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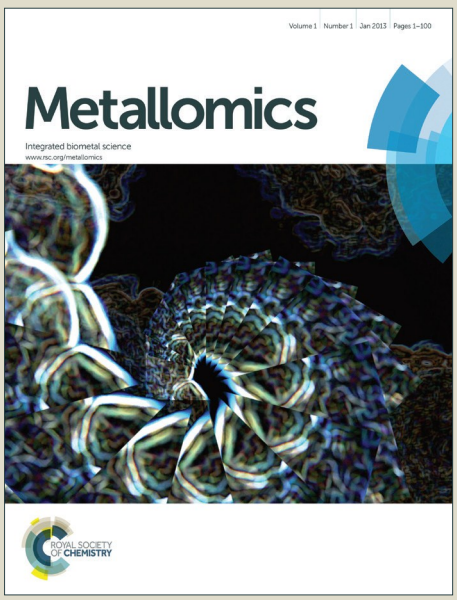

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ARTICLE

Molecular insight into arsenic toxicity via the genome-wide deletion mutant screening of *Saccharomyces cerevisiae*

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Arsenic is omnipresent in soil, air, food and water. Chronic exposure to arsenic is a serious problem to human health. In-depth understanding of this metalloid's toxicity is a fundamental step towards development of arsenic-free foods and measures for bioremediation. By screening the complete set of gene deletion mutants (4873) of *Saccharomyces cerevisiae*, this study uncovered 75 sensitive and 39 resistant mutants against arsenite [As(III)]. Functional analysis of the corresponding genes revealed the molecular details for its uptake, toxicity and detoxification. On the basis of the hypersensitivity of *yap3*Δ, the transcription factor, Yap3p, is for the first time linked to the cell's detoxification against As(III). Apart from confirming the previously described role of the mitogen-activated protein kinase (MAPK) Hog1 pathway in combating arsenic toxicity, the results show that the regulatory subunits (Ckb1p and Ckb2p) of protein kinase CK2 are also involved in the process, suggesting possible crosstalk between the two key protein kinases. The sensitivity to As(III) conferred by deletion of the genes involved in protein degradation and chromatin remodelling demonstrates protein damage is the key mode of toxicity for the metalloid. Furthermore, the resistant phenotype of *fps1*Δ, *snf3*Δ and *pho81*Δ against As(III) links arsenic uptake with the corresponding plasma membrane-bound transporters-aquaglyeroporin (Fps1p), hexose (Snf3p) and phosphate transporters. The molecular details obtained in this screen for As(III) uptake, detoxification and toxicity provide the basis for future investigations into arsenic-related problems in the environment, agriculture and human health.

Introduction

Arsenic is naturally present in ground and drinking water, air and foods. Its mining and industrial usage leads to the increased contamination of water and crops. Rice, the staple food of Asia, often contains an elevated level of arsenic, due to pollution and its natural prevalence in ground waters originating from the river systems of the Himalayas[1,2]. The metalloid ion exists in multiple oxidation states, but mainly trivalent [As(III)] or pentavalent [As(V)]. Both As(III) and As(V) are toxic. As(III) is predominantly present as $As(OH)_3$ in aqueous environments, mimicking the structure of glycerol, a common metabolite in microorganisms and mammalian cells. Therefore, it can enter the cell via aquaglyceroporin transporter (Fps1p)[3], which explains the higher toxicity of As(III) over As(V) and the other organic arsenic derivatives such as mono-, di- and trimethylated arsenicals.

Although it is historically and publically known that a high dose of arsenic is lethal, it is the chronic exposure to arsenic through drinking water and food, dubbed as "a silent assassin", that is insidious to the health of millions of people around the world. Chronic arsenic exposure is associated with cancer of the bladder, lungs, skin and prostate, as well as neurodegenerative and cardiovascular diseases[4,5]. As a consequence, the application of arsenic in certain medicines, which are used in treating acute promyelocytic leukaemia[6] and infectious diseases such as syphilis and trypanosomiasis[7], unavoidably has adverse side-effects on the health of patients.

The underlying molecular mechanism of arsenic toxicity in living organisms is still a subject of active research. Most investigations deploy model organisms such as *Saccharomyces cerevisiae*, a highly useful model for basic science and toxicology, and a pioneering organism in functional genomics and systems biology[8]. The findings from this system are relevant to human beings due to the genomic homology between the two organisms. They share thousands of orthologous genes, accounting for about one-third of the yeast genome[9,10], and there exists a high level of conservation

between the cellular processes of yeast and those of mammalian cells[11,12]. The publication of the yeast genome sequence led to the advent of complete collections of yeast gene deletion mutants such as the collection from EUROSCARF employed in this study. By observing the phenotype of a gene deletion mutant under a given condition, the function of that gene is revealed. Numerous genome-wide screens have been carried out until the present with a range of treatments such as oxidants and metal ions[13,14,15], and the functionality of many genes has been annotated in the process. Most importantly, the functions of ensembles of closely-related genes, in response to a particular stressor, can be revealed.

Two genome-wide screens were done with sodium arsenite in the past, mainly using the deletion mutant set of haploid strain BY4741[16,17]. Both screens primarily skewed towards uncovering the sensitive phenotype. Methodologically, both studies carried out the screening by spotting the individual mutants onto agar plates. The dosage ranges employed were conspicuously divergent, with one study using 0, 75, 100 and 150 μM and the other using 0, 750 and 1000 μM sodium arsenite. Despite certain congruent findings, the involvement of sulphur metabolism and glutathione biosynthesis in counteracting arsenite toxicity was identified in one study [17], but not in the other. Therefore, we believe it is still worthwhile to explore the As(III) toxicity by another genome-wide mutant screening. In this study, we set out to screen a homozygous diploid deletion set obtained from the European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF), using a 96-well plate-based liquid assay. By this approach, we previously screened the deletion collection with aluminium sulphate[14-15] and acquired novel data compared to a similar study using agar plates[18]. We expect that new findings can be achieved for further understanding of arsenic toxicity in this screen, using the 96-well plate-based liquid assay.

