Genome-wide screening identifies new genes required for stress-induced phase 2 detoxification gene expression in animals

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Abstract

Background: Phase 2 detoxification enzymes provide a vital defence against reactive oxygen species, including xenobiotic metabolites, which cause the oxidative damage involved in drug toxicity and many diseases. Hence, there is great interest in understanding how levels of these enzymes are regulated. CnC transcription factors, such as mammalian Nrf2, drive the expression of phase 2 enzymes and are activated as an important conserved response to oxidative stress and xenobiotics. For instance, the Caenorhabditis elegans Nrf2 orthologue, SKN-1, is activated in response to arsenite by the stress-activated p38-related kinase, PMK-1, leading to increased expression of phase 2 enzymes. Here we have used a genome-wide screening approach to identify other C. elegans genes that are required for stress-induced increases in phase 2 detoxification gene expression.

Results: Taking advantage of the elevated phase 2 gene expression in a mutant lacking the peroxidase PRDX-2, we have identified many new genes that are required for stress-induced expression of gcs-1, a phase 2 enzyme critically required for glutathione synthesis. Significantly, these include genes previously implicated in resistance to ionizing radiation, longevity and responses to pathogenic infection. Many of these new candidate activators of gcs-1 are also required for the stress-induced intestinal expression of other phase 2 genes. However, intriguingly, our data suggest other factors may be specifically required for the stress-induced expression of gcs-1. Notably, we demonstrate that the candidate activator TIR-1(SARM1) and the MAPKKK NSY-1(Ask1) are required for the arsenite-induced activation of PMK-1. However, our data suggest that the majority of candidates participate in novel mechanisms to promote gcs-1 expression. For example, the E4 ubiquitin ligase UFD-2(UBE4B) is dispensable for PMK-1 activation but important for maintaining nuclear levels of SKN-1, the stress-induced expression of multiple SKN-1-target genes and oxidative stress resistance.

Conclusions: Here we present the first functional, genome-wide analysis identifying genes that are required for activation of phase 2 detoxification genes in an animal. Our study identifies potential new regulators of Nrf2, reveals that additional mechanisms promote the stress-induced expression of specific phase 2 detoxification genes and provides new insight into the relationships between these universally important stress defences, oxidative stress resistance and aging.

Keywords: Nrf2, p38, peroxiredoxin, arsenite, signal transduction, glutathione, aging

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Background

Phase 2 conjugation reactions are vital for the biotransformation of lipophilic molecules to more soluble, excretable substances. By counteracting reactive metabolites of xenobiotics and oxygen (reactive oxygen species or ROS), phase 2 reactions provide important protection against the ROS-induced oxidative tissue damage that is associated with many diseases and aging. In addition, phase 2 reactions target diverse chemical substrates, thus presenting a significant barrier to both the activity and toxicity of therapeutic drugs [1]. Phase 2 reactions are also involved in the biosynthesis of hormones and inflammatory mediators [2]. Enzymes supporting phase 2 detoxification reactions, such as those involved in the biosynthesis of glutathione (e.g., γ-glutamylcysteine synthetase or GCS) and its conjugation to substrates (e.g., glutathione S-transferases or GSTs), are rapidly induced following exposure to stressful stimuli. This response is highly conserved, reflecting the key role of phase 2 detoxification reactions in protecting cells against ROS-induced damage. Indeed, there is growing evidence that increased levels of phase 2 enzymes have beneficial effects by preventing adverse drug reactions, protecting against carcinogens and promoting longevity [1,3,4]. On the other hand, increased phase 2 defences can also have negative consequences, for example, rendering tumour cells resistant to treatment [5,6].

Given the medical significance of phase 2 reactions, there is substantial interest in elucidating how these important stress and drug defences are regulated. Studies of the mechanisms regulating inducible phase 2 gene expression have focused upon the induction of phase 2 detoxification genes that are required for the stress-induced expression of multiple phase 2 genes and arsenite resistance [5,7-9]. However, our data suggest that the majority of candidate genes may promote arsenite-induced gcs-1 expression by alternative mechanisms, outside or downstream of the PMK-1/SKN-1 pathway. Indeed our study reveals unanticipated, additional complexity in the regulation of phase 2 detoxification systems and stress resistance in animals.

Results

Genome-wide RNAi screening identifies genes regulating gcs-1p::gfp expression

To identify new activators of phase 2 gene expression, particularly genes that are required for the stress-induced intestinal expression of these genes, we performed a genome-wide RNAi screen for genes required for the elevated intestinal expression of gcs-1p::gfp in the prdx-2 mutant background [14]. As intestinal expression of gcs-1p::gfp was assessed in the progeny of RNAi-treated worms (Figure 1), this prevented us from examining expression of gcs-1 and other phase 2 detoxification genes. Several signalling pathways regulate levels of active SKN-1. For example, phosphorylation of SKN-1 by the stress-activated mitogen-activated protein kinase (MAPK), PMK-1, is important for stress-induced increases in nuclear SKN-1 that result in increased phase 2 gene expression and oxidative stress resistance [11]. In contrast, insulin signalling and GSK-3-mediated phosphorylation both inhibit SKN-1 [4,12], as does interaction with the WD40-repeat protein WDR-23 [13]. However, the identification of genes that influence gcs-1 expression by SKN-1-independent mechanisms [14] suggests the existence of additional mechanisms for regulating phase 2 detoxification gene expression.

