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The glutathione system and the related thiol network in *Caenorhabditis elegans*

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ABSTRACT

Advances in the field of redox biology have contributed to the understanding of the complexity of the thiol-based system in mediating signal transduction. The redox environment is the overall spatiotemporal balance of oxidation-reduction systems within the integrated compartments of the cell, tissues and whole organisms. The ratio of the reduced to disulfide glutathione redox couple (GSH:GSSG) is a key indicator of the redox environment and its associated cellular health. The reaction mechanisms of glutathione-dependent and related thiol-based enzymes play a fundamental role in the function of GSH as a redox regulator. Glutathione homeostasis is maintained by the balance of GSH synthesis (*de novo* and salvage pathways) and its utilization through its detoxification, thiol signalling, and antioxidant defence functions via GSH-dependent enzymes and free radical scavenging. As such, GSH acts in concert with the entire redox network to maintain reducing conditions in the cell. *Caenorhabditis elegans* offers a simple model to facilitate further understanding at the multicellular level of the physiological functions of GSH and the GSH-dependent redox network. This review discusses the *C. elegans* studies that have investigated glutathione and related systems of the redox network including; orthologs to the protein-encoding genes of GSH synthesis; glutathione peroxidases; glutathione-*S*-transferases; and the glutaredoxin, thioredoxin and peroxiredoxin systems.

1. Introduction

Redox systems are regulated under non-equilibrium steady states that maintain the reducing conditions of the cell via electron transfer [1,2]. They provide central energy currencies to support metabolism and coordinate the overall organization of living organisms, including cell structure and function [3–5]. The cellular redox state is determined by the activity of relative ratios of cellular and extracellular redox couples. The two major redox systems, the pyridine nucleotide (NADH/NAD^+, NADPH/$NADP^+$) and thiol systems differ considerably in organization but both co-ordinate the cellular redox environment [6]. The thiol system includes the low molecular-weight thiol/disulfide couples such as glutathione (GSH/GSSG) and cysteine/cystine [7], and the oxidized/reduced states of redox enzymes that have cysteine residues at their active sites such as thioredoxins, glutaredoxins, and peroxiredoxins [8]. All these play major roles in the maintenance of the cellular redox environment. The electron carriers NADH and NADPH support metabolic requirements and maintain the redox status of the cell [9]. The NADH/NAD^+ redox couple assists in ATP synthesis, while the NADPH/$NADP^+$ system supports the maintenance of the thiol system which includes recycling of GSSG back to GSH [6]. Current evidence for the central role of GSH in thiol signalling suggests that the cellular redox environment is governed by the enzymatically-controlled reactions that facilitate the turnover of GSH rather than by the thermodynamic control of the GSH/GSSG couple [10]. This position is supported by *in vivo* studies, which used genetically encoded redox sensors to investigate the spatial patterning of cellular redox potential in *Caenorhabditis elegans* [11]. It was concluded that small changes in the GSH/GSSG ratio is amplified to large effects on redox potential through the oxidation/reduction of cysteine residues within proteins, rather than changes in the GSH/GSSG ratio directly affecting the cellular redox potential [11].

In this review, we focus on studies that have investigated glutathione synthesis and the related thiol systems that support the use of *C. elegans* as a suitable model organism for investigations of the thiol redox network.

Abbreviations: C. elegans, *Caenorhabditis elegans*; Grx, glutaredoxin; GSH, reduced glutathione; GSSG, disulfide glutathione; GST, glutathione-*S*-transferase; Prx, peroxiredoxin; Trx, thioredoxin; TrxR, thioredoxin reductase

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2. Glutathione

Glutathione (GSH) is the most abundant low molecular-weight thiol found in almost all eukaryotic organisms, most gram-negative bacteria and a few gram-positive bacteria [12,13]. GSH is often referred to as the major antioxidant of the cell, though the functions of GSH extend beyond its antioxidant properties to many other essential cellular processes, including detoxification of xenobiotic compounds, modulation of cell proliferation, transport and storage of cysteine and maintenance of redox status [14–17]. Cellular GSH is maintained by the *de novo* and salvage synthesis pathways [18]. The GSH:GSSG ratio is used as the primary indicator of cellular redox status due to the GSH pool being approximately three to four orders of magnitude higher in abundance than other major redox couples, such as the pyridine nucleotide and related thiol couples [7,19]. Mechanisms that involve GSH as a cofactor in enzymatic reactions may be more crucial to the regulatory role GSH has on the redox environment than the capacity of the GSH/GSSG couple to act as a redox buffer [20].

3. Thiol-based redox signalling

Thiol-based signalling occurs as part of normal cellular processes in response to elevated free radical and ROS production, which can affect many essential cellular processes including; phosphorylation pathways, gene transcription, cytoskeletal organization and ion channel activity [21–23]. Protein thiols undergo reversible and irreversible post-translational oxidative modifications such as, via the direct reaction with H_2O_2 that results in various oxidation states of cysteine residues including, sulfenic (−SOH), sulfinic ($-SO_2H$), or the irreversible sulfonic acid ($-SO_3H$) [24–26]. Protein *S*-glutathionylation is recognized as an efficient regulator of redox signal transduction largely due to the high cellular concentrations of GSH and the reversibility of the reaction catalyzed by the glutaredoxin enzyme family [27–29]. Many intracellular redox responses involved in signalling and protein function are regulated by *S*-glutathionylation, which forms disulfide bonds between GSH and the cysteine residues of proteins [30–32]. Protein *S*-glutathionylation can either activate or inhibit the activity and function of a range of structural proteins and enzymes [27,28]. Some enzymes can be *S*-glutathionylated at more than one cysteine residue, allowing for their activities to be modulated in a site-selective manner [28]. *S*-Glutathionylation can also prevent the irreversible oxidation of cysteine residues and thereby conserve the functions of the redox signalling pathways [33].

The reversible oxidation of protein thiols can act as 'redox switches' that control a range of functions of the protein, including regulation of protein activity, stabilization of protein structure, formation of protein activation complexes, and control of protein distribution [2,34]. The majority of cysteine residues support redox-signalling, largely by non-radical two-electron transfer [35]. A recent report has also discussed the possible role that free-radicals have in signal transmission of thiol-based signalling [21]. In the thiol network, hydrogen peroxide (H_2O_2) acts as a second messenger in transmitting the signal irrespective of which oxidant was responsible for its initiation [21]. The specific activities of the GSH-utilising enzymes and other related antioxidant systems which include, glutaredoxins, thioredoxins and peroxiredoxin systems are critical to GSH's influence on the thiol signalling system (Fig. 1) [8,10,36,37].

The abundance of cysteine residues in proteins increases with organism complexity, ranging from ~0.50% in archaea to ~2.25% in mammals [38], and is postulated to reflect the evolution of cysteine's signalling and control functions [39,40]. The percentage of cysteine in the *Caenorhabditis elegans* proteome is 1.97%, which is similar to the more complex eukaryotes [38]. Phylogenetic analysis of the protein-encoding genes of the major redox systems have shown that *C. elegans* thiol systems possess a considerable amount of similarity with human isoforms [41]. Accordingly, *C. elegans* has been used extensively to investigate processes related to redox biology, including the role of oxidative stress in aging and disease models (for reviews see Refs. [42–45]). This significant level of evolutionary conservation with higher eukaryotes suggests that *C. elegans* should be a suitable model to explore signalling mechanisms in the thiol proteome.

