Yap8p activation in *Saccharomyces cerevisiae* under arsenic conditions

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Received 29 January 2004; revised 7 April 2004; accepted 11 April 2004

Available online 22 April 2004

Edited by Ned Mantei

Abstract Yap8p, a member of the *Saccharomyces cerevisiae* Yap family, is activated in response to arsenic. Both the mechanisms by which this activation takes place and its regulation have not yet been identified. In this report, we show that Yap8p is not activated at the transcriptional level but, rather, its nuclear transport is actively regulated and dependent on the exportin chromosome region maintenance protein. In addition, it is shown that Cys^{132}, $Cys^{137}$and Cys^{274} are essential for Yap8p localization and transactivation function both of which are required for its biological activity.
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Keywords: Arsenic stress; YAP8 (ACR1, ARR1); YAP1

1. Introduction

Saccharomyces cerevisiae arsenic compounds resistance (*ACR*) cluster was isolated as loci that increase tolerance to arsenite [1]. The three contiguous genes identified as *ACR1* (*YAP8*), *ACR2* and *ACR3* encode a positive regulator, an arsenate-reductase and a plasma membrane arsenite efflux protein, respectively [2–5]. *ACR1*, also designated *YAP8*, belongs to the yeast AP-1 like factor (YAP) family of transcription factors [6]. Members of the YAP family (Yap1p to Yap8p) share a conserved bZIP DNA-binding domain, each regulating a specific set of genes involved in multi-drug resistance [7]. Yap8p could mediate arsenic stress responses by regulating the expression of *ACR2* and *ACR3*. Yeast cadmium factor 1 (Ycf1p) is also involved in the arsenite detoxification and known for conferring resistance to a variety of drugs through sequestering the glutathione *S*-conjugates into the vacuole [8]. Yap1p [9], the prototypical *YAP* gene family, which regulates the yeast oxidative stress response regulon, also regulates the expression of *YCF1*. Yap1p primary control lies in a redox-regulated Crm1-dependent nuclear export [10–12]. Redox signals disrupt the Yap1p–chromosome region maintenance protein (Crm1p) interaction through a redox-dependent alteration of the Yap1p C-terminal nuclear export signal (NES), thereby promoting Yap1 nuclear accumulation.

Data presented here show that arsenic compounds activate Yap8p at the level of both its nucleo-cytoplasmic shuttling and its transactivation potential. Regulation of Yap8p nucleo-cytoplasmic shuttling involves arsenic-sensitive Crm1p-dependent nuclear export and the Yap8 cysteine residues located in positions 132, 137 and 274.

2. Materials and methods

2.1. Growth conditions

Yeast strains were grown in complete YPD (1% yeast extract, 2% bactopeptone, 2% glucose) or selective media (SC or SD: 0.67% ammonium sulfate-yeast nitrogen base without amino acids [Difco], 2% glucose), supplemented with the appropriate selective amino acids. Early exponential phase cells ($A_{600} = 0.4$–0.5) were induced by the addition of 2 mM Na_2HAsO or $NaAsO_2$ and samples collected at the indicated time point. Samples for RNA and protein extraction were washed and stored at −80 °C. Phenotypic growth assays were carried out by spotting 5 μl of an early exponential phase diluted culture (approximately 2×10^3 cells) in medium containing increasing concentrations of 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 [13] (see Table 1).

2.2. Plasmids and constructs

To express the *YAP8* gene the corresponding chromosomal region was amplified by PCR. The product was first cloned using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen) and the *Kpn*I fragment, encoding the entire *YAP8* ORF plus 923 bp upstream and 643 bp downstream, sub-cloned into the CEN vector YEplac33 [18] to generate construct YCp*YAP8*. To create the *YAP8-TAPtag* chimera *Xho*I and *Nco*I restriction sites were introduced by PCR, the product was first cloned into the vector pBS1479 and subsequently sub-cloned into the pRS416 [19]. The fusion *GFP-YAP8* (pRS *cp-GFP-HA-YAP8*) was obtained by replacing the *Pvu*II/*Sal*I *YAP4* gene in the plasmid pRS *cp-GFP-HA-YAP4* [20]. The *lexA-YAP8* constructions were generated by cloning the respective *Sma*I/*Kpn*I PCR products into the YCp91 expression vector [6]. Site-directed mutagenesis of *YAP8* cysteines was performed by PCR amplification of entire plasmids using complementary primers containing the desired mutation. All constructs were sequenced using the ABI Prism DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and ABI Prism 373A Automatic Sequencer (Perkin Elmer). The fusion proteins were functional since they complement *yap8* mutant.

