Redox regulation, gene expression and longevity

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Lifespan can be lengthened by genetic and environmental modifications. Study of these might provide valuable insights into the mechanism of aging. Low doses of radiation and short-term exposure to heat and high concentrations of oxygen prolong the lifespan of the nematode *Caenorhabditis elegans*. These might be caused by adaptive responses to harmful environmental conditions. Single-gene mutations have been found to extend lifespan in *C. elegans*, *Drosophila* and mice. So far, the best-characterized system is the *C. elegans* mutant in the *daf-2*, insulin/IGF-I receptor gene that is the component of the insulin/IGF-I signaling pathway. The mutant animals live twice as long as the wild type. The insulin/IGF-I signaling pathway regulates the activity of DAF-16, a FOXO transcription factor. However, the unified explanation for the function of DAF-16 transcription targets in the lifespan extension is not yet fully established. As both of the Mn superoxide dismutase (MnSOD) isoforms (*sod-2* and *sod-3*) are found to be targets of DAF-16, we attempted to assess their functions in regulating lifespan and oxidative stress responsivity. We show that the double deletions of *sod-2* and *sod-3* genes induced oxidative-stress sensitivity but do not shorten lifespan in the *daf-2* mutant background, indicating that oxidative stress is not necessarily a limiting factor for longevity. Furthermore, the deletion in the *sod-3* gene lengthens lifespan in the *daf-2* mutant. We conclude that the MnSOD systems in *C. elegans* fine-tune the insulin/IGF-I-signaling based regulation of longevity by acting not as anti-oxidants but as physiological-redox-signaling modulators. **Geriatr Gerontol Int 2010; 10 (Suppl. 1): S59–S69.**

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Introduction

The lifespan of metazoans is determined by genetic and environmental factors. A variety of environmental conditions are known to extend lifespan: caloric restriction in a wide range of organisms,[1] low temperature in some poikilothermic animals[2] and low oxygen concentrations in the nematode *Caenorhabditis elegans*.[3] The mechanisms by which each condition slows the aging rate have not yet been fully elucidated. Function of caloric restriction has been postulated as hormonal changes, altered

gene expression, lowered metabolic rate and a reduced generation rate of mitochondrial reactive oxygen species (ROS). Lifespan could also be lengthened by environmental perturbations. Hormesis is a phenomenon occurring when agents that are harmful in high doses or over long periods actually produce beneficial effects, such as lifespan extension, when used at low doses or over short periods. *C. elegans* shows lifespan-extension hormesis when exposed to low doses of radiation,[4] or short-term heat,[5] hyperoxia[6] or hyperbaric oxygen.[7] These treatments are associated with adaptive resistance to lethal thermal or oxidative stress, and the gene expression of stress-defense proteins.[5,6,8]

Recently, lifespan-extension mutants of the nematode *C. elegans* have been extensively isolated, and the gene network responsible for its longevity has been unraveled.[9] Two main classes of lifespan-extension mutants have been reported; one class is related to the activity of

the mitochondrial electron transport chains, such as *clk-1*[10] and *isp-1,*[11] and the other is related to hormonal mechanisms, especially an insulin/IGF-I signaling pathway, such as *daf-2*[12,13] and *age-1.*[14,15]

Hormesis and oxygen

Lifespan hormesis

Hormesis is a phenomenon that occurs when agents that are harmful in high doses or over long periods, actually produce beneficial effects, such as lifespan extension, when used in low doses or over short periods. *C. elegans* shows lifespan-extension hormesis when exposed to low doses of radiation[4] or short-term heat exposure.[5] We showed that long-term hyperoxia shortened the lifespan of *C. elegans.*[16] During the course of this investigation, we noticed slight extension of lifespan by normoxic incubation after hyperoxia.[6,16] Darr and Fridovich showed an adaptive induction of anti-oxidant defense in *C. elegans.*[8]

Lifespan extension by short-term exposure to hyperoxia

The wild-type strain that was reared under normoxic conditions was exposed to 90% oxygen for 2 days from a 6-day adult age. The lifespan was measured after it was returned to normoxic conditions until the end of life. Figure 1 shows that a two-day exposure to 90% oxygen slightly, but nevertheless significantly, increases in mean and maximum lifespan.[6] It is well known that one of the best criteria of the aging process is an exponential acceleration of mortality rate with chronological age, which is formulated by the Gompertz equation. The acceleration rate, that is the Gompertz component, is a parameter of aging rate and has been estimated in *C. elegans.*[17] The Gompertz analysis of the survival data

Lifespan hormesis of high concentration of oxygen
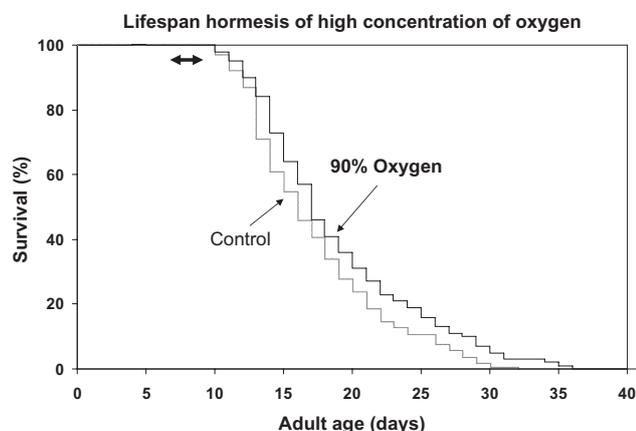
Figure 1 The wild-type *C. elegans* was exposed to 90% oxygen for 2 days from a 6-day adult age. The oxygen-exposed animals lived longer than the unexposed animals.

of control and hyperoxia-exposed animals was carried out in three different ways. In any analyses, the mortality rate of the hyperoxia-exposed animals was found to be smaller than that of the control animals, indicating that short-term exposure to hyperoxia slowed the aging rate.[6]

Adaptive response to oxidative stress

To test whether hyperoxia exposure can increase lethal oxidative stress resistance, the wild-type strain was pre-exposed to 90% oxygen for 2 days, and subsequently treated with 50 mmol paraquat (PQ) under 98% oxygen, compared with animals that were treated with 50 mmol PQ under 98% oxygen without pre-exposure. Exposure to 90% oxygen increased oxidative stress resistance.[6] This indicated that exposure to 90% oxygen induced an adaptive response for protection against oxidative stress, supporting the observation of Darr and Fridovich.[8] To test whether hyperoxia can induce the gene expression of anti-oxidant enzymes, we measured the gene expression of three types of SOD and catalase after exposure to 90% oxygen and found that 90% oxygen exposure induces the gene expression of *sod-1*, *sod-2*, *sod-3* and catalase. Exposure of *C. elegans* to hyperoxia adaptably increases resistance to subsequent lethal oxidative stress and increases expression of anti-oxidant defense enzymes, such as SOD and catalase. The most plausible explanation is that an increase in the ability to withdraw ROS induced by hyperoxia could reduce normally occurring oxidative stress that could be the cause of aging.