4. *Caenorhabditis elegans* as a model organism

C. elegans has become a prominent model for the study of various aspects of biology due to the ease of genetic manipulation, its completely traced embryonic [46] and post-embryonic cell lineages [47], its short larval lifecycle (~3 days), and median adult lifespan (~2–3 weeks) [48]. After embryonic development and hatching, the life cycle of the worm includes a series of four larval stages (L1-L4) before reaching adulthood. Another intermediary stage (dauer) begins to form late in the L1 stage in response to environmental stresses, such as overcrowding or high temperatures which results in the worm entering a state of arrested development [49].

The majority of *C. elegans* are hermaphrodites with only a small proportion, generally around 0.1%, of males produced each generation [50]. The small size of the nematode (~1 mm), along with the predominance of self-fertilizing hermaphrodites, makes it a valuable animal model for its ease in maintaining isogenic populations. Breeding males with hermaphrodites enables genetic crossing and is another key advantage of *C. elegans* as a genetic model. The nematode is optically transparent throughout all stages of its development and lifespan; from zygote to adult [51]. This has allowed the use of fluorescent reporters to investigate the localization patterns of gene expression and their protein products at tissue and subcellular levels *in vivo* [52]. RNA interference (RNAi) is another well-established method for the temporal silencing of genes to investigate gene function in *C. elegans* [53]. More recently, CRISPR/Cas9 gene editing has provided a tool for generating heritable modifications of the *C. elegans* genome [54]. The advent of this technique has allowed for targeted generation of small insertions or deletions (indels), or integration of larger DNA fragments into the genome including single copy exogenous genes, such as those that encode for fluorescent reporters [55]. Application of established and emerging genetic manipulation techniques are key to the success and the continued relevance of *C. elegans* as a valuable multicellular model organism.

5. Glutathione synthesis in *C. elegans*

5.1. De novo GSH synthesis pathway

The identification of the GSH cycle thiol intermediates in *C. elegans* indicated that it has a GSH metabolic network similar to that present in mammals [56]. In whole worm homogenates, the GSH concentrations have been reported to range between 10 and 40 nmol/mg protein [57–62], with the GSH:GSSG ratio of young adult wild-types being approximately 70:1 [57,63]. In mammals, the first reaction of the *de novo* GSH synthesis pathway is catalyzed by glutamate cysteine ligase (GCL), also known as γ-glutamylcysteine synthetase (GCS), which contains all the substrate and cofactor binding sites to produce γ-glutamylcysteine (γ-GC) [64]. The second reaction is catalyzed by glutathione synthetase to condense γ-GC with glycine to produce GSH [65].

In *C. elegans*, the *gcs-1* gene encodes for the ortholog of the mammalian GCLC (catalytic) subunit. Based on the predicted amino acid sequence homology, *C. elegans* GCS-1 has a 54% identity to the human GCLC ortholog [41]. The expression of *gcs-1* is regulated by the SKN-1 transcription factor in both constitutive and stress-induced conditions in various *C. elegans* tissues [66]. SKN-1 is a functionally conserved ortholog of Nrf2 that controls the regulation of approximately 300 genes under non-stressed conditions [67,68]. It is also involved in many stress response pathways, and is a central regulator of the nematode's

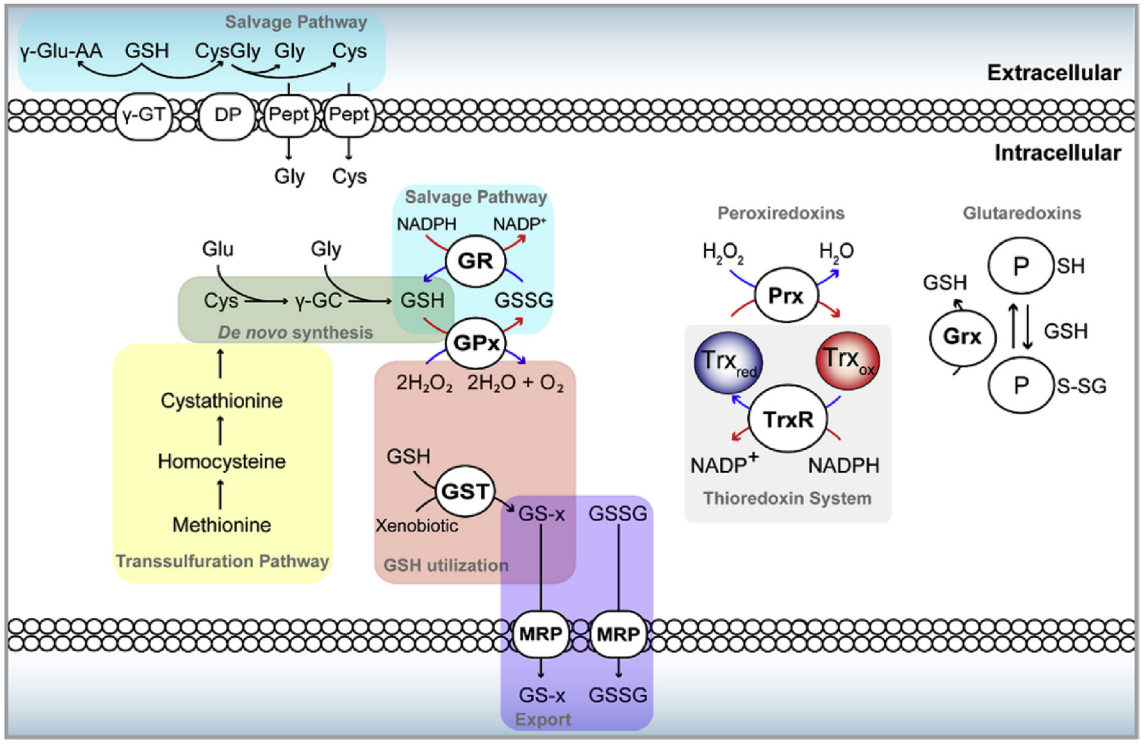

Fig. 1. The network of the cellular thiol redox system in multicellular eukaryotes. Glutathione (GSH) is synthesized in the cytosol via the *de novo* pathway (green box). The GSH salvage pathway (light blue boxes) occurs intracellularly via the reduction of GSSG by glutathione reductase (GR), and extracellularly through γ-glutamyl transferase (γ-GT) mediated degradation of exogenous GSH. The γ-GT activity generates cysteinylglycine (CysGly) and a gamma-glutamyl amino acid (γ-Glu-AA). CysGly is hydrolyzed by dipeptidases (DP) with the released cysteine (Cys) and glycine (Gly), which are taken up by peptide transporters (Pept) and become available for *de novo* GSH synthesis. The transsulfuration pathway provides an alternate source of cysteine via methionine. GSH is utilized by several major GSH-dependent enzymes; glutathione peroxidases (GPx) to reduce hydroperoxides; glutathione S-transferases (GST) to detoxify xenobiotic compounds by the formation of GSH conjugates (GS-x). These GSH-conjugates, and GSSG, can be exported from the cell via multidrug resistance proteins (MRP). Glutaredoxins (Grx) are primarily responsible for the de-glutathionylation of cysteine residues (PS-SG) present on proteins (P) and they can also reduce protein disulfide bonds, providing a backup for the thioredoxin system (not depicted in figure). The NADPH-dependent thioredoxin system (Trx/TrxR) functions by providing reducing equivalents for peroxiredoxins (Prx).