We have previously found that in the absence of the 2-Cys peroxiredoxin, PRDX-2, which detoxifies peroxides, expression of phase 2 detoxification genes (e.g., gcs-1) and resistance to arsenite-induced oxidative stress are increased. For example, a stress-inducible gcs-1p::gfp transcriptional reporter is expressed at significant levels in the intestine of prdx-2 mutant C. elegans even under normal growth conditions. Notably, this increased gcs-1 expression and arsenite resistance are only partially dependent on SKN-1 [14]. Hence, to identify new regulators of stress-induced phase 2 gene expression, we have conducted a genome-wide RNA interference (RNAi) screen to identify genes required for the elevated intestinal gcs-1p::gfp expression in a prdx-2 mutant. As predicted, a significant number of these genes are also required for the increased intestinal expression of gcs-1p::gfp in wild-type animals under stress conditions (arsenite exposure). Partial characterization of the most robust candidates reveals that our screen has identified new activators of PMK-1 and SKN-1, which are important for the arsenite-induced expression of multiple phase 2 genes and arsenite resistance. However, our data suggest that the majority of candidate genes may promote arsenite-induced gcs-1 expression by alternative mechanisms, outside or downstream of the PMK-1/SKN-1 pathway. Indeed our study reveals unanticipated, additional complexity in the regulation of phase 2 detoxification systems and stress resistance in animals.
Figure 1 Results of RNAi screening. RNAi screening identified genes that are (1) synthetic lethal with prdx-2 or (2) required for stress-induced, intestinal expression of a phase 2 detoxification transcriptional reporter gene (gcs-1p::gfp). First, 16,255 RNAi clones were screened in prdx-2 gcs-1p::gfp background, which contains increased, detectable ‘low’ levels of intestinal GFP (indicated by arrows for representative animals and illustration) in addition to wild-type levels of constitutive pharyngeal GFP expression (indicated with ‘PP’). Of the 951 RNAi clones that produced embryonic lethal (emb), lethal (let) or sterile (ste) phenotypes and hence too few prdx-2 mutant progeny for gcs-1p::gfp to be scored, 237 had not previously been reported to affect growth or reproduction in screens of wild-type animals. Following re-screening of these 237 genes three times, 70 RNAi clones repeatedly produced emb, let and/or ste phenotypes (Additional file 1: Table S1). Of the 15,304 wells containing at least 40 viable progeny in the initial screen, 50 RNAi clones were scored that increased intestinal gcs-1p::gfp expression, such that more than 50% of animals in the well had a ‘high’ level of gcs-1p::gfp expression throughout the intestine (see representative animal and illustration) (Additional file 1: Table S2). Another 355 RNAi clones reduced gcs-1p::gfp expression, such that more than 50% of animals in these wells had no detectable intestinal gcs-1p::gfp expression (‘None’; see representative animal and illustration) (Additional file 1: Table S3). These 355 RNAi clones were re-screened three times and subjected to secondary screens: (i) 21 clones were discovered to reduce the intestinal expression of non-phase 2 gene F09E5.3::gfp (Table S3); (ii) 24 clones reduced intestinal gsf-4p::gfp expression (Additional file 1: Table S3); (iii) 90 genes reproducibly required for As3+-induced intestinal expression of gcs-1p::gfp in prdx-2 mutant (Table 1); and (iv) 16 of these clones reduced arsenite-induced intestinal gcs-1p::gfp expression in wild-type worms (Table 1). GFP, green fluorescent protein; RNAi, RNA interference.

whether RNAi targeting of essential genes affected gcs-1p::gfp expression. However, incidentally, this allowed the identification of 70 genes that may be specifically required for development or reproduction in the absence of prdx-2 (see Additional file 1: Table S1 and Additional file 2: Figure S1).

Although the purpose of this screen was to identify genes that were required for the elevated intestinal expression of gcs-1p::gfp in prdx-2 mutant animals, in the course of screening, 50 RNAi clones were noted that increased the expression of gcs-1p::gfp still further (see Additional file 1: Table S2). These included RNAi targeting genes encoding E01A2.1, a glutamate-cysteine ligase regulatory subunit and WDR-23, a WD40-repeat protein that negatively regulates SKN-1 [13]. Both of these genes were identified by previous screens for inhibitors of phase 2 detoxification gene expression, confirming that our screening conditions were effective at identifying regulators of gcs-1p::gfp [13,17]. RNAi targeting glutathione synthetase (M176.2) also increased gcs-1p::gfp expression, providing further evidence that feedback control acts to increase GCS-1 levels when glutathione synthesis is inhibited [17].
Genes required for \textit{gcs-1p::gfp} expression overlap with genes required for resistance to ionizing radiation and longevity

It was found that 355 RNAi clones (approximately 2.3\% of those screened) prevented intestinal \textit{gcs-1p::gfp} expression (Figure 1 and see Additional file 1: Table S3). Secondary screens determined that 21 of these genes were also required for the intestinal expression of a transcriptional GFP reporter of a non-phase 2 gene, indicating a broader role in intestinal gene expression (see Additional file 1: Table S3). Accordingly, these genes were eliminated as candidate phase 2 gene regulators. Importantly, this analysis indicated that the majority of targeted genes are not generally required for intestinal gene expression or GFP stability.

Although the function of more than one-third of the identified genes is unknown, bioinformatic analysis revealed the remainder to represent a broad range of biological processes (see Additional file 2: Figure S2). To identify possible functional overlaps, we compared the results of our screen with published RNAi screens of the same RNAi library. This revealed a statistically significant overlap ($P = 0.0039$) between the genes we have identified and those found to be required for resistance to ionizing radiation (IR) (see Additional file 1: Table S4) [18]. This suggests that the role of these particular genes in IR resistance might be to up-regulate phase 2 detoxification gene expression to protect against free radicals that are generated by IR. A significant overlap ($P = 0.0008$) was also found between genes required for \textit{gcs-1p::gfp} expression and those required for the increased lifespan of insulin receptor (\textit{daf-2}) mutants (see Additional file 1: Table S4) [19]. This supports other studies that have suggested a positive correlation between the expression of phase 2 detoxification systems and lifespan [3,4]. Indeed, consistent with the vital role of \textit{gcs-1} in arsenite resistance [20], six of the seven genes required for both the longevity of \textit{daf-2} mutants [19] and intestinal \textit{gcs-1p::gfp} expression were important for the resistance of wild-type animals to arsenite (Figure 2A). This suggests that, as well as the increased lifespan, these genes may be important for the increased stress resistance associated with reduced insulin signalling. However, of the four genes previously identified as required for resistance to IR [18], only \textit{dnj-22} was also important for arsenite resistance (Figure 2A) and \textit{cand-1} RNAi actually increased arsenite resistance (Figure 2A). This suggests that alternative mechanisms to promote stress resistance can compensate for the reduced induction of \textit{gcs-1}.

Identification of new genes required for arsenite-induced \textit{gcs-1} expression and resistance to arsenite

Next, we examined whether candidate regulators of \textit{gcs-1} affected the expression of a second phase 2 transcriptional reporter gene, \textit{gst-4p::gfp}, which is detectably expressed in the hypodermis/body wall muscle and intestine of wild-type animals under normal conditions [21]. Strikingly, of the 355 RNAi clones initially identified, only 24 reduced the intestinal level of \textit{gst-4p::gfp} expression (see Additional file 1: Table S3). This provided further corroboration that genes identified by our screen are not generally required for intestinal gene expression or GFP stability. However, quantitative analysis of a subset of 24 candidates, identified 13 additional clones that significantly reduced intestinal \textit{gst-4p::gfp} expression in \textit{prdx-2} mutant animals (see Additional file 2: Figure S3). This suggests that approximately half of the genes identified as important for intestinal expression of \textit{gcs-1}, might also be important for the elevated expression of other phase 2 genes in animals lacking PRDX-2. Notably, none of these RNAi clones prevented pharyngeal \textit{gcs-1p::gfp} or hypodermal \textit{gst-4p::gfp} expression. Although the effectiveness of RNAi may be reduced in the pharynx, this suggests that they are not required for housekeeping levels of phase 2 gene expression in other tissues.

