Surveillance-Activated Defenses Block the ROS–Induced Mitochondrial Unfolded Protein Response

Eva D. Runkel1,2, Shu Liu2, Ralf Baumeister1,2,3,4*, Ekkehard Schulze2,4

1 Spemann Graduate School of Biology and Medicine, Albert-Ludwigs-University of Freiburg, Freiburg, Germany, 2 Laboratory for Bioinformatics and Molecular Genetics, Faculty of Biology, Albert-Ludwigs-University of Freiburg, Freiburg, Germany, 3 Center for Biochemistry and Molecular Cell Research, Faculty of Medicine, Albert-Ludwigs-University of Freiburg, Freiburg, Germany, 4 Centre for Biological Signaling Studies (BIOSS), Albert-Ludwigs-University of Freiburg, Freiburg, Germany

Abstract

Disturbance of cellular functions results in the activation of stress-signaling pathways that aim at restoring homeostasis. We performed a genome-wide screen to identify components of the signal transduction of the mitochondrial unfolded protein response (UPRmt) to a nuclear chaperone promoter. We used the ROS generating complex I inhibitor parquat to induce the UPRmt and we employed RNAi exposure post-embryonically to allow testing genes whose knockdown results in embryonic lethality. We identified 54 novel regulators of the ROS–induced UPRmt. Activation of the UPRmt, but not of other stress-signaling pathways, failed when homeostasis of basic cellular mechanisms such as translation and protein transport were impaired. These mechanisms are monitored by a recently discovered surveillance system that interprets interruption of these processes as pathogen attack and depends on signaling through the JNK-like MAP-kinase KGB-1. Mutation of kgb-1 abrogated the inhibition of ROS–induced UPRmt, suggesting that surveillance-activated defenses specifically inhibit the UPRmt but do not compromise activation of the heat shock response, the UPR of the endoplasmic reticulum, or the SKN-1/Nrf2 mediated response to cytosolic stress. In addition, we identified PIFK-1, the orthologue of the Drosophila PI 4-kinase four wheel drive (FWD), and found that it is the only known factor so far that is essential for the unfolded protein responses of both mitochondria and endoplasmic reticulum. This suggests that both UPRs may share a common membrane associated mechanism.

Introduction

In order to survive, organisms have to deal with an adverse environment either by avoiding unfavorable or toxic conditions, or by dealing with the consequences of such exposure. The nematode C. elegans for this purpose has developed a number of survival strategies. First, the sensory capabilities of this soil-dwelling animal enable the detection of probably hundreds of adverse mechanical, thermal and chemical stimuli. These neurons are wired to interneurons that serve as a neuronal processor with analytical power, which in turn couples to a motor response to search for or avoid certain environmental conditions. Second, mechanisms have been established in C. elegans to prevent uptake, to inactivate detrimental chemicals, or to repair the consequences of toxin exposure [1–4].

As part of an avoidance strategy to minimize future encounter of a toxin, it was recently reported that C. elegans surveys pathways typically disrupted by pathogens or toxins to engage in defenses. Experimental inactivation of genes in these pathways was sufficient to stimulate an aversion behavior in which the animals avoid normally attractive bacteria [3]. In this study, a large number of genes were found suggesting that this surveillance system (cSADDs) monitors the activity of core cellular components, including translation, energy metabolism, and protein degradation, and triggers food aversion, innate immunity and detoxification defenses upon detection of perturbations.

Unfolded protein responses (UPRs) are evoked when unfolded or misfolded proteins exceed the chaperone folding capacity of the cell. In eukaryotes, individual UPR pathways have evolved for distinct subcellular compartments, such as the endoplasmic reticulum (ER) or the cytosol [for review, see [5,6]]. To restore protein homeostasis, the UPRs signal from the stressed subcellular compartment to the nucleus and initiate an upregulation of a discrete set of compensatory genes, among them compartment-specific chaperones (for review, see [7,8]). In the nematode C. elegans, reporter gene fusions of the promoters of the respective chaperones have been applied to study the UPR pathways [9].

The cytosolic UPR, also known as heat shock response, is initiated by stress interfering with the cytosolic protein folding environment [heat, e.g.] and activates genes including the cytosolic chaperone gene ksp-16.2 [10,11]. In the endoplasmic reticulum (ER), protein folding stress can be experimentally evoked by the...
**Author Summary**

Cellular respiration takes place in the mitochondria. Reactive oxygen species (ROS) are a damaging byproduct of cellular respiration. In adverse conditions, when the load of ROS becomes critical for mitochondrial function, a stress-response pathway, the unfolded protein response of the mitochondria (UPRmt), is triggered. This can be monitored by the activation of the hsp-6 gene. We used the model organism Caenorhabditis elegans to screen for genes required for the activation of hsp-6 and found 54 novel candidates. Surprisingly, most of the genes we identified serve basic cellular functions and are not plausible candidates for regulatory functions. However, this group of genes was recently shown to trigger a cellular surveillance–mediated stress response, sensing pathogen invasion and toxin attack, and forcing the animals to escape from environmental hazards. This mechanism partially requires signaling through the kinase KGB-1. In worms in which KGB-1 was inactivated, UPRmt was not longer interrupted by downregulation of the cellular surveillance activating genes. We suggest this surveillance system as a regulator of the UPRmt that prevents its activation when pathogen attack is sensed.

Administration of tunicamycin, an inhibitor of protein glycosylation [12], that triggers an unfolded protein response (UPRER) to upregulate the transcription of the ER-specific chaperone gene hsp-4 [13] and results, among others, in a general blockade of translation.

Cytosolic oxidative stress elicits responses that in higher eukaryotes activate the phase II detoxification system that is triggered by the transcription factor SKN-1/Nrf2. In C. elegans, this pathway cross-talks with the DAF-2/Insulin/IGF receptor pathway, signaling to its main effector, the transcription factor DAF-16/FOXO [14]. A number of genes have been identified that are differentially regulated by SKN-1, DAF-16, or a combination of both ([15–20], for review see [21]).

Besides the UPR of the cytosol and the ER, more recently an unfolded protein response specific to mitochondria has been described ([22–25], for review see [26,27]). The unfolded protein response of the mitochondria (UPRmt) is initiated by several modes of mitochondrial stress and activates the expression of nuclear genes, among them hsp-6 and hsp-60 encoding mitochondrial chaperones [22]. Many of the described UPRmt inducing stressors interfere directly with the mitochondrial protein folding environment: Inducing stress signals include the downregulation of the mitochondrial chaperone genes hsp-6 and hsp-60, or knockdown of sypg-7 encoding a mitochondrial protease [22], or genes encoding components of the ETC which function in a cell non-autonomous way [28]. A temperature-sensitive mutation, zc32, whose corresponding gene is still enigmatic, was phenotypically characterized and shown to conditionally activate the UPRmt [23]. Several molecular components of the UPRmt pathway have been proposed and suggested a mechanistic model (for review, see [26,27]) in which, as a first step, accumulated unfolded or misfolded proteins are cleaved by the ClpP protease in the mitochondrial matrix [24]. Partly through the HAF-1 ABC transporter, the bZip transcription factor ATFS-1 is activated, whose nuclear targeting in turn directly induces the transcription of the mitochondrial chaperone genes hsp-6 and hsp-60 [25,29]. The homeobox transcription factor DVE-1 and the ubiquitin-like protein UBL-5 are also part of this UPRmt model and induce, independently of ATFS-1, mitochondrial chaperone expression upon peptide efflux from HAF-1 [23–25,30,31]. Recently, a much simpler mechanism was suggested by the same researchers. Under non-stress conditions, atfs-1 mRNA in the cytosol generates a transcription factor which, by default, is transported via the TIM-TOM import complexes into the mitochondria and there is proteolytically inactivated. Stress that alters the mitochondrial membrane potential blocks protein import of ATFS-1, resulting in its cytosolic accumulation and subsequent nuclear transport, where it can activate hsp-6 and hsp-60 genes [29].

