Proteomic Analysis Identifies Ribosome Reduction as an Effective Proteotoxic Stress Response*

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Background: Misfolded proteins are a ubiquitous and clinically relevant threat to cells.

Results: Arsenite stress in yeast leads to increased protein degradation and reduced protein production.

Conclusion: Reduction in ribosome abundance is a novel, rapid, effective, and reversible stress response against misfolded proteins.

Significance: These results provide the basis for further characterization of a potentially important stress response pathway.

Stress responses are adaptive cellular programs that identify and mitigate potentially dangerous threats. Misfolded proteins are a ubiquitous and clinically relevant stress. Trivalent metalloids, such as arsenic, have been proposed to cause protein misfolding. Using tandem mass tag-based mass spectrometry, we show that trivalent arsenic results in widespread reorganization of the cell from an anabolic to a catabolic state. Both major pathways of protein degradation, the proteasome and autophagy, show increased abundance of pathway components and increased functional output, and are required for survival. Remarkably, cells also showed a down-regulation of ribosomes at the protein level. That this represented an adaptive response and not an adverse toxic effect was indicated by enhanced survival of ribosome mutants after arsenic exposure. These results suggest that a major source of toxicity of trivalent arsenic derives from misfolding of newly synthesized proteins and identifies ribosome reduction as a rapid, effective, and reversible proteotoxic stress response.

All cells utilize stress responses to identify and mitigate potentially toxic threats. Misfolded proteins are a ubiquitous threat that may harm cells through multiple mechanisms. First, misfolding may result in the loss of the affected protein’s normal function. Second, protein misfolding can lead to gain-of-function toxicities related to promiscuous protein-protein interactions, often mediated by exposed hydrophobic residues and sometimes culminating in protein aggregation (1). Increasingly, protein misfolding is understood to be the primary cause of many common and rare human diseases, chief among these being various neurodegenerative diseases (2). The causes of protein misfolding are numerous, and include heat, oxidative damage, aging, inherited mutation, heavy metals, abnormal amino acid analogs, chemical inhibitors of the ribosome, errors in protein synthesis, and others (3).

Metalloids are chemical elements with properties of both metals and non-metals. Arsenic is a ubiquitous metalloid and a well established environmental toxin and carcinogen. The basis for its toxicity, however, remains unclear. Proposed mechanisms for arsenic toxicity include inhibition of cellular respiration, oxidative damage, and DNA damage among others (4–8). Recent evidence for the trivalent form of arsenic has suggested protein misfolding as an important and physiologically relevant consequence of this compound, particularly at lower concentrations (8–12).

Here, we have employed a newer proteomic approach, tandem mass tag (TMT)-based mass spectrometry (MS) (13) to investigate the cellular response to trivalent arsenic. We find that arsenic induces a profound reorganization of the cellular proteostasis machinery at the protein level. This reorganization appears to reflect a shift from a protein anabolic state to a protein catabolic state. Specifically, both major degradation pathways, the proteasome and autophagy, are up-regulated. By contrast, components of pathways controlling protein synthesis, particularly the ribosome itself, are broadly down-regulated. This reduction in ribosome abundance, rather than representing a toxic effect of arsenic, actually protects cells against its toxicity. Our findings suggest that ribosome reduction may serve as a rapid, effective, and reversible proteotoxic stress response.

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5 The abbreviations used are: TMT, tandem mass tag; YPD, yeast extract/potato/dextrose; SILAC, stable isotope labeling of cells in culture; TAP, tandem affinity purification.
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TABLE 1
Yeast strains
Strains from the Research Genetics (RG) Collections are available through ThermoFisher Scientific.

| Name          | Genotype                          | Source       |
|---------------|-----------------------------------|--------------|
| BY4741        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | RG collection|
| DW168         | MATa ade1 leu2−3.112 lys5 trp1::HisG ura3−3.2 lacl::ADE1 hym::ADE1 ade3::GAL::HO GFP::ATG8::URA3 | This study   |
| atg8Δ        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 atg8::KAN | RG collection|
| rprΔ         | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rpr::KAN | RG collection|
| sMB100       | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 atg8::KAN rpr::ClonNAT | This study   |
| Rpl16B-TAP   | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RPL16B::RPL16B-TAP (HIS3) | RG collection|
| Rpl31b-TAP   | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RPL31B::RPL31B-TAP (HIS3) | RG collection|
| rpl20αΔ      | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rpl20α::KAN | RG collection|
| rpl39Δ       | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rpl39::KAN | RG collection|
| rpl199Δ      | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rpl199::KAN | RG collection|
| rps16Δ       | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rps16::KAN | RG collection|
| rps16bΔ      | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rps16b::KAN | RG collection|
| slx9Δ        | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 slx9::KAN | RG collection|