2.3. Northern-blot analysis

RNA procedures were performed according to [15]. RNA was isolated from early log-phase cultures ($A_{600} = 0.4$–0.5) that were either

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Abbreviations: ACR, arsenic compounds resistance; YAP, yeast AP-1 like factor; YCF, yeast cadmium factor; Crm1p, chromosome region maintenance protein

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doi:10.1016/j.febslet.2004.04.019

Table 1
S. cerevisiae strains used in this study

Strain	Genotype	Source
FY1679	*MATα his3-200 ura3-52 GAL2*	Winston et al. [14]
FY*Δ1*	*Matα his3-200 ura3-52 GAL2 yap1::KAN*	Nevitt et al. [15]
BY 4743	*MATa/α his3Δ1/his3Δ1 leuΔ0/leuΔ0 lys2Δ0/LYS2 met15Δ0/MET15 ura3Δ0/ura3Δ0 YPR199c::kanMX4/YPR199c::kanMX4* (isogenic to FY strains)	EUROSCARF
BY*Δ8*	*MATa his3Δ1 leuΔ0 met15 Δ0lys2Δ0 ura3Δ0 YPR199c::kanMX4*	This study
BY*Δ1Δ8*	*MATa yap1::HIS3 leuΔ0 met15Δ0 lys2Δ0 ura3Δ0 YPR199c::kanMX4*	This study
FT4	*MATa ura3-5 trp1-Δ63 his3-Δ200 leu2::PET56*	Tzamarias and Struhl [16]
mcy8	*Matα his3 can1-100 ade2 leu2 trp1 yap1::URA3 ura3::(3XSV40AP1-lacZ) crm1::HIS3-pmet3-CRM1*	Kuge et al. [11]
L40a	*MATa ade2 trp1-901 leu2-3 112 his3-200 LYS::lexA-His3 URA3::lexA op-lacZ*	Marcus et al. [17]

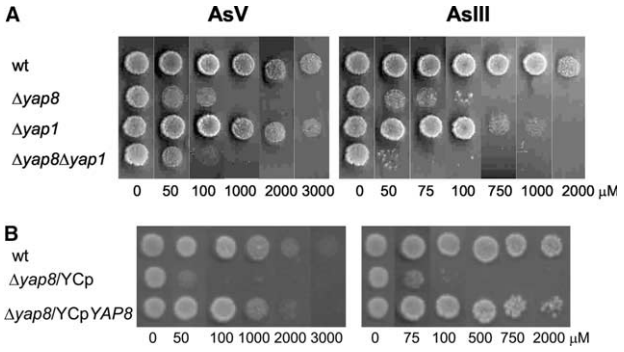

Fig. 1. *yap8* and *yap1* deleted strains show distinct sensitivity to arsenic. Tolerance of the *yap8* mutant is rescued by expressing *YAP8* in a CEN-based plasmid. Approximately 2×10^3 early exponential phase cells were spotted onto YPD (A) or SC (B) medium supplement with increased concentrations of arsenate (AsV) and arsenite (AsIII).

untreated or exposed to 2 mM Na_2HAsO or $NaAsO_2$. Approximately 40 μg of RNA were separated in formaldehyde gels, and transferred onto nylon membranes (Hybond XL, Amersham Pharmacia Biotech). The following intragenic PCR fragments were used as probes: 0.84 kb YAP8, 1.80 kb YAP1, 0.38 kb ACR2, 0.38 kb ACR3, 0.80 kb YCF1, 0.60 kb HSP26, 0.20 kb U3. mRNA levels were quantified (ImageQuant, Molecular Dynamics) and normalized against those of U3 internal loading control, a small nuclear RNA (SNR17A).

2.4. Protein extraction and Immunoblot analysis

Samples collected at the indicated time points were harvested by centrifugation at 4 °C. Protein extracts were prepared by the TCA acid lysis method and immunoblotted according to [15]. To measure intracellular levels of TAPtag-Yap8p and LexA-Yap fusion proteins, immunoblotting was performed with 100 and 50 μg of proteins, respectively. The LexA-hybrid proteins were detected using a monoclonal LexA antibody (Clontech) and anti-mouse IgG conjugated to alkaline phosphatase (Bio-Rad). Detection was performed using the AP Conjugate Substrate Kit (Bio-Rad).

