Mathematical modelling of arsenic transport, distribution and detoxification processes in yeast

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Summary

Arsenic has a dual role as causative and curative agent of human disease. Therefore, there is considerable interest in elucidating arsenic toxicity and detoxification mechanisms. By an ensemble modelling approach, we identified a best parsimonious mathematical model which recapitulates and predicts intracellular arsenic dynamics for different conditions and mutants, thereby providing novel insights into arsenic toxicity and detoxification mechanisms in yeast, which could partly be confirmed experimentally by dedicated experiments. Specifically, our analyses suggest that: (i) arsenic is mainly protein-bound during short-term (acute) exposure, whereas glutathione-conjugated arsenic dominates during long-term (chronic) exposure, (ii) arsenic is not stably retained, but can leave the vacuole via an export mechanism, and (iii) Fps1 is controlled by Hog1-dependent and Hog1-independent mechanisms during arsenite stress. Our results challenge glutathione depletion as a key mechanism for arsenic toxicity and instead suggest that (iv) increased glutathione biosynthesis protects the proteome against the damaging effects of arsenic and that (v) widespread protein inactivation contributes to the toxicity of this metalloid. Our work in yeast may prove useful to elucidate similar mechanisms in higher eukaryotes and have implications for the use of arsenic in medical therapy.

Introduction

Arsenic is prevalent in the environment and chronic exposure may cause cardiovascular diseases, neurological disorders, liver injury, and cancers of the skin, bladder, liver and lung. Despite its toxicity, arsenic is currently used in medical therapy as a treatment for acute promyelocytic leukaemia and it might also be applied for other haematological and solid cancers. Given this dual role as causative and curative agent of disease, there is a considerable interest in understanding arsenic toxicity and detoxification mechanisms (Soignet et al., 1998; Dilda and Hogg, 2007; Hughes et al., 2011).

Arsenic can exist in various inorganic and organic forms. For simplicity, we will herein refer to arsenic when the exact form is not known or biologically relevant. In nature, arsenic is mainly present as pentavalent arsenate [AsO$_4^{3-}$ or As$V$] and trivalent arsenite [As(OH)$_3$ or As$III$]. As$V$ is a phosphate analogue that disturbs energy-generation in cells by inhibiting oxidative phosphorylation. As$III$, the most toxic form of this metalloid, may bind to and interfere with protein activity, affect the pools of cellular antioxidants, trigger DNA damage and interfere with cytoskeletal functions (Shi et al., 2004; Aposhian and Aposhian, 2006; Kitchin and Wallace, 2008; Wysocki and Tamás, 2010; Hughes et al., 2011).

Several key proteins and mechanisms involved in arsenic toxicity and detoxification have been described in the eukaryotic model organism *Saccharomyces cerevisiae* (budding yeast). In several cases, similar mechanisms exist in higher eukaryotes (Wysocki and Tamás, 2010; Wysocki and Tamás, 2011). As$III$ enters *S. cerevisiae* through the aquaglyceroporin Fps1 and yeast cells lacking this protein (fps1Δ) are As$III$ resistant (Wysocki et al., 2001). Since Fps1 is a bidirectional channel, it can also mediate As$III$ efflux when the intracellular concentration exceeds that of the extracellular environment (Maciaszczyk-Dziubinska et al., 2010). The mitogen-activated protein kinase (MAPK) Hog1 is activated by As$III$ (Thorsen et al., 2006) and regulates cell cycle resumption during exposure...
(Migdal et al., 2008; Diner et al., 2011). Activated Hog1 can phosphorylate Fps1 and thereby downregulate Fps1-dependent AsIII transport (Thorsen et al., 2006; Mollapour and Piper, 2007). Consequently, deletion of HOG1 (hog1Δ) reduces Fps1 phosphorylation levels and increases Fps1-dependent AsIII influx and sensitivity (Thorsen et al., 2006). However, whether Hog1-mediated phosphorylation inactivates (or ‘closes’) Fps1 in response to AsIII to restrict AsIII influx and enhance tolerance, remains unknown. AsIII detoxification in S. cerevisiae involves two transport systems: the plasma membrane-localized exporter Acr3 and the vacuolar membrane-localized ABC-transporter Ycf1 (Wysocki et al., 1997; Ghosh et al., 1999). AsIII-exposed cells induce expression of the ACR3 gene, which results in increased AsIII export and enhanced tolerance (Wysocki et al., 1997; 2004). Intracellular AsIII can be conjugated to the thiol moiety of glutathione (GSH) followed by sequestration of the resulting AsIII-tri-glutathione complex As(GS)3 into vacuoles catalysed by Ycf1 (Ghosh et al., 1999). Cells lacking Acr3 (acr3Δ) are highly AsIII sensitive, whereas cells lacking Ycf1 (ycf1Δ) show moderate sensitivity. Cells deficient in both Acr3 and Ycf1 (acr3Δycf1Δ) display an additive hypersensitivity (Wysocki et al., 1997; 2001; Ghosh et al., 1999).

Besides acting as a chelating agent, GSH also protects cells from metal-induced oxidative damage due to its role in cellular redox control (Wysocki and Tamás, 2010). AsIII-exposed yeast cells strongly increase GSH biosynthesis and accumulate high amounts of cytosolic (Thorsen et al., 2007) and extracellular (Thorsen et al., 2012) GSH. Both mechanisms serve to decrease intracellular/cytosolic free arsenic levels to enhance tolerance (Thorsen et al., 2007; 2012). Consequently, cells devoid of glutathione biosynthesis are AsIII sensitive (Wysocki et al., 2004; Preveral et al., 2006; Thorsen et al., 2007).

Although much has been learned in recent years about the impact of arsenic on cells and the detoxification strategies used to acquire tolerance, several issues remain unresolved:

- How are transporters involved in AsIII tolerance regulated during exposure?
- How is arsenic distributed within cells? This question is important to explain toxicity mechanisms; yet, accurate measurement of cytosolic, vacuolar, protein-bound, and GSH-conjugated arsenic pools remains challenging.
- How do cells respond to chronic versus acute exposure?
- How efficient is vacuolar sequestration for AsIII detoxification and tolerance?

In this work, we combined arsenic transport assays in S. cerevisiae with mathematical modelling to seek answers to the questions above. Mathematical modelling has been proven to be useful in elucidating molecular mechanisms in yeast, e.g. for signalling (Schaber et al., 2006; Behar et al., 2008), cell cycle regulation (Csikasz-Nagy et al., 2009; Adrover et al., 2011) and especially in the HOG signal transduction system (Klipp et al., 2005; Schaber et al., 2010; 2011; 2012; Petelenz-Kurdziel et al., 2013). Modeling enables us to quantify formerly qualitative hypothesis. Moreover, a mathematical model forces the researcher to make hypotheses conceptually rigorous and allows for systematic testing of competing hypothesis, as will be demonstrated.

Our combined molecular and modelling analysis suggests that:

- Fps1 is controlled by Hog1-dependent and Hog1-independent mechanisms during arsenite stress.
- AsIII efflux through Acr3 quickly saturates upon AsIII stress.
- Ycf1 protein levels are upregulated in AsIII hyper-accumulating mutants (acr3Δ mutants).
- Protein-bound arsenite is the most abundant species during short-term (acute) exposure, whereas GSH-conjugated arsenic dominates during long-term (chronic) exposure.
- Widespread protein inactivation might contribute to arsenic toxicity.
- Increased GSH biosynthesis may protect the proteome against the damaging effects of arsenic.
- There is an export mechanism for AsIII out of the vacuole.

Results

Quantifying cellular arsenic levels, model construction and validation

AsIII uptake and efflux are linked to toxicity and detoxification respectively (Wysocki and Tamás, 2010; Wysocki and Tamás, 2011). To explain how various pathways contribute to intracellular arsenic accumulation, we combined quantitative time-course experiments with mathematical modelling. Yeast cells were first pre-treated with 0.1 mM AsIII for 24 h, and then exposed to extra 1.0 mM AsIII for 1 h (Experimental procedures). Subsequently, cells were washed and resuspended in AsIII-free medium. Samples were taken during the whole time-course and intracellular arsenic was determined. In this way, AsIII influx and efflux was measured in wild type, ycf1Δ (defective in vacuolar sequestration), hog1Δ (defective in Fps1 closure), acr3Δ (defective in export), acr3Δ hog1Δ (defective in both Fps1 closure and export), acr3Δ ycf1Δ (defective in both export and vacuolar sequestration), and gsh1Δ PRO2-1 (strongly diminished GSH levels) cells. These strains exhibited distinct AsIII accumulation profiles (Fig. 1). With a few exceptions, the accumulation profiles corresponded well with
sensitivity of these strains to AsIII (Supplementary Fig. S1). To assess whether these strains exhibit distinct intracellular distribution profiles we used mathematical models. Model development was guided by the principle of parsimony, i.e. we intended to obtain mathematical models that are as simple as possible and as complex as necessary to both explain the data and address our research questions described above. We implemented an ensemble of parsimonious mathematical models reflecting the uncertainty about the underlying molecular mechanisms of AsIII transport and intracellular distribution. These models were subsequently trained to explain the data by parameter estimation procedures. We used one part of the data to train the models and estimate the parameters, and another part of the data to validate the models by testing its predictive power using data not used to train the models. We used the measured data for AsIII uptake and efflux from most mutants, phosphorylated Fps1 and Hog1 phosphorylation/activation data to estimate model parameters (Supporting information: Methods). The AsIII uptake and efflux data from the GSH knock-down strain (gsh1Δ PRO2-1) were used for validating the predictive properties of the models (Fig. 4). This seemed reasonable, because one of the goals of this study was to analyse the role of GSH in detoxification. The credibility of results concerning the role of GSH increases, if the models are able to predict a GSH-related experiment, which was not used to train the model. We selected a model which was best supported by the available data and also had good predictive properties (Fig. 2, see Experimental procedures for the parameter estimation and model selection procedure). The best model was not only able to reproduce all experimental data well (Figs 1 and 3), but could also well predict the arsenic accumulation profile of GSH knock-down cells (gsh1Δ PRO2-1) (Fig. 4). Most of the model parameters (15 out of 20) were practically identifiable (Supplementary Fig. S2) indicating that the parameters are not arbitrary and supporting our intention of developing parsimonious models instead of over-fitted ones. Therefore, we were confident to use the best selected model for further analyses.

The best-ranked model can be found in Biomodels database under identifier MODEL1403280000 (Le Novere et al., 2006).
Activity and regulation of transporters during arsenite exposure

Fps1 is controlled by Hog1-dependent and Hog1-independent mechanisms during arsenite stress. We first used the model to explore regulation of Fps1 phosphorylation during As\textsubscript{III} exposure. The simulated time-course of phosphorylated Fps1 (Fps1-P) dynamics largely resembled extracellular arsenite (As\textsubscript{III}\textsubscript{ex}) dynamics, suggesting that Fps1 activity is regulated during As\textsubscript{III} exposure (compare Fig. 3B with Fig. 1A). Simulations also indicated a lower level of Fps1-P in \textit{hog1}\textsuperscript{Δ} cells than in wild type cells upon As\textsubscript{III} exposure by an almost constant amount of about 20% (Fig. 3B and C). Nevertheless, the dynamics of Fps1 phosphorylation upon As\textsubscript{III} stress was similar in wild type and \textit{hog1}\textsuperscript{Δ} cells. This suggests that Hog1 contributes to Fps1 phosphorylation in wild type cells, whereas in a \textit{hog1}\textsuperscript{Δ} mutant, an arsenic-dependent mechanism compensates for Hog1 loss (Supplementary Fig. S3). Thus, our data best support a model in which Fps1 is controlled by Hog1-dependent and Hog1-independent mechanisms during arsenite stress.

Acr3-mediated arsenite export quickly saturates. Members of the Acr3 family of arsenic transporters are

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**Fig. 2.** Alternative model structures. Dotted lines indicate alternative model components implemented in the candidate models. The dark dotted lines indicate alternatives present in the best-ranked model. Four different sources of variation were implemented, each of them able to adopt two different setups, consequently 16 different model combinations were generated: (1) binding of (GSH)\textsubscript{3} to As\textsubscript{III}/direct conversion of As\textsubscript{III} to As(GS)\textsubscript{3} through reaction 8 (v\textsubscript{8}); (2) Ycf1 concentration is/is not significantly upregulated in acr3\textsubscript{Δ} mutants during pre-incubation in 0.1 mM As\textsubscript{III}; (3) Michaelis–Menten (MM) or mass action (MA) rate laws for reaction 10 (v\textsubscript{10}); and (4) MM/MA rate laws for reaction 14 (v\textsubscript{14}).

