Trivalent arsenic inhibits the functions of chaperonin complex


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The microarray data were submitted to GEO (accession number GSE5973).
Running Title: Arsenic inhibits TRiC

Key Words: Arsenic; Chaperonin; YKOs

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Abstract

The exact molecular mechanisms by which the environmental pollutant arsenic works in biological systems are not completely understood. Using an unbiased chemogenomics approach in *Saccharomyces cerevisiae*, we found that mutants of the chaperonin complex TRiC and the functionally related prefoldin complex are all hypersensitive to arsenic compared to a wild-type strain. In contrast, mutants with impaired ribosome functions were highly arsenic-resistant. These observations led us to hypothesize that arsenic might inhibit TRiC function, required for folding of actin, tubulin, and other proteins post-synthesis. Consistent with this hypothesis, we found that arsenic-treatment distorted morphology of both actin and microtubules filaments. Moreover, arsenic impaired substrate folding by both bovine and Archaeal TRiC complexes *in vitro*. These results together indicate that TRiC is a conserved target of arsenic inhibition in various biological systems.
Summary

Arsenic is a ubiquitous environmental pollutant that causes severe health problems in humans. It is also used as an effective therapeutic agent against various diseases and infections. Using advanced genomic tools in the model organism yeast and biochemical experiments, we demonstrated that arsenic disturbs functions of the chaperonin complex required for proper folding and maturation of a large number of proteins. This mechanism of action by arsenic is conserved in various biological systems ranging from archaeal bacteria to mammals. Such an understanding should help exploring possible ways to overcome toxic effects caused by exposure to arsenic.
Introduction

Trivalent inorganic arsenic is among the most significant environmental hazards affecting the health of millions of people worldwide (NORDSTROM 2002). Particularly, inorganic trivalent arsenic [As(III)] in underground drinking water and some mining environments is recognized as the cause of various cancers affecting the skin, lung, urinary tract, bladder, liver, and kidney (TAPIO and GROSCHE 2006), as well as being implicated in several other disorders such as diabetes, hypertension, neuropathy, and vascular diseases (TSENG 2004). Interestingly, As(III) is also an effective therapeutic agent against cancer and human pathogens. A number of models have been proposed to explain the biological effects of As(III), including stimulation of reactive oxygen species (ROS) production (MILLER et al. 2002; TAPIO and GROSCHE 2006) and inhibition of tubulin polymerization (LI and BROOME 1999; RAMIREZ et al. 1997). However, exactly how As(III) disturbs biological systems is still not clear.

The eukaryotic chaperonin TRiC (TCP1-Ring Complex; also called CCT) is a ~900 kDa complex consisting of two apposed heterooligomeric protein rings. Each ring, constituted by eight homologous subunits (encoded by the essential CCT1-CCT8 genes in budding yeast), contains a central cavity in which unfolded polypeptide substrates attain a properly folded state in an ATP-requiring reaction (BUKAU and HORWICH 1998; GUTSCHE et al. 1999). TRiC is required for the proper folding of an important subset of cytosolic proteins, including cytoskeleton components, cell cycle regulators, and tumor suppressor proteins (SPIESS et al. 2004). Some of these protein substrates are themselves encoded by essential genes; thus TRiC is indispensable for eukaryotic cell survival. Many TRiC substrates are subunits of oligomeric complexes and their assembly into
functional multisubunit complexes also requires TRiC (SPIESS et al. 2004). Assembly of such macromolecular complexes in some cases eliminates the accumulation of toxic subunits such as free β-tubulin molecules, which can bind to γ-tubulin and thereby disrupt the formation of mitotic spindles in the yeast S. cerevisiae (ARCHER et al. 1995). Folding of yeast actin, α-tubulin, and β-tubulin and their oligomerization require TRiC and GimC (also known as prefoldin), a non-essential protein complex of six distinct but structurally related subunits of 13-23 kDa (GEISER et al. 1997; VAINBERG et al. 1998). Mutational loss of GimC function substantially reduces actin and tubulin folding efficiency although it does not cause obvious growth defects in yeast. However, deletion of various GimC subunits strongly reduces the viability of conditional-lethal alleles of TRiC subunits under permissive conditions (SIEGERS et al. 1999).

To elucidate the mechanisms of inorganic As(III)’s action(s) in a eukaryotic system, we first took an unbiased functional chemogenomics approach in yeast to systematically probe for the genetic determinants of arsenic-sensitivity. These genetic and subsequent biochemical results point to the conclusion that As(III) inhibits the yeast TRiC complex. This mechanism of action is apparently conserved because the activities of both a mammalian TRiC complex and an archaeal TRiC-like chaperonin are significantly inhibited by arsenic in vitro. Given that mammalian TRiC and some of its substrates are implicated in tumor suppression, angiogenesis, and neuropathy (BOUHOUCHE et al. 2006; LEE et al. 2003; SPIESS et al. 2004), TRiC is likely an important protein mediator of As(III)’s effects on human health.
Materials and Methods

Profiling of the sensitivity of genome-wide deletion mutants to arsenic and individual validation were carried out as described in Supplementary Methods online. As$_2$O$_3$ (Sigma-Aldrich, St. Louis, MO) was first dissolved in NaOH as a 400 mM stock solution of sodium arsenite.

