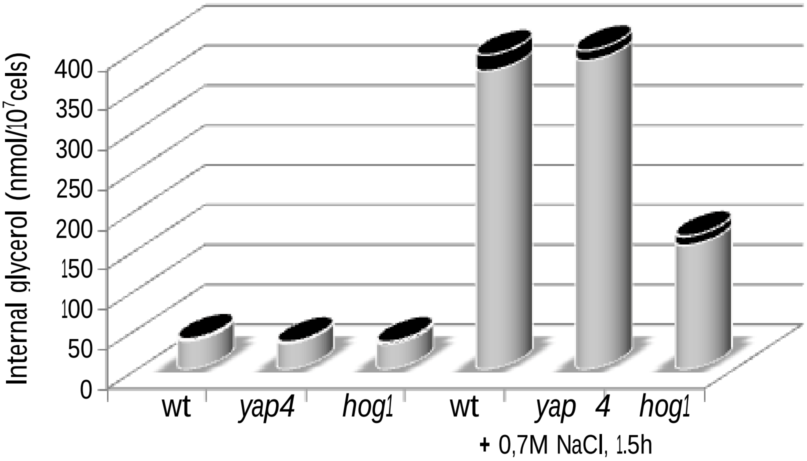


Figure 2.7: Internal glycerol accumulation is unaffected by the lack of Yap4.

Wild type and mutant cells were grown to early-exponential phase and either unchallenged (left) or exposed to 0.7 M NaCl (right) and samples collected after 1.5h. Glycerol content was quantified as described in *Material and Methods* (Annex B, page 139).

Analysis of *YAP4* expression under oxidative stress

As already referred, *YAP4* possesses several *cis*-elements important for its regulation under osmotic stress, particularly the two most proximal STREs (Nevitt *et al*., 2004a). However, *YAP4 cis*-elements also include an YRE (Yap-response element) making it putatively regulated by Yap1 and therefore responsive to oxidative stress. Therefore, we analysed *YAP4* expression upon H_2O_2 exposure in wt and mutant strains for Yap1, Hog1 and Msn2 and observed that *YAP4* is induced under oxidative stress and is regulated by Yap1 and Msn2 under this condition (Fig. 2.8). Moreover we also showed that under oxidative stress, the *YAP4 cis*-element YRE and the most proximal STRE are essential, supporting the interplay between Yap1 and Msn2 in *YAP4* (Nevitt *et al*., 2004b).

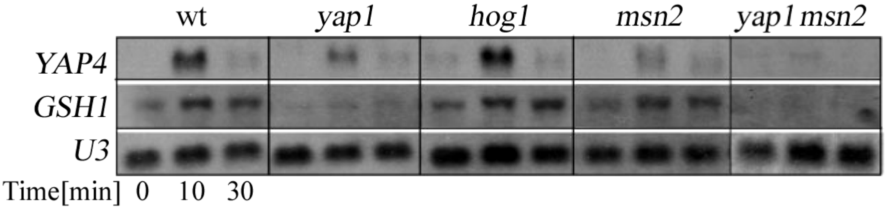


Fig. 2.8: *YAP4* induction under oxidative stress is dependent on Yap1 and Msn2.

Mid-log phase cells from wt and the mutant strains were upshifted to 1.5mM diamide, harvested at the indicated time points and the mRNAs analysed by Northern analysis. The mRNA levels for *U3*, a small nuclear RNA, were used as internal loading controls (Nevitt *et al*., 2004b).

Analysis of Yap4 induction upon oxidative stress

Having characterized the induction patterns of the *YAP4* mRNAs under oxidative stress and its regulation by Yap1 and Msn2, it became important to analyse whether this mRNA increase reflects an enhancement in Yap4 protein levels. Upon stress induction with 0.3mM H_2O_2, a *yap4* strain carrying an integrated *YAP4TAP* version shows induction kinetics similar to that observed for the mRNAs, being highly and transiently induced, with a maximum at 20min and then decreasing until basal levels around 90min (Fig. 2.9). Through an improved SDS-PAGE resolution, it can be detected the appearance of three, instead of the two previously shown Yap4 isoforms that are resolved into a single band after treatment with CIP (Fig. 2.9, last lane).

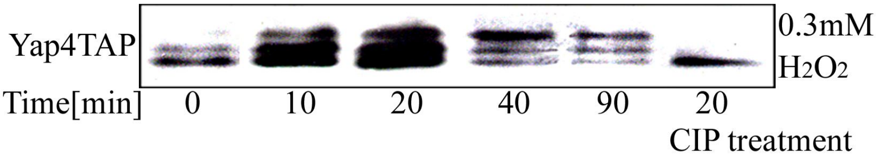


Figure 2.9: Yap4 is transiently induced and phosphorylated under oxidative stress.

The *yap4* mutant strain transformed with *YAP4TAP* was grown until mid-log phase, challenged with 0.3mM H_2O_2 and harvested at the indicated time points. Protein extraction, separation, transfer and immunoblotting were performed as described in *Material and Methods* (Annex B, page 139). CIP - calf intestinal phosphatase.

Taking into account that Yap4 is a constitutively nuclear resident protein, we thought that the phosphorylation step could be relevant for its post-translational activation and/or stability. Yap4 contains several putative phosphorylation motifs, including several putative PKA sites. In order to evaluate the effect of an overactive PKA, Yap4 levels were monitored in a *bcy1* strain (Toda *et al*., 1987) lacking the PKA regulatory subunit and thus possessing constitutively PKA activity. This strain shows a severe depletion of Yap4 levels (Fig. 2.10) and therefore Yap4 is negatively regulated by PKA. This result validates the regulation of *YAP4* gene by both Msn2 and Yap1 as PKA negatively regulates the expression of STRE genes controlled by Msn2 (Smith *et al*., 1998) and also Yap1 (Fernandes *et al*., 1997). For this reason, we do not know yet whether PKA negatively regulates Yap4 by affecting its phosphorylation or it is simply a reflection of its negative effect on Msn2 and Yap1.

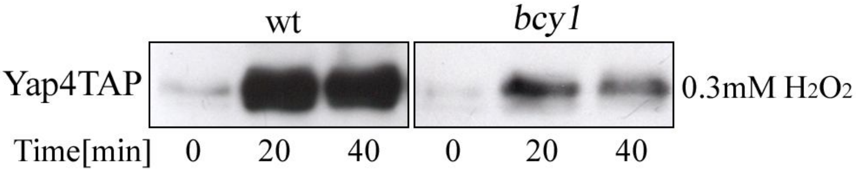


Figure 2.10: Yap4 levels are severely affected in a *bcy1* strain.

Yap4 levels are severely affected in the *bcy1* strain in which PKA is constitutively active. *YAP4TAP* was integrated in y*ap4* and *bcy1* mutant strains and mid-log phase cells were harvested after induction with 0.3mM H_2O_2 at the indicated time points and analysed by western blot. Protein extraction, separation, transfer and immunoblotting were performed as described in *Material and Methods* (Annex B, page 139).

Although several putative PKA phosphorylation sites exist along Yap4, these residues also represent sites for phosphorylation by other kinases. Site-directed mutagenesis of selected sites was performed in order to identify the residues involved in this posttranslational event. Fig. 2.11 illustrates the results obtained from this study. Mutation of serine at position 196 completely abolishes Yap4 phosphorylation, while mutation of S89 and T241 affects Yap4 phosphorylation and levels, respectively. These results suggest that these residues may be involved in maintaining protein stability, e.g. by protecting against targeted degradation and provide evidence for a post-translational regulation of Yap4 in the yeast response to oxidative stress.

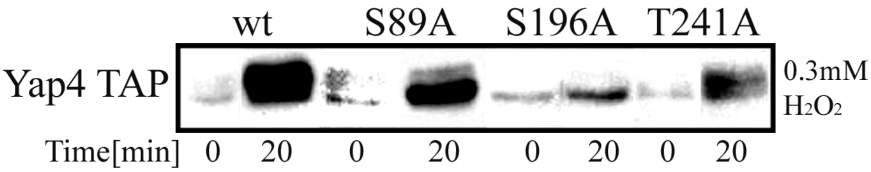


Figure 2.11: S196A mutation prevents Yap4 phosphorylation and S89 and T241 residues affect protein phosphorylation and levels under oxidative stress.

The *YAP4* residues indicated were mutated to alanine by site-directed mutagenesis and the mutated versions were integrated in *yap4* strain. Mid-log phase cells were harvested after induction with 0.3mM H_2O_2 at the indicated time points and analysed by western blot. Protein extraction, separation, transfer and immunoblotting were performed as described in *Material and Methods* (Annex B, page 139).

Conclusions

Upon several forms of stress, yeast cells activate different pathways in order to cope with the damage eventually produced and adapt to the new conditions imposed (Tamás and Hohmann., 2003). In particular, under osmotic stress, the HOG pathway is activated resulting in Hog1 activation and accumulation of Hog1 in the nucleus where it modulates the activity of target transcription factors, including Hot1 (Rep *et* al., 1999a), Sko1 (Pascual-Ahuir *et al*., 2001), Smp1 (de Nadal *et al*., 2003) or Msn2/4, involved in downstream signal amplification. Ultimately, one of the results of HOG pathway activation is an increase in the inner glycerol content to balance the osmotic pressure.

In this work it was shown that *YAP4* is responsive to osmotic stress and under such conditions its regulation involves both Hog1 and Msn2. This control of *YAP4* regulation is achieved via, at least, two STRE elements present in its promoter region.

The moderate osmotic phenotype of the *yap4* mutant along with the partial recovery of the *hog1* mutant by the overexpression of *YAP4* suggests that the role of Yap4 in the osmotic stress could be related to osmoprotection, eventually regulating the inner concentration of glycerol. However, as our results show, the internal glycerol content does not appear to be altered in the *yap4* null strain.

At the protein level, our results showed the Yap4 is induced and phosphorylated upon osmotic and hyperosmotic shock and this kinetics reflects its gene regulation. Upon an osmotic shift, Yap4 levels increase and return to basal levels faster than under a hyperosmotic shock, suggesting the mechanisms involved in Yap4 regulation are associated to the intensity of the stress imposed and result in different Yap4 levels.

YAP4 is regulated by Hog1. However, although Yap4 protein levels are significantly affected in the *hog1* mutant, phosphorylation is conserved and therefore the Hog1 kinase is not responsible for Yap4 phosphorylation.

Further studies are needed to clarify Yap4 role in the osmotic stress.

In this work we also presented evidences showing that *YAP4* is also responsive to oxidative and other stress conditions, besides the osmotic stress that we previously reported (Nevitt *et al.*, 2004). The presence of YRE, STREs and possibly HSEs (although not consensus HSEs) in its promoter region suggest that *YAP4* regulation can be modulated by combination of different transcription regulators in a stress-specific manner. Indeed, in contrast to the Hog1 and Msn2 regulation of *YAP4* in the osmotic stress through its two most proximal STREs *cis*-elements (Nevitt *et al.*, 2004a), the response to oxidative stress requires the concerted action of both Yap1 and Msn2 on the YRE and most proximal STRE. This observation thus provides evidence for the existence of an integrated cascade of several transcriptional activators. Moreover, this regulation is reflected at the protein level, being Yap4 highly and transiently regulated and also phosphorylated upon oxidative stress. Moreover, Yap4 is negatively regulated by PKA, probably through phosphorylation at S196, shown to abolish Yap4 phosphorylation when mutated to alanine.

The fact that *YAP4* is responsive to such a wide plethora of environmental insults suggests an important role in the response to stress. Indeed, previous work has revealed that its overexpression is capable of relieving cellular sensitivity to a wide range of conditions and drugs, including salt (Mendizabal *et al.*, 1998; Nevitt *et al.*, 2004), cisplatin (Furuchi *et al.*, 2001) and quinoline ringcontaining antimalarial drugs (Delling *et al.*, 1998). The fact that the adduct-forming chemotherapeutic agent cisplatin acts at the level of transcriptional repression, by interfering with the TATA box via competing with the TATA binding protein (TBP), essential for the RNA Poli II initiation (Vichi et al., 1997), may suggest a role for Yap4 within the realm of the basal transcriptional machinery. Allied to the richness of *cis*-elements found within the *YAP4* promoter, there is the potential for an integrative circuit whereby multiple stress response pathways converge to modulate global gene expression.

Chapter III

Role of Yap4 phosphorylation

Results and Discussion

This chapter is based on the manuscript submitted to *Yeast*:

Pereira J *et al*., (2008). Yap4 PKA-dependent phosphorylation affects protein stability but not its localization

(A copy of this manuscript can be found at the end of the thesis)

Abstract

Yap4 is a nuclear resident transcription factor induced in *Saccharomyces cerevisiae* when exposed to several stress conditions, including mild hyperosmotic and oxidative stress, temperature shift, metal exposure or stationary phase. Here, we report that Yap4 is a PKA dependent phosphoprotein. In order to ascertain whether Yap4 is directly or indirectly phosphorylated by PKA, we searched for stress and PKA-related kinases that could phosphorylate Yap4. We show that phosphorylation is independent of the kinases Rim15, Yak1, Sch9, Slt2, Ste20, Ptk2 and Mck1. Furthermore, we show that its nuclear localization is independent of its phosphorylation state. Yap4 has several putative phosphorylation sites, but only the mutation of residues T192 and S196 impairs its phosphorylation under different stress conditions. Yap4 phosphorylation seems to be required for stability of the protein as the non-phosphorylated form has a shorter half-life compared to the phosphorylated one. However, this difference is not reflected in the ability of the non-phosphorylated forms of Yap4 to partially rescue the *hog1* severe sensitivity phenotype, which is not affected.

Introduction

The budding yeast *Saccharomyces cerevisiae* responds to various environmental cues by reprogramming gene expression in a highly coordinated manner via several interdependent pathways. Transcription factors, many of which contain phosphorylation sites, are important key-players in this process. This post-translational modification is usually associated to an important level of control by kinases either activating or repressing a given factor. It is well known that PKA is the effector kinase of the cAMP pathway, which monitors carbon source availability and regulates the expression of genes necessary for cell growth (Zurita-Martinez *et al*., 2005). PKA negatively regulates the stress response through Msn2/4 phosphorylation. Many other kinases related to PKA play a role in the yeast stress response. For example, Yak1 is part of a glucose-sensing system involved in the growth control in response to glucose availability. It regulates by phosphorylation of its N-terminal domain, the subcellular distribution of Bcy1, the regulatory subunit of PKA, thus controlling PKA activity (Griffioen *et al*. 2001). Also it was recently shown that PKA and TOR pathways are implicated in the regulation of the genes that are required for proper nutrient regulation and cell growth (Slattery *et al*., 2008). These two signalling cascades converge on the Rim15, a protein kinase required for the entry into stationary phase (G0) and to response to nutrients (Pedruzzi *et al*., 2003; Rohde *et al*., 2008; Swinnen *et al*., 2006). PKA controls entry into stationary phase by repressing Rim15 activity through its phosphorylation (Reinders *et al*., 1998). It has been also described that TOR, Sch9 and PKA are able to regulate common targets, including the expression of genes bearing STRE elements and genes encoding the ribosomal proteins (Rohde *et al*., 2008). Moreover, both Sch9 and PKA are involved in the adaptation to nutrient availability (Roosen *et al*., 2005) as well as in the cooperative regulation of autophagy (Yorimitsu *et al*., 2007). Additionally, Sch9 was shown to be a chromatin-associated transcriptional activator of osmostress-responsive genes (Pascual-Ahuir *et al*., 2007).

The Yap family is formed by eight b-ZIP transcription factors of which Yap1 is the best characterized as the major regulator in oxidative stress (Rodrigues-Pousada *et al*, 2004). Yap8, another member of the family, is involved in the arsenic compound detoxification through the regulation of the genes *ACR2* and *ACR3* encoding respectively an arsenate reductase and a plasma membrane arsenite efflux-protein. (Menezes *et al*., 2004; Menezes *et al*., 2008). Yap4 belongs also to this family and it has been previously shown to be highly induced under several forms of stress (Nevitt *et al*., 2004b; Salin *et al*., 2008). Its expression is regulated under osmotic stress by Hog1/Msn2 and under oxidative stress by Msn2 and Yap1 (Nevitt *et al*., 2004a).

We are interested in investigating the role of Yap4 phosphorylation in the yeast stress response since it is a highly phosphorylated protein under all stress conditions so far tested (Nevitt *et al*., 2004a and b). Our results show that Yap4 levels increase in response to osmotic and oxidative stress, heat and arsenic exposure, and that Yap4 is phosphorylated in a PKA-dependent manner. Using site directed mutagenesis we have mutated several residues potentially phosphorylated by PKA and other kinases (see Table I). Mutation of the residues T192 and S196 prevents Yap4 phosphorylation under the different stress conditions imposed and does not affect Yap4 nuclear localization but does substantially reduce the half-life of the protein.

Results and Discussion

Yap4 is induced and phosphorylated under different stress conditions

We showed previously that the steady state populations of mRNAs encoding Yap4 were highly enhanced after various forms of stress with a correspondent increase of the protein levels. The protein is also phosphorylated after an osmotic shock (Nevitt *et al*., 2004a) and under oxidative stress conditions (Nevitt *et al*., 2004b). Fig. 3.1 shows clearly three different forms of Yap4 with different mobility shifts on the SDS-PAGE gel. From our previous results using alkaline phosphatase treatment (Nevitt *et al*., 2004a and b), we can associate the faster migrating isoform as non-phosphorylated one and the other two bands as the phosphorylated isoforms. This pattern of phosphorylation is maintained under other forms of stress, such as heat, oxidative and arsenic exposures as can be seen in Fig. 3.5D (page 95). Yap4 is highly induced at 20min and it decreases at 40 and 90min of treatment. However, when cells reach stationary phase (180 and 240min), a subsequent increase is observed.

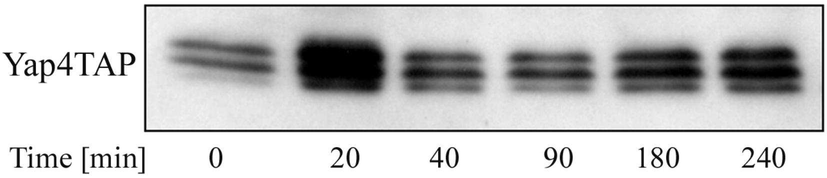


Figure 3.1: Kinetics of Yap4 phosphorylation.

The *yap4* mutant encoding an integrated *YAP4TAP* tag version was grown until mid log phase and harvested after induction with 0.4M NaCl at the time points indicated. Proteins were extracted and analysed by western blot (see *Material and Methods* in Annex B, page 139).

Prediction of Yap4 structural features

We used NetPhos and NetPhosK programmes to identify potential residues responsible for Yap4 phosphorylation (Blom *et al.*, 2004). The Yap4 putative phosphorylation sites are diagrammed in Fig. 3.2 and the potential kinases involved in the phosphorylation of these residues are shown in Table I. The predictions were also conducted using the ScanProsite programme, which also retrieved the predictions about Yap4 bipartite NLS and LZ (Hulo *et al.*, 2007). The first stretch of the NLS (nls1) spans the residues R193-V208 and the second one (nls2) the residues K242 -R258. The DBD prediction was obtained previously by alignment of Yap4 amino acid sequence with the remaining Yap members (Fernandes *et al.*, 1997) (Fig. 3.2). The large majority of consensus phosphorylation sites are putative PKA phosphorylation sites although other kinases were also found to recognize some of these elements (Table I).

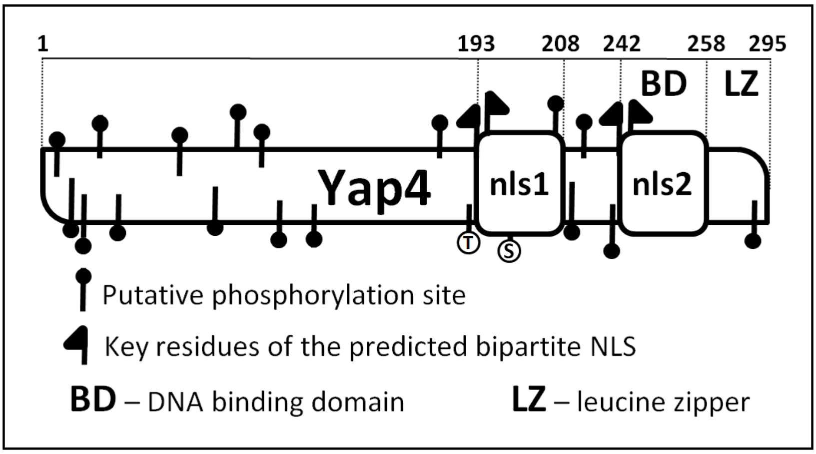


Figure 3.2: Schematic representation of predicted Yap4 structural domains.

The putative phosphorylation sites, BD, LZ and bipartite NLS were retrieved respectively from NetPhos and NetPhosK (Blom *et al.*, 2004), ScanProsite (Hulo *et al.*, 2007) and PSORT II programmes (psort.ims.u-tokyo.ac.jp) and by alignment with the remaining Yap members; (Fernandes *et al.*, 1997). The highlighted “T” and “S” putative phosphorylation sites are respectively T192 and S196.

Table I: Potential kinases involved in Yap4 phosphorylation.

	YAP4 residues																	
	T	S	T	T	S	S	T	S	S	T	T	T	T	S	T	S	S	T
Kinases	24	27	28	34	41	59	77	89	99	122	136	189	192	196	199	210	214	241
CaMII			■					■	■	■	■			■		■		
CKI	■							■					■		■		■	
CKII								■										
GSK3					■	■						■	■		■	■		
PKA			■					■	■	■	■			■		■		■
PKC		■					■						■					■
PKG				■					■					■		■		

Identification of kinases responsible for Yap4 phosphorylation

We showed previously that Hog1 controls Yap4 protein levels, but not its phosphorylation (Nevitt *et al*, 2004a) and that PKA regulates negatively Yap4, as its levels are severely affected in a *bcy1* strain (Nevitt *et al*., 2004b). The crosstalk between the PKA pathway and the General Stress Response is widely known to involve PKA and the transcription factor Msn2. In fact, PKA regulates negatively the GSR by controlling Msn2 phosphorylation status and consequently its nuclear localization. As such, transcription of Msn2-dependent stress responsive genes, like *YAP4* decrease in normal growing cells and are enhanced under stress conditions (Nevitt *et al*., 2004a).

We have therefore evaluated whether, besides Yap4 levels, PKA also affects its phosphorylation. We analysed Yap4 phosphorylation in the *tpk1 tpk2 tpk3 yak1* mutant, as the null PKA mutant (*tpk1 tpk2 tpk3*) is not viable (Smith *et al*., 1998).

As shown in Fig. 3.3A, under osmotic stress, Yap4 levels are enhanced and its phosphorylation is lost in the mutant *tpk1 tpk2 tpk3 yak1* even in the absence of stress, thereby revealing that Yap4 phosphorylation is driven by PKA. This result also shows that the different subunits of PKA can phosphorylate Yap4, as this modification is maintained for all single PKA catalytic mutants. This functional redundancy was already observed for other PKA targets (Ptacek *et al*., 2005).

We also questioned whether PKA was directly responsible for the phosphorylation of Yap4 or if other intermediate kinases were participating in it. Indeed, it is well known that Yak1 is phosphorylated by PKA, which in turn phosphorylates the regulatory subunit of PKA (Griffioen *et al*., 2001). We have therefore tested the effect of Yak1 in Yap4 phosphorylation. As the results illustrated in Fig. 3.3A show, Yak1 is not affecting Yap4 phosphorylation. Furthermore, Sch9 and Rim15 that act in PKA-related pathways do not impair Yap4 phosphorylation (Fig. 3.3A and B).

The analysis of Yap4 amino acid sequence reveals several consensus sequences for PKC1-dependent phosphorylation (Table I), suggesting that Yap4 could also be phosphorylated in a PKC1-dependent manner. Bermejo *et al*. (2008) reported that cell wall damage caused by zymolyase requires the sequential activation of Hog1 and Slt2. Stronger evidence linking Yap4 and the PKC pathway was found by Basmaji *et al*. (2006). Using as a bait in a two-hybrid assay Knr4 (Smi1), a yeast protein implicated in coordinating cell wall synthesis with bud emergence, they found among the 10 interacting proteins, Yap4 and the three components of the PKC1 pathway Pkc1, Slt2 and Rlm1. Since Slt2 is the MAP kinase of the PKC1 pathway, we also assayed Yap4 phosphorylation in the *slt2* mutant. The results illustrated in Fig. 3.3C show that it is not involved in Yap4 phosphorylation under different stress conditions.

Ptacek *et al*. (2005) proposed a phosphorylation map where the phosphorylation events that occur in yeast are indicated. According to this database (see http://networks.gersteinlab.org/phosphorylome/) the kinases Ste20, Ptk2 and Mck1 are able to phosphorylate Yap4 *in vitro*. We put forward the question of whether these proteins could be good candidates as an intermediate kinase between PKA and Yap4. However, in every stress conditions tested, Yap4 phosphorylation was not impaired in any of the *mck1*, *ste20* or *ptk2* mutants (Fig. 3.3D). The fact that, unlike S196, T192 is a consensus phosphorylation site for Gsk3 and not PKA, and mutation of both residues abolishes Yap4 phosphorylation, led us to investigate the *mck1* mutant. Mck1 is one of the four yeast orthologs of the mammalian Gsk3 kinase (Hirata *et al*., 2003), and it is plausible to assume that in the *mck1* mutant, the other orthologs (Rim11, Mrk1 and Yol128c) are phosphorylating Yap4, this being the reason why we do not see impairment of its phosphorylation.

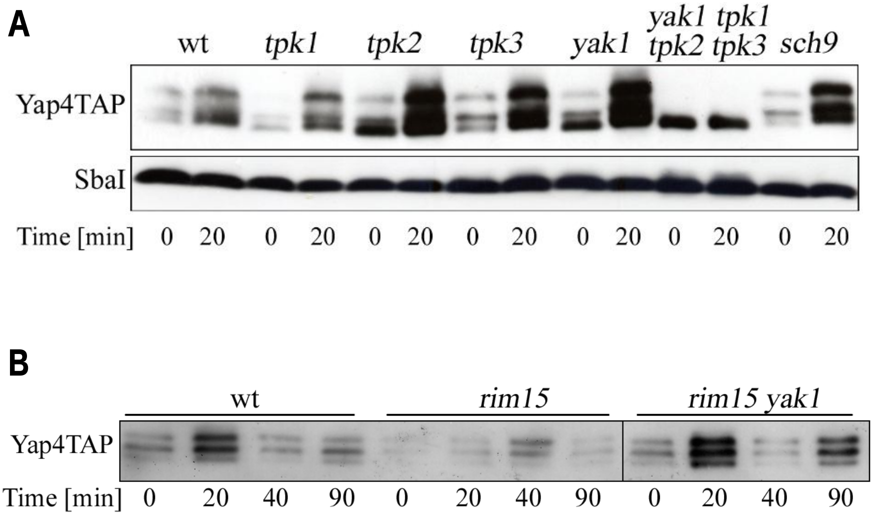


Fig. 3.3: Yap4 phosphorylation is dependent on PKA (continue in the next page)**.**

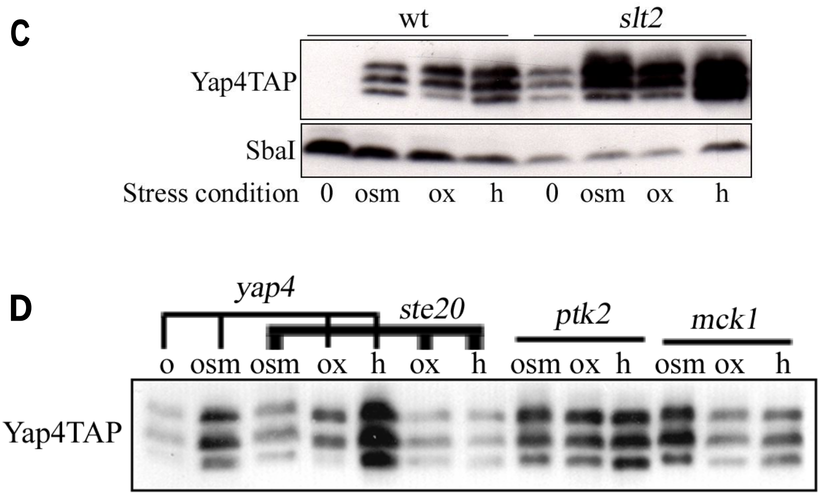


Fig. 3.3: Yap4 phosphorylation is dependent on PKA (continuation of the previous page).

Kinase-defective mutants were assayed for Yap4 phosphorylation under different stress conditions. Yap4 phosphorylation analysis in the mutants *tpk1 tpk2 tpk3 yak1*, *tpk1*, *tpk2*, *tpk3*, *yak1* and in *sch9* (A), *rim15* and *rim15 yak1* (B), *slt2* (C) and *ste20*, *ptk2* and *mck1* (D). All the mutants were tested under osmotic (**osm -** 20min 0.4M NaCl), oxidative (**ox -** 30min 0.3mM H_2O_2) and heat (**h -** 30min 37°C) stress. Protein extraction, separation, transfer and immunoblotting were performed as described in *Material and Methods* (Annex B, page 139).

Detection of the phosphorylated residues in Yap4

The prediction of multiple phosphorylation sites in Yap4 led us to perform site-directed mutagenesis in order to evaluate their contribution to phosphorylation. We investigated whether protein phosphorylation profile changed according to the stress condition. We decided therefore to mutate to alanine the residues potentially phosphorylated by PKA as indicated in Table I. As can be seen in Fig. 3.4A, S196A is the only mutation that abolishes Yap4 phosphorylation. All the other PKA dependent residues are left unaltered (Fig. 3.4B). This result does not support Budovskaya *et al.*, (2005) predictions about the conservation of PKA-consensus serine phosphorylation in several yeast related species. These authors assumed that a higher degree of evolutionary conservation of those sites would correspond to functional phosphorylation sites *in vivo*. They identified Yap4 S99 and S210 as putative PKA phosphorylation sites, but our data show that Yap4 phosphorylation is not impaired in the ^{S99A}Yap4 and ^{S210A}Yap4 mutants (Fig. 3.4B).

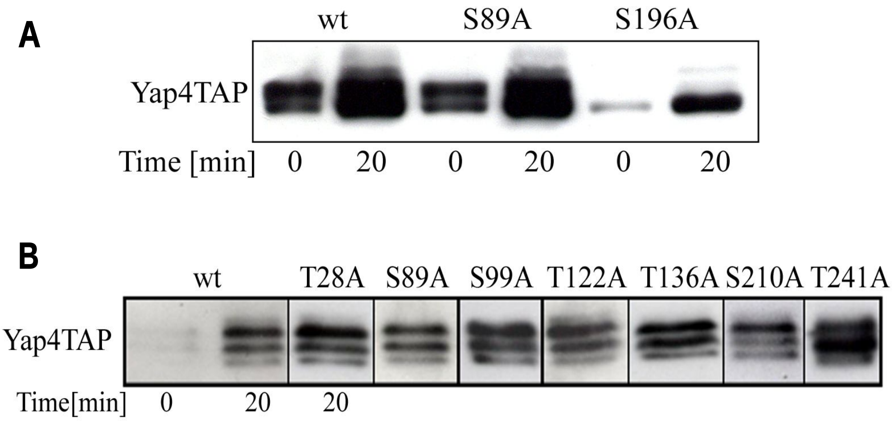


Fig. 3.4: S196A mutation abolishes Yap4 phosphorylation.

The *YAP4* residues potentially phosphorylated by PKA indicated in Table I were mutated to alanine by site-directed mutagenesis and the TAPtagged mutated versions were integrated in *yap4* strain. Mid-log phase cells were harvested after induction with 0.4M NaCl at the indicated time points and analyzed by western blot. (A) Mutations in Yap4 residues that did not affect its phosphorylation. (B) S196A abolishes Yap4 phosphorylation (see *Material and Methods* in Annex B, page 139).

Previously we obtained evidences that S89A and T241A mutations affected Yap4 phosphorylation and levels. However, using higher resolution SDS-PAGE gels, we could not observe significative differences between these mutants and the native protein (see also Fig. 3.5, page 94). Western blots revealed the presence of three bands suggesting that Yap4 protein is phosphorylated at least in two different residues. As T192 and S196 are flanking the basic residues R193 and

K194 of nls1, we assumed that phosphorylation of T192 together with S196 could modulate Yap4 import. We have therefore mutated T192 and this indeed affects Yap4 phosphorylation, as can be seen in Fig. 3.5A. Similar result is obtained using the double mutant ^{T192AS196A}Yap4 (Fig. 3.5A). In fact the kinetics of both phosphorylated and non-phosphorylated Yap4 are very similar (Fig. 4.5B).We decided to test other putative phosphorylation sites indicated in Table I, as we did before for PKA consensus sites, but none of them, besides T192A, affect Yap4 phosphorylation (Fig. 3.5C). We also evaluated if mutation of different Yap4 residues could impair its phosphorylation under different stress conditions. Upon an osmotic, oxidative, heat and arsenic challenges, the pattern of phosphorylation reveals for all the stresses that the same residues (T192 and S196) are preventing Yap4 phosphorylation (Fig. 3.5D). As a control, we used a predicted PKA-dependent mutant, T241A, whose phosphorylation is not affected.

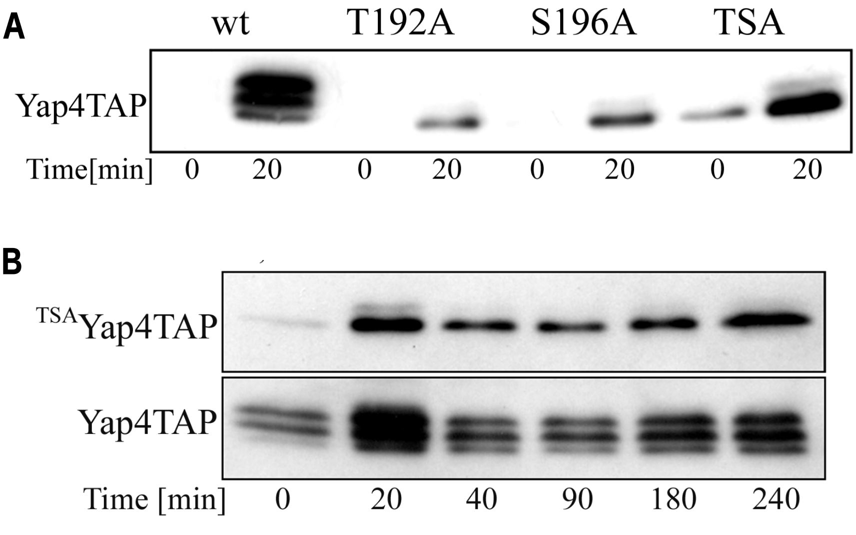
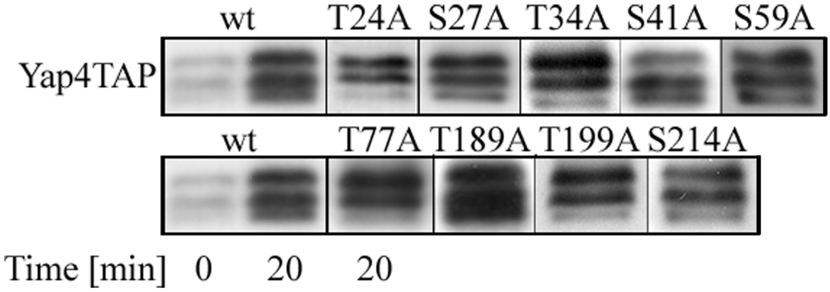


Fig. 3.5: T192A, S196A and T192AS196A mutations abolish Yap4 phosphorylation under different stress conditions (continue in the next page).