Experimental Procedures

Yeast Strains and Culture

Yeast strains are listed in Table 1. Standard techniques were used for strain constructions and transformations. Yeasts were cultured at 30 °C. YPD medium consisted of 1% yeast extract, 2% Bacto-peptone, and 2% dextrose. Details of strain constructions are available on request.

Proteomic Analysis

Sample Processing—Exponential phase yeast cultures were harvested, resuspended in lysis buffer (50 mM HEPES, pH 8.2, 8 mM urea, 75 mM NaCl, and protease inhibitors (Roche Applied Science)), and lysed by bead beating. Lysates were normalized by protein concentration, reduced with DTT (5 mM), and alkylated with iodoacetamide (10 mM). Excess iodoacetamide was precipitated with 0.4% TFA. Peptides were desalted using tC18 Sep-Pak solid-phase extraction cartridges (Waters).

Tandem Mass Tag Labeling—Desalted peptides were resuspended in 100 μl of 200 mM HEPES, pH 8.5. One hundred μg of peptides from each sample were labeled with TMT reagents (ThermoFisher Scientific). TMT reagents were dissolved in anhydrous acetonitrile and added to the peptides to achieve a final acetonitrile concentration of ~30% (v/v). After incubation at room temperature for 1 h, the reaction was quenched with hydroxylamine to a final concentration of 0.3% (v/v). The TMT-labeled samples were pooled at a 1:1:1:1:1 ratio. The sample was vacuum centrifuged to near dryness and again subjected to tC18 solid-phase extraction.

Off-line Basic pH Reversed-phase Fractionation—We fractionated the pooled TMT-labeled peptide sample using basic pH reversed-phase HPLC. We used an Agilent 1100 pump equipped with a degasser and a photodiode array detector (set at 220- and 280-nm wavelengths; ThermoFisher Scientific). Peptides were subjected to a 50-min linear gradient from 5–35% acetonitrile in 10 mM ammonium bicarbonate, pH 8, at a flow rate of 0.8 ml/min over an Agilent 300Extend-C18 column. The peptide mixture was fractionated into a total of 96 fractions, which were consolidated into 24, of which only 12 nonadjacent samples were analyzed. Samples were vacuum-centrifuged to near dryness. Each eluted fraction was acidified with 1% formic acid and desalted via StageTips (ThermoFisher Scientific), dried via vacuum centrifugation, and reconstituted in 4% acetonitrile, 5% formic acid for liquid chromatography (LC)-MS/MS processing.

Liquid Chromatography and Tandem Mass Spectrometry—Mass spectrometry data were collected using an Orbitrap Fusion mass spectrometer coupled to a Proxeon EASY-nLC II LC pump (Thermo Fisher Scientific). Peptides (~1 μg/analysis) were fractionated on a 75-μm-inner diameter microcapillary column packed with ~0.5 cm of Magic C4 resin (5 μm, 100 Å; Michrom Bioresources) followed by ~35 cm of GP-18 resin (1.8 μm, 200 Å; Sepax).

Peptides were separated using a 2-h gradient of 6–26% acetonitrile in 0.125% formic acid at a flow rate of ~350 nl/min. Each analysis used the multi-notch MS3-based TMT method (14). The scan sequence began with an MS1 spectrum (Orbitrap analysis; resolution 120,000; mass range 400–1400 m/z; automatic gain control target 2 × 10^5; maximum injection time 100 ms). Precursors for MS2/MS3 analysis were selected using a top speed of 2 s. MS2 analysis consisted of collision-induced dissociation (quadrupole ion trap analysis; automatic gain control 4 × 10^5; normalized collision energy 35; maximum injection time 150 ms). After acquisition of each MS2 spectrum, we collected an MS3 spectrum using a recently described method in which multiple MS2 fragment ions were captured in the MS3 precursor population using isolation waveforms with multiple frequency notches (14). MS3 precursors were fragmented by high energy collision-induced dissociation and analyzed using the Orbitrap (normalized collision energy 55; automatic gain control 5 × 10^4; maximum injection time 150 ms, resolution was 60,000 at 400 Thomson units).