Yeast strains and plasmids  Individual yeast deletion mutant strains used in this study were all obtained or derived from the yeast knockout mutants constructed by the Saccharomyces genome deletion project (Giaever et al. 1999). The DAmP alleles of TUB2, CCT1 and CCT2 were obtained from Open Biosystems (Breslow et al. 2008). The Cdc55p-3HA expression strain was a gift from Dr. Katja Siegers. Plasmids expressing ACT1 and TUB2 from a centromere-based plasmid were constructed by in vivo homologous recombination into YCplac33 (CEN, URA3) (Gietz and Sugino 1988). The CCT1, CCT2, CCT3, CCT4, CCT5, CCT6, CCT7, TUB1, and TUB3 overexpression plasmids were similarly constructed into YEplac195 (2µ, URA3) (Gietz and Sugino 1988). Either YCplac33 or YEplac195 was used as the vector control.

Immunofluorescence  A 100 ml culture of the wild-type diploid yeast BY4743a/α was grown in YPD at 30°C until mid-log phase and split. Sodium arsenite was added into one part of the culture at a final concentration of 1 mM. The other part served as a non-treatment control. Both were incubated at 30°C for 3 additional hours. Immunofluorescence analyses of actin and microtubule morphology were performed as
previously described (RIEDER et al. 1996). Actin was stained with rhodamine-phalloidin and microtubule with an anti-α-tubulin antibody.

**Immunoprecipitation and western blotting** The yeast strain expressing Cdc55p-3HA was grown in 100 ml YPD at 30°C until mid-log phase. The culture was split and sodium arsenite was added into one aliquot at a final concentration of 1 mM. The other aliquot served as a non-treatment control. Both were subsequently incubated at 30°C for 1 hour before cells were harvested. Cell homogenization and immunoprecipitation were carried out essentially as described (PAN and HEITMAN 2002). An anti HA and anti-actin antibody was used to immunoprecipitate Cdc55p-3HA and actin, respectively. Cdc55p-3HA, actin and TRiC on the western blots were detected with anti-HA, anti-actin, and anti-Cct5p antibodies.

**In vitro Actin folding Assay** The actin-folding assay was carried out as described by Frydman and Hartl (FRYDMAN and HARTL 1996). Briefly, 0.25 μM TRiC were incubated in buffer A (20 mM Hepes-KOH [pH 7.5], 100 mM KOAc, 5 mM MgCl₂, 1 mM DTT, 10 % glycerol, and 1% PEG 8000). Subsequently [³⁵S]-actin, which was denatured in 6 M guanidin/Hcl, was rapidly diluted 1:100 to a final concentration of 30 μM into the reaction mix. After incubation for 10 min at 4°C and centrifugation at 14,000 x g for 10 min to remove aggregated actin, the reaction was supplemented with 1 mM ATP and incubated for 40 minutes at 30°C to allow time for ATP-dependent actin re-folding by the chaperonin. 1 mM arsenite was added as indicated in the figure. Generation of native [³⁵S]-actin was determined by native gel electrophoresis using folded [³⁵S]-actin as a
control as described previously (FRYDMAN and HARTL 1996). The gel was exposed on a phosphor storage screen (Kodak, USA), and scanned in a Typhoon 9410 imager (GE Healthcare, USA).

Archaeal chaperonin folding assays. Purification of the Archaeal chaperonin from Mm-Cpn was carried out by conventional chromatography essentially as described (KUSMIERCZYK and MARTIN 2003). Rhodanese folding by the Archaeal chaperonin Mm-Cpn was assayed as described (WEBER and HAYER-HARTL 2000). In brief, 0.25 µM protein was incubated in Cpn-buffer supplemented with 20 mM sodium thiosulfate. Purified rhodanese was denatured in 6 M guanidinium/HCl containing 5 mM DTT and rapidly diluted 1:100 to a final concentration of 30 µM into the reaction mix. After incubation for 5 min at 37°C, the reaction was started by addition of 2 mM ATP and allowed to proceed for 50 min at 37°C. In order to detect the presence of re-folded rhodanese, 10 µl of the reaction were withdrawn and applied to a rhodanese activity assay performed as described (WEBER and HAYER-HARTL 2000).
Results