By phenotypic screening of the complete collection of homozygous deletion mutants of *S. cerevisiae*, we aim to uncover the sensitive and resistant mutants in response to As(III), using two predetermined benign and sub-lethal doses of sodium arsenite. A sensitive mutant means that deletion of the gene impairs the yeast's response to As(III) and therefore the deleted gene plays a role in combatting As(III) toxicity. Conversely, a resistant mutant against the metalloid reveals that the gene deletion is conducive to the yeast's survival under As(III) treatment and hence the deleted gene is likely involved in As(III) uptake or in synergistic actions which enhance As(III) toxicity. Here we report the comprehensive gene lists associated with the yeast cell's sensitivity or resistance in response to As(III) exposure. A detailed molecular mechanism for the metalloid's uptake, detoxification and toxicity is proposed.

Experimental

Yeast strains and chemicals

All *S. cerevisiae* strains used in this study were from EUROSCARF (Institute of Molecular Biosciences, Frankfurt, Germany), including the wild type BY4743, (*MATa*/*MATα* *his3*Δ0/*his3*Δ0; *leu2*Δ/*leu2*Δ0; *met15*Δ0/*MET15*; *LYS2*/*lys2*Δ0; *ura3*Δ0/*ura3*Δ0), and the complete set of 4,873 homozygous deletion mutants for non-essential genes. All chemicals were from Sigma-Aldrich.

As(III) toxicity screening

Deletion mutants as well as the wild type strain (BY4743) from EUROSCARF library plates, frozen in YEPD (10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} D-glucose) and 15% glycerol, were transferred to plates containing minimal medium (pH 4.3) (20 g L^{-1} D-glucose, 1.7 g L^{-1} yeast nitrogen base without ammonium sulphate, 5 g L^{-1} ammonium sulphate and 0.79 g L^{-1} complete supplement mixture which contained 10 mg L^{-1} adenine, 50 mg L^{-1} L-arginine, 80 mg L^{-1} L-aspartic acid, 20 mg L^{-1} L-histidine HCl, 50 mg L^{-1} isoleucine, 100 mg L^{-1} L-leucine, 50 mg L^{-1} L-lysine HCl, 20 mg L^{-1} L-methionine, 50 mg L^{-1} L-phenylalanine, 100 mgL^{-1} L-threonine, 50 mg L^{-1} L-tryptophan, 50 mg L^{-1} L-tyrosine, 140 mg L^{-1} L-valine, 20 mg L^{-1} uracil) and incubated at 30 °C for 3½ h to allow growth to reach an optical density at 600 nm (OD_{600}) of 0.045 prior to addition of sodium arsenite ($NaAsO_2$) for screening.

BY4743 parent wild type and the 4,873 deletion mutants were screened under two doses of sodium arsenite ($NaAsO_2$) (0.2 and 0.4 mM) and the control (an equal volume of sterile Milli Q water). These concentrations were chosen based on a prior titration with a range of $NaAsO_2$ concentrations (0.5-3.2 mM) using BY4743. The dose 0.2 mM was found to be benign to the wild type strain BY4743. The lower growth of mutants than BY4743 under this dose is regarded as a sensitive phenotype. On the other hand, the dose of 0.4 mM reduced the growth of BY4743 by 50%. Therefore, 0.4 mM $NaAsO_2$ was used here to uncover resistant mutants with growth higher than the wild type. OD_{600} at the time of treatment (T0) was measured using a 96-well plate reader (Multiskan EX, Thermo Electron, USA) then the cultures were incubated at 30 °C. At 16 h (T16) and 20 h (T20), cultures were re-suspended and OD_{600} measured. Measurement at T16 has been found, in our experience, to correlate with the greatest divergence between treatment and control. T20 is the time when cultures become stationary. In the final data analysis, only OD_{600} at T16 was used for each strain. The fold change in growth was calculated as a ratio by dividing OD_{600} at T16 by OD_{600} at T0. Each strain's phenotypic growth in this screen is expressed in a value calculated

by dividing the ratio of the mutant's growth over that of BY4743 in treatment by the ratio of mutant's growth over that of BY4743 in control. The ratio of the mutant growth over that of the wild type in treatment establishes the effect of arsenite on the growth of that mutant. For certain slow growing mutants, using such a ratio alone would result in false sensitive mutants. Therefore, the ratio in treatment is corrected with the ratio of mutant's growth over that of BY4743 in control. The cut-off value of 0.5, denoting 50% growth reduction under treatment, was used to select sensitive mutants, which were at the value 0.5 or lower. The cut-off value of 2, representing 200% greater growth than the wild type, was used for selecting resistant mutants, which were of 2 or higher.

The sensitive and resistant mutants identified from the initial screen were further screened for confirmation. The mutants were picked from EUROSCARF stock plates and screened against 0.2 mM $NaAsO_2$ for sensitivity and against 0.4 and 0.8 mM $NaAsO_2$ for resistance. The data was processed in the same manner as the initial screen.

Gene annotation of the screen data

Functions of the corresponding genes of the As(III) sensitive and tolerant mutants were annotated by using Saccharomyces Genome Database (http://www.yeastgenome.org), MIPS, Gene Ontology, BioGRID (http://www.thebiogrid.org) and FunSpec (http://funspec.med.utoronto.ca).

Results

The phenotypic growth of deletion mutants and the wild type under As(III) treatment was measured as optical density at 600 nm over a period of 16 hr, and the complete data set is in Electronic Supplementary Information 1. According to their growth profile, 75 mutants were found sensitive to As(III), whilst 39 mutants were resistant. The functional categories of the genes corresponding to the sensitive and resistant phenotypes are shown in Fig. 1. These genes are listed in Tables 1 and 2. The functional annotation for each gene is described in Electronic Supplementary Information 2 and 3.

The sensitive mutants against As(III) and their corresponding genes

By the means of web-based resources as described earlier and manual examination of the available annotation for each gene, the 75 genes of the corresponding deletion mutants were categorised into 10 groups on the basis of the functions of their encoded proteins (Table 1). Highly represented are the genes involved in signal transduction, transcriptional regulation, protein modification/transport/sorting, membrane-bound proteins/transporters, metabolism and stress response (Fig. 1A). Because deletion of these genes confers sensitivity, their presence is therefore necessary for combating arsenic toxicity.

Group A (Fig. 1 A) contains five genes related to protein translation. *CBS1* encodes a protein that regulates the translation of the mitochondrial gene *COB* which encodes apocytochrome b, a member of the electron transport chain. The sensitive phenotype of *cbs1*Δ against As(III) indicates the significance of *CBS1* in response to As(III). *NCL1* is a methyltransferase gene. The enzyme can methylate cytosine in tRNAs and therefore regulate protein translation. It was previously shown to be involved in oxidative stress response and mutants lacking Ncl1p are hypersensitive to oxidative stress[19]. The finding here demonstrates *NCL1* is also required against As(III) toxicity. The other three genes, *LHP1*, *NAB6* and *DEG1*, are relevant to RNA binding and modification. Their sensitive phenotype against As(III) points to the necessity of these genes for survival under As(III) exposure.

