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Abstract

Arsenic, a known human carcinogen, is widely distributed around the world and found in particularly high concentrations in certain regions including Southwestern US, Eastern Europe, India, China, Taiwan and Mexico. Chronic arsenic poisoning affects millions of people worldwide and is associated with increased risk of many diseases including arthrosclerosis, diabetes and cancer. In this study, we explored genome level global responses to high and low levels of arsenic exposure in *Caenorhabditis elegans* using Affymetrix expression microarrays. This experimental design allows us to do microarray analysis of dose-response relationships of global gene expression patterns. High dose (0.03%) exposure caused stronger global gene expression changes in comparison with low dose (0.003%) exposure, suggesting a positive dose-response correlation. Biological processes such as oxidative stress, and iron metabolism, which were previously reported to be involved in arsenic toxicity studies using cultured cells, experimental animals, and humans, were found to be affected in *C. elegans*. We performed genome-wide gene expression comparisons between our microarray data and publicly available *C. elegans* microarray datasets of cadmium, and sediment exposure samples of German rivers Rhine and Elbe. Bioinformatics analysis of arsenic-responsive regulatory networks were done using FastMEDUSA program. FastMEDUSA analysis identified cancer-related genes, particularly genes associated with leukemia, such as *dnj-11*, which encodes a protein orthologous to the mammalian ZRF1/MIDA1/MPP11/DNAJC2 family of ribosome-associated molecular chaperones. We analyzed the protective functions of several of the identified genes using RNAi. Our study indicates that *C. elegans* could be a substitute model to study the mechanism of metal toxicity using high-throughput expression data and bioinformatics tools such as FastMEDUSA.

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Introduction

Arsenic is a metalloid, which is distributed throughout the Earth crust in diverse complex forms with pyrites. Depending on the physicochemical conditions of the environment, arsenic can readily be dissociated from the complex, enter into ground water [1] and be taken up by microorganisms resulting in high levels of bio-availability [1,2]. In Asia, including India, Bangladesh, Vietnam, Thailand and China millions of people are exposed to arsenic. Two different oxidative states of arsenic, (III) and (V), are available in organic and inorganic forms that correlate with their cytotoxic potentials. Between these two states, compounds with (+3) oxidation state are more toxic to target cells and tissues due to several mechanisms including high affinity for protein thiols or vicinal sulfhydryl groups [3–8].

Chronic and/or acute high dose arsenic exposure can cause wide range of health problems including cancer, severe gastrointestinal toxicity, diabetes, cardiovascular disease and even death [5,8,9]. Arsenic is considered as a group 1 carcinogen, a categorical classification of an agent/mixture, which is definitely carcinogenic

to humans [10]. Since carcinogenic metals, including arsenic, tend to be weak mutagens, and they do not directly interact with DNA, several recent studies have suggested that epigenetic regulation may play a role in metal-induced carcinogenesis [11].

Although the metabolism of inorganic arsenic is quite well known, the precise mechanism of arsenic toxicity is not clearly understood. In mammals, a methylation pathway has been proposed for the metabolic processing of inorganic arsenicals. In this pathway, arsenite (iAs^{III}) is sequentially converted to monomethylarsonic acid (MMA^V) and dimethylarsinic acid (DMA^V) in both humans and laboratory animals including mice and rats. The intermediate arsenicals, MMA^{III} and DMA^{III} , also produced in this pathway, are highly toxic and suspected to be responsible for arsenic toxicity [12]. While some steps in this pathway are strictly chemical reactions, others are enzymatically catalyzed. However, work to date has identified one methyltransferase that is clearly a participant in this pathway. Arsenic (+3 oxidation state) methyltransferase (AS3MT)1 catalyzes conversion of iAs to methylated products. AS3MT homologs have not been identified in *C. elegans* genome [13]. Other aspects of arsenic

metabolism in *C. elegans* remain to be seen. Arsenic causes oxidative stress, apoptosis and mutagenesis [14–16]. Oxidative stress through generation of reactive oxygen species due to arsenic exposure [17–20] have been reported in tumor cell lines [21] as well as in normal human cells [22,23].

While arsenic is mostly documented as an inducing factor in cancers or several other diseases, there is extensive evidence that one form of arsenic, As₂O₃, has a potential antitumor effect *in vitro* and *in vivo* [24–26]. United States Food and Drug Administration (US-FDA) approved As₂O₃ for the treatment of Acute Promyelocytic Leukemia (APL). It's well established that As₂O₃ can completely cure ~80–90% of newly diagnosed APL patient [24–26].

C. elegans, a model organism that is less complex than the mammalian system while still sharing high genomic homology, provides an excellent model to elucidate the mechanisms of heavy metal toxicity [27]. This soil nematode has been used in toxicology studies, revealing molecular mechanisms of heavy metal toxicity [28], [29], [30]. Therefore, the *C. elegans* model system is valuable for the investigation of metal toxicity and may be particularly useful for examining gene-environment interactions. Several toxicity endpoints are well documented in the nematode, including growth rate, lifespan, reproduction, and feeding [31,32]. Acute toxicity can also be assessed in the nematode using altered gene expression levels, as well as behavioral endpoints, such as locomotion, and head thrashing [33–37]. Several cellular stress response systems such as the glutathione (GSH), metallothioneins (MTs), heat shock proteins (HSPs), as well as a variety of pumps and transporters are found to work to detoxify and excrete metals in *C. elegans* [27]. Previously, whole genome *C. elegans* DNA microarray and RNAi analysis were used to explore global changes in this nematode to understand mechanisms involved in resistance to cadmium toxicity [38].

In this study we used *C. elegans* whole genome expression microarrays to examine global changes in the nematode transcription profile upon arsenic exposure. Bioinformatics analysis of regulatory networks was done using FastMEDUSA. We analyzed the protective functions of several of the identified genes using RNAi. Molecular players previously associated with arsenic exposure in higher organisms were identified at a global level, confirming the effectiveness of the study. Moreover, we identified evolutionary conserved genes which were not previously associated with arsenic exposure, but associated with carcinogenesis.

Materials and Methods

Bacterial strains, media and culture conditions

Eight RNAi bacterial strains were used in this study including: *sdz-8*, *fin-1*, *hsp-70*, *numr-1*, *aip-1*, *gst-37*, *gcs-1*, and L4440 (empty vector control) [39].

C. elegans strains and maintenance

Strains N2, NL2099 *rif-3(pk1426)*, VC1642 *dnj-11(gk1025)*, and VC392 *dac-1(gk211)* were acquired from *Caenorhabditis* Genetics Center (CGC). Strains were maintained at 22°C. The wild type Bristol strain N2, was cultured in *C. elegans* habitation media (CeHM) in tissue culture flasks on a platform shaker [40]. Nematodes were bleached (0.5M NaOH, 1% Hypochlorite) to collect eggs which were incubated in M9 media for 24 hours to bring them to synchronized L1 stage and then transferred to *C. elegans* habitation media (CeHM).

Arsenic treatment for microarray experiments

Synchronized L1 stage animals were collected by spinning at 800 rpm for five minutes and transferred to Sodium arsenite containing (0.03% and 0.003% w/v) CeHM media and incubated at 22°C for 6 hours.

RNA Isolation

After arsenic treatment animals were collected and washed in M9 buffer, RNA was extracted using TRIzol reagent (Invitrogen). Residual genomic DNA was removed by DNase treatment (Ambion, Austin, TX). Three independent RNA isolations were performed with each condition for microarray analysis.

