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Arsenic Toxicity to *Saccharomyces cerevisiae* Is a Consequence of Inhibition of the TORC1 Kinase Combined with a Chronic Stress Response

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The conserved Target Of Rapamycin (TOR) growth control signaling pathway is a major regulator of genes required for protein synthesis. The ubiquitous toxic metalloid arsenic, as well as mercury and nickel, are shown here to efficiently inhibit the rapamycin-sensitive TORC1 (TOR complex 1) protein kinase. This rapid inhibition of the TORC1 kinase is demonstrated in vivo by the dephosphorylation and inactivation of its downstream effector, the yeast S6 kinase homolog Sch9. Arsenic, mercury, and nickel cause reduction of transcription of ribosome biogenesis genes, which are under the control of Sfp1, a TORC1-regulated transcriptional activator. We report that arsenic stress deactivates Sfp1 as it becomes dephosphorylated, dissociates from chromatin, and exits the nucleus. Curiously, whereas loss of *SFP1* function leads to increased arsenic resistance, absence of *TOR1* or *SCH9* has the opposite effect suggesting that TORC1 has a role beyond down-regulation of Sfp1. Indeed, we show that arsenic activates the transcription factors Msn2 and Msn4 both of which are targets of TORC1 and protein kinase A (PKA). In contrast to TORC1, PKA activity is not repressed during acute arsenic stress. A normal level of PKA activity might serve to dampen the stress response since hyperactive Msn2 will decrease arsenic tolerance. Thus arsenic toxicity in yeast might be determined by the balance between chronic activation of general stress factors in combination with lowered TORC1 kinase activity.

INTRODUCTION

The transition metal arsenic has a long history of human exploitation as both a poison and a medicine. In more recent times Ehrlich's discovery of the antisyphilitic drug arsphenamine (also known as salvarsan) by systematic chemical modification of arsenic derivatives marked the beginning of modern pharmaceutical research. Arsenic trioxide (ATO) is used today in cancer treatment (Evens *et al.*, 2004; Lu *et al.*, 2007; Wang and Chen, 2008).

Exposure to arsenic evokes a broad spectrum of cellular reactions in *Saccharomyces cerevisiae* (Tamas and Wysocki, 2001; Haugen *et al.*, 2004; Jin, 2008; Thorsen *et al.*, 2007) and in higher eukaryotes (Salnikow and Zhitkovich, 2008). A number of mechanisms exist for detoxification, probably because arsenic has always been widespread in the environment. These involve reduction of influx through the aquaglyceroporin Fps1p (Wysocki *et al.*, 2001; Thorsen *et al.*, 2006); sequestration into the vacuole in the form of glutathione conjugates, metallothionein, and other metal/protein complexes; and active extrusion (Ghosh *et al.*, 1999). In yeast, genome-wide analysis of the transcription patterns in response to arsenic revealed a complex network of transcription factors controlling the expression of several hundred genes (Haugen *et al.*, 2004; Wysocki *et al.*, 2004; Thorsen *et al.*, 2007). Mitogen-activated protein kinases mediate protective responses involving AP-1- and AP-1-like transcription factors in higher eukaryotes and in fungi (Cavigelli *et al.*, 1996; Rodriguez-Gabriel and Russell, 2005; Thorsen *et al.*, 2006).

The mechanisms by which arsenic might influence signaling pathways other than MAP kinase systems are beginning to emerge. In addition to specific responses (e.g., oxidative stress), arsenic leads to the up-regulation of general stress genes, many of which are targets of the Msn2 and Msn4 transcriptional activators. Msn2 and Msn4 are partially redundant transcriptional activators responsible for the induction of genes in response to several types of stress (Martinez-Pastor *et al.*, 1996; Görner *et al.*, 1998; Gasch, 2007). Activity and phosphorylation status of Msn2 is regulated by protein kinase A (PKA) as a crucial nutrient and carbon source mediator (Santangelo, 2006) and the action of phosphatase PP1. Interestingly however, among all adverse environmental conditions only acute glucose starvation causes dephosphorylation of Msn2 by inactivation of PKA and activation of PP1 (Görner *et al.*, 1998, 2002; De Wever *et al.*, 2005; Garmendia-Torres *et al.*, 2007).

Arsenic also causes the rapid down-regulation of many genes that promote growth, a large number of which encode ribosomal proteins (RPs). Ribosome biogenesis is a major consumer of cellular energy and RNA polymerase II activity

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Table 1. Strains used in this study

Strain	Genotype	Source
BY-4741a	*MAT***a** *leu2 ura3 his3 met15 can1*	EUROSCARF
BY sfp1	*MAT***a** *sfp1::kanMX4*	EUROSCARF
BY-1541	*MAT***a** *SFP1-GFP::HIS3*	Marion *et al.* (2004)
RL285-16B (BYα)	*MAT*$\boldsymbol{\alpha}$ *LYS MET HIS ura3 LEU*	This study
YJU519 (BYα)	*MAT*$\boldsymbol{\alpha}$ *SCH9-2D3E; LYS MET HIS ura3 LEU*	This study
W303-1A	*MAT***a** *leu2 ura3 his3 trp1 ade2 can1*	K. Nasmyth (University of Oxford, United Kingdom)
DH303a	*MAT***a** *leu2 ura3 HIS3 TRP1 ade2 can1*	This study
DH303 msn2	*MAT***a** *msn2::HIS3 TRP1*	This study
DH303 msn4	*MAT***a** *msn4::TRP1 HIS3*	This study
W303 msn2msn4	*MAT***a** *msn2::HIS3 msn4::TRP1*	Görner *et al.* (1998)
W303 tpk123msn2msn4	*MAT***a** *tpk1::URA3 tpk2::HIS3 tpk3::TRP1 msn2::HIS3 msn4::TRP1*	Görner *et al.* (2002)
W303 bcy1	*MAT***a** *bcy1::LEU2*	Görner *et al.* (2002)
YHL38 (W303a)	*MAT***a** *SFP1-TAP::URA3*	This study
YHL69 (W303a)	*MAT***a** *tor1::natMX*	This study
YSS100 (W303a)	*MAT***a** *sfp1::HIS IFH1-13MYC::TRP1*	This study
YHL156 (W303a)	*MAT***a** *sfp1::HIS tor1::natMX IFH1-13MYC::TRP1*	This study
TB50a	*MAT***a** *leu2 ura3 rme1 trp1 his3*	M. Hall (University of Basel, Basel, Switzerland)
RL295-8B (TB50a)	*MAT***a** *SCH9-3E ura3 LEU2 TRP1 HIS3*	This study
YAS053-1 (TB50a)	*MAT***a** *sch9:kanMX4 gln3::KanMX gat1::HIS3*	Urban *et al.* (2007)
RL284-3D (TB50a)	*MAT***a** *TCO89-TAP::TRP1 TOR1-1*	This study
RL176-1C (TB50α)	*MAT*$\boldsymbol{\alpha}$ *TCO89-TAP::TRP1 mycTOR1KD*	This study