longevity and healthspan [67,69]. In response to oxidative stress, the p38 mitogen-activated protein kinase (p38 MAPK) pathway in *C. elegans* has been shown to upregulate *gcs-1* expression in the intestinal cells via PMK-1-mediated phosphorylation of SKN-1, which leads to the accumulation of SKN-1 into the nuclei [70]. In the absence of oxidative stress, SKN-1 is phosphorylated by the activity of glycogen synthase kinase-3 (GSK-3), which prevents SKN-1 from accumulating in the intestinal cell nuclei, resulting in lower targeted expression of *gcs-1* [71].

In the heterozygote mutant *gcs-1*(*ok436*) strain, GSH levels were shown to be ~70% compared to the wild-type [72]. Homozygosity for *gcs-1*(*ok436*) null allele is lethal at the L2 larval stage [11]. The dynamic temporal impact of the *gcs-1* gene has been shown in a study where RNA*i* targeting *gcs-1* was conditionally induced in worms either immediately after hatching or at the start of adulthood [58]. When the *gcs-1* gene was knocked down immediately after worm hatching, no difference was observed in median lifespan, though a higher incidence of vulval rupture and a significantly lower resistance against paraquat exposure was observed [58]. Conversely, knockdown of *gcs-1* at the beginning of adulthood, showed an increase in lifespan and paraquat stress resistance without the vulval rupture phenotype. This observation of increased lifespan and stress resistance may have been afforded by the compensatory increase (~2-fold) in expression levels of several genes of the thioredoxin system (*trx-1*, *trxr-1*, *trxr-2*) and glutathione *S*-transferase gene, *gst-4* [58]. Other studies have shown that, whilst *gcs*-1 mRNA levels do not significantly change up to day 12 adults compared to young adults, induction of *gcs-1* expression in response to paraquat exposure diminishes in older worms [73], suggesting that changes in *skn-1* induction in response to oxidative stress may decline during aging.

In mammals, heterodimer formation between the GCLC and GCLM (modifier) subunits results in the GCL holoenzyme complex which has increased catalytic efficiency over the GCLC subunit alone [74]. The *E01A2.1* gene in *C. elegans* encodes the ortholog of the mammalian GCLM regulatory subunit [68,75]. Under conditions of arsenite-induced stress, the *E01A2.1* gene is upregulated by the SKN-1 transcription factor [68,76]. An early investigation of the protein-protein interactions of *C. elegans* using yeast two-hybrid (Y2H) screens, showed that the E01A2.1 protein interacts directly with the GCS-1 protein [77]. A later RNA*i* screen of a library of 11,511 genes determined that the silencing of a total of 37 *C. elegans* genes, one of which included *E01A2.1*, induced *gcs-1* expression [78], suggesting that the down regulation of the modifier subunit increases the expression of the catalytic subunit (*gcs-1*) *in vivo*.

Others have shown that RNA*i* knockdown of the *E01A2.1* gene in wild-type worms had no effect on lifespan under 1.5 mM paraquat challenge, whereas it decreased the lifespan of the long-lived *daf-2*(*e1370*) mutant [79]. The authors reported that the *daf-2*(*e1370*) L4 larvae had GSH levels 14-fold higher than the wild-type [79]. Microarray analysis of the genes involved in GSH synthesis, including the salvage synthesis pathway, did not show any significant changes in the abundance of mRNA levels in the *daf-2*(*e1370*) worms that could explain the high GSH levels [79]. It was postulated that the discrepancy between the high GSH levels and no observable difference in the transcript levels of the genes involved in GSH synthesis may be due to regulatory mechanisms at the post-transcriptional level [79].

The enzyme involved in the second step in the *de novo* GSH

synthesis pathway, glutathione synthetase, is encoded by the *gss-1* gene in *C. elegans*, which shares 39% identity with the human isoform [41]. Initial work predicted several SKN-1 binding sites to be present in the *gss-1* gene promoter region [66]. Later studies have shown that the *gss-1* gene is upregulated by SKN-1 in response to oxidative stress following arsenite [68] and benzo-α-pyrene exposure [80]. RNA*i* knockdown of *gss*-1 has no effect on the lifespan of the wild-type or *daf-2* mutants under non-stressed conditions [79]. Knockdown of *gss*-1 has also been shown to increase the fluorescent intensity of the *gcs-1*::GFP reporter, indicating a regulatory control mechanism exists to increase gene expression for the first step of the GSH biosynthesis pathway when the second step becomes compromised [81].

5.2. GSH salvage synthesis pathways

The enzymatic recycling of GSH from GSSG by glutathione reductase maintains the cellular GSH:GSSG ratio [37]. In *C. elegans*, the *gsr-1* gene encodes for the glutathione reductase enzyme, which produces two protein isoforms (GSR-1a and GSR-1b) [82]. Similar to the genes of the *de novo* GSH synthesis pathway, expression of *gsr-1* is modulated by the SKN-1 transcription factor [57]. The GSR-1b isoform is located in the cytoplasm, whereas the GSR-1a isoform possesses an additional 14 amino acid *N*-terminal extension which is predicted to include a mitochondrial targeting sequence (MTS) [82]. Isoform-specific rescue of the embryonic lethal phenotype of *gsr-1(tm3574)* mutants, indicated that lethality is prevented by expressing the GSR-1b cytoplasmic form and not the GSR-1a mitochondrial form, demonstrating that GSR-1b is essential for embryonic development [82]. RNA*i* knockdown of the *gsr-1* gene in wild-type worms has been reported to decrease lifespan under non-stressed conditions [57]. The potential role of *gsr-1* in lifespan is supported by findings that demonstrate homozygous *gsr-1* mutants with maternally contributed GSR-1 are relatively short-lived compared to wild-types [82]. However, other researchers have observed no difference in lifespan when the *gsr-1* gene was knocked down [59]. The *gsr-1* gene is vital in the stress response against several oxidants including juglone, cumene hydroperoxide, diamide, *tert*-butyl hydroperoxide and paraquat [57,79,82,83].

Extracellular degradation of GSH by the membrane-bound γ-glutamyl transferase (γ-GT) involves the transfer of GSH's γ-glutamyl moiety to a free amino acid, releasing the dipeptide cysteinylglycine in the process. The cysteinylglycine is hydrolyzed by dipeptidases located on the outer surface of the cell membrane to generate cysteine and glycine which are then taken up by the cell [15] where they contribute to the substrate pool for the GSH *de novo* synthesis pathway (Fig. 1). The detection of cysteinylglycine in *C. elegans* initially suggested the presence of the γ-GT ectoenzyme [56], with subsequent work reporting at least six γ-GT genes [57]. In eukaryotes, GSH can be exported from the cell as glutathione-*S*-conjugates and disulfide forms mainly via multidrug resistance proteins (MRPs) [84,85]. Experimental approaches are yet to determine if the mechanisms of cellular efflux of glutathione-*S* conjugates and GSSG in *C. elegans* are analogous to mammalian systems.

