Review Article

ROS in Aging *Caenorhabditis elegans*: Damage or Signaling?

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Many insights into the mechanisms and signaling pathways underlying aging have resulted from research on the nematode *Caenorhabditis elegans*. In this paper, we discuss the recent findings that emerged using this model organism concerning the role of reactive oxygen species (ROS) in the aging process. The accrual of oxidative stress and damage has been the predominant mechanistic explanation for the process of aging for many years, but reviewing the recent studies in *C. elegans* calls this theory into question. Thus, it becomes more and more evident that ROS are not merely toxic byproducts of the oxidative metabolism. Rather it seems more likely that tightly controlled concentrations of ROS and fluctuations in redox potential are important mediators of signaling processes. We therefore discuss some theories that explain how redox signaling may be involved in aging and provide some examples of ROS functions and signaling in *C. elegans* metabolism. To understand the role of ROS and the redox status in physiology, stress response, development, and aging, there is a rising need for accurate and reversible *in vivo* detection. Therefore, we comment on some methods of ROS and redox detection with emphasis on the implementation of genetically encoded biosensors in *C. elegans*.

1. Oxidative Stress Theory of Aging and Correlation of Oxidative Stress with Age

More than fifty years ago, Harman [1] postulated in his free-radical theory of aging that aging results from the accumulation of molecular damage caused by byproducts of the normal oxidative metabolism, called reactive oxygen species (ROS). The discovery of the superoxide dismutase enzyme, which detoxifies the superoxide anion [2], and the detection of the ROS hydrogen peroxide (H_2O_2) *in vivo* [3] further gave credibility to the free-radical theory of aging. Harman refined his theory to highlight the role of mitochondria in aging since mitochondria are considered as the main source of ROS [4]. Because there are many ROS that are not free-radicals and that can also cause oxidative damage, the free-radical theory of aging is now referred to as the oxidative stress theory of aging [5]. This new name for the theory also implies that oxidative stress can occur due to an imbalance between ROS production and removal. This imbalance leads to a progressive accumulation of oxidative damage with age, resulting in a decline of the functional efficiency of various cellular processes. Since its formulation, the oxidative stress theory of aging has been the most broadly tested theory of aging. Despite the tremendous effort to verify this theory, experimental studies do not support or remain inconclusive on whether oxidative damage is responsible for aging [6–10]. During the last years, many studies have yielded new insights on the role of ROS in aging. Many of these studies have been performed using the model organism *Caenorhabditis elegans* and will be discussed in this paper.

As organisms age, a number of behavioral, reproductive, morphological, and biochemical changes occur [11]. Previous studies have shown an age-related increase of oxidative damage in a variety of molecules (DNA, proteins, and lipids) in organisms ranging from invertebrates to humans [5, 12–14]. This finding is in accordance with the oxidative stress hypothesis of aging. In *C. elegans*, there is an increase in single-strand DNA breaks, 5-methylcytosine [15], and protein carbonyls accumulate with age [16, 17]. However, as in many other invertebrates [18, 19], in subsequent tests on extensively purified DNA of *C. elegans*, no cytosine methylation was detected during development and senescence [20].

Melov et al. [21] reported an increase in mitochondrial DNA deletions with age. However, these results could not be confirmed in our research group [22].

One way to investigate the role of ROS in aging is to examine oxidative stress resistance in long-lived strains. Two major interventions that greatly influence lifespan are the regulation of insulin/IGF-1 signaling (IIS) and dietary restriction. The IIS pathway is an evolutionary conserved pathway that regulates aging in organisms ranging from nematodes to mammals. In *C. elegans*, it comprises an insulin-like receptor *(daf-2)*, which negatively regulates a downstream FOXO transcription factor, DAF-16. A reduction of function of *daf-2* doubles the lifespan of *C. elegans*, and this lifespan extension is fully dependent on *daf-16*. Many of the transcriptional targets of DAF-16 are also activated in the dauer larvae, a special developmental stage of *C. elegans* that can survive harsh environmental conditions for several months, while the average lifespan of an adult *C. elegans* is about 2-3 weeks. The IIS pathway and pathways related to IIS and/or DAF-16 are induced by various environmental cues (nutrients, heat, UV, heavy metal, and oxidative stress) [23–26]. These pathways promote oxidative stress resistance and longevity and regulate the expression of ROS detoxification genes. Although oxidative stress resistance is increased in these long-lived strains, this does not mean that increased stress resistance is an essential prerequisite for longevity. To test this, the expression of *sod* genes was reduced in long-lived *daf-2* mutants. Although isolated mitochondria of *daf-2* mutants produce more superoxide compared to wild type [27], the loss of *sod* genes has little or no effect on *daf-2* longevity and in some cases, it even further increases lifespan of *daf-2* [28–31]. Thus, increased antioxidant defenses in *daf-2* do not significantly contribute to the extended lifespan of *daf-2* mutants.

A reduction of food intake by 40–60% without malnutrition, called dietary restriction (DR), has remarkable benefits for health and lifespan in many different species, including *C. elegans* [32]. It was long believed that DR would reduce metabolic rate and thereby decrease ROS production and ROS-induced damage, resulting in extended lifespan [33]. Measurement of the oxygen consumption and heat production rather shows an increase in metabolism in food-restricted worms, however [34, 35]. ROS production in worms imposed to at least one type of DR is not different from wild type [27]. Dietary restriction enhances SOD and catalase activity [36] and increases *sod* expression (*sod-1*, *sod-2*, *sod-4*, and *sod-5*), regulated by the FOXA transcription factor PHA-4 [37]. Although diet-restricted worms show increased antioxidant defense and oxidative stress resistance [36, 38], *sod* deletion does not reduce lifespan in two different types of DR [39, 40]. This suggests that enhanced antioxidant defense may not be essential for DR-induced longevity.