**Fig. 3.** Comparison of model simulation with cell signalling data. Solid lines show model simulations and (x) marks show the experimental data.

A. Fitted Hog1 phosphorylation (Hog1PP) data by best-ranked model. The Hog1PP percentage in response to As\textsubscript{III} stress is compared to Hog1PP at 5 min after 0.4M NaCl stress (Supporting information: Scaling data). The data are derived from Thorsen et al. (2006).

B and C. Fps1 phosphorylation data in wild type and \textit{hog1}\textsuperscript{Δ} mutant fitted by best-ranked model. Fps1-P data [mean ± SD (n ≥ 8)] is scaled to Fps1-P at 1 h after 1.0 mM As\textsubscript{III} stress (Supporting information: Scaling data).

**Fig. 4.** GSH knock-down experiment and prediction. Solid lines show model simulations and (x) marks show the experimental data [mean ± SD (n = 4)]. GSH knock-down mutant (\textit{gsh1}\textsuperscript{Δ} PR02-1) As\textsubscript{III} influx-efflux data are excluded from parameter estimation. The best-ranked model can predict the corresponding experiment.
present in every kingdom of life, but their transport properties are not fully characterized. Models containing Michaelis–Menten kinetics for export of free intracellular As(III) (As(III)in) ranked first (Table 1), and simulations showed that As(III)in export via Acr3 is saturated upon 0.1 mM As(III) exposure with a half-saturation constant of \(9.74 \times 10^{-4} \, \text{μmol l}^{-1}\) (Supplementary Fig. S4). The corresponding parameter value is very small which turns the As(III) export practically into a zero-order kinetics. However, the half-saturation constant was not practically identifiable (Supplementary Fig. S2).

Ycf1 levels increase in arsenite hyper-accumulating mutants. The model also provided insight into Ycf1 regulation. The data best support a model in which Ycf1 levels are higher in acr3Δ mutants (except in acr3Δycf1Δ) than in wild type and other strains (Model No. 2 in Table 1, Ycf1 column). Hence, cells that hyper-accumulate arsenic (see Fig. 1) increase Ycf1 levels, possibly to enhance vacuolar sequestration.

Evidence for a vacuolar export mechanism
We noted that wild type and ycf1Δ cells had similar arsenic accumulation profiles (Fig. 1A and B), yet ycf1Δ is As(III) sensitive showing a longer lag phase upon As(III) stress (Supplementary Fig. S1). We hypothesized that Ycf1-activity is masked by Acr3. Indeed, acr3Δycf1Δ mutants accumulated substantially less arsenic than acr3Δ (Fig. 1D and E) indicating that vacuolar arsenite (vAs(GS)3) constitutes a significant amount of the total intracellular arsenic pool in acr3Δ cells. Model and experimental data show a rapid decrease in intracellular arsenic levels for all strains when cells are resuspended in As(III)-free medium (Fig. 1).

Importantly, albeit with slower kinetics compared to acr3Δycf1Δ, the acr3Δ mutant can export most intracellular arsenic (Fig. 1D and E) despite a substantial amount being present in the vacuole. Likewise, experimental results show that arsenic export is faster in acr3Δhog1Δ than in acr3Δ cells (Fig. 1E and F) although the model predicts an even higher amount of vacuolar As(III) in acr3Δhog1Δ than in acr3Δ cells (compare Fig. 5F and D). This decrease in

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Models are ranked according to Akaike Information Criterion corrected for small sample size (AICc). GSH knock-down (gsh1Δ PRO2-1) influx–efflux data are not used for parameter estimation, but it is used for calculating the objective value (wSSR). Top eight models have (GSH)3 binding in their structure and top four models use MM kinetics for As(III) export through Acr3. Abbreviations: n, number of data points; k, number of parameters; wSSR, weighted sum of squared residuals; AICc, Akaike Information Criterion corrected for small sample size; AICw, Akaike weights. MA is Mass Action kinetics and MM is Michaelis–Menten kinetics.
AsIIItot cannot be explained by dilution due to cell proliferation as cell numbers stayed constant during the time when arsenic export occurred (Supplementary Fig. S5). Instead, the data support a model in which the decrease in intracellular arsenic in $acr3\Delta hog1\Delta$ cells is explained by a mechanism that channels arsenic out of the vacuole.

Proteins are the main target of arsenite during short-term (acute) stress

In situ measurement of intracellular arsenic pools is challenging; yet, the question of how arsenic is distributed within cells is important to explain toxicity mechanism. We therefore used the model to explore subcellular distribution of arsenic in yeast. Interestingly, model simulations suggest that 77%–91% of the increase in AsIIItot during acute AsIII exposure (i.e. addition of 1.0 mM AsIII) is explained by an increase in protein-bound arsenite (AsIIIprot) (Fig. 5). This feature is not only the case for different mutants, but also for different stress regimes as AsIIIprot increased more than other arsenic pools in response to AsIII in concentrations ranging from 0.5 to 2.0 mM (Supplementary Fig. S6). Hence, the model suggests that proteins are the primary targets for AsIII under acute exposure. In addition, we conducted a sensitivity analysis of the model parameters by systematically changing each parameter and analysing for its impact on model simulations (see Sensitivity Analysis in Supporting information).

Glutathione-conjugated arsenite is the most abundant arsenite species during long-term (chronic) stress

Experimental data showed that initial intracellular arsenite concentrations after 24 h of pre-incubation varied between two orders of magnitude, especially for the $acr3\Delta$ mutants (Fig. 1D–F). In those mutants, AsIIIex and AsIIIin concentrations equilibrate by passive flux through Fps1 and, thus, are assumed to initially be the same for all these mutants. Consequently, at the initial equilibrium after 24 h of pre-incubation, the protein bound arsenite (AsIIIprot), as a function of free intracellular arsenite (AsIIIin), is also the same for the $acr3\Delta$ mutants. Thus, the large variation of initial total intracellular arsenic in those mutants can only be accommodated with our model family by varying initial concentrations of GSH-conjugated arsenite.
conjugated As\(^{\text{III}}\) species by different amounts of initial GSH. Accordingly, in the best-ranked model (No. 2) the GSH-conjugated As\(^{\text{III}}\) species As(GS)\(_3\) and vAs(GS)\(_3\) are the most abundant As\(^{\text{III}}\) species after long-term/chronic exposure (i.e. initial concentrations after 24 h pre-incubation with 0.1 mM As\(^{\text{III}}\)), unless GSH is strongly downregulated (as in \(_{\text{gsh}}\)\(_1\) \(_{\text{ΔPRO2-1}}\) cells) (Fig. 6).

Model analysis also suggested that the concentrations of GSH-conjugated As\(^{\text{III}}\) species saturate as a function of As\(^{\text{III}}\) in due to limited total amount of GSH, whereas As\(^{\text{IIIprot}}\) increases linearly with As\(^{\text{III}}\) in due to the unlimited protein pool (Supplementary Fig. S10). This also explains why As\(^{\text{IIIprot}}\) becomes the most prominent arsenite species after acute stress. However, the concentrations of GSH-conjugated As\(^{\text{III}}\) species increase linearly with cellular GSH levels (Supplementary Fig. S11). Thus, As(GS)\(_3\) should increase as long as GSH is upregulated upon arsenite exposure. To test this idea, we modified the model such that the cellular GSH level continuously increased during As\(^{\text{III}}\) exposure (Supporting information: Modified Model Changes). The modified model simulations showed that GSH upregulation can result in As(GS)\(_3\) being the most abundant As\(^{\text{III}}\) species after 24 h treatment with 1.0 mM As\(^{\text{III}}\) (Fig. 7). In addition, sensitivity analysis of best-ranked model suggested that As\(^{\text{IIIin}}\)-GSH conjugation rates (\(k_8\) and \(k_9\)) are the most sensitive model parameter affecting total As\(^{\text{III}}\) level change in acr3\(_3\) cells (Supplementary Figs S7 and S8).

To test the model predictions (GSH upregulation during As\(^{\text{III}}\) exposure and GSH concentration in acr3\(_3\) mutants compared to wild type after chronic exposure), we conducted a dedicated experiment. A qualitative assay showed that a 6 h exposure to 0.1 mM As\(^{\text{III}}\) resulted in higher GSH levels in all strains tested. Moreover, acr3\(_3\) mutants produced more GSH than wild type cells and mutants expressing a functional Acr3 (Supplementary Fig. S9). Thus, experimental data confirmed the model predictions.

Taken together, our modelling framework combined with experimental assays suggests that GSH is upregulated during long-term exposure and sequesters most As\(^{\text{IIIin}}\) in GSH-conjugated As\(^{\text{III}}\) species, whereas under acute stress most arsenite binds to protein.

**Glutathione protects the proteome against the damaging effects of arsenic**

The above model analysis suggested that enhanced GSH biosynthesis may protect the proteome from the damaging effects of arsenic. As\(^{\text{III}}\) may impact the proteome in two ways: (i) it can bind to proteins thereby interfering with protein activity (Kitchin and Wallace, 2008; Hughes \textit{et al}., 2011), and (ii) it can disrupt protein function by triggering misfolding and aggregation of newly synthesized proteins (Jacobson \textit{et al}., 2012). To experimentally address the prediction that GSH protects the proteome against arsenic-induced damage, we moni-

![Fig. 6. Concentration of different As\(^{\text{III}}\) species after chronic exposure.](image1)

**Fig. 6.** Concentration of different As\(^{\text{III}}\) species after chronic exposure. A. Intracellular concentration (ng per 10\(^6\) cells) of different As\(^{\text{III}}\) species is simulated for the wild type and mutants having the ACR3 gene (ACR3\(^{+}\) mutants). B. As\(^{\text{III}}\) species concentration for acr3\(_3\) mutants.

![Fig. 7. Simulation of As\(^{\text{III}}\) species with continuous GSH production.](image2)

**Fig. 7.** Simulation of As\(^{\text{III}}\) species with continuous GSH production. Intracellular concentration (ng per 10\(^6\) cells) of different As\(^{\text{III}}\) species is simulated for 24 h and wild type cells using a modified model with continuous GSH production, plotted using area plots. GSH conjugation gradually surpasses protein binding in the presence of 1.0 mM of As\(^{\text{III}}\) during 24 h of simulation. No cell division is considered for the 24 h simulation, which is a reasonable assumption, because of the sensitivity of cells to high As\(^{\text{III}}\) dose (1.0 mM). Ycf1 level was assumed fix during simulation.
tored protein aggregation in living cells by following the subcellular distribution of Hsp104 (a major disaggregating chaperone) coupled to GFP (green fluorescent protein) as marker for aggregate formation. Hsp104–GFP was evenly distributed throughout the cytosol in unexposed cells, whereas AsIII triggered Hsp104–GFP redistribution to distinct foci that represent sites of protein aggregation (Supplementary Fig. S12) (Jacobson et al., 2012). Quantifying protein aggregation by counting the fraction of cells with Hsp104–GFP foci revealed that about 75% of wild type cells contained aggregates after 1 h of AsIII exposure (Fig. 8A). After 3 h, most wild type cells had cleared the cytosol from protein aggregates.

In contrast, cells defective in GSH biosynthesis (gsh1Δ cells) still showed extensive protein aggregation (>75% of cells contained aggregates) after 3 h of exposure (Fig. 8A). To test whether it is the AsIII-chelating property of GSH that protects the proteome from aggregation, we enabled synthesis of the AsIII-chelating molecule phytochelatin in S. cerevisiae cells that normally do not synthesize this molecule (Clemens et al., 1999; Wysocki et al., 2003). For this, we transformed wild type cells with a plasmid harbouring the PCS1 gene (from Schizosaccharomyces pombe) encoding phytochelatin synthase or an empty vector as a control. Next, we quantified AsIII-induced protein aggregation of the transformants as described above. Indeed, cells expressing the PCS1 gene (and hence capable of synthesizing phytochelatin (Wysocki et al., 2003)) had less aggregates than those not expressing PCS1 (Fig. 8B). Taken together, these experimental results support the model prediction that GSH protects the proteome against the damaging effects of arsenic, probably by chelating this metalloid.

**Discussion**

In this study, we combined mathematical modelling and experimental data to explore several issues related to arsenite transport, intracellular distribution and detoxification processes in yeast.