The conservation of GSH synthesis between *C. elegans* and mammals, including the transcriptional control by the Nrf2 ortholog, SKN-1, make *C. elegans* a useful multicellular model organism in studies aimed at exploring the mechanisms of glutathione homeostasis in humans (Fig. 2).

5.3. In vivo monitoring of glutathione homeostasis in C. elegans

In eukaryotes, the redox environment differs throughout the various organelles and sub-cellular compartments with the intracellular glutathione redox couple distributed heterogeneously throughout the cytosol [86,87]. Recent advances have seen the physical transparency of the nematode utilized in the development of redox biosensors that allow the visualization of redox status, which facilitates the resolution of tissue-specific differences in real-time [88]. The genetically encoded Grx1-roGFP2 fluorescent biosensor was the first redox-sensitive ratiometric reporter to enable *in vivo* measurement of the GSH:GSSG ratio in *C. elegans* [89]. Using the genetically encoded Grx1-roGFP2 sensor, it was shown that the GSH:GSSG ratio shifts to more reducing conditions during larval development [89]. In a later study, an alternative encoded redox sensor, roGFP1-R12 was used to investigate the oxidation state of protein thiols in several tissue types including, the intestine, pharynx and the PLM mechanosensory neurons [11]. Using roGFP1-R12 transgenic worms, the authors concluded that the GSH/GSSG couple amplifies small changes in its oxidation state to large changes in redox potential by the oxidation of cysteine residues in proteins, rather than having a direct buffering role on the cellular redox potential [11]. The authors show that significant differences in the glutathione redox potentials exist between isogenic worm populations and suggest that this may be due to the variation in abundance of GSSG [11].

Due to the high intracellular concentrations of GSH (1–10 mM) in aerobic organisms, GSH biosynthesis pathways must function efficiently to maintain adequate levels of this important low molecular-weight thiol. Reports describing the role of GSH in maintaining the cellular redox environment often understate the activities of the GSH-dependent enzyme network and related thiol systems. The two major GSH-dependent enzyme families present in *C. elegans* are the glutathione peroxidases and glutathione *S*-transferases. These and other key oxidoreductase enzymes, including glutaredoxins, thioredoxins and peroxiredoxins are also discussed in the context of GSH's role in redox signalling in the *C. elegans* model.

6. Glutathione peroxidases in *C. elegans*

The glutathione peroxidase (GPx) family utilize GSH for the reduction of H_2O_2 and a variety of organic hydroperoxides to water or to the corresponding alcohol [90]. They are categorized into two subfamilies; the selenium subfamily containing a selenocysteine (SeCys) in the catalytic triad of amino acids (Trp-Glu-SeCys) and the non-selenium subfamily containing a cysteine (Trp-Glu-Cys) [91]. Sequence analysis of the eight *C. elegans* GPx genes (*gpx-1* to *gpx-8*) revealed that none contain a selenocysteine [92]. Selenocysteine-containing GPxs have been the subject of many mammalian studies, yet the physiological roles of the non-selenium GPxs remain largely unclear. Though the majority of *C. elegans* GPx isoforms contain the Trp-Glu-Cys catalytic triad, the predicted amino acid sequence of GPX-4 lacks the Trp residue, and GPX-8 lacks both the Trp and Cys [92]. It was concluded that the absence of cysteine at the catalytic site indicates that the GPX-8 protein is likely not acting as a glutathione peroxidase [92].

Glutathione peroxidase activity in worms has been shown to decline when exposed to high glucose concentrations [62], paraquat [93] and the neurotoxin 6-hydroxydopamine (6-ODHA) [94], yet increase in response to copper exposure ($CuSO_4$) [95]. In addition to their notable absence of selenium, the structures of the GPx genes differ considerably in the worm compared to human isoforms. Sequence identity analysis of the predicted amino acid sequences has indicated that four of the *C. elegans* GPx isoforms (GPX-1, GPX-2, GPX-6 and GPX-7) share between 43 and 47% identity to the human phospholipid hydroperoxide glutathione peroxidase (GPx4) [92]. A single strain generated with deletions for the four phospholipid hydroperoxide GPx isoforms had no difference in median lifespan, though their age-specific mortality rates and levels of lipid hydroperoxides were higher when compared to the wild-types [92]. Each of the four *C. elegans* phospholipid hydroperoxides GPx genes are reported to be expressed primarily in the intestine, with *gpx-2* also expressed in several neurons located in the head and tail [92]. The expression levels of *gpx-6* were also observed to increase in starved worms at several of the larval stages tested, suggesting it may have a role in dietary restriction [92]. In an RNA*i* screening study of 162 genes that are exclusively expressed in the intestine of the worm, *gpx-1* was the only RNA*i*-targeted gene shown to increase expression

