

Review Article

The Redox System in *C. elegans*, a Phylogenetic Approach

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Oxidative stress is a toxic state caused by an imbalance between the production and elimination of reactive oxygen species (ROS). ROS cause oxidative damage to cellular components such as proteins, lipids, and nucleic acids. While the role of ROS in cellular damage is frequently all that is noted, ROS are also important in redox signalling. The “Redox Hypothesis” has been proposed to emphasize a dual role of ROS. This hypothesis suggests that the primary effect of changes to the redox state is modified cellular signalling rather than simply oxidative damage. In extreme cases, alteration of redox signalling can contribute to the toxicity of ROS, as well as to ageing and age-related diseases. The nematode species *Caenorhabditis elegans* provides an excellent model for the study of oxidative stress and redox signalling in animals. We use protein sequences from central redox systems in *Homo sapiens*, *Drosophila melanogaster*, and *Saccharomyces cerevisiae* to query Genbank for homologous proteins in *C. elegans*. We then use maximum likelihood phylogenetic analysis to compare protein families between *C. elegans* and the other organisms to facilitate future research into the genetics of redox biology.

1. Introduction

Molecular oxygen is necessary for the survival of most complex multicellular organisms. The necessity of oxygen comes from its role in aerobic respiration, a process of extracting energy from food that is approximately 19 times more efficient than its anaerobic counterpart. In eukaryotes, aerobic respiration is carried out in the mitochondria (descendant of an aerobically respiring bacterium) by a series of electron transfer reactions that are coupled to the generation of a proton gradient. This proton gradient is used to generate the cellular fuel adenosine triphosphate (ATP). The residual energy of the spent electrons is consumed in the reduction of molecular oxygen (O_2) to water (H_2O). Aerobic respiration cannot occur without this last step, but the reliance on oxygen as the final electron acceptor poses a continual threat of oxidative damage to aerobically respiring organisms.

The threat posed by oxygen comes largely from its conversion to the free radical superoxide ($O_2^{\bullet-}$) rather than water [1]. Superoxide is a highly reactive short-lived ROS. Detoxification of superoxide and other ROS is performed

by antioxidants, which convert ROS to less reactive molecules. The antioxidant enzyme superoxide dismutase (SOD) converts superoxide to water and hydrogen peroxide (H_2O_2), which is another ROS and a potent oxidising agent (see Figure 1) [2]. Under normal conditions, antioxidants help to prevent oxidative damage by using electrons to reduce ROS, thus inhibiting ROS from oxidising other molecules. However, an imbalance between ROS production and detoxification can result in oxidative stress. Numerous studies have found that high ROS levels are damaging to DNA, RNA, proteins, and lipids [3–6]. Additionally, oxidative (ROS) damage is thought to be one of the major causes of ageing, according to the free-radical theory of ageing [7]. However, the free-radical theory seems to conflict with recent findings regarding the role of ROS in redox signalling, findings that have unveiled an additional mechanism for oxidative toxicity besides simply macromolecular damage.

ROS are now known to do more than indiscriminately damage macromolecules; they function as important signalling molecules (reviewed by D’Autréaux and Toledano [8]). For instance, superoxide and H_2O_2 are part of a second messenger system involved in controlling subcellular redox

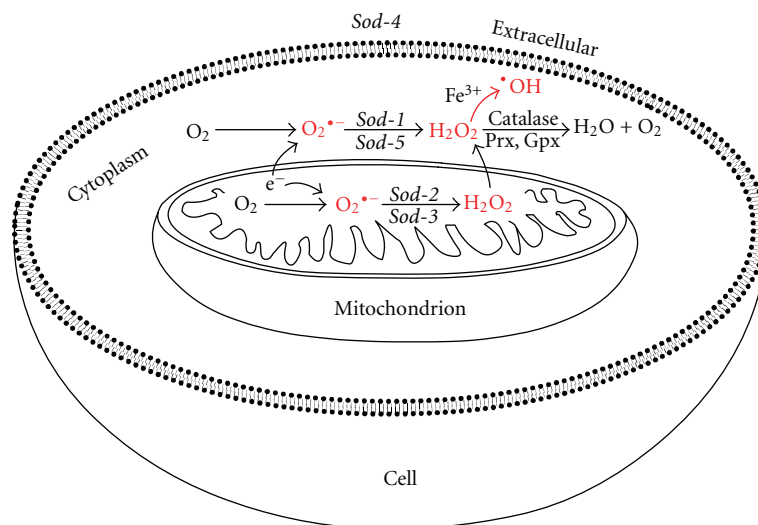


FIGURE 1: Simplified pathways of ROS production and antioxidant detoxification in a *C. elegans* cell. ROS molecules superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\bullet OH$) are depicted in red. Antioxidants convert ROS to less reactive molecules. The five different superoxide dismutase enzymes convert superoxide to H_2O_2 and are depicted in their respective cellular compartments. Catalase, peroxidorexin (Prx), and glutathione peroxidase (Gpx) convert H_2O_2 to H_2O and O_2 . Rapid detoxification of H_2O_2 is necessary as it can become oxidised to form the potent free-radical $\bullet OH$. ROS can be damaging to DNA, RNA, proteins, and lipids, and high ROS levels can cause oxidative stress. Some antioxidant genes, such as catalase and *sod-3*, can be upregulated in response to oxidative stress.

states; modulating protein activation and turnover; regulating gene expression; and mediating extracellular signalling [9]. For this crucial messaging system to function, the levels of superoxide/ H_2O_2 must be maintained at concentrations far below the level of toxicity. Therefore, ROS are unlikely to cause macromolecular damage under normal *in vivo* conditions. Consistent with this idea, recent studies have found that mitochondria produce superoxide/ H_2O_2 at levels much lower than those previously estimated [10–12].

Jones [13] recently proposed the “redox hypothesis” as an alternative to the free-radical theory of ageing that accommodates recent discoveries in redox signalling. This hypothesis states that changes to redox state, rather than oxidative damage, cause aging and age-related diseases. The redox state of a cell, cellular compartment, or molecular system is a measure of the availability of chemically reactive electrons. In the reducing state, such electrons are more abundant whereas in the oxidising state they are less abundant. With regards to oxidative stress, the redox hypothesis suggests that an increase in ROS levels can be deleterious if the resulting oxidative shift in redox state causes a disruption to redox signalling.

To date much of what has been discovered in redox biology has resulted from work in *E. coli*, *S. cerevisiae*, mammals, and plants. While unicellular organisms such as *E. coli* and *S. cerevisiae* offer obvious advantages as model organisms due to their relative simplicity, short generation times, ease of culturing and maintenance, and so forth, they cannot be used to study systems/mechanisms unique to multicellularity in general, and animals in particular. Although not to the same extent, *C. elegans* offers similar advantages to working with the simple unicellular systems above, with the addition of being a multicellular, metazoan

system allowing for much more of what is discovered in this organism to be extrapolated to research in other animals, including humans. This article seeks to scope out the redox proteins/systems of *C. elegans* as a resource for future work on redox signalling and oxidative stress within this model organism. In addition, this article will briefly explain the function of each protein family and how it relates to redox signalling and oxidative stress, with specific mention of what has been discovered in *C. elegans*.

2. Redox State, Redox Signalling, and Oxidative Stress

2.1. Redox-Sensitive Cysteine Switches. The majority of available reactive electrons in a biological redox system are found in cysteines (as in the abundant tripeptide glutathione). Cysteine is an amino acid with a thiol (sulfur) group that is easily oxidized. When oxidised, two thiols in close proximity to one another can bond to form a disulfide. Formation of disulfides is important in protein folding and maintaining protein structure, however, a small fraction of thiols have another function: redox-sensitive switches (see Figure 2) [14–18]. These redox-sensitive thiol switches are generally found at the surface or in the active sites of proteins. Change from a thiol state to a disulfide can alter a protein’s shape and function. The propensity for a redox-sensitive switch to be in one state or the other (thiol or disulfide) is dependent on the redox state of the cellular compartment and/or redox system to which it belongs. Therefore, the activity and conformation of a large number of proteins can be altered by changes in redox state [13].