(Warner, 1999). Consequently, the regulation of ribosomal protein gene transcription constitutes a key mechanism by which protein synthesis and cell growth is regulated in response to the environment. Recent work has begun to reveal *trans*-acting factors involved in RP gene activation and regulation. The forkhead-like protein Fhl1 and an interacting factor Ifh1 constitute one axis of RP gene regulation (Martin *et al.*, 2004; Schawalder *et al.*, 2004; Wade *et al.*, 2004; Rudra *et al.*, 2005). A second regulator that localizes to RP gene promoters is the split Zn-finger transcription factor Sfp1 (Fingerman *et al.*, 2003; Jorgensen *et al.*, 2004; Marion *et al.*, 2004). RP gene expression is regulated by PKA through both Fhl1 (Martin *et al.*, 2004) and Sfp1 (Jorgensen *et al.*, 2004). Sfp1 is strongly regulated by the target of rapamycin (TOR) kinase, a conserved serine/threonine kinase of the phosphatidylinositol kinase–related kinase family that functions in all eukaryotes as a central growth regulator (Wullschleger *et al.*, 2006). Yeast and other eukaryotes have two TOR-containing complexes, TORC1 and TORC2, which have different targets and distinct physiological functions (Loewith *et al.*, 2002). Rapamycin inhibits TORC1 activity by the formation of a ternary complex with the peptidyl-prolyl *cis*-trans isomerase Fpr1 (FKBP12; Heitman *et al.*, 1991). Inhibition of TORC1 causes a loss of Sfp1 from RP gene promoters and its movement from the nucleus to the cytoplasm (Marion *et al.*, 2004). Furthermore, the Sch9 protein kinase, a yeast S6 kinase homolog, has been implicated as a major and direct downstream target of TORC1 for control of both stress- and growth-related transcription (Pedruzzi *et al.*, 2003; Jorgensen *et al.*, 2004; Kaeberlein *et al.*, 2005; Urban *et al.*, 2007), and indeed TOR activity has also been shown to modulate Msn2 intracellular localization (Beck and Hall, 1999; Santhanam *et al.*, 2004).

In this study we investigated the function of the TORC1 and PKA signaling pathways and their downstream targets Sfp1, Sch9, and Msn2/4, in the arsenic stress response of the budding yeast *S. cerevisiae*. We report that repression of RP genes after trivalent arsenic exposure is correlated with and most likely a consequence of rapid inactivation of TORC1 (within several minutes), whereas PKA activity is sustained. Inhibition of TORC1 leads to reduced Sfp1 activity and RP gene transcription, which nevertheless serves to promote cell growth under conditions of continuous low-level arsenic exposure. Conversely, our data suggest that a second consequence of reduced TORC1 activity, namely the chronic Msn2/4-dependent induction of stress-related genes, is a major contributor to arsenic toxicity.

MATERIALS AND METHODS

Plasmids and Yeast Strains

Strains used in this study are summarized in Table 1. To generate the DH303 isogenic series, fragments obtained by PstI digestion of pJJ246 containing the *TRP1* gene and EcoRI/SalI digestion from plasmid pJJ217 (Jones and Prakash, 1990) containing the *HIS3* genes were integrated by homologous recombination into DH303, DH303 *msn2*, and DH303 *msn4.pHSE2-LacZ* has been described previously (Sorger and Pelham, 1987).

RNA and DNA Methods

Fluorescently labeled cDNA was synthesized from 15 μg of total RNA and a T20VN primer using 200 U Superscript II (Invitrogen, Carlsbad, CA) and Cy3-CTP or Cy5-CTP (GE Healthcare, Waukesha, WI). Labeled cDNAs were pooled and RNA was hydrolyzed for 20 min in 10 N NaOH at 65°C, neutralized with acetic acid, and purified by the Cy Scribe GFX purification kit (GE Healthcare). Microarrays (obtained from Microarray Centre, Toronto, Ontario, CAN) had PCR fragments of 6144 predicted *S. cerevisiae* ORFs spotted in duplicate. Hybridization was performed for 14–16 h in DigEasyHyb solution (Roche Diagnostics, Basel, Switzerland) with 0.1 mg/ml salmon sperm DNA (Sigma, St. Louis, MO) as a carrier solution at 37°C. After hybridization, microarrays were washed three times in 1× SSC, and 0.1% SDS at 50°C for 10 min, followed by 1 min in 1× SSC and 0.1× SSC at RT and a 5-min 500 rpm spin to dryness. The microarrays were scanned using an Axon GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA). The image data were quantified using the GenePix Pro4.1 software (Molecular Devices). Raw data are available under the ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) accession no. E-MEXO-1075.

For individual microarrays the intensity of the two fluorescent channels were normalized to the mean of ratio of medians of all unflagged features using the Genepix Pro4.1 normalization option. Values of not found features were excluded from further analysis. Mean ratios were calculated for features with at least four values. Genes labeled as dubious ORFs in SGD were also removed from analysis. The remaining values were normalized. The filtered values used for further analysis are available as supplementary file.

Cluster analysis (Eisen *et al.*, 1998; Nadon and Shoemaker, 2002; http://rana.stanford.edu/software) was performed using the cluster3 and visualized with TreeView (Saldanha, 2004; http://jtreeview.sourceforge.net). Significant associations to either gene ontology (GO)-terms or transcription factors were obtained with the T-Profiler (http://www.t-profiler.org/; Boorsma *et al.*, 2005). Values of genes associated with the most significant terms were visualized by Cluster analysis using complete linkage and correlation as similarity metric. The hierarchical cluster results were confirmed by K-means clustering. For analysis of the effect of the absence of transcription factors for As(III) gene regulation the ratios of the wild type versus the mutant were calculated and the log2-transformed values and graphically included in the cluster analysis by setting their column weight value to zero.

Green Fluorescent Protein Fluorescence Microscopy

Green fluorescent protein (GFP) was visualized directly in living cells carrying plasmids p*ADH1MSN2*-GFP or p*ADH1MSN4*-GFP and EY*sfp1* P_{sfp1}-GFP without fixation on object slides (at 30°C). Stress conditions were applied as specified for individual experiments. DAPI (2 μg/ml) added 10 min before microscopy was used to localize nuclei. In living cells DAPI also stains other nucleic acids apart from nuclear DNA, resulting in a characteristic background. Images were recorded on a Zeiss Axioplan 2 fluorescence microscope (Thornwood, NY) with a Spot Pursuit camera (Visitron Systems, Puchheim, Germany). Quantification of Sfp1-GFP localization was done by counting ~50 cells from three independent experiments ($p < 2 \times 10^{-4}$).

Chromatin Immunoprecipitation

For immunoprecipitations 20 μl rabbit IgG (Sigma, I5006) coupled magnetic Epoxy beads (Dynabeads M-270; Dynal Biotech, Invitrogen) was added, and tubes were rotated for 3 h at 4°C. Immunoprecipitates were washed with lysis buffer. Both an aliquot of sonicated cleared extract (input) and the immunoprecipitated material were de-cross-linked in TE (Tris-EDTA) plus 1% SDS for at least 8 h at 65°C. Quantitation of immunoprecipitated DNA was obtained by real-time PCR using SYBR Green detection (Bianchi *et al.*, 2004) on an Applied Biosystems ABI Prism 7700 machine (Foster City, CA). Primers used were RPL2B-5′ (CAGAGAGGTCGTCCCAGTCT) and RPL2B-3′ (GCAGAATCCACCAGGAGTGT) for *RPL2B* promoter and HL228 (GAATTGAGAGTTGCCCCAGA) and HL229 (AGAAGGCTGGAACGTTGAAA) for the *ACT1* gene. For each data point, results were obtained from at least two and up to four experiments.

Chemical Fragmentation

Chemical fragmentation analysis was performed as described (Urban *et al.*, 2007) with the following exceptions. Experiments were done in W303-1A strain background transformed with plasmid pRS416 containing *SCH9-5HA* (pJU676). Cultures were grown to midlog phase at 30°C in SC-Ura (pH 6.0, 2% glucose, and 0.2% Gln), inhibitors (or drug vehicles) were added for 30 min before harvesting. Antibody 12CA5 was used for the detection of SCH9-5HA.