**Activity and regulation of transporters**

The model provided novel understanding of transporter activity and regulation. First, we explored whether Hog1 contributes to Fps1 inactivation (‘closure’) upon AsIII stress as a mechanism to restrict AsIII influx and enhance tolerance. The model was able to explain Fps1 phosphorylation, AsIII influx–efflux and Hog1 phosphorylation data. The dynamics of Fps1 phosphorylation shows a significant analogy to AsIIIex dynamics, suggesting a quick response (i.e. ‘closure’) of Fps1 that may result in enhanced tolerance. The model suggested that Hog1 is the main Fps1 regulator, but that in the absence of Hog1 (hog1Δ cells) another arsenic-dependent mechanism can regulate Fps1 phosphorylation/activity. Moreover, sensitivity analysis of Fps1 phosphorylation suggested that the dephosphorylation reaction rate (k7) is the most sensitive model parameter affecting Fps1-P level change during AsIII exposure and after cell wash for both wild type and acr3Δ cells (Supplementary Figs S13 and S14), suggesting that dephosphorylation plays an important role in Fps1 regulation. Identification of the kinase(s) and phosphatase(s) involved in Fps1 phosphorylation and dephosphorylation will be important to further elucidate how cells limit arsenic influx and toxicity. The identifiability analysis supported the calibration of Fps1 phosphorylation parameters (Supplementary Fig. S2).

Second, the model suggested a quickly saturating Michaelis–Menten kinetics for AsIII export via Acr3. This finding is supported by the recent demonstration that Acr3 is an arsenite/proton antiporter characterized by Michaelis–Menten-type saturation kinetics (Maciaszczyk...
Dziubinska et al., 2011) and adds to our understanding of the transport properties of this widespread family of arsenite exporters.

Third, the best-ranked model had higher Ycf1 levels in cells lacking Acr3 (except for acr3Δycf1Δ) than in cells expressing Acr3. This model prediction is in agreement with experimental data showing that expression of the YCF1 gene is hyper-induced in mutants with elevated AsIII concentrations (Wysocki et al., 2004). Under the same experimental conditions, YCF1 expression is not induced by arsenic in wild type cells (Wysocki et al., 2004). These experimental data further support the model.

**Evidence of a vacuolar arsenic export mechanism**

The relevance of vacuolar sequestration of metal-glutathione conjugates as a detoxification mechanism in yeast is inferred from the metal sensitivity of mutants defective in this process (reviewed in Wysocki and Tamás, 2010). It has been postulated that vacuolar sequestration of As(GS)3 is particularly relevant for arsenic detoxification given that the vacuole is acidic and that the As(GS)3 complex is more stable at low pH in vitro (Canovas et al., 2004; Rey et al., 2004). Yet, deletion of YCF1 (ycf1Δ) only caused moderate AsIII sensitivity, visible as a longer lag phase while the growth rate is unaffected (Supplementary Fig. S1). Moreover, YCF1 gene expression is not induced in wild type cells during AsIII exposure (Wysocki et al., 2004). Hence, the relative importance of this pathway for AsIII detoxification remains unclear. Here, we provide evidence that AsIII is not stably retained in the vacuole; acr3Δ and acr3Δhog1Δ cells diminished intracellular arsenic despite having a significant fraction of the total cellular arsenic present in the vacuole (Figs 1 and 5D–F). How does vacuolar AsIII leave yeast cells? Vacuolar AsIII could be exported via exocytosis or it could first enter the cytosol and then be exported out of cells. It has been shown that Fps1 can mediate AsIII efflux when the intracellular concentration exceeds that of the extracellular environment (Maciaszczyk-Dziubinska et al., 2010). Moreover, deletion of YOG1 increases Fps1-mediated AsIII transport (Thorsen et al., 2006). Assuming that most AsIII leaves acr3Δhog1Δ cells through hyper-activated Fps1 (Maciaszczyk-Dziubinska et al., 2010) argues that vacuolar AsIII is not well retained in the vacuole is unexpected given that vacuolar sequestration of glutathione-conjugates is a conserved detoxification mechanism in yeasts and plants (reviewed in Wysocki and Tamás, 2010). Nevertheless, vacuolar degradation of glutathione-conjugates via γ-glutamyl-transpeptidase activity has been described in Arabidopsis (Grzam et al., 2007; Ohkama-Ohtsu et al., 2007), and a similar degradation pathway appears to exist in yeast (Ubiyovk et al., 2006; Wunschmann et al., 2010). Whether this pathway is responsible for As(GS)3 catabolism is currently unknown. The protein responsible for vacuolar arsenic export and the form of arsenic that this protein recognizes is not known. Assuming that acr3Δhog1Δ cells export arsenic through hyper-active Fps1, it is possible that arsenic enters the cytosol from the vacuole as As(OH)3, which is the form recognized by Fps1. Interestingly, S. cerevisiae possesses an uncharacterized Fps1 homologue encoded by the YFL054c gene. Whether Yfl054c is localized to the vacuolar membrane and mediates vacuolar arsenic export remains to be investigated.

**What is the role of Ycf1 for detoxification?**

If arsenic is not stably retained in the vacuole, what is then the importance of Ycf1-mediated sequestration of As(GS)3? The vacuole probably contributes to arsenite tolerance by keeping the AsIII, steady-state concentration below a certain (critical) level, exemplified by a higher AsIII sensitivity of acr3Δycf1Δ compared to acr3Δ (Supplementary Fig. S1). We previously speculated that conjugation of AsIII to GSH is rate-limiting and that a basal level of Ycf1 in the vacuolar membrane is sufficient for tolerance (Wysocki et al., 2004). Supporting this hypothesis, simulations suggested that vacuolar arsenite (vAs(GS)3) increases along with As(GS)3 increase (Fig. 7). This hypothesis also explains why overexpression of YCF1 (driven by the strong GAL1 promoter) does not improve AsIII resistance of yeast (Preveral et al., 2006). The steady-state concentration of AsIII in and consequently AsIII in cells diminished intracellular arsenic despite having a significant fraction of the total cellular arsenic present in the vacuole (Figs 1 and 5D–F). How does vacuolar AsIII leave yeast cells? Vacuolar AsIII could be exported via exocytosis or it could first enter the cytosol and then be exported out of cells. It has been shown that Fps1 can mediate AsIII efflux when the intracellular concentration exceeds that of the extracellular environment (Maciaszczyk-Dziubinska et al., 2010). Moreover, deletion of YOG1 increases Fps1-mediated AsIII transport (Thorsen et al., 2006). Assuming that most AsIII leaves acr3Δhog1Δ cells through hyper-activated Fps1 (Maciaszczyk-Dziubinska et al., 2010) argues that vacuolar AsIII is not well retained in the vacuole is unexpected given that vacuolar sequestration of glutathione-conjugates is a conserved detoxification mechanism in yeasts and plants (reviewed in Wysocki and Tamás, 2010). Nevertheless, vacuolar degradation of glutathione-conjugates via γ-glutamyl-transpeptidase activity has been described in Arabidopsis (Grzam et al., 2007; Ohkama-Ohtsu et al., 2007), and a similar degradation pathway appears to exist in yeast (Ubiyovk et al., 2006; Wunschmann et al., 2010). Whether this pathway is responsible for As(GS)3 catabolism is currently unknown. The protein responsible for vacuolar arsenic export and the form of arsenic that this protein recognizes is not known. Assuming that acr3Δhog1Δ cells export arsenic through hyper-active Fps1, it is possible that arsenic enters the cytosol from the vacuole as As(OH)3, which is the form recognized by Fps1. Interestingly, S. cerevisiae possesses an uncharacterized Fps1 homologue encoded by the YFL054c gene. Whether Yfl054c is localized to the vacuolar membrane and mediates vacuolar arsenic export remains to be investigated.

**Protein-binding and glutathione-conjugation of arsenite during acute and chronic arsenite exposure**

Model simulations suggested that 77%–91% of the increase in AsIII in during acute AsIII exposure (i.e. addition of 1.0 mM AsIII) is explained by an increase in protein-bound arsenite (AsIII-protein) in wild type cells and all mutants (Fig. 5). In addition, sensitivity analysis of the best-ranked model suggested that k2 (AsIII-protein association rate constant) and k3 (AsIII-protein dissociation rate constant) are the most sensitive model parameters affecting cellular arsenic levels during AsIII exposure and after cell wash in wild type. Hence, the sensitivity analysis emphasizes that proteins are the main targets of AsIII in wild type cells during acute and chronic arsenite exposure.
AsIII exposure. Since arsenic-binding can inhibit protein activity (Kitchin and Wallace, 2008; Hughes et al., 2011), our model suggests that widespread protein inactivation may be a major toxicity mechanism. Moreover, we recently demonstrated that AsIII disrupts protein function and causes toxicity by triggering misfolding and aggregation of newly synthesized proteins (Jacobson et al., 2012). Thus, AsIII inhibits protein activity in two ways; by direct binding to folded proteins and by interfering with folding of nascent polypeptides. This work also provided insight into the dynamics of subcellular arsenic distribution. GSH plays an important role in AsIII detoxification and tolerance, and GSH biosynthesis is stimulated during AsIII exposure (Thorsen et al., 2007). For the sake of simplicity, we assumed that cellular GSH content does not increase during 1 h of AsIII stress; however, we included the possibility of higher initial GSH levels for acr3Δ mutants in some models. Including this possibility was reasonable given the observed hyper-activation of Yap1 in mutants with elevated intracellular AsIII and that Yap1 controls expression of GSH biosynthesis genes (Wysocki et al., 2004; Thorsen et al., 2007). These models, in fact, were ranked best, because only they could accommodate large variations in initial intracellular arsenic concentrations in acr3Δ mutants. This prediction was experimentally confirmed (Supplementary Fig. S9). The model predicted higher concentration of GSH-conjugated than protein bound AsIII species after 24 h AsIII exposure (pre-incubation), except when GSH production is down-regulated (i.e. in gsh1Δ PRO2-1 cells) (Fig. 6). This finding suggests that enhanced GSH biosynthesis may protect the proteome from the damaging effects of AsIII. In support of this prediction, we experimentally demonstrated that GSH biosynthesis-deficient cells accumulated more aggregated/damaged proteins than wild type cells, whereas cells with increased AsIII-chelating capacity showed decreased levels of aggregated/damaged proteins (Fig. 8).

Correlation between intracellular arsenic distribution and toxicity

Correlating the predicted distribution of intracellular arsenic and the AsIII-sensitivity of different mutants can expand our understanding of arsenic toxicity mechanisms. Our results indicate that cells with a higher proportion of protein-bound arsenic (hog1Δ, acr3Δ, acr3Δ hog1Δ, acr3Δ ycf1Δ) are more arsenite sensitive (Supplementary Fig. S1) (also Thorsen et al., 2006), supporting the notion that widespread protein inactivation contributes to the toxicity of this metalloid. In contrast, our results do not show a direct correlation between increased GSH conjugation and AsIII sensitivity (growth and simulation of AsIII in acr3Δ and acr3Δ ycf1Δ cells in Supplementary Figs S1 and S5). This result challenges GSH depletion as a major arsenic toxicity mechanism in yeast. Curiously, the GSH knock-down strain is not very AsIII sensitive despite most of the intracellular arsenic appears protein-bound (Supplementary Fig. S1). However, the total amount of intracellular arsenic is lower in this strain than in the corresponding wild type (compare Fig. 1A with Fig. 4). Hence, gsh1Δ PRO2-1 cells might compensate for the lack of GSH with other (unknown) tolerance mechanisms.

To conclude, we selected a model out of an ensemble of designed models, which represented simplified mechanisms of AsIII accumulation in yeast cells. This simplified model has been instrumental to provide novel insights into several aspects of arsenic transport, intracellular distribution and detoxification processes, which could partly be confirmed experimentally. Because arsenic toxicity and detoxification mechanisms appear conserved in various eukaryotes, this work in yeast may prove useful to elucidate similar mechanisms in other organisms and have implications for the use of arsenic in medical therapy.

Experimental procedures

Experimental strains and growth conditions

The S. cerevisiae strains and plasmids used in this study are described in Supplementary Table S1. Yeast strains were grown at 30°C in minimal synthetic complete (SC) medium (0.67% yeast nitrogen base) and 2% glucose as a carbon source. Sodium arsenite (NaAsO2) was obtained from Sigma-Aldrich.

