Role of Yap8 and Yap1 b-ZIP transcription factors in arsenic stress

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This work is dedicated to my family

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Foreword:

The work of Bobrowicz et al pointed toward a role of Acr1/Yap8 as a regulator of the arsenic detoxification genes in yeast *S. cerevisiae.* The work on Chapter 2 provides molecular evidence that Yap8 is the transcriptional regulator responsible for the activation of *ACR2* and *ACR3* in the response to arsenic stress and elucidates the mechanisms by which Yap8 is activated under this condition. Also the involvement of Yap1 as a regulator of *YCF1* is presented in this chapter. In Chapter 3 it is shown that arsenic exposure generates oxidative stress in yeast cells and that Yap1 has a role in activating the expression of the genes involved in antioxidant defences. In Chapter 4, the DNA binding properties of Yap8 were studied as well as the importance of the Mediator complex in Yap8 transmission of the stress signal to the basal transcription machinery.

Thesis publications

- Menezes R., **Amaral, C**., Batista-Nascimento, L., Santos, C., Ferreira, R.B., Devaux, F., Eleutherio, E. C.A. and Rodrigues-Pousada, C. (2008)Contribution of Yap1 towards S. cerevisiae adaptation to arsenic mediated oxidative stress *Biochem. J.414: 301-311*
- Rodrigues-Pousada, C., Nevitt, T., Menezes, R., Azevedo, D. Pereira, J., (2004) **Amaral, C.;** Yeast activator proteins and stress response: an overview . *FEBS Letters* 567: 80-85
- Menezes, R., **Amaral, C.** Delaunay, A., Toledano, M., and Rodrigues-Pousada, C.(2004) Yap8p activation in *Saccharomyces cerevisiae* under arsenic conditions. *FEBS Letters* 566: 141-146
- Amaral, C., Ropio, J., Silva, A. Menezes, R., and Rodrigues-Pousada, C. Yap8-DNA interaction and Mediator requirement for Yap8 activity *in preparation*

Other publications not included in the thesis:

- Pereira,J., Pimentel,C. **Amaral, C**., Menezes R., Rodrigues-Pousada (2009) Yap4 PKA-and Gsk3-dependent phosphorylation affects its stability but not its nuclear localization. Yeast 26(12): 641-53

Abstract:

Arsenic compounds are highly toxic substances; nevertheless they are used in the treatment of acute promyelocytic leukaemia. Therefore it is pressing to gain knowledge on its toxicity and detoxification mechanisms. The cellular entry pathways have been discovered and by transcriptome analysis it is known that arsenic activates the transcription of genes activated by, among others, Rpn4, Met4 and Yap1.

Yap1 and Yap8 belong to the Yap family, which is composed of eight transcription factors, each with a determined function in the response to stress. Nevertheless Yap1 is involved in the response to oxidative stress and multi-drug stress, including arsenic, Yap2 in the response to cadmium, Yap4 may have a role in the response to several stresses and Yap8 is involved in the response to arsenic, as its major regulator. Its role is due to the fact that Yap8 is important for the expression of the detoxification genes *ACR2* and *ACR3*. In this work we show that its deletion abrogates the expression of these two genes. We also studied Yap8 activation mechanism, proving that having conserved cysteines with Yap1 allows it to be regulated by a similar mechanism. Yap8 is modified in these conserved cysteines, which masks the nuclear localization signal preventing it from interacting with the nuclear exportin Crm1, becoming nuclear. The study of Yap1 nuclear localization in an Δ*orp1* strain, responsible for the signal transduction under oxidative stress, indicates that arsenic behaves as a thiol-reactive chemical. We also prove, by mutation to alanine that the cysteines are relevant for Yap8 nuclear localization and are also relevant for transactivation potential.

In chapter3 we demonstrate that a mechanism of arsenic toxicity is through the production of Reactive Oxygen Species (ROS) since there is accumulation of lipidic peroxidation by-products, protein carbonylation and intracellular ROS production after arsenic stress. Genes related to anti-oxidative defences are up-regulated. The glutathione levels decrease after one hour of arsenic stress which may be a consequence of arsenic stress, because arsenic detoxification requires the use of glutathione by Arr2 and Ycf1, this disturbs the redox homeostasis causing oxidative stress. To compensate it, expression analysis show an up-regulation of the sulphur metabolism related genes. The oxidative stress responsive genes related to oxidative stress that are dependent on Yap1, are up-regulated, particularly in a Δ*yap8* strain. The particular sensitivity of a Δ*yap8* strain, and higher anti-oxidative gene response, may be explained due to a higher accumulation of arsenic as shown by atomic absorption, which occurs because *ACR3* is not transcribed. It was shown that Yap1 and Yap8 have distinct roles in the response to stress and Yap1 is the mediator of the response to oxidative stress caused by arsenic.

In Chapter 4 we studied the amino acids mutations in Yap8: K21A, R22A, A23T, Q25A, L26N R27A, S29A, N31A, K35A, K35E, R36A, and K37A and show that K21A, R22A, A23T, R27A, K35E, R36A and Q25A are relevant for Yap8 interaction with the DNA. The double mutation Yap8L26N-N31R conferred a slight capacity for Yap8 to recognize a Yap1 binding site despite the fact that the respective single mutations are not capable of doing so.

After the binding to DNA the signal transduction from Yap8 to the transcription machinery occurs through the Mediator complex. Mutant strains in the tail subunits were studied by growth in

medium with arsenic compounds which revealed that the subunits Med2, Med3, Med15 and Med16 and Med14 are required for the yeast response to arsenic stress. The assays of Yap8 transactivation potential show that all the subunits are required for its full activity. It was also studied the expression of Yap8 target gene *ACR2* and the results indicate that Med16 is the subunit more relevant for the transcription of this target gene followed by the subunits Med2, Med3 and Med15.

Resumo:

O elemento arsénico nos seus diferentes compostos, é altamente tóxico sendo, no entanto, usado no tratamento de leucemia promielocítica aguda. A sua presença no ambiente como contaminante e o seu uso como medicamento leva a necessidade do estudo dos mecanismos relevantes para a sua destoxificação e quais os seus mecanismos de acção. As vias de entrada nos diferentes tipos celulares são já conhecidos e análises do transcriptoma mostraram que genes regulados por factores de transcrição como o Rpn4, Yap1 e Met4 estão activados.

O Yap1 e Yap8 pertencem a família Yap, família de factores de transcrição, composta por oito membros. Cada membro regula a resposta a diferentes tipos de stress, no entanto o Yap1 está não só envolvido na resposta ao stress oxidativo mas também a variadas drogas incluindo o arsénico. Yap2 está envolvido na resposta ao cádmio, enquanto a actividade do Yap4 deverá estar relacionada com diversos stresses. O Yap8 está envolvido na resposta ao arsénico sendo o seu principal regulador. O seu papel deve-se ao facto de ser o regulador da transcrição de *ACR2* e *ACR3*. Neste estudo é demonstrado que a deleção do *YAP8* impede a transcrição destes dois genes. Foi também estudado o mecanismo de activação desta proteína, provando-se que as cisteinas conservadas com o Yap1 permitem um mecanismo de regulação semelhante ao deste. O que significa que a modificação destas cisteinas mascara o sinal de localização nuclear impedindo a sua interacção com a exportina nuclear Crm1, tornando-o nuclear. O estudo da localização sub-celular do Yap1 numa estirpe Δ*orp1*, proteína envolvida na transdução do sinal de stress oxidativo, indica que o arsénico se comporta como um químico que

reage com tiols. A mutação destas cisteinas para alaninas impede a localização nuclear do Yap8 e é também determinante para o potencial de transactivação deste.

No capítulo 3 demonstramos que um dos mecanismos de toxicidade do arsénico ocorre via produção de espécies reactivas de oxigénio. O que é verificado devido a acumulação de produtos da peroxidação lipídica, carbonilação de proteínas e produção de espécies reactivas de oxigénio intra-celularmente. Análises do transcriptoma indicam o aumento de expressão de genes envolvidos na resposta ao stress oxidativo. Os níveis de glutationa diminuem após uma hora de stress por arsénico o que pode ser uma consequência dos processos de eliminação do arsénico uma vez que Acr2 e Ycf1 precisam de glutationa. O gasto de glutationa na forma reduzida causa desequilíbrio da homeostase redox podendo causar stress oxidativo. Os genes alvo do Yap1 são transcritos activamente, particularmente numa estirpe Δ*yap8*. A maior sensibilidade desta estirpe deve-se ao facto de acumular mais arsenito como é mostrado por absorção atómica, esta acumulação ocorre porque nesta estirpe o gene *ACR3* não é expresso. Foi demonstrado que Yap1 e Yap8 têm papéis distintos na resposta ao stress e Yap1 regula a resposta ao stress oxidativo causado por arsénico.

No capitulo 4 é mostrado que das mutações no Yap8: K21A, R22A, A23T, Q25A, L26N, R27A, S29A, N31A, K35A, K35E, R36A e K37A sendo as mais relevantes para a interacção com o DNA: K21A, R22A, A23T, R27A, K35E, R36A e Q25A. No entanto as únicas mutações com fenótipo de sensibilidade a compostos de arsénico são R22A, R27A, R36A e Q25A. A mutação dupla Yap8L26N-N31R confere a capacidade ao Yap8 de reconhecer

ligeiramente a sequencia de DNA reconhecida pelo Yap1. Isto apesar das mutações isoladas não terem qualquer efeito no Yap8.

Após ligação ao DNA, o Yap8 interage com a maquinaria basal de transcrição, o que ocorre através do complexo Mediador. Estirpes mutantes nas subunidades da cauda do Mediador foram estudadas por crescimento em meio com compostos de arsénico revelando que as subunidades Med2, Med3, Med15, Med16 e Med14 são necessárias para a resposta da levedura ao stress por arsénico. O estudo do potencial de transactivação mostra que todas as subunidades são importantes para a total actividade de Yap8. As subunidades mais importantes são as reveladas pelos estudos fenotípicos com excepção da Med14, o que indica que esta subunidade está envolvida na resposta ao arsénico de forma independente do Yap8. Foi também estudada a expressão do gene *ACR2* e os resultados indicam que a subunidade que afecta mais pronunciadamente a transcrição do seu gene alvo é Med16, mas havendo também um papel relevante por parte das subunidades Med2, Med3 e Med15.

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Symbols and abbreviations

AP-1 activator protein 1
ARE AP-1 recognition element
As(V) arsenate
As(III) arsenite
AT 3-amino-1,2,4-triazole
bp base pairs
b-ZIP basic DNA-binding domain and leucine zipper
c-CRD C-terminal cysteine rich domain
DABCO 1,4-Diazabicyclo[2.2.2]octaneAPI 4',6'-diamidino-2-phenylindole, dihydrochloride
DEPC di-ethyl pyrocarbonate
DNA deoxyribonucleic acid
EDTA ethylenediaminetetraacetic acid
11bp 11bp sequence of the *ACR2/ACR3* promoter
GFP green fluorescent protein
GPXs glutathione peroxidases
GSH reduced glutathione
HSE heat shock element
HSF1 heat shock factor
GSSG oxidized glutathione
kb kilo base pairs
kDa kiloDalton
M molar
Min minutes
RNA ribonucleic acid
n-CRD N-terminal cysteine rich domain
NES nuclear export signal
NLS nuclear localization signal
nm nanometers
$O.D._{600nm}$ optical density at 600nm
ORF open reading frame
ºC degree Celsius
PCR polymerase chain reaction
PKA protein kinase A
RNA ribonucleic acid
ROS reactive oxygen species
S. cerevisiae *Saccharomyces cerevisiae*
S. pombe *Schizosaccharomyces pombe*
SC synthetic complete medium
SD synthetic minimal medium
STRE stress responsive element
13bp 13bp sequence of the *ACR2/ACR3* promoter
Y Yap recognition element

Amino acids

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

Nucleotide bases

A adenine
C cytosine
G guanine
T thymine

Chapter I

Introduction

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Introduction

1.1 Yeast as a biological model - scientific relevance

The budding yeast *Saccharomyces cerevisiae* is a simple eukaryote model organism that has the advantage of robust growth on minimal media and easy handling due to the fact that it does not cause pathogenesis in the health of Humans. Its genome was completely sequenced by an international consortium in which Claudina Rodrigues-Pousada's laboratory actively participated in [2-5]. It was the first eukaryotic genome to be sequenced, showing the presence of approximately 6,000 genes.

It contains several genes with high homology to human genes, approximately 30% human disease map-based genes have yeast homologues [6]. One such example is the yeast cadmium factor, Ycf1, that possesses similarity to human Mrp1 (42.6% identity, 63.3% similarity) and the cystic fibrosis transmembrane conductance regulator (Cftr) (31% identity, 56.7% similarity), members of the ATP binding cassette (ABC) [7]. Mutations in *CFTR1* that modulates chloride ion transport across epithelial membranes lead to cystic fibrosis disease [8]. Another example of yeast genes with similarity to the ones of humans is the *PAT1/PXA2* and *PAT2/PXA1,* each encodes two peroxisomal half ABC transporters orthologues of Ald1 and Ald2, Pat1/Pat2 dimerize to form an ABC transporter required for the import of very long-fatty acids into the peroxisome [9-11]. Mutations in *ALD* gene cause adrenoleukodystrophy (X-) disease, in which there is accumulation of long chain fatty acids as they are not metabolized [12].

Yeast is in itself also a tool since it has been used to rapidly study protein-protein interaction, by the designated two hybrid technique [13]. This application has suffered several modifications that allowed the study of transactivation potential of transcription factors and their interaction with DNA, for example. Another important use of yeast as a tool is for heterologous expression of relevant human proteins, allowing again, rapid test of various candidate drugs [14].

1.2 Transcriptional factors involved in Yeast Stress responses

All organisms must have the resources to adapt to an unbalanced environment. The cellular mechanisms are divided in general and specific stress response. The general stress response has conserved mechanisms across nature. *S. cerevisiae* developed also such a basic response common to all stresses. Two of the intervenient transcription factors are heat shock factor, Hsf1 and Msn2/Msn4. When cells are subjected to a mild stress they develop resistance to a subsequent lethal stress, *e.g.,* cells become less sensitive to the imposed stronger stress due to the accumulation of compatible solutes [15]. *HSF1* is an essential gene since its deletion causes lethality [16]. In yeast it is transcribed constitutively and forms homotrimers, unlike in higher eukaryotes where the trimerization occurs only after stress [17]. Each monomer binds one Heat Shock element (HSE) 5'nGAAn, and the yeast HSF1 binds constitutively to the sequence but does not activate transcription [18].

Msn2 and Msn4 are regulated by, among others, the cAMP-dependent protein kinase PKA. Under low PKA activity the protein

is nuclear localized and if hyperphosphorylated localizes in the cytoplasm [19]. They recognize the DNA sequence 5'CCCT designated Stress Responsive Element (STRE) [20].

1.3. Arsenic stress

1.3.1 Arsenic stress in Yeast

Arsenic (As) is a transition metal and a well-known carcinogen, due to its ability to induce oxidative stress, to diminished DNA repair, alter DNA methylation patterns, enhance cell proliferation and possible suppression of p53 [21]. Nevertheless, the arsenic-containing drugs are used as chemotherapeutic agents to combat infectious diseases caused by pathogenic parasites as well as cancer, including arsenic trioxide in the treatment of acute promyelocytic leukaemia [22,23]. Given the toxicity effects posed by these compounds it is pressing to elucidate the mechanisms that constitute the basis of their tolerance.

In the yeast, *Saccharomyces cerevisiae,* arsenate enters the cells through the phosphate transporters Pho84 and Pho87 [24] and the arsenite transport is facilitated through hexose permeases [25] although when glucose is present it is mainly transported through the aquaglyceroporin Fps1 [26]. In the absence of Fps1, the 17 hexose permeases and Acr3 (to eliminate the extrusion system) there is only residual uptake of arsenite. The transport is however resumed when the permeases Hxt1, Hxt3, Hxt4, Hxt5, Hxt7 and Hxt9 are expressed individually [25].

In yeast the studied arsenite detoxifying mechanisms are the Arsenic Compounds Resistance (ACR) pathway and the Yeast Cadmium Factor (Ycf1) - mediated efflux (see figure 1). The ACR cluster is essential for the adaptation to environments containing

high levels of arsenic. It is composed by the transcription factor *YAP8* (*ARR1*/*ACR1*) that controls the expression of the other two genes of the cluster [27, 28]. These genes are *ACR2* and *ACR3* [29] which encode an arsenate reductase [30] responsible for the reduction of arsenate to arsenite, and an efflux protein that detoxifies arsenite respectively [31]. Ycf1 catalyses the ATP driven uptake of several drugs conjugated with glutathione, into the vacuole, including arsenic in the arsenite form (Fig.1.2) [32]

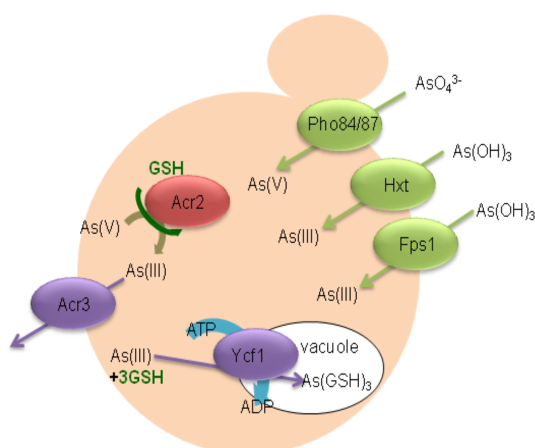

Figure1.1 Arsenic entry and detoxification pathways in *Saccharomyces cerevisiae.* Adapted from [1].

Schizosaccharomyces pombe resistance mechanisms to metals such as Cd^{2+} and Cu^{2+} rely on the formation of phytochelatins, peptides synthesized from glutathione, enzymatically [33], in plant these phytochelatins also confer resistance to arsenic (see section 1.3.3). A screen of *S. pombe* mutants revealed two genes that confer sensitivity to arsenic and cadmium: the p38 orthologue Spc1/Sty1 and the Cnt5 a novel

member of the centaurin ADP ribosylation factor GTPase activating protein (Arf GAP). This protein accumulates in the membrane and septum after arsenic stress where it may regulate membrane trafficking or maintain membrane integrity [34].

The *S. cerevisiae* orthologue of the human p38 is Hog1, the MAPkinase (mitogen-activated protein kinase) of the HOG (high osmolarity glycerol) pathway that is activated under arsenic exposure. As(III) leads to the Hog1 activation by phosphorylation in a Ssk1 and Pbs2 dependent way. Deletion of *hog1* leads to the increase uptake of arsenite which may be due to the deregulation of the As(III) transporter, Fps1. Activated Hog1 is one of the kinases responsible for Fps1 phosphorylation in the residue thr231, which reduces As(III) influx, when compared to a un-phosphorylated Fps1 [35]. Nevertheless Sotelo *et al* determined, by RT-PCR, that *ACR3* and *YCF1* are expressed at lower levels in a *hog1* mutant when compared to the wild type [36]. In contradiction, results from Thorsen *et al* indicate that Hog1 does not interfere with As(III) efflux by Acr3 [35].

Expression analysis showed that, under arsenite stress the categories of genes significantly enriched are: glutathione, methionine, sulphur and seleno amino acid metabolism, cell communication and heat shock response as detected through the KEGG (Kyoto Encyclopedia of Genes and Genomes) and the last two, through Simplified Gene Ontology (biological process). Network mapping based on the yeast-interaction network uncovered by the Active Modules algorithm, revealed seven significant neighbourhoods in the regulatory network, centred on nodes directed by Fhl1, Pre1, Yap1, Yap2/Cad1, Hsf1, Msn2 and Msn4.

The arsenic profile response is similar to the response seen in Cd^{2+} stressed cells, being the sulphur diverted to the glutathione production [37]. Nevertheless *cad1* transcriptome, in arsenite stress, did not show significant changes relative to the wild type. Their results also showed a down regulation of ribosomal proteins probably through control of Fhl1. Transcription profiles show that the transcription factors mediating the response to arsenic are Yap1, Arr1 and Rpn4. This relates arsenic stress to oxidative stress and protein damage, given Rpn4 is involved in regulation of proteasome activity [38]. Arsenite transcriptome analysis by Thorsen *et al*, showed that the leucine zipper Met4, responsible for the regulation of sulphur amino acid pathway and glutathione synthesis, for example in response to Cd^{2+}, is involved in the response to arsenite[37]. Under arsenite stress it is observed an increase in glutathione synthesis and a slight decrease in the incorporation of sulphate into proteins, in the higher arsenite concentration probably to shuttle all the sulphate to the production of glutathione. The results also show that the transcriptome and proteome profile are shifted towards the production of Cys and probably to the *de novo* synthesis of glutathione and sulphur assimilation, all the sulphate is therefore diverted to produce this protective peptide. This is directed by the cooperation of transcription factors Yap1 and Met4 [39]. Another transcriptome analysis by Jin *et al*, under arsenic stress, shows that the cell senses arsenic and is capable of shutting down the arsenic entry through the hexose permeases. The decrease in glucose may decrease PKA activity and therefore Msn2/Msn4 will be nuclear localized [40]. Hsf1 is another general stress response transcription factor that regulates several targets most of them are molecular chaperones [38]. The cell also increases iron uptake and formation

of iron-sulphur clusters as arsenic may remove iron from proteins [41,42]. As was observed before, there is also an up-regulation of genes involved in sulphur uptake and glutathione biosynthesis [38, 39] There is also an enrichment in genes involved in protein proteolysis, protein folding, detoxification of oxygen reactive species and telomere maintenance. The activator MAPK Hog1, together with other proteins from the HOG pathway, was also reported as required for the response to arsenic through analysis of the growth of deleted mutant strains [41] .

The Target of Rapamycin (TOR) is a central controller of the response to nutrients at the growth and metabolic level. Tor proteins are Ser/Thr kinases, conserved in eukaryotes, found in two complexes TORC1 and TORC2. TORC1 regulates several mechanisms such as protein synthesis, ribosome biogenesis, transcription and autophagy [43]. TOR kinase1, part of the TOR complex1, is inhibited under arsenic stress. TORC1 inactivation leads to down regulation of several genes, among them genes that code for ribosomal proteins. The TORC1 deactivation also prevents inhibition, of Msn2/Msn4. There is a fine regulation by PKA and TORC in order to maintain the required levels of Msn2/Msn4 under arsenite stress. PKA is active but the zinc fingers are localized in the nucleus due to down regulation of TORC1 [44].

High-throughput study, of mitochondrial genes showed that arsenic responsive genes are regulated by, for example, Rtg3, Abf1, Smp1, Reb1, Leu3, Hap5, Mcm1 and Ndd1. Search for human orthologues in deletion study revealed two of the affected genes are *SOD2* (see section 1.4) and the *RNR4* orthologue of *RRM2* which encodes for M2 subunit of enzyme ribonucleotide

reductase, involved in maintaining the deoxynucleotide triphosphate pool, therefore this enzyme is relevant to DNA repair and synthesis, this study reveals that mutation in this gene sensitizes the cells to arsenic [45].

1.3.2 Arsenic stress in other organisms

In bacteria, resistance to arsenic is mediated by the resistance operons *ars.* It has a conserved organization and regulation. It is composed by three genes *ArsRBC*. Each operon is regulated by an arsenite/antimonite responsive repressor which is encoded by the first gene of the operon, *ArsR*. The structure of the repressor is modified by the binding of arsenic or antimonite, therefore releasing the promoter [46]. The operon also contains: *arsB* and *arsC*. The first is highly conserved and confers resistance to arsenite and antimonite, transporting it to the extracellular milieu [47]. The ArsC is an arsenate reductase and there are two unrelated families of this gene [48]. Operons containing 5 coding units were reported that include two additional genes *arsD* and *arsA,* as in *Escherichia coli* plasmid R773. ArsA is allosterically modified by arsenite or antimonite and binds to ArsB converting it in a primary ATP-driven arsenite pump [49]. The metalochaperone ArsD transports to ArsA, increasing its efficiency to extrude arsenite [50]. Entry of arsenate occurs through the phosphate transporters Pit and Pst [51] whereas arsenite is transported into the cell by the aquaglyceroporin, GlpF [47].

Homologues of the yeast hexose transporter Glut1 in mammals can carry arsenite [52]. The human aquaglyceroporins (AQP) are also responsible for the entry of arsenite, from the four

studied, AQP7 and AQP9 are the most efficient transporters [53]. A null mice in AQP9 is more sensitive to arsenic and excretes lower amounts of this compound showing that it is also involved in arsenite extrusion [54]. Arsenate transport occurs, with low efficiency, through the intestinal sodium phosphate carrier NaPi-IIb, other transporters must therefore exist [55]. Arsenite export in mammalian cells occurs through Mrp1, an ABC binding cassette that transports several drugs, probably as GSH conjugates [56]. Another protein that may be involved in the resistance to arsenic is a multi-drug resistance P-glycoprotein of the superfamily of ATP binding cassette transporters Mdr1 [57]. Mouse mutants in this protein show accumulation of arsenite (Fig.1.2) [58].

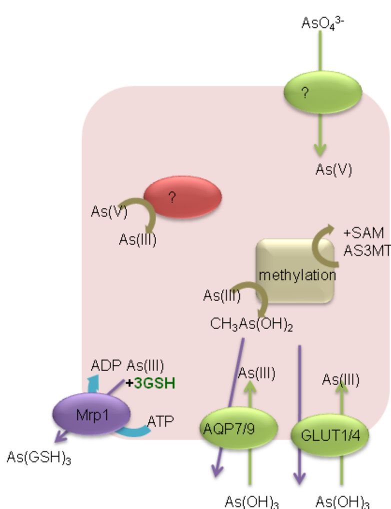

Figure 1.2 Arsenic entry and detoxification pathways in mammals. Adapted from [1].

The transport of arsenite in plants is made through Nodulin 26-like Intrinsic Proteins (NIPs). The expression of some of these proteins in the yeast *Δfps1* mutant confers sensitivity to As(III) and Sb(III) to the mutant strain indicating that Fps1 and nodulins have

the same role, *e.g.* transport of As(III) into the cells, although they also participate in extrusion [59]. To study arsenate sensitivity in *Arabidopsis thaliana* several mutants were tested and a gene conferring resistance in a GSH-independent way was identified, *ARS1*. The mutant has higher phosphate uptake than the wild type [60]. In plants arsenite is captured inside the cell by metallothionein protein and phytochelatins that accumulate in the vacuole. Phytochelatins are synthesized from glutathione by phytochelatin synthetase [61]. Plants also have an ABC transporter, without homology to Ycf1, that carries As(III)-GSH conjugates into the vacuole [62] and an arsenate reductase with moderate homology with the yeast Acr2 is also present [63].