Analysis of Sfp1 Phosphorylation

Overnight cultures of strain YHL89 (*SFP1*-TAP) were diluted in YPAD, grown to midlog phase, and harvested by centrifugation, and the pellets immediately frozen in liquid nitrogen. Pellets were resuspended in an equal volume of lysis buffer (100 mM HEPES-KOH, pH 8.0, 10% glycerol, 10 mM EGTA, 0.1 mM EDTA, 0.4% NP-40, 600 mM NaOAc, 1 mM PMSF, 1 mM DTT, 1× protease inhibitor cocktail [Roche, Indianapolis, IN] and 0.1× phosphatase inhibitor mix [PPi: 10 mM NaF, 10 mM NaN3, 10 mM *p*-nitrophenylphosphate, 10 mM $Na_2P_2O_4$, and 10 mM β-glycerophosphate]). Cells were lysed by vortexing two times for 1 min with zirconia/silica beads using a Minibeadbeater-8 machine (Biospec Products, Bartlesville, OK). Cell lysates were cleared by centrifugation (10 min, 3000 rpm, +4°C) and diluted with lysis buffer to a protein concentration of 5 mg/ml. For immunoprecipitations 40 μl rabbit IgG (Sigma, I5006) coupled magnetic Epoxy beads (Dynabeads M-270; Dynal Biotech, Invitrogen) were added, tubes were rotated for 3 h at 4°C, and then the beads were washed three times with lysis buffer. Proteins were eluted with 1× SDS-PAGE sample buffer and incubated at 65°C for 10 min and separated by SDS-PAGE. The quantitative ProQ Diamond Phosphoprotein Gel Stain (Invitrogen) and SYPRO Ruby protein stain (Bio-Rad, Hercules, CA) in-gel stains were used in accordance with the manufacturer's protocol. The fluorescent intensity of protein bands was visualized and quantified using an Ettan DIGE Imager (GE Healthcare) and accompanying ImageQuant TL software (GE Healthcare). For quantification, Sfp1 phosphoprotein levels were normalized to total Sfp1 protein levels and to the background on each lane and presented as relative phosphorylation normalized to untreated wild-type sample.

Phospho Msn2 Analysis

Phosphorylation status of Msn2 was determined by Western blots using a purified antiserum raised against phosphorylated peptides and loading of Msn2 was confirmed with an anti-Msn2 serum, both described by De Wever *et al.* (2005).

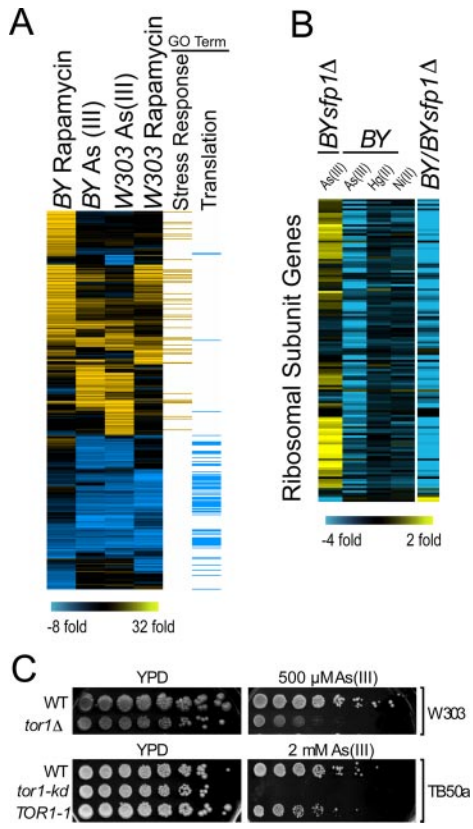

Figure 1. Transcript profile of arsenic stress. (A) Comparison of transcript profiles from arsenic-stressed (0.5 mM As(III) 30 min) BY4741 and W303-1A cells to rapamycin-treated cells (30 min, 200 ng/ml). Hierarchical clustering of those genes changed at least once <-4- or >4-fold. Right panel shows genes associated to GO terms "translation" and "response to stress". Supplemental Figure S2B shows a comparison of reported microarray data of rapamycin-treated cells. (B) Regulation of 137 ribosomal protein genes by arsenic (0.5 mM As(III)), mercury (30 μM Hg(II)), and nickel (1.5 mM Ni(II)) in wild-type compared with *sfp1*Δ mutant cells; all treatments were for 30 min. The BY/BYsfp1 lane shows the color coded quotient of treated wild type and sfp1 mutant. Data to view clustering figures with TreeView are available as Supplemental Files. (C) Tor1 mutant strains have a growth defect during chronic arsenic stress. *tor1*Δ is a derivative from *W303-1A*, the kinase-dead *tor1*-KD and the rapamycin-insensitive *TOR1-1* are derivatives from TB50a. Growth was recorded after a 3-5-d incubation at 30°C.

RESULTS

A Role for TORC1 and Sfp1 in the Transcriptional Response to Arsenic Stress

Whole genome mRNA transcript profiles after treatment with trivalent arsenic [hereafter referred to as As(III)] were in some aspects, such as bulk repression of RP genes and classes of stress genes, similar to those from rapamycin-treated cells both from reported profiles (Hardwick *et al.*, 1999) and our data. This result implicated the TOR nutrient-signaling pathway as a potential As(III) target. To further strengthen this interpretation arsenic stress profiles were generated after 30-min treatment with 0.5 mM As(III) and compared with those of 200 ng/ml rapamycin (Figure 1A). This concentration of As(III) caused only a slight increase in doubling time in exponential cultures. Transcript profiling experiments were done in the two strain backgrounds of the two isogenic sets used (see Table 1). Notably, BY4741 cells

Table 2. T-Profiler analysis (http://www.t-profiler.org/) of transcription factor–binding motifs from As(III)-treated cells

		W303				W303msn2msn4			
Motif	Name	t-value	E-value	Mean	ORFs	t-value	E-value	Mean	ORFs
GGTGGCRA	RPN4	5.83	8.10e-07	0.501	70	4.15	4.80e-03	0.396	62
AGGGG	MSN2-4	4.40	1.60e-03	0.086	643	1.08	1.00e+00	−0.028	569
CCCCT	MSN2-4	1.79	1.00e+00	0.013	662	-0.61	1.00e+00	−0.084	599
TTCTRGAA	HSF1	3.32	1.20e-01	0.214	104	2.74	5.90e-01	0.188	89
TTASTAA	YAP1	2.12	9.90e-01	0.038	432	1.13	1.00e+00	−0.017	378
ACGCGT	MBP1	0.78	1.00e+00	0.007	185	1.62	1.00e+00	0.04	176
CACGTK	PHO4	0.20	1.00e+00	−0.03	504	−5.11	4.70e-05	−0.346	231
CGATGAG	PAC	−3.43	<1.0e-15	−0.232	176	−2.29	9.60e-01	−0.231	139
AAAATTT	rRPe	−6.45	<1.0e-15	−0.181	891	−6.19	8.80e-08	−0.231	784
CCRTACA	RAP1	−8.87	<1.0e-15	−0.521	187	−9.14	<1.0e-15	−0.644	176

Raw data used for this analysis is available as supplementary files: W303_all.txt, W303_24_all.txt, and BY_all.txt, BY_sfp1_all.txt.

have a higher basal resistance against arsenic and gave a more pronounced response of induced genes (139 genes > 4-fold and 523 > 2-fold) than W303-1A (49 > 4-fold and 202 > 2-fold; see Figure 1A and Supplemental Files). In contrast, a similar number of genes was down-regulated in both strains (338 > 2-fold in BY4741 and 312 in W303-1A). T-Profiler analysis (Boorsma *et al.*, 2005) revealed significantly enriched GO terms (Tables 2 and 3), indicating the induction of regulons of the transcription factors Hsf1, Yap1, Rpn4, and Msn2/4, as well as repression of most ribosomal protein genes. The transcript patterns were also in agreement with previous studies (Haugen *et al.*, 2004; Thorsen *et al.*, 2007). Clustering analysis of our As(III) with rapamycin data further suggested similar regulation of genes associated with characteristic GO terms such as translation and stress response (Figure 1A, right panels). Significant GO terms were verified by GO-term enrichment (http://db.yeastgenome.org/cgi-bin/GO/goTermFinder.pl/; Supplemental Table ST1).