Microarray Analysis

For each experimental condition, RNA was isolated from three biological replicate samples. cRNA was synthesized from 10 µg of total RNA, and samples were hybridized to the *C. elegans* GeneChip (Affymetrix, Santa Clara, CA) by the US Food and Drug Administration/CFSAN/DMB Microarray Facility following the manufacturers instruction. The chip represents 22,500 transcripts of the expressed *C. elegans* genome based on the December 2005 genome sequence. The data were processed using Partek Genomics Suite, version 6.5 (Copyright © 2010 Partek Inc., St. Louis, MO, USA). The robust multichip averaging (RMA) algorithm was used to normalize and summarize the probe data into probeset expression values. The RMA algorithm performs background correction, normalization, and summarization using PM-only probes (Figure S1). A gene usually maps to several probesets. To convert probeset-level expression data to gene-level, we picked the highest-intensity probeset of each gene. We used ANOVA to compute differentially expressed genes between experimental treatment groups and control using the log transformed normalized intensity values generated from application of the RMA algorithm. We used the FDR for multiple comparison correction. Genes were considered differentially expressed if they had a p-value ≤ 0.05 after correction. The microarray data have been deposited in the GEO repository. Accession number is GSE39012.

Functional Enrichment Analysis

Genes showing a significant change in expression by microarray analysis ($FDR < 0.05$) were analyzed using 'stats' R package of R software (R Development Core Team [2012]: A language and environment for statistical computing. R foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org>). Genes were compared against a 21,249 *C. elegans* gene database to identify over-represented Gene Ontology terms. Statistical analysis was performed using chi-square test and the Yates' continuity correction. Significant functional terms were defined as $p < 0.05$.

qRT-PCR

cDNA was synthesized from 5 µg of total RNA using random hexamers and SuperScript II reverse transcriptase (Invitrogen). Real time PCR was performed using SYBR Advantage quantitative PCR premix (Clontech) and gene-specific oligonucleotide primers on the LightCycler (Roche). Primers for qRT-PCR are listed on Table S1. Relative fold-changes for transcripts were calculated using the comparative $C_T(2^{-\Delta\Delta C_T})$ method [41]. Cycle thresholds of amplification were determined by Light Cycler software (Roche). All samples were run in triplicates and normalized to GAPDH.

RNA Interference

E. coli DH5 α bacterial strains expressing double-stranded *C. elegans* RNA [42] were grown in LB broth containing ampicillin (100 $\mu\text{g}/\text{ml}$) at 37°C and plated onto NGM containing 100 $\mu\text{g}/\text{ml}$ ampicillin and 3 mM isopropyl 1-thio- β -D-galactopyranoside (IPTG). RNAi-expressing bacteria were allowed to grow overnight at 37°C. Synchronized L1 stage NL2099 (*rif-3*) strains were used for RNAi experiments for the functional validation of the differentially expressed genes identified through microarray. NL2099 (*rif-3*) worms were exposed to fresh RNAi expressing bacterial lawn on NGM agar plates for 48 hours, then washed with M9 and plated on sodium arsenite containing NGM plates with *E. coli* OP50 bacterial lawn, and incubated at 22°C (See '*C. elegans* survival assays for arsenic exposure following RNAi' section below). L4440 RNAi which contains the RNAi plasmid only was included as a control in all experiments.

C. elegans survival assays for arsenic exposure following RNAi

Sodium arsenite containing (0.03%) nematode growth media (NGM), in 6-cm Petri plates, were prepared for survival assays. The plates contained a lawn of OP50 bacteria as a food source. Plates were incubated overnight at room temperature before animals were added. Worms (L1 stage), treated with RNAi bacteria for 48 hours, were transferred to sodium arsenite containing NGM plates with OP50 bacterial lawns and incubated at 22°C. Around 20–30 L4 stage worms were added to each plate. Total 75 to 100 animals were scored for each condition every 24 h for survival and transferred to fresh bacterial lawns every day to avoid overgrowth by progeny. Assay was continued up to ten days. Animal survival was plotted using Kaplan-Meier survival curves and analyzed by log rank test using Graph Pad Prism (Graph Pad Software, Inc., La Jolla, CA). Survival curves resulting in *p* values of <0.05 relative to control were considered significantly different.

FastMEDUSA analysis

We used FastMEDUSA [43] to elucidate transcription factors (TFs) that putatively regulate the genome-level responses to high and low levels of arsenic exposure in *C. elegans*. FastMEDUSA applies a machine learning algorithm called boosting to train a predictive model from expression and promoter sequences of genes in a number of experimental conditions. FastMEDUSA uses a list of candidate TFs, the promoter sequences of all the genes and a matrix of discrete expression data as input. To discretize gene expression data, we computed fold change of expression signal of a gene in a sample to the gene's median expression across reference samples. A gene in a sample was called upregulated if the fold change ≥ 1.5 and downregulated if the fold change is ≤ -1.5 . Genes having inconsistent expression calls across technical replicates were filtered out. We obtained the list of candidate TFs in *C. elegans* from EDGEDb [44], and obtained 1,000 bp promoter sequence of genes from BioMart [45].

FastMEDUSA potentially builds a different model at each run as it contains some stochastic steps. Thus, we ran FastMEDUSA five times using a different random seed value at each run on the Biowulf cluster at the National Institutes of Health. For each FastMEDUSA run, we computed significance score of TFs as following. First, we computed prediction score for the upregulated genes in the experimental condition based on the original FastMEDUSA model. Then, we remove the TF from the FastMEDUSA model and recomputed the prediction score for the same gene set. The difference between the prediction scores give the significance score of the TF (details in [46]). We selected

top 20 TFs with highest significance score. Then we selected top ten consensus significant TFs that were selected as significant in at least four out of five runs. To find significant TF-gene associations, we computed the significance score for each TF-gene pair. We selected TF-gene associations that had a significance score ≥ 1 for at least four out of five runs and generated a network of these associations by using Cytoscape [47].

Results and Discussion

Arsenic exposure induced genome-wide gene expression changes in *C. elegans*

Arsenic induced global gene expression has been poorly explored. To study the global gene expression pattern after acute arsenic exposure, we performed a microarray study where wild type L4 stage *C. elegans* (N2) was exposed to sodium arsenite in two different concentrations (0.03% and 0.003% w/v) in CeHM media for 6 hours. Differentially expressed genes were identified (considering fold change (+/-) 1.2 fold, FDR = 0.05 and $P < 0.05$). *C. elegans* gave a strong global gene expression response to sodium arsenite where about one fifth of the genome (4731 genes) was differentially expressed upon high dose (0.03% w/v) exposure. Low dose (0.003% w/v) sodium arsenite led to differential expression in 218 genes, 179 of those were common between the two exposures (Fig. 1). Microarray data were confirmed using qRT-PCR to measure the expression levels of a set of selected genes (Fig. S2).

Comparison of gene expression changes between high and low levels of sodium arsenite exposure

We exposed worms to two different concentration of sodium arsenite to evaluate the genomic responses to different levels of arsenic exposure. This experimental design allows us to do microarray analysis of dose-response relationships of global gene expression patterns. High dose (0.03%) exposure caused stronger global gene expression changes in comparison with low dose (0.003%) exposure (Fig. 1, Table S2). Two hundred and four genes were up regulated four fold and higher upon high dose exposure, and forty nine genes were up regulated four fold and up upon low dose exposure. Forty six of these were common between these lists (Table 1). Forty three of forty six commonly upregulated genes show dose-response relationship where high levels of sodium arsenite led to higher gene expression levels in *C. elegans* (Table 1). At eight hours exposure we did not observe anatomical level changes in tissue structure, and lethality (data not shown).