Genome microarray analysis of *Arabidopsis thaliana* showed that As(V) stress represses the genes involved in phosphate starvation as expected because As(V) can behave as a phosphate analogue. Other genes were shown to be involved in antioxidative stress defences as for example the superoxide dismutases Cu/ZnSOD although the Fe/SOD was repressed [64].

1.4 Oxidative stress defences

Arsenic stress may cause oxidative stress as shown in several transcriptome studies that indicate that there is up-regulation of oxidative stress related genes [38, 39, 65]. This may be caused by the removal of iron from proteins that will then participate in Fenton reaction producing hydroxyl radical and anion [42]. Another cause of oxidative stress derived from arsenic is the consumption of glutathione by Acr2 and Ycf1 (fig.1).

The antioxidant defences catalyze the reduction of reactive oxygen species (ROS), through electronic transfer, usually via hydrogen. They can be divided in thiol and non-thiol containing

defences. In each case there are the nonenzymatic mechanisms such as glutathione and enzymatic ones such as superoxide dismutases, catalases, and peroxidases.

The tripeptide glutamate-cysteine-glycine GSH is synthesized by the action of γ-glutamylcysteine synthetase (Gsh1) which catalyses the limiting step of GSH formation: the condensation of Cys onto the γ-Carbon of Glu. *GSH1* expression is regulated by Yap1 and Met4 [66]. The addition of Gly is made by glutathione synthetase (Gsh2) [67]. GSH redox properties come from its Cys residue which low redox potential allows it to donate electrons to other Cys, therefore becoming oxidized enabling it to dimerize to the disulfide GSSG. This disulfide is reduced back to GSH by the action of the NADPH dependent glutathione reductase (Glr1) which expression is also regulated by Yap1 [68].

S. cerevisiae possesses two apparently redundant isoforms from the thioredoxin system, Trx1 and Trx2 and a thioredoxin reductase Trr1 in the cytoplasm, there is also a mitochondrial system composed of Trx3 and Trr2 [69]. Thioredoxins are small proteins with two redox active Cys within the conserved active site motif, Cys-Gly-Pro-Cys, that becomes oxidized to an intramolecular disulfide, typically in a thiol-dissulfide exchange reaction with a dissulfide bonded substrate. The flavoenzyme NADPH dependent Trr reduces thioredoxin back to the active dithiol form [70]. Trx2 expression is controlled by Yap1 [71] and Skn7 [72].

Other thiol protective systems are peroxiredoxins, ubiquitous proteins from the peroxidase family. Peroxiredoxins have in the N-terminal region a conserved Cys [73], which is the primary site of H_2O_2 oxidation. One of the first peroxidoxins identified was Tsa1, a thiol specific-antioxidant, capable of reducing hydrogen peroxide to water [74]. Another peroxidase like protein is

the Orp1/Gpx3 that catalyzes the formation of the regulatory disulphide bond in Yap1 (see point 1.6).

Glutaredoxins are small proteins that are reduced by glutathione unlike thioredoxins that are reduced by NADPH. This protein family may have two domains, the thioredoxin-like domain and a glutaredoxin one, as is the case of Grx3 and Grx4, or a single glutaredoxin domain as is the case of Grx5 [75]. They are divided in two families according to its active site. It may have two Cys (Grx1 and Grx2) or one in the active site (Grx3, Grx4 and Grx5) [76].

Non-thiol containing antioxidative defences is for example the superoxide dismutase and catalase. Yeast, like other eukaryotes, has two superoxide dismutases, (SOD1 and SOD2) that catalyse the dismutation of superoxide to oxygen and hydrogen peroxide. Both enzymes are encoded in the nucleus being the MnSOD2 localized in the mitochondrial matrix [77] and the CuZnSOD1 localizes in the cytoplasm and in the mitochondrial inter-membrane space [78]. Mutations of *SOD1*, in humans are associated with a subset of familial amyotrophic lateral sclerosis (ALS) [79].

1.5. Yap family history

The genome of the budding yeast *Saccharomyces cerevisiae* contains 14 members of transcription factors with a b-ZIP structure. Eight of these factors form a family, the Yeast Activator Protein family that comprises Yap1 to Yap8 with significant sequence similarity to the true yeast AP-1 protein, Gcn4, at the DNA binding domain. Within the Gcn4 basic domain, five residues, Asn235, Ala238, Ala239, Ser242 and Arg243, are responsible for the base-specific contacts in Gcn4 and Jun/Fos. The characteristics that distinguish the members of the Yap family

from Gcn4 are the amino acids that make contact with the DNA. Indeed, Ala239 is replaced by a Gln and Ser242 is replaced by Phe or Tyr. Furthermore, there are two family-specific residues, Gln234 and Ala241 (Fig.1.3). The consensus binding site of the members of the Yap family binding site was characterized as TTAC/GTAA for Yap1-Yap4 and termed the Yap site or Yap Response Element (YRE) [80]. So far the corresponding binding site for Yap5-Yap7 has not been characterized, and although Yap8 binds to the variant TGATTAATAATCA [81 and Amaral *et al.,* unpublished results], the existence of other binding sequences cannot be excluded. Yap1 has high homology with Yap2; Yap4 with Yap6 and Yap5 with Yap7, being Yap8 the most divergent member at the level of the DNA binding domain [82].

The Yap family has been implicated in a variety of stress responses including oxidative, osmotic, metal and metalloid, drug and heat stress [83]. Yap1, the first member of the Yap family to be described, was initially identified by its ability to bind and activate the SV-40 AP-1 recognition element (ARE: TGACTAA). Based on this ARE-binding capacity, this factor was purified as a 90kDa protein and the corresponding gene cloned by screening a λgt11 library with a monoclonal antibody raised against Yap1 [84]. Subsequently, this gene was also found in multicopy transformants resistant to the iron chelators 1, 10-phenantroline and 1-nitroso-2-naphtol as well as to a variety of drugs, including cycloheximide, the locus being historically designated *PAR1/SNQ3/PDR4 [85-87]*. Besides *YAP1*, a second gene, *YAP2*, was also described, to confer resistance to 1, 10-phenantroline in transformed cells overexpressing a multicopy yeast library. This gene encodes a 45kDa protein that also binds ARE *cis*-acting element [88,89], *YAP2* sequence was also found in the Data Bank designated as

Cad1 due to the acquisition of cadmium resistance in cells overexpressing this gene.

1.6. The role played by Yap family members in stress response

Yap1, the best studied member of the family, is known to respond to oxidative stress [90]. Its expression is mainly accomplished at the level of sub-cellular localization [91]. Indeed, Yap1 shifts to the nucleus upon oxidative stress in a mechanism that is not regulated at the level of nuclear import because interaction with the importin PseI is not stress-dependent [92]. Kuge *et al.* demonstrated that nuclear retention of Yap1 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 active protein. In addition, three conserved cysteine residues (Cys598, Cys620 and Cys629) were identified as being important for this post-translational regulation [91]. Delaunay *et al.* showed *in vivo* that the Cys, Cys303 and Cys598 located in the N-terminus (named as n-CRD) and in the c-CRD respectively, are oxidized by in response to H_2O_2, forming an intramolecular disulfide bond that compromises the binding of the exportin Crm1 to the non-canonical NES (SDIDVDGLCSEL) leading to Yap1 nuclear retention [90].

However, Yap1 is not oxidized directly by H_2O_2. Rather, the oxidant receptor peroxidase 1 (Orp1, also known as Gpx3), acts as the sensor together with the Yap1 binding protein (Ybp1). This protein forms a pre-complex with Yap1 and it is crucial for the mechanism of the sensing redox status of cells. The signal transduced to Yap1 is performed through the formation of an intermolecular disulfide bond between Cys36 of Orp1 and the Yap1

Cys598 that is then resolved into the previously described Yap1 Cys303-Cys598 intramolecular bridge [93]. In contrast, Yap1 response to diamide is Orp1/Gpx3-independent and does not involve the n-CRD [94]. This is consistent with the notion that it possesses two redox centers. Indeed, the electrophile N-ethylmaleimide (NEM) and the quinone menadione, both an electrophile and a superoxide anion generator, have been shown to modify c-CRD cysteines independently of Gpx3, leading to Yap1 nuclear accumulation. Mass spectrometry analyses further revealed that NEM and possibly menadione binds directly to the Yap1 c-CRD thus occluding the NES [95].

The family member with highest homology with Yap1 is Yap2. In the laboratory, taking advantage of the homology in the c-CRD domain, that contains four conserved cysteines, the Yap1 c-CRD was swapped by Yap2 c-CRD. This constructs were fused to GFP and were used to transform yeast strains to monitor the sub-cellular localization as well as the specificity to activate Yap1 target genes. Despite the similarities of the Yap2 c-CRD with Yap1 c-CRD it was shown that the Yap1 with Yap2 c-CRD is only capable of responding to Cd but not to hydrogen peroxide. These data showed a specificity of Yap2 c-CRD towards the cadmium response. The determinants for cadmium response are in the c-CRD of Yap2 that has a role in the transcriptional activation of *FRM2* the only Yap2 target that was found so far [96]. The Yap2 transactivation domain is also stimulated upon treatment with cadmium but not H_2O_2 [80].

YAP4 is induced under osmotic stress and this does not occur in the absence of Hog1 and Msn2 [97]. The regulation was confirmed by deletions of STRE (CCCT) localized in Yap4 promoter revealing that it is controlled by the zinc finger

transcription factor Msn2. *YAP4* gene expression is also induced under several other stress conditions. Under oxidative stress conditions *YAP4* expression is regulated by Yap1 and Msn2 [98]. It was also shown that Yap4 is phosphorylated under several stress conditions by the protein kinases PKA and Gsk3 in residues Thr192 and Ser196. Moreover Yap4 protein levels are reduced in the *gsk3* mutant [99]. ChIP-chip assays showed that Yap4 binds to several targets upon osmotic stress, some of them are also recognized by Yap6 and Sko1 [100].

Yap5 regulates, the transcription of Ccc1 a vacuolar transporter responsible for accumulation of iron in the vacuole, in this way controlling the cytoplasmic iron levels [101]. It was reported that this protein is nuclear resident. However, the authors used a multicopy vector to overexpress the GFP-Yap5 fusion protein and this can lead to artefactual localizations. Indeed we found a canonic Nuclear Export Signal (NES) in this protein. Yap5 contains two cyteines rich domains in the C-terminus both with 4 cysteines. The mutation of 4 in the n-CRD and 3 residues in the C-CRD abrogates its transcriptional activity [101]. Cell cycle studies by ChIP-chip showed that Yap5 might have several other possible targets namely *YCL033C* a methionine-R-sulfoxide reductase that protects iron-sulphur clusters from oxidative inactivation. They also show that Yap5 is regulated by SBF (Swi4-Swi6 cell cycle box binding factor) [102].

Yap3 and Yap7 are the less studied members of the Yap family. Yap3, like Yap1, Yap2, Yap5 and Yap8 has two CRD domains. *FCR3* gene, the *Candida albicans YAP3* orthologue confers resistance to fluconazole and 4-NQO (4-Nitroquinoline 1-oxide) in *pdr1pdr3* double mutant strain of *S. cerevisiae* [103].

Yap7 is described as nuclear resident protein since it has no NES or Cys residues [104].

Yap8/Acr1/Arr1 is responsible for the regulation in response to arsenic compounds of *ARR2* and *ARR3* [27, 28]. Mutants in Cys 132, 274 to Ala and double mutant, no longer activates transcription of the *ARR3*-lacZ, under arsenic or antimony stress [28]. By band-shift analysis it was shown that Yap8 requires more than the TTAATAA sequence, it requires a 13bp TGATTAATAATCA for efficient binding. It was also shown that orthologues of Yap8 also have the same 13bp palindromic sequence in the Acr3 orthologue Y. Di *et al* characterized an additional level of Yap8 control, by demonstrating that under arsenite stress the protein is stabilized through the averting of Yap8 degradation by the proteasome involving ubiquitin-conjugating enzyme Ubc4. Moreover, in this work the authors show by co-immunoprecipitation that Yap8 homodimerizes [105].

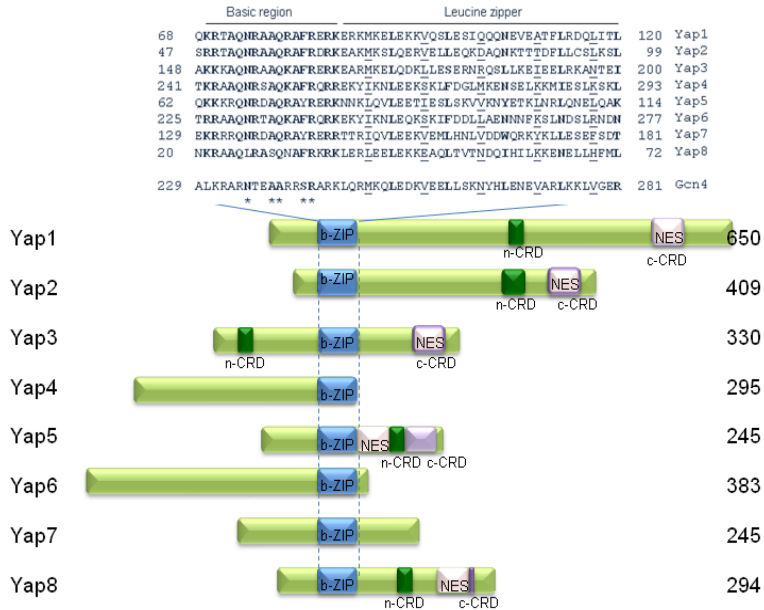

Figure 1.3 Structural features of the Yap family members

In the upper panel is the alignment of the Yap basic region and leucine zipper, together with Gcn4. The lower panel is a schematic representation of the entire Yaps and its cysteine rich domains and (n-CRD and c-CRD) and the nuclear export signal (NES). The sequence of the NES motifs was assigned by comparison with Yap1 sequence (Yap2, Yap3 and Yap8) or by means of the NetNES1.1 software (http://www.cbs.dtu.dk/services/NetNES/). Adapted from Rodrigues-Pousada et al, in press.

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Chapter 2

Yap8 activation in *Saccharomyces cerevisiae* under arsenic conditions

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2.1 Author's contribution to the article

Bobrowicz *et al* have shown that Acr1/Yap8 is a regulator of genes involved in arsenic detoxification in the budding yeast *S. cerevisiae* [1]. However, the regulation and activity of Yap8 in cells exposed to arsenic compounds was not investigated. In chapter 2 the mechanisms responsible for Yap8 regulation and activity are detailed. I performed analysis of the RNA levels of Yap8 potential target genes *ACR2* and *ACR3* encoding respectively an arsenate reductase and an arsenite efflux extrusion pump. Using wild type, Δ*yap8*, Δ*yap1* mutant strains Northern analyses were performed in order to evaluate *ACR2* and *ACR3* dependence on Yap8 and Yap1. We show that the main regulator is Yap8 although Yap1 also

contributes, to a less extent, to the full activation of these genes. I also performed Northern analysis to reveal if the expression of *YAP1* and *YAP8* is altered under arsenic stress. The Heat shock protein *HSP26* was used as an indicator of general stress response in the cell. In contrast to *YAP1* mRNAs that increase under stress, *YAP8* mRNAs levels remain constant during the treatment.

Yap1 is regulated at the level of its sub-cellular localization, and due to the presence of conserved Cys in Yap8 it was studied its regulation using the construction GFP-Yap8. In order to do this I performed fluorescence microscopy of cells with the GFP-Yap8 construction and of cells with GFP-Yap1. After adding arsenate or arsenite, Yap8 accumulates in the nucleus as well as Yap1. Both proteins are therefore regulated at the level of sub-cellular localization. Tamás's group showed that Yap8 is constitutively localized in the nucleus, either in unstressed and stressed cells. Nevertheless, in order to clarify these results I cloned the GFP construction in a multi-copy plasmid and transformed it in a W303 strain obtaining the same result as this group. These results may be due to the existence of an artefact because of the use of overexpression vectors. Yap1 is regulated via its interaction of the nuclear export signal (NES) with Crm1. When cells are subjected to oxidative stress the C-terminal cysteines of Yap1 form a disulfide bond masking the NES and thus the protein remains in the nucleus upon stress. To study Yap8 localization and to evaluate if Crm1 is also involved in Yap8 activity I have used a strain containing Crm1 under the control of the *MET* promoter. I show that Yap8 requires the presence of Crm1 to be exported from the nucleus. I also demonstrated that the cysteines conserved in Yap1 and Yap8 are important for nuclear localization of both proteins. The sub-cellular localization was analysed using the fluorescence microscope of the

mutated cysteines GFP-Yap8 versions. The overall results show that i.) like Yap1, Yap8 is only localized in the nucleus after stress ii.) Crm1 is the exportin responsible for Yap1 and Yap8 shuttle control between nucleus and cytoplasm and iii.) the Yap8 Cys residues are relevant for Yap8 activity.

2.2 Abstract

Yap8, a member of the *Saccharomyces cerevisiae* Yap family, is activated in response to arsenic. Both the mechanisms by which this activation takes place and its regulation have not yet been identified. In this report, we show that Yap8 is not activated at the transcriptional level but, rather, its nuclear transport is actively regulated and dependent on the exportin chromosome region maintenance protein. In addition, it is shown that Cys132, Cys137 and Cys274 are essential for Yap8 localization and transactivation function both of which are required for its biological activity.

2.3 Introduction

Saccharomyces cerevisiae arsenic compounds resistance (ACR) cluster was isolated as loci that increase tolerance to arsenite [1]. The three contiguous genes identified as *ACR1 (YAP8), ACR2* and *ACR3* encode a positive regulator, an arsenate-reductase and a plasma membrane arsenite efflux protein, respectively [2-5]. *ACR1*, also designated *YAP8*, belongs to the yeast AP-1 like factor (YAP) family of transcription factors [6]. Members of the YAP family (Yap1 to Yap8) share a conserved b-ZIP DNA-binding domain, each regulating a specific set of genes involved in multi-drug resistance

[7]. Yap8 could mediate arsenic stress responses by regulating the expression of *ACR2* and *ACR3*. Yeast cadmium factor 1 (Ycf1p) is also involved in the arsenite detoxification and known for conferring resistance to a variety of drugs through sequestering the glutathione S-conjugates into the vacuole [8]. Yap1 [9], the prototypical YAP gene family, which regulates the yeast oxidative stress response regulon, also regulates the expression of *YCF1*. Yap1 primary control lies in a redox-regulated Crm1-dependent nuclear export [10-12]. Redox signals disrupt the Yap1–chromosome region maintenance protein (Crm1p) interaction through a redox dependent alteration of the Yap1 C-terminal nuclear export signal (NES), thereby promoting Yap1 nuclear accumulation. Data presented here shows that arsenic compounds activate Yap8 at the level of both its nucleo-cytoplasmic shuttling and its transactivation potential. Regulation of Yap8 nucleo cytoplasmic shuttling involves arsenic-sensitive Crm1p-dependent nuclear export and the Yap8 Cys residues located in positions 132, 137 and 274.

2.4 Materials and methods

2.4.1. Growth conditions

Yeast strains were grown in complete YPD (1% yeast extract, 2% bactopeptone, 2% glucose) or selective media (SC or SD: 0.67% ammonium sulfate-yeast nitrogen base without amino acids [Difco], 2% glucose), supplemented with the appropriate selective amino acids. Early exponential phase cells (A600 = 0:4–0:5) were induced by the addition of 2 mM Na_2HAsO_4 or $NaAsO_2$ and samples collected at the indicated time point. Samples for RNA and protein extraction were washed and stored at 80 ºC. Phenotypic growth

assays were carried out by spotting 5 µL of an early exponential phase diluted culture (approximately $2x10^3$ cells) in medium containing increasing concentrations of Na_2HAsO_4 or $NaAsO_2$. Growth was recorded after 2 days at 30 ºC. The bacterial Escherichia coli strain XL1-Blue *recA1endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F0proAB lacIqZDM15Tn10* (Tetr)] (Stratagene) was used as the host for routine cloning purposes. Standard methods were used for genetic analysis, cloning and transformation [13](see Table 2.1).

2.4.2. Plasmids and constructs

To express the *YAP8* gene the corresponding chromosomal region was amplified by PCR. The product was first cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) and the *Kpn*I fragment, encoding the entire *YAP8* ORF plus 923 bp upstream and 643 bp downstream, sub-cloned into the CEN vector YEplac33 [18] to generate construct YCpYAP8. To create the YAP8-TAPtag chimera *Xho*I and *Nco*I restriction sites were introduced by PCR, the product was first cloned into the vector pBS1479 and subsequently sub-cloned into the pRS416 [19]. The fusion GFP-YAP8 (pRS cp-*GFP-HA-YAP8*) was obtained by replacing the *Pvu*II/*Sal*I *YAP4* gene in the plasmid pRScp-*GFP-HA-YAP4* [20]. The lexA-*YAP8* constructions were generated by cloning the respective *Sma*I/*Kpn*I PCR products into the YCp91 expression vector [6]. Cloning in the multi-copy vector was done by inserting the GFP-Yap8 fusion from pRS cp-*GFP-HA-YAP8* in yEPlac195 cut with *Sac* I and *Sal* I. Site-directed mutagenesis of *YAP8* cysteines was performed by PCR amplification of entire plasmids using complementary primers containing the desired mutation. All constructs were sequenced using the ABI Prism DyeDeoxy Terminator Cycle Sequencing Kit

(Applied Biosystems) and ABI Prism 373A Automatic Sequencer (Perkin Elmer). The fusion proteins were functional since they complement Δ*yap8* mutant.

Table 2.1 *S. cerevisiae* strains used

Strain	Genotype	Source
FY1679	*MATα his3-200 ura3-52 GAL2*	Winston *et al.* [14]
FY*Δ1*	*Matα his3-200 ura3-52 GAL2 yap1::KAN*	Nevitt *et al*. [15]
BY 4743	*MATa/α his3Δ1/his3Δ1 leuΔ0/leuΔ0 lys2Δ0/LYS2 met15Δ0/MET15 ura3Δ0/ura3Δ0 YPR199c::kanMX4/YPR199c::kanMX4* (isogenic to FY strains)	EUROSCARF
BY*Δ8*	*MATa his3Δ1 leuΔ0 met15Δ0 lys2Δ0 ura3Δ0 YPR199c::kanMX4*	This study
BY *Δ1Δ8*	*MATa yap1::HIS3 leuΔ0 met15Δ0 lys2Δ0 ura3Δ0 YPR199c::kanMX4*	This study
FT4	*MATa ura3-5 trp1-Δ63 his3-Δ200 leu2::PET56*	Tzamaris and Struhl [16]
mcy8	*Matα his3 can1-100 ade2 leu2 trp1 yap1::URA3 ura3:: (3XSV40AP1-lacZ) crm1::HIS3-pmet3-CRM1*	Kuge *et al*. [11]
L40a	*MATa ade2 trp1-901 leu2-3,112 his3-200 LYS::lexA-His3 URA3::lexA op-lacZ*	Marcus *et al.* [17]

2.4.3 Northern-blot analysis

RNA procedures were performed according to [15]. RNA was isolated from early log-phase cultures (OD_{600}0,4–0,5) that were either untreated or exposed to 2 mM As(V) (Na_2HAsO_4) or As(III) ($NaAsO_2$). Approximately 40 µg of RNA were separated in formaldehyde gels, and transferred onto nylon membranes (Hybond XL, Amersham Pharmacia Biotech).

The following intragenic PCR fragments were used as probes: 0.84 kb *YAP8*, 1.80 kb *YAP1*, 0.38 kb *ACR2*, 0.38 kb *ACR3*, 0.80 kb

YCF1, 0.60 kb *HSP26*, 0.20 kb U3. mRNA levels were quantified (Image-Quant, Molecular Dynamics) and normalized against those of *U3* internal loading control, a small nuclear RNA (*SNR17A*).

2.4.4 Protein extraction and Immunoblot analysis

Samples collected at the indicated time points were harvested by centrifugation at 4 ºC. Protein extracts were prepared by the TCA acid lysis method and immunoblotted according to [15]. To measure intracellular levels of TAPtag-Yap8 and LexA-Yap fusion proteins, immunoblotting was performed with 100 and 50 µg of proteins, respectively. The LexA-hybrid proteins were detected using a monoclonal LexA antibody (Clontech) and anti-mouse IgG conjugated to alkaline phosphatase (Bio-Rad). Detection was performed using the AP Conjugate Substrate Kit (Bio-Rad).

2.4.5. Fluorescence microscopy

Cells transformed with pRS *cp-GFP-HA-YAP8* were grown to early log phase and induced with either 2 mM Na_2HAsO_4 or $NaAsO_2$, at the indicated time points. 4,6-Diamino-2-phenylindole (DAPI) was added as a DNA marker at a final concentration of 20 µg/ml, 5 min before microscopy. After washing with phosphate-buffered saline(PBS), cells were resuspended in DABCO solution (75% glycerol, 0.25xPBS and 200 mM diazabicyclooctane, Sigma–Aldrich). Our own experience and the one of Delaunay and co-workers [21] have shown that DABCO does not affect GFP-Yap8 localization. Fluorescence of live yeast cells was visualized by a Leica DMBR fluorescence microscope.

2.4.6. β-Galactosidase activities

Cells expressing LexA fusions were replica-plated onto SD medium containing 2 mM Na_2HAsO_4 or $NaAsO_2$ and after two days covered with 10 ml of 0.5 M sodium phosphate buffer (pH 7.0); 0.2% (w/v) SDS; 2% (v/v) dimethyl formamide containing 100 mg X-gal/ml and 0.5% (w/v) agarose at 70ºC. Plates were analyzed after 30 min – 24 h incubation at 30ºC. For quantitative b-galactosidase measurements, cells were harvested in early log phase, resuspended in lacZ buffer (Sodium phosphate buffer, pH7, KCl 1Mm, Mg_2SO_4), permebialized with chloroform, vortexed two times for 10s with an interval on ice for min and assayed for enzyme activity after incubation with *ortho*-Nitrophenyl-β-galactoside (ONPG) at 0.6mg/ml, reaction was stopped by addition of 150µl Na_2CO_3 1M after the appearance of the first yellow colour. Cells co-expressing the Lex-Yap fusions and a reporter cassette bearing the *lacZ* gene driven by a promoter containing the Lexa binding sequence were grown in the presence and absence of arsenic.

