The Thioredoxin TRX-1 Modulates the Function of the Insulin-Like Neuropeptide DAF-28 during Dauer Formation in Caenorhabditis elegans

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Abstract

Thioredoxins comprise a conserved family of redox regulators involved in many biological processes, including stress resistance and aging. We report that the C. elegans thioredoxin TRX-1 acts in ASJ head sensory neurons as a novel modulator of the insulin-like neuropeptide DAF-28 during dauer formation. We show that increased formation of stress-resistant, long-lived dauer larvae in mutants for the gene encoding the insulin-like neuropeptide DAF-28 requires TRX-1 acting in ASJ neurons, upstream of the insulin-like receptor DAF-2. Genetic rescue experiments demonstrate that redox-independent functions of TRX-1 specifically in ASJ neurons are needed for the dauer formation constitutive (Daf-c) phenotype. GFP reporters of trx-1 and daf-28 show opposing expression patterns in dauers (i.e., trx-1 is up-regulated and daf-28 is down-regulated), an effect that is not observed in growing L2/L3 larvae. In addition, functional TRX-1 is required for the down-regulation of a GFP reporter of daf-28 during dauer formation, a process that is likely subject to DAF-28-mediated feedback regulation. Our findings demonstrate that TRX-1 modulates DAF-28 signaling by contributing to the down-regulation of daf-28 expression during dauer formation. We propose that TRX-1 acts as a fluctuating neuronal signaling modulator within ASJ neurons to monitor the adjustment of neuropeptide expression, including insulin-like proteins, during dauer formation in response to adverse environmental conditions.

Introduction

Thioredoxins comprise a conserved family of proteins characterized by the so-called thioredoxin fold and the highly conserved cysteine–glycine–proline–cysteine (CGPC) catalytic active site (reviewed in [1,2]). The two cysteines in the active site of thioredoxins are required for the reversible reduction of disulfide bonds in many target proteins. The functions of thioredoxins are extensive and mostly depend on their disulfide oxidoreductase attributes; in general, they can act as electron donors for metabolic enzymes, as antioxidants, or as redox regulators of signaling molecules and transcription factors (reviewed in [1]).

In some cases, thioredoxins have been reported to execute their functions in a redox-independent manner (reviewed in [2,3]). For instance, the regulation of phage T7 DNA polymerase activity by E. coli Trx1 [4,5], the cytokine function of human truncated thioredoxin (Trx80) [6], or the regulation of apoptosis signaling kinase 1 (ASK1) by human Trx1 [7], among others, are all functions carried out independently of their oxidoreductase activity. More recently, thioredoxins have also been shown to promote folding of proteins independently of their redox activities (reviewed in [3]).

In the nematode Caenorhabditis elegans, the gene trx-1 encodes a thioredoxin found to be expressed in the ASJ sensory neuron pair [8,9]. These neurons are implicated in the regulation of aging, the response to stress conditions and the control of a state of developmental arrest called the dauer larva [10,11,12]. Previously, we and others have shown that trx-1 deletion shortens lifespan and increases sensitivity to oxidative stress, though it itself does not affect dauer formation [8,9]. These findings implicate TRX-1 in processes that regulate aging and stress resistance, which raises the question whether it is involved in mechanisms implicated in the regulation of dauer formation.

The C. elegans dauer larva has evolved as a tightly-regulated, long-lived and stress-resistant developmental stage only triggered when the animals encounter harsh environmental conditions [13].
However, under circumstances in which genes involved in dauer development become inactivated due to mutations, animals arrest as dauer even under favorable conditions (dauer formation constitutive or Daf-c phenotype) [14]. Among the known daf-c genes whose functions are linked to ASJ, four have previously been well characterized for their role in the dauer formation pathway: daf-11 (abnormal dauer formation-1), daf-28, tax-2 (abnormal chemosens-2) and tax-4 (Figure S1; [12,15,16,17,18]). Moreover, mutations in these four daf-c genes have also been reported to extend lifespan [19,20,21], providing an additional link between dauer formation and aging. daf-11 encodes a transmembrane guanylyl cyclase, while tax-2 and tax-4 encode subunits of a cyclic guanosine monophosphate (cGMP)-gated ion channel; these three genes are expressed in subsets of amphid sensory neurons, including ASI and ASJ [15,22]. DAF-11 regulates dauer formation by modulating the levels of cGMP in these neurons, to which TAX-2/TAX-4 respond [22], which then regulate the expression of transforming growth factor-beta (TGF-beta) [23] and insulin-like neuropeptides [18,24] (Figure S1). There are ~40 insulin-like neuropeptides in C. elegans (www.wormbase.org), and several, including DAF-28, have been reported to be produced in ASJ, among other neurons [18,24]. To gain insight into whether TRX-1 plays a role in dauer formation in C. elegans, we analyzed at the cellular and genetic level its interaction with daf-c genes co-expressed in ASJ neurons.

We have identified the C. elegans thioredoxin TRX-1 as a novel modulator of the insulin-like neuropeptide DAF-28 during dauer formation. We found that trx-1 suppresses the Daf-c phenotype of all daf-28 insulin-like mutant alleles tested and that this suppression requires a functional DAF-2 insulin-like receptor. Genetic rescue experiments demonstrated that redox-independent functions of transgenic TRX-1 provided specifically in ASJ neurons can restore the suppression exerted by trx-1 deletion on the Daf-c phenotype of daf-28 mutants. The suppression observed at the genetic level is also manifest at the cellular level specifically during dauer formation: GFP reporters of trx-1 and daf-28 display opposing expression patterns in dauers, which is in contrast to what is observed in growing L2/L3 larvae. Moreover, functional TRX-1 is required for the down-regulation of a GFP reporter of daf-28 during dauer formation, a mechanism that is likely mediated by DAF-28-dependent feedback regulation. Our data suggest that TRX-1 contributes to the regulation of insulin-like neuropeptide expression, in particular DAF-28, during dauer formation in response to a changing environment.

