Dayong Wang

Molecular Toxicology in *Caenorhabditis elegans*

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Preface

Model animal *Caenorhabditis elegans* has been successfully used in toxicological study of various environmental toxicants or stresses. The basic knowledge system on model organism toxicology has been gradually established in *C. elegans*. With engineered nanomaterials as typical environmental toxicants, this has been described in the published book *Nanotoxicology in Caenorhabditis elegans*. In *C. elegans*, besides the cellular and developmental processes, the molecular events and the related signaling pathways are conserved to a great degree with those in mammals and in human beings. More importantly, *C. elegans* has been proven to be very sensitive to environmental exposures and can be employed to assess the potential toxicity of environmental toxicants at environmentally relevant concentrations. As a classic model animal, *C. elegans* has the well-described genetic and developmental backgrounds and the rich and available genetic resources based on the past half of century study. These backgrounds provide the strong basis for our establishing the knowledge system of molecular toxicology in nematodes. During the past 20 years, a large amount of data on the molecular toxicology has been obtained in *C. elegans*.

C. elegans is a tiny model animal. With the aid of a series of sensitive sublethal endpoints, it has the potential for understanding the response to environmental toxicants or stresses or the toxicity induction by environmental toxicants or stresses at the whole animal level. Therefore, the introduced knowledge system on the molecular toxicology in this book has been mainly organized at the whole animal level in nematodes.

In this book, we have raised three important concerns. The first concern is the definition and the association between molecular basis for toxicity in environmental toxicant- or stress-exposed nematodes and the molecular basis for transgenerational toxicity of environmental toxicants and stresses. The second concern is the definition and the association between protective molecular response to environmental toxicants and stresses and molecular basis for toxicity induction of environmental toxicants or stresses. The third concern is the definition and the association between molecular signals and epigenetic signals required for the regulation of toxicity of environmental toxicants or stresses.

Based on these concerns, in Chaps. 1 and 2, we first introduced the molecular basis for oxidative stress and reduced lifespan caused by environmental toxicants or stresses. In Chaps. 3, 4, 5, 6, 7 and 8, we introduced some important signaling pathways (oxidative stress-related, MAPKs, insulin and the related, development-related, cell death and DNA damage-related, and metabolism-related) involved in the regulation of toxicity of environmental toxicants or stresses. In Chaps. 9, 10, 11 and 12, we introduced the important roles of protective response signals, G-protein-coupled receptors and ion channels and downstream cytoplasmic signals, specific molecular signals, and epigenetic regulation signals in regulating the toxicity of environmental toxicants or stresses. In Chap. 13, we introduced and discussed the values and the limitations of normally used strategies to screen and to identify new genetic loci involved in the regulation of toxicity of environmental toxicants or stresses. Finally, in Chaps. 14 and 15, we introduced the molecular basis for adaptive response and transgenerational toxicity induced by environmental toxicants or stresses.

Nanjing, China Dayong Wang

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Chapter 1
Molecular Basis for Oxidative Stress Induced by Environmental Toxicants in Nematodes

Abstract Oxidative stress plays an important role in the toxicity induction of environmental toxicants or stresses in nematodes. Usually, this is the first cellular mechanism needed to be clarified for the toxicity formation of certain environmental toxicants or stresses. We here systematically introduced both the molecular machinery for the activation of oxidative stress and the response signals with the functions to defend against the oxidative stress in nematodes. Moreover, we explained the molecular basis for the induction of oxidative stress in nematodes exposed to environmental toxicants.

Keywords Molecular basis · Oxidative stress · Environmental toxicant · *Caenorhabditis elegans*

1.1 Introduction

Oxidative stress is normally considered to play a pivotal role in the toxicity induction of environmental toxicants or stresses in organisms, including the nematodes. That is, induction of oxidative stress acts as an important cellular mechanism for the toxicity formation of environmental toxicants or stresses in nematodes. Usually, the first cellular mechanism for the toxicity formation of certain environmental toxicants or stresses needed to be clarified is to determine the association between the toxicity formation of examined environmental toxicant or stress and the induction of oxidative stress.

With the concern on the important role of oxidative stress in toxicity induction of environmental toxicants or stresses, we here first introduced the evidence for the direct association of oxidative stress with toxicity of environmental toxicants in nematodes. Moreover, we systematically introduced both the molecular machinery for the activation of oxidative stress and the response signals with the functions to defend against the oxidative stress in nematodes. Finally, we introduced and explained the molecular basis for induction of oxidative stress in nematodes exposed to environmental toxicants.

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1.2 Evidence for the Direct Association of Oxidative Stress with Toxicity of Environmental Toxicants

1.2.1 Induction of Oxidative Stress in Targeted Organs of Environmental Toxicants

With the heavy metal of Hg as an example of environmental toxicants, the toxic effects of Hg exposure on development of male nematodes were examined. Acute exposure to Hg (from L3 larvae for 24 h) at the concentration of 0.5 mg/L significantly reduced the number of rays formed surrounding the tail [1]. Acute exposure to Hg at the concentration of 9.8 mg/L further severely decreased the number of rays [1]. Moreover, acute exposure to Hg at the concentration of 19.3 or 29.5 mg/L severely reduced both the number of rays and the size of fan in male nematodes [1]. In nematodes exposed to Hg at the concentration of 39.7 mg/L, no rays and no obvious fan could be observed in male nematodes [1]. Meanwhile, the obvious induction of reactive oxygen species (ROS) production was detected in the fans of tails in male nematodes after exposure to Hg at concentrations more than 0.5 mg/L (Fig. 1.1) [1]. The strong induction of ROS production was also observed in the intestine of male nematodes exposed to Hg at concentrations more than 0.5 mg/L (Fig. 1.1) [1]. These results imply the close correlation of induction of ROS production in the fans of tails with the formation of abnormal male-specific structures in nematodes after Hg exposure.