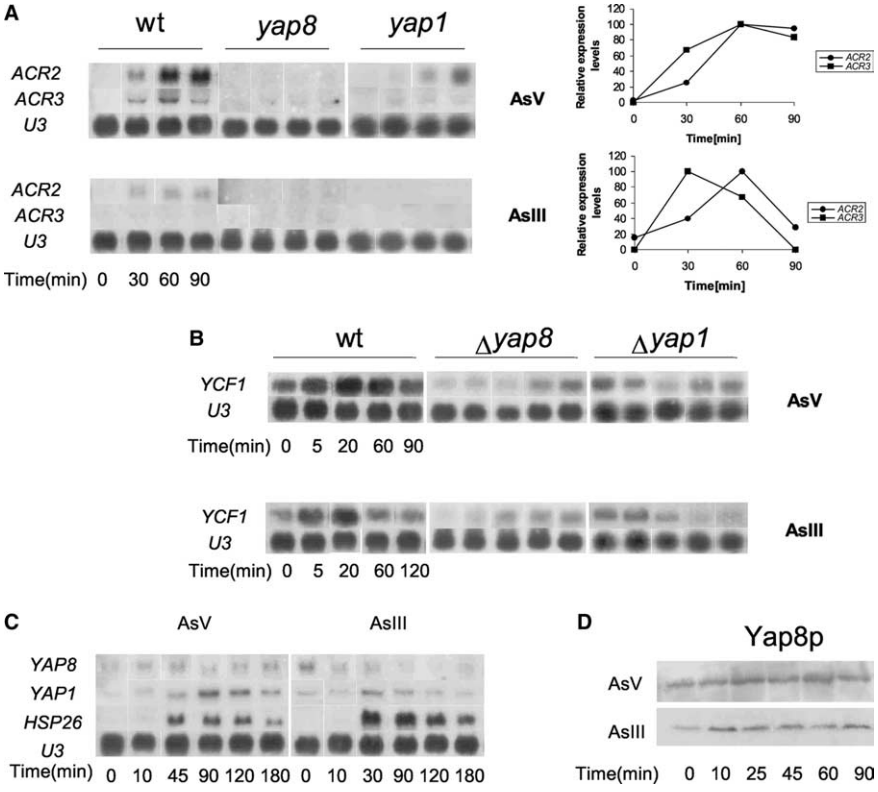

Fig. 2. Induction of genes involved in arsenic stress response. Early exponential phase cells were up-shifted to 2 mM of AsV or AsIII and harvested at the indicated time-points. RNAs and proteins were extracted as described. Northern-blot analysis of (A) *ACR2* and *ACR3*, (B) *YCF1* and (C) *YAP8* and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

2.5. Fluorescence microscopy

Cells transformed with pRS *cp-GFP-HA-YAP8* were grown to early log phase and induced with either 2 mM Na_2HAsO or $NaAsO_2$, at the indicated time points. 4,6-Diamino-2-phenylindole (DAPI) was added as a DNA marker at a final concentration of 20 μg/ml, 5 min before microscopy. After washing with phosphate-buffered saline (PBS), cells were resuspended in DABCO solution (75% glycerol, 0.25× 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 GFP-Yap8 localization. Fluorescence of live yeast cells was visualized by a Leica DMBR fluorescence microscope.

2.6. β-Galactosidase activities

Cells expressing LexA fusions were replica-plated onto SD medium containing 2 mM Na_2HAsO or $NaAsO_2$ and after two days covered with 10 ml of 0.5 M sodium phosphate buffer (pH 7.0); 0.2% (w/v) SDS; 2% (v/v) dimethyl formamide containing 100 mg X-gal/ml and 0.5% (w/v) agarose at 70 °C. Plates were analyzed after 30 min – 24 h incubation at 30 °C. For quantitative β-galactosidase measurements, cells were harvested in early log phase, permeabilized with chloroform and assayed for enzyme activity.

