Selenoprotein TRXR-1 and GSR-1 are essential for removal of old cuticle during molting in *Caenorhabditis elegans*

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Selenoproteins, in particular thioredoxin reductase, have been implicated in countering oxidative damage occurring during aging but the molecular functions of these proteins have not been extensively investigated in different animal models. Here we demonstrate that TRXR-1 thioredoxin reductase, the sole selenoprotein in *Caenorhabditis elegans*, does not protect against acute oxidative stress but functions instead together with GSR-1 glutathione reductase to promote the removal of old cuticle during molting. We show that the oxidation state of disulfide groups in the cuticle is tightly regulated during the molting cycle, and that when *trxr-1* and *gsr-1* function is reduced, disulfide groups in the cuticle remain oxidized. A selenocysteine-to-cysteine TRXR-1 mutant fails to rescue molting defects. Furthermore, worms lacking SELB-1, the *C. elegans* homolog of *Escherichia coli* SelB or mammalian EFsec, a translation elongation factor known to be specific for selenocysteine in *E. coli*, fail to incorporate selenocysteine, and display the same phenotype as those lacking *trxr-1*. Thus, TRXR-1 function in the reduction of old cuticle is strictly selenocysteine dependent in the nematode. Exogenously supplied reduced glutathione reduces disulfide groups in the cuticle and induces apolysis, the separation of old and new cuticle, strongly suggesting that molting involves the regulated reduction of cuticle components driven by TRXR-1 and GSR-1. Using dauer larvae, we demonstrate that aged worms have a decreased capacity to molt, and decreased expression of GSR-1. Together, our results establish a function for the selenoprotein TRXR-1 and GSR-1 in the removal of old cuticle from the surface of epidermal cells.

The life cycles of nematodes, arthropods, tardigrades, onychophoruns, nematomorphs, kinorhynchs, and priapulids all involve distinct stages separated by molts. However, despite the fact that together these phyla contain the most abundant and diverse group of animals presently living, much remains to be learned about the molecular mechanisms by which molting occurs and is regulated. In insects, molting and metamorphosis is triggered by ecdysone, whose synthesis is modulated in response to environmental, developmental, and physiological cues (1, 2). However, precisely how ecdysone leads to the shedding of the old cuticle is not yet understood. In *Caenorhabditis elegans*, molting occurs at the end of each of the four larval stages of the reproductive life cycle. It also occurs upon entry into and exit from the dauer developmental diapause. Both cholesterol (3) and nuclear hormone receptors (4) are required for molting in *C. elegans*, suggesting that, although ecdysone itself is not produced, molting may be regulated by a steroid-derived hormone. A number of different tissues have been implicated in the control of molting, suggesting that, as it is in insects, the process is likely to be under complex regulation (5). Genome-wide screens for molting mutants have led to the identification of genes encoding proteins involved in a variety of different processes, and established a hierarchy for their function (5).

Thioredoxin reductases are NADPH-dependent oxidoreductases that, together with thioredoxins, form the thioredoxin system (6, 7). Mammalian thioredoxin reductases are homodimeric selenoproteins: The single selenocysteine residue is in a C-terminal Gly-Cys-Sec-Gly motif (8), the cysteine and selenocysteine residues of which constitute one of two redox active sites in the enzyme (9, 10). Selenocysteine has been shown to be required for the ability of rat thioredoxin reductase 1 (TrxR1) to reduce hydrogen peroxide in vitro (11). However, the significance of selenocysteine for activity in vivo is presently unclear. TrxR1 and TrxR2 are essential for mouse embryogenesis (12, 13). Mouse embryos lacking TrxR2 have defects in cell growth, programmed cell death, and heart function (13). However, the function of selenium-containing thioredoxin reductases in other animal models has not been reported. Thioredoxin reductase activity is induced in epidermal cells in mammals in response to exposure to UV light (14, 15), but the function of the enzyme in epidermal cells is not well understood.

Results

Disulfide Groups in the Cuticle Are Reduced During Molting. The cuticle in *C. elegans* and other nematodes is a highly ordered matrix composed of collagens, cuticulins, glycoproteins, and lipids (16). Collagens, which make up a substantial fraction of the cuticle, are highly cross-linked, in part through nonreducible di- and trityrosine cross-links and in part through disulfide bonds that link cysteine residues in different collagen molecules (16). Treatment with reducing agents leads to permeabilization of the cuticle and the release of soluble collagens (17). To investigate whether cross-linking through disulfides changes during molting, we stained worms with Alexa Fluor 488 C_5-maleimide, a fluorescently-labeled maleimide derivative that reacts in vivo with accessible (extracellular) thiol (–SH) groups but not with

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oxidized sulfur atoms within disulfides (18). Worms at intermolt stages of the cycle (i.e., those that were not molting) showed staining of the pharyngeal lumen and the buccal cavity (and weakly of the intestinal lumen) but not of the cuticle (Fig. 1 *A–D*). Pretreatment of intermolt worms with the reducing agent DTT allowed high levels of cuticle staining, demonstrating that reduction of cuticle disulfides causes increased staining (Fig. 1 *E* and *F*). Exposure of worms at ecdysis to the label without pretreatment with DTT led to strong staining of the cuticle (Fig. 1 *G* and *H*). Cuticles shed at the L1, L2, L3, and L4 molts were all stained (Fig. S1 *A–D*). Treatment of detached cuticle with *N*-ethylmaleimide, which reacts specifically with thiols in biological systems, completely blocked staining (Fig. 1 *I* and *J*). Thus, disulfide groups in the cuticle are actively reduced during molting.

Diamide is a small diffusible molecule that rapidly and specifically oxidizes thiol groups in vivo and in vitro (19). We found that, when L3 larvae that had recently completed the L2 molt were exposed to 18 mM diamide, they were able to continue growing. The divisions of six 6 Pn.p cells that form the vulval equivalence group (P3.p-P8.p), for example, were normal (Fig. S1 *E* and *F*). However, such worms arrested at the L3 molt with defects in apolysis, the separation of old and new cuticle ($n = 10$; Fig. S1*G*). When late L2 larvae were exposed to diamide, they arrested at the L2 molt ($n = 10$), and P3.p-P8.p failed to divide (Fig. S1*H*). That diamide can cause a block in molting in *C. elegans* without preventing growth strongly suggests that reduction of either cuticle components or a regulator of molting is necessary for molting to occur.

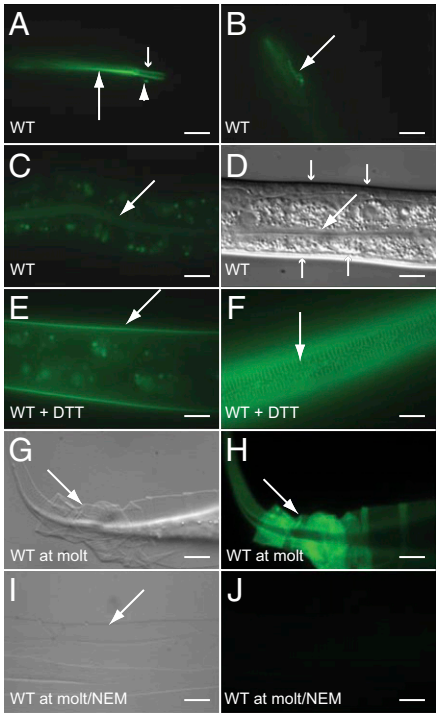

Fig. 1. Disulfide groups in the cuticle are reduced during molting in *C. elegans*. Micrographs of worms stained with Alexa Fluor 488 C_5-maleimide viewed with DIC (*D*, *G*, and *I*) or fluorescence (*A–C*, *E*, *F*, and *H*) optics. The large and small arrows in *A* indicate the lumen of the pharynx and buccal cavity, respectively. The arrowhead in *A* and the arrow in *B* indicate one of a pair of cell or neuronal exensions lying adjacent to the buccal cavity stained by the dye. The large arrows in *C* and *D* indicate the lumen of the intestine. The small arrows in *D* indicate the cuticle, which is not appreciably stained by the dye in nonmolting worms under normal conditions. (*E* and *F*) Nonmolting worms that had been incubated with 5 mM DTT for 30 min before staining with the dye. (*G* and *H*) Worms at molt that had not been incubated with DTT. The arrows indicate cuticle. (*I* and *J*) Molted cuticle that had been incubated with *N*-ethylmaleimide before staining. (Scale bars, 10 μm.)

Thioredoxin Reductase 1 and Glutathione Reductase Are Essential for Molting. Thioredoxin reductases are proposed to be required for the reduction of oxidized disulfides in metazoans. *C. elegans* contains two thioredoxin reductase homologs, TRXR-1 and TRXR-2, but only TRXR-1 is a selenoprotein (20–22). We generated a *trxr-1* deletion allele, *sv47* (Fig. 2*A*) that is very likely null for *trxr-1* function: no TRXR-1 protein was detected on Western blots of extracts from *sv47* mutants (Fig. 2*B*). Worms homozygous for *sv47* were not obviously different from the wild-type strain, N2, when grown under standard laboratory conditions: They grew normally and had normal life spans and brood sizes (Fig. S2*A* and Table S1). They did not show increased sensitivity to oxidative stress (Fig. S2 *B–L*) or defects in molting. *trxr-1*(*sv47*) worms that were also mutant for a deletion of *trxr-2*, *tm2047*, were also viable and fertile and did not arrest during molting. Thus, in an otherwise wild-type genetic background, thioredoxin reductases are dispensable for growth, development, and molting in *C. elegans*.