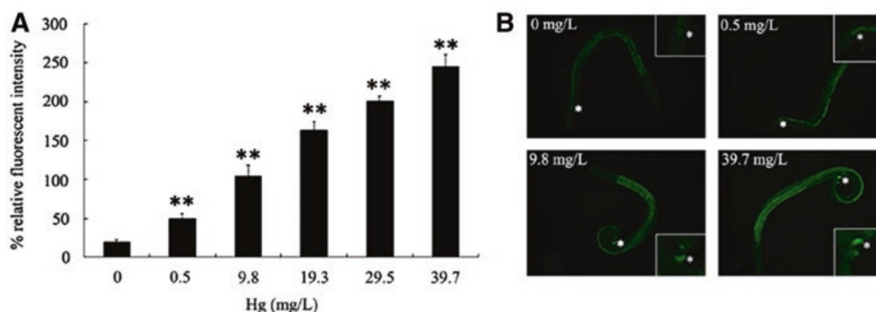

Fig. 1.1 ROS production in male nematodes exposed to Hg [1]. (**a**) ROS production in tails of male nematodes exposed to Hg at different concentrations. Bars represent means ± SEM. $^{**}P < 0.01$ vs 0 mg/L. (**b**) Representative images of ROS production. Asterisks indicate the position of male tails

1.2.2 Pharmacological Evidence for the Association of Oxidative Stress and Toxicity Formation of Environmental Toxicants

Antioxidant administration is a powerful strategy used for decreasing the free radical-induced oxidative damage. To confirm the direct association between the induction of oxidative stress and the toxicity of Hg exposure on the development of male-specific structures, the male nematodes were pretreated with vitamin E (200 mg/mL), a potent antioxidant, for 24 h at the L2-larval stage. After that, the male nematodes were exposed to Hg (9.8 mg/L) for 24 h at the L3-larval stage. Vitamin E treatment alone cannot induce the obvious ROS production in male tails [1]. Pretreatment with the vitamin E could obviously prevent the induction of severe ROS production in fans in tails of nematodes exposed to Hg (9.8 mg/L) (Fig. 1.2) [1]. Meanwhile, pretreatment with the vitamin E could obviously prevent the formation of high percentage of abnormal males and severe deficit in male-specific structures in tails in nematodes exposed to Hg (9.8 mg/L) (Fig. 1.2) [1]. This pharmacological data provides an important evidence for the direct association between the induction of oxidative stress and the Hg toxicity on development of male-specific structures in nematodes.

1.2.3 Association of Induction of Oxidative Stress in Intestine and Toxicity Formation in Other Targeted Organs

Besides the direct induction of oxidative stress in the targeted organs, another possibility also exists. That is, a close association of induction of oxidative stress in the intestine and toxicity formation in other targeted organs may be formed in nematodes. With multiwalled carbon nanotubes (MWCNTs), carbon-based engineered nanomaterials (ENMs), as an example, prolonged exposure (from L1-larvae to adult day 1) to MWCNTs at concentrations more than 0.1 μg/L could induce the significant intestinal ROS production (Fig. 1.3) [2]. Meanwhile, prolonged exposure to MWCNTs at concentrations more than 0.1 μg/L also significantly reduced the brood size (Fig. 1.3) [2]. Nevertheless, we did not detect the obvious induction of ROS production in reproductive organs of nematodes [2]. In nematodes, the reproductive organs such as spermatheca are the important secondary targeted organs for MWCNTs (Fig. 1.3) [2]. Therefore, the induction of oxidative stress in the intestine may potentially further contribute to the toxicity formation of environmental toxicants in other targeted organs in nematodes.