Protective function of the subset of the genes up-regulated against arsenic treatment was evaluated using RNAi

We wanted to test whether knocking down the upregulated genes will affect the sodium arsenite induced lethality in *C. elegans*. Four out of seven genes tested, caused statistically significant increase in lethality upon sodium arsenite exposure when knocked-out via RNAi, suggesting that these genes may have stress response function against arsenic (Fig. 2). Among these genes, *aip-1* encodes an AN-1-like zinc finger-containing protein homologous to arsenite-inducible RNA-associated protein (AIRAP), which is conserved among *C. elegans*, *Drosophila*, and mammals. AIP-1 is a predicted RNA binding protein that may function in ubiquitin-mediated proteolysis following arsenite treatment. AIP-1 is expressed at high levels in hypodermal and intestinal cells of *C. elegans* following arsenic exposure, and previously shown to protect *C. elegans* and mammalian cells from arsenite toxicity [48]. Our *aip-*

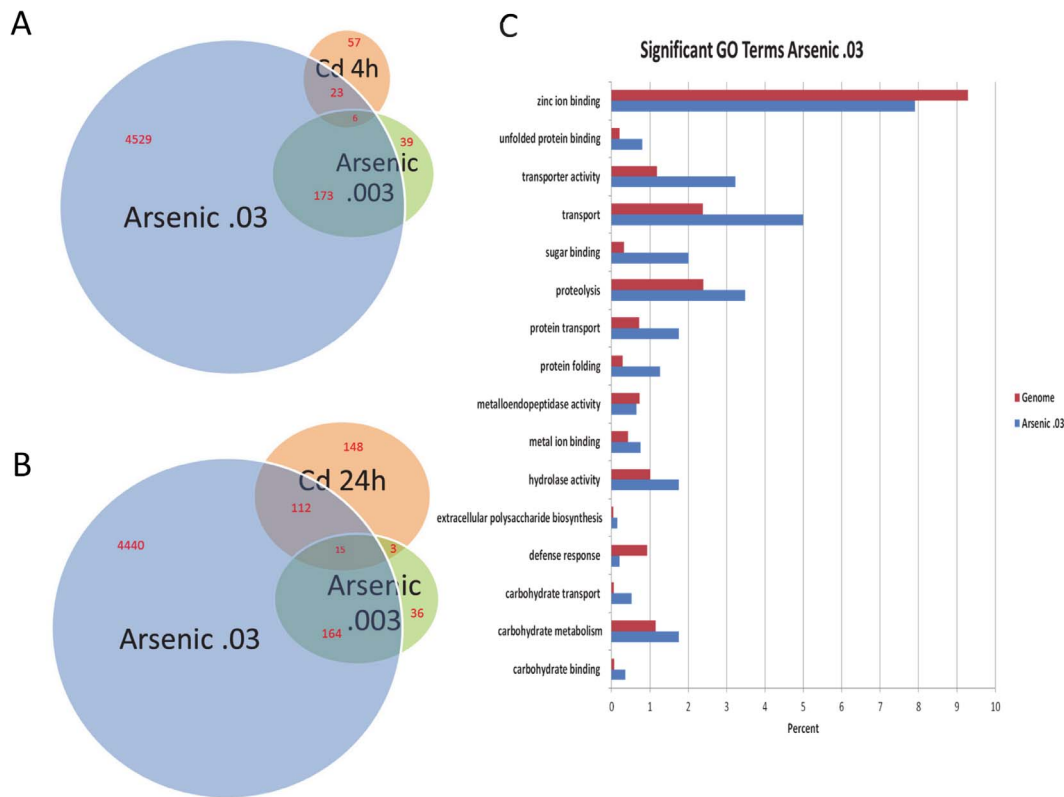


Figure 1. Genomic-wide expression profile comparisons of *C. elegans* genes regulated by arsenic and cadmium. (A) A Venn diagram illustrating number of genes expressed at low and high dose arsenic exposure, 4-hour cadmium exposure, and overlap among these data. (B) A Venn diagram illustrating number of genes expressed at low and high dose arsenic exposure, 24-hour cadmium exposure, and overlap among these data. (C) Enrichment of gene ontology categories for genes differentially expressed at high dose arsenic exposure. doi:10.1371/journal.pone.0066431.g001

I RNAi results agree with the previously published data (Fig. 2A). *gcs-1* encodes the *C. elegans* ortholog of gamma-glutamylcysteine synthetase heavy chain (GCS(h)), which is predicted to function as a phase II detoxification enzyme that catalyzes the rate-limiting first step in glutathione biosynthesis, in a conserved oxidative stress response pathway [49]. Inoue et al [50] showed that the *Caenorhabditis elegans* PMK-1 p38 MAPK pathway regulates the oxidative stress response via the CNC transcription factor SKN-1, leading to phosphorylated SKN-1 accumulation in intestine nuclei, where SKN-1 activates transcription of *gcs-1*. SKN-1 also regulates expression of AIP-1 [51]. We found that most of the *C. elegans* Glutathione S-transferases (GSTs), which are important detoxifying enzymes, responded to arsenic exposure (Table 2). Among these genes, *gst-37*, previously defined as a acrylamide responsive gene in *C. elegans* using expression microarrays [52]. *gst-37* RNAi experiments resulted in increased lethality in arsenic exposure conditions (Figure 2B). In our microarray data several *hsp* (heat-shock protein) genes found to be responsive to sodium arsenite (Table 2). HSP-70 is a member of the *hsp70* family of molecular chaperones, involving in general stress response, including response to heat and cadmium exposure, in *C. elegans* [30,53]. We found that *hsp-70* RNAi leads to increased lethality in arsenic exposure conditions (Figure 2D). Arsenic toxicity leads to induced HSP70 in other systems including *Xenopus laevis* embryos [54], and broiler chickens [55]. Oxidative stress, the central component of heat shock response, is also induced by arsenic [56].

Oxidative stress- response genes are induced due to sodium arsenite exposure

Our microarray data revealed that genomic response of *C. elegans* to sodium arsenite exhibits characteristics of global oxidative stress response (Fig. 3). Oxidative stress from arsenic exposure might result from production of Reactive Oxygen Species (ROS), such as superoxide, hydrogen peroxide, or hydroxyl radical by arsenicals, or from release of iron from ferritin or through induction of heme oxygenase. Increased biosynthesis of defensive enzymes responsive to oxidative stress has been described in both prokaryotes and eukaryotes. We compared our arsenic-response microarray data with previously published *C. elegans* global stress-response results. *C. elegans*' response to both, paraquat- induced stress [57] (Fig. 3A, Table S4), and hyperbaric oxygen-induced stress [58] (Fig. 3B, Table S5) showed significant overlap with our arsenic-response microarray data. We performed Gene Ontology (GO) Term enrichment analysis on our high dose arsenic response data (Figure 1C) along with paraquat and hyperbaric oxygen stress data (Figure 3C and 3D). General stress related GO categories, such as 'unfolded protein binding', 'protein folding', 'protein transport', and proteolysis were found to be enriched under high dose arsenic exposure conditions (Fig. 1C). Some of the protein folding, and transport related GO term enrichments were also present in paraquat stress data but not in hyperbaric oxygen stress data (Fig. 3C, D), suggesting that arsenic and paraquat result in similar functional responses in *C. elegans*. Interestingly, expression of zinc ion binding gene classes was depleted in all of these stresses