3. Results and discussion

3.1. YAP8 is required for resistance to arsenic compounds and for a regulated expression of the ACR2, ACR3 and YCF1 genes

A strain lacking the *YAP8* gene (Δ*yap8*) is highly sensitive to arsenate and arsenite at very low concentrations (100 μM) (Fig. 1A). In comparison, the isogenic wild-type strain tolerates up to 2 mM of both drugs. The Δ*yap8* arsenic phenotype is rescued by reintroducing *YAP8* on a centromeric plasmid (Fig. 1B). A strain lacking *YAP1* is also hypersensitive to both drugs (Fig. 1A). A strain lacking both *yap1* and *YAP8* is even more sensitive to arsenic compounds than either of the single mutant strains (Fig. 1A), indicating that both *YAP1* and *YAP8* are independently required for the cell tolerance to arsenic compounds.

The expression of *ACR2*, *ACR3* and *YCF1* is induced by arsenic stress (Figs. 2A and B), with arsenate having a much potent effect than arsenite. This induction is decreased or even abolished in Δ*yap8* (Fig. 2A). Induction of these genes by arsenic compounds is also diminished in Δ*yap1*, consistent with the notion that both *ACR3* [22] and *YCF1* [9] are known Yap1-target genes. The requirement of both *YAP1* and *YAP8* for arsenic stress tolerance can thus be rationalized by their function of regulating the arsenic stress-induction of genes important for arsenic compounds detoxification.

3.2. Yap8p is redistributed to the nucleus upon arsenic treatment

We explored the mechanism of Yap8 activation by arsenic compounds. Transcriptional activation is not a major determinant in this activation since *YAP8* mRNA and protein levels are constitutive and not induced upon arsenic treatment (Figs. 2C and D). In contrast, *YAP1* mRNA levels are slightly induced by arsenic compounds (Fig. 2C) indicating that Yap1 might participate in the arsenic stress response.

Since Yap1p is regulated at the level of a Crm1-dependent nuclear export [10,12,23] we investigated whether a similar mechanism could apply to Yap8p by monitoring the cell distribution of a GFP-Yap8 fusion protein. Yap8 appeared predominantly localized in the cytoplasm under non-stress conditions and redistributed into the nucleus within 10 min after arsenic treatment (Fig. 3A). Arsenate had the effect of keeping Yap8 longer in the nucleus than arsenite (data not shown), probably due to the delayed detoxification of this compound that needs a reduction step to arsenite.

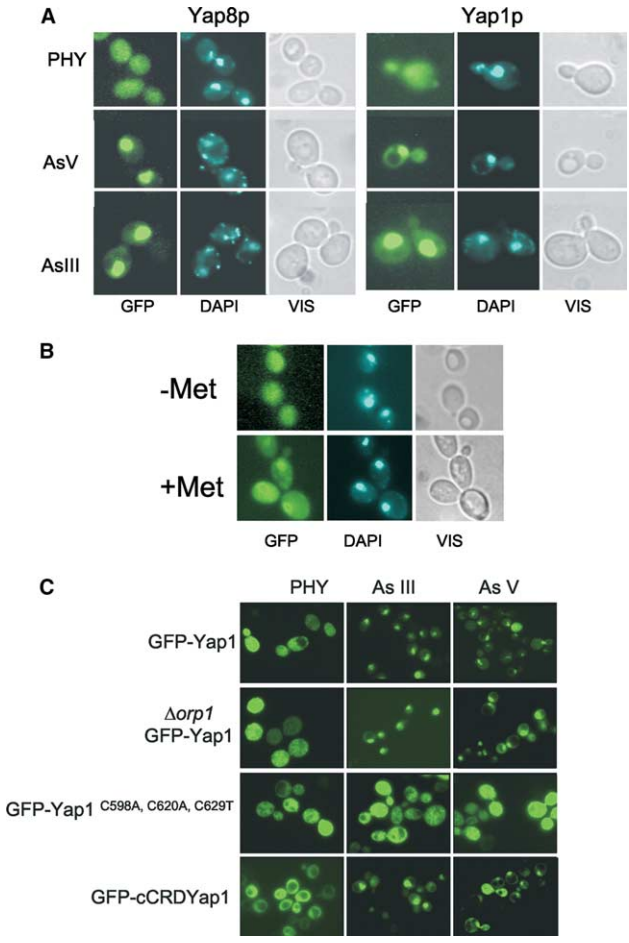

Fig. 3. Yap8p and Yap1p are relocated to the nucleus upon arsenic treatment. (A) Cells grown to early exponential phase were induced 10 min with 2 mM of arsenate and analysed under a fluorescence microscope. (B) Cells expressing the *CRM1* driven by the *MET3* promoter were grown to early exponential phase in the presence or absence of methionine. PHY, physiological conditions (C) Δyap1 or Δorp1 strains expressing GFP-Yap1, or its mutant derivatives were induced 10 min with 2 mM of AsIII or AsV and analyzed by fluorescence microscopy.