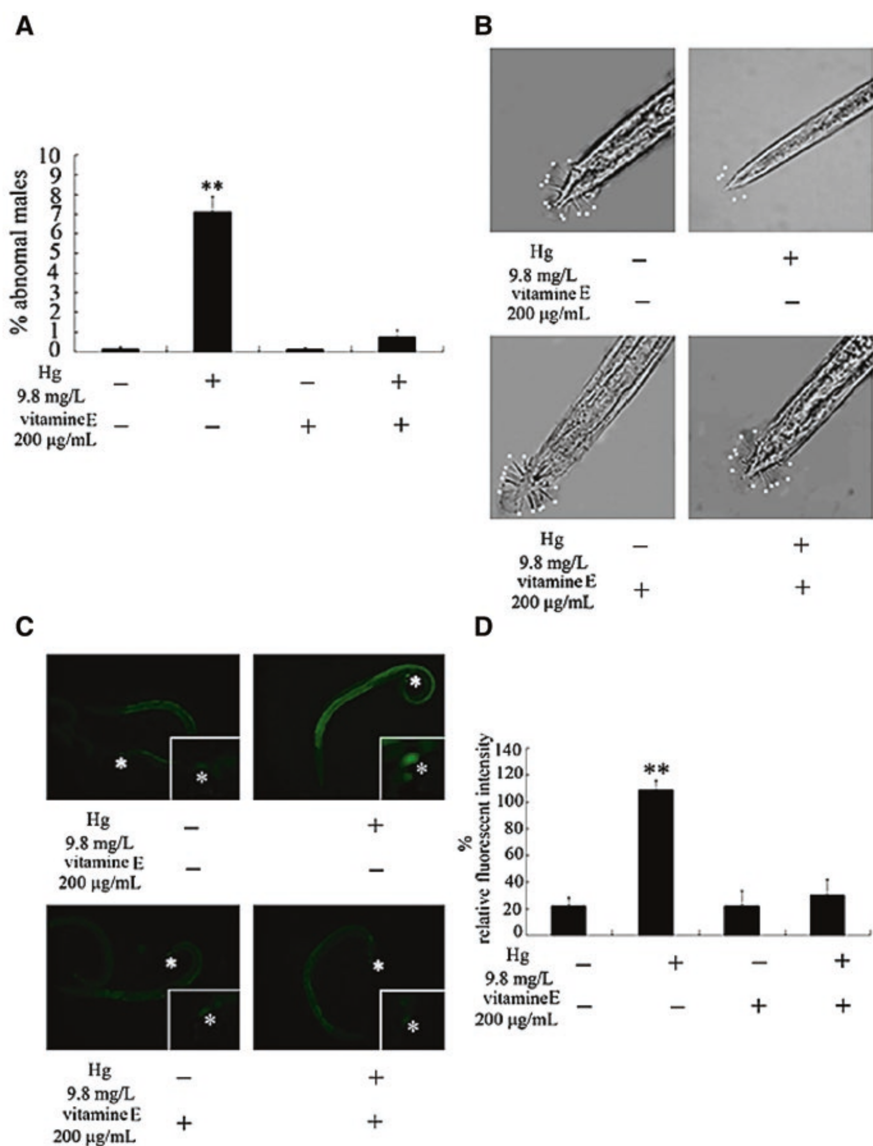

Fig. 1.2 Effects of vitamin E pretreatment on the development of male-specific structures in nematodes exposed to Hg [1]. (**a**) Effects of vitamin E pretreatment on the formation of abnormal males induced by Hg exposure. (**b**) Effects of vitamin E pretreatment on the development of male-specific structures induced by Hg exposure. (**c**) Representative images of ROS production. Asterisks indicate the position of male tails. (**d**) Effects of vitamin E pretreatment on the ROS production induced by Hg exposure. Bars represent means ± SEM. $^{**}P < 0.01$

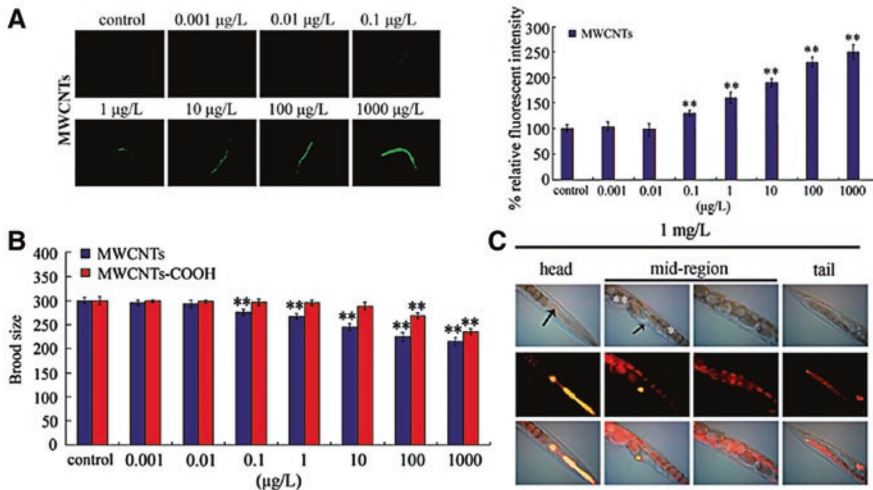

Fig. 1.3 Toxicity of MWCNTs on nematodes after prolonged exposure [2]. (**a**) Toxicity of MWCNT exposure in inducing intestinal ROS production. (**b**) Toxicity of MWCNT exposure in reducing brood size. (**c**) Distribution and translocation of MWCNTs labeled with Rho B. Arrowheads indicate the pharynx and the spermatheca, respectively, at the head region or mid-region of nematodes. The intestine (*) and the embryos (**) in the mid-region are also indicated. Prolonged exposure was performed from L1-larvae to adult day 1. Bars represent mean ± SEM. $^{**}P < 0.01$ vs control

1.3 Molecular Machinery for the Activation of Oxidative Stress in Nematodes

The superoxide anion ($O^{\cdot 2}$) can generate several types of toxic ROS, which will further lead to the toxic effects on nematodes at different aspects. In organisms, the major source of superoxide anion is the electron transport in mitochondrion. Electron transport is normally mediated by five complexes (complexes I–V) embedded in the inner membrane of the mitochondrion.

1.3.1 Role of GAS-1 in the Activation of Oxidative Stress

In nematodes, *gas-1* encodes a subunit of mitochondrial complex I. Mutation of *gas-1* did not affect the physical structure in mitochondria [3]. In contrast, mutation of *gas-1* decreased the complex I-dependent mitochondrial metabolism as indicated in alterations in the rates of both oxidative phosphorylation and electron transport [3]. The rates of oxidative phosphorylation were significantly decreased in *gas-1* mutant nematodes [4]. The *gas-1* mutant shows the hypersensitive property to oxygen and shortened lifespan [4].

In *gas-1* mutant nematodes, the significantly elevation in mitochondrial matrix oxidant burden was detected, which may be closely associated with the limited superoxide scavenging capacity and the decreased mitochondria content, as well as the membrane potential [5].

In nematodes, *gas-1* is mainly expressed in the mitochondria of neuromuscular system [3]. Importantly, it has been shown that GAS-1 can act at the presynaptic to regulate different biological processes [3].

In nematodes, *nuo-6* encodes another subunit of mitochondrial NADH dehydrogenase (ubiquinone) complex (complex I). However, mutation of *nuo-6* decreased the electron transport and increased the longevity [6]. The generation of superoxide was elevated in *nuo-6* mutant nematodes, although the overall ROS levels were not and the oxidative stress was low in *nuo-6* mutant nematodes [6]. More importantly, it is considered that this elevation is required for the increase in longevity, since this could be abolished by the antioxidants and phenocopied by mild treatment with prooxidant paraquat [6]. The increased superoxide generation may act as a signal in young *nuo-6* mutant to trigger the changes of gene expression so as to prevent or attenuate the effects of the subsequent aging [6]. That is, the superoxide generated in the *nuo-6* mutant may act as a protective signal in response to the damage during the aging process.