**Results**

**trx-1 has a novel synthetic dauer formation constitutive (Daf-c) phenotype**

To investigate whether trx-1 plays a role in dauer formation, we first constructed double mutant combinations with different alleles of daf-11, since this gene is predicted to regulate dauer formation upstream of both the TGF-beta and insulin-like signaling (IS) pathways (Figure S1; [18,23]). Previously, it has been suggested that ASJ neurons are required for the daf-11 Daf-c phenotype, since laser ablation of ASJ suppresses the Daf-c phenotype of a daf-11(sa195) mutant [12]. We tested whether trx-1 is also required for the daf-11 Daf-c phenotype. The trx-1(ok1449) allele used in this study is a null mutation: a deletion in the coding region preventing translation of the protein (Table S1; [9]). trx-1(ok1449) enhanced the Daf-c phenotype of all daf-11 alleles tested at 15°C (Figure 1A and Table 1). Even at 25°C, two of the three daf-11 alleles tested showed a significant increase in dauer formation (Figure 1B and Table 2). Thus, trx-1 has a novel synthetic Daf-c phenotype. In addition, the slight suppression of the daf-11(sa195) Daf-c phenotype exerted by trx-1(ok1449) at 25°C (Figure 1B and Table 2), partially phenocopies the effect of killing ASJ in a daf-11(sa195) single mutant background [12]. Together, our findings indicate that most of the Daf-c phenotype of daf-11 mutants is TRX-1-independent, while a small fraction requires TRX-1. This dual effect of the trx-1 mutation on daf-11 for dauer formation is reminiscent, although not identical, to that reported for mutations in the cGMP-gated ion channel genes tax-2 and tax-4 [16]. tax-2 and tax-4 mutants show weak Daf-c phenotypes, which are epistatic to the strong daf-11 Daf-c phenotypes [16]. trx-1 deletion did not affect the weak Daf-c phenotype caused by tax-4(p6738) (Figures 1A and 1B; Tables 1 and 2). Since mutations in tax-4 essentially remove the input from daf-11 signaling for dauer formation [16], trx-1 deletion could not replicate the synthetic interaction observed with daf-11 mutants. These results further support the notion of tax-4 acting downstream of daf-11 for dauer formation [16], with trx-1 acting mostly independently of daf-11 in the dauer formation pathway. We speculate that for the most part TRX-1 and DAF-11 are affecting a separate set of neurons for dauer formation (e.g., TRX-1 in ASJ and DAF-11 in ASI) [this work; [23]], while they share some modulatory functions in ASJ neurons.

**trx-1 deletion enhances the Daf-c phenotype of mutations in the TGF-beta signaling pathway**

There is evidence that DAF-11 acts upstream of the IS and TGF-beta signaling pathways (Figure S1; reviewed in [25,26]). To determine if trx-1 interacts with any of these two genetic pathways for dauer formation, we first scored for dauers in double mutants containing trx-1(ok1449) and daf-c mutations in the TGF-beta signaling pathway. Previously, strong synergy was identified between daf-c mutations in several genes of the IS pathway, including daf-2 and daf-28, and mutations in genes of the parallel TGF-beta signaling pathway, represented here by tax-7, daf-1 and daf-8 [21,27]. We observed a marked increase in dauer formation in all three trx-1/TGF-beta double mutant combinations tested at 15°C (Figure 1A and Table 1). A straightforward interpretation of this synthetic enhancement observed is that the Daf-c phenotype of mutations in the TGF-beta signaling pathway is TRX-1-independent. Since TGF-beta single mutants already form nearly 100% dauers at 25°C ([28]; data not shown), trx-1;TGF-beta double mutants did not display any further increase in dauer formation at that temperature (data not shown). We conclude that TRX-1 acts independently of the TGF-beta signaling pathway for dauer formation.

**TRX-1 function is needed for the Daf-c phenotype of daf-28 mutants**

To investigate the interaction of trx-1 with the IS pathway for dauer formation, we first analyzed double mutants of trx-1 with daf-28, trx-1 suppressed the Daf-c phenotype of the two daf-28 alleles tested (Figures 1A and 1B; Tables 1 and 2), regardless of their nature (sa191 is a dominant-negative allele; tm308 is predicted to be null) (Table S1). These results indicate that the suppression is not allele-specific and that the Daf-c phenotype of daf-28 mutants requires TRX-1 function for dauer formation. Our findings suggest that loss of TRX-1 function likely increases insulin-like signaling upon mutation of daf-28.