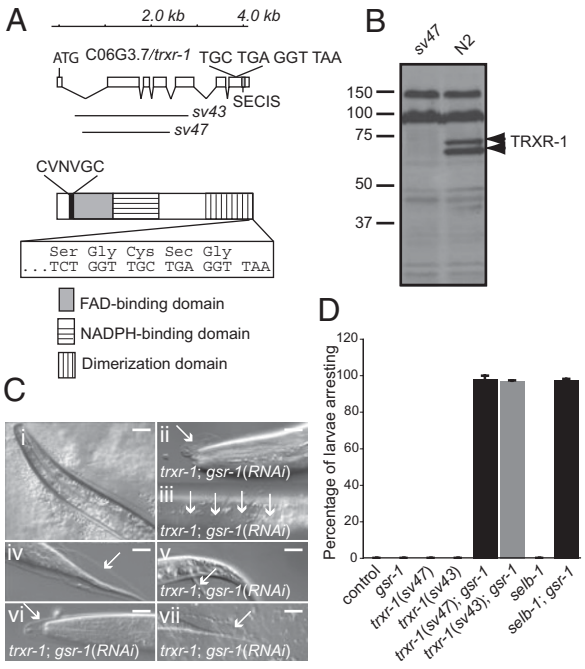

Fig. 2. *C. elegans* TRXR-1 is required together with GSR-1 glutathione reductase for molting. (*A Upper*) The exon intron structure of the *trxr-1* gene. Boxes represent exons, and lines represent introns. The sequence of the last four codons is shown, as is the position of the selenocysteine insertion sequence (SECIS) element in the 3′ untranslated region. The lines underneath denote the regions deleted in the *sv43* and *sv47* mutant alleles. (*A Lower*) Domain structure of the TRXR-1 protein. The sequence of the N-terminal redox active site is shown above, and the sequence of the C-terminal site is shown boxed below. (*B*) Western blot of *C. elegans* protein extracts probed with an antibody raised against TRXR-1. N2 indicates wild-type control. The upper bands result from nonspecific cross-reactivity of the antibody. (*C*) Micrographs of molting worms viewed with DIC optics. Old cuticle (indicated by arrows) associated with the buccal cavity (*ii*), midbody (*iii*), and rectum (*v*) is shown. The other panels show cuticle associated with either the head or tail regions. (*i* and *iv*) Wild-type control worm at molt. The complete genotype of the *trxr-1* worms was *trxr-1*(*sv47*); *rrf-3*(*pk1426*); *gsr-1*(*RNAi*). (Scale bars, 10 μm.) (*D*) Graph showing percentage of larvae that arrest growth before becoming adults. All worms were homozygous for *rrf-3* (*pk1426*).

Work in yeast (23) and *Drosophila* (24) has indicated that enzymes with redox functions can have overlapping functions in vivo. Therefore, to test whether TRXR-1 functioned together with other proteins to regulate molting, we performed RNAi in a *trxr-1*(*sv47*) background against a panel of genes encoding proteins with known or proposed redox functions (Table S2). RNAi of the single glutathione reductase gene in the worm, *gsr-1*, was found to cause a highly penetrant growth arrest specifically in the absence of *trxr-1* function (Fig. 2*D*). No synthetic effects were observed with any of the other genes tested. Inspection of the arrested *trxr-1*; *gsr-1*(*RNAi*) larvae under high magnification revealed that they had invariably arrested during molting (Fig. 2*C*). The arrest did not occur specifically at one molt, but occurred at any one of the four larval molts: 71% of *trxr-1*(*sv47*); *gsr-1*(*RNAi*) animals arrest at the L1 molt; 18% arrest at the L2 molt, 9% arrest at the L3 molt, and 2% arrest at the L4 molt. The majority of the *trxr-1*; *gsr-1*(*RNAi*) animals that arrested were surrounded by partially detached old cuticle, suggesting that apolysis and ecdysis were defective. When arrested *trxr-1*; *gsr-1*(*RNAi*) worms were stained with C_5-maleimide, they were either not appreciably labeled or were labeled only at low levels (Fig. 3 *B*, *C*, *E*, *F*, and *H*). Furthermore, in worms in which low levels of labeling could be seen, the pattern was abnormal: It was markedly punctate (Fig. 3*H*) rather than smooth (Fig. 3*G*). However, when cuticle from *trxr-1*; *gsr-1*(*RNAi*) worms was first treated with DTT, it was labeled by C_5-maleimide (Fig. 3*I*), implying that cuticle in these worms is not lacking thiol groups. We conclude that worms with decreased *trxr-1* and *gsr-1* function are defective in their ability to reduce cuticle components during molting.

Reduced Glutathione Induces Apolysis. In yeast, oxidative refolding of proteins containing disulfide bonds requires protein disulfide isomerase (25), which contains a thioredoxin domain. Furthermore, mutants in *C. elegans pdi-2*, which encodes a protein disulfide isomerase, have been shown to have defects in cuticle synthesis and molting (26). We found that PDI-2 and a second protein disulfide isomerase in *C. elegans*, PDI-3, appear to be required for proper protein folding because RNAi of the *pdi-2* or *pdi-3* genes resulted in induction of the unfolded protein response (UPR; Fig. S3 *E* and *F*). However, the UPR was not induced in *trxr-1*(*sv47*); *gsr-1*(*RNAi*) animals (Fig. S3 *G* and *H*). Furthermore, whereas cuticle synthesis is severely disrupted in *pdi-2* mutants (26), this was not the case for *trxr-1*(*sv47*); *gsr-1* (*RNAi*) animals. First, formation of both the annuli and the alae on the L1 cuticle appear normal in animals lacking *trxr-1* and *gsr-1* activity (Fig. S3 *I*–*L*). In animals with aberrant cuticle secretion, L1 alae are absent or flattened and the annuli are disrupted (26). Analysis of the cuticle by electron microscopy failed to reveal any defects in *trxr-1*; *gsr-1*(*RNAi*) larvae (Fig. S3 *M* and *N*). Furthermore, the expression of GFP reporters for both QUA-1, a cuticle protein required for molting (27), and for LON-3, a cuticle collagen, was normal (Fig. S3 *O*–*R*). These observations argue against a model in which the molting defect seen in *trxr-1*; *gsr-1* animals is the result of a defect in the proper folding of cuticle proteins within the secretory pathway.

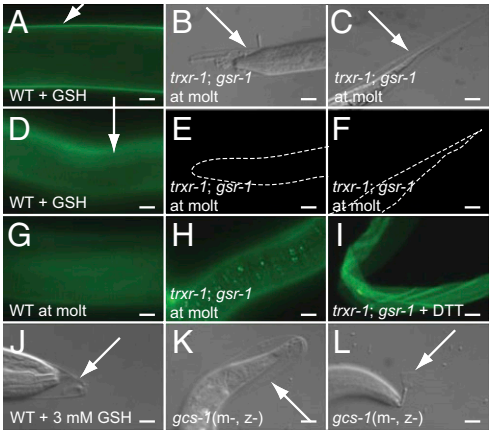

Fig. 3. TRXR-1 and GSR-1 are required for the reduction of disulfide groups in the cuticle during molting, which is promoted by GSH. (*A*–*I*) Micrographs of worms stained with Alexa Fluor 488 C_5-maleimide viewed with DIC (*B* and *C*) or fluorescence (*A* and *D*–*I*) optics. The exposure time for the image in *H* was 1 s; for all other fluorescence images, exposure time was 100 ms. (*A* and *D*) Intermolt worms that had been incubated with 5 mM GSH for 30 min at room temperature before staining with the dye. (*B*, *C*, and *E*–*H*) Worms at molt that had not been incubated with GSH. The arrows indicate cuticle. (*H*) Note the punctate staining seen in some *trxr-1*; *gsr-1*(*RNAi*) worms at longer exposure times. (*I*) Cuticle from *trxr-1*; *gsr-1*(*RNAi*) worms that had been incubated with DTT before staining. (*J*–*L*) Partially detached cuticle in worms exposed to either 3 mM GSH (*J*) or that lacked maternal (m^-) and zygotic (z^-) activity of *gcs-1* (*K* and *L*). (Scale bars, 10 μm.)

In both *Saccharomyces cerevisiae* and *Drosophila*, the thioredoxin system has been shown to promote the reduction of glutathione (23, 24). Treatment of worms with GSH at a concentration of 3 mM, the concentration within eukaryotic cells, led to apolysis, the separation of old and new cuticle (Fig. 3*J*). All of the worms ($n = 20$) were affected but, at this concentration, the separation observed was confined to the cuticle in the head region; cuticle posterior of pharynx was not obviously detached. Treatment of worms with 10 mM GSH, however, led to detachment of the cuticle along the entire length of the worm ($n = 20$; Fig. S4 *A*–*C*). Such worms were very fragile and frequently burst during mounting for microscopy, an observation that suggests that GSH can induce apolysis even before the new cuticle is properly formed. Even at low concentrations, GSH treatment permitted strong staining by C_5-maleimide (Fig. 3 *A* and *D*). Thus, extracellularly administered GSH can promote the reduction of disulfide groups in the cuticle, and reduction of cuticle components leads to detachment of the old cuticle. Consistent with the possibility that *trxr-1*; *gsr-1*(*RNAi*) animals arrest because they have reduced levels of GSH, exogenously supplied GSH promoted the separation and removal of cuticle from arrested *trxr-1*; *gsr-1*(*RNAi*) larvae ($n = 10$; Fig. S4 *D*–*G*). Furthermore, a defect in glutathione synthesis blocked molting: Animals with reduced zygotic and maternal activity of *gcs-1*, which encodes the enzyme that catalyses the first step in the synthesis of glutathione, arrested at the molt ($n = 40$; Fig. 3 *K* and *L*).