2. Evaluation of the Oxidative Stress Theory in *C. elegans*

Conform to the oxidative stress theory of aging, oxidatively damaged biomolecules accumulate with age in *C. elegans*.

Moreover, it is often found that manipulations that slow down aging, such as dietary restriction, hormesis (discussed below), or reduced IIS, also increase oxidative stress resistance. Although such observations provide correlative evidence for the oxidative stress theory of aging, they do not allow concluding that this theory is correct. Long-lived strains are resistant to other types of stresses than oxidative stress, such as heat, UV, and pathogenic bacteria. Various studies show that enhanced oxidative stress resistance is not essential for the extended lifespan in long-lived strains [38]. The most conclusive approach to test the causal relation between ROS and aging is to change the antioxidant defense system and to examine the effect on aging. Antioxidant defenses can be altered by genetic (mutation or overexpression of antioxidant enzymes) or pharmacological interventions (SOD/catalase mimetics, vitamins).

2.1. Pharmacological Interventions. Numerous studies have examined the effect of exogenous antioxidant compounds on lifespan in *C. elegans* [41]. Various noncatalytic antioxidants, such as vitamin E and C, trolox, α-tocopherol, and N-acetylcysteine, affected lifespan differently in distinct studies [42–47]. In some cases, these compounds increased oxidative stress resistance without changing the lifespan [48]. The variability in the outcome of these reports may be explained by differences in dose, experimental conditions, and method of delivery. In *C. elegans*, the uptake of many drugs is rather poor as they are excluded by the thick cuticle [49]. The use of liposomes ameliorates the uptake of hydrophilic antioxidants, such as ascorbic acid, N-acetylcysteine, and reduced glutathione, and prolongs lifespan, while conventional delivery methods do not [50]. It should be noted that this lifespan extension was dose-independent and that liposomes can affect the fat metabolism of the worms, however.

The effect of the SOD/catalase mimetics EUK-8 and EUK-134 on lifespan in *C. elegans* has been tested by various research groups. Administration of these compounds increases SOD activity *in vivo*, primarily in the mitochondria, and it also enhances paraquat resistance [51–53]. EUK-8 and EUK-134 treatment extended lifespan in one study [54]. However, other groups detected no increase in lifespan for doses that protect the worm against paraquat [51, 53, 55]. In fact, they even found a dose-dependent decrease in lifespan at higher doses of EUK-8 and EUK-134. This toxic effect might be due to enhanced ROS production when EUK-8 is administered at high doses [56]. Thus, EUK-8 and EUK-134 clearly exhibit antioxidant activity *in vivo* and can enhance oxidative stress resistance, but without causing lifespan extension.

2.2. Genetic Interventions. *C. elegans* contains numerous antioxidant genes, and the effect on aging by deleting or overexpressing many of these genes has been examined recently. While most eukaryotes contain 2 or 3 different superoxide dismutase (*sod*) genes, *C. elegans* possesses at least six isoforms [64]. *sod-1* and *sod-5* encode for the cytosolic CuZnSODs [65, 66], while the *sod-4* gene expresses

TABLE 1: Overview of the effect *sod* knockdown/out or overexpression of *C. elegans* superoxide dismutases on ROS levels, oxidative damage, and lifespan under stressed and unstressed condition (paraquat or hyperoxia). OE: overexpression; ND: not determined; PQ: paraquat; J: juglone; DR: dietary restriction.

	sod gene	ROS levels	Oxidative damage	Effect on lifespan	Effect on stress survival	Effect on lifespan long-lived worms
			Knockdown/out			
MnSOD	*sod-2*	Increased [57] or no effect [27]	Increased [30, 40]	No effect [29–31, 39] or increased [27, 40, 57]	Decreased (PQ) [29–31, 40] (J) [40] or no effect (hyperoxia) [29]	No effect (*daf-2*) [29, 30, 40] (DR) [39] or decreased (*daf-2*) [31] or increased (*clk-1*) [30, 40]
	sod-3	Increased [57]	ND	No effect [29, 31, 39, 40] or increased [57]	Decreased (pathogenic bacteria) [58] (PQ-J) [40] or no effect (PQ-hyperoxia) [29, 31] or increased [39]	No effect (*daf-2*) [29] (DR) [39, 40] or increased (*daf-2*) [31] or decreased (*isp-1*) [40]
	sod-2; sod-3	Increased [59] or decreased [60]	No effect [29, 60]	No effect [29, 31] or increased [40]	Decreased (PQ-hyperoxia) [29, 31, 40] (J) [40]	Increased (*daf-2*) [31]
CuZnSOD	*sod-1*	Increased [61]	Increased [30] or no effect [29]	Decreased [29, 39, 61] or no effect [30, 39, 40]	Decreased (PQ) [29, 30, 39, 40, 61] (J) [40] (hyperoxia) [29]	Decreased (*daf-2*) [29] or no effect (DR) [39] or increased (*daf-2*) [30]
	sod-4	ND	ND	No effect [29, 31, 39, 40]	No effect [29] or increased [39]	Increased (*daf-2*) [29] or no effect (DR) [39]
	sod-5	ND	ND	No effect [29, 39, 40]	No effect [29, 39]	No effect (*daf-2*) [29] (DR) [39]
	sod-1; sod-4; sod-5	ND	ND	Decreased [29]	ND	ND
SOD	*sod-12345*	ND	No effect [62]	No effect [62]	Decreased [62]	ND
			OE			
MnSOD	*sod-2*	ND	No effect [63]	Increased [63]	ND	ND
CuZnSOD	*sod-1*	Increased [63]	Increased [63]	Increased [29, 63]	Decreased (PQ) [29]	ND

two extracellular CuZnSODs that are products of alternative splicing [67] and are either membrane bound or extracellular. *sod-2* and *sod-3* encode MnSOD enzymes that are localized in the mitochondrial matrix [68, 69]. SOD-1 and SOD-2 contribute to most of the SOD activity during normal development [29, 65], whereas the secondary SODs *sod-3* and *sod-5* are clearly upregulated in dauers [29, 70], in *daf-2* mutants [71] and under oxidative stress [72]. Very recently, a number of groups has examined the effect of *sod* deletion and overexpression on lifespan and oxidative stress resistance in *C. elegans* [27, 29–31, 39, 40, 57, 60, 61, 63]. An overview of these studies is represented in Table 1.

Loss of *sod-1* lowers the resistance to oxidative stress [29, 30, 39, 40, 61]. Although SOD-1 contributes to 80% of the total SOD activity in *C. elegans* [29], its deletion only slightly reduces lifespan [29, 39, 61] or even not at all in other studies [30, 39, 40]. Reduced *sod-1* expression increases ROS levels and oxidative damage in some studies [30, 61] but it did not in another one [29]. These contradicting results may reflect that the impact of *sod-1* reduction on oxidative stress is relatively subtle and difficult to detect. In contrast to *sod-1*, loss of *sod-4* or *sod-5* does not alter the lifespan under stressed or unstressed conditions [29, 31, 39, 40]. Unexpectedly, *sod-4* deletion prolongs lifespan in *daf-2* mutants [29]. Conform to the findings in mammalian studies [73],

it has been proposed that *sod-4* may stimulate IIS through the H_2O_2-dependent inactivation of the phosphatases [6]. This suggests that CuZnSOD is involved in the redox regulation of IIS. Consistent with this statement, mutation of *sod-4* or *sod-5* enhances dauer larva formation [29]. To resume, loss of CuZnSOD has little negative effect on oxidative stress and lifespan and it may regulate IIS via redox signaling.

While the loss of *sod-2*, *sod-3* or both, reduces oxidative stress resistance in most cases [29–31, 39, 40], it does not shorten lifespan under unstressed conditions [29–31, 39, 40], and in some studies it even increases lifespan [27, 40, 57]. Diminished MnSOD enhances ROS levels and oxidative damage to a minor extent in some reports [30, 40, 57, 59] but not in others [27, 29, 60]. MnSOD knockout/down experiments demonstrated that MnSOD is not required for lifespan extension in long-lived *daf-2* mutants and diet-restricted worms [29–31, 39, 40]. In contrast, abolished *sod-2* expression largely extends the lifespan of long-lived *clk-1* mutants [30, 40]. Consistent with this, *sod-2* mutation results in a Clk phenotype, that is, slow growth and defecation rate, and reduced and delayed fecundity [29, 40]. Thus, MnSOD is required for oxidative stress resistance, but it does not limit the lifespan of wild type or long-lived strains. The fact that loss of MnSOD enhances oxidative stress and concurrently prolongs lifespan in certain studies, contradicts a causal role for mitochondrial superoxide in the aging process.

Worms completely devoid of both Mn- and CuZnSOD have been recently obtained and are highly sensitive to multiple stresses (oxidative stress, heat, and osmotic stress), they develop slower and have a reduced brood size [62]. Remarkably, this total loss of SOD activity has no effect on lifespan at all. This lack of effect on lifespan can be explained by a counterbalance between superoxide toxicity on the one hand and an adaptive response to reduced superoxide detoxification on the other hand [62]. Most importantly, it shows that oxidative stress *per se* has no profound effect on aging in *C. elegans*.

If the oxidative stress hypothesis of aging is correct, an increase in SOD-1 should result in a decrease in oxidative damage and an extended lifespan. Overexpression of *sod-1* indeed prolongs lifespan [29, 63]. But instead of decreasing oxidative stress as expected by the oxidative stress hypothesis, *sod-1* overexpression does not reduce lipid oxidation and glycation and actually increases ROS levels and protein oxidation levels [63], and it increases paraquat sensitivity [29]. On the other hand, the lifespan extension of *sod-1* over-expressors is DAF-16- and partially HSF-1-dependent and *sod-1* overexpression enhances *hsp-4* and *hsp-6* expression, suggesting an unfolded protein response (UPR) [63]. This idea is further confirmed by the observation that knocking down genes involved in UPR, partly suppresses longevity of *sod-1* overexpressors. The exact mechanism by which *sod-1* overexpression increases lifespan through DAF-16 remains unclear, however. Like *sod-1*, *sod-2* overexpression also prolongs lifespan in a DAF-16-dependent manner, but it does not change the protein carbonylation levels [63]. These results imply that overexpression of *sod-1* or *sod-2* extends lifespan, not by reducing oxidative stress, but instead by activating longevity-related transcription factors, such as

DAF-16. Therefore, lifespan extension by SOD overexpression does not support the oxidative stress hypothesis. The fact that *sod-1* overexpression increases oxidative damage and prolongs lifespan at the same time, even contradicts this hypothesis.

C. elegans contains three catalase enzymes with highly similar sequences in a tandem array: *ctl-3*, *ctl-1*, and *ctl-2* [74]. The peroxisomal catalase, CTL-2 [75], contributes to ~80% of the total catalase activity and a knockout of *ctl-2* can reduce mean lifespan by 16% while decreasing egg-laying capacity [74]. Remarkably, protein carbonyl levels increase more slowly with age in *ctl-2* mutants compared to wild type [74]. Moreover, overexpression of all three catalase genes reduces lifespan [29].

C. elegans contains three peroxiredoxins, two 2-Cys peroxiredoxins *prdx-2* (*CePrx2*), and *prdx-3* (*CePrx1*), and one 1-Cys peroxiredoxin *prdx-6* (*CePrx3*). *Prdx-3* is probably mitochondrial, and only deletion of *prdx-2* results in an altered phenotype, displaying a reduced size, fertility, and oxidative stress resistance [76]. At 25°C, the lifespan of *prdx-2* mutants is not reduced, but at 15°C and 20°C *prdx-2* knockout worms live shorter than wild-type worms [76–78]. *prdx-2* is expressed in pharyngeal neurons and in the reproductive system [76, 78]. Interestingly, overexpression of *prdx-2* in the intestine increases oxidative resistance but it does not prolong lifespan [78], indicating that the oxidative stress resistance obtained by tissue-specific *prdx-2* expression does not determine lifespan.

Analysis of the *C. elegans* genome reveals four glutare-doxins (GLRXs), at least eight thioredoxins (TRXs), and two thioredoxin reductases (TRXRs). Deletion of the cytosolic *trx-1*, expressed in specific pharyngeal neurons and the intestine, decreases lifespan with 19% and increases paraquat sensitivity [79, 80]. Furthermore, *trx-1* deletion partially suppresses lifespan extension in *daf-2* mutants and completely suppresses longevity induced by two forms of dietary restriction [81]. Overexpression of *trx-1* results in a moderate lifespan increase [80]. Deletion of mitochondrial *trx-2* and/or *trxr-2*, expressed in muscles, intestine, and neurons, does not affect lifespan in wild type and *daf-2* mutants and has no effect on heat shock and oxidative stress resistance [82]. Deletion of the methionine sulfoxide reductase, encoded by *msra-1*, reduces paraquat resistance and shortens lifespan of wild type and *daf-2* mutant worms [83].

C. elegans contains 57 genes that encode for glutathione-S-transferase (GST). *gst-4* is upregulated by oxidative stress, and although its overexpression enhances oxidative stress resistance, it does not extend lifespan [84]. GST-10 catalyzes the detoxification of the lipid peroxidation end-product HNE. *gst-10* overexpressors are more resistant to various forms of stress (heat, UV, and oxidative stress) and have an increased lifespan [85]. RNAi of 5 of the 26 tested *gst* genes causes an increase in HNE-mediated damage, while RNAi of only 2 of these *gst* genes (*gst-5* and *gst-10*) decreases lifespan [86]. This suggests that an increase in HNE-induced damage is not sufficient to reduce lifespan.

Iron-catalyzed ROS generation can increase protein damage and reduce oxidative stress resistance without affecting

lifespan [87]. Similarly, overexpression of the iron storage protein ferritin, *ftn-1*, reduces protein carbonylation and enhances oxidative stress resistance, but does not increase lifespan. These results suggest that high iron levels can increase oxidative stress, but iron levels under standard culture conditions do not limit lifespan. Moreover, deletion of *ftn-1* even increases lifespan and dauer formation of *daf-2* mutants, indicating a role for ferritin in IIS.

In general, deletion of some but not all antioxidant genes shortens lifespan to a minor extent. In some cases, loss of antioxidant enzymes can increase oxidative stress sensitivity without affecting lifespan and in some reports, it can even enhance longevity. Overexpression of a few antioxidant genes can moderately increase lifespan, but not necessarily increases oxidative stress resistance. In other instances, overexpression of antioxidant enzymes reduces lifespan. Therefore, genetic interventions in antioxidant defenses do not generally support the oxidative stress theory of aging in *C. elegans*.

Does this limited effect of antioxidants on aging in *C. elegans* support studies in higher model organisms, such as *Drosophila melanogaster* and mice (for review, see [7, 88])? Similar to worms, mice display a slightly reduced lifespan upon loss of CuZnSOD [89], while in the fruit fly, loss of CuZnSOD dramatically reduces lifespan [90, 91]. Heterozygous CuZnSOD^{+/−} *Drosophila* has an unaltered lifespan, however [90]. Unlike *C. elegans*, loss of MnSOD is lethal to mice and flies [92–94], but heterozygotic MnSOD mutation has no effect on aging in mice. Interestingly, these heterozygous mice show a 100% increase in tumor incidence [95]. Thus, antioxidant genetic alteration can induce oxidative stress and pathology without affecting lifespan. Overexpression of CuZnSOD, MnSOD, catalase, and glutathione peroxidase does not prolong lifespan in mice, although oxidative stress resistance is increased [88, 96]. It was verified with various parameters, such as mean and maximum lifespan, body weight, and fecundity, that the mice were maintained under optimal health conditions, to avoid stress conditions which may affect lifespan of mice with altered antioxidant defense. In flies, lifespan is not changed or increased in SOD overexpressors, depending on the study [97–102]. This discrepancy between studies can be a result of artifacts, such as transformation method or genetic background [103, 104]. To conclude, like in *C. elegans*, antioxidant studies in other model organisms do not generally support the oxidative stress theory of aging.

2.3. Redox Signaling Theories on Aging in C. elegans. Since evidence against the oxidative stress theory of aging is accumulating, a few theories have been recently proposed to explain the correlation between ROS and aging. One alternative for the oxidative stress theory of aging is the redox stress hypothesis, which proposes to include a signaling role for ROS in the aging process. It states that functional loss during aging is caused by a progressive pro-oxidizing shift in the redox state of the cell. This leads to overoxidation of redox-sensitive proteins and consequently the disruption of redox-regulated signaling mechanisms [10]. Figure 1 represents an overview of signaling pathways regulated by ROS that we describe below in more detail.

While high concentrations of the $O_2^{\bullet-}$ generators juglone and paraquat are lethal, a life-long exposure to low concentrations of these compounds can extend lifespan [27, 105, 106]. Exposure to low doses of juglone enhances an antioxidant defense (increased GSH levels, SOD, catalase activity, and *sod-3* and *hsp-16.2* expression). In accordance with these findings, DAF-16, SIR-2.1, and 14-3-3, proteins involved in stress response, are required for the lifespan extension in worms exposed to low concentration of juglone [106]. In short, these results indicate that increased ROS production promotes a stress response, mediated by DAF-16, SIR-2.1 and the redox-dependent transcription factor SKN-1, and thereby extends lifespan. This phenomenon is called hormesis [107] and has also been observed in worms with decreased glucose metabolism, where it is described as mito-hormesis more specifically [46]. Reduced glycolysis increases mitochondrial ROS production, which in turn increases oxidative stress resistance and catalase activity, prolonging lifespan [46]. However, pretreatment with antioxidants and vitamins prevents this lifespan extension, demonstrating that the increased ROS formation is an essential signal to activate lifespan extension. Consistent with these findings and the redox stress hypothesis, inhibition of glycolysis enhances stress resistance in *C. elegans* by activating the pentose phosphate pathway, which is crucial for maintaining the reducing cytosolic NADPH concentration [108]. More recently, it was found that impairment of *daf-2* reduces glucose uptake and induces a transient drop in ATP levels, thereby activating the energy sensor AAK-2. In its turn, AAK-2 mediates the generation of a transient ROS signal that ultimately promotes L-proline catabolism and partially extends lifespan. As a result, oxidative phosphorylation is enhanced and ROS levels are increased. This increase in ROS levels induces adaptive response that is partially mediated by the Nrf/SKN-1 transcription factor, resulting in enhanced stress resistance and extended lifespan [109].

Mitohormesis also plays a role in longevity of mutants with an impaired mitochondrial metabolism, or *mit* mutants. Some *mit* mutants (*clk-1*, *isp-1*, and *nuo-6*) show increased ROS production [27, 105, 110], an enhanced *sod* expression [30, 111, 112], and catalase activity [113]. While the increase in antioxidant defenses is dispensable for their long lifespan [30, 74], the increase in ROS levels is a prerequisite for *isp-1* and *nuo-6* [27] longevity, perhaps by provoking an adaptive response. The hypoxia-inducible transcription factor HIF-1 is activated by mild increases in ROS and HIF-1 is required for the extended lifespans of *clk-1* and *isp-1* mutants [105]. Thus, HIF-1 appears to link respiratory stress in the mitochondria to a nuclear transcriptional response that promotes longevity. Recently, it was shown that inhibition of mitochondrial respiration triggers the mitochondrial unfolded protein response (UPR[mito]), increasing *hsp-6* expression [112]. This response is needed for the longevity in *mit* mutants and acts through unidentified signaling molecules, maybe ROS, between different tissues [114]. Like in *mit* mutants, UPR is essential for the longevity in *sod-1* overexpressors [63].
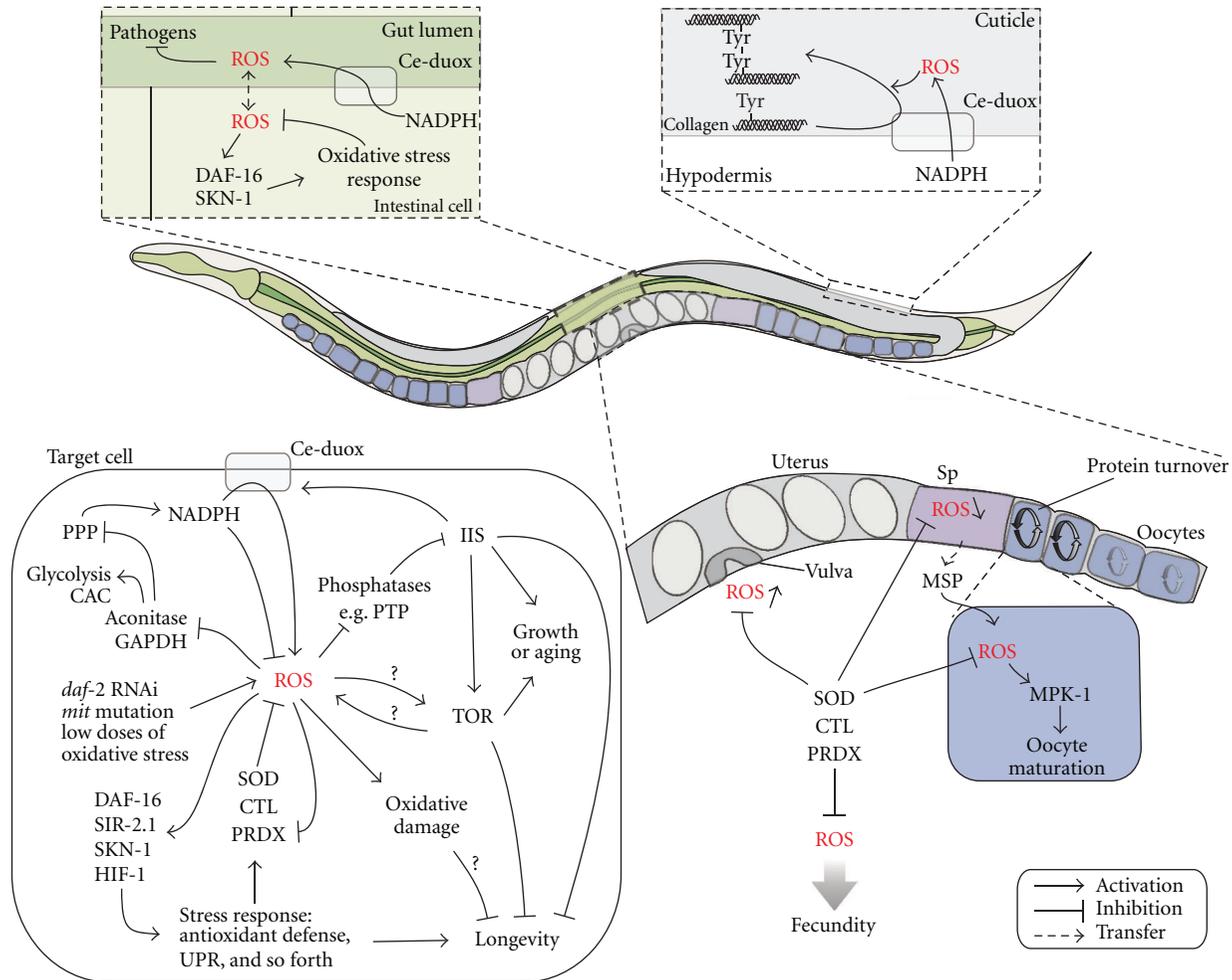
Figure 1: Schematic representation of signaling pathways regulated by ROS in *C. elegans*. PPP: pentose phosphate pathway; CAC: citric acid cycle; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; Sp: spermatheca.

To conclude, according to the (mito)hormesis theory, ROS can be regarded as a molecular signal that triggers a hormetic effect, inducing an overall increase in stress resistance and longevity. The specific antioxidant response may be important to maintain a reduced environment that remains sensitive to subsequent ROS signals.

Mammalian studies have reported that ROS (H_2O_2) activates the regulator of cell growth and proliferation, TOR, and its target S6 kinase [115, 116]. Moreover, TOR activation increases ROS production [117], whereas TOR inhibition reduces ROS production [118]. According to the TOR-centric model of aging, these multiple links between ROS and TOR indicate that ROS is a messenger molecule rather than a toxic byproduct, that accumulates life-limiting damage [119]. This TOR-centric model postulates that TOR is necessary during development, but that it is not switched off in adults. As a result, cells become hypertrophic and hyperactive. This hyperfunction causes cellular damage and age-related diseases [120]. According to this model, TOR limits lifespan by accelerating age-related diseases before oxidative damage accumulation can cause death.

2.4. Mechanisms of Redox Signaling in C. elegans.

While the theories above can explain how ROS influence aging in another way than by causing oxidative damage, they do not provide details on how ROS can transfer a signal in a cellular environment. A few specific mechanisms of ROS or redox signaling has been described in *C. elegans* and are discussed below.

Protein-tyrosine phosphatases (PTP) contain a reduced cysteine residue that is a well-characterized target of ROS. PTP is oxidized by H_2O_2 to the sulfenic (–SOH) inactive form, which can be reversed by cellular thiols. The oxidized sulfenic acid rapidly reacts with amide nitrogen of its protein backbone generating inactive sulfenyl-amide, to prevent further irreversible oxidation to sulfonic (–SO_2H) and sulfinic species (–SO_3H). Inactivation of phosphatases can also occur by glutathionylation. Reactivation of the glutathiolated PTP may be catalyzed by glutaredoxin (GLRX) [73]. Other potential redox-sensitive phosphatases are the PTEN homolog DAF-18, the PP2A homolog PPTR-1, and the MKP-1 homolog VHP-1 [73].

In mammalian studies, insulin is found to stimulate H_2O_2 production by NADPH oxidase [121, 122]. H_2O_2 inactivates phosphatases which in turn negatively regulate insulin signaling. This leads to a positive feedback loop: ROS generated in response to insulin facilitate insulin signaling. In *C. elegans*, loss of *sod-4* enhances longevity and loss of either *sod-4* or *sod-5* enhances dauer formation in *daf-2* mutants [29], providing evidence for redox-sensitive regulation of IIS in *C. elegans*. Moreover, *sod-5* is specifically expressed in neurons involved in dauer formation in a *daf-16*-dependent manner [6, 96]. SOD-4 and SOD-5 generate extracellular and cytosolic H_2O_2, respectively, which can inactivate phosphatases, such as protein tyrosine phosphatase (PTP), thereby promoting IIS [73]. Therefore, SOD-4 and SOD-5 may facilitate a rapid exit from the dauer larvae stage to ensure further development and offspring during optimal conditions.

Another study that indicates a possible signaling function for ROS in *C. elegans*, describes redox changes in a limited set of proteins upon H_2O_2 treatment. A short-term H_2O_2 treatment causes immediate and reversible behavioral changes, such as reduced mobility, pharyngeal pumping, and reproduction, as well as decreased growth rate, and decreased ATP levels [77]. By using the redox proteomic technique OxICAT, 40 different proteins with redox-sensitive cysteines were identified which are involved in mobility and feeding (oxidative inactivation of MYO-2), protein translation (oxidative inactivation of EFT-1), protein homeostasis (oxidative activation of HSP-1), and ATP regeneration. Proteins involved in glycolysis are oxidatively inactivated, thus, redirecting glucose to the pentose phosphate pathway, which results in enhanced NADPH levels. These observations coincide with the finding that reduced glycolysis enhances oxidative stress resistance [46, 108]. Thus, oxidative stress induces oxidative modifications of specific redox-sensitive proteins to reduce energy consuming processes which are not essential for survival, such as protein synthesis and movement, thereby saving energy to restore redox homeostasis.

2.5. Functional Roles for ROS in C. elegans. Rather than being purely harmful, it has been established that ROS can also play an important role in metabolism. Here we provide a few examples of how ROS contribute to *C. elegans* metabolism, and therefore may be crucial for maintaining a normal lifespan.

Most organisms, including humans, *D. melanogaster*, and plants, produce ROS in phagocytic and nonphagocytic cells via NADPH oxidase in response to microbial infection [123–125]. *C. elegans* also produces extracellular ROS by a dual NADPH oxidase (CeDuox-1) in response to exposure to gut-infecting pathogenic bacteria *Enterococcus faecalis* or yeast [58, 126–128]. These studies suggest that *C. elegans* produces ROS in the intestine in response to pathogens while an oxidative stress response mediated by DAF-16 and the redox sensitive transcription factor SKN-1 is induced to protect neighbouring tissues. [58].

Another function for ROS in *C. elegans* metabolism, mediated by NADPH oxidase, is cuticle biogenesis. *C. elegans* encodes two dual oxidases, Ce-Duox1 (*bli-3*) and Ce-Duox2, which contain a NADPH oxidase domain and a peroxidase domain. The NADPH oxidase domain generates extracellular superoxide which dismutates spontaneously or by SOD-4 to H_2O_2. This peroxide is then used by the peroxidase domain for the cross-linking of tyrosine residues in collagen to create the cuticle of the worm [129].

The redox state of the cell has also been shown to regulate physiological processes during development, such as proliferation and differentiation [130]. Generally, a more reducing environment is associated with proliferation and an oxidizing environment initiates differentiation [131]. Consistent with mammalian studies, where GSH content is high during gametogenesis and fertilization [132], we find that in the spermatheca, where the oocytes are fertilized, GSSG/GSH levels are low [133]. Moreover, *in vivo* H_2O_2 levels are low and *sod-1* is highly expressed in the spermatheca (unpublished data). It was also reported that protein carbonylation levels are low in spermatheca as they are abruptly reduced by enhanced proteasome activity in the ovaries at the time of oocyte maturation [134]. Before oocytes can be fertilized in the spermatheca, they need to mature, a process that is characterized by their transition into the meiotic metaphase I [135]. The major sperm protein (MSP) secreted by the sperm in the spermatheca promotes ROS production in the most proximal oocytes, which augments MPK-1 activity, essential for this oocyte maturation. SOD-1 is found to inhibit MPK-1 activation [136]. Thus, ROS can act as a secondary messenger for oocyte maturation necessary for fertilization, while these oocytes are cleared from oxidative damage by enhancing proteasomal activity [134].

3. ROS and Redox Detection

A major challenge in establishing the exact function of ROS in metabolism and aging is their accurate detection. Because redox signaling acts through small, local, and transient changes, redox detection should ideally be selective, sensitive, instantaneous, reversible, compartment-specific, noninvasive, and applicable *in vivo*. Most conventional redox measurements are nonspecific, disruptive (which may create oxidation artifacts), irreversible (which precludes dynamic measurements), and some probes can generate ROS by themselves upon radiation (e.g., H_2DCF-DA) [137–139]. These exogenous chemical absorbent, fluorescent or luminescent probes need to be taken up by the biological model. This uptake is rather poor and variable in *C. elegans*, making comparison between samples complicated. In addition, the uptake of these chemical reporter molecules may not be identical for each subcellular compartment. Recently, small-molecule probes were designed to be targeted to the mitochondria to ensure the probe uptake into the mitochondria [140–142]. An extensive review on these chemical probes can be found elsewhere [137–139].

Many of the limitations of conventional redox probes were overcome by the development of genetically encoded

redox sensors based on the green fluorescent protein GFP. Generally, these chimeric proteins contain a regulatory domain that will specifically and reversibly bind the ROS or metabolite of interest, resulting in a conformational change altering the fluorescent properties of the biosensors. As a result, the fluorescent properties of the molecules are a direct measure of the levels of the ROS or metabolite *in vivo*. A major advantage of these genetically encoded sensors is that they can be targeted to specific tissues, cells or subcellular locations [143]. Hence, when choosing an accurate promoter for expression of the biosensor, real-time and *in vivo* analysis ranging from a specific single cell to a whole organism can be easily performed. Because of its genetic amenability and its transparency, *Caenorhabditis elegans* is ideally suited for such approaches, while its size allows analysis by both microplate fluorometry and confocal microscopy. A few examples of genetically encoded sensors that we successfully implemented to measure H_2O_2 levels and GSH in *C. elegans* will be further discussed.

H_2O_2 has emerged as a widespread, physiologically relevant and selective signaling molecule [144–146], and it is relatively, abundant (1–100 nM) [145] and stable compared to other ROS. These features allow H_2O_2 to serve as an important second messenger. HyPer is a H_2O_2 (hydrogen peroxide)-specific intracellular biosensor. This sensor consists of a circularly permutated yellow fluorescent protein (cpYFP) inserted into the H_2O_2-sensitive regulatory domain of the *Escherichia coli* transcription factor OxyR (OxyR-RD) [147]. HyPer is a H_2O_2-sensitive, selective, and reversible biosensor, and its fluorescence ratio reflects the balance between H_2O_2-mediated oxidation and a GLRX/GSH-mediated reduction. Like most cpYFP's, HyPer fluorescence is influenced by pH (6–10): acidification decreases the 500 nm/420 nm excitation ratio [147]. HyPer has already been used in various cell culture [147–151] and zebra-fish [152] studies. Using HyPer, we identified local cells or tissues with distinct *in vivo* H_2O_2 levels in *C. elegans*. For example, we found that, consistent with the role of hypodermal cells in cuticle biogenesis, H_2O_2 levels are particularly high in these cells [133] (Figure 2).

The redox state of the cell is determined by the redox state of multiple redox pairs in the cell. Because of the high intracellular GSH concentration (1–11 mM) and the high GSH/GSSG ratio (≥ 100) [131], the redox state of the glutathione couple is a good proxy for the total redox state of the cell. The new generation of redox-sensitive sensors are the redox-sensitive GFP's (roGFP), such as roGFP2. These biosensors are ratiometric by excitation, thus minimizing measurement errors due to variable *in vivo* probe concentrations and photobleaching [153]. They are able to detect small changes in GSSG within a highly reduced glutathione pool (GSH/GSSG ≥ 100) [131]. The fusion of human glutaredoxin-1 (Grx1) to roGFP2 makes the redox response of the probe faster and more glutathione specific [154, 155]. Ubiquitous overexpression of this sensor in *C. elegans* shows a reduced state (low levels of GSSG/GSH) of the spermatheca (Figure 3).

Consistent with the redox hypothesis of aging, both Hyper and Grx-roGFP2 strains show an increase in H_2O_2
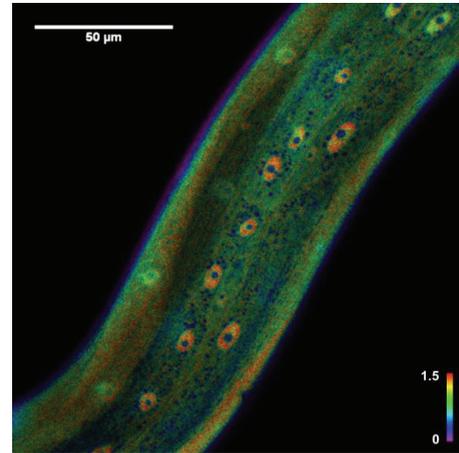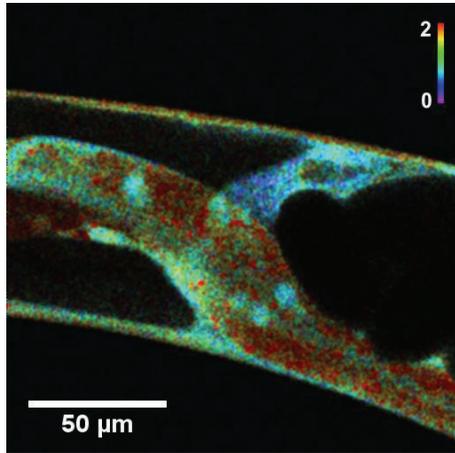

(a)
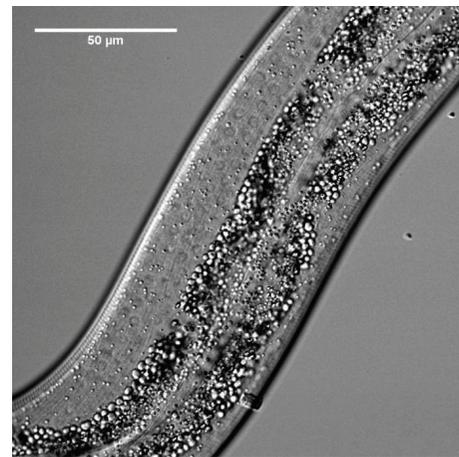
(b)

FIGURE 2: (a) Intensity normalized ratio image of a HyPer transgenic worm. Color represents oxidized/reduced HyPer ratio values, while color saturation represents fluorescence intensities. Hypodermal nuclei clearly show increased levels of H_2O_2. (b) Corresponding brightfield image. Construction of transgenic strains, confocal microscopy, and image analysis was performed as described in [133].

and GSSG/GSH with age. In dietary restricted populations, this increase is attenuated [133].

The fusion of the yeast peroxidase Orp1 (also known as Gpx3) to roGFP2 converts roGFP2 into a specific probe for H_2O_2 [156]. H_2O_2-specific oxidation of Orp1 induces the formation of a disulfide bridge in roGFP2. roGFP2-Orp1 can be selectively and reversibly oxidized by H_2O_2 [156] and reduced by thioredoxin (TRX) or glutaredoxin (GLRX) [155]. Like all other roGFP-based probes, this probe is independent of pH (5.5–8.0) [157]. roGFP2-Orp1 has been already applied in cell cultures [156] and *D. melanogaster* [158].

The last few years, many more *in vivo* ROS, redox, and metabolic sensors have been developed [147, 154, 156, 159–164], and their use and limitations have been reviewed recently [155]. It is very likely that this booming research

(a)
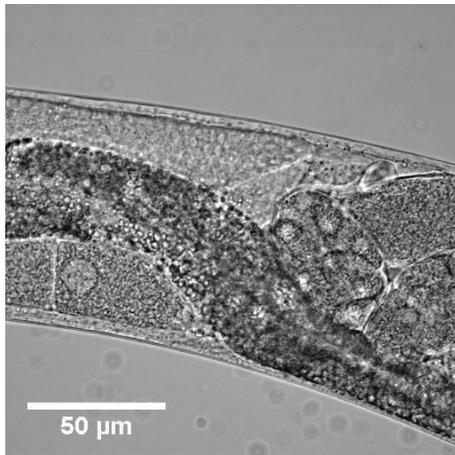
(b)

FIGURE 3: (a) Intensity normalized ratio image of a Grx1-roGFP2 transgenic worm. Color represents oxidized/reduced Grx1-roGFP2 ratio values, while color saturation represents fluorescence intensities. Spermatheca show clearly reduced GSSG/GSH ratios. (b) Corresponding brightfield image. Construction of transgenic strains, confocal microscopy, and image analysis was performed as described in [133].

field will yield more accurate, sensitive, and selective redox sensors in the near future.

4. General Conclusion

In recent years, there is mounting evidence against the oxidative stress theory of aging in *Caenorhabditis elegans*. Many intervention studies altering its antioxidant system have been performed, and most of them failed to support this theory. Gradually, it has become clear that a general increase in oxidative stress does not limit lifespan in this model organism. However, in the stressful conditions of its natural habitat oxidative stress resistance might be important to ensure a normal lifespan and reproduction. Although it cannot be ruled out that specific targets (proteins, lipids, or nucleic acids) accumulate some type of damage over age,

and thereby contribute to the aging process, a global rise in oxidative damage is clearly not a major factor determining lifespan. In contrast, there is a growing body of evidence that ROS and redox signaling may be important in the aging process, and that ROS may exert essential functions in metabolism. However, to fully understand the mechanisms of ROS and redox signaling, it is crucial that the molecular details, the localization, and the regulation of the specific reactive oxygen species involved can be analyzed accurately. This will allow a critical validation of the latest aging theories that try to explain the correlation between ROS and aging. To this end, the recent development and implementation of genetically encoded biosensors is a promising tool that we believe will be highly valuable to further explore ROS and redox biology in aging *C. elegans*.

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