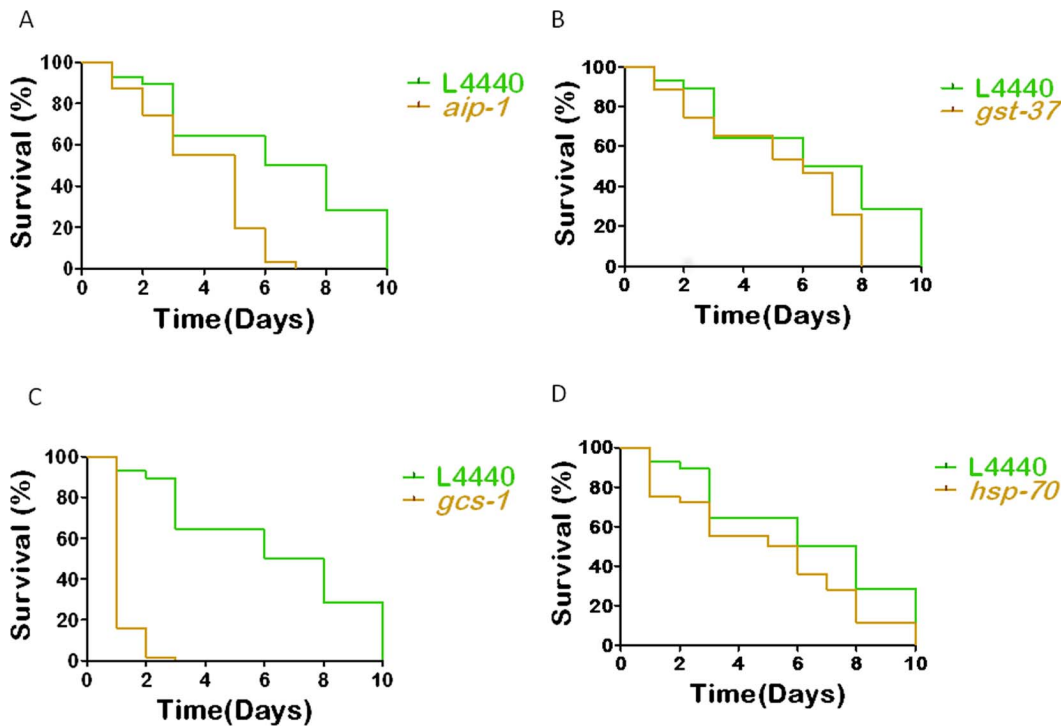


Figure 2. Arsenic induced genes mediate stress response. (A) *aip-1*, $p < 0.0001$, (B) *gst-37*, $p = 0.0085$, (C) *gcs-1*, $p < 0.0001$ and (D) *hsp-70*, $p = 0.04$, RNAi results in lethality in *C. elegans* exposed to sodium arsenite. doi:10.1371/journal.pone.0066431.g002

(Fig. 1C, 3C–D). The essential trace element zinc is broadly required in cellular functions, and disturbances in zinc homeostasis cause a range of health problems that include growth retardation, immunodeficiency, neuronal and sensory dysfunctions [59].

Glutathion S-transferases (GSTs) are essential detoxifying enzymes that constitute up to 10% of cytosolic protein in some mammalian organs, and catalyze the conjugation of reduced glutathione on a wide variety of substrates [60,61]. This activity detoxifies endogenous compounds such as peroxidised lipids [62]. GSTs may also bind toxins and function as transport proteins [63]. *C. elegans* genome possesses a large number of GST genes. We found that sixty seven percent of (thirty three of forty nine) the *C. elegans* *gst* genes are differentially expressed upon arsenic exposure (Table 2). Other genes encoding antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase are differentially expressed in arsenic-exposed *C. elegans* (Table 2). Lynn et al. [64] reported that arsenite activates NADH oxidase to produce superoxide, which then causes oxidative DNA damage. We found that putative NADH oxidase encoding gene F56D5.3 is upregulated 43 fold in arsenic exposed *C. elegans* (Table 2). Recent studies revealed an association between sonic hedgehog signaling and oxidative stress in several different tissues including rat brain and mouse bone marrow [65,66]. Twenty of the fifty eight hedgehog related genes of *C. elegans*, found to be differentially expressed upon arsenic exposure (Table 2). Functions of sonic hedgehog signaling genes in arsenic toxicity and protection remain to be seen.

Arsenic-induced perturbations in iron metabolism may lead to oxidative stress

Almost all cells utilize iron as a cofactor for essential biochemical activities, such as oxygen transport, energy metabo-

lism and DNA synthesis. However, iron catalyses the propagation of ROS and generation of highly reactive radicals through fenton chemistry, hence, free iron is potentially toxic to cells [67,68]. Much of the excess intracellular iron is stored in the cytosol, bound to ferritin. Very little is known about the interaction of the species of arsenic with free iron at the cellular level. Release of iron from ferritin is an under investigated possible mechanism of arsenic induced oxidative stress. It has been shown that arsenic species can cause release of iron from horse spleen ferritin *in vitro* [69]. Iron administration into HeLa cells leads to increased ferritin mRNA levels [70]. We found that ferritin encoding genes of *C. elegans*, *fhn-1*, and *fhn-2* are upregulated upon sodium arsenite exposure (table 2). There is strong experimental support suggesting a protective role for ferritin against oxidative stress. Both transcriptional and posttranscriptional mechanisms have been implicated in ferritin induction by oxidants, such as ROS, and nitric oxide [71,72]. *C. elegans* homologs of iron transporter ferroportin, *fhn-1.1* and *fhn-1.2* are also differentially expressed against sodium arsenite (Table 2). Sideroflexins are recently discovered mitochondrial multiple transmembrane proteins with unknown function, which are associated with iron accumulation in mitochondria [73]. We found that *C. elegans* sideroflexin genes *sfxn-2* and *sfxn-5* are downregulated upon arsenic exposure. Altogether, our data suggest that arsenic may induce perturbations in proteins involved in iron metabolism.

Genomic response to arsenic versus cadmium in *C. elegans*

Heavy metals such as copper, zinc, cadmium, and metalloids such as arsenic, are major environmental toxicants that are associated with a variety of human diseases. In spite of extensive research on the pathogenesis of human diseases which are linked

Table 1. List of genes up-regulated (>4 times) in both high and low dose arsenic exposure.