TRXR-1 Requires Selenocysteine for Function in Vitro and in Vivo. Previous work has suggested that *trxr-1* is the only gene to encode selenoprotein in *C. elegans* (20–22). Consistent with this suggestion, when *trxr-1*(*sv47*) homozygous mutant worms [or those homozygous for a second allele, *trxr-1*(*sv43*) (Fig. 2*A*)] were grown on *Escherichia coli* bacteria radioactively labeled with ^{75}Se, no label was detected in protein extracted from the mutants (Fig. 4*A* and Fig. S5*A*). Because the *trxr-1* mutants lacked the closely spaced doublet of bands seen in wild type, *trxr-1* encodes two proteins, both of which contain selenocysteine.

In prokaryotes, archaea, and eukaryotes, selenocysteine is incorporated into selenoproteins by the alternate decoding of UGA stop codons by a selenocysteine tRNA, $tRNA^{[Ser]Sec}$ (28). Among other factors, incorporation also requires a *cis*-acting sequence in the mRNA, selenocysteine insertion element, and a dedicated translation elongation factor, SelB or EFsec (28). In rats, in the absence of selenium the UGA codon can code for cysteine in place of selenocysteine (29). To test the importance of selenocysteine for TRXR-1 function in *C. elegans*, we generated an *E. coli* strain lacking SelD selenophosphate synthetase, an enzyme that activates selenium for specific incorporation into

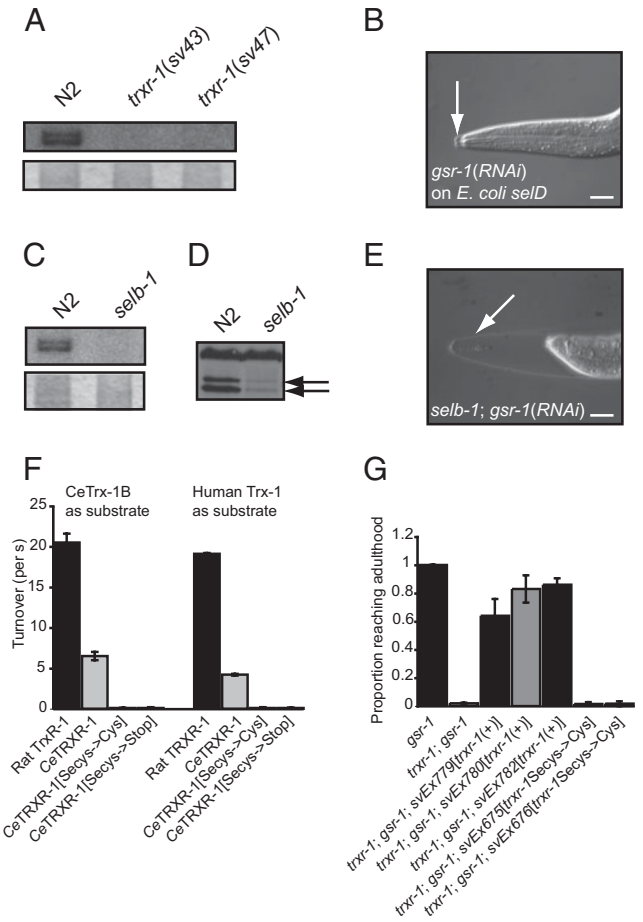

Fig. 4. TRXR-1 is a selenoprotein; incorporation of selenium requires *selb-1*, and is necessary for function. (*A Upper*) Autoradiogram of a gel containing protein extracts of worms raised on ^{75}Se-labeled *E. coli*. (*A Lower*) The Coomassie-stained gel. (*B*) Nomarski micrograph of an arrested worm grown on *selD* mutant *E. coli* that expressed *gsr-1* dsRNA. (Scale bars, 10 μm.) (*C Upper*) Autoradiogram of a gel containing protein extracts of worms raised on ^{75}Se-labeled *E. coli*. (*C Lower*) The Coomassie-stained gel. (*D*) Western blot of *C. elegans* protein extracts probed with an antibody raised against TRXR-1. (*E*) Micrograph of a *selb-1* mutant worm grown on *E. coli,* wild type with respect to *selD*, that expressed *gsr-1* dsRNA. Arrows indicate old cuticle. (*F*) Graph showing the activities of wild-type and mutant TRXR-1 proteins in catalyzing thioredoxin-mediated reduction of insulin in vitro (*Materials and Methods*). (*G*) Graph indicating the ability of wild-type or mutant *trxr-1* transgenes to rescue the growth arrest defect displayed by *trxr-1*(*sv47*); *gsr-1* (*RNAi*) larvae. All strains were homozygous for *rrf-3*(*pk1426*).

bacterial selenoproteins and selenium-containing tRNA species, and fed worms these bacteria. Genotypically *trxr-1*(+) worms were able to develop in to fertile adults on the *E. coli selD* deletion strain. However, when *gsr-1* was knocked down by RNAi, worms raised on this strain arrested growth during molting (Fig. 4*B*). To study further the requirement for selenocysteine for thioredoxin reductase function in vivo, we generated a deletion mutant of the single SelB/EFsec homolog in *C. elegans*, *selb-1* (ref. 30; Fig. S5*B*). Consistent with a requirement for SELB-1 for selenocysteine incorporation, no radiolabeled proteins were detected in protein extracts from *selb-1*(*sv36*) mutant worms grown on ^{75}Se-labeled *E. coli* wild type with respect to *selD* (Fig. 4*C* and Fig. S5*C*). The *sv36* mutants showed no obvious defects under standard laboratory growth conditions (Fig. S2*A* and Table S1). However, when subjected to *gsr-1*(*RNAi*), they arrested growth during molting displaying phenotypes identical to those of *trxr-1*; *gsr-1*(*RNAi*) animals (Figs. 2*D* and 4*E*).

The TRXR-1 protein present in *selb-1* mutants is predicted to lack the last two amino acids, Sec and Gly (Fig. 2*A*). The truncation apparently affects TRXR-1 stability or synthesis because levels of the protein are strongly reduced in *selb-1* mutants (Fig. 4*D*). In the context of the full-length protein, replacement of selenocysteine with cysteine dramatically reduced the ability to complement defects in vivo (Fig. 4*G*) without affecting protein stability (Fig. S5*D*). Replacement of selenocysteine with cysteine also dramatically reduced the ability of *C. elegans* TRXR-1 to reduce the *C. elegans* or human thioredoxin 1 in vitro (Fig. 4*F* and Fig. S5*F*). We conclude that selenocysteine incorporation is required for TRXR-1 activity in vivo and in vitro.

***trxr-1* and *gsr-1* Are Expressed and Required in Cells That Secrete Cuticle.** Expression of *trxr-1*::*gfp* reporter was seen in the hypodermis, in the pharynx and within the nervous system (Fig. 5*C* and Fig. S6 *A*, *B*, and *E*). A *gsr-1*::*gfp* reporter was strongly expressed in the hypodermis and the pharynx (Fig. 5 *A* and *B*) but not in the nervous system. Expression of a *selb-1* cDNA under the control of either pharyngeal- or hypodermal-specific promoters (but not of a neuronal-specific promoter) partially rescued the *selb-1*(*sv36*); *gsr-1*(*RNAi*) arrest phenotype (Fig. 5*E*), implying that *selb-1* (and consequently *trxr-1*) activity in the pharynx and hypodermis promotes molting.

Molting Efficiency Is Reduced in Old Worms. The redox activity of selenoproteins, in particular of thioredoxin reductases, has led to the suggestion that these proteins inhibit the changes associated with aging and promote longevity (31). However, the oxidative damage theory of aging remains controversial (32). To examine whether molting is less efficient in older *C. elegans* worms, we investigated whether aged dauer larvae have a reduced capacity to undergo the postdauer molt. When 1-wk-old dauer larvae

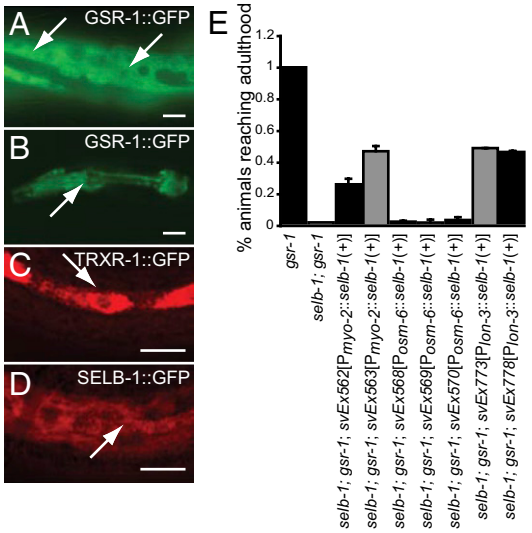

Fig. 5. Expression patterns of *gsr-1*::*gfp*, *trxr-1*::*gfp* and *selb-1*::*gfp* transgenes and focus for *selb-1* activity. (*A–D*) Micrographs of worms with the indicated transgenes viewed with fluorescence (*A* and *B*) or confocal fluorescence (*C* and *D*) optics. The arrows in *A*, *C*, and *D* indicate GFP fluorescence in the hypodermis; arrows in *B* indicate GFP fluorescence in the pharynx. The worms in *C* and *D* were fixed and stained with an anti-GFP antibody. (Scale bars, 10 μm.) (*E*) Graph indicating the ability of seven transgene arrays to rescue the growth arrest defect displayed by *selb-1*; *gsr-1* (*RNAi*) larvae. The arrays contain a *selb-1* cDNA under the control of different tissue-specific promoters. The *myo-2* promoter is active in the pharynx. The *osm-6* and *lon-3* promoters are active in ciliated sensory neurons and the hypodermis, respectively. All strains in *E* were homozygous for *rrf-3* (*pk1426*).