Mutations in proteins of the mitochondrial electron transport chain (ETC) typically distort electron transfer to oxygen and, thus, generate reactive oxygen species (ROS). Recently, it was suggested that, in addition to recognizing protein misfolding stress, ROS in a parallel pathway may generate a signal to downregulate translation initiation via the GCN-2 dependent phosphorylation of eIF2α [32]. Thus, in analogy to the UPRER, it was proposed that activating the UPRmt has two consequences: Downregulation of translation, and selective activation of expression of chaperone genes.

Paraquat is a non-selective contact herbicide that in experimental research is frequently used to provoke the generation of reactive oxygen species in the cell, since it accepts electrons in the electron transport chain (ETC) at the inner mitochondrial membrane and transfers them to molecular oxygen, generating the superoxide anion [33–36]. Paraquat administration, among others, induces the mitochondrial manganese superoxide dismutase gene sod-3 [22], which is known to respond to increased ROS [37], and also the UPRmt responsive gene hsp-60 [22]. The onset of the UPRmt reporter upon paraquat-mediated accumulation of ROS may be due to consecutive protein damage, such as irreversible protein carbonylations [38]. It was shown in recent years that a moderate elevation of ROS generated in the mitochondria, such as in a loss of function mutant of the ETC component ISP-1 [34,39], leads to an increase in lifespan [34,40]. This effect can also be mimicked by low concentrations of paraquat [34,40,41]. Thus, administration of paraquat/ROS may have either detrimental or beneficial consequences for a cell or an organism.

Here, we investigate the retrograde signaling to the hsp-6 promoter initiated by an increase in mitochondrial ROS, which we trigger by low doses of paraquat. Genome-scaled RNAi screening revealed, among others, ATFS-1 as essential for the retrograde mitochondrial stress response to ROS, similar to its role in UPRmt. We also found that HAF-1 is dispensable for the paraquat induced signaling, suggesting that a peptide efflux via HAF-1 is not required after ROS induced mitochondrial stress to induce the hsp-6 promoter. We identified 54 additional genes whose downregulation prevented the activation of hsp-6. 87% of them were previously shown to encode components of cellular surveillance monitored pathways, or were found in protein complexes involved in surveillance monitored pathways (eSADDs). We postulate that cellular surveillance serves as a master regulator, activation of which inhibits the onset of the paraquat induced UPRmt. pif-1 encodes a novel PI 4-kinase, downregulation of which blocks the UPRmt independently of the surveillance pathway and may, therefore act downstream of it. Our model suggests that C. elegans uses decisions at several levels to protect itself from external and internal stress.

**Results**

**Paraquat induces hsp-6 reporter expression**

Expression of the mitochondrial chaperone gene hsp-6 is induced upon treatment of C. elegans with paraquat (Figure 1A), which has been considered to activate oxidative stress.
[34,39,42,43] and the mitochondrial unfolded protein response (UPR\textsuperscript{mt}) [22]. We devised a new protocol for paraquat administration which allowed the detection of essential embryonic genes involved in the UPR\textsuperscript{mt}, which could not have been found in previous screens to identify components of the UPR\textsuperscript{mt}. In this protocol L3 stage animals were cultured with paraquat for two days. To monitor stress resistance pathways we used a previously described \textit{hsp-6::gfp} reporter strain which carries the \textit{zcl13} transgene containing about 1.7 kb of the 5' flanking region and the first 10 codons of \textit{hsp-6} fused to GFP [22]. We performed a concentration series and observed that \textit{hsp-6::gfp} induction peaked around 0.5 to 1.7 mM and faded with increasing concentrations of paraquat correlating with increased toxicity (Figure S1). To lower the possible impact of toxicity, we performed subsequent experiments using the lower concentration of 0.5 mM paraquat. This induced the \textit{hsp-6} reporter 41-fold (Figure 1B). Visual inspection by stereomicroscopy revealed that GFP expression started one day after paraquat exposure.

In addition to the expression of \textit{hsp-6}, activated UPR\textsuperscript{mt} can also be monitored by GFP expression from the promoter of \textit{hsp-60} [22]. Comparing the fluorescence intensity of both reporters after paraquat exposure showed that at 0.5 mM \textit{hsp-6::gfp} was induced significantly while \textit{hsp-60::gfp} was not (Figure S2). At a paraquat concentration of 2.0 mM, both reporters were significantly induced, as published [22]. \textit{hsp-6::gfp} induction, however, was twenty times stronger (Figure S2). We concluded that \textit{hsp-6::gfp} is the more sensitive reporter for monitoring paraquat exposure, and, thus, performed subsequent experiments with this reporter.

**Figure 1.** Paraquat induces \textit{hsp-6} and its reporter in a ROS–dependent manner. A. Quantitative analysis (by qRT-PCR) of endogenous \textit{hsp-6} mRNA in wild type (N2) worms exposed to 0.5 mM paraquat from early L3 stage on for 4 h, 24 h and 30 h, presented as fold induction. Dots indicate single experiments; mean plus SD. B. Quantification of GFP fluorescence intensity in the \textit{hsp-6} reporter strain (\textit{Phsp-6::gfp}) after two days of exposure to 0.5 mM paraquat from early L3 stage on. Paraquat significantly increased (p<0.0001) \textit{hsp-6} reporter expression. Columns represent pooled normalized values of three independent experiments plus standard error of the mean (SEM). Numbers in or on columns indicate the number of analyzed animals (\textit{n\textsubscript{total}}=152). ***: p<0.0001; Mann Whitney test. C. The paraquat-triggered induction of the \textit{hsp-6} reporter (\textit{Phsp-6::gfp}) was decreased by the addition of the ROS scavenger N-acetyl-L-cysteine (NAC). Columns represent normalized values plus standard error of the mean (SEM). Numbers in columns indicate the number of analyzed animals (\textit{n\textsubscript{total}}=40). **: p<0.0001; Unpaired t test with Welch's correction. doi:10.1371/journal.pgen.1003346.g001

Consequently to increase the amount of ROS [33,36], as with paraquat, both rotenone, which targets the ubiquinone of complex I, or to coenzyme Q to cytochrome C. Both substances have been shown to increase mitochondrial ROS can activate the reporter. We exposed the \textit{hsp-6} reporter strain from early L3 stage on for two days to 0.25 μM rotenone, which targets the ubiquinone of complex I, or to 0.25 μM antimycin A, which prevents electron transfer from coenzyme Q to cytochrome C. Both substances have been shown to increase the amount of ROS [33,36]. As with paraquat, both substances were found to significantly increase the fluorescence of \textit{hsp-6::gfp}.

**ETC impairment by ROS activates \textit{hsp-6::gfp}**

The UPR\textsuperscript{mt} was so far primarily investigated with stressors that seem to cause unfolded protein stress by directly interfering with mitochondrial proteostasis (such as the knockdown of mitochondrial chaperones or the inactivation of mitochondrial proteases [22–24,27]). Paraquat, in contrast, is a compound primarily known to cause oxidative stress impairing the ETC [42–46]. We wondered whether the induction of \textit{hsp-6::gfp} is specific for paraquat or whether also other conditions known to increase mitochondrial ROS can activate the reporter. We exposed the \textit{hsp-6} reporter strain from early L3 stage on for two days to 0.25 μM rotenone, which targets the ubiquinone of complex I, or to 0.25 μM antimycin A, which prevents electron transfer from coenzyme Q to cytochrome C. Both substances have been shown to increase the amount of ROS [33,36]. As with paraquat, both substances were found to significantly increase the fluorescence of \textit{hsp-6::gfp}.
toxins caused an induction of the *hsp-6* reporter (Figure 3A) being in line with the idea that an increase in mitochondrial ROS induces *hsp-6*.

Genetic interference with the ETC by the introduction of a missense mutation in the *isp-1* allele *qm150* increases mitochondrial superoxide [34]. Supporting our previous findings, *hsp-6:GFP* was induced as well. GFP was constitutively expressed in the *isp-1*(qm150) mutant through all developmental stages and adulthood (Figure 3A). A similar result was observed for the *nec-1(*kn1) mutant, which carries a mutation in the cytochrome b of the mitochondrial respiratory chain complex II (data not shown).