Data Analysis—Mass spectra were processed using a SEQUEST-based in-house software pipeline (15). Spectra were converted to mzXML using a modified version of ReAdW.exe. Database searching included all entries from the yeast SGD (Saccharomyces Genome Database). This database was concatenated with a database composed of all protein sequences in the reversed order. Searches were performed using a 50-ppm pre-
cursur ion tolerance for total protein level analysis. The product ion tolerance was set to 0.9 Da. The relatively wide mass tolerance window (50 ppm) was chosen to maximize sensitivity in conjunction with SEQUEST searches and linear discriminant analysis (15, 16). TMT tags on lysine residues and peptide N termini (+229.163 Da) and carbamidomethylation of cysteine residues (+57.021 Da) were set as static modifications, whereas oxidation of methionine residues (+15.995 Da) was set as a variable modification.

For TMT-based reporter ion quantitation, we extracted the signal-to-noise ratio for each TMT channel and found the closest matching centroid to the expected mass of the TMT reporter ion. Peptide-spectrum matches were identified, quantified, and collapsed to a 1% peptide false discovery rate (FDR) and then collapsed to a final protein-level FDR of 1%. Protein assembly was guided by principles of parsimony to produce the smallest set of proteins necessary to account for all observed peptides. Proteins were quantified by summing reporter ion counts across all matching peptide-spectrum matches using in-house software, as described previously (17). Peptide-spectrum matches with poor quality, MS3 spectra with more than eight TMT reporter ion channels missing, MS3 spectra with TMT reporter-summed signal to noise ratio that is <200 or no MS3 spectra were excluded from quantitation (17).

**Immunoblot Analysis**

For measurement of ribosome levels, exponential phase cultures were treated with sodium arsenite (1 mM) for the indicated times. Extracts were prepared by resuspending cell pellets in 1× Laemmli loading buffer and boiling for 5 min. For analysis of the GFP-Atg8 reporter protein, exponential phase cultures were treated with sodium arsenite (1 mM) for the indicated times. Extracts were prepared by a lithium acetate/NaOH method. Briefly, cell pellets were treated with 2 M lithium acetate on ice for 5 min followed by 0.4 M NaOH for 5 min. Cells were then resuspended in 2× Laemmli loading buffer and boiled for 5 min. The following antibodies were used: anti-rabbit IgG (GE Healthcare) for TAP tag analysis, anti-Pgk1 (Invitrogen), anti-GFP (Abcam), and anti-phospho-eIF2-α-Ser51 (Cell Signaling Technology).

**Phenotypic Analysis**

Overnight cultures in YPD at 30 °C were normalized by culture density and spotted in 3-fold serial dilutions onto YPD plates lacking or containing the indicated drug and cultured at 30 °C.

**Results**

**Proteomic Analysis of Arsenite Toxicity**—We sought to better understand the nature of arsenite toxicity by using a newer proteomic approach to characterize, in a comprehensive way, the cellular response to this compound. In recent years, this type of proteomic analysis has frequently been achieved using the method of stable isotope labeling of cells in culture (SILAC). Here, we have employed a newer methodology known as tandem mass tag-based mass spectrometry. TMT-MS analysis has several advantages over SILAC. First, because there are currently 10 distinct protein labels used for analysis, TMT-MS allows for the simultaneous quantitation of up to 10 independent cultures. Second, because labeling occurs post-translationally rather than at the time of protein synthesis, peptide labeling for quantitation can be done at the end of the experimental intervention, allowing for cultures to be grown under otherwise identical conditions and without the need for different media conditions as in SILAC. Finally, methodologic advances in peptide fragmentation and analysis in the TMT-MS method potentially allow for greater proteomic coverage and thus more comprehensive analysis (Ref. 17; see “Experimental Procedures” for details).