Determination of intracellular arsenic during influx–efflux

Intracellular arsenic was measured essentially as described previously (Thorsen et al., 2006). Briefly, cells were first exposed to 0.1 mM AsIII for ~24 h (chronic exposure) before addition of 1.0 mM AsIII (acute exposure). The pre-exposure is required to correctly assess the contribution of Acr3 to AsIII efflux (Ghosh et al., 1999; Thorsen et al., 2006). Cultures were incubated for 1 h with 1.0 mM AsIII to allow intracellular accumulation, then washed and resuspended in AsIII-free medium to allow AsIII efflux. Cells were collected at the indicated time points, washed in ice-cold water and pelleted by centrifugation. Cell pellets were resuspended in water, boiled for 10 min, centrifuged, and the supernatants were collected. The arsenic content of each sample was determined using a graphite furnace atomic absorption spectrometer (SIMAA 6000; Perkin-Elmer) as described previously (Wagner and Boman, 2004). Arsenic influx–efflux measurements were performed at least twice from independent cell cultures.

Microscopy

Yeast cells expressing Hsp104-GFP were grown to mid-log phase in SC medium containing appropriate amino acid requirements for plasmid selection or in rich YPD (1% yeast extract, 2% peptone, and 2% glucose) medium. To induce

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PCS1 expression, 2% galactose was used. Cells were washed twice with water or PBS and the GFP signals were observed in living cells using a Leica DM RXA (Leica Microsystems) fluorescence microscope equipped with 100× HCX PL Fluor 1.30 objective and appropriate fluorescence light filter sets. Images were captured with digital camera [Hamamatsu C4742-95 (Hamamatsu Photonics)] and QFluoro software, and processed with Photoshop CS (Adobe Systems). To quantify protein aggregation we use several images taken from the same culture and also from independent cultures. In each image we count the total number of cells and the number of cells that contain HSP104-GFP foci to calculate the fraction of cells that show Hsp104-GFP foci. For each condition/mutant, we count several hundreds of cells by visual inspection.

**Data processing**

Experimental data were scaled in various ways to be comparable to the model simulations (Supporting information: Scaling Data).

**Model formulation**

In order to model processes that are relevant for As\textsuperscript{III} toxicity and detoxification in yeast, 16 different mathematical models were implemented as ordinary differential equations (ODEs). The models are highly simplified representations of the biochemical network underlying As\textsuperscript{III}-mediated signalling, complex formation and influx–efflux. The mathematical formulation of the described processes and all estimated parameters and initial conditions are detailed in the Supplementary Tables S2–S6, whose structure is explained in Supporting information: Models Setup Summary. For simplicity and for the lack of appropriate data, mass action kinetics was used in most reactions. Following the principle of parsimony for model development, only state variables that we felt were absolutely necessary was included in the model and all processes were reduced to a minimum. Still, there are processes and state variables where it was not obvious, whether they would be necessary or not to explain the data. Those we subjected to a systematical model selection analysis detailed below. We now separately address the main variables and processes considered in detail. Reactions numbers refer to Fig. 2.

**Fps1-mediated arsenite influx, protein binding**

As\textsuperscript{III} influx–efflux through Fps1 was considered as a passive diffusion process (Reaction v\textsubscript{1} in Fig. 2) regulated by the phosphorylation state of Fps1. The phosphorylated form of Fps1 (Fps1-P) is considered as the inactive/closed state of the channel (Thorsen et al., 2006). Both binding and dissociation of As\textsuperscript{III} to/from proteins was implemented using mass action kinetics [reactions v\textsubscript{2} and v\textsubscript{3}]. Protein-bound arsenite (As\textsuperscript{III}_\textsubscript{prot}) is not available for sequestration to the vacuole or efflux via Fps1 or Acr3.

**Hog1 and Fps1 regulation**

The MAPK Hog1 becomes dually phosphorylated and, thereby, activated in response to As\textsuperscript{III} exposure (Thorsen et al., 2006), which is considered here as a single constitutive reaction (Reaction v\textsubscript{4}). Hog1 dephosphorylation is also implemented as a single reaction (Reaction v\textsubscript{5}). Due to the uncertainty about the Fps1 regulation mechanisms, we distinguished three different Fps1 phosphorylation mechanisms within the model. First, basal Fps1 phosphorylation, which is independent of As\textsuperscript{III} stress (Reaction v\textsubscript{6} in Fig. 2). Second, Hog1-dependent Fps1 phosphorylation (Reaction v\textsubscript{6} in Fig. 2). Third, As\textsuperscript{III}₆₆₆₆-dependent Fps1 phosphorylation (Reaction v\textsubscript{6} in Fig. 2). Fps1 dephosphorylation was also assumed as mass action kinetics (Reaction v\textsubscript{7}). Fps1 and Hog1 phosphorylation data are detailed in Supplementary Table S7.

**Arsenite-glutathione conjugation**

As\textsuperscript{III}_\textsubscript{in} can conjugate with three GSH molecules to form As(GS)\textsubscript{3} (Delnomedieu et al., 1994). For simplicity, we introduced the species (GSH)_\textsubscript{n}, which represents three GSH molecules and we can use a simple rate law for As\textsuperscript{III}_\textsubscript{in}-GSH conjugation. As\textsuperscript{III}_\textsubscript{in}-GSH conjugation was implemented in two different ways in reaction v\textsubscript{8} (Fig. 2): first, As\textsuperscript{III}_\textsubscript{in} directly converts to As(GS)\textsubscript{3} (Fig. 2), i.e. independent of the GSH concentration. Such a mechanisms would disregard possible effects of GSH depletion or upregulation; second, we explicitly consider a binding reaction of (GSH)_\textsubscript{n} to As\textsuperscript{III}_\textsubscript{in}. Such a formulation allows considering effects of GSH depletion (or rather saturation of As(GS)\textsubscript{3}) formation), as well as different initial As(GS)\textsubscript{3} values through different initial (GSH)\textsubscript{3} values and corresponding steady-state concentration.

We allowed for three different initial (GSH)\textsubscript{3} concentrations, depending on the considered mutant:

1. (GSH)\textsubscript{3-wt-0} : was used for wild type and ycf1Δ and hog1Δ mutants
2. (GSH)\textsubscript{3-acr3Δ-0} : was used for acr3Δ and acr3Δycf1Δ mutants
3. (GSH)\textsubscript{3-acr3Δhog1Δ-0} : was used for acr3Δhog1Δ mutant.

(GSH)\textsubscript{3-acr3Δ} is equal to cellular GSH level in Muller (1996) and the other (GSH)\textsubscript{3} initial concentrations were estimated from experimental data (Supplementary Table S4). No (GSH)\textsubscript{3} production or degradation reaction was considered in the models, assuming that cellular GSH content is not significantly altered during 1 h incubation with high As\textsuperscript{III} concentration. As(GS)\textsubscript{3} dissociation is considered as mass action kinetics (Reaction v\textsubscript{9}).

**Ycf1-mediated vacuolar sequestration**

Experimental data indicate that YCF1 gene expression is not significantly upregulated in response to As\textsuperscript{III} exposure (Wysocki et al., 2004; Thorsen et al., 2007), except in cells that hyperaccumulate As\textsuperscript{III}, i.e. in acr3Δ mutants (Wysocki et al., 2004). Based on this information, two alternative setups were considered in acr3Δ mutants (except acr3Δycf1Δ mutant) with same or different Ycf1 level comparing to wild type (Supplementary Table S4 and Models Setup Summary). In all of these models, the Ycf1 level is supposed to be constant during 1 h of 1.0 mM As\textsuperscript{III} stress. In both cases, mass action and Michaelis–Menten kinetics were used alternatively for
vacular sequestration of As(GS)₃ and lead to 4 different combinations of v₁₀ reaction (v₁₀-a,v₁₀-b,v₁₀-c,v₁₀-d) which are detailed in Supplementary Table S3. To design the model such that it starts in steady state, we assumed an efflux process for vAs(GS)₃ out of vacuole (Reaction v₁₁), which, however, can become negligible depending on the estimated parameters.

**ACR3 transcription and Acr3-mediated arsenite efflux**

Due to the lack of experimental data on Acr3 concentrations in the cell, we combined ACR3 translation and transcription in a single reaction (Reaction v₁₂ in Fig. 2). Acr3 is constitutively degraded through reaction v₁₃ (Fig. 2). Acr3-mediated As³⁻ₐₙ export was implemented in reaction v₄ in two different forms, using either mass action (MA) or Michaelis–Menten (MM) kinetics (Fig. 2).

**Initial value of free intracellular arsenite**

We assumed that cells reach a steady state during 24 h of pre-incubation with 0.1 mM As³⁻. We suppose that the concentration of intracellular arsenite (As³⁻ₐₙ) equals As³⁻ concentration in the medium (As³⁻ₐₐ) in acr3 mutants. This is based on the assumption that diffusion through Fps1 channel is passive and merely concentration gradient-dependent and that Fps1 is the only As³⁻ influx–efflux pathway in acr3 mutants (Wysocki et al., 2001; Liu et al., 2004; Maciaszczyk-Dziubinska et al., 2010). For wild type cells and all ACR3 mutants (Wysocki et al., 2001; Maciaszczyk-Dziubinska et al., 2010) data well (Fig. 4). We conducted a local sensitivity analysis for the best approximating model ΔΔₐ and ΔΔₐ media. This is based on the assumption that diffusion through Fps1 channel is passive and merely concentration gradient-dependent and that Fps1 is the only As³⁻ influx–efflux pathway in acr3 mutants (Wysocki et al., 2001; Liu et al., 2004; Maciaszczyk-Dziubinska et al., 2010). For wild type cells and all ACR3 mutants (Wysocki et al., 2001; Maciaszczyk-Dziubinska et al., 2010). To calculate wSSR of both the fitted data (Figs 1 and 3) and the predicted data (Fig. 4). This way, we included both the exploratory and the predictive properties of the models in the ranking. The best-ranked model (Fig. 2) was able to predict the GSH knock down strain (gsh₁Δ PRO2-1) data well (Fig. 4). We conducted a Profile Likelihood-based identifiability analysis (Raue et al., 2009; Schaber and Klipp, 2011) using COPAS (Schaber, 2012) (Supporting information: Methods). Most parameters (12 out of 20) were practically identifiable (Supplementary Fig. S2). There was only one structurally non-identifiable parameter (vmax₃₋ₐₐ), which was previously determined by a preliminary identifiability analysis and set to 1.0. Later, we conducted a local sensitivity analysis for the best approximating model (No. 2) to identify how the concentration of Fps1-P and cellular arsenic level change at the end of simulation with respect to local parameters change, using COPAS (Supporting information: Methods). The best-ranked model can be found in BioModels database under identifier MODEL1403280000 (Le Novere et al., 2006).

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**Supporting information**

Additional supporting information may be found in the online version of this article at the publisher’s web-site.
Mathematical modeling of arsenic transport, intracellular distribution and detoxification processes in yeast

Supporting Information

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Scaling Data

As\textsuperscript{III} influx-efflux data

To study the effect of GSH knockdown on As\textsuperscript{III} uptake and efflux, \textit{S. cerevisiae} strains derived from YPH98 (Spector \textit{et al.}, 2001) were used (wild type (Y252) and gsh1\textDelta-PRO2-1) and not from W303-1A as in the rest of the study. Assuming similar As\textsuperscript{III} dynamics, we rescaled the measured intracellular arsenite of the Y252 strain (As\textsuperscript{III}\textsubscript{gsh1\textDelta-PRO2-1}) by equating the maxima of the two different wild type measurements:

\[
\text{As}_{\text{gsh1\textDelta-PRO2, Scaled}}^\text{III} = \frac{\text{As}_{\text{gsh1\textDelta-PRO2}}^\text{III, max-W303-1A}}{\text{As}_{\text{max-Y252}}^\text{III}}
\]

Hog1 phosphorylation data

Hog1 phosphorylation data was extracted from Thorsen \textit{et al.} (Thorsen \textit{et al.}, 2006), Figure 3-B. We used the ImageJ software (1.44p version, National Institute of Mental Health, Bethesda, Maryland, USA) to quantify corresponding western blots. Hog1 phosphorylation levels were scaled to phosphorylated Hog1, 5 min after addition of 0.4M NaCl, assuming that this value is the maximum Hog1 phosphorylation level (Table S7).

Fps1 phosphorylation data

Fps1 phosphorylation data was derived from Thorsen \textit{et al.} (Thorsen \textit{et al.}, 2006) (SI Table S7). We assumed that phosphorylated Fps1 levels are at basal level before As\textsuperscript{III} stress, two hours after washing the cells in As\textsuperscript{III} free medium, both for wild type and hog1\textDelta cells. All Fps1 phosphorylation data were scaled to the Fps1 phosphorylation value in wild type cells one hour after As\textsuperscript{III} addition, which was set to 100%.