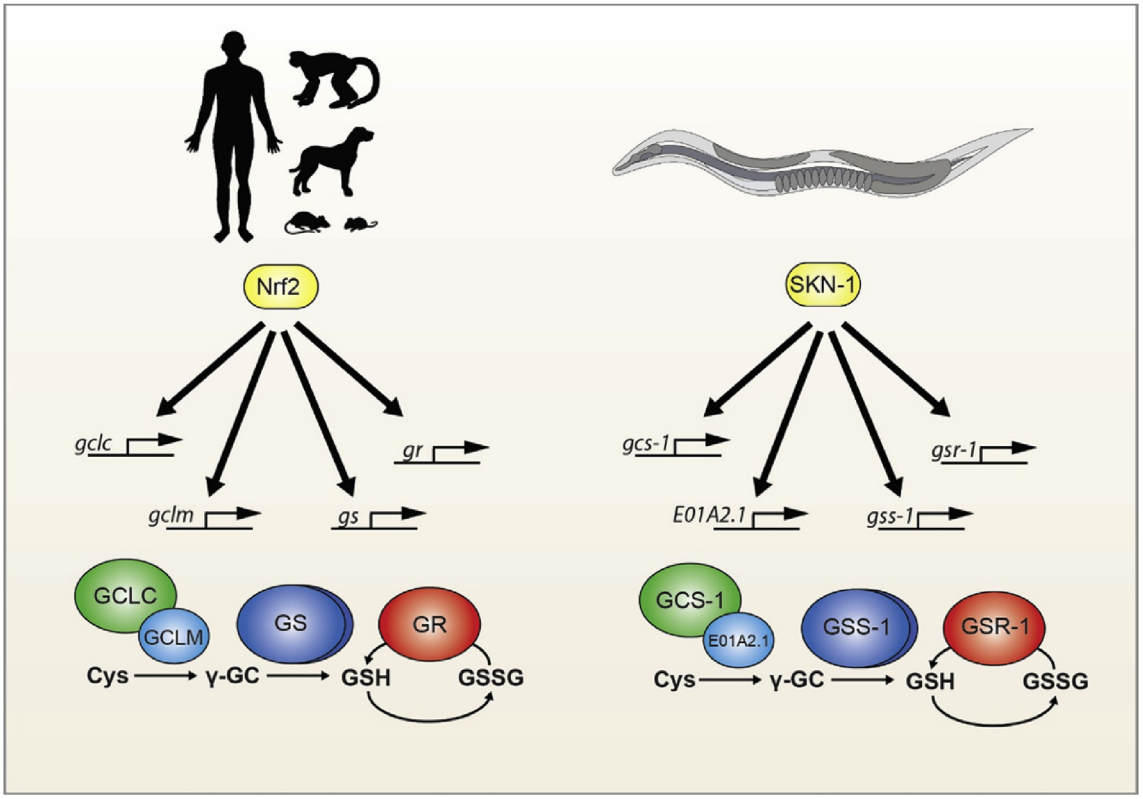

Fig. 2. Homology of the GSH synthesis pathways in mammals and *Caenorhabditis elegans.* In mammals, the *de novo* synthesis pathway is comprised of the glutamate cysteine ligase catalytic (GCLC) and modifier subunits (GCLM), and glutathione synthetase (GS); with glutathione reductase (GR) involved in recycling intracellular GSSG to GSH. In worms, the respective orthologs are designated as glutamate cysteine synthetase heavy (GCS-1) and light (E01A2.1) subunits, glutathione synthetase (GSS-1) and glutathione reductase (GSR-1). Genes of the *de novo* (*gcs-1*, *E01A2.1* and *gss-1*) and recycling pathway (*gsr-1*) are transcriptionally upregulated by the SKN-1 (Nrf2 ortholog) under constitutive and stress-induced conditions.

and activity of PEPT-1 [96]. PEPT-1 is a transporter, located on the apical membrane of the enterocyte, responsible for the uptake of di- and tripeptides [97]. This suggests that lower *gpx-1* transcript levels may stimulate uptake of dietary glutathione and its precursors.

The non-phospholipid hydroperoxide GPx genes (*gpx-3*, *gpx-4*, *gpx-5, gpx-8*) encoded in the *C. elegans* genome are reported to share putative homology with the human GPx3 and GPx5 isoforms [92]. Few studies have reported on the function of the non-phospholipid hydroperoxide GPx genes in *C. elegans*. However, *gpx*-5 has been shown to play a role in arsenite stress defence [57], and is potentially involved in synaptic remodelling [98], and circadian-stress tolerance in *C. elegans* [99]. The absence of selenocysteine at the active site of any of the *C. elegans* GPx isoforms suggests that the major mechanistic peroxidase activity of this enzyme family differs to that in mammals.

7. Glutathione-*S*-transferases in *C. elegans*

Glutathione *S*-transferases (GSTs) are a functionally diverse family of enzymes that utilize GSH in conjugation reactions [100]. GSTs represent a major class of enzymes that are involved in phase II detoxification in *C. elegans* [101]. The *C. elegans* genome contains 56 validated and putative GST genes [102] with most categorized within the sigma subfamily, though alpha, kappa, pi, zeta, and omega subfamilies are also present [103,104]. Changes in expression levels of specific GSTs in response to certain xenobiotic compounds suggest substrate-specific roles of GSTs in xenobiotic detoxification [80,105,106]. The correlation between GST activity and longevity has been described among evolutionary divergent species, including *C. elegans* and *Drosophila melanogaster* [107,108]. Several proteomic studies have indicated that *C. elegans* GST isoforms have a multitude of functions, and are involved in cell signal pathways via direct protein-protein interactions under H_2O_2-stressed and non-stressed conditions [109–112].

7.1. Sigma and pi GST subfamilies

The most rigorously investigated GST gene in *C. elegans* is *gst-4*, which shares sequence similarity with the human sigma subfamily. An early study demonstrated that *gst-4* expression increases in response to paraquat challenge [113]. Overexpression of *gst-4* was later shown to increase stress-resistance against juglone and paraquat exposure, without any effect on lifespan under normal growth conditions [114]. With the advent of *gst-4*::GFP reporter strains [115], many studies have investigated the effect that certain compounds have on the expression of *gst-4* (see Table 1). Most compounds elicit an increase in the intensity of the *gst-4*::GFP reporter in a stress-dependent manner. In contrast, 25 μM doses of folic acid treatment have been reported to increase the expression of *gst-4*, resulting in an increase in stress resistance [116]. Expression patterns of the *gst-4*::GFP reporters have been shown to localize in the intestine [117], body wall muscles, hypodermis, cells surrounding the pharynx, and the pharynx in L3 larvae only [114]. Among these tissues, the intestine is a prominent site of increased *gst-4*::GFP induction in response to exposure to chemical and oxidative stress [117–120].

Transcriptional activation of the *gst-4* gene is often used as an indicator of SKN-1 activity; with several inducers of *gst-4* including H_2O_2 and sodium azide being shown to be *skn-1* dependent [118]. SKN-1 also controls the induction of several other GST genes [66,68,69]. Induction of *gst-4* has also recently been shown to be upregulated independent of SKN-1, by the EOR-1 transcription factor that mediates the effects of the epidermal growth factor (EGF) pathway involved in regulating cell growth and differentiation [121]. The authors concluded that the discovery that *gst-4* transcriptional regulation is not exclusively controlled

Table 1
Compounds that affect *gst-4* (glutathione-*S*-transferase-4) expression in *C. elegans*.

Compound	*gst-4* expression	Refs
acrylamide	↑	[129,190]
aspirin	↑	[191]
fluoxetine[a]	suppressed	[192]
folic acid	↑	[116]
3β-hydroxy-urs-12-en-28-oic acid	↑	[106]
juglone	↑	[119,193,194]
lithium compounds (LiCl, Li_2CO_3)[b]	suppressed	[195]
microplastic particles	↑	[120]
mianserin[a]	↑	[192]
mirtazapine	↑	[192]
3-nitropropionic acid	↑	[196]
paraquat	↑	[113]
quinolinic acid	↑	[196]
tributyltin	↑	[117]

[a] Co-treatment with fluoxetine suppressed the mianserin-induced increase of the *gst*-4::GFP reporter, though had negligible effect on reporter activity when treated with fluoxetine alone.
[b] Expression levels measured using microarray analysis.

by the SKN-1 transcription factor should serve as a cautionary note for work that utilizes the *gst-4*::GFP reporter as an indicator of SKN-1 activity [121].

An RNA*i* screening study of 27 of the 44 then identified *C. elegans* GST genes showed that knockdown of five, which included one pi (*gst-10*) and four sigma isoforms (*gst-5*, *gst-6*, *gst-8*, and *gst-24*), sensitized the worms to exposure to the lipid peroxidation product, 4-hydroxyneoneal (4-HNE) [122]. Of the five, knockdown of *gst-5* and *gst-10*, resulted in a shortened lifespan [122]. The pi class gene, *gst-10*, is involved in catalyzing the conjugation of GSH to 4-HNE in *C. elegans*, suggesting a functional role of this pi class GST in dealing with cellular stress [122–124]. Another pi class GST homologue, *gst-1*, has also been shown to protect against dopaminergic neuron degeneration in *C. elegans* [125], which may have implications for understanding the progression of Parkinson's Disease. In a later RNA*i* screening study, over 40 GST genes were tested for survival against juglone and arsenite exposure [57]. For juglone, *gsto-1* was the only GST gene that showed a significant decrease in survival when knocked down. Though the mean survival against arsenite treatment for the majority of the silenced GST genes showed a modest decrease, the knockdown of only *gst-32* and *gst-44* significantly decreased survival [57].