Although identified RNAi clones were likely to include any that can rescue the defects of the \textit{prdx-2} mutant, we predicted our screen to have also identified genes required for the increased intestinal expression of phase 2 detoxification genes under stress conditions, here mimicked by loss of the peroxidase PRDX-2 [14]. To test this hypothesis, we examined whether candidate genes were also required for the arsenite-induced expression of \textit{gcs-1p::gfp} in wild-type animals. Although high throughput RNAi screens for \textit{C. elegans} have intrinsically high false negative rates [22], we reasoned that less reproducible effects were also likely to indicate RNAi clones with more marginal effects on intestinal \textit{gcs-1p::gfp}. Hence, we chose the 90 most robust RNAi clones that ablated intestinal \textit{gcs-1p::gfp} expression in \textit{prdx-2} mutants in each of four separate screenings (see Additional file 1: Table S3). Importantly, the reproducibility of these clones also indicates that the RNAi treatment was consistently effective. Excitingly, more than half of these RNAi clones (53) prevented arsenite-induced, intestinal \textit{gcs-1p::gfp} expression in at least one of three trials, indicating that our screen had successfully identified new candidate activators of arsenite-induced phase 2 gene expression (see Additional file 1: Table S5). As the most robust of these candidates, the 16 RNAi clones that prevented arsenite-induced, intestinal \textit{gcs-1p::gfp} expression in each of three trials, were selected for further analysis (Figure 1 and Table 1).

First, we examined whether these 16 potential new activators of arsenite-induced \textit{gcs-1} expression were required for arsenite-induced increases in endogenous \textit{gcs-1} expression. Analysis of the \textit{gcs-1p::gfp} reporter line suggests that the constitutive, pharyngeal expression, which is
unaffected by these clones, is likely to make a substantial contribution to total gcs-1 mRNA levels. Nevertheless, the increased intestinal expression of gcs-1 following arsenite treatment is reflected in an approximately 2.5-fold increase in total gcs-1 mRNA [8]. Hence, we examined whether RNAi clones targeting a subset of 12 genes, representative of each functional category (Table 1), affected the total levels of gcs-1 mRNA in arsenite-treated animals. These RNAi clones all reduced total gcs-1 mRNA levels in arsenite-treated wild-type animals, with seven out of twelve clones producing a statistically significant decrease. In many cases, gcs-1 mRNA levels were reduced to 40% to 50% of vector control levels, approximating gcs-1 mRNA levels in untreated animals and thus indicating that induction by arsenite was greatly impaired (Figure 2B upper panel). Thus mRNA analysis is consistent with the reporter screens, strongly suggesting that the majority of genes in Table 1 are important for the arsenite-induced...
### Table 1 Activators of stress-induced *gcs-1p::gfp* expression and their effects on stress-induced phase 2 gene expression, stress resistance, PMK-1 and SKN-1

#### Functional classification of gene targeted by RNAi

| Gene expression | Human orthologue | Proposed function | Intestinal *gst-4p::gfp* expression | mRNA (Figure 2B) | Arsenite resistance (Figure 2A,C) | PMK-1 activation (Figure 4) | Nuclear levels of SKN-1 S393A::GFP (Figure 6A) |
|-----------------|------------------|-------------------|------------------------------------|------------------|----------------------------------|-----------------------------|----------------------------------|
| *C17G10.1*     | OGFOD1           | Regulates translation | -                                 | ↓                | ↓                               | -                           | ↑                               |
| *C35C5.1 sdc-2*| GOLGA6L5         | Sex determination/dosage compensation | -                                 | ND              | ND                              | ↓***                        | ↑                               |
| *F47A4.2 dpy-22*| MED12            | Mediator subunit   | -                                 | -               | ND                              | -                           | -                               |
| *C16A3.8 thoc-2*| THOC2            | Transcriptional elongation | -                                 | ↓***             | ↓***                            | ↓*                         | ↑                               |
| *K04G7.11*     | SYF2             | Pre-mRNA-splicing factor | -                                 | ↓*              | ND                              | -                           | ↑                               |
| **Protein homeostasis** |                          |                   |                                    |                  |                                  |                             |                                  |
| *Y102A5A.1 cand-1* | CAND1            | Cullin-associated NEDD8-dissociated | -                                 | ↑***             | -                               | ↑*                         | -                               |
| *B0025.2 csn-2* | CSN2             | Cop9 signalosome subunit | ↑                                 | ↓***             | ↓*                              | -                           | -                               |
| *T05H10.5 ult-2*| UBE4B            | E4 ubiquitin conjugating enzyme | ↓                                 | ↓*              | ND                              | -                           | -                               |
| **Signal transduction** |                          |                   |                                    |                  |                                  |                             |                                  |
| *F13B10.1 tir-1* | SARM1            | Pathogen responses | -                                 | ↑***             | ↓*                              | -                           | -                               |
| *T28H11.2 smr-1*| Serpentine receptor | -                   | -                                 | -               | ND                              | -                           | -                               |
| *ZK7923.3 inx-9*| Innexin          | -                  | -                                 | ND              | ND                              | -                           | -                               |
| **Growth/metabolism** |                          |                   |                                    |                  |                                  |                             |                                  |
| *C06A8.1 mthf-1* | MTHFR            | Methylene tetrahydrofolate reductase | -                                 | ↓***             | ↓**                            | ↓*                         | -                               |
| *C34F1.3 AMPD2* |                   | AMP deaminase 2    | -                                 | ↓*              | ND                              | -                           | -                               |
| **Structural** |                          |                   |                                    |                  |                                  |                             |                                  |
| *F46E10.11 hpo-26* |                   | Hypersensitive to pore-forming toxin | -                                 | ↓***             | ND                              | ND                         | ↑                               |
| **Transport/trafficking** |                          |                   |                                    |                  |                                  |                             |                                  |
| *R11A5.1 apb-3* | AP3B1/2          | Adaptin           | -                                 | ↓**             | ↑***                            | -                           | ↑                               |
| **Unknown function** |                          |                   |                                    |                  |                                  |                             |                                  |
| *F22F7.4*      |                   | -                  | -                                 | -               | -                               | -                           | -                               |