To study the cellular response to arsenic, we cultured wild-type budding yeast in rich media in triplicate. We treated the cultures with sodium arsenite and then sampled each culture at 0, 1, and 4 h. Protein was extracted, proteolytically processed, subjected to labeling with TMT probes, and analyzed by mass spectrometry (Fig. 1A). The raw data were then curated both manually and by computer-assisted methods. Data were obtained for 4,564 proteins, of a total of ~6,000 predicted in this organism. In terms of proteome coverage, this represents, to our knowledge, the most exhaustive proteomic analysis to date. Furthermore, the fact that the TMT-MS method allowed for triplicate measurements over time from single cultures resulted in a high degree of reproducibility which greatly facilitated analysis (supplemental Table 1 and below).

To ensure that we were studying a specific cellular response to arsenic and not merely a nonspecific toxicity or cellular death pathway, we evaluated growth and viability of cells under these conditions. Over a 4-h arsenic treatment, cells continued to grow, albeit with a markedly reduced growth rate (Fig. 1B). If these cells were terminally damaged, one would expect them to be unable to recover after removal of arsenic or to do so only very slowly. Rather, we found that within 1 h of arsenic washout, the cells were able to resume growth with a normal doubling time (Fig. 1B), indicating that any observed cellular changes under these conditions are likely to be physiologically relevant and specific to arsenic toxicity.

**Arsenic Results in a Massive Conversion from a Protein Anaerobic to Catabolic State**—Of the 4,564 proteins detected, ~75–80% showed no change in protein levels in response to arsenic (supplemental Table 1). Of the ~1,000 proteins that did show a change in protein abundance, ~20% currently have no known function, leaving ~800 proteins available for review. Surprisingly, a significant percentage of these proteins clustered into a small number of functional categories. For example, the proteins Arr1–3 protect against arsenic by promoting its efflux (18). Although the transcription factor Arr1 was not represented in our data set, Arr2 and Arr3 were strongly up-regulated (Fig. 1C and supplemental Table 1), as previously shown (19) and served as internal controls. A second, much larger group of proteins involved in sulfur-related metabolism was also strongly up-regulated. Although this group will not be discussed further, pathways of sulfur metabolism, including homocysteine and glutathione synthesis, are believed to function in arsenic detoxification (20).

The abundance of proteins in cells is established by pathways of protein synthesis and degradation, sometimes referred to as proteostasis. Our analysis indicated a massive reorganization of
FIGURE 1. Proteomic analysis of arsenic-induced toxicity. A, experimental design of the proteomic analysis of arsenic mediated toxicity. Triplicate wild-type cultures were treated with sodium arsenite (1 mM) and sampled at 0, 1, and 4 h. Extracts were prepared, standardized by protein amount, proteolytically digested, labeled with TMT probes, and subject to mass spectrometric analysis by LC-MS/MS. BPRP refers to basic pH reversed-phase chromatography. B, growth of wild-type cells during treatment with sodium arsenite (1 mM) and after drug wash-out, as indicated. C, major cellular pathways showing increased or decreased protein abundance after arsenic treatment. The numbers of co-regulated pathway components are listed in parentheses.
proteostasis pathways in response to arsenic. Broadly, these changes appeared to effect a shift from a conventional protein anabolic state to a protein catabolic state. Specifically, both major protein degradation pathways, the proteasome and autophagy, showed significant and uniform increases in the protein abundance of their components (Fig. 1C). Molecular chaperones, which function in protein folding and may assist in protein degradation, were also up-regulated (Fig. 1C), consistent with toxicity due to misfolded proteins. Strikingly, components of the ribosome showed the opposite effect and were strongly and uniformly down-regulated at the protein level. This effect was not limited to the ribosome proper, as numerous proteins involved in ribosome biogenesis were similarly affected. Finally, some of the most dramatically down-regulated proteins were related to the import of amino acids and glucose (Fig. 1C). Overall, this signature appeared consistent with a cellular response designed to broadly decrease protein synthesis.

Up-regulation of the Proteasome by Arsenic—The proteasome is a 2.5-MDa complex consisting of 33 core subunits with a large number of additional interacting proteins as well a number of dedicated assembly chaperones (3). It functions to destroy proteins that have been previously covalently modified by the small protein ubiquitin and represents the major pathway of selective intracellular protein degradation in eukaryotes. The proteasome consists of two major assemblies, the core particle and the regulatory particle. The core particle houses the proteolytic active sites within a central cylindrical chamber, whereas the regulatory particle, which sits at either end of the core particle, orchestrates the recognition, unfolding, deubiquitination, and insertion of substrates into the core particle (21).