Group B (Fig. 1A) contains genes that are involved in signal transduction. The presence of *HOG1*, *PBS1*, *SSK1* and *SSK2* highlights the prominence of mitogen-activated protein kinase (MAPK) signalling pathway. *HOG1* was originally found to be involved in high osmolarity glycerol (HOG) response. It encodes a MAPK. The gene, *PBS2*, is for the MAPK kinase (MAPKK), which phosphorylates and hence activates MAPK. The kinase (MAPKKK) further upstream of Pbs2p is encoded by *SSK2*. Ssk2p interacts with Ssk1p, leading to autophosphorylation and activation of Ssk2p which phosphorylates Pbs2p. This screening data set shows that the deletion mutants of *HOG1*, *PBS2*, *SSK1* and *SSK2* are highly sensitive to As(III). The functions of the other genes, *PDE2*, *REG1*, *ARL1*, *LTE1*and *SLT1*, are diverse and detailed in Supplementary Information 2. Their deletion renders the mutants sensitive to As(III) toxicity.

The composite of group C (Fig. 1A) reflects the essentiality of protein folding (*PAC2*, *PAC10*, *PFD1*, *YKE2*, *RBL2* and *CIN2*), transport (*ARL1*, *LST7*, *LST4*, *VPS13*, *SEC22* and *VPS71*) and degradation (*BUL1*) in response to As(III). This is of no surprise as the ability of As(III) to compromise protein folding by coordinating to the cysteine residues of unfolded proteins has been shown[20]. The misfolded proteins, due to the toxic effect of As(III) need to be transported to the proteasome for degradation. Interruption of this process by gene deletion would be detrimental to cell survival. Therefore, the deletion mutants in this group showed sensitivity to As(III).

Group D represents genes related to the regulation of transcription. *ARR1* is required for transcription of the a membrane bound exporter of As(III) encoded by *ARR3*. The sensitivity of *arr1*Δ along with the sensitive phenotype associated with *YAP1*and *RPN4* demonstrates their critical role in response to As(III). The hypersensitivity of *YAP3* deletion mutant highlights that a third member of the YAP

family, together with *YAP1* and *ARR1* (also known as *YAP8*), is required for regulating As(III) detoxification. Group E includes the genes encoding membrane-bound proteins. The presence of *ARR3* is expected as Arr3p is an As(III) exporter, essential for the yeast cell's defence mechanism against As(III). Also within this group are genes related to iron homeostasis (*FET5*, *FIT1* and *FIT3*). Their sensitive phenotype demonstrates the significant role of iron homeostasis in response to As(III) exposure. This finding is reminiscent of the similar discoveries for the importance of iron homeostasis in the yeast cell's resistance to cobalt and nickel[21,22]. The sensitivity of mutants in Group F indicates certain metabolic processes are important in response to As(III). For instance, *TPS1* and *TPS2* are involved in trehalose biosynthesis. It is known that trehalose plays a role in stress response and supressing protein aggregation[23]. And it was demonstrated that these two genes are involved in the cell's resistance to nickel because they were up-regulated in a nickel-resistant *S. cerevisiae* mutant[22]. This study demonstrates that their deletion confers sensitivity to As(III).

The genes related to the maintenance of genomic stability in Group G indicate the need for chromatin remodelling in response to As(III) insult. Of the genes involved in chromatin remodelling (*SWC3*, *RXT3*, *NGG1*, *SAS4*, *ADA2*, *VPS72*, *RSC1*, *VPS71*), three genes (*SWC3*, *VPS72*, *VPS71*) are part of the SWR1 complex which exchanges histone variant H2AZ (Htz1p) for chromatin-bound histone H2A, important for regulating gene expression[24]. The sensitive phenotype of these mutants indicates these genes are a part of molecular mechanisms involved in dealing with arsenic exposure.

The resistant mutants against As(III) and their corresponding genes

The resistant mutants, as listed in Table 2, are those with two-fold or greater growth than the wild type BY4743 against 0.4 mM $NaAsO_2$. The detailed annotation of the genes is provided in Supplementary Information 3.

The corresponding genes of the resistant mutants are over-represented by ribosomal genes and genes involved in protein translation (Fig. 1B). Their encoded proteins are components of translational machinery in the cell. Deletion of these genes would slow down protein synthesis and hence cell growth. This could allow the cell to adapt and survive the As(III) insult and therefore lead to the resistant phenotype. The presence of signalling genes (*CKB1*, *CKB2*, *PHO4*, *PHO81*) on the list of deletion mutants resistant to arsenite indicates possible signalling pathways regulating the entry of As(III). *CKB1* and *CKB2* encode the regulatory components (Ckb1p and Ckb2p) of the tetrameric, pleotropic serine/threonine protein kinase CK2. Since it was previously shown that CK2 does not form tetramers in the absence of either of the regulatory subunits[25], the resistant phenotype of *ckb1*Δ and *ckb2*Δ demonstrates the likelihood that the tetrameric holoenzyme CK2 is associated with As(III) uptake and toxicity. Ckb1p and Ckb2p could also be directly involved in regulating the uptake of As(III) by interacting with the other signalling proteins such as those in Hog1p MAPK pathway. Both *PHO4* and *PHO81* are related to phosphate transport, with the former encoding a transcription factor Pho4p required in phosphate limited environments, and the latter coding for a protein that regulates phosphate transporters. The increased tolerance to As(III) of these two mutants demonstrates that phosphate transporters could serve as the route of As(III) entry. Similarly, the resistance associated with *fps1*Δ and *snf3*Δ is linked to their roles as membrane-bound transporters for glycerol (Fps1p, known as aquaglyceroporin) and hexose (Snf3p). Ask10p is a positive regulator of Fps1p. Deletion of *ASK10* would abolish the positive effect of Ask10p and result in deactivation of aquaglyceroporin, and hence the resistance to As(III) shown by *ask10*Δ.