2.5 Results and discussion

2.5.1. YAP8 is required for resistance to arsenic compounds and for a regulated expression of the ACR2 and ACR3 genes.

A strain lacking the *YAP8* gene (Δ*yap8*) is highly sensitive to arsenate and arsenite at very low concentrations (100 µM) (Fig. 2.1A). In comparison, the isogenic wild-type strain tolerates up to 2 mM of both drugs. The Δ*yap8* arsenic phenotype is rescued by reintroducing *YAP8* on a centromeric plasmid (Fig. 2.1B). A strain lacking *YAP1* is also hypersensitive to both drugs (Fig. 2.1A). A strain lacking both Δ*yap1* and Δ*yap8* is even more sensitive to

arsenic compounds than either of the single mutant strains (Fig. 2.1A), indicating that both *YAP1* and *YAP8* are independently required for the cell tolerance to arsenic compounds. The expression of *ACR2* and *ACR3* is induced by arsenic stress (Figs. 2.2A and B), with arsenate having a much potent effect than arsenite. This induction is decreased or even abolished in Δ*yap8* (Fig. 2.2A). Induction of these genes by arsenic compounds is also diminished in Δ*yap1*, consistent with the notion that both *ACR3* [22] and *YCF1* [9] are known Yap1-target genes. The requirement of both *YAP1* and *YAP8* for arsenic stress tolerance can thus be rationalized by their function of regulating the arsenic stress-induction of genes important for arsenic compounds detoxification.

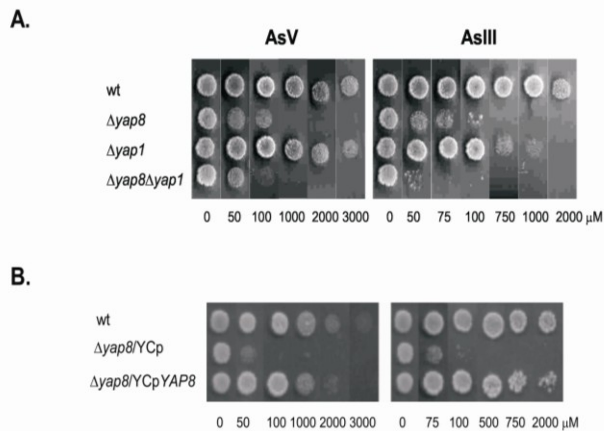
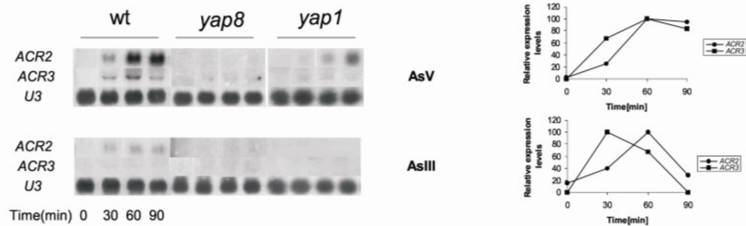
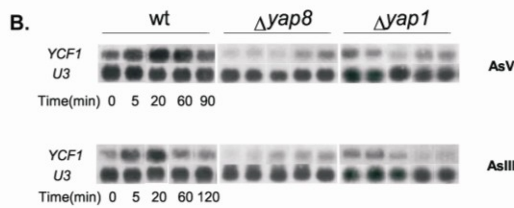


Figure. 2.1. Δ*yap8* and Δ*yap1* deleted strains show distinct sensitivity to arsenic. Tolerance of the Δ*yap8* mutant is rescued by expressing *YAP8* in a CEN-based plasmid. Approximately $2x10^3$ early exponential phase cells were spotted onto YPD (A) or SC (B) medium supplement with increased concentrations of arsenate (AsV) and arsenite (AsIII).





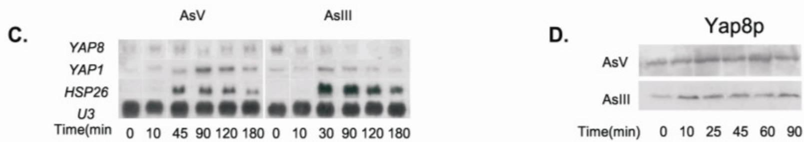


Figure. 2.2. Induction of genes involved in arsenic stress response. Early exponential phase cells were up-shifted to 2 Mm of AsV or AsIII and harvested at the indicated time-points. RNAs and proteins were extracted as described. Northern-blot analysis of (A) *ACR2* and *ACR3*, (B) *YCF1*, (C) *YAP8* and *YAP1*. *U3* mRNA was used as internal loading control and the HSP26 as an inducing control of arsenic stress. (D) Cells expressing the fusion YAP8-TAPtag were assayed for immunoblot.

2.5.2 Yap8 is redistributed to the nucleus upon arsenic treatment.

We explored the mechanism of Yap8 activation by arsenic compounds. Transcriptional activation is not a major determinant

in this activation since *YAP8* mRNA and protein levels are constitutive and not induced upon arsenic treatment (Figs. 2.2C and D). In contrast, *YAP1* mRNA levels are slightly induced by arsenic compounds (Fig. 2.2C) indicating that Yap1 might participate in the arsenic stress response.

Since Yap1 is regulated at the level of a Crm1-dependent nuclear export [10, 12, 23] we investigated whether a similar mechanism could apply to Yap8 by monitoring the cell distribution of a GFP-Yap8 fusion protein. Yap8 appeared predominantly localized in the cytoplasm under non-stress conditions and redistributed into the nucleus within 10 min after arsenic treatment (Fig. 2.3.1A). Arsenate had the effect of keeping Yap8 longer in the nucleus than arsenite (data not shown), probably due to the delayed detoxification of this compound that needs a reduction step to arsenite. We next investigated the involvement of Crm1p in Yap8 cell redistribution using a strain carrying *CRM1* under control of a methionine-repressible promoter [11]. In non-stressed cells, GFP-Yap8 appeared in the nucleus in the presence of methionine but was cytoplasmic when methionine was absent (Fig. 2.3.1B). These data establish that Crm1 mediates Yap8 nuclear export and suggest that this export is regulated by arsenic stress.

We analysed the sub-cellular localization of GFPYap8 encoded by a multi-copy plasmid and verified that it is constitutively nuclear in a W303 background (Fig.2.3.2).

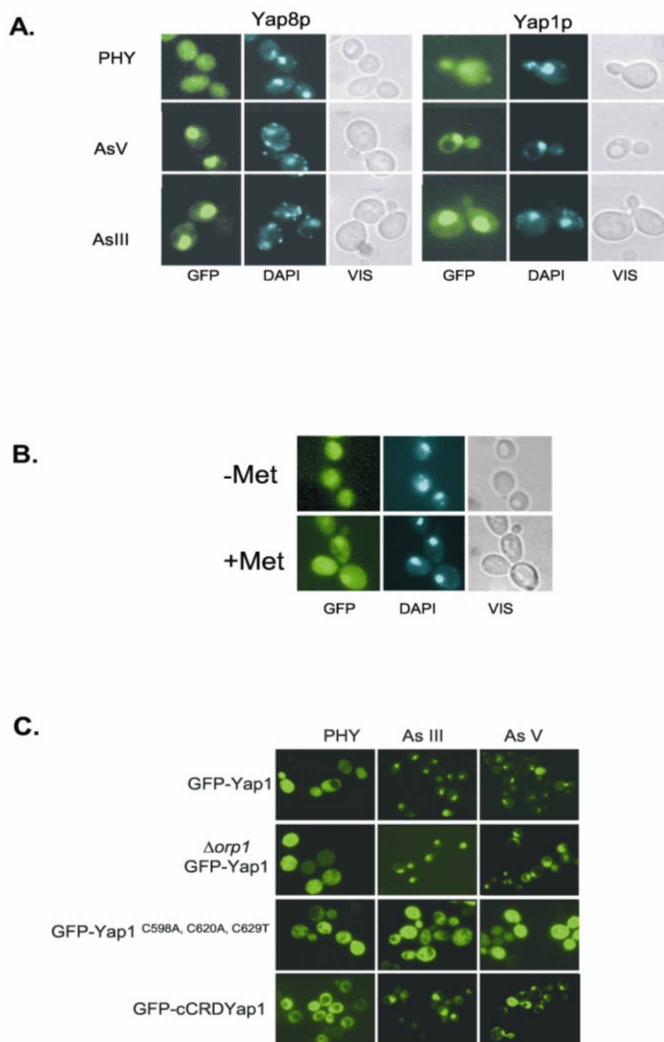


Figure. 2.3.1 Yap8 and Yap1 are relocated to the nucleus upon arsenic treatment. (A) Cells grown to early exponential phase were induced 10 min with 2 mM of arsenate and analysed under a fluorescence microscope.
(B) Cells expressing the *CRM1* driven by the *MET3* promoter were grown to early exponential phase in the presence or absence of methionine. PHY, physiological conditions (C) Δ*yap1* or Δ*orp1* strains expressing GFP-Yap1, or its mutant derivatives were induced 10 min with 2 mM of AsIII or AsV and analyzed by fluorescence microscopy

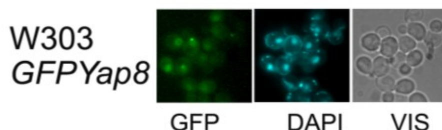


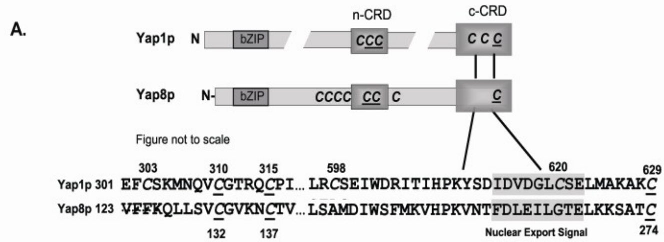
Figure 2.3.2 Yap8 subcellular localization under physiological conditions when over-expressed in YEplac195. W303 strain was transformed with the fusion *GFP-HA-YAP8* and cells were collected as described in Materials and Methods and analyzed in fluorescence microscopy.

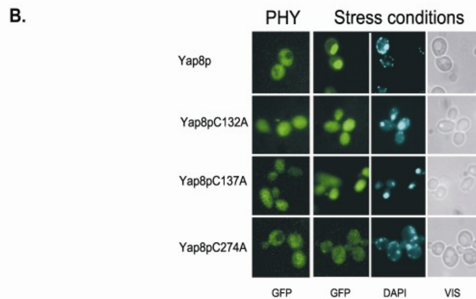
2.5.3. C132, C137 and C274 are essential for Yap8 nuclear translocation.

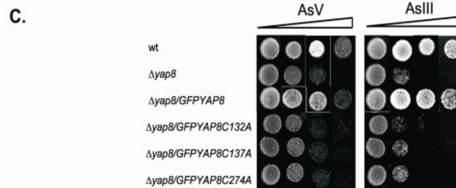
In analogy to Yap1, which can respond to Cys-reactive chemicals through its modification at specific Cys residues [12, 23, 24], we investigated the requirement of Yap8 Cys residues in Yap8 activation by arsenic compounds Yap8 and Yap1 share 19% identity with two domains of higher homology, one being in the C-terminus of these proteins (Fig. 2.4A). The corresponding Yap1 C-terminal domain, also called c-CRD for C-terminal Cys-rich domain carries the Crm1-cognate NES comprising a leucine-rich hydrophobic amino acid stretch embedded in a three-repeat Cys motif (Cys598, Cys620, Cys629) [10]. Yap8 also contains a putative NES in the corresponding domain, previously identified by a short leucine-rich hydrophobic amino-acid sequence flanked by positively charged residues (amino acids 240–282) [22]. However, this Yap8 C-terminal domain only contains one Cys residue (C274) that appears conserved with Yap1 Cys629. The second domain of high homology corresponds to the Yap1 n-CRD that also carries a three-repeat Cys motif (Cys303, Cys310, Cys315). In the corresponding region Yap8 carries two cysteines (C132 and C137) that are conserved with Yap1 Cys310 and Cys315, respectively.

Taking into account the structural similarities between Yap8 and Yap1 we anticipated that Yap8 conserved cysteines might play a role in the regulation of Yap8 nuclear export. Yap8 C132, C137 and C274 were individually and simultaneously substituted with alanine residues. These mutations all impaired the nuclear relocalization of Yap8 in response to arsenate (2 mM) (Fig. 2.4B) and the ability of Yap8 to restore resistance to arsenic compounds to the Δ*yap8* strain (Fig. 2.4C). In contrast, the GFP-Yap8 clearly restores the As-phenotype of the yap8 mutant. These Cys substitutions do not affect the stability of Yap8 as shown by Western blot (Fig. 2.4D). We suggest that arsenic compounds inhibit Crm1-dependent Yap8 nuclear export by binding to these Cys residues thereby inhibiting Yap8–Crm1 interaction through a change of conformation. Fig. 2.5 Yap8 transactivation function is stimulated upon arsenate treatment. The function of transactivation can be another level of control for a transcription factor. We thus investigated whether arsenic compounds could modulate the Yap8 transactivation function using a one-hybrid strategy [25]. A LexA-Yap8 fusion protein, containing an artificial NLS, was able to activate transcription of the *lacZ* reporter gene from a LexA operator and this activation was increased upon arsenate treatment. Arsenite had only a slight or no effect on lacZ gene activation by LexA-Yap8 (Fig. 2.5A). Interestingly, the transactivation potential of LexA-Yap8 under arsenate is of the same order of magnitude as LexA-Yap2p under cadmium [6]. The ability of the LexA-Yap8 fusion to rescue the Δ*yap8* arsenic phenotype demonstrates that this fusion retains the Yap8 intrinsic molecular function in arsenic resistance (Fig. 2.5B). We next sought mapping the Yap8 transactivation domain by testing various Yap8 truncations in the one-hybrid assay (see diagram of Fig. 2.5A). None of the tested constructs could activate

lacZ transcription thus indicating that only full-length Yap8 retains the function of transactivation. We also tested the possible requirement of Cys132, Cys137 and Cys274 in this function. Interestingly, alanine substitution of each of these cysteines abolished lacZ gene expression by the Lex-Yap8 fusion indicating that these residues are also important for the Yap8 function of transactivation (Fig. 2.5A). However, as can be seen in Fig. 2.5B, LexA-Yap8 fusion mutated in the Cys137 partially restores the As-phenotype of the yap8 mutant. It is therefore plausible that Cys137 does not play a relevant physiological role in the Ya8p activity as play the Cys132 and Cys274.







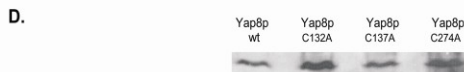


Figure. 2.4. (A) Comparison of Yap8 and Yap1. (B) Yap8 cysteines (underlined) were substituted by alanine and nuclear redistribution monitored by fluorescence microscopy after 10 min induction with 2 mM AsV. (C) Phenotypic analysis of the Δ*yap8* mutant expressing the *GFP-YAP8* chimera and its mutated versions. (D) Δ*yap8* cells expressing YAP8-TAPtag fusion and its Cys mutants were induced 10 min with 2 mM AsV and assayed by immunoblotting.

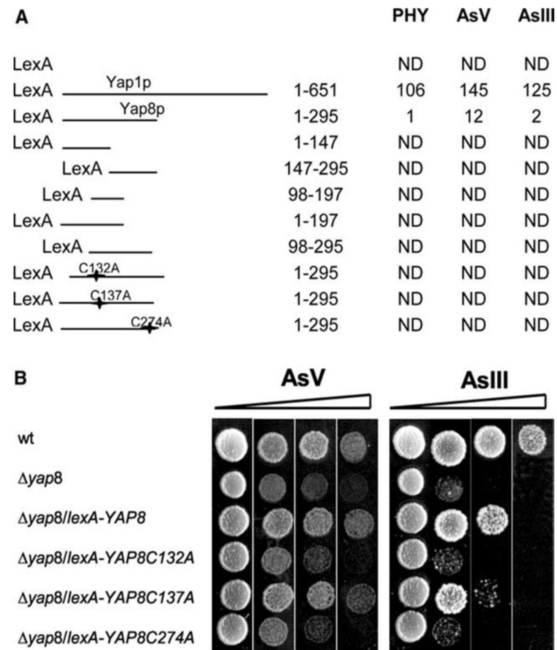


Figure 2. 5. Yap8 and Yap1 transactivation potential. (A) Cells co-expressing the Lex-Yap fusions and a reporter cassette bearing the lacZ gene driven by a promoter containing the LexA binding sequence were grown in the presence and absence of arsenic. Values of β-galactosidase activities (average of six independent transformants) are normalized to the A600 of cells and accurate to_15%. PHY, physiological conditions; N.D. not detected. (B) Arsenic sensitivity of the yap8 mutant expressing, lexA-YAP8 and its mutated versions.

2.5.4. Yap1 activation by arsenic compounds is distinct from H_2O_2 activation and similar to activation by thiol-reactive chemicals.

A GFP-Yap1 fusion potently accumulated in the nucleus upon treatment with arsenic compounds (Fig. 2.3C), indicating that Yap1 activation by these chemicals is exerted at the level nuclear export as for its activation by H_2O_2 and by thiol-reactive chemicals [24]. Yap1 cannot be activated by H_2O_2 in cells lacking Orp1/Gpx3, a GPx-like peroxidase that acts as the sensor of the pathway [12]. In

cells lacking Orp1, GFP-Yap1 still potently accumulated in the nucleus upon treatment with arsenic compounds indicating a mechanism distinct from H_2O_2. Further, an NLS-GFP-c-CRD fusion protein [24] also accumulated in the nucleus upon arsenite and arsenate treatment while GFP-Yap1 carrying substitution of all three c-CRD cysteines was unresponsive to these chemicals. Thus, Yap1 activation by arsenic compounds involves c-CRD cysteines and therefore uses a mechanism similar to that activated by thiol-reactive chemicals. In contrast to Yap8, arsenic compounds did not significantly modulate Yap1 transactivation function.

2.6. Conclusion

In conclusion, Yap8 is an important regulator of genes involved in arsenic compounds detoxification. Activation of Yap8 involves an arsenite- and arsenate-sensitive Crm1-dependent nuclear export. Yap8 Cys132, Cys137, and Cys274 are crucial in this regulated export. Arsenic compounds probably regulate Yap8 nuclear export by modifying its Cys residues and hence the NES, in analogy with the activation of Yap1 by these same compounds and by other thiol-reactive chemicals. Thus, Yap8 and Yap1 share a conserved mechanism of activation by arsenic compounds. A recent paper also identified Yap8 as a regulator of the *S. cerevisiae* arsenite and arsenate response. This study also suggested a regulation of Yap8 at the level of its transactivation function, consistent with our results (Fig. 2.5). Surprisingly these authors found Yap8 constitutively nuclear [26] which contrast with the data shown here. The reason for this discrepancy could be related to unknown genetic differences in the *S. cerevisiae* strains used and the fact they had

the recombinant protein in higher amounts as we tried to mimic in figure 3.2. These differences cannot be attributed to Ybp1, a protein required for Yap1 activation by H_2O_2 and known for being mutated in some *S. cerevisiae* strains [27], since activation of both Yap8 and Yap1 by arsenic compounds is independent of this factor (data not shown).

2.7 Acknowledgements:

We must express our gratitude to Dr. Hollenberg (Heirich-Heine Duesseldorf University), Dr. Kuge (Tohoku University) and Dr. Oliver (Manchester University) for having kindly provided yeast strains and plasmids. Tracy Nevitt is also acknowledged for dissecting the BY4743 strain and for reading the manuscript. This work was supported by grants from FCT to C.R.-P. (POCTI 34967) and fellowships to R.A.M. (SFRH/BPD/11438/2002).

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Chapter 3

Contribution of Yap1 towards *S. cerevisiae* adaptation to arsenic mediated oxidative stress

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3.1 Author's contribution to the article

Arsenic compounds are highly toxic and potent human carcinogens. These compounds are wide spread in nature resulting from geological formations and from human activities such as, the use of pesticides that contribute to their accumulation in the environment. Despite of its toxicity they can bring benefits to human health through the use to treat promyelocytic leukaemia and parasitic infections. The mechanisms underlying arsenic tolerance are far from being known. In the previous chapter I described the

mechanisms of Yap8 activation and in the present one it is investigated how the redox homeostasis is disturbed by arsenic compounds.

In order to have a general picture of gene expression, under arsenate, I carried out microarray transcriptional profiling and treated the results using t-profiler. It was observed a differential expression of several genes, as for instance the up-regulation of those involved in oxidative stress. This was the first suggestion indicating the production of Reactive Oxygen species (ROS) by arsenic compounds. Many of the up-regulated genes are known to be Yap1 target genes. From those I tested *SOD1*, *GSH1* and *TRX2* genes in the wild type and the Δ*yap8* strains. Curiously enough, the expression of these genes in the Δ*yap8* revealed very high levels that can be interpreted as due to the defective arsenic extrusion system in this strain as shown in chapter 2. I observed that Yap1 and Yap8 are nuclear resident proteins during the whole period of 24h of continuous exposure as I registered, with the help of L. Batista-Nascimento. Several indicators of oxidative stress were determined such as intracellular oxidation and lipid peroxidation, protein carbonylation as well as the GSSG/GSH ratio. Glutathione is a major anti-oxidative defence and its levels decrease when in the presence of arsenate, although its levels quickly increase back to normal levels even in Δ*yap1* strain. The production of glutathione has as limiting step, the reaction catalyzed by the enzyme Gsh1, the expression of which is regulated by Yap1 and Met4. Oxidised glutathione is reduced by Glr1, described as a Yap1 target, therefore the increase of reduced glutathione in the Δ*yap1* strain can only be explained by *GSH1* expression being induced by the transcription factor Met4, under

this condition. This was clarified by the Northern analysis of *GSH1* expression in the Δ*yap1* strain. It was observed a low expression of *GSH1* even in the absence of Yap1 transcription factor. The results shown in chapter 3 indicate that i.) arsenic compounds induce the formation of ROS ii.) Yap1 is essential in preventing oxidation of cellular components iii.) Yap1 and Yap8 factors remain in the nucleus during prolonged exposure to arsenate and iv.) genes involved in oxidative stress are highly induced in arsenic stress particularly in a Δ*yap8* strain.

3.2 Abstract

In the budding yeast *Saccharomyces cerevisiae*, arsenic detoxification involves the activation of Yap8, a member of the Yap (yeast AP-1-like) family of transcription factors, which in turn regulates *ACR2* and *ACR3*, genes encoding an arsenate reductase and a plasma-membrane arsenite-efflux protein respectively. In addition, Yap1 is involved in the arsenic adaptation process through regulation of the expression of the vacuolar pump encoded by *YCF1* (yeast cadmium factor 1 gene) and also contributing to the regulation of *ACR* genes. Here we show that Yap1 is also involved in the removal of ROS (reactive oxygen species) generated by arsenic compounds. Data on lipid peroxidation and intracellular oxidation indicate that deletion of *YAP1* and *YAP8* triggers cellular oxidation mediated by inorganic arsenic. In spite of the increased amounts of As(III) absorbed by the Δ*yap8* mutant, the enhanced transcriptional activation of the antioxidant genes such as *GSH1* (γ-glutamylcysteine synthetase gene), *SOD1*

(superoxide dismutase 1 gene) and *TRX2* (thioredoxin 2 gene) may prevent protein oxidation. In contrast, the *yap1* mutant exhibits high contents of protein carbonyl groups and the GSSG/GSH ratio is severely disturbed on exposure to arsenic compounds in these cells. These results point to an additional level of Yap1 contribution to arsenic stress responses by preventing oxidative damage in cells exposed to these compounds. Transcriptional profiling revealed that genes of the functional categories related to sulphur and methionine metabolism and to the maintenance of cell redox homoeostasis are activated to mediate adaptation of the wild-type strain to 2 mM arsenate treatment.

3.3 Introduction

Arsenic (As) is a highly toxic metalloid widely distributed in Nature and mostly found in drinking water. The first step of inorganic As(V) removal from the cytoplasm consists of its two electron reduction to As(III) using glutathione as the source of reducing potential [1]. Chronic exposure to this compound is generally associated with an increased risk of multiple cancers, vascular diseases, developmental anomalies and neurological disorders [2–4]. To counteract the deleterious effects caused by arsenic compounds, almost all living organisms have developed mechanisms to eliminate it. In the budding yeast *Saccharomyces cerevisiae*, resistance to arsenic is achieved through the activation of the transcriptional regulator Yap8 [yeast AP-1-like transcription factor Yap8, also called Acr1 (arsenic compound resistance protein 1)] [5], which in turn, induces the expression of an arsenate reductase and a plasma-membrane arsenite-efflux protein encoded by the

genes *ACR2* and *ACR3* respectively [6-10]. In addition, the *YCF1* gene product, yeast cadmium factor 1, also facilitates the vacuolar extrusion of glutathione-conjugated arsenite molecules [11]. Although Yap8 is the main regulator of arsenic stress responses, Yap1 is also involved to a lesser extent through *YCF1* activation under these conditions and contributes to the full activation of enzymes encoded by the *ACR* genes [12]. Both regulators belong to the Yap family of b-ZIP (basic domain/leucine zipper) transcription factors, formed by eight members [10], which modulates the activation of specific genes in response to various stress (for a review, see [5]).Arsenic toxicity and carcinogenicity in animals has been suggested to be probably due to the generation of an oxidative stress, thus provoking a deleterious effect by this metal [13]. Yeast mutants in genes related to several mitochondrial processes, which show sensitive phenotypes to arsenic compounds, were recently identified. A total of 20 specific-As(V)-sensitive mutants were found, from which 13 genes have orthologues in humans[14]. On the other hand, high-throughput arsenite-triggered changes in transcriptional profiling [15,16] indicate that cell antioxidant defences are up-regulated in yeast. Furthermore, other investigators have shown a dose-dependent increase in the levels of peroxidation of membrane lipids as a consequence of arsenite exposure [17]. Yap1, the best-characterized member of the Yap family and the major regulator in oxidative stress, is involved in arsenic stress responses. These facts together led to the hypothesis that arsenic induces oxidative stress in which Yap1 plays a major role. It is indeed known that arsenite As(III) can react with the thiol groups of proteins, inhibiting many biological pathways, whereas the pentavalent form As(V) of arsenic is a phosphate analogue interfering with phosphorylation

reactions [1]. Although the toxic effect of both oxidation states, As(V) and As(III), appears to be very similar, the elimination of As(V) requires its reduction to As(III) using the redox potential of GSH and thus interfering with the GSH pool of the cell [1]. As recently pointed by other investigators [14], tolerance to either arsenate or arsenite also involves specific sets of mitochondrial genes. We decided to evaluate, using biochemical and molecular approaches, the damage caused by As(V) and As(III). We show that oxidative stress is generated as an effect of arsenic exposure in strains defective in the arsenic-extrusion machinery and in the antioxidant defence system. By measuring the GSH/GSSG ratios, we provide evidence indicating that arsenic compounds trigger the disruption of the redox equilibrium (being the homoeostasis rapidly achieved through the enhancement of GSH generation). Transcriptional profiling of the wild-type strain under exposure to arsenate reveals the induction of many Yap1-dependent genes and genes involved in sulphur metabolism. Our results show that the antioxidant defences are up-regulated in the mutant Δ*yap8*, which absorbs increased amounts of arsenite, in comparison with the parental strain. Since the status of protein carbonylation is not changed in the wild-type and Δ*yap8* strains, we conclude that the activation of the antioxidant system under arsenic stress prevents the accumulation of oxidized proteins. Consistent with this notion, the Δ*yap1* mutant displays high levels of protein oxidation.