Sixteen RNAi that prevented any detectable intestinal *gcs-1p::gfp* expression in *prdx-2* mutant animals and in more than 50% of wild-type animals treated with 1 mM arsenite for 90 min (N2 *gcs-1p::gfp*) on each of three occasions on which they were screened, following quantitative analysis, but which did not reduce intestinal expression of a non-phase 2 reporter gene, *F09E5.3::gfp* (Additional file 1: Table S3). These genes were placed into categories based on gene ontology [15]. Where RNAi affected *gst-4p::gfp* expression in wild-type (Additional file 1: Table S3) or *prdx-2* mutant animals (Additional file 2: Figure S3) the effect is indicated by an arrow. Where RNAi produced a greater than 10% change in *gcs-1* or *gst-7* mRNA levels in arsenite-treated animals (Figure 2B), arsenite resistance (Figure 2A,C), PMK-1 phosphorylation in arsenite-treated animals (Figure 4) or levels of SKN-1 S393A::GFP in intestinal nuclei (Figure 6A) this is indicated. Statistically significant differences from control animals are indicated (*P* < 0.05; **P** < 0.01; ***P** < 0.001). ND, not determined; RNAi, RNA interference; WT, wild type.
expression of gcs-1 (Figure 2B upper panel). Notably, only ten of these sixteen RNAi's reduced the intestinal expression of gst-4p::gfp in either wild-type or prdx-2 mutant animals (Table 1, Additional file 1: Table S3 and Additional file 2: Figure S3). Hence, to further test the generality of the role of candidate genes in phase 2 gene expression, we investigated whether the subset of 12 RNAi affected the arsenite-induced expression of another phase 2 gene, gst-7, which is also induced two- to threefold by arsenite [8]. This revealed that RNAi targeting five of these genes (tir-1, ufd-2, thoc-2, mthf-1 and F22F7.4) significantly reduced gst-7 mRNA levels in arsenite-treated animals (Figure 2B lower panel). However, RNAi targeting of other candidate genes either had little effect, or in two cases, csn-2 and apb-3, actually significantly increased gst-7 mRNA levels (Figure 2B lower panel). Together with our reporter analysis (Table 1, see Additional file 2: Figure S3), this suggests that while some genes are more broadly required for stress-induced phase 2 detoxification gene expression, others may be specifically required for the stress-induced expression of gcs-1 (Figure 2B,C).

Next we examined whether these 12 genes were important for arsenite resistance. Notably, RNAi targeting of four genes (sdc-2, thoc-2, K04G7.11 and tir-1) significantly increased the sensitivity to arsenite toxicity (Figure 2A,C and see Additional file 2: Figure S4). However, in most cases arsenite resistance was unaffected or only slightly reduced (Figure 2C). Moreover, similar to cand-1 RNAi (Figure 2A), RNAi targeting of a second regulator of protein homeostasis, csn-2, actually increased arsenite resistance (Figure 2C). This contrasted with the effects of other RNAi clones identified by our screen, which was consistent with the increased intestinal gst-4p::gfp and gst-7 mRNA levels observed in csn-2 RNAi-treated animals (Table 1 and see Additional file 1: Table S3) and the previously reported role of CSN-2 in regulation of phase 2 detoxification genes and peroxide resistance [17]. CSN-2 is a component of the Cop9 signalosome (CSN), which, as a multisubunit complex, influences protein turnover by removing the ubiquitin-like modifier NEDD8 from Cullin-Ring ubiquitin ligases [23]. In agreement with these previous studies, our data suggest that CSN-2, and other CSN subunits, suppress basal phase 2 gene expression under normal growth conditions (Table 1) but are required for stress-induced increases in gcs-1 expression (e.g., arsenite or loss of prdx-2) (Table 1, Figure 3A,B). Notably, although gcs-1 induction is impaired in CSN-deficient animals (Figures 3A,B, 2B upper panel), the high basal gcs-1 expression and increased expression of other phase 2 detoxification genes, e.g., gst-7 (Figure 2B lower panel, see Additional file 2: Figure S5), is sufficient to confer increased oxidative stress resistance (Figures 2C and 3C) [17]. This suggests that for survival following exposure to an acutely toxic level of arsenite, basal levels of phase 2 detoxification gene expression may be more important than the ability to induce gcs-1 (Figure 2). Strikingly, despite their increased resistance to acute oxidative stress, under normal growth conditions CSN-deficient animals were short-lived (Figure 3D).

The role of genes required for arsenite-induced gcs-1p::gfp expression in arsenite-induced activation of PMK-1

Having established that candidate genes were required for arsenite-induced gcs-1 expression (Figure 2B upper panel and see Additional file 1: Table S3) but had different effects on the arsenite-induced expression of other phase 2 genes (Figure 2B lower panel and see Additional file 2: Figure S3) and/or arsenite resistance (Figure 2A,C), we proceeded to investigate the mechanisms by which selected candidate genes (Table 1) promoted stress-induced gcs-1 expression. First, we examined whether these genes contributed to known mechanisms involved in arsenite-induced phase 2 gene expression. An important response to arsenite is the activation of PMK-1 by SEK-1-mediated phosphorylation. Following activation, PMK-1 phosphorylates SKN-1 increasing nuclear SKN-1 levels and promoting gcs-1 expression [11]. Hence, we tested whether genes required for arsenite-induced activation of gcs-1p::gfp (Table 1) were required for PMK-1 activation. Strikingly, only RNAi targeting the Toll/interleukin-1 receptor domain protein, TIR-1, reduced the arsenite-induced activation of PMK-1 (Figure 4). This suggests that the other genes are required for alternative, PMK-1-independent, mechanisms to activate gcs-1p::gfp. Indeed, there was even an indication that many RNAi clones caused an increase in PMK-1 phosphorylation, perhaps reflecting a compensatory activation of PMK-1 when other signalling pathways are disrupted (Figure 4).

TIR-1 and NSY-1 are required for arsenite-induced activation of the p38-related MAPK, PMK-1

Previous studies have shown that TIR-1 and the MAPKKK NSY-1 are both required for the activation of PMK-1 in response to pathogenic infection but not for responses to arsenite [11,24,25]. Hence, to confirm our RNAi data suggesting that TIR-1 is required for arsenite-induced activation of PMK-1 (Figure 4), we examined the phosphorylation of PMK-1 in wild-type, tir-1 and nsy-1 mutant C. elegans. Consistent with previous studies, treatment of wild-type animals with 5 mM arsenite caused a rapid increase in the level of PMK-1 phosphorylation that was maximal by 5 min (Figure 5A) [14]. In contrast, there was no detectable phosphorylation of PMK-1 in tir-1 or nsy-1 mutant C. elegans after 5 min arsenite treatment. This suggests that TIR-1 and NSY-1 are both required for the arsenite-induced activation of PMK-1. Indeed, although nsy-1 was not identified by our initial genome-wide screen, both tir-1 and nsy-1 RNAi significantly reduced
the level of gcs-1p::gfp induced by arsenite in wild-type and prdx-2 mutant animals (Figure 5B). Moreover, consistent with the important role of PMK-1 activation and gcs-1 expression in resistance to arsenite [11,20], tir-1 and nsy-1 mutant animals were significantly more sensitive to arsenite toxicity than wild-type animals (Figure 5C). Thus, here we have identified that TIR-1 and NSY-1, which are required for PMK-1 activation during infection, are also important for activation of PMK-1 in response to arsenite. Importantly, the inability of tir-1 RNAi treatment to increase further the arsenite-sensitivity of pmk-1 mutant animals (Figure 5D) suggests impaired PMK-1 activation underlies the arsenite-sensitivity of TIR-1-deficient animals (Figure 5).