Gene name	Brief description	Fold-Change (03% vs No Treatment)	p-value	Fold-Change (003% vs No treatment)	p-value
<i>numr-1</i>	unknown	140.416	4.44E-09	25.1465	5.73E-08
<i>W06H8.2</i>	unknown	91.5165	2.23E-09	25.2296	1.67E-08
<i>gst-30</i>	glutathione S-transferase	64.4519	2.75E-10	12.8297	5.18E-09
<i>T19D12.3</i>	unknown	63.3912	2.67E-09	16.5752	2.76E-08
<i>ftn-1</i>	ferritin heavy chain	55.3853	2.60E-08	5.7706	3.57E-06
<i>hsp-70</i>	heat shock protein 70	53.7595	8.41E-09	14.8787	8.61E-08
<i>F55G11.2</i>	unknown	46.5611	1.05E-08	23.5514	3.39E-08
<i>gst-38</i>	glutathione S-transferase	44.4153	2.61E-10	25.1488	6.92E-10
<i>F56D5.3</i>	NADH oxidase	42.8716	4.55E-07	9.13127	1.04E-05
<i>clcc-3</i>	unknown	42.2267	1.34E-07	10.7623	1.986E-06
<i>hsp-16.41</i>	heat shock protein	37.3042	5.15E-09	21.7315	1.36E-08
<i>gst-12</i>	glutathione S-transferase	32.4796	2.51E-08	13.1085	1.52E-07
<i>hsp-16.2</i>	heat shock protein	31.7514	6.75E-08	15.6115	2.66E-07
<i>gst-25</i>	glutathione S-transferase	29.8987	2.85E-09	6.60598	9.57E-08
<i>dod-17</i>	unknown	27.2977	1.12E-08	9.87008	1.01E-07
<i>Y38E10A.13</i>	unknown	26.7901	2.41E-06	4.21829	0.0002776
<i>clcc-163</i>	C-type lectin	26.6706	3.41E-09	6.12515	1.19E-07
<i>aip-1</i>	AN-1-like zinc finger-containing protein	20.8251	1.36E-08	4.29368	1.08E-06
<i>F44E5.4</i>	Hsp70 family	18.5558	2.86E-09	6.50486	4.08E-08
<i>clcc-2</i>	C-type lectin	17.5573	1.56E-08	5.14494	4.39E-07
<i>gst-16</i>	glutathione S-transferase	16.8344	3.30E-09	8.24937	1.89E-08
<i>F43E2.5</i>	methionine sulfoxide-S-reductase (MsrA)	16.6723	5.69E-09	5.49549	1.14E-07
<i>hsp-16.1</i>	heat shock protein HSP16-1	14.9708	1.10E-07	6.90193	8.15E-07
<i>sdz-8</i>	alcohol dehydrogenase	14.765	1.73E-10	6.55788	1.49E-09
<i>ZK742.4</i>	predicted NADH:flavin oxidoreductase	14.2453	9.39E-09	7.42734	5.04E-08
<i>H20E11.2</i>	unknown	14.2209	6.13E-09	7.25052	3.53E-08
<i>gst-5</i>	glutathione S-transferase	12.8611	3.93E-10	6.47925	2.56E-09
<i>C32H11.4</i>	unknown	12.2617	1.28E-11	9.75833	2.26E-11
<i>Y52E8A.3</i>	unknown	11.9775	1.94E-09	10.165	2.92E-09
<i>hsp-16.48</i>	heat shock protein	11.9585	8.18E-09	7.00906	3.49E-08
<i>F49F1.6</i>	containing a signal sequence and ShK toxin domains	10.8365	1.28E-08	4.10812	2.90E-07
<i>cdr-4</i>	glutathione S-transferase	10.6628	1.14E-08	4.46537	1.76E-07
<i>Y40B10A.2</i>	unknown	9.86761	5.76E-10	4.30897	8.49E-09
<i>C55A6.6</i>	alcohol dehydrogenase	9.78048	5.79E-08	5.28538	3.77E-07
<i>ugt-13</i>	ugt family	9.63811	1.88E-06	7.71078	3.47E-06
<i>clcc-143</i>	C-type lectin	9.15882	1.75E-08	4.8121	1.36E-07
<i>nit-1</i>	Nitrilase	7.82259	7.22E-08	4.76861	3.72E-07
<i>gst-4</i>	glutathione S-transferase	7.60112	9.16E-09	5.85187	2.09E-08
<i>C17H12.6</i>	unknown	6.8668	1.65E-07	6.55322	1.91E-07
<i>clcc-9</i>	C-type lectin	6.4249	1.88E-06	6.405	1.90E-06
<i>dod-24</i>	unknown	5.66907	6.82E-09	4.92816	1.13E-08
<i>gst-7</i>	glutathione S-transferase	4.92498	7.38E-08	4.22807	1.34E-07
<i>C32H11.3</i>	unknown	4.57737	3.54E-07	4.55978	3.60E-07
<i>C12C8.2</i>	cystathionine gamma-lyase orthologous to human CTH	4.22098	2.29E-07	4.30506	2.11E-07
<i>gst-20</i>	glutathione S-transferase	4.16523	3.23E-07	4.9671	1.61E-07
<i>F55G1.9</i>	carboxylate reductase	4.09065	4.49E-09	4.61329	2.75E-09

doi:10.1371/journal.pone.0066431.t001

Table 2. Oxidative stress and iron metabolism related gene expression is altered upon arsenic exposure in *C. elegans*.

Gene class	Gene symbol	P-value	Fold change
Glutathione S-Transferase	<i>gst-1</i>	7.05368E-08	3.67142
	<i>gst-2</i>	1.23816E-06	16.4022
	<i>gst-3</i>	0.000598368	2.02115
	<i>gst-4</i>	9.15939E-09	7.60112
	<i>gst-5</i>	3.92978E-10	12.8611
	<i>gst-6</i>	6.35087E-08	3.62039
	<i>gst-7</i>	7.37605E-08	4.92498
	<i>gst-8</i>	0.000617048	2.08152
	<i>gst-9</i>	1.05337E-06	10.5567
	<i>gst-10</i>	1.08689E-05	1.58882
	<i>gst-12</i>	2.51366E-08	32.4796
	<i>gst-13</i>	2.19643E-08	7.52696
	<i>gst-14</i>	3.17455E-06	5.83786
	<i>gst-16</i>	3.30181E-09	16.8344
	<i>gst-19</i>	4.29809E-08	-8.44166
	<i>gst-20</i>	3.22626E-07	4.16523
	<i>gst-22</i>	2.83887E-05	1.85819
	<i>gst-23</i>	0.00140456	-1.38266
	<i>gst-25</i>	2.85006E-09	29.8987
	<i>gst-26</i>	1.01402E-08	-7.46037
	<i>gst-27</i>	6.8661E-11	-3.00299
	<i>gst-28</i>	4.10692E-06	-3.23139
	<i>gst-29</i>	0.00104296	-1.92675
	<i>gst-30</i>	2.74896E-10	64.4519
	<i>gst-31</i>	6.48964E-07	9.26347
	<i>gst-36</i>	0.00580448	1.21481
	<i>gst-38</i>	2.6131E-10	44.4153
	<i>gst-39</i>	1.262E-08	3.78938
	<i>gst-40</i>	4.63406E-09	9.47291
	<i>gst-42</i>	1.24648E-07	-2.85235
	<i>gstk-1</i>	0.000165975	-1.60882
<i>gsto-2</i>	2.68578E-06	4.99268	
<i>gsto-3</i>	1.13627E-07	5.62192	
Superoxide dismutase	<i>sod-1</i>	0.000634797	1.35592
	<i>sod-2</i>	0.00234654	-1.27465
	<i>sod-4</i>	0.000138537	2.49845
Glutathione diSulfide Reductase	<i>C46F11.2</i>	4.28943E-09	3.27018
Flavin-containing MonoOxygenase	<i>fmo-1</i>	0.000092521	1.52425
	<i>fmo-2</i>	9.40436E-07	3.03595
	<i>fmo-3</i>	0.000199546	-1.34887
	<i>fmo-5</i>	5.92737E-05	-1.60891
Glutamate Synthase	<i>W07E11.1</i>	0.00131395	-1.54529
Catalase	<i>ctl-1/ctl-3</i>	1.96717E-06	2.33936
	<i>ctl-2</i>	3.94594E-07	-1.81633
Glutathione Peroxidase	<i>C11E4.1</i>	9.01179E-05	-2.21564
	<i>F26E4.12</i>	0.000013568	2.04773
	<i>R05H10.5</i>	9.17508E-06	4.41822
	<i>R03G5.5</i>	4.31118E-05	-1.34898
Heat Shock Protein	<i>hsp-1</i>	5.78737E-05	1.52653

Table 2. Cont.