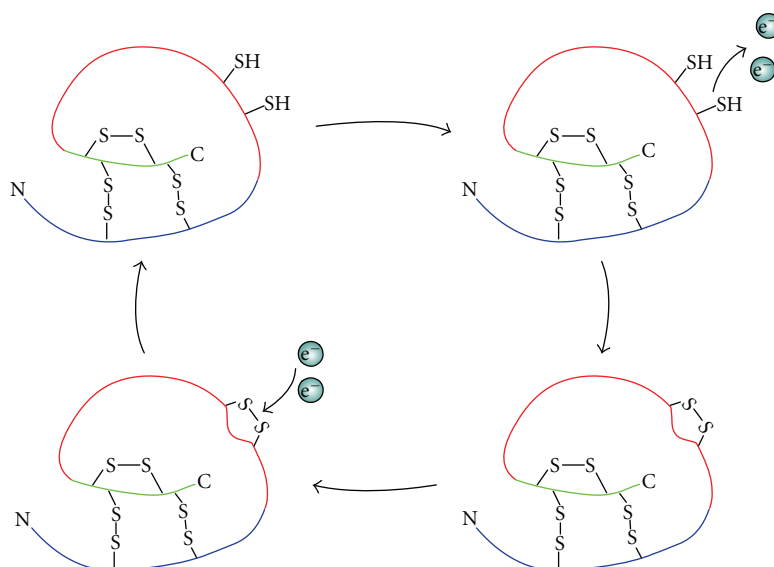


FIGURE 2: Cartoon of protein with redox-sensitive thiol switch. Structural disulfides can be seen on the inside of this protein, protected from redox reactions (those attached to the green and blue segments). A redox-sensitive thiol/disulfide switch is depicted on the protein surface (attached to the red segment). When reduced the redox-sensitive switch is in its dithiol state, however, when these thiols become oxidised (i.e., electrons are removed by an oxidant such as ROS) they bond to form a disulfide, altering the structure and/or function of the protein.

2.2. The Redox State as a Signal. Redox signalling relies on oxidants and reductants that react preferentially with redox sensitive cysteines. Methionine also contains a redox active sulfur and is used in redox signalling [19], but this occurs to a lesser extent and is not discussed in this article. The most important oxidants that participate in signalling-related modification of cysteine residues are hydrogen peroxide (H_2O_2), a reactive oxygen species, and nitric oxide (NO), a reactive nitrogen species [20]. The focus of this article will be H_2O_2 as the signalling role of NO has been reviewed extensively [21]. Although a potent oxidant, the signalling role of H_2O_2 is primarily limited to redox-sensitive cysteine and methionine residues [22–24]. The fact that much of the cellular H_2O_2 is formed via the dismutation of superoxide ($O_2^{\bullet-}$) by SOD enzymes means that the amount of superoxide produced directly contributes to the levels of H_2O_2 in a cell or cellular compartment [9]. This gives superoxide an important indirect role in redox signalling. In animals, superoxide is primarily generated by NADPH oxidase (NOX) [25], Coenzyme Q_{10} [9, 26], and Complex I and III [12] of the mitochondrial electron transport chain.

The difference in redox states between organelles results from differences in the ratio between H_2O_2 and other thiol oxidants and various disulfide reductants/antioxidants. Because the redox state can alter protein conformation and reactivity, it can be used to activate or inactivate protein function. For example, the redox state of two different cellular compartments regulates DNA binding of the nuclear factor erythroid 2-related factor 2 (Nrf-2) transcription factor [27]. Nrf-2 is activated in the cytoplasm by an oxidative signal that results in translocation to the nucleus. However, in order to bind to DNA in the nucleus, a redox-sensitive cysteine must

be reduced. This demonstrates specificity in redox signalling between different cellular compartments, for which the redox state of the compartment must be appropriate to its role (see Figure 3) [28].

2.3. Major Redox Systems: Glutathione, Thioredoxin, and Cysteine. Two central thiol/disulfide couples work in the reduction of protein disulfides as counterparts to H_2O_2 and other oxidising agents to control redox state: the glutathione/glutathione disulfide (GSH/GSSG) couple (mediated through glutaredoxins) and the active site dithiol/disulfide of thioredoxins (Trx_{red}/Trx_{ox} ; see Figure 4) [29]. Glutathione and thioredoxins each interact with a different subset of proteins thus forming distinct redox systems. The redox state of one of these systems may differ from the other even in the same cellular compartment [27, 30–34]. As well as glutathione and thioredoxin working as reducers of protein disulfides, a third thiol/disulfide couple, cysteine/cystine, has also been proposed by Jones et al. [32] as a possible oxidiser of protein dithiols used in redox regulation and signalling. Changes to the ratios of these three redox couples have been observed in various disease states, and it is possible that a gradual loss of redox state homeostasis over time contributes to ageing and age-related diseases [13].

2.4. Redox State versus Transient Local Redox Signalling. The redox state of the various redox systems in a cell or cellular compartment must normally reside within a narrow range, not only to maintain the constitutive signals resulting from the homeostatic redox state itself, but also to allow for meaningful thresholds, where a change in redox state outside

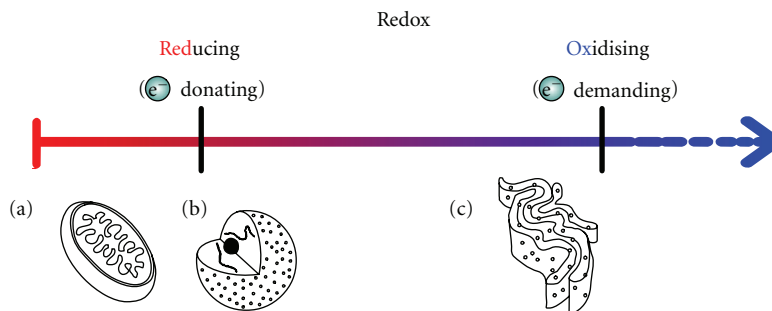


FIGURE 3: Redox state of between different cellular compartments. Redox state is a measure of the amount of electrons available for redox reactions. In the reducing state electrons are donated, whereas in the oxidising state electrons are removed. The mitochondria and nucleus are reducing (a and b). The endoplasmic reticulum is more oxidising (c).

the typical range of a cellular compartment can be used to signal a change in metabolism, environment, or stress. In addition to global signals at the level of an entire organelle, generation of H_2O_2 with no measurable effect on overall redox state of the various redox systems in a cell or cellular compartment may still have a very real effect on proteins in close proximity to the site of generation, resulting in a transient local signal—an idea explored more fully by Dwivedi and Kemp [35]. An example of modulation of signalling by transient local changes rather than a global shift in redox state is altered protein phosphorylation [36] resulting, for example, from the inactivation of protein tyrosine phosphatases [37, 38], MAP kinase phosphatases [39], and PTEN [40]. Within the redox hypothesis paradigm, much of the toxicity of oxidative stress could result from an oxidative shift in redox state within one or more cellular compartments. This shift would likely disrupt transient redox signalling as well as perturb the regular function of redox regulated proteins within these compartments. The end result could still be pathological oxidative damage to cellular components even though the cause could be indirect.

3. Phylogenetic Analysis

3.1. Thiol/Disulfide Redox Regulators

3.1.1. Thioredoxin and Related Proteins. Thioredoxin (TRX) was first discovered in *Escherichia coli* as a hydrogen donor for ribonucleotide reductase [41, 42]. Since the initial characterisation, TRX proteins have been recognized as more general disulfide reductases that are found in all phylogenetic domains of life. TRX proteins have a distinct structure that encompasses the active site dithiol known as the “thioredoxin fold.” This domain is also found in a variety of related proteins including glutaredoxin, protein disulfide isomerase, peroxiredoxin, and glutathione S-transferase [43]. Each of these protein families is discussed in other sections of this article. The characteristic CGPC active site dithiol motif can be oxidised to a disulfide to release electrons that are used to reduce redox-sensitive disulfides within a wide range of target proteins [44].

TRX proteins were long thought to be primarily involved in restoration of redox-sensitive disulfides to their reduced state after being oxidised by ROS. In particular, ROS scavengers such as peroxiredoxin require the activity of TRX proteins for their regeneration. However, the role of TRX as a disulfide reductase is now known to be important for immune signalling [45], regulating transcription factors [46], and modulating cellular signalling [47].