Candidate RNAi that reduces levels of SKN-1 in intestinal nuclei has broad effects on arsenite-induced phase 2 detoxification gene expression

Next, we focused on determining how other candidate genes promoted arsenite-induced gcs-1 expression. The arsenite-induced expression of gcs-1 is highly dependent on the SKN-1 transcription factor [8]. Although PMK-1-dependent phosphorylation is important for accumulation of SKN-1 in intestinal nuclei, SKN-1 activity is also regulated by other mechanisms. For instance, phosphorylation of SKN-1 by GSK-3 or the insulin-regulated AKT/SGK kinases directly inhibits SKN-1 activity [4,12]. Accordingly, we investigated whether other genes identified by our screen might be required for the activation...
of SKN-1 by a PMK-1-independent mechanism. As the basal levels of SKN-1::GFP in intestinal nuclei are normally undetectably low, to test this hypothesis we examined whether candidate genes (Table 1) affected the nuclear abundance of a constitutively active form of SKN-1 in which the GSK-3 phosphorylation site had been mutated; SKN-1S393A::GFP [12]. Unexpectedly, RNAi targeting several genes, including K04G7.11 and apb-3, substantially increased nuclear SKN-1S393A::GFP levels (Figure 6A).

Moreover, whereas wild-type SKN-1::GFP was undetectable in the intestinal nuclei of control animals, these RNAi clones also caused wild-type SKN-1::GFP to be detected in the intestinal nuclei of a significant number of animals (Figure 6B). Thus, paradoxically, loss of K04G7.11 and apb-3 prevents arsenite-induced increases in intestinal gcs-1p::gfp expression (Table 1) and gcs-1 mRNA levels (Figure 2B), despite increased levels of nuclear SKN-1 (Figure 5A). This suggests both genes act downstream

**Figure 4** Effect of genes required for stress-induced activation of gcs-1 on arsenite-induced PMK-1 activation. Western blot analysis of RNAi and vector-control-treated animals before and following 5 min exposure to 5 mM arsenite revealed that out of 14 of the most robust activators of gcs-1p::gfp (Table 1), only tir-1 RNAi reduced the level of PMK-1 phosphorylation. (A) Mean percentage difference between the level of PMK-1 phosphorylation in arsenite-treated control and RNAi-treated animals following analysis of quantitative densitometry data obtained from at least two independent experiments. ***indicates that tir-1 RNAi significantly reduced PMK-1 phosphorylation compared with vector control (Student’s T test, P = 0.00056). The effects of other RNAi clones on arsenite-induced PMK-1 phosphorylation were not statistically significant (P > 0.05). (B) Representative Western blots of those quantitatively analysed in (A), RNAi, RNA interference.
of SKN-1, or through parallel SKN-1-independent pathways, to promote gcs-1 expression. Notably, apb-3 and K04G7.11 RNAi did not prevent the arsenite-induced expression of four other phase 2 genes, gst-7, gst-4, dhs-8 and sdz-8 (Figure 2B and 6C). Indeed, consistent with the increased levels of nuclear SKN-1, gst-7, gst-4, dhs-8 and sdz-8 mRNA levels were even slightly increased in apb-3 and K04G7.11 RNAI-treated animals (Figure 6C). This indicates apb-3 and K04G7.11, like csn-2, and potentially other genes identified by our screen (Figure 6A), are not universally important for the arsenite-induced expression of all phase 2 genes but specifically required for arsenite-induced increases in gcs-1 mRNA expression.

Although the reductions in PMK-1 activity associated with tir-1 RNAi (Figure 4) were insufficient to inhibit nuclear accumulation of the hyperactive SKN-1$^{S393A}$-GFP mutant (Figure 6A), excitingly, RNAi clones targeting thoc-2 and ufd-2 significantly reduced the levels of SKN-1$^{S393A}$::GFP in intestinal nuclei, indicating that UFD-2 and THOC-2 are required for nuclear SKN-1 abundance (Figure 6A). SKN-1 activity is essential for the C. elegans transcriptional response to arsenite [8]. Indeed, in contrast to apb-3 and K04G7.11, thoc-2 and ufd-2 were important, not only for the arsenite-induced expression of gcs-1, but of several other SKN-1-regulated phase 2 genes (Figure 6C). Indeed, ufd-2 RNAi produced a statistically significant reduction in the total mRNA levels for all five phase 2 genes that we investigated.

To test whether the associated reduction in SKN-1 activity could underlie the reduced arsenite-induced phase 2 gene expression in these UFD-2-deficient animals (Figure 6C), we examined whether wdr-23 RNAi, which increases SKN-1 activity and intestinal expression of gcs-1::gfp (Figure 6D) [13,26], bypassed the requirement for UFD-2. The inability of ufd-2 RNAi to reduce intestinal gcs-1::gfp expression in wdr-23 RNAI-treated animals (Figure 6D) suggests that loss of WDR-23 increases SKN-1 levels sufficiently to restore normal expression of phase 2 genes to UFD-2-deficient animals. These data are consistent with UFD-2 promoting arsenite-induced phase 2 gene expression and oxidative stress resistance by increasing SKN-1 activity. Moreover, despite normal levels of PMK-1 phosphorylation, but consistent with the reduced SKN-1 activity and arsenite tolerance associated with ufd-2 RNAi, ufd-2 (tm1380) mutant animals (predicted null) were also more sensitive to arsenite toxicity than wild-type animals (Figure 6E and see Additional file 2: Figure S6).
Discussion

Conserved MAPK (p38 and PMK-1) and CnC transcription factors (Nrf2 and SKN-1) are vital for stress-induced increases in phase 2 detoxification gene expression and accordingly protect both normal and tumour cells from xenobiotic/drug-induced oxidative damage. Although work has focused on the regulation of p38 (PMK-1)/Nrf2 (SKN-1) activity, here we provide evidence that the regulation of stress-induced increases in phase 2 gene expression in animals is much more complex than previously appreciated.
Our RNAi screening in *C. elegans* has uncovered new genes that are required for the elevated expression of a phase 2 detoxification gene (*gcs-1*) under stress conditions (Table 1 and see Additional file 1: Table S3). Partial characterization of some of the most robust candidate genes has identified new genes required for the arsenite-induced activation of PMK-1 and SKN-1. However, despite PMK-1’s critical role in promoting arsenite-induced gene expression [8,11], our data suggest that many of these new activators of *gcs-1* expression participate in novel mechanisms to promote phase 2 gene expression that are downstream or independent of PMK-1 (Figure 7). For example, UFD-2 is required for SKN-1 activity but not PMK-1 activation (Figures 4B and 6, see Additional file 2: Figure S6). Moreover, our screening has revealed genes, such as *mthf-1* and *F22F7.4*, that are important for arsenite-induced increases in the expression of multiple phase 2 genes without apparently affecting either PMK-1 or SKN-1 activity. This raises the intriguing possibility that a significant number of genes identified here may contribute to the stress-induced activation of phase 2 genes by mechanisms outside of the canonical PMK-1/ SKN-1 pathway.