**Suppression of the daf-28 Daf-c phenotype by trx-1(ok1449) depends on DAF-2 insulin-like receptor signaling**

To test whether the ability of trx-1(ok1449) to suppress the Daf-c phenotype of daf-28(sa191) depends on functional DAF-2, we first
Figure 1. Redox-independent functions of TRX-1 in ASJ neurons modulate DAF-28 signaling during dauer formation. Dauer formation at 15°C (A) and 25°C (B) is shown for each of the daf-c mutant alleles tested in a trx-1(+) wild-type background (white bars) or in combination with a trx-1(ok1449) deletion null mutant allele (black bars). (C) Rescue of the suppression of the Daf-c phenotype in trx-1(ok1449); daf-28(sa191) transgenic animals (patterned bars) expressing eitherPtrx-1::trx-1::GFP, Pssu-1::trx-1::GFP,Ptrx-1::trx-1(SGPS)::GFP, Pgpa-4::trx-1::GFP, Pges-1::trx-1::GFP orPtrx-1::GFP. The cellular site of expression for each transgenic extrachromosomal array is outlined above the graph in panel C. The non-transgenic control represents the average of all assays performed with non-transgenic progeny that segregated from the same transgenic parents (dotted line). The molecular identity of all mutant alleles presented here are shown in Table S1.

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analyzed dauer formation in double mutants containing the trx-1(ok1449) deletion and hypomorphic (partial loss-of-function) mutant alleles of daf-2 or pdk-1. The latter encodes a homologue of the mammalian Akt/PI3K kinase PDK1, involved in transducing DAF-2 signals on to the DAF-16 FOXO transcription factor [10]. Genes encoding a homologue of the mammalian PTEN tumor suppressor (trx-1) and the triple mutant: 2 assays. N: total (pooled) number of animals.

To investigate whether wild-type TRX-1 can restore the suppression of the daf-28(sa191) Daf-c phenotype caused by trx-1 deletion at 25°C, the transgenic Ptrx-1::trx-1::GFP (see Materials and Methods) was used to transform trx-1(ok1449); daf-28(sa191) animals. Transgenic lines expressing this transgene restored the Daf-c phenotype back to trx-1(ok1449); daf-28(sa191) single mutant animals, at both 15 and 25°C (Figure 1A and 1B; Tables 1 and 2), suggesting that the suppression is no longer effective when daf-2 is mutated. Therefore, although genetic interactions using non-null mutants (cf. Table S1) need to be interpreted with some caution, these data demonstrate that the ability of trx-1(ok1449) to suppress the Daf-c phenotype of daf-28(sa191) depends on functional DAF-2.

Redox-independent functions of transgenic TRX-1 specifically in ASJ neurons restore the suppression of daf-28(sa191) by trx-1(ok1449)

To investigate whether wild-type TRX-1 can restore the suppression of the daf-28(sa191) Daf-c phenotype caused by trx-1 deletion at 25°C, the transgenic Pssw-1::trx-1::GFP (see Materials and Methods) was used to transform trx-1(ok1449); daf-28(sa191) animals. Transgenic lines expressing this transgene restored the Daf-c phenotype back to daf-28(sa191) single mutant levels (Figure 1C and Table 3). Since the GFP-tagged transgene can be visualized in ASJ, this is in agreement with the hypothesis that wild-type trx-1 acts in ASJ neurons to modulate DAF-28 signaling during dauer formation. To confirm this notion, the expression of wild-type trx-1 genomic DNA fused to GFP was driven from another ASJ neuron-specific promoter, swu-1 [33]. Consistent with our hypothesis, this transgene Pswu-1::trx-1::GFP also rescued the suppression of the Daf-c phenotype of daf-28(sa191) by trx-1(ok1449) (Figure 1C and Table 3).
TRX-1 contributes to the down-regulation of daf-28 expression during dauer formation, a process likely controlled by DAF-28-mediated feedback regulation

Previously, it was shown that Pdaf-28::GFP expression in dauers is down-regulated by conditions of starvation and exposure to dauer pheromone [18]. To test whether trx-1 expression responds to these dauer-promoting signals, we analyzed Ptrx-1::GFP expression in both wild-type natural dauers, which are induced by a combination of both signals, and in daf-c mutant dauers, represented here by daf-11 and daf-2. Since trx-1 suppresses the Daf-c phenotype of daf-28 mutants (cf. above), one could expect that the response of Ptrx-1::GFP levels to dauer-promoting signals might be opposite to that of Pdaf-28::GFP. Consistent with our hypothesis, Pdaf-28::GFP was down-regulated and Ptrx-1::GFP was up-regulated in wild-type natural dauers, and in daf-11 and daf-2 mutant dauers (Figures 2A and 2C). In contrast, these opposing expression levels observed in dauers were not seen in well-fed, growing L2/L3 larvae of the corresponding genotypes (Figure S2), suggesting a possible regulatory effect of TRX-1 on DAF-28 (or vice versa) only during dauer formation. Therefore, to test whether TRX-1 function contributes to the down-regulation of daf-28 expression during dauer formation, we analyzed Pdaf-28::GFP expression in trx-1 mutant dauers. If the down-regulation of daf-28 expression during dauer formation was independent of TRX-1, we would expect trx-1 mutant dauers to express Pdaf-28::GFP at levels comparable with those of wild-type natural dauers (cf. Figure 2A), indicating that the down-regulation still persists even after loss of trx-1. Interestingly, we observed that Pdaf-28::GFP expression in trx-1 mutant dauers is not down-regulated, but is in fact not significantly different from what is seen in well-fed, growing L2/L3 wild-type larvae (Figures 2B and 2D). These findings suggest that the up-regulation of TRX-1 specifically during dauer formation is, at least in part, causative of a reduction in DAF-28 signaling. Moreover, the up-regulation ofPtrx-1::GFP expression in daf-28 mutant dauers (Figures 2B and 2D), is further increased when compared with that seen in wild-type natural dauers (cf. Figures 2A and 2C). This result suggests that trx-1 up-regulation during dauer formation is likely subject to DAF-28-mediated feedback regulation. Together, these results are consistent with a model (Figure 3) in which TRX-1 modulates DAF-28 signaling by contributing to the down-regulation of daf-28 expression exclusively during dauer formation, a modulatory process that is likely controlled by DAF-28-dependent feedback regulation.