Gene class	Gene symbol	P-value	Fold change
	<i>hsp-12.1</i>	2.25089E-05	-2.3433
	<i>hsp-12.2</i>	0.00731473	-1.24178
	<i>hsp-16.1/hsp-16.11</i>	1.09759E-07	14.9708
	<i>hsp-16.2</i>	6.75136E-08	31.7514
	<i>hsp-16.41</i>	5.15451E-09	37.3042
	<i>hsp-16.48/hsp-16.49</i>	8.17801E-09	11.9585
	<i>hsp-17</i>	1.89686E-05	2.13635
	<i>hsp-25</i>	0.0102672	-1.22664
	<i>hsp-3</i>	0.00126013	-1.48891
	<i>hsp-4</i>	0.000494313	1.39174
	<i>hsp-43</i>	8.36411E-06	2.87926
	<i>hsp-6</i>	7.8582E-07	4.9375
	<i>hsp-70</i>	8.41166E-09	53.7595
	<i>F44E5.4</i>	2.85528E-09	18.5558
Hedgehog-like Proteins	<i>grl-10</i>	2.49496E-05	-1.94499
	<i>grl-14</i>	2.3101E-07	2.2902
	<i>grl-4</i>	0.00135253	1.45752
	<i>grl-7</i>	2.50635E-05	-1.86634
	<i>wrt-6</i>	-1.33577	2.49E-03
	<i>wrt-1</i>	-1.38953	1.43E-04
	<i>grd-12</i>	-1.41453	1.18E-02
	<i>wrt-8</i>	-1.46887	8.54E-03
	<i>grd-11</i>	-1.4916	4.22E-03
	<i>grd-2</i>	-1.56007	1.09E-03
	<i>grd-5</i>	-1.58758	9.18E-06
	<i>qua-1</i>	-1.49428	6.25E-04
	<i>hog-1</i>	-1.61787	3.75E-05
	<i>wrt-3</i>	-1.84712	6.93E-04
	<i>wrt-4</i>	-1.95916	6.27E-07
	<i>wrt-2</i>	-2.25742	5.30E-05
	<i>grd-10</i>	-2.55	3.99E-05
	<i>grd-3</i>	-2.9124	1.51E-05
	<i>grd-1</i>	-3.13208	4.69E-05
	<i>wrt-10</i>	-4.24045	3.56E-08
Transcription Factor	<i>skn-1</i>	0.000524389	2.10743
NADH Oxidase	<i>F56D5.3</i>	4.55012E-07	42.8716
Mitochondrial Iron Transporter Sideroflexin	<i>sfxn-2</i>	4.77799E-05	-2.22696
	<i>sfxn-2</i>	0.000478497	-2.32737
	<i>sfxn-5</i>	8.57816E-06	-1.976
Ferritin	<i>ftn-1</i>	2.59928E-08	55.3853
	<i>ftn-2</i>	5.85469E-06	2.36642
Ferroportin	<i>fpn-1.1</i>	7.19801E-09	6.49655
	<i>fpn-1.2</i>	7.73361E-06	-2.11996

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to environmental heavy metal and metalloid exposure, the fraction of the molecular mechanisms of pathogenesis induced by these agents, that shared, is not known at the genomic level. We compared *C. elegans*' response to cadmium and arsenic using previously published microarray dataset [38]. Using the same

threshold for both cadmium and arsenic response datasets (1.5 fold $p < 0.0001$), we found a significant overlap between affected genes (Fig. 1A,B, Fig. S3, Table S3). We performed Gene Ontology (GO) term enrichment analysis on cadmium microarray expression data. Some of the protein folding, and transport related GO

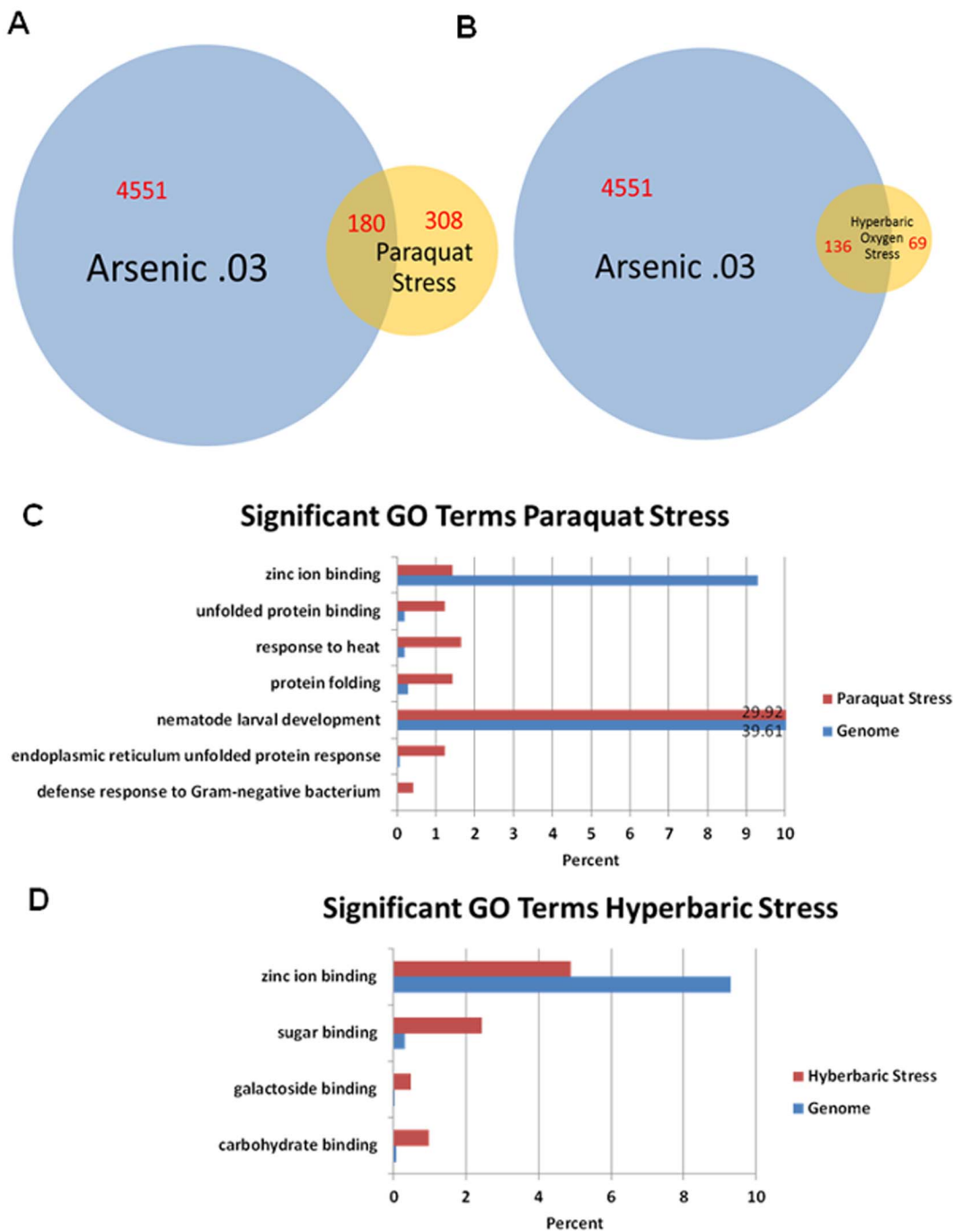


Figure 3. Genome-wide expression profile comparisons of *C. elegans* genes regulated by arsenic and oxidative stress. (A) A Venn diagram illustrating number of genes expressed at high dose arsenic exposure, paraquat exposure, and overlap among these data. (B) A Venn diagram illustrating number of genes expressed at high dose arsenic exposure, hyperbaric oxygen exposure, and overlap among these data. (C) Enrichment of gene ontology categories for genes differentially expressed at paraquat exposure. (D) Enrichment of gene ontology categories for genes differentially expressed at hyperbaric oxygen exposure. doi:10.1371/journal.pone.0066431.g003

term enrichments were present in cadmium response data (Fig. S3A, B). Expression of zinc ion binding gene classes was depleted in cadmium data, similar to our findings regarding arsenic, paraquat stress, and hyperbaric oxygen stress data. GO class of ‘nematode larval development’ was found to be enriched in cadmium response data but not in arsenic response data,

suggesting that different developmental consequences may arise against arsenic and cadmium in *C. elegans*.