C



D

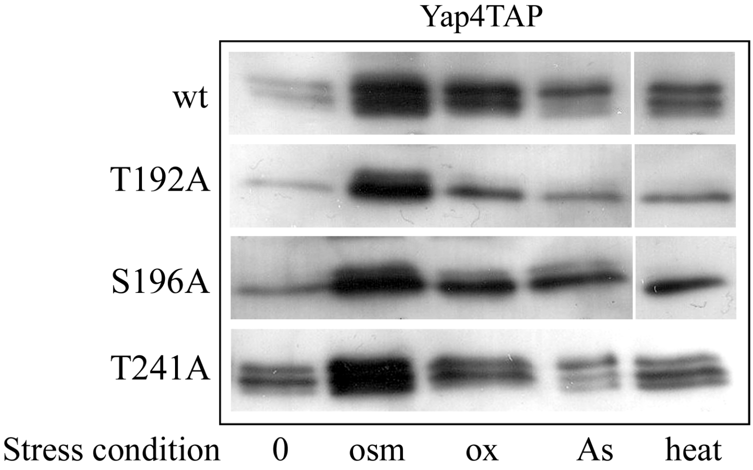


Fig. 3.5: T192A, S196A and T192AS196A mutations abolish Yap4 phosphorylation under different stress conditions (continuation of the previous page).

The Yap4 residues indicated were mutated to alanine by site-directed mutagenesis and the TAPtagged mutated versions were integrated in *yap4* strain. Mid-log phase cells were harvested after induction with 0.4M NaCl at the indicated time points and analyzed by western blot. (A) Mutations T192A, S196A and T192AS196A impair Yap4 phosphorylation under osmotic stress. (B) The remaining mutations in the consensus residues for the kinases indicated in Table I did not produce any modification in Yap4 phosphorylation. (C) T192A, S196A and T192AS196A (TSA) mutations abolish Yap4 phosphorylation under different stress conditions. Mid-log phase cells expressing the indicated *YAP4* mutations were harvested after induction with 0.4M NaCl (**osm**, 20min), 0.3mM H_2O_2 (**ox**, 30min), 2mM Na_2HAsO_4 (**As**, 30min) and after 30min of growth at 37°C (**h**). Protein extraction, separation, transfer and immunoblotting were performed as described in *Material and Methods* (Annex B, page 139).

Yap4 nuclear localization is independent of its phosphorylation

Many important transcription factors contain phosphorylation sites within or adjacent to a classic NLS. Several studies show that the nuclear localization of these proteins can be regulated by phosphorylation at these sites. Msn2 localization, for example, is controlled by the level of phosphorylation of its NLS, (Gorner *et al*., 1998 and 2002; Garmendia-Torres *et al*., 2007, De Wever *et al*., 2005).

In Yap4, the two residues affecting its phosphorylation (T192 and S196) are just flanking the key residues R193 and K194 of Yap4 predicted bipartite NLS (Fig. 3.2, page 88), suggesting that phosphorylation of T192 and S196 could be a mechanism of activation of Yap4 NLS. We therefore hypothesized that phosphorylation of Yap4, a nuclear resident protein, might regulate its nuclear import. In order to test this hypothesis, Yap4, ^{T192A}Yap4 and ^{S196A}Yap4 single mutants and ^{T192AS196A}Yap4 double mutant were fused to GFP and analysed for their localization in the nucleus.

The results shown in Fig. 3.6 reveal that the phosphorylated form of Yap4 as well as the single mutants GFPT^{192A}Yap4, and GFP^{S196A}Yap4 and the double mutant GFP^{T192AS196A}Yap4 localize into the nucleus, independent of its phosphorylation state. This observation indicates that Yap4 phosphorylation is not responsible for Yap4 localization. This result was further substantiated by the fact that in the PKA null mutant *ptk1 ptk2 ptk3 yak1*, in which Yap4 is not modified, it is still localized in the nucleus (Fig. 3.6E).

Based on this information we assessed the physiological consequences of the absence of phosphorylation. As Fig. 3.7 indicates, overexpression of the non-phosphorylated Yap4 mutants (^{T192A}Yap4, ^{S196A}Yap4 and

^{T192AS196A}Yap4) rescues the growth phenotype of the *hog1* mutant under osmotic stress that we previously reported (Nevitt *et al.*, 2004a). It seems therefore that Yap4 physiological function is not affected by the absence of its phosphorylation at least at this level.

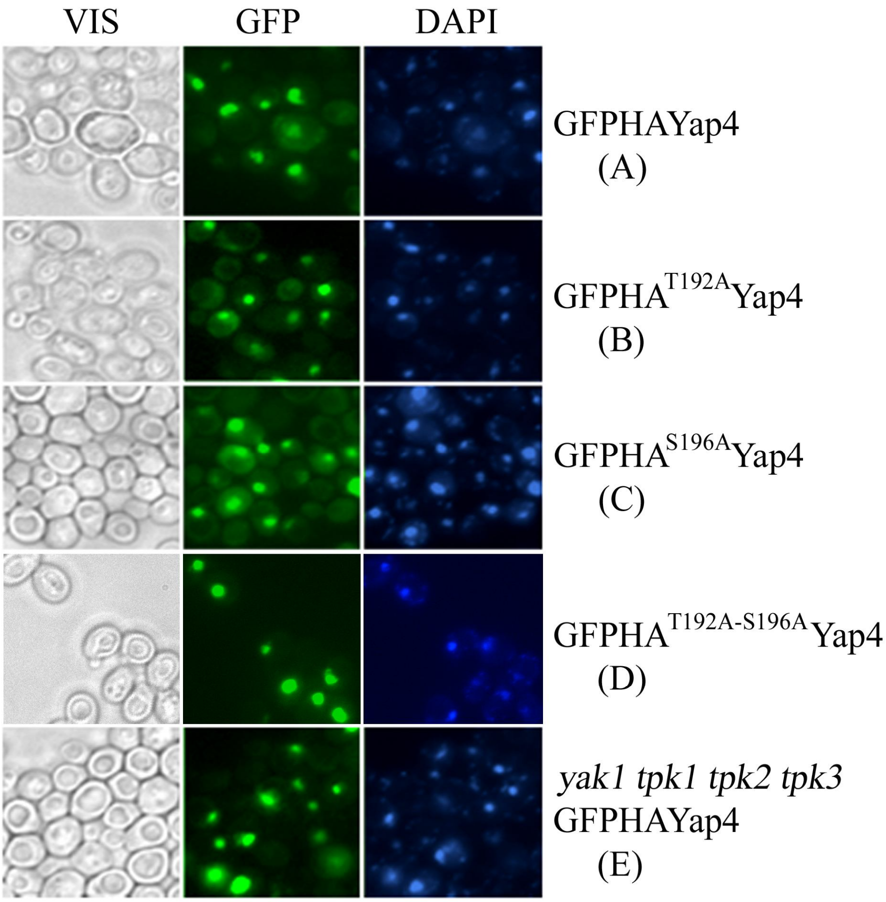


Fig. 3.6: Non-phosphorylated Yap4 localizes in the nucleus.

yap4 mutant cells expressing episomal wild type and non-phosphorylated versions of GFPYap4 (GFP^{T192A}Yap4, GFP^{S196A}Yap4 and GFP^{T192AS196A}Yap4) and the PKA null mutant cells *tpk1 tpk2 tpk3 yak1* expressing episomal GFPYap4 were harvested at mid-log phase and observed under fluorescent microscopy as described in *Materials and Methods* (Annex B, page 139).

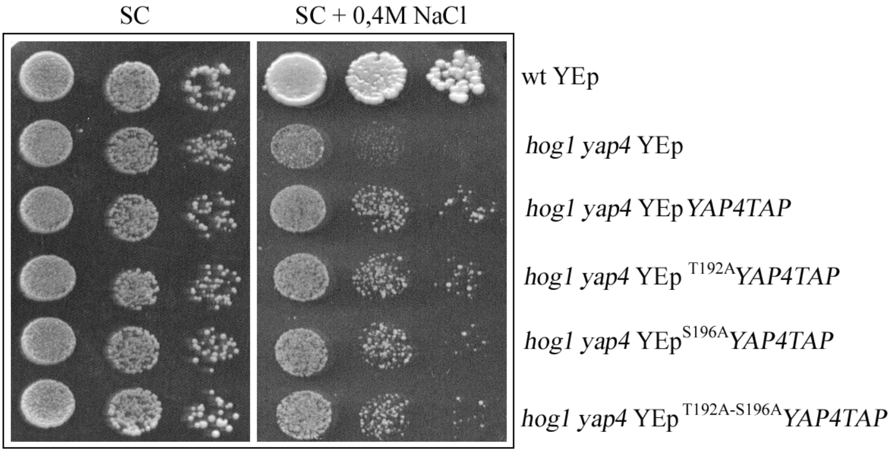


Fig. 3.7: Absence of phosphorylation does not compromise Yap4 ability to rescue the *hog1* osmosensitive phenotype.

The double mutant *hog1 yap4* was transformed with YEp356R expressing *YAP4* wild type and mutant alleles. Mid-log phase cells were serially diluted and spotted onto SC medium supplemented or not with 0.4M NaCl and incubated at 30ºC for 2 days.

Absence of Yap4 phosphorylation does not seem to affect transcription of target genes

Previously we observed that *DCS2* transcription was dependent on Yap4 (Nevitt *et al*., 2004), and this dependence was more accentuated when cells were grown in a medium supplemented with glycerol as a carbon source (our unpublished results, see Chapter II, page 69). Therefore we also evaluated *DCS2* expression in the absence of Yap4 phosphorylation upon osmotic stress and glucose starvation, growing cells with a medium supplemented with glycerol. As Fig. 3.8 shows, Dcs2 expression does not seem to be affected by loss of Yap4 phosphorylation.

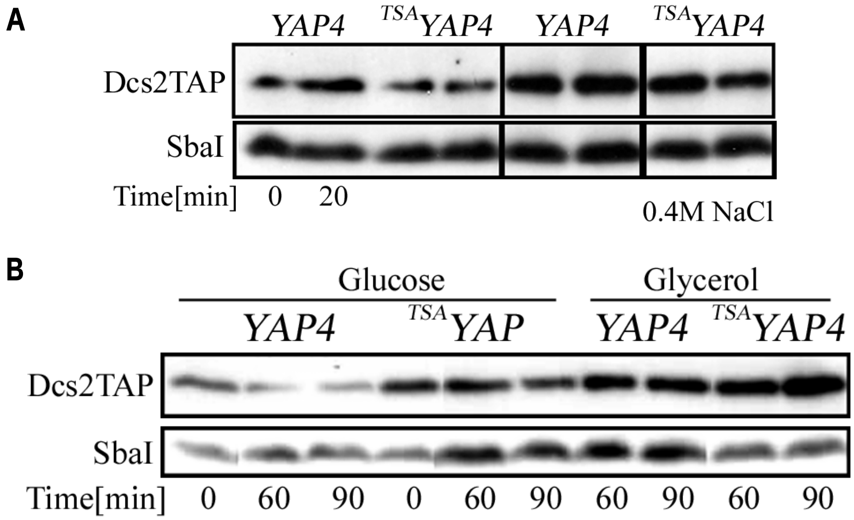


Figure 3.8: Lost of Yap4 phosphorylation does not seem to affect the expression of Dcs2.

YAP4 was deleted in a strain containing a tagged *DCS2* (*DCS2TAP*). Then, wild type *YAP4* or ^{T192AS196A}*YAP4* (^{TSA}*YAP4*) was integrated and cells were grown to early-exponential phase and exposed to 0.4M NaCl (A) or alternatively early-exponential phase growing cells were shift to a rich medium where glucose was substituted by glycerol (B). Samples were harvested at the indicated time points indicated. Protein extraction, separation, transfer and immunoblotting were performed as described in *Material and Methods* (Annex B, page 137).

The expression of the hexose transporter *HXT5* was also found by us to be diminished in the *yap4* mutant strain (our unpublished results). Therefore, we also evaluated if Yap4 phosphorylation affects the expression of this glucose transporter. Our results show that *HXT5* expression is significantly increased in a strain expressing the non-phosphorylated Yap4 (Fig. 3.9), suggesting that Yap4 phosphorylation could modulate the expression of its target genes.

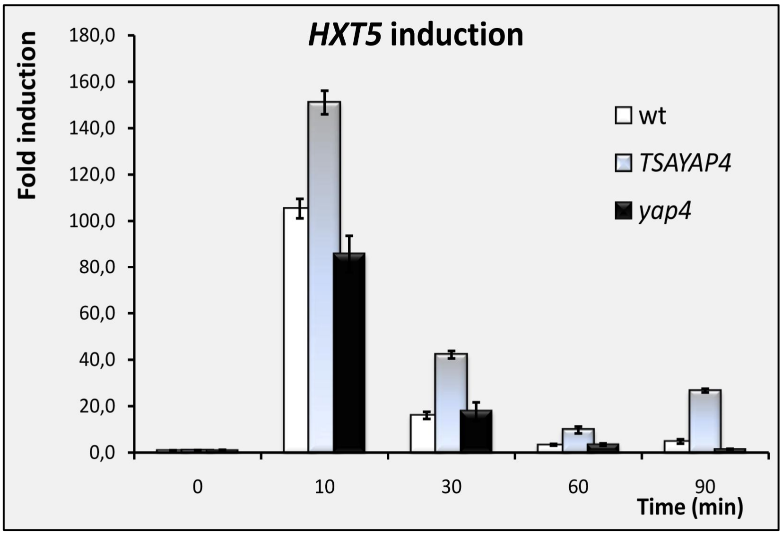
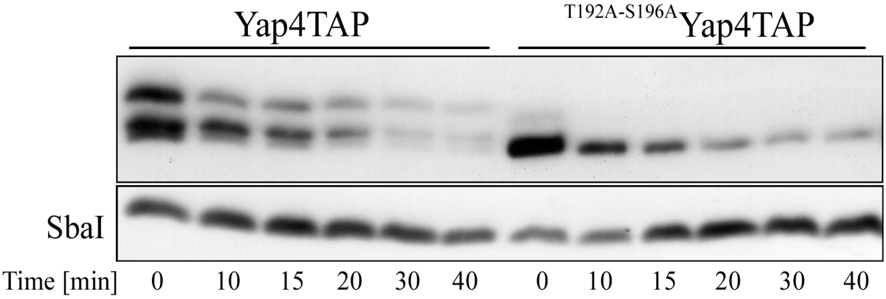


Figure 3.9: Lost of Yap4 phosphorylation increases the expression of *HXT5*.

Wild type *YAP4* or ^{T192AS196A}*YAP4* (^{TSA}*YAP4*) was integrated in a *yap4* mutant strain and cells were grown to early-exponential phase and induced with 0.4M NaCl. The mRNAs were extracted and *HXT5* expression was measure by real-time PCR as described in *Materials and Methods* (Annex B, page 139).

Yap4 protein stability is dependent on its phosphorylation

It is well known that the activity of many transcription factors is modulated by post-translational modifications and phosphorylation is one of the most frequent event. This modification can affect their activity through its stability, cellular localization, protein-protein interaction, DNA-binding activity, transcriptional activity (Holmberg *et al*., 2002). Protein phosphorylation can represent therefore a critical process for modulating the life-span of a protein. In order to evaluate whether the phosphorylation of Yap4 could affect its stability, we have performed experiments to measure the half-life time of the non-phosphorylated and phosphorylated forms of Yap4. Cell extracts were obtained from *yap4* mutant expressing integrated tagged versions of the *YAP4* and ^{T192AS196A}*YAP4* mutant, both untreated and treated with cycloheximide, an inhibitor of protein synthesis (Belle *et al.*, 2006). As can be seen in Fig. 3.10, Yap4 stability is significantly affected by the abolishment of phosphorylation with values of T½ for the wild-type of 12.1 ± 1.1 min and 7.9 ± 0.7 min for the ^{T192AS196A}Yap4 mutant (a 35% decrease). These values are comparable for other Yap members (Belle *et al.*, 2006). Our data were normalized against SbaI (ortholog of p23 in mammalian cells), which is a good control since its half-life is of 280 min (Belle *et al.*, 2006). Our results show therefore that phosphorylation of Yap4 can modulate the half-life of Yap4 protein.



T½	12.1 ± 1.1 min	7.9 ± 0.7 min

Fig. 3.10: Yap4 stability is partially dependent on phosphorylation.

The half-life (**T½**) of Yap4TAP and ^{T192AS196A}Yap4TAP was measure and the respective values are indicated in the box below. ^{T192AS196A}Yap4 is 35% less stable than its wild type counterpart. Protein levels of the co-chaperone SbaI, which has a half-life of 280 min, were used as loading control (Mollapour *et al.*, 2007). The time points indicated are relative to the addition of cycloheximide after cells have been induced for 20 min with 0.4M NaCl. This western blot is representative of three independent assays and the half-life and respective standard deviation calculations were performed using those assays as described in Belle *et al.*, 2006. Protein extraction, separation, transfer and immunoblotting were performed as described in *Material and Methods* (Annex B, page 139).

Conclusions

In this work we evaluated the potential role of Yap4 phosphorylation in its regulation. We showed that Yap4 phosphorylation is PKA-dependent. We further analyse the existence of an intermediate kinase between PKA and Yap4. We test the effect of Yak1 in Yap4 phosphorylation, as well as of Sch9 and Rim15, that act in PKA related pathways. Our results clearly show that Yap4 phosphorylation in not impaired in any of this kinase mutants' strain. Slt2, the MAPK of the PKC pathway, also does not affect Yap4 phosphorylation. The same result was obtained for the kinases Ste20, Ptk2 and Mck1, predicted to phosphorylate Yap4 *in vitro*. However, our data does not exclude the existence of other kinases phosphorylating Yap4 as mutation of Yap4 residues T192 and S196 to alanine abolishes its phosphorylation, but T192 is not a predicted PKA phosphorylation site. Absence of Yap4 phosphorylation was shown not to abolish its ability to rescue the severe osmosensitivity of the *hog1* mutant strain, but the stability of the protein is compromised, exhibiting a 35% decrease of its half-life.

The function of the transcription factor Yap4 is not yet completely understood. It is possible that its phosphorylation may be required for interaction with the basal transcriptional machinery. One the other hand, Yap4 can function as a docking protein to bind several factors involved in transcription. More experiments are being carried out to support these assumptions.

Chapter IV

Determinants of Yap4 localization

Results and Discussion

This chapter is based on ongoing work and some results included in the manuscript:

Pereira J *et al*., (2008). Yap4 PKA-dependent phosphorylation affects protein stability but not its localization

(submitted to *Yeast*)

(A copy of this manuscript can be found in the end of the thesis)

Summary

Yap4 is a nuclear resident protein and its import into the nucleus, unlike Yap1, does not involve the importin Pse1.

Yap4 has two different predicted NLSs, one a classic N-terminal NLS, that we show not to be functional, and the other a bipartite C-terminal NLS. The first strech of the bipartite NLS contains two residues whose mutation was shown to abolish Yap4 phosphorylation, suggesting that Yap4 may be phosphorylated within its NLS, what would interfere with its import. Here we also show that deletion of Yap4 leucine zipper impairs its nuclear localization, suggesting that Yap4 dimerization precedes its import. Further studies are being carried out to characterize Yap4 import into the nucleus.

Introduction

A fundamental difference between prokaryotic and eukaryotic cells resides in the compartmentalisation of the genetic material in a specific organelle, the nucleus, individualized by the nuclear membrane. This membrane is crossed by several complex structures, the nuclear pore complexes (NPC), which allow the traffic of molecules between the nucleus and the cytoplasm. NPC prevents passive diffusion of macromolecules larger than 40 kDa, although, in some particular cases proteins smaller than 20-30kDa, such as histones, are actively assisted by carriers to cross the NPC (Lange *et al*., 2007 and references therein). In fact, the transport of macromolecules through the NPC is mediated and the classical nuclear import pathway is one of the best-characterized systems for this transport. In this pathway, a protein containing a basic nuclear localization signal (NLS) is imported by a heterodimeric import receptor. This import receptor is formed by an adaptor protein, the importin, which directly binds the classical NLS of the target protein, and also the β-karyopherin (importin β) that mediates interactions with the nuclear pore complex (reviewed in Lange *et al*., 2008).

The classical NLS (cNLS) for nuclear protein import is composed by only one (monopartite) or two (bipartite) stretches of basic amino acids. Monopartite cNLSs are exemplified by the SV40 large T antigen NLS (^{126}PKKKRRV^{132}) and bipartite cNLSs are exemplified by the nucleoplasmin NLS (^{155}KRPAATKKAGQAKKKK^{170}) (Lange *et al*., 2008). However, there are plenty of examples in the literature of proteins possessing NLSs with longer amino acid regions rather than a short peptide motif like the classical NLS. Isoyama *et al*. (2001), for example, showed that Yap1 import into the nucleus involves the importin β

family member Pse1, but the Yap1 NLS, located in its N-terminal region, is a bipartite non-classical NLS, composed by the amino acid stretches spanning from amino acid 5 to 16 (^{5}TAKRSLDVVSPG^{16}) and from amino acid 50 to 59 (^{50}KKKGSKTSKK^{59}). Moreover, basic residues K7 and R8 are essential for the NLS as their mutation to alanine is enough to block the nuclear import. Interestingly, the regulation of Yap1 activity does not happen during its import, which is constitutive, but rather occurs at the level of nuclear export. Yap1 also possesses a NES with which the exportin Crm1 interacts. However, upon oxidative stress, the interaction between Yap1 and Crm1 is prevented and Yap1 accumulates in the nucleus (reviewed in Rodrigues-Pousada *et al*., 2004).
Isoyama *et al*., (2001) suggest that the fact that Yap1 NLS is located next to the DNA binding domain, a feature that is shared with many other transcription factors (including Gal4 DNA binding domain, the basic leucine-zipper domain including the CAAT/enhancer-binding protein and v-jun, basic helix-loop-helix domain, homeodomain, and high-mobility group domain), could protect their DNA binding domains and inhibit the DNA binding activity until these factors reach an appropriate DNA binding site. The binding of the transcription factor to DNA may accelerate the dissociation of the receptor-cargo interaction.

In numerous cases, protein import into the nucleus is regulated by phosphorylation and Harreman *et al.* (2004) review three possible mechanisms through which this could happen. First, phosphorylation could cause a conformational change of the protein, which reveals or masks an NLS sequence. This is the case of the growth regulatory protein STAT (signal transducers and activators of transcription) where phosphorylation causes the protein to dimerize creating an NLS. A second mechanism involves the release or binding of an NLS masking protein upon phosphorylation, as it occurs in the p65 subunit of NF-κB. In this case, the inhibitor I-κB is bounded to NF-κB and phosphorylation of this sub-unit leads to its degradation, unmasking the NF-κB NLS. In these two hypotheses, phosphorylation can occur in any site within the protein. However, when phosphorylation occurs within or adjacent to the NLS, this may modulate the affinity of an NLS for its import receptor importin α. To challenge this last mechanism, Harreman *et al*. (2004) demonstrate that mimicking phosphorylation at a site adjacent to an NLS decreases the binding affinity of the NLS for importin α. Moreover, they showed that the cell cycle-dependent nuclear import of *Saccharomyces cerevisiae* transcription factor Swi6 correlates with a phosphorylation dependent change in affinity for importin α, being the phosphorylated form of Swi6 predominantly cytoplasmic and the hypophosphorylated form more abundant in the nucleus. This seems also to be the case of v-Jun oncoprotein, where phosphorylation may directly modulate interactions with the NLS receptor, or Pho4, whose NLS phosphorylation reduces its affinity for the importin Pse1, preventing Pho4 import into the nucleus (Kaffman et al., 1998).

These observations from Harreman *et al*. (2004) suggest that the phosphorylation of a residue within the NLS implies the addition of a negatively charged phosphate group inside this basic region and this may weak the electrostatic interactions between the NLS and the importin α that are necessary for the import process. Obviously, depending on the extension of the phosphorylation and the distance of the phosphorylated residue to the NLS, this negative modulation of nuclear localization through phosphorylation may be in some cases only partial, not totally impairing protein import, as Harreman *et al.*(2004) verified in their model with Swi6.

This seems to be also the case of the regulation of Msn2 localization through phosphorylation of its NLS. In fact, Msn2 is heavily phosphorylated in its NLS in a PKA-dependent manner and this form is conserved mainly in the cytoplasm, while the unphosphorylated Msn2 predominantly localises in the nucleus (Gorner *et al.*, 1998 and 2002; Garmendia-Torres *et al.*, 2007).

The Yap family is heterogeneous regarding to the localization of its members in the cell. Traffic of Yap1, Yap2 and Yap8 is tightly regulated as they possess both NLS and NES, being shuttled in and out of the nucleus under normal growth conditions and at least for Yap1, only the nuclear export is modulated. Upon specific stress conditions the interaction between their NES and the exportin Crm1 is blocked and they accumulate in the nucleus (reviewed in Rodrigues-Pousada *et al.*, 2004). Yap4 and Yap6, however, are nuclear resident proteins and are predicted to have NLS but not any NES.

Here we reported that, unlike Yap1, Pse1 does not mediate Yap4 import. Furthermore, the prediction of Yap4 NLS retrieved two candidate stretches, one N-terminus and another C-terminus. This last NLS is conserved along the Yap family and contains two residues that abolish Yap4 phosphorylation when are mutated, suggesting a putative mechanism of modulation of Yap4 localization through phosphorylation. The data obtained show that Yap4 LZ is also important for Yap4 localization, although it does not possess any NLS activity, indicating that Yap4 dimerization may precede its translocation into the nucleus. Ongoing work will further characterize Yap4 import into the nucleus.

Results and Discussion

Yap4 is imported into the nucleus independently of Pse1

It is known that the nuclear import of Yap1, the well-studied member of the Yap family, is mediated by the importin Pse1 (Isoyama *et al*., 2001). To verify whether Yap4 localization would also be mediated by this importin we have used the conditional *pse1-1* mutant. If this hypothesis is correct, the blocking of Pse1 would lead to a Yap4 cytoplasmic distribution, allowing us to follow its phosphorylation. However, we observed that Yap4 is not transported to the nucleus exclusivelly by this importin as in the conditional mutant *pse1-1* Yap4 is still present in the nucleus (Fig. 4.1, midle panel).

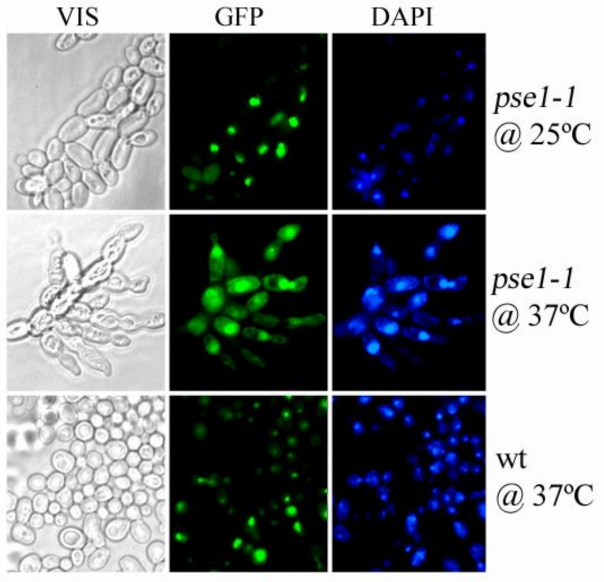


Fig. 4.1: Yap4 import into the nucleus is not mediated by the importin Pse1.

Wt and *pse1-1* conditional mutants cells were transformed with episomal GFPHAYap4 harvested at mid-log phase (growth under permissive (25ºC) and non-permissive temperature (37ºC) and observed under fluorescent microscopy as described in *Materials and Methods* (Annex B, page 139).

Determinants for Yap4 nuclear localization

In contrast to Yap1, Yap2 and Yap8, which are only localized in the nucleus after stress, Yap4 is a nuclear resident protein. Predictions based on its amino acid sequence using the ScanProsite (Hulo *et al*., 2007) and PSORT II (//psort.ims.u-tokyo.ac.jp) programmes and alignment with the remaining Yap members (Fernandes *et al*., 1997) indicates two putative Yap4 NLS, one N-terminus and other C-terminus (for a detailed view of the alignment of whole Yap family, see Annex A, page 135). One of the predicted Yap4 NLS is the N-terminal amino acid sequence KPKK that Goodin *et al*. (2001) identified in the plant nucleorhabdovirus Sonchus Yellow Net Virus as a NLS, showing to be enough to import into the nucleus the chimera GFP-GST in yeast. The C-terminus NLS is a non-classic bipartite one, composed by two short stretches of basic amino acids separated by a nonconserved sequence of 32 residues. Those stretches spans the residues ^{193}RKNSATTNLPSEERRRV^{208} (nls1) and the residues ^{243}KRAAQNRSAQKAFRQRR^{258} (nls2), being this last one coincident with the Yap4 DNA-binding domain (Fig. 4.2), a feature that is conserved among all Yap members, even Yap3, 5 and 8 whose predicted NLSs are not bipartite (please consult Annex A and Table II for a detailed view, page 135). Strikingly, residues T192 and S196, which were shown to abolish Yap4 phosphorylation, are just flanking the first two basic residues of the nls1 (R193K194), what, according to the observations of Harreman *et al.* (2004), strongly suggest that phosphorylation of these two residues would modulate Yap4 nuclear import.

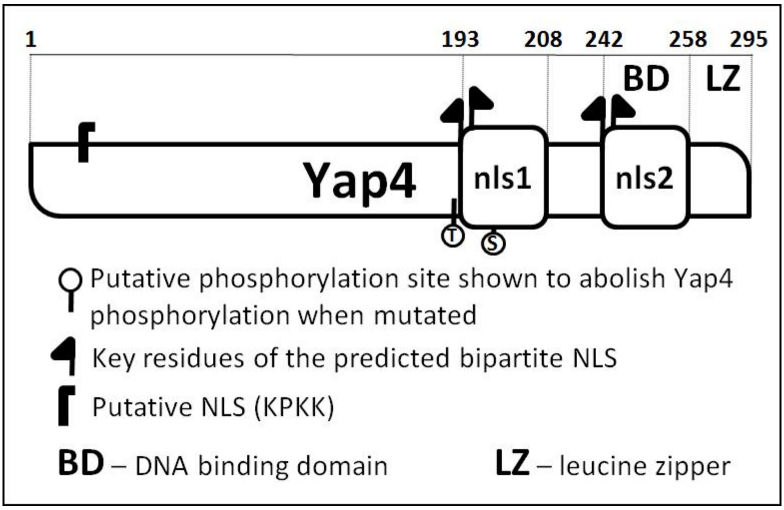


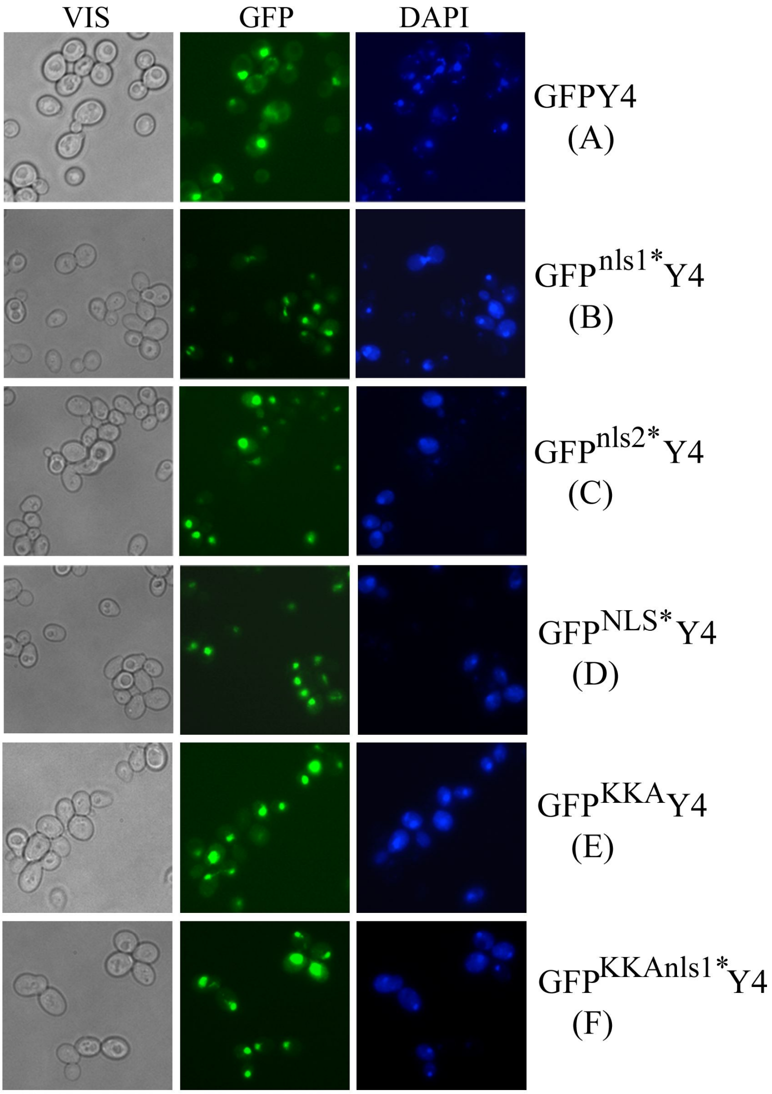
Fig. 4.2: Schematic representation of predicted Yap4 structural domains.

The BD, LZ and bipartite NLS were retrieved ScanProsite (Hulo *et al.*, 2007) and PSORT II programmes (psort.ims.u-tokyo.ac.jp) and by alignment with the remaining Yap members; (Fernandes *et al.*, 1997).The highlighted “T” and “S” putative phosphorylation sites are respectively T192 and S196, shown to abolish Yap4 phosphorylation when mutated to alanine. For a detailed view of the conservation of NLS features, please consult Annex A, page 131).

Our first approach to study these determinants of Yap4 localization was to mutate the basic residues of its predicted NLSs. In the case of Yap1, Isoyama *et al*. (2001) mutated the basic residues of the first stretch of its NLS, K7 and R8, to alanine, and this was enough to abrogate the NLS activity. We thus mutate the basic residues of both Yap4 NLS stretches (K31AK32A in the N-terminal predicted NLS and R192AK193A (nls1) plus K242AR243A (nls2) in the C-terminal predicted NLS). However, these mutations, even when combinated in the same construct, were not enough to prevent Yap4 nuclear localization (Fig. 4.3).

Remarkably, although nuclear, the ^{R193AK194AK242AR243A}Yap4 and ^{K31AK32AR193AK194A}Yap4 mutants are no longer phosphorylated (Fig. 4.4). This result suggests that the conformational changes imposed by the mutations surrounding T192 and S196, shown to abolish Yap4 phosphorylation when mutated to alanine (Pereira *et al*., submitted to *Yeast*, see Fig. 3.5, page 93), may prevent the kinase access or recognition of its binding sites in Yap4.

At this point, we decided to evaluate how these mutations were affecting Yap4 function in the stress response, verifying if they were still able to relieve *hog1* severe osmosensivity as the wild type version (Nevitt *et al*., 2004). As Fig. 4.5 shows, ^{R193AK194A}Yap4 mutant is still able to partially rescue *hog1* osmosensitive phenotype. However, that is not the case of the mutants ^{K242AR243A}Yap4 and ^{R193AK194AK242AR243A}Yap4. In these two cases, the fact two residues within the basic region of binding to the DNA (K242 and R243) were mutated, certainly affects Yap4 ability to bind to the *cis*-elements of the genes it regulates.



nls1* = R193A+K194A; nls2* = K242A+R243A; NLS* = nls1* + nls2*
KKA = K31A+K32A; KKnls1* = KKA + nls1*

Figure 4.3: Mutation of Yap4 basic residues of its predicted NLSs does not prevent its nuclear localization.

Cells transformed with episomal *GFPHAYAP4* versions containing the indicated mutations were harvested at mid-log phase and observed under fluorescent microscopy as described in *Materials and Methods* (Annex B, page 143). The * in the NLS notation means that the indicated key basic residues within each NLS were mutated.