Oxygen concentrations and lifespan

Lifespan is known to be affected by environmental conditions: food concentrations,[1] temperature in some poikilothermic animals[2] and oxygen concentrations in the nematode *C. elegans*.[3] We showed that the lifespan of *C. elegans* was decreased with increasing environmental oxygen concentrations.[3,6] In contrast, low oxygen concentrations lengthened the lifespan.[3] The Gompertz component, a parameter of aging rate, increased with an increase in oxygen concentrations and decreased under 1% oxygen.[3,6] These results suggest that the environmental oxygen concentration is a lifespan determinant. Because ROS are thought to arise in organisms with a dependence on the oxygen concentration,[18] these findings suggest that ROS-mediated oxidative stress is involved in the determination of lifespan.

When the animals were exposed to a high concentration of oxygen at the earlier phase of the adult lifespan, oxygen-induced reduced lifespan shortening was not observed.[16] Furthermore, a split of exposure to the high concentration of oxygen reduced the oxygen-induced

reduced lifespan. These results suggest that there exists a mechanism that repairs the oxygen-induced damage leading to reduced lifespan.

Lifespan extension mutants

Single-gene mutations have been found to extend lifespan in *C. elegans*,[9] *Drosophila* and mice.[4] So far, the best-characterized system is an insulin/IGF-1 signaling pathway that regulates the lifespan of *C. elegans*. Insulin/IGF-I signaling is mediated by the DAF-2 insulin/IGF-I receptor. The *C. elegans daf-2* mutants that reduce the activity of DAF-2 remain youthful and active much longer than the wild-type animals and live more than twice as long.[12] The lifespan-extension phenotype of the *daf-2* is suppressed by mutations in *daf-16*, indicating that *daf-16* is negatively regulated by DAF-2 signaling and is the major downstream effector. The *daf-16* encodes a FOXO transcription factor.[19,20]

Binding of insulin/IGF-I-like ligands to the DAF-2 insulin/IGF-I receptor controls insulin/IGF-I signaling. There are at least 38 genes (*ins*) encoding insulin/IGF-I-like peptides in *C. elegans*.[21,22] Many of these genes are divergent insulin superfamily members, and as the specific ligand has not yet been identified, it is possible that these members might have complex and redundant roles. The *daf-28* gene encodes insulin-like peptide. A dominant-negative allele of the *daf-28* mutant lives 10% longer than wild-type animals. A phenotype of the *daf-28* mutant is rescued by the *ins-4* or *ins-6* transgene, suggesting a redundant nature.[22] Some *ins* genes are expressed in sensory neurons.[22,23] Environmental cues such as food, pheromones and temperature might affect insulin/IGF signaling through different expression and the secretion of various INS peptides. The mutation of *age-1*, which encodes the phosphoinositide-3-OH (PI3) kinase catalytic subunit, doubles the lifespan in *C. elegans*.[14,15]

The current model of insulin/IGF-I signaling is as follows (Fig. 2): the DAF-2 insulin/IGF-I receptor transduces signals by activating AGE-1. AGE-1 PI3 kinase phosphorylates PIP2 to generate the second messenger PIP3. In contrast, DAF-18 PTEN dephosphorylates PIP3 and thus antagonizes the action of AGE-1. Thus, the PIP3 level is determined by a balance between generation by AGE-1 PI3 kinase and degradation by DAF-18 PTEN. PIP3 activates 3-phosphoinositide-dependent kinase-1 (PDK-1), which in turn phosphorylates and activates AKT-1/AKT-2 Ser/Thr kinase. AKT-1/AKT-2 phosphorylates and inactivates the DAF-16 transcription factor to be sequestered from the nucleus to the cytoplasm. In this state, adults age rapidly. On the contrary, when DAF-2 signaling is reduced, DAF-16 is eventually translocated to the nucleus to promote transcription of target genes. In fact, disrupting AKT-consensus phosphorylation sites in

A model of pathway for lifespan determination
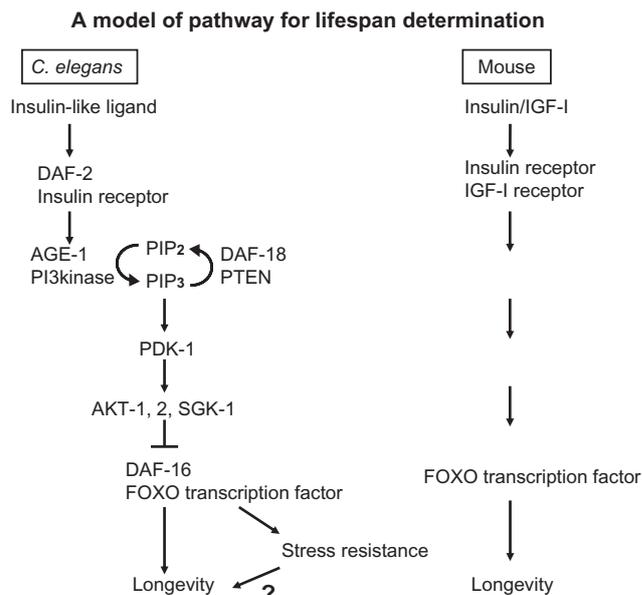
Figure 2 The insulin/IGF-1 signaling regulates stress resistance and longevity in *C. elegans* and mammals.

DAF-16 causes nuclear accumulation, although the nuclear accumulation is not sufficient for lifespan extension.[24]

C. elegans worms grow through four larval stages (L1-L4) before reaching maturity. However, when the food supply is limited and the population density is high at the L1 stage, animals become dauer larvae after the L2 stage. The dauer larva is a developmentally arrested dispersal stage and lives up to several months, greatly exceeding the normal adult lifespan of about 3 weeks under stressful environmental conditions.[25] It seems that the dauer stage is non-aging, because the post-dauer lifespan is not affected by a prolonged dauer stage of up to 2 months.[26] The dauer larva is more resistant to a variety of environmental stresses, including hypoxia, heat, desiccation and oxidative stress, and has increased levels of SOD and catalase.[27] We showed that the expression of the Mn superoxide dismutase (MnSOD) gene (*sod-3*) is higher in the dauer larvae than in the adults.[28] As dauer larvae live much longer than adults, some genes expressing altered levels in dauer state might be the key to longevity. By using serial analysis of gene expression (SAGE), Jones *et al*. found that the expression of *tts-1* (transcribed telomere-like sequence), a variant histone H1 and a nucleosome assembly protein possibly relating to the structure or stability of chromatin is high in dauer larvae. These results suggest that the chromatin structure might change to be more stable in the dauer state than in the growing state.[29] Holt and Riddle examined gene expression profiles of carbohydrate metabolism in dauer larvae by using SAGE. A high gene expression of pyruvate kinase, alcohol

dehydrogenase, a putative cytosolic fumarate reductase, two pyruvate dehydrogenase components and succinyl CoA synthetase α subunit implies that anaerobic metabolism is prominent in dauer larvae.[30]