We next wondered whether a compound causing oxidative stress not obviously linked to mitochondrial or ETC dysfunction can also activate *hsp-6:GFP*. The neurotoxin acrylamide triggers cytosolic phase II antioxidant responses in a SKN-1 dependent manner [47,48]. Its mode of ROS production has not been associated with mitochondrial function [49]. We cultured early L3 larvae of the *hsp-6* reporter strain for two days with 2.1 mM acrylamide, and observed only a slight 1.5-fold induction of the *hsp-6* reporter, whereas paraquat induced the *hsp-6* reporter 25 times more effectively (Figure 3B). The same acrylamide concentration, however, failed to activate the phase II response, monitored by the induction of *gst-4:GFP*. Our results suggest that *hsp-6:GFP* responds to both mutants and substances that are considered to increase mitochondrial ROS.

The ROS scavenger NAC reduces paraquat mediated *hsp-6* induction

To analyze whether the increase in mitochondrial ROS is causative for the induction of the *hsp-6* reporter, we compared the response of paraquat treated *hsp-6* reporter animals with those in which paraquat treatment was paired with the addition of 10 mM of the ROS scavenger N-acetyl-L-cysteine (NAC) [50,51]. We observed a 75% reduction in the intensity of *hsp-6:GFP* fluorescence in the presence of NAC (Figure 1C). This suggests that paraquat induced ROS is coupled to *hsp-6:GFP* induction. With this experiment we cannot distinguish whether the UPRmt is induced directly by the increased level of ROS or through a secondary protein damage caused by ROS. In the latter case, NAC treatment may also ultimately prevent protein misfolding by scavenging ROS and thus reducing *hsp-6:GFP* induction. Since the ROS scavenger NAC reduced *hsp-6:GFP*, rather than increased the induction, we consider it unlikely that paraquat induced ROS serves as a signal rather than a toxin, as has been proposed recently [32]. We conclude that ROS increase is a primary causative element in the induction of *hsp-6:GFP*, which, however, may also evoke the response of *hsp-6* through an increase in mitochondrial unfolded proteins.

Paraquat treatment alters mitochondrial morphology

It is possible that 0.5 mM paraquat increases ROS production and signaling to *hsp-6* directly or by affecting the integrity or functions of mitochondrial proteins. An effect of paraquat (0.1 mM) on protein oxidative damage has been shown, even though the abundance of mitochondria was not affected [34]. An altered mitochondrial morphology has been used as an argument for high levels of protein stress in the organelle [24]. Mitotracker is a compound that was used before in experiments to stain mitochondrial unfolded proteins.

We applied paraquat at

Paraquat does not induce unfolded protein responses in the ER or cytosol

Next, we investigated whether 0.5 mM paraquat induced stress responses in cellular compartments other than the mitochondria. Thus, we tested whether reporters of the unfolded protein response of the ER (*hsp-4:GFP*) [10] or of the cytosol (*hsp-16.2:GFP*) [9] also responded to paraquat treatment. We applied paraquat at
early L3 stage and analyzed reporter fluorescence two days later. While both reporters were induced by their respective specific triggers tunicamycin and heat stress, no significant induction was observed with paraquat (Figure 4B, 4C). We conclude that 0.5 mM paraquat does not activate the unfolded protein response in the ER or in the cytosol.

Induction of \textit{hsp-6} by paraquat is independent of key regulators of other ROS stress responses

While paraquat does not activate cytosolic UPR, it is known to activate the cytosolic oxidative stress reporter \textit{gst-4::gfp} [52]. In \textit{C. elegans}, several key regulators of cytosolic oxidative stress responses have been described before. The transcription factors SKN-1 and DAF-16 are crucial for the induction of the phase II oxidative stress response and the defense against oxidative damage, respectively [53,54]. Hypoxia inhibits respiration and activates HIF-1 by elevating the levels of ROS [40]. Therefore, expression of \textit{hsp-6} by paraquat could be dependent on these regulators. We therefore tested paraquat-induced expression of \textit{hsp-6::gfp} in loss-of-function mutants of \textit{skn-1(zu67)}, \textit{daf-16(mu86)} and \textit{hif-1(ia4)}. We found that none of the mutants prevented the induction of \textit{hsp-6} upon 0.5 mM paraquat exposure (Figure 5). This suggests that the response to paraquat triggers a pathway that does not require the transcription factors SKN-1, DAF-16 and HIF-1, and probably also not the pathways in which these factors are effectors namely the cytosolic stress response, insulin signaling, and the heat shock response.

A genome-scaled RNAi screen identifies 55 genes required for paraquat triggered \textit{hsp-6::gfp} induction

To identify essential components of the paraquat mediated induction of \textit{hsp-6::gfp}, we screened the ORFeome RNAi library (Open Biosystems) [55] for suppressors. Synchronized L1 larvae were allowed to develop by feeding on the respective RNAi bacteria for one day. Then, they were exposed to paraquat in order to bypass the paraquat-hypersensitive L1/early L2 stage and to benefit from the enhanced paraquat inducibility of the \textit{hsp-6} reporter in the L3 larval stage. After two days of incubation with paraquat we screened for worms that had failed to induce the \textit{hsp-6} reporter assuming that the respective RNAi clone downregulated a factor essential for \textit{hsp-6} induction (Figure S3).

We confirmed 55 genes whose knockdown led to an evident impairment of \textit{hsp-6::gfp} induction (Table 1). The majority of these also showed morphological, behavioral or developmental abnormalities, among them impaired movement and developmental delay or arrest, which in each case appeared independent of paraquat administration.

Based on GO term analysis [56] the genes of all screening positives were assigned to functional groups. We found two subunits of a vacuolar H$^+$ ATPase (Functional Group: ATP synthesis coupled proton transport), several proteasomal regulatory subunits (Functional Group: Cellular protein catabolic process (Proteolysis)), and several subunits of the cytosolic chaperonin complex, the orthologue of human TRiC/CCT (TCP-1 Ring Complex) (Functional Group: Cellular protein metabolic process...
We also detected several genes encoding proteins involved in intracellular protein transport, including two nuclear transport factors (Functional Group: Intracellular protein transport). Furthermore, the screen revealed three genes encoding small nuclear ribonucleoproteins ( Functional Group: mRNA splicing). A large group, 26 genes, encode proteins of both ribosomal subunits (20 out of 55 screening positives) and additional genes (6 out of 55 screening positives) whose products have been associated with the translation of proteins (Functional Group: Protein translation). The mechanisms through which knockdown of these genes prevents hsp-6 reporter induction is not an attenuation of the general translation, since the inhibitory effect could not be mimicked if translation was attenuated by other means (Figure S4).

Two genes (Functional Group: Regulation of transcription, DNA dependent) encode transcription factors, one being the GATA type transcription factor ELT-2, which is required for intestinal cell differentiation and maintenance [57,58], and the other being ATFS-1, the bZip transcription factor involved in UPR<sup>mt</sup> [25]. The detection of the latter in the unbiased screen confirmed our observation that ATFS-1, in line with its previously described role in UPR<sup>mt</sup> signaling [25,32], is also involved in the hsp-6::gfp induction by paraquat (see Figure 2). One functional group was assigned to two genes whose products are putatively involved in signaling (Functional Group: Signaling). Seven genes were not clustered in groups (Table 1). We quantified the hsp-6 reporter induction by paraquat for three screening positives, rpl-36, atfs-1, and the PI 4-kinase gene pifk-1 (F35H12.4). RNAi against each gene significantly prevented hsp-6::gfp induction (Figure 6).