We next investigated the involvement of Crm1p in Yap8 cell redistribution using a strain carrying *CRM1* under control of a methionine-repressible promoter [11]. In non-stressed cells, GFP-Yap8 appeared in the nucleus in the presence of methionine but was cytoplasmic when methionine was absent (Fig. 3B). These data establish that Crm1 mediates Yap8 nuclear export and suggest that this export is regulated by arsenic stress.

3.3. C^{132}, C^{137} and C^{274} are essential for Yap8p nuclear translocation

In analogy to Yap1, which can respond to cysteine-reactive chemicals through its modification at specific cysteine residues [12,23,24], we investigated the requirement of Yap8 cysteine residues in Yap8 activation by arsenic compounds.

Yap8p and Yap1p share 19% identity with two domains of higher homology, one being in the C-terminus of these proteins (Fig. 4A). The corresponding Yap1 C-terminal domain, also called c-CRD for C-terminal cysteine-rich domain carries the Crm1-cognate NES comprising a leucine-rich hydrophobic amino acid stretch embedded in a three-repeat cysteine motif (Cys^{598}, Cys^{620}, Cys^{629}) [10]. Yap8 also contains a putative NES in the corresponding domain, previously identified by a short leucine-rich hydrophobic amino-acid sequence flanked by positively charged residues (amino acids 240–282) [22]. However, this Yap8 C-terminal domain only contains one cysteine residue (C^{274}) that appears conserved with Yap1 Cys^{629}. The second domain of high homology corresponds to the Yap1 n-CRD that also carries a three-repeat cysteine motif (Cys^{303}, Cys^{310}, Cys^{315}). In the corresponding region Yap8p carries two cysteines (C^{132} and C^{137}) that are conserved with Yap1p Cys^{310} and Cys^{315}, respectively. Taking into account the structural similarities between Yap8p and Yap1p we anticipated that Yap8p conserved cysteines might play a role in the regulation of Yap8 nuclear export. Yap8 C^{132}, C^{137} and C^{274} were individually and simultaneously substituted with alanine residues. These mutations all impaired the nuclear relocalization of Yap8p in response to arsenate (2 mM) (Fig. 4B) and the ability of Yap8 to restore resistance to arsenic compounds to the *Δyap8* strain (Fig. 4C). In contrast, the GFP-Yap8p clearly restores the As-phenotype of the *yap8* mutant. These cysteine substitutions do not affect the stability of Yap8 as shown by Western blot (Fig. 4D). We suggest that arsenic compounds inhibit Crm1-dependent Yap8 nuclear export by binding to these cysteine residues thereby inhibiting Yap8–Crm1 interaction through a change of conformation.