The formation of the dauer larva in *C. elegans* is normally triggered by food limitation and/or pheromones.[25,31,32] The switch between development and dauer diapause is regulated by both the nervous and endocrine systems through the recognition and transduction of environmental cues through the TGF-β,[33] cGMP,[34] serotonin[35] and insulin/IGF-1[13] signaling pathways. In this regard, the TGF-β and insulin/IGF-1 signaling pathways converge on a steroid hormone pathway.[36] By genetic analysis of mutants showing "<u>da</u>uer larva <u>f</u>ormation abnormal" Daf phenotype, a number of genes that regulate dauer formation have been identified.[25] Among the mutants that show the constitutive formation of dauer larvae, those that down-regulate insulin/IGF-1 signaling, such as the *daf-2* mutants but not a TGF-β signaling such as the *daf-7* (TGF-β) mutants, also manifest an adult-lifespan extension phenotype. This suggests that the insulin/IGF-1 signaling pathway initiates the program for long survival in the dauer state and that the ectopic activation of this program in the adult stage by its mutations might extend adult lifespan.[12] We discovered that the crude pheromone extract of *C. elegans* culture medium extends the adult lifespan in *C. elegans*.[32] This extension does not occur in the *daf-16* mutant animal that suppresses the expansion of lifespan caused by reducing the insulin/IGF-1 signaling indicating relevancy of the insulin/IGF-1 signaling in the lifespan extension caused by the extract.

Insulin/IGF-I signaling pathway and stress resistance

To investigate the relationship between lifespan extension and oxidative-stress resistance, we screened the oxidative-stress resistance phenotype in various mutants.[6,28] We examined the survival period of each mutant under experimentally induced, acute oxidative stress. We used paraquat, an intracellular superoxide O_2^- generator, under hyperoxia for acute oxidative stress. The *daf-2* mutants with an extended lifespan survived for a longer period of time than wild-type animals in the presence of paraquat under hyperoxic or normoxic conditions. The *daf-2* mutant is also more resistant to menadione, another intracellular O_2^- generator, under hyperoxia than wild-type animals. The mutants in the TGF-β pathway and cGMP pathway do not show oxidative-stress resistance. The oxidative-stress resistance seen in the *daf-2* mutants is suppressed by mutations in *daf-18* or *daf-16* indicating that *daf-16* and *daf-18* act downstream of *daf-2* to confer oxidative-stress resistance, as well as extended lifespan.

Vanfleteren[37] and Larsen[38] showed that the lifespan extension mutant *age-1* is more resistant to oxidative stress in old age than wild-type animals at the same age. We showed that the *age-1* mutants of young adults also display the oxidative-stress resistance.[6] The oxidative-stress resistance in *age-1* mutants is suppressed by *daf-16* mutation, indicating that *daf-16* is located downstream of *age-1* in the pathway for regulating oxidative-stress resistance.

In contrast, oxidative-stress resistance in two alleles of *age-1* mutants (*m333* and *mg44*) is not fully suppressed by *daf-18* mutation, indicating that *daf-18* does not act downstream of *age-1*.[6] *daf-16* and *daf-18* act downstream of *daf-2* in the insulin/IGF-I signaling pathway for oxidative-stress resistance.[28] Taken together, we postulate the following pathway for oxidative-stress resistance:

$$daf\text{-}2\text{-} > daf\text{-}18\text{-} > age\text{-}1\text{-} > daf\text{-}16\text{-} >$$
Oxidative-stress resistance

This pathway is essentially identical to the pathway regulating longevity,[39] suggesting a strong association between lifespan extension and oxidative-stress resistance. However, Dorman and Canyon showed that the *daf-18* mutation suppressed the lifespan-extension phenotype of another allele of *age-1 (hx546)* indicating that *daf-18* acts downstream of *age-1*.[40] Such differences could be attributed to the differences in severity of the *age-1* alleles used.

Two alleles of *age-1* mutants (*m333* and *mg44)* display the Daf phenotype, which is completely suppressed by *daf-18* or *daf-16* mutations, indicating the following pathway for dauer formation:

$$daf\text{-}2\text{-} > age\text{-}1\text{-} > daf\text{-}18\text{-} > daf\text{-}16\text{-} > \text{dauer formation}$$

Thus, oxidative-stress resistance is closely associated with longevity but not with dauer formation. The PIP3 level is maintained under a balance between generation by AGE-1 PI3 kinase and degradation by DAF-18 PTEN, which could determine the impact of this pathway. The loss or reduction of function mutations in *age-1* could reduce PI3 kinase activity to drop this second messenger level. When DAF-18 is reduced, only the pre-existing pool of the second messenger might be insufficient to inhibit longevity and oxidative-stress resistance, but sufficient to inhibit dauer formation. Dillin *et al*. found that the inactivation of *daf-2* during adulthood by RNAi extends lifespan and increases oxidative-stress resistance.[41] Because dauer formation is switched in the early larval stage, the insulin/IGF-I pathway controls the dauer switch and oxidative-stress resistance/longevity independently.

There are several genes in *C. elegans* that encode SOD enzymes: *sod-1* encodes cytosolic CuZnSOD,[38] *sod-2* and *sod-3* each encodes mitochondrial MnSOD,[42–44] and *sod-4* encodes extracellular CuZnSOD.[45] We showed
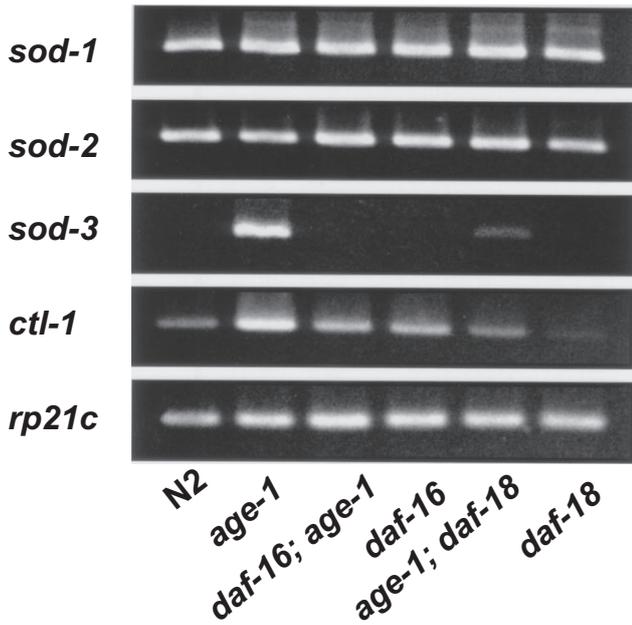
Figure 3 The gene expression of superoxide dismutases and catalase in *C. elegans* longevity mutants. RT–PCR analysis of the mRNA level of *sod-1*, *sod-2*, *sod-3* and *ctl-1* was carried out in *age-1* and/or *daf-16* and *daf-18* mutants.

