Regulation of the arsenic-responsive transcription factor Yap8p involves the ubiquitin-proteasome pathway

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Summary

Toxic metals are ubiquitous in the environment and all organisms possess systems to evade toxicity and acquire tolerance. The *Saccharomyces cerevisiae* AP-1-like protein Yap8p (systematic name YPR199c; also known as Acr1p and Arr1p) confers arsenic tolerance by stimulating enhanced transcription of the arsenic-specific detoxification genes *ACR2* and *ACR3*. Here, we report that Yap8p is regulated at the level of degradation. We show that Yap8p is stabilized in arsenite-exposed cells in a time- and dose-dependent manner. Yap8p degradation proceeds through the ubiquitin-proteasome pathway and is dependent on the ubiquitin-conjugating enzyme Ubc4p. Further, we show that mutants that are defective in the ubiquitin-proteasome pathway display increased Yap8p levels and elevated expression of the Yap8p gene-target *ACR3*. Yap8p forms homodimers in vivo but dimerization is not regulated by arsenite. Instead, arsenite-stimulated Yap8p stabilization and transcriptional activation of *ACR3* requires critical cysteine residues within Yap8p. Collectively, our data is consistent with a model where Yap8p is degraded by the ubiquitin-proteasome pathway in untreated cells, whereas arsenite-exposure results in Yap8p stabilization and gene activation. In this way, regulated degradation contributes to Yap8p control.

Key words: Arsenite, Yap8p, Ubiquitin-proteasome pathway, Protein degradation, Yeast

Introduction

Toxic metals are ubiquitously present in nature and all organisms have developed mechanisms to evade toxicity and to acquire tolerance. These mechanisms include increased metal export from the cell, sequestration within internal organelles, chelation by metal-binding proteins and peptides, and reduction of uptake. The activity of tolerance and detoxification systems may be controlled at transcriptional and/or post-transcriptional levels (Rosen, 2002; Rosen, 1999; Tamás et al., 2005; Tamás and Wysocki, 2001). Several prokaryotic metal-responsive transcription factors have been characterized at the molecular, biophysical and structural levels. However, less is known about eukaryotic metal-responsive transcription factors and most information available concerns factors controlled by nutrient metals (Rutherford and Bird, 2004; van Bakel and Wijmenga, 2005). Three transcription factors contribute to nonessential metal tolerance in *Saccharomyces cerevisiae* (budding yeast); Yap1p, Met4p and Yap8p (systematic name YPR199c; also known as Acr1p and Arr1p). These proteins control expression of various detoxification systems and display distinct mechanisms of regulation. Yap1p, the most well-characterized member of the yeast AP-1 family, is a central regulator of responses elicited by oxidants, by chemicals with electrophilic properties, and by metals including arsenic, cadmium, antimony and mercury (Tamás et al., 2005; Toledano et al., 2004). All these agents appear to control Yap1p through regulated nuclear export by stress-induced modifications of conserved cysteine residues. These modifications involve disulphide-bond-formation in response to peroxides (Delaunay et al., 2000; Kuge et al., 2001; Kuge et al., 1997) or direct binding of thiol-reactive chemicals (Azevedo et al., 2003) and possibly also of metals. Such thiol modifications are likely to produce a conformational change resulting in Yap1p nuclear retention, as recently established by structural analysis of oxidized Yap1p (Wood et al., 2004). Met4p controls the assimilation of sulphate into sulphur-containing amino acids and glutathione. Met4p regulation is complex and involves the ubiquitin-proteasome pathway: depending on the growth condition, Met4p is either inactivated by ubiquitylation followed by degradation or inactivated by oligo-ubiquitylation without degradation (Kaiser et al., 2000; Rouillon et al., 2000). In response to cadmium and possibly also to arsenic, both mechanisms of inhibition are overridden to enable Met4p activation by rapid removal of inhibitory ubiquitin moieties as well as by inhibition of Met4p degradation through dissociation of the SCF^{Met30} ubiquitin ligase (Barbey et al., 2005; Yen et al., 2005).

The AP-1-like protein Yap8p specifically contributes to arsenic tolerance. It has been firmly established that Yap8p mediates arsenic-induced expression of *ACR2* (*ARR2*), encoding a cytosolic arsenate [As(V)] reductase, and *ACR3* (*ARR3*), encoding a plasma membrane arsenite [As(III)] efflux protein. Cells lacking *YAP8* fail to induce expression of these genes and display strong arsenic sensitivity (Bobrowicz and Ulaszewski, 1998; Haugen et al., 2004; Menezes et al., 2004; Wysocki et al., 2004). How Yap8p is regulated is less clear. We

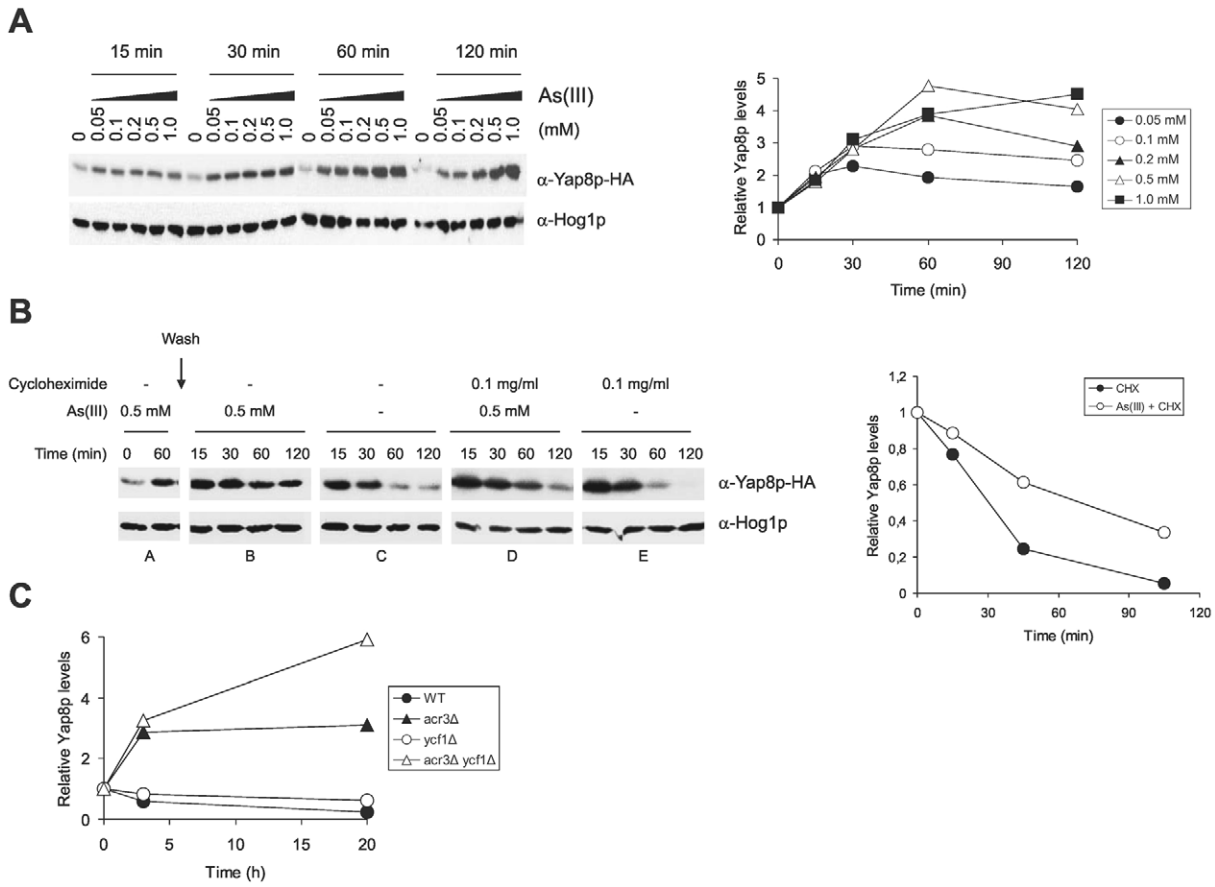

Fig. 1. Yap8p is stabilized in the presence of arsenite. (A) Yap8p-HA was expressed from the constitutive *TPI1* promoter in the *yap8Δ* mutant (W303-1A background) and samples were taken for western blot analysis at the time-points indicated prior to and after exposing cells to increasing concentrations of sodium arsenite [As(III)]. The concentrations used are indicated above the blot. Hog1p was used as loading control and Yap8p protein levels were quantified and normalized to the Hog1p level of each lane. (B) Cells expressing Yap8p-HA were exposed to 0.5 mM As(III) for 1 hour (panel A), then washed and placed in growth medium with or without As(III) (panels B-E). Cycloheximide (0.1 mg/ml) was added as indicated and Yap8p-HA levels were monitored by western blot analysis. Yap8p protein levels in the presence of cycloheximide (CHX) were quantified and normalized to the Hog1p level as described above. (C) Yap8p-HA levels correlate with the cytosolic As(III) concentration. Yap8p protein levels were quantified as described above in wild-type, *acr3Δ*, *ycf1Δ* and *acr3Δ ycf1Δ* cells treated with 10 μM As(III) for the indicated time.

previously demonstrated that Yap8p resides predominantly in the nucleus by monitoring a GFP-Yap8p fusion protein as well as by detecting a genomic copy of Myc-tagged Yap8p in nuclear extracts (Wysocki et al., 2004). Chromatin immunoprecipitation assays further strengthened the notion that at least a portion of Yap8p is nuclear because Yap8p was found to be associated with the *ACR3* promoter in both untreated and As(III)-exposed cells (Wysocki et al., 2004). By contrast, Menezes et al. found the majority of GFP-Yap8p in the cytoplasm, and also that As(III)-treatment triggered its nuclear accumulation (Menezes et al., 2004). The cause of the discrepancy between these studies is not clear but could be due to the use of different strains and/or expression systems. Alternatively, Yap8p might consist of both a cytosolic fraction and a nuclear fraction, as shown for the yeast Jun-like transcription factor Gcn4p (Pries et al., 2002).

Our finding that Yap8p is associated with the *ACR3* promoter both in the absence and presence of arsenite (Wysocki et al., 2004) suggests that Yap8p regulation is neither exerted at the level of localisation nor at the level of As(III)-stimulated binding to the *ACR3* promoter. Correct Yap8p function requires cysteine residues that are conserved in several fungal AP-1 proteins; the Yap8p-C132A, C137A and C274A mutants were unable to induce As(III)-instigated *ACR3* expression and, consequently, failed to promote cellular arsenic tolerance. On the basis of these data, these cysteines were suggested to be implicated in Yap8p transactivation function (Menezes et al., 2004; Wysocki et al., 2004).

Environmental or chemical stress may result in protein destabilization and the ubiquitin-proteasome pathway provides a mechanism to remove damaged proteins. In addition, the ubiquitin-proteasome pathway regulates a broad range of cellular processes including metabolic adaptations, cell cycle progression, differentiation and also signalling and gene regulation. Ubiquitylation of protein substrates proceeds by a step-wise process involving three enzymes; ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3). Poly-ubiquitylated proteins are then targeted to the 26S proteasome for degradation (Ciechanover, 2005; Glickman and Ciechanover, 2002; Varshavsky, 2005).

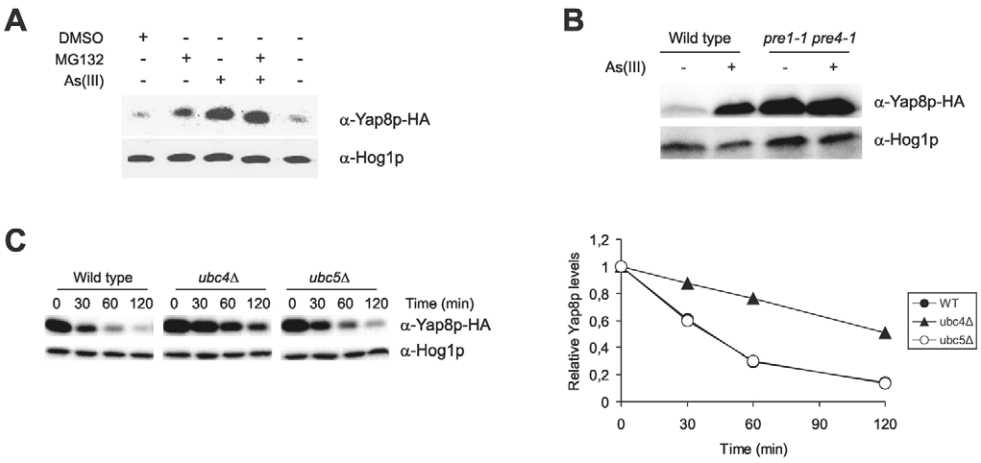

Fig. 2. Yap8p degradation involves the ubiquitin-proteasome pathway. (A) The *erg6Δ* mutant (BY4741 background) was transformed with a plasmid expressing Yap8p-HA. Proteasome activity was reduced by adding MG132 (0.1 mM) to cell cultures and Yap8p-HA levels were monitored as above. Since MG132 was dissolved in DMSO, an equal volume of this solvent was added to the control sample. A long exposure time was needed to visualize Yap8p-HA in the control sample of the *erg6Δ* strain. (B) Yap8p levels increase in the *pre1-1 pre4-1* mutant that is defective in the β-type subunits of the catalytic 20S core of the proteasome. The Yap8p-HA plasmid was transformed into wild type (WCG4a background) and the *pre1-1 pre4-1* mutant, and Yap8p-HA levels were monitored by western blot analysis. (C) The rate of Yap8p degradation is reduced in cells lacking the ubiquitin-conjugating enzyme Ubc4p. Cells (in BY4741 background) expressing Yap8p-HA were exposed to 0.5 mM As(III) for 1 hour, then washed and placed in growth medium without As(III). Cycloheximide (0.1 mg/ml) was added and Yap8p-HA levels were monitored by western blot analysis. Yap8p protein levels were quantified and normalized to the Hog1p level of each lane.

The goal of the present study was to get insight into Yap8p regulation in response to arsenite. We demonstrate that, Yap8p is degraded by the ubiquitin-proteasome pathway in the absence of As(III), and As(III)-exposure results in Yap8p stabilization and target gene activation.

Results

Yap8p is stabilized in arsenite-exposed cells

To get insight into Yap8p regulation, we generated two epitope-tagged versions of this transcription factor: the Yap8p-HA fusion protein was expressed from the constitutive *TPI1* promoter [*TPI1* expression is unaffected by As(III); data not shown] from a centromeric plasmid whereas Myc_9-Yap8p (nine tandem repeats of *myc* fused to the gene encoding Yap8p) was expressed from the native *YAP8* promoter on a 2μ plasmid. Both fusion proteins fully complemented the As(III) sensitivity of the *yap8Δ* mutant (data not shown). We next monitored Yap8p by western blot analysis prior to and during exposure to a range of As(III) concentrations. As shown in Fig. 1A, Yap8p-HA levels were low in unexposed cells whereas As(III) treatment resulted in increased protein levels. More Yap8p-HA accumulated when cells were incubated with higher As(III) concentrations and an increase over time was also observed (Fig. 1A). In fact, elevated Yap8p-HA levels were detected already at a very low concentration [0.05 mM As(III)] at which growth of wild-type cells is unaffected. Similar results were obtained irrespective of whether the protein was expressed from the constitutive *TPI1* promoter (Fig. 1A) or the native *YAP8* promoter (data not shown).

Previous studies revealed that *YAP8* mRNA levels are not significantly altered in response to As(III) (Haugen et al., 2004; Menezes et al., 2004) and we confirmed this finding also for the W303-1A strain background (data not shown). Hence, the dose-dependent increase in Yap8p levels observed (Fig. 1A) is probably not a result of altered *YAP8* gene expression. To test whether Yap8p half-life is affected by As(III), we first exposed *yap8Δ* cells expressing Yap8p-HA to 0.5 mM As(III) for 1 hour to increase protein levels (Fig. 1B, panel A), then washed and transferred the cells to As(III)-free growth medium and monitored Yap8p-HA by western blot analysis. Interestingly, Yap8p-HA levels diminished when cells were transferred to As(III)-free medium and returned to pre-exposure level (Fig. 1B, panel A, sample at 0 minutes) after 60 minutes (Fig. 1B, panel C). By contrast, Yap8p-HA remained at an elevated level in control cells where As(III) exposure persisted (Fig. 1B, panel B). To distinguish whether Yap8p is regulated at the translational or post-translational level, we monitored Yap8p-HA in the presence of cycloheximide. We observed a similar rate of decrease in Yap8p-HA levels when cells were transferred to As(III)-free medium in the presence of the protein synthesis inhibitor (Fig. 1B, panel E). Quantification of Yap8p protein levels indicated that the half-life of Yap8p increased two- to three-fold in response to As(III) (Fig. 1B). We conclude that the abundance of Yap8p is regulated at the post-translational level.

We next monitored Yap8p-HA in the detoxification-defective *acr3Δ* and *ycf1Δ* mutants. The *acr3Δ* mutant cannot mediate As(III) efflux and therefore accumulates As(III) over time, whereas *ycf1Δ* cannot catalyse vacuolar As(III) uptake, resulting in elevated cytosolic As(III) levels. Note that Acr3p plays a more prominent role in As(III) detoxification than Ycf1p (Ghosh et al., 1999; Wysocki et al., 1997; Wysocki et al., 2001). Yap8p-HA was largely unaffected by a low As(III) concentration [10 μM As(III)] in the wild type that effectively detoxifies As(III) (Fig. 1C). Likewise, Yap8p-HA was not affected in *ycf1Δ*. By contrast, Yap8p-HA levels increased significantly both in *acr3Δ* and *acr3Δ ycf1Δ* cells (Fig. 1C). Hence, Yap8p stability appears to correlate with the intracellular As(III) levels reported for these strains (*acr3Δ ycf1Δ*>*acr3Δ*>*ycf1Δ*>wild type) (Ghosh et al., 1999; Wysocki et al., 1997; Wysocki et al., 2001). Taken together, our data suggest that Yap8p escapes degradation in response to As(III) and is stabilized in a dose-dependent manner.

Yap8p is degraded by the ubiquitin-proteasome pathway

To identify the pathway mediating Yap8p degradation, we first

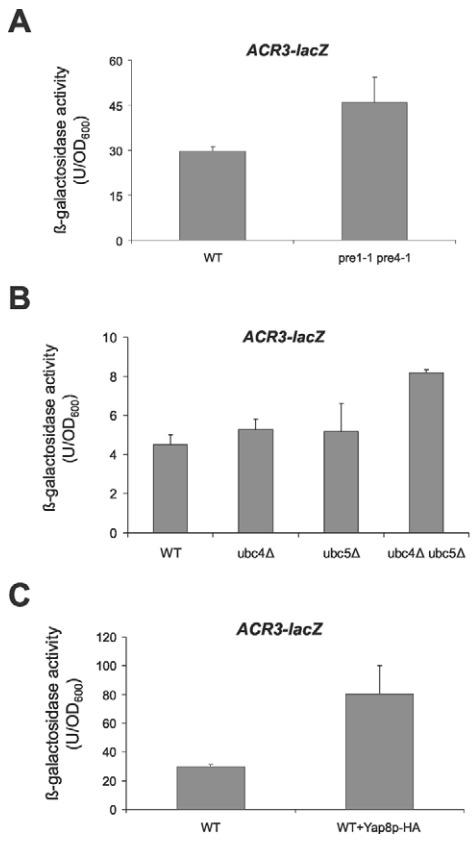

Fig. 3. Yap8p stabilization contributes to increased transcriptional activity. An *ACR3*-promoter-*lacZ* fusion construct was transformed into wild-type cells and into ubiquitin-proteasome pathway mutants. Transformants were assayed for β-galactosidase activity as described in Materials and Methods. The results are the average of three independent experiments and the error bars represent s.d. (A) β-galactosidase activity measurements in the *pre1-1 pre4-1* mutant (WCG4a background). (B) β-galactosidase activity measurements in *ubc4Δ* and *ubc5Δ* mutants (DF5 background). (C) β-galactosidase activity measurements in wild-type cells (WCG4a background) transformed with an empty vector or with a plasmid carrying Yap8p-HA.

monitored Yap8p-HA in cells that had been treated or not with the proteasomal inhibitor MG132 (Fig. 2A). For this, we used *erg6Δ* cells to allow uptake of the drug (Lee and Goldberg, 1996). Treatment with 0.1 mM MG132, which reduces proteasome activity by about 70% (Lee and Goldberg, 1996), resulted in significantly increased Yap8p-HA levels (Fig. 2A). As(III) treatment (0.5 mM) gave rise to somewhat higher Yap8p-HA levels than MG132 alone, whereas Yap8p-HA levels were not further elevated when cells were treated with both MG132 and As(III).

We next analysed Yap8p-HA stability in a *pre1-1 pre4-1* mutant that is defective in the β-type subunits of the catalytic 20S core of the proteasome and exhibits reduced proteasome activity (Hilt et al., 1993). Immunological detection evidenced strongly enhanced Yap8p-HA levels in the *pre1-1 pre4-1* mutant already in the absence of As(III) (Fig. 2B). As(III)-exposure (0.2 mM) did not increase Yap8p-HA levels further in *pre1-1 pre4-1* whereas a strong increase was observed in the wild type (Fig. 2B). These results indicate that Yap8p is stabilized in cells with defective proteasome activity. We also monitored Yap8p-HA in a *pep4Δ* mutant that is defective in the vacuolar protein degradation pathway. Since no obvious difference in Yap8p-HA levels were observed between wild-type and *pep4Δ* (data not shown) cells we conclude that this pathway is not involved in Yap8p degradation.

To expand this analysis, the Yap8p-HA plasmid was introduced into mutants lacking various ubiquitin-conjugating E2 enzymes. We exposed the transformants for 1 hour to As(III) to stabilize Yap8p-HA, transferred the cells to As(III)-free medium and monitored Yap8p-HA degradation by western blot analysis in the presence of cycloheximide. The rate of Yap8p-HA degradation was clearly reduced in *ubc4Δ* and the half-life of Yap8p-HA increased about fourfold (Fig. 2C). Ubc4p mediates selective degradation of short-lived proteins together with Ubc5p (Seufert and Jentsch, 1990). However, Yap8p-HA degradation was not affected in *ubc5Δ* (Fig. 2C). The stronger contribution of Ubc4p to Yap8p-HA degradation is consistent with *UBC4* being more expressed than *UBC5* in exponentially growing cells (Seufert and Jentsch, 1990). Finally, Yap8p-HA degradation was not altered in *ubc7Δ* or in heterozygous *UBC1*/*ubc1Δ* and *UBC3*/*ubc3Δ* diploids (*UBC1* or *UBC3* deletion is lethal in haploids) (data not shown). Collectively, these data demonstrate that Yap8p degradation proceeds through the ubiquitin-proteasome pathway under non-stress conditions.

Yap8p stabilization contributes to increased transcriptional activity

We next asked whether Yap8p stabilization contributes to increased transcriptional activity. To this end, we transformed wild-type cells and mutants defective in Yap8p degradation with a plasmid containing an *ACR3* promoter fused to the *lacZ* reporter gene (Wysocki et al., 2004) and determined β-galactosidase activity (Fig. 3). Interestingly, β-galactosidase activity was higher in the *pre1-1 pre4-1* mutant than in wild-type cells under normal conditions, i.e. in the absence of As(III) (Fig. 3A). We next monitored *ACR3-lacZ* expression in *ubc4Δ* and *ubc5Δ* mutants. β-galactosidase activity was clearly elevated in the *ubc4Δ ubc5Δ* double mutant compared with the corresponding wild-type strain, whereas activity was not affected in the two single mutants (Fig. 3B). Note that the experimental conditions used in Fig. 3B are different from those in Fig. 2C. We measured β-galactosidase activity in the absence of As(III) (Fig. 3B), and Yap8p-HA degradation after removal of As(III) (Fig. 2C). We also noticed that the increase in Yap8p-dependent *ACR3-lacZ* expression is moderate (about 50%) in ubiquitin-proteasome pathway mutants (Fig. 3) compared with the strong increase in Yap8p-HA levels in *pre1-1 pre4-1* cells (Fig. 2B) and the reduced rate of Yap8p-HA degradation in *ubc4Δ* cells (Fig. 2C). Nevertheless, stabilized Yap8p appears to display increased transcriptional activity, possibly due to increased steady-state levels. The fact that ectopic Yap8p-HA expression in wild-type cells stimulated *ACR3-lacZ* expression in the absence of As(III) is consistent with this notion (Fig. 3C).

Cysteine residues are required for As(III)-induced Yap8p stabilization

Yap8p has eight cysteine residues, three of which (C132, C137 and C274) are conserved in several fungal AP-1 proteins (Fig.

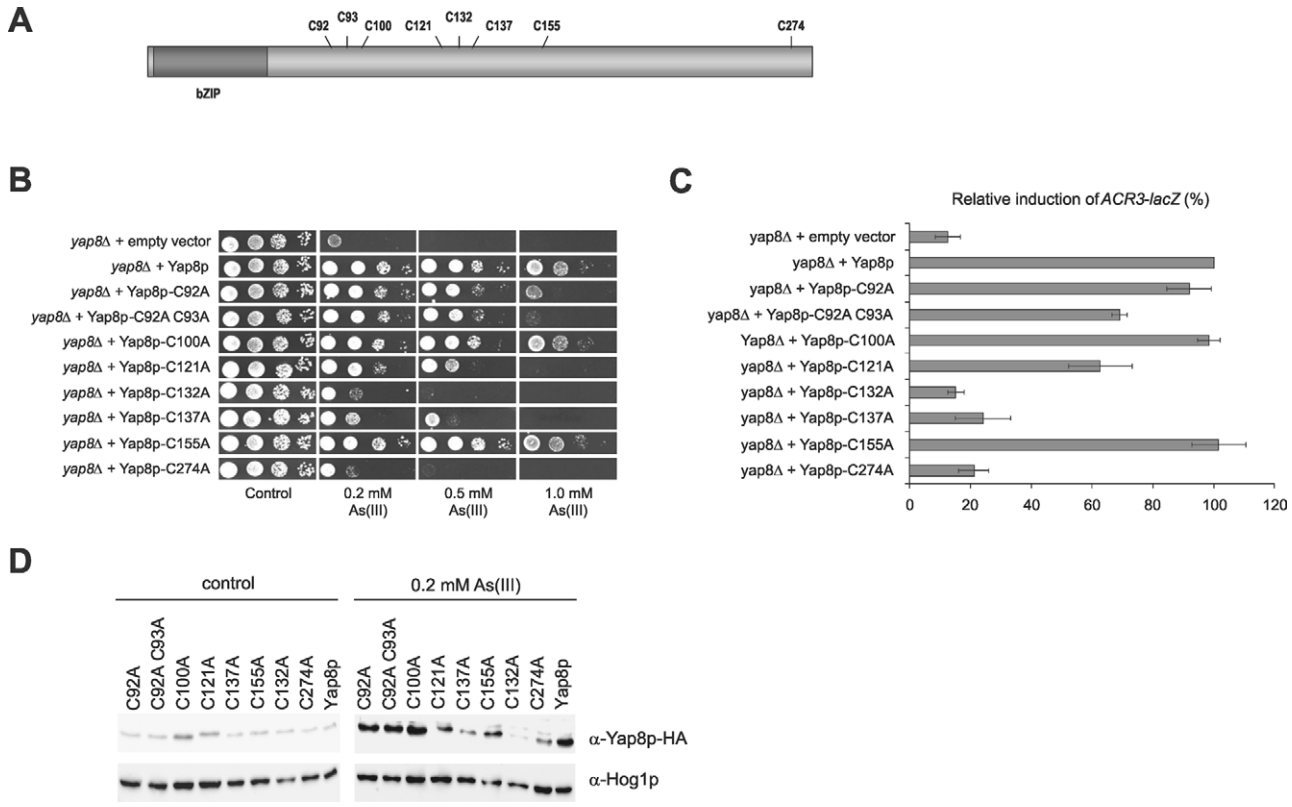

Fig. 4. Analysis of Yap8p cysteine mutants. (A) Cartoon of Yap8p with the bZIP DNA-binding domain and cysteine residues indicated. (B) Phenotypes of Yap8p cysteine to alanine mutations. Plasmids containing wild-type or mutant forms of Yap8p-HA were transformed into W303-1A *yap8Δ*. Transformants were grown in liquid medium and tenfold serial dilutions of the cultures were spotted on agar plates containing sodium arsenite. Growth was scored after 2 days at 30°C. (C) β-galactosidase assays. The W303-1A *yap8Δ* mutant was co-transformed with a plasmid containing the indicated version of Yap8p-HA and a plasmid containing the *ACR3*-promoter-*lacZ* fusion gene. Transformants were assayed for β-galactosidase activity as described in Materials and Methods. *ACR3-lacZ* induction levels were calculated by comparing β-galactosidase activities in untreated and As(III)-exposed cells (0.1 mM As(III); 6 hours). The induction level of *ACR3-lacZ* in the presence of wild-type Yap8p-HA was set to 100 and the *ACR3-lacZ* induction levels in the presence of the Yap8p mutants are given relative to that of wild-type Yap8p-HA. Error bars represent ± s.d. (D) Yap8p-C132A, C137A and C274A fail to stabilize in the presence of As(III). Plasmids containing wild-type or mutant forms of Yap8p-HA were transformed into W303-1A *yap8Δ* and Yap8p-HA protein levels were monitored by western blot analysis prior to and 1 hour after addition of 0.2 mM As(III).

4A) (Toone et al., 2001). C132, C137 and C274 have previously been shown to be important for proper Yap8p function since mutation of either residue affected the ability of Yap8p to stimulate *ACR3* expression and to confer arsenic tolerance (Menezes et al., 2004; Wysocki et al., 2004). To gain more insight into the molecular role of these residues, we changed all the eight cysteines into alanine and analysed the function of the mutated proteins. Plasmids containing wild type or mutant forms of Yap8p-HA were transformed into *yap8Δ* and growth of the transformants was scored in the presence of As(III). The different mutations affected Yap8p function to various degrees (Fig. 4B): the C132A, C137A and C274A mutations severely reduced Yap8p function; C121A, C92A and the C92A C93A double mutant had intermediate effects whereas the C100A and C155A mutations did not impair Yap8p function (Fig. 4B). We also tested the ability of the Yap8p mutants to induce *ACR3-lacZ* expression. β-galactosidase measurements demonstrated that the mutants conferring the most severe phenotypes (C132A, C137A, C274A) were strongly hampered in their ability to trigger *ACR3-lacZ* expression (Fig. 4C). In fact, mutation of these cysteine residues resulted in an almost complete loss of Yap8p function. Yap8p mutants conferring an intermediate phenotype were partially defective in As(III)-induced *ACR3-lacZ* expression; Yap8p-C121A and C92A C93A retained about 60% of wild-type Yap8p activity (Fig. 4C).

We next monitored protein stability of the Yap8p mutants (Fig. 4D). In the absence of As(III), all mutated proteins were detected at levels similar to wild-type Yap8p. However, in cells treated with 0.2 mM As(III) for 1 hour, the Yap8p mutants that were most severely affected in function (i.e. C132A, C137A and C274A) were not stabilized but instead remained at low levels. Also the 'intermediate mutant' C121A appeared somewhat affected in As(III)-mediated stabilization (Fig. 4D). A lack of As(III)-instigated stabilization may contribute to the inability of these Yap8p mutants to properly stimulate *ACR3-lacZ* expression.

Yap8p functions as a homodimer in vivo

We next expressed the Yap8p cysteine mutants in wild-type

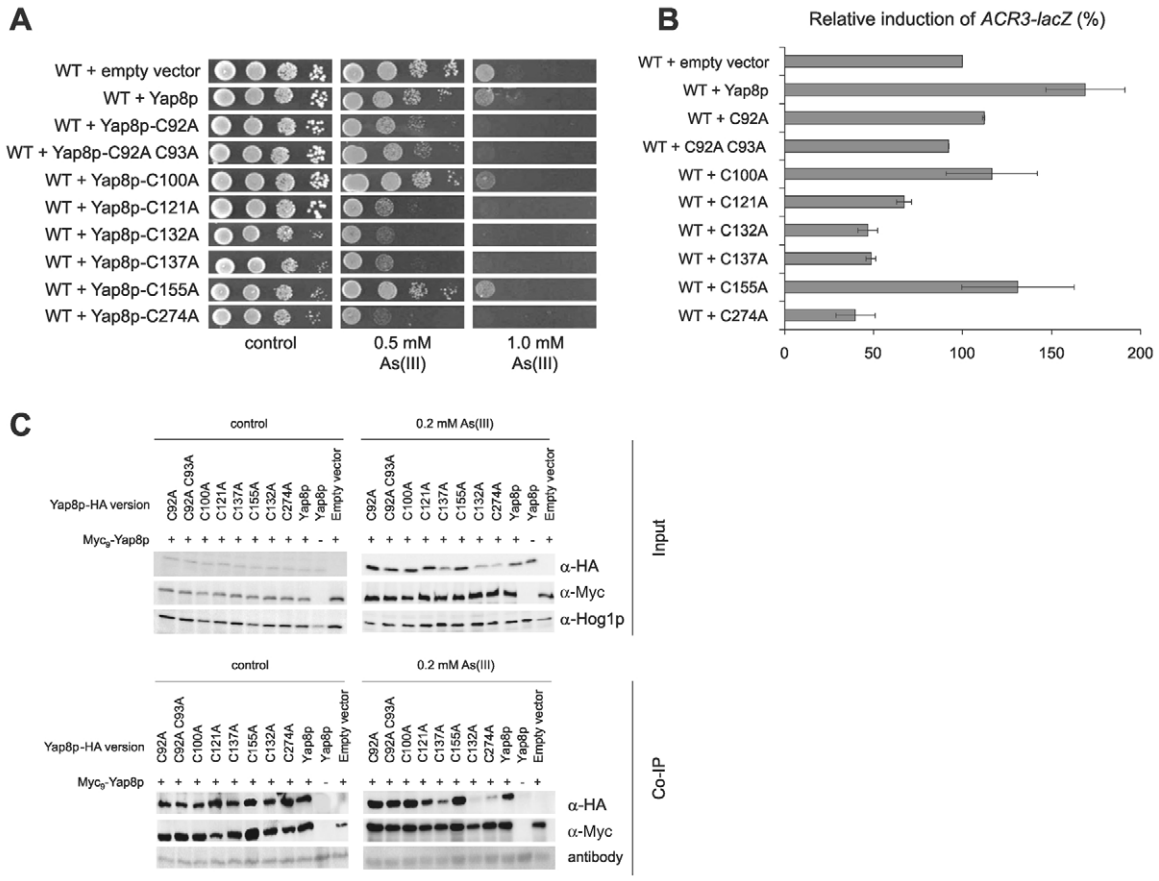

Fig. 5. Yap8p functions as a homodimer in vivo. (A) Phenotypes of wild-type W303-1A cells expressing Yap8p-HA cysteine to alanine mutants. Plasmids containing wild-type or mutant forms of Yap8p-HA were transformed into wild-type cells and growth of the transformants was scored as above. (B) β-galactosidase assays. The W303-1A wild type was co-transformed with a plasmid containing the indicated version of Yap8p-HA and a plasmid containing the *ACR3*-promoter-*lacZ* fusion gene. Transformants were assayed for β-galactosidase activity as described in Materials and Methods. *ACR3-lacZ* induction levels were calculated by comparing β-galactosidase activities in untreated and As(III)-exposed cells (0.1 mM As(III); 4 hours). The induction level of *ACR3-lacZ* in the wild-type strain with the empty vector was set to 100 and the *ACR3-lacZ* induction levels in the presence of the indicated Yap8p versions are given relative to that of wild-type cells with the empty vector. Error bars represent ± s.d. (C) Yap8p forms homodimers in vivo. Co-IP assays were performed with cells co-transformed with two epitope-tagged versions of Yap8p (Myc_9-Yap8p and Yap8p-HA). Myc_9-Yap8p was immunoprecipitated with an anti-Myc antibody, and the presence of wild-type or mutant forms of Yap8p-HA in the precipitates was detected using an anti-HA antibody. Cells were either untreated or exposed to 0.2 mM As(III) for 1 hour as indicated. The upper panel shows expression levels of wild-type and mutant forms of Yap8p-HA in cells coexpressing Myc_9-Yap8p (Input) whereas the lower panel shows Co-IP. Proteins were separated by SDS-PAGE and the presence of Yap8p was monitored with anti-Myc and anti-HA antibodies.

cells that contain a genomic copy of *YAP8*. Importantly, wild-type cells expressing specific Yap8p mutants were more As(III) sensitive than those expressing wild-type Yap8p ectopically or containing an empty vector (Fig. 5A). The As(III)-sensitivity of these transformants was furthermore accompanied by reduced *ACR3-lacZ* expression (Fig. 5B). In particular, ectopic expression of Yap8p-C132A, C137A and C274A strongly sensitized wild-type cells and reduced *ACR3-lacZ* expression to about half of that of control cells. Again, Yap8p-C121A and, to a lesser extent, C92A C93A produced intermediate effects (Fig. 5A,B). We also noticed that Yap8p overexpression resulted in increased *ACR3-lacZ* expression in the wild-type in response to As(III). A possible explanation for the observed dominant-negative effect of the cysteine mutants is that Yap8p functions as a homodimer and overexpression of the mutant form perturbs proper dimer formation. To address this, we coexpressed two differently tagged versions of Yap8p (Myc_9-Yap8p and Yap8p-HA) and performed co-immunoprecipitation (Co-IP) assays. Myc_9-Yap8p was immunoprecipitated using anti-Myc antibody and the presence of Yap8p-HA in the precipitates was determined using anti-HA antibody. As shown in Fig. 5C, Myc_9-Yap8p was able to co-precipitate Yap8p-HA. Conversely, when Yap8p-HA was immunoprecipitated using anti-HA antibodies, Myc_9-Yap8p was detected in the precipitates (data not shown). The amount of Yap8p-Yap8p interaction was not much affected by As(III) (Fig. 5C and data not shown). Hence, Yap8p forms homodimers in vivo and homodimerization is not regulated by As(III). This finding is in agreement with the fact that mammalian AP-1 consists of homo- and heterodimers of the Jun, Fos or ATF family members, and with a recent study demonstrating Yap8p homodimerization in vitro (Newman and Keating, 2003).

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source/Reference
W303-1A	*MAT**a** ura3-1 leu2-3/112 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal0*	(Thomas and Rothstein, 1989)
RW117	W303-1A *yap8Δ::loxP*	(Wysocki et al., 2004)
RW104	W303-1A *acr3Δ::loxP-kanMX-loxP*	(Wysocki et al., 2001)
BY4741	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
BY4741 *erg6Δ*	BY4741 *erg6Δ::kanMX4*	EUROSCARF
BY4741 *ubc4Δ*	BY4741 *ubc4Δ::kanMX4*	EUROSCARF
BY4741 *ubc5Δ*	BY4741 *ubc5Δ::kanMX4*	EUROSCARF
WCG4a	*MAT**a** ura3 leu2-3,112 his3-11,15 CanS Gal+*	Dieter H. Wolf
YHI29/14	*MAT**a** ura3 leu2-3,112 his3-11,15 CanS Gal+ pre1-1 pre4-1*	Dieter H. Wolf
DF5	*MAT**a** his3-Δ200, leu2-3, 2-112, lys2-801, trp1-18(am), ura3-52*	Matthias Peter
YND185	DF5 *ubc4Δ::HIS3 ubc5Δ::LEU2*	Matthias Peter
YND187	DF5 *ubc4Δ::KAN*	Matthias Peter
YND188	DF5 *ubc5Δ::KAN*	Matthias Peter
IRW100	W303-1A *ycf1Δ::loxP-kanMX-loxP*	(Wysocki et al., 2001)
RW105	W303-1A *ycf1Δ::loxP acr3Δ::loxP-kanMX-loxP*	(Wysocki et al., 2001)

Table 2. Plasmids used in this study

Plasmid	Description	Source/Reference
pCGFY1	pRS314 (CEN, *URA3*), His_6-FLAG-Yap1p controlled by *GAL1* promoter	(Owsianik et al., 2002)
pMiT004	His_6-FLAG-tagged Yap8p in pCM188 (CEN, *URA3*, *tetOff-CYC1* promoter)	(Wysocki et al., 2004)
YEp195-GFP-*YAP8*	GFP-*YAP8* behind native promoter in YEp*lac*195	(Wysocki et al., 2004)
pGAL-HF-Yap8p	pRS314 (CEN, *URA3*), His_6-FLAG-Yap8p controlled by *GAL1* promoter	This work
pYX122	CEN, *HIS3*, *TPI1* promoter	
pYX-TPI-Yap8p-HA	Yap8p-HA controlled by *TPI1* promoter in pYX122	This work
Yap8p-HA-C92A	C92A point mutation in pYX-TPI-Yap8p-HA	This work
Yap8p-HA-C92A C93A	C92A C93A point mutation in pYX-TPI-Yap8p-HA	This work
Yap8p-HA-C100A	C100A point mutation in pYX-TPI-Yap8p-HA	This work
Yap8p-HA-C121A	C121A point mutation in pYX-TPI-Yap8p-HA	This work
Yap8p-HA-C137A	C137A point mutation in pYX-TPI-Yap8p-HA	This work
Yap8p-HA-C155A	C155A point mutation in pYX-TPI-Yap8p-HA	This work
Yap8p-HA-C132A	C132A point mutation in pYX-TPI-Yap8p-HA	This work
Yap8p-HA-C274A	C274A point mutation in pYX-TPI-Yap8p-HA	This work
YEp195-Myc_9-Yap8p	Myc_9-Yap8p controlled by the endogenous *YAP8* promoter in YEplac195 (2μ, *URA3*)	This work
pHB1-MYC_9	*E. coli* expression vector for the generation of Myc_9-tagged fusion proteins	Stephen J. Elledge
pRW11	*ACR3-lacZ*, 2μ, *LEU2* in YEp363	(Wysocki et al., 2004)
pEM19	*ACR3-lacZ*, CEN, *URA3* in pSEYC102	(Wysocki et al., 2004)

We next performed Co-IP assays to explore dimerization between wild-type (Myc_9-Yap8p) and mutant forms of Yap8p (Yap8p-HA). We found that wild-type and mutant forms of Yap8p were able to dimerize in the absence of As(III) and that wild-type and mutant forms of Yap8p were present at comparable levels (Fig. 5C, Co-IP). However, when cells were treated with As(III), less of the Yap8p-C132A, C137A and C274A mutants were detected in the precipitates than of wild-type Yap8p-HA. Western blot analysis confirmed that wild-type and mutant forms of Yap8p-HA were expressed at similar levels in cells coexpressing Myc_9-Yap8p in the absence of As(III), and that Yap8p-C132A, C137A and C274A were not stabilized in As(III)-treated cells (Fig. 5C, input). Hence, the fact that less of Yap8p-C132A, C137A and C274A were detected in the precipitates of As(III)-exposed cells is probably due to less protein present in the input since these mutants are defective in As(III)-instigated stabilization. We conclude that Yap8p dimer-formation is not affected by changing crucial cysteines into alanine, at least not in the absence of As(III).

Discussion

The arsenic-responsive transcription factor Yap8p is central for the adaptation of yeast cells to the presence of this toxic metalloid. Here, we describe a new mode of Yap8p regulation that involves stabilization in response to As(III). We found that Yap8p is present at low levels in untreated cells, due to degradation by the ubiquitin-proteasome pathway. In As(III)-challenged cells, Yap8p escapes degradation and stimulates enhanced transcription of its target gene *ACR3*. Whereas the ubiquitin-conjugating enzyme Ubc4p was shown to be implicated in Yap8p degradation, the ubiquitin ligase that acts on Yap8p remains to be identified.

Proper Yap8p function requires crucial cysteine residues and the mutational analysis performed here confirmed previous studies implicating Yap8p-C132, C137 and C274 in this process (Menezes et al., 2004; Wysocki et al., 2004). Further, additional cysteines that affect Yap8p function were discovered: the Yap8p-C121A, C92A and C92A C93A mutants had reduced ability to stimulate *ACR3-lacZ* expression and to confer tolerance. Hence, these residues appear also to be important for Yap8p to appropriately respond to As(III). Importantly, we demonstrated that Yap8p-C132A, C137A and C274A (and to some extent also C121A) did not stabilize in As(III)-treated cells. Defective As(III)-induced stabilization may contribute to the failure of these Yap8p mutants to stimulate *ACR3-lacZ* expression. These results, together with the findings that Yap8p levels and *ACR3-lacZ* expression are enhanced in ubiquitin-proteasome pathway mutants suggest that, Yap8p is regulated at the level of As(III)-induced stabilization and stabilization may contribute to increased

transcriptional activity. The fact that overexpression of Yap8p stimulated *ACR3-lacZ* expression in the absence of As(III) lends further support to this notion.

Two scenarios may explain how As(III)-exposure leads to Yap8p stabilization: the activity of the ubiquitin-proteasome pathway might be reduced in response to As(III) or, alternatively, As(III) might act on Yap8p directly such that Yap8p becomes less prone to degradation. The following observations lend support to the latter: first, expression of genes encoding functions in protein degradation, including subunits of the proteasome, is strongly induced by As(III) (Haugen et al., 2004); second, specific Yap8p cysteine mutants do not stabilize in the presence of As(III) (this work); third, direct monitoring of proteasome activity using the well-described proteasome substrate ubiquitin-proline-β-galactosidase (Johnson et al., 1995; Lee and Goldberg, 1996) indicated higher proteasome activity in As(III)-treated cells (data not shown). As(III) affects proteins in at least two ways: due to its high reactivity with sulphhydryl groups it can form metal-thiol bonds with vicinal cysteines (Delnomdedieu et al., 1993). In addition, As(III) triggers increased intracellular levels of superoxide which, in turn, may lead to formation of other reactive oxygen species such as hydroxyl radicals and hydrogen peroxide (Shi et al., 2004). We explored Yap8p stabilization in response to peroxide (*tert*-butylhydroperoxide) and a superoxide generating agent (paraquat) and found Yap8p levels unchanged (data not shown). Similarly, As(III)-induced Yap8p stabilization was unaffected in cells overexpressing *CTT1* (encoding catalase), *SOD1* or *SOD2* (encoding superoxide dismutases) (data not shown). Although these results do not exclude that As(III)-induced oxidative modifications contribute to Yap8p stabilization, it makes such a mechanism less likely. Instead, it is tempting to speculate that As(III)-binding induces a conformational change within Yap8p such that it becomes less prone to degradation. Because C132, C137 and C274 are required both for Yap8p stabilization and induction of *ACR3-lacZ* expression, these cysteines are probable sites for As(III)-binding.

How important is Yap8p stabilization for increased transcriptional activity? Quantitative assessment of the contribution of stabilization to Yap8p activity is not straightforward. The *pre1-1 pre4-1* and *ubc4Δ ubc5Δ* mutants are As(III) hypersensitive; these mutants only tolerate very low amounts of As(III) and it is not possible to interpret *ACR3-lacZ* expression changes in response to such low As(III) concentrations (data not shown). Nonetheless, several observations indicate that stabilization does not account for full Yap8p activation. First, ectopic overexpression of Yap8p or increasing Yap8p levels in ubiquitin-proteasome pathway mutants resulted in moderately elevated *ACR3-lacZ* expression levels whereas the increase in Yap8p-dependent *ACR3-lacZ* expression in response to As(III) was much more important. Second, certain mutations within Yap8p, such as Yap8p-C92A C93A, reduced *ACR3-lacZ* expression without affecting As(III)-mediated stabilization. Hence, full activation of Yap8p probably involves (an) additional mechanism(s). A recent study suggested that Yap8p is activated at the level of its transactivation function, by monitoring a LexA-Yap8p fusion protein (Menezes et al., 2004). Curiously, these authors found that *lacZ*-reporter-gene expression was stimulated by LexA-Yap8p in response to pentavalent arsenate [As(V)]; however, they did not find any clear evidence for enhanced LexA-Yap8p transactivation function in response to trivalent arsenite [As(III)] (Menezes et al., 2004). Hence, whereas As(III) contributes to enhanced Yap8p transactivation function remains to be firmly established.

Our previous work showed that Yap8p predominantly resides in the nucleus (Wysocki et al., 2004) whereas Menezes et al. found the majority of Yap8p in the cytoplasm (Menezes et al., 2004). The data from these studies could be interpreted such that Yap8p consists of both a cytosolic and a nuclear fraction, as shown for Gcn4p (Pries et al., 2002). Interestingly, Gcn4p is, in part, regulated at the level of stabilization in response to amino acid starvation (Kornitzer et al., 1994). Moreover, Gcn4p stability depends on its subcellular localization; the smaller cytosolic Gcn4p fraction appears to be relatively stable, whereas the larger nuclear fraction is less stable (Pries et al., 2002). Whether different Yap8p fractions would display different stability is currently unknown. Because Yap8p is present in both untreated and treated cells, nuclear Yap8p may bind to the *ACR3* promoter at any time, also in the absence of As(III) (Wysocki et al., 2004). However, Yap8p is likely to associate with or dissociate from DNA both in the presence and absence of As(III). Although we currently do not know whether the (strength of) Yap8p-DNA association is affected by As(III), the present study revealed a reduced rate of Yap8p degradation in As(III)-exposed cells. Moreover, stabilized Yap8p displayed increased transcriptional activity, possibly due to increased steady-state protein levels. It is reasonable to assume that there is a balance between Yap8p DNA-binding, stabilization and degradation, and gene-target activation. Interestingly, binding of As(III) to specific cysteines within the *Escherichia coli* arsenite-responsive repressors ArsD and ArsR has been shown to produce conformational changes that result in the release of these repressors from DNA followed by induction of the *ars* operon (Li et al., 2001; Shi et al., 1996). To conclude, we have shown here that regulated degradation contributes to Yap8p control. However, more work is clearly needed for a full understanding of the mechanisms of Yap8p regulation. In particular, elucidation of As(III)-binding to Yap8p and the consequences thereof on protein conformation, stability and activity awaits purification and detailed biochemical, biophysical and structural characterization of Yap8p.

Materials and Methods

Yeast strains and growth conditions

Yeast strains used in this study are described in Table 1. Yeast cells were grown in rich YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or in minimal YNB medium (0.67% Yeast Nitrogen Base) supplemented with complete supplement mix (BIO 101) and 2% glucose as carbon source. Sodium arsenite (Sigma) was added directly to the growth medium and plate-growth assays were performed as described previously (Wysocki et al., 2001).

Construction of plasmids and YAP8 mutants

The plasmids used in this study are listed in Table 2. His_6-FLAG-*YAP8* was excised from plasmid pMiT004 (Wysocki et al., 2004) using *Xba*I/*Sal*I and inserted into plasmid pCGFY1 (Owsianik et al., 2002), thereby replacing His_6-FLAG-*YAP1* and producing plasmid pGAL-HF-Yap8p. Cysteine to alanine mutations within Yap8p were carried out by using the QuickChange site-directed mutagenesis kit (Stratagene) with plasmid pGAL-HF-Yap8p as template. Wild-type and mutant forms of *YAP8* were amplified by PCR and the resulting fragments were inserted into *Nco*I/*Apa*I-digested pYX122 vector generating in-frame fusions of *YAP8* with the HA-tag at the C-terminal end of Yap8p. The C132A and C274A mutations were amplified using GFP-Yap8-C132A and GFP-Yap8-C274A as templates (Wysocki et al., 2004). The Myc_9-tag (nine tandem repeats of Myc) was amplified from

plasmid pHB1-MYC9 by PCR and the resulting fragment was used to replace the GFP-tag of YEp195-GFP-*YAP8* (Wysocki et al., 2004) to create plasmid YEp195-Myc_9-Yap8p where Myc_9 was fused to the N-terminal end of Yap8p. All plasmids were confirmed by sequencing. The sequences of primers used for PCR and site directed mutagenesis reactions will be provided upon request.

Protein extraction and western blot analysis

Exponentially growing cells (in YNB medium) were either not treated or exposed to different concentrations of sodium arsenite; samples for western blot analyses were taken at the time-points indicated in the figures. Total protein was extracted based on the TCA method as described previously (Delaunay et al., 2000) and protein concentrations were determined using a kit (Bio-Rad). Proteins were separated by SDS-PAGE and blotted onto nitrocellulose filters (Amersham). The filters were probed with primary anti-Myc antibody (9E10: Roche) or anti-HA antibody (Sigma) to detect the epitope-tagged versions of Yap8p. All filters were probed with anti-Hog1p antibody (yC-20: Santa Cruz Biotechnology, CA) as a loading control. The filters were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies, then with ECL Plus western blotting detection reagent (Amersham) and finally visualized using LAS-100 image reader (Fuji Film). Yap8p protein levels were quantified using the Multi Gauge software (Fuji Film) and normalized to the Hog1p level of each lane.

Co-immunoprecipitation assays

The *yap8Δ* mutant was co-transformed with plasmids YEp195-Myc_9-Yap8p and pYX-TPI-Yap8p-HA or with the pYX-TPI-Yap8p-HA plasmids containing the *YAP8* cysteine to alanine mutations. Exponentially growing cells were either not treated or treated with sodium arsenite and then broken with glass beads in buffer A [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 15 mM EDTA, 15 mM EGTA, 2 mM dithiothreitol, 0.1% Triton X-100, complete protease inhibitor mixture (Roche)]. Protein extracts (~3 mg) were incubated overnight with 20 μl of Protein A-Sepharose beads (Sigma) and 0.5 μg of anti-HA antibody or with anti-mouse IgG–agarose (Sigma) and 0.5 μg of anti-Myc antibody. Beads were washed extensively with buffer A and finally resuspended in 40 μl of SDS loading buffer. To detect Yap8p-HA and Myc_9-Yap8p, anti-HA and anti-Myc antibodies were used as described above.

β-galactosidase activity measurements

Exponentially growing cells (in YNB medium) expressing the *ACR3* promoter-*lacZ* fusion gene (Wysocki et al., 2004) were either not treated or exposed to sodium arsenite. β-galactosidase activity assays were performed at least three times on permeabilized cells as previously described (Guarente, 1983). The values are given with standard deviation (s.d.).

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