**Identification of new genes required for the activation of specific or multiple phase 2 genes**

SKN-1 is generally important for the stress-induced expression of multiple phase 2 genes. Consistent with this, the new activators of PMK-1 and SKN-1 that we have identified, are important for the activation of multiple phase 2 genes (Table 1 and Figure 6). Indeed, like SKN-1 [8], UFD-2 is also required for intestinal *gst-4* expression under non-stress conditions (Table 1 and Additional file 2: Table S3). However, our data suggest that many of the genes identified here may be specifically important for the stress-induced expression of *gcs-1* (Figures 2B and 6C, and Table 1 and see Additional file 2: Figure S3). Studies of yeast have revealed that transcriptional responses to oxidative stress involve the activation of parallel signalling pathways and transcription factors to coordinate the expression of distinct and overlapping sets of phase 2 genes [27]. Excitingly, our data suggest that alternative pathways, involving some of the genes identified here, may also contribute to the regulation of different subsets of phase 2 genes in animals. For instance, unexpectedly, we have identified several genes (e.g., *apb-3* and *csn-2*) that are required for stress-induced increases in *gcs-1* even in the presence of high levels of nuclear SKN-1. This suggests that arsenite-induced *gcs-1* expression also involves other regulators acting downstream of PMK-1 to promote SKN-1 activity specifically at this promoter. In addition to participating in the glutathionylation of toxic electrophiles, glutathione has a key role as a cell redox buffer. Hence, the presence of these additional regulatory mechanisms may be important for allowing *gcs-1* expression and glutathione biosynthesis to be regulated independently from other phase 2 genes (Figure 7).
High basal levels of phase 2 detoxification gene expression protect against acute stress conditions but do not necessarily extend lifespan

RNAI targeting subunits of the CSN increase the expression of phase 2 reporter genes in wild-type animals [17]. Hence, we were surprised when our screen identified csn-2 amongst the genes required for the elevated gcs-1p::gfp and gst-4p::gfp expression in the prdx-2 mutant background. However, our further analysis has revealed that, despite high basal levels of phase 2 gene expression and increased levels of nuclear SKN-1 (Figure 6A) [17], CSN-deficient animals are unable to effectively increase the expression of gcs-1 in response to arsenite (Figures 2B upper panel and 3A,B, and Table 1). Despite this attenuated response to arsenite, CSN-deficient animals are more resistant to both arsenite (Figure 2C and 3C) and another oxidative stress-causing agent, tert-butyl peroxide [17]. Indeed, analysis of other genes identified here indicates that the inability to induce gcs-1 expression (Figure 2B upper panel), does not necessarily correlate with a lower arsenite tolerance (Figure 2C). Accordingly, we propose that in conditions of acute arsenite toxicity, basal levels of gcs-1 expression may be more important for survival than the ability to increase gcs-1 mRNA levels. This suggests that approaches to increase the basal levels of phase 2 gene expression may be effective as a means to prevent the acute toxicity associated with particular drugs [1].

Despite their increased oxidative stress resistance, CSN-deficient animals are short-lived (Figure 3D). This is consistent with increasing evidence that there is not necessarily a direct correlation between resistance to environmental oxidative stress and longevity. For example, prdx-2 mutant animals have high basal expression of phase 2 detoxification genes and are more resistant to arsenite but age prematurely and are short-lived [14]. It is possible that the short lifespan of CSN-deficient and PRDX-2-deficient animals reflect unrelated functions of the CSN and PRDX-2. However, these findings do support other studies suggesting that constitutively high levels of phase 2 detoxification can be deleterious [4,14,28]. Thus, although increased SKN-1 activity can extend lifespan [4,13], the ability to regulate phase 2 detoxification gene expression in response to changes in the environment may also be important.

TIR-1 and NSY-1 are required for PMK-1-dependent responses to oxidative stress and pathogens

Our analysis of the most robust regulators of gcs-1 expression identified TIR-1 as important for arsenite-induced activation of the p38-related MAPK, PMK-1. TIR-1 and the MAPKKK NSY-1 are required for activation of PMK-1 in response to pathogens and for the resistance of C. elegans to infection [25]. Here we have shown that TIR-1 is also required for the induction of phase 2 detoxification gene expression and resistance to the oxidative stress-causing agent, arsenite. This indicates that arsenite and pathogens may activate similar signalling pathways/defence mechanisms and raises the intriguing possibility that the expression of phase 2 detoxification genes may contribute to the role of TIR-1 in protecting against pathogenic infection. This is consistent with recent studies revealing that SKN-1-dependent expression of phase 2 detoxification genes is important for C. elegans resistance to bacterial infection [29,30].

Identification of genes that interact with prdx-2

Our screen has also uncovered genetic interactions with prdx-2. For instance, in the course of screening we identified 70 genes that may specifically be required for the development or reproduction of animals lacking PRDX-2 (see Additional file 1: Table S1). Consistent with a functional overlap between these genes and the peroxidase prdx-2, functional analysis revealed that many encoded stress defence proteins (Additional file 2: Figure S1). Indeed, the 11 RNAi clones that produced the most severe phenotype, preventing the survival or growth of prdx-2 mutant C. elegans, included three targeting genes encoding peroxide-detoxifying enzymes: two catalases (ctl-1 and ctl-2) and the mitochondrial 2-Cys peroxiredoxin (prdx-3). This suggests an essential role for peroxide-detoxifying enzymes that is partially redundant with prdx-2 (see Additional file 1: Table S1).