that the level of *sod-3* mRNA in *daf-2* mutants is higher than that in wild-type animals.[28] The levels of mRNA transcripts of *sod-1*, *sod-2*, and catalase in the *daf-2* are similar to those in wild-type animals.[28] The level of *sod-3* mRNA in *daf-2* mutants increases as it develops from the egg to the L2 larval stage coinciding with increases in oxidative-stress resistance. The elevated level of *sod-3* mRNA in the *daf-2* mutants is suppressed by *daf-16* and *daf-18* mutation.[28] Figure 3 shows that the level of *sod-3* mRNA in the *age-1* mutants is higher than that in wild-type animals.[6] The elevated level of *sod-3* mRNA in *age-1* mutants is suppressed by *daf-16* mutation but is not fully suppressed by *daf-18* mutation (Fig. 3). These results provide further evidence that the insulin/IGF-I signaling pathway regulates extended lifespan, oxidative-stress resistance and *sod-3* expression in a similar way. These results suggest that the extended lifespan is correlated with the efficient withdrawal of ROS generated in mitochondria during normal metabolism.

Another class of the lifespan-extension mutations, *clk-1*, does not show oxidative-stress resistance or increased expression of *sod-3*. However, *clk-1* mutation largely promotes oxidative-stress resistance and an increased expression of *sod-3* in the *daf-2* mutant background,[28] as well as extended lifespan.[10] In contrast, mutation in *isp-1* cannot promote an extended lifespan of *daf-2* and itself causes oxidative-stress resistance and increased expression of *sod-3*.[11] Insulin/IGF-I signaling

seems to regulate the expression of *sod-3* in concert with mitochondrial energy metabolites.

Redox signaling in lifespan regulation

With respect to the role of MnSOD in organismal aging, the seemingly conflicting observations so far reported suggest that MnSOD overexpression extends lifespan in *Drosophila*[46] and rodents,[47] shortens lifespan when catalase is co-overexpressed[48] and has little effect on lifespan[49] in *Drosophila*. In the mouse, heterozygous MnSOD knockouts that show about half of the wild-type MnSOD activity indicate no changes in their lifespan despite an increase in nuclear and mitochondrial oxidative-DNA-damage accumulation and cancer incidence.[50]

To further clarify the roles of MnSOD in *C. elegans* aging, we explored the effects of the deletion mutations for the individual MnSOD genes, *sod-2* and *sod-3*, on oxidative-stress responsivity and lifespan in the wild-type and long-lived *daf-2* mutant.[28] We assessed oxidative-stress responsivity by using hyperoxia and PQ under hyperoxia. The *sod-2/sod-3* double deletion mutant animals at adult stage all died within 2 days under hyperoxia (98% oxygen), whereas the wild type, *sod-2* and *sod-3* single deletion mutant adults all survived longer than 10 days under hyperoxia. The *sod-2* deletion mutant was found to be more sensitive to PQ-hyperoxia than the wild type and the *sod-3* deletion mutant, but less sensitive to PQ-hyperoxia than the *sod-2; sod-3* double mutant (Fig. 4a). These results indicate that SOD-2 enzyme plays a more important role for anti-oxidant defense than SOD-3, and that SOD-2 and SOD-3 cooperatively exert an anti-oxidant-defense function.
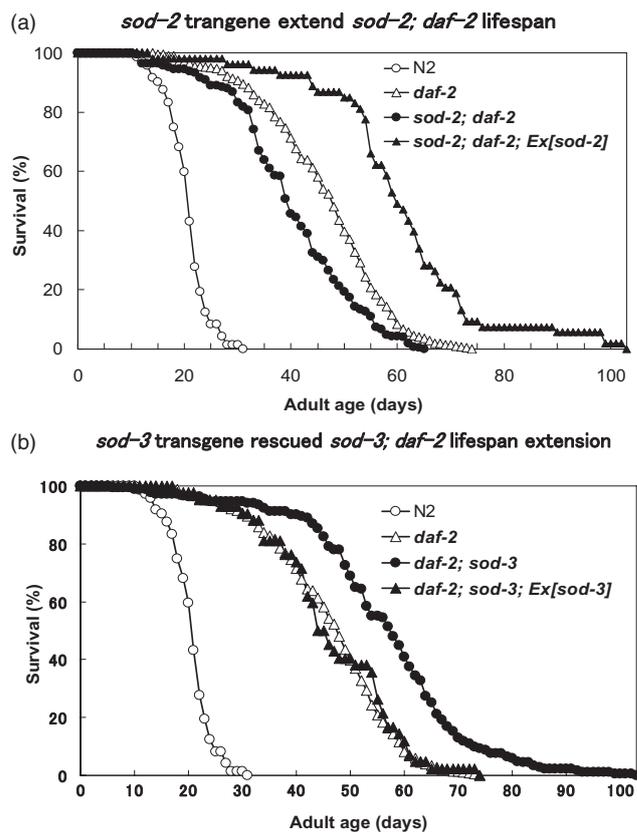
In order to elucidate a role of MnSOD in resistance to oxidative stress (Oxr phenotype) in the *daf-2* mutants,[28] we measured the PQ-hyperoxia sensitivities of the *sod-2* and/or *sod-3* deletions in the *daf-2* mutant.[51] Neither the *sod-2* nor *sod-3* deletion mutation alone suppressed Oxr in the *daf-2* mutant (Fig. 4b). However, the *sod-2; daf-2; sod-3* triple mutant showed oxidative-stress sensitivity and, therefore, did not show Oxr (Fig. 4b). These results show that Oxr in the *daf-2* mutant requires at least one functioning MnSOD isoform and is, therefore, totally ameliorated by the two MnSOD double deletions.