Mutants of the chaperonin pathway are As(III)-hypersensitive

Haploinsufficiency of a target gene could lead to increased sensitivity to the cognate anti-proliferation cytotoxin or drug (Giaever et al. 1999; Lum et al. 2004). To probe molecular mechanisms of As(III) actions in yeast, we investigated As(III)-sensitivity of genome-wide heterozygous diploid yeast knockout (YKO) mutants using a TAG-array based analysis (Giaever et al. 1999; Lum et al. 2004) followed by individual validation. We found that 33 heterozygous diploid YKOs were significantly more sensitive than an isogenic wild-type strain to 450 µM sodium arsenite (Fig. 1A and Supplementary Table 1). Because As(III) completely inhibits yeast cell growth at higher concentrations, we reasoned that it might inactivate at least one essential protein or protein complex. 15 of these 33 As(III)-hypersensitive mutants were heterozygous for essential genes, including 5 (CCT1, CCT4, CCT5, CCT7, and CCT8) encoding subunits of the TRiC complex and 2 more (SPC97 and TUB4) that are directly involved in microtubule biogenesis and function (Fig. 1A and Supplementary Table 1). We directly tested three other heterozygous YKOs (CCT2, CCT3, and CCT6) of TRiC that were missed in the initial array-based screen due to low microarray hybridization signal intensities and found that they were also more sensitive to As(III) than a control strain (Fig. 1B). Yeast cells overexpressing one of the TRiC complex genes, CCT1, were also partially arsenic-resistant on solid medium (Fig. 1C). Such an effect was also observed in liquid culture. Under unperturbed conditions, the growth rates of hoΔ mutants carrying an empty vector and overexpressing TCC1 in a liquid synthetic medium were at 113 minutes/division and 115 minutes/division, respectively. In the presence of 450 µM of
As(III), their growth rates were 181 minutes/division and 137 minutes/division, respectively. These results together suggest that As(III) might inhibit TRiC complex function.

Synthetic lethality interactions were previously observed between CCT1 partial loss of function alleles and deletion mutations of the GimC complex, which acts as a co-chaperone in TRiC-mediated actin and tubulin folding (SIEGERS et al. 1999). Thus, if As(III) inhibits TRiC, GimC deletion mutants should be arsenic-hypersensitive. To test this prediction and to further extend the study of cellular response to As(III), we systematically investigated genome-wide haploid YKOs for As(III)-sensitivity using dSLAM, a barcode microarray-based method for detecting gene-compound and gene-gene interactions (PAN et al. 2004). Upon individual validation, we found that 191 haploid YKOs were sensitive to As(III) at 400 µM or lower (Fig. 2A and Supplementary Table 2). The nine most sensitive ones directly affected either As(III) efflux (arr1Δ and arr3Δ) or the GimC complex (gim3Δ, gim4Δ, gim5Δ, pac10Δ, pfd1Δ, yke2Δ, and yml094c-aΔ, which deletes part of GIM5) (Data not shown) and all GimC mutants were more sensitive than the ARR mutants when tested at 40 µM As(III) (Fig. 2B and data not shown), further supporting the model that arsenic inhibits TRiC.

Most other As(III)-sensitive mutants were susceptible only to relatively high concentrations (Supplementary Table 2). These included additional mutants affecting actin and microtubule biogenesis and those affecting peroxisome biogenesis, mitotic chromosome segregation, cell cycle progression, histone modification, ergosterol biosynthesis, oxidative stress response, DNA repair, and others (Fig. 2A and Supplementary Table 2), consistent with results of recently performed screens using the
haploid or homozygous diploid knockout mutants (DILDA et al. 2008; JIN et al. 2008; THORSEN et al. 2009). That mutants defective in oxidative stress response and DNA repair were sensitive to As(III) also agrees with a previous finding that As(III) stimulates the production of reactive oxygen species (ROS) (TAPIO and GROSCHE 2006), which damage DNA molecules. The arsenic-sensitive phenotypes of some of these other mutants are likely related to TRiC inhibition because CCT1 overexpression partially suppressed arsenic-sensitivity in various mutants, including those of oxidative stress response and DNA repair (Fig. 2C). Some of the affected pathways are likely required for buffering the effects of arsenic inhibition of TRiC. Others might well reflect additional independent bona fide arsenic targets in yeast.

**Slowed protein synthesis confers As(III) resistance**

We also identified 109 arsenic-resistant haploid YKOs and about 62.4% of them affected either ribosomal protein genes or those involved in ribosomal biogenesis (Fig. 3A and Supplementary Table 3). A similar connection between mutations in ribosomal biogenesis and arsenic-resistance was also recently observed by others (DILDA et al. 2008). Although ribosomal proteins are essential, some are encoded by duplicated genes in yeast and deleting one copy is non-lethal. Interestingly, arsenic-resistance of the ribosomal protein mutants largely correlated with their fitness defects under normal conditions (data not shown). We suspected that these mutants might have compromised capacity in protein synthesis, which leads to As(III)-resistance. To test this hypothesis, we investigated the effects of inhibiting protein synthesis with a sublethal concentration of cycloheximide. Similar to the *rpl19bΔ* and *rps23bΔ* mutations that affect ribosomal
protein genes, treatment with cycloheximide conferred yeast cells resistance to As(III) (Fig. 3B). This relationship between protein synthesis and arsenic cytotoxicity is consistent with the model that As(III) inhibits TRiC, which facilitates the folding of newly synthesized polypeptides and their subsequent assembly into oligomeric complexes (SPIESS et al. 2004). Inhibition of TRiC by As(III) likely has two distinct types of effects: 1) production of insufficient quantities of correctly folded substrates and assembled complexes that are essential for viability; and 2) accumulation of incorrectly folded substrates or unassembled subunits that are toxic.