A major proteotoxic stress response centers upon the transcription factor Rpn4, which recognizes the promoters of all known proteasome genes (22). Because Rpn4 is also a substrate of the proteasome with an extremely short half-life (23), the abundance of Rpn4 reflects the functional capacity of proteasomes within cells. Under conditions that overwhelm proteasome function, such as the presence of misfolded proteins, Rpn4 levels rise and stimulate transcription of proteasome components until proteasome function is restored, resulting in Rpn4 destruction and normalization of this homeostatic pathway (23). In the presence of arsenic, Rpn4 levels rapidly rise to ~20-fold above the basal level but then begin to normalize, even over a relatively short 4-h window (Fig. 2A). This induction is sufficient to result in an elevated protein abundance of nearly all proteasome subunits, including both core particle components (Fig. 2B) and regulatory particle components (Fig. 2C). Unlike Rpn4, most proteasome subunits show long half-lives, thus their levels tend to plateau or rise slowly over time. Cuz1 is a recently described arsenic-inducible proteasome-in-
Armed with proteasomal function for surviving arsenic toxicity is now reasonably well established. Several mutants of the ubiquitin-proteasome pathway show decreased survival in the presence of arsenic. Indeed, rpn4Δ mutants are among the most severe in this regard (Refs. 12 and 24 and Fig. 3F), likely reflecting its role as a master regulator of proteasome function.

**Up-regulation of Autophagy by Arsenic**—In addition to the proteasome, cells harbor a second major pathway of protein degradation known as autophagy. Although the role of autophagy in responding to nutrient starvation is best understood, in recent years autophagy has been implicated in diverse cellular processes, including responses to misfolded proteins (25–27). Our proteomic analysis indicated a broad up-regulation of autophagy components at the protein level. Indeed, only one autophagy protein showed a decrease in protein abundance (supplemental Table 1). Within this category, one of the most highly up-regulated proteins was Atg8 (Fig. 3A), which is significant as Atg8 is a key regulator of autophagy and is required for autophagosome formation (25). Other components of the pathway, for example Atg19 and Atg33, showed varying degrees of up-regulation (Fig. 3B–C), whereas numerous control proteins, including Act1, showed no effect (Fig. 3D).
general, autophagy components tended to show a progressive increase in protein levels over the course of the experiment.

A role for autophagy in response to arsenic toxicity has not been previously characterized in yeast. To determine whether an increase in protein levels of autophagy components translated into a functional increase in autophagy within the cell, we utilized a well established assay of autophagy function employing an N-terminally tagged GFP-Atg8 construct that was genomically encoded and expressed from the endogenous locus (28). This construct is known to complement the atg8Δ mutant. Upon stimulation of autophagy, the GFP-Atg8 protein is delivered to the lysosome where the C-terminal part of the protein representing Atg8 is destroyed. By contrast, the GFP moiety is relatively resistant to proteolysis and accumulates over time (28). We treated cells with sodium arsenite and monitored for the production of the protease-resistant GFP fragment by immunoblot. We found that there was a time-dependent increase in the production of this free GFP fragment (Fig. 3E), consistent with an increase in autophagy. Total levels of GFP-Atg8 also increased over time, consistent with the proteomic data (Fig. 3, A and E), whereas a control protein, Pgg1, showed no change (Fig. 3E).

Finally, we sought to determine whether this increase in autophagy was physiologically relevant for arsenic toxicity. To do so, we utilized an atg8 null mutant. Surprisingly, this mutant showed no defect in survival in the presence of sodium arsenite (Fig. 3F). However, when this mutant was combined with the rpm4Δ mutant, a strong synthetic growth defect was apparent (Fig. 3F). These results indicate that a functional autophagy pathway is required for survival against arsenic toxicity and that defects in autophagy can be at least partially compensated by induction of proteasome-mediated degradation pathways.