### Table 3. Percent dauer formation at 25°C of trx-1(ok1449); daf-28(sa191) double mutants expressing the indicated transgene.

| Transgene | Transgenic line | Transgenic animals | Non-transgenic animals |
|-----------|-----------------|--------------------|------------------------|
|           |                 | % ± SEM            | N                      |
|           |                 |                    | % ± SEM                | N                      |
| Pgpa-4:trx-1::GFP | 1 | 53 ± 14 | 424 | 34 ± 9 | 531 |
| Pgpa-4:trx-1::GFP | 2 | 36 ± 10 | 502 | 38 ± 9 | 472 |
| Pgpa-4:trx-1::GFP | 3 | 34 ± 9  | 450 | 28 ± 7 | 616 |
| Pges-1:trx-1::GFP | 1 | 40 ± 12 | 518 | 37 ± 6 | 503 |
| Pges-1:trx-1::GFP | 2 | 46 ± 13 | 501 | 39 ± 6 | 507 |
| Pges-1:trx-1::GFP | 3 | 43 ± 8  | 474 | 30 ± 12 | 470 |
| Pef-1:GFP | 1 | 43 ± 8  | 474 | 30 ± 12 | 470 |
| Pef-1:GFP | 2 | 48 ± 7  | 339 | 30 ± 10 | 479 |
| Pef-1:GFP | 3 | 34 ± 8  | 455 | 31 ± 9  | 541 |

1Mean percentage of dauer larvae ± standard error of the mean (SEM); 2 assays per transgenic line, except for Ptrx-1::GFP and Pges-1::trx-1::GFP: 3 assays. N: total (pooled) number of animals.

2p<0.001 by chi-squared test.

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Figure 2. TRX-1 contributes to the down-regulation of daf-28 insulin-like gene expression during dauer formation. Quantification of Ptrl-1::GFP and Pdaf-28::GFP expression in dauers and well-fed, growing L2/L3 larvae in ASJ or ASI neurons. Average fluorescence intensity in ASJ or ASI neurons, normalized to that of well-fed, growing L2/L3 wild-type larvae, is shown for wild-type dauers and for daf-2 and daf-11 mutant dauers in (A), and for trl-1 and daf-28 mutant dauers in (B). Two independent transgenic lines were examined for each of the two transcriptional Ptrl-1::GFP and Pdaf-28::GFP reporters, and the results were very similar; one transgenic line was tested for daf-28(tm2308) dauers. The data derived from one transgenic line are presented. Each bar represents the average relative fluorescence intensity of 28–34 animals ± standard error of the mean (SEM). ** p < 0.01 relative to L2/L3 wild-type larvae, by Student’s t test (two-tailed, two-sample unequal variance); na: not assayed; ns: not significant. Shown in (C) and (D) are representative color-inverted images of animals assayed in (A) and (B), respectively, revealing the differences in GFP intensity in ASJ or ASI neurons (boxed). The tip of the head points to the left in all panels.

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Discussion

Here, we have identified the thioredoxin TRX-1 as a novel modulator of the insulin-like neuropeptide DAF-28 during dauer formation in C. elegans. We found that trx-1 suppresses the Daf-c phenotypes of all daf-28 insulin-like mutant alleles tested, and that this suppression is dependent on a functional DAF-2 insulin-like receptor (Figures 1A and 1B; Tables 1 and 2). Genetic rescue experiments showed that redox-independent functions of TRX-1 specifically in ASJ neurons are needed for the Daf-c phenotype caused by defects in daf-28 function (Figure 1C and Table 3). The suppression observed at the genetic level phenocopied that seen at the cellular level specifically during dauer formation: Ptrx-1::GFP and Pdaf-28::GFP showed opposing expression patterns in dauers (Figures 2A and 2C), which were not observed in growing L2/L3 larvae (Figure S2). In addition, Pdaf-28::GFP down-regulation during dauer formation required functional TRX-1, a mechanism that is likely controlled by DAF-28-dependent feedback regulation (Figures 2B and 2D). Taken together, our results suggest a model in which TRX-1 contributes to the adjustment of daf-28 insulin-like expression, and consequently its function, during dauer formation (Figure 3).