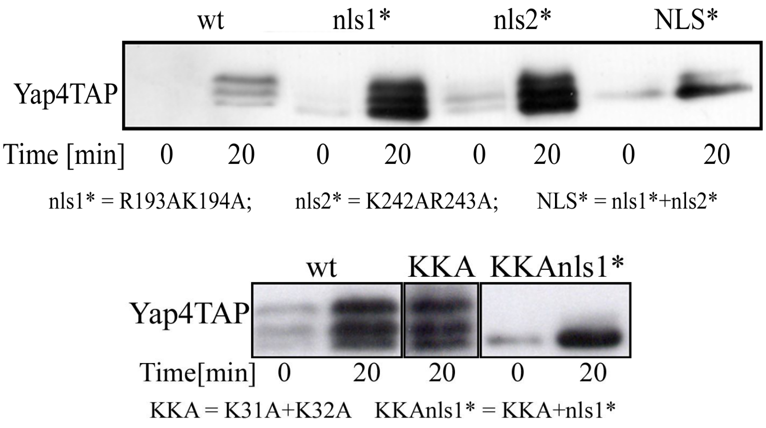


Fig. 4.4: Yap4 phosphorylation is impaired in the ^{R193AK194AK242AR243A}Yap4 and ^{K31AK32AR193AK194A}Yap4 mutants.

Analysis of protein expression and phosphorylation of the Yap4 NLS key residues mutants. Mid-log phase cells expressing the indicated *YAP4* mutations were harvested after induction with 0.4M NaCl and western blots were performed as described *in Material and Methods* (Annex B, page 139).

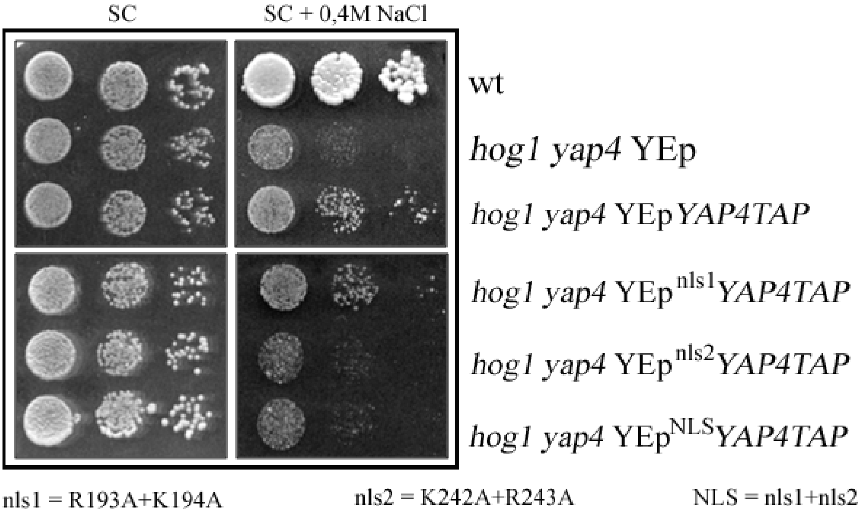


Fig. 4.5: Mutation of Yap4 NLS key residues compromises its ability to rescue the *hog1* osmosensitive phenotype.

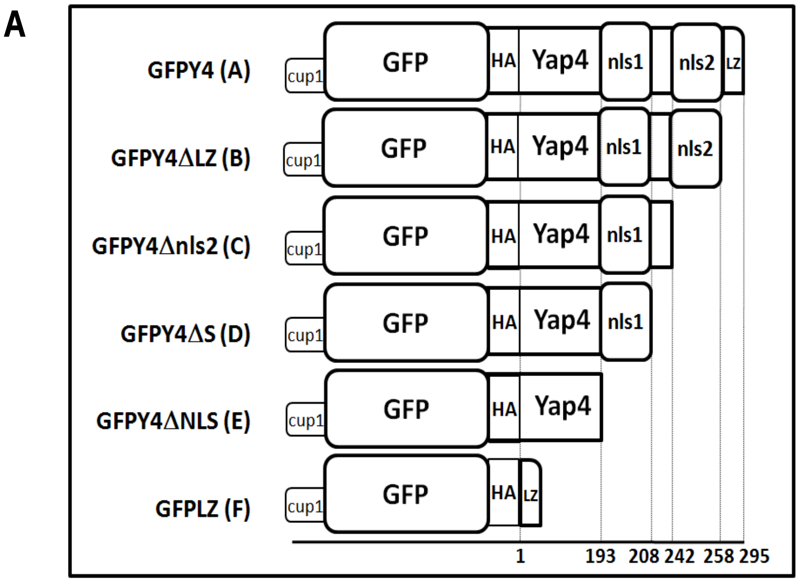
The double mutant *hog1 yap4* was transformed with YEp356R expressing *YAP4* wild type and mutant alleles. Mid-log phase cells were serially diluted and spotted onto SC medium supplemented or not with 0.4M NaCl and incubated at 30ºC for 2 days.

The fact that Yap4's nuclear localization was not abolished either in the KKA or in the KKnls1 mutant (Fig. 4.3, panel E and F, respectively, page 116) rules out the predicted Yap4 N-terminal NLS as an effective NLS, but not the C-terminal NLS as in this case both stretches possess more basic residues besides that the four that were mutated (Fig. 4.3, panel A to D, page 119). To clarify this issue, we obtained successive truncations of Yap4 fused to GFP and under *CUP1* promoter as diagrammed in Fig. 4.6A. All the constructs were assessed for stability by Western blotting and all found to be stable (Fig. 4.6B).

As Fig. 4.6C shows, GFP analysis revealed that Yap4 is localized to the nucleus (A) but after removal of the leucine zipper Yap4 nuclear localization is impaired (B). Subsequent deletions C, D, and E, which do not contain the LZ, are also all absent in the nucleus, a fact that suggests a role for LZ in the Yap4 import. In order to confirm these results we used a construct containing only the LZ domain fused to GFP. As can be seen in Fig. 4.6B (construct F), although LZ is required for nuclear localization of Yap4, it is not sufficient to drive GFP into the nucleus.

This result suggests that Yap4 dimerization could precede its nuclear localization and once LZ is deleted, Yap4 is not able to dimerize and cannot be translocated to the nucleus. A similar mechanism was described for nuclear translocation of Fos that is highly favoured by a previous heterodimerization with Jun through their leucine zippers (Chida *et al*., 1999). However, according to *in silico* data of Deppmann *et al*. (2006), and unlike the remaining Yap members, Yap4, Yap6 and Yap8 are not predicted to homodimerize or heterodimerize between them. These authors postulate that other partners not yet identified could bind these two proteins.

However, to our knowledge there are so far no experimental evidences to support these data. Further analyses are needed to clarify this putative mechanism of protein homo or heterodimerization prior to Yap4 nuclear translocation.



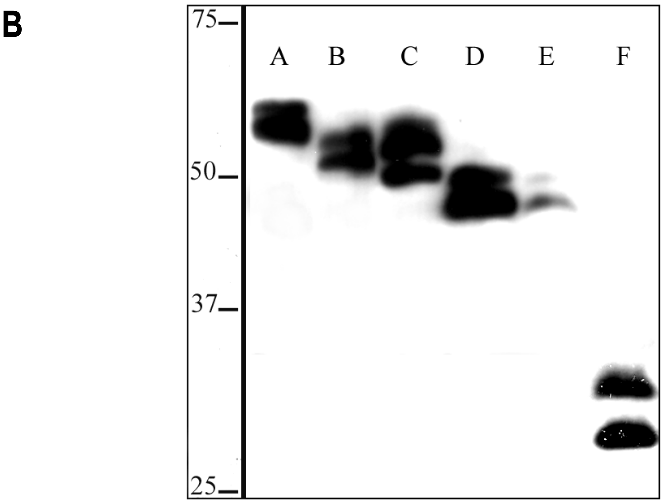


Fig. 4.6: Yap4 deletion analysis (continue next page).

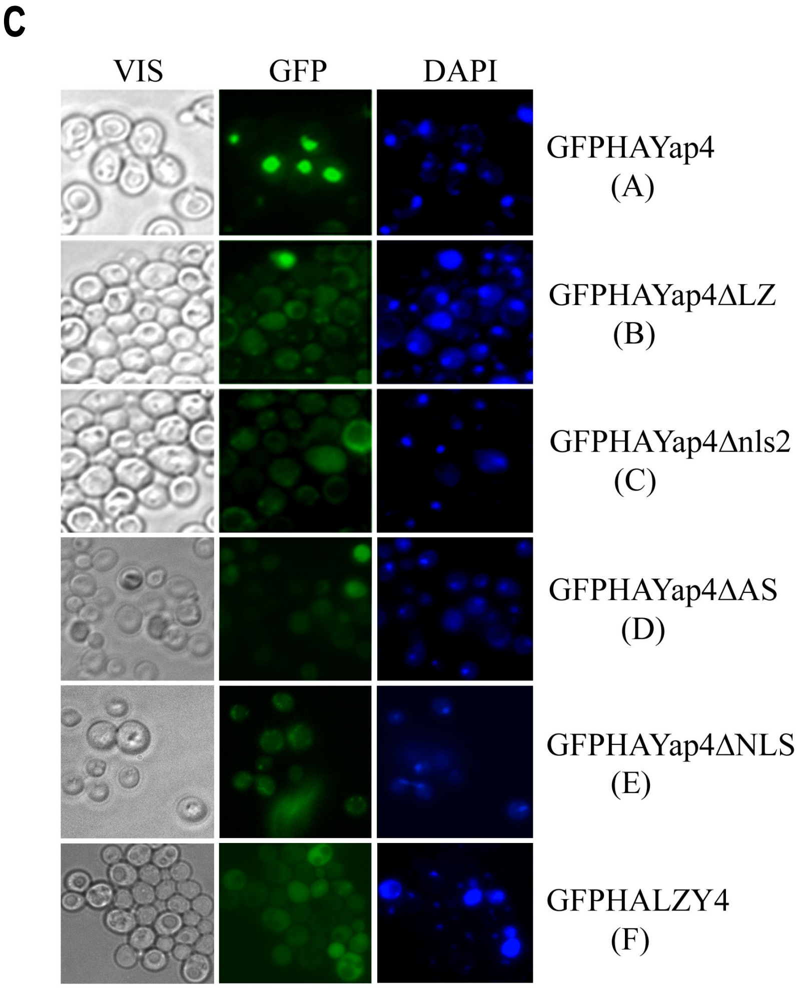


Fig. 4.6: Yap4 deletion analysis (*continuation*)

(A) The truncations were obtained as described in *Materials and Methods* (Annex B, page 139). In the lower panel is indicated the relative position of the residues of Yap4 bordering the different truncations. (B*)* Stability of the GFPHAYap4 truncations. *yap4* mutant cells were transformed with the *GFPYAP4* truncations and western blots were performed as described *in Material and Methods* (Annex B, page 139), except the antibody used in the western blot was anti-HA-Peroxidase, High Affinity 3F10 (Roche). (C) Cells transformed with episomal *GFPYAP4* versions were harvested at mid- log phase and observed under fluorescent microscopy.

A – GFPHAYap4, **B** – GFPHAYap4ΔLZ, **C** – GFPHAYap4Δnls2,
D – GFPHAYap4ΔS, **E** – GFPHAYap4ΔNLS, **F** – GFPHALZ

Conclusions

In this chapter we present our data about the nuclear translocation of Yap4. Pse1, the importin that mediates Yap1 import, is not involved in Yap4 traffic to the nucleus. As a nuclear resident protein, Yap4 should possess a NLS. The analysis of its amino acid sequence reveals two putative NLSs, being the N-terminus not a functional one. The bipartite C-terminal NLS is conserved within the members of the Yap family and contains two residues whose mutation was shown to abolish Yap4 phosphorylation. This suggests that Yap4 phosphorylation could interfere with its NLS activity. However, our data show that nuclear import of Yap4 is conserved in the absence of its phosphorylation. In addition, deletion of the leucine zipper impairs Yap4 nuclear localization, although the LZ does not have any NLS activity. Taking together, these results suggest that Yap4 dimerization may precede its import and possibly its phosphorylation only occurs in the nucleus. Further studies are being performed to evaluate this hypothesis.

Chapter V

Final conclusions and perspectives

Final conclusions and perspectives

Yeasts possess many different pathways and players involved in the adaptation to its ever-change environment. In this work we showed that *YAP4* has a broader role in maintaining cell homeostasis than previously considered. Our data show that *YAP4* is responsive to several stress conditions, including osmotic, oxidative, metal and stationary phase stress and heat shock. Under osmotic stress, *YAP4* is controlled by the HOG pathway and General Stress Response through its effectors Hog1 and Msn2, respectively. Under oxidative stress, *YAP4* is regulated by Yap1 and Msn2. Both these two mechanisms involved *YAP4* consensus *cis*-elements, more exactly the two most proximal STREs under osmotic stress and the YRE and the most proximal STRE under oxidative stress.

The protein encoded by *YAP4* is highly and transiently induced and phosphorylated upon exposure to these stress conditions. This led us to characterize the role of phosphorylation in Yap4 function. *YAP4*, a STRE-dependent gene, is negatively regulated by PKA via Msn2. Moreover, Yap4 phosphorylation is also PKA-dependent. Fig. 5 presents a model of regulation of Yap4 that summarizes our data.

Yap4 phosphorylation seems to have opposite effects. On one hand it affects Yap4 stability reducing it by 35% in the absence of phosphorylation. On the other hand, the expression of the Yap4 target gene *HXT5* is increased in a strain expressing a non-phosphorylated Yap4 version. Yap4 is a nuclear resident protein and its traffic is not affected either in a PKA null strain, in which the native Yap4 is not phosphorylated, or in a strain expressing a non-phosphorylated Yap4 version. Our data also strongly suggest that this modification involves the residues T192 and S196 that were shown to abolish Yap4 phosphorylation, when mutated to alanine. The fact that these two residues are within the NLS would therefore interfere with its activity, modulating Yap4 import into the nucleus. However our results do not support this assumption. Taking together these results suggest that Yap4 phosphorylation is a nuclear event.

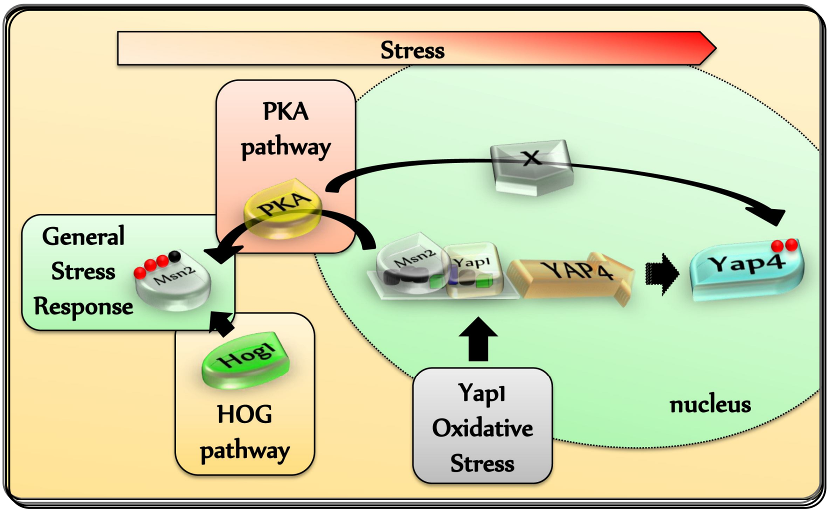


Fig. 5: Model of the different mechanisms regulating Yap4.

Yap4 regulation involves different players. Under osmotic stress, *YAP4* is regulated by the HOG pathway and General Stress Response through its effectors Hog1 and Msn2. Under oxidative stress, *YAP4* is regulated by Msn2 and Yap1. *YAP4* is also under the control of the PKA pathway at two different levels: PKA negatively regulates the expression of *YAP4* through the phosphorylation of Msn2 and also mediates, directly or indirectly, the phosphorylation of Yap4, possible in the nucleus.

It would be therefore relevant to know the importin involved in this traffic to evaluate this hypothesis. So far we only know that, unlike Yap1, Yap4 import is not affected in a compromised-Pse1 strain. Alternatively, a glutamate mutant for the residues T192 and S196 will be accessed for its nuclear localization. This approach will mimic Yap4 phosphorylation, allowing us to observe its effect in the nuclear import of Yap4.

Until now, Yap4 did not show any severe phenotype associated to its deletion. Previous work associating *YAP4* with resistance to cisplatin and other antimalarial drugs (Furuchi *et al*., 2001; Delling *et al*., 1998), as well as salt tolerance of the *ena1* mutant (Mendizabal et al., 1998) and our own work, showing the relieve of the *hog1* severe osmosensivity phentotype (Nevitt *et al*., 2004a) was performed under *YAP4* over-expression conditions. The *yap4* mutant strain does not exhibit any phenotype associated to these conditions, except a mild osmosensibility (Nevitt *et al*., 2004a). However, in a recent EMBO meeting about gene transcription in yeast (21-26 June, Sant Feliu de Guixols, Spain) B. F. Pugh (The Pennsylvania State University, USA) present genome-wide ChIP-on-Chip data revealing that upon a temperature shift, Yap4, Yap6, Xbp1 and other regulators, are found constitutively bound to the chromatin as sequence-specific regulators necessary for the proper assembly of the pre-initiation complex (PIC). This clearly imply Yap4 at the level of gene transcription and may explain why Yap4 is involved in a large repertoire of stress conditions without representing any drastic change to the yeast cell when it is deleted. One of its roles could be the fine tuning of gene expression upon a stress challenge, favoring the assembly of the PIC necessary for the proper gene transcription by the basal transcription machinery. Yap4 phosphorylation could be important at this level, affecting the DNA recognition. The work presented in this thesis regarding the characterization of Yap4 represents a contribution to further develop our understanding of Yap4 function. Our results point out to a role of this protein as a sequence-specific cofactor of the basal machinery of transcription. We believe that immunoprecipitation of Yap4 together with two-hybrid screening will give us important clues on its role.

Annex

Annex A

Alignment of YAP family

This alignment was obtained using the DIALIGN software program for multiple sequence alignment developed by Morgenstern *et al*., 2004.

```
YAP1   1msvstakrsl dvvspgslae fegsksrhde ienehrrtgt rdgeDSEQPK
YAP2   1m--------- ---------- ---------- ---------- ----------
YAP3   1mt-------- ---------- ---------- ---------- ----------
YAP4   1mlmqikmdnh pfnfqpilas hsmtr----- ---------- ----DSTKPK
YAP5   1mal------- ---------- ---------- ---------- ----------
YAP6   1---------- ---------- ---------- ---------- ----------
YAP7   1mrqrrsvvav svkpkgfklg h--------- ---------- -------KQG
YAP8   1mak------- ---------- ---------- ---------- ----------
       Experimental verified NLS (Isoyama et al., 2001) ******

YAP1  51Kkgsktskkq d--------- ---------- ---------- ----------
YAP2   2---------- ---------- ---------- ---------- ----------
YAP3   3--------PS NMDDNTSgfm kfinpqcqee dccirnslfq edskcikqqp
YAP4  32KMTDTAFVPS PPV------- ---------- ---------- ----------
YAP5   4---------- -PL------- ---------- ---------- ----------
YAP6   1-------MQN PPL------- ---------- ---------- ----------
YAP7  25SMSTTSPPPS SPDGNVStsg p--------- ---------- ----------
YAP8   4---------- ---------- ---------- ---------- ----------
        ********** *******

YAP1  62---------- ---------- ---------- ---------- ----------
YAP2   2---------- ---------- ---------- ---------G NILRKGQQIY
YAP3  45dllseqtapf piledqcpal nldrsnndll lqnnisfpkG SDLQAIQLTP
YAP4  45---------- ---------- ---------- ---------- ----------
YAP5   6---------- ---------- ---------- ---------- ----------
YAP6   7---------- ---------- ---------- ---------- ----------
YAP7  46---------- ---------- ---------- ---------- ----------
YAP8   4---------- ---------- ---------- ---------- ----------
                                                  * **********

YAP1  62---------- ---------- ---------- ---------- ----------
YAP2  13LAGDMKKQML LNKDGTpkrk vg-------- -------RPG ----------
YAP3  95ISGDYSTYVM ADNNNNDNDS YSNTNYFSKN NGISPSSRSP S---------
YAP4  45--------GF IKEENKADLH TISVVASNVT LPQIQLPKIA tleepgyesr
YAP5   6--------IK PKESEESHLA LLSKIHVSKN WKLPPRLPHR A---------
YAP6   7--------IR PDMYNQGSSS MATYNASEKN LNEHPSPQIA Q---------
YAP7  46---------- ---------- --SAIKLSKN WELPQRLKPG ----------
YAP8   4---------- ---------- ---------- ---PRGRKGG ----------
        ********** ********** ********** ********** *
```

```
YAP1  62---------- ---------- ---------- ---------- --------L-
YAP2  38---------- ---------- ---------- ---------- -----RKRI-
YAP3 136---------- ---------- ---------- ---------- --VAHNENV-
YAP4  87tgsltdlsgr rnsvnigalc edvpntagph iarpvtinnl ipPSLPRLN-
YAP5  39---------- ---------- ---------- ---------- --AQRRKRVh
YAP6  40---------- ---------- ---------- ---------- --PSTSQKL-
YAP7  64---------- ---------- ---------- ---------- -----RKPks
YAP8  11---------- ---------- ---------- ---------- -----RKPS-
                                                      *******

YAP1  63---------- DP-------- ---------- ---------- ----------
YAP2  42---------- DS-------- ---------- ---------- ----------
YAP3 143---------- PD-------- ---------- ---------- ----------
YAP4 136---------- TYQLRPQLSD THLNCHFNSN P--------- -------YTT
YAP5  47rlhedyetee ND-------- ---------- ---------- ----------
YAP6  47---------- PYRINPTTTN GDTDISVNSN Piqpplpnlm hlsgpsdYRS
YAP7  69krgdasa--- ---------- ---------- ---------- ----------
YAP8  15---------- LT-------- ---------- ---------- ----------
                   ********** ********** *                 ***

YAP1  65---------- ---------- ---------- ---------- ----------
YAP2  44---------- ---------- ---------- ---------- ----------
YAP3 145---------- ---------- ---------- ---------- ----------
YAP4 160ASHAPFESSY ttastftsq- ---------- ---------- ----------
YAP5  59---------- ---------- ---------- ---------- ----------
YAP6  87MHQSPIHPSY iipphsnerk qsasynrpqn ahvsiqpsvv fppksysisy
YAP7  76---------- ---------- ---------- ---------- ----------
YAP8  17---------- ---------- ---------- ---------- ----------
        **********

YAP1  65---------- ---------- ---------- ---------- ----------
YAP2  44---------- ---------- ---------- ---------- ----------
YAP3 145---------- ---------- ---------- ---------- ----------
YAP4 179-------PAA SYFPSNSTPA TRKNSATTNL PSEERRRVSV SLSEQVFneg
YAP5  59---------- ---------- ---------- ---------- ----------
YAP6 137apyqinpPLP NGLPNQSISL NKEYIAEEQL STLPSRNTSV TTAPPSFqns
YAP7  76---------- ---------- ---------- ---------- ----------
YAP8  17---------- ---------- ---------- ---------- ----------
               *** ********** ********** ********** *******
```

```
YAP1  65---------- ---------- ---------- ---------- ----------
YAP2  44---------- ---------- ---------- ---------- ----------
YAP3 145---------- ---------- ---------- ---------- ----------
YAP4 222eryNN----- ---------- ----DGQLIG KTGKPLRN-- ----------
YAP5  59---------- ---------- ---------- ---------- ----------
YAP6 187adtaknsadn ndnndnvtkp vpdkDTQLIS SSGKTLRN-- ----------
YAP7  76---NN----- ---------- ----DGsski kkvqtsnqkd qmttkdhene
YAP8  17---------- ---------- ---------- ---------- ----------
           **                    ****** ********

YAP1  65---------- ---------- --ETKQKRTA QNRAAQRAFR ERKERKMKEL
YAP2  44---------- ---------- --EAKSRRTA QNRAAQRAFR DRKEAKMKSL
YAP3 145---------- ---------- --DSKAKKKA QNRAAQKAFR ERKEARMKEL
YAP4 241---------- ---------- -----TKRAA QNRSAQKAFR QRREKYIKNL
YAP5  59---------- ---------- --EELQKKKR QNRDAQRAYR ERKNNKLQVL
YAP6 225---------- ---------- -----TRRAA QNRTAQKAFR QRKEKYIKNL
YAP7 104gakghegksd degngsgden gvDSVEKRRR QNRDAQRAYR ERRTTRIQVL
YAP8  17---------- ---------- --PPKNKRAA QLRASQNAFR KRKLERLEEL
                                ******** ********** **********
                                ******** ********** **********
                                   ***** ********** **********
                                   ***** ********** **********
                                    **** ********** **********
```

DNA-binding Domain

```
YAP1  93 KKVQSLESI QQQNEVEATF LRDQLITLVN ELKKYRPETR NDSKVLEYLa
YAP2  72 ERVELLEQK DAQNKTTTDF LLCSLKSLLS EITKYRAKNS DDERILAFL-
YAP3 173QDKL------ ---- LESER NRQSLLKEIE ELRKANTEIN AENRLLl---
YAP4 266EEKSKLFDGL MKENSELK-- ---------- ---------- ----------
YAP5  87EETIESLSKV VKNYEtkl-- ---------- ---------- ----------
YAP6 250EQKSKIFDDL LAENNNFK-- ---------- ---------- ----------
YAP7 154EEKVEMLHNL VDDWQr---- ---------- ---------- ----------
YAP8  45EKKEAQLtvt ---------- ---------- ---------- ----------
        ********** ********** ********** ********** *********
        ********** * *
        ********
        ****
        ***
```

Leucine zipper

```
YAP1 143rrdpnlhfsk nnvnhsnsep idtpnDDIQE NVKQKmnftf QYPLDNDNDN
YAP2 121---------- ---------- -----DDLQE QQKREnekgt stavskaa--
YAP3 209---------- ---------- ---------- ---------- ----------
YAP4 284---------- ---------- ---------- ---------- --KMIESLKS
YAP5 105---------- ---------- ---------- ---------- ----------
YAP6 268---------- ---------- ---------- ---------- --SLNDSLRN
YAP7 170---------- ---------- ---------- ---------- KYKLLESEFS
YAP8  55---------- ---------- ---------- ---------- ----------
                                   ***** *****      **********
```

```
YAP1 193DNSKNVGKQL PSPNDPSHSA PMPINqtqkk lsdatdsssa tldslsnsnd
YAP2 144-------KEL PSPNsden-- -MTVNTSIEV QPHTQ----- ----------
YAP3 209---------- ---------- ---------- ---------- ----------
YAP4 292KLKE------ ---------- ---------- ---------- ----------
YAP5 105---------- ---------- ---------- ---------- ----------
YAP6 276---------- ---------- ---------- ---------- ----------
YAP7 180DTKENLQKSI ALNNELQKAL PLIVNTPFQQ QPENP----- ----------
YAP8  55---------- ---------- ---------- ---------- ----------
        ********** ********** ********** *****

YAP1 243vlnntpnsst smdwldnviy tnrfvsgddg snsktknlds nmfsndfnfe
YAP2 169---------- ---------- ---------- ---------- ----------
YAP3 209---------- ---------- ---------- ---------- ----------
YAP4 296---------- ---------- ---------- ---------- ----------
YAP5 105---------- ---------- ---------- ---------- ----------
YAP6 276---------- ---------- ---------- ---------- ----------
YAP7 215---------- ---------- ---------- ---------- ----------
YAP8  55---------- ---------- ---------- ---------- ----------

YAP1 293nqfdeqvsef cskmnqvcgt rqcpiPKKPI SALDkevfas ssilssNSPA
YAP2 169---------- ---------- -----ENEKV mwnigsw--- ------NAPS
YAP3 209---------- ---------- ---------- ---------- ----------
YAP4 296---------- ---------- ---------- ---------- ----------
YAP5 105---------- ---------- ---------- ---------- ----------
YAP6 276---------- ---------- ---------- ---------- ----------
YAP7 215---------- ---------- -----PDNPI SILE------ ----------
YAP8  55---------- ---------- ---------- ---------- ----------
                                   ***** ****             ****

YAP1 343LTNTWEShsn itdntpanvi atdatkyens fsgfgrlgfd msanhyVVND
YAP2 185LTNSWDSppg nrtgavtigd esingsempd fsldl----- ------VSND
YAP3 209---------- ---------- ---------- ---------- ----------
YAP4 296---------- ---------- ---------- ---------- ----------
YAP5 105---------- ---------- ---------- ---------- ----------
YAP6 276---------- ---------- ---------- ---------- ----------
YAP7 224---------- ---------- ---------- ---------- ----------
YAP8  55---------- ---------- ---------- ---------- ----------
        *******                                           ****

YAP1 393NSTGstdstg stgnknkk-- ---------- ---------- ----------
YAP2 224RQTGlealdy dihnyfpqhs erltaekidt sacqceidqk ylpyeteddt
YAP3 209---------- ---------- ---------- ---------- ----------
YAP4 296---------- ---------- ---------- ---------- ----------
YAP5 105---------- ---------- ---------- ---------- ----------
YAP6 276---------- ---------- ---------- ---------- ----------
YAP7 224---------- ---------- ---------- ---------- ----------
YAP8  55---------- ---------- ---------- ---------- ----------
        ****
```

```
YAP1 411---------- ---------- ---------- ---------- ---NNNNSDD
YAP2 274lfpsvlplav gsqcnnicnr kcigtkpcsn keikcdlits hllNQKSLAS
YAP3 209---------- ---------- ---------- ---------- ----------
YAP4 296---------- ---------- ---------- ---------- ----------
YAP5 105---------- ---------- ---------- ---------- ----------
YAP6 276---------- ---------- ---------- ---------- ----------
YAP7 224---------- ---------- ---------- ---------- ----------
YAP8  55---------- ---------- ---------- ---------- ----------
                                                       *******


YAP1 418VLPFISESPF DMNQVTNFFS PGSTGIGNNA ASntnpsllq sskedipfin
YAP2 324VLPVAASHTK TIRTQSEAIE HISSAISNGK AS-------- ----------
YAP3 209---------- ---------- ---------- ---------- ----------
YAP4 296---------- ---------- ---------- ---------- ----------
YAP5 105---------- ---------- ---------- ---------- ----------
YAP6 276---------- ---------- ---------- ---------- ----------
YAP7 224---------- ---------- ---------- ---------- ----------
YAP8  55---------- ---------- ---------- ---------- ----------
        ********** ********** ********** **

YAP1 468anlafpDDNS TNIQLQPFSE SQSQNKFDYD MFFRDSsKEG NNLFGEFLED
YAP2 356---------- ---------- ---------- ---------- ----------
YAP3 209---------- ---------- ---------- -------RSG NENFSKDIED
YAP4 296---------- ---------- ---------- ---------- ----------
YAP5 105---------- ---------- ---------- ---------- ----------
YAP6 276------DNNI LIAQHEAIRN AITMLRSEYD VLCNEN---- ----------
YAP7 224---------- ---------- ---------- ---------- ----------
YAP8  55---------- ---------- ---------- ---------- ----------
              **** ********** ********** ****** *** **********

YAP1 518DDDDKKAANM SDDESSLIKN Q--------- ---------- ----------
YAP2 356---------- ---------- ---------- ---------- ----------
YAP3 222DTNYKYSFPT KDEf------ ---------- ---------- ----------
YAP4 296---------- ---------- ---------- ---------- ----------
YAP5 105------- NR LQNELQAKES ENHALKQKLE TLTLKQASVP AQdpilqnLI
YAP6 306--------NM LKNENSIIKN EHNMSRNENE NLKLENKRFH AEyirmied-
YAP7 224---------- ---------- ---------- ---------- --------MV
YAP8  55---------- ---------- ---------- ---------- ----------
        ********** ********** ********** ********** **      **
```

```
YAP1 539---------- ---------- ---------- ---------- ----------
YAP2 356---------- ---------- ---------- ---------- ----------
YAP3 236---------- ---------- ---------- ---------- ----------
YAP4 296---------- ---------- ---------- ---------- ----------
YAP5 147ENFKPMKAIP IKyntaikrh qhstelpssv kcgfcndntt cvckeletdh
YAP6 347---------- ---------- ---------- ---------- ----------
YAP7 226ENFKPIGAVS LK-------- ---------- ---------- ----------
YAP8  55---------- ---------- ---------- ---------- ----------
        ********** **

YAP1 539--------LI NEEPELPKQY LQSVpgn--- ---------- ----------
YAP2 356---------- ---------- ---------- ---------- ----------
YAP3 236---------- ---------- ---------- ---------- ----------
YAP4 296---------- ---------- ---------- ---------- ----------
YAP5 197rksDDGVATE QKDMSMPHAE CNNKDNPNGL CSNCTNIDKS CIDirsiih-
YAP6 347---------- ---------- ---------- ---------- ----------
YAP7 238---------- ---------- ---------- ---------- ----------
YAP8  55-- NDQIHIL KKENELLHFM LRSLLTERNM PSDERNISKA CCEekpptcn
           ******* ********** ********** ********** ***
YAP1 558---------- ---------- ---------- ---------- ----------
YAP2 356---------- ---------- ---------- ---------- ----------
YAP3 236---------- ---------- ---------- ---------- ----------
YAP4 296---------- ---------- ---------- ---------- ----------
YAP5 246---------- ---------- ---------- ---------- ----------
YAP6 347---------- ---------- ---------- ---------- ----------
YAP7 238---------- ---------- ---------- ---------- ----------
YAP8 102tldgsvvlss tynsleiqqc yvffkqllsv cvgknctvps plnsfdrsfy

YAP1 558---------- ---------- ---------- -----ESEIS QKNGSSLQNA
YAP2 356---------- ---------- ---------- ---------- ----------
YAP3 236---------- ---------- ---------F TSMVLESKLN HKGKYSLKDN
YAP4 296---------- ---------- ---------- ---------- ----------
YAP5 246---------- ---------- ---------- ---------- ----------
YAP6 347---------- ---------- ---------- ----IENTKR KEQEQRDEIE
YAP7 238---------- ---------- ---------- ---------- -KGK------
YAP8 152pigctnlsnd ipgysflnda mseihtfgdF NGELDSTFLE FSGTEIKEPN
                                       * ********** **********

YAP1 573DKINNGNDND NDndvvpske gs-------- --------LL RCSEIWDRIT
YAP2 356---------- ---------- ---------- ---------- -CYHILEEIS
YAP3 257EIMKRNTQYT DEA------- ---------- -----GRHVL TVPATWEYLY
YAP4 296---------- ---------- ---------- ---------- ----------
YAP5 246---------- ---------- ---------- ---------- ----------
YAP6 363QLKKKIRSLE EIV------- ---------- -----GRHsd sat-------
YAP7 241---------- ---------- ---------- ---------- ----------
YAP8 202NFITENTNAI ETAaasmvir qgfhprqyyt vdafgGDVLL SAMDIWSFMK
        ********** ***                        ***** **********
```

```
YAP1 607   THPKYSDIDV DGLCSELMAK AKCSERG-VV INAEDVQLAL NKHMn-
YAP2 365   SLPKYSSLDI DDLCSELIIK AKCTDdckIV VKARDLQSAL VRQLl-
YAP3 285   KLSEERDFDV TYVMSKLQGQ ECCHTHGpay prslidflve eatlne
YAP4 296   ---------- ---------- ---------- ---------- ------
YAP5 246   ---------- ---------- ---------- ---------- ------
YAP6 384   ---------- ---------- ---------- ---------- ------
YAP7 241   ---------- --------LK AHC------- ---------- ------
YAP8 252   VHPKVNTFDL EILGTELKKS ATCSNfdili slkhfikvfs skl---
           ********** ********** ******* ** ********** ****
```

Legend: The shadowed residues correspond to the predicted NLSs, which are also indicated in the Table II. The residues inside the box correspond to the experimental verified NLS for Yap1 (Isoyama *et al.*, 2001). The underlined bold residues correspond to the DNA-binding domain. These predictions were obtained using PSORT II server (\\psort.ims.u-tokyo.ac.jp) and data from Fernandes *et al*. (1998).