β-tubulin contributes to As(III) toxicity

One such TRiC substrate is β-tubulin, which causes growth defects in yeast cells when overexpressed and not dimerized with α-tubulin (ARCHER et al. 1995). Presumably, β-tubulin polypeptides produced at lower rates (resulting from slowed protein translation) in ribosomal protein mutants or cycloheximide-treated cells were more compatible with the capacity of arsenic-crippled TRiC complex. As a result, unfolded polypeptides and/or unassembled toxic free β-tubulin molecules might have been accumulated at lower levels, mitigating As(III) toxicity. To test this hypothesis, we investigated whether β-tubulin contribute to As(III) toxicity by genetically perturbing the relative ratio between α- and β-tubulins. We first modestly overexpressed TUB2 (the β-tubulin gene) from a centromeric plasmid under control of its endogenous promoter and tested its effects on the growth and arsenic-sensitivity of HO/hoΔ (a surrogate wild-type) and CCT6/cct6Δ heterozygous diploid mutants. Modest overexpression of TUB2 made the HO/hoΔ mutant slightly yet reproducibly more sensitive to As(III) (Fig. 3D). It also
noticeably hampered growth of the CCT6/cct6Δ mutant in the absence of As(III) and even more so in its presence (Fig. 3D). In comparison, modest overexpression of ACT1, which encodes another important cytoskeletal substrate of TRiC, actin, had no effect (Fig. 3C). In agreement with these results, deleting TUB3, a non-essential gene encoding α-tubulin, caused As(III)-hypersensitivity (Fig. 2B and 3C). Overexpression of TUB3 from a high copy plasmid also suppressed As(III) toxicity in cin2Δ, gim3Δ, and pfd1Δ mutants (Fig. 3C), which are defective in tubulin folding and dimerization. Similar results were observed when TUB1, an essential α-tubulin gene highly homologous to TUB3, was overexpressed (data not shown). In these mutants, overexpression of α-tubulin likely allowed more efficient folding and/or incorporation of β-tubulin molecules into non-toxic heterodimers due to the increased abundance of available α-tubulin molecules. These results together indicated that yeast cells with a relatively high β/α-tubulin ratio are sensitized to As(III) and those with a low β/α-tubulin ratio are more resistant to the drug. Thus unfolded and/or folded yet free β-tubulin molecules apparently contribute to As(III) cytotoxicity.

**Arsenic unlikely directly inhibits microtubule function in yeast**

It has been proposed that As(III) directly binds to tubulins and disrupts their functions in mammalian cells (Li and Broome 1999). In particular, As(III) was shown to directly bind to β-tubulin isolated from a human breast cancer cell line and structural modeling suggested the amino acid residue Cys12 is key to such a physical interaction (Zhang et al. 2007). This model of arsenic action would be consistent with most of the genetic evidence described above. However, it is not consistent with other genetic
evidence. We found that a tub2-DAmP mutant (BRESLOW et al. 2008), which presumably expresses TUB2 at a lower level as compare to a wild-type due to mRNA instability, responds to As(III) and the well-known microtubule poison benomyl very differently. Consistent with the idea that benomyl directly binds to and inhibits microtubules, the tub2-DAmP was hypersensitive to this drug (Fig. 3E). The latter result also implies that TUB2 expression is indeed reduced in the DAmP allele. In contrast to the hypothesis that As(III) directly affects microtubules, the tub2-DAmP strain was slightly more resistant to As(III) than the wild-type (Fig. 3E), a highly reproducible phenotype. A TUB2/tub2Δ heterozygous diploid mutant was similarly hypersensitive to benomyl but slightly more resistant to As(III) as compared to a HO/hoΔ control strain (data not shown). In contrast to the tub2-DAmP mutant, both the cct1-DAmP and cct2-DAmP mutants were hypersensitive to both As(III) and benomyl. These results suggest that As(III) unlikely inhibit yeast growth by binding to microtubules as benomyl does. Of course, it is still possible that arsenic binds to yeast microtubule but has a different effect than benomyl. However, the Cys12 residue critical for arsenic binding to human β-tubulin is not conserved in its yeast orthologue. The genetic results are also more consistent with the model that As(III) inhibits the TRiC complex, which is required to compensate for the loss of microtubule functions in the presence of benomyl due to its essential functions in microtubule biogenesis (SIEGERS et al. 2003; URSIC et al. 1994).

**Arsenic affects TRiC function in vivo**

Defects in TRiC were previously shown to distort morphological organization of both actin and microtubule filaments (SIEGERS et al. 2003; URSIC et al. 1994). If As(III)
inhibits TRiC, we expected that arsenic treatment of wild-type yeast cells would have similar effects, and this was exactly what we observed. Actin filaments are typically polarized to emerging buds under normal conditions (Fig. 4A) (Adams and Pringle 1984; Kilmartin and Adams 1984). In the presence of As(III), such polarization disappeared (Fig. 4A). Arsenic-treatment also distorted the mitotic microtubule structures (Fig. 4A). Similar observations were recently made in another study (Jin et al. 2008), although the exact microtubule morphology change caused by arsenic-treatment shown there was different from what we saw. Such difference was likely caused by different experimental protocols. In this other study, the intrinsic fluorescence of GFP tagged α-tubulin was observed, whereas we used immuno-staining. Despite this, the fact that arsenic-treatment affects the morphology of both actin and microtubule filaments strongly support the model that As(III) inhibits TRiC functions in vivo.