were stimulated to exit dauer by being placed on food, they invariably began molting within 13 h (n = 20) and took, at most, only 15 min to complete ecdysis. In contrast, of 20 3-mo-old dauer larvae placed on food, none completed the molt within 24 h. Furthermore, 5 of the 20 larvae arrested during the molt (Fig. S6 *G* and *H*) and subsequently died without maturing into adults. Thus, molting efficiency dramatically decreases with age in wild-type dauer larvae. The reduced ability to undergo the molt correlated with a decrease in the amount of reduced thiols in the cuticle: Unlike wild-type intermolt animals, in which the cuticle is not strongly stained by C_5-maleimide, appreciable fluorescence is seen when young dauer larvae are stained with the dye (Fig. S6 *I* and *J*). The level of fluorescence, however, is dramatically reduced in old dauer larvae (Fig. S6 *K* and *L*). Consistent with earlier global studies in which a reduction in *gsr-1* expression was seen with age in dauer larvae (33), we found that young dauer larvae showed robust expression of a *gsr-1*::*gfp* reporter in hypodermal cells, but fluorescence of the reporter was almost not detectable in aged larvae (Fig. S6 *M* and *N*). *trxr-1* and *gsr-1* are together required for the postdauer molt: Whereas knockdown of *gsr-1* by RNAi in *trxr-1*(+) animals did not prevent 3-d-old dauer larvae from developing into normal fertile adults (n = 66), *gsr-1*(*RNAi*) in a *trxr-1* mutant background caused a 100% penetrant growth arrest during the postdauer molt (n = 128; Fig. S6 *O*–*Q*). Similar effects were seen with *selb-1*: 99% (n = 145) of 3-d-old *selb-1*(*sv36*); *gsr-1*(*RNAi*) dauer larvae arrested at the molt when stimulated to exit dauer (Fig. S6*R*).

Discussion

Selenoproteins are found in organisms in all three categories of life: archaea, prokaryotes, and eukaryotes. Although extensive work has been done on the functions on eukaryotic selenoproteins in vitro, few reports exist on the functions of selenoproteins in general or of thioredoxin reductases in particular in animal models. *C. elegans* represents an attractive system with which to address the importance of selenocysteine and thioredoxin reductase function in vivo.

Collagens and other components of the cuticle are known to be cross-linked via disulfide groups both in *C. elegans* and larger metazoans, including mammals. However, that disulfide groups are reduced during molting has not previously been demonstrated. Our results indicate that the thioredoxin and glutathione systems are required for the reduction of disulfide groups in the cuticle during molting. Earlier work identified BLI-3 (a dual oxidase), MLT-7 (a heme peroxidase), and PDI-2 (a putative protein disulfide isomerase) as important for molting (26, 34). BLI-3 and MLT-7 act by promoting the oxidative cross-linking of collagens via dityrosine groups rather than the reduction of disulfides. PDI-2, on the other hand, has two separate functions in cuticle formation: Alone, it acts as a dithiol oxidase to catalyze the formation of disulfides in cuticle collagens; in complex with DPY-18/PHY-1 or PHY-2, it acts as a prolyl hydroxylase to catalyze the hydroxylation of proline residues in collagens, a modification important for the stability of the triple helical fold. It is noteworthy that although *bli-3*, *mlt-7* or *pdi-2* mutants display molting defects, they also show severe defects in cuticle synthesis; many arrest as embryos, and the larvae that escape embryonic arrest are severely Dpy or otherwise malformed (26, 34). The observations that the UPR is not activated in *trxr-1*; *gsr-1* larvae, and that the cuticle in such larvae is not severely defective, argue against a model in which the molting defect in *trxr-1*; *gsr-1* larvae is an indirect result of defects in cuticle synthesis or secretion.

Our analysis, together with earlier studies (20–22), strongly suggests that *trxr-1* is the only gene in *C. elegans* encoding selenoprotein. Thus, the six or more dedicated proteins and RNAs thought to be required for the insertion of selenocysteine in metazoans appear to exist in *C. elegans* for the sole purpose of decoding a single codon. *Caenorhabditis briggsae* TRXR-1 is also predicted to be a selenoprotein (22). Because the common ancestor of *C. elegans* and *C. briggsae* is thought to have lived between 40 and 100 million years ago, there appears to be considerable evolutionary pressure to maintain selenocysteine in TRXR-1. One possibility is that, in the wild, neither TRXR-1 nor GSR-1 alone provides sufficient reducing power during molting and, therefore, that both proteins are necessary under conditions found in natural habitats. Notably, the two tissues in which thioredoxin reductase functions to promote molting (the hypodermis and the pharynx) are exposed to molecular oxygen. Molting might require extra reducing power to overcome the oxidizing environment of the atmosphere.

That *trxr-1* and *gsr-1* have overlapping functions during molting is consistent with the possibility that the thioredoxin and glutathione systems have a common target or targets. Although glutathione and thioredoxin reductases are related to one another in sequence, extensive biochemical analyses have revealed that glutathione reductases are unable to reduce thioredoxins in vitro. It is noteworthy, however, that the *Drosophila* thioredoxin system can catalyze the reduction of GSSG by NADPH in a two-step, thioredoxin-dependent reaction (24). *S. cerevisiae* strains lacking both the thioredoxins present in this organism, Trx1 and Trx2, have increased levels of GSSG (23). Thus, the genetic redundancy between *trxr-1* and *gsr-1*, and the observation that GSH can reduce disulfides in the cuticle, suggests that TRXR-1 and GSR-1 might act in molting by increasing GSH concentrations at the expense of GSSG. GSH can be exported from cells by certain proteins in the ATP-binding cassette (ABC) family, including ABCG2 (35), and thus could potentially exert its molting-promoting functions extracellularly (e.g., together with secreted glutaredoxins). However, other models are also consistent with our results. Although isomerization of disulfides has not, to date, been shown to be important for molting, TRXR-1 and GSR-1 could potentially act to promote the isomerization of disulfides by facilitating the reductive step in isomerization.

Thioredoxin reductases are widely thought to be required for cell proliferation (possibly by supporting ribonucleotide reduction) and to provide protection against oxidative stress. However, worms lacking *trxr-1* display no obvious developmental defects under laboratory growth conditions and are not hypersensitive to acute oxidative stress. It is possible that other unrelated proteins are able to reduce thioredoxins in *C. elegans*. Alternatively, thioredoxins may play no or only minor roles in protecting against acute oxidative stress or supporting cell proliferation in this organism.

Although still controversial (32), the oxidative damage theory of aging remains one of the theories most often cited in modern aging studies. Through their ability to affect redox status, thioredoxin reductases have been suggested to be important for the ability of cells and organisms to inhibit processes that occur during aging. However, experimental evidence supporting a role for thioredoxin reductases in inhibiting such processes is presently lacking. One of the most visible signs of aging in many animals is a change in quality of the skin. The observation that the efficiency of molting is reduced in aged dauer worms, and that TRXR-1 (together with GSR-1) promotes molting, highlights a function for TRXR-1 in epidermal cells in *C. elegans*. Thioredoxin reductase activity is induced in mammalian epidermal cells by agents (such as UV light) that cause age-associated changes to skin (14, 15). Thus, a function for thioredoxin and glutathione reductases in the removal of old and damaged cuticle may have been conserved in evolution.

Materials and Methods

Isolation of *C. elegans* Deletion Mutants. *selb-1*(*sv36*), *trxr-1*(*sv43*), and *trxr-1* (*sv47*) were isolated by screening a deletion library of wild-type N2 worms mutagenized with ethyl methanesulfonate. The sequences of the primers

used for identifying and backcrossing the mutants are described in *SI Materials and Methods*.

^{75}Se Labeling of *C. elegans* Worms. Worms were labeled with ^{75}Se by feeding them *E. coli* bacteria that had been grown in the presence of ^{75}Se. A 1.3-mL culture of *E. coli* strain OP50 was grown overnight in LB medium supplemented with cysteine to a final concentration of 100 μg/mL and 20 μCi of ^{75}Se. Two hundred microliters of the overnight culture was spread onto an NGM agar *C. elegans* culture plate and allowed to grow for 24 h at room temperature (~21 °C). After this time, the plate was seeded with five L4 hermaphrodite worms of the appropriate strain. The worms were harvested shortly before they had consumed all of the bacteria.

Staining of Worms with Alexa Fluor 488 C_5-Maleimide. Worms were washed individually in 50 μL of M9 buffer in a depression slide and then left in the buffer to cool on ice for 15 min. One microliter of dye stock solution was added to give a final concentration of 5 μM. The worms were left for 15 min on ice, washed in ice-cold M9, and then mounted for microscopy.

Isolation of the *E. coli* HT115(DE3) Lacking selD. The plasmids and the sequences of the primers used create the *selD* deletion mutation in the background of *E. coli* HT115(DH3) are given in *SI Materials and Methods*.