Inhibition of TORC1 by rapamycin causes dephosphorylation (H. Lempiäinen and D. Shore, unpublished) and nuclear export of the transcription factor Sfp1 (Marion *et al.*, 2004). Inhibition of Sfp1 by arsenic stress was predicted previously based on the global repression of RP genes (Haugen *et al.*, 2004; Thorsen *et al.*, 2007). To verify this directly, we compared the mRNA profile of BY4741 wild-type cells to the corresponding BY*sfp1*Δ mutant cells. In stark contrast to the wild type, we found almost no down-regulation of RP genes in the *sfp1*Δ mutant strain (Figure 1B, compare *BY* to *BYsfp1*Δ). Changes of expression levels of genes coding for the large and small ribosomal subunit under arsenic treatment are shown in Figure 1B. Therefore, Sfp1 has a central role for down-regulation of RP gene transcription during arsenic stress.

Table 3. GO-enrichment analysis

Category	BY	BYsfp	W303	W303_msn2/4.
Heat shock protein activity	2.704	0.651	2.287	—
Aryl-alcohol dehydrogenase activity	2.596	1.927	2.447	2.15
Proteasome core complex	1.768	0.992	1.118	0.937
Endopeptidase activity	1.182	0.398	0.64	0.596
Protein folding	1.176	0.375	0.903	
Ribosome	−0.363	0.222	−1.192	−1.244
Structural constituent of ribosome	−0.523	0.224	−1.36	−1.342
Cytosolic ribosome (sensu Eukarya)	−0.588	0.296	−1.939	−1.787

Mean expression of genes associated to the respective terms. Restrictions: E-value of <0.05 and t-value <−4 or >4, values are log2 of normalized transcript differences (mean of ratios). —, overlaps with Msn2/4 regulon.

From these profiles we noted that Sfp1 was also required for full induction of some genes. Strikingly, the levels of Hsf1-dependent heat shock genes were threefold reduced on average (Supplemental Figure S1A). However, Hsf1 function for heat stress signaling was not compromised in the *sfp1*Δ mutant. Thus, as predicted activity of a heat-shock element (HSE)-driven *lacZ* reporter under heat stress was unaffected by the absence of Sfp1, whereas induction by arsenic stress was abolished in the mutant (our unpublished results). Furthermore, we also noticed reduced induction of Yap1- and Rpn4-dependent genes in *sfp1*Δ mutant cells (Supplemental Figure S1, B and C, respectively). The mechanism(s) underlying these effects are at present unknown but could be specific to an arsenic stress signal or an indirect consequence of Sfp1 loss.

Because the microarray data suggested a role of TORC1 in the arsenic stress response, we looked at the survival of different Tor1 mutants using colony-forming ("drop") assays on plates containing As(III). We found that mutants lacking full Tor1 function either in a strain deleted for *TOR1* (W303 *tor1*Δ) or a *tor1-kDa* mutant with impaired kinase activity ($Tor1^{D2275A}$), had lower viability in the presence of As(III) (Figure 1C). In contrast, the *TOR1–1* allele, which confers rapamycin resistance, did not significantly change arsenic sensitivity. From this, one may conclude that TORC1 activity is necessary for an appropriate adaptive response to As(III).

Arsenic Stress Promotes Sfp1 Chromatin Dissociation, Dephosphorylation, and Cytosolic Relocalization

In unstressed cells growing in nutrient rich medium Sfp1 is concentrated in the nucleus and is associated with RP gene promoters (Jorgensen *et al.*, 2002; Marion *et al.*, 2004). Inhibition of TORC1 kinase by rapamycin causes release of Sfp1 from promoters and its cytoplasmic redistribution (Marion *et al.*, 2004). We therefore investigated if As(III) exposure triggers a similar response. To follow Sfp1 localization, we used cells expressing a GFP-tagged version of Sfp1 from the endogenous *SFP1* locus. Within 20 min after arsenic addi-

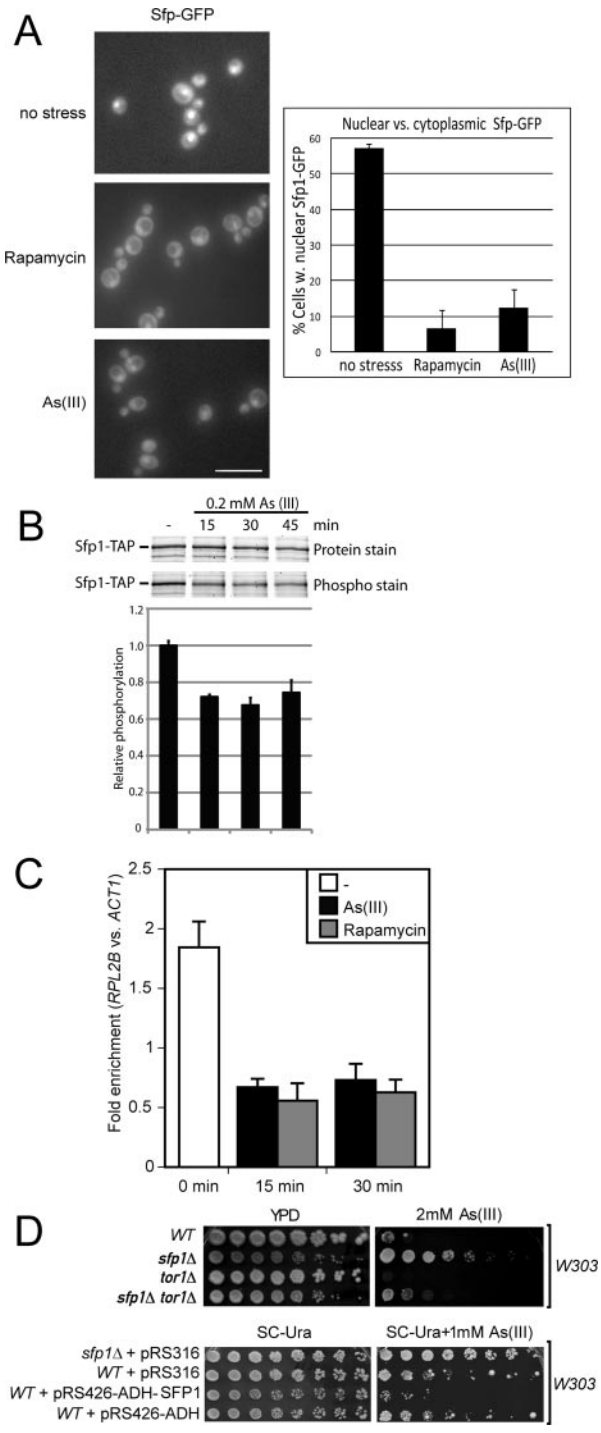

Figure 2. Regulation of Sfp1 by As(III) stress. (A) Localization of Sfp1-GFP. Cells carrying a *SFP1*-GFP genomic fusion gene were grown to exponential phase and treated with 0.5 mM As(III) and rapamycin (200 ng/ml) for 20 min. GFP-fluorescence images were taken from cells without fixation. Bar, 25 μm. Evaluation of Sfp1-GFP localization is indicated in the bar graph. (B) In-gel phosphoprotein stain of Sfp1 phosphorylation. Sfp1-TAP was purified and separated and stained as described in *Materials and Methods.* Bottom panel shows phosphorylation of Sfp1 normalized to Sfp1 protein levels at the indicated time points as an average of three independent experiments. (C) Binding of Sfp1 to RP gene promoter in As- and rapamycin-treated cells. Sfp1 recruitment to *RPL2B* promoter was analyzed by ChIP in a strain carrying a TAP-tagged endogenous allele of *SFP1*. Samples from unstressed cells (t = 0 min), from

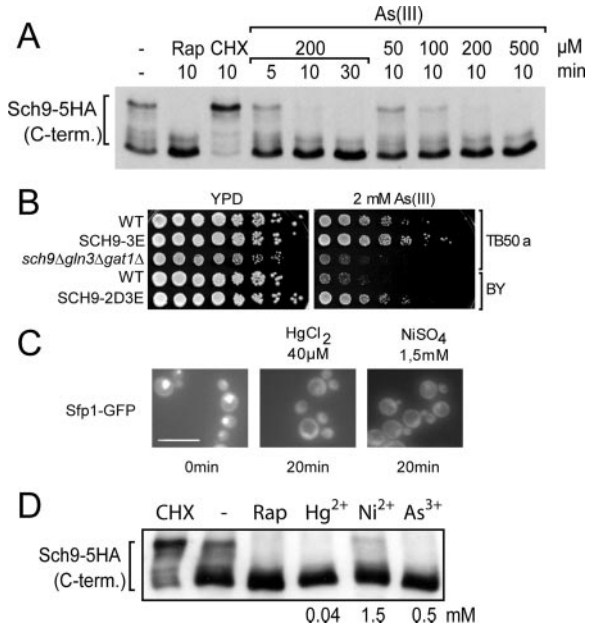

Figure 3. TORC1 kinase inhibition by arsenic. (A) Inhibition of TORC1 kinase activity by As(III). Chemical fragmentation analysis of the C-terminus of Sch9 demonstrates dephosphorylation in response to rapamycin (Rap, 200 ng/ml) and As (As(III), 200 or 500 μM) and hyperphosphorylation in response to cycloheximide (CHX, 25 μg/ml). Treatment times are indicated. (B) Growth properties of the indicated *SCH9* mutant strains, together with their isogenic parent wild-type strains on As(III) plates. The *sch9*Δ mutants were stabilized by the simultaneous deletion of *GLN3* and *GAT1.* Fivefold serial dilutions of overnight cultures were spotted onto containing the indicated amounts of As(III). Growth was recorded after a 3-5-d incubation at 30°C. Relevant strain backgrounds (W303-1A, BY4741) are indicated. (C) Sfp1-GFP localization of cells treated with 40 μM $HgCl_2$ and 1.5 mM $NiCl_2$ for 20 min. Bar, 25 μm. (D) Chemical fragmentation analysis of Sch9 after 20-min treatment at 30°C with to 40 μM $HgCl_2$ and 1.5 mM $NiCl_2$.

tion we observed a clear reduction of the nuclear fluorescence signal (Figure 2A and Supplemental Figure S2A). Quantification showed that arsenic and rapamycin caused a similar cytoplasmic accumulation of Sfp1-GFP. To address the Sfp1 phosphorylation status in vivo, we used an in-gel phosphoprotein stain to measure the phosphorylation of a partially purified Sfp1 TAP-tagged protein. Arsenic caused a clear reduction of Sfp1 phosphorylation to about ~75% (Figure 2B, bottom), which was visible 15 min after arsenic addition and persisted for at least 30 min. Using chromatin immunoprecipitation (ChIP), we determined the association of a Sfp1 TAP-tag fusion protein with the *RPL2B* promoter. Arsenic treatment reduced the Sfp1-TAP ChIP signal within 15 min to levels similar to what was observed after rapamycin treatment (Figure 2C). Arsenic and rapamycin thus have similar effects both on chromatin binding and subcellular location of Sfp1.

We therefore tested the consequence of an *SFP1* deletion for cell viability during chronic As(III) exposure by drop

As(III)-(0.5 mM) and rapamycin-(200 ng/ml) treated cells at the indicated time points (t = 15 min or 30 min). (D) Growth properties of the indicated *SFP1* mutant strains, together with their isogenic parent wild-type strains on arsenic plates. Fivefold serial dilutions of overnight cultures were spotted onto rich (YPD) or synthetic medium (SC-URA) plates containing the indicated amounts of As(III). Growth was recorded after a 3-5-d incubation at 30°C.

assays on plates (Figure 2D). To our surprise we found that the *sfp1* mutation had the opposite effect compared with *tor1* mutations, rendering cells more resistant. However, the *tor1 sfp1* double mutant showed an intermediary phenotype. These genetic results point to a separate function of Tor1 during chronic As(III) stress apart from regulation of Sfp1. Additionally, high-level expression of Sfp1 reduced resistance (Figure 2D, bottom). Although, these genetic data are incompatible with a simple linear pathway connecting relationship of Tor1 and Sfp1 during arsenic stress, they do show that both are required for an optimal response.

TORC1 Kinase Is Inhibited by Arsenic

In an attempt to find an explanation for the apparently contradictory results obtained with *sfp1* and *tor1* deletion strains, we investigated whether the TORC1 kinase activity is changed by As(III) treatment. We measured the in vivo phosphorylation state of a C-terminal fragment of Sch9, which has been shown recently to be a bona fide TORC1 substrate (Urban *et al.,* 2007). Arsenic addition caused a rapid dephosphorylation of Sch9, similar to that observed after rapamycin treatment (Figure 3A). Although we cannot rule out the possibility that As(III) induces the activity of phosphatase(s) directed against Sch9, these data were consistent with the notion that As(III) causes a rapid inactivation of the TORC1 kinase pathway. Because Sch9 is a primary target of TORC1, we investigated its function during chronic arsenic stress (Urban *et al.,* 2007). Cells lacking Sch9 were sensitive to As(III) (Figure 3B). Significantly, two TORC1-independent "phospho-mimetic" mutants of Sch9 (SCH9-3E and SCH9-2D3E; Urban *et al.,* 2007) conferred resistance to As(III) (Figure 3B). The resistance phenotype of these mutants is consistent with a function of Tor1 separate from Sfp1 regulation during arsenic stress as shown above.

According to microarray data, mercury at a low concentration (0.04 mM) and nickel (at 1.5 mM) had an repressive effect comparable to As(III) (0.5 mM) on global RP gene transcription (Figure 1B and our unpublished results). We therefore tested if these metal ions also cause inhibition of TORC1. Under these same conditions Sfp1 rapidly accumulated in the cytoplasm within 20 min (Figure 3C), and TORC1 activity decreased, as judged by the phosphorylation status of Sch9 (Figure 3D). Therefore As(III) and certain other toxic metal ions cause repression of TORC1 activity, leading to Sfp1 dephosphorylation, loss of chromatin contact, cytoplasmic sequestration, and finally, reduction of ribosomal protein gene transcription.

Msn2 and Msn4 Are Activated by Arsenic Stress

Arsenic stress could also be an inhibitor of PKA activity, because Sfp1 is both TORC1 and PKA regulated (Jorgensen *et al.,* 2004; Marion *et al.,* 2004). Furthermore, the transcription factors Msn2 and Msn4 are also inhibited by PKA (Smith *et al.,* 1998), and both As(III) and rapamycin induce a cluster of Msn2/4 target genes (Figure 1A, Tables 2 and 3 and Supplemental Files). We confirmed this role of Msn2/4 for gene activation by transcript profiling of a *msn2*Δ*msn4*Δ double deletion strain. Induction of 57 of the total 202 As(III)-induced genes was reduced by more than 1.4-fold in the *msn2*Δ*msn4*Δ mutant (Figure 4A, compare *W303* with *W303m24*Δ). Twenty-four of these 57 genes ($P < \sim 2 \times 10^{-16}$) were previously reported to be up-regulated by Msn2 overexpression (Chua *et al.,* 2006), indicating that many of these are direct target genes of Msn2/4. To test if Msn2/4 were directly activated by arsenic stress, we investigated the intracellular localization of Msn2- and Msn4-GFP fusion proteins. In unstressed cells both Msn2 and Msn4 are predominantly cytoplasmic, but both Msn2- and Msn4-GFP rapidly accumulated in the nucleus for at least 20 min after As(III) stress (Figure 4B), similar to other stress types (Görner *et al.,* 1998).

Activation of Msn2 is either a consequence of reduced PKA activity or environmental stress (Durchschlag *et al.,* 2004). To differentiate between the two possibilities during As(III) stress, we determined the phosphorylation state of Msn2 using antibodies directed against PKA phosphorylation sites (serines 288, 582, 620; De Wever *et al.,* 2005; Figure 4C). Glucose starvation has been shown to cause a strong reduction of the phosphorylation status of the serine 620 PKA site in Msn2 (Görner *et al.,* 2002). Because arsenate competes with phosphate for binding sites of glycolytic substrates, thereby inhibiting glycolysis (Jung and Rothstein, 1965), we suspected that arsenic stress might mimic aspects of glucose depletion. However, the signals obtained with phospho-specific antibodies indicated that As(III) stress leads to sustained phosphorylation of the tested sites (Figure 4C). Such an effect was also observed during heat stress (Görner *et al.,* 2002 and W. Reiter, unpublished results). This sustained phosphorylation of Msn2 could result from continued PKA activity, the inactivation of one or more phosphatases, or both. In any case, these results indicated that As(III) stress, unlike acute glucose starvation, does not abolish PKA activity.

To analyze the importance of Msn2/4 for growth in the presence of arsenic, we tested mutants lacking Msn2, Msn4 or both (Figure 5A). Deletion of either *MSN2* or *MSN4* had no effect on sensitivity, whereas the *msn2*Δ*msn4*Δ double mutant displayed increased tolerance. Therefore, Msn2/4 reduce growth during arsenic stress. This was supported by the enhanced arsenic sensitivity upon overexpression of either *MSN2* or *MSN4* under the control of the *ADH1* promoter. Mutants lacking the PKA regulatory subunit (*bcy1*Δ) have constitutively high PKA activity and low activity of Msn2/4. These mutants displayed higher resistance to arsenic. Comparing the arsenic resistance of the *msn2*Δ*msn4*Δ double mutant strain to a mutant additionally lacking PKA activity (*tpk1*Δ*tpk2*Δ*tpk3*Δ *msn2/4*Δ) demonstrates a role of PKA in parallel to its function as a regulator of Msn2/4 (Figure 5B). This might involve the inhibitory action of PKA on the Rim15 kinase and its downstream transcription factor Gis1 (Roosen *et al.,* 2005).

As shown above, TORC1 function for arsenic resistance is upstream of the Sch9 kinase and the Sfp1 transcription factor. Other TORC1 downstream targets may also play a role in arsenic resistance, among which might be the Msn2/4 stress response factors. Strikingly, we found that deletion of both *MSN2* and *MSN4* completely alleviates the arsenic sensitivity of *tor1*Δ mutants (Figure 5C). The genetic interaction seen with these mutants displays clearly the connection between TORC1-based nutrient signaling and Msn2/4 stress signaling during a chronic stress.

DISCUSSION

Arsenic in the form of trivalent arsenite As(III) causes major metabolic adjustments and evokes a highly complex transcriptional response. We analyze here the contribution of the nutrient signaling protein kinases Target Of Rapamycin Complex 1 (TORC1) and PKA to the cellular arsenic response. We show rapid inactivation of TORC1 kinase but not of PKA. This effect modulates the activity of downstream factors such as Sch9, Sfp1, and Msn2/4 and has complex consequences for cell growth and survival in response to chronic arsenic exposure. Interestingly, our genetic data suggest that As(III) toxicity is caused in part by interference between signaling pathways.

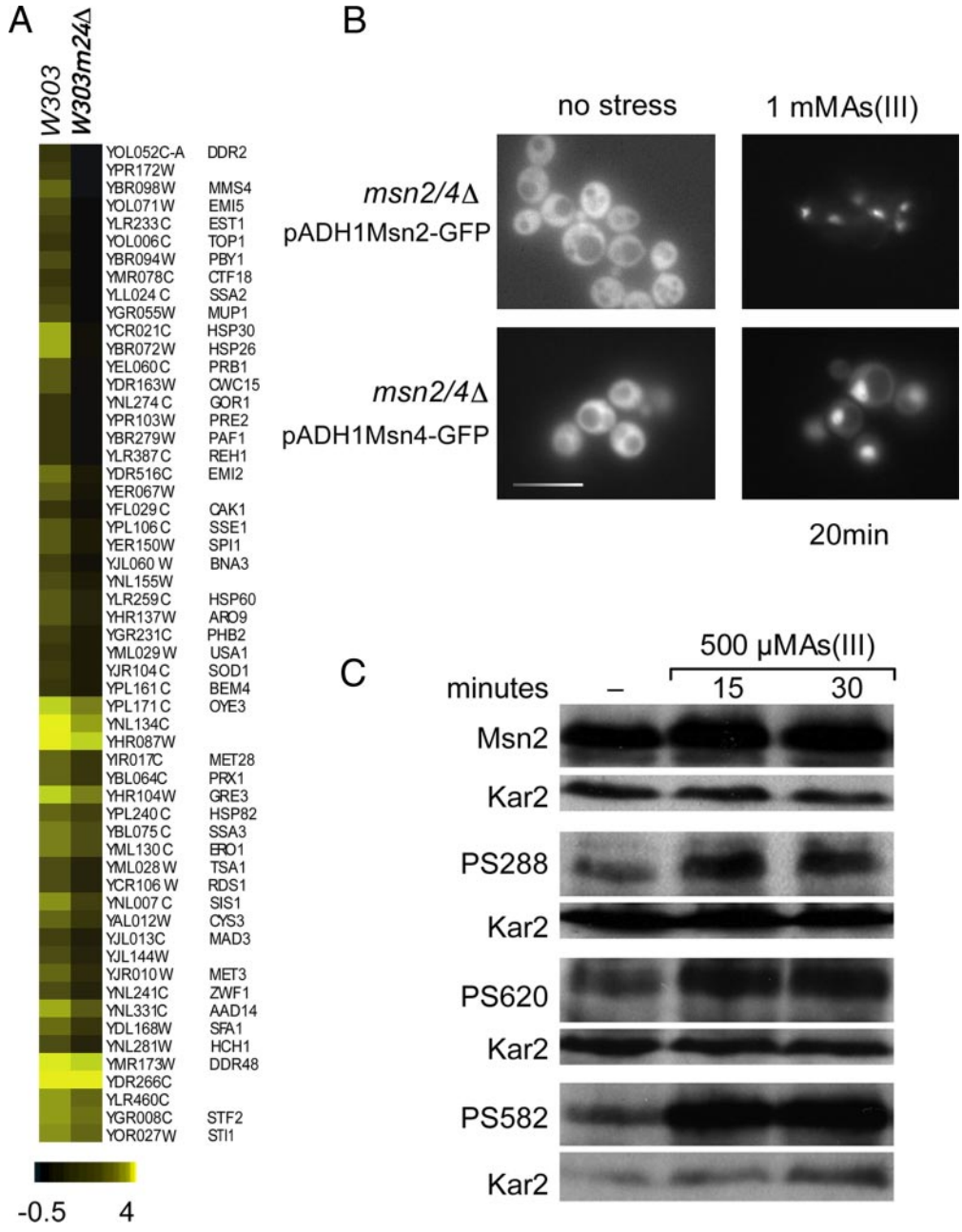

Figure 4. Function of Msn2/4 and the PKA pathway in arsenic response. (A) Hierarchical clustering (Eisen *et al.*, 1998) of 56 As(III)-induced genes, which were influenced more than 1.4-fold by the absence of Msn2 and Msn4 in the *msn2*Δ*msn4*Δ double mutant strain. (B) Localization of Msn2-GFP in arsenic-stressed cells. W303 *msn2msn4* cells transformed with *pADH1-MSN2*-GFP and *pADH1-MSN4-GFP* were grown to exponential phase in synthetic medium and treated for 20 min with 1 mM As(III) at 30°C. GFP-fluorescence images were taken from cells without fixation. Bar, 25 μm. (C) Phosphorylation status of PKA sites of Msn2. W303-1A cells were grown in YPD medium to exponential phase and stressed by addition of 1 mM As(III). Samples for total protein extracts were taken at 15-min intervals and analyzed on Western blots with phosphorylation-specific antibodies directed against S620, S582, and S288. Msn2 and Kar2 levels served as a loading control.

Arsenic Inhibits TORC1

Our biochemical evidence indicates clearly that arsenic inhibits the rapamycin-sensitive TORC1 kinase. First, cells treated with rapamycin and the metalloid arsenic share a similar global transcriptional pattern. Second, interaction of the TORC1 mediator Sfp1 with a ribosomal protein gene promoter was reduced by As(III) to the same low level as observed after rapamycin treatment. Third, coincident with As(III) exposure, Sfp1 phosphorylation was decreased, and its localization was shifted from almost exclusively nuclear to a more cytoplasmic distribution. Fourth, we found that the phosphorylation status of Sch9, a bone fide in vivo target of TORC1 (Urban *et al.*, 2007), was rapidly reduced upon As(III) stress. Moreover, cells treated with mercury and nickel ions also displayed a similar shift of localization of Sfp1 and reduced Sch9 phosphorylation and RP gene repression, indicating that the response to arsenic observed here is part of a more general metal ion stress response.

The mechanism by which arsenic inhibits TORC1 activity is currently not known to us. Arsenic could, for example,

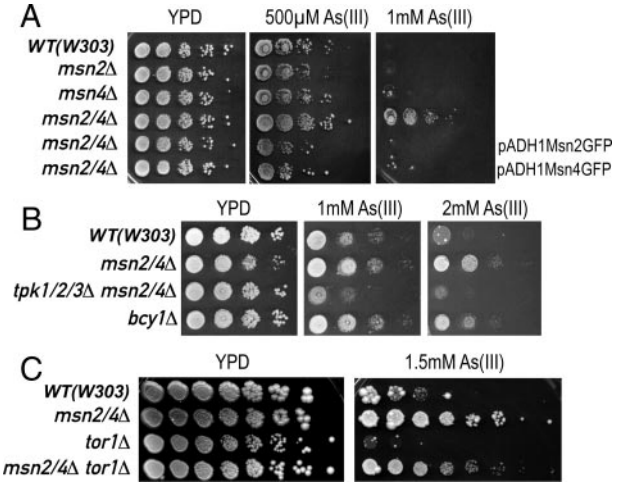

Figure 5. Growth of Msn2/4 mutants on As(III) solid medium representing chronic arsenic stress. Cultures were grown to saturation and spotted in 5- or 10-fold dilution series. (A) Comparison of DH303 series cells. (B) Hyperactive PKA (*bcy1*) confers resistance to As(III). PKA targets beside Msn2 are involved in As(III) resistance. (C) Double deletion of Msn2 and Msn4 alleviates the arsenic sensitivity of *tor1*Δ mutants. Plates were photographed after 2–4 d at 30°C.

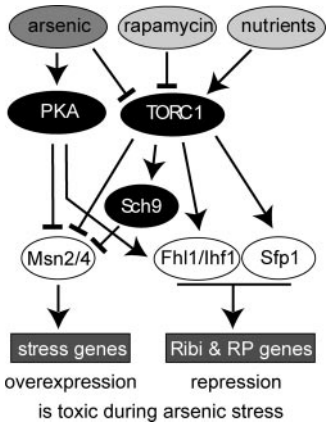

Figure 6. Schematic model of connections between signaling pathways and substrates.

affect the stability of the TORC1 complex and/or upstream signaling components. In *S. cerevisiae*, TOR signaling has been proposed to respond to metabolic signals originating from nitrogen and carbon sources (Schmidt *et al.*, 1998; Beck and Hall, 1999; Kuruvilla *et al.*, 2001; Crespo *et al.*, 2002). Glutamine deprivation inhibits TORC1 (Crespo *et al.*, 2002). Arsenic stress causes cells to channel sulfur into increased glutathione biosynthesis, as suggested by phenotypic profiling (Haugen *et al.*, 2004) and later by direct measurement (Thorsen *et al.*, 2007). The glutamate incorporated into glutathione is connected to glutamine pools by glutamate synthase (GOGAT) encoded by *GLT1*. It remains to be shown if increased glutathione consumption by the formation of $As(GS)_3$ conjugates results in a significant drop of glutamine levels. On the other hand, glutathione deprivation by arsenic might also cause other side effects such as oxidative stress, which also reduces TORC1 activity (Urban *et al.*, 2007).

The Complex Role of TORC1 in Arsenic Resistance

The down-regulation of RP gene expression signifies a major shift of cellular activity because it consumes a large proportion of the synthetic capacity of rapidly growing cells (Warner, 1999). Many different stress conditions cause reduction of RP gene synthesis (Jorgensen *et al.*, 2004; Marion *et al.*, 2004), presumably to save resources and to allow a shift of the gene transcription capacity of RNA Pol II to genes encoding proteins with protective functions, such as heat-shock proteins (HSPs). This was also observed earlier for arsenic (Chang *et al.*, 1989). The other side of the coin is that high RP gene transcription facilitates ribosome biogenesis, high capacity for protein synthesis and thus enhanced proliferation, when resources are available (Hartwell *et al.*, 1974; Hartwell and Unger, 1977; Warner, 1999; Jorgensen *et al.*, 2004). Therefore, dynamic control of RP gene transcription is important in a competitive environment. Our data indicate that inhibition of TORC1 by As(III) leads to the rapid inactivation of Sfp1 and a consequent reduction of RP gene expression. A similar loss of RP gene promoter binding is observed for Ifh1 upon As(III) treatment (data not shown), again paralleling the effect of rapamycin (Schawalder *et al.*, 2004). Interestingly, Sfp1 null mutants showed a higher capacity to survive under conditions of chronic arsenic stress. This result might be viewed as contradictory to the lower resistance seen with the *TOR1* null mutants. However, we see in microarrays that RP gene expression does not change in Sfp1 mutants. Therefore, these mutant cells are desensitized against RP gene repression. We speculate that in the wild-type strains chronic arsenic stress might drive many cells toward a terminal arrest condition caused in part by reduced RP gene expression. The arsenic sensitive phenotype of *tor1* mutant cells might be due to other targets of TORC1 such as Sch9. Furthermore, the transient inhibition of Sfp1 due to TORC1 inhibition as here reported with arsenic stress might be part of a general adaptive response. Consistent with this, overexpression of Sfp1 was detrimental to growth under arsenic stress. Nevertheless, increased survival of *SFP1* deletion mutants under chronic arsenic stress comes with the cost of a significantly reduced growth rate.

Unlike *SFP1* deletion, the absence of either Tor1 or Sch9 rendered cells more sensitive to arsenic. Consistent with an active role of the Tor1-Sch9 axis in counteracting As(III) stress, the phosphomimetic Sch9 mutants (*SCH9-3E*, *SCH9-2D3E*), whose activity is TORC1 independent, confer As(III) resistance. Because all three proteins (Sfp1, Tor1, and Sch9) function to activate RP genes, these observations indicate that Tor1 and Sch9 have function(s) related to the response to As(III) that are not shared with Sfp1. We suggest that one such function is the inhibition of Msn2/4, because deletion of these two genes completely reverses the As(III)-sensitivity phenotype of *tor1* cells (Figure 6). Consistent with this idea, previous work has implicated TORC1 as a negative regulator of Msn2/4 (Beck and Hall, 1999), at least partly acting through Sch9, which has recently been shown to down-regulate several Msn2/4 target genes (Urban *et al.*, 2007) and also directly regulates Rim15, a protein kinase involved in Msn2/4 regulation (Wanke *et al.*, 2008). TORC1 may well have other functions that promote growth in the presence of As(III). For example, TORC1 is involved in vacuolar-sorting functions (Aronova *et al.*, 2007) and might be important for vacuolar sequestration of glutathione conjugates resulting from As(III) exposure (Ghosh *et al.*, 1999). In any event, our genetic data indicate that inhibition of TORC1 signaling has two counteracting consequences regarding cell growth under conditions of chronic As(III) stress: inhibition of Sfp1 and derepression of Msn2/4. Although the former promotes growth

at sublethal doses of As(III), continued expression of the stress factors Msn2/4 blocks growth. Moreover, arsenic might also trigger a general stress response, activating Msn2/4 and inactivating TORC1 in parallel. This scenario is in line with our genetic data showing that *msn2/4* and *tor1* mutants are independent. However, the fact that rapamycin as a rather specific inhibitor of TORC1 also activates Msn2, favors a sequential model in which TORC1 acts upstream to inactivate Msn2/4.

PKA and Arsenic Resistance

Arsenic-treated cells rapidly activated Msn2 and Msn4, raising the possibility that PKA becomes inhibited. This was also suggested recently (Jin *et al.*, 2008). Under optimal growth conditions PKA phosphorylates and inhibits both transcription factors, and inactivation of PKA is sufficient to activate Msn2 (Smith *et al.*, 1998). Furthermore, PKA activity is tightly regulated by short-term changes of glucose availability, with the presence of glucose resulting in high PKA activity. A connection between arsenite and PKA might be suggested by reduced glycolytic energy production similar to glucose depletion conditions (Jung and Rothstein, 1965). Acute glucose depletion triggers the rapid dephosphorylation of Msn2, whereas other Msn2 activating conditions such as heat stress do not lead to dephosphorylation (Görner *et al.*, 2002; De Wever *et al.*, 2005). As(III) stress (similar to heat stress;Görner *et al.*, 2002) slightly increased the Msn2 phosphorylation level on PKA sites. In principle this effect may also be the result of the inactivation of a phosphatase. In fact, PP2A was shown to be required for rapamycin- and stress-triggered nuclear retention of Msn2 (Santhanam *et al.*, 2004), which in turn is connected to TORC1 via its repressor Tap42 (Duvel *et al.*, 2003; Kuepfer *et al.*, 2007). PP2A is also involved in Msn2 regulation (W. Görner and C. Schüller, unpublished observations). Taken together, the phosphorylation status of Msn2 indicated sustained PKA activity and/or possibly reduced PP2A activity during As(III) stress.

In any case, PKA activity stays in a range that does not hinder down-regulation of RP genes. Hyperactive PKA prevents cytoplasmic accumulation of Sfp1 and also activates Fhl1/Ihf1, thus promoting RP gene transcription (Martin *et al.*, 2004; Schawalder *et al.*, 2004; Schmelzle *et al.*, 2004). Because in strains with zero PKA activity the shift of Sfp1 localization is not disturbed (Warner, 1999; Marion *et al.*, 2004), PKA activity below a threshold is most probably a precondition for inhibition of Sfp1. Sustained PKA activity is beneficial during chronic As(III) stress. It prevents hyperactivation of Msn2/4, which is detrimental for cell growth (Durchschlag *et al.*, 2004). This is supported by the increased As(III) resistance of mutants lacking Msn2 and Msn4. Second, additional repression of RP gene expression by reduced PKA activity through inactivation of Fhl1-Ifh1 might cause a pronounced shut-down of RP gene transcription, leading to irreversible stasis. In line with this notion, we find enhanced resistance of mutants with constitutive high PKA activity (*bcy1*Δ) and increased arsenic stress sensitivity in cells with low (or no) PKA activity. Part of this phenotype can be attributed to inactivation of Msn2 and Msn4 by high PKA, but also to other targets, because its inactivation increases sensitivity in cells lacking Msn2 and Msn4 (*tpk1/2/3*Δ*msn2/4*Δ vs. *msn2/4*Δ). In fact, PKA and TORC1 kinases also regulate the Rim15 kinase, which is required for entry into the stationary phase (Pedruzzi *et al.*, 2003).

Our results indicate a strong contribution of RP gene transcription in the arsenic stress response and a delicate balance of responses regulated by PKA and TOR (Figure 6). Dynamic regulation of RP gene expression has a role in the adaptation of growth and division according to environmental conditions. Arsenic seems to drive this response system into a dead-end situation where chronic activation of stress genes leads to severe growth inhibition. Our data indicate that although PKA may have an important influence on RP gene transcription under some conditions, TORC1 activity plays a key role even when PKA levels are high (Marion *et al.*, 2004; Zurita-Martinez and Cardenas, 2005; Chen and Powers, 2006), arguing against models in which PKA acts downstream of TOR to regulate ribosome biogenesis (Schmelzle *et al.*, 2004). One key question that remains is the mechanism by which arsenic inhibits TORC1. The answer to this may have important implications for understanding the effects of As(III) on humans, given the high degree of conservation of TORC1.

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