We next investigated whether As(III) might interfere with the physical interaction between TRiC and its substrates. In addition to the major substrates actin and tubulins, TRiC is required for folding of cytoplasmic proteins such as Cdc55p in yeast (Siegers et al. 2003). We found that As(III) reproducibly and significantly reduced physical interaction between TRiC and Cdc55p in co-immunoprecipitation assays (Fig. 4B), further indicating that As(III) directly affects TRiC functions. However, arsenic had no effect on the physical interaction between TRiC and β-tubulin (data not shown) and its effect on the interaction between TRiC and actin was inconclusive. Out of three independent co-immunoprecipitation experiments, we found that As(III) partially reduced TRiC binding to actin in one experiment and did not see any effect in the other two (Data
not shown). Thus, As(III) inhibits TRiC binding to some substrates (i.e. Cdc55p) but not to others in vivo (β-tubulin).

**Arsenic inhibits TRiC activity in vitro**

Eukaryotic pyruvate dehydrogenase (PDH), which consumes pyruvate to form acetyl-CoA while reducing NAD$^+$ to NADH, has long been considered a primary target for arsenic (SCHILLER et al. 1977). A possible consequence of arsenic inhibition of PDH is disruption of energy production and lowered intracellular ATP/ADP ratio. TRiC folding of substrates absolutely requires ATP hydrolysis (SPIESS et al. 2004) and As(III) might inhibit TRiC function indirectly by inactivating PDH and thereby damping intracellular ATP concentration. However, we consider this unlikely because PDH is nonessential in yeast. In addition, we did not observe a synthetic lethality or slow growth interaction between a PDH ($pda1\Delta$) mutation and a GimC mutation ($pfd1\Delta$) (data not shown).

We next tested the possibility that As(III) directly inactivates TRiC using a well-characterized in vitro assay that monitors the ability of purified chaperonin to fold chemically-denatured $^{35}$S-actin (MEYER et al. 2003). We found that addition of 1 mM As(III) to a folding reaction using purified bovine TRiC significantly inhibited actin folding. Such inhibition was observed both when the inhibitor was added before and shortly after substrate binding to TRiC. Here binding of TRiC to actin was not inhibited by As(III) (Fig. 4C). Yet actin folding activity was still inhibited, suggesting that As(III) inactivates TRiC after substrate binding. Similarly, As(III) inhibited substrate folding by a TRiC-like Archaeal complex, the MM-cpn chaperonin from *Methanococcus*
maripaludis, which is ~40% identical to its human counterpart (Fig. 4D). Together, these results support the idea that As(III) directly inhibits the folding activity of TRiC and TRiC-like chaperones.

We also found that inhibition of TRiC by arsenic in vitro was reversible because its activity was recovered after gel filtration (data not shown), ruling out direct covalent modification of TRiC by As(III) as a mechanism of action. Such reversibility was also observed with arsenic inhibition of yeast growth; nearly 100% of yeast cells regained colony formation capacity after being incubated in the presence of inhibitory concentration of the drug (> 2 mM) for over 24 hours at 30°C (data not shown).

**Detoxification mutations sensitize TRiC defective cells toward As(III)**

Arsenic concentrations required for inhibiting growth of wild-type yeast cells are much higher than needed for arsenic chemotherapy and toxicity in human cells. To mitigate such difference, we next investigated whether arsenic inhibition of TRiC at clinically achievable doses could inhibit growth of relatively healthy yeast cells. To do this, we first performed a genome-wide synthetic As(III)-hypersensitivity screen to identify secondary mutations that further sensitize a *pfdlΔ* mutant, which does not have obvious growth defect on its own but does exhibit partial defect in TRiC-mediated actin and tubulin folding (Geiser et al. 1997; Vainberg et al. 1998), to 5 µM of As(III) (Fig. 5A). We identified and individually confirmed a large number of such mutations (Supplementary Table 4). Among them, both *arr1Δ* and *arr3Δ* rendered the *pfdlΔ* mutant highly sensitive to 5 µM of As(III) without affecting its growth rate under normal conditions (Fig. 5B and Supplementary Table 4). Growth of the *arr1Δ pfdlΔ* and *arr3Δ*
pfld1Δ double mutants was also significantly inhibited by a lower concentration of arsenic (2 μM) (Fig. 5B). Thus, at clinically effective doses, arsenic inactivation of TRiC indeed significantly inhibited the growth of otherwise healthy yeast cells. These results indicate that, at the mechanistic level, arsenic modes of actions may well be the same in both yeast and human, even though the wild-type yeast cells seem to be more resistant to As(III) than human cells. Interestingly, Arr1p is a transcription factor required for expression of Arr3p, an arsenite transporter that actively pumps the drug out of yeast cells (GHOSH et al. 1999). Mammalian genomes do not seem to encode an Arr3p-like arsenic transporter, the absence of which might allow for more effective accumulation of intracellular As(III).