Generation and Use of anti-CeTRXR-1 Antibodies. An anti-peptide antibody was raised in rabbits against a peptide with the sequence NH_2–CTLEKKEGDEEKQAS–$CONH_2$, corresponding to the residues 650–663 of *C. elegans* TRXR-1 protein. Immunizations were performed by Agrisera, Vännäs, Sweden. Western blots were probed with affinity purified anti-TRXR-1 antibody at a 1:1,000 dilution.

Measurements of Enzymatic Activities in Vitro. The Trx-insulin and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) assays were used to determine the activities of purified wild-type and mutant TRXR-1 proteins in vitro. The assays were performed as described by Arnér et al. (36).

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Supporting Information

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SI Materials and Methods

Nematode Strains. *C. elegans* strains were cultivated as described by Brenner (1). The mutant alleles and *gfp* transgenes used in this work were *selb-1*(*sv36*) *I* (this work), *rrf-3*(*pk1426*) *II* (2), *gcs-1*(*ok436*), *trxr-1*(*sv43*) *III*, *trxr-1*(*sv47*) *III* (this work), *zIs356*[*daf-16::gfp rol-6*(*su1006*)] (3), *zcIs4*[*hsp-4*::*gfp*] (4), *muIs84*[pAD76 (*sod-3*::*gfp*)] (5), *svEx267*[P_{gsr-1}::*gsr-1*::*gfp*], *svEx277*[P_{trxr-1}::*gfp*], *svEx285*[P_{selb-1}::*selb-1*::*gfp*], *svEx741*[*gcs-1*::*gfp*] (this work), *chEx1677*[*qua-1*::*gfp*] (6), *svIs36*[P_{lon-3}::*lon-3*::*gfp*]. *svIs36* was generated from the extrachromosomal array, *svEx90* (7). The *gcs-1*::*gfp* plasmid is described in An et al. (8). *gcs-1*(*ok436*) was generated by the *C. elegans* gene knockout consortium. All strains were derived from the N2 wild-type strain (1).

Isolation of *sv36*, *sv43,* and *sv47 C. elegans* Deletion Mutants. A deletion library of wild-type N2 worms mutagenized with EMS was screened for deletions in the *selb-1* and *trxr-1* genes. Pooled genomic DNA samples representing a total of 400,000 haploid genomes were used as templates for nested PCR reactions. For *selb-1*, the first round of PCR was performed with primers with the sequences 5′-GGA CCT CTC AAT CTA GGC ATTC-3′and 5′-CCT TCG ATT TTT CCA CGT GGA C-3′. The second round was performed with primers with the sequences 5′-TTA ACA CGT CGA ATC GCC GAA C-3′ and 5′-GTT TCG GCC TTG AAC ATT CCA G-3′. One mutation, *selb-1*(*sv36*), was isolated. During backcrossing, nematodes were genotyped by single worm duplex PCR. The sequences of the primers used were 5′-TTA TCG ATT GCC CTG GGC ATT C-3′, 5′-CTG GGA CAG TGA TAC GTG GAG-3′and 5′-GAA ATT CGC GCC AAT TTC TTT TC-3′. To screen for mutations in the *trxr-1* gene, PCR was performed first with primers having the sequences 5′-GAT GGC ATG AAA TCT TCT ACC-3′and 5′-GGA GGC GCA TCA GAC CA GAG-3. The second round of PCR was performed with primers with the sequences 5′-GAC AGC CGA GAC AAC AAG AAG-3′and 5′-CTC TACTAG GCA CCA CCG A-3. Two deletion alleles were isolated, *sv43* and *sv47*. The sequences of the primers used to genotype worms during backcrossing of *sv43* were 5′-GAT GGC ATG AAA TCT TCT ACC-3′, 5′-CAT TTA TGA CGT AAC AAC TG-3′and 5′-CAT TCA GGA GTT TGT CGA AC-3′. The sequences of the primers used to genotype worms during backcrossing of *sv47* were 5′-GTC ACT ATC TTG TAT TTC CG-3′, 5′-TTG AGT TCT AAA TTG AAG GC-3′and 5′-TCA GAA TCG TGT TAT ATT CC-3′. The *sv47* deletion removes base pairs 721–2383 of sequence, where 1 is the A of the *trxr-1* start codon. Analysis of the mRNA produced from the *sv47* allele by RT-PCR revealed that it is generated by the splicing of exon 1 directly to exon 5. The exon 5 sequence is out of frame, and the message is predicted to encode a severely truncated protein of just 29 amino acids. The *sv43* deletion removes base pairs 370–2764. In the message generated, exon 1 sequences are spliced to a cryptic splice site in intron 1 at position 249 so that the message has 121 bases of intron sequence between exon 1 and sequences in exon 5. An in-frame stop codon is generated by the splicing event, and the exon 5 sequence is out of frame: the message encodes a protein of 58 amino acids. The *sv36* deletion removes base pairs 853–2427 of genomic *selb-1* sequence. In the message generated, exon 1 is spliced directly to exon 6 resulting in disruption of the reading frame. The message encodes a protein of 143 amino acids that lacks motifs at the C terminus known to be essential for the ability of *E. coli* SelB to bind to selenocysteine insertion elements (SECIS; ref. 9).

RNAi. To perform RNAi (10), 10-mL liquid cultures of *E. coli* HT115(DE3) bacteria in 2× YT containing 50 μg/mL carbenicillin and 12.5 μg/mL tetracycline were established from frozen cultures and grown for 5–6 h at 37 °C. Small amount of the cultures were spread onto NGM agar worm culture plates supplemented with 50 μg/mL carbenicillin and 1 mM IPTG. The plates were left for 12 h or more for the bacterial lawn to grow and then seeded with five L4 larval worms of the appropriate genotype. The plates were left for 24 h at 20 °C after which time the worms were transferred to fresh RNAi plates. After a further 8 h, the parental worms were moved to fresh plates, allowed to lay eggs for 12 h, and then removed. Only eggs and larvae from the last set of plates were used in phenotypic tests. Larvae with reduced zygotic and maternal activity of *gcs-1* were generated by placing *gcs-1*(*ok436*)/*mIn1* hermaphrodies on *gcs-1* RNAi plates. The *gcs-1*(*ok436*) homozygotic progeny were examined for molting defects. When their mothers are grown on normal plates, *gcs-1* (*ok436*) homozygotes are maternally rescued and grow up to become sterile adults. *gcs-1*(*RNAi*) with N2 worms [or with the *rrf-3* (*pk1426*) strain] did not cause obvious defects at a high penetrance.

^{75}Se Labeling of *C. elegans* Worms. Worms were labeled with ^{75}Se by feeding them *E. coli* bacteria that had been grown in the presence of ^{75}Se. The protocol described by Buettner et al. (11) was used with minor modifications. Briefly, a 1.3 mL culture of *E. coli* strain OP50 (1) was grown overnight in LB medium supplemented with cysteine to a final concentration of 100 μg/mL and 20 μCi of ^{75}Se. A total of 200 μL of the overnight culture was spread onto an NGM agar *C. elegans* culture plate and allowed to grow for 24 h at room temperature (~21 °C). After this time, the plate was seeded with five L4 hermaphrodite worms of the appropriate strain. The worms were allowed to grow until they had just consumed all of the bacteria, and then washed off the plate with M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 85 mM NaCl, 1 mM Mg_2SO_4). After at least three washes in M9 buffer, the worms were left at room temperature for 45 min to allow the digestion of any bacteria remaining in their intestines. The worms were then washed twice in ice-cold protein lysis buffer (150 mM NaCl, 50 mM Tris.Cl, pH 8.0, Complete protease inhibitor mix containing EDTA; Roche Diagnostics). The worms were broken open by sonication in this buffer.

Isolation of the *E. coli* HT115(DE3) Lacking selD. A protocol developed by Datsenko and Wanner was used to create the *selD* mutant strain (12). HT115(DE3) [Genotype F-, *mcrA*, *mcrB*, IN (*rrnD-rrnE*)1, *rnc14*::Tn10(DE3 lysogen: lavUV5 promoter -T7 polymerase)] was first transformed with pKD46, a plasmid encoding the λ Red recombinase under the control of an arabinose-inducible promoter (12). pKD3 plasmid DNA, which contains the chloramphenicol resistance marker (Cm^R) DNA was used as a template in PCR with the primers 5′-AAT TCA GAC ACT CTC ACT TAT CAC TTC ACG GAA TGA GGG TGT AGG CTG GAG CTG CTT C-3′and 5′-CGC GCG CCA GAC TCG GTT TTT CGG CAA TAA ACA ACC GCA TCA TAT GAA TAT CCT CCT TAG-3′. The PCR product, which contained Cm^R flanked by *E. coli selD* sequences, was digested with DpnI and then transformed in the presence of arabinose into HT115 (DE3) harboring pKD46. The colonies were selected on plates containing chloramphenicol and tetracycline at 37°C, at which temperature pKD46 cannot replicate. PCR with the primers 5′-ACG TCC CGG ACC CGA CGC CG-3′ and 5′-GCC TTT CCG GTG CGG TTT GG-3′ was used to screen the colonies for the

presence of the transformed fragment integrated at the genomic *selD* locus. To eliminate the Cm^R marker, cells grown at 37°C were transformed with pCP20, which encodes FLP recombinase under the control of a thermal-inducible promoter, confers resistance to carbenicillin (Cb^R), and is temperature sensitive for replication (12). Transformants were initially checked by PCR for the elimination of the Cm^R fragment and then for sensitivity to Cm and Cb. Finally, to generate a strain for RNAi of *C. elegans gsr-1*, a plasmid from the Ahringer library encoding double-stranded RNA from *gsr-1* was transformed into the HT115(DE3) Δ*selD* strain.