Next, we examined the lifespan of the MnSOD-deletion mutants.[51] The *sod-2* and *sod-3* single and double deletions did not appear to significantly affect the lifespan. These data indicate that the MnSOD do not participate in lifespan determination and suggest that the sensitivity to oxidative stress does not necessarily correlate with lifespan in *C. elegans*. To explore the possible involvement of the MnSOD in the lifespan extension phenotype (Age) of the *daf-2* mutants, we examined the lifespan of the MnSOD-deletions in the
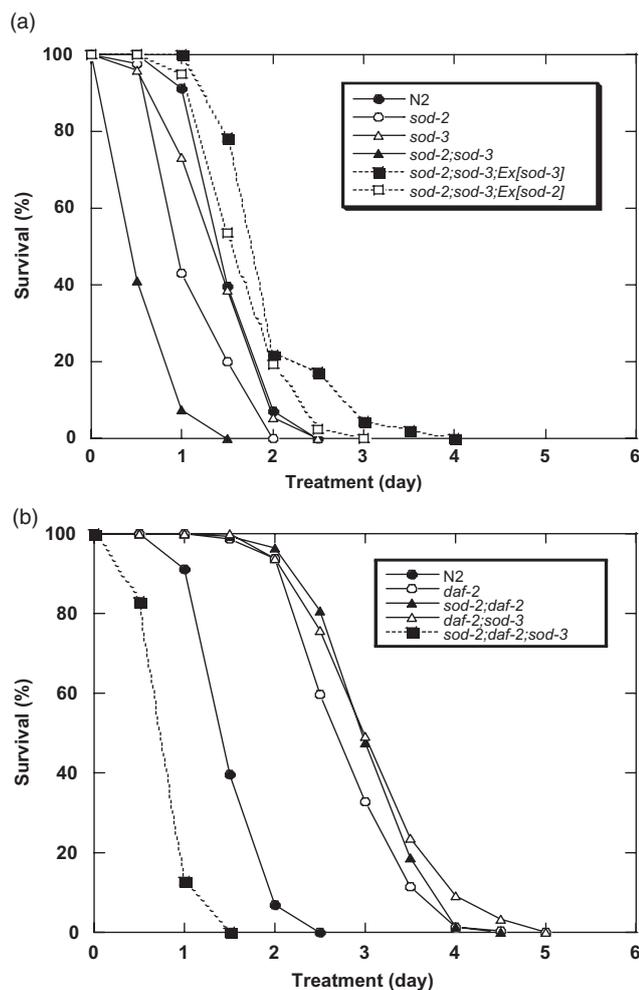
Figure 4 Oxidative-stress responsivity of *sod-2/sod-3* deletion mutants. (a) Oxidative-stress responsivity of *sod-2* and/or *sod-3* deletion mutants in the background of wild-type *C. elegans*. (b) Oxidative-stress responsivity of *sod-2* and/or *sod-3* deletion mutants in the background of *daf-2* mutation of *C. elegans*. Survival of adult animals is plotted as a function of treatment period with paraquat.


Figure 5 Lifespan of *sod-2/sod-3* deletion mutants. (a) Lifespan of *sod-2* deletion mutants in the background of *daf-2* mutation of *C. elegans*. (b) Lifespan of *sod-3* deletion mutants in the background of *daf-2* mutation of *C. elegans*.

daf-2 mutant. The *sod-2* and *sod-3* single and double deletions did not abolish Age of the *daf-2* mutant. Because the *sod-2/sod-3* double deletions eliminated Oxr in *daf-2*, as mentioned above (Fig. 4b), these results indicate that Oxr is not causally linked to Age in the *daf-2* mutant. Interestingly, however, the deletions of the *sod-2* and/or *sod-3* deletions appeared to cause fluctuations in the degree of the Age phenotype in the *daf-2* mutant (Fig. 5a,b). Specifically, the *sod-2; daf-2* double mutant showed a shorter lifespan than that of the *daf-2* mutant counterparts (Fig. 5a). In contrast, the *daf-2; sod-3* double mutant had a slightly but significantly longer lifespan than that of the *daf-2* mutant (Fig. 5b). The introduction of a wild-type *sod-2* transgene extended the lifespan of the *sod-2; daf-2* mutant, (Fig. 5a) whereas a wild-type *sod-3* transgene shortened

the lifespan of the *daf-2; sod-3* mutant (Fig. 5b), indicating that SOD-2 and SOD-3 actually function as lifespan modulators in the *daf-2* mutant. In addition, the *sod-2; daf-2; sod-3* triple mutant lived longer than the *daf-2* mutant, similarly to the *daf-2; sod-3* double mutant, showing that *sod-3* is epistatic to *sod-2* in Age phenotype.

Similar effects of *sod-2* and *sod-3* deletions on Daf-c: abnormal dauer formation, the other *daf-2* phenotype, were observed in the *daf-2* mutants.[51] Because the Age did not correlate with Oxr in the *sod-2* and/or *sod-3* deletion mutants under the *daf-2* mutant background, it is likely that the MnSOD in *C. elegans* function in longevity, by acting not as anti-oxidant-defense enzymes but as regulatory-signaling modulators. Taken together, the MnSOD are likely to constitute a redox regulation pathway to modulate the longevity and o developmental switching processes similarly under the control of the insulin-like signaling.

From our observations that *sod-3* is epistatic to *sod-2* in the Age and Daf-c in the *daf-2* mutants, we propose a working model whereby SOD-2 regulates the function of SOD-3, and whereby SOD-3 more directly modulates both longevity and development under insulin-like

Redoxregulation of longevity by MnSOD
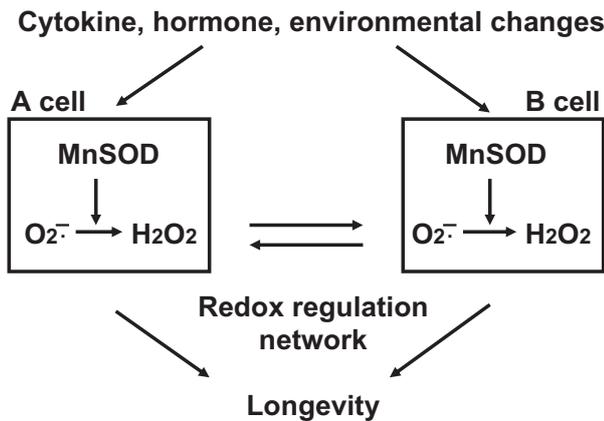
Figure 6 Mn superoxide dismutase (MnSOD) induced in various cells by diverse stimuli such as cytokines, hormones and physical agents might be involved in signal transduction between these cells, resulting in the formation of a signaling network to regulate longevity.
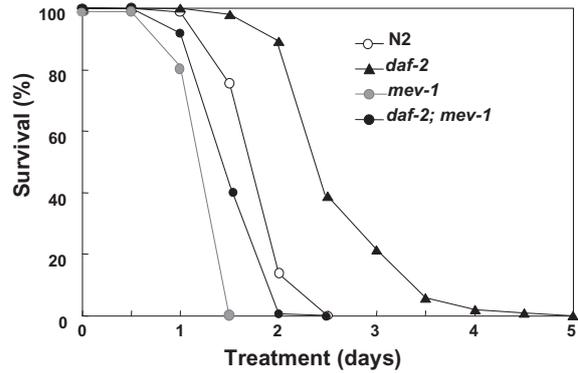
signaling control. Hu *et al*. reported that superoxide is a mediator of signal transduction during homeostasis and in developmental processes in multicellular systems.[47] SOD-2 and SOD-3 express in different cells in the head of *C. elegans,*[51] suggesting that two MnSOD isoforms expressed in different cells are involved in signal transduction between these cells regulating longevity and development.