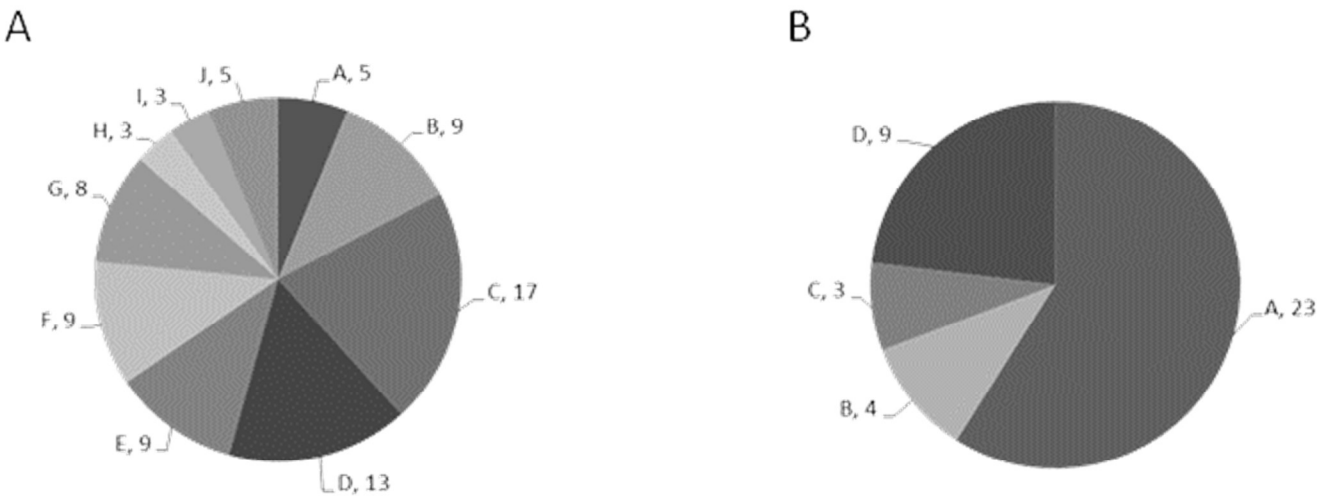

Fig. 1 Functional categories of the genes corresponding to the sensitive and resistant phenotypes and number of genes associated with each category. (A) Functional categories of the genes in 75 sensitive deletion mutants. The functional classifications are based on gene ontology and molecular function in SGD, where **A** represents genes related to protein translation, **B** for signal transduction, **C** for protein modification, transport and sorting, **D** for transcriptional regulation, **E** for membrane-bound proteins/transporters, **F** for metabolism, **G** for genomic maintenance and stability, **H** for structural proteins, **I** for cell cycle and **J** for uncharacterised or dubious ORFs. The details for the functional groups (A-J) are provided in Table 1. (B) Functional category of the genes in 39 resistant deletion mutants, where **A** represents genes related to protein translation, **B** for signal transduction, **C** for membrane bound or associated proteins/ transporters and **D** for uncharacterised or dubious ORFs. The details for the functional groups (A-D) are provided in Table 2.

Table I. Functional analysis of the genes deleted in the sensitive mutants

Functional category	No. of genes	Genes involved in As(III) tolerance
Protein translation	5	CBS1, NCL1, DEG1, LHP1, NAB6
Signal transduction	9	HOG1, PBS2, SSK1, SSK2, SLT2, PDE2, REG1, ARL1, LTE1
Protein modification, transport and sorting	17	BUL1, PFD1, LST7, LST4, PAC10, RBL2, CSF1, PAC2, CIN2, SEC22, YKE2, TUB3, VPS71, ARL1, VPS72, VPS13, ARL1
Transcriptional regulation	13	SAS4, YAP1, ARR1, YAP3, RPN4, STP1, ADA2, RSC1, NGG1, MED1, IRA2, MNE1, URE2
Membrane bound proteins/transporters	9	ARR3, TAT1, LAS21, SEC22, FIT3, FET5, FIT2, MAL31, YPK9
Metabolism	9	TPS1, IDP1, ERG4, GPM2, TPS2, SLC1, CYS3, ELO3, OPI3
Genomic maintenance and stability	8	HNT3, VPS71, SWC3, VPS72, RXT3, RTT107, ADA2, NGG1
Structural proteins	3	SEH1, TUB3, JNM1
Cell cycle	3	JNM1, SHE1, KAR9
Uncharacterised or dubious	5	BSC1, YDL057W, API2, YOR338W, YDL050C

Table 2. Functional analysis of the genes deleted in the resistant mutants

Functional category	No. of genes	Genes involved in As(III) sensitivity
Protein translation	23	RPS9B, RPS16A, RPS14A, RPS18A, RPL29, TMA22, RPS1B, RPS21B, TMA23, RPS28B, RPL37A, RPL6B, RPS29A, RPL43A, RPL16B, RPS29B, RPS17B, RPL13B, RPL37B, BUD31, RPS22B, RPL36B, URM1
Signal transduction	4	CKB1, CKB2, PHO4, PHO81
Membrane associated	3	FPS1, ASK10, SNF3
Uncharacterised	9	YLL044W, YIL014C-A, YLR444C, YLR111W, YGL088W, YDR417C, YLR402W, YLR184W, YML009C-A

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Discussion

This genome-wide screen of the individual deletion mutants for 4,873 non-essential genes reveals both sensitive and resistant mutants against arsenite. The sensitive mutants demonstrate that the deleted genes are essential to combat As(III) toxicity. The term "essential gene" here is used to describe the requirement of a given gene under As(III) exposure, it has no relevance to the other term, "non-essential genes" used in yeast genetics to describe the genes whose deletion results in cells viable in normal growth conditions. As demonstrated by the sensitive mutants, 75 genes are required for yeast cell's survival upon exposure to As(III). The number of resistant mutants (39) uncovered in this study is striking, compared to the finding of five resistant mutants in another genome-wide screen against As(III)[16].