3.4 Material and Methods

3.4.1 Strains, plasmids and growth conditions

The yeast strains used in the present study were: BY4741 *MAT a*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0 (*EUROSCARF), BY4741 *Δyap1 MAT a*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0;YML007w::kanMX4 (*EUROSCARF), BY*Δyap8 MAT a;his3Δ1; leuΔ0; met15Δ; lys2Δ0; ura3Δ0; YPR199c::kanMX4* [12], BY *Δyap1Δyap8 MAT a; his3Δ1; leuΔ0; met15Δ0;lys2Δ0; ura3Δ0; YPR199c::kanMX4; YML007w::HIS* [12] and FT4 *yap1 MAT a; ura3-52; trp1Δ63; his3-Δ200; leu2::PET56*;*yap1Δ* [18]. The complete coding region of *YAP8* gene was deleted by the micro homology PCR method [19] to create the strain FT4 Δ*yap8 MAT a; ura3-52; trp1Δ63; his3-Δ200; leu2::PET56;yap8::KAN*. Deletion was confirmed by PCR analysis of genomic DNA using upstream and downstream primers. To overexpress *YAP8*, the corresponding chromosomal region was amplified by PCR using the primers 5_-CCATTGTAGGAGAGTAACCT-3 and 5_-CATCGAATACTCCACATCGATC-3_. The product was first cloned using the Zero Blunt® TOPO® PCR cloning Kit (Invitrogen) and the XbaI/BamHI fragment was subcloned into the 2 *μ* vector YEplac195 [20]. The construct was sequenced using the ABI Prism Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and ABI Prism 373A Automatic Sequencer (PerkinElmer). The plasmid overexpressing YAP1 was available in our laboratory [18]. The CEN plasmids expressing the myc-tagged *YAP1* and GFP (green fluorescent protein)-tagged *YAP1* and *YAP8* versions were obtained from Dr M.B. Toledano's group [21], from Kuge and colleagues [22] or was available in our laboratory [12] respectively. Strains were grown in complete YPD [1% yeast extract, 2% (w/v)

bactopeptone and 2% (w/v) glucose] or selective media [SC (synthetic complete) or SD (minimal synthetic defined): 0.67%ammonium sulfate/yeast nitrogen base without amino acids (Difco) and 2% (w/v) glucose] supplemented with the appropriate selective amino acids. Early-exponential-phase cells [attenuance (*D*600) 0.4–0.5] were stressed by the addition of 2 mM As(V) (Na_2HAsO) or As(III) ($NaAsO_2$) and samples were collected at the indicated time points. Phenotypic growth assays were carried out by spotting 5 *μ*l of an early-exponential-phase sequentially diluted culture (approx. 2000–20 cells) in selective medium containing up to 2 mM Na_2HAsO or $NaAsO_2$. Growth was recorded after 2 days at 30◦C. The bacterial *Escherichia coli* strain XL1-Blue *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F_*proAB lacI*q*ZDM15 Tn10* (Tetr)] (Stratagene) was used as the host for routine cloning purposes. Standard methods were used for genetic analysis, cloning and transformation [23].

3.4.2 Intracellular oxidation, lipid peroxidation and protein carbonylation

Measurements of intracellular oxidation and lipid peroxidation were performed with 50 mg (dry weight) of mid-exponential phase cells grown under physiological conditions or exposed to arsenate or arsenite stress. Intracellular oxidative stress generated by arsenic compounds was monitored by measuring changes in fluorescence resulting from the oxidant-sensitive probe DCFDA (2 ,7-dichlorofluorescein diacetate) [24]. A fresh ethanol stock solution of DCF-DA was added to the culture to a final concentration of 10 *μ*M and cells were incubated for 2000 *g* for 5 min at 22◦C and washed twice with distilled water. The cell pellets were resuspended in 500 *μ*l of water and lysed by vortex mixing in the presence of 1.5 *g* of

glass beads. The extracts obtained after centrifugation at 15 000 *g* for 5 min were diluted and fluorescence was measured using a Photo Technology International spectrofluorimeter set at 15 min to allow uptake of the probe. Cells from each aliquot were cooled on ice, harvested by centrifugation at an excitation wavelength of 504 nm and an emission wavelength of 524 nm with a slit width of 5 nm. The effect of deletions alone was measured under physiological conditions and the results under arsenic stress were expressed as the relation between the fluorescence of stressed and unstressed cells. Lipid peroxidation was determined by quantifying TBARS (thiobarbituric acid-reactive substances). After 24 h incubation with As compounds, cells were cooled on ice, harvested by centrifugation and washed twice with 20 mM Tris/HCl buffer, pH 7.4. The pellets were resuspended in 500 *μ*l of the same buffer containing 10% (w/v) trichloroacetic acid and 1.5 g of glass beads were added. The samples were lysed using six cycles of 20 s agitation on a vortex-mixer, followed by 20 s on ice. Lipid peroxidation of cell extracts was monitored spectrophotometricaly at 532 nm in an EDTA/thiobarbituric acid/NaOH solution as described in [25], which determines the accumulation of TBARS in cells. The control data for each mutant strain grown under physiological conditions was expressed as the amount of MDA (malondialdehyde)/mg of cell dry weight formed and the results under arsenic stress are expressed as the relation between the values of stressed and non-stressed cells. Several authors have recently used this technique [26, 27]. All these experiments were carried out at least three times, with no fewer than three replicate measurements in each experiment and the results are presented are means+−S.D. To detect the presence of carbonyl groups introduced in proteins as a result of arsenic stress, an OxyBlotTM

protein oxidation detection kit (Intergen) was used. The samples were analysed by immunoblotting and processed as described in [28], using rabbit anti-dinitrophenol antibody as the primary antibody. As a loading control, levels of the co-chaperone Sba1 were measured [29].

3.4.3 Glutathione Determination

Measurements of thiols were performed by the spectrophotometric GR–DTNB [glutathione reductase–5,5-´dithiobis-(2-nitrobenzoic acid)] recycling method originally described by Griffith[30]. The wild-type and mutant strains grown to early exponential phase were induced with 2 mM As(V), and samples collected at the indicated time points were extracted in a solution consisting of 0.1 M HCl and 1 mM EDTA, pH 1.35 [28]. The kinetics of TNB [5-thio-2-nitrobenzoate] formation was monitored photometrically at 405 nm. GSSG concentrations were determined in the same extracts after a 30 min incubation of the supernatant with 2-vinylpyridine at room temperature (20–22◦C) to derivatize GSH [30]. The concentration was determined by reference to a standard prepared in HCl and was expressed as nmol of glutathione/mg cell dry weight. Measurements were carried out three times, with three replicates in each experiment and results are means+−S.D.

3.4.4 Determination of As (III) retention by atomic absorption

Analysis of the capacity of *S. cerevisiae* wild-type cells and *Δyap1* and *Δyap8* mutant versions to absorb and accumulate As(III) was determined using atomic-absorption spectrophotometry, as previously described [31]. Arsenite, to a final concentration of 2 mM, was added to the medium containing 1 mg (dry weight) of mid

exponential-phase cells, and the culture was incubated at 28◦C in a rotating bath for 4 h and 24 h. For measuring residual As(III) present in the medium, 5 ml aliquots were centrifuged at 2000 *g* for 5 min at 22◦C and the supernatant was collected and subjected to atomic-absorption spectrophotometry using a PerkinElmer 3100 atomic-absorption spectrometer. As(III) absorption was calculated by determining the difference in metalloid content between the control medium without cells and the test medium containing cells. Percentages of As(III) absorption were calculated by use of the following equation: Absorption (%) = [(initial concentration–final concentration)⁄initial concentration] × 100The measurements were carried out three times, with three internal replicates and the results presented are means+−S.D.

3.4.5 Protein extraction and Immunoblot analysis

Δyap1 mutant cells transformed with the plasmid encoding *c-myc–YAP1* [21] were grown to early exponential phase and induced or not with 2 mM As(V). Samples collected at the indicated time points were harvested by centrifugation at 4 ◦C and the protein extracts were prepared by the trichloracetic acid-lysis method and immunoblotted as described in [32]. To follow the kinetics of the recombinant *c*-Myc-Yap1 protein under arsenic stress, immunoblotting was performed with 50 *μ*g of proteins that were probed with the 9E10 anti-*c*-Myc monoclonal antibody.Sba1 (p23), encoded by the *SBA1* gene [33], was used as a loading control [29, 34]. Detection was performed using an ECL® (enhanced chemiluminescence) Western-blotting reagent kit(Amersham Pharmacia).

3.4.6 Fluorescence microscopy

FT4 Δ*yap8* and FT4 Δ*yap1* strains transformed with pRS encoding *cp-GFP-HA-YAP8* [12] or *cp-GFP-HA-YAP1* [22] respectively (where cp is centromeric plasmid and HA is haemagglutinin), were grown to early exponential phase and induced with either 2 mM As(V) or As(III) at the indicated time points. DAPI (4 ′ ,6-diamidino-2-phenylindole) was added as a DNA marker at a final concentration of 5 *μ*g/ml, 5 min before microscopy. After washing with PBS, cells were resuspended in a solution of 200 mM DABCO (1,4-diazadicyclo[2.2.2]octane) in 75% (v/v) glycerol and 0.25×PBS (Sigma–Aldrich). Both we and Delaunay et al. [21] have shown that DABCO does not affect the localization of the GFP fusions. GFP signals were analysed in living cells with a Leica DMRXA fluorescent microscope equipped with a Roper Scientific Micro-Max cooled CCD(charge-coupled device) camera and MetaMorph software (Universal Imaging Inc.).

3.4.7 Northern Blot, Real time PCR and Microarray analysis

RNA procedures were performed as described in [32]. RNA was isolated from cultures that were either untreated or exposed to 2mM Na_2HAsO or $NaAsO_2$ at the indicated time points. For Northern-blot analysis, approx. 40 *μ*g of total RNA was separated in formaldehyde gels and transferred on to nylon membranes (Hybond XL;Amersham Pharmacia Biotech). Intragenic PCR fragments of *GSH1* and *SNR17A*/small nucleolar RNA *U3* were used as probes. For real-time quantitative PCR the RNA samples were treated with DNase (TURBO DNAse-free; Ambion) according to the manufacturer's instructions. cDNAs were synthesized by reverse transcription from 0.5 *μ*g of total RNA, using 50 pmol of

(dT)15, 1 mM dNTP and 5 units of Transcriptor Reverse transcriptase as described by the manufacturer (Roche). cDNA amplification was quantitatively analysed by incorporation of SYBR Green I (LightCycler FastStart DNA Master SYBR Green I; Roche) into double-stranded DNA, according to the manufacturer's instructions, on a Roche LightCycler II Instrument, using the *ACT1* (actin) gene as a loading control. The fold change was determined by the $2^{\Delta\Delta}$CT method [35]. The primers used were as follows: *GSH1*: 5'-GCTGCTGGTAAAAGAGACAATG-3' and 5'-ACTCACATCGTTAGCCTCACAA-3'; *TRX2*: 5'-GGTCACTCAATTAAAATCCGCTTC-3' and 5'CGACGACTCTGGTAACCTCCTTAC3'; *SOD1*: 5'-AGCCAACCACTGTCTCTTACGA-3' and 5'-ACACCATTTTCGTCCGTCTTTA-3'; and *ACT1*: *5'*-CTA TTG GTA ACG AAA GAT TCAG-3' and 5'-CCT TAC GGA CAT CGA CAT CA-3'. For transcript profiling, total RNA was purified using the RNAeasy kit (Qiagen), followed by the RNA clean-up procedure. A 10 *μ*g portion of RNA was used to generate labelled cDNA, which was hybridized on the DNA arrays as described in [36]. We used arrays containing probes for most of the yeast open reading frame, obtained from the plate-forme transcriptome of the IFR36 (www.transcriptome.ens.fr). Slides were read using a Genepix 4000B scanner from Axon. The images were analysed with the Genepix pro 6.0 software. Data were normalized using global lowess followed by print tip group median from Goulphar software [37]. Complete microarray data are available as Supplementary Table S1 at http://www.BiochemJ.org/bj/414/bj4140301add.htm. The gene-ontology analyses were performed by submitting the whole set of microarray results to the t-profiler tool [38] using default parameters. Redundant or meaningless functional

categories were hidden (Table 1). The results presented in Table 1 and Supplementary Tables 3.S1 and S2 (at http://www.BiochemJ.org/bj/414/bj4140301add.htm) are from three independent experiments. Only genes measured at least twice were kept for further functional analyses.

3.4.8 Statistical analysis

The results reported in the present study are the averages for at least three independent experiments, with three replicates in each experiment, and are expressed as the mean+−S.D. Statistical differences among treatments were analysed by one-way ANOVA with Tukey's HSD (honest significant difference) multiple comparisons test (α =0.05) using STATISTICA for Windows (StatSoft Inc., Tulsa, OK, U.S.A.).

3.5 Results

3.5.1 YAP8 overexpression does not alleviate ∆yap1 sensitivity under As(V).

It has been suggested that Yap1 and Yap8 play distinct and well defined roles in the arsenic stress response by regulating distinct sets of genes [15,16]. In order to evaluate the physiological relevance of this specificity, we performed growth complementation assays (Fig. 3.1). The ∆*yap8* mutant reveals a severe growth sensitive phenotype to both arsenate and arsenite, which is rescued by the overexpression of *YAP8*. The ∆*yap1* mutant shows a mild growth-sensitive phenotype under arsenate conditions. In contrast, under arsenite treatment this phenotype is more accentuated. The overexpression of *YAP8* in the ∆*yap1* mutant

partially restores the growth-sensitivity of this strain under arsenite treatment, a finding consistent with the notion that Yap1 and Yap8 exert specific roles in arsenic stress responses. On the other hand, *YAP1* overexpression is not able to rescue the sensitive phenotype of the Δ*yap8* mutant strain either under As(V) or As(III) conditions (Fig. 3.1). Altogether, these results suggest some level of specificity of *YAP1* and *YAP8* in arsenic detoxification.

Table 3.1 Gene-ontology analyses of the arsenate transcriptional response
T-profiler [38] was used to conduct gene-ontology analyses from the global set of microarray data (see the Experimental values). The T-values and E-values are directly taken from the t-profiler output. The T-test is to assess whether the means of two groups are statistically different from each other. E-value is a parameter that describes the number of hits one can 'expect' to see by chance when searching a database of a particular size. It decreases exponentially as the score (S) of the match increases. Redundant or meaningless gene-ontology categories were hidden. Examples of genes belonging to each category are indicated, together with their fold induction.

Category	T-value	E-value	Genes
Heat shock protein activity	10.46	$<1.0\times10^{-15}$	*HSP42 (11.2), SSA4 (11.0, HSP26 (7.5). SSE2 (5.9), HSP12 (5.5), HSP78 (5.1, HSP30 (5.1), SSA3 (3.5), SSA1 (3.0), HSP60 (2.2),HSP104 (2.2). . . .*
Sulphur metabolism	6.61	5.3×10^{-8}	*CYS3 (5.4), MET16 (5.2), MET10 (4.9), MET22 (4.0), HOM3 (4.0), MET6 (3.7), STR3 (3.7), MET28 (3.5), MET32 (3.3), MET2 (2.8),MET8 (2.7), CYS4 (2.1). . . .*
Response to stimulus	5.89	5.4×10^{-6}	*CUP1-2 (19.4), CUP1-1 (18.8), HSP42 (11.2), SSA4 (11.0), ARR2 (9.7), HSP26 (7.5), GRE2 (6.6), HSP12 (5.5), HSP78 (5.1), HSP30(5.1), TRX2 (4.5), MXR1 (4.4), MET22 (4.0), FLR1 (3.7), SSA3 (3.5), HSP82 (3.5), TTR1 (3.4), DDR2 (3.0), POS5 (2.5), STF2 (2.4),ATR1 (2.3), TSL1 (2.3), UBC4 (2.2), HSP104 (2.2), SNG1 (2.1), RDS1 (2.1), GRE3 (2.0), GPX2 (2.0). . . .*
Oxireductase activity	5.67	2.0×10^{-5}	*OYE3 (14.2), ARR2 (9.7), AAD6 (8.3), AAD16 (7.1), AAD14 (6.8), GRE2 (6.6), SER33 (5.5), MET16 (5.2), MET10 (4.9), TRX2 (4.5),MXR1 (4.4), OYE2 (4.0), TTR1 (3.4), PRX1 (3.1), YPR1 (2.9), TSA2 (2.8), MET8 (2.7), SER3 (2.5), ADE3 (2.4), ZWF1 (2.3), FMO1(2.2), DLD3 (2.1), ALD4 (2.1), GRE3 (2.0), GPX2 (2.0) . . .*
Response to oxidative stress	4.70	3.6×10^{-3}	*HSP12 (5.5), TRX2 (4.5), MXR1 (4.4), FLR1 (3.7), TTR1 (3.4), POS5 (2.5), GPX2 (2.0), GLR1 (1.9), GRX1 (1.8), TRX1 (1.7) . . .*
Proteasome complex	4.31	2.2×10^{-2}	*UBC4 (2.2), RAD6 (1.9), RPN8 (1.9), RPN4 (1.9), SCL1 (1.8), UBP6 (1.7), PRE8 (1.7) . . .*

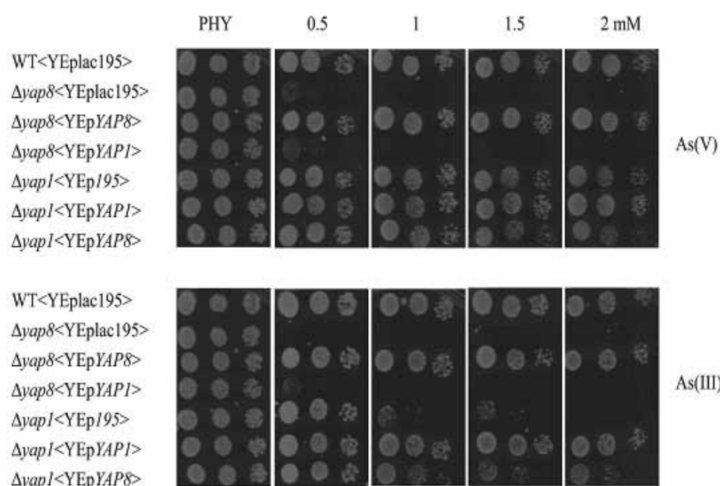


Figure 3.1: Arsenic-sensitivity phenotypes and respective recovery by overexpression of *YAP1* and *YAP8*
Wild-type (WT),*Δyap1* and *Δyap8* mutant strains were transformed with YEplac195 (empty vector), YEpYAP1 or YEpYAP8 and serially diluted cultures were spotted on selective medium supplemented with increasing concentrations of arsenate or arsenite (up to 2 mM). Plates were grown for 2 days at 30◦C. A representative experiment is shown. Abbreviation: PHY, physiological conditions.

3.5.2 Induction of antioxidant defences by arsenate

In order to characterize global changes in cells subjected to arsenate treatment, we performed microarray analysis. We therefore compared the transcriptome of cells submitted to a 30 min exposure to 2 mM of arsenate with the one of cells mock-treated with water. We made a global gene-ontology search using the t-profiler software [38] to identify the cellular pathways that were affected by arsenate (see Table 1 and Supplementary Tables 3.S1 and S2 in (http://www.BiochemJ.org/bj/414/bj4140301add.htm). This analysis indicated that arsenate up-regulated genes involved in protein folding, sulphur and methionine metabolism (mainly target genes of the transcription factor Met4p), redox homoeostasis (including most of the

target genes of Yap1) and proteasome activity (including the proteasome gene transcriptional regulator *RPN4*). This pattern is indeed very characteristic of the oxidative stress response and is similar to what has been described in the case of cell exposure to arsenite [15,16] or cadmium [39]. Furthermore, the cellular pathway of response to stimulus, including the genes *ACR2* and *ACR3*, is also shown to be induced by arsenate treatment.

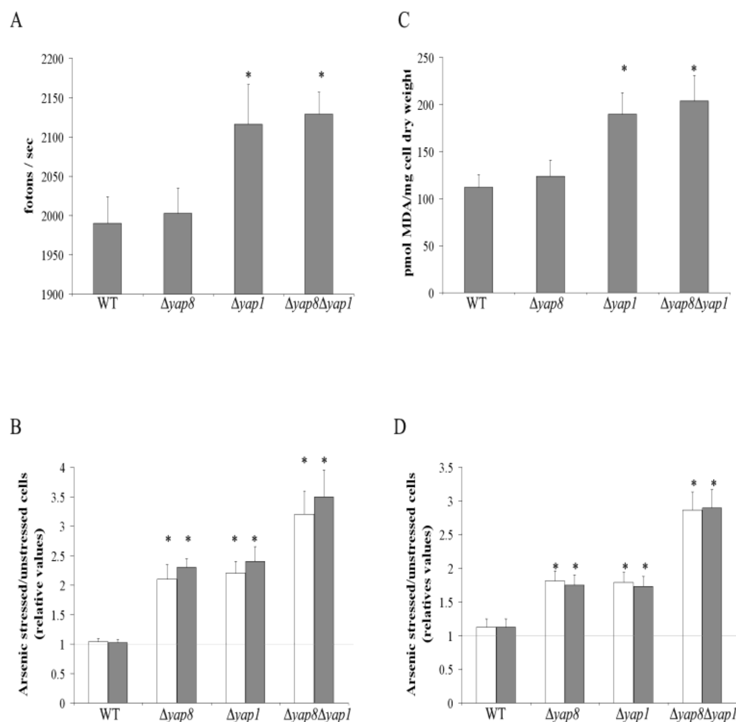


Figure 3.2: Increase in intracellular oxidation and lipid peroxidation caused by arsenic stress

(**A**) Changes in DCF-DA fluorescence caused by deletions were monitored under physiological conditions. The values are expressed as photons/s. (**B**) Control and mutant strains were stressed with 2 mM arsenate or arsenite for 24 h and the enhancement of the intracellular oxidation was expressed as ratio of the fluorescences of stressed and non-stressed cells. (**C**) The effect of deletions in the lipid-peroxidation content was measured as pmol of MDA/mg cell dry weight by the TBARS method. (**D**) The enhancement of lipid peroxidation was measured as the ratio of the levels observed for stressed and non-stressed cells. The horizontal line corresponding to a ratio of 1.00 in (**B**) and (**D**) indicates the relative value under non-stressed conditions. Values are means+− S.D. for three independent experiments. ∗∗P <0.01. WT, wild-type; white bars, arsenate; black bars, arsenite.

3.5.3 Arsenic treatment generates oxidative damage in the yap1 mutant

Once having established that As(V) induces expression of genes involved in redox homoeostasis, we adopted several biochemical approaches to verify whether 2mM arsenate or arsenite treatment was associated with an oxidative environment in yeast cells. Intracellular oxidation was determined using the probe DCFDA, which is sensitive to ROS (reactive oxygen species). Time course experiments had been previously carried out in order to determine the maximum levels of intracellular oxidation, which occurred at 24 h of arsenic treatment (results not shown). The results in Fig. 3.2(A) reveal that deletion of *YAP1*, but not of *YAP8*, interferes with the redox state of the cytoplasm under physiological conditions. The direct exposure of wild-type cells to either arsenate or arsenite does not lead to any detectable increase in the intracellular oxidation levels (Fig. 3.2B), the level being identical in the absence of either *YAP8* or *YAP1* but increasing, however, when compared with the parental strain. This value is still more accentuated in the case of the double mutant. Furthermore, under physiological conditions, increased peroxidation levels of cellular lipids, as compared with the wild-type strain, were detected in the ∆*yap1* mutant through the formation of MDA (Fig. 3.2C). Exposure of wild-type cells to both arsenate and arsenite stress does not cause any significant increase in lipid peroxidation compared with the unstressed cells (Fig. 3.2D), a finding that is in good agreement with the intracellular oxidation results. In single and double mutants these levels are increased about 1.6- and 2.6-fold respectively. Taken together, these results show that arsenic compounds generate oxidative stress under conditions where the arsenate

extrusion system and/or the antioxidant machinery are deficient. Protein carbonyl content is an indicator of oxidative stress and is by far the most commonly used biomarker of protein oxidation [40]. To monitor the possible oxidative effects of arsenic treatment at the protein level, the changes in protein carbonyl status during exposure of yeast cells to both arsenate and arsenite were monitored up to 4 h. The resulting OxyBlotsTM show that the amount of carbonylated proteins does not vary significantly in the wild-type strain and the mutant Δ*yap8*, although in the latter the levels of oxidized proteins are slightly increased at all time points studied (3. 3). A more severe effect was, however, observed in the Δ*yap1* strain. When these cells are treated with arsenate, the protein carbonyl content is slightly enhanced compared with the untreated cells. By contrast, exposure to arsenite caused a strong increase in the protein oxidation levels. This effect is even more severe during the first hour of treatment. Furthermore, additional carbonylated proteins, most of high molecular mass, were observed in the Δ*yap1* strain. These findings suggest that the effects of oxidative stress generated by arsenic compounds lead to protein oxidation only in the absence of Δ*yap1*.

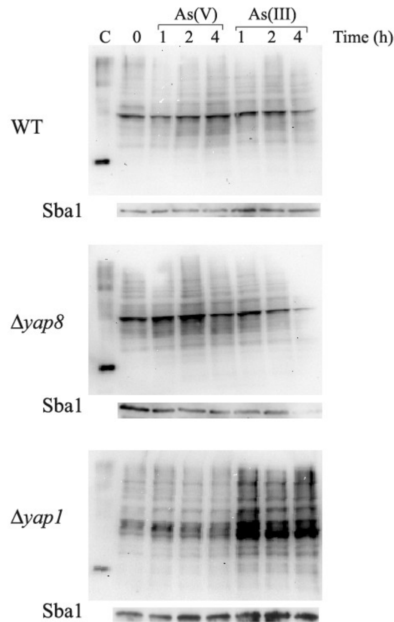


Figure 3.3 Immunological detection of protein carbonyl groups in the Saccharomyces cerevisiae wild-type (WT) and Δyap1 and Δyap8 mutant strains
The carbonyl groups introduced in proteins as a consequence of arsenate and arsenite treatment were derivatized to 2,4-dinitrophenylhydrazone (DNP) and were immunoblotted with anti-DNP antibodies. Sba1 was used as an internal loading control. C, OxyBlotTM Protein Standard (Intergen). A representative experiment is shown.