In contrast to *C. elegans*, MnSOD has been shown to have only a single form in mammals, but it is noteworthy that this gene is induced in a number of mammalian cell types in response to a variety of stimuli, including TNF-α,[52] NGF,[53] VEGF,[54] IL-1,[55] interferon-γ,[56] insulin,[57] thyroid hormone,[58] endotoxin-like LPS[59] and hyperoxia,[55] possibly resulting in heterogeneities of its cellular expressions. Thus, we hypothesize that these heterogeneities might participate in formation of a signaling network to regulate diverse complex biological processes, including longevity regulation (Fig. 6).
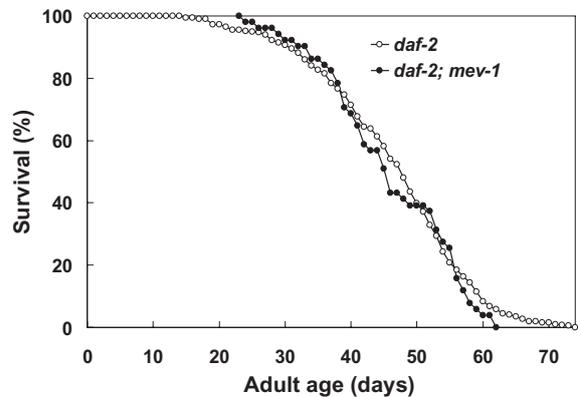
Uncoupling between aging and oxidative stress

As described above, the sensitivity to oxidative stress does not necessarily correlate with lifespan in *C. elegans* mutants. In order to further examine the relationship between oxidative stress sensitivity and aging, we used a mutation that enhances oxidative-stress sensitivity other than the MnSOD deletions.[51] A mutant in the *mev-1* gene, which encodes the cytochrome b subunit of the succinate dehydrogenase (complex II) of the electron-transport chain shows an elevated generation of ROS, an increased sensitivity to oxidative stress and a

Figure 7 Oxidative-stress responsivity and lifespan of *mev-1* and/or *daf-2* mutants. (a) The *mev-1* mutant is sensitive to oxidative-stress, even in the *daf-2* mutant background. (b) The *mev-1* mutant did not have a short lifespan in the *daf-2* mutant background.

reduced lifespan.[60] We analyzed the effects of the *mev-1* mutation on oxidative-stress responsivity and lifespan under the *daf-2* mutant background. The *daf-2; mev-1* double mutant animals showed severe oxidative-stress sensitivity similar to the *mev-1* mutant counterparts.[51] (Fig. 7a), indicating that the *mev-1* mutation also ameliorated Oxr in *daf-2*. In contrast, the *daf-2; mev-1* double mutant animals still lived as long as the *daf-2* mutant (Fig. 7b).[51] These results indicate the uncoupling between aging and oxidative stress.

The free radical hypothesis of aging postulates that senescence is a result of an accumulation of molecular oxidative damage caused by ROS that are produced as by-products of normal metabolic processes.[61,62] A logical prediction based on this hypothesis is that the oxidative-stress sensitivity should inversely correlate with longevity. The present results that the *sod-2* deletion and *sod-2/sod-3* double deletions caused oxidative-stress sensitivity but had no effect on lifespan in *C. elegans* conflict with this prediction. Furthermore, it has been implicated that oxidative-stress resistance induced

by reduction in the insulin-like signaling is causally linked to lifespan extension in *C. elegans*,[6,28,37,38] *Drosophila*[63] and mice.[64] The present findings that the *sod-2/sod-3* double deletions and *mev-1* mutation ablated Oxr but did not abolish Age in the *daf-2* mutant do not support this implication. The SOD mimetics has been reported to induce oxidative-stress resistance but does not lengthen the lifespan of *C. elegans*[65] and *Drosophila*,[66] also arguing against the free radical hypothesis of aging. Alternatively, the ROS-induced damaging processes that are not affected by these mutations or mimetics might lead to senescence.

Cellular aging and oxidative stress

Cultured normal cells generally have a finite replicative lifespan as originally shown by Hayflick and Moorhead.[67] Human diploid fibroblasts lose the capacity to replicate after vigorous proliferation and enter a viable but non-proliferative state of senescence. Senescent cells are characterized by the upregulation of cyclin-dependent kinase inhibitors, p21[SDI1/WAF1/CIP1,68] p16[INK4a69] and hypo-phosphorylated Rb.[70] Human fibroblasts grown in hypoxia have an extended replicative lifespan.[71]

Mouse embryonic fibroblasts (MEF) senesce after vigorous proliferation, and then grow into immortal cells in normoxia (20% oxygen), whereas human normal fibroblasts never achieve immortality. Parrinello *et al.* found that MEF do not senesce in hypoxia (3% oxygen) and that MEF accumulate more DNA damage in normoxia than hypoxia, and more damage than human fibroblasts in normoxia.[72] These results suggest that human cells have a superior ability to prevent or repair oxidative DNA damage compared with murine cells.

Stress-induced premature senescence (SIPS) occurs when cells are grown in hyperoxia,[73] and treated with hyperbaric oxygen[74] and H_2O_2,[75] with the appearance of several biomarkers of replicative senescence. Hyperoxia (40% oxygen) increases the rate of telomere shortening from 90 bp per population doubling (normoxia) to more than 500 bp per population doubling (hyperoxia),[76] suggesting that telomere shortening causes premature senescence. In contrast, Gorbunova *et al.* indicated that the overexpression of the catalytic subunit of telomerase cannot prevent SIPS, but can protect from stress-induced apoptosis and necrosis. This finding suggests that SIPS might not be a result of telomere shortening.[77]

As shown in Figure 8, the addition of N-acetylcysteine, which increases the level of cellular reduced glutathione (GSH) known as ROS scavenger, into culture medium extends the replicative lifespan of human embryonic fibroblasts.[78] In contrast, the addition of L-buthionine-(R,S)-sulfoximine, a specific inhibitor of GSH synthetase, shortens the replicative lifespan.[78] These results suggest that cellular ROS levels are a determinant of replicative lifespan.