Down-regulation of Ribosome Components by Arsenic—The ribosome is among the largest of cellular assemblies and consists of 137 subunits, broadly organized into a large and a small subunit (29). Of these 137 subunits, data from 97 were available for analysis. Strikingly, 74 of these subunits showed a significant reduction in protein abundance after treatment with arsenic. This included components of both the large (Fig. 4, A and B) and small (Fig. 4, C and D) subunits of the ribosome. Of the remaining 23 subunits, only one subunit, Rps31, showed an increase in abundance (Fig. 4E). Even in this case, the significance of this change was uncertain as Rps31 encodes a fusion protein (30). Thus, the slight increase in abundance of this protein might have more to do with ubiquitin function than ribosome function. In addition to cytoplasmic ribosomes, cells also contain mitochondrial ribosomes that are composed of distinct subunits. Of the ~78 components of the mitochondrial ribosome, 44 were represented in this data set. In contrast to the cytoplasmic ribosome, the overwhelming majority of these proteins showed no change in protein abundance (supplemental Table 1 and data not shown), further indicating the specificity of the effects observed here.

We sought to verify these proteomic results using conventional immunoblotting techniques. Consistent with the proteomic data, two ribosome components, Rpl16A and Rpl31B, showed a reduction in protein levels after arsenic treatment, whereas an unrelated protein, Pgg1, was unchanged (Fig. 4, F–G). These results indicate a broad and significant down-regulation of the ribosome under arsenic stress. Given that ribosomes are among the most abundant proteins within cells (31), the extent of down-regulation is perhaps even more striking.

Ribosome Reduction Represents an Adaptive Cellular Response to Arsenic Toxicity—The observed reduction in ribosome protein levels after arsenic exposure could in principle reflect a toxic consequence of arsenic, an adaptive cellular response to mitigate arsenic toxicity, or an unrelated phenomenon. To address these possibilities, we characterized the effect of arsenic on several null mutants of ribosome components. If ribosome reduction were simply a toxic effect of arsenic, one might expect such mutants to be particularly sensitive to arsenic exposure. By contrast, if ribosome reduction were part of a cell survival pathway, mutation of ribosome components might protect cells against this toxic insult. We examined five unrelated mutants of the ribosome, representing both large and small ribosome components, and found that these mutants actually showed greater survival against arsenic toxicity than did the wild-type strain (Fig. 5A). Indeed, a previous unbiased genome-wide phenotypic screen for arsenic-resistant mutants found that ribosome mutants represented the single largest class of resistant mutants (8). Interestingly, those mutants that showed a stronger basal growth defect (e.g. rpl20aΔ and rpl39aΔ) showed a corresponding stronger resistance to arsenic (Fig. 5A), implying that the same cellular property, i.e. reduced ribosome function, was responsible for both their slow growth in the absence of drug and their enhanced growth in the presence of drug. The protective effect of ribosome mutations against arsenic toxicity could also be visualized early after exposure for at least some of the mutants, including rpl19bΔ. Under standard growth conditions in liquid media, this mutant showed a mild growth defect (Fig. 5B). By contrast, when cultured in the presence of arsenic, the strains showed the opposite relationship: rpl19bΔ grew at a slightly faster rate than wild-type despite its basal growth defect, and this difference could be detected within just a few hours (Fig. 5B).

Stress responses are typically reversible to allow for a normalization of cell function once the threat has been removed or neutralized. We sought to determine if this was the case for ribosome modulation. We treated logarithmically growing cells with sodium arsenite for four hours to reduce ribosome levels. We then washed out the drug and monitored ribosome levels by immunoblot for two ribosome components, Rpl16A and Rpl31B (Fig. 5, C and D). We observed that not only did cells restore their ribosome levels after arsenic wash-out, the time course of this process appeared to coincide with the time course of general recovery of growth after arsenic washout (compare with Fig. 1B). That is, there was only a modest increase in ribosome levels during the first hour after drug washout followed by more robust recovery thereafter. In total, these results are consistent with a model in which ribosome reduction is a rapid, effective, and reversible stress response against arsenic toxicity.

Defects in Ribosome Biogenesis Protect against Arsenic Toxicity—We considered the possibility that the enhanced survival of ribosome mutants to arsenic-mediated toxicity (Fig. 5A) could in fact reflect an arsenic-induced inhibitory activity on protein synthesis that did not require a reduction in ribo-
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**Figure A:**
![Graph showing relative abundance of Rpl35b](image)

**Figure B:**
![Graph showing relative abundance of Rpl21b](image)

**Figure C:**
![Graph showing relative abundance of Rps16b](image)

**Figure D:**
![Graph showing relative abundance of Rps29b](image)

**Figure E:**
![Heatmap and bar graph showing protein abundance after arsenite treatment](image)

**Figure F:**
![Western blot of Rpl16A-TAP and Pgk1](image)

**Figure G:**
![Western blot of Rpl31B-TAP and Pgk1](image)
some abundance. Although this possibility would not exclude an important role for ribosome reduction in this proteotoxic stress response, the broad proteomic changes in ribosome biogenesis factors suggested that new ribosome biosynthesis did indeed play a key role in this stress response. Sixty proteins involved in ribosome biogenesis showed a decrease in protein.