The hypersensitivity shown by *arr1*Δ and *arr3*Δ evidently demonstrates that arsenic-specific detoxification genes, *ARR1* (also known as *ACR1*) and *ARR3* (*ACR3*), are at the forefront of survival mechanism against As(III). *ARR3* encodes a plasma membrane-bound As(III) transporter to efflux the toxic metalloid from the cytosol to the extracellular space[26,27,28], whilst *ARR1* encodes the transcription factor to regulate the expression of Arr3p in response to As(III) insult[29]. The deletion mutant of *ARR2*, which is the gene for As(V) reductase, did not show sensitivity. This is due to the As(III) salt used in the screen, therefore As(V) was not the dominant arsenic species although some arsenite ions could be oxidised to arsenate. The hypersensitive phenotype of *yap3*Δ uncovered here is novel, suggesting the necessity of this transcription activator gene in response to arsenic toxicity. The finding is conceivable since the other members of the Yap family are heavily involved in regulating molecular responses against oxidative stress (Yap1p) and cadmium toxicity (Yap2p)[30,31,32]. In fact, Arr1p also belongs to the Yap family, and is referred to as Yap8p[33]. This discovery of Yap3p in regulating arsenic detoxification is significant in answering the question of how the cell senses the toxic metalloid. As Yap1p possesses two redox centres, one for reactive oxygen species and the other for thiol-reactive chemicals, such as heavy metals[32], we think that Yap3p, as well as Yap1p, may act as an arsenic sensor and regulate the downstream detoxification response. This involves upregulating the expression of vacuolar ATPase Ypk9p, as deletion of its corresponding gene (*YPK9*) resulted in sensitivity to As(III), in addition to its previously observed sensitivity to cadmium, manganese, nickel and selenium[34]. Based on the higher sensitivity of *yap3*Δ than *yap1*Δ, it is likely that Yap3p plays a more direct role in combating As(III) toxicity, whilst Yap1p might be more involved in regulating the antioxidant response. In this context, it is worth bringing up an interesting finding in plant that a tobacco gene, Ntcyc07, enhances As(III) tolerance in *S. cerevisiae* via interaction with Arr1p to promote the expression of *ARR3* and decrease the expression of As(III)-uptake related gene *FPS1*[35,36]. However, there is no existing homologue of Ntcyc07 in yeast.

The hypersensitive phenotype of MAPK deletion mutant *hog1*Δ under 0.2 mM As(III) is in agreement with the previous genome-wide screens [16-17]. However, further details were revealed in this exercise by the sensitivity of *pbs2*Δ, *ssk1*Δ, *ssk2*Δ, *ckb1*Δ and *ckb2*Δ. *PBS2* and *SSK2* encode MAPKK and MAPKKK respectively, upstream of MAPK for its phosphorylation and activation, while the protein encoded by *SSK1* regulates Ssk2p. Hence, deletion of *PBS2*, *SSK1* and *SSK2* would disrupt Hog1p activity and its signalling pathway, which explains their sensitivity to the metalloid. In contrast, deletion of *CKB1* or *CKB2*, the two regulatory subunits of protein kinase CK2, results in resistance against As(III). In *ckb1*Δ or *ckb2*Δ mutants, the tetrameric protein kinase CK2 cannot be formed[25]. That the absence of tetrameric holoenzyme CK2 gives rise to resistance to arsenite suggests the tetrameric CK2 is involved in As(III) uptake. Moreover, the individual subunits in *ckb1*Δ (Cka1p, Cka2p and Ckb2p) and *ckb2*Δ (Cka1p, Cka2p and Ckb1p) can function independently. We therefore reason that it is likely Ckb2p in *ckb1*Δ and Ckb1p in *ckb2*Δ that account for their resistant phenotypes, because neither *cka1*Δ nor *cka2*Δ displayed a pronounced resistant phenotype. The finding again highlights the involvement of protein kinase CK2 in metal toxicity, in addition to our previous finding that the catalytic subunit Cka2p is closely related to aluminium toxicity[15]. Interestingly, *ckb2*Δ did show a high level of resistance against As(III) in the genome-wide screen conducted by Zhou *et al.*[16], but it was not explicitly highlighted. Considering that both Hog1 MAPK pathway and CK2 are involved in regulating arsenic toxicity, it is possible that they may be linked. This means that Ckb1p or Ckb2p may regulate MAPK, MAPKK or MAPKKK. Indeed, according to the protein-protein interaction database (www.thebiogrid.org), *CKB1* and *PBS2* interact at the genetic level.

Protein damage is the key mode of action for As(III) toxicity. This is demonstrated by the sensitive phenotype of

*bul1*Δ and the other mutants involved in protein transport and sorting. Because *BUL1* encodes a ubiquitin-binding protein for protein ubiquitination[37], it is therefore required by the cell to clear the damaged protein. Chemically, trivalent arsenite readily reacts with the thiols of cysteine residues in proteins, resulting in misfolding and loss of protein activity[38]. Such toxicity can also be extended to free cysteine and glutathione, which are the part of sulphur metabolism. In fact, our data demonstrate deletion of *CYS3* confers sensitivity to As(III). Cys3p (cystathionine γ-lyase) catalyses the formation of cysteine from cystathionine, hence its deletion interrupts sulphur metabolism and in turn affect the level of cysteine and glutathione.

Metal toxicity starts with the prerequisite step, cellular uptake of metal ions. Disruption of the uptake process should lead to resistance. This notion is supported by this study. Firstly, as mentioned in the outset of this study, trivalent arsenite [As(III)] structurally resembles glycerol, and enters the cell via the membrane-bound glycerol channel Fps1p[39]. Our finding that *fps1*Δ is indeed resistant to arsenite serves as a valuable quality control, demonstrating the validity of this screen. Furthermore, this screen revealed that *ask10*Δ is highly resistant to As(III) toxicity. *ASK10* encodes Ask10p (also known as Rgc2p), which is an activator of Fps1p[40]. Because Ask10p is required for the opening of Fps1p, its deletion abolishes Fps1p channel activity, therefore leading to reduced arsenite uptake and in turn resistance. In conjunction with the previously discussed *HOG1* pathway, it is conceivable that in the wild type yeast cell, upon arsenite exposure, Hog1p is activated and inhibits the Fps1p channel via inactivating Ask10p.