Discussion

By using a combination of functional genomic, genetic and biochemical studies, we have investigated the genetic determinants of arsenic susceptibility in yeast and elucidated a mechanism of action by arsenic common to both eukaryotes and Archaea. Our data suggest that arsenic inhibits the function(s) of the chaperonin complex. Similar functional genomic studies have been recently reported (DILDA et al. 2008; JIN et al. 2008; THORSEN et al. 2009). Although there was considerable overlap among all of these studies regarding the lists of arsenic-sensitive and -resistant mutants identified, our study is distinctly different from these others, which studied only the haploid and/or homozygous diploid knockout mutant libraries that lack mutants of essential genes. In contrast, we first studied the genome-wide heterozygous diploid mutants, which directly and dramatically revealed the essential TRiC complex as a candidate target of As(III) via
drug-induced haploinsufficiency. We subsequently corroborated this finding by identifying As(III)-hypersensitive and -resistant haploid mutants. In particular, we have put an emphasis on the most sensitive haploid knockout mutant(s) with the premise that they likely affect functions most closely related to the arsenic target(s). That all 6 mutants of the GimC complex exhibited the highest sensitivity towards low concentrations of As(III) (Fig. 2B and Supplementary Table 2) further supported the idea that TRiC is the target. More importantly, we provided further evidence that As(III) inhibits TRiC functions both in vivo and in vitro. Such a conclusion is also consistent with a previous observation made in mouse Swiss 3T3 cells that treatment with 30 μM of arsenic leads to distorted organization of both actin and microtubule filaments (LI and CHOU 1992). Lastly, with subtle modification of the previously described dSLAM methodology (PAN et al. 2004), we also demonstrated that it is possible to systematically identify genome-wide double mutations that confer hypersensitivity to a given drug in a high throughput manner. This will allow for expansion in the elucidation of genetic interaction networks involved in drug response to beyond monogenic traits.

Despite that As(III) concentrations used in most of our experiments were relatively high, our results are likely relevant to arsenic effects on human health. Firstly, our results with the apparently healthy arr1Δ pfdlΔ and arr3Δ pfdlΔ double mutants (Fig. 5B) have demonstrated that low concentrations of arsenic can inhibit cell growth in yeast. That human cells are more susceptible might also be due to stress-induced apoptosis that is basically lacking in yeast cells. Secondly, both yeast and human cells accumulate high levels of arsenic after exposure to low concentrations and this is more obvious in human cells than in yeast. It was shown that ~10^6 wild-type yeast cells
exposed to 160 µM of arsenic for 2 hours accumulate ~0.163 nmol of the drug (DILDA et al. 2008), equivalent to a final intracellular concentration of ~2.3 mM, assuming an average volume of a haploid yeast cell of ~70 fl (SHERMAN 2002). Similarly, ~10^6 human APL cells (NB4) exposed to 20 µM of arsenic for 4 hours accumulated ~0.73 nmol of the drug (DILDA et al. 2008), equivalent to an intracellular concentration of ~0.7 mM, assuming an average volume of of ~1,040 fl for an NB4 cell (MIOSSEC-BARTOLI et al. 1999). As(III) concentrations within cells of certain human organs and tissues or within some intracellular compartments might be even higher. Thus our observation of TRiC inhibition by 1 mM of As(III) in vitro could be relevant in vivo even when mammalian cells are exposed to relatively low levels of As(III). Thirdly, our in vitro assays might have under-detected the potency of arsenic inhibition of TRiC. In the protein-folding assays, we had to include 1 mM of DTT, which is absolutely required for substrate folding by TRiC and MM-cpn (Frydman, unpublished observations). The presence of DTT in the folding assays likely at least partially reversed the inhibitory effects of arsenic on TRiC and MM-cpn. Unfortunately, we could not test the effects of lower concentrations of As(III) on TRiC activities due to this technical limitation of the assay.

Among the heavy metals that interact with thiol groups, As(III) inhibition of TRiC seems to be specific. One piece of supporting evidence is that mutants of the prefoldin complexes, which are hypersensitive to As(III), did not exhibit sensitivity toward Cd^{2+} when compared to a wild-type strain (Data not shown). However, currently it is not clear how As(III) inhibits the TRiC complex at the biochemical level. It did not seem to inhibit TRiC’s substrate binding (Fig. 4C) or its ATPase activity. In fact, arsenic
stimulated the ATPase activity of TRiC by ~2-fold (data not shown). Given the similar inhibitory effects observed when As(III) was added both before and shortly after substrate binding (Fig. 4C), it is possible that it blocks a late step of the process, for example, release of correctly folded products from the chaperonin complex. This might gradually lead to accumulation of unproductive TRiC complex and reduction in its overall productivity. This might partly explain why arsenic reproducibly inhibits TRiC binding to Cdc55 but not actin and tubulin. Possibly, most TRiC molecules within arsenic treated cells are stuck with the more abundant substrates such as actin and tubulin.