1.3.2 Role of MEV-1 in the Activation of Oxidative Stress

Mitochondrial succinate–ubiquinone reductase (complex II) functions to catalyze the electron transport from succinate to ubiquinone. This complex II contains succinate dehydrogenase (SDH), flavin protein, iron–sulfur protein, and two other subunits containing cytochrome *b*560. In nematodes, *mev-1* encodes an ortholog of succinate dehydrogenase cytochrome b560 subunit of the complex II that is required for the oxidative phosphorylation. SDH, the catalytic component of complex II, is normally anchored to the inner membrane of mitochondrion with the cytochrome *b*560. Nevertheless, the SDH activity in *mev-1* mitochondrial fraction was identical to that of wild-type nematodes [7]. In contrast, the complex II activity in the *mev-1* membrane fraction was significantly reduced by more than 80% [7]. These results imply that mutation of *mev-1* may affect neither the SDH anchoring to the mitochondrial membrane nor the SDH activity. The MEV-1 may potentially compromise the ability of complex II to participate in the electron transport. That is, cytochrome *b*560, the *mev-1* gene product, can participate directly into the transporting electrons from SDH to CoQ. The cytochrome *b*560 has three membrane-spanning domains, and the substitution of glutamic acid for glycine could affect the ability of iron to accept and relinquish the electrons.

The ability of complex II to catalyze the electron transport from succinate to ubiquinone is compromised in *mev-1* mutant nematodes, which may result in the indirect increase in the superoxide levels and the oxygen hypersensitivity, as well as

the premature aging [8]. At least for the longevity control, the evidence was raised that the MEV-1 may govern the aging rate by modulating the cellular response to oxidative stress [8]. In *mev-1* mutant nematodes, an overproduction of superoxide anion from the mitochondria and a reciprocal reduction in glutathione content were detected [8]. Although the superoxide anion is normally produced at complexes I and III in the electron transport system under normal conditions, the mutation of *mev-1(kn1)* could increase the superoxide anion production at the complex II itself (as indicated by an attendant decrease in glutathione levels) rather than at the complexes I and III [8]. Additionally, the *mev-1* mutant nematodes had metabolic changes as indicated by the lactate level with twofold higher than that in wild-type nematodes [8]. In nematodes, the Cyt-1/ceSDHC may play an important role not only in the energy metabolism but also in the superoxide anion production.

1.3.3 Role of ISP-1 in the Activation of Oxidative Stress

In organisms, the mitochondrial complex III functions to catalyze the electron transfer from ubiquinol to cytochrome c. The complex III contains three subunits, cytochrome b, the iron–sulfur protein, and cytochrome c1, to catalyze the redox reactions. In nematodes, *isp-1* encodes a “Rieske” iron–sulfur protein subunits of complex III of the mitochondrial respiratory chain. The prolines are important structurally to make the peptide backbone locally rigid, and *isp-1(qm150)* is a point mutation at residue 225 that changes the proline into a serine [9]. This mutation may affect the properties of the iron–sulfur center directly through a local distortion of the structure and the redox potential. The electron transfer from ubiquinol at its binding site on cytochrome b to cytochrome c1 is involved in the conformation of head of the ISP carrying the 2Fe-2S group [9].

In the *isp-1* mutant nematodes, the decrease in electron transport, the reduction in oxygen consumption, and the resistance to oxidative stress were observed [6, 9]. In *isp-1* mutant nematodes, the generation of superoxide was elevated [6]. The increased superoxide generation in *isp-1* mutant nematodes was also abolished by the antioxidants and phenocopied by mild treatment with the prooxidant paraquat [6]. Moreover, an increased lifespan was detected in the *isp-1* mutant nematodes [9]. Nevertheless, the effects of ISP-1 and DAF-2, an insulin receptor, on longevity were not additive in nematodes [9].

In nematodes, the elevated ROS levels in *isp-1* mutant nematodes may induce an activation of multiple stress response pathways [10]. These pathways include those of mitochondrial unfolded protein response, SKN-1-mediated stress response, and hypoxia response [10]. Mutation of *isp-1* could further increase the expression of specific antioxidant enzymes, such as the superoxide dismutase genes *sod-3* and *sod-5* [10]. Meanwhile, mutation of *sod-3* or *sod-5* decreased the lifespan and exacerbated the slow physiologic rates in *isp-1* mutant nematodes [10].

1.3.4 Role of CLK-1 in the Activation of Oxidative Stress

1.3.4.1 Mitochondrial CLK-1

In nematodes, *clk-1* encodes a ubiquinone (UQ) (coenzyme Q) biosynthesis protein COQ7. UQ is a lipophilic redox-active molecule and an electron carrier in the mitochondrial electron transport chain. Normally, the electron transfer via the UQ involves the formation of semi-ubiquinone radicals and the generation of superoxide radicals upon reaction with oxygen. In contrast, the UQ in the reduced form can serve as a lipid-soluble antioxidant to protect the cells from lipid peroxidation. In the *clk-1* mutant nematodes, no detectable levels of UQ could be observed [11]. Meanwhile, the UQ biosynthesis intermediate, demethoxyubiquinone (DMQ9), was present at a high level in *clk-1* mutant nematodes [11]. In the *clk-1* mutant nematodes, the DMQ9 may act as an electron carrier in the respiratory chain, since the activities of NADH–cytochromec reductase and succinate–cytochromec reductase were similar to those in wild-type nematodes [11].

In the *clk-1* mutant nematodes, the rates of oxidative phosphorylation were decreased; however, the lifespan was increased [12]. Moreover, it has been reported that mutation of *sod-2* resulted in the increase in lifespan in *clk-1* mutant nematodes; however, mutation of either of the two cytoplasmic *sod* genes, *sod-1* or *sod-5*, decreased the lifespan of *clk-1* mutant nematodes [12]. Additionally, the increase in mitochondrial superoxide levels by mutation of *sod-2* or treatment with paraquat could still cause the increase in lifespan in *clk-1; sod-1* double mutants [12]. These results imply that the elevated ROS in the mitochondria can act to increase lifespan, whereas the elevated ROS in the cytoplasm may decrease the lifespan of nematodes.