7.2. Kappa GST subfamily

Unlike most vertebrates, which encode only one GST kappa gene, *C. elegans* encodes for two; *gstk-1* and *gstk-2* [104,126]. GSTK-1 is expressed in the intestine, rectal gland cells, body wall muscles and epidermis and contains a *C*-terminal peroxisomal-targeting sequence which directs its localization to the peroxisome [126]. The GSTK-2 is expressed in the pharynx, body wall muscles and the intestine and is localized in the mitochondria of these tissues [126]. RNA*i* knockdown of *gstk-1* and *gstk-2* were reported to have no effect on several phenotypes including, reproduction, development, motility and lifespan [126]. However, simultaneous double RNA*i* knockdown of both genes resulted in a significant decline in oxygen consumption and *cis*-vaccenic acid content which did not occur when the genes were silenced individually [126]. The authors concluded that both genes are involved in respiration and lipid metabolism [126].

7.3. Omega GST subfamily

Several isoforms of the GST omega subfamily are encoded in the *C. elegans* genome, including *gsto-1*, *gsto-2*, *gsto-3* and the putative GST omega genes *gst-44* and *C02D5.4* [102]. Overexpression and RNA*i* studies have shown *gsto-1* to be implicated with increased stress resistance and to be exclusively expressed in the intestine [127]. Intestinal expression of *gsto-1* was initially shown to be regulated by the ELT-2 transcription factor [127]. *Gsto-1* is upregulated under transient hypoxic conditions leading to an extension of lifespan mediated by the mechanistic Target of Rapamycin (mTOR) signalling pathway; a longevity pathway associated with dietary restriction [128].

The GST superfamily performs a diverse range of functions and have considerable variation in expression patterns in *C. elegans* [129]. While there remains much to be elucidated about the substrate-specificities, expression patterns and the relative levels of the many GST isoforms in *C. elegans*, research into the dynamic roles of this enzyme superfamily in detoxification and signalling will further our understanding on how the redox environment is mediated by enzymatically-controlled GSH-dependent redox processes.

8. Glutaredoxins in *C. elegans*

Glutaredoxins can serve as a backup for the thioredoxin system by catalyzing the reversible reduction of target protein disulfides [130]. These relatively small enzymes, which range between 9 and 15 kDa [131], are also regarded as the main catalysts of reversible protein deglutathionylation [28]. Depending on the number of cysteine residues in the active site of the enzyme, glutaredoxins are classified as either monothiol or dithiol forms [132]. For the *C. elegans* glutaredoxin genes, five have been annotated; *glrx-3*, *glrx-5*, *glrx-10*, *glrx-21*, *glrx-22* [57]. Analysis of the glutaredoxin sequences indicates that the GLRX-3 and GLRX-5 are monothiol forms, while the GLRX-10, GLRX-21 and GLRX-22 are dithiols [41]. Interestingly, while GLRX-5, GLRX-10, GLRX-21 and GLRX-22 are formal glutaredoxins ranging between 100 and 140 amino acids, GLRX-3 is a much larger isoform at 345 amino acids. Based on phylogenetic analysis, the *C. elegans* GLRX-3 isoform has been postulated to be an ortholog of the mammalian GLRX3 PICOT (protein kinase C-interacting cousin of thioredoxin) [41]. Except for *glrx-5* which is predicted to be a mitochondrial glutaredoxin [133], the other annotated glutaredoxins are expected to be located in the cytosol. This is particularly interesting as the National BioResource Project (NBRP) reports the *glrx-5*(*tm3867*) deletion allele to be the only lethal or sterile glutaredoxin mutant strain [134], while the cytoplasmic glutaredoxin mutants including, *glrx-10*(*tm4634*) [135], *glrx-21*(*tm2921*) [136], and *glrx-22*(*tm3743*) are all reported as viable.

Only a few published reports are available that investigate the role of the dithiol *glrx-10* and *glrx-21* genes in *C. elegans*. In a transgenic *C. elegans* model of Parkinson's Disease which expresses two pathogenic mutations in the human leucine-rich repeat kinase 2 (LRRK2) gene, loss of the *glrx-10* gene has been shown to lead to an increase in the degeneration of dopaminergic neurons [135,137]. Rescue of the *glrx-10* function mutants by re-expression of the active form of the wild-type *C. elegans glrx-10* gene partially afforded protection in dopaminergic neurons [135]. Two separate studies have shown that loss-of-function of *glrx-21* results in selenium-induced decline of motility [136] and reproduction [138]. Both studies suggested that GLRX-21 is required for the partial protection afforded by GSH during selenium-induced impairment [136,138].

To date, there remains only these limited reported studies of glutaredoxins in *C. elegans*, which provides opportunities for future work to characterize the function of these genes. A recent report has demonstrated that *C. elegans* exhibit changes in protein *S*-thiolation patterns (i.e. *S*-glutathionylation and *S*-cysteinylation) of targeted cysteine residues [139]. This evidence suggests that glutaredoxins may provide an evolutionary conserved mechanism for catalyzing the reversal of *S*-glutathionylation in *C. elegans*. Functional characterization of the *C. elegans* GLRX isoforms may offer new insights into the role of *S*-glutathionylation to determine how central this post-translational modification is in the context of cellular redox signalling *in vivo*.

9. Thioredoxin systems in *C. elegans*

The thioredoxin system is comprised of thioredoxins (Trx) and NADPH-dependent thioredoxin reductases (TrxR) [140]. Thioredoxins are small (~12 kDa) ubiquitous oxidoreductases that contain a highly specific thiol-disulfide active site to co-ordinate the regulation of the cellular redox environment, which is largely achieved by the supply of reducing equivalents for peroxiredoxins [141]. Oxidized thioredoxins (Trx_{ox}) are reduced (Trx_{red}) by thioredoxin reductases (TrxR), utilising NADPH as a cofactor [7]. The *C. elegans* thioredoxin system is composed of five thioredoxin genes (*trx-1* to *trx-5*) and two thioredoxin reductase genes (*trxr-1* and *trxr-2*) [142].