**FIGURE 4. Down-regulation of ribosomal proteins by arsenic.** A–D, relative protein abundance of selected ribosome subunits as determined by proteomic analysis at 0, 1, and 4 h after treatment with sodium arsenite (1 mM). Ribosome large subunit proteins Rpl35b (panel A) and Rpl21b (panel B) are shown as well as small subunit proteins Rps16b (panel C) and Rps29b (panel D). Error bars represent S.D. from triplicate cultures. In addition, all differences between untreated and treated samples were statistically significant by Student’s t test (p < 0.01). E, left panel, clustering diagram showing relative protein abundance of ribosome subunits in triplicate at 0, 1, and 4 h after treatment with sodium arsenite (1 mM), as indicated. Right panel, distribution of ribosome subunits by change in protein levels after arsenic treatment.

**FIGURE 5. Ribosome reduction protects against arsenic-induced toxicity.** A, growth of wild-type yeast and five otherwise unrelated ribosome mutants (rpl20aΔ, rpl39Δ, rpl19bΔ, rps17aΔ, and rps16bΔ) in the presence or absence of sodium arsenite (1.5 mM) as indicated. Plates were cultured at 30 °C for 3 days. B, growth in liquid media (YPD) of wild-type and rpl19bΔ in the presence or absence of sodium arsenite (1 mM) as indicated. Error bars represent S.D. from two independent cultures. Error bars do not overlap at the 2-, 4-, 6-, and 8-h time points in the no drug panel and at the 4-, 6-, 8-, and 10-h time points in the sodium arsenite panel. C and D, ribosome protein levels after wash-out of sodium arsenite for two subunits, Rpl16a-TAP (panel C) and Rpl31b-TAP (panel D). Upper panels, anti-TAP antibody; lower panels, anti-Pgk1 antibody (loading controls). Treatment was with 1 mM sodium arsenite for 4 h before wash-out.

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abundance after arsenic treatment (Fig. 1C and supplemental Table 1). By contrast, only a single ribosome biogenesis protein showed an increase in protein abundance (supplemental Table 1). We selected for further study the protein Slx9, which has well established functions in ribosome biogenesis but not in other aspects of ribosome function or protein synthesis (32, 33). Slx9 showed a strong reduction in protein levels after arsenic treatment (Fig. 6A). Our data predict that a slx9Δ mutant, assuming that its negative effect on ribosome biogenesis was sufficient to significantly impair protein synthesis, would show enhanced survival against arsenic-mediated toxicity. Indeed, the slx9Δ mutation protected cells against trivalent arsenic (Fig. 6B). Although these results do not exclude additional regulatory controls on protein synthesis within this stress response (see below), they support a model in which reduction in ribosome abundance plays a key role in addressing arsenic-mediated proteotoxicity.

Ribosome Reduction in Response to Cadmium Chloride-induced Proteotoxicity—To determine whether the ribosome reduction response was specific to trivalent arsenic, we employed the divalent heavy metal cadmium chloride, which has been established as a cause of proteotoxicity for 20 years (34). We found that ribosome levels were reduced after treatment with cadmium chloride, similar to arsenic (Fig. 7A). Thus, ribosome reduction is not specific to arsenic but is operational under at least two distinct proteotoxic stress conditions.

Trivalent Arsenic Induces Multiple Pathways of Inhibition of Protein Synthesis—Some previously described proteotoxic stress responses are known to inhibit protein synthesis. Most notably, the unfolded protein response can inhibit protein synthesis through a mechanism distinct from ribosome reduction: phosphorylation of the translation initiation factor eIF2-α antagonizes protein synthesis (35). We treated cells with sodium arsenite, as before, and monitored eIF2-α phosphorylation using a well established phospho-specific antibody. We observed strong induction of eIF2-α phosphorylation (Fig. 7B) after arsenic treatment. These results suggest that, in response to arsenic, cells induce multiple pathways that negatively regulate protein synthesis. Thus, ribosome reduction appears to be one part of a broad and complex stress response against arsenic-mediated proteotoxicity.