Glutathione–modulating agents & *in vitro* lifespan of human fibroblasts
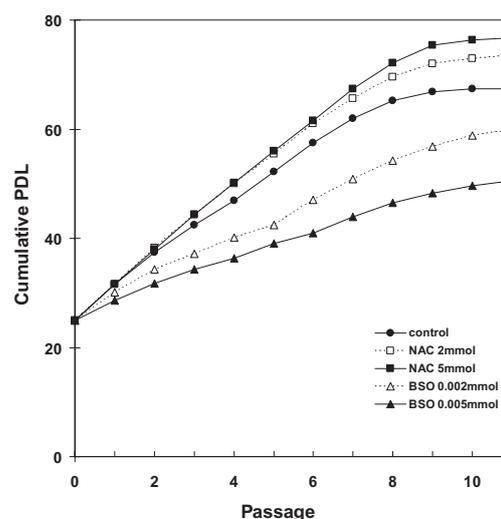
Figure 8 Human diploid fibroblasts at 25 population doubling level (PDL) were serially cultivated in a medium containing 2 mmol or 5 mmol N-acetylcysteine (NAC) or 2 mmol or 5 mmol L-buthionine-(R,S)-sulfoximine (BSO). The cumulative PDL is shown.

The inactivation of cytosolic CuZnSOD (SOD1) by RNAi induces premature senescence in human fibroblasts depending on p53 induction.[79] In human fibroblasts with inactivated p53, the SOD1 RNAi is without effect, suggesting that oxidative DNA damage mediates premature senescence. The overexpression of extracellular CuZnSOD decreases the intracellular peroxide content, slows the telomere-shortening rate, and extends the replicative lifespan under normoxia and hyperoxia.[80]

The overexpression of an activated V12Ras induces premature senescence in human fibroblasts and induces increased mitochondrial ROS generation.[81] In hypoxia, the overexpression of the V12Ras cannot induce premature senescence or increase the level of p21 that is related to the senescent phenotype. These findings suggest that ROS play a role in the regulation of the replicative lifespan.

Conclusion

In the past decades, gene-manipulation studies have shown that gene networks exist for the determination of lifespan in diverse species. There appear to be common and different features among species. ROS appear at various points in the aging processes, and play a variety of roles, including the manifestation of oxidative stress and the mediation of signal transduction. The precise role of ROS in the aging process is not yet clear. DNA microarray technology allows us to show global gene expression profiles governing the lifespan and aging

rate, although these studies are just beginning. Using the data from DNA microarray analysis, painstaking studies are needed to clarify the aging mechanism to analyze how various gene products work in concert to regulate the aging rate.

Conflicts of interest

None.

References

1 Weindruch RH, Walford RL, Fligiel S, Guthrie D. The retardation of aging in mice by dietary restriction: longevity, cancer, immunity, and lifetime energy intake. *J Nutr* 1986; **116**: 641–654.

2 Rockstein M, Chesky JA, Susman ML. Comparative biology and evolution of aging. In: Finch CE, Hayflick L, eds. *The Handbook of the Biology of Aging*. New York: Van Nostrand Reinhold Compan, 1977; 3–34.

3 Honda S, Ishii N, Suzuki K, Matsuo M. Oxygen-dependent perturbation of life span and aging rate in the nematode. *J Gerontol* 1993; **48**: B57–B61.

4 Johnson TE, Hartman PS. Radiation effects on life span in *Caenorhabditis elegans*. *J Gerontol* 1988; **43**: B137–B141.

5 Lithgow GJ, White TM, Melov S, Johnson TE. Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress. *Proc Natl Acad Sci USA* 1995; **92**: 7540–7544.

6 Honda Y, Honda S. Life span extensions associated with up-regulation of gene expression of anti-oxidant enzymes in *Caenorhabditis elegans*; Studies of mutation in the *age-1*, PI3 kinase homologue and short-term exposure to hyperoxia. *J Amer Age Assoc* 2001; **24**: 179–186.

7 Cypser JR, Johnson TE. Multiple stressors in *Caenorhabditis elegans* induce stress hormesis and extended longevity. *J Gerontol* 2002; **7**: B109–B114.

8 Darr D, Fridovich I. Adaptation to oxidative stress in young, but not in mature or old, *Caenorhabditis elegans*. *Free Radic Biol Medical* 1995; **18**: 195–201.

9 Guarente L, Kenyon C. Genetic pathways that regulate ageing in model organisms. *Nature (London)* 2000; **408**: 255–262.

10 Lakowski B, Hekimi S. Determination of life-span in *Caenorhabditis elegans* by four clock genes. *Science* 1996; **272**: 1010–1013.

11 Feng J, Bussiere F, Hekimi S. Mitochondrial electron transport is a key determinant of life span in *Caenorhabditis elegans*. *Dev Cell* 2001; **1**: 633–644.

12 Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R. A *C. elegans* mutant that lives twice as long as wild type. *Nature (London)* 1993; **366**: 461–464.

13 Kimura KD, Tissenbaum HA, Liu Y, Ruvkun G. *daf-2*, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. *Science* 1997; **277**: 942–946.

14 Friedman DB, Johnson TE. A mutation in the *age-1* gene in *C. elegans* lengthens life and reduces hermaphrodite fertility. *Genetics* 1988; **118**: 75–86.

15 Morris JZ, Tissenbaum HA, Ruvkun G. A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in *Caenorhabditis elegans*. *Nature (London)* 1996; **382**: 536–539.

16 Honda S, Matsuo M. Lifespan shortening of the nematode *Caenorhabditis elegans* under higher concentrations of oxygen. *Mech Age Dev* 1992; **63**: 235–246.

17 Johnson TE. Increased life-span of *age-1* mutants in *Caenorhabditis elegans* and lower Gompertz rate of aging. *Science* 1990; **249**: 908–912.

18 Freeman BA, Crapo JD. Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. *J Biol Chem* 1981; **256**: 10986–10992.

19 Lin K, Dorman JB, Rodan A, Kenyon C. *daf-16*: an HNF-3/forkhead family member that can function to double the life-span of *Caenorhabditis elegans*. *Science* 1997; **278**: 1319–1322.

20 Ogg S, Paradis S, Gottlieb S *et al.* The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. *Nature* 1997; **389**: 994–999.

21 Kawano T, Ito Y, Ishiguro M *et al.* Molecular cloning and characterization of a new insulin/IGF-like peptide of the nematode *Caenorhabditis elegans*. *Biochem Biophys Res Comm* 2000; **273**: 431–436.

22 Pierce SB, Costa M, Wisotzkey R *et al.* Regulation of DAF-2 receptor signaling by human insulin and *ins-1*, a member of the unusually large and diverse *C. elegans* insulin gene family. *Genes Dev* 2001; **15**: 672–686.

23 Li W, Kennedy SG, Ruvkun G. *daf-28* encodes a *C. elegans* insulin superfamily member that is regulated by environmental cues and acts in the DAF-2 signaling pathway. *Genes Dev* 2003; **17**: 844–858.

24 Lin K, Hsin H, Libina N, Kenyon C. Regulation of the *Caenorhabditis elegans* longevity protein DAF-16 by insulin/IGF-1 and germline signaling. *Nat Genet* 2001; **28** (2): 139–145.

25 Riddle DL, Albert PS. Genetic and environmental regulation of dauer larva development. In: Riddle DL, Blumenthal T, Meyer BJ, Priess JR, eds. *C. elegans II*. New York: Cold Spring Harbor Laboratory Press, 1997; 739–768.