C. elegans neuropeptides are classified into three classes: the insulin-like proteins, the FMRF (Phe-Met-Arg-Phe)-amide peptides (referred to as FLPs) and the neuropeptide-like proteins or NLPs (reviewed in [40]). In addition to DAF-28, it might be possible that TRX-1 also modulates other neuropeptides co-expressed in ASJ neurons (Figure 3), such as the insulin-like proteins INS-9 and INS-1, or NLP-3 and FLP-21 ([24,41]; reviewed in [40]). If the effect of TRX-1 on dauer formation was solely dependent on DAF-28 signaling, we would expect that overexpression of trx-1 in the likely null mutant daf-28(tm2308) results in no enhancement of the Daf-c phenotype. Interestingly, the observation that overexpression of wild-type trx-1::GFP in ASJ was sufficient to enhance the weak Daf-c phenotype of the likely null mutant daf-28(tm2308) (Table S2), suggests that TRX-1 in part affects dauer formation independently of DAF-28, and potentially independently of DAF-2 (Figure 3). Moreover, the fact that trx-1 deletion, like daf-28(u191) [21], enhanced the Daf-c phenotype of TGF-beta pathway mutations and of most daf-11 mutations, whereas trx-1 deletion suppressed the Daf-c phenotype of daf-28 mutations (Figures 1A and 1B; Tables 1 and 2), also supports the notion that TRX-1 to some extent modifies dauer formation independently of DAF-28, and possibly also of DAF-2 (Figure 3). Furthermore, because wild-type animals overexpressing wild-type trx-1::GFP in ASJ were not induced to form dauers at 25°C (Table S2), other pathways and/or neurons might act in parallel to contribute, together with TRX-1, to the modulation of DAF-28 signaling. In fact, the cGMP pathway has previously been suggested to regulate DAF-28 signaling during dauer formation (Figure S1; [18]).

ASJ neurons have been shown to regulate dauer formation in concert with other sensory neurons [11,12], despite their discrete synaptic connectivity (ASK is the only sensory neuron in synaptic contact with ASJ; www.wormatlas.org). The localized function of TRX-1 in ASJ neurons for dauer formation (Figure 1C; Table 3) and the effect of TRX-1 on daf-28 expression (Figures 2B and 2D) support a model wherein TRX-1 modulates (the levels of) neurosecretory signals emanating from ASJ neurons to regulate dauer formation in a cell non-autonomous fashion. Although our results do not exclude that TRX-1 affects other neurons through direct synaptic connections, the control it exerts on neurosecretory signaling would allow TRX-1 to function locally in ASJ neurons and affect dauer formation remotely. Such a model would explain the observation that deletion of trx-1 modifies the Daf-c phenotypes of mutations in genes not expressed in ASJ neurons (Figure 1A; Table 1) (e.g. daf-7 is only expressed in ASI neurons) [42]. Similarly, TRX-1 could contribute to the down-regulation of daf-28 expression in ASI, either through ASJ-derived DAF-28 or other TRX-1-modified neuropeptides released from ASJ neurons.

Previously, mammalian Trx1 has been proposed to participate in the redox regulation that mediates insulin secretion via NADPH as a signaling molecule [43]. However, we have shown in this report that TRX-1 function in dauer formation does not require its redox activity (Figure 1C and Table 3), suggesting that TRX-1 contributes to the down-regulation of daf-28 expression during dauer formation via mechanisms other than redox regulation of neuropeptide production and/or release.

It has recently been found in mammals that key neuronal lipid metabolites act as hypothalamic signaling mediators that monitor energy status and contribute to maintaining organism metabolic homeostasis (reviewed in [44]). We propose that the thioredoxin

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![Diagram](image-url)
TRX-1 might similarly act as a neuronal modulator in ASJ neurons to monitor metabolic status and thus adjust neuropeptide expression, including the insulin-like neuropeptide DAF-28, during dauer formation in response to adverse conditions (Figure 3). In this context, ASJ neurons would monitor the choice between reproductive development and dauer formation by responding to intra-neuronal fluctuations of TRX-1 (Figure 3). In turn, these TRX-1 fluctuations would be triggered in response to signals from the environment and peripheral tissues that reflect the animal’s global energy status. Interestingly, the *C. elegans* ASI neurons have been suggested to mediate responses to nutrient availability through neuroendocrine signals that promote adult longevity by sensing similar energy-monitoring molecules within them [45]. Moreover, ASJ neurons have been proposed to influence dauer formation through sulfated neuroendocrine signals [33]. Thus, together with previous observations by others, our findings anticipate a neuroendocrine network in the nematode involved in adjusting the animal’s global energy status in response to a changing environment, both during development and adulthood. Future work is needed to identify and dissect the components of such a neuroendocrine network that, including TRX-1, are involved in regulating mechanisms designed to monitor energy homeostasis in varying environmental conditions in the nematode, and most likely also in higher organisms.

Materials and Methods

Nematode strains and culture conditions

The standard methods used for culturing *C. elegans* were described previously [46]; reviewed in [47]. Strains and transgenes used in this work are summarized in Table 3S. All strains were grown at 20°C, except for *daf-c* mutant strains, which were grown at 15°C.