Many of the RNAi clones that caused a reproducible loss of intestinal gcs-1p::gfp expression in prdx-2 mutant worms did not affect the induction of gcs-1p::gfp by arsenite in wild-type animals. These will include any RNAi clones that specifically rescue the defects responsible for the elevated intestinal gcs-1p::gfp expression in prdx-2 mutant animals or that are more specifically required for the induction of gcs-1 in response to other stresses, e.g., peroxide. Further studies to distinguish between these possibilities may uncover stress-specific pathways to up-regulate phase 2 detoxification genes. Alternatively, by identifying genes required for Prx-specific effects on signalling, this may improve our understanding of how signalling functions contribute to the conserved role of Prx in longevity [31].

Genes identified by this screen may include potential therapeutic targets for prevention or treatment of cancer and age-associated diseases

The important role that phase 2 detoxification enzymes play in protecting against drug and stress-induced cell damage has stimulated interest in developing ‘chemopreventive’ agents to increase their levels and prevent the toxic and carcinogenic effects associated with this oxidative damage. Conversely, phase 2 detoxification enzymes afford tumour cells important protection against
chemotherapeutic agents, including arsenic (metalloid)-based drugs [5]. Our study provides important insight into the genes and pathways involved in up-regulating one of these enzymes in *C. elegans*. The conservation of stress response pathways suggests that human orthologues of these genes may provide new targets for approaches to manipulate the levels of phase 2 enzymes, to either protect cells or potentiate the toxicity of chemotherapeutic drugs. For instance, the human orthologue of UFD-2, UBE4B, has been proposed as a new target for chemotherapy, following the discovery that UBE4B promotes p53 degradation [32]. If the function of UFD-2 is conserved and UBE4B is important for Nrf2 activity, this raises the possibility that increased levels of phase 2 detoxification enzymes may also contribute to the survival of cancer cells. This would also indicate potential unwanted side effects of inhibition of UBE4B, including increased risk of adverse drug reactions.

In addition to the implications for chemotherapeutic interventions, our study supports the notion that the ability to up-regulate these defences in response to stress is important for longevity. As such it also provides new avenues to explore in the quest to delay or prevent the onset of age-associated diseases.

**Conclusions**

The regulation of the expression of phase 2 detoxification enzymes in animals is more complex than previously appreciated, involving both general and gene-specific mechanisms.

**Methods**

*Caenorhabditis elegans* strains

All strains were maintained at 15°C using standard methods [33]. N2 Bristol (wild-type), VE1: prdx-2(ak169) II, LD172: N2;ldIs003 [gcs-1p::gfp], VE4: prdx-2(ak169) ldIs003 [gcs-1p::gfp] II, BC14910 dpy-5(e907)/dpy-5(e907); sEx14910 [rCes905E3.3::GFP + pCeh361], CL2166: N2 dvIs19 [pAF15 (gst-4p::gfp-ns) III, VE12: prdx-2(ak169) dvIs19 [pAF15 (gst-4p::gfp-ns)] III, AU3: nsy-1(ag3) II, IG685: tir-1(tm3036) III, KU25: pmk-1(km25) IV, PP198 ufd-2(tm1380) II, LD1257: N2 ldEx010 [SKN-1op::GFP], LD1255: N2 ldEx014 [SKN-1op::GFP] and LD1252: N2 ldEx020 [SKN-1p::GSL24::GFP].

RNA interference

RNAi experiments were carried out essentially as described previously [34]. RNAi clones were grown in Luria Broth (LB) liquid media containing 50 μg/ml ampicillin overnight then diluted to OD₆₀₀ = 1.0 and induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). This was used to seed plates, which were left at room temperature for 2 days to induce double-stranded RNA synthesis before worms were added.

Genome-wide RNAi screening

Genome-wide RNAi screening was performed using a commercially available RNAi feeding library (MRC Geneservice, Cambridge, UK) [34]. Single colonies were inoculated into 800 μl of LB media containing 50 μg/ml ampicillin in 96-well deep well plates, covered with sterile, breathable film and incubated at 37°C on a shaking platform overnight. Then 1 mM IPTG was added to each well and bacteria harvested by centrifugation at 4°C, resuspended in 500 μl LB containing 100 μg/ml ampicillin and 1 mM IPTG then 20 μl spotted onto individual wells of 24-well plates containing RNAi agar. RNAi plates were incubated for 2 days at room temperature, then approximately ten synchronized L1-stage animals dispensed into each well. After incubation at 15°C for 7 days, larval stage F1 progeny were scored for intestinal GFP fluorescence using a Discovery V8 Zeiss fluorescent stereomicroscope. For each batch of RNAi clones tested, empty vector (pL4440) was included as a negative control. Clones that gave rise to less than approximately 40 viable progeny were scored lethal or sterile, as appropriate, and excluded from analysis of reporter gene expression. Clones that affected reporter expression were identified by comparing intestinal GFP fluorescence with empty vector controls. All RNAi clones that reduced intestinal gcs-1p::gfp expression to undetectable levels, i.e. ‘none’ (Figure 1), in more than 50% of the prdx-2 mutant worms in the initial screen were re-screened three more times and also screened three times in wild-type animals for loss of gst-4p::gfp. For analysis of arsenite-induced intestinal gcs-1p::gfp expression, after 7 days of incubation on RNAi plates, worms were washed off screen plates in M9 buffer into the corresponding well of a 24-well plate in which the RNAi agar contained 1 mM sodium arsenite (Sigma S-7400, Poole, UK). Worms were incubated at 15°C for 90 min then reduced intestinal gcs-1p::gfp expression compared with empty vector control was assessed as described above. All RNAi clones that were analysed further were sequenced to confirm the identity of the targeted gene.

Comparative analysis of genes identified in different genome-wide RNA interference screens

The statistical significance of the observed overlap between different gene lists obtained in different genome-wide RNAi screens was calculated using cumulative hypergeometric probability [35].

Scoring of green fluorescent protein reporter gene expression

Intestinal expression of GFP reporter genes was scored similarly to that previously described [12]. ‘None’ indicates that no GFP was detected in intestinal cells, ‘low’ indicates that GFP was detected in the nuclei of a few (≤7) anterior or posterior intestinal cells, ‘medium’
indicates that GFP was detected in some (>7) but not all intestinal cells throughout the length of the intestine and 'high' indicates that GFP was detected in all cells throughout the intestine (see Figure 1 for representative images and illustrations). For more sensitive analysis of the effect of specific RNAi clones on intestinal GFP expression (in Table 1, Additional file 2: Figures S3 and S5 and Figure 5), animals were mounted on an agarose pad and scored under the 40× objective on a Zeiss Axioskop. Statistically significant differences between groups (P values) were determined using a chi² test (Microsoft Excel).