Apart from the Fps1p-mediated arsenite uptake, this study uncovered two additional entry paths for the metalloid, i.e., via glucose transporter (*SNF3*) and phosphate transporters. The underlying reason for these transporters to take up arsenic as "mistaken identity" instead of their intended cargoes is also due to the chemistry of arsenite. Apart from the polyhydroxylated form, $As(OH)_3$, analogous to glycerol, polymerisation of three $As(OH)_3$ is predicted to form a six-membered ring structure which seems to be similar to the six-membered ring structure of hexose sugars[41]. Therefore, arsenite can gain entry into the cell via glucose transporters. This explains the finding here that deletion mutant *snf3*Δ displayed tolerance to arsenite. The fact, that mammalian glucose transporter, GLUT1, also facilitates arsenite entry into the cell[42], demonstrates the commonality between the yeast model and mammals. The structural similarity amongst arsenate (AsO_4) and phosphate (PO_4) explains the involvement of phosphate transporters in arsenate uptake. The entry of arsenate by phosphate transporters is deduced according to the resistant phenotype of *pho4*Δ and *pho81*Δ, which encode transcription factor Pho4p and its regulator - cyclin-dependent kinase inhibitor Pho81p[43]. Deletion of both genes would lead to lower expression of phosphate transporters, therefore the resistant phenotype against arsenite exposure. A recent study in *Candida albicans* indicates that Pho4p transcription factor can differentiate among As(III), As(V) and phosphate, and therefore trigger presumably specific responses[44]. This may explain the much higher tolerance for *Candida* species against As(III)[45]. The involvement of phosphate transporters implies that some of the applied As(III) during the treatment were oxidised to the pentavalent form and those As(V) ions could be taken up via these transporters, contributing to cytotoxicity. The significance of these uncovered arsenic transporters and their regulators such as Fps1p, Snf3p, Pho4p, Pho81p and Ask10p is relevant not only to our understanding of arsenic toxicity, but they are also potentially useful in bioremediation of the arsenic-contaminated environment as demonstrated by Shah et al.[46].

Deletion of ribosomal genes and those related to ribosomal biogenesis confers arsenite resistance. In yeast, there are 78 ribosomal proteins encoded by 137 ribosomal genes, with 59 of these proteins encoded by a pair of paralogous genes[47,48]. Forty-six proteins are in the 60S large subunit and 32 in the 40S small subunit of the yeast ribosome. Deletion of ribosomal genes slows down the protein translation process, and in turn cell growth. The resistance against arsenite shown by 19 deletion mutants of ribosomal genes, representing approximately 14% of 137 ribosomal genes, demonstrates that slowed protein synthesis confers resistance to adversary arsenite exposure. This notion is further supported by the findings of Pan *et al*.[49] and Dilda *et al*.[50]. Additionally, the ribosomal proteins could serve as the targets of arsenicals in wild type strains[51]. Since such damaged proteins would cause cytotoxicity, their deletion is in fact beneficial in an arsenic-rich environment.

Although previous studies using yeast deletion mutants have led to a significant advance in our understanding of the metal uptake, toxicity mechanisms and cellular detoxification, the molecular details of these key aspects are still to be defined. So scarce is the knowledge of how toxic metals, like arsenic, are sensed by the cell and how the sensing is linked to specific cellular responses. As the mammalian cell senses growth factors via their receptors, the yeast cell has transporters/receptors (transceptors) to sense its nutrients (e.g. phosphate). Such sophisticated built-in systems are the result of lengthy adaptation and evolution. In contrast to nutrients, organisms such as yeast do not have such innate transceptors *per se* to sense toxic metals like arsenite. Rather, the cell has to organise its defence upon an insult. This study highlights the uptake routes for arsenic as "mistaken identity" via glycerol channel, hexose transporter and phosphate transporters. The phenotype of three YAP transcription factor genes (*ARR1*/*YAP8*, *YAP1*, *YAP3*) demonstrates their critical role in organising cellular defences, likely by sensing intracellular As(III) and activating specific genes for exporting As(III) to the extracellular space and other

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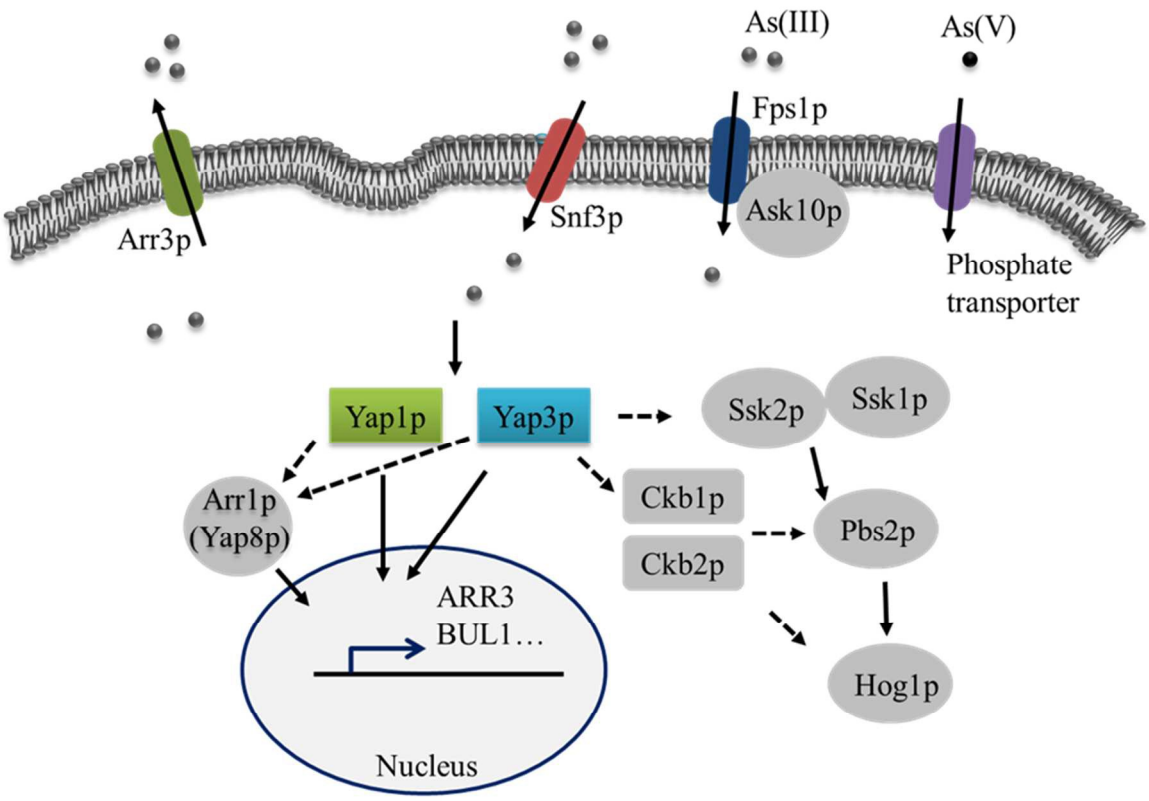

Fig. 2 The proposed mechanistic response against arsenite in *S. cerevisiae*. Upon exposure to As(III) (grey spheres), the metalloid enters the cell via glycerol transporter Fps1p and hexose transporter Snf3p. Some As(III) might be oxidised to As(V) (black sphere) which then enters the cell through phosphate transporters. The sudden increase of intracellular arsenite can attack Yap1p and Yap3p, which initiates downstream regulation, including activation of gene expression for *ARR3*, *BUL1* and the other detoxification genes as well as activation of Hog1p MAPK pathway which can in turn deactivate Fps1p, and also possibly trigger interaction between regulatory subunits (Ckb1p and Ckb2p) of protein kinase CK2 with the components of MAPK pathway. --- dashed line denotes possible interactions.

detoxification processes. The Hog1pathway, possibly in conjunction with CK2, is essential to shut down uptake transporters like Fps1p. This overall mechanistic network is illustrated in Fig. 2.