3.5.4 YAP1 deletion interferes with the redox equilibrium under arsenate

Our transcriptome analysis showed that arsenate up-regulates genes in the cellular pathway of sulphur and methionine metabolism. Furthermore, it has been shown that arsenite-exposed cells channel a large part of assimilated sulphor into glutathione biosynthesis [16]. Yap1 is an important regulator of *GSH1*, which encodes the enzyme responsible for catalysing the condensation of

cysteine on to the γ -carbon atom of glutamate in the limiting step of glutathione biosynthesis. This prompted us to evaluate how the requirement of Yap1 couples with the induction of GSH biosynthesis mediated by arsenate and arsenite. It was observed, by measuring the GSH and GSSG contents in the wild-type and mutant strains subjected to arsenate stress, that in all strains the GSH levels diminish about 2-fold during the first 1 h of treatment with 2 mM As(V) (Fig. 3.4A). These values rise gradually over time, reaching physiological levels after 4 h. A similar pattern was observed under As(III) treatment (results not shown). The GSSG/GSH ratio increases after 1 h of treatment and reflects the decrease in GSH (compare Fig.s 3.4A and 4.4B). Notably, the double mutant *Δyap1Δyap8* takes a longer time to recover the GSH levels of the parental strain. Our results show that the redox equilibrium is disrupted in strains bearing *YAP1* deletions, although homoeostasis is rapidly achieved through the enhancement of GSH generation.

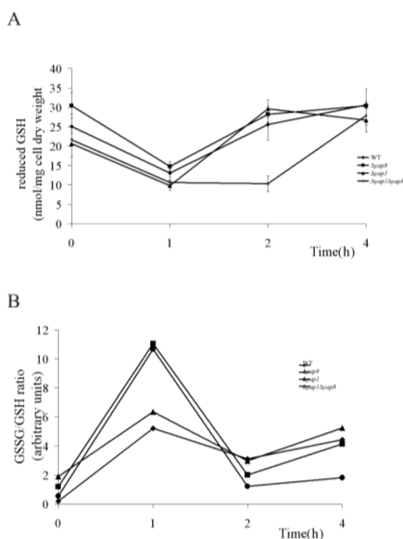


Figure 3.4: Antioxidant defences during arsenic treatment
(**A**) The wild-type (WT) and *Δyap1*, *Δyap8* and *Δyap1Δyap8* mutant cells were grown to mid-exponential phase and were exposed to 2 mM arsenate. Extracts were obtained at the time points indicated and GSH levels were determined as described in the Experimental section.
Values are means+−S.D. for three independent experiments. (**B**) Levels of GSSG were determined in the same extracts after its derivatization to GSH and the GSSG/GSH ratio was calculated.∗∗P <0.01.

3.5.5 Yap1 is maintained in the induced state in a long-term arsenate exposure

The biochemical assays clearly show that the wild-type strain, in contrast with the mutant ∆*yap1*, does not suffer the deleterious effects of oxidative stress on exposure to inorganic arsenic. Indeed, the microarrays revealed that many of the antioxidant defences, as well as the arsenic detoxification system, were induced under As(V) treatment (see Supplementary Tables 3.S1 and S2 in (http://www.BiochemJ.org/bj/414/bj4140301add.htm)) to facilitate cell adaptation. In order to analyse the c-Myc–Yap1 protein levels in cells induced or not with arsenate, Westernblot analyses were

performed. As Fig. 3.5(A) shows, the levels of Yap1 were rather higher in arsenate-treated cells than in cells grown under physiological conditions. Yap1 protein levels peak at about 45min. after arsenate addition and a slight induction is maintained even up until 12h of treatment. Furthermore, localization assays revealed that GFP–Yap1, as well as GFP–Yap8, are accumulated in the nucleus until 24 h of arsenate treatment (Fig. 3.5B).

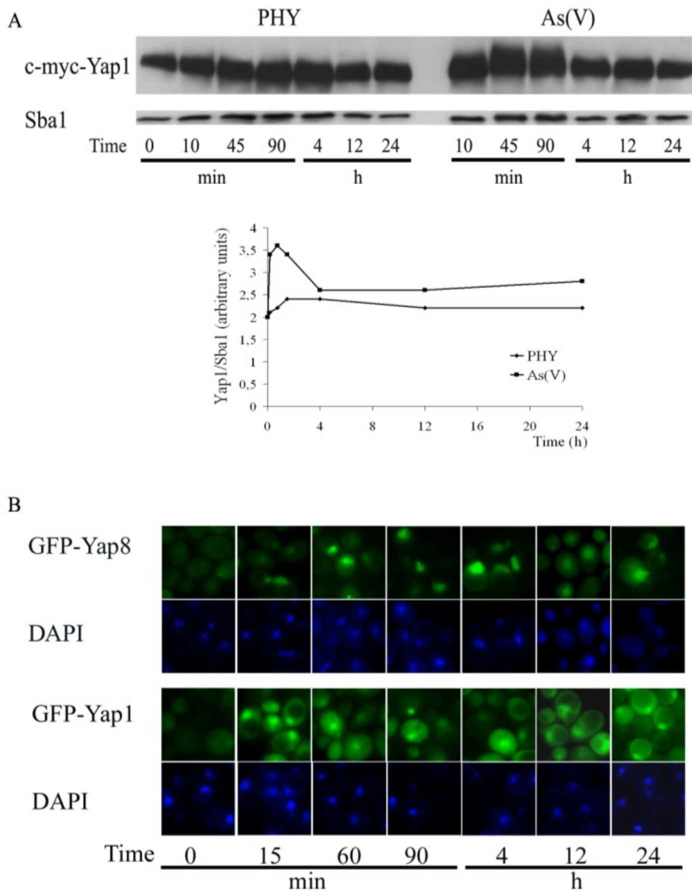


Figure 3.5: Yap1 is activated in a long-term arsenate exposure

(**A**) Strain BY (EUROSCARF) Δ*yap1* mutant cells transformed with a CEN-plasmid encoding the fusion c-myc–YAP1 were grown under physiological conditions or in the presence of 2 mM arsenate and sample were harvested at the indicated time points. Protein extraction, separation, transfer and immunoblotting were performed as described in the Experimental section. The protein levels for Sba1 were used as an internal loading control against which all protein levels were normalized (lower panel). (**B**) Kinetics of Yap1 and Yap8 nuclear localization. *Δyap1* and *Δyap8* mutants expressing the fusions *GFP–YAP1* and *GFP–YAP8* respectively, were induced with 2 mM arsenate and analysed for GFP staining at the indicated time points. Representative experiments are shown.

3.5.6 GSH1, *SOD1* and *TRX2* are highly induced under arsenic treatment

To evaluate whether Yap1 activation reflects the induction of the antioxidant cell defences such as *GSH1, SOD1* and *TRX2,* we monitored their transcriptional activation by real-time PCR. Fig. 3.6 reveals that, in the wild-type strain, these genes are highly induced by 2 mM arsenite or arsenate. Strikingly, transcriptional activation of *GSH1*, *SOD1* and *TRX2* is even higher in the Δ*yap8* mutant than in the wild-type strain. Under conditions of exposure to arsenate, all of these genes display a first peak of induction at 90 min, although after 24 h incubation with the metalloid the mRNA levels keep increasing in Δ*yap8* (see Fig. 3.6A). The pattern of mRNA induction in the wild-type and Δ*yap8* strains is very similar up to 4 h exposure to arsenite. However, after this point a strong transcriptional activation of the three genes is observed only in the Δ*yap8* mutant (Fig. 3.6B). Expression of *SOD1* and *TRX2* is completely abolished in the Δ*yap1* mutant (results not shown), a finding consistent with the fact that Yap1 regulates them. Some level of Yap1-independent *GSH1* induction was observed (Fig. 3.6C), suggesting that other factors might be regulating its expression under arsenic stress.

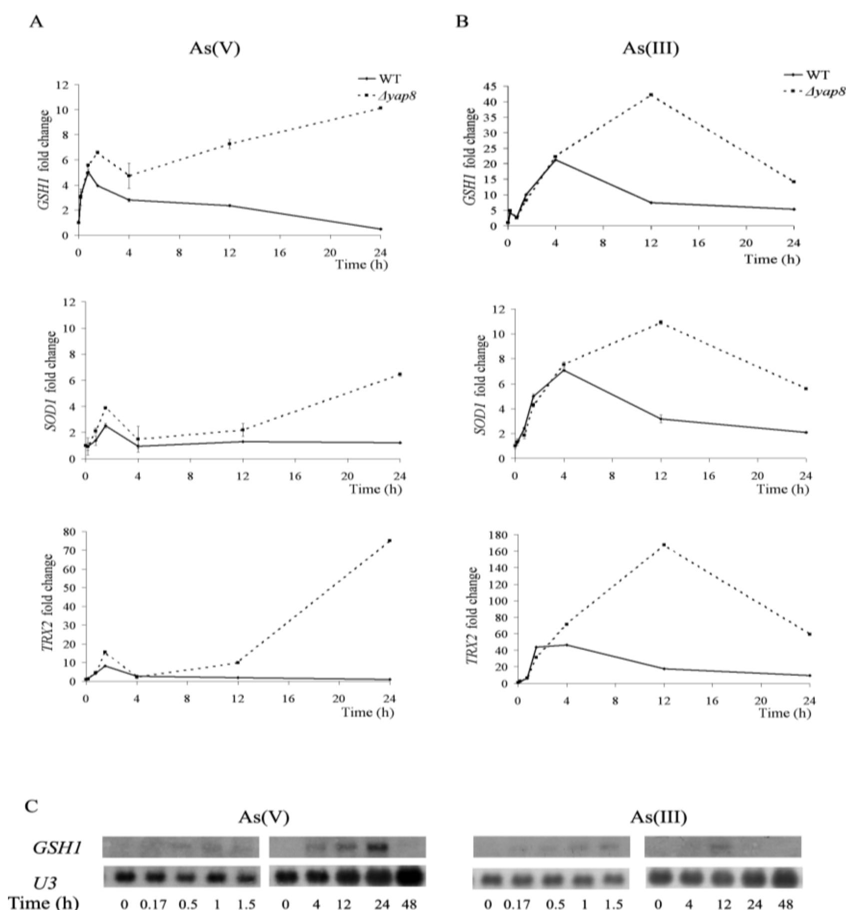


Figure 3.6: Effect of As(V) and As(III) on the transcriptional activation of *GSH1*, *SOD1* and *TRX2* in the wild-type (WT) and *Δ*yap8 mutant strains
Cells upshifted to arsenate (**A**)- or arsenite (**B**)-supplemented medium were harvested at the indicated time points. RNA extraction and real-time PCR were performed as described in the Experimental section. (**C**) *GSH1* expression in *the Δyap1* mutant was monitored by Northern blotting as described in the text. The mRNA levels for small nuclear RNA *U3* were used as an internal loading control. Representative experiments are shown.

3.5.7 The ∆yap8 mutant absorbs increased levels of As (III)

As Yap8 regulates As(III) detoxification, we hypothesized that the strong transcriptional activation of the antioxidant genes in the mutant ∆*yap8* was caused by arsenite accumulation. In order to

verify this, we used atomic-absorption spectroscopy. After adding 2 mM arsenite to wild-type and Δ*yap8* cultures, the residual amounts of the metalloid in the supernatant at the time points indicated in Fig. 3.7 were determined. We also performed measurements in the Δ*yap1* mutant in order to evaluate the contribution of Yap1 to arsenite detoxification. As Fig. 7.7 shows, arsenite absorption in *S. cerevisiae* wild-type cells is very low (around 5%). The Δ*yap8* mutant absorbs higher levels of As(III) (8%) than those observed in the wild-type. Increased As(III) absorption in the Δ*yap8* mutant may explain why the antioxidant genes are more activated in this strain than in the wild-type (see Fig. 3.6). Surprisingly, Δ*yap1* As(III) absorption is slightly decreased compared with the wild-type, suggesting that either the uptake is compromised in this strain, that extrusion is enhanced or that both events are occurring.

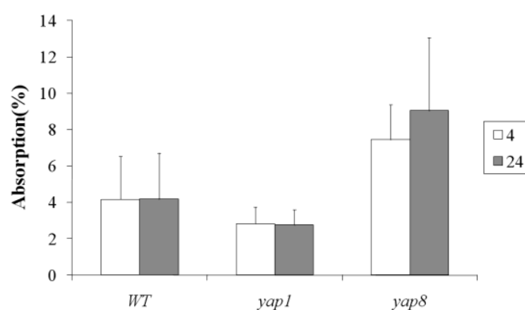


Figure 3.7 Effect of *YAP1* and *YAP8* deletion on As(III) absorption
Atomic-absorption analyses of As(III) were carried out in buffer solution containing 1 mg (dry weight) of *S. cerevisiae* wild-type (WT) or mutant cells and the amount of As(III) absorbed was estimated by determining the difference between the initial As(III) added (2 mM) and the residual As(III) present in the medium after 4 (white bars) and 24 (black bars) h. The percentage of As(III) absorbed was calculated by determining the ratio of As(III) taken up by cells and the initial As(III). Values are means+−S.D. for three independent experiments. ∗∗P <0.01.

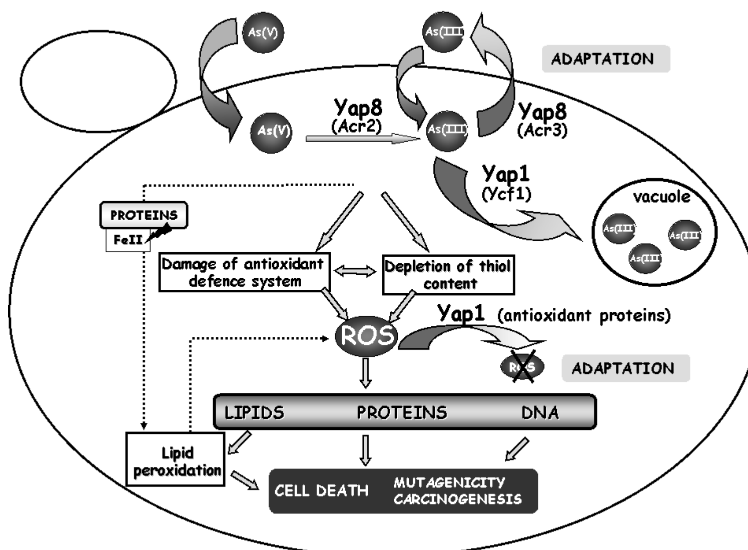
3.6 Discussion

It has been accepted that, among the various modes of action for arsenic carcinogenesis in human cells, the oxidative stress relevance is the one that assumes that ROS can directly or indirectly damage DNA and proteins [13,41]. Indeed, the property of the organic arsenicals to inhibit the GSH and thioredoxin reductases, as well as to bind GSH [42,43], perturbs the redox equilibrium of the cytoplasm, leading to the accumulation of ROS. Here we report that Yap1, the major regulator of oxidative stress response in *S. cerevisiae*, is involved in the removal of ROS generated by arsenic compounds. Our data on lipid peroxidation, together with those on intracellular oxidation, indicate that Δ*yap1* and Δ*yap8* mutant cells are more oxidized than those of the wild-type strain upon treatment with arsenic compounds (3. 2). The increased lipid peroxidation levels that we observed upon As(III) exposure is consistent with the notion that arsenite has the ability to release Fe(II) from its complexes with proteins, potentially stimulating the peroxidation of cellular lipids in yeast [44] (Scheme 3.1). This may, in turn, give rise to the highly reactive superoxide radical. Moreover, enhanced lipid peroxidation levels are also in agreement with the assumption that its induction by arsenite may be responsible for the toxic effect of this compound in eukaryotic cells [17]. Both Δ*yap1* and Δ*yap8* strains exhibit high levels of lipid peroxidation and intracellular oxidation; however, Δ*yap1* is the only one displaying high contents of oxidized proteins (Fig. 3.3). The antioxidant genes *GSH1*, *SOD1* and *TRX2* are induced in the wild-type strain their induction being even higher in the Δ*yap8* mutant (see Fig.s 3.6A and 3.6B). The antioxidant machinery is therefore activated by Yap1, which prevents protein damage in these strains.

Redox-inactive toxic metals such as arsenic react with GSH, the reduced form of glutathione, which is the major antioxidant reserve of the cell [45]. We have in fact observed that, during the first 1 h of treatment with arsenate, the levels of GSH are decreased in all strains (Fig. 3.4), with a consequent increase in the oxidized form (GSSG). As GSSG is formed, cells must, in order to maintain redox homoeostasis, induce the generation of GSH. This occurs either via its recycling through the glutathione reductase Glr1 or synthesis *de novo*, in which the activity of Gsh1 is essential both pathways in the wild-type strain (see Table 1 and Supplementary Tables 3.S1 and S2 (http://www.BiochemJ.org/bj/414/bj4140301add.htm)). We show that genes grouped in functional categories related to sulphur metabolism, sulphur amino-acid biosynthesis and metabolism, as well as *GLR1*, are induced when this strain is treated with 2 mM arsenate for 30 min (Table 1). Indeed, it had already been shown that arsenite-exposed cells channel a large part of assimilated sulphur into glutathione biosynthesis [16]. In the case of Δ*yap1* the recycling pathway is impaired, since *GLR1* is a Yap1 target. Interestingly, even in cells harbouring *YAP1* deletion, the GSH levels upon 2 h of exposure to arsenic compounds increased, a finding consistent with the observation that *GSH1* mRNA levels were not completely abolished in the Δ*yap1* mutant treated with both arsenate and arsenite (Fig. 3.6C). Similarly, under glutathione depletion [46] and cadmium injury [47], *GSH1* has been shown to be regulated by both Yap1 and Met4. Furthermore, the fact that induction of *GSH1* is only partially decreased in Δ*yap1* cells treated with the superoxide-anion generator menadione [48] might suggest that treatment of yeast cells with arsenic compounds also leads to the formation of superoxide radicals, which, in turn, could trigger the Yap1-independent basal transcription of *GSH1*. Yap1

contributes, therefore, to arsenic stress responses by relieving the deleterious effects of ROS in the cells (Scheme 3.1) through at least the transcriptional activation of antioxidant enzymes encoded by genes such as *TRX2, GSH1* and *SOD1* (Fig. 3.6). Indeed, as suggested by others [17], the enhanced *SOD1* transcription is one of the most important factors responsible for triggering the adaptation process to mitigate the toxic effect of arsenite in eukaryotic cells. Genes involved in protein folding are also induced by arsenate stress, a phenomenon that is common to many forms of stress (see Table 1 and Supplementary Tables 3.S1 and S2 (http://www.BiochemJ.org/bj/414/bj4140301add.htm)). The absence of a significant increase in intracellular oxidation, lipid peroxidation and protein carbonylation, as well the changes in the GSSG/GSH ratio, in the wild-type cells reflects the ability of the wild-type strain to counteract the direct and/or indirect deleterious effects of the metal and to adapt to the stress condition (see Scheme 1). Phenotypic assays reveal, indeed, that the wild-type cells are able to grow in the presence of 2 mM arsenate and arsenite (Fig. 3.1). Furthermore, *YAP8* and *YAP1* are both necessary to trigger the adaptation response, exerting a complementary role, though at different levels. Because *YAP8* is the key regulator of arsenic stress responses by controlling the expression of the arsenite efflux protein encoded by *ACR3*, its absence leads to the accumulation of As(III), enhancing the generation of ROS, as suggested from the atomic-absorption, lipid peroxidation and intracellular-oxidation assays (see Fig.s 3.2, 3.3 and 3.7 and Scheme 3.1). The *YAP1* gene is shown to be essential in preventing protein oxidation (Fig. 3.3). On the other hand, Yap1 also contributes to the regulated expression of *ACR2* and *ACR3*, through recognizing the *cis*-element TGATTAATAATCA positioned

in the divergent promoter of these genes (results not shown). It also regulates the expression of the vacuolar pump encoded by *YCF1*, which composes a parallel arsenite detoxification pathway by catalyzing the ATP-driven uptake of As(III)–GSH conjugates into the vacuole [1,49]. It is noteworthy that the Δ*yap1* mutant absorbs lower levels of arsenite than does the wild-type strain. Assuming that arsenite vacuolar extrusion mediated by Ycf1 might be at least partially compromised in this mutant, a plausible explanation for the low values observed could be related to an impaired uptake of the metal. It was in fact shown that Hog1 kinase, which in turn modulates the uptake of As(III) dependent on the aquaglyceroporin Fps1, is activated by the metalloid [50]. It is possible that, in the Δ*yap1* strain, this process is not fully operating. In conclusion, we have identified an oxidative pathway dependent on Yap1 involved in the response of *S. cerevisiae* to arsenic compounds.



Scheme 3.1 Contribution of Yap1 and Yap8 to arsenic stress responses
Yap8 is the key regulator of this response by mediating the efficient removal of arsenite from the cytoplasm. Yap1 activity is also required at this level, its major contribution being the induction of the antioxidant defenses in order to scavenge the ROS generated as a secondary effect of arsenic exposure.

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Chapter 4

Yap8-DNA interaction and Mediator requirement for Yap8 activity

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4.1 Author's contribution to the article

I did most of the work included in Chapter 4 as described below.

The resistance to arsenic compounds in yeast is achieved by the action of Ycf1 and also of Acr2 (arsenate reductase) and Acr3 (arsenite extrusion pump). The main regulator of the transcription of the genes *ACR2* and *ACR3* is Yap8. Using a modified one hybrid strategy I showed that Yap8 recognizes a 13bp sequence

existent in its target gene promoter. In order to study the DNA binding domain of the transcription factor Yap8 I mutated residues in its basic region and used the modified one hybrid strategy to study if the mutants maintained the DNA binding capacity of the native Yap8. I also analysed the mutant capacity to recover the sensitivity phenotype to arsenic compounds of the Δ*yap0* strain. After the DNA recognition, transcription factors may recruit the complex Mediator, in order to study this interaction, we used the two hybrid strategy. I performed the two hybrid assays with the help of Ana Rita Silva and Joana Ropio. I made the Northern analysis of the RNA levels of *YAP8* and Yap8 target gene *ACR2* in the absence of the tail subunits of the Mediator complex. We analysed the behaviour of the mutants in the tail subunits in the presence of arsenic compounds therefore I made the phenotypes of the W303 strains.

4.2 Abstract

Yap8 is a transcription factor that controls expression of arsenic resistance genes: *ACR2* and *ACR3* through recognition of the 13bp-upstream activating sequence (UAS) TGATTAATAATCA. It has in the DNA binding domain a Leu in position 26 instead of the Asn that is conserved in all members of the Yap family. In order to study if this change is involved in preventing the binding of Yap8 to the canonic Yap element, we mutated this Leu to Asn. The results show that L26N mutant did not activate transcription through the canonical Yap site, but the mutant recognizes the 13bp sequence recognized by the native Yap8. The double mutation Leu26 to Asn and Asn31 to Arg is the only one to alter Yap8 DNA binding specificity revealing the recognition of TTACTAA. To study the

importance of amino acid residues in the vicinity of the core basic region some residues (Lys21, Arg22, Arg27, Lys35, Arg36, Lys37) were mutated to Ala. The results showed that the corresponding amino acid residues required for Pap1 DNA recognition were also important for Yap8 interaction with the DNA: Lys21, Arg22, Arg27, Lys35, Arg36.

After binding the promoter of its target genes, activators trigger the recruitment of the basal transcriptional machinery. It is known that the subunits of the tail domain interact directly with the activator; we evaluated which were the subunits from this domain i) to mediate arsenic tolerance, ii) to contribute to Yap8 transactivation activity and iii) to activate transcription of *ACR2*. Phenotypic analysis using Mediator tail mutants showed that the subunits Med2, Med3, Med14, Med15 and Med16 are important for resistance to arsenic. The analysis of Yap8 transactivation function and transcriptional activation of *ACR2* in the respective mutants revealed that these subunits are the most important for these functions.

4.3 Introduction

The basic leucine zippers are transcription factors that contain a basic region and a region rich in leucines, hydrophobic and non-polar residues at specific positions [2]. The basic region allows the recognition of the DNA, that together with the leucine zipper forms stable α-helices after binding to DNA as is the case of the yeast transcription factor Gcn4 [3]. The DNA sequence recognized by Yap1 to Yap3 is TTACTAA that is designated the Yap binding site. However, Yap1 binds also the optimal *cis*-element of AP-1 (TGACTCA) and of SV40 (TGACTAA) with different affinities [1].

Inspection of the promoter region of the Yap8 target genes (*ACR2* and *ACR3*) revealed the presence of the sequence TGATTAATAATCA in their promoter to which Yap8 binds. This 13bp sequence is the minimum required, as mutation of the flanking bases prevents Yap8 binding [4].

In all the Yap family b-Zip transcription factors, there is a conserved sequence NXXAQXXFR, in the DNA binding region. These conserved residues interact with the DNA, making direct or water mediated interactions with the base pairs [5]. Taking advantage of the Pap1 structure, a Yap1 orthologue in *S. pombe*, we studied the interaction between Yap8 and its recognized DNA sequence. Asn86 in Pap1 and Asn235 in Gcn4 are very important for its interaction with DNA although they bind with different specificity. The Asn of Pap1 forms one direct bond to DNA and one hydrogen bond, via a water molecule, with the adenine that is base paired with the first thymine of the sequence: GGTTACGTAACC. It also forms a water mediated hydrogen bond with the second guanine. In Gcn4 the Asn interacts with the first bases of the recognized sequence: TG of TGAC. In Yap8 this Asn that is conserved in the Yap family and Pap1 is replaced by a Leu in the position 26 see fig.4.1. The specific transcription factors, such as Yap8, regulate transcription activation. Nevertheless, other effectors such as the acetyl-transferase and the complex that interacts with the TATA binding protein: SAGA, the nucleosome-remodelling complexes SWI/SNF and the signal transducer from the activator, the Mediator, are required [6].

The Mediator comprising in yeast 25 subunits, transmits the signal of the enhancer or inhibitor to the basal transcriptional machinery. Each subunit of the complex is clustered together with other subunits forming four modules: tail, head, middle complex and

Cdk8. The Cdk8 is a repressor complex, the head and the middle modules interact with the basal transcriptional machinery and the tail with the activator [7]. ChIP experiments show that the Mediator not only is associated to the promoter of activated genes but also to the promoter of several constitutively transcribed genes [8]. Improved purifications of the Mediator and ChIP experiments show that the Mediator is recruited to the Upstream Activating sequence independently of the polymerase II [9, 10] . It binds the polymerase II at the opposite side of the DNA binding cleft [11] and phosphorylation of the C–terminal element may disrupt the interaction [12]. *In vitro* studies indicate that only polymerase II, TFIIB and TFIIF leave the promoter when initiating the elongation leaving the Mediator behind, suggesting that the Mediator together with the general transcription factors TFIIA, TFIID, TFIIH and maybe TFIIE, might be involved in promoting re-initiation [13].

We studied Yap8 recognition sequence inserting the sequence to be studied in the promoter of the *HIS3* reporter gene. We demonstrated that Yap8 requires a minimum of 13bp of a palindromic sequence existent in its target gene promoter. Yap8 DNA binding element was analysed through mutations of specific bases and its binding to DNA was studied using the same assay. In an attempt of converting Yap8 DNA binding region in the one of Yap1 we performed mutations in this region. Only a double mutation was capable of giving Yap1 like DNA binding characteristics to Yap8 L26N-N31R. Relevant Yap8 amino acid residues(see Table S4.2) for DNA interaction: Lys35, Arg36 and Lys37 were mutated to Ala. The mutants were studied using the *HIS3* reporter assay and then phenotypic analysis was determined through the sensitivity to arsenic compounds. After getting the information from both assays, the most relevant mutations for Yap8

DNA binding were shown to be R22A, Q25A, R27A and R36A. We also studied Yap8 requirements of the Mediator for its activity using strains with deleted genes encoding Mediator tail subunits. All the subunits are relevant for Yap8 full activity, but the most relevant ones are Med16, Med15, Med2 and Med3.

```
                 Basic region                Leucine zipper
68   QKRTAQNRAAQRAFRERKERKMKELEKKVQSLESIQQQNEVEATFLRDQLITL  120  Yap1
47   SRRTAQNRAAQRAFRDRKEAKMKSLQERVELLEQKDAQNKTTTDFLLCSLKSL   99  Yap2
148  AKKKAQNRAAQKAFRERKEARMKELQDKLLESERNRQSLLKEIEELRKANTEI  200  Yap3
241  TKRAAQNRSAQKAFRQRREKYIKNLEEKSKLFDGLMKENSELKKMIESLKSKL  293  Yap4
62   QKKKRQNRDAQRAYRERKNNKLQVLEETIESLSKVVKNYETKLNRLQNELQAK  114  Yap5
225  TRRAAQNRTAQKAFRQRKEKYIKNLEQKSKIFDDLLAENNNFKSLNDSLRNDN  277  Yap6
129  EKRRRQNRDAQRAYRERRTTRIQVLEEKVEMLHNLVDDWQRKYKLLESEFSDT  181  Yap7
20   NKRAAQLRASQNAFRKRKLERLEELEKKEAQLTVTNDQIHILKKENELLHFML   72  Yap8
80   SKRKAQNRAAQRAFRKRKEDHLKALETQVVTLKELHSSTTLENDQLRQKVRQL  153  Pap1
```

Figure 4.1 Alignment of Yap family members form *S.cerevisiae* and Pap1 from *S. pombe*, in red the divergent amino acid residuein Yap8 in a conserved region. In the basic region conserved amino acid residuesin boldface. In the leucine zipper conserved residues at position of the coiled coil (boldface) hydrophobic residues at position of the coiled coil (underlined) are indicated. Adapted from [1]

4.4 Materials and Methods

All products are from Sigma brands unless stated otherwise.

4.4.1 Strains and plasmids used

Yeast strains were grown at 30ºC in YPD medium (1%(w/v) yeast extract, 1%(w/v) bactopeptone both from Difco, 2%(w/v) glucose and 2%(w/v) of agar if solid, also from Difco). Standard liquid cultures were incubated with orbital shaking (200rpm) at 30ºC. For plasmid selection 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) supplemented with non-essential amino acids was used.

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 30mg/mL kanamycin or 100µg/mL ampicillin.

Strains used are described in table 4.1. Plasmids used were: pLF35, Yap1 in YEplac112 (2 µm, *TRP*)[1]. Yap8 YEplac112 resulting from the digestion of Yap8 pRS416 (Batista-Nascimento, L. unpublished) by *Bam*HI and *Kpn*I was cloned in YEplac112. pLF98 containing *HIS3* reporter based on Vishy A1 and A5 was used [1]. The following vectors were based on the pFL98 having instead of the Yap site, the 13bp and the 11bp sequences. lexYap8 was used [14]. Yap8 bZIPb and Yap8His in pET30a(+), primers used for cloning in table 4.S1. To construct the plasmid containing the binding site we have proceeded as follows: the primers were made with cohesive ends for *Eco*RI and *Bam*HI and, 300pmol of the primers were used at a concentration of 30pmol/µL each, that were annealed at 65ºC for 5min and kept at 16ºC until the annealed primers were ligated in the cut pLF98. The promoter of the *HIS3* gene was sequenced to confirm the introduction of the new binding sites.

4.4.2 Protein expression

For protein labelling with ^{15}N, 120mL of a saturated culture of Yap8bZipb transformed in BL21 (DE3) were used to inoculate 1200mL of M9 (0.05M $Na_2HPO_4.12H_2O$, 0.02M KH_2PO_4, 8.6mM NaCl) supplemented with 2mM of $MgSO_4$, 0.2mM $CaCl_2.2H_2O$, 0.4% $C_6H_{12}O_6$, 0.1% $^{15}NH_4Cl$ and 30µg/mL kanamycin (final concentrations), until an $O.D._{600}$ of 0.5 and induced with IPTG 0.5M

(Promega) until an O.D.$_{600}$ of 1, at 37ºC. Cells were collected by centrifugation at 5000*g* for 5min and washed with Tris-HCl, 20mM pH7.5. Cells were broken by 3 cycles in a French Pressure Cell at 900psi. The recombinant protein was purified from the extract by metal-affinity chromatography (Ni-NTA – His-Bind), 2 columns with 1.5ml settled volume (Novagen) according to the manufacturer's instructions excepting that we used solutions with Tris-HCl pH 7.5, 20mM, binding buffer composed of Tris, 5Mm imidazole, 0.5M NaCl; the wash buffer is - Tris, 60mM Imidazole, 0.5M NaCl; and the elution buffer - Tris, 1M imidazole, 0.5M NaCl ; strip buffer - Tris buffer, 0.5M NaCl, 100mM EDTA.

Table 1.

S. cerevisiae and *E.coli* strains used in this study

Strain	Genotype	Source
Xl1-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*q*Z*Δ*M15* Tn*10* (Tetr)].	Stratagene
BL21(DE3)	F^- *ompT hsd*S_B($r_B^-m_B^-$) *gal dcm* (DE3)	Invitrogen
CL18-1 (*med2*)	*MATa his3 leu2 ura3 ade2 trp1med2Δ::HIS3MX6*	Laurent Kuras
CL7-1 (*med3*)	*MATa his3 leu2 ura3 ade2 trp1 can1-100 med3Δ::HIS3MX6*	Laurent Kuras
CL10-1 (*med5*)	*MATa his3 leu2 ura3 ade2 trp1 can1-100 med5Δ::HIS3MX6*	Laurent Kuras
DY3168 (*med14*)	*MATa his3 leu2 ura3 ade2 trp1 can1-100 lys2 med14-100*	Laurent Kuras
CC939-1A (*med15*)	*his3 leu2 ura3 ade2 trp1 can1-100 lys2 med15*Δ::*LEU2trp1med15Δ::HIS3MX6*	Laurent Kuras
BY4742 (WT)	*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
BY4742 (*med1*)	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* YPR070w::kanMX4	EUROSCARF
BY4742 (*med2*)	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* YDL005c::kanMX4	EUROSCARF
BY4742 (*med3*)	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* YGL025c::kanMX4	EUROSCARF
BY4742 (*med5*)	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* YGL151w::kanMX4	EUROSCARF
BY4742 (*med15*)	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* YOL051w::kanMX4	EUROSCARF
BY4742 (*med16*)	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* YNL236w::kanMX4	EUROSCARF
Δyap0	*Δhis Δleu Δtrp Δura,YAP1 to YAP8* deleted	Batista-Nascimento, unpublished

4.4.3 Growth in media containing stress agents: Plate sensitivity assays and DNA binding assays

Plates were prepared by mixing the indicated amounts of stress agents to 10mL of the warm agar media while still liquid. Cells were grown to mid-log phase and diluted so that 5µL contained approximately 5000 cells. It was assumed that an $O.D._{600nm}$ of 1 represents approximately $2x10^7$ cells/mL and cells were diluted in PBS (137mM NaCl, 2,7mM KCl, 4,3mM Na_2HPO4, 1,47mM KH_2PO4) to an $O.D._{600}$ of 0.05. 5µL of each strain was spotted onto the test plates respectively with 5000, 500 and 50 cells per spot. For the binding assay in liquid medium, the cells were grown in liquid culture until an $O.D._{600nm}$=0.4-0.5 and either 2mM Na_2HAsO_4 was added for 30min. or the cells were centrifuged at 2600*g* and resuspended in medium in the absence of histidine and with 10mM AT.

4.4.4 Site-directed mutagenesis using Pfu Turbo (Stratagene)

Site-directed mutagenesis was performed according to mutagenesis kit (Stratagene). Complementary primers for site-directed mutagenesis were designed and are indicated in Supplemental material table 4.S1.

4.3.5 β-galactosidase assay

Strains expressing the *lacZ* gene, under the control of the lexA binding sequence, and Yap8lexA construction were spotted onto SD medium containing 1mM Na_2HAsO_4 or $NaAsO_2$ and after 1h were covered with agarose solution 0.5% (w/v) boiled in 10 ml of 0.5 M sodium phosphate buffer (pH 7.0) to which was added 0.2% (w/v) SDS; 2% (v/v) dimethyl formamide containing 100mg X-

gal/ml. Plates were analyzed after 6h incubation at 30ºC. For quantitative β-galactosidase measurements, in liquid culture, cells were stressed for 40min. with Na_2HAsO_4, harvested in early log phase, resuspended in lacZ buffer (Sodium phosphate buffer, pH7, KCl 10mM, Mg_2SO_4 1mM), permeabilized with chloroform, vortexed two times for 10s with an interval on ice for 2min. and assayed for enzyme activity after incubation with *ortho*-Nitrophenyl-β-galactoside (ONPG) at 0.6mg/ml, reaction was stopped by addition of 150µl Na_2CO_3 1M after the appearance of the first yellow colour. Absorbance was measured at 420, 550 and 600nm.

4.4.6 Binding assay with the Yap8 protein

The Yap8His was expressed in *E.coli* BL21(DE3) after 0.5Mm IPTG for 7h induction, cells were collected, washed and the pellet was frozen. The cells were resuspended in Tris buffer, 0.5M NaCl. and broken by 3-5 passages through the French Press. The protein was purified from the cell extract with Ni-NTA – His-Bind resin (Novagen) with 200µL of slurry, in an 1.5mL eppendorf according to the manufacturer's instructions with the difference that the solutions were made with Tris HCl, pH7.5.After the third wash with the washing buffer, the DNA was incubated as 20ng of linearized plasmid containing the reporter gene *HIS3* under the control of the sequence to be studied, in 400uLTris for 5min. After centrifugation the pellet was washed two times with Tris solution. The eluted solution was used for PCR amplification of *HIS3*.

4.4.7 RNA extraction and analysis

Cells were grown in SD complete medium until O.D.$_{600nm}$=0.4-0.5 and stressed for 1h with 1mM Na_2HAsO_4. Cells were centrifuged for 2min at 15339g and the pellet was frozen at -80ºC. RNA extraction was performed. The pellet was resuspended in 500µL TES buffer (10mM TrisHCl, 10mM EDTA, 0.5% SDS). 500µL of acidic phenol (Invitrogen)(acid pH): chlorophorm(5:1) was added for the selective removal of DNA and proteins and the sample was incubated at 65ºC for 1h, being vortexed every 10min..The mixture was transferred to a new eppendorf and centrifuged at maximum speed for 10min, 500µL of acidic phenol (Invitrogen)(acid pH): chlorophorm (5:1) was added to the upper aqueous phase, the sample was vortexed and centrifuged at 15339g for 10min.. The upper aqueous phase was carefully removed, transferred into a fresh tube with 500 µL of chlorophorm:isoamylethanol (25:1) and was vortexed and centrifuged again. The total RNA was finally precipitated by adding 1/10 volume of 3M Sodium acetate, pH5.2 and 2.5 volumes of absolute ethanol overnight at -20ºC or 1-2h at -80ºC. The sample was centrifuged at 15339g at 4ºC for 30min., washed with 70% ethanol, 15min., and finally resuspended in water (1000x the volume of the initial culture) treated with DEPC (diethyl pyrocarbonate) in order to eliminate RNases. RNA concentration and purity were evaluated by measuring the spectrophotometric absorbance of the 1 to 600 diluted samples at O.D.$_{260nm}$ and O.D.$_{280nm}$ and assuming that a 40µg/mL RNA solution gives an O.D.$_{260nm}$ of 1 and the ratio O.D.$_{260nm}$/O.D.$_{280nm}$ for a good purity RNA solution should range between 1.8-2.0. Northern Blot and probe hybridization was performed as described in [14] except that the membrane used was positively charged nylon from Roche.

4.4.8 Reverse transciptase reaction and RT-PCR

cDNA was synthesized by random hexanucleotide-primed or oligo d(T) primed reverse transcription from 0.05 µg/µl RNA. 20µg of RNA were treated with DNase (Ambion TURBO DNA-free) according to manufactures instructions. In brief, 20µg of RNA were treated in 50µl with 3U DNase for 30minutes at 37ºC. Reaction was stopped by incubating with 10µl Inactivating reagent for 2 minutes at room temperature. Samples were centrifuged at 10000g for 1.5 min. at room temperature. RNAs were quantified and their integrity was checked by loading 1µg in a gel. The cDNA synthesis was performed in 10µl reaction with 0,5µgRNA, 1mMdNTP, 5U Transcriptor Reverse transcriptase(Roche), 1x transcriptor reaction buffer. 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 using the DNA intercalating compound SYBR Green (LightCycler Fast Start DNA Master SYBR Green I, Roche) in a Roche LightCycler II instrument. Specific primers were used (see table S4.1). 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, 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 (empty vector) minus ΔC_t of the experimental sample.

4.5 Results and Discussion

4.5.1. Yap8 recognizes a 13bp sequence in the promoter of ACR2 and ACR3

Previously to the publication by Ilina *et al*, showing that Yap8 binds to the 13bp sequence TGATTAATAATCA located in the promoter of *ACR2* and *ACR3*. We used an *in vivo* assay described by Fernandes *et al* to study the sequence recognized by Yap8. The Yap genes were cloned in the multicopy plasmid that was expressed in cells concomitantly transformed with the reporter gene *HIS3*. The Upstream Activating Sequence (UAS) of the *HIS3* promoter was replaced by: Yap response element Y-TA**TTACTAA**TC, the palindromic sequence: 13bp site-TGA**TTAATAA**TCA and the 11bp site GA**TTAATAA**TC as illustrated in diagram 4.1. Recognition of the artificial *HIS3* promoter by the overexpressed Yap protein enables grow of the transformed Δ*his3* strain in the medium without histidine and in medium without histidine and with the competitive inhibitor of His3 enzyme product, the compound 3-amino-1,2,4-triazole (AT).

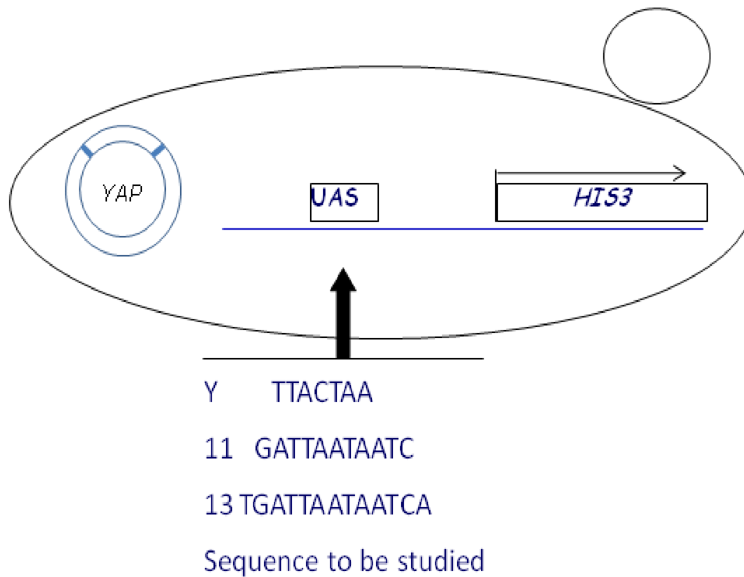

Diagram 4.1 Binding assay scheme.
Plasmids expressing the indicated Yap proteins were introduced in a Δ*his3* strain, encoding *HIS3* alleles under the control of the indicated upstream activated sequence (UAS) (Y-Yap recognition element, 13bp-13bp sequence of the *ACR2/ACR3* promoter and 11bp- 11bp sequence of the *ACR2/ACR3* promoter. Y - Yap site; site recognized by Yap1 to Yap3). The resulting strains were analyzed in selective medium as described in Materials and Methods

This assay showed that Yap8 only recognizes a sequence with a minimum of 13bp as was also shown by [4] and it does not recognize the Yap response element (Fig.4.2).

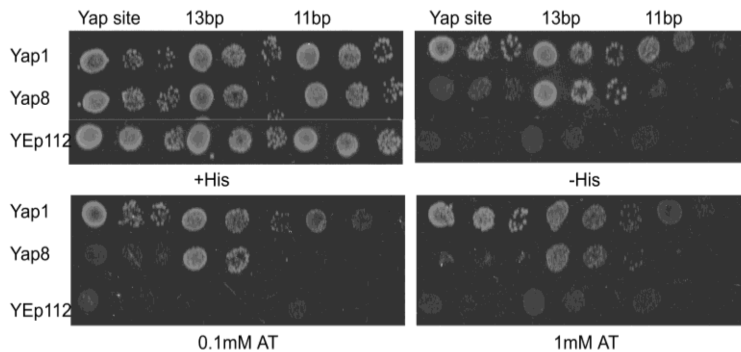

Figure 4.2 Yap1 and Yap8 activate transcription through the 13bp-site. FT4 strain was co-transformed with the *HIS3* reporter plasmid and empty vector YEplac112, or with the vector encoding *YAP1* or Y*AP8.* Serially diluted cultures were spotted onto selective medium supplemented as indicated. Plates were incubated for 2 days at 30º C., AT- 3-amino-1,2,4-triazole.

The abovementioned transformants were grown in liquid medium, stressed with hydrogen peroxide or arsenic compounds and RNA was extracted to quantify by RT-PCR the *HIS3* RNA levels in strains harbouring the Yap site or the 13bp sequence in order to evaluate that the results obtained are due to the transcription of the reporter gene. We can observe that Yap1 recognizes the Yap sequence and activates transcription of the *HIS3* even in the absence of restrictive conditions (medium with histidine). When AT was added there was a slight decrease in the amount of *HIS3* gene RNA (Fig.4.3). There was no expression of the reporter gene when Yap8 was over-expressed in the presence of the Yap site in the *HIS3* promoter. This corroborates the results obtained in the plate assay showing that Yap8 does not recognize the Yap site. Reporter gene expression driven by the 13bp site is mediated by Yap1 under arsenate conditions as expected, that can be explained by the fact

that Yap1 contributes to the expression of *ACR2* and *ACR3* (see Chapter 2, Fig.2.2) In the case of Yap8, the expression of the reporter gene is increased under arsenate stress. *HIS3* levels are greater in the case of Yap1 than of Yap8, a fact that can be explained due to the increased expression and transactivating potential of Yap1 under arsenic stress, in contrast to Yap8 (see chapter 2 Fig. 2.2 (C) and Fig.2.5.1)

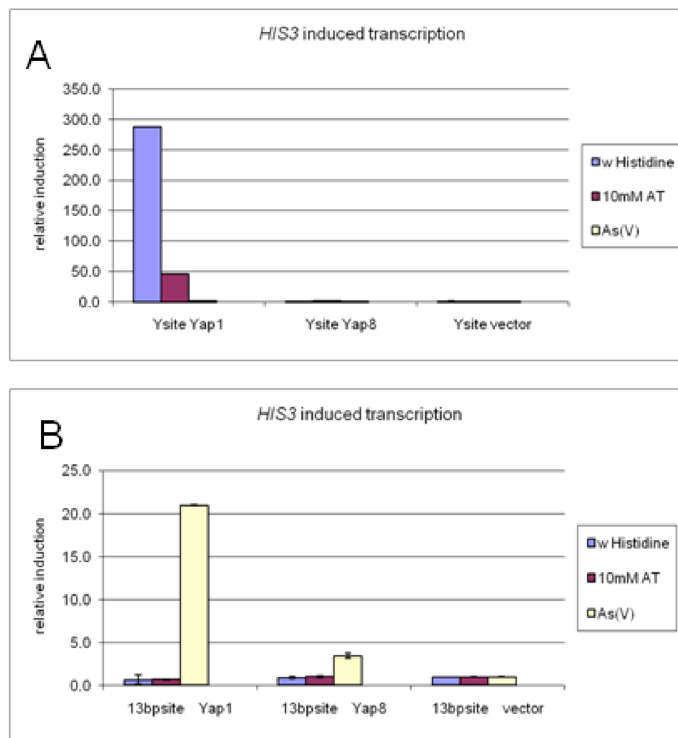

Figure 4.3 *HIS3* mRNA levels in strains over-expressing *YAP1* or *YAP8* and the reporter constructions A-Yap site, B-13bp site. RNA was extracted and quantified by RT-PCR as described in Materials and Methods

Binding of His tagged Yap8 to the 13bp site was also analysed using an *in vitro* binding assay. The purified protein, remaining in

the resin, was incubated with the DNA, and then washed and the eluted fractions were used as templates for PCR using specific *HIS3* primers. It was detected an enrichment of *HIS3* gene in the eluted fraction of the protein incubated with 13bp, as shown in Fig.4.4, demonstrating that the 13bp site is the preferred Yap8 recognition element.

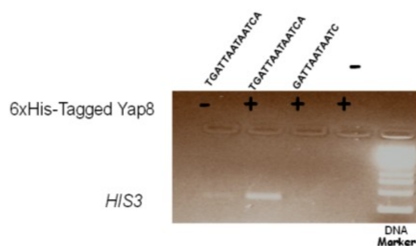

Figure 4.4 Yap8His binding to DNA
Yap8His was purified and incubated with 20ng of the target DNA sequences. Specific binding was monitored by PCR using the eluted fractions.

4.5.2 Mutation of Yap8 Leu26 to the conserved Asn

There is a conserved sequence in the DNA binding domain of the Yap b-Zip transcription factors NXXAQXXFR in which the residues interacting with the DNA remain unchanged. A striking difference between the DNA binding domain of Yap1/Pap1 and the one of Yap8, is an Asn residue that is replaced by a Leu in Yap8. To study if the Asn is important for DNA recognition that could explain the distinct DNA binding specificities of Yap8, we mutated the Yap8 Leu26 to Asn. This Leu26 is in the hydrophobic region and it is in the same vicinity as Asn86 in Pap1 (Fig4.S.1 and Fig4.S.2). The corresponding amino acid residues in Yap1, Yap8 and Pap1 have shared positions (Fig4.S.1, Fig4.S.2 and Fig4.S.3). A binding assay was done as described in diagram 4.1 and the results can be seen

in Fig.4.5. The mutant L26N behaves as the wild type Yap8 that recognizes only the 13bp sequence. Although the amino acid residue residuesAsn is conserved in the Yap family, this modification is not sufficient to alter Yap8 DNA binding properties.

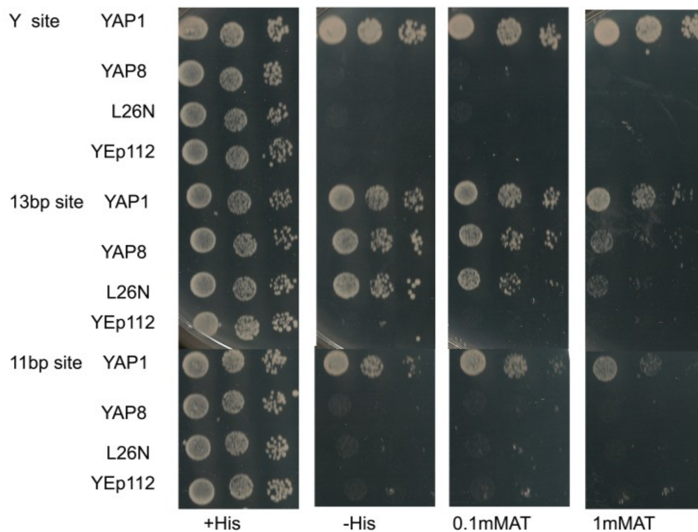

Figure 4.5 DNA binding specificity of L26N
FT4 strain was co-transformed with the *HIS3* reporter plasmid and the empty vector YEplac112 or with the vector encoding *YAP1* or *YAP8*. Serially diluted cultures were spotted onto selective medium supplemented as indicated. Plates were incubated for 2 days at 30º C. Phy – physiological conditions, AT- 3-amino-1,2,4-triazole

In order to evaluate if the L26N can replace Yap1, wild-type and mutant versions of Yap8 were overexpressed in the *Δyap0*, a strain deleted in all *YAP* genes (Batista-Nascimento et al, unpublished). Phenotypic analysis reveals that the mutant version L26N, behaves as the wild type Yap8, when exposed to cadmium and oxidative stress and not as Yap1 (Fig.4.6).

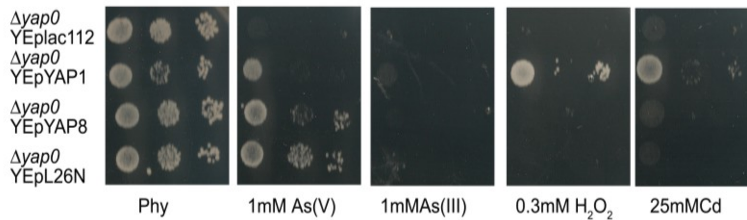

Figure 4.6 L26N behaves as wild-type Yap8 under various stress conditions
Wild type and *Δyap0* mutant strains were transformed with the empty vector or with the vector encoding the L26N. Serially diluted cultures were spotted onto selective medium supplemented with different concentrations of arsenate or arsenite. Plates were incubated for 2 days at 30º C. Phy – physiological conditions.

4.5.3 Converting Yap8 DNA binding domain element in Yap1 DNA binding element

To study the Yap8 DNA binding specificity we mutated residues in the DNA binding region to the corresponding residues in Pap1 and Yap1: Ala23 to Thr, Asn31 to the basic Arg, Ser29 to Ala, Lys35 to the acidic Glu and as a control Gln25 to Ala. The latter was used as control since it was already described that it is essential for DNA recognition. Using the binding assay described above, it can be observed that none of the mutants recognizes the canonical sequence (Fig.4.7). They were chosen due to their divergence with Yap1. According to Fig 4.S.2 and Fig 4.S.3, Ala23 is located in the polar region near Gln30 and the corresponding amino acid residue in Pap1, Lys83 binds the DNA. The corresponding amino acid residue to Asn21 in Pap1, Arg91 also binds to the DNA. Ser29 is also in the same position as Ala89, in a basic area. Lys35 is near three nonpolar residues and the corresponding amino acid residue in Yap1 Glu83, probably does not contact DNA since it is acidic, Lys95 in Pap1 does not bind DNA.

The mutant A23T recognizes the Yap8 DNA binding sequence TGA**TTAATAA**TCA with less affinity than wild-type Yap8. This showed that the Ala, despite being uncharged and small, is important either for the overall configuration of the DNA recognition amino acid residue sequence or the fact that the substitution by a polar and large amino acid residue disturbs the interaction with the DNA.

We also analysed the capacity of mutant proteins to recover the sensitivity phenotype of the *Δyap0* strain to arsenic compounds, oxidative stress and cadmium (Fig.4.8). With the exception of Q25A which unlike the wild type Yap8 confers no resistance to arsenic compounds, all the other mutants behave as the wild type Yap8.

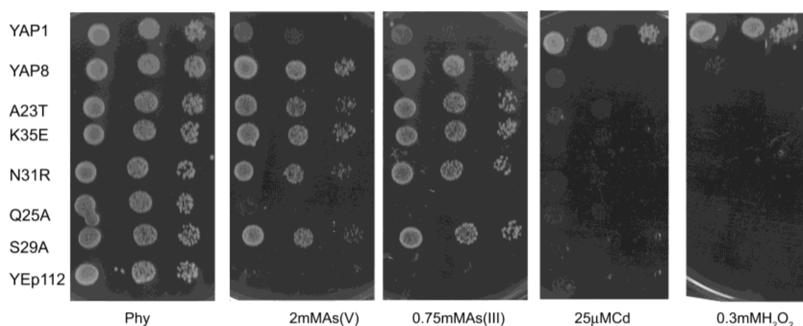

Figure 4.8 The single Yap8 mutants behave as the wild type proteins. The *Δyap0* mutant strain was transformed with the vectors encoding the wild type *YAP1*, *YAP8* or the respective single mutants. Serially diluted cultures were spotted onto selective medium supplemented with different concentrations of chemicals. Plates were incubated for 2 days at 30º C. Phy – physiological conditions

The single mutations were not sufficient to confer to Yap8 the properties of Yap1 DNA binding region, and therefore we decided to generate a double mutant of Leu26 and Asn31 to polar Asn and

basic Arg respectively, corresponding to the amino acid residue in equivalent position in Yap1 (Fig.4.9). We used these two amino acid residues because, besides their polarity, Asn and Arg are longer than the amino acid residues they replaced, that may allow a greater proximity to DNA. These mutations, were capable of conferring only a little effect on Yap8 regarding the Yap1 DNA binding properties. It is not ruled out that other amino acid residues would be required to have a very stable DNA-interaction.

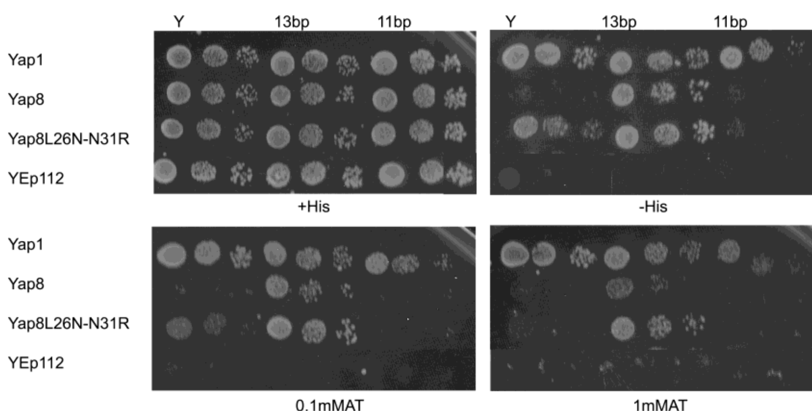

Figure 4.9 The double mutant Yap8L26N-N31R recognizes the canonical Yap site and activates transcription of the *HIS3* reporter gene.
FT4 strain was co-transformed with the *HIS3* reporter plasmid and the empty vector YEplac112 or with the vector encoding Yap8L26N-N31R. Serially diluted cultures were spotted onto selective medium supplemented as indicated. Plates were incubated for 2 days at 30º C.

4.5.4 Amino acid residues surrounding the core residues of the Yap8 DNA binding element

The Yap family and Pap1 DNA binding region contains the following sequence KRXAQNRXAQRAFRXRK, (x any amino acid), several residues that have a relevant function in DNA recognition (fig. 4.1). Our hypothesis is that the residues next to the core

element NXXAQXXFR are important for Yap8 function as it recognizes a minimum of 13bp sequence. The amino acid residues out of the conserved core element were analyzed by replacing the basics Lys21, Arg22 and also the polar Asn31, located inside the core sequence, to Ala. Lys21 is placed between neutral hydrophilic residues and Arg22 between a neutral and a hydrophobic one in regions that appear to be relevant for interaction with the DNA (Fig 4.S.2).

Binding assays as depicted in diagram 4.1 show that Arg22 has probably an important role in the binding to DNA, as well as Lys21, although to a lesser degree (fig.4.10). In summary, the assays of these mutations K21A, R22A and N31A showed that Arg22 and Lys21 seem to play an important role in the recognition of DNA.

Figure 4.10 R22A and K21A do not recognize the13bp sequence
FT4 strain was co-transformed with the *HIS3* reporter plasmid and the empty vector YEplac112 or with the vector encoding *YAP1* or *YAP8*. Serially diluted cultures were spotted onto selective medium supplemented as indicated. Plates were incubated for 2 days at 30º C.

Phenotypic analyses show that from the three analysed amino acids, Arg22 is the most relevant in Yap8 function as its mutation leads to a non complementation of the *Δyap0* sensitivity phenotype to arsenic stress. In Pap1 the Arg82 corresponding to Arg22, of Yap8 interacts with the flanking guanine (<u>G</u>TTAC). Although binding assays revealed that Lys21 is required for DNA binding, its

phenotypic assay did not correlate with the previous result. Indeed, this mutant confers resistance to As (V) and As (III) (Fig.4.11). Asn31 appears to have no relevant role in the binding to DNA since its mutation to Ala did not prevent the identification of the Yap8 recognized sequence of 13bp. The corresponding amino acid residue in Pap1, the basic Arg91, makes an interaction mediated by water with the phosphate backbone [5]. The fact that Asn is smaller than Arg91 in Pap1 and it is non-polar may explain its non-relevant role in DNA recognition.

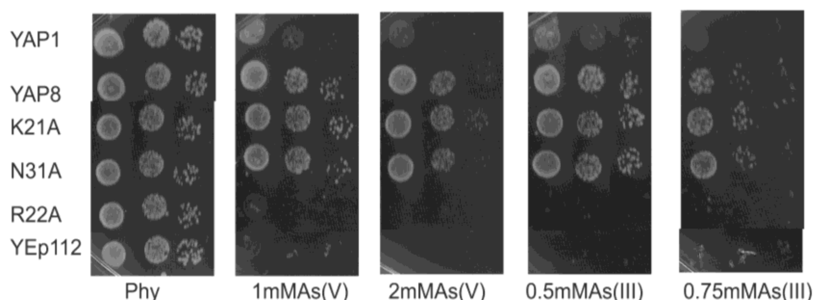

Figure 4.11 R22A does not behave as the wild type protein
The *Δyap0* mutant strain was transformed with the vectors encoding the wild type YAP1, YAP8 or the respective single mutants. Serially diluted cultures were spotted onto selective medium supplemented with different concentrations of arsenate or arsenite. Plates were incubated for 2 days at 30º C. Phy – physiological conditions.

Another set oof mutations was made: Arg27, Lys35, Arg36 and Lys37 were replaced by Ala to study the next to Yap8 Leu26 and the…XRK region binds to DNA. Arg27 is in similar vicinity as the corresponding amino acid residue in Pap1. The Lys35 and Lys37 were used to study amino acid residues that in Pap1 do not bind the DNA, trying to understand why Yap8 only recognizes, a sequence with at least 13bp. Lys35 corresponds to Yap1 acidic

residue Glu83 (see section 4.5.3). Arg36 is near Gln25, which indicates that is in an area close to DNA interaction. (Fig4.S.2)
The DNA binding assay showed that Arg27 and Arg36 are the most relevant amino acid residues of this set (Fig.4.12) for Yap8 binding upon the DNA.

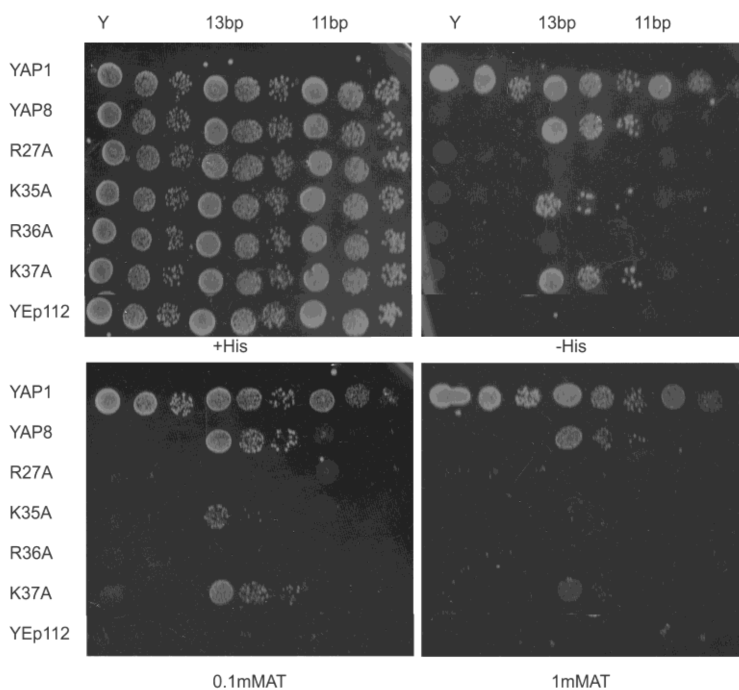

Figure 4.12 R27A and R36A do not recognize the 13bp sequence
FT4 strain was co-transformed with the *HIS3* reporter plasmid and the empty vector YEplac112 or with the vector encoding *YAP1* or *YAP8*. Serially diluted cultures were spotted onto selective medium supplemented as indicated. Plates were incubated for 2 days at 30º C.

Phenotypic analyses (fig.4.13) show that mutations in Arg27 and Arg36 disrupt the normal behaviour of the protein since the mutants do not totally complement the sensitivity to arsenic compounds of

the *Δyap0*. This was expected since in Pap1 the corresponding Arg87 and Arg96 make contacts with the phosphate backbone in Pap1 [5].

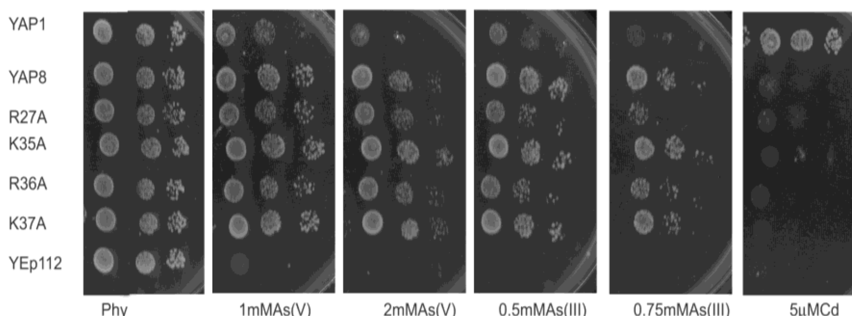

Figure 4.13 R27A and R36A do not completely recover *Δyap0* strain sensitivity phenotype to arsenic compounds
The *Δyap0* mutant strain was transformed with the vectors encoding the wild type *YAP1*, *YAP8* or the respective single mutants. Serially diluted cultures were spotted onto selective medium supplemented with different concentrations of arsenate or arsenite. Plates were incubated for 2 days at 30º C. Phy – physiological conditions.

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4.5.5.1 Yap8 signal transduction to the basal transcriptional machinery: Importance of the tail subunits

The Mediator complex is composed of four domains named tail, middle, head and Cdk8 of which the tail interacts with the transcription factor. To understand the relevance of these proteins in the yeast cellular response to arsenic stress, phenotypic analysis of deleted mutants in the tail subunits were made. It was also used as a control the mutant in the middle subunit, Med1 that due to its localization in the interior of the complex will probably not interact with Yap8.

From the phenotypic analysis (Fig 4.14), under arsenic stress, we observed a growth defect in the BY *Δmed1* strain even under

physiological conditions, reason why it was not possible to determine its sensitivity to arsenic compounds. Analysing the results with all the mutant strains, one can see sensitivity to arsenite particularly in the growth of Δ*med15* and Δ*med16* strains and to a less degree in the phenotype of Δ*med2* and Δ*med3.* It is shown that *Δmed5* shows a sensitivity higher than the one of the wild type at 2mM of As(III). Upon arsenate conditions the same pattern is observed with the exception that at 2mM there is no sensitivity of the Δ*med5* mutant. This is expected since Guglielmi *et al,* showed the localization of this subunit in the middle domain [7]. However other work proposed that this subunit belongs to the tail domain, interacting with Med15 and Med16 [15].

The W303 strain is much more sensitive to arsenite than to arsenate, the two mutant strains, Δ*med1* and Δ*med5,* show sensitivity to arsenic compounds comparable to the wild type. The mutants in *MED*2, 3, 14 and 16 are more sensitive than the wild type exposed to arsenate and arsenite. These strains appear to have a small growth defect under physiological conditions; nevertheless the sensitivity to arsenic is more evident. The high sensitivity to arsenic of the W303 strain can be explained by the fact that this strain possesses a mutated Ybp1 in one of the alleles, a protein required for the activation of Yap1 by Gpx3/Orp1 [16]. Nevertheless the subunits relevant for arsenic resistance are the same as for BY4742 as well as the subunit 14 observed in W303. It should be noted that the use of W303 to evaluate the importance of Med14 was due to the fact that the corresponding mutant in BY4742 is lethal.

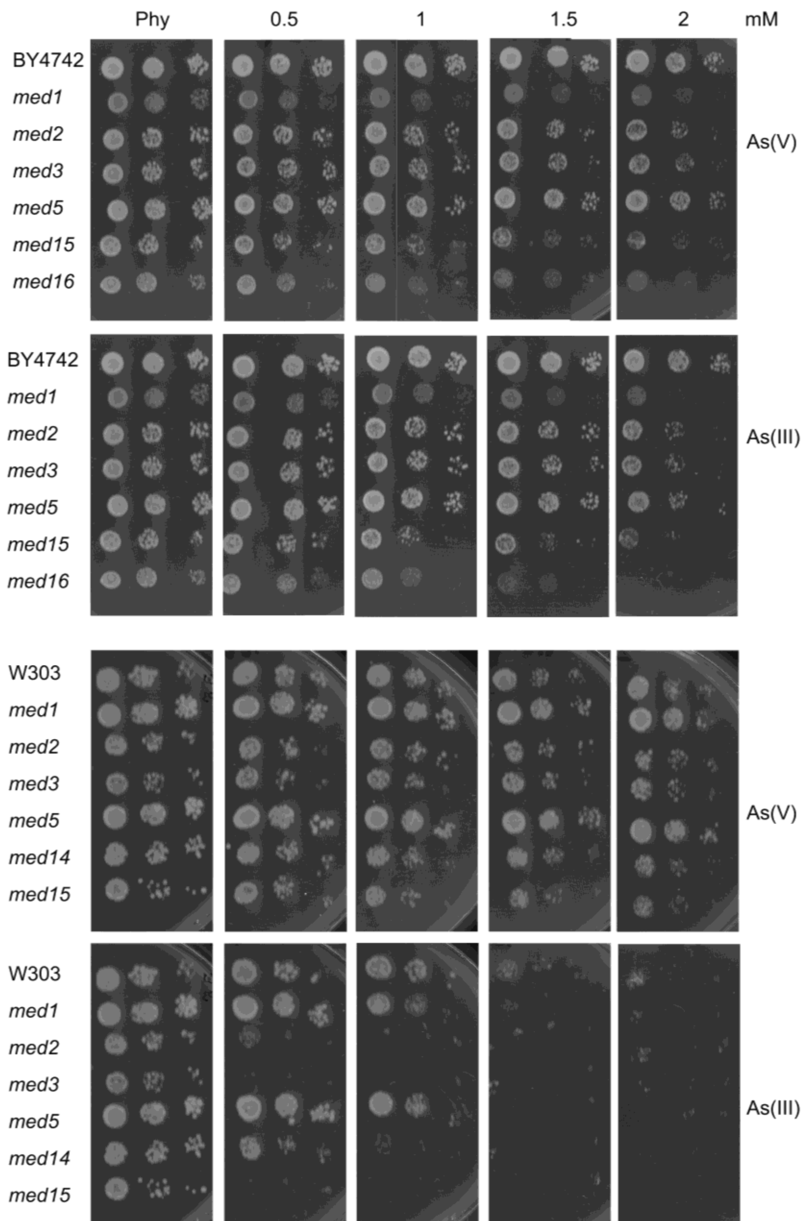


Figure 4.14 phenotypic analyses in arsenic compounds of strains deleted for the tail subunits of the Mediator as described in materials and methods. Plates were incubated for 2 days at 30º C. Phy- physiological conditions

4.5.5.2 Yap8 requires the tail subunits of the Mediator complex for full activity

The transactivation potential of Yap8 in the absence of the Mediator subunits was also studied using a lexAYap8 construction (fig.4.15) as described in Material and Methods. The β-galactosidase assay, upon arsenate and arsenite, stress revealed that the most relevant subunits for Yap8 activity, as a transcription factor, are Med2, Med3 and to a lesser degree Med15 and Med16. In the W303 strain the same results were obtained, except for Med3 and the subunit 14.

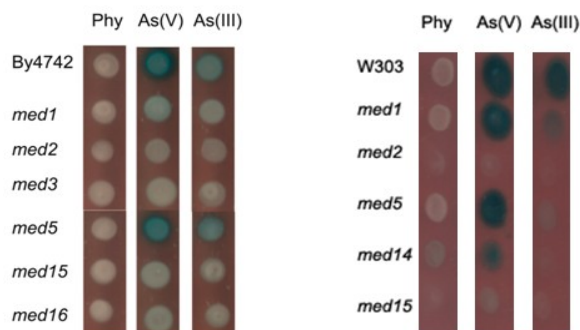
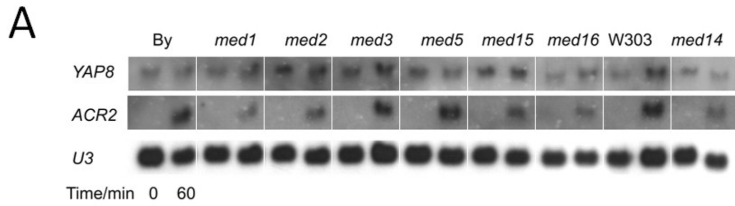


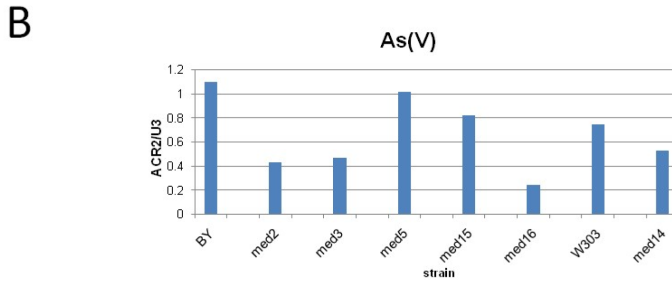
Figure 4.15 β-Galactosidase assay of the transactivation potential of Yap8 in the mutants of the tail subunits of the Mediator strains.
The assay was performed after 1h stress in solid media as described in materials and methods. Phy-physiological conditions

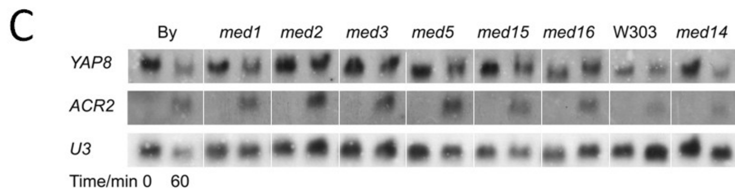
4.4.5.3 Influence of the mediator in the transcription of ACR2.

Yap8 target gene, *ACR2,* was also studied in the absence of the tail subunits by Northern analysis (fig.4.16). The results show that the steady state population of *YAP8* mRNAs is maintained constant in all the *med* mutant strains indicating that the Mediator tail

subunits do not interfere individually with the expression of *YAP8.* The densitometry of the *ACR2* signals under arsenate stress indicated, as well as for the β-galactosidase activity, that all subunits are relevant for Yap8 activity as regulator of *ACR2.* Subunit 2, 3, 15 and particularly 16 were the most important for the transcription of *ACR2.* Under arsenite stress, all the subunits are required for full transcription of *ACR2* being the most relevant Med16. As shown in Fig.4.15 β-galactosidase activity indicates that Yap8 does not require Med14 subunit to activate transcription under arsenic stress.







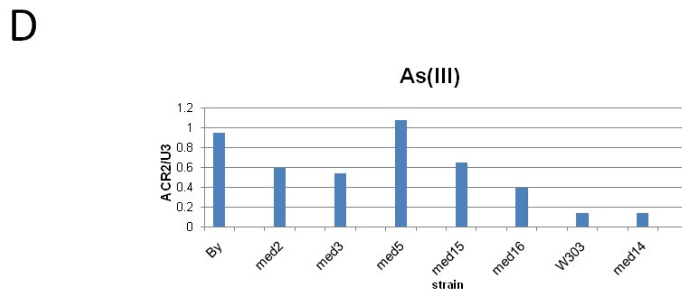


Figure 4.16 Induction of *YAP8* and *ACR2*
Early exponential phase cells were treated for 1h with (A) 1mM As(V) stress, (C)1mM As(III), *U3* RNA was used as loading control (B) densitometry of the Northern results for the membrane with 1mM As(V), (D) densitometry of the Northern results for the membrane with 1mM(III), the densitometries were performed using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health,Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2009).

From what was shown in the different experiments (see above), it is possible to deduce that the subunit interacting most probably with Yap8 is Med16, with a role for Med2, 3 and Med15 that cannot be neglected. This could also be explained by the absence of Med16, due to its localization in the Mediator complex that would lead to the absence of the Mediator subunits 2, 3 and 15 as previously described [19]. The decrease of *ACR2* levels may be the result of the absence of three subunits, and not exclusively of Med16. It is plausible to accept that a subunit binds directly with Yap8 but the others such as Med2, Med3 and Med15 may either be interacting with the activator or can be involved in the correct structure of the tail to interact. This study can be only investigated accurately by two-hybrid system and co-immunoprecipitations assays.

In order to analyse the interaction of the b-ZIP with the DNA by Nuclear Magnetic Resonance (NMR) a construction was made based on the structure predicted by 3DJIGSAW [20], from amino acid residue11 to 60. For unknown reasons the protein had a very low content of helix as verified by CD-spectropolarimeter. It is known that the structure of the basic region is only stabilized after the binding of the DNA [21]. We only verified a small increase in the helix content. The same spectrum was obtained either in the presence or in the absence of the histidine tail in the protein.

4.6 Conclusions

In conclusion, from the studied mutations, the most important for DNA binding in Yap8 are K21A, R22A, A23T, R27A, K35A, K35E, R36A and Q25A. Nevertheless the only mutants that showed a

sensitivity phenotype to arsenic compounds are R22A, Q25A, R27A and R36A. Some correlation between the results of the DNA binding assay and the mutant's capacity to recover the *Δyap0* sensitivity to arsenic compounds takes place for some of the residues. The fact that for others such correlation does not exist may be due to the use of a multi-copy plasmid that may mask subtle growth deficiencies of the *Δyap0* strain transformed with the Yap8 mutants.

From what is shown in the phenotypical analysis, study of Yap8 transactivation potential in the absence of the tail Mediator subunits and transcriptional activation of *ACR2* in the absence of the tail subunits, it is possible to hypothesize that the subunit interacting, most probably with Yap8 is Med16, with a role for Med2, 3 and Med15.

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Chapter 5

Discussion and Perspectives

Arsenic compounds (As) are at the origin of several damaging processes in the cell, leading to several diseases such as cancer. It has been proposed that the generation of reactive oxygen species (ROS) is the first line of arsenic toxicity. In the chapter 3 it is shown that under arsenate stress there is the up-regulation of genes that regulate the response to oxidative stress such as *HSP12, TRX2* and *MRX1* (see table 3.1). The fact that glutathione decreases after the first hour (fig.3.4) may be caused by direct reaction of arsenite, that is used as a reducer by Acr2 [1] and Ycf1[2]. The decrease in glutathione may cause oxidative stress since it is the cellular major anti-oxidative buffer [3]. Nevertheless, reduced glutathione levels increase in the wild type after 2h by Glr1 activity and increased synthesis *de novo* as indicated by the up regulation of genes related to sulfur uptake, glutathione biosynthesis and glutathione reduction, when under arsenic stress (see table 3.1 and fig. 3.6) [4-6]. In a Δ*yap1* strain there is also increase of reduced glutathione, once *GLR1* expression is under the control of Yap1 this increase can only be due to the activity of *GSH1* of which expression is controlled by Yap1 but also by Met4 [7]. Northern analysis of *GSH1* expression in a Δ*yap1* showed that there is expression of this gene in the mutant strain, therefore most probably involving another transcription factor in the response to arsenic [5]. Arsenate may also cause oxidative stress through the removal of iron from proteins that may enter Fenton reactions that transforms hydrogen peroxide in hydroxyl radical and hydroxyl anion [8] Transcriptome analyses under arsenic stress show an up-regulation of iron

transporters and of genes involved in Iron-Sulfur clusters synthesis [6], indicating the cell is sensing a low iron content in proteins [9]. The presence of intracellular ROS was directly measured through the probe DCF-DA and it was also measured the presence of ROS by-products after reaction with cellular components such as lipids and proteins (fig.3.2, 3.3) There was an increase in ROS, after arsenic stress, particularly in a Δ*yap1* strain. The results also show that Yap1 is active during prolonged arsenate stress and its oxidative stress related target genes are up-regulated after arsenic stress (fig.3.5 and 3.6). Together these results indicate that arsenic causes the production of deleterious ROS and Yap1 is essential for its detoxification.

The budding yeast *Saccharomyces cerevisiae* possesses a cluster of ACR genes that is essential for the adaptation to environments containing high levels of arsenic. It is composed of the transcription factor *YAP8* that controls the expression of the other two genes of the cluster [10, 11]. These genes are *ACR2* and *ACR3* [12] which encode an arsenate reductase [1] responsible for the reduction of arsenate to arsenite, and an arsenite efflux protein [13], respectively. The results shown in this thesis reveal that Yap8 is responsible for the activation of *ACR2* and *ACR3*. It is also shown that Yap8 is activated by the same mechanism as Yap1, through reaction of arsenite molecules with Cys (Cys 132, 137 and 274), masking the Nuclear Export Signal (NES) which prevents its interaction with the nuclear exportin Crm1, and leading to its nuclear accumulation. The mechanism of activation through Cys modification also occurs in mammalian cells, namely by modification of Keap1 Cys, the protein that anchors the basic leucine zipper Nrf2 to the cytoplasm, regulating its activity [14]. The

study of Yap1 nuclear localization in an Δ*orp1* strain, essential for the signal transduction under oxidative stress, shows that Yap1 is not activated through oxidation of its cysteines. Using a construction with only the C-terminal of Yap1 we saw that only these cysteines are required for Yap1 activation. These results indicate that arsenic behaves as a thiol-reactive chemical. This is corroborated by results obtained in the laboratory using the alkylating agent AMS as in [15] by (Menezes et al, unpublished). These cysteines are also important for Yap8 transactivation potential that is only active upon arsenic stress, a fact that led us to put forward the hypothesis that arsenic modification of its cysteines induces a conformational change of its structure. It would be interesting to perform a structural study before and after the addition of arsenic to the purified protein in order to observe such conformational changes. However, from the results obtained for the transactivation potential, that requires the entire protein for the full Yap8 activity, it would be, therefore, ideal to use the entire protein instead of the C-terminal domain.

The conformational change occurring upon arsenic stress, should improve Yap8 interaction with the transcriptional machinery for which the interaction with the Mediator complex will be required. Our studies, in chapter 4, of the mediator requirement for Yap8 activity show that the tail subunits that may interact with Yap8 are the subunits Med2, Med3, Med15 and Med16. Confirmation of these results is required using the two-hybrid system or co-immunoprecipitation. After clearly demonstrating which is the Mediator subunit or subunits interacting with Yap8 it would be interesting to randomly mutate residues, testing those preventing

interaction with the mediator and revealing therefore the interacting domain.

The other domain to be studied is the b-ZIP DNA binding domain of which the first interactions have been determined. These studies show that the most relevant mutations for DNA interaction are K21A, R22A, A23A, R27A, K35A, R36A and Q25A. Additional studies to specify which amino acid interacts with which DNA base should be made, particularly to clarify why Yap8 has a Leu in place of the Asn that is conserved in all Yap family members and the Yap1 orthologue, Pap1, in *S. pombe* (Fig.4.1). The most appropriate study would be the crystallization and X-ray of the protein, with the DNA, in order to be able to compare with the data obtained for Pap1. Nevertheless protein modeling and NMR may also give important clues about the overall structure of the DNA binding domain and the amino acids interacting with the DNA.

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Supplemental Material

3.S1- Genes induced more than 2-fold upon arsenate treatment (FI-fold induction)

ORF	NAME	Log_2 (FI)	FUNCTION
YHR053C	*CUP1-1*	4.23	copper binding metallothionein
YHR055C	*CUP1-2*	4.28	copper binding metallothionein
YDR171W	*HSP42*	3.48	Small cytosolic stress-induced chaperone
YGR142W	*BTN2*	3.08	Gene/protein whose expression is elevated in a btn1 minus/Btn1p lacking yeast strain.
YCL026C-B	*HBN1*	4.05	Hypothetical ORF
YPL171C	*OYE3*	3.83	NADPH dehydrogenase
YCL026C-A	*FRM2*	3.01	Protein involved in the integration of lipid signaling pathways with cellular homeostatis
YCR021C	*HSP30*	2.35	Protein induced by heat shock, ethanol treatment, and entry into stationary phase; located in plasma membrane
YPR200C	*ARR2/ ACR2*	3.28	Arsenate reductase required for arsenate resistance; converts arsenate to arsenite which can then be exported from cells by Arr3p
YBR072W	*HSP26*	2.90	heat shock protein 26
YOL151W	*GRE2*	2.72	NADPH-dependent methylglyoxal reductase (D-lactaldehyde dehydrogenase); stress induced (osmotic, ionic, oxidative, heat shock and heavy metals); regulated by the HOG pathway
YFL056C	*AAD6*	3.05	aryl-alcohol dehydrogenase (putative)
YLR327C	*TMA10*	3.03	Hypothetical ORF
YPR167C	*MET16*	2.37	3'phosphoadenylylsulfate reductase
YER052C	*HOM3*	2.00	aspartate kinase (L-aspartate 4-P-transferase) (EC 2.7.2.4)
YPL250C	*ICY2*	2.75	Protein that interacts with the cytoskeleton and is involved in chromatin organization and nuclear transport, interacts genetically with TCP1 and ICY1
YFR030W	*MET10*	2.30	sulfite reductase alpha subunit
YDR258C	*HSP78*	2.36	heat shock protein 78
YLR092W	*SUL2*	2.41	high affinity sulfate permease
YMR090W		2.90	Hypothetical ORF
YOR298C-A	*MBF1*	2.40	multiprotein bridging factor
YAL012W	*CYS3*	2.44	cystathionine gamma-lyase
YML131W		2.35	Hypothetical ORF
YFL057C	*AAD16*	2.83	Putative aryl-alcohol dehydrogenase with similarity to P. chrysosporium aryl-alcohol dehydrogenase; mutational analysis has not yet revealed a physiological role
YNL277W	*MET2*	1.51	homoserine O-trans-acetylase
YIL074C	*SER33*	2.47	3-phosphoglycerate dehydrogenase
YOL064C	*MET22*	2.01	3'(2')5'-bisphosphate nucleotidase
YLR216C	*CPR6*	2.58	cyclophilin 40

YBR169C	*SSE2*	2.55	HSP70 family
YNL331C	*AAD14*	2.77	aryl-alcohol dehydrogenase (putative)
YGL184C	*STR3*	1.87	cystathionine beta-lyase
YBR008C	*FLR1*	1.89	major facilitator transporter
YDL159W-A	*YDL159W-A*	2.6	Hypothetical ORF identified by homology. See FEBS Letters [2000] 487:31-36.
YHR179W	*OYE2*	1.99	NAPDH dehydrogenase (old yellow enzyme), isoform 2
YFL014W	*HSP12*	2.45	heat shock protein 12
YBR101C	*FES1*	1.76	Hsp70 nucleotide exchange factor
YGR209C	*TRX2*	2.18	thioredoxin
YGR055W	*MUP1*	2.15	high affinity methionine permease
YML130C	*ERO1*	1.67	Glycoprotein required for oxidative protein folding in the endoplasmic reticulum
YHR199C	*AIM46*	2.17	The authentic, non-tagged protein was localized to the mitochondria
YPL240C	*HSP82*	1.79	heat shock protein 90
YER175C	*TMT1*	1.66	Trans-aconitate Methyltransferase 1
YER042W	*MXR1*	2.15	peptide methionine sulfoxide reductase
YCL040W	*GLK1*	1.74	glucokinase
YER092W	*IES5*	2.14	Protein that associates with the INO80 chromatin remodeling complex under low-salt conditions
YGL037C	*PNC1*	2.04	nicotinamidase
YPR154W	*PIN3*	1.53	[PSI+] induction
YIR017C	*MET28*	1.79	transcriptional activator in the Cbf1p-Met4p-Met28p complex
YOR027W	*STI1*	1.59	heat shock protein also induced by canavanine and entry into stationary phase
YGR211W	*ZPR1*	1.42	zinc finger protein
YHL036W	*MUP3*	1.57	very low affinity methionine permease
YER067W		1.36	Hypothetical ORF
YOR303W	*CPA1*	1.69	Small subunit of carbamoyl phosphate synthetase, which catalyzes a step in the synthesis of citrulline
YNL260C		1.27	Protein required for cell viability
YAL005C	*SSA1*	1.60	heat shock protein of HSP70 family
YOL032W	*OPI10*	1.13	Protein with a possible role in phospholipid biosynthesis
YLR364W		1.43	Hypothetical ORF
YOR130C	*ORT1*	1.07	Ornithine transporter of the mitochondrial inner membrane, exports ornithine from mitochondria as part of arginine biosynthesis; human ortholog is associated with hyperammonaemia-hyperornithinaemia-homocitrullinuria (HHH) syndrome
YGR161C	*RTS3*	1.77	Hypothetical ORF
YGR250C		1.00	Hypothetical ORF
YNL191W		1.21	Hypothetical ORF
YLR108C	*DUG3*	1.83	Probable glutamine amidotransferase
YER091C	*MET6*	1.87	vitamin B12-(cobalamin)-independent isozyme of methionine synthase (also called N5-methyltetrahydrofolate homocysteine methyltransferase or 5-methyltetrahydropteroyl triglutamate

			homocysteine methyltransferase)
YIL117C	*PRM5*	1.60	Pheromone-regulated protein, predicted to have 1 transmembrane segment; induced during cell integrity signalling
YDR261W-A		1.60	TyA Gag protein; the main structural constituent of virus-like particles (VLPs)
YDR210W-A		1.55	TyA Gag protein; the main structural constituent of virus-like particles (VLPs)
YOR302W		1.75	CPA1 uORF , Arginine attenuator peptide, regulates translation of the CPA1 mRNA
YHL021C	*AIM17*	2.13	The authentic, non-tagged protein was localized to the mitochondria
YCL020W		1.55	TyA Gag protein; the main structural constituent of virus-like particles (VLPs)
YGR197C	*SNG1*	1.10	Involved in nitrosoguanidine resistance
YDR368W	*YPR1*	1.54	homologous to the aldo-keto reductase protein family
YLR410W-A		1.53	TyA Gag protein; the main structural constituent of virus-like particles (VLPs)
YHR122W		1.02	Protein required for cell viability
YOL052C-A	*DDR2*	1.59	Multistress response protein,Jexpression isJactivated byJa variety of xenobiotic agents and environmental or physiological stressesJJJ
YBR054W	*YRO2*	1.28	Putative plasma membrane protein of unknown function, transcriptionally regulated by Haa1p; green fluorescent protein (GFP)-fusion protein localizes to the cell periphery and bud
YGR161W-A		1.47	TyA Gag protein; the main structural constituent of virus-like particles (VLPs)
YBL101W-A		1.61	TyA Gag protein; the main structural constituent of virus-like particles (VLPs)
YOR343W-A		1.52	TyA Gag protein; the main structural constituent of virus-like particles (VLPs)
YDR516C	*EMI2*	1.37	Early Meiotic Induction
YGL114W		1.01	Hypothetical ORF
YFL002W-B		1.49	TyA Gag protein; the main structural constituent of virus-like particles (VLPs)
YDR513W	*TTR1*	1.77	glutaredoxin
YOR192C-A		1.39	TyA Gag protein; the main structural constituent of virus-like particles (VLPs)
YBL075C	*SSA3*	1.81	heat shock protein of HSP70 family
YPL274W	*SAM3*	1.18	high affinity S-adenosylmethionine permease
YNL241C	*ZWF1*	1.22	glucose-6-phosphate dehydrogenase
YGR204W	*ADE3*	1.24	C1-tetrahydrofolate synthase

YPR201W	*ARR3/ ACR3*	1.39	Arsenite transporter of the plasma membrane, required for resistance to arsenic compounds; transcription is activated by Arr1p in the presence of arsenite
YIL168W	*SDL1*	1.37	L-serine dehydratase
YER081W	*SER3*	1.35	3-phosphoglycerate dehydrogenase
YPL106C	*SSE1*	1.30	HSP70 family
YER150W	*SPI1*	1.15	strongly expressed during stationary phase, and trancription is dependent on MSN2/MSN4.
YPR067W	*ISA2*	1.23	Iron Sulfur Assembly -- IscA/NifA homolog
YOR020C	*HSP10*	1.28	heat shock protein 10
YOR007C	*SGT2*	1.30	small glutamine-rich tetratricopeptide repeat containing protein
YGR136W	*LSB1*	1.19	LAs17 Binding protein
YML128C	*MSC1*	1.31	Protein of unknown function, green fluorescent protein (GFP)-fusion protein localizes to the endoplasmic reticulum; msc1 mutants are defective in directing meiotic recombination events to homologous chromatids
YEL071W	*DLD3*	1.09	D-lactate dehydrogenase
YBL064C	*PRX1*	1.64	also called mTPx I, a mitochondrial isoform of thioredoxin peroxidase (EC 1.11.1.-)
YLR178C	*TFS1*	1.28	lipid binding protein (putative) supressor of a cdc25 mutation
YHR018C	*ARG4*	1.00	argininosuccinate lyase
YGR008C	*STF2*	1.25	ATPase stabilizing factor
YMR038C	*LYS7*	1.15	copper chaperone for superoxide dismutase Sod1p
YBL078C	*ATG8*	1.29	similar to LC3, a microtubule-associated protein from rat
YLR259C	*HSP60*	1.16	chaperonin
YGL062W	*PYC1*	1.42	pyruvate carboxylase
YOL058W	*ARG1*	1.12	arginosuccinate synthetase
YGR172C	*YIP1*	1.06	Golgi integral membrane protein; binds to the transport GTPases Ypt1p and Ypt31p
YHR104W	*GRE3*	1.03	aldose reductase
YOR343W-B		1.51	TyB Gag-Pol protein; proteolytically processed to make the Gag, RT, PR, and IN proteins that are required for retrotransposition
YLR064W		1.02	Hypothetical ORF
YML100W	*TSL1*	1.19	similar to TPS3 gene product trehalose-6-phosphate synthase/phosphatase complex 123 kDa regulatory subunit
YBR082C	*UBC4*	1.15	ubiquitin conjugating enzyme e2
YCL042W		1.55	Hypothetical ORF
YCR005C	*CIT2*	1.09	citrate synthase

Table 4.S1 Primers used in this study

Primer Name	Sequence
His3fw	ATC ACA CCA CTG AAG ACT GC
His3rv	AAC ATC GTT GGT ACC ATT GG
ACT1up	CTA TTG GTA ACG AAA GAT TCA G
ACT1down	CCT TAC GGA CAT CGA CAT CA
13bp.His3fw	GAT CCT GAT TAA TAA TCA G
13bp.His3rv	AAT TCT GAT TAT TAA TCA G
11bp.His3fw	GAT CCG ATT AAT AAT CG
11bp.His3rv	AAT TCG ATT ATT AAT CG
Q25Y8A fw	AGA GCT GCG GCA CTT AGA GCA
Q25Y8A rv	TGC TCT AAG TGC CGC AGC TCT
A23Y8T fw	AAA TAA GAG AAC TGC GCA ACT T
A23Y8T rv	A AGT TGC GCA GTT CTC TTA TTT
N31Y8A fw	GCA TCC CAA GCA GCA TTT AGa
N31Y8A rv	TCT AAA TGC TGC TTG GGA TGC
K21Y8A fw	CCT AAA AAT GCG AGA GCT GCG
K21Y8 rv	CGC AGC TCT CGC ATT TTT AGG
R22Y8A fw	AAA AAT AAG GCA GCT GCG CAA
R22Y8A rv	TTG CGC AGC TGC CTT ATT TTT
K35Y8A fw	GCA TTT AGA GCA CGA AAG TTG
K35Y8A rv	CAA CTT TCG TGC TCT AAA TGC
K37Y8A fw	AGA AAA CGA GCG TTG GAA AGA
K37Y8A rv	TCT TTC CAA CGC TCG TTT TCT
R27Y8A fw	GCA ACT TGC AGC ATC CCA AA
R27Y8A rv	TTT GGG ATG CTG CAA GTT GC
R36Y8A fw	CAT TTA GAA AAG CAA AGT TGG
R36Y8A rv	TTC CAA CTT TGC TTT TCT AAA
Yap8bZipNdeI fw	GGAATTC CATATG AGG AAG CCT TCA CTT AC

Yap8bZipXhoI rv	CGG ctc gag ACC CTG AAA ATA TAA ATT TTC CTT CTT TAA TAT GTG AATTt
Yap8.NdeI.fw	GAA TCA TAT GGC AAA ACC GCG TGG AAG AA
Yap8.XhoI.rv	CCC CTCGAG TAATT TTG ACG AAA AGA CC

Table 4.S2 Information obtained for Yap8 mutants in the DNA binding domain

Y8mutant	Y8 sequence	Phenotype in arsenic	Pap1 interaction with DNA	Yap8 possible interaction with DNA
K21A	does not recognizes	recovers		
R22A	does not recognize	no recovery	R82 interacts with Guanosine (GTTAC) directly and via water molecule	
A23T	does not recognize	recovers		
Q25A	does not recognize	no recovery	Q85 contacts phosphate groups	? contacts phosphate groups
L26N	recognizes	recovers	N86 interacts with adenosine that is base paired with the first thymine, directly and by hydrogen bond via water molecule and with guanosine via a water molecule .Also forms a water mediated contact with the guanine(GTTAC)	
R27A	does not recognize	recovers partially	R87 contacts phosphate groups	?contacts phosphate groups
S29A	recognizes	recovers	A89 interacts with 1st thymine (TTA)	
N31A	recognize	recovers	R91 interacts with phosphate via water hydrogen bond	
N31R	recognize	recovers	R91 interacts with phosphate via water and hydrogen bond	
K35E	does not recognize	recovers		
K35A	recognizes slightly	recovers		?no interaction with DNA
R36A	does not recognize	recovers partially	R96 contacts phosphate groups	?contacts phosphate groups
K37A	recognizes	recovers	does not interact with DNA	

Figures

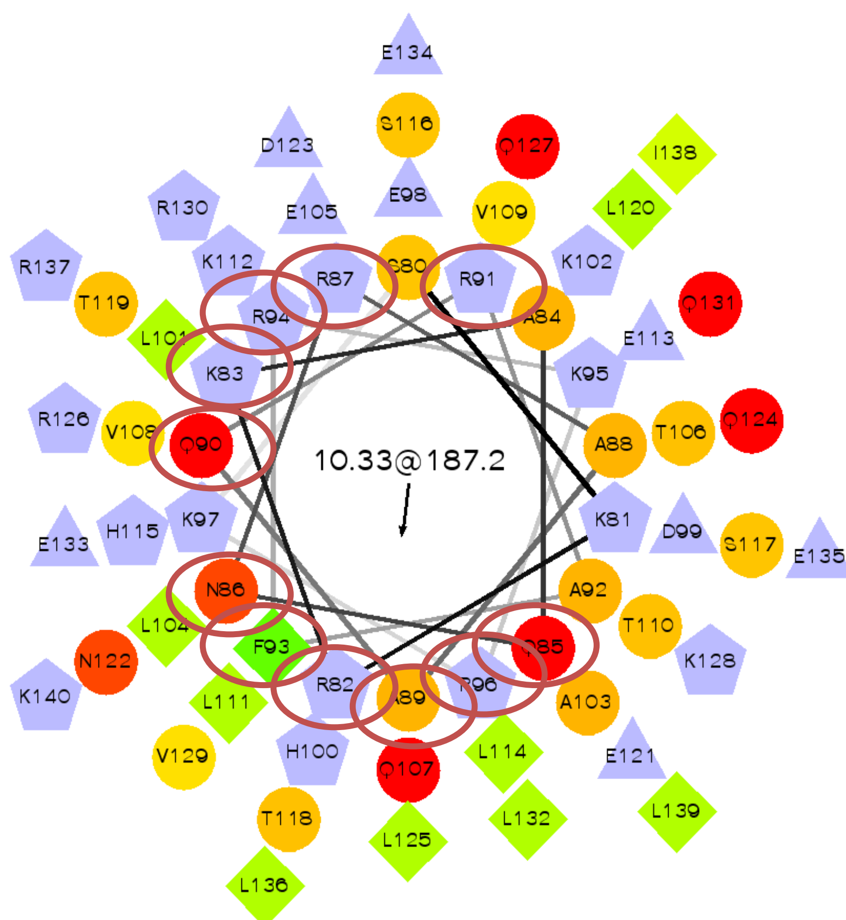

Figure 4.S.1 helical representation of Pap1 from residues 79-140 as depicted by Wheel (http://rzlab.ucr.edu/scripts/wheel)
The hydrophilic residues are represented as circles, hydrophobic residues as diamonds, potentially negatively charged as triangles, and potentially positively charged as pentagons. Hydrophobicity is color coded: the most hydrophobic residue is green, and the amount of green is decreasing proportionally to the hydrophobicity, with zero hydrophobicity coded as yellow. Hydrophilic residues are coded red with pure red being the most hydrophilic (uncharged) residue, and the amount of red decreasing proportionally to the hydrophilicity. The potentially charged residues are light blue. The amino acids that interact with the DNA have a red circle as described in Fujii *et al*.

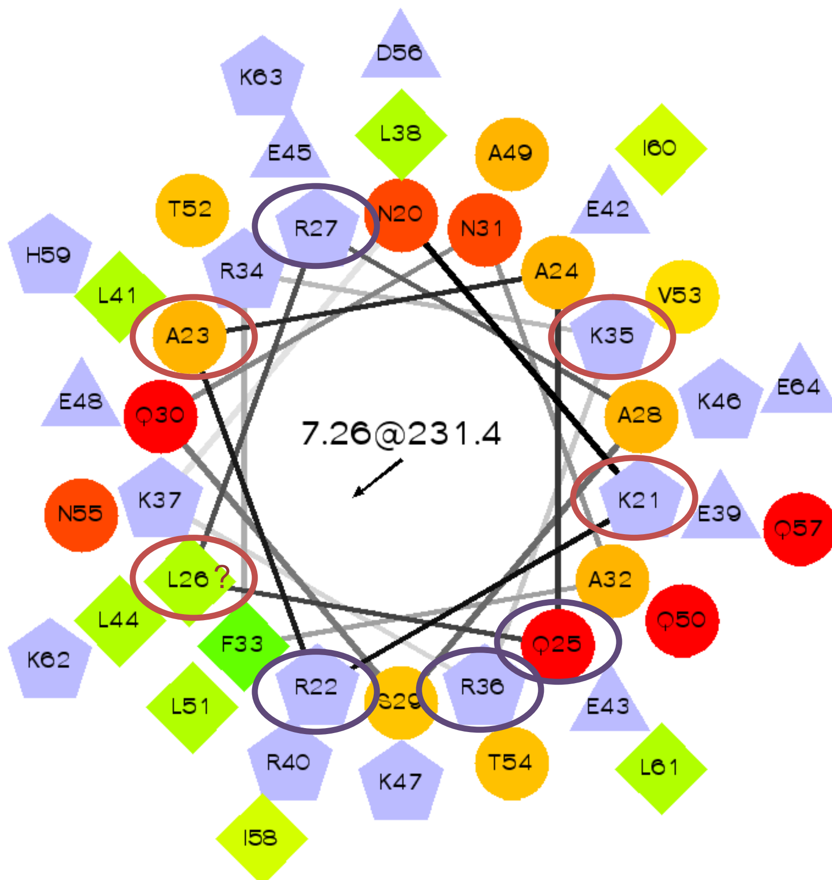

Figure 4.S.2 helical representation of Yap8 from residues 20-64 as depicted by Wheel (http://rzlab.ucr.edu/scripts/wheel)

Circles represent hydrophilic residues, diamonds hydrophobic residues, triangles potentially negatively charged, and pentagons potentially positively charged. Colour codes: green corresponds to the most hydrophobic residue, and the amount of green is decreasing proportionally to the hydrophobicity, with yellow as zero hydrophobicity. Pure red being the most hydrophilic (uncharged) residue, and the amount of red decreasing proportionally to the hydrophilicity. Light blue is for all potentially charged residues. The residues that may interact with the DNA have a red circle, residues also positive by phenotypic analysis have a violet circle.

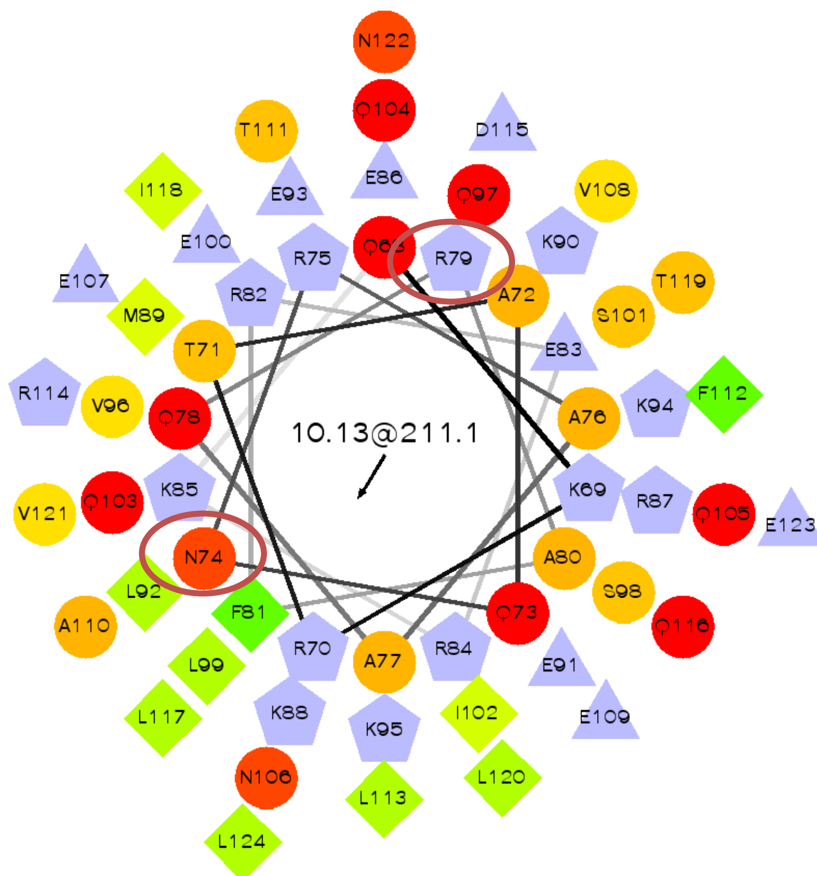

Figure 4.S.3 helical representation of Yap1 from residues 68-124 as depicted by Wheel (http://rzlab.ucr.edu/scripts/wheel)
Circles represent hydrophilic residues, diamonds hydrophobic residues, triangles potentially negatively charged, and pentagons potentially positively charged. Colour codes: green corresponds to the most hydrophobic residue, and the amount of green is decreasing proportionally to the hydrophobicity, with yellow as zero hydrophobicity. Pure red being the most hydrophilic (uncharged) residue, and the amount of red decreasing proportionally to the hydrophilicity. Light blue is for all potentially charged residues. The residues that may interact with the DNA have a red circle.