Robinson et al. reported arsenic- and cadmium-induced toxicogenomic response in mouse embryos undergoing neurulation. They examined the dose-dependent effects of arsenics and cadmium on gene expression in association with increased embryotoxicity in C57BL/6J mouse embryos, and identified

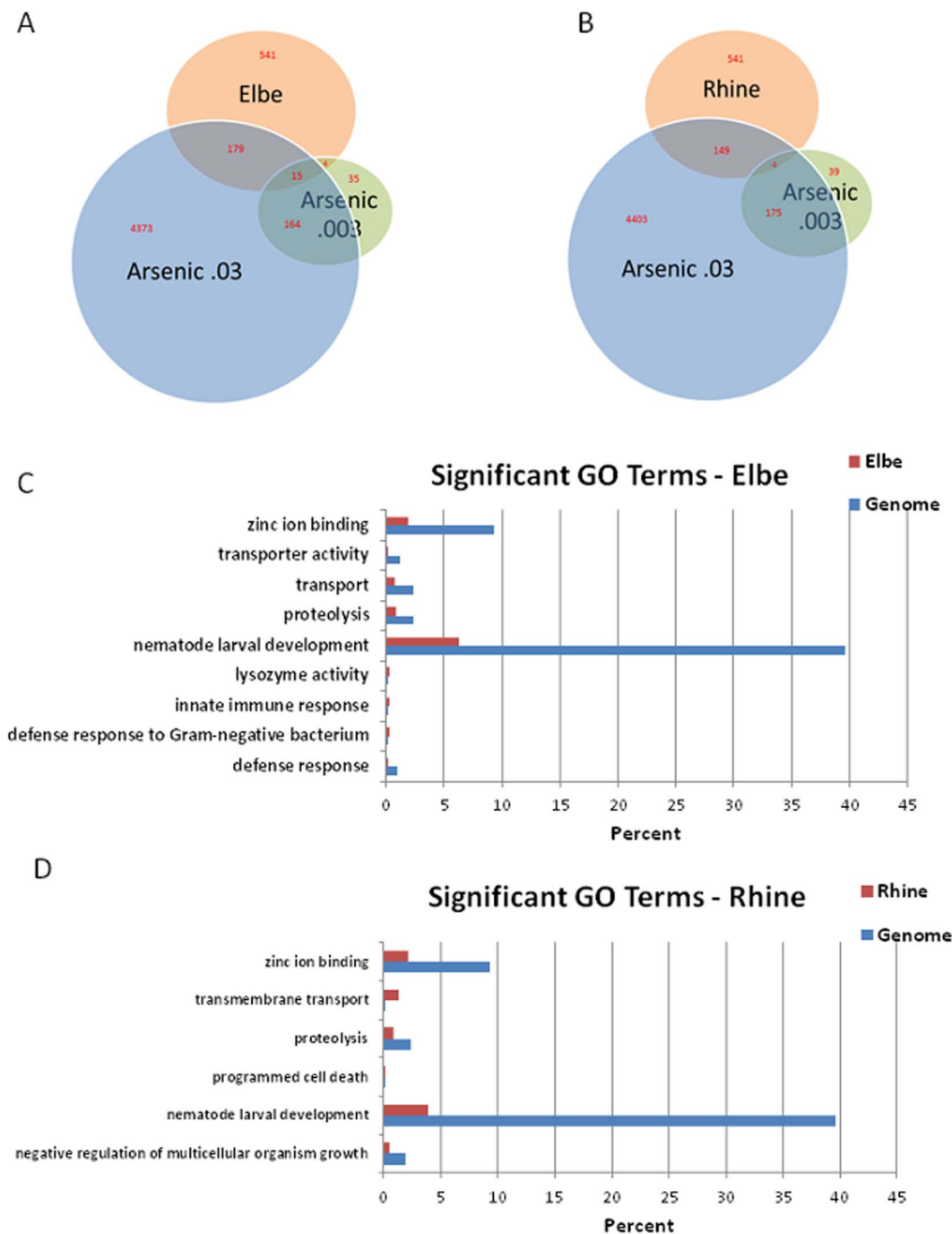


Figure 4. Genome-wide expression profile comparisons of *C. elegans* genes regulated by arsenic and river sediment toxicants. (A) A Venn diagram illustrating number of genes expressed at low and high dose arsenic exposure, Elbe River sediment exposure, and overlap among these data. (B) A Venn diagram illustrating number of genes expressed at low and high dose arsenic exposure, Rhine River sediment exposure, and overlap among these data. (C) Enrichment of gene ontology categories for genes differentially expressed at Elbe River sediment exposure. (D) Enrichment of gene ontology categories for genes differentially expressed at Rhine River sediment exposure. doi:10.1371/journal.pone.0066431.g004

overlapping and non-overlapping metal-induced gene expression alterations [74]. They found that 1960 and 775 genes identified to be significantly altered by arsenic and cadmium, respectively (F-test, $p < 0.0001$), and 116 of these genes overlapping between these two populations. Understanding genomic level responses to different heavy metals will help to resolve shared mechanisms of heavy metal-induced diseases.

Genomic responses of *C. elegans* to environmental contamination can be used as an ecotoxicogenomics tool

Arsenic is ubiquitous throughout the earth crust in different complex forms with pyrites [75], can easily dissociate from the complex and enter into ground water [1], and be taken up by microorganisms resulting in high levels of bio-availability [1,2].

Table 3. Transcription factors that are predicted to be involving in the response to arsenic exposure by FastMEDUSA.

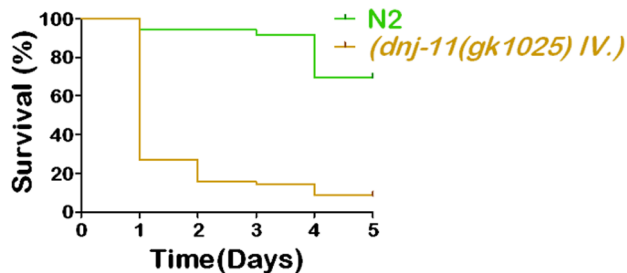
Gene name	Description	Anatomic expression pattern
<i>dac-1</i>	Ortholog of SKI/SNO/DAC family of proteins	nervous system, hypodermal seam cells
<i>nhr-35</i>	Nuclear hormone receptor	Intestine
<i>C32D5.1</i>	Putative DNA binding domain	Unknown
<i>nhr-45</i>	Nuclear hormone receptor	Unknown
<i>dnj-11</i>	Ortholog of the mammalian ZRF1/MIDA1/MPP11/DNAJC2 family	pharynx, intestine, muscle, nervous system, reproductive system
<i>mxl-2</i>	Max-Like protein X - bHLH-Zip family protein	hypodermis, intestine
<i>R06C1.6</i>	Uncharacterized protein	Unknown
<i>nhr-61</i>	Nuclear hormone receptor	Unknown
<i>zip-4</i>	Putative C/EBP protein, divergent orthologue to human CEBPA-Mutated in acute myeloid leukemia	Weak general expression and higher expression in pharynx and somatic gonad
<i>ztf-22</i>	Zinc finger putative Transcription Factor family	Nervous system, head muscles, intestine

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Because of these properties, arsenic is considered as an important environmental toxin. Menzel et al. used *C. elegans* as a bio-monitor to characterize sediment toxicity of German rivers Rhine and Elbe [76]. In that study, *C. elegans* were exposed to sediments of three German rivers, Danube, Rhine, Elbe; Danube being the cleanest, and Elbe is the most contaminated among the three rivers, based on chemical properties of the sediments, including arsenic levels. Using expression profile of *C. elegans* exposed to Danube sediment as a reference, they identified that 748 and 694 transcripts were

significantly altered in Elbe and Rhine exposed animals, respectively. We wanted to address the question of how an expression profile identified against a particular pollutant, in our case arsenic, would correlate with an expression profile identified against contaminated river sediments. We found bigger overlap between global arsenic response and response to Elbe, which is the most contaminated river in this study, with higher arsenic levels (Fig. 4, Table S6, S7). These results indicate that *C. elegans* may be used as an environmental bio-monitor, and meta-analysis of publicly available *C. elegans* expression microarray data will provide a platform to gain insights into complex environmental issues.