26 Klass M, Hirsh D. Non-ageing developmental variant of *Caenorhabditis elegans*. *Nature (London)* 1976; **260**: 523–525.

27 Anderson GL. Superoxide dismutase activity in dauer larvae of *Caenorhabditis elegans* (Nematoda: Rhabditidae). *Can J Zool* 1981; **60**: 288–291.

28 Honda Y, Honda S. The *daf-2* gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in *Caenorhabditis elegans*. *FASEB J* 1999; **13**: 1385–1393.

29 Jones SJ, Riddle DL, Pouzyrev AT *et al.* Changes in gene expression associated with developmental arrest and longevity in *Caenorhabditis elegans*. *Genome Res* 2001; **11**: 1346–1352.

30 Holt SJ, Riddle DL. SAGE surveys *C. elegans* carbohydrate metabolism: evidence for an anaerobic shift in the long-lived dauer larva. *Mech Age Dev* 2003; **124**: 779–800.

31 Jeong PY, Jung M, Yim YH *et al.* Chemical structure and biological activity of the *Caenorhabditis elegans* dauer-inducing pheromone. *Nature* 2005; **433**: 541–545.

32 Kawano T, Kataoka N, Abe S *et al.* Lifespan extending activity of substances secreted by the nematode *Caenorhabditis elegans* that include the dauer-inducing pheromone. *Biosci Biotechnol Biochem* 2005; **69**: 2479–2481.

33 Ren P, Lim CS, Johnsen R *et al.* Control of *C. elegans* larval development by neuronal expression of a TGF-b homolog. *Science* 1996; **274**: 1389–1391.

34 Birnby DA, Link EM, Vowels JJ *et al.* A transmembrane guanylyl cyclase (DAF-11) and hsp90 (DAF-21) Regulate a common set of chemosensory behaviors in *Caenorhabditis elegans*. *Genetics* 2000; **155**: 85–104.

35 Sze JY, Victor M, Loer C *et al.* Food and metabolic signalling defects in a *Caenorhabditis elegans* serotonin-synthesis mutant. *Nature* 2000; **403**: 560–564.

36 Motola DL, Cummins CL, Rottiers V *et al.* Mangelsdorf DJ. Identification of Ligands for DAF-12 that Govern Dauer Formation and Reproduction in *C. elegans*. *Cell* 2006; **124**: 1209–1223.

37 Vanfleteren JR. Oxidative stress and ageing in *Caenorhabditis elegans*. *Biochem J* 1993; **292**: 605–608.

38 Larsen PL. Aging and resistance to oxidative damage in *Caenorhabditis elegans*. *Proc Nat Acad Sci USA* 1993; **90**: 8905–8909.

39 Larsen PL, Albert PS, Riddle DL. Genes that regulate both development and longevity in *Caenorhabditis elegans*. *Genetics* 1995; **139**: 1567–1583.

40 Dorman JB, Albinder B, Shroyer T *et al.* The *age-1* and *daf-2* genes function in a common pathway to control the lifespan of *Caenorhabditis elegans*. *Genetics* 1995; **141**: 1399–1406.

41 Dillin A, Crawford DK, Kenyon C. Timing requirements for insulin/IGF-1 signaling in *C. elegans*. *Science* 2002; **298**: 830–834.

42 Giglio MP, Hunter T, Bannister JV *et al.* The manganese superoxide dismutase gene of *Caenorhabditis elegans*. *Biochem Mol Biol Int* 1994; **33**: 37–40.

43 Suzuki N, Inokuma K, Yasuda K *et al.* Cloning, sequencing and mapping of a manganese superoxide dismutase gene of the nematode *Caenorhabditis elegans*. *DNA Res* 1996; **3**: 171–174.

44 Hunter T, Bannister WH, Hunter GJ. Cloning, expression, and characterization of two manganese superoxide dismutases from *Caenorhabditis elegans*. *J Biol Chem* 1997; **272**: 28652–28659.

45 Fujii M, Ishii N, Joguchi A *et al.* A novel superoxide dismutase gene encoding membrane-bound and extracellular isoforms by alternative splicing in *Caenorhabditis elegans*. *DNA Res* 1998; **5**: 25–30.

46 Sun J, Folk D, Bradley TJ *et al.* Induced overexpression of mitochondrial Mn-superoxide dismutase extends the life span of adult *Drosophila melanogaster*. *Genetics* 2002; **161**: 661–672.

47 Hu D, Cao P, Thiels E *et al.* Hippocampal long-term potentiation, memory, and longevity in mice that overexpress mitochondrial superoxide dismutase. *Neurobiol Learn Mem* 2007; **87**: 372–384.

48 Bayne AC, Mockett RJ, Orr WC *et al.* Enhanced catabolism of mitochondrial superoxide/hydrogen peroxide and aging in transgenic *Drosophila*. *Biochem J* 2005; **391**: 277–284.

49 Orr WC, Mockett RJ, Benes JJ *et al.* Effects of overexpression of copper-zinc and manganese superoxide dismutases, catalase, and thioredoxin reductase genes on longevity in *Drosophila melanogaster*. *J Biol Chem* 2003; **278**: 26418–26422.

50 Remmen HV, Ikeno Y, Hamilton M *et al.* Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. *Physiol Genomics* 2003; **16**: 29–37.

51 Honda Y, Tanaka M, Honda S. Modulation of longevity and diapause by redox regulation mechanisms under the insulin-like signaling control in *Caenorhabditis elegans*. *Exp Gerontol* 2008; **43**: 520–529.

52 Wong GHW, Goeddel DV. Induction of manganous superoxide dismutase by tumor necrosis factor: possible protective mechanism. *Science* 1988; **242**: 941–944.

53 Bedogni B, Pani G, Colavitti R *et al.* Redox regulation of cAMP-responsive element-binding protein and induction of manganous superoxide dismutase in nerve growth factor-dependent cell survival. *J Biol Chem* 2003; **278**: 16510–16519.

54 Abid MR, Tsai JC, Spokes KC *et al.* Vascular endothelial growth factor induces manganese-superoxide dismutase expression in endothelial cells by a Rac1-regulated NADPH oxidase-dependent mechanism. *FASEB J* 2001; **15**: 2548–2550.

55 Lee CY, Pastore JN, Tang G *et al.* Cellular distribution of pulmonary Mn and CuZn superoxide dismutase: effect of hyperoxia and interleukin-1. *J Histochem Cytochem* 1994; **42**: 1201–1205.

56 Kifle Y, Monnier J, Chesrown SE *et al.* Regulation of the manganese superoxide dismutase and inducible nitric oxide synthase gene in rat neuronal and glial cells. *J Neurochem* 1996; **66**: 2128–2135.

57 Bertrand F, Desbois-Mouthon C, