BJ Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

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

Regina A. Menezes*, Catarina Amaral*, Liliana Batista-Nascimento*, Claudia Santos†, Ricardo Boavida Ferreira †‡, Fréderic Devaux §, Elis C.A. Eleutherio‖ and Claudina Rodrigues-Pousada*

*Genomics and Stress Laboratory, Instituto de Tecnologia Química e Biológica, Avenida da República, 2780-157 Oeiras, Portugal

†Disease and Stress Biology Laboratory, Instituto de Tecnologia Química e Biológica, Avenida da República, 2780-157, Oeiras, Portugal

‡Departamento de Botânica e Engenharia Biológica, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, 1349, Lisboa, Portugal

§Laboratoire de Génétique Molèculaire, CNRS UMR 8541, Ecole Normale Supérieure, 46 rue d'Ulm, 75230 Paris CEDEX 05, France

‖Laboratório de Investigação de Fatores de Estresse (LIFE), Departamento de Bioquímica, Instituto de Química - UFRJ, 21949-900, Rio de Janeiro, RJ, Brazil

Corresponding Author: Claudina Rodrigues-Pousada, Genomics and Stress Laboratory, Instituto de Tecnologia Química e Biológica, Av. da República, Apt. 127, 2781-901 Oeiras, Portugal, Phone: 00351 214 469 624, Fax: 00351 214 433 644, claudina@itqb.unl.pt

Running Title: Yap1 and Yap8 in arsenic stress

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

In the budding yeast *Saccharomyces cerevisiae* arsenic detoxification involves the activation Yap8, a member of the Yap family of transcription factors, which in turn regulates *ACR2* and *ACR3*, encoding an arsenate reductase and a plasma membrane arsenite efflux-protein, respectively. In addition, Yap1 is involved in the arsenic adaptation process through regulating the expression of the vacuolar-pump encoded by *YCF1* and also contributing to the regulation of *ACR* genes. Here we show that Yap1 is also involved in the removal of ROS 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 *yap8* mutant, the enhanced transcriptional activation of the antioxidant genes such as *GSH1, SOD1* and *TRX2* may prevent protein oxidation. In contrast, the y*ap1* mutant exhibits high contents of protein carbonyl groups and the GSSG:GSH ratio is severely disturbed upon 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 with sulphur and methionine metabolism and with the maintenance of the cell redox homeostasis are activated to mediate adaptation of the wild type strain to 2 mM arsenate treatment.

Keywords: Arsenic stress; oxidative stress; *YAP8 (ACR1, ARR1); YAP1;* transcriptional regulation

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

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 in a 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 *Saccharomyces cerevisiae*, resistance to arsenic is achieved through the activation of the transcriptional regulator Yap8 (Acr1) [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 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 less extent through *YCF1* activation under these conditions and contributing to the full activation of enzymes encoded by the *ACR* genes [12]. Both regulators belong to the Yap (Yeast AP-1) family of bZIP 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 likely 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. Twenty 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 upregulated 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 sulphydryl 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 have 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 homeostasis 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 *yap8*, which absorbs increased amounts of arsenite, in comparison to the parental strain. Since

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Stage 2(a) POST-PRINT

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

the status of protein carbonylation is not changed in the wild type and *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 *yap1* mutant displays high levels of protein oxidation.

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

EXPERIMENTAL

Strains, plasmids and growth conditions

The yeast strains used in this work are: 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 microhomology PCR method [19] to create the strain FT4 *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'CCATTGTAGGAGAGTAACCT3' and 5'CATCGAATACTCCACATCGATC3'. The product was first cloned using the Zero Blunt® TOPO® PCR cloning Kit (Invitrogen) and the *Xba*I/*Bam*HI fragment sub-cloned into the 2 μ vector YEplac195 [20]. The construct was sequenced using the ABI Prism DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and ABI Prism 373A Automatic Sequencer (Perkin Elmer). The plasmid overexpressing *YAP1* was obtained from [18]. The CEN plasmids expressing the myc-tagged *YAP1* and GFP-tagged *YAP1* and *YAP8* versions were obtained from [21], [22] and [12], respectively. 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 (A_{600}=0.4–0.5) were stressed by the addition of 2 mM As(V) (Na_2HAsO) or As(III) ($NaAsO_2$) and samples collected at the indicated time points. Phenotypic growth assays were carried out by spotting 5 μl of an early exponential phase sequentially diluted culture (approximately 2×10^3 to 2×10 cells) in selective medium containing up to 2mM 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* (Tet^r)] (Stratagene) was used as the host for routine cloning purposes. Standard methods were used for genetic analysis, cloning and transformation [23].

Intracellular oxidation, lipid peroxidation and protein carbonylation

Measurements of intracellular oxidation and lipid peroxidation were performed with 50 mg (dry weight) of mid-log 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 2', 7'-dichlorofluorescein diacetate (DCF-DA) [24]. A fresh ethanol stock solution of DCF-DA was added to the culture to a final concentration of 10 μM and cells were incubated 15 min to allow uptake of the probe. Cells from each aliquot were cooled on ice, harvested by centrifugation and washed twice with distilled water. The cell pellets were resuspended in 500 μL of distilled water and lysed by vortexing in the presence of 1.5 g of glass beads. The extracts obtained after centrifugation at 13,000 rpm for 5 min were diluted and fluorescence was measured using a Photo

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Stage 2(a) POST-PRINT

Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

Technology International (PTI) spectrofluorimeter set 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 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 µL of the same buffer containing 10% trichloroacetic acid and 1.5 g of glass beads were added. The samples were lysed by 6 cycles of 20 s agitation on a vortex mixer followed by 20 s on ice. Lipid peroxidation of cell extracts was followed spectrophotometricaly at 532 nm in an EDTA/thiobarbituric acid/NaOH solution according to the methodology of [25], which determines the accumulation in cells of products reacting with 2-thiobarbituric acid (the so-called TBA-reactive products). The control data for each mutant strain grown under physiological conditions was expressed as the amount of malondialdehyde (MDA)/mg cell dry weight formed and the results under arsenic stress were expressed as the relation between the values of stressed and nonstressed cells. Several authors currently use 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 data presented are mean arithmetic values with their respective standard deviation values. To detect the presence of carbonyl groups introduced in proteins as a result of arsenic stress, an Oxyblot® protein oxidation detection kit (Intergen) was used. The samples were analysed by immunoblotting and processed as described in [28], using rabbit anti-DNP antibody as the primary antibody. As a loading control, Sba1 levels were measured [29].

Glutathione Determination

Measurements of thiols were performed by the spectrophotometric GR-DTNB recycling method originally described by Griffith (1980). The wild type and mutant strains grown to early log phase were induced with 2mM As(V) and samples collected at the indicated time points were extracted in a buffer solution prepared with 0.1 N HCl, 1 mM EDTA [28]. Kinetics of TNB 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 to derivatize GSH [30]. The concentration was determined by reference to a standard prepared in HCl and was expressed as nmol glutathione/mg cell dry weight. Measurements were carried out three times, with three replicates in each experiment and the data presented are mean arithmetic values with their respective standard deviation.

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-log cells, and the culture was incubated at 28ºC in a rotating bath for 4 h and 24 h. For measuring residual As(III) present in the medium, 5 mL aliquots were centrifuged and the supernatant was collected and subjected to atomic absorption spectrophotometry using the Atomic Absorption Spectrometer, Perkin Elmer 3100. As(III) absorption was inferred by determining the difference in metalloid content between the control medium without

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

BJ Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

cells and the test medium containing cells. Percentages of As(III) absorption were calculated through the equation:

Absorption (%) = [(initial concentration – final concentration) / initial concentration] X 100. The measurements were carried out three times, with three internal replicates and the data presented are mean arithmetic values with their respective standard deviation.

Protein extraction and Immunoblot analysis

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

Fluorescence microscopy

FT4 *yap8* and FT4 *yap1* strains transformed with pRS *cp-GFP-HA-YAP8* [12] or pRS *cp-GFP-HA-YAP1* [22], respectively, were grown to early log phase and induced with either 2mM As(V) or As(III), at the indicated time points. 4,6-Diamino-2-phenylindole (DAPI) was added as a DNA marker at a final concentration of 5 μg/ml, 5 min before microscopy. After washing with phosphate-buffered saline (PBS), cells were resuspended in DABCO solution (75% (v/v) glycerol, 0.25X PBS 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 localization of the GFP fusions. GFP signals were analyzed in living cells with a LEICA DMRXA fluorescent microscope equipped with a Roper Scientific Micro-Max cooled CCD camera and MetaMorph software (Universal Imaging, Inc.).

Northern Blot, Real time PCR and Microarray analysis

RNA procedures were performed according to [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, approximately 40μg of total RNA were separated in formaldehyde gels, and transferred onto nylon membranes (Hybond XL, Amersham Pharmacia Biotech). Intragenic PCR fragments of *GSH1* and *U3* were used as probes. For real-time quantitative PCR the RNA samples were treated with DNase (Ambion TURBO DNAse-free) according to the manufacturer's instructions. cDNAs were synthesized by reverse transcription from 0,5 μg total RNA, using 50 pmol $(dT)_{15}$, 1mM dNTP and 5U Transcriptor Reverse transcriptase according to the Roche manufacturer's. cDNA amplification was quantitatively analyzed by incorporation of SYBR Green I (LightCycler FastStart DNA Master SYBR Green I, Roche) into dsDNA according to the manufacturer's instructions on a Roche LightCycler II Instrument, using the *ACT1* 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'-

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Stage 2(a) POST-PRINT

Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

GCTGCTGGTAAAAGAGACAATG-3' and 5'-ACTCACATCGTTAGCCTCACAA-3', *TRX2* 5'- GGTCACTCAATTAAAATCCGCTTC -3' and 5'-CGACGACTCTGGTAACCTCCTTAC-3', *SOD1* 5'-AGCCAACCACTGTCTCTTACGA-3' and 5'-ACACCATTTTCGTCCGTCTTTA-3' and *ACT1 5'*-CTA TTG GTA ACG AAA GAT TCA G-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. Ten micrograms of RNA were used to generate labelled cDNA which were hybridised on the DNA arrays, as described in [36]. We used arrays containing probes for most of yeast ORF, 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 [37]. Complete microarray data are available at the journal web site (supplementary table S1). The Gene Ontology (GO) 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 data presented in Table 1 and Supplementary Tables S1 and S2 result from three independent experiments. Only genes measured at least twice were kept for further functional analyses.

Statistical analysis

The results reported in this work are the averages of at least three independent experiments, with three replicates in each experiment, and are expressed as the mean ± standard deviation. Statistical differences among treatments were analysed by one-way analysis variance (ANOVA) with Tukey's HSD (honest significant difference) multiple comparisons test (α=0.05) using STATISTICA for Windows (StatSoft, Inc., Tulsa, OK, USA).

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

BJ Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

RESULTS

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 set of genes [15, 16]. In order to evaluate the physiological relevance of this specificity we performed growth complementation assays (Figure 1). The *yap8* mutant reveals a severe sensitive growth 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 alleviates the growth sensitivity of this strain under arsenite treatment, a finding consistent with the notion that Yap1 and Yap8 exerts specific roles in arsenic stress responses. On the other hand, *YAP1* overexpression is not able to rescue the sensitive phenotype of the *yap8* mutant strain neither under As(V) nor As(III) conditions (Figure 1). Altogether, these results suggest some level of specificity of *YAP1* and *YAP8* in arsenic detoxification.

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 minutes 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 Table S1 and S2 of Supplementary Materials.). This analysis indicated that arsenate up-regulated genes involved in protein folding, sulphur and methionine metabolism (mainly target genes of Met4p), redox homeostasis (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 was 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.

Arsenic treatment generates oxidative damage in the *yap1* mutant

Once established that As(V) induces expression of genes involved in redox homeostasis we carried out 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 DCF-DA that is sensitive to reactive oxygen species. Time-course experiments were previously carried out in order to determine the maximum levels of intracellular oxidation, which occurred at 24 h arsenic treatment (data not shown). The results of Figure 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 (Figure 2 B), being identical in the absence of either *YAP8* or *YAP1* and increasing however when compared to 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 to the wild type strain, were detected

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Stage 2(a) POST-PRINT

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

in the *yap1* mutant through the formation of MDA (Figure 2 C). Exposure of wild type cells to both arsenate and arsenite stress does not cause any significant increase in lipid peroxidation compared to the unstressed cells (Figure 2 D), which is in good agreement with the intracellular oxidation results. In single and double mutants these levels are increased about 1.6-fold and 2.6-fold increased, respectively. Taken together, these data 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 a biomarker of oxidative stress and by far the most commonly used marker 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 followed up to 4 h. The resulting oxyblots show that the amount of carbonylated proteins does not vary significantly in the wild type strain and the mutant *yap8*, although in the latter the levels of oxidised proteins are slightly increased in all time points studied (Figure 3). A more severe effect was however observed in *yap1*. When these cells were treated with arsenate the protein carbonyl content is slightly enhanced compared to the untreated cells. In 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 weight, were observed in the *yap1* strain. These data suggest that the effects of oxidative stress generated by arsenic compounds lead to protein oxidation only in the absence of *yap1*.

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 sulfur into glutathione biosynthesis [16]. Yap1 is an important regulator of *GSH1*, which encodes the enzyme γ- glutamylcysteine synthetase responsible for catalysing the condensation of cysteine onto the gamma carbon 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 hour of treatment with 2 mM As(V) (Figure 4 A). These values rise gradually over the time reaching physiological levels after 4 h. A similar pattern was observed under As(III) treatment (results not shown). The ratio oxidized/reduced glutathione (GSSG:GSH) increases at 1 h treatment as reflecting the decrease of GSH (compare Figures 4 A and B). Notably, the double mutant *Δyap1Δyap8* takes longer times to recover the GSH levels of the parental strain. Our results show that the redox equilibrium is disrupted in strains bearing *YAP1* deletions, although homeostasis is rapidly achieved through the enhancement of GSH generation.

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 to the mutant *yap1*, does not suffer the deleterious effects of oxidative stress upon 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)

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Stage 2(a) POST-PRINT

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

treatment (see Tables S1 and S2 of Supplementary Materials) to facilitate cell adaptation. In order to analyse the cmyc-Yap1 protein levels in cells induced or not with arsenate western blot analyses were performed. As illustrated in Figure 5 A, the levels of Yap1 were rather higher in arsenate-treated cells than in cells grown under physiological conditions. Yap1 protein levels peak around 45 min upon arsenate addition and a slight induction is maintained even up to 12 h treatment. Furthermore, localization assays revealed that GFP-Yap1, as well as GFP-Yap8, are accumulated in the nucleus until 24 h arsenate treatment (Figure 5B).

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. The results in Figure 6 reveal 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 *yap8* mutant than in the wild type strain. Under arsenate conditions 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 *yap8* (see Figure 6A). The pattern of mRNA induction in the wild type and *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 *yap8* mutant (Figure 6 B). Expression of *SOD1* and *TRX2* is completely abolished in the *yap1* mutant (data not shown), consistent with the fact that Yap1 regulates them. Some level of Yap1-independent *GSH1* induction was observed (Figure 6 C), suggesting that other factors might be regulating its expression under arsenic stress.

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. After adding 2 mM arsenite to wild type and *yap8* cultures the residual amounts of the metalloid in the supernatant at the time points indicated in Figure 7 were determined. We also performed measurements in the *yap1* mutant in order to evaluate the contribution of Yap1 to arsenite detoxification. As shown in Figure 7, arsenite absorption in *S. cerevisiae* wild type cells is very low (around 5 %). The *yap8* mutant absorbs higher levels of As(III) (8%) than those observed in the wild type. Increased As(III) absorption in the *yap8* mutant may explain the reason why the antioxidant genes are more activated in this strain than in the wild type (see Figure 6). Surprisingly, *yap1* As(III) absorption is slightly reduced compared to the wild type suggesting that either the uptake is compromised in this strain or the extrusion is enhanced or both events are occurring.

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Stage 2(a) POST-PRINT

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

DISCUSSION

It has been assumed that, among the various modes of action for arsenic carcinogenesis in human cells, the oxidative stress relevance is the of one which 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 reactive oxygen species generated by arsenic compounds. Our data on lipid peroxidation together with intracellular oxidation indicate that *yap1* and *yap8* mutant cells are more oxidized than those of the wild type strain upon treatment with arsenic compounds (Figure 2). The increased lipid peroxidation levels, 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] (diagrammed in Figure 8). This may in turn give rise to 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 *yap1* and *yap8* strains exhibit high levels of lipid peroxidation and intracellular oxidation, however *yap1* is the only one displaying high contents of oxidized proteins (Figure 3). The antioxidant genes *GSH1*, *SOD1* and *TRX2* are induced in the wild type strain being their induction even higher in *yap8* mutant (see Figure 6 A and B). The antioxidant machinery is therefore activated by Yap1, which prevents protein damage in these strains.
Redox-inactive toxic metals such as arsenic react with the reduced form of glutathione, the major antioxidant reserve of the cell [45]. We have in fact observed that during the first hour of treatment with arsenate the levels of GSH are reduced in all strains (Figure 4) with the consequent increase of its oxidized form. As far as GSSG is formed, in order to maintain redox homeostasis, cells must induce the generation of reduced glutathione. This occurs either via its recycling through the glutathione reductase Glr1 or synthesis *de novo*, in which the activity of Gsh1 is essential. The transcriptomic analyses fully supports the activation of both pathways in the wild type strain (see Table 1 and Supplementary Material). We show that genes grouped in functional categories related with sulfur metabolism, sulfur amino acid biosynthesis and metabolism, as well as *GLR1*, is induced when this strain is treated with 2 mM arsenate for 30 min (Table 1). Indeed, it was already shown that arsenite-exposed cells channel a large part of assimilated sulfur into glutathione biosynthesis [16]. In the case of *yap1* the recycling pathway is impaired since *GLR1* is a Yap1 target. Interestingly even in cells harbouring *YAP1* deletion the GSH levels upon 2h exposure to arsenic compounds increased, consistent with the fact that *GSH1* mRNA levels were not completely abolished in the *yap1* mutant treated with both arsenate and arsenite (Figure 6 C). 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 reduced in *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 (Figure 8) through at least the transcriptional activation of antioxidant enzymes encoded by genes such as *TRX2, GSH1* and *SOD1* (Figure 6). Indeed as suggested by others [17], the enhanced *SOD1*

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Stage 2(a) POST-PRINT

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

BJ Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

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 fact that is common to many forms of stress (see Table 1 and Supplementary Materials).

The absence of a significant increase in the 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 diagram in Figure 8). Phenotypic assays reveal, indeed, that the wild type cells are able to grow in the presence of 2 mM arsenate and arsenite (Figure 1). Furthermore, *YAP8* and *YAP1* are both necessary to trigger the adaptation response, exerting a complementary role though at different levels. Being *YAP8* 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 Figures 2, 3 and 7 and diagram in Figure 8).

The *YAP1* gene is shown to be essential to prevent protein oxidation (Figure 3). On the other hand, Yap1 also contributes to the regulated expression of *ACR2* and *ACR3*, through recognizing the cis-element GATTAATAATCA positioned in the divergent promoter of these genes (data not shown). It also regulates the expression of the vacuolar pump encoded by *YCF1*, which composes a parallel arsenite detoxification pathway by catalysing the ATP driven uptake of As(III)-GSH conjugates into the vacuole [1, 49]. It is interesting the fact that the *yap1* mutant absorbs lower levels of arsenite compared to 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 with 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 *yap1* strain this process is not fully operating.

In conclusion, we identified an oxidative pathway dependent on Yap1 involved in the response of *S. cerevisiae* to arsenic compounds.

Acknowledgments – We are grateful to Prof. Peter Piper (University of Sheffield) for providing us with the anti-Sba1 antibody and to Dr. Delmo Santiago Vaitsman (UFRJ, Rio de Janeiro, Brazil) for measuring As(III) atomic absorption. This project was financially supported by FCT, POCTI to CRP. The plate-forme transcriptome IFR36 is funded by the Reseau Nationale des Génopoles. LIFE Laboratory is supported by CAPES/PROCAD and FAPESP (grant 04-10067/6). RAM and CA were supported by FCT fellowships.

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

REFERENCES

1 Bhattacharjee, H. and Rosen, B. P. (2007) Arsenic Metabolism in Prokaryotic and Eukaryotic Microbes. In Microbiology of Heavy Metals (Nies , D. H. and Silver, S., eds.). pp. 371-406, Springer-Verlag, Berlin Heildelberg

2 Rosen, B. P. (2002) Transport and detoxification systems for transition metals, heavy metals and metalloids in eukaryotic and prokaryotic microbes. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. **133**, 689-693

3 Davison, K., Cote, S., Mader, S. and Miller, W. H. (2003) Glutathione depletion overcomes resistance to arsenic trioxide in arsenic-resistant cell lines. Leukemia. **17**, 931-940

4 Patrick, L. (2003) Toxic metals and antioxidants: Part II. The role of antioxidants in arsenic and cadmium toxicity. Altern. Med. Rev. **8**, 106-128

5 Rodrigues-Pousada, C., Nevitt, T. and Menezes, R. (2005) The yeast stress response. Role of the Yap family of b-ZIP transcription factors. The PABMB Lecture delivered on 30 June 2004 at the 29th FEBS Congress in Warsaw. Febs J. **272**, 2639-2647

6 Wysocki, R., Bobrowicz, P. and Ulaszewski, S. (1997) The Saccharomyces cerevisiae ACR3 gene encodes a putative membrane protein involved in arsenite transport. J. Biol. Chem. **272**, 30061-30066

7 Ghosh, M., Shen, J. and Rosen, B. P. (1999) Pathway of AS(III) detoxification in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA. **96**, 5001-5006

8 Mukhopadhyay, R., Shi, J. and Rosen, B. P. (2000) Purification and Characterization of Acr2p, the Saccharomyces cerevisiae Arsenate Reductase. J. Biol. Chem. **275**, 21149-21157

9 Mukhopadhyay, R. and Rosen, B. P. (2001) The Phosphatase C(X)5R Motif Is Required for Catalytic Activity of the *Saccharomyces cerevisiae* Acr2p Arsenate Reductase. J. Biol. Chem. **276**, 34738-34742

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

11 Li, Z. S., Szczypka, M., Lu, Y. P., Thile, D. J. and Rea, P. A. (1996) The yeast cadmiun factor protein (*YCF1)* is a vacuolar glutathione S-conjugate pump. J. Biol. Chem. **271**, 6509-6517

12 Menezes, R. A., Amaral, C., Delaunay, A., Toledano, M. and Rodrigues-Pousada, C. (2004) Yap8p activation in Saccharomyces cerevisiae under arsenic conditions. FEBS Lett. **566**, 141-146

13 Kitchin, K. T. and Ahmad, S. (2003) Oxidative stress as a possible mode of action for arsenic carcinogenesis. Toxicol. Lett. **137**, 3-13

14 Vujcic, M., Shroff, M. and Singh, K. K. (2007) Genetic determinants of mitochondrial response to arsenic in yeast Saccharomyces cerevisiae. Cancer Res. **67**, 9740-9749

15 Haugen, A. C., Kelley, R., Collins, J. B., Tucker, C. J., Deng, C., Afshari, C. A., Brown, J. M., Ideker, T. and Van Houten, B. (2004) Integrating phenotypic and expression profiles to map arsenic-response networks. Genome Biol. **5**, R95

16 Thorsen, M., Lagniel, G., Kristiansson, E., Junot, C., Nerman, O., Labarre, J. and Tamas, M. J. (2007) Quantitative transcriptome, proteome, and sulfur metabolite profiling of the Saccharomyces cerevisiae response to arsenite. Physiol. Genomics. **30**, 35-43

17 Samokhvalov, V. A., Museikina, N., Mel'nikov, G. V. and Ignatov, V. V. (2003) [Arsenite-induced lipid peroxidation in Saccharomyces cerevisiae]. Mikrobiologiia. **72**, 308-311

18 Fernandes, L., Rodrigues-Pousada, C. and Struhl, K. (1997) Yap, a Novel Family of Eight bZIP Proteins in Saccharomyces cerevisiae with Distinct Biological Functions. Mol. Cell. Biol. **17**, 6982-6993

19 Guldener, U., Heck, S., Fielder, T., Beinhauer, J. and Hegemann, J. H. (1996) A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res. **24**, 2519-2524

20 Gietz, R. D. and Sugino, A. (1988) New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene. **74**, 527-534

21 Delaunay, A., Isnard, A. D. and Toledano, M. B. (2000) H2O2 sensing through oxidation of the Yap1 transcription factor. Embo J. **19**, 5157-5166

22 Kuge, S., Jones, N. and Nomoto, A. (1997) Regulation of yAP-1 nuclear localization in response to oxidative stress. Embo J. **16**, 1710-1720

23 Ausubel, F. M., Katagiri, F., Mindrinos, M. and Glazebrook, J. (1995) Use of Arabidopsis thaliana defense-related mutants to dissect the plant response to pathogens. Proc. Natl. Acad. Sci. U S A. **92**, 4189-4196

24 Davidson, J. F., Whyte, B., Bissinger, P. H. and Schiestl, R. H. (1996) Oxidative stress is involved in heat-induced cell death in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U S A. **93**, 5116-5121

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

BJ Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

25 Steels, E. L., Learmonth, R. P. and Watson, K. (1994) Stress tolerance and membrane lipid unsaturation in Saccharomyces cerevisiae grown aerobically or anaerobically. Microbiology. **140 (Pt 3)**, 569-576

26 Almeida, T., Marques, M., Mojzita, D., Amorim, M. A., Silva, R. D., Almeida, B., Rodrigues, P., Ludovico, P., Hohmann, S., Moradas-Ferreira, P., Corte-Real, M. and Costa, V. (2008) Isc1p Plays a Key Role in Hydrogen Peroxide Resistance and Chronological Lifespan through Modulation of Iron Levels and Apoptosis. Mol. Biol. Cell. **19**, 865-876

27 Gomes, D. S., Pereira, M. D., Panek, A. D., Andrade, L. R. and Eleutherio, E. C. (2008) Apoptosis as a mechanism for removal of mutated cells of Saccharomyces cerevisiae: the role of Grx2 under cadmium exposure. Biochim. Biophys. Acta. **1780**, 160-166

28 Santos, C., Gaspar, M., Caeiro, A., Branco-Price, C., Teixeira, A. and Ferreira, R. B. (2006) Exposure of Lemna minor to arsenite: expression levels of the components and intermediates of the ubiquitin/proteasome pathway. Plant Cell Physiol. **47**, 1262-1273

29 Mollapour, M. and Piper, P. W. (2007) Hog1 mitogen-activated protein kinase phosphorylation targets the yeast Fps1 aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. Mol. Cell. Biol. **27**, 6446-6456

30 Griffith, O. W. (1980) Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal. Biochem. **106**, 207-212

31 Adamis, P. D., Gomes, D. S., Pereira, M. D., Freire de Mesquita, J., Pinto, M. L., Panek, A. D. and Eleutherio, E. C. (2004) The effect of superoxide dismutase deficiency on cadmium stress. J. Biochem. Mol. Toxicol. **18**, 12-17

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

33 Fang, Y., Fliss, A. E., Rao, J. and Caplan, A. J. (1998) SBA1 encodes a yeast hsp90 cochaperone that is homologous to vertebrate p23 proteins. Mol. Cell. Biol. **18**, 3727-3734

34 Truman, A. W., Millson, S. H., Nuttall, J. M., Mollapour, M., Prodromou, C. and Piper, P. W. (2007) In the yeast heat shock response, Hsf1-directed induction of Hsp90 facilitates the activation of the Slt2 (Mpk1) mitogen-activated protein kinase required for cell integrity. Eukaryot. Cell. **6**, 744-752

35 Livak, K. J. and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. **25**, 402-408

36 Fardeau, V., Lelandais, G., Oldfield, A., Salin, H., Lemoine, S., Garcia, M., Tanty, V., Le Crom, S., Jacq, C. and Devaux, F. (2007) The central role of PDR1 in the foundation of yeast drug resistance. J Biol. Chem. **282**, 5063-5074

37 Lemoine, S., Combes, F., Servant, N. and Le Crom, S. (2006) Goulphar: rapid access and expertise for standard two-color microarray normalization methods. BMC Bioinformatics. **7**, 467

38 Boorsma, A., Foat, B. C., Vis, D., Klis, F. and Bussemaker, H. J. (2005) T-profiler: scoring the activity of predefined groups of genes using gene expression data. Nucleic Acids Res. **33**, 592-595.

39 Fauchon, M., Lagniel, G., Aude, J. C., Lombardia, L., Soularue, P., Petat, C., Marguerie, G., Sentenac, A., Werner, M. and Labarre, J. (2002) Sulfur sparing in the yeast proteome in response to sulfur demand. Mol. Cell. **9**, 713-723

40 Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A. and Colombo, R. (2003) Protein carbonyl groups as biomarkers of oxidative stress. Clin. Chim. Acta. **329**, 23-38

41 Shi, H., Shi, X. and Liu, K. J. (2004) Oxidative mechanism of arsenic toxicity and carcinogenesis. Mol. Cell. Biochem. **255**, 67-78

42 Lin, S., Cullen, W. R. and Thomas, D. J. (1999) Methylarsenicals and arsinothiols are potent inhibitors of mouse liver thioredoxin reductase. Chem. Res. Toxicol. **12**, 924-930

43 Styblo, M., Serves, S. V., Cullen, W. R. and Thomas, D. J. (1997) Comparative inhibition of yeast glutathione reductase by arsenicals and arsenothiols. Chem. Res. Toxicol. **10**, 27-33

44 Ahmad, S., Kitchin, K. T. and Cullen, W. R. (2000) Arsenic species that cause release of iron from ferritin and generation of activated oxygen. Arch. Biochem. Biophys. **382**, 195-202

45 Ercal, N., Gurer-Orhan, H. and Aykin-Burns, N. (2001) Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. Curr. Top. Med. Chem. **1**, 529-539

46 Wheeler, G. L., Trotter, E. W., Dawes, I. W. and Grant, C. M. (2003) Coupling of the transcriptional regulation of glutathione biosynthesis to the availability of glutathione and methionine via the Met4 and Yap1 transcription factors. J. Biol. Chem. **278**, 49920-49928

47 Dormer, U. H., Westwater, J., McLaren, N. F., Kent, N. A., Mellor, J. and Jamieson, D. J. (2000) Cadmium-inducible expression of the yeast GSH1 gene requires a functional sulfur-amino acid regulatory network. J. Biol. Chem. **275**, 32611-32616

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Stage 2(a) POST-PRINT

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

48 Stephen, D. W., Rivers, S. L. and Jamieson, D. J. (1995) The role of the YAP1 and YAP2 genes in the regulation of the adaptive oxidative stress responses of Saccharomyces cerevisiae. Mol. Microbiol. **16**, 415-423

49 Dilda, P. J., Perrone, G. G., Philp, A., Lock, R. B., Dawes, I. W. and Hogg, P. J. (2008) Insight into the selectivity of arsenic trioxide for acute promyelocytic leukemia cells by characterizing Saccharomyces cerevisiae deletion strains that are sensitive or resistant to the metalloid. Int. J. Biochem. Cell. Biol. **40**, 1016-1029

50 Thorsen, M., Di, Y., Tangemo, C., Morillas, M., Ahmadpour, D., Van der Does, C., Wagner, A., Johansson, E., Boman, J., Posas, F., Wysocki, R. and Tamas, M. J. (2006) The MAPK Hog1p modulates Fps1p-dependent arsenite uptake and tolerance in yeast. Mol. Biol. Cell. **17**, 4400-4410

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

Table 1. GO analyses of the arsenate transcriptional response. T-profiler [38] was used to conduct GO analyses from the global set of microarray data (see methods). The T-values and E-values are directly taken from the t-profiler output. Redundant or meaningless GO 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	<1.0e-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	5.3e-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	5.4e-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	2.0e-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	3.6e-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	4.31	2.2e-2	*UBC4* (2.2), *RAD6* (1.9), *RPN8* (1.9), *RPN4* (1.9),

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

complex			*SCL1* (1.8), *UBP6* (1.7), *PRE8* (1.7)…

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

LEGENDS

Figure 1: Arsenic sensitivity phenotypes and respective recovery by overexpression of *YAP1* and *YAP8*. Wild type, *Δyap1* and *Δyap8* mutant strains were transformed with YEplac195 (empty vector), YEp*YAP1* or YEp*YAP8* and serially diluted cultures were spotted onto selective medium supplemented with increasing concentrations of arsenate or arsenite (up to 2 mM). Plates were grown for 2 days at 30ºC. A representative experiment is shown. WT – wild type, PHY – physiological conditions.

Figure 2: Increase of intracellular oxidation and lipid peroxidation caused by arsenic stress. A) Changes in DCFH fluorescence caused by deletions were followed under physiological conditions. The values are expressed as photons/sec. B) Control and mutant strains were stressed with 2 mM arsenate or arsenite during 24 h and the enhancement of the intracellular oxidation was expressed as a relation between the fluorescence of stressed and nonstressed cells. C) The effect of deletions in the lipid peroxidation content was measured as pmol of MDA/mg cell dry weight by the method of TBARS. D) The enhancement of lipid peroxidation was measured as a relation between the levels observed for stressed and nonstressed cells. The bold line in panels B and D indicates the relative value under nonstressed condition. Values are means ± SD of three independent experiments. **$p<0.01$. WT – wild type, white bars - arsenate, black bars - arsenite.

Figure 3: Immunological detection of protein carbonyl groups in the *Saccharomyces cerevisiae* wild type 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-dinitrophenyl-hydrazone (DNP) and were immunobloted with anti-DNP antibodies. The Sba1 is used as an internal loading control. C – OxyBlot TM Protein Standard (Intergen). A representative experiment is shown.

Figure 4: Antioxidant defences during arsenic treatment. A) The wild type, *Δyap1*, *Δyap8* and *Δyap1Δyap8* mutant cells were grown to mid-exponential phase and were exposed to 2 mM arsenate treatment. Extracts were obtained at the time points indicated and GSH levels were determined as described in the experimental section. Values are means ± SD of three independent experiments. B) GSSG levels were determined in the same extracts after its derivatization to GSH and the GSSG/GSH ratio was inferred. **$p<0.01$. WT – wild type.

Figure 5: Yap1 is activated in a long-term arsenate exposure. A) BY *yap1* mutant cells transformed with a CEN-plasmid encoding the fusion *cmyc-YAP1* were grown under physiological conditions or in the presence of 2 mM arsenate and samples 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 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.

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Biochemical Journal Immediate Publication. Published on 25 Apr 2008 as manuscript BJ20071537

Figure 6: Effect of As(V) and As(III) on transcriptional activation of *GSH1*, *SOD1* and *TRX2* in the wild type and *Δyap8* 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 Blot as described. The mRNA levels for *U3* were used as an internal loading control. WT –wild type. Representative experiments are shown.

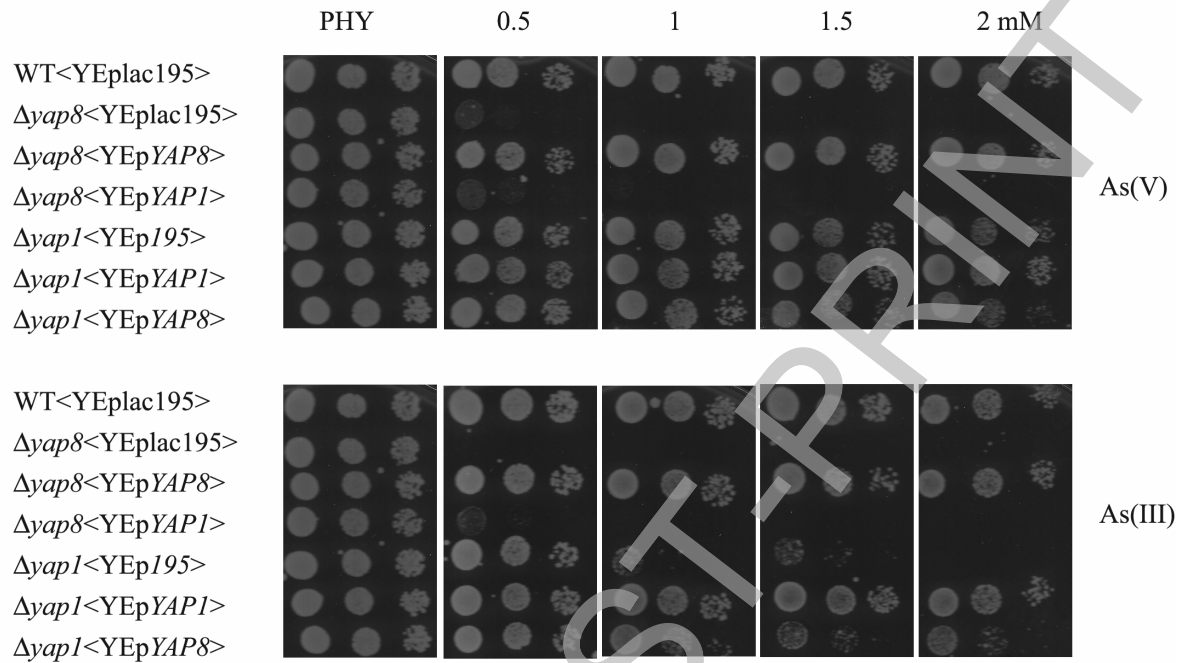
Figure 7: Effect of *YAP1* and *YAP8* deletion on As (III) absorption. Atomic absorption analyses of As(III) were carried out in buffer solution containing 1 mg (dry weight) of *Saccharomyces cerevisiae* wild type or mutant cells and the amount of As(III) absorbed by was estimated by determining the difference between the initial As(III) added (2 mM) and the residual As(III) present in the medium after 4 (white bars) and 24 h (black bars). The percentage of As(III) absorbed was inferred by determining the relation between As(III) uptaken by cells and the initial As(III). Values are means ± SD of three independent experiments. **p<0.01. WT – wild type.

Figure 8: Schematic representation of the 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 defences in order to scavenge the ROS generated as a secondary effect of arsenic exposure.

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

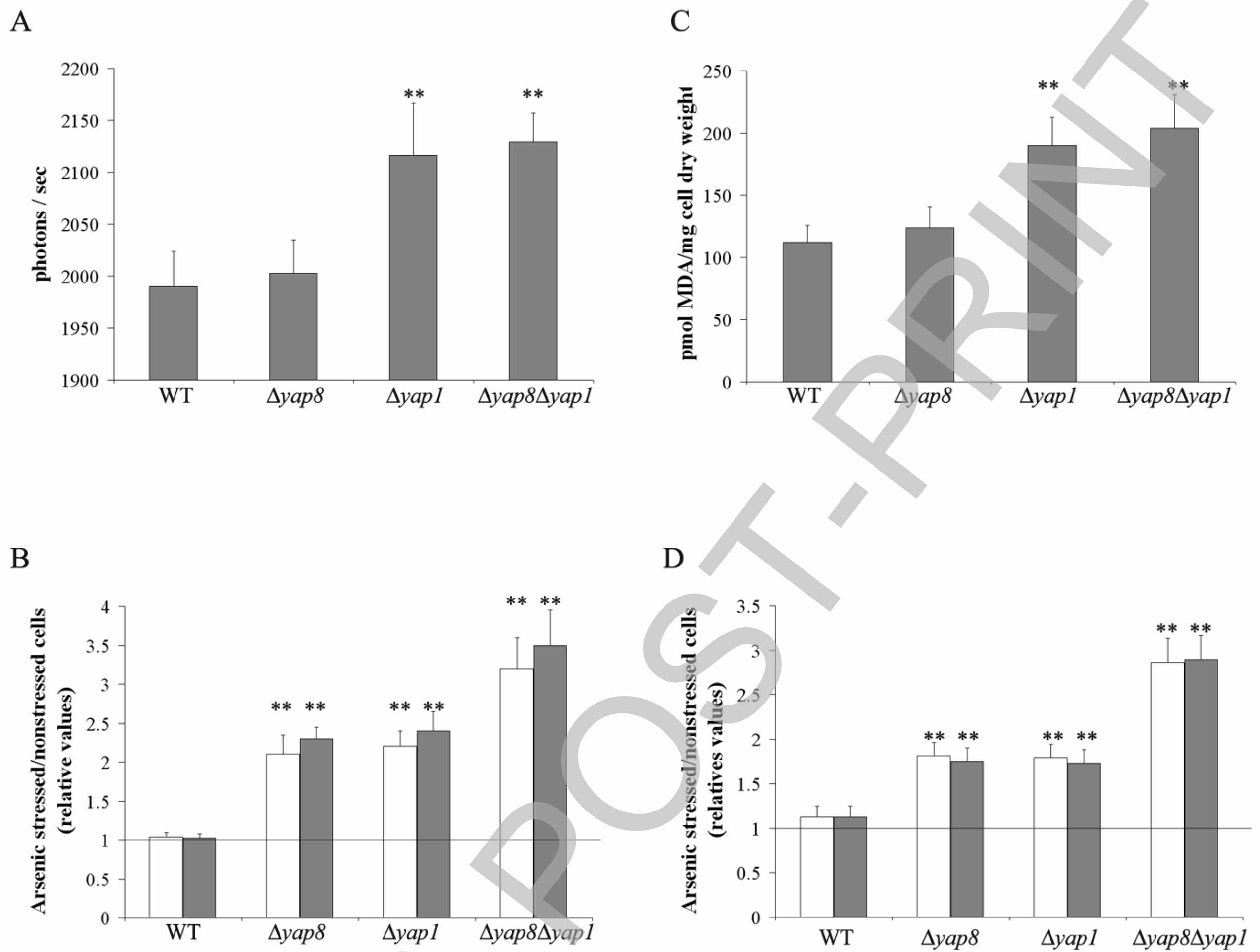


Menezes *et al*-Fig.1

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

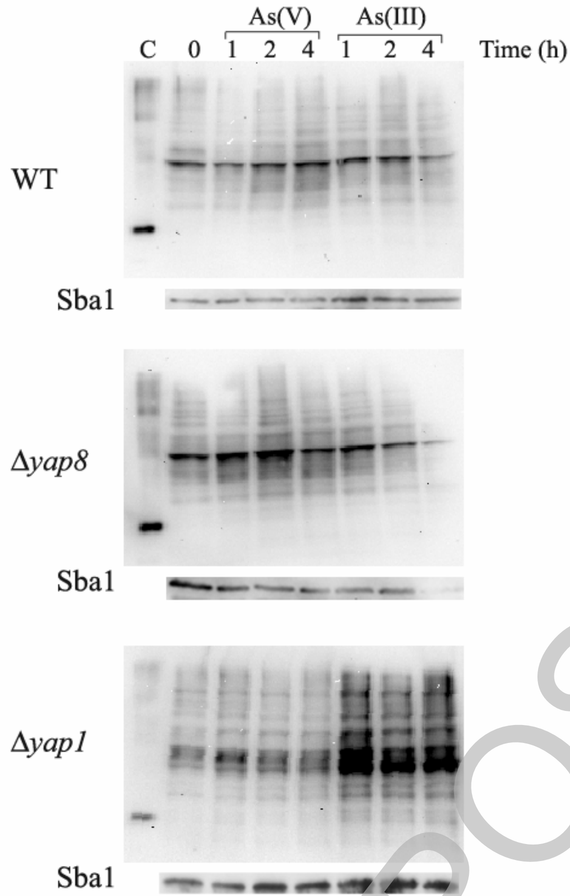


Menezes *et al*-Fig.2

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

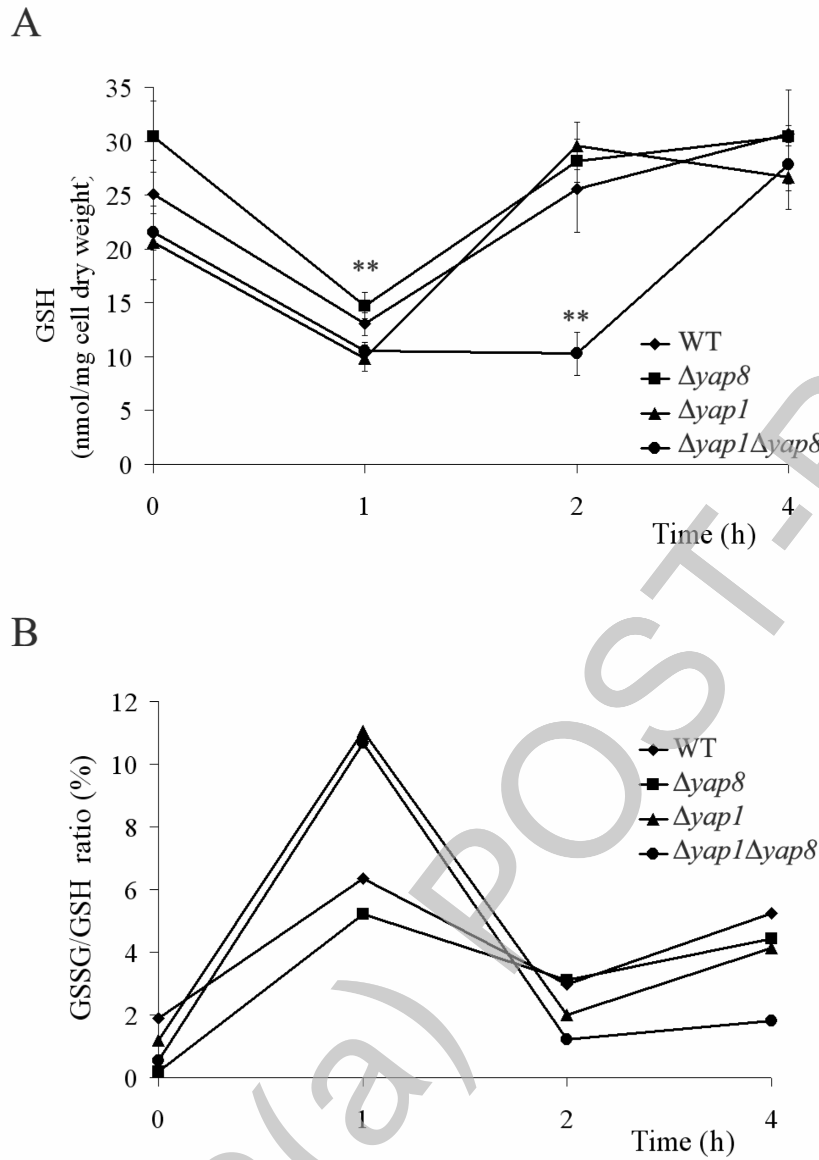


Menezes *et al*-Fig.3

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

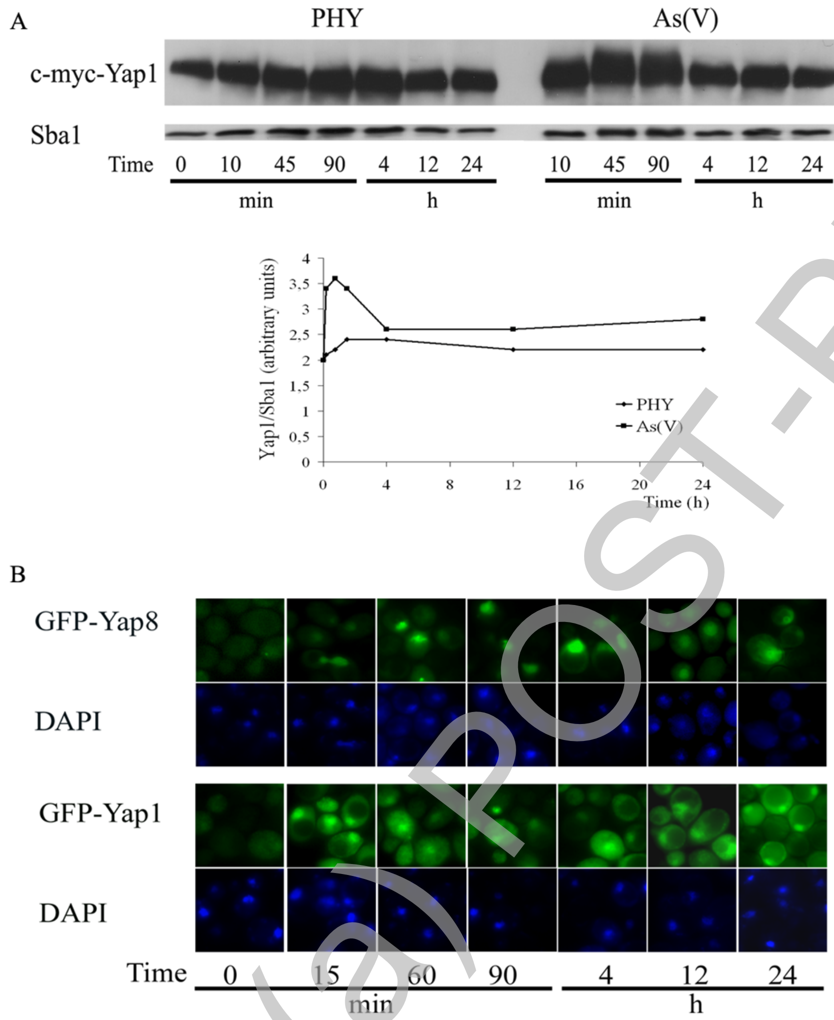


Menezes *et al*-Fig.4

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

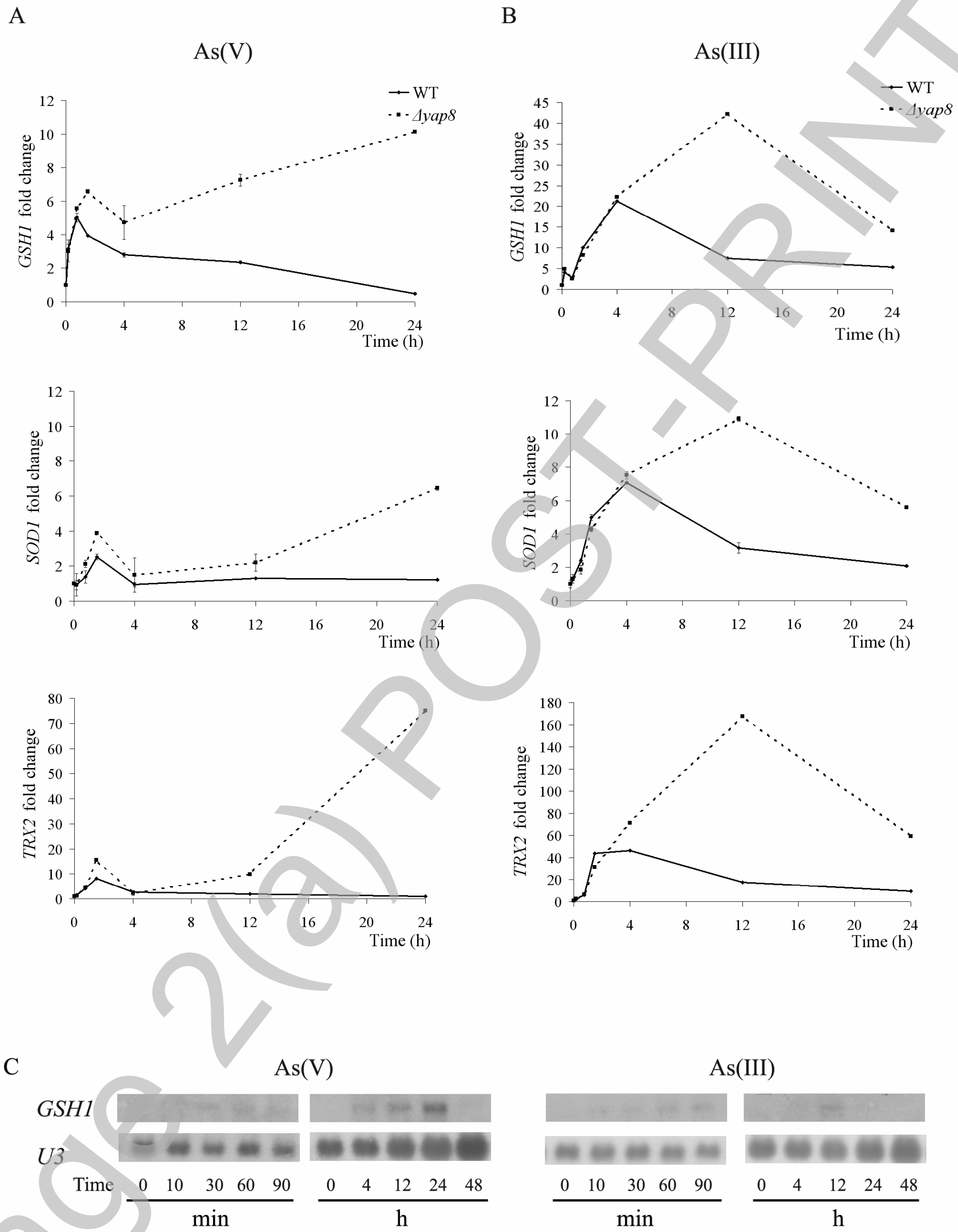


Menezes *et al*-Fig.5

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

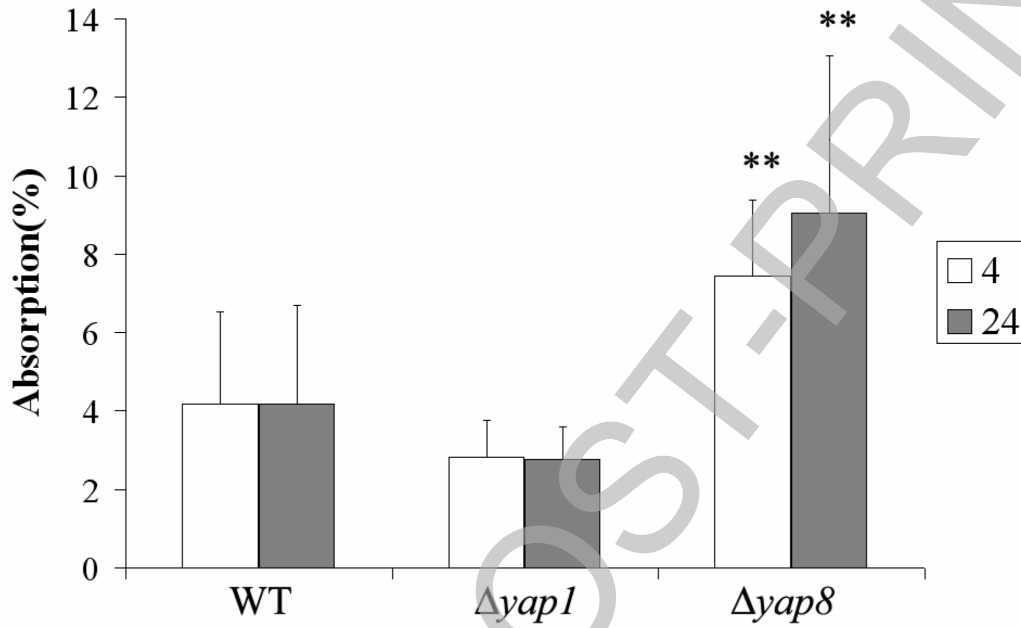


Menezes, *et al*-Fig.6

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society

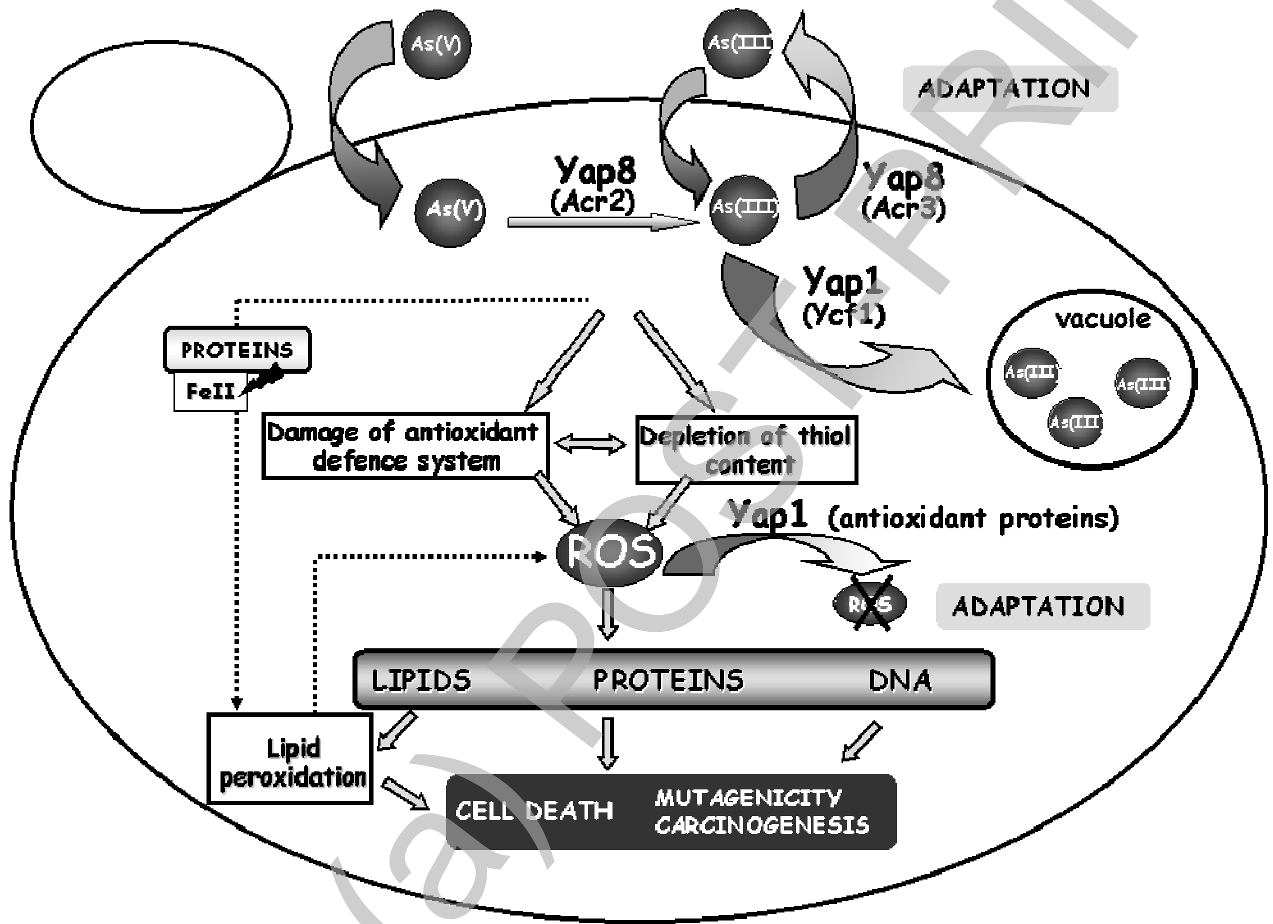


Menezes *et al*-Fig7

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society



Menezes *et al*-Fig.8

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071537

Stage 2(a) POST-PRINT

Licenced copy. Copying is not permitted, except with prior permission and as allowed by law.
© 2008 The Authors Journal compilation © 2008 Biochemical Society