Table II – Summary of the predicted NLSs for the Yap family members

	Predicted NLS	
Yap1	^{36}RRTGTRDGEDSEQPKKK^{53}	^{69}KRTA QNRAAQRAFR ER^{85}
(true NLS$^{\#}$)	(^{5}TAKRSLDVVSPG^{16}	^{50}KKKGSKTSKK^{59})
Yap2	^{18}KKQMLLNKDGTPKRKVG^{34}	^{48}RRTAQNRAAQRAFRDRK^{64}
Yap3	^{149}KKKAQNRAAQKAFRERKE^{166}	
Yap4	^{193}RKNSATTNLPSEERRR^{208}	^{242}KRAAQNRSAQKAFRQRR^{258}
¤	^{29}KPKK^{32}	
Yap5	^{63}KKKRQNRDAQRAYRERKN^{80}	
Yap6	^{226}RRAAQNRTAQKAFRQRK^{242}	^{351}KRKEQEQRDEIEQLKKKI^{368}
Yap7	^{69}KRGDASANNDGSSKIKK^{85}	^{130}KRRRQNRDAQRAYRERRT^{148}
Yap8	^{7}RKGGRKPSLTPPKNKRAAQLRASQNAFRKRK^{38}	

true NLS experimentally verified by Isoyama *et al.*, 2001

¤ additional putative NLS

Annex B

Material and Methods

1. *Escherichia coli*

2. *Saccharomyces cerevisiae*

4. Purification and analysis of deoxyribonucleic acids

4.3 Recombinant DNA techniques

4.5 Polymerase chain reaction (PCR)

5. Purification and analysis of ribonucleic acids

6. Protein extraction and western blotting

6.1 Protein extraction

1. *Escherichia coli*

1.1 Strains

The standard *Escherichia coli* strains *XL-1 Blue* and *XL10 GOLD* (STRATAGENE) were routinely used in this work for cloning and amplification.

1.2 Media, growth and maintenance conditions

E. coli was grown at 37ºC in LB broth (1% (m/v) NaCl, 0.5% (m/v) yeast extract, 1% (m/v) bactotryptone) or on LB 2% (m/v) agar.

Selection of recombinant clones was performed by growth in the presence of 100µg/mL of the antibiotic ampicilin or 35mg/mL kanamycin.

Stock cultures were maintained in LB agar media at 4°C for less than one week or at -80ºC in LB containing 15% (m/v) glycerol.

1.3 Transformation of *E. coli* by the $CaCl_2$ method

An overnight culture of *E. coli* cells was grown in LB broth at 37ºC with shaking and used to inoculate 500mL of fresh medium next morning. Cells were grown at 37ºC with agitation until 0.4-0.6 OD_{600nm} was reached. The culture was chilled in an ice bath and then centrifuged at 5000rpm in a JA-10 rotor for 10min at 4ºC. After decanting the supernatant, the pellet was resuspended in 200mL of ice-cold 100mM $CaCl_2$ and the sample was centrifuged as before and resuspended in 40mL of ice-cold 100mM $CaCl_2$, 15% (v/v) glycerol. The cell suspension was divided in 200μL aliquots and either used immediately or frozen in liquid nitrogen and kept at -80°C for later use. Up to 50μL of plasmid DNA (containing less than 200ng DNA) or ligation mixture were used to transform 200μL of competent cells. The transformation mixture was incubated in ice for 30min and then heat-shocked at 42°C for 30 seconds. 800μL of SOC medium was added to each transformation tube and incubated for 1h at 37°C with agitation before plating onto appropriate selective media.

Media for bacteria growth were sterilized in an autoclave at 121°C for 20min.

2. *Saccharomyces cerevisiae*

2.1 Strains

S. cerevisiae strains used in this study are listed in Table III, annex C (page 165).

2.2 Media formulation

Yeast strains were grown in YP medium supplemented with 2% glucose or SC medium [0.67% yeast nitrogen base without amino acids, 0.6% casamino acids (Difco)] supplemented with glucose and the appropriate selective amino acids. Standard liquid cultures were incubated with orbital shaking (200rpm) at 30°C.

Selection of auxotrophic markers:

SD medium (0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose and 2% (w/v) bacto-agar for solid media) was used; nutritional requirements consisting of Lα-amino acids and bases (uracil, adenine), were added from 100X concentrated stock solutions to a final concentration of 50mg/L Lα-tryptophane, 100mg/L for Lα-histidine, Lα-lysine and Lα-leucine, 25mg/L adenine and uracil.

SC medium (0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose and 2% (w/v) bacto-agar for solid media) supplemented with non-essential amino acids from a 25X concentrated stock solution:

aminoacid	**Conc (g/L)**	**aminoacid**	**Conc (g/L)**
Lα-arginine-HCl	0.50	Lα-valine	3.75
Lα-phenylanine	1.25	Lα-isoleucine	0.75
Lα-methionine	0.50	Lα-aspartic acid	2.50
Lα-glutamic acid	2.50	Lα-lysine-HCl	0.75
Lα-tyrosine	0.75		

Bases (uracil, adenine) and other auxotrophic requirements were added from a 100X concentrated stock solution for the selective growth of strains:

aminoacid	**Conc (g/L)**	**aminoacid**	**Conc (g/L)**
Lα-lysine-HCl	1.00	Lα-hystidine	1.00
Lα-threonine	2.00	Lα-leucine	1.00
Lα-serine	4.00	Adenine	0.25
Lα-tryptophane	0.50	Uracil	0.25

CAA - same as SD but with the addition of casaminoacids (hydrolyzed casein) at a final concentration of 6 g/L which contains all amino acids, with exception of tryptophane, adenine and uracil.

Media for yeast growth were sterilized in an autoclave at 121ºC for 20min. Glucose containing media as well as glucose solutions (made up to a 40% solution) were autoclaved at 110ºC for 35min; a 10X concentrated solution of casaminoacids was autoclaved at 121ºC for 35min, to eliminate traces of tryptophane present; both solutions were added to the media before use. Amino acids and base stock solutions were filter-sterilized using a 20μm pore size filter.

2.3 Growth and maintenance conditions

Yeast strains were grown at 30°C in solid media or liquid media in an orbital shaker at 200rpm.

Stress conditions were applied as described in each experiment and were 0.4M NaCl (Nevitt *et al.,* 2004b), 0.3mM H_2O_2 (Delaunay *et al.*, 2000), 2mM Na_2HAsO_4 (Menezes *et al.*, 2008) and growth at 37ºC. Samples were collected at the indicated time points by cold centrifugation at 5000rpm. Samples for protein extraction were washed with TCA 20% and stored at -80ºC.

Standard methods were used for genetic analysis (Guthrie and Fink, 1991), cloning (Sambrook *et al.*, 1989; Ausubel *et al.*, 1995) and transformation (Gietz *et al.*, 1995).

S. cerevisiae stock cultures were maintained in YPD agar plates at 4ºC or frozen at -80ºC in YPD containing 40% (v/v) glycerol.

2.4 Growth in media containing stress agents: Plate sensitivity assays

Plates were prepared by mixing the indicated amounts of stress agents to 20mL of the warm agar media while still liquid (45-55°C). Cells were grown to mid-log phase and diluted so that 5µL contained approximately 5000 cells. For this purpose it was assumed that an OD_{600nm} of 1 represents approximately $2x10^7$ cells/mL. 5µL of each strain was spotted onto the test plates.

2.5 Transformation of lithium acetate-treated *S. cerevisiae* cells.

A yeast culture grown until stationary phase was diluted to 0.1 OD_{600nm} in 20mL of fresh YPD medium, incubated at 30°C with agitation until an OD_{600nm} of 0.6-0.8 was reached. Cells were collected at 5000rpm in a microcentrifuge for 1min and washed with 10mL sterilized water, centrifuged again and resuspended in 1mL 0.1M lithium acetate/TE (10mM Tris pH7.5, 1mM EDTA). Cells were harvested again and resuspended in 400µL of 0.1M lithium acetate/TE. 50µL aliquots were used to transform up to 5µg of plasmid DNA. 5µL of sheared salmon sperm DNA at 10mg/mL was added and mixed to the cell suspension (sheared salmon sperm DNA prepared by sonication and then boiled for 30min and frozen at -20°C). 300µL of 40% (w/v) PEG 3350/0.1M lithium acetate/TE (10mM Tris pH7.5, 1mM EDTA) was added to each tube and mixed and cells were incubated at 30°C for 30min and finally heat-shocked at 42°C for 15min. 800µL sterile water was added and the cell suspension was centrifuged at 5000rpm for 1min at room temperature (RT). The pellet was resuspended in 200µL of water, plated on selective media and left at 30°C for 2-4 days.

3. Vectors and plasmids used in this study

All vectors and plasmids used in this study are listed in Table V (page 169).

4. Purification and analysis of deoxyribonucleic acids

4.1. Plasmid DNA purification from *E. coli*

Preparation of plasmid DNA stocks and routine purification of plasmid DNA for current work was made using commercial plasmid Midi-Prep columns (for up to 100µg plasmid DNA) and plasmid Mini-Prep columns (for up to 20µg plasmid DNA), according to the manufacturer's instructions.

4.2 Genomic and plasmid DNA purification from *S. cerevisiae*

A 5mL yeast culture was grown in YPD or selective media at 30ºC with agitation until late log phase was reached. The cells were centrifuged in 2mL tubes at 15000rpm in a microcentrifuge for 1min. at RT and washed once with sterilized water. The sample was centrifuged again as before and resuspended in 200µL of breaking buffer [2% (m/v) triton X-100, 1% SDS, 100mM NaCl, 10mM Tris.HCl, pH 8.0, 1mM EDTA], 0.3g glass beads (size 425-600nm) were added and 200µL of phenol/chloroform/isoamylic alcohol (25:24:1). The mixture was incubated at RT with shaking for 10min. Subsequently 200µL of TE were added and then it was centrifuged at 15000rpm for 10min at RT. 360µL of the supernatant was removed and precipitated with 1mL of ethanol, the sample was vortexed, allowed to sit for 5min at RT and then centrifuged at 15000rpm for 10min at RT. The pellet was then washed with 70% ethanol and centrifuged again. After decanting the ethanol, the sample was centrifuged briefly to collect residual liquid, which was removed with a pipette for shorter drying time. The pellet was air dried for 10min and resuspended in 50μL TE. 1μL was used for subsequent 50μL PCR reaction volumes.

4.3 Recombinant DNA techniques

Restriction enzyme hydrolysis of DNA, ligation of DNA restriction fragments and agarose gel electrophoresis of were performed essentially as described in Ausubel *et al*. (1995).

4.3.1 Gene Cloning

To express or overexpress a given gene the latter was inserted into the desired plasmid. The gene of interest was PCR amplified from genomic DNA. Proof reading DNA polymerases (Pfx –Invitrogen; Pfu Turbo – Stratagene or KOD – Novagen) were used to reduce the mis-incorporation of nucleotides. The PCR product was digested with the appropriate restriction endonuclease, gel purified (Commercial gel extraction systems from Invitek, Genomed or Promega) as directed by the manufacturer, and ligated into a vector similarly prepared. Ligated products were transformed and amplified in *E. coli*, and clones were verified by the subsequent digestion of plasmid DNA and/or PCR verification of the insert. The fidelity of the cloned gene was verified by DNA sequencing in both strains.

4.3.2 Gene disruption by PCR

All genes were disrupted by a one step PCR procedure to replace the entire open reading frame with the appropriate marker as follows. The following set of oligomers were designed for each gene: oligomer A1 (5') is an 18-25 base sequence 150-250bp upstream of the ATG; A2 (3') 18-25 base intragenic sequence close to the translation stop codon (TAA/TGA), A3 (5') intragenic sequence of 18-25 base close to the ATG; A4 (3') 18-25 base sequence 150-250bp after the stop codon; S1 (5') contains 45 bases immediately upstream of the ATG plus the following kanamycin-specific sequence 5'-CAGCTGAAGCTTCGTACGC- 3'; S2 (3') is composed of 45 bases immediately downstream of the stop codon followed by the kanamycin-specific sequence 5'-GCATAGGCCACTAGTGGATCTG-3'. Standard PCR conditions were used to amplify the deletion cassette. The PCR product was purified Yeast cells were then transformed with 0.5-2μg of the DNA product. Selection of transformants was accomplished by plating on YPD containing the antibiotic Geneticin (Invitrogen).

Positive knockouts were confirmed by PCR amplification using a following oligomer combination: A1 (gene-specific to the promoter region, sense strand), A2 (gene-specific to the coding region, anti-sense strand) and K2 (kanamycin-specific, anti-sense strand); A3 (gene-specific to the coding region, sense strand), A4 (gene-specific to the downstream untranslated region, anti-sense strand) and K3 (kanamycin-specific, sense strand). Oligomers were designed such that different sizes are obtained for the wild type and disrupted amplifications.

4.4 Marker removal

Marker removal is possible due to the loxP sequences that flank the deletion cassette that, upon overexpression of a plasmid-borne recombinase, promote homologous recombination and cassette removal.

A pre-culture of the transformant is prepared overnight and a fresh 10mL culture is grown until OD_{600} 0.7 is reached. Competent cells are prepared as indicated above and the cells are transformed with the plasmid pSH47 containing the recombinase, and plated onto uracil-deficient medium. After the appearance of positive clones, one or more is used to inoculate fresh YPD and cultured at 30ºC with agitation for 5-6h. The culture is harvested by centrifuging at 5000rpm for 1min, and the medium decanted; the pellet is then resuspended in YP Galactose (2%) and the expression of the recombinase induced for 30min. The culture is harvested and plated onto YPD plates overnight. As soon as colonies appear replica plates are made onto fresh YPD and YPD containing Geneticin for selection of cells that have lost the kanamycin cassette. Positive colonies are streaked onto fresh medium and PCR-tested with A1-A4 and K2-K3. Loss of pSH47 can be induced by replica plating onto 5-fluorotic acid containing YPD plates (5-FOA YPD) which is toxic to uracil utilizing strains. These strains are now competent for further gene disruption with the KanMX module.

4.5 Polymerase chain reaction (PCR)

4.5.1 PCR using Taq polymerase

PCR using Taq polymerase was used to check gene disruptions or constructs. Routinely, a PCR reaction mix was set up containing 10pmol of each oligomer, 100ng of *S. cerevisiae* total genomic DNA or 1-10ng of plasmid DNA, 1X Taq DNA polymerase buffer (as provided by the enzyme supplier), 0.5-1.5mM $MgCl_2$, 200μM of each deoxynucleotide (dATP, dCTP, dTTP and dGTP) and 2.5U of commercial Taq DNA polymerase. The reaction volume was brought up to 50μL with sterile distilled water. To carry out the amplification reaction the thermocycler was set to perform the first cycle at 94ºC for 5min followed by 2-30 cycles of 94ºC for 30 seconds, 50-55ºC for 30 seconds and 72ºC for 1min and a final cycle of 5min at 72ºC. About 1/10 of the reaction volume was used to examine on a 1% (w/v) agarose gel stained with ethidium bromide (0.5μg/mL).

4.5.2 PCR using Pfx

Amplification of fragments used, either to clone a gene or to mutate certain nucleotides, require a higher fidelity enzyme. In these cases, the proof-reading enzyme Pfx (Invitrogen) was used. The PCR reaction was set in a similar way as previously described for Taq with slight differences: 5 units of enzyme, 300μM of dNTPs and 1mM $MgSO_4$ instead of $MgCl_2$ was used as co-factor. The temperature of extension used was 68ºC instead of 72ºC, since this is the optimal temperature for Pfx activity.

4.5.3 Site-directed mutagenesis using Pfu Turbo (Stratagene)

Site-directed mutagenesis was performed according to QuikChange® II Site-Directed Mutagenesis Kit (Stratagene). Complementary oligomers for site-directed mutagenesis were designed as indicated in Table IV (page 167).

4.5.4 *YAP4* truncations

GFPHA*YAP4* was amplified from the pRS314 plasmid containing this chimera under the control of cup1 promoter, kindly given by Furuchi (Furuchi *et al.*, 2001), and recloned in pRS416. The obtained pRS416GFPHA*YAP4* was then used as template for all forward truncations. This initial amplification was performed with the universal oligomers M13. To obtain the following truncations in *YAP4*, we used oligomer M13 paired with the different truncation oligomers indicated in Table III (page 167). Finally, to obtain the construction pRS416GFPHALZY4 we amplified the template with oligomers GFPHArev and LZY4fwd containing each one a Bgl*II* restriction site, digest the PCR product with Bgl*II* and religate the plasmid. The polymerase used in all PCR procedures was the proof reading KOD DNA Polymerase (Novagen® Toyobo). All constructs were confirmed by sequencing in both strands of the DNA.

4.6 DNA probe labelling for hybridization procedures

DNA was labelled by the random sequence nonamers method. For that DNA was labelled with 32P-dCTP (Amersham) using the Megaprime

DNA Labelling System (Amersham) according to the instruction manual. The probe was subsequently purified from unincorporated nucleotides using G50 Columns (G50 was saturated with TE buffer and left overnight at 4ºC with occasional shaking, 1mL syringe was filled with saturated G50 and centrifuged at 3000rpm for 3min at RT, filled with more G50 and centrifuged again in the same conditions until the column was compact), the probe was then eluted from the column by a centrifugation of 3000rpm for 5min at RT. The specific activity of the probe was determined in a scintillation counter by mixing 1µL of the purified labelled DNA with 10mL scintillation liquid. In order to optimize the signal to noise ratio in the hybridization experiments, probes were used with a specific activity ranging between 108-109dpm/µg.

5. Purification and analysis of ribonucleic acids

5.1 Total RNA isolation from *S. cerevisiae*

Yeast cells from an overnight culture were diluted to 0.1 OD_{600nm} in 10mL of fresh media and allowed to grow until 0.45 OD_{600nm}. Cells were harvested by centrifugation at 4000rpm for 2-3min. at 4ºC, washed with sterile water and resuspended in 400µL TES buffer (10mM TrisHCl, 10mM EDTA, 0.5% SDS). 400µL of acidic phenol (pH 4.5) was added for the selective removal of DNA and proteins and the sample was incubated at 65ºC for 1h, being vortexed every 15min. after which the mixture was cooled down on ice and then centrifuged at 15000rpm for 10min. 400µL of acid phenol was added to the upper aqueous phase, the sample was vortexed and incubated again at 65ºC for 15min, cooled down on ice and then centrifuged at 15000rpm for 10min. The upper aqueous phase was carefully removed, transferred into a fresh tube and two phenol/chloroform (1:1) extractions were performed. The aqueous phase was then extracted with chloroform and total RNA was finally precipitated by adding 1/10 volume of 3M NaAc, pH5.2 and 2.5 volumes of absolute ethanol overnight at -20°C or 1-2h at -80°C. The sample was centrifuged at 15000rpm at 4°C for 45min, washed twice with 70% ethanol, 15min, and finally resuspended in water treated with DEPC (diethyl pyrocarbonate) in order to eliminate RNases. For DEPC treatment, 1mL of DEPC was added to 1L of ddH2O (double distilled water) and stirred overnight in a fume hood. DEPC treated water was autoclaved twice at 121°C for 20min.

RNA concentration and purity were evaluated by measuring the spectrophotometric absorbance of the samples at OD_{260nm} and OD_{280nm} and assuming that a 40μg/mL RNA solution gives an OD_{260nm} of 1 and the ratio OD_{260nm}/OD_{280nm} for a good purity RNA solution should range between 1.9-2.0.

5.2 Northern blot analysis of *S. cerevisiae* total RNA

Total RNA from *S. cerevisiae* was run on a 1.5% formaldehyde-agarose gel [sterile bi-distilled H_2O, 1X MOPS buffer (0.2M MOPS, 50mM sodium acetate, 5mM EDTA, pH7.0] and 1.1X formaldehyde 37%). The volume of each RNA sample corresponding to 30μg was taken to 10μL with water, 2μL of 5X MOPS buffer, 10μL formamide and 3.5μL formaldehyde was added and incubated at 65°C for 15min. 2μL of gel loading dye (50% (v/v) glycerol, 1mM EDTA, 0.4% (w/v) bromophenol blue, 0.4% (w/v) xylene cyanol) was added to each sample, mixed and kept on ice until loading of the gel. The electrophoresis was run at 2.5

V/cm in 1X MOPS buffer. After the run, the gel was washed twice in sddH2O, for 30min to remove the formaldehyde. The RNA was transferred overnight onto a nylon membrane (Schleicher and Schuell) by capillary blotting. After 16h, the membrane was removed and fixed by baking at 80ºC for 2h. The membrane was pre-hybridized at 65ºC for at least 1h in hybridization buffer (0.25 M phosphate buffer, 7% (w/v) SDS, 1mM EDTA, 1% (w/v) BSA (bovine serum albumin, Sigma). The pre-hybridization solution was discarded and replaced by an equal volume of hybridization solution; the labelled denatured probe was added and incubated overnight at 65ºC. Following hybridization the membranes were washed twice with wash buffer (20mM phosphate buffer, 0.1% (w/v) SDS and 1mM EDTA), the first wash done at 65ºC and the second at RT until the background signal had disappeared. Finally, the membrane was wrapped in plastic film, placed in an autoradiograph cassette between two screens and exposed to an appropriate Biomax (KODAK MR) X-ray film at -80ºC.

5.3 Real Time PCR analysis

20mg of RNA were treated with DNase (Ambion TURBO DNA-free) according to manufactures instructions. In brief, reaction was made in 50µl with 3U DNase for 30min at 37ºC. Reaction was stopped by incubating with 10µl Inactivating reagent for 2 min at room temperature. Samples were centrifuged at 10000g for 1.5 min at room temperature. RNA was quantified and integrity was checked by loading 1µg in a gel.

The cDNA synthesis was performed in 10µl reaction with 0.5µg RNA, 5pmol/ml $(dT)_{15}$ 1mMdNTP, 5U Transcriptor Reverse transcriptase, 1x transcriptor reaction buffer (Roche). The mixture was incubated at 25ºC for 10min, then 55ºC for 90min and 85ºC for 5min.

Real time detection of the PCR product was done by means of the DNA intercalating compound SYBR Green (LightCycler Fast Start DNA Master SYBR Green I, Roche catalogue number 03003230001) in a Roche LightCycler II instrument. Specific oligomers for *HXT5* were used (see Table IV, page 167). Duplicates were used for each sample and the *ACT1* gene was used as loading control. The fold change was determined by the $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The ΔC_t of the control and experimental samples was calculated from the threshold cycle of the target gene minus the threshold cycle of the reference gene (*ACT1*). The $\Delta\Delta C_{t=}(Ct_{target} - Ct_{reference})_{test} - (Ct_{target} - Ct_{reference})_{control}$ was calculated by subtracting the ΔC_t of the control sample (not incubated with NaCl) minus ΔC_t of the experimental sample.

6. Protein extraction and western blotting

6.1 Protein extraction

6.1.1 Protein extraction with TCA

At the indicated conditions and time points, 20mL of cell suspension were harvested by centrifugation at 5000rpm for 1min at 4ºC, washed with 20% (w/v) TCA (trichloroacetic acid) and quickly frozen at -80ºC after a spin down to reject the supernatant. Pellets were then resuspended in 400µL TCA 20% and half the pellet volume of glass beads (size 425-600nm) and lysed by vortexing 3min. The supernatant was transferred to a new tube and pelleted for 1min at 14000rpm. The pellet was then 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 1.0M Tris (pH 8.0) and heated for 5min at 95ºC. Finally, the solution was centrifuged again for 3min at 14000rpm and the supernatant proteins were quantified using the Bradford method (Bradford, 1976) with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hemel Hempstead, Herts, UK). All equipment and reagents were pre-cooled before use.

6.1.2 Protein extraction for phosphatase treatment

Protein extracts for the phosphatase treatment were performed as described in Delaunay *et al*. (2000) and previously for the protein extraction with TCA, with minor changes. The TCA-precipitated pellet of protein extracts were washed three times with 200µL of cold acetone, pelleted 3min at 14000rpm and dried in a speed vac. Proteins were then solubilised with 100µL Iodoacetamide buffer (75mM iodoacetamide, 1%(w/v) SDS, 100mM Tris/HCl pH 8.0, 1mM EDTA and complete protease inhibitors (Boehringer Mannheim)) during 20min at RT. Then proteins were centrifuged 10min at 14000rpm and dialyzed overnight with Slide-A-Lyzer® Mini Dialysis Units (10000 MWCO, Pierce). Finally, 10% of the dialyzed protein volume of PMSF was add to the dialyzed proteins and the mixture was incubated with the CIP (calf intestinal phosphatase; New England Biolabs) for 45min at 37ºC. At the end, dephosphorylated proteins were boiled for 5min with Laemli buffer and pelleted 5min at 14000rpm.

Unless indicated, all equipment and reagents were pre-cooled before use.

6.2 Protein electrophoresis by SDS-PAGE

1mm 12% (w/v) polyacrylamide running gels were prepared by mixing the different components in the following proportions: 10.6mL of acrylamide/bis-acrylamide [(33.5:0.2)%(w/v)], 15mL of 1M Tris pH 8.8, 264µL of 20% (w/v) SDS, 3.5mL of distilled water, 40µL of recently prepared 10% (w/v) APS (ammonium persulfate), 20µL of TEMED. The final percentage of acrylamide in the running gel ranged from 10% to 15% (w/v), varying the resolving capacity of the gel. The running gel was poured in a 12*10cm or 12*20cm (height*width) vertical SDS-PAGE apparatus, avoiding any air bubbles, to a height 1cm bellow the end of the comb. The gel was carefully overlaid with water and left to polymerize. After polymerization, water was completely removed and the stacking gel was prepared by mixing the components in the following proportions: 2.7mL of acrylamide/bis-acrylamide [(30.04:0.8) %(w/v)], 2.55mL of 1M Tris pH 6.8, 40µL of 20% (w/v) SDS, 5mL of distilled water, 40µL of recently prepared 10% (w/v) APS, 15µL of TEMED. The stacking gel was poured on top of the running gel, an appropriate well comb was introduced and the gel was left to polymerize overnight. The gel wells were washed with 1X SDS-PAGE running buffer (prepared as a 10X concentrated stock solution consisting of 250mM Tris, 2M glycine, 1% (w/v) SDS) before loading of the samples. 50µg of total protein extract were loaded and electrophoresis was carried out at an incremental voltage, starting from 85V till reach 150V in 1X SDS-PAGE running buffer until the loading stain reached the end of the gel. Alternatively, electrophoresis was carried overnight at 60V (17h running). After disassembly of the electrophoresis apparatus, the stacking gel was cut out and discarded.

6.3 Semi-dry transfer of protein to a solid support

After electrophoresis of the protein samples, the running gel was immediately soaked in transfer buffer (20mM Tris, 150mM glycine, 20% (v/v) methanol) for 10min. Six pieces of 3MM paper (Whatman) of the same size of the gel were pre-wetted in the transfer buffer and placed over the anode of the Semi-dry Trans-Blot SD Transfer Cell (Biorad). A nitrocellulose membrane (Hybond™-ECL™ (GE Healthcare)) pre-wetted 10min with water and another 10min with transfer buffer was put on top of the filter paper avoiding any air bubbles. The gel was placed on top of the nitrocellulose membrane again avoiding any air bubbles and sandwiched with six more pre-wetted sheets of 3MM paper. The cathode in the lid of the transfer apparatus finally covered the blot sandwich. The current was set to 25V and transfer was carried out for 20min. Prior to probing blots for the presence of an antigen, efficient transfer of proteins was insured by staining the nitrocellulose filter with Ponceau S. A 10X concentrated stock solution of Ponceau S (2% (w/v) Ponceau S, 30% (w/v) TCA, 30% (w/v) sulfosalicylic acid) was diluted 1:10 with water and used to incubate the filter with agitation for 5min at room temperature. The membrane was then washed for 1-2min with water until clear visualization of the protein bands was obtained and finally rinsed with several changes of blocking solution (5% low fat dry milk in PBS) until the stain was washed away, prior to membrane blocking and immunodetection.

6.4 Immuno-detection of immobilized protein samples

After transfer, blocking of nonspecific adsorption of the immunological reagents to the nitrocellulose filter was carried out. For this, the membrane was immersed in the blocking solution incubated at room temperature for a minimum of 30min with slow agitation. The filter was rinsed twice with the same solution and then incubated with gentle shaking with the required primary antibody diluted in PBS solution containing 1% Tween-20 and 0.1% low fat dry milk. The dilution factor for the antibody (ranging from 1:1000 to 1:5000) and the length of incubation (ranging from 1 hour at room temperature to overnight at 10ºC) were dependent on the specific antibody being used. The filter was thoroughly washed at room temperature with slow agitation for three times 10min each using 100mL PBS solution supplemented with 1% Tween-20. For the non-conjugated antibodies, the membrane was then incubated in PBS solution containing 1% Tween-20 and 0.1% low fat dry milk and 1:5000 dilution of the proper secondary antibody (horseradish peroxidase-conjugated anti-mouse IgG (Amersham, Pharmacia) or horseradish peroxidase-conjugated anti-rabbit IgG (BIORAD)), for a minimum of 60min. The filter was washed again with PBS solution supplemented with 1% Tween-20 three times 10min each with gentle shaking and finally detection was carried out using the ECL Plus chemiluminescent detection system (Amersham Pharmacia) according to the manufacturer’s instructions. The filter was then exposed to autoradiography films (Hyperfilm™ ECL, Amersham) for a range of times in order to estimate optimal irradiation time and finally developed manually.

7. Protein stability assay

Yeast mid log phase cells were subjected to an osmotic shift with 0.4M NaCl during 20min and then treated with cycloheximide 100μg/mL, collected at the desired time points, washed with TCA 20% and frozen at -80ºC before protein extraction. Protein half-life was quantified as described in Belle *et al.* (2006).

8. GFP analysis of yeast cells by fluorescence microscopy

yap4 mutant strains transformed with the indicated episomical GFPHA*YAP4* versions were grown to early exponential phase. DAPI (4´,6-diamidino-2-phenylindole) was added as a DNA marker at a final concentration of 5µg/ml, 5min before microscopy. After washing with PBS, cells were resuspended in a solution of 200mM DABCO (1,4-diazadicyclo[2.2.2]octane) in 75% (v/v) glycerol and 0.25×PBS (Sigma–Aldrich). GFP signals were analyzed in living cells with a Leica DMRXA fluorescent microscope equipped with a Roper ScientificMicro-Max cooledCCD (charge-coupled device) camera and MetaMorph software (Universal Imaging Inc.).

9. Intracellular glycerol content

To measure the internal glycerol levels, the assay was performed as described previously (Rep *et al*, 1999a) with minor modifications. Cells were harvested by centrifugation for 1min at 2700g, washed and resuspended in cold water. After measuring the OD_{600}, samples were heated for 15min at 95ºC. 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.

Annex C - List of strains used

Table III

Strain	**Genotype**	**Source**
FY1679	Mat*a ura3-52/ura3-52 trp1Δ63/TRP1 leu2Δ1/LEU2 his3Δ200/HIS3 GAL2/GAL*	Winston *et al.*, 1995
yap4	Mat*a yap4 ura3-52/ura3-52 trp1Δ63/TRP1 his3Δ200/HIS3 GAL2/GAL*	This study
W303-1A	MAT*a leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC mal0*	Thomas and Rothstein (1989)
hog1	MAT*a HOG1*::*TRP1*	S. Hohmann
hog1yap4	MAT*a HOG1*::*TRP1 YAP4*::*KanMX*	Nevitt *et al.* (2004a)
slt2	MAT*a SLT2*::*TRP1*	Peter Piper (Sheffield University)
slt2 yap4	MATa *SLT2*::*TRP1 YAP4*::kanMX	This study
MLY41	MAT*a ura3-52* (Σ1278b background)	Zurita-Martinez and Cardenas (2005)
SZy2a	MAT*a YAK1*::hygB ura3-52,	
tpk1	MAT*a ura3-52 TPK1*::kanMX	
tpk2	MAT*a ura3-52 TPK2*::kanMX	
tpk3	MATa ura3-52 *TPK3*::kanMX	
SZy9a	*YAK1*::hygB *TPK1*::KanMX, *TPK2*::nat, *TPK3*::KanMX	
sch9	MLY265 MAT*a ura3-52 SCH9*::kanMX	
MY 1	*leu2*::PET56 *gcn4 gal2*	Malys *et al.* (2004)
rim15	MY 2872 *RIM15*::KAN	
rim15 yak1	MY 3297 *RIM15*::*KAN YAK1*::LEU2	
DCS2TAP	PTC197 - MAT*a DCS2-TAP-URA3 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	
yap4DCS2TAP	PTC197 *DCS2TAP YAP4::kanMX*	Pereira *et al.* (unpublished work)
By4741	Mata *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
ste20	Matα *YHL007c::KanMX4*	
ptk2	Matα *YJR059w*::KanMX4	
mck1	Mata *YNL307c::KanMX4*	
PSY580	MAT*a ura3-52 leu2 1 trp1 6*	Pamela Silver (Claude Jacq)
PSY1201	MAT*a ura3-52 leu2Δ1 trp1Δ63 pse1-1*	

Annex D - List of oligomers used

Table IV

Oligomer		Sequence
Site-directed mutagenesis		
T24A	Fwd	5’ – CATTCAATGGCAAGAGACAGTAC – 3’
	Rev	5’ – GTACTGTCTCTTGCCATTGAATG – 3’
S27A	Fwd	5’ – ATGACAAGAGACGCTACAAAGCCA – 3’
	Rev	5’ – TGGCTTTGTAGCGTCTCTTGTCAT – 3’
T28A	Fwd	5' – GACAAGAGACAGTGCAAAGCCAAAAAAAATGACG – 3'
	Rev	5' – GTCATTTTTTTTGGCTTTGCACTGTCTCTTGTCATTG – 3'
K31AK32A	Fwd	5’ – TGACAAGAGACAGTACAAAGCCAGCAGCAATGACGGATACCGCTTTCGTGC – 3’
	Rev	5’ – GCACGAAAGCGGTATCCGTCATTGCTGCTGGCTTTGTACTGTCTCTTGTCA – 3’
T34A	Fwd	5’ – CAAAAAAAATGGCGGATACCGCTTT – 3’
	Rev	5’ – AAAGCGGTATCCGCCATTTTTTTTG – 3’
S41A	Fwd	5’ – ACGGATACCGCTTTCGTGCCAGCCCCTCCTGTAG – 3’
	Rev	5’ – CTACAGGAGGGCTTGGCACGAAAGCGGTATCCGT – 3’
S59A	Fwd	5’ – TGCATACAATTGCGGTAGTTGCTTC – 3’
	Rev	5’ – GAAGCAACTACCGAAATTGTATGCA – 3'
T77A	Fwd	5’ – CAAAGATTGCAGCACTTGAAG – 3’
	Rev	5’ – CTTCAAGTGCTGCAATCTTTG – 3’
S89A	Fwd	5’ – CAGAACTGGCGCACTTACAGATCTTTC – 3’
	Rev	5’ – GAAAGATCTGTAAGTGCGCCAGTTCTG – 3’
S94A	Fwd	5’ – GTTCTTCCAGCAAGATCTGTAAG – 3’
	Rev	5’ – CTTACAGATCTTGCTGGAAGAAC – 3’
S99A	Fwd	5' – GGAAGAAGAAATGCTGTTAATATAGGAGC - 3'
	Rev	5' – GCTCCTATATTAACAGCATTTCTTCTTCC – 3'
T122A	Fwd	5' – CAAGGCCGGTGGCAATAAACAATTTGATTC – 3'
	Rev	5' – GAATCAAATTGTTTATTGCCACCGGCCTTG – 3'
T136A	Fwd	5’ – CTTTACCAAGACTGAACGCATACCAGCTTAG – 3'
	Rev	5' – CTAAGCTGGTATGCGTTCAGTCTTGGTAAA – 3'