Transgene injection constructs and germline transformation

The *Ptrx-1::trx-1::GFP* translational fusion construct (cf. Figure 1C; Tables 3 and S2) and the *Pdaf-28::GFP* transcriptional fusion construct (cf. Figures 2 and S2) were previously reported [9,48]. For the *Ptrx-1::GFP* transcriptional fusion construct (cf. Figures 1C, 2, and S2; Table 3), ~1 kb of sequence upstream of the *trx-1* b splice variant, plus the first 65 nucleotides of the coding sequence were amplified by PCR and cloned in-frame with GFP into the pPD95.77 vector. The *Ptrx-1::trx-1(1SGPS)::GFP* translational fusion construct (cf. Figure 1C and Table 3) was obtained by site-directed mutagenesis of the *trx-1* active site, as specified in the QuickChange II Site-Directed Mutagenesis Kit (Stratagene), using the *Ptrx-1::trx-1::GFP* translational fusion construct as a template. The *Psgu::trx-1::GFP*, *Ppca-4::trx-1::GFP* and *Pgex-1::trx-1::GFP* translational fusion constructs (cf. Figure 1C and Table 3) included ~0.7 kb (sgu-1) or ~2.5 kb (pca-4 and ges-1) of promoter sequence upstream of the respective start codon fused to the full-length *trx-1* genomic DNA fragment, in-frame with GFP, into the pPD95.77 vector. For the experiments shown in Figures 2 and S2, 80 ng/μl of *Ptrx-1::GFP* or 50 ng/μl of *Pdaf-28::GFP* were coinjected with 20 ng/μl of the injection marker *Pelt-2::mCherry* (a gift from Gert Jansen, Rotterdam), into *tx-1(ok1449); daf-28(sa191)* or *tx-1(ok1449)* animals. These extrachromosomal arrays were then crossed into wild-type, *daf-11(ks67), daf-2(c1370), daf-28(sa191)* or *daf-28(tm2308)* animals. For dauer formation assays, 8–20 gravid hermaphrodites were allowed to lay eggs for a given time at the test temperature (Tables S4 and S5), and then removed. Dauers and non-dauers (L5 and L4 larvae and adult animals) on the agar and side of the plate were then counted at specific time points after egg-laying ended (Table S4). The scoring time points were selected for each genotype so that all animals had passed the pre-dauer or L2 stages at the time of scoring. Corresponding single and double mutant strains were always assayed in parallel. In all cases, dauers were discriminated from non-dauers based on the absence of pharyngeal pumping, intestinal re-organization and radial shrinkage of the body [13]. More than 165 animals were counted in a total of 2–3 independent assays per genotype. Dauers of selected key genotypes used in this study were subjected to morphological analysis using differential interference contrast (DIC) microscopy at an optical magnification of x800 or x1000, and also tested for resistance to 1% sodium dodecylsulfate (SDS) [13]. All dauers analyzed were identical to wild-type dauers in their morphological features (i.e. pharynx and alae) and SDS-resistance.

Analysis of dauer formation

For dauer formation assays, 8–20 gravid hermaphrodites were allowed to lay eggs for a given time at the test temperature (Tables S4 and S5), and then removed. Dauers and non-dauers (L5 and L4 larvae and adult animals) on the agar and side of the plate were then counted at specific time points after egg-laying ended (Table S4). The scoring time points were selected for each genotype so that all animals had passed the pre-dauer or L2 stages at the time of scoring. Corresponding single and double mutant strains were always assayed in parallel. In all cases, dauers were discriminated from non-dauers based on the absence of pharyngeal pumping, intestinal re-organization and radial shrinkage of the body [13]. More than 165 animals were counted in a total of 2–3 independent assays per genotype. Dauers of selected key genotypes used in this study were subjected to morphological analysis using differential interference contrast (DIC) microscopy at an optical magnification of x800 or x1000, and also tested for resistance to 1% sodium dodecylsulfate (SDS) [13]. All dauers analyzed were identical to wild-type dauers in their morphological features (i.e. pharynx and alae) and SDS-resistance.

Microscopy and fluorescence imaging

The average relative intensity of a transcriptional *Ptrx-1::GFP* or *Pdaf-28::GFP* reporter was assessed by quantifying GFP intensity in ASJ neurons of dauers or growing L2/L3 larval mutant for a
specific daf-2 gene as compared to growing L2/L3 wild-type larvae. Animals were visualized on a Zeiss Axiosplan fluorescence microscope at an optical magnification of x640. Worms were put into M9 buffer on a very thin 2% agarose pad containing an anesthetic (15 mM NaN₃). Previously, it had been shown that exposure to 20 mM NaN₃ or less during 60 min [55], ~30 mM NaN₃ for 20 min [56] or 30 mM NaN₃ for 5 min [57] can induce evident physiological changes in the worm. All animals assayed in our study have been exposed to only 15 mM NaN₃ for a maximum of 10–15 min prior to image acquisition, thereby avoiding the induction of evident physiological changes. A Hamamatsu CCD camera and Openlab software (Improvision) were used for image acquisition at the brightest focal plane and a fixed exposure time. Pixel intensity in the entire ASJ cell body was determined from captured images in the form of maximum gray values by using NIH ImageJ software. An exception was made for Pdaf-28::GFP expression intensity in dauers, which was measured from either ASJ or ASI, due to the fact that overall very low expression levels hindered correct neuron identification. Since the brightest expressing cell was always measured for every dauer, the data for Pdaf-28::GFP expression intensity in dauers, therefore, is necessarily a conservative (over-)estimate. In all cases, fold differences with respect to growing L2/L3 wild-type larvae were calculated to show the average relative intensity among the assayed animals. Wild-type or mutant dauers and growing L2/L3 daf-2 mutant larvae were always assayed in parallel with growing L2/L3 wild-type larvae at the test temperature. In brief, wild-type, trx-1(ok1449), daf-28(sa191) and daf-28(tm2308) dauers were collected from crowded starved plates maintained at 20 °C, while daf-11(kc67) and daf-2(e1370) dauers were picked from uncrowded well-fed plates grown at 25 °C; growing L2/L3 daf-2 mutant larvae were collected from uncrowded well-fed plates maintained at 20 °C. Growing L2/L3 wild-type larvae were always picked from uncrowded well-fed plates. Twenty-eight to thirty-six animals were analyzed per genotype and condition. Two independent extrachromosomal transgenic lines were examined for each of the two transcriptional Ptxr-1::GFP and Pdaf-28::GFP reporters, and the results were very similar. The data derived from one transgenic line are presented. Each bar represents the average relative fluorescence intensity of 28–36 animals ± standard error of the mean (SEM).