Except for ATFS-1 none of the 55 screening positives had been implicated in the UPR<sup>mt</sup> before. In order to test whether we found so far not described genes in the UPR<sup>mt</sup> pathway, we performed RNAi against all in the temperature-sensitive mutant
strain SJ52 [zc32; Phsp-60::gfp] used in a previous UPRmt screen. Since many of our screening positive RNAis caused larval arrest, the UPRmt screening protocol used by these authors was modified [23,24]. We added synchronized L1 larvae to the respective RNAi plates, shifted the plates from 15°C to the restrictive temperature of 25°C when worms had developed to L4 larvae or young adults, and analyzed GFP fluorescence after two additional days. This protocol allowed the analysis of the role of our embryonic or larval lethal screening positives according to the UPRmt model. 29 candidates interfered with the activation of the hsp-60::gfp reporter in the zc32 mutant, among them atfs-1 which we considered as confirmation of the quality of our protocol (Table 1), 24 candidates did not obviously alter hsp-60::gfp expression in the background of zc32. We quantified GFP intensity in zc32 mutant animals expressing hsp-60::gfp which were treated with atfs-1, pifk-1 and rpl-36 RNAi. Confirming a previous report, knockdown of atfs-1 attenuated hsp-60 reporter induction [25] (Figure S5). Induction of the reporter was also efficiently prevented by RNAi of pifk-1 (Figure S5). In contrast, downregulation of rpl-36, which encodes a protein of the large ribosomal subunit, rather increased GFP expression of the hsp-60 reporter compared to the vector control (Figure S5). These results suggest that atfs-1 and pifk-1 are required, whereas rpl-36 may be dispensable for the zc32 triggered mitochondrial stress response (Table 1). Since the identity of the zc32 mutation is still enigmatic, it is currently not possible to interpret the difference obtained in both experimental paradigms.

ATFS-1, PIFK-1, and RPL-36 are also required for isp-1(qm150)–mediated induction of hsp-6::gfp

Beside paraquat other ROS-generating compounds and ETC mutants induce hsp-6::gfp (see Figure 3A). Here we tested whether the block of hsp-6::gfp induction is either specific for paraquat or if it would also block hsp-6::gfp induction resulting from the ETC mutant isp-1(qm150), which is a generator of mitochondrial superoxide [34]. RNAi specifically impairing paraquat uptake or metabolism would not block the isp-1(qm150) mediated signaling. We tested RNAi of atfs-1, which is known to be part of the UPRmt, pifk-1, which we found as a potential new component of the mitochondrial stress signaling, and as a third candidate rpl-36, which is not essential for the zc32, but for the paraquat-triggered hsp-6::gfp induction. GFP expression of L1 larvae were placed on the respective RNAi plates and grown until adulthood. Analyses in
Table 1. 55 screening positives and their involvement in other cellular stress pathways.

<table>
<thead>
<tr>
<th>Gene Brief description</th>
<th>UPR\textsuperscript{m} (paraquat)</th>
<th>phase II response</th>
<th>UPR\textsuperscript{cr}</th>
<th>UPR\textsuperscript{m}</th>
<th>UPR\textsuperscript{m} (zc32)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ATP synthesis coupled proton transport</strong></td>
<td>vha-1 vacuolar H(+)-ATPase subunit</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>vha-2 vacuolar H(+)-ATPase subunit</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Cellular protein catabolic process (Proteolysis)</strong></td>
<td>rpn-7 19S proteasome, regulatory subunit</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>pas-4 20S proteasome, regulatory subunit</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>pas-7 205 proteasome, regulatory subunit</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Cellular protein metabolic process (Protein folding)</strong></td>
<td>cct-1 cytosolic chaperonin, subunit</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>cct-2 cytosolic chaperonin, subunit</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>cct-4 cytosolic chaperonin, subunit</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>cct-5 cytosolic chaperonin, subunit</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Intracellular protein transport</strong></td>
<td>snap-1 soluble NSF attachment Protein</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>sec-23 COPII subunit</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>apm-1 adaptor complexes medium subunit</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>apb-1 AP-1/AP-2/AP-4, beta subunit</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>imb-3 nuclear transport factor</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>imb-5 nuclear transport factor</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>mRNA splicing</strong></td>
<td>snr-1 small nuclear ribonucleoprotein/U1snRNP</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>snr-2 small nuclear ribonucleoprotein/U1snRNP</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>snr-6 small nuclear ribonucleoprotein/U1snRNP</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Regulation of transcription, DNA-dependent</strong></td>
<td>atfs-1 bZip transcription factor</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>elt-2 GATA-4/5/6 transcription factor</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Signaling</strong></td>
<td>F35H12.4 PI 4-kinase</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>off</td>
</tr>
<tr>
<td></td>
<td>Y47D3B.1 G-protein coupled receptor signaling</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Protein translation</strong></td>
<td>hel-1 helicase, exporting mRNA from the nucleus</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>phi-2 eIF-4A</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>phi-4 mRNA splicing factor</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>phi-19 polypeptide release factor 3</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>phi-21 peptide chain elongation factor</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Y65B4A.6 predicted ATP-dep. RNA helicase FAL1</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>rpl-14 large ribosomal subunit protein</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rpl-17 large ribosomal subunit protein</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>rpl-18 large ribosomal subunit protein</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>rpl-19 large ribosomal subunit protein</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>rpl-22 large ribosomal subunit protein</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>rpl-23 large ribosomal subunit protein</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>rpl-26 large ribosomal subunit protein</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>rpl-30 large ribosomal subunit protein</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>rpl-31 large ribosomal subunit protein</td>
<td>off</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
adulthood were possible since RNAi against these three genes did not cause larval arrest. During larval growth, GFP was continuously expressed on control plates. On the RNAi plates however, GFP fluorescence was strongly reduced. Downregulation of all three screening positives during postembryonic stages of \textit{L1} larvae were raised on the respective RNAi plates containing 0.4 \text{mM} paraquat, and the number of animals on each plate that reached adulthood at day 5 was counted. Without paraquat, all worms developed to become adults, except for \textit{rpl-36} (RNAi) of which 1.5\% did not reach adulthood within this time. Following paraquat exposure, about 50\% of the controls became adults until day 5. In contrast, none of the animals subjected to RNAi against \textit{atfs-1} and \textit{rpl-36} respectively reached adulthood in this time window, but 27\% of \textit{pifk-1} (RNAi) animals became adult. This data indicate that RNAi against all three genes increased, rather than decreased, paraquat sensitivity (Figure 8). Thus, for these three exemplary screening positives a relief of stress scenario can be ruled out. Furthermore, we conclude that the established UPR\textsuperscript{mt} component \textit{atsfs-1} contributes to a protective response in line with a previous report [32].

\textbf{Knockdown of screening positive genes increases paraquat sensitivity}

The expected function of a stress signaling pathway is to trigger a protective response. Screening positives may emerge for two different reasons: Either a specific stress signaling pathway could be blocked, or, alternatively, the level of stress could be reduced. In order to distinguish between these two alternatives we reasoned that a relief of stress would lead to paraquat hyposensitivity or resistance, whereas the disruption of a protective function would lead to an increased sensitivity to paraquat. We tested RNAi with three exemplary screening positives (\textit{atsfs-1}, \textit{rpl-36}, and \textit{pifk-1}) in a paraquat toxicity assay during larval development. Synchronized \textit{L1} larvae were raised on the respective RNAi plates containing 0.4 \text{mM} paraquat, and the number of animals on each plate that