Western Blotting with anti-CeTRXR-1 Antibodies. Samples for Western blot analysis were prepared by growing three plates for each strain until the worms had consumed nearly all of the bacteria. Worms were washed with M9 several times until there were no bacteria left. To the pellet of worms was added twice its volume of Nonidet P-40 lysis buffer (150 mM NaCl, 1% Nonidet P-40, 50 mM Tris[chemp]HCl pH 8) containing protease inhibitor mixture. The preparation was quickly frozen in liquid nitrogen. After thawing, the pellets were resuspended in SDS/PAGE sample buffer and then incubated for 10 min at 95 °C. Insoluble worm debris was cleared by centrifugation at 13,000 rpm in a microfuge. Protein samples were run in a 10% 0.75 mm SDS/PAGE gel at 120 V and then the blotted onto membrane for 1 h at 90 mA under semidry conditions. A 5% solution of nonfat dry milk blocking was used to block nonspecific binding. Western blots were probed with affinity purified anti-TRXR-1 antibody at a 1:1,000 dilution. Two bands are detected in extracts from wild-type worms, that are missing in the *trxr-1*(*sv47*) mutant. We do not at present know how the *trxr-1* gene gives rise to two proteins. RT-PCR analysis of *trxr-1* transcripts failed to provide evidence for alternative splicing or use of alternate promoters.

Previous work has shown that expression of a full length *C. elegans trxr-1* cDNA in mammalian cells leads to the expression of both isoforms (13). Because there are four in-frame ATG codons close to the start of the ORF, it is possible that the proteins result from translation initiation at two different start sites. Alternatively, TRXR-1 protein might be posttranslationally processed. The antibody also reacts nonspecifically with two more slowly migrating proteins present in all strains.

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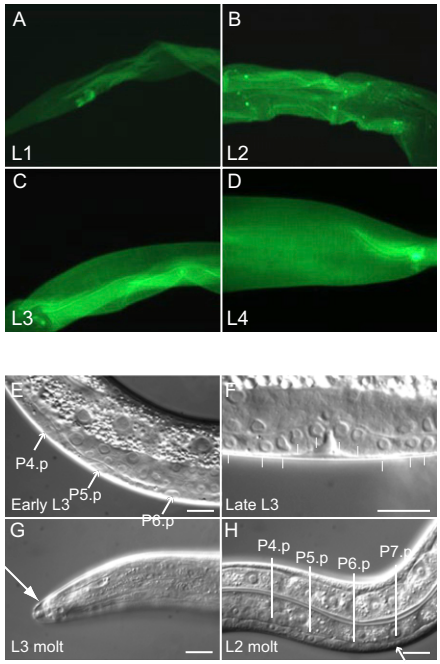

Fig. S1. Cuticle components are reduced during the L1–L4 molts, and inhibiting reduction inhibits molting. (*A*–*D*) Micrographs of shed cuticle from the indicated molts stained with Alexa C_5-maleimide. (*E*–*H*) Micrographs of worms treated with diamide viewed with DIC optics. Note that diamide prevents progression through the molt but does not block growth during the intermolt. (*E*–*G*) Images of the same worm at different time points that had been exposed to diamide directly after the L2 molt. Diamide did not prevent the divisions of cells in the vulval equivalence group (P3.p-P8.p) from occurring during the intermolt, but did cause a block at the L3 molt. P4.p, P5.p, and P6.p are indicated by arrows in *E*. The lines in *F* indicate descendents of P5.p, P6.p, and P7.p during their third round of division. The arrow in *G* indicates partially detached cuticle at the anterior of the worm arrested at the L3 molt. (*H*) A late L2 larval worm that had been exposed to diamide. P3.p-P8.p failed to divide, and the worm was blocked at the L2 molt.

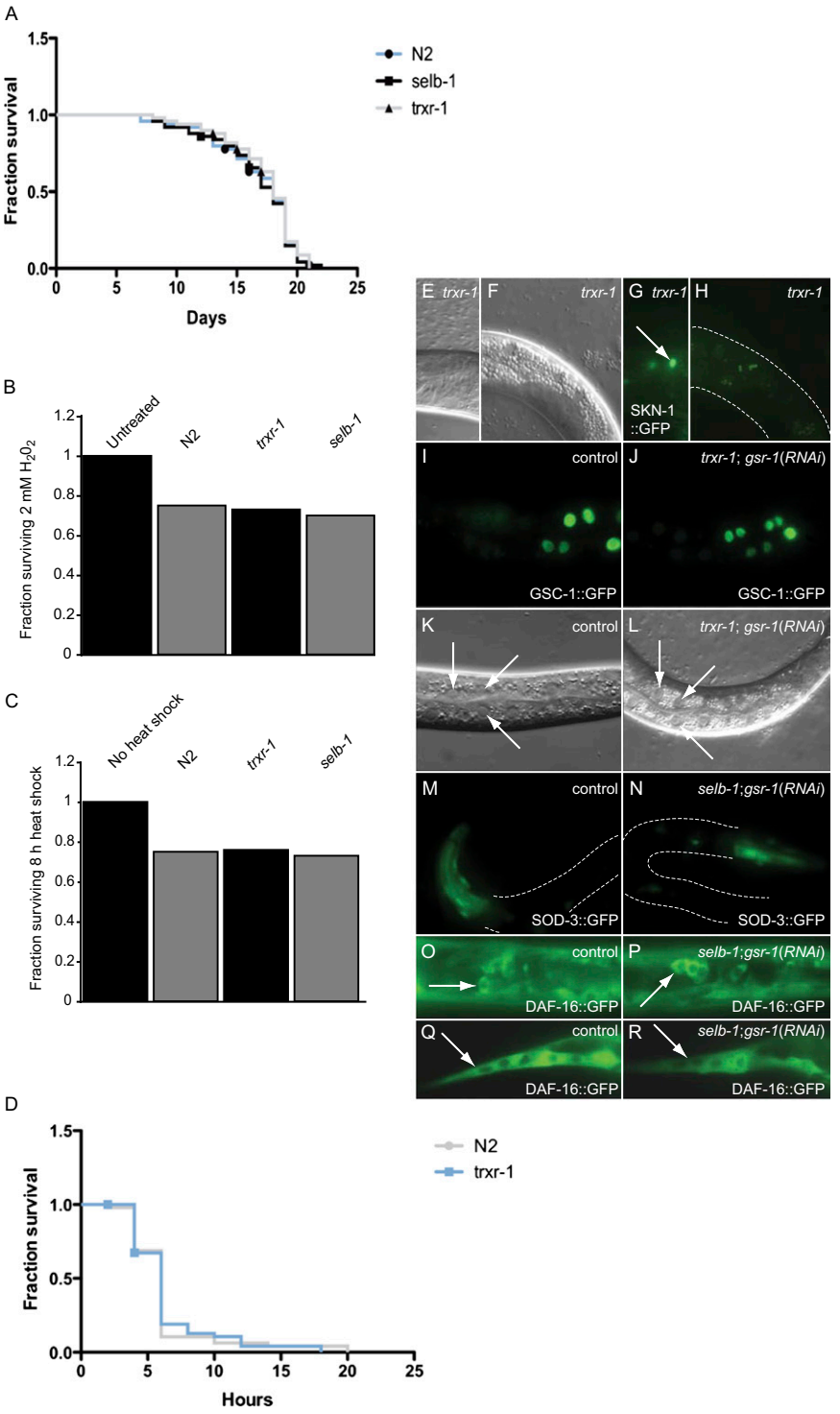

Fig. S2. *trxr-1* and *selb-1* mutants are not short lived, do not show increased sensitivity to acute oxidative stress, and do not display induction of molecular markers for the oxidative stress response. (*A*) Graph showing the proportions of *trxr-1* and *selb-1* worms grown at 20 °C surviving over time. Time 0 denotes the L4 to adult molt. (*B*) Fraction of *trxr-1* and *selb-1* worms surviving after incubation in 2 mM H_2O_2 for 2 h. (*C*) Fraction of worms surviving after heat shock for 8 h at 33 °C. (*D*) Fraction of worms surviving at the indicated times after incubation in 6 mM t-butyl peroxide. (*E*–*R*) Worms harboring markers for oxidative stress. (*E*, *F*, *K*, and *L*) Micrographs of worms viewed with DIC optics. (*G*–*J* and *M*–*P*) Micrographs of worms viewed with fluorescence optics. *trxr-1* denotes *trxr-1*(*sv47*), and *selb-1* denotes *selb-1*(*sv36*). The SKN-1::GFP marker is constitutively expressed in ASI sensory neurons; expression is induced in the intestine upon oxidative stress (1). (*G*) The constitutive expression in ASI (indicated by an arrow) is normal in *trxr-1* mutant animals (the weak signal is background autofluorescence seen at long exposure times). (*H*) Expression in the intestine is not induced. The GSC-1::GFP marker is expressed in posterior intestinal cells in unstressed animals and throughout the intestine in stressed animals (1). In both the control worm shown and the *trxr-1*; *gsr-1*(*RNAi*) animal, only the posterior intestinal cells express the marker. The arrows in *K* and *L* indicate more anterior intestinal nuclei that do not show appreciable GFP fluorescence. The worms shown harbored *svEx741*, an array made by injecting *gsc-1*::*gfp* DNA kindly provided by J. H. An and K. Blackwell (1). The SOD-3::GFP marker is widely expressed but most strongly in the pharynx (2). The level of expression is increased upon stress. The expression of the marker was not increased in *selb-1*; *gsr-1*(*RNAi*) animals, which have reduced *trxr-1* function (this study). SOD-3::GFP was encoded by *muIs84* (2). The DAF-16::GFP marker is predominantly cytoplasmic in unstressed animals, but pre-

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dominantly nuclear in animals exposed to oxidative stress (ref. 3; or those with reduced insulin signaling). No difference in the distribution of DAF-16::GFP was seen in *selb-1*; *gsr-1*(*RNAi*) mutant strain compared with the control. DAF-16::GFP was encoded by *zIs356* (3). The animals in *I–R* were homozygous for *rrf-3*(*pk1426*).