Sequence comparisons and phylogenetic analysis revealed that *C. elegans* possesses twenty proteins closely related in sequence and length to TRX proteins in yeast, humans and fruit flies. Seven of these twenty proteins contain the characteristic CGPC active site sequence required for TRX activity. Five of these CGPC containing proteins, TRX-1, TRX-2, TRX-4, Y45E10.A, and Y55F3AR.2, are closely related to proteins of known TRX activity in *S. cerevisiae*, *H. sapiens*, and *D. melanogaster*. Human cytosolic thioredoxin 1 (TRXN1) has roles in the activation of transcription factors activator protein-1 (AP-1) [48] and nuclear factor kappa B (NF κ B) [49, 50], as well as in immune signalling [45, 51]. Although orthology is unclear between human TRXN1 and *C. elegans* TRX proteins (Figure 5), both human TRXN1 and *C. elegans* TRX-1 are cytoplasmic [52]. TRX-1 is expressed in intestinal cells as well as the ASJ pair of neurons and modulates adult lifespan extension induced by dietary restriction [53]. Human mitochondrial TRXN2, for which there is likely orthology with *C. elegans* TRX-2, is part of a mitochondria-dependent superoxide/TRXN2/apoptosis signal-regulating kinase 1 (ASK-1) apoptosis signalling pathway [54]. In *C. elegans* interactions of TRX-1 and TRX-2 with exonuclease 3 (EXO-3) and *C. elegans* p53-like protein (CEP-1), appear to play a role in neural structure and function as well as ageing [55]. Some of the functions of TRX are redox independent. For example, TRX-1 modulates the activity of the insulin-like neuropeptide DAF-28 in ASJ sensory neurons in the head during *C. elegans* dauer formation. This function was retained even after the redox activity of the protein was disrupted by replacing the two Cys residues of its active site with two Ser residues [56]. In regard to thioredoxin-like (TRXL) proteins, the close relationship found between Y45E10.A and Y55F3AR.2 and the thioredoxin-like proteins of humans (TXNL1)

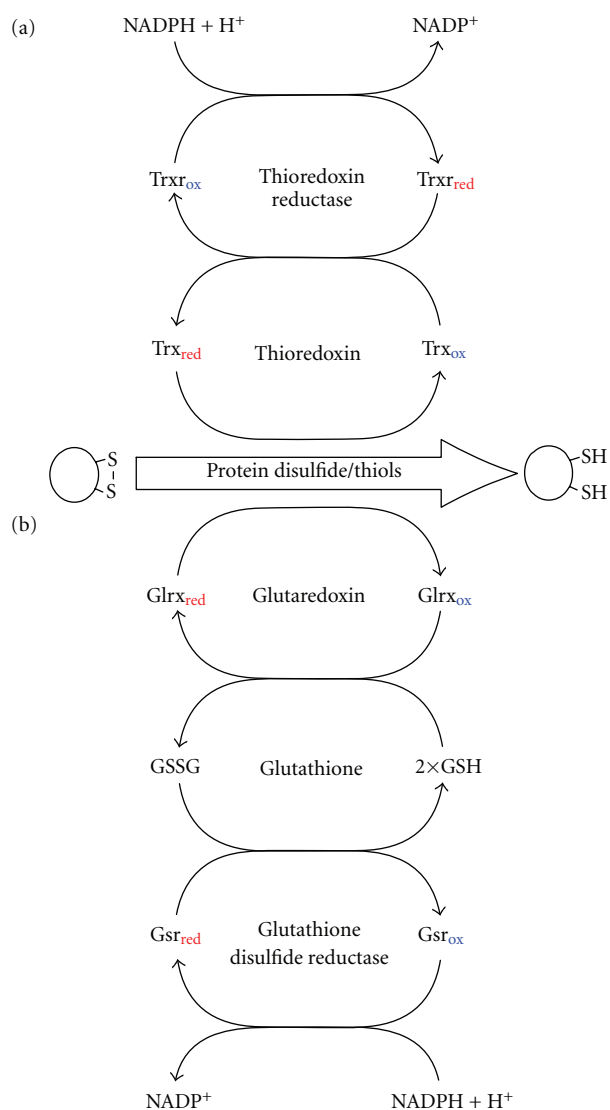


FIGURE 4: Glutathione-glutaredoxin and thioredoxin redox regulation systems. (a) Thioredoxin receives electrons from thioredoxin reductase and uses these to directly reduce protein disulfides. (b) Glutathione receives electrons from glutathione disulfide reductase; however, reduction of protein disulfides by glutathione is mediated through glutaredoxin. These two systems have similar functions, although there is evidence that indicates they each interact with different subsets of proteins. Arrows represent a change in redox state (loss or gain of electrons).

and *D. melanogaster* (TXL) suggests possible orthology. Functions of the TRXL proteins are yet to be determined.

The other two *C. elegans* homologs containing the CGPC sequence, TRX-3 and TRX-5, were found as part a clade containing nucleoredoxin (NXN) and related proteins. Humans possess a single NXN, which contains a CPPC active site, and two nucleoredoxin-like proteins (NXNL1 and NXNL2). NXN (reviewed in [57]) has been shown to function as a redox regulator of gene expression [58] and a negative regulator of toll-like receptor signalling [59]. It also sustains Wnt/ β -Catenin signalling [60]. Six out of the nine

C. elegans proteins within the NXN clade contain the CPPC NXN active site sequence, suggesting a possible expansion of the NXN subfamily. Proteins within this NXN clade are also closely related to the 16-Kilodalton class of thioredoxins described in the parasitic nematode *Brugia malayi* [61].

3.1.2. *Glutathione*. Reduced glutathione (GSH) is a tripeptide consisting of glycine, cysteine and glutamic acid. GSH synthesis is performed in a two-step ATP-dependent process. In the rate-limiting first step *gamma*-glutamylcysteine synthetase (GCS; see Table 1) synthesises *gamma*-glutamylcysteine from L-glutamate and cysteine. In the second-step glutathione synthetase (GSS; see Table 2) adds glycine to the C-terminal of *gamma*-glutamylcysteine. These enzymes are highly conserved in eukaryotes (Tables 1 and 2) and even in prokaryotes (not shown).

Glutathione plays an essential role in antioxidant defence as a source of electrons for antioxidant enzymes such as glutaredoxins and peroxidases [63]. The high (millimolar) concentrations of glutathione in the cell ensure an abundance of electrons for these antioxidant systems and thus provide a robust buffer against oxidative shifts in redox state [64]. GSH also serves as a reversible cysteine adduct. Glutathione S-transferases (GSTs) can form mixed disulfides between glutathione and redox-sensitive cysteine thiols of proteins. This activity can be used to regulate protein activity and under oxidizing conditions can prevent irreversible oxidation of thiols to sulfinic (SO₂H) and sulfonic acid (SO₃H) oxoforms [65, 66]. GST can also conjugate glutathione to xenobiotic compounds as part of a detoxification response [67] and to a fatty acid in the synthesis of prostaglandin hormone [68, 69].

The ratio of reduced glutathione to glutathione disulfide within a cellular compartment, that is, [GSH]²/[GSSG], determines its redox state. High [GSH]²/[GSSG] ratios such as those found in the mitochondria, cytoplasm, and nucleus ensure that the majority of redox-sensitive protein switches within these compartments are in the reduced (-SH) state [77]. Maintenance of the proper [GSH]²/[GSSG] ratio ensures redox homeostasis, whereas changes to this ratio provide a simple means to adjust the redox state between compartments as well as within compartments under different physiological conditions. For example, changes in redox state have been found to trigger responses associated with defence against particular biotic or abiotic stressors [78]. In plants, changes to the cellular glutathione pool have been shown to elicit pathogen resistance responses [79, 80]. These examples demonstrate that global changes to protein activity and widespread changes to signalling can be achieved quite readily by simply changing the redox set point within a cellular compartment.

3.1.3. *Glutathione Disulfide Reductase and Thioredoxin Reductase*. When oxidised, the reduced (thiol) states of glutathione and TRX enzymes are restored by glutathione disulfide reductase (GSR) and TRX reductase (TRXR), respectively, using electrons obtained from NADPH (Figure 4)

TABLE 1: Similarity between *C.e gamma*-glutamylcysteine synthetase and orthologues in *H.s.*, *S.c.*, and *D.m.*

Species name	Protein name	Function	% identity	E value
<i>H.s.</i>	GCLC	<i>gamma</i> -glutamylcysteine synthetase	350/643 (54%)	0.0
<i>D.m.</i>	GCLC	<i>gamma</i> -glutamylcysteine synthetase	281/498 (56%)	0.0
<i>S.c.</i>	GSH1	<i>gamma</i> -glutamylcysteine synthetase	254/672 (38%)	2e – 138

C.e.: *C. elegans*, *H.s.*: *H. sapiens*, *S.c.*: *S. cerevisiae*, *D.m.*: *D. melanogaster*.

TABLE 2: Similarity between *C.e* glutathione synthetase and orthologues in *H.s.*, *S.c.*, and *D.m.*

Species name	Protein name	Function	% identity	E value
<i>H.s.</i>	GSS	glutathione synthetase	185/470 (39%)	3e – 95
<i>D.m.</i>	CG32495	glutathione synthetase	180/491 (37%)	8e – 102
<i>S.c.</i>	GSH2	glutathione synthetase	147/494 (30%)	1e – 56

C.e.: *C. elegans*, *H.s.*: *H. sapiens*, *S.c.*: *S. cerevisiae*, *D.m.*: *D. melanogaster*.

[81]. The maximum like-likelihood tree of this family forms three clades (Figure 6). GSR is absent from *D. melanogaster* and has been substituted by a novel glutaredoxin-thioredoxin reductase fusion protein (TRXR-1) [82]. *S. cerevisiae* possess proteins with TRXR enzymatic activity, however, these proteins are evolutionarily unrelated to the animal forms and are thus not represented on the phylogenetic tree [83]. Interestingly, TRXR-1 is the only selenocysteine containing protein in *C. elegans*, and both TRXR-1 and GSR-1 are essential for proper moulting [84].

The third clade of this protein family is composed of dihydrolipoamide dehydrogenase (DLD) orthologs. DLD is similar in structure to both TRXR and GSR, however, its functions are quite different. DLD is a component of various protein complexes located within the mitochondrial matrix, including pyruvate dehydrogenase, alpha-ketoglutarate dehydrogenase, and the branched chain amino acid-dehydrogenase complexes as well as the glycine cleavage system. In these complexes DLD is required for regeneration of oxidised lipoamide from the reduced dihydrolipoamide cofactor. The pyruvate dehydrogenase and branched chain amino acid-dehydrogenase complexes (2-Oxo acid dehydrogenase complexes) are thought to play a role in redox regulation via the reduction of thioredoxins [85]. In addition, the alpha-ketoglutarate dehydrogenase complex has been found to be a generator of H₂O₂ [86, 87].

3.1.4. Glutaredoxin. Glutaredoxin (GLRX) uses electrons extracted from GSH to reduce redox-sensitive disulfides of a variety of proteins, thereby modulating enzyme activity [88]. GLRX can also carry out oxidative cysteine glutathionylation of proteins resulting in protein-glutathione mixed disulfides [78], and the reverse reaction, deglutathionylation (i.e., the reduction of the mixed disulfides), restoring the protein to its unmodified form. GLRX enzymes come in two forms: a monothiol form that contains a single cysteine in the active site and a dithiol form that contains two cysteines in the active site. These two forms differ in function and can be seen as distinct clades in the phylogenetic tree (Figure 7). The reduction of protein disulfides as well as the oxidative formation of protein-glutathione-mixed disulfides

are both catalysed via dithiol mechanisms, whereas reductive deglutathionylation is performed by a monothiol mechanism [89].

One of the GLRX clades contains only monothiol proteins with the CGFS active site sequences, whereas the other clade contains mostly dithiols with a variety of active site sequences, as well as a few proteins with a single cysteine active site. The mammalian GLRX3 (PICOT), likely ortholog of *C. elegans* GLRX-3, has been characterised as an iron-sulfur binding protein possibly regulated by ROS and reactive nitrogen species [90]. GLRX3 is essential for embryonic development, postembryonic growth, and heart function [91]. Human GLRX1 has a number of roles including the regulation of redox signal transduction and protein translocation [92], caspase-3 signalling in tumor necrosis factor- α -induced cell death [93], and angiotensin II redox signalling via glutathionylation of Ras [94]. *C. elegans* GLRX-10 is closely related to human GLRX1, both of which are nested within a subclade of dithiol GLRX enzymes, all of which except GLRX2 contain the CPYC active site sequence. F10D7.3 is somewhat similar to Grx6p and Grx7p from *S. cerevisiae*, although the difference in size and active site makes orthology unlikely. GLRX-21, GLRX-22, and ZC334.7 were found to group closely with *S. cerevisiae* Grx8p, but again differences in size (particularly the larger size of ZC334.7) and the sequence of their dithiol active sites makes orthology unlikely. To date very little work on GLRX proteins has been performed in *C. elegans*. Worth mentioning, however, is a paper published in 2010 which found that GLRX-21 functions in the prevention of selenium-induced oxidative stress [95].

3.1.5. Protein Disulfide Isomerase. The protein disulfide isomerase (PDI) protein family is composed of a large and diverse group of enzymes, most of which contain at least one TRX-like domain with a CxxC active site motif. PDI enzymes reside in the endoplasmic reticulum (ER) where their usual function is to catalyse protein folding. The active site cysteines of PDI are used in thiol-disulfide exchange between cysteine residues of the substrate proteins. This PDI thiol-disulfide exchange enables proteins to rapidly

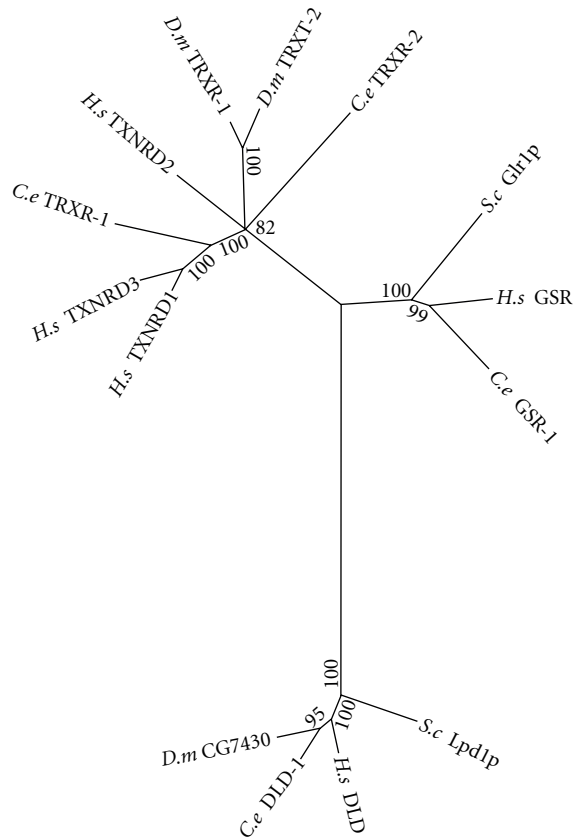


FIGURE 6: Maximum likelihood majority-rule bootstrap consensus tree of thioredoxin reductase (TRXR), glutathione disulfide reductase (GSR) and dihydroliipoamide dehydrogenase (DLD) proteins. *C. elegans* possess two TRXR: mitochondrial TRXR-1 and cytosolic TRXR-2. In addition *C. elegans* contain a single glutathione disulfide reductase (GSR) and the closely related DLD. See Figure 5 for details of how the sequences were identified and processed as well as how the phylogenetic analysis was carried out.

acquire the correct configuration of structural disulfide bonds required to achieve their native structure [96]. PDI functions in four different chemical reactions: (1) the oxidation of protein disulfides, using GSSG as the electron acceptor; (2) the reduction of protein disulfides, using GSH or NADPH as the electron donor; (3) the deglutathionylation of mixed disulfides; (4) the isomerization (rearrangement) of intramolecular disulfides. These functions of PDI proteins require the more oxidised redox state of the ER [70, 97]. In addition to passively relying on a more oxidised redox state, it has also been suggested that some PDI proteins may play a role in redox regulation [62].

In *C. elegans*, Karala et al. [98] analysed and compared the activities of PDI-1, PDI-2, and PDI-3 and found that all three displayed thiol-disulfide exchange activity, but that each showed a difference in reactivity towards various protein substrates. RNAi knockdown of the *pdi-2* and *pdi-3* genes results in an unfolded protein response, which suggests PDI-2 and PDI-3 are indeed required for proper protein folding [83]. Additionally, Winter et al. [99] studied

PDI-1, PDI-2, and PDI-3 and found that PDI activity is required for embryonic development and proper formation of the extracellular matrix.

Comparison of PDI sequences and phylogenetic analysis revealed a number of unnamed proteins with likely orthology to known human PDIs, as well as a few small gene expansion events both in *C. elegans* and human. The end result is 19 proteins in both *C. elegans* and human, but only 8 in *D. melanogaster* and 4 in *S. cerevisiae* (see Figure 8). Human P4HB (PDI/PDIA1), PDIA3 (ERp57), and PDIA4 (ERp72)—the probable orthologs of *C. elegans* PDI-2 (or PDI-1), PDI-3, and C14B9.2, respectively—all react readily with peptide dithiols *in vitro* to form disulfides [100]. *C. elegans* PDI-1 is peculiar in that the N-terminal active sites of all of its closely related homologs contain the characteristic PDI sequence CGHC, whereas in *C. elegans* the glycine has been replaced by a valine (CVHC). The similarity of *C. elegans* PDI-1 to human P4HB in size and sequence would suggest an orthologs relationship between these two proteins. However, human P4HB can be found as the beta subunit of prolyl 4-hydroxylase (P4H), a complex which hydroxylates proline to hydroxyproline in the production of collagen [101]. In *C. elegans*, the beta subunit of the P4H complex is PDI-2 [102, 103], making PDI-2 the more likely ortholog of P4HB, despite PDI-2 being ~100 amino acids shorter than *C. elegans* PDI-1 and human P4HB. Regarding the other PDI homologs, Ko and Chow [104] found that the DPY-11 protein of *C. elegans* that is a possible ortholog of human TMX1, is necessary for body and sensory organ morphogenesis, which they argue is due to its role in substrate modification in the hypodermis. In terms of redox signalling, human P4HB has been found to work antagonistically with TRXN1 in the regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)-dependent gene expression: TRXN1 activates the NF- κ B pathway, whereas P4HB expression suppresses NF- κ B activity in a dose-dependent manner [105]. Other possible PDI orthologous relationships involving *C. elegans* include human TMX3, *D. melanogaster* CG5027, and *C. elegans* ZK973.11; human TXNDC12 (ERp18) and *C. elegans* Y57A101.23; *D. melanogaster* CG4670 and *C. elegans* F47B7.2; human PDIA6 (p5), *C. elegans* TAG-320 and *C. elegans* Y49E10.4 (see Table 3). Although, whether any of these proteins participate in redox signalling remains to be investigated.

3.2. Superoxide/H₂O₂ Generation and Removal

3.2.1. NADPH Oxidase. The NADPH oxidase (NOX) system was first described as a system used by mammalian phagocytes in the production of superoxide as a response to infection by microorganisms [106]. The core enzyme of this microbial defence system is NOX2, which—under the regulation of its p22phox, p47phox, p40phox, p67phox, and RAC subunits—catalyses the formation of large amounts of superoxide, which in turn is converted to additional reactive oxygen species. The resulting high ROS levels results in the death of invading microbial pathogens. A total of seven NOX

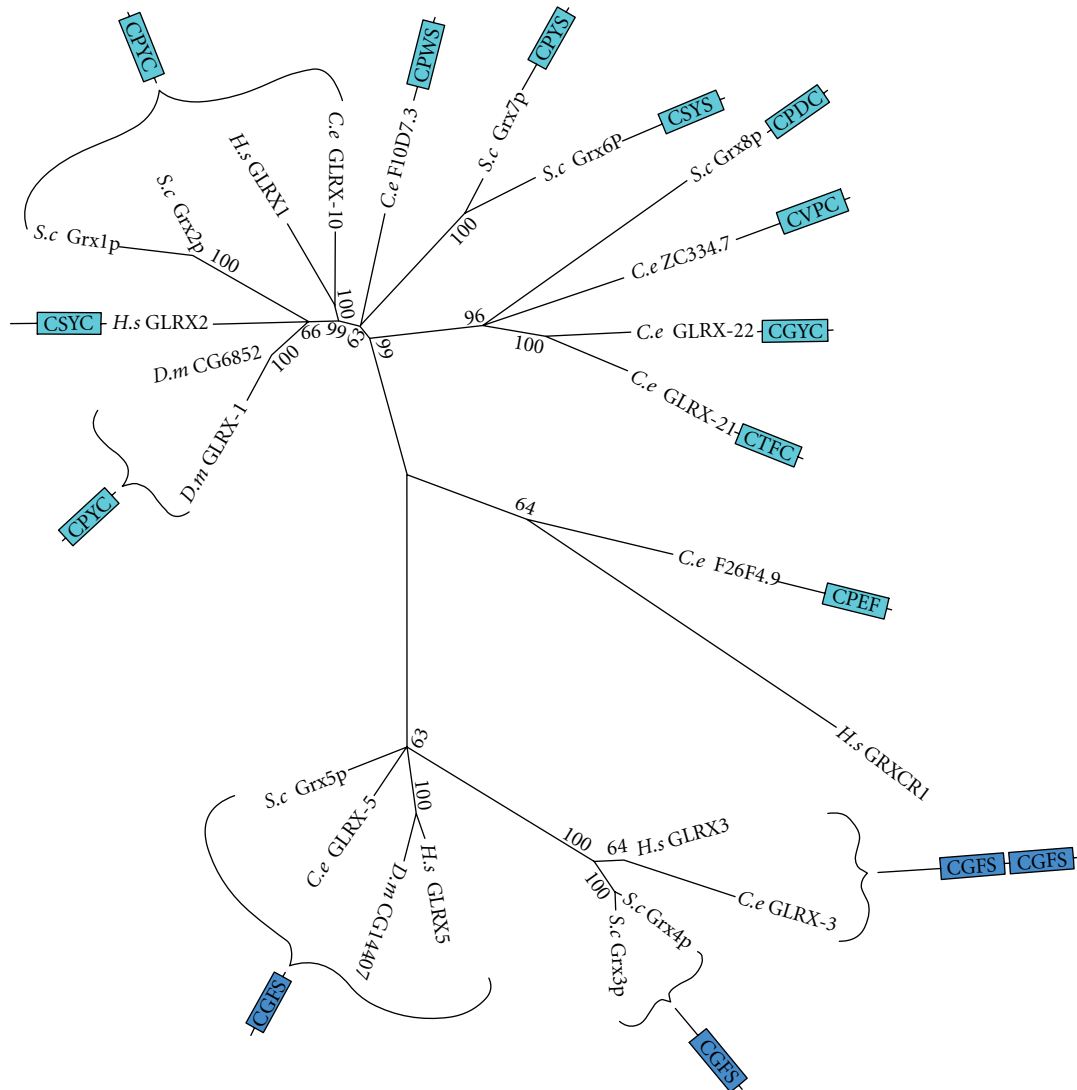


FIGURE 7: Maximum likelihood majority-rule bootstrap consensus tree of glutaredoxin (GLRX) proteins. Dark blue signifies the conserved active site of monothiol GLRX. Light blue signifies proteins of greater similarity to known dithiol GLRX, regardless of whether or not two cysteines are actually present in the region aligned to the active sites. See Figure 5 for details of how the sequences were identified and processed as well as how the phylogenetic analysis was carried out.

homologues exist in mammals, including NOX1 through 5 and the dual oxidases DUOX-1 and DUOX-2. Most of these homologs generate much lower levels of ROS than NOX2 and are found in a much wider range of cell types. Lambeth [25] presents a case for the importance of NOX proteins in the generation of ROS signals, but this hypothesis has not yet been rigorously tested.

Most important to a discussion of NOX activity in *C. elegans* is the function of DUOX, as the only two NOX homologs in *C. elegans*, DUOX-2 and BLI-3, are related to the DUOX proteins of humans and *D. melanogaster* (Figure 9). These proteins contain an additional peroxidase domain not found in the NOX1 through 5. DUOX serves a dual role in both the generation of superoxide and catalysis of reactions in the extracellular matrix using H_2O_2 . The subunits used to regulate NOX2 are not used in the regulation of

DUOX enzymes. Additionally, blastp searches do not reveal homology to any of the NOX2 regulating subunits in *C. elegans*. It is important to note the DUOX has not been implicated in ROS signalling, and there is evidence to suggest that in mammals they instead play a role in the biosynthesis of thyroid hormones in the extracellular matrix [107]. In *C. elegans*, the DUOX homolog BLI-3 functions in tyrosine cross-linking in the extracellular matrix [108, 109]. Further research may yet reveal additional mechanisms for the DUOX homologs in *C. elegans*.

3.2.2. Superoxide Dismutase. SOD proteins are generally regarded as antioxidants responsible for eliminating the ROS superoxide. An alternative view is that these enzymes generate H_2O_2 for use in redox signalling [110]. In this regard, the levels of SOD activity could be important in