T189A	Fwd	5’ – GCTTCATATTTTCCCTCAAATAGTAGCCCAGCTACGAGA – 3’
	Rev	5’ – TCTCGTAGCTGGGCTACTATTTGAGGGAAAATATGAAGC – 3’
T192A	Fwd	5’ – GTACCCAGCTGCGAGAAAAAATA – 3’
	Rev	5’ – ACTATTTTTTCTCGCAGCTGGGGTA – 3’
R193A-K194A	Fwd	5’ – CAAATAGTACCCCAGCTACGGCAGCAAATAGTGCCACGACTAAC – 3'
	Rev	5’ – GTTAGTCGTGGCACTATTTGCTGCCGTAGCTGGGGTACTATTTG – 3'
S196A	Fwd	5’ – GAGAAAAAATGCTGCCACGACTAAC – 3’
	Rev	5’ – GTTAGTCGTGGCAGCATTTTTTCTC – 3’
T199A	Fwd	5’ – AAAATAGTGCCACGGCTAACCTTC – 3’
	Rev	5’ – GAAGGTTAGCCGTGGCACTATTTTT – 3’
S210A	Fwd	5' – GAAAGACGTCGAGTTGCCGTTTCTCTTTC – 3'
	Rev	5' – GAAAGAGAAACGGCAACTCGACGTCTTTC – 3'
S214A	Fwd	5’ – TCCGTTTCTCTTGCAGAGCAGGTTT – 3’
	Rev	5’ – AAACCTGCTCTGCAAGAGAAACGGA – 3’
T241A	Fwd	5’ – CAAACCTTTAAGAAATGCTAAGAGAGCTGCCC – 3'
	Rev	5' – GGGCAGCTCTCTTAGCATTTCTTAAAGGTTTG – 3'
K242A-R243A	Fwd	5’ – CTTTAAGAAATACTGCGGCAGCTGCCCAAAATC – 3'
	Rev	5' – GATTTTGGGCAGCTGCCGCAGTATTTCTTAAAG – 3'
S288A	Fwd	5’ – GATTGAAGCATTAAAGTCG – 3’
	Rev	5’ – CGACTTTAATGCTTCAATC – 3’
Yap4 truncations		
GFPHArev		5’ – CATTAAAGATCTGTAATCTGGAACATCGTA – 3’
Y4ΔLZrev		5’ – GAATTCTTAACGACGCTGCCTAAA – 3’
Y4Δnls2rev		5’ – GAATTCTTAGTATTTCTTAAAGGTTTGCCT – 3
Y4ΔSrev		5’ – GAATTCTTAAACTCGACGTCTTTCCTCTGA – 3’
Y4ΔNLSrev		5’ – GAATTCTTACGTAGCTGGGGTACTATTTGA – 3’
LZY4fwd		5’ – CGTCGTAGATCTGAGAAATACATCAAATC – 3’
Real Time PCR		
HXT5fwd		5' - AGGTAAAGAAAGAATGCT CG – 3’
HXT5rev		5’ – AGTTTCCTGACTTCTCGTTG – 3’
ACT1fwd		5’ – CTATTGGTAACGAAAGATTCAG – 3’
ACT1rev		5’ – CCTTACGGACATCGACATCA – 3’

Annex E - List of plasmids used

Table V

name		source
Y4T	YIplac211 YAP4TAP	This study
^{X}Y4T	^{X}YAP4TAP	
X = S27A, T28A, T34A, K32AK33A, S41A, S54A, S89A, S99A, T122A, T136A, T189A, T192A, T192AS196A, R193AK194A,S196A, S199A, S210A, S214A, T241A, K242AR243A, R193AK194AK242AR243A, S288A		
GFPY4	pRS314 GFPHAYAP4	Furuchi *et al.* (2001)
GFP^{x}Y4	GFPHA^{x}YAP4	This study
X = T192A, S196A, T192AS196A, R193AK194A, K242AR243A, R193AK194AK242AR243A		
GFPY4ΔLZ	pRS416 GFPHAYAP4ΔLZ	This study
GFPY4Δnls2	GFPHAYAP4Δnls2	
GFPY4ΔS	GFPHAYAP4ΔS	
GFPY4ΔNLS	GFPHAYAP4ΔNLS	
GFPLZ	GFPHALZ	
YEpY4	YEp356R YAP4TAP	This study
YEp^{X}YAP4TAP	^{x}YAP4TAP	
X = T192AS196A, R193AK194A, K242AR243A, R193AK194AK242AR243A		

Annex F - Publications

Pereira J *et al*. (2008). Yap4 PKA-dependent phosphorylation affects protein stability but not its localization. Submitted to *Yeast*;

Nevitt T, **Pereira J**, Azevedo D, Guerreiro P and Rodrigues-Pousada C (2004). Expression of YAP4 in *Saccharomyces cerevisiae* under osmotic stress. *Biochem J* **379:** 367-374;

Nevitt T, **Pereira J** and Rodrigues-Pousada C (2004). YAP4 gene expression is induced in response to several forms of stress in *Saccharomyces cerevisiae*. *Yeast* **21**: 1365-1374;

Rodrigues-Pousada C, Nevitt T, Menezes R, Azevedo D, **Pereira J**, Amaral C (2004). Yeast activator proteins and stress response: an overview. *FEBS Letters* **567**: 80-85;

Yeast

Yap4 PKA-dependent phosphorylation affects its stability but not its nuclear localization

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Keywords:	Yap4 , phosphorylation, stability, nuclear localization

Title

Yap4 PKA-dependent phosphorylation affects its stability but not its nuclear localization

Authors

Jorge Pereira, Catarina Pimentel, Catarina Amaral, Regina A. Menezes and Claudina Rodrigues-Pousada

Running head: Yap4 phosphorylation

Genomics and Stress Laboratory,

Instituto de Tecnologia Química e Biológica,

Av. da República, Apt. 127,

2781-901 Oeiras, Portugal

Abstract

Yap4 is a nuclear resident transcription factor induced in *Saccharomyces cerevisiae* when exposed to several stress conditions, including mild hyperosmotic and oxidative stress, temperature shift, metal exposure or stationary phase. Here, we report that Yap4 is a PKA dependent phosphoprotein. In order to ascertain whether Yap4 is directly or indirectly phosphorylated by PKA, we searched for stress and PKA-related kinases that could phosphorylate Yap4. We show that phosphorylation is independent of the kinases Rim15, Yak1, Sch9, Slt2, Ste20, Ptk2 and Mck1. Furthermore, we show that its nuclear localization is independent of its phosphorylation state. This protein has several putative phosphorylation sites, but only the mutation of residues T192 and S196 impairs its phosphorylation under different stress conditions. However, phosphorylation seems to be required for stability of the protein as the non-phosphorylated form has a shorter half-life compared to the phosphorylated one. The ability of the non-phosphorylated forms of Yap4 to partially rescue the *hog1* severe sensitivity phenotype is not affected. In order to determine the sequences responsible for Yap4 localization we have made several constructs. Remarkably after removal of the leucine zipper (LZ) Yap4 is no longer in the nucleus thus suggesting that Yap4 dimerization is required for its nuclear import.

Keywords: Yap4 / phosphorylation / stability / nuclear localization

Symbols used in this work:

BR – basic region; CWI pathway - cell wall integrity pathway; LZ – leucine zipper; NLS – nuclear localization signal; PKA – protein kinase A; STRE – stress responsive element; TCA – trichloroacetic acid;

Introduction

The budding yeast *Saccharomyces cerevisiae* responds to various environmental cues by reprogramming gene expression in a highly coordinated manner via several interdependent pathways. Transcription factors, many of which contain phosphorylation sites, are important key-players in this process. This post-translational modification is usually associated to an important level of control by kinases either activating or repressing a given factor. It is well-known that PKA is the effector kinase of the cAMP pathway, which monitors carbon source availability and regulates the expression of genes necessary for cell growth (Zurita-Martinez *et al.*, 2005). PKA negatively regulates the stress response through Msn2/4 phosphorylation. However in this case Snf1 kinase may be also involved (De Wever *et al.*, 2005). Many other kinases related to PKA play a role in the yeast stress response. For example, Yak1 is part of a glucose-sensing system involved in the growth control in response to glucose availability. It regulates by phosphorylation of its N-terminal domain, the subcellular distribution of Bcy1, the regulatory subunit of PKA, thus controlling PKA activity (Griffioen *et al.* 2001). Also it was recently shown that PKA and TOR pathways are implicated in the regulation of the genes that are required for proper nutrient regulation and cell growth (Slattery *et al.*, 2008). These two signalling cascades converge on the Rim15, a protein kinase required for the entry into stationary phase (G0) and to response to nutrients (Pedruzzi *et al.*, 2003; Rohde *et al.*, 2008; Swinnen *et al.*, 2006). PKA controls entry into stationary phase by repressing Rim15 activity through its phosphorylation (Reinders *et al.*, 1998). It has been also described that TOR, Sch9 and PKA are able to regulate common targets, including expression of STRE and the ribosomal proteins genes (Rohde *et al.*, 2008) and both Sch9 and PKA are involved in the adaptation to nutrient availability (Roosen *et al.*, 2005) as well as in the cooperative regulation of autophagy (Yorimitsu *et* *al*., 2007). Additionally, Sch9 was shown to be a chromatin-associated transcriptional activator of osmostress-responsive genes (Pascual-Ahuir *et al.*, 2007).

The Yap family is formed by eight b-ZIP transcription factors of which Yap1 is the best characterized as the major regulator in oxidative stress (Rodrigues-Pousada *et al*, 2004). Yap8, another member of the family, is involved in the arsenic compound detoxification through the regulation of the genes *ARR2* and *ARR3* encoding respectively an arsenate reductase and a plasma membrane arsenite efflux-protein. (Menezes *et al*., 2004; Menezes *et al*., 2008). Yap4 belongs also to this family and it has been previously shown to be highly induced under several forms of stress such as oxidative, osmotic, temperature shift and metals (Nevitt *et al*., 2004b; Salin *et al*., 2008). Its expression is regulated under osmotic stress by Hog1/Msn2 and under oxidative stress by Msn2 and Yap1 (Nevitt *et al*., 2004a).

We are interested in investigating the role of Yap4 phosphorylation in the yeast stress response since it is a highly phosphorylated protein under all stress conditions so far tested (Nevitt *et al*., 2004a and b). Our results show that Yap4 levels increase in response to several environmental conditions, such as osmotic and oxidative stress, heat and arsenic exposure, and that Yap4 is phosphorylated in a PKA-dependent manner. Using site directed mutagenesis we have mutated several residues potentially phosphorylated by PKA and other kinases (see Table I). The residues T192 and S196 are important for this modification under the different stress conditions imposed, do not affect Yap4 nuclear localization but do substantially reduce the half-life of the protein.

Material and Methods

Yeast strains and growth conditions

S. cerevisiae strains used in this study are listed in Table II. Yeast strains were grown in YP medium supplemented with 2% glucose or SC medium [0.67% yeast nitrogen base without amino acids, 0.6% casamino acids (Difco, Le Pont de Claix, France)] supplemented with glucose and the appropriate amino acids. Standard liquid cultures were incubated with orbital shaking (200rpm) at 30ºC.

The stress conditions applied were 0.4M NaCl (Nevitt *et al.*, 2004b), 0.3mM H_2O_2 (Delaunay *et al.*, 2000), 2mM Na_2HAsO_4 (Menezes *et al.*, 2008) and growth at 37ºC. Samples were collected at the indicated time points as shown in the respective figures by cold centrifugation at 5000rpm. Samples for protein extraction were washed with TCA 20% and stored at -80ºC.

Standard methods were used for genetic analysis (Guthrie *et al.*, 1991), cloning (Sambrook *et al.*, 1989; Ausubel *et al.*, 1995) and transformation (Gietz et *al.*, 1995).

Site-directed mutagenesis

Site-directed mutagenesis was performed according to QuikChange® II Site-Directed Mutagenesis Kit (Stratagene, California, USA). Complementary primers for site-directed mutagenesis were designed as indicated in table III.

Western blot analysis

Protein extracts and western blot analysis were performed as described in Nevitt *et al.*, (2004b) with minor changes. 50µg of total protein extract were loaded and separated on a 12x10cm or 12x20cm (height x width) 12% SDS-PAGE apparatus and blotted onto a

Hybond™-ECL™ (GE Healthcare Limited, Buckinghamshire, UK) nitrocellulose membrane. When indicated, SbaI levels were used as loading control (Mollapour *et al.*, 2007). All the western blots analysis presented in this work are representative of at least three independent assays.

Protein stability assays

Yeast mid log phase cells were subjected to an osmotic shift with 0.4M NaCl during 20min and then treated with cycloheximide 100µg/mL, collected at the time points indicated in Figure 6, washed with TCA 20% and frozen at -80ºC before protein extraction. Protein half-life was quantified as described in Belle *et al.* (2006).

***YAP4* truncations**

GFPHAYAP4, under the control of *CUP1* promoter, was amplified from the pRS314 plasmid containing this chimera (Furuchi *et al.*, 2001), and recloned in pRS416. The obtained p*GFPHAYAP4* was then used as template for all the constructs. This initial amplification was performed with the universal primers M13. To obtain the subsequent truncations of *YAP4*, we used primer M13 paired with different oligonucleotides indicated in Table III. Finally, the construction p*GFPHALZY4* was obtained by PCR using the primers GFPHArev and LZY4fwd each containing a Bgl*II* restriction site. The amplified plasmid was digested with Bgl*II* and the *YAP4* truncated ORF ligated in frame. The proof reading KOD DNA Polymerase (Novagen®, Toyobo, Japan) was used in all PCR procedures and the recombinant molecules confirmed by sequencing of both DNA strands.

Fluorescence microscopy

yap4 mutant strains transformed with the indicated episomal *GFPHAYAP4* versions were grown to early exponential phase and DAPI (4´, 6-diamidino-2-phenylindole, Sigma-Aldrich, Missouri, USA) was added as a DNA marker at a final concentration of 5µg/ml, 5min before microscopy. After washing with PBS, cells were resuspended in a solution of 200mM DABCO (1, 4-diazadicyclo[2.2.2]octane (Sigma-Aldrich, Steinhem, Germany) dissolved in 75% (v/v) glycerol and 0.25PBS, which we found not to affect the localization of the GFP fusions. GFP signals were analysed in living cells with a DMRXA fluorescent microscope (Leica Microsystems Imaging Solutions, Cambridge, UK) equipped with a MicroMax cooledCCD (charge-coupled device, Roper Scientific, Georgia, USA) camera and MetaMorph software (Universal Imaging Inc., Pennsylvania, USA).

Results and Discussion

Yap4 is phosphorylated under different stress conditions

We showed previously that the steady state populations of mRNAs encoding Yap4 were highly enhanced after various forms of stress with a correspondent increase of the protein levels. The protein is also phosphorylated after an osmotic shock (Nevitt *et al,* 2004a) and under oxidative stress conditions (Nevitt *et al,* 2004b). Figure 1 shows clearly three different forms of Yap4 with different mobility shifts on the SDS-PAGE gel. From our previous results using alkaline phosphatase treatment (Nevitt *et al.*, 2004a), we can associate the faster migrating isoform as non-phosphorylated one and the other two bands as the phosphorylated isoforms. This pattern of phosphorylation is maintained under other forms of stress, such as heat, oxidative and arsenic exposures as can be seen in Figure 4B. Yap4 is highly induced at 20min and it decreases at 40 and 90min of treatment. However, when cells reach stationary phase (180 and 240min), a subsequent increase is observed.

Prediction of Yap4 structural features

We used NetPhos and NetPhosK programmes to identify the potential residues responsible for Yap4 phosphorylation (Blom *et al.*, 2004). The Yap4 putative phosphorylation sites are diagrammed in Figure 2 and the potential kinases involved in Yap4 phosphorylation are shown in Table I. These predictions were also base on analysis using the ScanProsite programme which also retrieved the predictions about Yap4 bipartite NLS and LZ (Hulo *et al.*, 2007). The first stretch of the NLS (nls1) spans the residues R193-V208 and the second one (nls2) spans the residues K242 -R258. The

DBD prediction was obtained previously by alignment of Yap4 amino acid sequence with the remaining Yap members (Fernandes *et al*., 1997) (see Figure 2).

The large majority of consensus phosphorylation sites are putative PKA phosphorylation sites although other kinases were also found to recognize some of these elements, as indicated in Table I.

Identification of kinases responsible for Yap4 phosphorylation

We showed previously that Hog1 controls Yap4 protein levels, but not its phosphorylation (Nevitt *et al*, 2004a) and that PKA regulates negatively Yap4, as its levels are severely affected in a *bcy1* strain (Nevitt *et al*., 2004b). The crosstalk between the PKA pathway and the General Stress Response is widely known to involve PKA and the transcription factor Msn2. In fact, PKA regulates negatively the General Stress Response by controlling Msn2 phosphorylation status and consequently its nuclear localization. As such, transcription of Msn2-dependent stress responsive genes, like *YAP4* decrease in normal growing cells and are enhanced under stress conditions (Nevitt *et al*., 2004a). We have therefore evaluated whether, besides Yap4 levels, PKA also affects its phosphorylation. We analysed Yap4 phosphorylation in the *tpk1 tpk2 tpk3 yak1* mutant, as the null PKA mutant (*tpk1tpk2 tpk3*) is not viable (Smith *et al.*, 1998). As shown in Figure 3A, under osmotic stress, Yap4 levels are enhanced and its phosphorylation is lost in the mutant *tpk1 tpk2 tpk3 yak1* even in the absence of stress, thereby revealing that Yap4 phosphorylation is driven by PKA. This result also shows that the different subunits of PKA can phosphorylate Yap4, as this modification is maintained for all single PKA catalytic mutants. This functional redundancy was already observed for other PKA targets (Ptacek *et al*., 2005).

We also questioned whether PKA was directly responsible for the phosphorylation of Yap4 or if other intermediate kinases were participating in it. Indeed, it is well known that Yak1 is phosphorylated by PKA which in turn phosphorylates the regulatory subunit of PKA (Griffioen *et al.*, 2001). We have therefore tested the effect of Yak1 in Yap4 phosphorylation. As the results illustrated in Figure 3A show, Yak1 is not affecting Yap4 phosphorylation. Furthermore, Sch9 and Rim15 that act in PKA-related pathways do not impair Yap4 phosphorylation (Figure 3A and B).

The analysis of Yap4 amino acid sequence reveals several consensus sequences for PKC1-dependent phosphorylation (Table I), suggesting that Yap4 could also be phosphorylated in a PKC1-dependent manner. Bermejo *et al.* (2008) reported that cell wall damage caused by zymolyase requires the sequential activation of Hog1 and Slt2. Since Slt2 is the MAP kinase of the PKC1 pathway, we also assayed Yap4 phosphorylation in the *slt2* mutant. The results illustrated in Figure 3C show that this kinase is not involved in Yap4 phosphorylation under different stress conditions.

Ptacek *et al.* (2005) proposed a phosphorylation map where the phosphorylation events that occur in yeast are indicated. According to this database (see http://networks.gersteinlab.org/phosphorylome/) the kinases Ste20, Ptk2 and Mck1 are able to phosphorylate Yap4 *in vitro*. We put forward the question of whether these proteins could be good candidates to be an intermediate kinase between PKA and Yap4. However, in every stress conditions tested, Yap4 phosphorylation was not impaired in any of the *mck1*, *ste20* or *ptk2* mutants (Figure 3D and Supplementary Figure 1). The fact that, unlike S196, T192 is a consensus phosphorylation site for Gsk3 and not PKA, and mutation of both residues abolishes Yap4 phosphorylation, led us to investigate the *mck1* mutant. Mck1 is one of the four mammalian Gsk3 kinase yeast orthologs (Hirata *et al.*, 2003), and it is plausible to assume that in the *mck1* mutant, the other orthologs

(Rim11, Mrk1 and Yol128c) are phosphorylating Yap4, this being the reason why we do not see impairment of its phosphorylation.

Detection of the phosphorylated residues in Yap4 protein

The prediction of multiple phosphorylation sites in Yap4 led us to perform site-directed mutagenesis in order to evaluate their contribution to phosphorylation. We investigated whether protein phosphorylation profile changed according to the stress condition. We decided therefore to mutate to alanine the residues potentially phosphorylated by PKA as indicated in Table I. As can be seen in Figure 4A, S196A is the only mutation that abolishes Yap4 phosphorylation. All the other PKA dependent residues are left unaltered (see as an example S89A in Figure 4A). This result does not support Budovskaya *et al*., (2005) predictions about the conservation of PKA-consensus serine phosphorylation in several yeast related species. These authors assumed that a higher degree of evolutionary conservation of those sites would correspond to functional phosphorylation sites *in vivo*. They identified Yap4 S99 and S210 as putative PKA phosphorylation sites, but our data show that Yap4 phosphorylation is not impaired in the ^{S99A}Yap4 and ^{S210A}Yap4 mutants (see Supplementary Figure 2).

Western blots revealed the presence of three bands suggesting that Yap4 protein is phosphorylated at least in two different residues. As T192 and S196 are flanking the basic residues R193 and K194 of nls1, we assumed that phosphorylation of T192 together with S196 could be a mechanism of activation of Yap4 NLS. We have therefore mutated T192 and this indeed affects Yap4 phosphorylation, as can be seen in Figure 4B. A similar type of result is also obtained using the double mutant ^{T192AS196A}Yap4 (Supplementary Figure 3). Under osmotic, oxidative, heat and arsenic stresses, the pattern of phosphorylation reveals for all the stresses that the same residues

(T192 and S196) are preventing Yap4 phosphorylation (Figure 4B). As a control, we used a predicted PKA-dependent mutant, T241A, whose phosphorylation is not affected.

Yap4 nuclear localization is independent of its phosphorylation

Many important transcription factors contain phosphorylation sites within or adjacent to a classic NLS. Several studies show that the nuclear localization of these proteins can be regulated by phosphorylation at these sites. Msn2 localization, for example, is controlled by the level of phosphorylation of its NLS, (Gorner *et al.*, 1998 and 2002; Garmendia-Torres *et al*., 2007, De Wever *et al.*, 2005).

In Yap4, the two residues affecting its phosphorylation (T192 and S196) are just flanking the key residues R193 and K194 of Yap4 bipartite NLS (Figure 2 and 3), suggesting that phosphorylation of T192 and S196 could be a mechanism of activation of Yap4 NLS. We therefore hypothesized that phosphorylation of Yap4, a nuclear resident protein, might regulate its nuclear import. In order to test this hypothesis, Yap4, ^{T192A}Yap4 and ^{S196A}Yap4 single mutants and ^{T192AS196A}Yap4 double mutant were fused to GFP and analysed for their localization in the nucleus. The results shown in Figure 7 reveal that the phosphorylated form of Yap4 as well as the single mutants GFP^{T192A}Yap4, and GFP^{S196A}Yap4 and the double mutant GFP^{T192AS196A}Yap4 localize into the nucleus, independent of its phosphorylation state. This result indicates that this post-translational modification is not responsible for Yap4 localization. This observation was further substantiated by the fact that in the PKA null mutant *ptk1 ptk2 ptk3 yak1*, in which Yap4 is not modified, it is still localized in the nucleus (Figure 5).

Based on this information we assessed the physiological consequences of the absence of phosphorylation. As figure 6 indicates, overexpression of the non-phosphorylated Yap4 mutants (^{T192A}Yap4, ^{S196A}Yap4 and ^{T192AS196A}Yap4) rescues the growth phenotype of the *hog1* mutant under osmotic stress that we previously reported (Nevitt *et al.*, 2004a). It seems therefore that Yap4 physiological function is not affected by the absence of its phosphorylation at least at this level.

It is known that the nuclear import of Yap1, the well-studied member of the Yap family, is mediated by the importin Pse1 (Isoyama *et al.*, 2001). To verify whether Yap4 localization would also be mediated by this importin we have used the conditional *pse1-1* mutant. If this hypothesis is correct the blocking of Pse1 would lead to a Yap4 cellular distribution allowing us to follow its phosphorylation. However, we observed that Yap4 is not transported to the nucleus by this importin (Supplementary Figure 4).

Determinants for Yap4 nuclear localization

In contrast to Yap1, Yap2 and Yap8, which are only localized in the nucleus after stress, Yap4 is a nuclear resident protein. This transcription factor possesses a bipartite NLS containing two short stretches of basic amino acids separated by a nonconserved sequence of 32 residues, both stretches spanning the residues R193-V208 (nls1) and the residues K242-R258 respectively (nls2) (see Figure 2). It is still unclear what the sequences essential for Yap4 localization are. To address this issue we tested a number of constructs of Yap4 protein fused to GFP which were expressed under the *CUP1* promoter, as diagrammed in Figure 8A. All the constructs were assessed for stability by Western blotting and all found to be stable (See Supplementary Figure 5).

As Figure 8B shows, GFP analysis revealed that Yap4 is localized in the nucleus (A) but after removal of the leucine zipper Yap4 nuclear localization is impaired (B). Subsequent deletions (C, D, and E) which do not contain the LZ are also all absent in the nucleus, a fact that suggests a role for LZ in the Yap4 import. In order to confirm these results we used a construct containing only the LZ domain. As can be seen in Figure 8B (construct F), although LZ is required for nuclear localization of Yap4, it is not sufficient to drive GFP into the nucleus. This result suggests that Yap4dimerization could precede its nuclear localization and once LZ is deleted, Yap4 is not able to dimerize and cannot be translocated to the nucleus. A similar mechanism was described for nuclear translocation of Fos that is highly favoured by a previous heterodimerization with Jun through their leucine zippers (Chida *et al.*, 1999). However, according to *in silico* data of Deppmann *et al*. (2006), and unlike the remaining Yap members, Yap4, Yap6 and Yap8 are not predicted to homodimerize or heterodimerize between them. Those authors postulate that other partners not yet unidentified could exist. However, to our knowledge there are so far no experimental evidences to support these data. Further analyses are needed to clarify this putative mechanism of protein homo or heterodimerization prior to its nuclear translocation.

Yap4 protein stability is dependent on its phosphorylation

It is well known that the activity of many transcription factors is modulated by post-translational modifications and phosphorylation is one of the most frequent event. This modification can affect their activity through its stability, cellular localization, protein-protein interaction, DNA-binding activity, transcriptional activity (Holmberg *et al.*, 2002). Protein phosphorylation can represent therefore a critical process for modulating the life-span of a protein. In order to evaluate whether the phosphorylation of Yap4 could affect its stability, we have performed experiments to measure the half-life time of the non-phosphorylated and phosphorylated forms of the protein. Cell extracts were obtained from *yap4* mutant expressing integrated versions of the *YAP4* gene and the ^{T192AS196A}*YAP4* mutant, both untreated and treated with cycloheximide, an inhibitor of protein synthesis (Belle *et al.*, 2006). As can be seen in Figure 6, the results show that Yap4 stability is significantly affected by the abolishment of phosphorylation with values of $T_{½}$ for the wild-type of 12.1 ± 1.1 min and 7.9 ± 0.7 min for the ^{T192AS196A}Yap4 mutant (a 35% decrease). These values are comparable for other Yap members (Belle *et al.*, 2006). Our data were normalized against the Sba1 (ortholog of p23 in mammalian cells) which is a good control since its half-life is of 280 min (Belle *et al.*, 2006). Our results show therefore that phosphorylation of Yap4 can modulate the half-life of Yap4 protein.

Conclusions

In this work we evaluated the potential role of Yap4 phosphorylation in its regulation. We showed that Yap4 phosphorylation is PKA-dependent. We further analyse the existence of an intermediate kinase between PKA and Yap4. We test the effect of Yak1 in Yap4 phosphorylation, as well as of Sch9 and Rim15, that act in PKA related pathways. Our results clearly show that Yap4 phosphorylation in not impaired in any of this kinase mutants' strain. Slt2, the MAPK of the PKC pathway, also does not affect Yap4 phosphorylation. The same result was obtained for the kinases Ste20, Ptk2 and Mck1, predicted to phosphorylate Yap4 *in vitro*. Mutation of Yap4 residues T192 and S196 to alanine abolishes its phosphorylation. Our data does not exclude however the existence of other kinases phosphorylating Yap4 as T192 is not a predicted PKA phosphorylation site. Deletion of different domains of Yap4 revealed that removal of the LZ prevents nuclear localization, but the LZ *per se* does not contain any signal for nuclear localization. This suggests that dimerization of Yap4 may precede its nuclear import. The stability of the protein is however affected by the absence of the phosphorylation, exhibiting a 35% decrease of its half-life.

The function of the transcription factor Yap4 is not yet completely understood. It is possible that its phosphorylation may be required for interaction with the basal transcriptional machinery. One the other hand, Yap4 can function as a docking protein to bind several factors involved in transcription. More experiments are being carried out to support these assumptions.

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Legends:

Figure 1: Kinetics of Yap4 phosphorylation.

Yeast *yap4* cells containing the integrated *YAP4*TAP tag versions were grown until mid log phase and harvested after induction with 0.4M NaCl at the time points indicated. Proteins were extracted and analysed by western blot.

Figure 2: Schematic representation of predicted Yap4 structural domains

The putative phosphorylation sites, BD, LZ and bipartite NLS was retrieved respectively from NetPhos and NetPhosK programmes (Blom *et al*., 2004), ScanProsite programme (Hulo *et al*., 2007) and by alignment with the remaining Yap members; (Fernandes *et al*., 1997). The highlighted "**T**" and "**S**" putative phosphorylation sites are respectively T192 and S196.

Figure 3: Yap4 phosphorylation is dependent on PKA.

Kinase-defective mutants were assayed for Yap4 phosphorylation under different stress conditions. Yap4 phosphorylation analysis in the null PKA mutant *tpk1 tpk2 tpk 3 yak1*, in the single PKA mutants *tpk1*, *tpk2* and *tpk3*, in *yak1* and in *sch9* (A), as well as in the *rim15*and *rim15 yak1* (B), in *slt2* (C) and *ste20*, *ptk2* and *mck1* (D).

Figure 4: T192A, S196A and T192AS196A mutations abolish Yap4 phosphorylation under different stress conditions.

The residues of Yap4 indicated in Table I were mutated to alanine by site-directed mutagenesis, the mutated versions were integrated in *yap4* strain and protein extracts were analysed by western blot. A) Effect of mutations S89A and S196A in Yap4 phosphorylation under osmotic stress conditions. B) Mid-log phase cells expressing the indicated *YAP4* mutations were harvested after induction with 0.4M NaCl (**osm**, 20min.), 0.3mM H_2O_2 (**ox**, 30min), 2mM Na_2HAsO_4 (**As**, 30min.) and after 30min of growth at 37ºC (heat).

Figure 5: Non-phosphorylated Yap4 localizes in the nucleus.

yap4 mutant cells expressing episomal wild type and non-phosphorylated versions of GFPYap4 (GFP^{T192A}Yap4, GFP^{S196A}Yap4 and GFP^{T192AS196A}Yap4) and the PKA null mutant cells *tpk1 tpk2 tpk3 yak1* expressing episomal GFPYap4 were harvested at mid-log phase and observed under fluorescent microscopy.

Figure 6: Absence of phosphorylation does not compromise Yap4 ability to rescue the *hog1* osmosensitive phenotype.

The double mutant *hog1 yap4* was transformed with YEp356R expressing *YAP4* wild type and mutant alleles. Mid-log phase cells were serially diluted and spotted onto SC medium supplemented or not with 0.4 M NaCl and cells were incubated at 30ºC for 2 days. This result is representative of three independent assays.

Figure 7: Truncation of Yap4.

(A) The five truncations referred were obtained as described in *Materials and Methods*. In the lower panel is indicated the relative position of the residues of Yap4 bordering the different truncations. (B*)* Cells transformed with episomal GFPYap4 versions indicated were harvested at mid-log phase and observed under fluorescent microscopy.

Figure 8: Yap4 stability is partially dependent on phosphorylation.

The half-life (**T**$_{½}$) of Yap4TAP and ^{T192AS196A}Yap4TAP was measure and the respective values are indicated in the box below. ^{T192AS196A}Yap4 is 35% less stable than its wild type counterpart. Protein levels of the co-chaperone SbaI, which has a half-life of 280 min, were used as loading control (Mollapour *et al*., 2007). The time points indicated are relative to the addition of cycloheximide after cells have been induced for 20 min with 0.4M NaCl. This western blot is representative of three independent assays and the half-life and respective standard deviation calculations were performed using those assays.

Tables

Table I: Potential kinases involved in Yap4 phosphorylation.

	Yap4 residues																		
Kinases	**T24**	**S27**	**T28**	**T34**	**S41**	**S59**	**T77**	**S89**	**S99**	**T122**	**T136**	**T189**	**T192**	**S196**	**T199**	**S210**	**S214**	**T241**	**S288**
CaMII			■					■	■	■	■			■		■			
CKI	■							■					■		■		■		
CKII								■											
GSK3					■	■						■	■		■	■			
PKA			■					■	■	■	■			■		■		■	
PKC		■					■						■					■	■
PKG				■					■					■		■			

Table II: Strains used in this work

Strain	**Genotype**	**Source**
FY1679	*Mata ura3-52/ura3-52 trp1Δ63/TRP1 leu2Δ1/LEU2 his3Δ200/HIS3 GAL2/GAL*	Winston *et al*., 1995
yap4	*Mata yap4 ura3-52/ura3-52 trp1Δ63/TRP1 his3Δ200/HIS3 GAL2/GAL*	This study
W303-1A	*MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC mal0*	Thomas and Rothstein (1989)
hog1	*MATa HOG1::TRP1*	S. Hohmann
hog1yap4	*MATa HOG1::TRP1, YAP4::KAN*	Nevitt *et al*. (2004a)
slt2	*MATa SLT2::TRP1*	Peter Piper (University of Sheffield)
slt2 yap4	*MATa SLT2::TRP1 YAP4::kanMX*	This study
MLY41	*MATa ura3-52* (Σ1278b background)	Zurita-Martinez and Cardenas (2005)
SZy2a	*MATa YAK1::hygB ura3-52*	
tpk1	*MATa ura3-52 TPK1::kanMX*	
tpk2	*MATa ura3-52 TPK2::kanMX*	
tpk3	*MATa ura3-52 TPK3::kanMX*	
SZy9a	*YAK1::hygB TPK1::KanMX, TPK2::nat, TPK3::KanMX*	
sch9	*MLY265 MATa ura3-52 SCH9::kanMX*	
MY 1	*leu2::PET56 gcn4 gal2*	Malys *et al*. (2004)
rim15	*MY 2872 RIM15::KAN*	
rim15 yak1	*MY 3297 RIM15::KAN YAK1::LEU2*	
By4741	*Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0;*	EUROSCARF
ste20	*Matα YHL007c::KanMX4*	
ptk2	*Matα YJR059w::KanMX4*	
mck1	*Matα YNL307c::KanMX4*	
PSY580	*MATa ura3-52 leu2 1 trp1 6*	Seedorf *et al*. (1997)
PSY1201	*MATa ura3-52 leu2Δ1 trp1Δ63 pse1-1*	

Table III: Primers used in this work

Primer		Sequence
S89A	Fwd	5’ – CAGAACTGGCGCACTTACAGATCTTTC – 3’
	Rev	5’ – GAAAGATCTGTAAGTGCGCCAGTTCTG – 3’
T192A	Fwd	5’ – GTACCCAGCTGCGAGAAAAAAATA – 3’
	Rev	5’ – ACTATTTTTTCTCGCAGCTGGGGTA – 3’
R193A-K194A	Fwd	5’ – CAAATAGTACCCCAGCTACGGCAGCAAATAGTGCCACGACTAAC – 3'
	Rev	5' – GTTAGTCGTGGCACTATTTGCTGCCGTAGCTGGGGTACTATTTG – 3'
S196A	Fwd	5’ – GAGAAAAAATGCTGCCACGACTAAC – 3’
	Rev	5’ – GTTAGTCGTGGCAGCATTTTTTCTC – 3’
T241A	Fwd	5' – CAAACCTTTAAGAAATGCTAAGAGAGCTGCCC – 3'
	Rev	5' – GGGCAGCTCTCTTAGCATTTCTTAAAGGTTTG – 3'
K242A-R243A	Fwd	5' – CTTTAAGAAATACTGCGGCAGCTGCCCAAAATC – 3'
	Rev	5' – GATTTTGGGCAGCTGCCGCAGTATTTCTTAAAG – 3'
GFPHArev		5’ – CATTAAAGATCTGTAATCTGGAACATCGTA – 3’
Y4ΔLZrev		5’ – GAATTCTTAACGACGCTGCCTAAA – 3’
Y4Δnls2rev		5’ – GAATTCTTAGTATTTCTTAAAGGTTTGCCT – 3
Y4ΔSrev		5’ – GAATTCTTAAACTCGACGTCTTTCCTCTGA – 3’
Y4ΔNLSrev		5’ – GAATTCTTACGTAGCTGGGGTACTATTTGA – 3’
LZY4fwd		5’ – CGTCGTAGATCTGAGAAATACATCAAATC – 3’

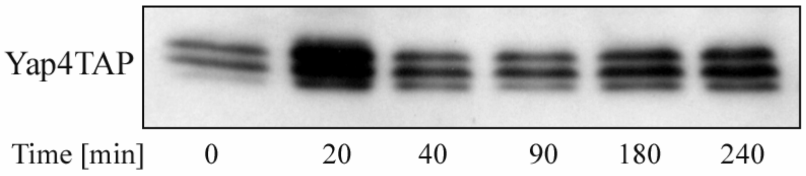
Table IV: Plasmids used in this work

name		**source**
Y4T	YIplac211 YAP4TAP	This study
^{X}Y4T	^{X}YAP4TAP	
X = S27A, T28A, T34A, S41A, S54A, S89A, S99A, T122A, T136A, T189A, T192A, S196A, S199A, S210A, S214A, T241A, S288A, T192AS196A, R193AK194A, K242AR243A, R193AK194AK242AR243A		
GFPY4	pRS314 GFPHAYAP4	Furuchi *et al*. (2001)
GFP^{x}Y4	GFPHA^{x}YAP4	This study
X= T192A, S196A, T192AS196A, R193AK194A, K242AR243A, R193AK194AK242AR243A		
GFPY4ΔLZ	pRS416 GFPHAYAP4ΔLZ	This study
GFPY4Δnls2	GFPHAYAP4Δnls2	
GFPY4ΔS	GFPHAYAP4Δς	
GFPY4ΔNLS	GFPHAYAP4ΔNLS	
GFPLZ	GFPHALZ	
YEpY4	YEp356R YAP4TAP	This study
YEp^{X}YAP4TAP	^{x}YAP4TAP	
X= T192AS196A, R193AK194A, K242AR243A, R193AK194AK242AR243A		

Figures

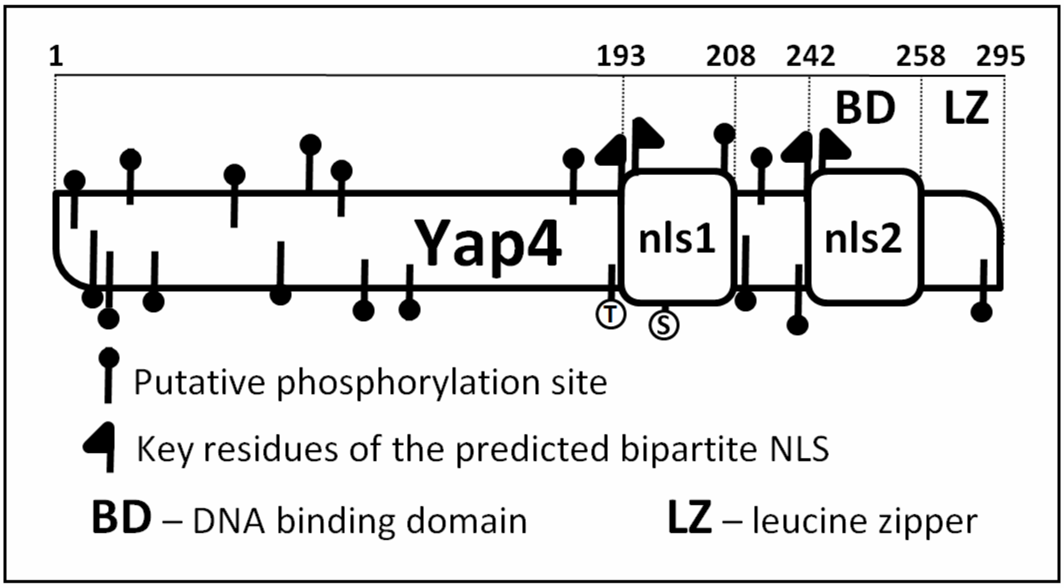
Pereira *et al.*, 2008

Figure 1

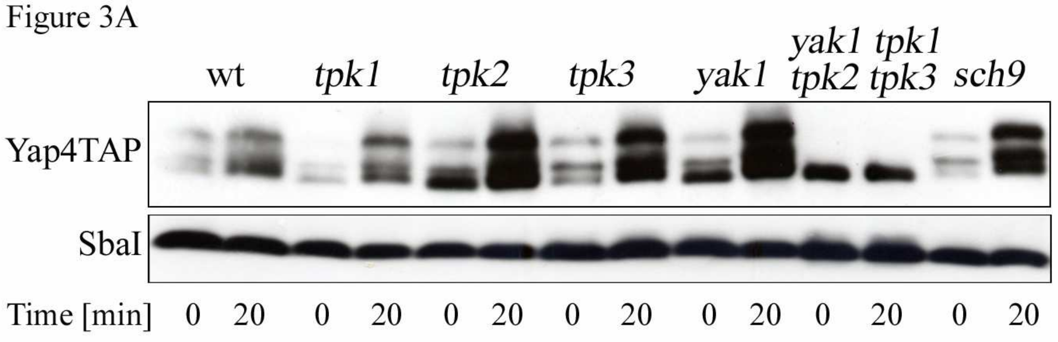


Pereira *et al.*, 2008

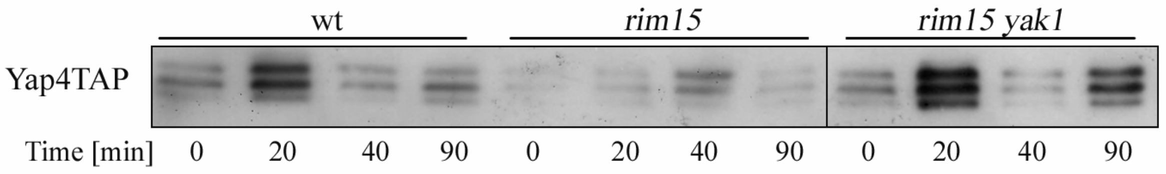
Figure 2



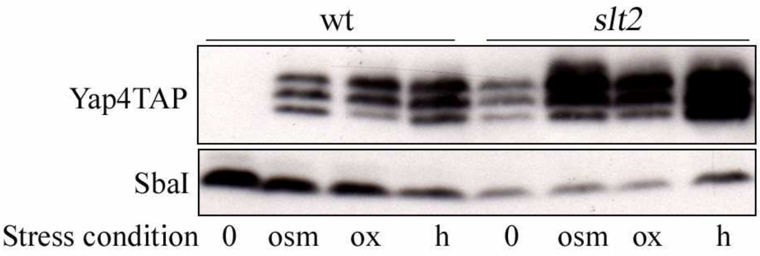
Pereira *et al.*, 2008
Figure 3A



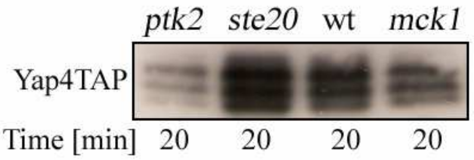
Pereira *et al.*, 2008
Figure 3B



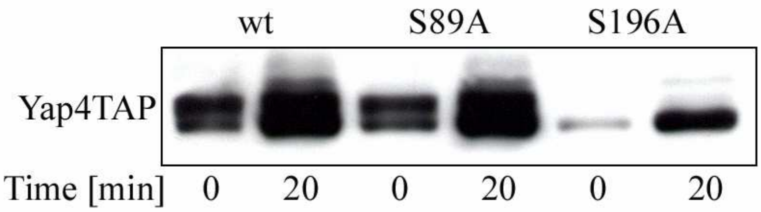
Pereira *et al.*, 2008
Figure 3C



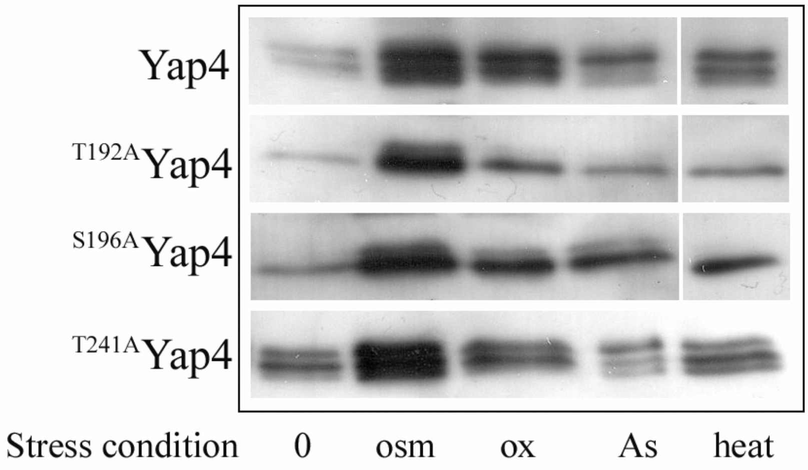
Pereira *et al.*, 2008
Figure 3D



Pereira *et al.*, 2008
Figure 4A

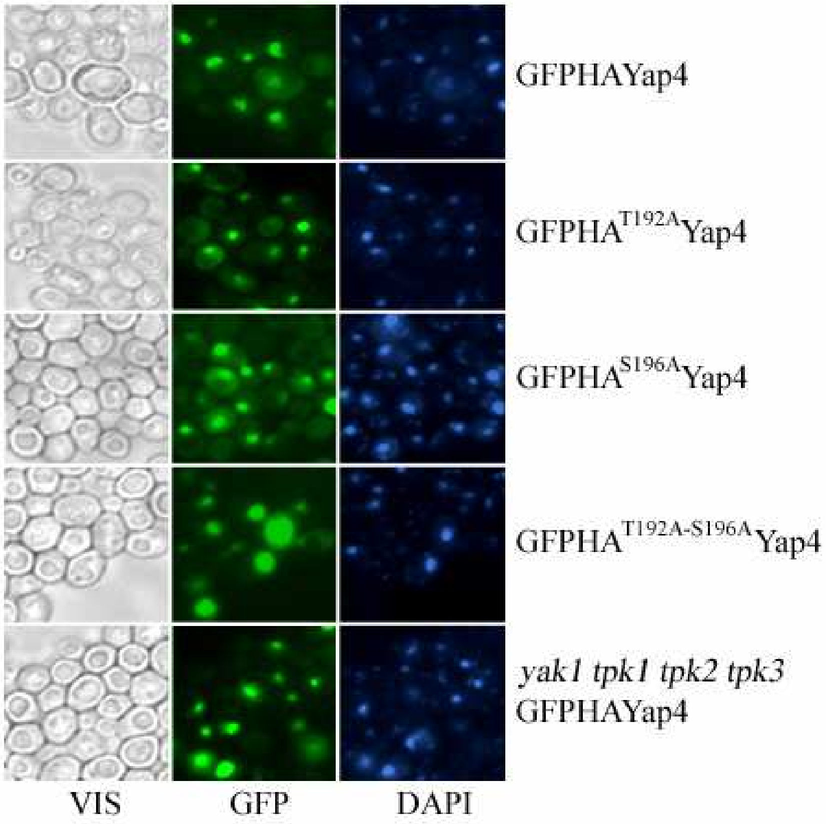


Pereira *et al.*, 2008
Figure 4B



Pereira *et al.*, 2008

Figure 5



Pereira *et al.*, 2008
Figure 6

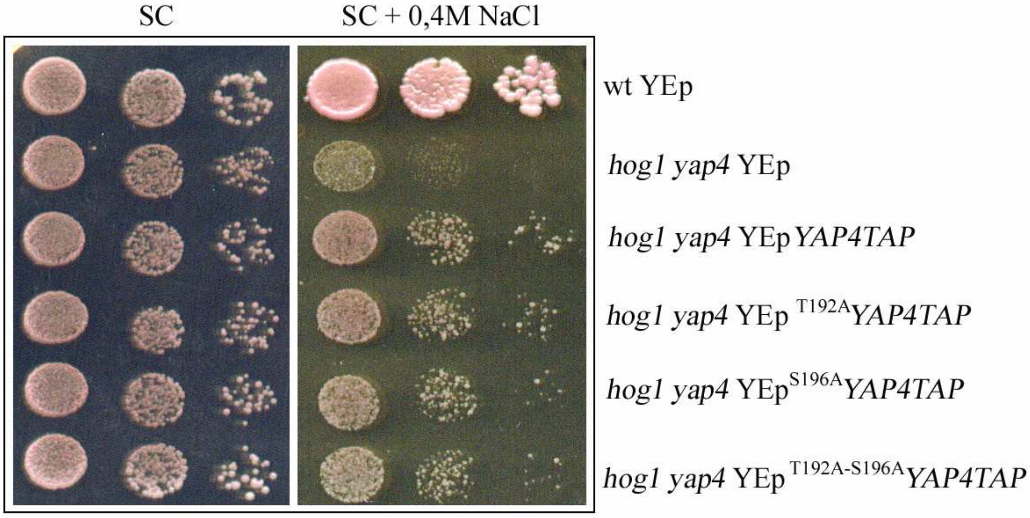


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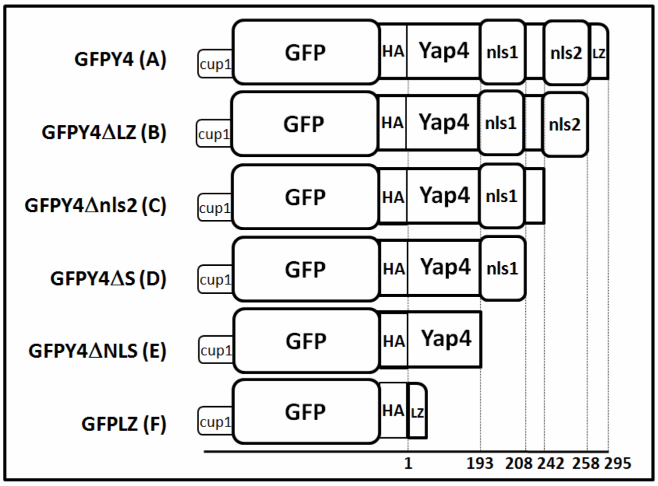
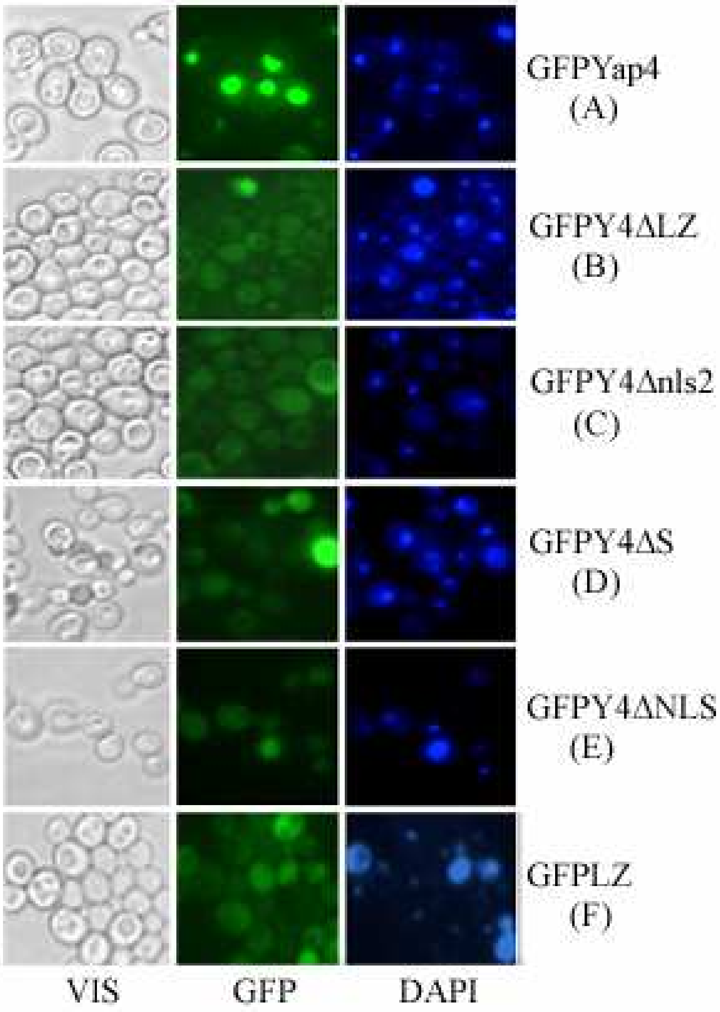
Fig. 7A

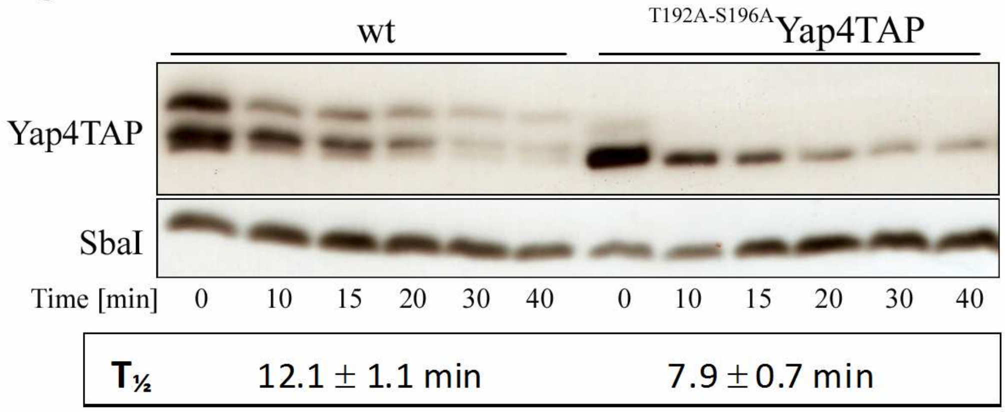


Pereira *et al.*, 2008
Figure 7B



Pereira *et al*., 2008

Figure 8



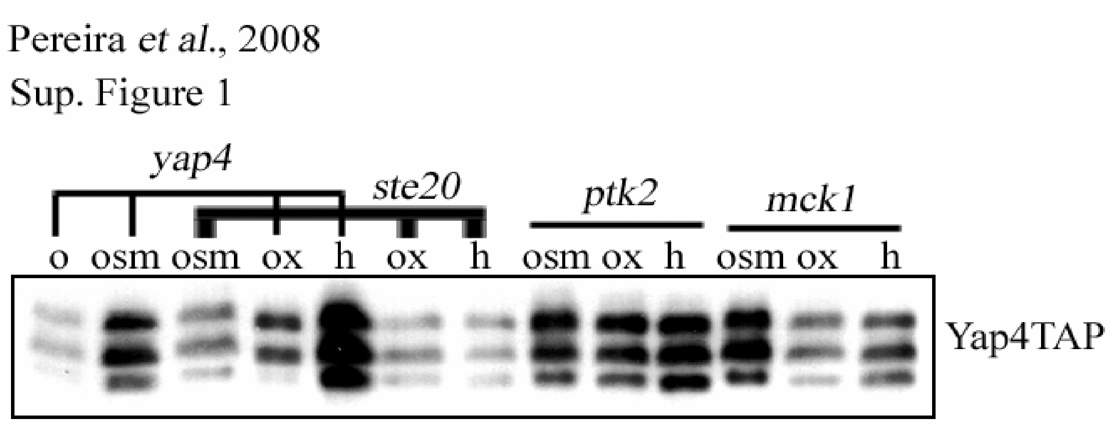
Pereira *et al*., 2008

Supplementary Material

Table I: Additional primers used in this work

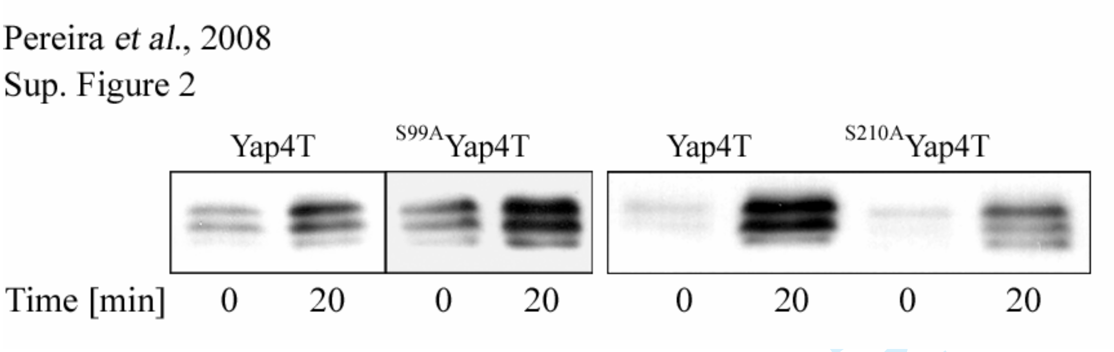
Primer		Sequence
T24A	Fwd	5’ – CATTCAATGGCAAGAGACAGTAC – 3’
	Rev	5’ – GTACTGTCTCTTGCCATTGAATG – 3’
S27A	Fwd	5’ – ATGACAAGAGACGCTACAAAGCCA – 3’
	Rev	5’ – TGGCTTTGTAGCGTCTCTTGTCAT – 3’
T28A	Fwd	5' – GACAAGAGACAGTGCAAAGCCAAAAAAAATGACG – 3'
	Rev	5' – GTCATTTTTTTTGGCTTTGCACTGTCTCTTGTCATTG – 3'
T34A	Fwd	5’ – CAAAAAAAATGGCGGATACCGCTTT – 3’
	Rev	5’ – AAAGCGGTATCCGCCATTTTTTTTG – 3’
S41A	Fwd	5’ – ACGGATACCGCTTTCGTGCCAGCCCCTCCTGTAG – 3’
	Rev	5’ – CTACAGGAGGGCTTGGCACGAAAGCGGTATCCGT – 3’
S59A	Fwd	5’ – TGCATACAATTGCGGTAGTTGCTTC – 3’
	Rev	5’ – GAAGCAACTACCGAAATTGTATGCA – 3'
T77A	Fwd	5’ – CAAAGATTGCAGCACTTGAAG – 3’
	Rev	5’ – CTTCAAGTGCTGCAATCTTTG – 3’
S94A	Fwd	5’ – GTTCTTCCAGCAAGATCTGTAAG – 3’
	Rev	5’ – CTTACAGATCTTGCTGGAAGAAC – 3’
S99A	Fwd	5’ – GGAAGAAGAAATGCTGTTAATATAGGAGC - 3'
	Rev	5’ – GCTCCTATATTAACAGCATTTCTTCTTCC – 3’
T122A	Fwd	5' – CAAGGCCGGTGGCAATAAACAATTTGATTC – 3'
	Rev	5' – GAATCAAATTGTTTATTGCCACCGGCCTTG – 3'
T136A	Fwd	5' – CTTTACCAAGACTGAACGCATACCAGCTTAG – 3'
	Rev	5' – CTAAGCTGGTATGCGTTCAGTCTTGGTAAA – 3'
T189A	Fwd	5’ – GCTTCATATTTTCCCTCAAATAGTAGCCCAGCTACGAGA – 3’
	Rev	5’ – TCTCGTAGCTGGGCTACTATTTGAGGGAAAATATGAAGC – 3’
T199A	Fwd	5’ – AAAATAGTGCCACGGCTAACCTTC – 3’
	Rev	5’ – GAAGGTTAGCCGTGGCACTATTTTT – 3’
S210A	Fwd	5' – GAAAGACGTCGAGTTGCCGTTTCTCTTTC – 3'
	Rev	5' – GAAAGAGAAACGGCAACTCGACGTCTTTC – 3'
S214A	Fwd	5’ – TCCGTTTCTCTTGCAGAGCAGGTTT – 3’
	Rev	5’ – AAACCTGCTCTGCAAGAGAAACGGA – 3’
S288A	Fwd	5’ – GATTGAAGCATTAAAGTCG – 3’
	Rev	5’ – CGACTTTAATGCTTCAATC – 3’

Supplementary Figures



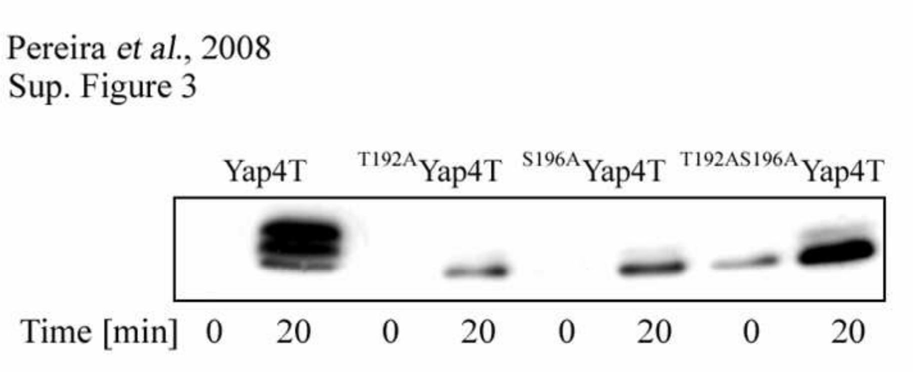
Supplementary Figure 1: Yap4 phosphorylation is independent Ste20, Ptk2 and Mck1.

Kinase mutants *ste20*, *ptk2* and *mck1* were assayed for Yap4 phosphorylation under the stress conditions indicated: **osm -** 0.4M NaCl, 20min; **ox** – 0.3mM H_2O_2, 30min; **h** – 37ºC, 30min. This western blot is representative of at least three independent assays.



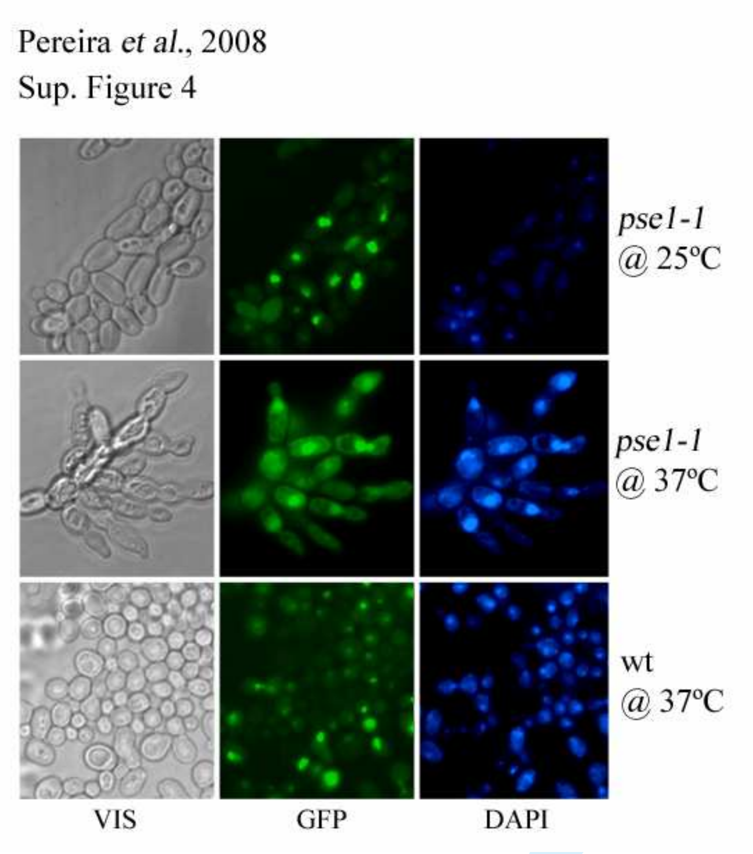
Supplementary Figure 2: Yap4 phosphorylation is not impaired in ^{S99A}Yap4 and ^{S210A}Yap4.

S99 and S210 *YAP4* residues were mutated to alanine by site-directed mutagenesis and integrated in a *yap4* strain. Mid-log phase cells were harvested after induction with 0.4M NaCl at the indicated time points and analysed by western blot. This result is representative of at least three independent assays.



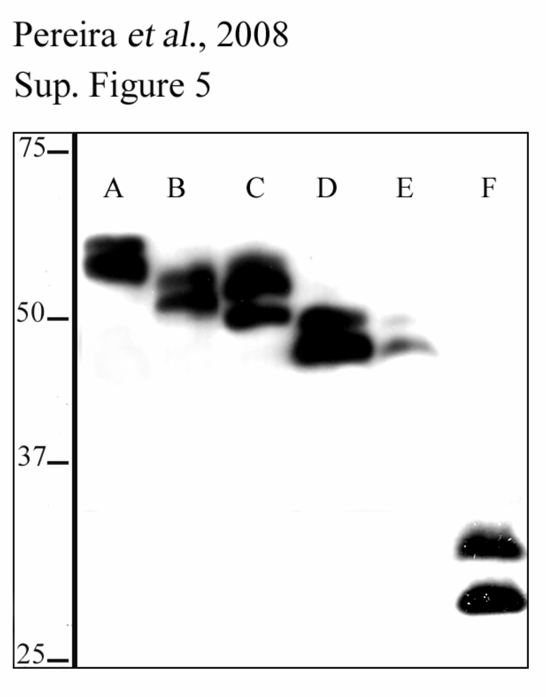
Supplementary Figure 3: Yap4 phosphorylation is abolished in the single mutants ^{T192A}Yap4 and ^{S196A}Yap4 and in the double mutant ^{T192AS196A}Yap4.

The indicated residues of *YAP4* were mutated to alanine by site-directed mutagenesis and integrated in a *yap4* strain (for details see Material and Methods). Mid-log phase cells were harvested after induction with 0.4M NaCl at the indicated time points and analysed by western blot. This western blot is representative of at least three independent assays.



Supplementary Figure 4: Yap4 import into the nucleus is not mediated by the importin Pse1.

Wt and *pse1-1* conditional mutants cells were transformed with episomal GFPHAYap4 harvested at mid-log phase (growth under permissive (25ºC) and non-permissive temperature (37ºC) and observed under fluorescent microscopy as described in *Materials and Methods*.



Supplementary Figure 5: Protein expression of the GFPHAYap4 truncations. *yap4* mutants were transformed with *YAP4* truncations indicated and western blots were performed as described in *Material and Methods*, except the antibody used in the western blot was anti-HA-Peroxidase, High Affinity 3F10 (Roche).

A – GFPHAYap4, B – GFPHAYap4ΔLZ, C – GFPHAYap4Δnls2,

D – GFPHAYap4ΔS, E – GFPHAYap4ΔNLS, F – GFPHALZ.

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

Tracy NEVITT*, Jorge PEREIRA*, Dulce AZEVEDO*, Paulo GUERREIRO*† and Claudina RODRIGUES-POUSADA*[1]

*Stress and Genomics Laboratory, Institute of Chemical and Biological Technology, Av. da República, Apt. 127, 2781-901 Oeiras, Portugal, and †Escola Superior de Tecnologia da Saúde de Lisboa, Av. D. João II, Lt. 4.69.01, Parque das Nações, 1990-096 Lisboa, Portugal

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

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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.

[1] To whom correspondence should be addressed (e-mail claudina@itqb.unl.pt).

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

MAT, mating type; KAN, kanamycin resistance.

Strain	Genotype	Source
FY1679	MATα his3-200 ura3-52 GAL2	[24]
yap1	MATα yap1::KAN	This study
yap4	MATα yap4::KAN	This study
yap1/yap4	MATα 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	MATα 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	MATα msn2::HIS3	Dr F. Estruch
msn4	MATa msn4::TRP1	Dr F. Estruch
msn2/msn4	MATa msn2::HIS3, msn4::URA3	Dr F. Estruch
msn2/yap4	MATα msn2::HIS3, yap4::KAN	This study
hot1	MATa hot1::KAN	[36]
msn1	MATα 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 μl 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: ΔSTRE$_{430}$, 5′-GACGCA-ATGCGTGAGTTGATTTCCCGAG-3′ (that alter the STRE sequence from GGAGGGG to TGAGTTG); ΔSTRE$_{716}$, 5′-GT-AAAACTTGTTGTACGGAGAGTTGTCGAGAAAAG-3′ (that alter the STRE sequence from GAGGGG to GAGTTG); ΔYRE, 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 μg 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 [α-^{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′-CCCACAT-GTTGGCTCACTTACAGATC -3′ and 5′-CCCACATGTATTCT-TTTAATTTCGACTTTA-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 21 000 ***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.

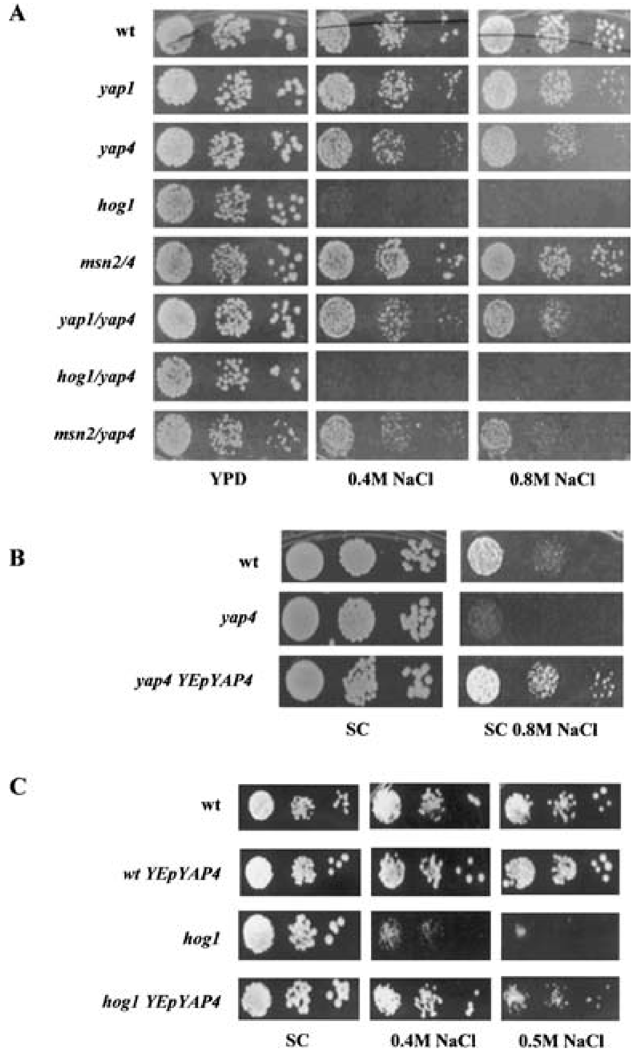

Figure 1 Mutant strain *yap4* shows a salt-sensitive phenotype

(**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

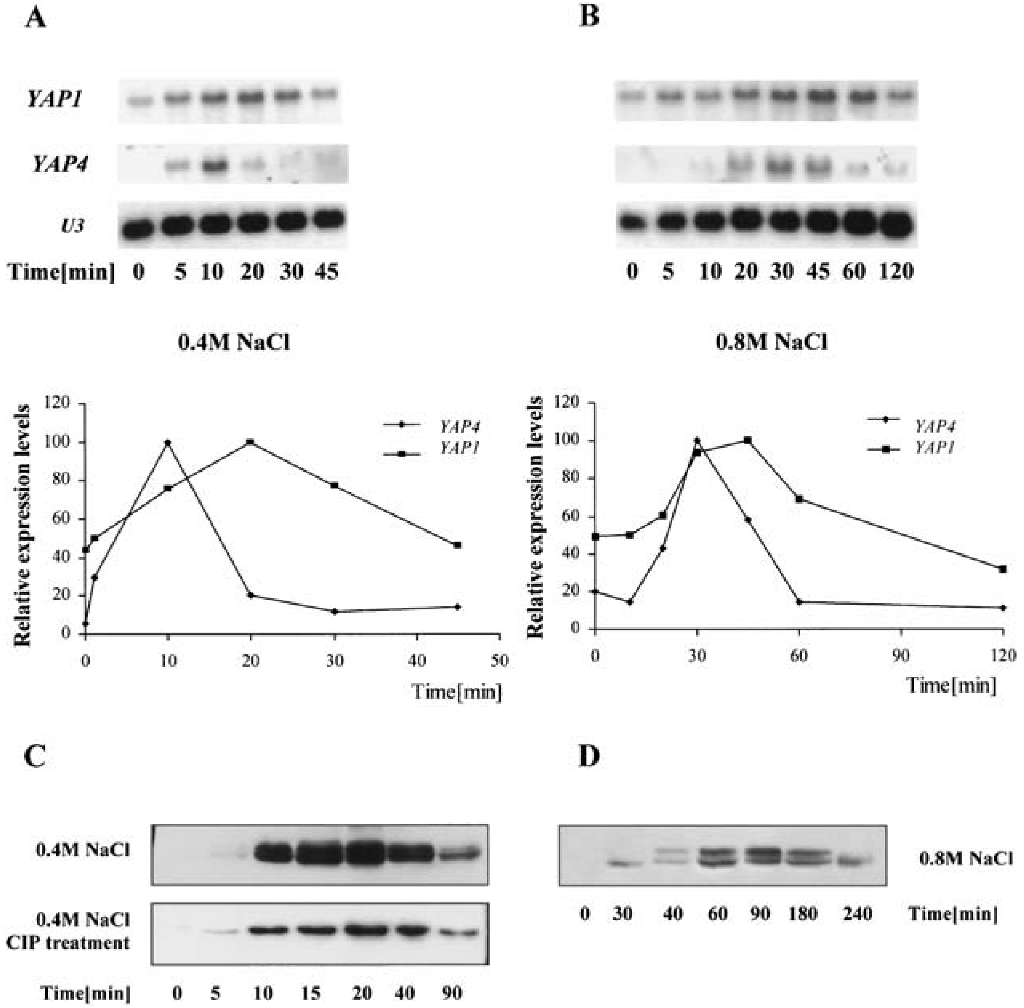

Figure 2 Induction of *YAP4* and *YAP1* under conditions of (A) mild (0.4 M NaCl) and (B) moderate (0.8 M NaCl) osmotic stress

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.

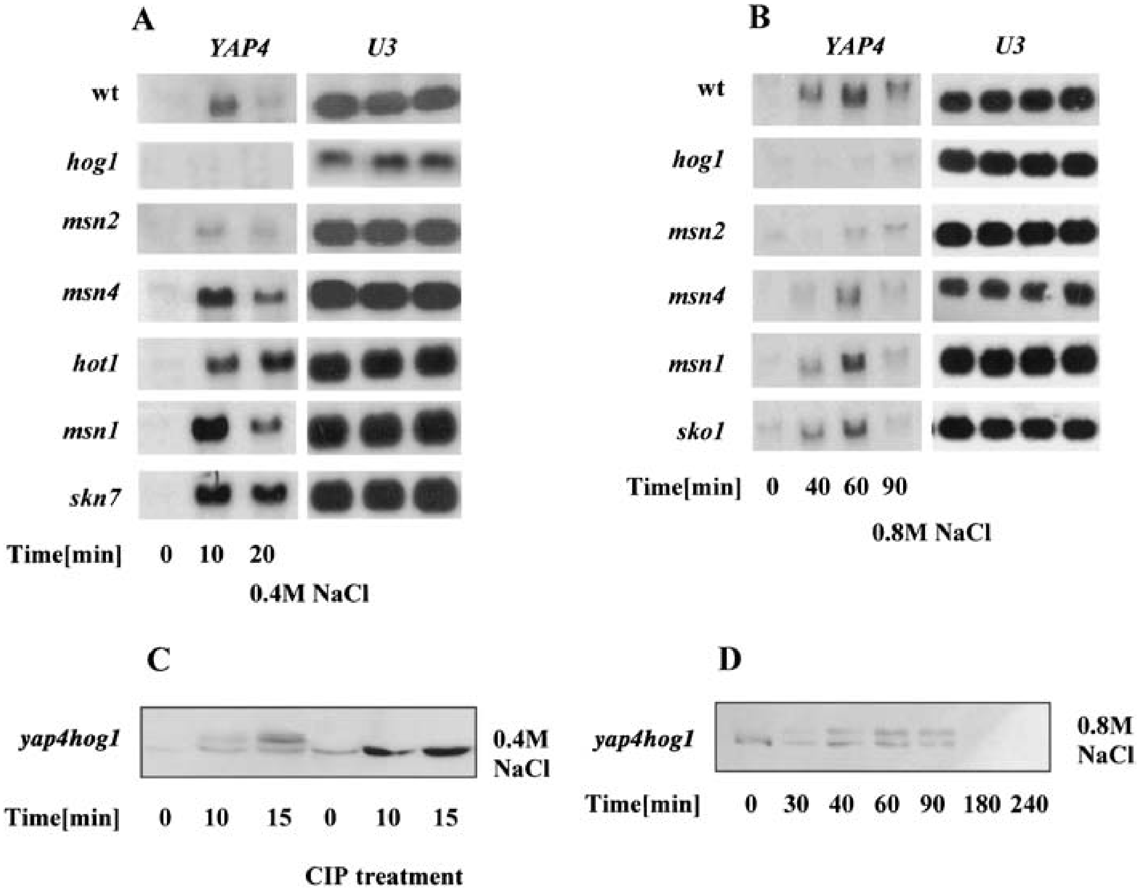

Figure 3 Epistasis analyses using strains deleted in Hog1p and Hog1-target transcription factors

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

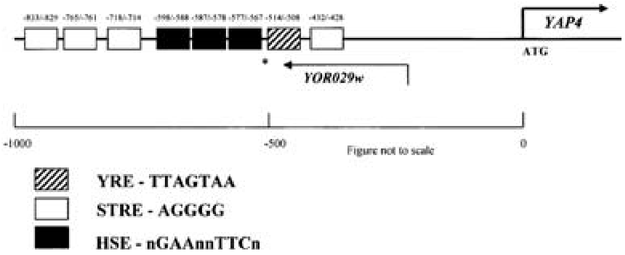

Figure 4 Schematic representation of the YRE, HSE and STRE elements present in the *YAP4* upstream region

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).

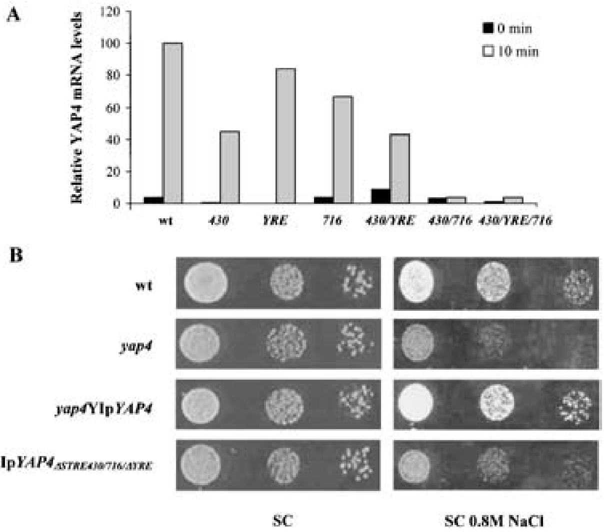

Figure 5 Regulation of *YAP4* gene is mediated by STREs located in its promoter region

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 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).

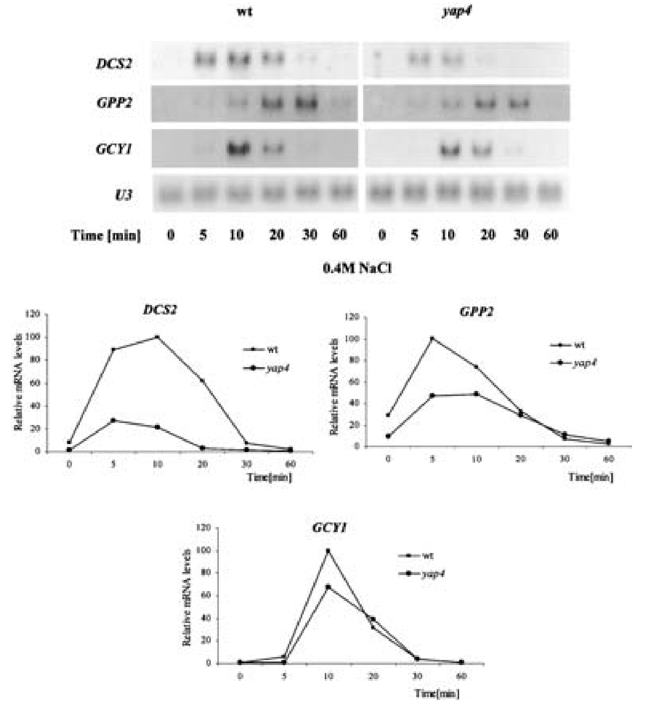

Figure 6 Expression analysis of *DCS2*, *GPP2* and *GCY1* genes in the wild-type (wt) and *yap4* mutant strains

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).

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

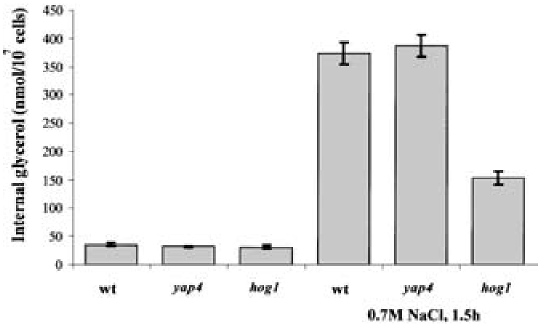

Figure 7 Internal glycerol accumulation is unaffected by the lack of Yap4p

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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Research Article

YAP4 gene expression is induced in response to several forms of stress in *Saccharomyces cerevisiae*

Tracy Nevitt, Jorge Pereira and Claudina Rodrigues-Pousada*
Stress and Genomics Laboratory, Instituto de Tecnologia Química e Biológica, Av. da República, Apt. 127, 2781-901 Oeiras, Portugal

**Correspondence to:
Claudina Rodrigues-Pousada,
Stress and Genomics Laboratory,
Instituto de Tecnologia Química
e Biológica, Av. da República,
Apt. 127, 2781-901
Oeiras, Portugal.
E-mail: claudina@itqb.unl.pt*

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Abstract

Exposure of *Saccharomyces cerevisiae* to several environmental insults, including conditions of oxidative, heavy metal, metalloid and heat stress, induces the expression of the *YAP4* gene, previously shown to play a role in the response to hyperosmotic stress. Expression analyses in several mutant strains under pro-oxidant conditions have determined that *YAP4* is regulated by the transactivators Yap1p and Msn2p. Mutation of either the Yap1p-response element (YRE), located at −517 bp from the ATG, or the most proximal stress response element (STRE) at −430 bp, is shown to strongly compromise *YAP4* gene expression under these conditions. Furthermore, these two mutations in combination lead to a severe depletion of detectable mRNA levels, indicating interplay between the transcription factors Yap1p and Msn2p in the regulation of *YAP4* transcription. Transcriptional activation of this gene reflects a concomitant increase in Yap4p protein levels that appear phosphorylated upon stress and negatively regulated by protein kinase A. Yap4p amino acid residues Ser89, Ser196 and Thr241 are shown to be required for protein phosphorylation and/or protein stability. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords: *YAP4 (CIN5)*; **Yap1p; Msn2p; YRE; STRE; oxidative stress**

Introduction

The budding yeast *Saccharomyces cerevisiae*, by virtue of its rapidly fluctuating environment, has developed effective stress sensing and response mechanisms. This is accomplished through the action of several transcription factors whose role in the stress response may be widespread, such as Msn2/4p, or stress-specific, such as Yap1p. Accumulating evidence now suggests that regulatory networks involving multiple factors exist to modulate changes in global gene expression.

The general stress response pathway is activated upon exposure to many forms of stress, including osmotic and oxidative stress, heat, low pH and nutrient starvation amongst others (Estruch, 2000). This response, mediated by the two zinc finger transcription factors, Msn2p and Msn4p, acts via a consensus *cis*-regulatory element termed the stress response element (STRE-C_4T) present on target gene promoters. Previous studies have demonstrated that a significant number of genes induced by oxidative stress shows Msn2/4p-dependence (Gasch *et al*., 2000; Lee *et al*., 1999), pointing towards an overlap between this and the Yap1p regulon (Hasan *et al*., 2002). Similarly, such co-regulation has also been described between Msn2/4p and Hsf1p under various conditions of cellular stress (Amoros *et al*., 2001).

Response and adaptation to conditions of oxidative stress requires the regulated expression of a large number of genes involved in antioxidant protection and thiol redox homeostasis (Toledano *et al*., 2003). Several studies have demonstrated that Yap1p, a b-Zip protein belonging to the yeast Yap family of transcriptional regulators (Rodrigues-Pousada *et al*., 2004), plays a central role in sensing and modulating gene expression in response to several forms of oxidative stress (Alarco *et al*., 1997; Delaunay *et al*., 2000; Gasch *et al*., 2000; Lee *et al*., 1999; Dumond *et al*., 2000; Nguyen *et al*., 2001; Wemmie *et al*., 1994;

Azevedo *et al.*, 2003). Yap1p-responsive genes typically contain within their promoter region a Yap-response element (YRE-TTAC/GTAA) encoding for most cellular proteins with antioxidant function and several membrane-associated drug efflux pumps (Toledano *et al.*, 2003).

To counteract the positive regulatory effect exerted by Yap1p and Msn2p is the Ras-cAMP-PKA pathway, which has been previously shown to negatively regulate both Yap1p (Fernandes *et al.*, 1997) and STRE-driven induction (Gorner *et al.*, 1998; Marchler, 1993).

Other regulators of oxidative stress, many of which act as Yap1p co-regulators, include Skn7p (Lee *et al.*, 1999), Sko1p (Rep *et al.*, 2001), Hsf1p (Trott and Morano, 2003) and Yap2p (our unpublished results), itself a member of the YAP family.

YAP4, the fourth Yap family member, has recently been described as a downstream component of the HOG pathway involved in the *Saccharomyces cerevisiae* response to hyperosmolarity (Nevitt *et al.*, 2004). This gene contains within its promoter region one consensus YRE sequence positioned at −514 bp from the ATG, four STRE sequences located at −432 bp, −714 bp, −761 bp and −821 bp, as well as multiple heat shock elements (HSEs). In this study we show that *YAP4* is transcribed and translated under several stress conditions. Under oxidative stress *YAP4* expression is dependent on Yap1p and Msn2p. Deletion of the YRE and most proximal STRE leads to a complete abrogation of *YAP4* mRNAs as well as of Yap4p protein levels. Furthermore, we show that Yap4p levels are greatly reduced in a *bcy1* strain bearing a constitutively active PKA. This protein is phosphorylated and several residues are identified that, when mutated, prevent Yap4p phosphorylation and/or affect protein stability. Taken together, our data suggest that, under conditions of oxidative stress, *YAP4* is regulated at the transcriptional as well as post-translational levels.

Materials and methods

Yeast 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* genes were deleted by the microhomology PCR method described by Güldener *et al.* (1996). Deletion was confirmed by PCR analysis of genomic DNA, using upstream and downstream primers. The *hog1* and *skn7* mutant strains in the W303-1A background were a kind gift from Dr S. Hohmann (University of Göteborg) whilst the *msn2* and *msn4* single and double

Table 1. *S. cerevisiae* strains and plasmids

Strain	Genotype	Source
W303-1A	MAT**a** leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC mal0	Thomas and Rothstein (1989)
W303-1B	MATα leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC mal0	Thomas and Rothstein (1989)
hog1	MAT**a** hog1::TRP1	S. Hohmann
sko1	MAT**a** sko1::KAN	This study
yap1	MAT**a** yap1::KAN	This study
yap4	MAT**a** yap4::KAN	This study
yap1yap4	MAT**a** yap1, yap4::KAN	This study
msn2	MATα msn2::HIS3	F. Estruch
msn4	MAT**a** msn4::TRP1	F. Estruch
msn2msn4	MAT**a** msn2::HIS3, msn4::URA3	F. Estruch
msn2yap1	MATα msn2::HIS3, yap1::KAN	This study
skn7	MAT**a** skn7::KAN	S. Hohmann
yap4yap4	MAT**a** yap4::KAN, YIpYAP4	This study
yap4YAP4$_{\Delta STRE430}$	MAT**a** yap4::KAN, YIpYAP4$_{\Delta STRE430}$	This study
yap4YAP4$_{\Delta STRE716}$	MAT**a** yap4::KAN, YIpYAP4$_{\Delta STRE716}$	This study
yap4YAP4$_{\Delta YRE}$	MAT**a** yap4::KAN, YIpYAP4$_{\Delta YRE}$	This study
yap4YAP4$_{\Delta STRE430/716}$	MAT**a** yap4::KAN, YIpYAP4$_{\Delta STRE430/716}$	This study
yap4YAP4$_{\Delta STRE430/YRE}$	MAT**a** yap4::KAN, YIpYAP4$_{\Delta STRE430/YRE}$	This study
yap4YAP4$_{\Delta STRE716/YRE}$	MAT**a** yap4::KAN, YIpYAP4$_{\Delta STRE716/YRE}$	This study
yap4YAP4$_{\Delta 430/716/YRE}$	MAT**a** yap4::KAN, YIpYAP4$_{\Delta STRE430/716/YRE}$	This study

mutants a kind gift from F. Estruch (University of Valencia). Yeast strains were grown in YP medium supplemented with 2% glucose or SC medium [0.67% yeast nitrogen base without amino acids, 0.6% casamino acids (Difco)] supplemented with glucose and the appropriate selective amino acids. Standard liquid cultures were incubated with orbital shaking (200 r.p.m.) at 30 °C.

Stress conditions were applied by exposure to the following stress inducers, the concentrations of which have been previously described: 0.3 mM H_2O_2 (Delaunay *et al.*, 2000), 1.5 mM diamide (Gasch *et al.*, 2000), 1 mM cadmium (Azevedo *et al.*, 2003), 50 mM arsenate (Bobrowicz *et al.*, 1997) or 37 °C heat shock, and samples collected at the indicated time points by cold centrifugation at 5000 rpm. Samples for RNA and protein extraction were washed and stored at −80 °C.

Standard methods were used for genetic analysis (Guthrie and Fink, 1991), cloning (Sambrook *et al.*, 1989; Ausubel *et al.*, 1995) and transformation (Gietz *et al.*, 1995).

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

Site-directed mutation of *YAP4 cis*-elements were generated by first cloning the entire *YAP4* ORF with its 1043 bp upstream region into the YIplac211 (Gietz and Sugino, 1988) *Hin*dIII site. Mutations were subsequently generated by PCR amplification of the entire construct, using complementary primers containing the desired mutation as follows: ΔSTRE$_{430}$ 5′-GACGCAATGCGTGAGTTGATTTCCCGAG-3′, which alters the STRE sequence from GGAGGGG to TGAGTTG; ΔSTRE$_{716}$ 5′-GTAAAACTTGTTGTACGGAGAGTTGTCGAGAAAAG-3′, which alters the STRE sequence from GAGGGG to GAGTTG; ΔYRE 5′-CGTTAATTTGTAAGTTATGCAACCAAGTGC-3′, which changes 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 over mutant constructs.

Construct sequencing

All constructs generated in this study were sequenced using the ABI Prism DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and ABI Prism 373A Automatic Sequencer (Perkin-Elmer).

Northern blot analysis

Total RNA was extracted by the hot acid phenol method (Ausubel *et al.*, 1995) from early log-phase cultures that were either untreated (control) or exposed to 0.3 mM H_2O_2 or 1.5 mM diamide. Approximately 30 μg RNA was loaded per lane, separated in formaldehyde gels (Ausubel *et al.*, 1995) and transferred onto nylon membranes (MagnaCharge; Schleicher & Shuell; Hybond XL, Amersham Pharmacia). Probes [an intragenic *YAP4* PCR, an intragenic *U3* PCR fragment (a small nuclear, RNA SNR17A, used as the internal loading control), an intragenic *YAP1* PCR fragment and an intragenic *GSH1* PCR fragment (Wu *et al.*, 1994)] were radiolabelled with αP^{32}-ATP by random priming (MegaPrime; Amersham-Pharmacia), G50-purified and hybridized overnight in Church hybridization buffer (0.25 M sodium phosphate, pH 7.5, 7% sodium dodecyl sulphate, 1 mM EDTA, 1% BSA) at 65 °C. Radioactive blots were washed (20 mM sodium phosphate, pH 7.5, 0.1% sodium dodecyl sulphate, 1 mM EDTA) and the signal detected by exposure to radio-sensitive film (Biomax MR; Kodak) or to a Molecular Dynamics phosphor screen and the signal detected by a Storm 860 Scanner. Messenger RNA levels were quantified (ImageQuant, Molecular Dynamics) and normalized against those of *U3*.

Generation of YAP4TAPTag, protein extraction and Western analysis

TAPtag cloning

AflIII restriction sites were introduced into the desired *YAP4* fragment by PCR amplification of pRS416*YAP4*, a centromeric vector (Sikorsky and Hieter, 1989) containing the entire *YAP4* ORF cloned in this study as described above, using primers 5′-CCC ACA TGT TGG CTC ACT TAC AGA TC-3′ and 5′-CCC ACA TGT ATT CTT TTA ATT TCG ACT TTA-3′. The amplified *YAP4* fragment was then *Afl*III-digested and cloned into the pBS1479 (Rigaut *et al.*, 1999) *Nco*I site, placing it in-frame with the TAPtag plasmid sequence. The *YAP4*– TAPtag fragment was then digested with *Mls*I and *Xba*I and re-cloned into pRS416*YAP4*.

Site-directed mutagenesis

Site-directed mutagenesis was performed according to QuikChange® II Site-Directed Mutagenesis Kit (Stratagene). Complementary primers for site-directed mutagenesis were designed as follows: **S89A** CAGAACTGGC**GCA**CTTACAGATCT-TTC; **S196A** GAGAAAAAAT**GCT**GCCACGAC-TAAC; and **T241A** CCTTTAAGAAAT**GCT**AA-GAGAGCTG: the mutagenized bases are shown in bold.

Protein extracts

Samples collected at the indicated time points were harvested by centrifugation at 4 °C, washed with 20% TCA and lysed by vortexing. The supernatant was centrifuged and the pellet resuspended in Laemli buffer (62.5 mM Tris–Cl, pH 8.7, 2% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue), neutralized with Tris 1.0 M, pH 8.0, and heated for 5 min at 95 °C. Finally, the solution was centrifuged and the supernatant proteins were quantified by Bradford (1976) using the Bio-Rad protein assay reagent. All operations and reagents were pre-cooled before use.

Western blot analysis

Samples were separated by SDS 10% polyacrylamide gel electrophoresis, transferred onto a PROTRAN® (Schleicher & Schuell) nitrocellulose transfer membrane and incubated with anti-TAPtag antibody (PAP rabbit antibody, Sigma; 1 : 1.000 dilution in PBS-Tween 0.1%, 1% milk). Chemiluminescent detection was enhanced with ECL+ Western Blotting Detection System (Amersham Pharmacia).

Protein extracts of *yap4* strains expressing TAP-tagged Yap4p were subjected to calf intestinal phosphatase incubation for 45 min at 37 °C prior to SDS–PAGE and immunoblot (Delaunay *et al.*, 2000).

Results and discussion

YAP4 is induced in response to several stress conditions

Yeast cells are regularly exposed to fluctuations in their environmental milieu. Adaptation to new conditions requires the rapid remodelling of gene expression programmes and several genes have been described as being responsive to a variety of stress conditions and signalling pathways. Within this context, this work describes the regulation of *YAP4* gene expression that is stimulated under diverse environmental stresses. The expression profile for *YAP4* under stress conditions imposed by the oxidants H_2O_2 and diamide, the heavy metal cadmium, arsenic and heat shock are shown in Figures 1 and 2B (details regarding inducer concentrations can be found in Materials and methods). Samples were collected at the indicated time points and Northern analyses performed on the total RNA extracts. These results clearly show that *YAP4* is induced under several forms of stress, whose diverse nature of inducing properties comprise pro-oxidants, metals, metalloids and heat. However, unlike that observed under hyperosmolarity (Nevitt *et al.*, 2004), no phenotype can be observed for the *yap4* mutant growing under these stress conditions.

Yap1p and Msn2p mediate activation of *YAP4* expression under oxidative conditions

In *Saccharomyces cerevisiae* the response to oxidative exposure is both rapid and transient (Gasch *et al.*, 2000) involving the Yap1-mediated regulation of gene transcription. The presence of a consensus YRE in the *YAP4* promoter presented a strong indication of its participation in the yeast Yap1p-regulated oxidative stress response. Having demonstrated that *YAP4* gene expression is activated under conditions of oxidative stress (Figure 1), it became important to assess the involvement of Yap1p in the regulation of *YAP4* gene expression. To this end, expression analyses were carried out in the *yap1* mutant strain as well as in several mutant strains, as indicated in Figure 2. Our results reveal a 50% reduction of *YAP4* mRNA levels in the *yap1* mutant when compared to the wild-type strain (Figure 2B). Although these results indicate that Yap1p is indeed regulating *YAP4* gene expression under conditions of oxidative stress, it furthermore suggests that other independent factors also contribute towards this regulation. A relevant candidate for consideration is Msn2/4p, due to its central role in the general stress response. The presence of STRE sites in the *YAP4* promoter, as well as its dependence on Msn2p under osmostress (Nevitt *et al.*, 2004),

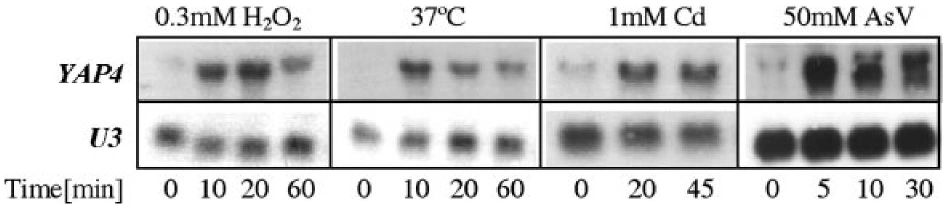

Figure 1. *YAP4* induction profile under 0.3 mM H_2O_2 and heat stress (37 °C), 1 mM cadmium and 50 mM arsenate. Exponentially growing cultures were exposed to the above conditions and changes in *YAP4* gene expression analysed by Northern analysis as described in Materials and methods

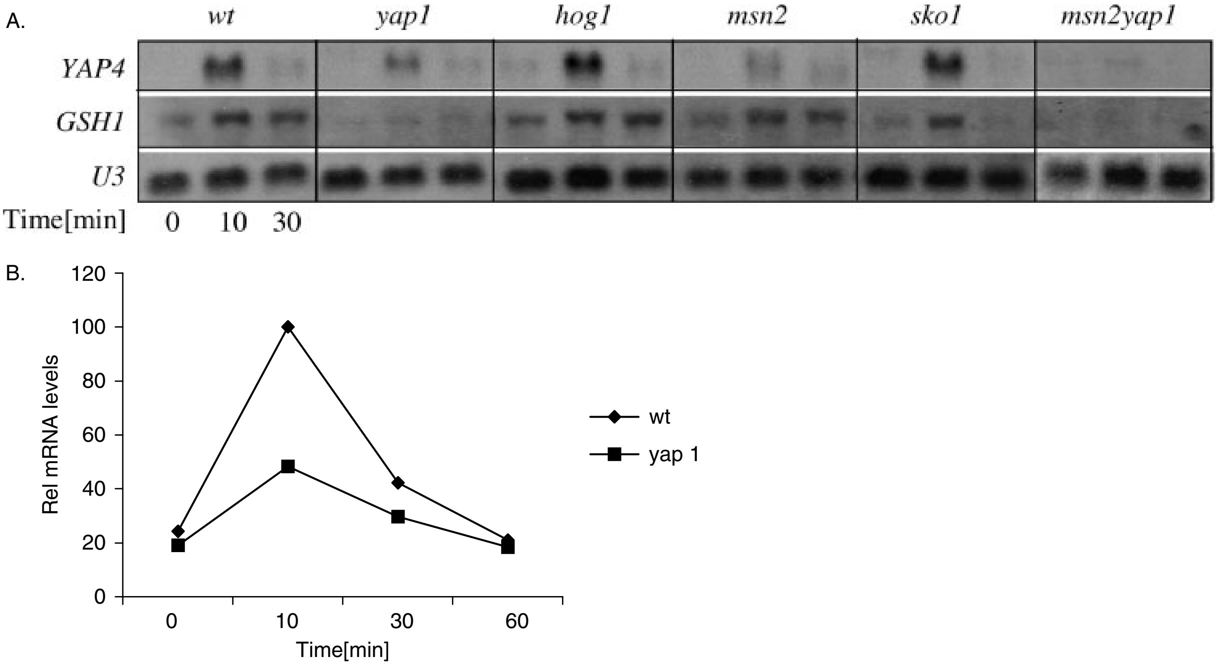

Figure 2. (A) *YAP4* induction under oxidative stress is dependent on Yap1p and Msn2p. Northern analyses on wild-type and mutant strains were performed as described in the legend to Figure 1. (B) Densitometric representation of *YAP4* induction in the wild-type and *yap1* mutant strain upon exposure to 1.5 mM diamide

further supports the hypothesis that these elements may contribute towards the regulation of *YAP4* under oxidative stress. From the data obtained, we observe that whilst *YAP4* induction is almost depleted in the *msn2* strain, showing a 75% reduction in mRNA levels (Figure 2A), this does not occur in the *msn4* mutant strain, which maintains almost wild-type levels (data not shown). The fact that there is a total depletion of *YAP4* gene expression in the *msn2/yap1* double mutant indicates that these two transcription factors are involved in the synergistic regulation of the *YAP4* gene. In contrast to the results observed upon an upshift in osmolarity, *YAP4* expression in the *hog1* mutant strain is identical to the wild-type (Figure 2A), indicating that under oxidative stress the mechanisms of *YAP4* expression are differentially regulated. The fact that *YAP4* expression appears unaffected by the deletion of *SKN7* (data not shown) furthermore suggests that it can be placed under the set of Yap1p-regulated genes that do not require Skn7p co-regulation (Toledano *et al.*, 2003). Sko1p has been implicated in the response to both osmotic (Proft *et al.*, 2001) and oxidative stress (Rep *et al.*, 2001). Similar to what is observed under hyperosmolarity (Nevitt *et al.*, 2004), Sko1p does not appear to regulate *YAP4* mRNA levels under oxidative stress (Figure 2A). The fact that no phenotype is associated to the *yap4* mutant under oxidizing conditions generated by H_2O_2 is most probably due to the central role of Yap1p as a transcriptional activator under these conditions that may mask the possible regulatory effects of Yap4p on the fine-tuning of gene regulation.

Role of the YRE and STRE cis-elements in YAP4 expression under oxidative stress

YAP4 chromosomal features upstream of the ATG include one YRE, which forms the binding site for the YAP family of transcription factors, and four STREs recognized by Msn2p and Msn4p, as well as at least three consensus HSEs (Figure 3). This region includes the as-yet uncharacterized neighbouring ORF (*YOR029w*) of unknown function. *YOR029w* contains elements such as long stretches of Ts characteristic of promoter regions and appears as a hypothetical protein in the database without any similarity to other classified ORFs. Sequence comparisons with other hemiascomycetes reveal no significant homology with annotated ORFs (**http://cbi.labri.fr/Genolevures/publications.php#FEBS2000**). Due to its low CAI value (0.07), it is unlikely that this ORF is expressed at all.

Using directed mutagenesis, the two most proximal STREs (−430 and −716 bp) as well as the YRE (−517 bp), were mutated both singly and in combination, as shown in the legend to Figure 4, and integrated back into the genome. *YAP4* gene expression was then monitored by Northern analysis. Under oxidative stress conditions our results, depicted in Figure 4, show that the most proximal STRE (−430 bp) is responsible for approximately 80% of the observed wild-type *YAP4* gene expression levels.

Mutation of the YRE reduces these levels by approximately 50–60%. The more distal $STRE_{716}$, when mutated in conjunction with a deletion of the $STRE_{430}$, reveals an expression profile similar to that observed in the single $STRE_{430}$ mutant (data not shown). Furthermore, the double mutation of YRE and $STRE_{716}$ shows approximately the same expression level as that observed for the single YRE mutant (data not shown). However, the lack of all three elements ($STRE_{430}$, $STRE_{716}$, YRE_{517}) leads to an almost complete abolishment of *YAP4* mRNAs. These results suggest that under oxidizing conditions the two most proximal elements, namely $STRE_{430}$ and YRE, are sufficient to synergistically mediate a full *YAP4* expression profile. Furthermore, abolition of the YRE leads to a decrease in expression levels similar to that observed in the *yap1* mutant strain (Figure 2B), suggesting that Yap1p is involved in *YAP4* regulation by binding to the YRE.

Analysis of Yap4p phosphorylation

Having determined the induction patterns of the *YAP4* mRNAs under the various conditions and strain backgrounds, it became important to analyse whether this mRNA increase reflects an enhancement in Yap4p protein levels. A *yap4* strain carrying an episomally-expressed TAP-tagged Yap4p shows induction kinetics similar to that observed for its mRNAs (Figure 5A). Upon stress induction with 0.3 mM H_2O_2, one can observe the appearance of several bands, which are converted to a single band after treatment with calf intestinal phosphatase (CIP) (Figure 5A, lane 6) indicating that Yap4p is phosphorylated. Taking into account that Yap4p is a constitutively

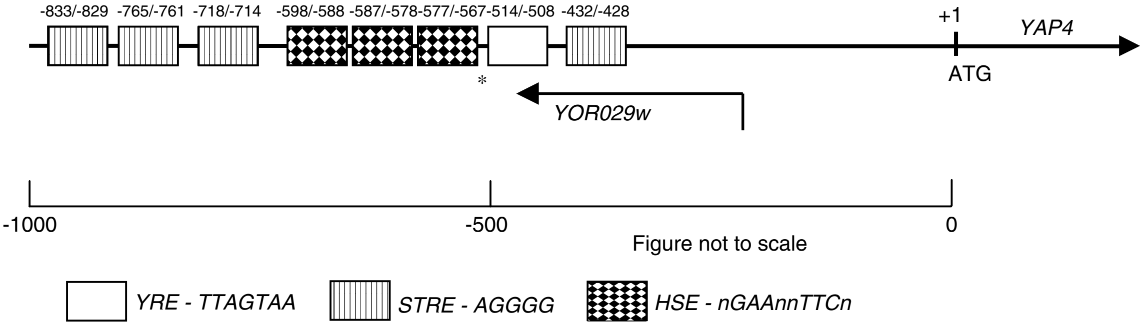

Figure 3. Schematic illustration of the *cis*-elements present in the *YAP4* upstream promoter region. An as-yet unidentified ORF, *YOR029w*, found within the *YAP4* promoter region, is illustrated (adapted from Nevitt *et al*., 2004)

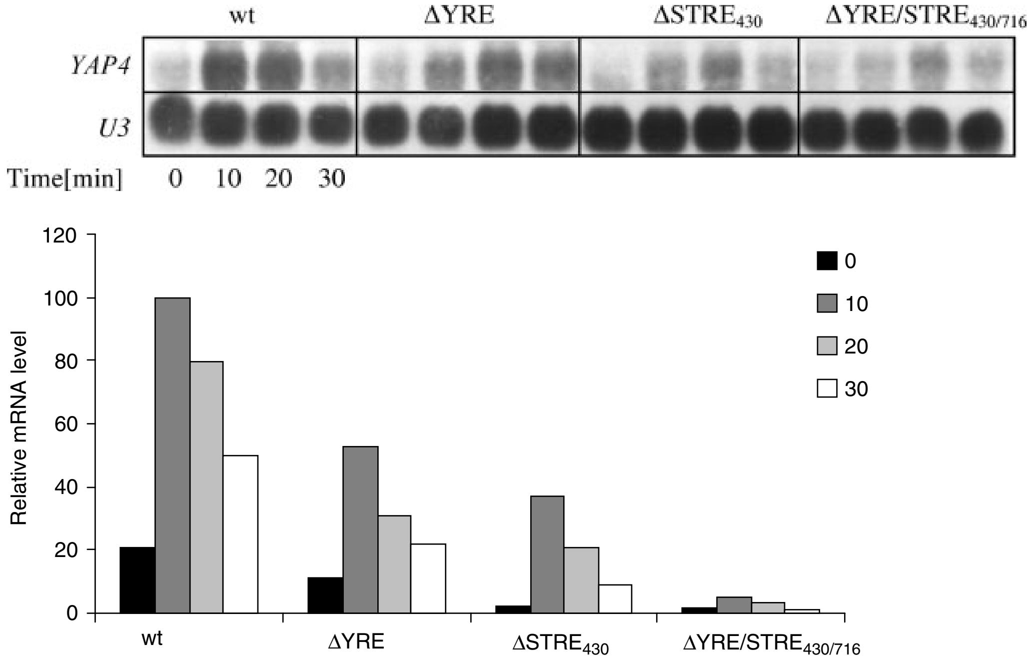

Figure 4. Regulation of *YAP4* under oxidative stress is mediated by at least two *cis*-elements located in it promoter region. The *yap4* mutant strain was transformed with an integrative plasmid (as described in Materials and methods) containing the *YAP4* gene in which the YRE and/or the most proximal STRE site has been mutated in the promoter. *YAP4* was induced with 0.3 mM H_2O_2, followed by Northern analysis and mRNA quantification by densitometric analysis

nuclear protein, this phosphorylation step is most probably relevant for its post-translational activation and/or stability. Yap4p contains several putative phosphorylation motifs, including several putative PKA sites. In order to evaluate the effect of an overactive PKA, Yap4p protein levels were monitored in a *bcy1* strain (Toda *et al.*, 1987) lacking the PKA regulatory subunit. This strain shows a severe depletion of Yap4p protein levels (Figure 5B). However, whether this is a direct effect due to Yap4p phosphorylation by this protein kinase, or whether it is simply a reflection of its negative effect on Msn2p and Yap1p, is currently being evaluated. Although several putative PKA phosphorylation sites exist along the length of Yap4p, these residues also represent sites for phosphorylation by other kinases. Site-directed mutagenesis of selected sites was performed in order to identify the residues involved in this post-translational event (Figure 5C). Figure 5D illustrates the results obtained from this study. Mutation of serine in position 89 greatly diminishes phosphorylated Yap4p, concomitantly reducing protein levels. Mutation of threonine at position 241 decreases protein levels but retains Yap4p proportional phosphorylation. In contrast, the mutation of serine at position 196 decreases both the levels of phosphorylated protein and the total amount of cellular Yap4p. These results suggest that these residues may be involved in maintaining protein stability, e.g. by protecting against targeted degradation and provide evidence for a post-translationally regulated Yap4p role in the yeast response to oxidative stress.