**Supporting Information**

**Figure S1** A speculative model describing the genetic pathways that regulate dauer formation. Not all genes known to act in these pathways are shown. Solid lines represent active regulation, and dashed lines represent inactive regulation. Arrows indicate positive regulation and crossbars indicate repressive regulation. See text for details and references.

**References**

1. Lillig CH, Holmgren A (2007) Thioredoxin and related molecules—from biology to health and disease. Antioxid Redox Signal 9: 25–47.
2. Meyer Y, Buchanan BB, Vignols F, Reichheld JP (2009) Thioredoxins and Glutaredoxins: Unifying Elements in Redox Biology. Annu Rev Genet 43: 335–367.
3. Berndt G, Lillig CH, Holmgren A (2008) Thioredoxins and glutaredoxins as facilitators of protein folding. Biochim Biophys Acta 1783: 641–650.
4. Huber HE, Russel M, Model P, Richardson CC (1986) Interaction of mutant thioredoxins of Escherichia coli with the gene 5 protein of phage T7. The redox capacity of thioredoxin is not required for stimulation of DNA polymerase activity. J Biol Chem 261: 15006–15012.
5. Russel M, Model P (1986) The role of thioredoxin in filamentous phage assembly. Construction, isolation, and characterization of mutant thioredoxins. J Biol Chem 261: 14997–15005.
6. Pellekai K, Avila-Carillo J, Gurumath R, Bengtsson A, Scheynius A, et al. (2003) Truncated thioredoxin (Trx0) exerts unique mitogenic cytokine effects via a mechanism independent of thiol oxidoreductase activity. FEBS Lett 539: 143–148.

**Author Contributions**

Conceived and designed the experiments: JCFG AC JA AMV PS. Performed the experiments: JCFG. Analyzed the data: JCFG JA AMV PS. Contributed reagents/materials/analysis tools: AC AMV. Wrote the paper: JCFG JA AMV PS.

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7. Liu Y, Min W (2002) Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner. Circ Res 90: 1259–1266.

8. Jee C, Vazquez I, Lee J, Park BJ, Ahn J (2003) Thioredoxin is related to life span regulation and oxidative stress response in Caenorhabditis elegans. Genes Cells 10: 1203–1210.

9. Miranda-Vizuete A, Fierro Gonzalez JC, Gahmon G, Burghoorn J, Navas P, et al. (2006) Lifespan decrease in a Caenorhabditis elegans mutant lacking TRX-1, a thioredoxin expressed in ASJ sensory neurons. FEBS Lett 580: 484–490.

10. Alcedo J, Kenyon C (2004) Regulation of C. elegans longevity by specific gustatory and olfactory neurons. Neuron 41: 45–55.

11. Bargmann CI, Horvitz HR (1991) Control of larval development by chemosensory neurons in Caenorhabditis elegans. Science 253: 1243–1246.

12. Schackwitz WS, Inoue T, Thomas JH (1996) Chemosensory neurons function in parallel to mediate a pheromone response in C. elegans. Neuron 17: 719–728.

13. Cassada RC, Russell RL (1975) The dauer larva, a post-embryonic development variant of the nematode Caenorhabditis elegans. Dev Biol 46: 326–342.

14. Swanson MM, Riddle DL (1981) Critical periods in the development of the Caenorhabditis elegans dauer larva. Dev Biol 84: 27–40.

15. Coburn GM, Bargmann CI (1996) A putative cyclic nucleotide-gated channel is required for sensory development and function in C. elegans. Neuron 17: 695–706.

16. Coburn GM, Mori I, Ohshima Y, Bargmann CI (1998) A cyclic nucleotide-gated channel inhibits sensory axon outgrowth in larval and adult Caenorhabditis elegans: a distinct pathway for maintenance of sensory axon structure. Development 125: 249–258.

17. Komatsu H, Mori I, Rhee JS, Akaike N, Ohshima Y (1996) Mutations in a cyclic nucleotide-gated channel lead to abnormal thermosensation and chemosensation in C. elegans. Neuron 17: 707–718.

18. Li W, Kennedy SG, Ruvkun G (2003) daf-28 encodes a C. elegans insulin superfamily member that is regulated by environmental cues and acts in the DAF-2 signaling pathway. Genes Dev 17: 844–850.

19. Apfeld J, Kenyon C (1999) Regulation of lifespan by sensory perception in Caenorhabditis elegans. Nature 402: 804–809.

20. Hahn JH, Kim S, Park YK (2009) Endogenous cGMP regulates adult longevity via the insulin signaling pathway in Caenorhabditis elegans. Aging Cell 8: 473–483.

21. Malone EA, Inoue T, Thomas JH (1996) Genetic analysis of the roles of daf-28 and age-1 in regulating Caenorhabditis elegans dauer formation. Genetics 143: 1193–1205.

22. Birnby DA, Link EM, Vowels JJ, Tian H, Colacurcio PL, et al. (2000) A membrane guanylyl cyclase (DAF-11) and Hsp90 (DAF-21) regulate a common set of chemosensory behaviors in Caenorhabditis elegans. Genetics 155: 103–114.