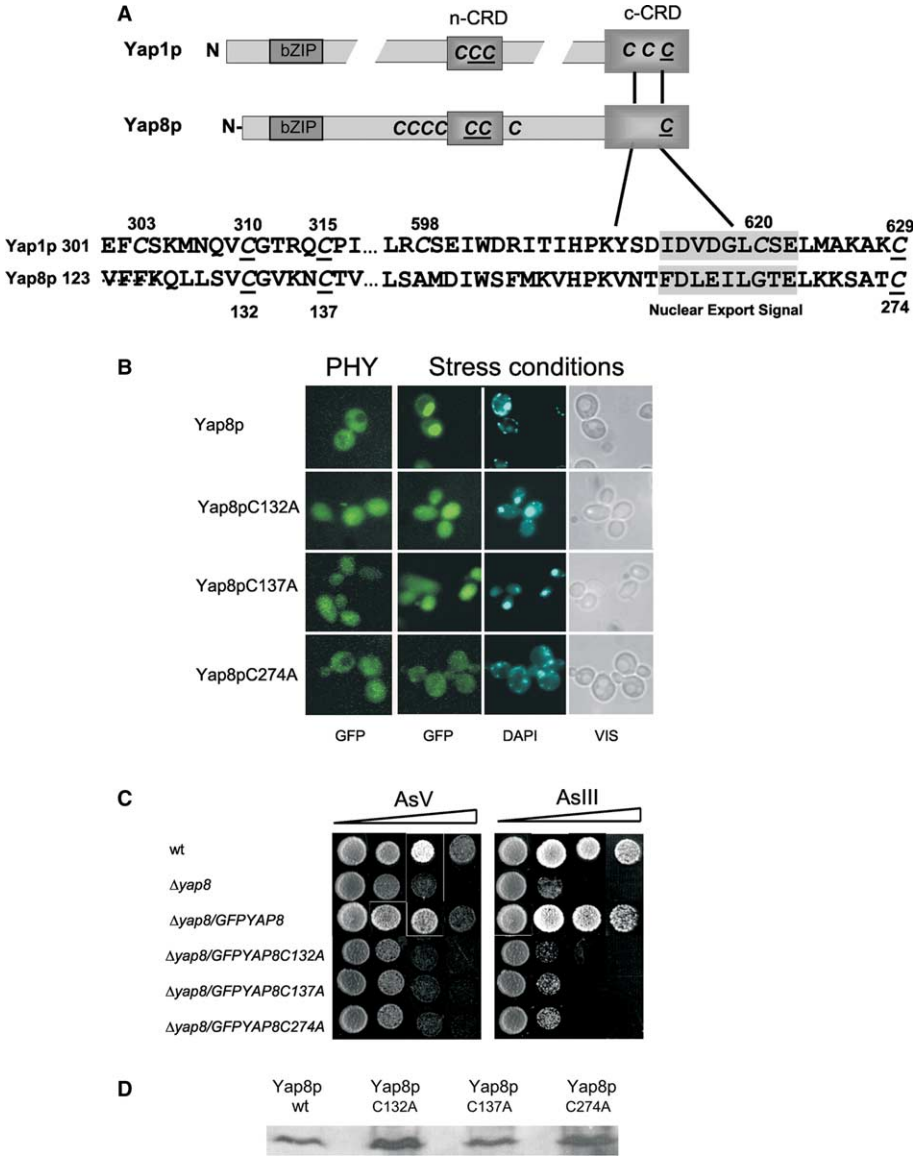

Fig. 4. (A) Comparison of Yap8p and Yap1p. (B) Yap8p cysteines (underlined) were substituted by alanine and nuclear redistribution monitored by fluorescence microscopy after 10 min induction with 2 mM AsV. (C) Phenotypic analysis of the *yap8* mutant expressing the *GFP-YAP8* chimera and its mutated versions. (D) Δ*yap8* cells expressing *YAP8-TAPtag* fusion and its cysteine mutants were induced 10 min with 2 mM AsV and assayed by immunoblotting.

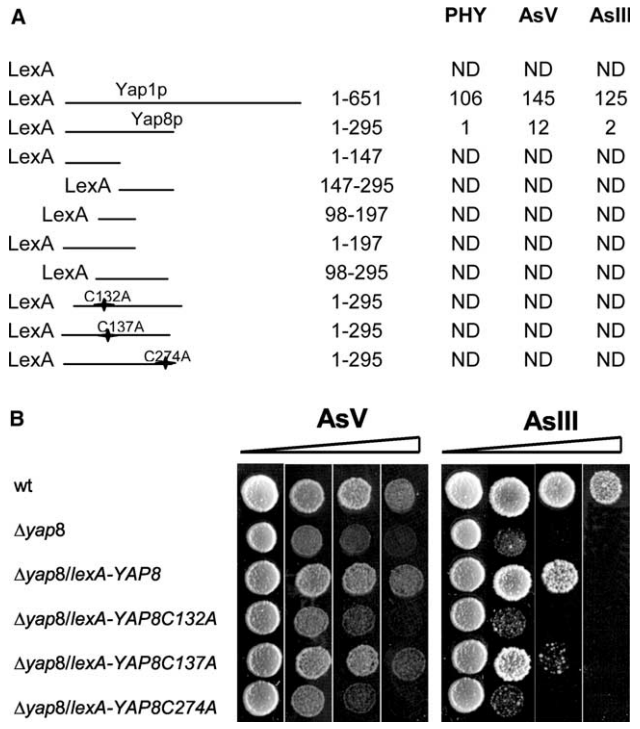

Fig. 5. Yap8p and Yap1p transactivation potential. (A) Cells co-expressing the Lex-Yap fusions and a reporter cassette bearing the *lacZ* gene driven by a promoter containing the LexA binding sequence were grown in the presence and absence of arsenic. Values of β-galactosidase activities (average of six independent transformants) are normalized to the A_{600} of cells and accurate to ± 15%. PHY, physiological conditions; N.D. not detected. (B) Arsenic sensitivity of the *yap8* mutant expressing, lexA-*YAP8* and its mutated versions.

3.4. *Yap8p transactivation function is stimulated upon arsenate treatment*

The function of transactivation can be another level of control for a transcription factor. We thus investigated whether arsenic compounds could modulate the Yap8 transactivation function using a one-hybrid strategy [25]. A LexA-Yap8 fusion protein, containing an artificial NLS, was able to activate transcription of the *lacZ* reporter gene from a LexA operator and this activation was increased upon arsenate treatment. Arsenite had only a slight or no effect on *lacZ* gene activation by LexA-Yap8 (Fig. 5A). Interestingly, the transactivation potential of LexA-Yap8p under arsenate is of the same order of magnitude as LexA-Yap2p under cadmium [6]. The ability of the LexA-Yap8 fusion to rescue the Δ*yap8* arsenic phenotype demonstrates that this fusion retains the Yap8 intrinsic molecular function in arsenic resistance (Fig. 5B). We next sought mapping the Yap8 transactivation domain by testing various Yap8 truncations in the one-hybrid assay (see diagram of Fig. 5A). None of the tested constructs could activate *lacZ* transcription thus indicating that only full-length Yap8 retains the function of transactivation. We also tested the possible requirement of Cys^{132}, Cys^{137} and Cys^{274} in this function. Interestingly, alanine substitution of each of these cysteines abolished *lacZ* gene expression by the Lex-Yap8 fusion indicating that these residues are also important for the Yap8 function of transactivation (Fig. 5A). However, as can be seen in Fig. 5B, LexA-Yap8 fusion mutated in the Cys^{137} partially restores the As-phenotype of the *yap8* mutant. It is therefore plausible that Cys^{137} does not play a relevant physiological role in the Ya8p activity as play the Cys^{132} and Cys^{274}.

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

A GFP-Yap1 fusion potently accumulated in the nucleus upon treatment with arsenic compounds (Fig. 3C), indicating that Yap1 activation by these chemicals is exerted at the level nuclear export as for its activation by H_2O_2 and by thiol-reactive chemicals [24]. Yap1 cannot be activated by H_2O_2 in cells lacking Orp1/Gpx3, a GPx-like peroxidase that acts as the sensor of the pathway [12]. In cells lacking Orp1, GFP-Yap1 still potently accumulated in the nucleus upon treatment with arsenic compounds indicating a mechanism distinct from H_2O_2. Further, an NLS-GFP-c-CRD fusion protein [24] also accumulated in the nucleus upon arsenite and arsenate treatment while GFP-Yap1 carrying substitution of all three c-CRD cysteines was unresponsive to these chemicals. Thus, Yap1 activation by arsenic compounds involves c-CRD cysteines and therefore uses a mechanism similar to that activated by thiol-reactive chemicals.

In contrast to Yap8p, arsenic compounds did not significantly modulate Yap1 transactivation function.

4. Conclusion

In conclusion, Yap8p is an important regulator of genes involved in arsenic compounds detoxification. Activation of Yap8p involves an arsenite- and arsenate-sensitive Crm1-dependent nuclear export. Yap8 Cys^{132}, Cys^{137}, and Cys^{274} are crucial in this regulated export. Arsenic compounds probably regulate Yap8 nuclear export by modifying its cysteine residues and hence the NES, in analogy with the activation of Yap1 by these same compounds and by other thiol-reactive chemicals. Thus, Yap8 and Yap1 share a conserved mechanism of activation by arsenic compounds. A recent paper also identified Yap8 as a regulator of the *S. cerevisiae* arsenite and arsenate response. This study also suggested a regulation of Yap8 at the level of its transactivation function, consistent with our results (Fig. 5). Surprisingly these authors found Yap8 constitutively nuclear [26] which contrast with the data shown here. The reason for this discrepancy is not clear, but could be related to unknown genetic differences in the *S. cerevisiae* strains used. These differences cannot be attributed to Ybp1, a protein required for Yap1 activation by H_2O_2 and known for being mutated in some *S. cerevisiae* strains [27], since activation of both Yap8 and Yap1 by arsenic compounds is independent of this factor (data not shown).

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

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