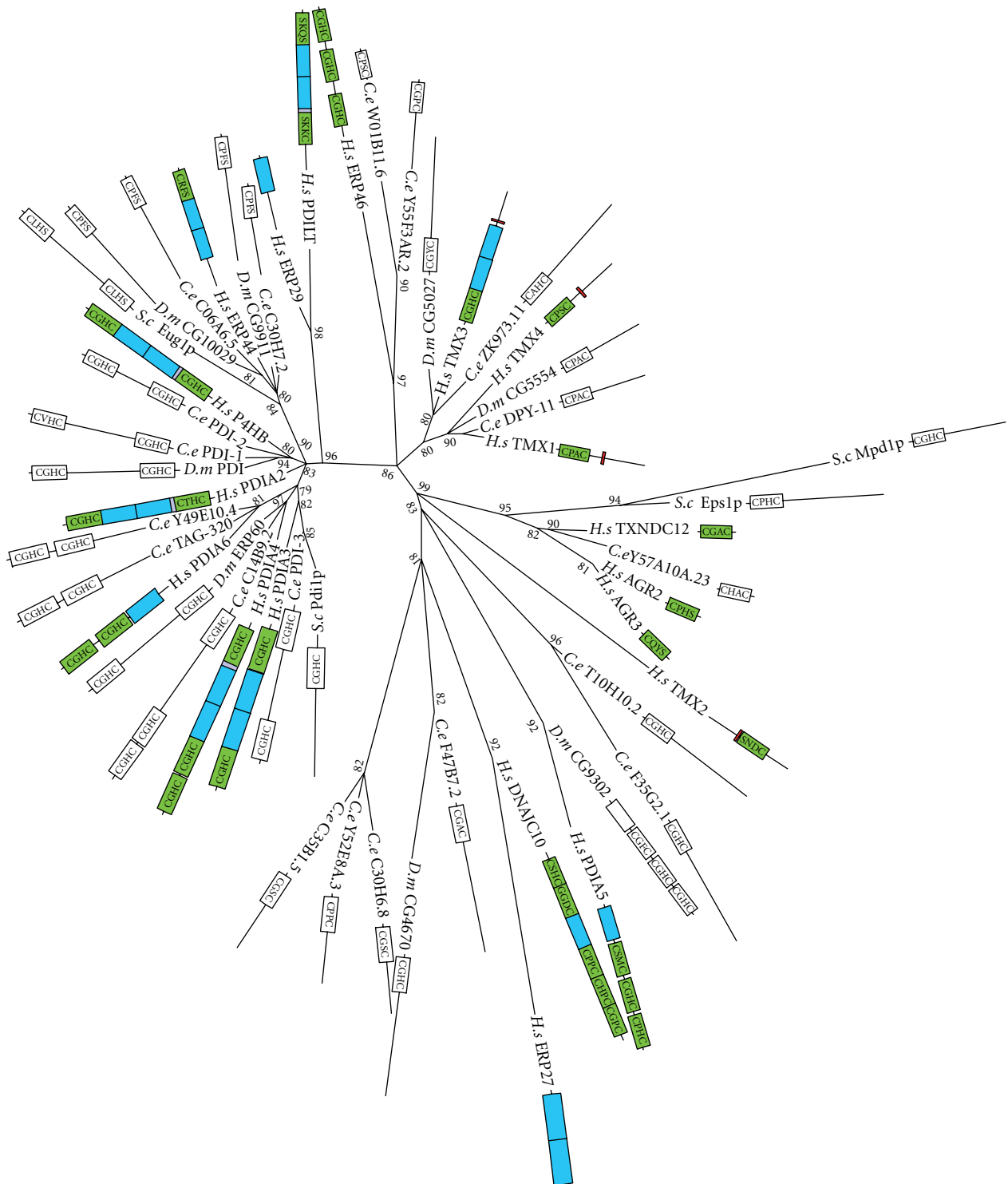


FIGURE 8: Maximum likelihood majority-rule bootstrap consensus tree of protein disulfide isomerase (PDI) proteins. Green boxes are likely TRX-like fold in human PDI; blue are likely noncatalytic thiodoxin domains (according to Hatahet and Ruddock [62]). White boxes show CxxC catalytic motifs, in *C. elegans* and *D. melanogaster*. Although domain structure cannot be inferred from sequence alone, it is likely that proteins that share similar sequences, catalytic motifs, and length also share a similar domain structure and in some cases are likely orthologous. See Figure 5 for details of how the sequences were identified and processed as well as how the phylogenetic analysis was carried out.

TABLE 3: Characterised protein disulfide isomerase and their *C. elegans* orthologs.

PDI homolog	Known functions	<i>C. elegans</i> ortholog (paralogs)
AGR2	Unknown	None
AGR3	Unknown	None
<i>ERp27</i>	<i>Unknown</i>	(<i>C30H6.8, C35B1.5,</i>
<i>DNAj10</i>	<i>ER-associated protein degradation [70]</i>	<i>F47B7.2, Y52E8A.3)</i>
ERp29	Specialist “escort” chaperone [71] Thyroglobulin processing [72]	None
ERp46	Some P4HB functional redundancy Protective role against hypoxia [70]	(W01B11.6, Y55F3AR.2)
ERp44	Possible role in late-stage oligomerization reactions Possible role in late-stage, thiol-dependent, protein quality-control system or trafficking [70]	C06A6.5, C30H7.2
P4HB	Thiol-disulfide exchange catalysis Role in protein folding Beta subunit of P4H complex [70]	PDI-2, PDI-1
PDIA2	Expressed in pancreas and brain [73, 74] Thiol-disulfide exchange catalysis similar to P4HB Specific function still unclear	None
PDIA3	Involved in MHC class I folding as part of MHC I peptide-loading complex [75] Oxidative folding of glycoproteins	PDI-3
PDIA4	Thiol-disulfide exchange catalysis similar to P4HB Some PDIA3 redundancy Can form part of a complex with P4HB, PDIA6, ERdj3, BiP, CypB, HSP40, GRP94, GRP170, UDP glucosyltransferase, and SDF2-L1 [76]	C14B9.2
PDIA5	Unknown	(F35G2.1, T10H10.2)
PDIA6	Likely catalyze thiol-disulfide exchange similar to P4HB Likely role in protein folding	TAG-320, Y49E10.4
PDILT	Unknown	None
TMX1	ER transmembrane PDI Unknown function	DPY-11
TMX2	ER transmembrane PDI Unknown function	None
TMX3	ER transmembrane PDI Protein dithiol-disulfide oxidant	ZK973.11
TMX4	ER transmembrane PDI Unknown function	None
TXNDC12	Unknown	Y57A10A.23

Italic rows are human proteins that share the same *C. elegans* paralogs.

regulating H₂O₂ levels. A signalling role for SOD goes well beyond the popular view that SOD is responsible for the complete removal of superoxide from cellular compartments for the sole purpose of preventing oxidative damage.

Much of the recent research on SOD enzymes has focused on their possible role in the ageing process, in experiments designed to test the free-radical theory of ageing. In some cases, decreasing levels of SOD have been shown to shorten the lifespan of yeast [111–114], fruit flies [115], and mice [116], but this is not uniformly the case. In fact, an analysis of the entire *sod* gene family in *C. elegans* revealed that both increasing and decreasing expression of the *sod* genes had little effect on lifespan [117]. When *sod* gene expression

is experimentally increased, lifespan is either unaltered or decreased [117–120]. A report from Y. Honda and S. Honda [121] showed that increased expression of *sod-1* and *sod-2* extended the lifespan of *C. elegans*, but that this was not due to decreased oxidative damage. While results are inconsistent between species and are not even consistent between experiments on a single species, it is clear that the view of SOD as an eliminator of ROS that would otherwise limit lifespan is much too simplistic.

Two distinct classes of SOD enzymes exist within eukaryotes: copper/zinc SOD (Cu/Zn SOD), found in the cytosol or extracellular matrix [122]; manganese SOD (Mn SOD), found in the mitochondria [123]. Phylogenetic analysis

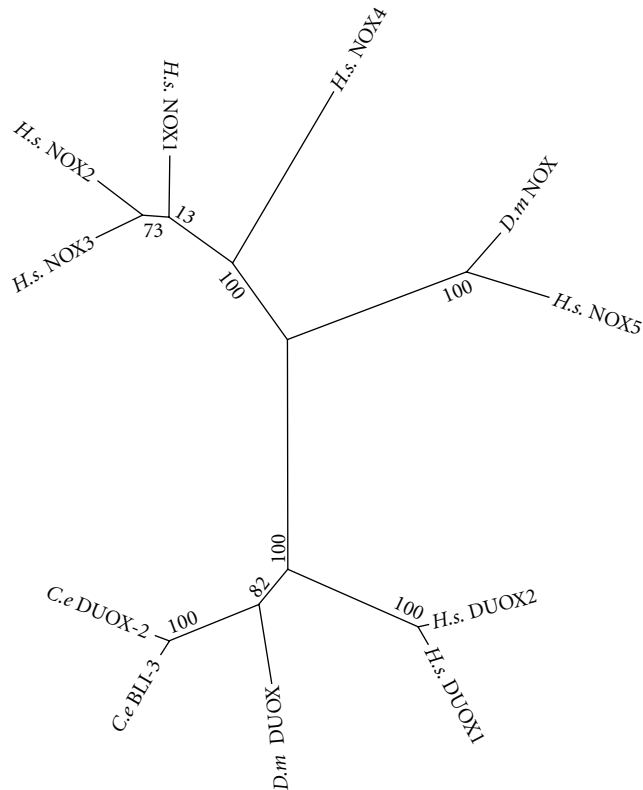


FIGURE 9: Maximum likelihood majority-rule bootstrap consensus tree of NADPH oxidase (NOX)/dual oxidase (DUOX) proteins. See Figure 5 for details of how the sequences were identified and processed as well as how the phylogenetic analysis was carried out.

clearly shows two main clades corresponding to Mn and Cu/Zn enzymes (Figure 10). Unlike the other three species in the analysis, each of which has only a single Mn SOD, there has been a duplication of the gene in *C. elegans*, *sod-2* and *sod-3*. The same is true of the human Cu/Zn *sod-1*, in that human and the other two species have a single gene that corresponds to a pair of genes in *C. elegans*, *sod-1* and *sod-5*. The last *sod* gene in *C. elegans*, *sod-4*, corresponds to a single gene in each of human and *D. melanogaster*. It is an extracellular Cu/Zn SOD [124], with a possible function is *daf-2* signalling [117].

3.2.3. Glutathione Peroxidase. The glutathione peroxidases (GPX) were first characterised as a family of proteins that reduce H_2O_2 to H_2O using GSH as the electron donor [125]. Humans and *C. elegans* both contain a large number of GPX proteins (8 and 7, resp.) compared to yeast (3 proteins) and *D. melanogaster* (only 2 proteins). Most of these appear to have arisen independently by gene duplications within the two taxonomic lineages (Figure 11). While five of the eight humans GPX contain a selenocysteine in their active site, not a single selenocysteine is found in any of the *C. elegans* GPX homologs.

Despite the relatedness of *C. elegans* proteins with proteins of known GPX activity, Vanfleteren [126] was unable to detect any GPX activity in *C. elegans* tissue. However, the

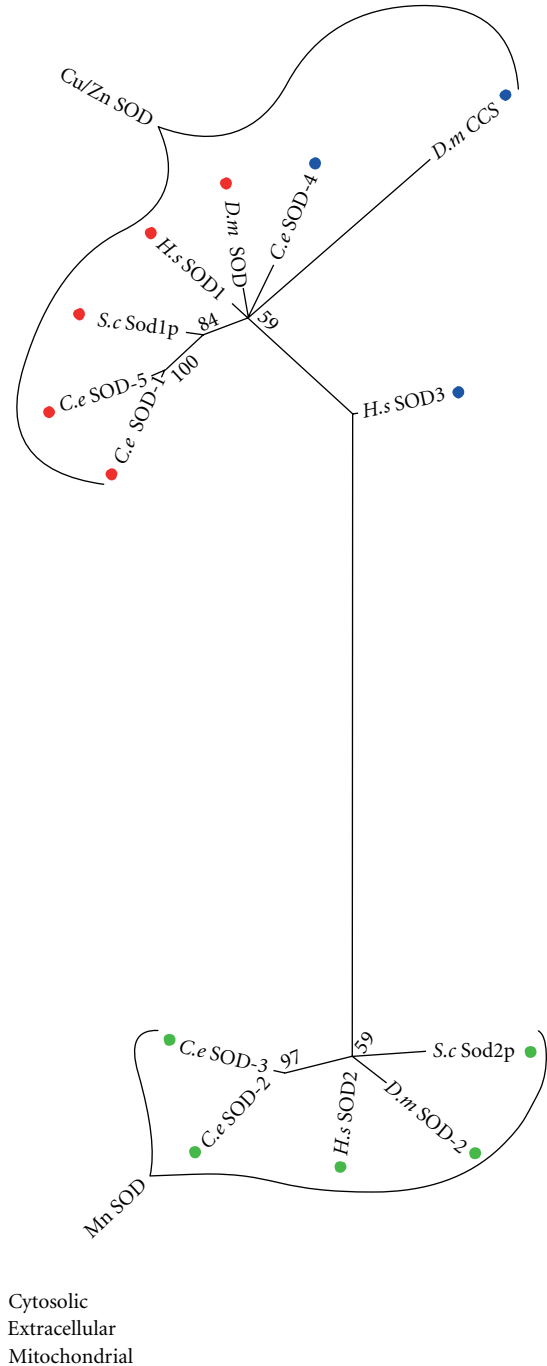


FIGURE 10: Maximum likelihood majority-rule bootstrap consensus tree of superoxide dismutase (SOD) proteins. See Figure 5 for details of how the sequences were identified and processed as well as how the phylogenetic analysis was carried out.

in vitro assay used in this article only included GSH as a reducing substrate. Use of GSH appears to be limited to GPX enzymes that contain selenocysteine; the cysteine-containing GPX homologs of *C. elegans* likely use a peroxiredoxin-like mechanism with thioredoxin as their reducing substrate [127].

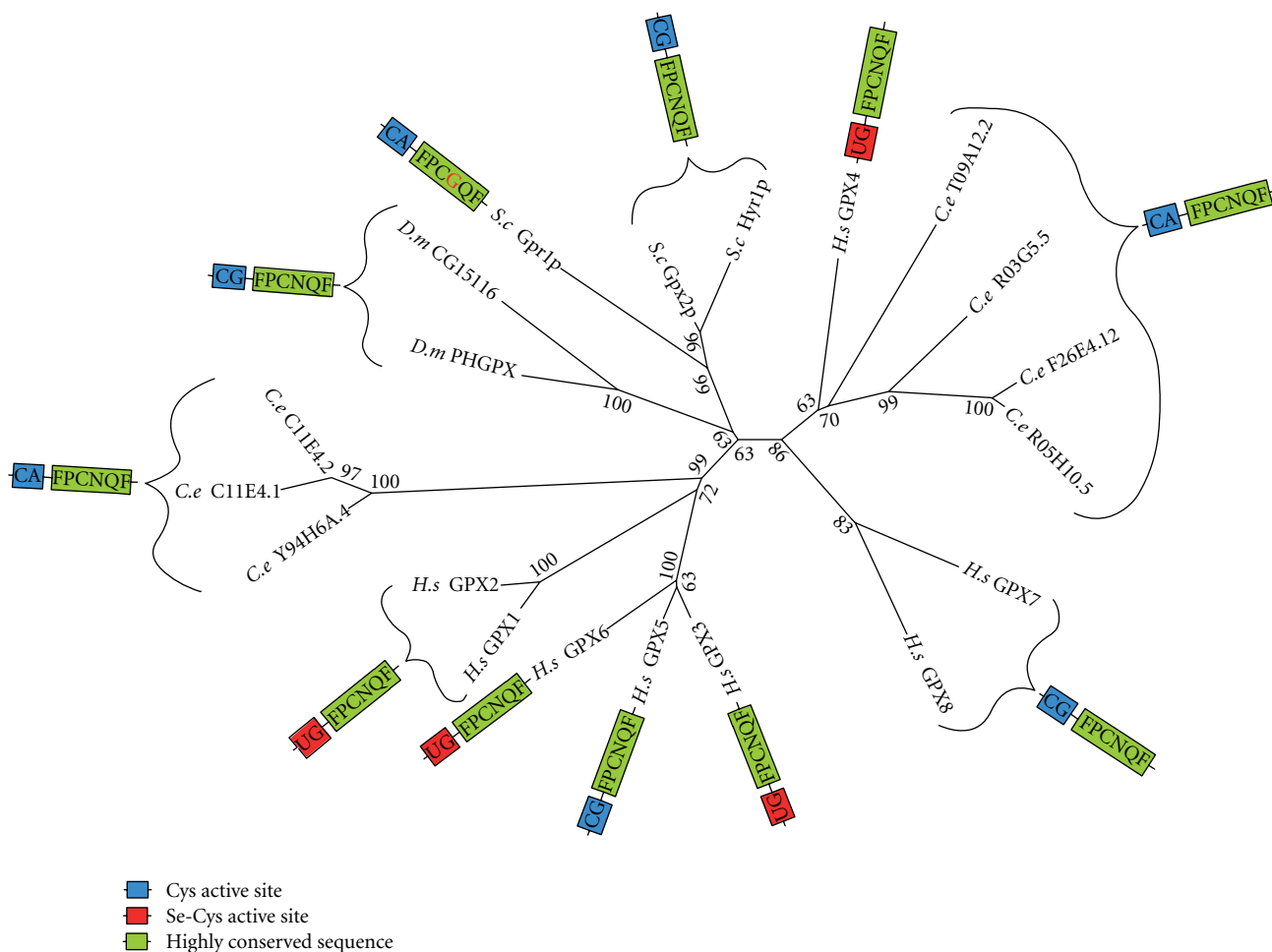


FIGURE 11: Maximum likelihood majority-rule bootstrap consensus tree of GPX proteins. See Figure 6 for details on the phylogenetic analysis.

Numerous studies have implicated GPX proteins in redox signalling. For example, Faltin et al. [128] found that ROS signalling used in the early stage shoot organogenesis of plants is regulated by the GPX homolog PHGPx. In mammals, ROS regulation of lipopolysaccharide (LPS) signalling is modulated by GPX1 [129], while GPX1 deficiency is found to enhance proinflammatory cytokine-induced redox signalling [130]. Conversely, high levels of catalase and GPX activity have been found to diminish H_2O_2 signalling in human alveolar macrophages [131]. The *C. elegans* protein F26E4.12, a homolog of human GPX4 and plant PHGPx, regulates the peptide transporter PEPT-1 [132].

3.2.4. Peroxiredoxin. Peroxiredoxins (PRDX) are found as homodimers in which the active site cysteines align in close proximity and form intermolecular dithiols/disulfides. PRDX disulfides function in the reduction of H_2O_2 to H_2O for both antioxidant defence and mediation of ROS signalling [133, 134]. Active site disulfides formed in the reduction of H_2O_2 are reduced back to dithiols by the thioredoxin redox system. PRDX comes in three forms: "Typical" 2-Cys, "Atypical" 2-Cys, and 1-Cys.