The *trx-1* gene is specifically expressed in the ASJ neurons [143]. Discovery of an ASJ motif, a functional *cis*-regulatory promoter region, has been reported to regulate the ASJ-specific gene expression of *trx*-1 by binding SPTF-1, an ortholog to the *Sp* family zinc-finger transcription factor [144]. Mutant worms that lack the *trx-1* gene are more vulnerable to paraquat-induced oxidative stress [145] and have decreased lifespans [143,145]. TRX-1 has been shown to regulate lifespan extension in genetic and nutrient-based models of dietary restriction possibly via its upregulation in ASJ neurons [146]. Interestingly, *trx-1* was later shown to regulate SKN-1 nuclear localization in a cell non-autonomous manner, with loss of *trx-1* from ASJ neurons promoting the nuclear localization of intestinal SKN-1 [147]. TRX-1 has also been implicated to potentially have a mechanistic role in dauer formation via the down-regulation of the insulin-like DAF-28 signalling neuropeptide in ASJ neurons [148]. TRX-1 has also been shown to have a role in *C. elegans* avoidance behavior by coordinating a dynamic *trans*-nitrosylation/de-nitrosylation response to nitric oxide produced by *Pseudomonas aeruginosa* [149]. Recent studies have reported that *trx-1* is involved in protection against methylmercury toxicity of dopaminergic neurons in males, but not hermaphrodites [150]. However, *trx-1*, in combination with *trxr-1*, can provide protection against methylmercury in aging hermaphrodites [151].

The *trx-2* and *trxr-2* genes comprise the mitochondrial thioredoxin system and are upregulated upon induction of the mitochondrial unfolded protein response (UPR^{mt}) [152,153]. Characterization of the intestine-specific TRX-3 have shown that the *trx-3*(*tm2820*) mutant has no significant difference in reproductive capacity, longevity, and resistance to stress (including heat-treatment, juglone and paraquat exposure) compared to the wild-type, though they exhibit a decrease in physical size and a shorter timing of their defecation cycle [154]. Overexpression of TRX-3 protected against pathogen infection, which suggested that TRX-3 may have a role in the worm's innate immune response [154]. The lesser studied *C. elegans* thioredoxin genes, *trx-3*, *trx-4* and *trx-5* have been shown to afford no significant protection in dopaminergic neurons against methylmercury toxicity [151]. However, there is evidence to suggest that *trx-5* may have a possible protective role against dopaminergic neuron loss in worms treated with the neurotoxin, 6-ODHA [142].

In addition to the five annotated thioredoxin genes (*trx-1* to *trx-5*), there are four other genes, which encode for proteins with highly conserved thioredoxin domains that contain the CGPC (Cys-Gly-Pro-Cys) active site [154]. These include the two annotated *dnj-27* and *png-1* genes and the uncharacterized *Y55F3AR.2* and *txl-1* genes [154]. DNJ-*27* is an ortholog of the mammalian endoplasmic reticulum (ER) localized ERdj5 protein, with both containing four thioredoxin-like domains [155]. The *dnj-27* gene is highly expressed in the pharynx and vulva, with lower levels of expression in body wall muscles, intestine, gonadal sheath cells, rectum and hypodermis [155]. The *dnj-27* gene has been shown to provide a protective role against pathological phenotypes in several *C. elegans* neurodegenerative disease models [155]. The *png-1* gene encodes for a bifunctional enzyme that possesses deglycosylation enzyme activity in addition to its oxidoreductase activity [156,157]. The *N*-terminal thioredoxin domain of the PNG-1 protein is understood to be unique to *C. elegans* [156]. Higher eukaryotic organisms possess a PUB domain at the *N*-terminus, which is implicated to mediate protein-protein interactions in the ubiquitin-proteasome pathway [158]. *In vivo C. elegans* studies have shown that mutations in the thioredoxin domain of the *png-1* gene results in an increase in axon branching defects [159]. Further investigations of the lesser studied thioredoxin genes should be of interest for future *C. elegans* studies.

Protein structure studies of the two *C. elegans* thioredoxin reductases have determined the TRXR-1 (~74 kDa) to be 667 amino acids and the TRXR-2 (~55 kDa) to be 503 amino acids [160]. The TRXR-1 protein is located in the cytosol and is highly expressed in the pharynx, hypodermis, rectal epithelial cells, intestine, nervous system [161] and vulva [162], whilst the mitochondrial thioredoxin reductase, TRXR-2, is expressed in the intestine and in several neurons located in the head [152]. Both the *trxr-1* and *gsr-1* (glutathione reductase) genes work in conjunction during *C. elegans* larval development by the reduction of disulfide bonds to carry out precise removal of the cuticle from the surface of epidermal cells [161].

Interestingly, *trxr-1* is the only selenoprotein that has been detected in *C. elegans* [161,163]. Having only one selenoprotein has made *C. elegans* a valuable *in vivo* model to investigate dose-dependent beneficial and toxicological properties of selenium-based compounds [63,164–166]. The CRISPR/Cas9 system has been used to generate targeted mutations of the *trxr-1* selenocysteine [167]. Two such *trxr-1* variants; one with a point mutation where the selenocysteine residue was replaced by a cysteine and the other with a premature stop codon, showed resistance during development when exposed to the chemotherapeutic agent, cisplatin [167]. The *trxr-1* gene affords partial protection from degeneration of dopaminergic neurons against the neurotoxin 6-hydroxydopamine (6-OHDA) [142]. Studies using the nematode could provide insights into the physiological roles of selenocysteine-containing proteins based on investigations focusing on the *trxr-1* selenoprotein.

10. Peroxiredoxins in *C. elegans*

Peroxiredoxins (Prx) are a class of thiol peroxidases that scavenge organic and inorganic peroxides. They are highly abundant proteins involved in the conversion of the majority of cellular H_2O_2 to water [168,169]. *C. elegans* contain three known peroxiredoxin genes, *prdx-2*, *prdx-3*, and *prdx-6* [44]. Both *prdx-2* and *prdx-3* encode for typical 2-Cys peroxiredoxins, with *prdx-6* encoding for a 1-Cys peroxiredoxin [170]. Expression patterns of *prdx-2* have been reported in the I4 (pharyngeal) and I2 (sensory) interneurons [170], intestine, epithelial cells, muscle (pharyngeal, vulval and body wall), and various neurons in the head and tail [171].

In *C. elegans*, PRDX-2 is highly abundant, constituting ~0.5% of the total protein and is involved in protecting protein thiol groups from H_2O_2-induced oxidative modifications [172]. In *prdx-2*(*gk169*) mutants, loss of peroxiredoxin activity leads to an increase in *de novo* GSH synthesis compared to wild-types, with an ~2-fold increase in *gcs*-1 mRNA and in GSH levels [173], possibly indicating a compensatory response by the *de novo* GSH synthesis network. Loss-of-function *prdx-2*(*gk169*) mutants have a shorter lifespan compared to wild-types at 15 °C [172,173] and 20 °C [173], with no difference observed at 25 °C [172], indicating a dynamic temperature-dependent response for the PRDX-2 enzyme. This is supported by observations that transient changes in housing temperature during development increase lifespan through a PRDX-2-dependent stress response [174]. Moreover, survival rates of *prdx-2*(*gk169*) mutants exposed to 10 h heat-shock treatments at 35 °C show a marked decline in lifespan when compared to wild-types [175].