A



B

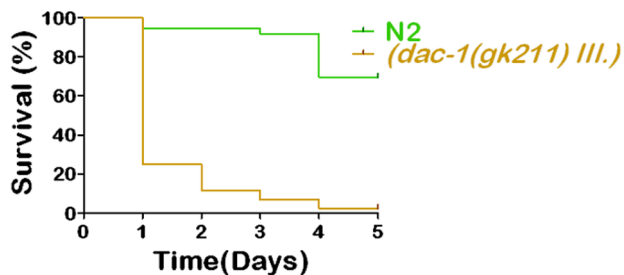


Figure 5. Lethality assays of knock-downs of the FastMedusa identified arsenic responsive regulatory genes. (Assays are performed on arsenic exposure conditions.) (A) *dnj-11(gk1025)*, $p < 0.0001$, (B) and *dac-1(gk211)*, $p < 0.0001$. doi:10.1371/journal.pone.0066431.g005

Discovering transcription factors of genomic response to arsenic exposure in *C. elegans* using FastMEDUSA

We utilized FastMEDUSA to compute TFs involved in the transcriptional response against high (0.03%) and low (0.003%) concentrations of arsenic in *C. elegans* (see Materials and Methods). We predicted ten consensus-significant TFs associated with high concentration of arsenic (Table 3). As the transcriptional response of *C. elegans* to low concentrations of arsenic was very minimal, FastMEDUSA did not find any significant TFs associated with this condition. We also predicted significant TF-gene associations based on FastMEDUSA models and plotted them in a network (Figure S4, Table S8).

Two of the predicted significant TFs, *dnj-11* and *dac-1*, were tested for their contribution to arsenic stress response. We found that loss of function of these genes using RNAi exhibited increased lethality suggesting that these genes induce the stress response in *C. elegans* (Fig. 5). *dnj-11* encodes a protein containing DnaJ and Myb domains that is orthologous to the mammalian ZRF1/MIDA1/MPP11/DNAJC2 family of ribosome-associated molecular chaperones (Wormbase). MPP11 was identified as a leukemia-associated antigen, and expression of this gene is up-regulated during leukemic blasts in patients [77]. In rats, MPP11 homolog MIDA1 was identified to induce humoral immune responses in glioma, and immunization with MIDA1 containing plasmid resulted in a significant suppression of tumor growth in immunized animals [78,79].

Chromosomal defects involving MPP11 are associated with primary head and neck squamous cell tumors [80]. Interestingly, a recent study revealed that As_2O_3 had anti-cancer effects on both cultured oral squamous cell carcinoma (OSCC) cells and OSCC xenografts by inhibiting cell growth, suppressing angiogenesis and

inducing apoptosis [81]. There is extensive evidence that arsenic trioxide (As_2O_3), has a potential role of antitumor effect *in vitro* and *in vivo* [24–26]. US Food and Drug Administration approved As_2O_3 for the treatment of Acute Promyelocytic Leukemia (APL). It's well established that As_2O_3 can cure ~80–90% of newly diagnosed APL patients [24–26]. Precise molecular mechanism of the therapeutic effect of As_2O_3 is not known. Our results suggest a molecular mechanism for the therapeutic effect of As_2O_3 , such that, As_2O_3 may regulate MPP11 expression, which may stimulate immune responses lead to killing of leukemic blast cells, and squamous cell carcinoma cells.

dac-1 encodes the *C. elegans* ortholog of Dachshund, a transcriptional regulator of the SKI/SNO/DAC family of proteins first described in *Drosophila*. The altered expression of DACH1, a *Drosophila* Dachshund homolog, has been associated with tumor progression and metastasis in human breast, prostate, ovarian and endometrial cancers [82–86]. Another arsenic response gene identified via FastMEDUSA is *zip-4*, a putative C/EBP protein, divergent orthologue to human CEBPA gene, which is mutated in acute myeloid leukemia [87]. We also identified several *C. elegans* nuclear receptors (NRs) as arsenic responsive genes using Fast MEDUSA. Nuclear receptors (NRs) encompass a family of transcription factors often regulated by small lipophilic molecules, such as steroids, retinoids, bile and fatty acids, that mediate endocrine control [88]. *C. elegans* has a large family of NRs, containing 284 of these receptors in its genome (wormbook). A large percentage of human cancers, particularly breast, prostate, and endometrial cancers, rely on steroid production for initial growth [89,90]. Our data suggest that induction of NRs via arsenic may contribute increased incidence of cancers in arsenic exposed human populations.

Supporting Information

Figure S1 Boxplots which depict A) row data, and B) data after normalization. PM only probe set signal was applied.
(TIF)

Figure S2 qRT-PCR results for selected high ranker genes.
(TIF)

Figure S3 GO term enrichments with cadmium in *C. elegans*. (A) Enrichment of GO categories for 4-hour cadmium exposure. (B) Enrichment of GO categories for 24-hour cadmium exposure.
(TIF)

Figure S4 Significant transcription factor- gene interactions in high arsenic condition. The color of the nodes represent the overall expression of the gene (green: down-regulated, red: up-regulated). The size of vertices is proportional

to their degree (i.e., number of edges incident on them). Each node is labeled with the corresponding gene or TF's name. Rounded squares represent transcription factors, and circles represent putative target genes of these transcription factors. The layout of the network was generated manually on Cytoscape.
(TIF)

Table S1 List of primers used for qRT-PCR.
(DOCX)

Table S2 List of genes differentially expressed in both high and low dose arsenic exposure (+/–1.5 fold).
(DOCX)

Table S3 List of genes differentially expressed in both, high dose arsenic, and cadmium 24-hour exposures (+/–1.5 fold).
(DOCX)

Table S4 List of genes differentially expressed in both, high dose arsenic, and paraquat exposures (+/–1.5 fold).
(DOCX)

Table S5 List of genes differentially expressed in both, high dose arsenic, and hyperbaric oxygen exposures (+/–1.5 fold).
(DOCX)

Table S6 List of genes differentially expressed in both, high dose arsenic exposure and Elbe River sediment exposures (+/–1.5 fold).
(DOCX)

Table S7 List of genes differentially expressed in both, high dose arsenic exposure and Rhine River sediment exposures (+/–1.5 fold).
(DOCX)

Table S8 Putative targets of top ten transcription factors that are predicted to be involving in the response to arsenic exposure by FastMEDUSA.
(DOCX)

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Author Contributions

Conceived and designed the experiments: SNS RS HNC. Performed the experiments: SNS IP JHL. Analyzed the data: SNS JL SB. Contributed reagents/materials/analysis tools: SB. Wrote the paper: SNS SB HNC.

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