Arsenite resistance assays

Five to ten L4-stage animals were transferred to plates seeded with the indicated RNAi bacterial clone. Then 30 to 40 L4-stage F1 progeny of RNAi-treated animals (or OP50-maintained N2 and uft-1 (tm1380) mutants) were transferred to plates containing the indicated concentration of sodium arsenite and incubated at 15°C. Viability was assessed at the indicated time points and animals were scored as dead and removed from the plate if pharyngeal pumping had ceased and they did not respond to gentle prodding with a platinum wire. P values were derived from log-rank survival analyses of individual experiments (Minitab 16) or Cox’s regression analysis of multiple experiments (Figure 2).

Analysis of lifespan

To analyse lifespan, 30 L4-stage hermaphrodites were placed onto the appropriate RNAi plate and allowed to lay eggs. Once the eggs developed to L4 stage, approximately 150 F1 progeny were transferred to fresh RNAi plates, so that there were 50 animals per plate. Once at the young adult stage, animals were transferred to RNAi plates containing 25 μM 5-fluoro-2'-deoxyuridine to prevent egg laying. Animals were transferred to new RNAi plates every few days throughout the experiment. Animals were incubated at 15°C and viability was assessed at least every 2 days at the same time of day. Animals were scored as dead and removed from the plate if pharyngeal pumping had ceased and they did not respond to gentle prodding with a platinum wire. Animals that died from bagging, ruptured vulva or crawled off the plate were censored. For statistical comparisons between control and RNAi-treated animals, P values were derived from a log-rank survival test (Minitab 16).

Immunoblotting

Approximately 3,000 synchronized L1-larval-stage wild-type/mutant or control/RNAi-treated worms were added to the appropriate plates and harvested at L4 larval stage, before or following treatment with 5 mM arsenite. As described previously [14], extracts were prepared and equal amounts of protein (coomassie) analysed using antibodies against the dual phosphorylated form of p38 (#9211, Cell Signaling Technology) to detect phosphorylated PMK-1 and anti-β-tubulin antibodies (E7, Developmental Studies Hybridoma Bank, Iowa City, Iowa) as a loading control. Quantitative densitometric analysis (area under the peak) of Western blot autoradiographs was conducted using ImageJ 1.44 to determine phosphorylated PMK-1 levels relative to tubulin loading for each lane. The level of phosphorylated PMK-1 in RNAi-treated or mutant samples was then determined relative to control samples on the same blot.

Analysis of gcs-1 and gst-7 mRNA expression

Wild-type (N2) animals were transferred to RNAi or control plates and RNA was extracted from approximately 10,000 synchronized L3-larval-stage progeny following exposure to 5 mM sodium arsenite for 30 min, which induced two- to threefold increases in gcs-1 and gst-7 expression and 6.6-, 10.6- and 5.5-fold increases, respectively, in gst-4, dhs-8 and sdz-8 mRNA levels [8]. RNA extraction was carried out using Trizol (Sigma) and gcs-1, gcs-7, gst-4, dhs-8, sdz-8 and act-1 mRNA levels were determined using Superscript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Paisley, UK) and Corbett Life Science Rotor-Gene 6000 system. mRNA levels were determined from a minimum of three replicate samples relative to act-1 mRNA (For primer sequences see Additional file 1: Table S6). These were then compared with the levels in vector-control-treated animals. Experiments were repeated at least three times and the statistical significance of differences between gcs-1, gcs-7, gst-4, dhs-8 or sdz-8 mRNA levels in RNAi-treated and control animals determined (Student’s T test).

Additional files

Additional file 1: Table S1. Genes required for growth, development or reproduction in the absence of prdx-2. Table S2. RNAi clones that increased intestinal gcs-1:p:gf expression in prdx-2 mutant. There were 50 clones that resulted in higher levels of nuclear GFP throughout the intestine of at least 50% of the RNAi-treated prdx-2 gcs-1:p:gf worms (as illustrated in Figure 1). Table S3. RNAi clones that reduced intestinal gcs-1:p:gf expression in genome-wide RNAi screen in prdx-2 mutants and their effect on the expression of a non-phase 2 intestinally expressed gene reporter f09e5.3p:gf [16] and a second phase 2 reporter gene ghs-4p:gf [21] in wild-type (N2) animals under normal growth conditions.
Table S4. Overlap between genes that are required for expression of gcs-1:p:gf in the prdx-2 mutant (Table S3) and those identified by published RNAi screens of the same RNAi library for different phenotypes.
Table S5. RNAi clones that prevented arsenite-induced intestinal gcs-1:p:gf expression in wild-type animals. Of the 90 RNAi clones that reduced intestinal gcs-1:p:gf expression in prdx-2 mutant animals under normal growth conditions on each of four occasions (Table S3), 53 also prevented an increase in intestinal gcs-1:p:gf expression in wild-type (N2) gcs-1:p:gf animals treated for 90 min with 1 mM arsenite. Table S6. Primer sequences used in quantitative PCR reactions.
Additional file 2: Figure S1. Functional analysis of genes for which RNAi produced a synthetic lethal phenotype with prdx-2 (Additional file 1: Table S1). Figure S2. Genes required for intestinal gcs-1:p:gf
expression in prdx-2 mutants represent a broad range of functional groups. Figure S3. The role of candidate genes identified by the screen in the regulation of gsp-4::gfp expression in prdx-2 mutant animals. Figure S4. The effect of selected candidate RNAi (Table 1 and Figure 2B,C) on the arsenite resistance of wild-type animals. Figure S5. The effect of arsenite-induced PMK-1 phosphorylation. csn-4 and csn-5 RNAi on intestinal expression of gsp-4::gfp in wild-type animals under normal growth conditions. Figure S6. UFD-2 is not required for arsenic-induced PrxK1 phosphorylation.

Abbreviations
CnC: Cap and Collar; CSN: C-terminal domain of the Cullin family; CRL: Cullin-Ring-Ligase; CSN: Cop9 signalosome; GCS: Glutamate-cysteine ligase; GI-ME-N: Glutathione S-transferase I; GSH: Reduced glutathione; GST: Glutathione S-transferase; HR: Hypersensitive response; KEAP1: Kelch-like ECH associated protein 1; Nrf2: Nuclear factor (erythroid-derived 2)-like 2; PARP: Poly(ADP-ribose) polymerase; PPR: Predicted protein phosphatase regulator; PRC: Polycomb repressive complex; PUM: Precursor messenger RNA; PXR: Peroxisome proliferator-activated receptor; RNAi: RNA interference; ROS: Reactive oxygen species; SKN-1: Nrf-related transcription factor; UFD-2: Ubiquitin receptor-2; UVR-6: UV response checkpoint; ZC3H13: Zn finger, C3H type 13.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
EV conceived and HC carried out the RNAi screen. HC, MO, EB, JW and EV performed the research and analysed the data. EV, HC, EB and MO wrote the manuscript. All authors read and approved the final manuscript.

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