As stated at the outset, *S. cerevisiae* is a useful model organism and can be related to human beings and the other species. This is clearly demonstrated by comparison of our findings to the datasets in the other organisms. The role of aquaglyceroporin (Fps1p) and glucose transporter (Snf3p) in As(III) transport is supported by the findings in mammalian cells and zebrafish[42, 52,53]. The zebrafish study also revealed the critical role of iron homeostasis in the organism's response to arsenic by showing that three genes (ferritin like, zgc:173594, and frh3) associated with iron homeostasis were markedly up-regulated[53]. This is in agreement with what we have found in the current study. Intriguingly, the supplementary data (Table S3) of Xu et al. contain the exciting finding that ck2b gene (protein kinase CK2 β subunit) was highly up-regulated upon exposure to arsenic[53]. This is indeed the confirmatory evidence from such a distant species for our finding that protein kinase CK2 is involved in regulating the cell's response to arsenic toxicity. Additionally, our recent data (unpublished) in mouse neuronal cells further support the finding here that CK2 regulates arsenic toxicity.

In conclusion, the data here provide additional details to the existing understanding of arsenic toxicity and cellular

detoxification. The fact that yeast cells have specific built-in molecules such as *ARR1* and *ARR3* for As(III) efflux strongly suggests such detoxification apparatus has been developed along the evolutionary timeline of thousands of years. A recent finding demonstrates, similarly, that human populations in the arsenic contaminated Andean highlands have acquired genetic adaptation by developing a particular detoxification mechanism[54]. Such commonality between these two species again reinforces the potential usefulness of this study. The uncovered genes relevant to the metalloid's detoxification and uptake provide valuable knowledge for dealing with arsenic-related health and environmental problems. The proposed regulatory mechanism for the yeast cell, in response to arsenic exposure, highlights the key findings of this screen, and further demonstrates the value of yeast deletion collections as tools in toxicological investigation. As demonstrated by the engineered arsenic-accumulating plant using bacterial detoxification genes [55], the yeast genes revealed here such as *CKB2* and *ASK10* could also be targeted in developing arsenic resistant yeast strains or plant varieties for bioremediation.

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References

1. E. Sohn, *Nature* 2014, *514*. S62-S63.
2. K. M. McCarty, H. T. Hanh, K.-W. Kim, *Rev. Environ. Health* 2011, *26*. 71-78.
3. M. Thorsen, Y. Di, C. Tängemo, M. Morillas, D. Ahmadpour, C. Van der Does, A. Wagner, E. Johansson, J. Boman, F. Posas, R. Wysocki, M. J. Tamás, *Mol. Biol. Cell* 2006, *17*. 4400-4410.
4. M. F. Naujokas, B. Anderson, H. Ahsan, H. V. Aposhian, J. H. Graziano, C. Thompson, W. A. Suk, *Environ. Health Persp.* 2013, *121*. 295-302.
5. A. H. Smith, C. M. Steinmaus, *Annu. Rev. Publ. Health* 2009, *30*. 107-122.
6. Z.-X. Shen, Z.-Z. Shi, J. Fang, B.-W. Gu, J.-M. Li, Y.-M. Zhu, J.-Y. Shi, P.-Z. Zheng, H. Yan, Y.-F. Liu, Y. Chen, Y. Shen, W. Wu, W. Tang, S. Waxman, H. de Thé, Z.-Y. Wang, S.-J. Chen, Z. Chen, *P. Natl. Acad. Sci. USA* 2004, *101*. 5328-5335.
7. F. Ravandi, *Leukemia* 2004, *18*. 1457-1459.
8. D. Botstein, G. R. Fink, *Genetics* 2011, *189*. 695-704.
9. A. H. Kachroo, J. M. Laurent, C. M. Yellman, A. G. Meyer, C. O. Wilke, E. M. Marcotte, *Science* 2015, *348*. 921-925.
10. K. P. O'Brien, M. Remm, E. L. L. Sonnhammer, *Nucleic Acids Res.* 2005, *33*. D476-D480.
11. J. A. Simon, A. Bedalov, *Nat. Rev. Cancer* 2004, *4*. 481-487.
12. W. H. Mager, J. Winderickx, *Trends Pharmacol. Sci.* 2005, *26*. 265-273.
13. G. W. Thorpe, C. S. Fong, N. Alic, V. J. Higgins, I. W. Dawes, *P. Natl. Acad. Sci. USA* 2004, *101*. 6564-6569.
14. N. M. Tun, P. J. O'Doherty, G. G. Perrone, T. D. Bailey, C. Kersaitis, M. J. Wu, *Metallomics* 2013, *5*. 1068-1075.
15. N. Tun, P. O'Doherty, Z. Chen, X. Wu, T. Bailey, C. Kersaitis, M. Wu, *Metallomics* 2014, *6*. 1558-1564.
16. X. Zhou, A. Arita, T. P. Ellen, X. Liu, J. Bai, J. P. Rooney, A. D. Kurtz, C. B. Klein, W. Dai, T. J. Begley, M. Costa, *Genomics* 2009, *94*. 294-307.
17. M. Thorsen, G. Perrone, E. Kristiansson, M. Traini, T. Ye, I. Dawes, O. Nerman, M. Tamas, *BMC Genomics* 2009, *10*. 105.
18. M. Kakimoto, A. Kobayashi, R. Fukuda, Y. Ono, A. Ohta, E. Yoshimura, *BioMetals* 2005, *18*. 467-474.
19. C. T. Y. Chan, Y. L. J. Pang, W. Deng, I. R. Babu, M. Dyavaiah, T. J. Begley, P. C. Dedon, *Nat. Commun.* 2012, *3*. 937-937.
20. D. Ramadan, P. C. Rancy, R. P. Nagarkar, J. P. Schneider, C. Thorpe, *Biochemistry* 2008, *48*. 424-432.
21. C. Alkim, L. Benbadis, U. Yilmaz, Z. P. Cakar, J. M. Francois, *Metallomics* 2013, *5*. 1043-1060.
22. G. Küçükgöze, C. Alkım, Ü. Yılmaz, H. İ. Kısakesen, S. Gündüz, S. Akman, Z. P. Çakar, *FEMS Yeast Res* 2013, *13*. 731-746.
23. M. D. Pereira, E. C. A. Eleutherio, A. D. Panek, *BMC Microbiol.* 2001, *1*. 11-11.
24. G. Mizuguchi, X. Shen, J. Landry, W.-H. Wu, S. Sen, C. Wu, *Science* 2004, *303*. 343-348.
25. K. Kubiński, K. Domańska, E. Sajnaga, E. Mazur, R. Zieliński, R. Szyszka, *Mol. Cell. Biochem.* 2007, *295*. 229-236.
26. P. Bobrowicz, R. Wysocki, G. Owsianik, A. Goffeau, S. Ułaszewski, *Yeast* 1997, *13*. 819-828.
27. R. Wysocki, P. Bobrowicz, S. Ulaszewski, *J. Biol. Chem.* 1997, *272*. 30061-30066.
28. E. Maciaszczyk-Dziubinska, D. Wawrzycka, E. Sloma, M. Migocka, R. Wysocki, *BBA-Biomembranes* 2010, *1798*. 2170-2175.
29. E. Maciaszczyk, R. Wysocki, P. Golik, J. Lazowska, S. Ulaszewski, *FEMS Yeast Res.* 2004, *4*. 821-832.
30. L. Fernandes, C. Rodrigues-Pousada, K. Struhl, *Mol. Cell. Biol.* 1997, *17*. 6982-93.
31. P. J. O'Doherty, V. Lyons, V. J. Higgins, P. J. Rogers, T. D. Bailey, M. J. Wu, *Free Radical Res.* 2013, *47*. 1054-1065.
32. D. Azevedo, L. Nascimento, J. Labarre, M. B. Toledano, C. Rodrigues-Pousada, *FEBS Lett.* 2007, *581*. 187-195.
33. C. Rodrigues-Pousada, R. A. Menezes, C. Pimentel, *Yeast* 2010, *27*. 245-258.
34. K. Schmidt, D. M. Wolfe, B. Stiller, D. A. Pearce, *Biochem. Bioph. Res. Co.* 2009, *383*. 198-202.