That TRiC is required for folding and maturation of as many as ~9-15% of all cytosolic proteins in mammals (THULASIRAMAN et al. 1999) might also at least partly explain the pleiotropic effects of arsenic on human health. For example, exposure to arsenic has been linked to cancers and neuropathy. The former might be related to the fact that TRiC is required for the assembly of the Von Hippel-Lindau (VHL) tumor suppressor complex (FELDMAN et al. 1999), which plays a positive role in stabilizing and activating p53 (ROE et al. 2006), a major tumor suppressor commonly mutated in various human cancers. Arsenic inhibition of TRiC might thus indirectly down-regulate p53 activity and cause cancers, a model consistent with the observation that arsenic inhibits p53 activation in response to DNA damage (TANG et al. 2006). In addition, mutating TRiC subunit genes CCT5 and CCT4 are directly implicated in sensory neuropathy in both human patients and in a rat model (BOUHOUCHE et al. 2006; LEE et al. 2003).

**Acknowledgements**
We thank Katja Siegers for providing the anti-Cct5p antibody and a yeast strain that expresses Cdc55p-3HA (SIEGERS et al. 2003). We are also grateful to David Drubin and Andy Hoyt for anti-α-tubulin antibodies and Pamela Meluh for an anti-β-tubulin antibody. We thank Robert Cohen and Pamela Meluh for helpful comments on the manuscript. This work was supported in part by NIH grants HG02432 and RR020839 to J.D.B, by NIH grant HG004840 to X.P, by NIH grant RR019409 to J.M.M, and by NIH grant GM74074 and a grant from the NIH Roadmap Initiative on Nanomedicine to J.F.

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Figure Legend

Figure 1. Genetic alterations in the TRiC complex affect arsenic-susceptibility. A, As(III)-sensitive heterozygous diploid YKOs identified by genome-wide mutant fitness profiling. Essential genes are colored in red and nonessential genes in black. The network diagram was created with Cytoscape 2.0 (SHANNON et al. 2003). B, Sensitivity of the heterozygous diploid YKOs of all 8 TRiC subunits to As(III) at 450 µM. An ho/hoΔ mutant phenotypically indistinguishable from a wild-type yeast, served as the control strain. C, An hoΔ mutant harboring a vector or a plasmid overexpressing a TRiC subunit as indicated was tested for growth on solid SC-Ura that either contained or lacked 1 mM As(III).

Figure 2. Arsenic-sensitive haploid YKOs and their genetic relationships with CCT1. A, As(III)-sensitive haploid deletion mutants identified by genome-wide mutant fitness profiling. A total of 191 were individually verified to be sensitive to As(III) at 400 µM. The number of mutants in each biological process affected and the corresponding percentage among all mutations identified were listed. This plot was derived from Supplementary Table 2. B, Growth of representative arsenic-sensitive haploid YKOs of indicated genotypes on a solid synthetic complete (SC) medium that either lacked or contained As(III) at 40 µM, 150 µM, and 800 µM. C, Partial suppression of the arsenic-sensitivity by CCT1 overexpression in various mutants.

Figure 3. The rate of protein synthesis and free β-tubulin modulate yeast arsenic-sensitivity. A, The distribution of arsenic-resistant haploid YKOs according to
biological processes affected. This plot was derived from Supplementary Table 3. **B,** Cells of indicated genotypes were grown in an SC medium that either contained or lacked 800 µM of As(III). Cycloheximide was used at 10 ng/ml. **C,** One of the α-tubulin genes TUB3 was overexpressed from a 2µ plasmid in mutants defective in microtubule biogenesis. Cells were grown on solid SC–Ura either in the presence or absence of As(III). As(III) concentration used was 600 µM for the hoΔ, tub3Δ, and cin2Δ mutants and 50 µM for the gim3Δ and pfd1Δ mutants. **D,** The β-tubulin gene TUB2 and the actin gene ACT1 were expressed in mutants of indicated genotypes from a centromere-based plasmid. Cells were grown on solid SC that lacked uracil (SC–Ura) either in the presence or absence of 200 µM of As(III). **E,** Strains of indicated genotypes were grown on solid YPD with or without As(III) (1 mM) or benomyl (20 µg/ml).

**Figure 4. As(III) inhibits TRiC functions.** **A,** Wild-type yeast (BY4743) cells were grown in liquid YPD either in the absence or presence of 1 mM of As(III) for 3 hours. Actin was stained with Rhodamine-phalloidin and microtubules were visualized with an anti-α-tubulin antibody. **B,** ATP-depleted cell lysates prepared from yeast cells expressing Cdc55-3HA grown in liquid YPD that either contained or lacked 1 mM As(III) were subjected to immunoprecipitation with anti-HA antibody. Western blots of the total cell extracts (5µg) and the immunoprecipitates (IP) from lysates containing 200µg total protein were analyzed with an anti-HA antibody and an antibody against one of the TRiC subunits Cct5. **C,** In vitro binding and folding of denatured [35S]-actin by bovine TRiC both in the presence and absence of 1 mM As(III) were assessed by native gel analysis followed by autoradiography. Three (2, 3, and 4) different schemes of
arsenic treatment were tested. Native \[^{35}\text{S}\]-actin samples incubated with or without As(III) were included as controls (right two lanes). The extent of As(III) inhibition in each condition was quantified for 3 independent experiments and expressed as \% Actin Folding relative to the untreated control. D, \textit{In vitro} Rhodanese folding by the \textit{M. maripaludis} TRiC-like chaperonin (Mm-cpn) was assessed in the presence and absence of 1 mM sodium arsenite as described (KUSMIERCZYK and MARTIN 2003).