\begin{table}[h]
\centering
\small
\begin{tabular}{|l|l|l|l|l|l|l|}
\hline
Gene & Brief description & UPR\textsuperscript{mt} (paraquat) & phase II response & UPR\textsuperscript{cyt} & UPR\textsuperscript{in} & UPR\textsuperscript{mt} (zc32) \\
\hline
\textit{rpl-33} & large ribosomal subunit protein & off & + & + & + & + \\
\textit{rpl-35} & large ribosomal subunit protein & off & + & + & + \\
\textit{rpl-36} & large ribosomal subunit protein & off & + & + & + \\
\textit{rpl-41} & large ribosomal subunit protein & off & + & + & + \\
\textit{rps-2} & small ribosomal subunit protein & off & + & + & n.d. \\
\textit{rps-7} & small ribosomal subunit protein & off & + & + & + \\
\textit{rps-8} & small ribosomal subunit protein & off & + & + & off \\
\textit{rps-14} & small ribosomal subunit protein & off & + & + & off \\
\textit{rps-17} & small ribosomal subunit protein & off & + & + & off \\
\textit{rps-26} & small ribosomal subunit protein & off & + & + & off \\
\textit{rps-27} & small ribosomal subunit protein & off & + & + & off \\
\hline
\textbf{Others} & & & & & & \\
\textit{act-3} & actin & off & + & + & + & off* \\
\textit{C14B1.2} & & off & + & + & + & off* \\
\textit{C18A3.3} & contains eukaryotic rRNA proc. domain & off & + & + & + \\
\textit{C23G10.8} & & off & + & + & + \\
\textit{pan-1} & predicted transmembrane protein & off & + & + & + & +* \\
\textit{W04A4.5} & contains HEAT domain & off & + & + & + & off \\
\textit{Y39B6A.42} & & off & + & + & + \\
\textit{control} & & + & + & + & + \\
\hline
\end{tabular}
\caption{Qualitative analyses of GFP induction (by stereomicroscopy) of the different stress reporters with their respective inducers. UPR\textsuperscript{mt} (paraquat): induction of the \textit{hsp-6} reporter (Phsp-6::gfp) with paraquat; phase II response: SKN-1 dependent induction of the \textit{gst-4} reporter (P\textit{gst-4::gfp}) with acrylamide; UPR\textsuperscript{cyt}: induction of the \textit{hsp-16.2} reporter (Phsp-16.2::gfp) with heat shock; UPR\textsuperscript{in}: induction of the \textit{hsp-4} reporter (Phsp-4::gfp) with tunicamycin; UPR\textsuperscript{mt} (zc32): induction of the \textit{hsp-60} reporter (Phsp-60::gfp) in the \textit{zc32} ts-mutant at the restrictive temperature. +: GFP induced; off: GFP not induced; n.d.: not determined; *: in two out of three experiments; assessed by qualitative compound microscopy. Group assignment of individual candidates is based on DAVID gene enrichment analyses [56]. doi:10.1371/journal.pgen.1003346.t001}
\end{table}
To get more detailed insights we quantified three candidate screening positives (afs-1, rpl-36, pifk-1) for each response. ATFS-1 was chosen as a known UPR\textsuperscript{mt} pathway component, pifk-1 emerged as a novel gene implicated in the UPR\textsuperscript{mt} and rpl-36 RNAi enhanced zc32 triggered mitochondrial stress signaling but abolished paraquat mediated induction of the hsp-6 reporter.

Acrylamide induces a SKN-1 dependent induction of gst-4::gfp [47,52,59]. RNAi knockdown of none of the 55 candidates blocked gst-4 expression in response to 2.1 mM acrylamide (Table 1). Quantification of three screening positives revealed that gst-4::gfp fluorescence was not suppressed by rpl-36 and afs-1 RNAi, suggesting that the inactivation of these genes does not interfere with the class II response. However, RNAi of pifk-1 reduced both the basal expression of gst-4::gfp and the acrylamide dependent induction of the gene. We suggest that either pifk-1 affects gst-4 expression in a general way, or that induction by acrylamide also involves pifk-1 function to some extent (Figure 9A).

We noticed that RNAi of occ-1, occ-5, pas-4, and pas-7 already resulted ingst-4 expression in the absence of acrylamide, confirming a previous report [48] (Table 1). Thus, for those four candidates that affect protein folding and turnover, we could not exclude that such constitutive activation of the class II detoxification system might reduce the ROS burden after paraquat administration. This would render the worms more resistant to paraquat, and could explain why hsp-6 is not induced in those four experiments. While the occ-1/5 RNAi mediated induction of gst-4 appeared to be independent of SKN-1, knockdown of the proteasomal subunit mitigates gst-4 expression via SKN-1 [48]. We anticipated, therefore, that such an indirect effect would be SKN-1 dependent, at least in case of RNAi against a proteasomal subunit gene. Therefore, we tested paraquat mediated hsp-6 induction in skn-1(zu67) mutant animals after RNAi with pas-4, and pas-7. Loss of function of SKN-1 did not reconstitute the paraquat mediated hsp-6 induction, which argues against such an indirect effect of the SKN-1 activating RNAi experiments. However, a SKN-1 independent relief of stress cannot be excluded.

Next, we tested whether the screening positives crosstalk with the cytosolic unfolded protein response. We heat-shocked L4 staged hsp-16.2::gfp reporter worms for 4 h at 34°C and observed fluorescence one day later. Qualitative assessment of GFP fluorescence revealed no obvious impairment in any of the RNAi experiments (Table 1). Quantification showed that RNAi against pifk-1 did not affect heat-shock induction of hsp-16.2::gfp, indicating that pifk-1 is not involved in this response. Knockdown of rpl-36 and afs-1, the two factors affecting the UPR\textsuperscript{mt} response, did not prevent, but significantly decreased hsp-16.2 induction to 34% and 58%, respectively (Figure 9B). This suggests that some crosstalk between the UPR\textsuperscript{mt} and the heat shock responses exists, or that between the UPR\textsuperscript{mt} and the heat shock responses exists, or that these genes have dual roles in both pathways. This would make sense, since noxious heat will also result in denaturation of mitochondrial proteins, which may also increase mitochondrial ROS production. Since most of the factors involved in UPR\textsuperscript{mt} are cytosolic signaling components [25], the same proteins could also help in activating the cytoplasmic heat shock response.

Next, a possible role of the screening positives in the induction of the unfolded protein response of the endoplasmic reticulum (UPR\textsuperscript{ER}) was tested. UPR\textsuperscript{ER} was triggered by incubation with 7.2 μM tunicamycin and monitored using the hsp-4::gfp reporter [9]. We found three screening positives (sha-1, snap-1, and sec-23) whose knockdown induced the hsp-4 reporter already in the absence of tunicamycin implicating that the loss of expression of those genes induces ER stress constitutively. All three candidates play a role in intracellular protein transport. With one exception, pifk-1, visual inspection revealed that none of the other RNAi treated screening positives prevented or strongly reduced hsp-4::gfp induction (Table 1). Quantification of afs-1, rpl-36 and pifk-1, respectively, showed that RNAi with afs-1 did not affect induction significantly, whereas rpl-36 reduced the induction to 49%, which proved to be significant (Figure 9C). Thus, it may be possible that affecting the balance of ribosomal protein expression interferes with the induction of unfolded protein responses in both ER and mitochondria. Interestingly, the observed strong
A

Figure 7. Knockdown of *rpl-36, atfs-1, and pifk-1* suppresses the *isp-1(qm150)*-mediated induction of the *hsp-6* reporter. The *isp-1(qm150)* mutant of mitochondrial superoxide [17] constitutively activated the *Phsp-6* reporter (*Phsp-6::gfp*). RNAi of all three tested genes suppressed (p<0.001) the constitutive *hsp-6* reporter gene induction. Representative micrographs (A) and quantification of GFP fluorescence intensity (B). *hsp-6* reporter worms carrying the *qm150* allele were analyzed for GFP expression after one week on the respective RNAi plates. Columns represent pooled values of three independent experiments plus standard error of the mean (SEM). Numbers in columns indicate the number of analyzed animals (n_total = 317). ***: p<0.001; Kruskal-Wallis test plus Dunn’s Multiple Comparison Test. Equal optical settings per row, scale bar 100 µm. (i): RNAi; L4440: empty vector control.

doi:10.1371/journal.pgen.1003346.g007

B

impairment of the UPR_{mt} upon knockdown of *pifk-1* (Table 1) was confirmed by qualitative analyses. The induction of *hsp-4::gfp* was reduced to 20% compared to control RNAi (Figure 9C). This is remarkable since at least to our knowledge PIFK-1 is the first protein which seems to be implied in signaling of UPRs in both organisms.

cSADDs suppresses the response of *hsp-6* to paraquat

We noticed that many of the screening positives we had identified are genes also identified in a recent publication by Melo et al. (2012). There, the authors report a cellular surveillance system, which they call cSADDs (cellular surveillance activated detoxification and defenses) that monitors basic cellular functions and, if compromised, generates specific behavioral, immune, and detoxification responses, respectively [3]. Downregulation of 36 of the 55 genes (65%) identified in our screen was identified to induce the cSADDs, including food aversion behavior [3], Table S1. In addition, of the remaining 19 genes twelve encode proteins belonging to either functional protein classes or protein complexes which activate the cellular surveillance system upon distortion [3]. Thus, in total 87% of the screening positives encode proteins belonging to processes or complexes that are monitored by the surveillance system.

Given that the cellular surveillance system is monitoring life-threatening conditions, such as toxin or pathogen exposure, we hypothesized that cSADDs may inhibit the onset of other stress responses that are evoked by milder, not life-threatening stresses, like the concentrations we have chosen for paraquat administration to induce the UPR_{mt}. Signaling from cellular surveillance partially requires the activity of a JNK signaling cascade, in which KGB-1 is an essential component. We hypothesized that interfering with the signaling of cSADDs by a mutation in *kgb-1* should at least partially release its inhibitory impact of the UPR_{mt}.