1.3.4.2 Nuclear CLK-1

Besides this, recently a distinct nuclear form of CLK-1 was further identified in nematodes. This nuclear CLK-1 may mediate a retrograde signaling pathway in response to the mitochondrial ROS by acting as a barometer of oxidative metabolism [13]. The nuclear CLK-1 can regulate both the mitochondrial ROS metabolism and the mitochondrial unfolded protein response by modulating the corresponding gene expression [13]. That is, the basal levels of ROS produced by the mitochondria may direct a pool of CLK-1 to the nucleus where it regulates the expression of genes associated with both the mitochondrial ROS metabolism and the mitochondrial unfolded protein response and lowers the ROS level [13]. The lowered ROS further results in the predominant localization of CLK-1 in the mitochondria and induces the return to basal ROS production, which will be helpful for maintaining the mitochondrial homeostasis [13]. Therefore, a respiratory enzyme exists in the nucleus to regulate both the mitochondrial stress responses and the longevity in nematodes.

1.4 Response Signals with the Functions to Defend Against the Oxidative Stress

1.4.1 Superoxide Dismutases (SODs)

The SOD activity can be detected in the extracts of nematodes, and the SODs have been well known to protect the cells or organisms from oxidative stress by catalytically removing the superoxide radical ($^{*}O^{2-}$). For example, dauer is a developmental state for larvae adapting the environmental stresses or starvation. The extracts of dauer larvae had 17.1 units SODase per milligram protein, whereas obligate larvae and young adults had 4.3 and 3.8 units SODase per milligram, respectively [14]. Additionally, the ratio of SODase to oxygen consumption was markedly increased in dauer larvae compared with that in young adults, implying that this elevated SODase might contribute to an increased resistance to certain environmental stresses [14].

1.4.1.1 SOD-1

In nematodes, *sod-1* encodes a cytoplasmic copper/zinc superoxide dismutase. The SOD-1 activity has been implicated in the increase in lifespan of dauer larvae, because its activity was the highest at this developmental stage compared with others [14]. In *sod-2* mutant, both the cytosolic $^{*}O^{2-}$ level and the mitochondrial $^{*}O^{2-}$ level were significantly increased [14], implying that both cytosolic SOD-1 and mitochondrial SOD-1 are required for the detoxification of $^{*}O^{2-}$ [15].

1.4.1.2 SOD-2 and SOD-3

In nematodes, *sod-2* and *sod-3* encode mitochondrial iron/manganese superoxide dismutases. Both SOD-2 and SOD-3 function to defend against the oxidative stress and to promote the normal lifespan [5].

Both *sod-2* expression and *sod-3* expression were diminished by mutation of *daf-16* encoding a FOXO transcriptional factor in the insulin signaling pathway. Nevertheless, the increased longevity was observed in *sod-2(ok1030)* or *sod-2(gk257)* mutant which has the decreased Mn-SOD scavenging capacity and increased mitochondrial matrix oxidant burden [5, 16]. In *sod-2* or *sod-3* mutant, the mild compensatory upregulation of other *sod* genes was even detected [5, 16]. Mutation of *sod-2* even increased the lifespan in *clk-1* mutant, but it clearly decreased the lifespan of *isp-1* mutant [16].

1.4.1.3 SOD-4

In nematodes, *sod-4* encodes an extracellular Cu^{2+}/Zn^{2+} superoxide dismutase. SOD-4 is expressed in the nervous system, intestine, and rectal gland cells. The *sod-4* expression was also significantly upregulated in dauers [14].

In nematodes, although the *sod* genes were not required for the longevity of *daf-2* insulin/IGF-1 receptor mutant, mutation of *sod-4* enhanced the longevity and the constitutive diapause in *daf-2* mutant [17].

1.4.1.4 SOD-5

sod-5 encodes a cytoplasmic Cu^{2+}/Zn^{2+} superoxide dismutase. The *sod-5* expression could be increased in *sod-1* mutant [5, 16], implying the possible functional compensation between SOD-1 and SOD-5. Both SOD-3 and SOD-5 may act as direct targets for DAF-16 due to the existence of DAF-16 binding element on the promoter regions for these genes.

1.4.2 Catalases (CATs)

In nematodes, *ctl-1*, *ctl-2*, and *ctl-3* encode the catalases, antioxidant enzymes that protect the cells from the damage of oxidative damage. CTL-1 is predicted to be a cytosolic catalase. CTL-2 is observed to be mainly located in the peroxisomes of intestinal epithelial cells. The altered peroxisome morphology was observed in *ctl-2* mutant nematodes [18] and suggests the possible changes in peroxisomal function, including increased ROS production.

CTL-1 and CTL-2 could be negatively regulated by DAF-2-mediated insulin signaling [18]. The *ctl-1* mutant has no obvious effect on either nematode aging or egg-laying capacity; however, the *ctl-2* mutant exhibits progeric phenotype and decreased egg-laying capacity [18]. Mutation of *ctl-2* could reduce the lifespan of long-lived *daf-2* or *clk-1* mutant and accelerates the onset of its egg-laying period [18].

1.4.3 Insulin Signaling

1.4.3.1 Role of Insulin Signaling in the Activation of Oxidative Stress

In the insulin signaling pathway, AGE-1 is the downstream target of insulin receptor DAF-2. Mutation of *age-1* was resistant to the oxidative stress [19]. Meanwhile, both the activity of (SOD) and the activity of catalase exhibited an age-dependent increase in *age-1* mutant [19], which suggests that the signaling cascade of

DAF-2-AGE-1 regulates the activation of oxidative stress by negatively regulating the SOD and catalase activities. It has been further found that the expression of *sod-3* encoding a Mn-SOD was increased by *daf-2* mutation, which is regulated by the insulin-like signaling pathway [20], suggesting that the DAF-2 may regulate the longevity and the oxidative stress by affecting Mn-SOD-associated antioxidant defense system. Nevertheless, in wild-type and *age-1* dauer larvae, only elevated levels of the SOD activity, but not of the catalase activity, could be detected [19].

The forkhead transcription factor DAF-16 is negatively regulated by the DAF-2-AGE-1 signaling cascade in the insulin signaling pathway. Under the normal conditions, the DAF-16 protein is normally located in the cytoplasm. However, under the oxidative stress or certain environmental stress, the DAF-16 would be translocated into the nuclei [21]. Additionally, the DAF-16 could be constitutively accumulated in the nuclei of *mev-1* or *gas-1* mutant nematodes even under the normal conditions, which could be recovered by the supplementation of the antioxidant coenzyme Q10 (Fig. 1.4) [21].

Besides the oxidative stress or the environmental stress, it has also reported that SOD-3, a superoxide dismutase regulated by DAF-16, could be induced in intestinal cells after the infection with pathogenic bacteria [22]. Moreover, both the SOD-3 and the CTL-2 were required for DAF-16-mediated resistance to infection with pathogenic bacteria [22]. Therefore, the nematodes may potentially respond to the pathogen infection by producing intestinal ROS while simultaneously inducing a DAF-16-dependent oxidative stress response to protect adjacent tissues from the damage from the infection [22].

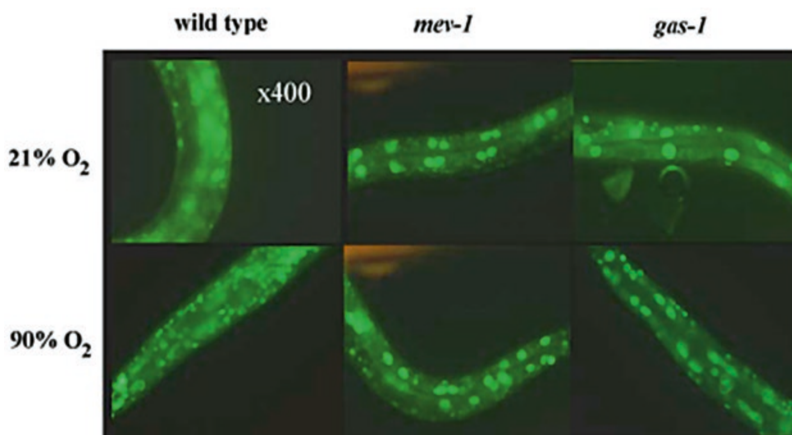

Fig. 1.4 Localization of DAF-16::GFP in wild type, *mev-1* and *gas-1* L2 larvae under atmospheric conditions (21% oxygen) or 90% oxygen [21]

1.4.3.2 Interaction Between β-Catenin/BAR-1 and DAF-16 in the Regulation of Oxidation Stress

In nematodes, β-catenin/BAR-1 is a transcriptional factor in the Wnt signaling pathway. It has been shown that mutation of *bar-1* could reduce the activity of DAF-16 in dauer formation and lifespan [23]. Moreover, BAR-1 was required for the oxidative stress-induced expression of SOD-3, a DAF-16 target [23]. The association of β-catenin with FOXO could be enhanced in cells exposed to the oxidative stress [23]. These observations demonstrate the important association of β-catenin with FOXO in the regulation of oxidative stress [23].

1.4.3.3 Identification of Upstream Regulator of DAF-2 in the Regulation of Oxidative Stress

In nematodes, mutation of *pcm-1* encoding a protein L-isoaspartyl methyltransferase induced a susceptibility to oxidative stress, since treatment with paraquat, a ROS generator, resulted in the more severe developmental delay at the second larval stage in *pcm-1* mutants than in wild-type nematodes [24]. This effect could be reversed by the administration with vitamin C, implying that the observed developmental delay and the egg-laying defects may be resulted from the oxidative stress [24]. Genetic interaction assay further indicated that mutation of *daf-2* could inhibit the Egl phenotype in *pcm-1* mutant treated with juglone, suggesting that the PCM-1 is involved in the control of cellular responses by acting an upstream regulator of DAF-2 in the insulin signaling pathway [24].

1.4.4 SKN-1/Nrf Signaling

1.4.4.1 Role of SKN-1 in the Regulation of Oxidative Stress

In nematodes, the SKN-1/Nrf plays a pivotal role in resisting the oxidative stress [25]. The *skn-1* mutants were sensitive to oxidative stress and showed the reduced longevity [25]. SKN-1 is expressed in both the ASI sensory neurons and the intestine. The SKN-1::GFP would be translocated and accumulated into the nucleus in response to the oxidative stress [25].

During the regulation of oxidative stress, the activity of SKN-1 is normally regulated by its phosphorylation modification. For the modification of SKN-1, it has been further suggested recently that SKN-1 could also be *O*-GlcNAcylated at Ser470 and Thr493 by *O*-GlcNAc transferase OGT-1. Under the condition of oxidative stress, SKN-1 was highly *O*-GlcNAcylated, which resulted in the decrease in GSK-3-mediated phosphorylation at Ser483 adjacent to the *O*-GlcNAcylated residues (Ser470 and Thr493) [26]. In nematodes, disruption of *O*-GlcNAc modification on SKN-1 could inhibit the SKN-1 accumulation in the intestinal

nuclei and decreased the activities of SKN-1 in modulating the lifespan and being against the oxidative stress [26]. That is, a cross talk between the phosphorylation and the *O*-GlcNAcylation for SKN-1 may exist in the regulation of oxidative stress and lifespan in nematodes.

1.4.4.2 Identification of Downstream Targets for Transcriptional Factor SKN-1 in the Regulation of Oxidative Stress

First of all, it has been well known that the SKN-1/Nrf can modulate the oxidative stress by regulating the phase II detoxification genes, such as *gcs-1* and *gst-4*, through constitutive and stress-inducible mechanisms during the postembryonic stages [25].

Besides this, it has been further suggested that the expressions of totally 810 genes could be controlled by the SKN-1/Nrf2 using the whole transcriptome RNA sequencing technique [27]. Among these genes, *nlg-1* encodes a synaptic cell adhesion molecule neuroligin, which acts as a direct target of SKN-1 [27]. Pharmacological treatments to induce the oxidative stress could increase the synaptic abundance of NLG-1 [27]. The increasing *nlg-1* dosage was correlated with the increased survival in response to the oxidative stress [27]. In contrast, genetic inactivation of *nlg-1* reduced the survival and suppressed the resistance of nematodes to oxidative stress [27]. That is, neuronal SKN-1 activation may potentially confer a protection mechanism for nematodes in response to environmental stresses by affecting the function of neuroligin [27].

1.4.4.3 Identification of Upstream Regulators for SKN-1 in the Regulation of Oxidative Stress

1.4.4.3.1 DAF-16

In nematodes, the *skn-1* expression could be activated by the DAF-16 in the insulin signaling pathway [28]. Nevertheless, the SKN-1 was required for the oxidative stress but not the increased lifespan induced by overexpression of DAF-16 [28]. Meanwhile, it was noted that the DAF-16 overexpression could rescue the short lifespan of *skn-1* mutants but not their susceptibility to oxidative stress [28]. Therefore, the function of SKN-1 in promoting longevity may through a different mechanism from that in protecting against the oxidative damage in nematodes.

1.4.4.3.2 PMK-1

PMK-1 is a p38 MAPK in the p38 MAPK signaling pathway, an integral part of the response of nematodes to a variety of environmental stresses. After exposure to the oxidative stress, the PMK-1 could phosphorylate the SKN-1 and causes the

translocation and accumulation of SKN-1 in the intestine nuclei, where SKN-1 activated the transcription of *gcs-1* encoding a phase II detoxification enzyme [29].

1.4.4.3.3 BLI-3

In nematodes, the ROS could be generated during infection by Duox1/BLI-3, a dual oxidase [30]. Meanwhile, bacterial pathogen infection increased the expression of SKN-1 in the intestine [30]. Mutation of *skn-1* decreased the resistance of nematodes to pathogen infection, whereas increasing SKN-1 activity would augment the resistance of nematodes to pathogen infection [30]. NSY-1, SEK-1, and PMK-1 in the p38 MAPK signaling pathway were all required for the activation of SKN-1 during the pathogen infection [30]. Moreover, it has been shown that the ROS produced by BLI-3 was the important source for SKN-1 activation via the p38 MAPK signaling pathway during the pathogen infection [30]. That is, ROS generation by BLI-3 may activate a protective SKN-1 response via the p38 MAPK signaling in pathogen-infected nematodes.

1.4.4.3.4 SKR-1/2 and WDR-23

In mammals, the Nrf2 is regulated in part by the redox sensor repressor protein of Keap1. In nematodes, new genes required for activation of the core SKN-1 target gene *gst-4* induced by treatment of juglone were identified using genome-wide RNAi screening [31]. Among these candidate regulators, mutation of *skr-1/2* encoding the homologs of yeast and mammalian Skp1 inhibited the induction of SKN-1-dependent detoxification genes and reduced the resistance of nematodes to prooxidants without decreasing the p38 MAPK activation [31]. Moreover, during the control of oxidative stress, SKR-1/2 further acted upstream of the WD40 repeat protein WDR-23, which binds to and inhibits the SKN-1 [31]. Therefore, the signaling cascade of SKR-1/2-WDR-23 is a novel p38 MAPK-independent signaling mechanism that activates the SKN-1 [31].

1.4.4.3.5 ELT-3

Using a transcription factor library to identify genes required for activation of SKN-1 target *gst-4* in *brap-2* mutants, ELT-3, a GATA transcription factor, was identified as a positive regulator of *gst-4p::gfp* expression [32]. In nematodes, the ELT-3 interacted with the SKN-1 to activate the *gst-4* transcription [32]. Moreover, ELT-3 was required for the lifespan extension of nematodes overexpressing the SKN-1 [32].

1.4.4.3.6 GSK-3

Under the normal conditions, phosphorylation by glycogen synthase kinase-3 (GSK-3) could prevent SKN-1 from the accumulation in nuclei [33]. If this inhibition was blocked, the background levels of p38 MAPK signaling were still required for the SKN-1 function. Therefore, GSK-3 inhibits SKN-1 activity in the intestine and influences redox conditions.

1.4.4.3.7 MDT-15

MDT-15, a subunit of the conserved mediator complex, was required for the oxidative stress responses [34]. More importantly, MDT-15 was required for SKN-1 expression [34]. MDT-15 was also required for the expression of genes in SKN-1-dependent and SKN-1-independent fashions downstream of insulin/IGF-1 signaling [34]. In nematodes, the MDT-15 directly binds to the SKN-1 through a region distinct from the classical transcription factor-binding KIX-domain [34].

1.4.4.3.8 MKK-4, IKKε-1, NEKL-2, and PDHK-2

Based on the RNAi suppression screen to identify additional kinases acting in the activation of SKN-1 in response to oxidative stress, four kinases, MKK-4, IKKε-1, NEKL-2, and PDHK-2, were further identified [35]. These kinases were required for the nuclear localization of SKN-1 in response to oxidative stress in nematodes [35]. Moreover, mutation of two of these kinase genes, such as *pdhk-2* and *nekl-2*, could result in a shorter lifespan and increased sensitivity to arsenite stress [35].