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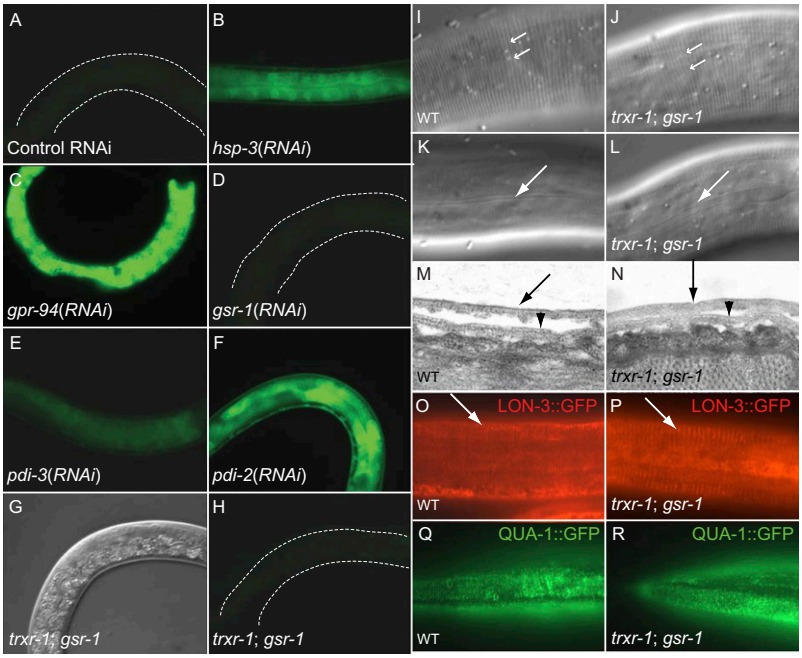

Fig. S3. The unfolded protein response is strongly induced in *pdi-2* mutant larvae but not in *trxr-1*; *gsr-1*(*RNAi*) larvae, which do not display cuticle synthesis defects. (*A–H*) Micrographs of L2 larvae harboring the *zcIs4* transgene array, which encodes *hsp-4*::*gfp*, a marker for the unfolded protein response (UPR; ref. 1). The larvae in *A–F* and *H* were viewed with fluorescence optics; the larva in *G* was viewed with DIC optics. *hsp-3*(*RNAi*) and *grp-94*(*RNAi*) are positive controls previously shown to cause induction of the UPR (2). Control RNAi in *A* was *zcIs4* worms cultured on HT115 (DE3), the *E. coli* RNAi strain, harboring empty vector, L4440. *pdi-2* or *pdi-3* encode proteins homologous to those in yeast that promote protein folding within the ER (3). *trxr-1*; *gsr-1* denotes *trxr-1* (*sv47*); *gsr-1*(*RNAi*). Note that whereas RNAi directed against *pdi-2* (and to a lesser extent *pdi-3*) caused a potent induction of *hsp-4*::*gfp*, no induction was seen in *trxr-1*; *gsr-1* animals. (*I–R*) Absence of cuticle synthesis defects in *trxr-1*; *gsr-1* larvae. (*I* and *J*) Annuli (indicated by arrows) in wild-type and *trxr-1*(*sv47*); *gsr-1* (*RNAi*) larvae. Annuli, circumferentially oriented parallel furrows in the cuticle, are disrupted in mutants with defects in cuticle synthesis or secretion (4). (*K* and *L*) Alae (indicated by arrows) in wild-type and *trxr-1*(*sv47*); *gsr-1*(*RNAi*) L1 larvae. Formation of alae, longitudinal ridges that run along either side of the animal in L1, dauer and adult worms is disrupted in animals with aberrant cuticle secretion (5). The alae are neither absent nor obviously abnormal in *trxr-1* mutants subjected to *gsr-1*(*RNAi*). (*M* and *N*) Transmission electron micrographs of cuticle in molting wild-type and *trxr-1*(*sv47*); *gsr-1*(*RNAi*) larvae. The arrows and arrowheads indicate the old and new cuticle, respectively. (*O* and *P*) Fluorescence micrographs of worms harboring a LON-3::GFP transgene stained with an anti-GFP antibody. LON-3 is a cuticle collagen (6, 7). LON-3::GFP in the mutants is localized to the annuli, indicated by arrows, as it is in wild type. (*Q* and *R*) Fluorescence micrographs of worms harboring a QUA-1::GFP transgene. QUA-1 is a cuticle protein that controls molting (8). With the exception of wild type, all strains were homozygous for *rrf-3*(*pk1426*).

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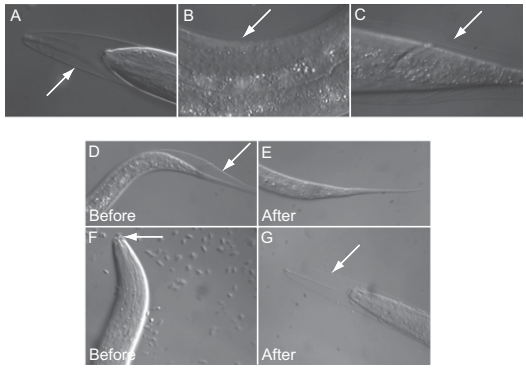

Fig. S4. GSH induces separation of the old and new cuticle in wild-type worms, and promotes apolysis and ecdysis in arrested *trxr-1*; *gsr-1* larvae. (*A*–*C*) Wild-type worm treated with 10 mM GSH for 45 min at 25 °C. (*D* and *F*) *rrf-3*(*pk1426*); *trxr-1*(*sv47*); *gsr-1*(*RNAi*) larvae arrested during molting. In *D*, extensive apolysis has occurred at the posterior of the worm; in *F*, the old cuticle remains attached to the new cuticle, except at the extreme anterior. (*E* and *G*) The same worms after treatment with 3 mM GSH for 45 min at 25°C. In *E*, the old cuticle has been completely removed; in *G*, GSH has induced separation of the old and new cuticle.