Over-oxidation of PRDX-2 has been contested to have a negligible effect on worm physiology, with the suggestion that *C. elegans* perhaps relies on degradation and subsequent clearance of over-oxidized PRDX-2; with *de novo* synthesis of PRDX-2 potentially playing a role in the physiological response [176]. The cyclic oxidation state of the PRDX-2

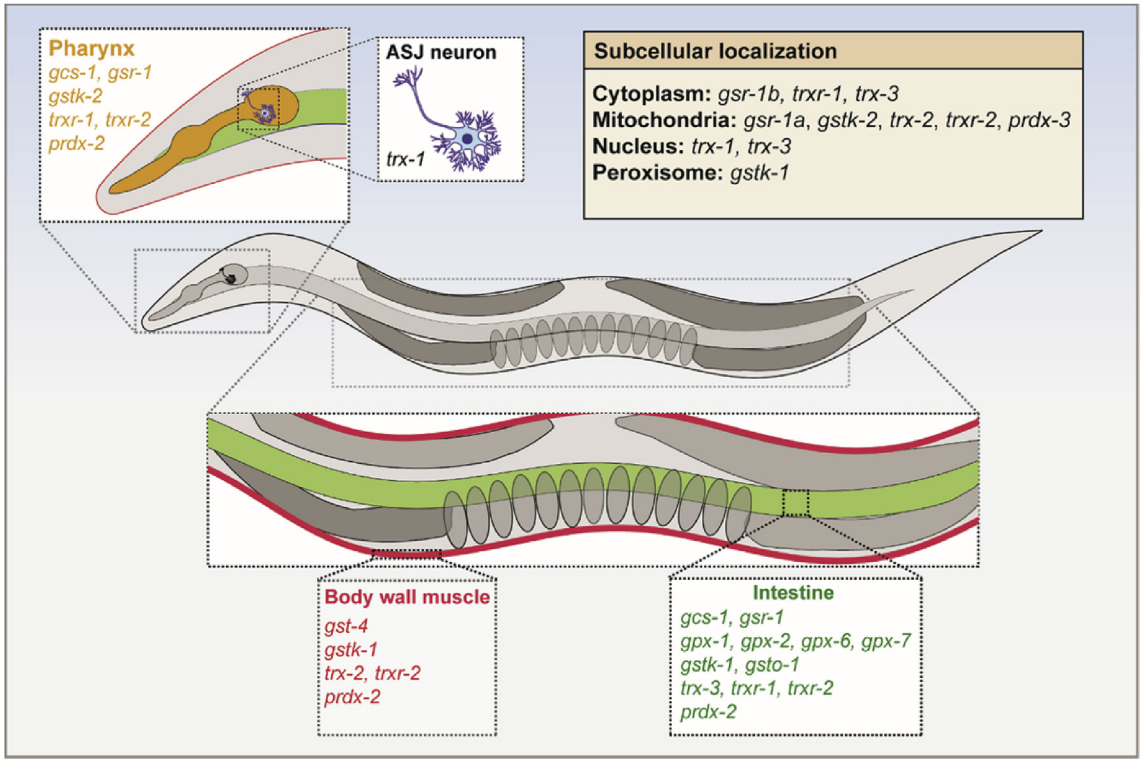

Fig. 3. Expression patterns and subcellular localization of select thiol-related systems in *Caenorhabditis elegans*. The tissue- and subcellular-specific differences in expression patterns of thiol-related genes in the worm. Generation of GFP reporter strains, allows the investigation of tissue, cell and organelle-specific gene expression patterns. Within the same tissue types and subcellular compartments (e.g. mitochondria), localized gene expression of the thiol-related system can control the redox environment of respective compartments. Note: the listed genes are not exhaustive of all known localization expression patterns of the thiol-related systems (see WormBase resource for comprehensive descriptions of all identified expression patterns).

protein possibly represents a circadian-rhythm mechanism in the worm [177] which is conserved in other eukaryotic models [178].

Several studies have shown that PRDX-2 is involved in hormetic responses. Exposure of worms to low doses of H_2O_2 (0.01–1 μM) resulted in an increased sensory response of the ASH neurons which required the PRDX-2-mediated p38/PMK-1 signalling cascade [179]. PRDX-2 is also involved in the hormetic response leading to lifespan extension, where worms treated with metformin, the antihyperglycemic drug used in the treatment of type II diabetes, led to an increase in endogenous levels of H_2O_2 [180]. Interestingly, both increased and loss of expression of PRDX-2 increased stress resistance against 5 mM arsenite exposure [173]. A recent study has reported that PRDX-2 is required for insulin secretion (in the form of the DAF-28 neuropeptide), leading to higher activity of the DAF-2 insulin signalling pathway and inhibition of the nuclear localization of SKN-1 and DAF-16 [181]. This later study provides an explanation for the paradoxical observation seen in the loss-of-function *prdx-2*(*gk169*) mutants that exhibit increases in arsenite stress resistance [181].

The lesser studied *prdx-3* gene is predicted to be localized in the mitochondria [182,183]. Though RNA*i*-targeted *prdx-3* knockdown worms had no difference in lifespan, they exhibited increased mitochondrial uncoupling, with lower ATP levels, motility, and reproduction, indicating that while *prdx-3* silencing does not affect lifespan under these conditions, it is important for healthspan [183]. Work on *C. elegans* peroxiredoxins have focused primarily on the typical 2-Cys peroxiredoxin, *prdx-2*. This is perhaps due to an early report that showed no significant difference in the longevity, development and progeny production phenotypes in the RNA*i* knockdown of the *prdx-3* (2-Cys) and the *prdx-6* (1-Cys) genes [170].

11. Conclusions

The anatomical simplicity of *C. elegans*, along with the ease of genetic modification and the ability to perform *in vivo* studies, has made it a successful research tool for investigating complex biological processes in multicellular organisms. In addition to the advantages of the nematode model itself, several open access resources including, WormBase [184], WormBook [185], and WormAtlas [186], provide comprehensive information on *C. elegans* genetics, biology and structural anatomy. Moreover, the two main strain repositories, the *Caenorhabditis* Genetics Center (CGC) and the National BioResource Project (NBRP), offer an extensive collection of mutant strains to service the *C. elegans* research community.

The advantage of studying redox systems in the simple nematode model is the capacity to investigate tissue and cell-specific differences in expression patterns *in vivo*. Most of what is understood about the gene expression patterns in *C. elegans* has been achieved through the generation of transcriptional and translational fluorescent reporter transgenes. Understanding the tissue and organelle-specific localization patterns of the thiol-related systems in *C. elegans* may provide insight into their roles in maintaining the redox environment in other multicellular organisms (Fig. 3).

It has recently been suggested that *C. elegans* could potentially be the subject of the first complete animal model of redox signal transduction [45]. Advances in proteomics techniques hold promise to further our understanding of how post-translational modifications influence signalling events of the thiol proteome [187]. Techniques such as OxiCat, which measures the reversible oxidation of cysteine residues have demonstrated the suitability of *C. elegans* for redox proteomic studies [172,188]. Others have shown that proteomic approaches that measure cysteine reactivity can be used as a predictor of protein functionality in *C. elegans* [189]. In terms of the current knowledge of

the reaction mechanisms of glutathione-dependent enzymes (for review see Ref. [37]), future efforts should employ *C. elegans* to gain further understanding of the *in vivo* functional role of GSH in the context of thiol-mediated redox signalling.

Declaration of interest

GDF and WJB declares no conflict of interest.

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