1.4.4.3.9 IRE-1

In nematodes, IRE-1, an endoplasmic reticulum (ER) transmembrane protein, has the function in maintaining the ER homeostasis by initiating unfolded protein response (UPRER) [36]. The ROS generated at the ER or by mitochondria could sulfenylate a cysteine within the IRE-1 kinase activation loop, which inhibited the IRE-1-mediated UPRER, initiated the p38 MAPK/SKN-1 antioxidant response, and increased the stress resistance and the lifespan [36]. That is, IRE-1 has an important function as a cytoplasmic sentinel that potentially activates p38 MAPK and SKN-1.

1.4.4.3.10 HCF-1

HCF-1, a host cell factor-1, is a regulator for DAF-16 in the regulation of both longevity and stress response [37]. Moreover, HCF-1 prevented the nuclear accumulation of SKN-1 and inhibited the transcriptional activation of SKN-1, as well as its targeted genes [37]. Additionally, the function of SKN-1 in enhancing oxidative stress resistance could be incurred by *hcf-1* mutation [37].

1.5 Systematic Identification of Novel Genes Required for the Regulation of Oxidative Stress

1.5.1 Functional Genomic Approach to Identify Novel Genes Required for the Regulation of Oxidative Stress

In order to perform this functional genome screen, synchronized L1-larvae of *rrf-3(pk1426)* strain were fed with bacteria expressing dsRNA corresponding to the examined genes [38]. Upon reaching to the L4 larval stage (day 0), the nematodes were treated with paraquat (80 mM), and then the survival of animals was monitored in every other day (from day 3 to day 15) [38]. The identified candidate genes are shown in Table 1.1. Based on this gene list, at least a partial functional overlap between the processes of ROS resistance and regulation of lifespan may exist. About ~30% of the aging genes identified here were involved in mitochondrial function [38]. These included those genes encoding components for the respiratory chain, mitochondrial ribosomes, ADP/ATP carriers, and molecules involved in mitochondrial protein synthesis (Table 1.1) [38].

1.5.2 Identification of Genes Required for the Regulation of Oxidative Stress or Stress Response Based on the Translocation of Environmental Toxicant

Besides the phenotypic analysis, another strategy to identify the genes required for the regulation of oxidative stress or stress response is based on the translocation of environmental toxicant, such as the graphene oxide (GO), a carbon-based nanomaterial [39]. Activation of oxidative stress is an important cellular contributor to GO toxicity formation in nematodes [40]. Based on the translocation pattern of GO/Rho B, seven genes were identified to be required for the GO toxicity and translocation of GO [39]. Among these seven genes, mutation of *hsp-16.48*, *gas-1*, *sod-2*, *sod-3*, or aak-2 resulted in the greater GO translocation into the body and toxicity on the functions of both primary targeted organs, such as the intestine, and secondary targeted organs, such as the neurons and reproductive organs (Fig. 1.5) [39]. In

Table 1.1 Paraquat resistance assay for the RNAi clones [38]

Predicted gene	Domain and function	Survival (%)					Mean survival (± SEM)	*P*-value	Relative resistance (%)
		Day 0	Day 3	Day 5	Day 7	Day 9			
Genes on Chrom III									
Vector	Negative control	100	64	3	0	0	4.28 (±0.13)	100	
daf-2	Positive control	100	90	77	64	27	8.32 (±0.36)	<0.0001	194
C40H1.5	Transthyretin-like family	100	66	26	9	4	5.02 (±0.28)	0.0164	117
Y111B2C.m	epc-1, polycomb enhancer protein	100	93	70	43	6	7.13 (±0.29)	<0.0001	167
Y76A2B.1	pod-1, coronin-like, actin-binding protein	100	55	24	7	0	4.68 (±0.20)	0.1031	109
Y76A2B.3	Long-chain acyl-coA synthetase	100	76	22	5	5	5.07 (±0.29)	0.0067	118
Y119D3_446.d	Glycolipid transferase	100	72	24	7	1	5.03 (±0.19)	0.0031	118
Y55D5A_391.b	daf-2, insulin/IGF-I receptor	100	71	18	18	7	5.23 (±0.31)	0.0083	122
Y71H2_390.d	SNAP-25 interacting protein, exocytosis	100	62	31	17	12	5.36 (±0.41)	0.0110	125
Y53G8A_1734.d	ral-1, Ras-like GTPase, related to Ral-1	100	82	39	9	2	5.56 (±0.25)	<0.0001	130
T28D6.4	Ankyrin repeat	100	91	60	38	12	6.95 (±0.35)	<0.0001	162
K12H4.5	Phosphatidylserine decarboxylase	100	92	71	41	11	7.27 (±0.25)	<0.0001	170
T28A8.6		100	70	24	7	4	5.02 (±0.29)	0.0137	117
C29F9.7	pat-4, integrin-linked kinase	100	76	55	38	17	6.73 (±0.40)	<0.0001	157
Y66A7A1		100	52	33	4	0	9.00 (±0.29)	0.0572	210
Y71H2_388.c	PP2A regulatory subunit (cytochrome C oxidase subunit)	100	82	48	2	0	5.57 (±0.20)	<0.0001	130
F54D8.2	Cytochrome c oxidase subunit VIa	100	70	41	22	3	5.62 (±0.27)	<0.0001	131
F56D2.1	Mitochondrial processing peptidase	100	55	17	3	0	4.46 (±0.20)	0.4303	104
K04G7.4	Nuo-4, NADH: ubiquinone oxidoreductase	100	78	55	4	0	5.06 (±0.23)	<0.0001	118
T20H4.5	Ubiquinone Fe-S protein	100	99	89	45	2	7.58 (±0.18)	<0.0001	177
T26A5.3	Tag-99, NADH: ubiquinone oxidoreductase	100	60	31	7	0	4.91 (±0.25)	0.0138	115

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