Methods

Parameter Estimation

Model implementation and parameter estimation was done with COPASI (version: 4.8) (Hoops \textit{et al.}, 2006). Model parameters were estimated using Evolutionary Programming. The weighted Sum of Squared Residuals (wSSR) was used as objective function.

\[
w\text{SSR} = \sum_{i=1}^{m} w_i \sum_{j=1}^{n} (\hat{y}_{i,j} - y_{i,j})^2
\]

with \(i=1,...,m\) as the number of experiments, and \(j=1,...,n\) as the data pointed for experiment \(i\). \(w_i\) represents the respective weight of experiment \(i\), set to the inverse of the average of the respective time series with a correction factor accommodating the different number of data points for some experiments. \(\hat{y}_{i,j}\) is the simulated value for data point number \(j\) within experiment \(i\) and \(y_{i,j}\) is the measured data point \(j\) within experiment \(i\). As\textsuperscript{III} uptake and efflux data for the wild type, \textit{ycf1\Delta, hog1\Delta, acr3\Delta, acr3\Delta hog1\Delta} and \textit{acr3\Delta ycf1\Delta} strains were used for parameter estimation (Fig. 1). In addition, Fps1 phosphorylation data (from wild type and \textit{hog1\Delta} mutant) and
Hog1 phosphorylation data (from wild type) were used for parameter estimation (Fig. 3).

**Model Selection**

Model selection was done using Modelmage software (Schaber et al., 2011). In order to select the most parsimonious mathematical model, which best approximates the data, we used the Akaike Information Criterion corrected for small sample sizes ($AIC_c$). $AIC_c$ is an information theoretic approach for model selection, based on Kullback-Leibler (K-L) concept of information lost when using a model to approximate full truth. The full truth includes an infinite number of parameters, which determine the systems output (Burnham & Anderson, 2010). The $AIC_c$ is described as follows:

$$AIC_c = 2k + n\left(\ln\left(\frac{2\pi \cdot \text{wSSR}}{n}\right) + 1\right) + \frac{2k(k+1)}{n-k-1},$$

where $K$, $n$ and $\text{wSSR}$ represent number of parameters, number of data points and the weighted sum of squared residuals, respectively. Finally, models were ranked according to $AIC_c$, where the model with the minimum $AIC_c$ score was ranked first.

The K-L confidence set comprised of all models for which their likelihood relative to the estimated K-L best model likelihood, be ≈ 1/8 (Burnham & Anderson, 2010). In order to select and compare the best approximating model(s) we calculated the Akaike weights ($AICw$) (Burnham & Anderson, 2002)

$$AICw_i = \frac{e^{-\frac{1}{2}\Delta_i}}{\sum_{i=1}^{R} e^{-\frac{1}{2}\Delta_i}},$$

where $\Delta_i = AIC_i - AIC_{\text{min}}$, with $AIC_i$ being the $AICc$ for model $i$, $i=1, ..., R$ according to ranking and $AIC_{\text{min}}$ the minimal $AICc$. The $AICw$'s can be considered as the weight of evidence in favour of a model given as a number between 0 and 1, i.e. the higher the weight, the closer the model is to the hypothetical true model (Burnham & Anderson, 2002). We considered those models as best approximating that had an $AICw > 0.125$.

**Sensitivity Analysis**

We conducted a local sensitivity analysis using COPASI and analyzed scaled (normalized) sensitivity, where the scaled sensitivity $S_{ij}$ of a certain output $o_i(p_j)$ (concentration of the molecular species of interest at the end of simulation run) with respect to a certain parameter $p_j$ change $\Delta p_j$ is calculated as:

$$S_{ij} = \frac{p_j}{\frac{\partial o_i}{\partial p_j}} \cdot \frac{o_i(p_j + \Delta p_j) - o_i(p_j)}{\Delta p_j} \approx \frac{o_i}{\Delta p_j}.$$

Where $\Delta p_j = 0.001 \cdot p_j$.

**Identifiability Analysis**

We conducted profile likelihood based identifiability analysis (Raue et al., 2009) using Copasi software as explained in literature (Schaber, 2012).
Four components were implemented differently leading to different candidate models. Each of these four components can adopt two possible setups. Thus, 16 different combinations were generated. The alternative model formulations are indicated by dashed components in Fig. 2. For a better overview we shortlist the components and their setups:

**A) As\(^{III}\)-GSH conjugation**
- As\(^{III}\)-GSH conjugation was modeled in two different forms.
  - I. \(\text{As}^{\text{III}}\) in directly converts to \(\text{As(GS)}_3\).
  - II. \(\text{As}^{\text{III}}\) in binds to \((\text{GSH})_3\) and produces \(\text{As(GS)}_3\), where initial \((\text{GSH})_3\) concentration was assumed fixed and was estimated from experimental data.

**B) YCF1 gene expression**
- Two sets of models were designed based on different assumptions about cellular Ycf1 concentrations:
  - I. Ycf1 concentration was assumed fixed after preincubation in 0.1mM \(\text{As}^{\text{III}}\) containing medium.
  - II. Ycf1 concentration was higher in \(\text{acr3}\Delta\) and \(\text{acr3}\Delta\text{hog1}\Delta\) mutants (not \(\text{acr3}\Delta\text{ycf1}\Delta\) mutant) after preincubation with 0.1mM \(\text{As}^{\text{III}}\).

**C) vacuolar sequestration of As\((\text{GS})_3\)**
- Two kinetics were tested for vacuolar sequestration.
  - I. Mass action kinetics.
  - II. Michaelis-Menten kinetics.

**D) As\(^{III}\) efflux through Acr3**
- Two kinetics were tested for As\(^{III}\) export through Acr3.
  - I. Mass action kinetics.
  - II. Michaelis-Menten kinetics.

Mathematical formulation of models is explained in Tables S2-S6. The order of mathematical details in these tables is explained below:

**Table S2:**
- This table lists the algebraic and ordinary differential equations of the master model.

**Table S3:**
- This table lists the rate laws for the reactions from Table S2 and details the differences between the model alternatives.

**Table S4:**
- This table lists the state variables and their initial conditions. As models are initially set to steady state, some initial conditions could be derived from those that are estimated. The latter are listed in Table S6.

**Table S5:**
- This table lists auxiliary variables and physical quantities including volume, Molar weight and cell surface calculation.
Table S6:
This table lists all estimated parameters including rate constants and initial conditions.

Theoretical Implementation of GSH Knockdown

In order to implement GSH knockdown in the model we used a knockdown factor, GSHknockdown-f. The GSH knockdown factor multiplies to initial GSH concentration in the set of models with GSH binding mechanisms. In the set of models which use direct conversion of As$^{\text{III}}$ to AsGS$_3$, this factor multiplies in the rate law. The mathematical notation is explained in the supplementary Table S5. This factor is derived from (Spector et al., 2001). In this paper authors state that $\Delta$gsh1PRO2–1 cell extracts could support the growth of Δgsh1 cells with the efficiency similar to that of wild type extracts diluted 150-fold. Thus, they conclude that the GSH produced in GSH knockdown mutant is 0.5%-1.0% of that of wild type. As they didn’t quantified the GSH content of the cells we quantified an autoradiogram in that paper (Fig.2.C), which is a more direct measure of cellular GSH content, both for Δgsh1PRO2–1 mutant and wild type cells. We quantified the autoradiogram and considered the 3 hour labeling intensity of Δgsh1 as background remove the background from 3 hour labeling intensity of wt and Δgsh1PRO2–1. The ratio of 3 hour labeling intensity of Δgsh1PRO2–1 over 3 hour labeling intensity of wt considered as GSH$_{\text{knockdown}}$-f. The GSH$_{\text{knockdown}}$-f is equal to 30% (0.3).

Modified Model Changes

A simple constant flux reaction is added to the best ranked model for GSH production and no GSH degradation is implemented.

$$k_{\text{GSH-production}} = 0.02 \, (\mu\text{mol} \cdot \text{lit}^{-1} \cdot \text{Sec}^{-1})$$

The initial concentration of state variables were set as below (Concentrations are in $\mu\text{mol} \cdot \text{lit}^{-1}$ unit):

<table>
<thead>
<tr>
<th>State Variable</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Acr3]$_0$</td>
<td>0.0004</td>
</tr>
<tr>
<td>[As$^{\text{III}}$]$_0$</td>
<td>10.1584</td>
</tr>
<tr>
<td>[As$^{\text{III}}$]$_{\text{prot}}$</td>
<td>142.7820</td>
</tr>
<tr>
<td>[As(GS)$_3$]$_0$</td>
<td>491.9193</td>
</tr>
<tr>
<td>[vAs(GS)$_3$]$_0$</td>
<td>154.5897</td>
</tr>
<tr>
<td>[Hog1]$_0$</td>
<td>0.1659</td>
</tr>
<tr>
<td>[Hog1PP]$_0$</td>
<td>0.0010</td>
</tr>
<tr>
<td>[Fps1]$_0$</td>
<td>0.0033</td>
</tr>
<tr>
<td>[Fps1P]$_0$</td>
<td>0.0266</td>
</tr>
<tr>
<td>[Ycf1]$_0$</td>
<td>0.0132</td>
</tr>
<tr>
<td>[GSH]$_0$</td>
<td>1600.0000</td>
</tr>
</tbody>
</table>

Steady State Calculations

The steady state concentration of As$^{\text{III}}$$_{\text{in}}$ and consequently As$^{\text{III}}$$_{\text{prot}}$ are independent of (GSH)$_3$, Ycf1, As(GS)$_3$ and vAs(GS)$_3$ concentrations. If we consider more complex interactions the steady state concentration of As$^{\text{III}}$$_{\text{in}}$ and consequently As$^{\text{III}}$$_{\text{prot}}$ would be affected by the mode of regulation of the other As$^{\text{III}}$ species. For example we included As$^{\text{III}}$ induced GSH upregulation (Ycf1 is considered constant) and derived steady state concentration of As$^{\text{III}}$$_{\text{in}}$ and other As$^{\text{III}}$ species with respect to vAs(GS)$_3$.  
(GSH)_{3\, ss} = \frac{k_9 \cdot k_{15} \cdot k_{11} \cdot v_{\text{As}(G\text{S})_{3\, ss}}}{k_8 \cdot k_{16} \cdot k_{10} \cdot G\text{S}_{\text{knockdown-factor}} \cdot \text{Ycf1}}

\text{As}_{\text{III\, in\,-ss}} = \frac{k_{16} \cdot k_9 \cdot k_{11} \cdot v_{\text{As}(G\text{S})_{3\, ss}}}{k_{15} \cdot k_8 \cdot k_{10} \cdot G\text{S}_{\text{knockdown-factor}} \cdot \text{Ycf1}}

\text{As}_{\text{III\, prot\,-ss}} = \frac{(k_2)^2 \cdot k_{16} \cdot k_9 \cdot k_{11} \cdot v_{\text{As}(G\text{S})_{3\, ss}}}{(k_3)^2 \cdot k_{15} \cdot k_8 \cdot k_{10} \cdot G\text{S}_{\text{knockdown-factor}} \cdot \text{Ycf1}}

\text{As}(G\text{S})_{3\, ss} = \frac{k_9}{k_8 \cdot \text{As}_{\text{III\, in\,-ss}} + \frac{k_{10} \cdot \text{Ycf1}}{k_{11}} + 1}

V_{\text{G\text{S}_{\text{H\,-pro}}} = k_{15} \cdot \text{As}_{\text{III\, in}} \cdot V_{\text{cell\,-vac}}

V_{\text{G\text{S}_{\text{H\,-deg}}} = k_{16} \cdot \text{G\text{S}} \cdot V_{\text{cell\,-vac}}

k_{15} = \text{As}_{\text{III\, induced \,G\text{S\, rate \,constant}}}

k_{16} = \text{G\text{S\, degradation \,rate \,constant}}

Thus, in case we include As$_{\text{III}}$ induced GSH upregulation (if enough experimental data available), As$_{\text{III}}$ steady state concentration would be dependent on vacuolar sequestration of arsenite. Consequently, we can use it to more clearly investigate the efficiency of the vacuole in arsenite tolerance acquisition.

**Sensitivity Analysis**

In order to determine most important parameters governing As$_{\text{III}}$ influx and efflux, and Fps1 phosphorylation we conducted local sensitivity analysis using best approximating model (SI: Methods).