Discussion

Proteotoxicity Is a Major Component of Toxicity for Trivalent Arsenic—The molecular basis for the toxicity of arsenic has remained incompletely understood and has been variously ascribed to inhibition of ATP production via glycolysis, direct inhibition of pyruvate dehydrogenase, DNA damage, oxidative damage, kinase inhibition, and inhibition of molecular chaperones (4–8). Increasing evidence suggests an alternate model for the toxicity of trivalent arsenic, which is that it induces widespread protein misfolding. Evidence in support of this model includes the following: 1) various mutants of the ubiquitin-pro-
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Ribosome Reduction as a Proteotoxic Stress Response—The data presented here suggest that reduction in ribosome abundance can serve as an effective proteotoxic stress response, a possibility that has not been previously reported. By reducing cellular ribosome levels, the generation of newly synthesized misfolded proteins may be limited, allowing protein degradation pathways to more effectively deal with existing misfolded proteins. Some previously described stress responses are known to regulate protein synthesis. However, the findings presented here appear to be distinct from these responses. The unfolded protein response, which is stimulated by misfolded proteins within the endoplasmic reticulum, also broadly down-regulates protein synthesis. In this case, however, a phosphorylation-based signaling pathway results in inhibition of the key translation initiation factor, eIF2α (35). The heat shock response also results in a general repression of protein synthesis, although in this case there is a switch in the mode of protein synthesis such that a small number of specific transcripts are favored, resulting in the production of the so-called heat shock proteins. Heat shock is additionally distinguished from arsenic in that, whereas chemical inhibition of protein synthesis by cycloheximide can prevent the formation of arsenic-induced protein aggregates, it fails to do so under heat shock (24), implying that the stress associated with arsenic is more closely related to newly synthesized proteins. Induction of eIF2-α phosphorylation in response to arsenic (Fig. 7B) indicates that cells employ multiple mechanisms to reduce total protein synthesis. We suspect that there may be still other mechanisms that cells employ to reduce protein synthesis after arsenic treatment. Further investigation will be needed to address this possibility.

An important question for future work concerns the mechanism of ribosome reduction in response to trivalent arsenic. In principle, this could be achieved by either a reduction in new ribosome production or an increase in post-translational degradation of ribosomes. A key observation in this regard is that microarray-based studies of the transcriptional response to arsenic have also noted broad reductions in the levels of mRNAs for ribosome components (7, 20). These studies did not examine ribosome protein levels or propose that ribosome reduction might be an adaptive cellular response. Nevertheless, one possibility is that the reduction in ribosome protein abundance is largely due to transcriptional changes. The broad and consistent decrease in the abundance of ribosome biogenesis factors (Figs. 1C and 6A) likely also plays a role in regulating ribosome abundance. The possibility that autophagy or the proteasome might contribute to this down-regulation via targeted degradation remains an important and non-mutually exclusive alternative.

Implications for Higher Organisms—In addition to its known roles as a toxin, arsenic is also a highly effective cancer therapy for acute promyelocytic leukemia (38). When given in combination with a second agent, all-trans-retinoic acid, cure rates are 90% or higher (38). Acute promyelocytic leukemia is caused by a cytogenetic translocation (t(15;17) that results in the novel fusion protein PML–RARα, upon which the cancer is dependent. Although its efficacy was known before its mechanism of action, it is now clear that arsenic treats this leukemia by covalently binding to the PML–RARα oncoprotein and causing it to misfold, which ultimately results in proteasome-mediated degradation of PML–RARα (10). The results presented here suggest that protein misfolding due to arsenic is unlikely to be limited in scope. The beneficial effect of PML–RARα misfolding may not reflect its specificity for arsenic modification but, rather, its singular importance for acute promyelocytic leukemia to propagate. Currently, we do not understand the molecular basis for arsenic-induced protein misfolding or whether certain biochemical or structural properties will predispose proteins to arsenic modification. A better understanding of how arsenic causes protein misfolding could allow for the purposeful targeting of other disease-causing proteins and ultimately lead to novel therapies.

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