To test this idea, we crossed the *hsp-6* reporter strain with the *kgb-1* mutant, which has been shown to partially suppress the surveillance mediated food avoidance [3]. Then, in the presence of 0.5 mM paraquat, as used in our screening protocol, *kgb-1* worms were grown on elt-2 (RNAi) bacteria, knockdown of which triggers cSADDs mediated aversion [3]. Whereas down-regulating elt-2 in *kgb-1* control strains eliminated the GFP induction as reported, the introduction of the *kgb-1* mutation released this inhibitory effect to some extent (Figure 10A). We observed the same recovery of *hsp-6::gfp* induction in the *kgb-1* mutant when worms were grown on *rpl-36* RNAi (Figure S6). We conclude that elt-2 and *rpl-36*, and the partially the other 46 genes whose RNAi activated cSADDs, contribute to cSADDs mediated inhibition of the mitochondrial stress response.

In line with this idea, loss of *kgb-1* would not affect the inhibitory effect of those screening positives that do not do not evoke cSADDs. ATFS-1, the transcription factor controlling *hsp-6* activation [25] and PIFK-1 (this study) have not been detected as activators of cSADDs [3]. We therefore first tested whether RNAi with these genes triggers the aversion phenotype, that served as a readout for cSADDs [3]. RNAi of neither of both genes induced food aversion, whereas RNAi of elt-2, used as a control as described [3], did (Table 2). Next, we tested whether in a *kgb-1* mutant paraquat triggered *hsp-6::gfp* induction is relieved, as we have observed using elt-2 RNAi. In line with our hypothesis, RNAi of *atfs-1* or *pifk-1*, both in the *kgb-1* control strain and in the *kgb-1* mutant, still blocked the paraquat-triggered *hsp-6::gfp* induction (Figure 10A, 10B).

From these data we conclude that we identified two functionally different groups of genes: (1) those, like *atfs-1* and *pifk-1*, that are involved in signaling from the mitochondria to the nucleus resulting in *hsp-6* induction, and (2) those that are involved in processes targeted by pathogen invasion and toxin attack, whose downregulation induces the cellular surveillance system and results in cSADDs, including behavioral, immune, and detoxification responses (Figure 10C).

Discussion

Mild stress induced by paraquat evoked the UPR_{mt} in a ROS-dependent manner

In this work we analyzed the response of *C. elegans* to a low, non-lethal concentration (0.5 mM) of the ROS generator paraquat by inducing the UPR_{mt}, visualized by expression of the *hsp-6::gfp* reporter gene. Whereas higher concentration of paraquat resulted in a dramatic impact on the development of *C. elegans*, including larval arrest or rapid death, the low concentration we used in our experiments only produced a slight delay of larval development, and even extended lifespan of the animals when applied at adult stage [40]. We show here that the established ROS scavenger NAC substantially reduced *hsp-6::gfp* induction in our protocol,
suggested to encode a key regulator of the UPRmt and activator of hsf-6, which was been proposed to be an essential component of the UPRmt after induction by ethidium bromide, zc32 and elk-1(qm30) [29].

Novel screen for genes required for paraquat induction of hsp-6 revealed preferentially cSADDs genes

We conducted a genome scaled screen employing postembryonic RNAi exposure to identify genes involved in the paraquat triggered UPRmt. This was the first systematic analysis of genes required for the paraquat/ROS induced UPRmt [22,29], and also the first protocol that allowed screening with genes with an embryonic lethal mutant phenotype [23].

Among the 55 genes we identified was atfs-1, previously suggested to encode a key regulator of the UPRmt and activator of hsp-6 and hsp-60 transcription [29]. None of the other 54 genes had previously been implicated in the ROS induced UPRmt. Most of them encode proteins involved in basic cellular functions, which include components of the protein degradation and protein folding pathways, as well as translation. Accordingly, RNAi knockdown of most of them caused a pronounced delay or arrest of larval development already in the absence of paraquat, which did not affect our screening due to the postembryonic application of RNAi, but would have prevented their identification in previously described screens.

The largest family of genes identified encodes components of the small and large ribosomal subunits, as well as several factors involved in protein translation. It has been suggested that stress of both mitochondria and the endoplasmic reticulum result in the downregulation of translation. Therefore, RNAi against the ribosomal proteins, in a simple model, may prevent general translation and, thus, result in a relief of stress. In agreement with such a model, Baker et al. [32] recently suggested that, upon protein misfolding, the activation of the kinase GCN-2 results in an inhibition of translation initiation. Thus, upon mitochondrial stress, blocking translation would reduce the load on the protein folding machinery, and thereby alleviate stress. For a number of reasons downregulation of general translation was not observed by us: First, because downregulation of ribosomal genes did not prevent the expression of other GFP reporter genes tested in this study (Figure 9, Figure S5). Second, because we found that downregulation of rpl-36, a representative member of this group of genes, showed an enhanced rather than reduced sensitivity to paraquat. Third, a general reduction of translation mediated by the ife-2(ak306) mutant in our hands was not sufficient to phenocopy the effects of RNAi against ribosomal genes. Intriguingly, we find that, in addition to genes for the ribosomal subunits, most genes identified in our screen overlap with a list of genes found in a recently published report addressing food avoidance behavior as part of cSADDs (see Table S1) [3]. There, the existence of a systemic surveillance system of basic molecular functions was proposed, which triggers defensive molecular and behavioral consequences that allows animals to detect invading pathogens or exposure to toxins. RNAi of core cellular activities, which include translation and protein turnover, induces detoxification and innate immune defense already in the absence of a pathogen or pathogenic toxin.

A total of 36 of the 55 genes (65%) identified in our screen have been linked to aversion behavior ([3], Table S1). In addition, of the remaining genes all but seven, including atfs-1 and pifk-1, encode proteins belonging to functional classes or protein complexes of which genes encoding other components have been identified in aversion behavior. Since the cellular surveillance system signals through an endocrine response which involves the activity of the JNK pathway, we tested whether inactivation of kgb-1 could release the inhibitory role of cSADDs. This we could show for elt-2 and rpl-36, but not for atfs-1 and pifk-1. The knockdown of the latter two genes did not trigger cSADDs, and therefore these genes are most likely specifically involved in UPRmt signaling.
Figure 9. The knockdown of rpl-36, atfs-1, or pifk-1 does not prevent non-mitochondrial stress responses. Worms were grown from L1 larval stage on the respective RNAi plates before being exposed to the respective stress and analyzed four days after L1. A. A reporter strain for the SKN-1 dependent phase II response (Pgst-4::gfp) was exposed to 2.1 mM acrylamide starting at early L3 stage. RNAi of rpl-36 and atfs-1 did not prevent, but pifk-1 (RNAi) significantly (p < 0.001) reduced reporter gene induction as compared to vector control. Columns represent pooled normalized values of four independent experiments plus standard error of the mean (SEM). Numbers in columns indicate the number of analyzed animals (n_{total} = 819). ***, p < 0.001; Kruskal-Wallis test plus Dunn’s Multiple Comparison Test; Mann Whitney test (comparison of vector with and without acrylamide). Equal optical settings, scale bar 200 μm. B. Cytosolic UPR (heat shock) reporter worms (Phsp-16.2::gfp) were exposed to 34°C for 4 h at L4. While the downregulation of none of the three screening positives prevented the heat shock response completely, the knockdown of rpl-36 and atfs-1 significantly decreased heat stress induced reporter expression (p < 0.001). Columns represent pooled normalized values of two independent experiments plus standard error of the mean (SEM). Numbers in or on columns indicate the number of analyzed animals (n_{total} = 354). ***, p < 0.001; Kruskal-Wallis test plus Dunn’s Multiple Comparison Test; Mann Whitney test (comparison of vector with and without heat shock). Equal optical settings, scale bar 100 μm. C. The UPRER reporter strain (Phsp-4::gfp) was raised from L1 stage RNAi plates (with 7.2 μM tunicamycin). UPRER.
induction was not blocked by any RNAi tested here, but \textit{pifk-1} (RNAi) and \textit{rpl-36} (RNAi) strongly impaired its induction (p<0.0001). Columns represent pooled normalized values of four independent experiments plus standard error of the mean (SEM). Numbers in or on columns indicate the number of analyzed animals (n\textsubscript{total} = 495). 

**Materials and Methods**

Transgenic and mutant \textit{C. elegans} strains

\textit{C. elegans} variety Bristol, strain N2 was used as wild type strain. All strains were maintained and raised at 20°C on NGM agar seeded with \textit{Escherichia coli} OP50 [67], unless otherwise indicated. The following strains were obtained from CGC: SJ1400: zcIs13[\textit{hsp-6::GFP}], SJ4005: zcIs4[\textit{hsp-4::GFP}], CL1626: dvIs19[\textit{af15gst-4::GFP::NLS}], ST66: ncs17[\textit{hsp-16.2::GFP}pBluescript], RB867: hsf-1(\textit{ok765})\textit{IV}, KX13: \textit{ifes-2}(\textit{ok306})\textit{X}, ZG31: \textit{hif-1}(\textit{ua4})\textit{IV}, CF1038: \textit{daf-16}(\textit{ma68})\textit{I}, EU1: \textit{skn-1}(\textit{zu67})\textit{IV}\textit{[\textit{unc-2}(\textit{n754})\textit{let-2}]}\textit{IV}, DP38: \textit{unc-119}(\textit{ed3})\textit{III}, MQ807: \textit{ivp-1}(\textit{qm150})\textit{IV}. The following strain was obtained by backcrossing SJ1400 seven times against laboratory N2: BR5194: zcIs13[\textit{hsp-6::GFP}]. The following strains were obtained by crossing the respective mutants (see above) with BR5194: BR6118: hsf-1(\textit{ok705})\textit{IV}, zcIs13[\textit{hsp-6::GFP}], BR6019: ifes-2(\textit{ok306})\textit{X}, zcIs13[\textit{hsp-6::GFP}], BR6097: hif-1(\textit{ua4})\textit{IV}, zcIs13[\textit{hsp-6::GFP}], BR6020: daf-16(\textit{ma68})\textit{I}, zcIs13[\textit{hsp-6::GFP}], BR6098: skn-1(\textit{zu67})\textit{IV}, zcIs13[\textit{hsp-6::GFP}], BR 6372: \textit{ivp-1}(\textit{qm150})\textit{X}, zcIs13[\textit{hsp-6::GFP}]. The reporter strain SJ4058: zcIs9[\textit{hsp-60::gfp}] was obtained from C. Benedetti. The UPR\textsuperscript{mt} reporter strain SJ52: [\textit{zcs2} II; \textit{hsp-60::gfp}] \textit{V} was kindly provided by C. Haynes.

RNA interference assays

**RNAi screening protocol.** Genome-scaled screening was performed in duplicated 96 well liquid culture plates using the ORFeome RNAi feeding library (Open Biosystems) [55]. Day 1:
Figure 10. cSADDs inhibits paraquat-mediated signaling to hsp-6 through KGB-1. A–B. In kgb-1(um3) mutant animals, which are cSADDs deficient, paraquat induced hsp-6 induction is not blocked by elt-2 RNAi. Thus, ROS induced UPRmt is activated in the absence of functional cSADDs. In contrast, kgb-1(um3) does not prevent inhibition of hsp-6 induction by atfs-1 and pifk-1 knockdown, suggesting that they function downstream of kgb-1 and the cSADDs. Columns represent normalized values plus standard error of the mean (SEM). Numbers in or on columns indicate the number of analyzed animals (n_{total} = 248). ***: p < 0.001, *: p < 0.05; Kruskal-Wallis test plus Dunn’s Multiple Comparison Test (A). Equal optical settings, scale bar 400 μm. (i): RNAi; L4440: empty vector control; +: wild type allele (B). C. Model: Genes activating the cSADDs (cellular surveillance system) inhibit the paraquat-triggered induction of the UPR^{mt}. doi:10.1371/journal.pgen.1003346.g010
RNAi bacteria from frozen glycerol stocks were inoculated in 40 μL LB supplemented with 12.5 μg/ml tetracycline and 12.5 μg/ml carbenicillin and grown at 28°C, 180 rpm overnight. Eggs were prepared from gravid C. elegans adults by alkaline sodium hypochlorite treatment and allowed to develop in M9 at 15°C overnight. Day 2: 1.0 mM IPTG was added and the incubation was continued for another 2 h at 37°C, 180 rpm. A suspension of synchronized C. elegans L1 larval was diluted to a concentration of 1 worm/μL in M9 supplemented with 10 μg/ml cholesterol, 50 μg/ml carbenicillin, 12 μg/ml tetracycline, 1 mM IPTG and 10 μg/ml fungizone. 20 μL of this suspension were distributed to 96 well plates after the bacterial culture had cooled down to room temperature. Day 3: paraquat (Sigma) was added to each well to a final concentration of 2.0 mM. Day 5: Plates were screened for non-GFP-expressing worms. Positive RNAi bacteria were recloned, sequenced and retested in at least one additional experiment.

### Table 2. Knockdown of ATFS-1 and PIFK-1 does not evoke aversion behaviour [3].

<table>
<thead>
<tr>
<th>Gene</th>
<th>Brief description</th>
<th>N\text{total}</th>
<th>Aversion Score (N\text{off}/N\text{total})</th>
</tr>
</thead>
<tbody>
<tr>
<td>afts-1</td>
<td>bZip transcription factor</td>
<td>223</td>
<td>0.01</td>
</tr>
<tr>
<td>pifk-1</td>
<td>PI 4-kinase</td>
<td>281</td>
<td>0.00</td>
</tr>
<tr>
<td>elt-2</td>
<td>positive control</td>
<td>32</td>
<td>0.72</td>
</tr>
<tr>
<td>L4440</td>
<td>negative control</td>
<td>309</td>
<td>0.00</td>
</tr>
</tbody>
</table>

N\text{total} = total number of analyzed animals. Aversion is determined by the ratio of worms outside the bacterial lawn (N\text{off}) and the total amount (N\text{total}) 48–58 hr of growth on RNAi bacteria. Aversion scores for afts-1, pifk-1 RNAi and L4440 represent mean value from three independent experiments.

doi:10.1371/journal.pgen.1003346.t002

RNAi on NGM plates. A 1:50 dilution of the respective RNAi bacterial overnight culture (37°C, 150 rpm) in LB medium supplemented with 12.5 μg/ml tetracycline and 12.5 μg/ml carbenicillin was grown for another 6 h at 37°C, 150 rpm. Bacteria were then seeded on NGM plates containing 1.0 mM IPTG and 25.0 mg/ml carbenicillin.

Stress induction on NGM agar plates

Eggs were prepared from the respective gravid C. elegans adults by exposure to alkaline sodium hypochlorite and allowed to hatch in M9 [67] overnight. Synchronized L1 larvae were placed on NGM agar plates seeded with the respective bacteria.

**Heat shock assay.** L1 larvae (ST66) were grown on the respective RNAi bacteria for two days, subjected to 34°C for 4 h and analyzed for GFP expression one day later.

**Paraquat/Acrylamide stress assays.** L1 larvae (BR5194, CL2166) were grown on the respective RNAi bacteria for 24 h, subjected to 0.5 mM paraquat (Sigma), 0.25 μM rotenone (Sigma), 0.25 μM antimycin A (Sigma), or 2.1 mM acrylamide (BioRad), respectively. Chemicals were added from aqueous stock solutions (for rotenone in 0.1% dimethylsulfoxide (DMSO)) onto the plates. An influence of DMSO on the investigated stress signaling has been ruled out. GFP expression was analyzed two days later.

**UPR\text{mt} assay.** L1 worms (SJ405) were immediately exposed to 7.2 μM tunicamycin (Sigma) after being placed on the respective RNAi bacteria and analyzed for GFP expression three days later.

**ze22 stress assay.** L1 larvae (SJ52) were grown on the respective RNAi bacteria at 15°C, subjected to the restrictive temperature of 25°C as soon as animals raised on control L4440 plates had developed to L4/young adults and analyzed for GFP expression two days after.

**NAC assay**

Day 1: N-acetyl-L-cysteine (NAC) (Sigma) aqueous stock solution (200.0 mM) was distributed to NGM agar plates to a final concentration of 15 mM. Gravid adults were left to lay eggs on NAC plates for 6 h. Day 3: Paraquat was added to the plates (0.5 mM). Day 5: GFP fluorescence was quantified.

**Paraquat resistance test**

Eggs were prepared from gravid C. elegans adults (N2) by exposure to alkaline sodium hypochlorite and allowed to hatch in M9 [67] over night. Synchronized L1 larvae were placed on NGM agar plates seeded with the respective bacteria and containing 0.4 mM paraquat. Worms were raised at 20°C for five days. Then the number of animals that reached the adult stage and the number of animals which still remained in larval stages were determined.

**Staining of mitochondria**

Lyophilized Mito Tracker stain (Mitotracker Deep Red FM, Invitrogen) was suspended in anhydrous dimethylsulfoxide to a stock solution of 1 M, which was diluted further in H2O to a working solution of 10 mM. Working solution was added to the worms on NGM agar plates to a final concentration of 100 nM 8 h prior to analyses.

**Microscopy and image analysis**

Live worms were analyzed for GFP expression either on NGM agar plates or in 96 well microtiter plates in liquid with a stereo microscope (SZX12, Olympus). Micrographs were taken from cold-immobilized animals on NGM plates using the stereo microscope and a Zeiss MRm2 CCD camera. For quantification micrographs were taken from sodium azide-immobilized animals with an Axioimager.Z1 compound microscope with an AxioCam MRm3 CCD camera; Axiovision software version 4.8.1 (Carl Zeiss AG, Germany) was used for image analysis. Mito Tracker stained mitochondria were analyzed with a Nikon Ti A1 confocal microscope and NIS-Elements AR 4.0 64-bit software using a 60× water immersion objective with a numerical aperture of 1.2.

**Food aversion assay**

RNAi. Assay plates were prepared as described [3]. Synchronized L1 staged N2 were placed in the middle of the bacterial lawn. Aversion was analyzed after 48 h and scored by the quotient of the number of animals residing outside the bacterial lawn (N_{off}) and the total amount of animals (N_{total}) aversion score (AV): N_{off}/N_{total}. Empty vector L4440 RNAi bacteria and elt-2 RNAi expressing bacteria were used as controls [3].

**Statistical analyses**

Statistical analyses were performed with GraphPad Prism 4 software using unpaired t test (with Welch’s correction if required), one-way analysis of variance (plus Tukey’s multiple comparison test), Mann-Whitney test or Kruskal-Wallis test (plus Dunn’s multiple comparison test), respectively. For the comparison of data sets with more than one parameter (RNAi and drug treatment) the background expression of the non-drug treated RNAi-fed cohort
Supporting Information

**Figure S1** Dose-response curve of paraquat and Phsp-60::gfp. Phsp-60::gfp reporter worms were exposed to different concentrations of paraquat (0–50 mM) for two days starting from early L3. 50 μM was lethal. GFP fluorescence intensity was analyzed with compound microscopy. A. Representative micrographs. B. Corresponding quantification. Columns represent pooled values of three independent experiments plus standard error of the mean (SEM). Numbers in or on columns indicate the number of analyzed animals (n\text{total} = 725). ***: p<0.001; Kruskal-Wallis test plus Dunn’s Multiple Comparison Test. (PDF)

**Figure S2** Phsp-60::gfp responds more sensitively to paraquat than Phsp-60::gfp. Quantification of GFP fluorescence intensity in the hsp-60 reporter strain (Phsp-60::gfp) and the hsp-60 reporter strain (Phsp-60::gfp) after two days of exposure to 0.5 mM and 2.0 mM paraquat, respectively. Exposure started at the early L3 stage. 0.5 mM Paraquat significantly increases (p<0.0001) hsp-60 reporter expression, but not Phsp-60::gfp. 2.0 mM paraquat induces both reporters. Columns represent mean plus standard error of the mean (SEM). Numbers in columns indicate the number of analyzed animals (hsp-60: n\text{total} = 30; hsp-60: n\text{total} = 29). ***: p<0.001; Kruskal-Wallis test plus Dunn’s Multiple Comparison Test. (PDF)

**Figure S3** Design of the genome-scaled RNAi screen. Microtiter plates including RNAi bacterial strains were grown overnight in duplicates. Each well contained RNAi bacteria specific for one C. elegans gene. The same day, eggs were prepared by bleaching gravid adults and allowed to further develop overnight in supplemented M9. The next day (Day 2), bacterial cultures were induced with IPTG. Subsequently, synchronized L1 larvae were added to the bacterial cultures and maintained at 20°C. At Day 3, paraquat was added. After two days (Day 5), plates were screened for worms that failed to increase GFP expression with a stereo fluorescence microscope. (PDF)

**Figure S4** Knockdown of translation associated genes abolishes hsp-6 induction without blocking translation. The largest group of screening positives corresponds to genes encoding ribosomal proteins or other factors implicated in protein translation. In the most trivial scenario RNAi against translation associated genes reduces translation and thus prevents GFP expression from the hsp-6 reporter (A). This idea is contradicted by experiments in which the induction of other GFP reporters (hsp-16.2::gfp, gst-4::gfp, hsp-4::gfp) is still possible, when translation associated genes were knocked down (Figure 9). (PDF)

**Figure S5** The effects of RNAi of rpl-36, atfs-1 and phk-1 on zc32 mediated activation of Phsp-60::gfp. Representative micrographs (A) and quantification of GFP fluorescence intensity (B). The UPR\textsuperscript{mt} reporter strain (zc32; Phsp-60::gfp) induces the UPR\textsuperscript{mt} upon shift to the restrictive temperature (25°C). Being raised on the respective RNAi plates from L1, worms were shifted from the permissive temperature (15°C) to 25°C as soon as the animals grown on control RNAi plates (L4440) had developed to L4/young adults. GFP fluorescence was analyzed after two days. While the induction of the UPR\textsuperscript{mt} was enhanced by rpl-36 RNAi (p<0.001), a complete block of the UPR\textsuperscript{mt} was observed by RNA interfering with the PI 4-kinase gene phk-1. This indicates the requirement of atfs-1 for the UPR\textsuperscript{mt} (p<0.001). Columns represent pooled normalized values of four independent experiments plus standard error of the mean (SEM). Numbers in columns indicate the number of analyzed animals (n\text{total} = 712). ***: p<0.001; Kruskal-Wallis test plus Dunn’s Multiple Comparison Test; Mann Whitney test (comparison of vector at 15°C and 25°C). Equal optical settings, scale bar 200 μm. (PDF)

**Figure S6** The cSADDs inhibit paraquat mediated signaling to hsp-6. The loss of the hsp-6::gfp induction in vgl-36(RNAi) is suppressed by mutant rpl-36(m3), indicating a KGB-1 mediated repression of UPR\textsuperscript{mt} by cSADDs. vgl-36(RNAi) was shown to be sufficient to induce cSADDs [3]. Columns represent normalized values plus standard error of the mean (SEM). ***: p<0.001; Kruskal-Wallis test plus Dunn’s Multiple Comparison Test. (PDF)

**Table S1** Knockdown of 36 of 55 screening positives were shown by Melo and Ruvkun, 2012 [3] to evoke aversion behavior. This table displays the subset of our screening positives which were recently shown to trigger aversion [3]. (DOCX)

**Acknowledgments**

We thank Cole Haynes for generously providing the UPR\textsuperscript{mt} reporter strain (SZ52) and advice in its handling. We thank Daniel Garcke for assistance during RNAi screening, Ruth Jeline and Simone Werner for assistance in stress assays, and Lydia Lieth for technical assistance. Special thanks goes to Rebecca Dickstein and Pamela Padilla from University of North Texas at Denton, and to Justine Melo from MGH/Harvard Medical School for helpful discussions.
Conceived and designed the experiments: EDR SB ES EDR. Performed the experiments: EDR LS ES RB. Wrote the paper: RB ES EDR.

References