35. Y. G. Mok, B. D. Lee, Y. J. Kim, C. E. Lee, D. G. Kim, J. H. Lee, J. K. Shim, Y. Meng, B. P. Rosen, J. S. Choi, H. S. Shin, S. K. Kim, J. S. Lee, S. Hwang, *FEBS Lett.* 2008, *582*. 916–924.
36. M. S. Lee, S. Hwang, *Plant Biotechnol. Rep.* 2012, *6*. 391-395.
37. T. Andoh, Y. Hirata, A. Kikuchi, *Mol. Cell. Biol.* 2000, *20*. 6712-6720.
38. M. F. Hughes, *Toxicol. Lett.* 2002, *133*. 1-16.
39. R. Wysocki, C. C. Chéry, D. Wawrzycka, M. Van Hulle, R. Cornelis, J. M. Thevelein, M. J. Tamás, *Mol. Microbiol.* 2001, *40*. 1391-1401.
40. S. E. Beese, T. Negishi, D. E. Levin, *PLoS Genet.* 2009, *5*. e1000738,
41. Z. Liu, E. Boles, B. P. Rosen, *J. Biol. Chem.* 2004, *279*. 17312-17318.
42. Z. Liu, M. A. Sanchez, X. Jiang, E. Boles, S. M. Landfear, B. P. Rosen, *Biochem. Bioph. Res. Co.* 2006, *351*. 424-430.
43. A. Kaffman, I. Herskowitz, R. Tjian, E. O'Shea, *Science* 1994, *263*. 1153-1156.
44. V. Urrialde, D. Prieto, J. Pla, R. Alonso-Monge, *Frontiers in Microbiol.* 2015, *6*. Article 118.
45. I. Berdicevsky, L. Duek, D. Merzbach, Y. S., *Environ. Pollut.* 1993, *80*. 41-44.
46. D. Shah, M. W. Y. Shen, W. Chen, N. A. Da Silva, *J. Biotechnol.* 2010, *150*. 101-107.
47. K. K. Steffen, M. A. McCormick, K. M. Pham, V. L. MacKay, J. R. Delaney, C. J. Murakami, M. Kaeberlein, B. K. Kennedy, *Genetics* 2012, *191*. 107-118.
48. R. J. Planta, W. H. Mager, *Yeast* 1998, *14*. 471-477.
49. X. Pan, S. Reissman, N. R. Douglas, Z. Huang, D. S. Yuan, X. Wang, J. M. McCaffery, J. Frydman, J. D. Boeke, *Genetics* 2010, *186*. 725-734.
50. P. J. Dilda, G. G. Perrone, A. Philp, R. B. Lock, I. W. Dawes, P. J. Hogg, *Int. J. Biochem. Cell Biol.* 2008, *40*. 1016-1029.
51. L. Szinicz, W. Forth, *Arch. Toxicol.* 1988, *61*. 444-449.
52. E. Maciaszczyk-Dziubinska, D. Wawrzycka, R. Wysocki, *Int. J. Mol. Sci.* 2012, *13*. 3527-3548.
53. H. Xu, S. Lam, Y. Shen, Z. Gong, *PLoS ONE* 2013, *8(7): e68737*.
54. C. M. Schlebusch, L. M. Gattepaille, K. Engström, M. Vahter, M. Jakobsson, K. Broberg, *Mol. Biol. Evol.* 2015, *32*. 1544-1555.
55. O. P. Dhankher, Y. Li, B. P. Rosen, J. Shi, D. Salt, J. F. Senecoff, N. A. Sashti, R. B. Meagher, *Nat. Biotechnol.* 2002, *20*. 1140-1145.