\textbf{Figure 5. A genome-wide screen identifies double mutants hypersensitive to low concentrations of arsenic.} A, A high efficiency \textit{pfd1}Δ::\textit{URA3} gene disruption cassette was transformed into a pool of haploid-convertible heterozygous diploid yeast knockout mutants. After sporulation, a pool of haploid double knockout mutants were derived from the heterozygous diploid double mutant pool in the presence of 5 \textmu M of As(III). A separate pool of haploid single and double mutants were also similarly derived either in the presence of absence of 5 \textmu M of As(III). Relative representation of each knockout mutation in both pools was compared by TAG-array analysis (PAN \textit{et al.} 2004). B, Haploid-convertible \textit{ARR1}/\textit{arr1}Δ::\textit{kanMX} \textit{PFD1}/\textit{pfd1}Δ::\textit{URA3} and \textit{ARR3}/\textit{arr3}Δ::\textit{kanMX} \textit{PFD1}/\textit{pfd1}Δ::\textit{URA3} diploid double mutants were sporulated and spotted on a haploid selection medium that either lacked both uracil and G418 (to select for \textit{Ura}+ single and double mutants), contained both uracil and G418 (to select for G418-resistant single and double mutants), or lacked uracil but contained G418 (to select for the double mutants). Cell growth under each condition was assessed both in the absence (control) and presence of 2 \textmu M or 5 \textmu M of As(III) and photographed.
Pan Fig. 1

A

Microtubule biogenesis
TRiC complex components

As(III) sensitivity
physical interaction

B

HO/hoΔ
CCT1/cct1Δ
CCT2/cct2Δ
CCT3/cct3Δ
CCT4/cct4Δ
CCT5/cct5Δ
CCT6/cct6Δ
CCT7/cct7Δ
CCT8/cct8Δ

C

control 1 mM

vector

2μ CCT1
2μ CCT3
2μ CCT4
2μ CCT5
2μ CCT6
2μ CCT7
1. Arsenic detoxification & uptake (3.7%)
2. Actin & microtubule biogenesis; chromosome segregation (14.7%)
3. DNA damage response & repair (14.7%)
4. Oxidative stress response (3.1%)
5. Histone modification & transcription regulation (11.5%)
6. Mitotic cell cycle progression (4.2%)
7. Peroxisome biogenesis (7.3%)
8. Ergosterol biosynthesis & cell wall integrity (4.7%)
9. Protein transportation & secretion (7.9%)
10. PKA signaling (2.6%)
11. Others (25.7%)

Pan Fig. 2
Pan Fig. 3

A

1. As(III) uptake (0.9%)
2. Ribosomal structure & biogenesis (62.4%)
3. mRNA transcription & processing (11.0%)
4. Others (25.7%)

B

control 800 μM

hoΔ rpl19bΔ rps23bΔ
cycloheximide (in hoΔ mutant)

C

vector 2μ TUB3

hoΔ
tub3Δ
cin2Δ
gim3Δ
pfd1Δ
control 600 μM
control 600 μM
control 50 μM
control 50 μM

D

control 200 μM

VECTOR ACT1 CEN TUB2 CEN

HO/hoΔ

CCT6/cct6Δ

vector ACT1 CEN TUB2 CEN

E

control As(III) Benomyl

hoΔ
tub2-DAmP
cct1-DAmP
cct2-DAmP

control

CCT6/cct6Δ

vector ACT1 CEN TUB2 CEN
Pan Fig. 4

(a) Actin and Microtubule control 1 mM cell extract

(b) Anti-HA cell extract

(c) Anti-CctS cell extract

(d) % Folded Rhodanese

Experiments:
1. TRiC + denatured 35S-actin + ATP
2. TRiC + denatured 35S-actin + ATP + As(III)
3. TRiC + As(III) + denatured 35S-actin + ATP
4. TRiC + denatured 35S-actin + ATP + As(III)

% Actin Folding: 100 21±6 31±5 30±3

N. Native 35S-actin
TRiC bound 35S-actin
N. Native 35S-actin
35S-actin

Chaperonin ATP As(III) - - + + + + - - + + + - - + + + +
Pan Fig. 5

(a) Haploid-convertible heterozygous diploid pool

ptd1::URA3

Heterozygous diploid double mutant pool

with or without 5 μM As(III)

Haploid conversion

single & double mutants

Tag-array Analysis

double mutants

(b) -uracil -G418 +uracil +G418

arr1Δ

arr2Δ

arr1Δ +G418

arr2Δ +G418

control

2 μM

5 μM