23. Murakami M, Koga M, Ohshima Y (2001) DAF-2/TGF-beta expression required for the normal larval development in C. elegans is controlled by a presumable guanylyl cyclase DAF-11. Mech Dev 109: 27–35.

24. Pierce SB, Costa M, Wisotzkey R, Devadhar S, Homburger SA, et al. (2001) Regulation of DAF-2 receptor signaling by human insulin and insulin-like peptides. J Biol Chem 276: 35672–35680.

25. Malone EA, Inoue T, Thomas JH (1996) Chemosensory neurons function in dauer formation in C. elegans. Genes Dev 10: 1438–1452.

26. Hu PJ (2007) Dauer. In: The C. elegans Research Community, ed. WormBook. doi/10.1895/wormbook.1.47.1, http://www.wormbook.org.

27. Caras M, Inoue T, Thomas JH (1996) Chemosensory neurons function in dauer formation in C. elegans. Genetics 130: 105–123.

28. Paradis S, Alton M, Toker A, Thomas JH, Ruvkun G (1999) A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in Caenorhabditis elegans. Genes Dev 13: 1438–1452.

29. Mihaylova VT, Botland C, Manjarrez L, Stern MJ, San H (1999) The PTEN tumor suppressor homolog in Caenorhabditis elegans regulates longevity and dauer formation in an insulin receptor-like signaling pathway. Proc Natl Acad Sci U S A 96: 7427–7432.

30. Paradis S, Kevan G (1998) The C. elegans PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway. Mol Cell 2: 825–837.

31. Padmanabhan S, Mukhopaithay S, Narasimhan SD, Tesz G, Czech MP, et al. (2009) A PPAR beta/alpha subunit regulates C. elegans insulin/IGF-1 signaling by modulating AKT-1 phosphorylation. Cell 136: 939–951.

32. Carroll RT, Duhoyk GR, Sedensky MM, Morgan PG (2006) Sulfated signal from ASJ sensory neurons modulates stomatid-dependent coordination in Caenorhabditis elegans. J Biol Chem 281: 35309–35316.

33. Muller EG (1995) A redox-dependent function of thio-redoxin is necessary to sustain a rapid rate of DNA synthesis in yeast. Arch Biochem Biophys 318: 356–361.

34. Pellicena-Pallé A, Stitzer GM, Salz HK (1997) The function of the Drosophila thioredoxin homologue encoded by the deadliale gene is redox-dependent and blocks the initiation of development but not DNA synthesis. Mech Dev 62: 61–65.

35. Tsunenari K, Wills J, Cock I, Perkins A, Orozco C, et al. (1995) Site-directed mutagenesis of human thioredoxin. Identification of cysteine 74 as critical to its function in the “early pregnancy factor” system. J Biol Chem 268: 22405–22410.

36. Ohlstein JE, Bargmann CI, Gassadka PY, Pocius G (1994) Site-directed mutagenesis of active site cysteines in human thioredoxin produces competitive inhibitors of human thioredoxin reductase and elimination of mitogenic properties of thioredoxin. J Biol Chem 269: 11714–11720.

37. Jansen G, Thijssen KL, Werner P, van der Horst M, Hazendonk E, et al. (1999) The complete family of genes encoding G proteins of Caenorhabditis elegans. Nat Genet 21: 414–419.

38. Aamodt EF, Chung MA, McGhee JD (1991) Spatial control of gut-specific gene expression during Caenorhabditis elegans development. Science 252: 579–582.

39. Li C, Kim K (2008) Neuropeptides. In: The C. elegans Research Community, ed. WormBook. doi/10.1895/wormbook.1.112.1, http://www.wormbook.org.

40. Nathoo AN, Moeller RA, Westland BA, Hart AC (2001) Identification of neuropeptide-like protein gene families in Caenorhabditis elegans and other species. Proc Natl Acad Sci U S A 98: 14000–14005.

41. Ren P, Lin CS, Johnsen R, Albert PS, Pilgrim D, et al. (1996) Control of C. elegans larval development by neuronal expression of a TGF-beta homolog. Science 274: 1389–1391.

42. Moll CC, Stinchcombe D, Ambros V (1991) Efficient gene transfer in C. elegans: extrachromosomal maintenance and integration of transforming sequences. EMBO J 10: 3959–3970.

43. Wicki SR, De Vries CJ, van Loo HW, Plasterk RH (2000) CHE-3, a cytosolic dimeric heavy chain, is a required for sensory cilia structure and function in Caenorhabditis elegans. Dev Biol 221: 295–307.

44. Edgley M, D’Souza A, Moulger G, McKay S, Shen R, et al. (2002) Improved detection of small deletions in complex pools of DNA. Nucleic Acids Res 30: e52.

45. Ahringer J (2006) Reverse genetics. In: The C. elegans Research Community, ed. Wormbook. doi/10.1895/wormbook.1.47.1, http://www.wormbook.org.

46. Massie MR, Lapacek EM, Boggs KD, Sine KE, White GE (2003) Exposure to the metabolic inhibitor sodium azide induces stress protein expression and thermotolerance in the nematode Caenorhabditis elegans. Cell Stress Chaperones 8: 1–7.

47. Kell A, Ventura N, Kahn N, Johnson TE (2007) Activation of SKN-1 by novel kinases in Caenorhabditis elegans. Free Radic Biol Med 43: 1560–1566.

48. An JH, Blackwell TK (2003) SKN-1 links C. elegans mesodermal specification to a conserved oxidative stress response. Genes Dev 17: 1082–1089.