Conclusions

In conclusion, this work describes the inducibility of the yeast *YAP4*-encoded transcriptional modulator by a panel of different forms of stress. The presence of at least three types of *cis*-elements associated to the stress response in its promoter region, viz. YRE, STREs and HSEs, suggest that its regulation can be modulated by a combination of transcriptional regulators in a condition-specific manner. Indeed, as described in Nevitt *et al.* (2004), the activation of *YAP4* in response to

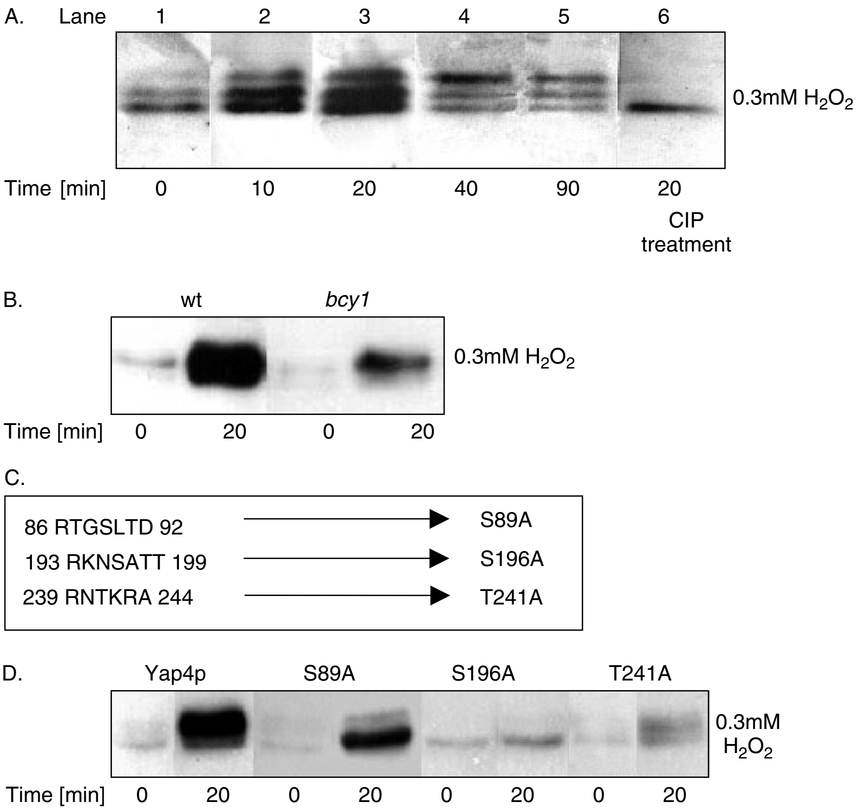

Figure 5. (A) Yap4p protein kinetics upon induction by 0.3 mM H_2O_2 performed on 30 µg total protein extracts, as described in Materials and methods. Yap4p phosphorylation can be observed upon exposure to stress and is abolished after CIP treatment. (B) Yap4p protein levels are negatively regulated by PKA. Yap4p protein levels were analysed using 60 µg wild-type and *bcy1* mutant total protein extracts. (C) Key residues are responsible for Yap4p phosphorylation and/or wild-type cellular protein levels. (D) Mutation analyses of the indicated Yap4p phosphorylation residues were performed as described in Materials and methods

hyperosmotic stress involves Msn2p acting via the two most proximal STREs. However, the response to oxidative stress imposed by H_2O_2 and diamide requires the concerted action of both Yap1p and Msn2p on the YRE and most proximal STRE, respectively. This observation thus provides evidence for the existence of an integrated of cascade of several transcriptional activators. Although the mechanisms that dictate the precise accessibility of the various transcription factors to the distinct *cis*-elements are poorly understood, there is growing evidence that the selective transcription of many genes is a widespread phenomenon (Claude Jacq, personal communication).

The fact that *YAP4* is responsive to such a wide plethora of environmental insults suggests an important role in the response to stress. Indeed, previous work has revealed that its overexpression is capable of relieving cellular sensitivity to a wide range of conditions and drugs, including salt (Mendizabal *et al.*, 1998; Nevitt *et al.*, 2004), cisplatin (Furuchi *et al.*, 2001) and quinoline ring-containing antimalarial drugs (Delling *et al.*, 1998). The fact that the adduct-forming chemotherapeutic agent cisplatin acts at the level of transcriptional repression, by denying the RNA polymerase II initiation complex access to the TATA box (Vichi *et al.*, 1997), may suggest a role for Yap4p within the realm of the basal transcriptional machinery. Allied to the richness of *cis*-elements found within the *YAP4* promoter, there is the potential for an integrative circuit whereby multiple stress response pathways converge to modulate global gene expression.

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Minireview

Yeast activator proteins and stress response: an overview ☆

Claudina Amélia Rodrigues-Pousada*, Tracy Nevitt, Regina Menezes, Dulce Azevedo,
Jorge Pereira, Catarina Amaral

*Stress and Genomics Laboratory, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Avenida da República,
Apt. 127, 2781-901 Oeiras Codex, Portugal*

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Abstract Yeast, and especially *Saccharomyces cerevisiae*, are continuously exposed to rapid and drastic changes in their external milieu. Therefore, cells must maintain their homeostasis, which is achieved through a highly coordinated gene expression involving a plethora of transcription factors, each of them performing specific functions. Here, we discuss recent advances in our understanding of the function of the yeast activator protein family of eight basic-leucine zipper *trans*-activators that have been implicated in various forms of stress response.

Keywords: Stress; *YAP1*; *YAP2*; *YAP4*; *YAP8*; Remaining Yaps; *Saccharomyces cerevisiae*

1. Introduction

The cellular stress response is evolutionarily conserved in all living organisms, a major role being attributed to the induced heat-shock proteins and other molecules that confer stress protection. The molecular responses elicited by the cells dictate whether the organism adapts, survives, or, if injured beyond repair, undergoes death. The regulation of stress response includes transcriptional, post-transcriptional and post-translational mechanisms, the former being the most extensively studied.

Transcriptional regulation is mediated by a pre-existing transcriptional activator, the heat-shock factor (HSF), which binds to arrays of a 5-bp heat-shock element (HSE; nGAAn) present upstream of all heat-shock genes. Consequently, heat-shock proteins are induced, the majority acting as molecular chaperones in protein refolding and protecting them from degradation and aggregation. The HSF is a modular protein consisting of a helix–turn–helix class DNA-binding domain (DBD), a leucine zipper domain, required for trimerization, and a carboxy-terminal transcription activation domain [1]. Both the HSF of *Saccharomyces cerevisiae* and that of the closely related yeast *Kluveromyces lactis* contain an unique transcription activation domain amino terminal to the DBD [2,3]. Although the heat-shock response is a well-conserved mechanism throughout evolution, metazoans possess three HSFs, each playing a role in heat shock and development [4]. Notwithstanding, a great number of findings have demonstrated that gene expression under stress conditions in *S. cerevisiae* also elicits HSF-independent mechanisms, of which the Msn2p and Msn4p-mediated general stress response has been the most extensively studied [5,6]. These two partially redundant zinc-finger transcription factors govern the majority of genes involved in a plethora of stress responses. Msn2p and Msn4p bind to the stress response element (STRE), a 5-bp sequence, C4T [5]. In addition, the two basic-leucine zipper (b-ZIP) transcription factors, Yap1p and Yap2p [7,8], along with six newly identified proteins, form a family of *trans*-regulators that have been implicated in various forms of stress response [9–12]. The large amount of data from the many laboratories that work within this field of research is reviewed in great detail elsewhere [13,14] and here, we review the main aspects of the stress response in which the yeast activator protein (Yap) factors have been shown to be involved.

2. Identification of the Yap1p and Yap2p transcriptions factors

Yap1p, the first member of the family of Yaps to be described, was initially identified by its ability to bind and activate the SV-40 AP-1 recognition element (ARE: TGACTAA). Based on its ARE-binding capacity, this factor was purified as 90 kDa protein and the corresponding gene was cloned by screening a λgt11 library with a monoclonal antibody against Yap1p [15]. Subsequently, this gene was also found in multicopy transformants resistant to the iron chelators 1,10-phenanthroline and 1-nitroso-2-naphtol [16] as well as to a variety of drugs including 4-nitroquinoline-*N*-oxide, *N*-methyl-*N*′-nitro-*N*-nitrosoguanine, triaziquone, sulfomethuron methyl and cycloheximide, the locus being historically designated *PAR1*/*SNQ3*/*PDR4* [17–19]. Besides *YAP1*, a second gene, *YAP2*, conferring resistance to 1,10-phenanthroline in transformed cells overexpressing a multicopy yeast library, was also described [8]. This gene encodes a 45-kDa protein that also binds the ARE *cis*-acting element. Sequence homologies identified it as *CAD1*, due to the acquisition of cadmium resistance in cells overexpressing a multicopy genomic library [20]. Later, it was

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☆ PABMB Lecture.

* Corresponding author. Fax: +351-21-443-36-44.
E-mail address: claudina@itqb.unl.pt (C.A. Rodrigues-Pousada).

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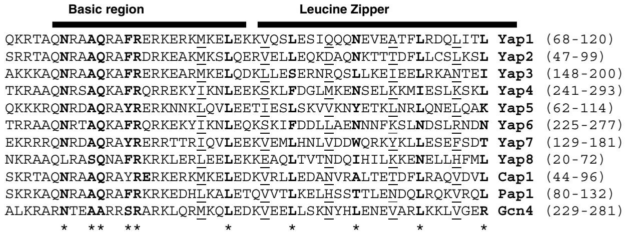
also shown that these cells gain resistance to cycloheximide [8,21].

The sequencing of the *YAP1* and *YAP2* genes revealed the presence of a b-ZIP-family domain in the N-terminus homologous to the true budding yeast AP-1 factor Gcn4p and to c-Jun, its mammalian counterpart. Furthermore, they also share two regions of similarity, one at the C-terminus (CR1) and an internal region (CR2) located close to the b-ZIP domain [8].

3. An extended YAP gene family

Biochemical and crystallographic analysis had previously defined the Gcn4p–DNA complex and the optimal AP-1 site (TGACTCA) [22,23]. Within the basic domain, five residues (corresponding to Asn235, Ala238, Ala239, Ser242 and Arg243 in Gcn4p) (see Fig. 1) responsible for the base-specific contacts in Gcn4p and Jun/Fos are most highly conserved [24–26]. Making use of a degenerate motif based on the sequences of a large number of basic regions in b-ZIP proteins from various organisms, the complete yeast genome was searched to identify *S. cerevisiae* b-ZIP proteins. The search revealed 14 proteins including, Gcn4p, Yap1p, Yap2p, Met28p [27], Sko1p [28] and Hac1p [29] that probably represent the complete set of budding yeast b-ZIP proteins [7]. Alignment of these sequences revealed a family of six newly identified proteins, Yap3p–Yap8p, containing conserved amino acid residues similar to those present in Yap1p and Yap2p. The features that distinguish this Yap family from Gcn4p are the amino acids that make contact with the DNA. Indeed, in position 238 a glutamine replaces an alanine and in position 242, a phenylalanine/tyrosine replaces a serine. Furthermore, there are two family-specific residues, namely, a glutamine in position 234 and an alanine in position 241 (Fig. 1). The Yap family binding site was thus subsequently characterized as TTAC/GTAA [7] for Yap1p–Yap4p. So far the corresponding binding site for Yap5p–Yap8p has not been characterized, although in the case of Yap8p, it appears to be TTAATAA [30]. We cannot, however, exclude the existence of other binding sites. Orthologues of Yap1p, but not of the remaining family members, have been found in other organisms including *Schizosaccharomyces pombe* (Pap1p) [31], *Candida albicans* (Cap1p) [32] and *K. lactis* (Klap1p) [33]. With reference to the structural similarities between the Yap family members, Yap1p shares the greatest homology with Yap2p and to a lesser extent with Yap3p; Yap4p is most homologous to Yap6p and Yap5p to Yap7p, whereas Yap8p is the least closely related family member. Approximately 15% of the genome contains one or more well-positioned consensus Yap response element (YRE) sequences within its promoter region, highlighting the potential regulatory effects of this family of transcription factors.



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QKRTAQNRAAQRAFRERKERKMKELEKKVQSLESIQQQNEVEATFLRDQLITL Yap1 (68-120)
SRRTAQNRAAQRAFRDRKEAKMKSLQERVELLEQKDAQNKTTTDFLLCSLKSL Yap2 (47-99)
AKKKAQNRAAQKAFRERKEARMKELQDKLLESERNRQSLLKEIEELRKANTEI Yap3 (148-200)
TKRAAQNRSAQKAFRQRREKYIKNLEEKSKLFDGLMKENSELKKMIESLKSKL Yap4 (241-293)
QKKKRQNRDAQRAYRERKNNKLQVLEETIESLSKVVKNYETKLNRLQNELQAK Yap5 (62-114)
TRRAAQNRTAQKAFRQRKEKYIKNLEQKSKIFDDLLAENNNFKSLNDSLRNDN Yap6 (225-277)
EKRRRQNRDAQRAYRERRTTRIQVLEEKVEMLHNLVDDWQRKYKLLESEFSDT Yap7 (129-181)
NKRAAQLRASQNAFRKRKLERLEELEKKEAQLTVTNDQIHILKKENELLHFML Yap8 (20-72)
SKRTAQNRAAQRAYRERKERKMKELEDKVRLLEDANVRALTETDFLRAQVDVL Cap1 (44-96)
SKRKAQNRAAQRAFRKRKEDHLKALETQVVTLKELHSSTTLENDQLRQKVRQL Pap1 (80-132)
ALKRARNTEAARRSRARKLQRMKQLEDKVEELLSKNYHLENEVARLKKLVGER Gcn4 (229-281)
      *  **  **         *      *      *      *      *
    235 238  242        253    260     267    274    281
```

Fig. 1. Alignment of the Yap b-Zip domains. Sequences of the eight *S. cerevisiae* Yap b-ZIP domains are compared with the corresponding regions from Gcn4p and Yap-like proteins from *S. pombe* (Pap1p), *Aspergillus nidulans* (meaBp) and *C. albicans* (Cap1p). In the basic region, the residues directly interacting with base pairs are in bold face and Yap-specific residues in large font. In the leucine zipper, the conserved leucines (or other residues) d of the coiled coil (bold-face) and hydrophobic (typically) residues at position a of the coiled coil (underlined) are indicated (adapted from [10]).

4. Yap1p, the major regulon in oxidative stress response

The oxidative stress response is designated as the phenomenon by which a cell responds to alteration in its redox state due to the generation of radical oxygen species (ROS) caused by the incomplete reduction of O_2 during respiration as well as to the exposure to a variety of chemicals and metals.

The role of Yap1p in the regulation of enzymes that protect against oxidative stress was first suggested when the *yap1* mutant was found to be hypersensitive to both H_2O_2 and t-BOOH as well as to chemicals that generate superoxide anion radicals (menadione, methylviologen and plumbagine). Such *yap1* mutants have reduced specific activities of several enzymes involved in oxygen detoxification such as superoxide dismutase, glucose-6-phosphate dehydrogenase and glutathione reductase [16]. Parallel *yap1* mutant studies further indicated sensitivity to methylglyoxal, cadmium [20,21] and cycloheximide, amongst others [21]. Later, Kuge et al. [34] gave the first and clear clue towards the role of Yap1p in this response mechanism through the identification of the Yap1p target, *TRX2*, showing that its induction by H_2O_2, t-BOOH, diamide and diethylmaleate (DEM) is Yap1p-dependent and mediated by two YREs present in its promoter. Furthermore, they also demonstrated that the *yap1*-deleted strain is hypersensitive to diamide and DEM. The identification of the second Yap1p target, *GSH1*, further established its role in cadmium detoxification pathways [35]. Subsequently, several other Yap1p-dependent genes involved in cadmium tolerance have been identified [36,37]. Studies by global analysis have also added a growing number of different Yap1p targets involved in the detoxification of ROSs [38,39]. One can find, among the proteins encoded by these genes, most cellular antioxidant defenses as well as those involved in thiol redox control (for details see [14]). Although several unpublished data have indicated a slight induction of *YAP1* upon exposure to stress, the control over Yap1p-mediated gene regulation is accomplished through its cellular localization. Kuge et al. [34] demonstrated that nuclear retention of Yap1p is mediated by the cysteine-rich domain located at the C-terminus of the protein (c-CRD). Removal of this region generates a constitutively nuclear, and hence, active protein. Furthermore, three conserved cysteine residues (C598, C620 and C629) were identified as important for this post-translational regulation. Yan et al. [40] then showed that nuclear export of Yap1p is mediated by the exportin, Crm1p, binding to the Yap1p nuclear export signal (NES) which overlaps with the c-CRD. Later, Delaunay et al. [41] demonstrated, in vivo, that two cysteines, C303 from the N-terminal CRD (n-CRD) and C598 from the C-terminal CRD (Fig. 2A), are

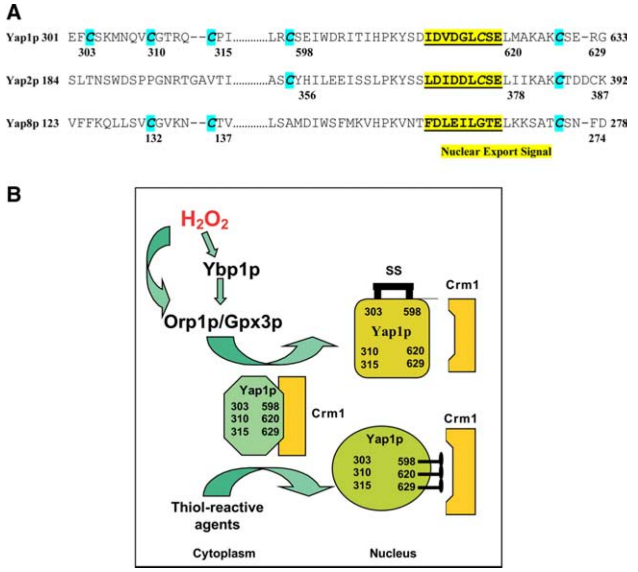

Fig. 2. (A) Alignment of the conserved cysteine residues of Yap1p, Yap2p and Yap8p. NES is underlined. (B) Schematic illustration of the two Yap1p redox centers. Under non-oxidizing conditions, Yap1p is exported from the nucleus via the exportin Crm1p. Upon H2O2, the formation of the C303–C598 disulfide bond in Yap1p via Gpx3p/Ybp1p masks the NES and Yap1p is retained in the nucleus, activating the target genes. Under thiol reactive agents, this mechanism does not operate and the drug binds directly to the Yap1p (adapted from [44,46]).

oxidized in response to H_2O_2, forming an intramolecular disulfide bond that masks the NES, thus compromising the binding of Crm1p and leading to its activation through nuclear retention. Furthermore, in vitro work performed by Wood et al. [42] has revealed that an additional intramolecular disulfide bond, namely between C310 and C629, is formed between the n-CRD and the c-CRD upon exposure to H_2O_2. These results hence indicate that Yap1p activity is regulated by post-translational mechanisms and raise the question of whether Yap1p oxidation occurs directly by the hydroperoxides or whether there is an intermediary molecule that may allow for its oxidation. Elegant experiments provided by the group of Toledano and co-workers [43] have demonstrated that Yap1p is not directly oxidized by H_2O_2. Rather, a glutathione peroxidase (GPx)-like protein (Gpx3p/Orp1p) fulfils the sensor function, transducing the oxidation signal to Yap1p through the creation of an intermolecular disulfide bond between the Gpx3p Cys36 and the Yap1p Cys598 that is then resolved into the previously characterized Yap1p C303–C598 intramolecular bridge. Furthermore, another protein, Ybp1p, has also been shown to interact with Yap1p in vivo upon exposure to H_2O_2 and to act in the same pathway as Gpx3p. Although its specific role is not understood, it is clear that interaction of this protein with Yap1p is required for Yap1p oxidation by Gpx3p and its contribution is currently being assessed [44]. In contrast, response to diamide does not involve the peroxidase, Gpx3p [43], nor is the C303 from the nCRD required for Yap1p activation [41,45]. Consistent with these results, Yap1p has been described to possess a further redox center [46]. Indeed, *N*-ethylmaleimide (NEM), an electrophile, and the quinone and menadione, both an electrophile and superoxide anion generator, were shown to modify the c-CRD cysteines independently of the Gxp3p pathway and this is sufficient to drive Yap1p translocation into the nucleus. Furthermore, mass spectrometry analyses revealed that, in contrast to H_2O_2, NEM, and possibly menadione, binds directly to the Yap1p c-CRD. A schematic illustration of these interactions with the consequent activation of Yap1p is given in Fig. 2B. These sensing mechanisms appear conserved in *S. pombe* as suggested by in vitro studies that indicate that DEM interacts irreversibly with Pap1p through at least two cysteine residues (Cys523 and Cys532) [47].

5. Yap2p/Cad1p the second member of the Yap family

Yap2p (Cad1p) is capable, when overexpressed, to confer resistance to stress agents such as 1,10-phenanthroline [8], cadmium [21], cerulenin and cycloheximide [20], suggesting a role for this transcription factor in the response to toxic compounds. DNA microarray analyses [48] indicate that Yap2p regulates a set of proteins involved in the stabilization and folding of proteins in an oxidative environment. Interestingly enough *YAP2* contains in its leader two small open reading frames, which were shown to play a role in its mRNA stability [49,50]. If this has a role in stress response, it remains to be elucidated.

Although Yap2p transcriptional activity was found to be stimulated upon cadmium treatment [7], the mechanism of activation of this transcription factor needs further investigation. The strong sequence homology between Yap2p and Yap1p in the C-terminal CRD (residues 570–650 in Yap1p and 330–409 in Yap2p) was used to further provide an insight into the function of Yap2p. Domain swapping of the Yap1p c-CRD by that of Yap2p has shown that the fusion protein is regulated by cadmium and not by H_2O_2 (Azevedo, Toledano and Rodrigues-Pousada, unpublished observations). Nuclear localization of the fusion protein correlates with both activation of Yap1p-specific target genes and growth in increasing concentrations of cadmium but not of H_2O_2. These data indicate, therefore, that the specificity towards H_2O_2 and cadmium resides in the carboxyl-terminal domain of Yap1p and Yap2p, respectively. It has also been found that treatment with cadmium activates the full-length Yap2 protein promoting its subsequent re-localization into the nucleus through a mechanism involving a regulated Yap2p–Crm1p interaction. Furthermore, the nuclear localization of the protein correlates with the Yap2p-dependent transcription of its target gene, *FRM2*, encoding a protein with strong homology with nitroreductase. A GFP fusion of the Yap2p c-CRD domain (amino acids 328–409) and the Gal4p DBD, that contains both the nuclear import signal within the Gal4p DBD and the Yap2p NES, is cadmium-responsive. This fusion is localized to the cytoplasm in untreated cells and redistributes to the nucleus in response to cadmium. Considering its high degree of homology to Yap1p, the role of the cysteine residues may prove relevant to Yap2p activation, possibly in a manner analogous to that observed for Yap1p. Given that overexpression phenotypes do not necessarily reflect a true biological function and that no phenotype has yet been associated to the *yap2* mutant, a role for Yap2p remains to be deciphered.

6. Yap4p and Yap6p in the response to osmotic stress

The fourth member of the family, Yap4p (Cin5p/Hal6p), is a 33-kDa protein and was initially characterized as a chromosome instability mutant [51]. Overexpression studies in the *ena1* mutant subsequently identified both *YAP4* (*HAL6*) and *YAP6* (*HAL7*) as genes that confer salt tolerance through a mechanism unrelated to the Na^+/Li^+ extrusion ATPase [12], whilst multicopy *YAP4* expression was shown to confer resistance to the antimalarial drugs, chloroquine, quinine and mefloquine [9,11]. Furuchi et al. [9] subsequently isolated these two genes in overexpression studies that imparted selective resistance to the chemotherapeutic agent cisplatin. Genomic DNA microarray analyses indicate a clear induction of *YAP4* and *YAP6* genes under conditions of oxidative and osmotic stress, heat, amongst others [39,52,53].

Although no further information has been obtained for *YAP6*, studies concerning the regulation of *YAP4* under conditions of hyperosmolarity have determined that not only is the null mutant osmo-sensitive but also that Msn2p-mediated *YAP4* induction occurs in a Hog1p-dependent manner through at least two STREs present in its promoter region [54]. Hyperosmotic stress leads to an arrest in cellular growth and to the altered transcription of genes involved in the stress response culminating in the adaptation of the yeast cell to the new environmental conditions. Crucial to this adaptation process is its capacity to increase the biosynthesis of glycerol, the cellular osmolyte, largely accomplished through the activity of the HOG MAP kinase pathway. Changes in external osmolarity and cell turgor activate a signal cascade that culminates with the phosphorylation of the Hog1p kinase and its translocation into the nucleus where it modulates gene expression through interaction with several transcription factors (for review [13]). The hypersensitivity of the *hog1* mutant strain to even mild hyperosmotic conditions is in part derived from a marked reduction of its intracellular glycerol content and is a clear reflection of its fundamental role in this response pathway. Indeed, studies have demonstrated that even *hog1* mutant strains can thrive in the presence of moderate hyperosmolarity when growth is performed at 37 °C, due to the concomitant activation, upon heat shock, of an alternative glycerol biosynthetic pathway via dihydroxyacetone [55]. The fact that *YAP4* overexpression clearly relieves the *hog1* osmo-sensitive phenotype has placed an added importance on the identification of its target genes (Fig. 3). Global analyses of the yeast genome and proteome are useful tools for the understanding of complex regulatory networks. Within this context, microarray analyses using the Yap4p mutant under conditions of hyperosmolarity were performed and amongst the data obtained, three genes have been validated as dependent on Yap4p to varying degrees. Two of these are involved in glycerol biosynthesis, namely *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 with reduction values corresponding to 40% and 50% of the maximum levels, respectively. Although the internal glycerol content appears unaffected in the *yap4* mutant, these findings suggest that the Yap4p contribution towards its biosynthesis may be at the level of the fine-tuning of its regulation [54]. In support of this hypothesis is the fact that previous studies have demonstrated that the effects of deleting *GPP2* are only fully observed by the concomitant deletion of its isoenzyme encoded by *GPP1* [56]. Furthermore, *DCS2*, a gene homologous to the *DCS1*-encoded decapping enzyme, shows 80% depletion in induction levels in the *yap4* mutant. Recently, *DCS1* has been described as an inhibitor of trehalase activity [57] that may have implications for osmo-response due to the role of trehalose as a superior cellular osmolyte providing protection against dehydration and desiccation [58,59]. The complete identification of the remaining *YAP4* target genes will greatly contribute towards the understanding of its functional role. Unpublished results (Nevitt, Pereira and Rodrigues-Pousada) also indicate that *YAP4* and *YAP6* are induced in response to several other stress conditions including oxidative stress, heat, and exposure to cadmium and arsenic compounds. Whilst *YAP4* regulation under oxidative stress has been shown to be dependent on Yap1p and Msn2p, mediated through the YRE and most proximal STRE, respectively, very little is known about its regulation under the remaining conditions. However, the *YAP4* promoter region contains multiple consensus HSE sites, which, together with the STREs may represent a general response switch. However, no phenotypes for the *yap4* mutant have yet been described for these stress conditions. The functional relevance of these data is not yet fully understood but it may represent a fine-tuning of regulation of gene expression under stress response.

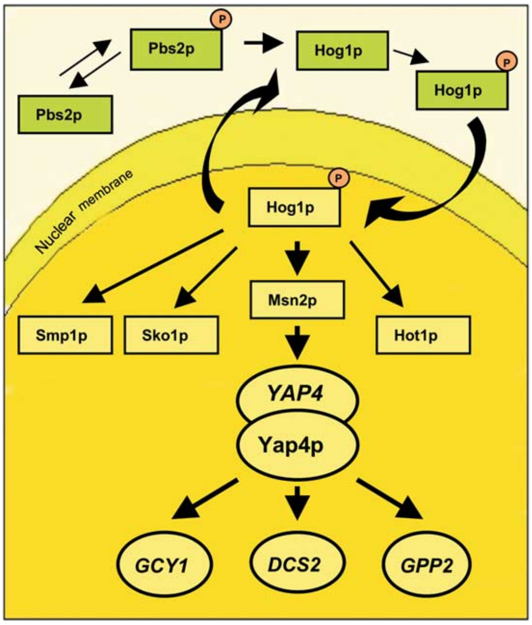

Fig. 3. Components of the HOG pathway. Upon exposure to osmotic stress, the nuclear accumulation of Hog1p activates downstream transcription factors. Msn2p is involved in the activation of *YAP4* and subsequently the encoded factor induces the targets three of which are represented (see text for further details).

7. Yap8p/Acr1p and its role upon arsenic conditions

The *YAP8* (*ACR1*) gene is located in chromosome XVI, in a cluster composed also by the genes *ACR2* and *ACR3* [10]. Yap8p, a 33-kDa protein, positively regulates these two genes, encoding an arsenate-reductase and a plasma membrane arsenite efflux protein, respectively, thus conferring resistance to arsenic compounds [60–62]. Yap8p also participates in the regulation of a parallel arsenite detoxification pathway by controlling the expression of the yeast cadmium factor 1 (*YCF1*) [63].

The activity of Yap8p is not regulated at the transcriptional level. Instead, it rapidly accumulates in the nucleus upon exposure to arsenic, indicating that its translocation is a regulated mechanism [64]. Repression of *CRM1* expression retains Yap8p in the nucleus. Analogous to that observed for Yap1p [40,41], under these conditions, Yap8p is no longer actively exported to the cytoplasm, which strongly suggests that Crm1p is the protein responsible in mediating its nuclear export. Yap8p function also depends on the activation of its transactivation potential after exposure to arsenic compounds. The cysteine residues located at positions 132, 137 and 274 (Fig. 2A) are essential for Yap8p nuclear accumulation as well as for the activation of its transactivation function [64]. It remains to be elucidated whether arsenite binds these cysteines directly or whether, similarly to Yap1p [43], they are modified as a result of the alteration of the cellular redox state. However, and in contrast to this, recent results indicate not only that Yap8p is a nuclear resident protein but also that cysteines 132 and 274 do not affect its localization, although the mutant strain displays arsenic sensitive phenotype [13]. Furthermore, Yap8p was shown to interact with the *ACR3* promoter in vivo and in particular with the sequence TTAATAA [13]. It should be noted that the use of different strains, namely W303-1A and FT4, might be responsible for this contradiction. Indeed, results obtained by Veal et al. [44] have demonstrated that W303 contains a mutant allele of *YBP1*, *ybp1-1*, encoding four amino acid substitutions. The result is increased sensitivity to peroxide stress, reduced H_2O_2-induced oxidation and nuclear accumulation of Yap1p compared with cells containing the *YBP1* gene. As *YBP1* is essential for Yap1p activation under oxidative stress, one may postulate that it might also be extended to Yap8p activation. On the other hand, it is not yet clear whether arsenic causes the induction of ROS and therefore it is possible that Ybp1p also plays an important role in arsenic detoxification.

8. Remaining Yap members: Yap3p, Yap5p and Yap7p

Of the remaining Yap proteins, very little is known. In fact, for Yap3p, there is virtually no response at the level of genomic microarray analysis to multiple forms of environmental insults and cellular stress. Yap5p has been shown not to bind the TTACTAA sequence and its activation potential is not induced by aminotriazole [7]. Microarray datasets available in the Saccharomyces Genome Database [65] show that it is strongly induced under amino acid starvation, nitrogen depletion and stationary and diauxic phases. However, Northern blot analysis or real-time PCR has not yet validated these results. Similarly, with respect to *YAP7* microarray analysis shows a marked repression under nitrogen depletion and stationary phase.

9. Concluding remarks

The existence of such an extended family of AP-1-like factors has so far only been described in the yeast *S. cerevisiae*. In fact, whilst homologues for *YAP1* exist in other eukaryotes none have been found for the remaining family members. In *S. pombe*, Pap1p shares 26% identity and 41% similarity with Yap1p. However, in contrast to what has been described for *S. cerevisiae*, multiple environmental insults including osmotic, oxidative, heat shock, and nitrogen and carbon starvation have all been put into one response pathway. This MAP kinase pathway is mediated by Sty1p, which itself shares 82% identity with the *S. cerevisiae* Hog1p and is similar to mammalian p38. This and other architectural features of the Sty1 pathway make it more analogous to mammalian stress activated signaling systems and may, in part, explain the apparent lack of Yap2p–Yap8p functional homologues in these organisms.

The existing data on the Yap family members support both a degree of functional overlap between them as well as distinct physiological roles. Indeed, it has been described that the double mutant *yap1yap2* shows increased sensitivity to cadmium (Azevedo, Toledano and Rodrigues-Pousada, unpublished results) and hydrogen peroxide stress (Costa and Moradas-Ferreira, unpublished results) than the single *yap1* mutant strain. Similarly, the *yap1yap8* double mutant is more sensitive to arsenic conditions than either single mutant [64]. Whilst the existence of these multiple cross-talking signaling pathways endows *S. cerevisiae* with an added flexibility with respect to resistance, it may furthermore explain why the deletion of often key components, as exemplified by the *msn2* or *hot1* mutants, does not always give rise to a sensitive phenotype. The challenge, therefore, remains in deciphering the functional role of the remaining Yaps and to determine the interactions that occur at the protein level between each other and other cellular proteins. The construction of a yeast strain deleted for all eight *YAP* genes may provide a future insight into this protein family, in particular one that would allow for the discrimination of each of their function. Whatever the outcome, results should be obtained from well-described strains since it is becoming clear that not only do different strains have different sensitivities to the stress imposed, but also that differences at the level of the fundamental mechanisms of gene expression are starting to emerge.

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