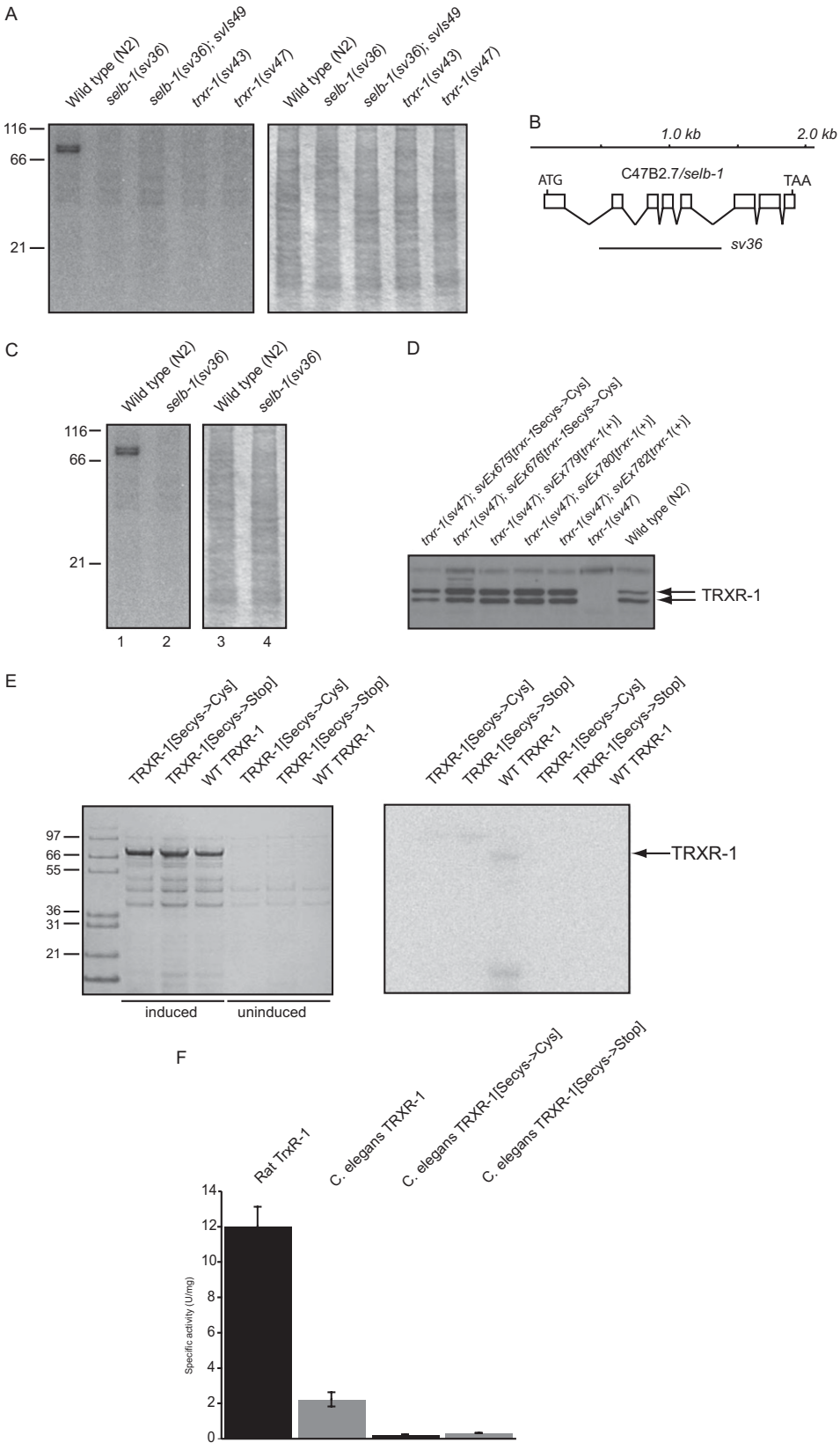

Fig. S5. TRXR-1 is the sole selenoprotein detected in *C. elegans* worms labeled with ^{75}Se, and production of *C. elegans* TRXR-1 proteins in vitro. (*A Left*) Autoradiogram of a gel containing protein extracts of worms raised on ^{75}Se-labeled *E. coli*. (*A Right*) The Coomassie-stained gel. (*B*) The *C. elegans selb-1* gene. Boxes represent exons, and lines represent introns. The line underneath denotes the region deleted in the *sv36* mutant allele. (*C Left*) Autoradiogram of a gel containing protein extracts of worms raised on ^{75}Se-labeled *E. coli*. (*C Right*) The Coomassie-stained gel. (*D*) Western blot of worms harboring transgenes encoding wild-type or mutant TRXR-1 probed with an antibody raised against TRXR-1. (*E*) Production of *C. elegans* TRXR-1 proteins in vitro. (*Right*) Coomassie-

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stained gel of protein extracts from *E. coli* strains harboring plasmids encoding wild-type or mutant TRXR-1. An *E. coli* SECIS element was present to the 3′ of the *trxr-1* coding region to allow incorporation of selenocysteine encoded by the UGA codon. *trxr-1* cDNA sequences were under the transcriptional control of the *E. coli lac* promoter. The three lanes on the left show protein extracts from cells in which *lac* promoter activity was induced with IPTG; those on the right are from untreated cells. All cells were grown in the presence of ^{75}Se. (*Right*) An autoradiogram of the gel shown at *Left*. The radioactive label is incorporated into wild-type TRXR-1 but not the proteins encoded by the cDNAs lacking the UGA codon. The protein produced corresponds in size to the larger of the two isoforms seen *C. elegans*. (*F*) Graph showing the activity of purified proteins in catalyzing the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to 5′-thionitrobenzoic acid (TNB; ref. 1). Error bars represent SEM.

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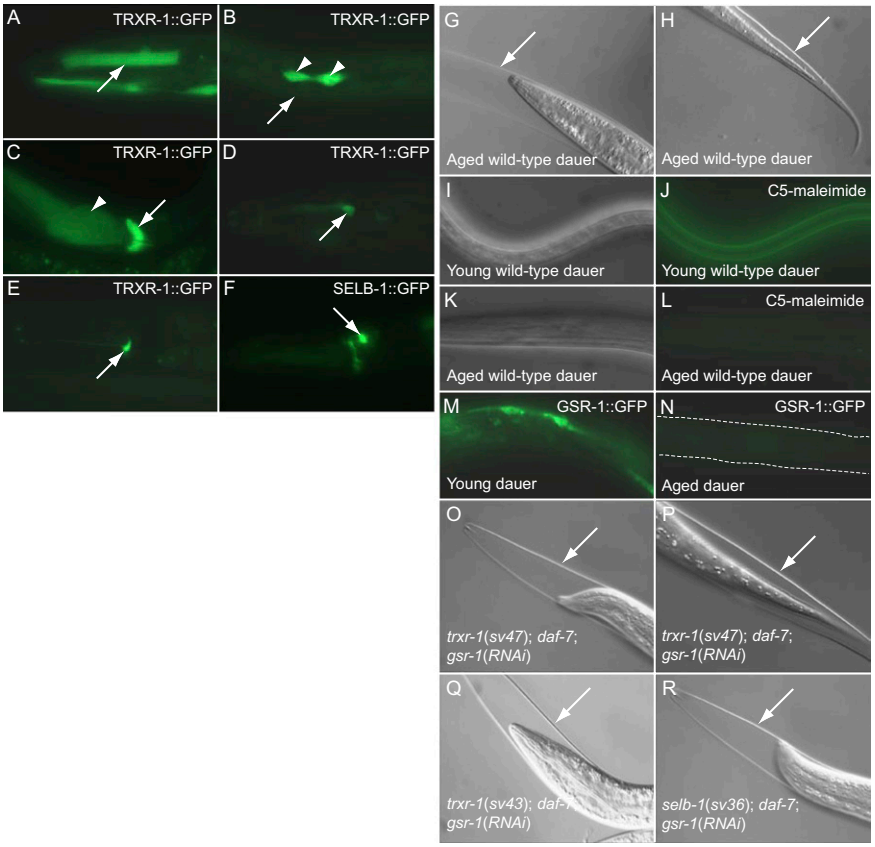

Fig. S6. TRXR-1::GFP and SELB-1::GFP reporters are expressed in tissues implicated in molting; age and reduced *trxr-1* and *gsr-1* function compromise the efficiency of the dauer molt. Micrographs of hermaphrodite worms were viewed with DIC (*G–I*, *K*, and *O–R*) or fluorescence (*A–F*, *J*, *L–N*) optics. (*A–F*) Micrographs of worms carrying GFP reporters. The arrow in *A* indicates expression of TRXR-1::GFP in pharyngeal muscle. The arrowheads in *B* indicate expression in seam cells, and the arrow indicates weak expression in the hypodermis. The arrow in *C* indicates expression in rectal epithelial cells; the arrowhead indicates expression in the posterior part of the intestine. The arrow in *D* indicates one of a pair of cells that lie in the anterior head region that express the reporter. Their position and morphology are consistent with them being amphid sheath cells. The arrows in *E* and *F* indicate expression in neurons within the nerve ring. Both markers are strongly expressed in a pair of neurons. (*G* and *H*) Three month-old dauer worm arrested at the dauer/L4 molt. Arrows indicate old cuticle. (*I–L*) Staining of the dauer cuticle with C_5-maleimide decreases with age. (*M* and *N*) Expression of GSR-1::GFP in dauer larvae decreases with age. (*O–R*) Larvae of the indicated genotypes arrested at the dauer/L4 molt. The worms in the bottom four panels were homozygous for *rrf-3*(*pk1426*) and were <4 d old. *daf-7* denotes *daf-7*(*e1372*ts). Worms containing *daf-7*(*e1372*ts) were raised at the restrictive temperature, 25°C, until they had entered dauer, and then moved to the permissive temperature, 15°C, at which temperature *daf-7*(*e1372*ts) single mutant worms exit dauer and develop into adults.

Table S1. Brood sizes of *trxr-1* and *selb-1* mutants

Genotype	Average brood size* ± SD
Wild type N2	306.5 ± 36.5
trxr-1(*sv47*)	273.6 ± 29.6
trxr-1(*sv43*)	292.9 ± 32.5
selb-1(*sv36*)	304.1 ± 26.6

*The total numbers of progeny of 15 worms of each genotype were determined.

Table S2. Genes tested for synthetic growth defects with *trxr-1*

Gene category	Gene name	Gene sequence designation	Slow growth/larval arrest*
Thioredoxin reductase	*trxr-2*	ZK637.10	No
Catalases	*Ctl-1*	Y34D9A.6	No
	Ctl-2	Y54G11A5.1	No
Superoxide dismutases	*sod-1*	C15F1.19	No
	sod-2	F10D11.2	No
	sod-4	F55H2.1	No
	sod-5	ZK430.1	No
Glutaredoxins	*glrx-10*	Y34D9A.6	NA†
	glrx-21	ZK121.1	No
Glutathione peroxidases		C11E4.1	No
		C11E4.2	No
		F26E4.12	No
		T09A12.2	No
		R03G5.5	No
		Y94H6A.4	NA†
Thioredoxins		Y44E3A.3	No
	trx-1	B0228.5	No
	trx-2	B0024.9	No
		K02H11.6	No
	trx-3	M01H9.1	No
Methionine sulfoxide reductase	*msra-1*	F43E2.5	No
Glutathione reductase	*gsr-1*	C46F11.2	Yes

*All genes were tested by conducting RNAi with the indicated gene in *rrf-3*(*pk1426*), *rrf-3*(*pk1426*); *trxr-1*(*sv47*) and *selb-1*(*sv36*); *rrf-3* (*pk1246*) mutant backgrounds. *rrf-3* mutations increase the sensitivity to RNAi (1). For *trxr-2*, a possible interaction was also tested by generating a *trxr-2*(*tm2047*); *trxr-1*(*sv47*) double mutant strain.
†Not applicable. RNAi of these genes caused larval arrest of the *rrf-3*(*pk1426*) control strain.

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