Phylogenetic analysis showed that *C. elegans* have two typical 2-Cys PRDX, 1-Cys PRDX, but does not possess an atypical 2-Cys PRDX homolog (Figure 12). Human PRDX1 and PRDX2, two of the typical 2-Cys PRDX homologs to *C. elegans* PRDX-2, might participate in both intra- and extracellular signalling cascades by regulating levels of H_2O_2 [23]. The human mitochondrial typical 2-Cys PRDX3, which is closely related to *C. elegans* PRDX-3, participates in the regulation of apoptotic signalling. Little research has been done on the PRDX proteins of *C. elegans*. What is known is that PRDX-2 is necessary for normal growth and egg production in *C. elegans*, which Isermann et al. [135] argue is likely due to its role in peroxide signalling. Interestingly, loss of PRDX-2 actually increases resistance to some oxidative stress causing agents but results in a decrease in lifespan [136].

3.2.5. Catalase. Catalase functions in the decomposition of H_2O_2 to H_2O and O_2 . Phylogenetic analysis shows a lineage specific expansion from one catalase to three in *C. elegans* (Figure 13). *C. elegans* CTL-1 is required for the extended

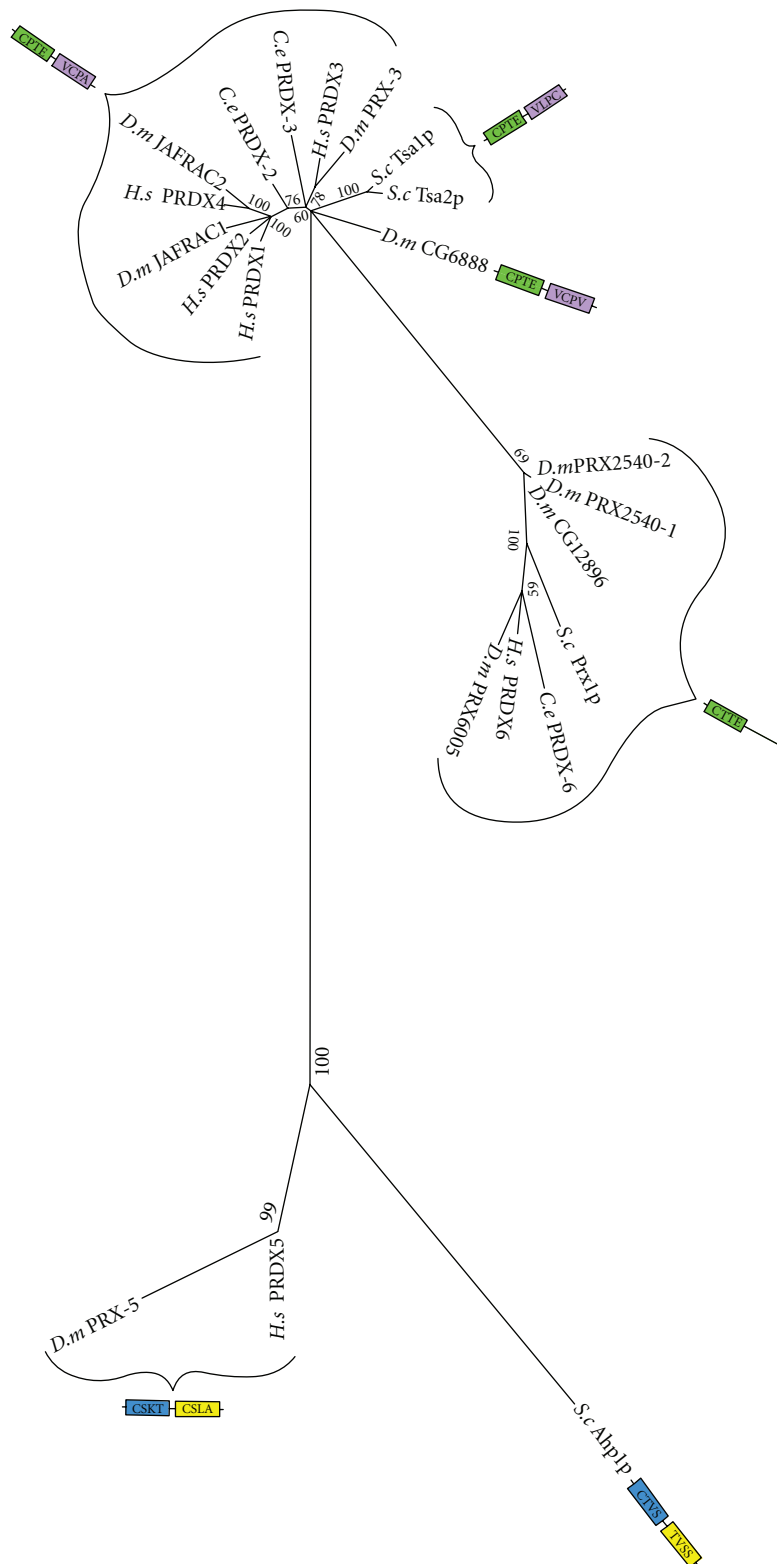


FIGURE 12: Maximum likelihood majority-rule bootstrap consensus tree of peroxiredoxin (PRDX) proteins. “Typical” 2-Cys PRDX is shown in green and purple; “Atypical” 2-Cys in blue and yellow; and 1-Cys in green. Active site cysteine regions highlighted in the same colour are those that aligned in the MSA. See Figure 5 for details of how the sequences were identified and processed as well as how the phylogenetic analysis was carried out.

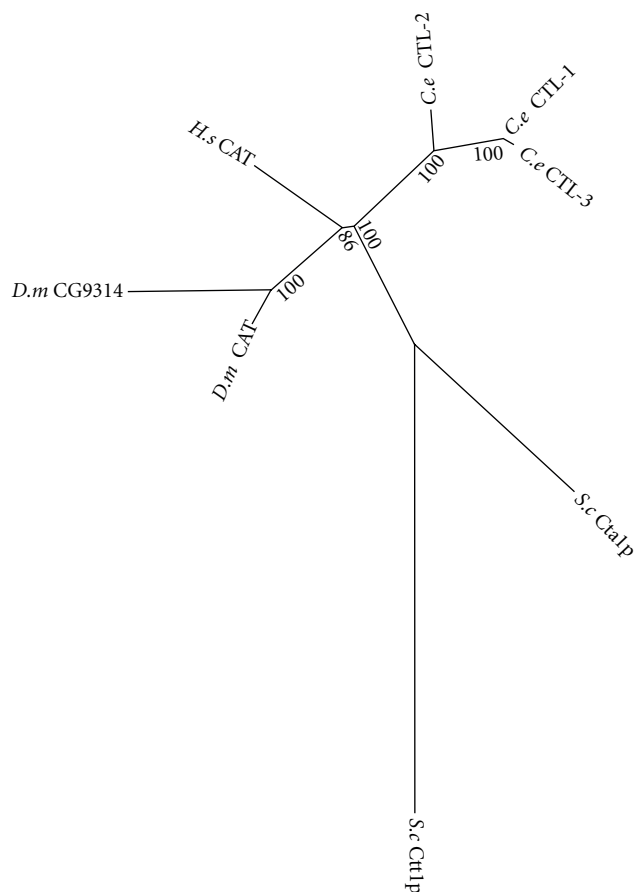


FIGURE 13: Maximum likelihood majority-rule bootstrap consensus tree of catalase proteins. See Figure 6 for details on the phylogenetic analysis.

adult lifespans of *daf-2*, *age-1*, and *clk-1* mutants [137]. CTL-2 is found in the peroxisomes of *C. elegans* [138] and a lack of this protein has been found to cause a progeric phenotype and adversely affect development/egg laying [139]. The *ctl-1* and *ctl-2* genes are both negatively regulated by DAF-2-mediated insulin signalling [140]. As such, DAF-2 signalling might result in an increase in the levels of H_2O_2 , and thus a more oxidizing state.

4. Conclusion

Reactive oxygen species are thought to play a role in many diseases of ageing, including Parkinson's disease, Alzheimer's disease, heart failure, and myocardial infarction. This makes understanding their dual roles as oxidative stressors and signalling molecules highly significant. Important to this understanding is a clear description of the protein families that contribute to the generation and metabolism of ROS. Most of the known protein families that participate in redox biology are discussed in this article, but it is likely that additional, undescribed families of redox proteins remain to be discovered. It is striking that of the protein families that have been compared in this study, most show clear

relationships between sequences, with no extreme examples of species-specific family expansion. *S. cerevisiae* frequently, and *D. melanogaster* sometimes, had smaller gene families than the other two species.

In addition to oxidative damage, higher ROS levels disrupt the regular function of redox regulators and their downstream effectors. It is, therefore, likely that at least some, if not many, of the toxic effects associated with oxidative stress are the result of disruption to redox signalling. Continued research into the various functions of the *C. elegans* redox proteins discussed in this article will help to achieve a better understanding of redox signalling, oxidative stress, and the relationship between these two biological phenomena. *C. elegans* and *H. sapiens* exhibited fairly conserved gene family structure, indicating that *C. elegans* will provide a medically relevant model of redox signalling.

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