**Sensitivity analysis of the cellular arsenic at the end of 1.0 mM As$_{\text{III}}$ exposure**

The sensitivity analysis of the cellular As$_{\text{III}}$ concentration up to the end of 1mM As$_{\text{III}}$ exposure with respect to model kinetic parameters suggests that in wild type cells, the protein binding reaction rate constant ($k_2$) is the most sensitive parameter of the model, whereas in acr3Δ mutant, the GSH conjugation reaction rate constant ($k_8$) is the most sensitive one (Fig. S7).

**Sensitivity analysis of the cellular arsenic after cell wash**

Sensitivity analysis of the cellular As$_{\text{III}}$ concentration after cell wash up to 2 hours with respect to model kinetic parameters for wild type cells, indicate that protein binding and dissociation rate constants ($k_2$ and $k_3$) are the most sensitive model parameters in terms of cellular As$_{\text{III}}$ after cell wash, whereas in acr3Δ cells the As$_{\text{III}}$-GSH conjugation rate constant ($k_9$), is the most sensitive model parameter (Fig. S8).

**Sensitivity analysis of Fps1 phosphorylation at the end of 1.0 mM As$_{\text{III}}$ exposure**

Sensitivity analysis of phosphorylated Fps1 concentration at the end of 1mM As$_{\text{III}}$ exposure with respect to perturbation in model kinetic parameters suggest that
both in wild type and acr3Δ mutant, Fps1 dephosphorylation rate constant (k_7) and
Hog1 dependent Fps1 phosphorylation rate constant (k_{6,Hog1}) are model’s most
sensitive parameters (Fig. S13).

**Sensitivity analysis of the Fps1 phosphorylation after cell wash**

Sensitivity analysis of the phosphorylated Fps1 concentration after cell wash up to 2
hours with respect to perturbation in model’s kinetic parameters, suggest that Fps1
dephosphorylation rate constant (k_7) is the most sensitive model parameter both in
wild type and acr3Δ mutant cells. Fps1 phosphorylation is much more sensitive to
Hog1-dependent phosphorylation (k_{6,Hog1}) during As^{III} exposure than after cell wash.
Also, Fps1 phosphorylation is more sensitive to perturbation in basal Fps1
phosphorylation rate constant (k_{6,basal}) after cell wash than As^{III} exposure (Fig. S14).

**Identifiability Analysis**

We conducted profile likelihood based identifiability analysis using Copasi software.
12 parameters out of 20 free parameters were practically identifiable. The Fps1
mediated As^{III} influx-efflux, protein binding and dissociation, Hog1
dephosphorylation, Basal Fps1 phosphorylation, Fps1 dephosphorylation, As^{III} in-GSH
conjugation and dissociation, vacuolar export of vAs(GS)_3, initial value of As^{III} inu, initial
concentration of GSH in acr3Δ and acr3Δhog1Δ are practically identifiable (Fig. S2).
Supplementary Figures

Supplementary Figure 1: Growth measurements of different strains under 0.1mM As\textsuperscript{III} stress. Growth of wild type and mutants in the absence (control) and presence of 0.1 mM As\textsuperscript{III} was monitored by measuring the optical density (OD) at 600 nm.
Supplementary Figure 2: Profile Likelihood based Identifiability analysis of model’s free parameters. 95% confidence region is calculated by 2 methods, likelihood contours (pink line) and likelihood ratio (green line). The minimum objective value reached is shown at bottom (violet line) and the estimated parameter value is shown by a bold dot (•). Results show that 15 parameters out of 20 free parameters are practically identifiable based on likelihood ratio definition. In primary analysis \( \text{vm}_{14} \) was determined structurally non-identifiable.
Supplementary Figure 3: Simulated Fps1 phosphorylation and dephosphorylation flux in wild type and hog1Δ mutant. Fps1 phosphorylation ($v_{6, \text{basal}}$, $v_{6, \text{Hog1PP}}$, $v_{6, \text{AsIII}}$) and dephosphorylation fluxes ($v_7$) are simulated using the best ranked model. (A) Simulation of Fps1 phosphorylation flux by basal phosphorylation branch ($v_{6, \text{basal}}$ in Figure 2). (B) Simulation of Fps1 phosphorylation flux by As$^{\text{III}}$ dependent phosphorylation branch ($v_{6, \text{AsIII}}$ in Figure 2). (C) Simulation of Fps1 phosphorylation flux by Hog1 dependent phosphorylation branch ($v_{6, \text{Hog1PP}}$ in Figure 2). (D) Fps1 dephosphorylation flux ($v_7$ in Figure 2).
Supplementary Figure 4: $\text{As}^{\text{III}}$ export flux through Acr3 (flux.v20).
$\text{As}^{\text{III}}$ flux through Acr3 is simulated after 1mM $\text{As}^{\text{III}}$ stress during exposure and 2 hours after cell wash. Simulation suggests rapid flux saturation over time (Top panel). This rapid saturation is because of Acr3 mediated $\text{As}^{\text{III}}$ flux saturation in low $\text{As}^{\text{III}}$ concentrations (Bottom panel).
Supplementary Figure 5: Growth measurements during 1.0 mM As$^{III}$ exposure and after cell wash. 1.0 mM As$^{III}$ was added to cells at t=0 min. At t=60 min, the cells were washed to remove extracellular As$^{III}$. Growth was monitored by measuring the optical density (OD) at 600 nm.
Supplementary Figure 6: Simulation of different arsenic species in response to increasing $\text{As}^{\text{III}}$ concentrations. $\text{As}^{\text{III}}_{\text{ex}}$ concentration increases from 0.5 to 2 mM (lighter colors and darker colors are output of lower and higher $\text{As}^{\text{III}}$ stress, respectively). Simulation for wild type cells was done using best approximating model. (A) Simulation of $\text{As}^{\text{III}}_{\text{in}}$ and $\text{As}^{\text{III}}_{\text{prot}}$. (B) Simulation of $\text{As(GS)}_3$ and $\text{vAs(GS)}_3$. 

Supplementary Figure 7: Sensitivity analysis of total cellular arsenic level during As$^{\text{III}}$ exposure.

Here we conducted the sensitivity analysis for total cellular arsenic level with respect to model kinetic parameters perturbation, both for wild type and acr3Δ mutant during As$^{\text{III}}$ exposure. The plot represents normalized sensitivity versus different model parameters. The normalized sensitivity is the change in the output with respect to parameter perturbation which are normalized to their values before each calculation step (i,j). This is expressed in the mathematical formula in the supplementary information section “Sensitivity Analysis”. The ordinates are dimensionless quantities. The higher sensitivity of a parameter means the lower robustness of the considered output with respect to the corresponding parameter.
Supplementary Figure 8: Sensitivity analysis of total cellular arsenic level cell wash.

Here we conducted the sensitivity analysis for total cellular arsenic level with respect to model kinetic parameters perturbation, both for wild type and acr3Δ mutant after cell wash. The plot represents normalized sensitivity versus different model parameters. The normalized sensitivity is the change in the output with respect to parameter perturbation which are normalized to their values before each calculation step (i,j). This is expressed in the mathematical formula in the supplementary information section “Sensitivity Analysis”. The ordinates are dimensionless quantities. The higher sensitivity of a parameter means the lower robustness of the considered output with respect to the corresponding parameter.
Supplementary Figure 9: Cells lacking ACR3 produce more GSH during As\textsuperscript{III} exposure than cells harboring a functional ACR3 gene. Cells lacking ACR3 produce more GSH during As\textsuperscript{III} exposure than cells harboring a functional ACR3 gene. Cross-feeding assay. Precultures of the strains above were grown for about 19h and then split into two halves; one half was treated with 0.1 mM As\textsuperscript{III} for 6h whereas the other half was left untreated (control). Thereafter, 10 OD units of cells were harvested, washed in 1ml ice-cold water and resuspended in 1 ml water. The cells were then broken by boiling, briefly centrifuged to remove cell debris and the supernatants collected. 5µl of each supernatant were spotted (undiluted 1:1, diluted 1:2, diluted 1:5) on top of a lawn of gsh\textsuperscript{1Δ} cells (the gsh\textsuperscript{1Δ} mutant cannot proliferate unless GSH is provided exogenously). After incubating the plates at 30°C for about 24h, a halo of proliferating gsh\textsuperscript{1Δ} cells was visible around the spotted supernatants. The size of the halo is an indirect measure of the GSH present in the supernatants, and thus the GSH produced by untreated and As\textsuperscript{III} treated cells. For a more thorough description of the glutathione cross-feeding assay, see Thorsen et al, 2012 (Thorsen et al., 2012).
Supplementary Figure 10: Log-Log plot of arsenic species steady state concentration versus As\textsubscript{III} in concentration in wild type cells. Calculations suggest that As\textsubscript{III}prot concentration linearly increase with As\textsubscript{III} in, whereas As(GS)\textsubscript{3} and vAs(GS)\textsubscript{3} concentrations saturate gradually from 10 μM As\textsubscript{III} in concentration.
Supplementary Figure 11: Log-Log plot of arsenic species steady state concentration versus total GSH concentration in wild type cells. Calculations suggest that As(GS)$_3$ and vAs(GS)$_3$ concentrations significantly increase upon cellular GSH upregulation, whereas As$^{III}_{prot}$ stays constant.
**Supplementary Figure 12: As^{III} triggers protein aggregation/Hsp104 redistribution.**

Hsp104–GFP localization was monitored by fluorescence microscopy in living wild type cells before (control) and after addition of 0.5 mM As^{III} to the cell culture. Hsp104-GFP foci (indicated by arrows) represent sites of protein aggregation (Jacobson et al., 2012).
Supplementary Figure 13: Sensitivity analysis of cellular Fps1-p level during As\textsuperscript{III} exposure. Here we conducted the sensitivity analysis for cellular Fps1-p level with respect to model kinetic parameters perturbation, both for wild type and acr3Δ mutant during As\textsuperscript{III} exposure. The plot represents normalized sensitivity versus different model parameters. The normalized sensitivity is the change in the output with respect to parameter perturbation which are normalized to their values before each calculation step (i,j). This is expressed in the mathematical formula in the supplementary information section “Sensitivity Analysis”. The ordinates are dimensionless quantities. The higher sensitivity of a parameter means the lower robustness of the considered output with respect to the corresponding parameter.
Supplementary Figure 14: Sensitivity analysis of cellular $\text{Fps1-p}$ level after cell wash. Here we conducted the sensitivity analysis for cellular $\text{Fps1-p}$ level with respect to model kinetic parameters perturbation, both for wild type and $\text{acr3Δ}$ mutant after cell wash. The plot represents normalized sensitivity versus different model parameters. The normalized sensitivity is the change in the output with respect to parameter perturbation which are normalized to their values before each calculation step $\left(i, j\right)$. This is expressed in the mathematical formula in the supplementary information section “Sensitivity Analysis”. The ordinates are dimensionless quantities. The higher sensitivity of a parameter means the lower robustness of the considered output with respect to the corresponding parameter.
References

### Table S1: Yeast strains and plasmids used in this study

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<tr>
<th>Name</th>
<th>Genotype/description</th>
<th>Source/reference</th>
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<tbody>
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<td>W303-1A</td>
<td>MATa ura3-1 leu2-3/112 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mol0</td>
<td>(Thomas &amp; Rothstein, 1989)</td>
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<td>(Wysocki et al., 2001)</td>
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<td>W303-1A hog1Δ::TRP1</td>
<td>S. Hohmann</td>
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<td>EDO1</td>
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<td>(Thorsen et al., 2006)</td>
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#### Plasmids

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<tr>
<th>Name</th>
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<tr>
<td>SpPCS1</td>
<td><em>S. pombe</em> PCS1 gene under control of GAL1 promoter in pYES2</td>
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<td>pYES2</td>
<td>2μ, GAL1 promoter, URA3</td>
<td>Invitrogen</td>
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</table>


Table S2: Ordinary differential equation system of the master model.

Rates in {} indicate options. Volumes are in liter (l) and concentrations in (μmol/l).

| ODE | \[ \begin{align*} 
A_s^{III}_{ex} &= \left\{ A_s^{III}_{ex0} + \left( [A_s^{III}_{ex}]_{shock} \cdot \left( 1 - e^{\frac{A_s^{III}_{ex-t1} - \text{Time}}{A_s^{III}_{ex-tm}}} \right) \right) \right\} \\
&\text{before washing out} \\
A_s^{III}_{ex-t1} &= 0 \quad \text{Stress time (Seconds)} \\
A_s^{III}_{ex-t2} &= 3600 \quad \text{Washing out time (seconds)} \\
\end{align*} \right. |
Table S3: Rate equations of the master model including different model alternatives.

Concentrations are denoted by [ ] and initial concentration by \([\text{[]}_0\). The auxiliary variables and parameters are described in Table S5. Volumes are in liter (l) and concentrations in (μmol/l). Bold parameters are free parameters that are estimated from data and their value is reported in Table S6.

<table>
<thead>
<tr>
<th>Rate</th>
<th>Rate law</th>
<th>Description</th>
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<tr>
<td>(V_1)</td>
<td>([\text{Fps1}] \cdot k_1 \cdot ([\text{As}^{\text{III}}<em>{\text{ex}}] - [\text{As}^{\text{III}}</em>{\text{in}}]))</td>
<td>Arsenite influx-efflux reaction.</td>
</tr>
<tr>
<td>(V_2)</td>
<td>(k_2 \cdot [\text{As}^{\text{III}}_{\text{in}}])</td>
<td>Protein binding reaction.</td>
</tr>
<tr>
<td>(V_3)</td>
<td>(k_3 \cdot [\text{As}^{\text{III}}_{\text{prot}}])</td>
<td>Protein bound arsenite dissociation.</td>
</tr>
<tr>
<td>(V_4)</td>
<td>(k_4 \cdot k_{0-4} \cdot [\text{As}^{\text{III}}_{\text{in}}] \cdot [\text{Hog1}])</td>
<td>Arsenic induced Hog1 phosphorylation.</td>
</tr>
<tr>
<td>(V_5)</td>
<td>(k_5 \cdot [\text{Hog1PP}])</td>
<td>Hog1PP dephosphorylation.</td>
</tr>
<tr>
<td>(V_6)</td>
<td>([\text{Fps1}] \cdot (k_{6-\text{As}^{\text{III}}} + k_{6-\text{Hog1PP}} \cdot [\text{Hog1PP}] + k_{6-\text{basal}}))</td>
<td>Fps1 phosphorylation reactions.</td>
</tr>
<tr>
<td>(V_7)</td>
<td>(k_7 \cdot [\text{Fps1P}])</td>
<td>Fps1-P dephosphorylation.</td>
</tr>
<tr>
<td>(V_{8-a})</td>
<td>(k_{0-8} \cdot k_8 \cdot \text{GSH}<em>{\text{knockdown-f}} \cdot [\text{As}^{\text{III}}</em>{\text{in}}] \cdot [\text{GSH}_3])</td>
<td>(\text{As}^{\text{III}})-glutathione conjugation. (GSH)_3 binding is considered.</td>
</tr>
<tr>
<td>(V_{8-b})</td>
<td>(k_{0-8} \cdot k_8 \cdot \text{GSH}<em>{\text{knockdown-f}} \cdot [\text{As}^{\text{III}}</em>{\text{in}}])</td>
<td>(\text{As}^{\text{III}})-glutathione conjugation. Direct (\text{As}^{\text{III}}) to (\text{As(GS)}_3) conversion.</td>
</tr>
<tr>
<td>(V_9)</td>
<td>(k_9 \cdot [\text{As(GS)}_3])</td>
<td>(\text{As(GS)}_3) dissociation.</td>
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<tr>
<td>(V_{10-a})</td>
<td>(\frac{k_{0-10} \cdot [\text{Ycf1}] \cdot V_{\text{max10}} \cdot [\text{As(GS)}<em>3]}{k</em>{\text{m10}} + [\text{As(GS)}_3]})</td>
<td>Vacuolar sequestration of (\text{As(GS)}_3). (First setup)</td>
</tr>
<tr>
<td>(V_{10-b})</td>
<td>(\frac{k_{0-10} \cdot V_{\text{max10}} \cdot [\text{As(GS)}<em>3]}{k</em>{\text{m10}} + [\text{As(GS)}_3]})</td>
<td>Vacuolar sequestration of (\text{As(GS)}_3). (Second setup)</td>
</tr>
<tr>
<td>(V_{10-c})</td>
<td>(k_{0-10} \cdot [\text{Ycf1}] \cdot k_{10} \cdot [\text{As(GS)}_3])</td>
<td>Vacuolar sequestration of (\text{As(GS)}_3). (Third setup)</td>
</tr>
<tr>
<td>(V_{10-d})</td>
<td>(k_{0-10} \cdot k_{10} \cdot [\text{As(GS)}_3])</td>
<td>Vacuolar sequestration of (\text{As(GS)}_3). (Fourth setup)</td>
</tr>
<tr>
<td>(V_{11})</td>
<td>(k_{11} \cdot [v\text{As(GS)}_3])</td>
<td>(v\text{As(GS)}_3) export out of vacuole.</td>
</tr>
<tr>
<td>(V_{12})</td>
<td>(k_{0-12} \cdot k_{12} \cdot [\text{As}^{\text{III}}_{\text{in}}])</td>
<td>Acr3 protein translation.</td>
</tr>
<tr>
<td>(V_{13})</td>
<td>(k_{13} \cdot [\text{Acr3}])</td>
<td>Acr3 protein degradation.</td>
</tr>
<tr>
<td>(V_{14-a})</td>
<td>(\frac{[\text{Acr3}] \cdot V_{\text{max14}} \cdot [\text{As}^{\text{III}}<em>{\text{in}}]}{k</em>{\text{m14}} + [\text{As}^{\text{III}}_{\text{in}}]})</td>
<td>(\text{As}^{\text{III}}) export through Acr3. (First setup)</td>
</tr>
<tr>
<td>(V_{14-b})</td>
<td>([\text{Acr3}] \cdot k_{14} \cdot [\text{As}^{\text{III}}_{\text{in}}])</td>
<td>(\text{As}^{\text{III}}) export through Acr3. (Second setup)</td>
</tr>
</tbody>
</table>
Table S4: State variables and their initial conditions.

Model’s state variables and their initial concentrations are listed below. \([I_0]\) indicates initial concentrations and concentrations are in (μmol/l). Bold parameters are free parameters that are estimated from data and their value is reported in Table S6.

<table>
<thead>
<tr>
<th>State variable (Compartment)</th>
<th>Initial Concentration</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acr3 (Cell)</td>
<td>(\frac{[\text{Fps1}]}{[\text{Acr3}]} \cdot \frac{([\text{As}^{III}]<em>{\text{in}} - [\text{As}^{III}]</em>{\text{out}})(k_{14} + [\text{As}^{III}]<em>{\text{in}})}{v</em>{14-a}, v_{14-b}})</td>
<td>Acr3 concentration in the cell. Initial concentration was calculated according to steady state assumption.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACR3⁺ mutants</td>
</tr>
<tr>
<td></td>
<td></td>
<td>acr3Δ mutants</td>
</tr>
<tr>
<td>Fps1 (Cell)</td>
<td>(\frac{[\text{Fps1}]<em>{\text{in}}}{[\text{Fps1}]} \cdot \frac{([\text{As}^{III}]</em>{\text{in}})}{k_{20} ([\text{As}^{III}]_{\text{in}})})</td>
<td>Total Fps1 concentration (Fps1 + Fps1P) was assumed fix and the corresponding value was extracted from <a href="http://yeastgfp.yeastgenome.org/">http://yeastgfp.yeastgenome.org/</a> which is 0.03 (μmol/l). Fps1 concentration is calculated according to steady state assumption.</td>
</tr>
<tr>
<td></td>
<td>(0.03)</td>
<td>Hog1 concentration is calculated according to steady state concentration.</td>
</tr>
<tr>
<td>Fps1P (Cell)</td>
<td>(\frac{k_{6-\text{basal}} \cdot [\text{As}^{III}]<em>{\text{in}}}{k</em>{7}})</td>
<td>Phosphorylated Fps1 (Fps1-P) concentration is calculated according to steady state assumption.</td>
</tr>
<tr>
<td>Hog1 (Cell)</td>
<td>(\frac{[\text{Hog1}]<em>{\text{in}}}{[\text{Hog1}]</em>{\text{in}}} \cdot \frac{k_{4}}{k_{5} + 1})</td>
<td>Total Hog1 concentration (Hog1 + Hog1PP) is extracted from <a href="http://yeastgfp.yeastgenome.org/">http://yeastgfp.yeastgenome.org/</a> which is 0.167 (μmol/lit). Initial concentration of Hog1 is calculated according to steady state assumption.</td>
</tr>
<tr>
<td>Hog1PP (Cell)</td>
<td>(\frac{[\text{Hog1PP}]<em>{\text{in}}}{[\text{Hog1PP}]</em>{\text{in}}} \cdot \frac{k_{4}}{k_{5} + 1})</td>
<td>Hog1PP initial concentration is calculated according to steady state assumption.</td>
</tr>
<tr>
<td>Ycf1 (Cell)</td>
<td>(0.013151)</td>
<td>Not Ycf1 upregulation</td>
</tr>
<tr>
<td></td>
<td>Ycf1 upregulation on models (ACR³ mutants and wild type)</td>
<td>hog1Δ mutants</td>
</tr>
<tr>
<td></td>
<td>(0.013151 - \Delta[\text{Ycf1}])</td>
<td>Ycf1 upregulation on models (acr3Δ mutants)</td>
</tr>
<tr>
<td>As³⁺ (Cell-Vac)</td>
<td>(\frac{[\text{As}^{III}]_{\text{in}}}{[\text{As}^{III} - \text{ACR3}^+]})</td>
<td>Initial arsenite concentration in V cellulare. This is estimated for wild type and is assumed to equal acr3Δ mutants.</td>
</tr>
</tbody>
</table>
\[
\text{As(GS)}_3 (\text{Cell-Vac}) \quad \begin{cases} 
  \frac{k_d \cdot \text{GSH}_{\text{knockdown-factor}} \cdot [\text{As}^{\text{III}}]_0}{k_0} & v_{\text{a-b}} \text{ is selected} \\
  \frac{k_d \cdot \text{GSH}_{\text{knockdown-factor}} \cdot [\text{As}^{\text{III}}]_0}{k_0} & v_{\text{a-c}} \text{ is selected} 
\end{cases} 
\]

Glutathione conjugated arsenite \([\text{As(GS)}_3]_0\) initial concentration is calculated according to steady state assumption.

\[
\text{vAs(GS)}_3 (\text{Vac}) 
\]

Vacuole-sequestered arsenite. Initial concentration is calculated according to steady state assumption.

\[
\text{As}^{\text{III}}_{\text{prot}} (\text{Cell-Vac}) \quad \frac{k_2 \cdot [\text{As}^{\text{III}}]_0}{k_3} \quad \text{Initial concentration of protein-bound arsenite is calculated using steady state assumption.}
\]

\[
(\text{GSH})_3 (\text{Cell-Vac}) \quad \begin{cases} 
  [\text{GSH}]_0 \cdot \text{acr3\Delta hog1\Delta} \\
  [\text{GSH}]_0 \cdot \text{acr3\Delta} \\
  [\text{GSH}]_0 \cdot \text{ACR3}^+ \quad \text{ACR3}^+ \text{ mutants and wild type} 
\end{cases} 
\]

Glutathione initial concentration. \([\text{GSH}]_0 \cdot \text{ACR3}^+\) is explained in Table S5.

\[
\text{As}^{\text{III}}_{\text{ex}} (\text{medium}) 100 \quad \text{As}^{\text{III}}_{\text{ex}} \text{ concentration in medium (Table S2).}
\]
Table S5: Auxiliary variables, physical quantities and their Definition/value.

Concentrations are denoted by [] and \([0]\) denotes the initial concentration. Bold parameters are free parameters that are estimated from data and their value is reported in Table S6. Volumes are in Liter, concentrations are in \(\mu\)mol/l.

<table>
<thead>
<tr>
<th>Variable/Parameter</th>
<th>Definition/Value</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k_{o-4})</td>
<td>(\begin{cases} 1 &amp; \text{HOG1}^+ \text{ mutants and wild type} \ 0 &amp; \text{hog1}Δ \text{ mutants} \end{cases})</td>
<td>Acting as a switch which removes Hog1 phosphorylation reaction.</td>
</tr>
<tr>
<td>(k_{o-8})</td>
<td>(\begin{cases} 1 &amp; \text{GSH}^+ \text{ mutants and wild type} \ 0 &amp; \text{GSH knockdown mutants} \end{cases})</td>
<td>Acting as a switch which is being used in GSH_damping_factor in GSH knockdown mutant ((gsh1Δ) PRO2-1).</td>
</tr>
<tr>
<td>(k_{o-10})</td>
<td>(\begin{cases} 1 &amp; \text{YCF1}^+ \text{ mutants and wild type} \ 0 &amp; \text{ycf1}Δ \text{ mutants} \end{cases})</td>
<td>Acting as a switch which cancels vacuolar sequestration.</td>
</tr>
<tr>
<td>(k_{o-14})</td>
<td>(\begin{cases} 1 &amp; \text{ACR3}^+ \text{ mutants and wild type} \ 0 &amp; \text{acr3}Δ \text{ mutants} \end{cases})</td>
<td>Acting as a switch which cancels Acr3 mediated As(\text{III}) export.</td>
</tr>
<tr>
<td>(Asngpermil_{tot})</td>
<td>(\left(\text{As(GS)}<em>3 + \text{As}^{\text{III}}</em>{\text{in \text{prot}}} \right) \cdot \text{AsMw} \cdot V_{\text{cell-vac}} \cdot 10^9 + \nu\text{As(GS)}<em>3 \cdot \text{AsMw} \cdot V</em>{\text{vac}} \cdot 10^9)</td>
<td>Total amount of arsenic (nanogram/10⁶cells) which is being fitted to experimental data.</td>
</tr>
<tr>
<td>Fps1Pfit</td>
<td>(\frac{100 \cdot \text{[Fps1P]}}{\text{Fps1P}_{\text{max}}})</td>
<td>A variable which is used to fit Fps1 phosphorylation data. Because phosphorylated Fps1 data is relative.</td>
</tr>
<tr>
<td>Hog1PPfit</td>
<td>(\frac{100 \cdot \text{[Hog1PP]}}{\text{Hog1}_{\text{sum}}})</td>
<td>A variable which is used to fit Hog1 phosphorylation data. Because phosphorylated Hog1 data is relative.</td>
</tr>
<tr>
<td>Hog1(_{\text{sum}})</td>
<td>([\text{Hog1}] + [\text{Hog1PP}])</td>
<td>Total Hog1 concentration (Hog1 + Hog1PP) is extracted from (\text{<a href="http://yeastgfp.yeastgenome.org%7D%5C">http://yeastgfp.yeastgenome.org}\</a>). Initial concentration of Hog1 is calculated according to steady state assumption. Two different species of Hog1 are considered in these models.</td>
</tr>
<tr>
<td>(GSH_{\text{knockdown-f}})</td>
<td>(\begin{cases} 0.2175 &amp; \text{GSH knockdown (}k_{o-8} = 0) \ 1 &amp; \text{GSH}^+\text{mutants and wild type (}k_{o-8} = 1) \end{cases})</td>
<td>A coefficient which impose the knockdown effect in the model for (gsh1Δ) PRO2-1 strain (Supplementary...</td>
</tr>
<tr>
<td>Expression</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>-------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>$[(\text{GSH})<em>3]</em>{0-\text{acr3}\Delta\text{hog1}\Delta}$</td>
<td>Initial glutathione concentration in $\text{acr3}\Delta\text{hog1}\Delta$ mutant.</td>
<td></td>
</tr>
<tr>
<td>$[(\text{GSH})<em>3]</em>{0-\text{acr3}}^+ \cdot [(\text{GSH})<em>3]</em>{0-\text{acr3}\Delta\text{hog1}\Delta}$</td>
<td>Initial glutathione concentration in $\text{acr3}\Delta$ and $\text{acr3}\Delta\text{ycf1}\Delta$ mutants.</td>
<td></td>
</tr>
<tr>
<td>435.424</td>
<td>Initial glutathione concentration in ACR3$^+$ knockout mutants and wild type is considered three times reported value in Perrone et al. (Perrone et al., 2005), as cells were pre-incubated for 24 hours in 0.1 mM As$^{3+}$ containing medium.</td>
<td></td>
</tr>
</tbody>
</table>

\[ V_{\text{cell}} = 5 \cdot 10^{-14} \quad \text{Saccharomyces cerevisiae volume measured in glucose medium 1\% (Vindelov & Arneborg, 2002).} \]

\[ V_{\text{vac}} = 2 \cdot 10^{-14} \quad \text{Saccharomyces cerevisiae vacuole volume approximated as 1/5 of cell volume (Vindelov & Arneborg, 2002).} \]

\[ V_{\text{cell-vac}} = 3 \cdot 10^{-14} \quad \text{Saccharomyces cerevisiae volume without vacuole volume.} \]

\[ V_{\text{medium}} = 5 \cdot e-11 \quad 1000 \text{ times cell volume (Schaber et al., 2010).} \]

\[ \text{AsMw} = 74.9216 \quad \text{Arsenite molar weight (grams).} \]

\[ \text{Surface}_{\text{cell}} = (36\cdot\pi)^{1/3} \cdot V_{\text{cell}}^{2/3} \quad \text{Cell surface approximation. Cell is considered as a sphere.} \]

\[ \text{Surface}_{\text{vac}} = (36\cdot\pi)^{1/3} \cdot V_{\text{vac}}^{2/3} \quad \text{Vacuole surface approximation. Vacuole is considered as a sphere.} \]

References:


Table S6: Reaction rate constants and model parameters.

$[.]_0$ indicates initial concentration (μmol/l). The volume is in litre (l), and the concentration is μmol/l, mass is in grams and time in seconds.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Description</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1$</td>
<td>0.00275</td>
<td>$\text{As}^{\text{III}}$ Influx-efflux rate through Fps1 channel.</td>
<td>Estimated</td>
</tr>
<tr>
<td>$k_2$</td>
<td>0.00876</td>
<td>$\text{As}^{\text{III}}$ -Protein binding rate constant.</td>
<td>Estimated</td>
</tr>
<tr>
<td>$k_3$</td>
<td>0.000645</td>
<td>$\text{As}^{\text{III}}_{\text{prot}}$ dissociation rate constant.</td>
<td>Estimated</td>
</tr>
<tr>
<td>$k_4$</td>
<td>0.063925</td>
<td>$\text{As}^{\text{III}}$ induced $\text{Hog1}$ phosphorylation rate constant.</td>
<td>Estimated</td>
</tr>
<tr>
<td>$k_5$</td>
<td>118.09</td>
<td>Dual phosphorylated $\text{Hog1}$, dephosphorylation rate constant.</td>
<td>Estimated</td>
</tr>
<tr>
<td>$k_6$-$\text{As}^{\text{III}}$</td>
<td>0.00018</td>
<td>$\text{As}^{\text{III}}$ induced Fps1 phosphorylation rate constant.</td>
<td>Estimated</td>
</tr>
<tr>
<td>$k_6$-$\text{Hog1}$</td>
<td>853.5</td>
<td>$\text{Hog1}$ induced Fps1 phosphorylation rate constant.</td>
<td>Estimated</td>
</tr>
<tr>
<td>$k_6$-basal</td>
<td>0.051</td>
<td>Basal Fps1 phosphorylation rate constant.</td>
<td>Estimated</td>
</tr>
<tr>
<td>$k_7$</td>
<td>0.0501</td>
<td>Phosphorylated Fps1, dephosphorylation rate constant.</td>
<td>Estimated</td>
</tr>
<tr>
<td>$k_8$</td>
<td>0.8665</td>
<td>$\text{As}^{\text{III}}$ -glutathione conjugation rate constant.</td>
<td>Estimated</td>
</tr>
<tr>
<td>$k_9$</td>
<td>19.93</td>
<td>$\text{As(GS)}_3$ dissociation rate constant.</td>
<td>Estimated</td>
</tr>
<tr>
<td>$k_{10}$</td>
<td>4.2e-06</td>
<td>Vacuolar sequestration of $\text{As(GS)}_3$ rate constant.</td>
<td>Estimated</td>
</tr>
<tr>
<td>$k_{11}$</td>
<td>2.4e-07</td>
<td>v$\text{As(GS)}_3$ vacuolar export rate constant.</td>
<td>Estimated</td>
</tr>
<tr>
<td>$k_{12}$</td>
<td>$\frac{k_{13} \cdot [\text{Acr3}]<em>0}{[\text{As}^{\text{III}}</em>{\text{in}}]_0}$</td>
<td>Acr3 expression rate constant which is calculated.</td>
<td>Calculated</td>
</tr>
<tr>
<td>$k_{13}$</td>
<td>1e-8</td>
<td>Acr3 degradation rate constant.</td>
<td>Estimated</td>
</tr>
<tr>
<td>$k_{m14}$</td>
<td>5.2e-06</td>
<td>Acr3 mediated $\text{As}^{\text{III}}$ export michaelis-constant.</td>
<td>Estimated</td>
</tr>
<tr>
<td>$V_{m14}$</td>
<td>1</td>
<td>Acr3 mediated, maximum $\text{As}^{\text{III}}$ export rate constant.</td>
<td>Set</td>
</tr>
<tr>
<td>$\text{As}^{\text{III}}_{\text{in-ACR3}}$</td>
<td>20.413</td>
<td>Initial concentration of free intracellular arsenite in ACR3$^+$ mutants and WT.</td>
<td>Estimated</td>
</tr>
<tr>
<td>Fps1$P_{\text{max}}$</td>
<td>0.03</td>
<td>Maximum phosphorylated Fps1 estimated from data.</td>
<td>Estimated</td>
</tr>
<tr>
<td></td>
<td>Value</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Δ[Ycf1]</td>
<td>15.622</td>
<td>Ycf1 increase in <em>acr3Δ</em> mutants relative to wild type cells.</td>
<td></td>
</tr>
<tr>
<td>[(GSH)₃]ᵣ₋acr3Δhog1Δ</td>
<td>6.9888</td>
<td>Initial glutathione increase, in <em>acr3Δhog1Δ</em> mutant relative to wild type.</td>
<td></td>
</tr>
<tr>
<td>[(GSH)₃]ᵣ₋acr3Δ</td>
<td>3.28633</td>
<td>Initial glutathione increase in <em>acr3Δ</em> and <em>acr3Δycf1Δ</em> mutants relative to wild type.</td>
<td></td>
</tr>
</tbody>
</table>

Reference:
Table S7: Fps1 and Hog1 phosphorylation data

Phosphorylated Fps1 was measured using western-blot for wild type and hog1Δ mutant. In addition, phosphorylated Hog1 was measured up to 60 minutes after 1mM As\textsuperscript{III} stress. The data are derived from Thorsen et al. (Thorsen et al., 2006).

<table>
<thead>
<tr>
<th>Time</th>
<th>% Hog1PP upon 1.0 mM As\textsuperscript{III} stress in wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.2</td>
</tr>
<tr>
<td>15</td>
<td>7.7</td>
</tr>
<tr>
<td>30</td>
<td>6.5</td>
</tr>
<tr>
<td>60</td>
<td>6.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>% phosphoFPS1 Control</th>
<th>% phosphoFPS1 upon 1.0 mM As\textsuperscript{III} stress</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td><strong>hog1Δ</strong></td>
</tr>
<tr>
<td>24.8</td>
<td>16.2</td>
</tr>
<tr>
<td>30.1</td>
<td>27.3</td>
</tr>
<tr>
<td>28.3</td>
<td>23.5</td>
</tr>
<tr>
<td>26.1</td>
<td>23.6</td>
</tr>
<tr>
<td>16.5</td>
<td>15</td>
</tr>
<tr>
<td>29.4</td>
<td>20.1</td>
</tr>
<tr>
<td>17.9</td>
<td>10</td>
</tr>
<tr>
<td>22.3</td>
<td>18.7</td>
</tr>
<tr>
<td>16</td>
<td>13</td>
</tr>
<tr>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td><strong>WT+As</strong></td>
<td><strong>hog1Δ+As</strong></td>
</tr>
<tr>
<td>45.6</td>
<td>39.6</td>
</tr>
<tr>
<td>35.8</td>
<td>28.8</td>
</tr>
<tr>
<td>34.5</td>
<td>24.1</td>
</tr>
<tr>
<td>30.1</td>
<td>25.4</td>
</tr>
<tr>
<td>38.4</td>
<td>29.9</td>
</tr>
<tr>
<td>49.9</td>
<td>33.4</td>
</tr>
<tr>
<td>39.1</td>
<td>38.8</td>
</tr>
<tr>
<td>35.5</td>
<td>32.1</td>
</tr>
</tbody>
</table>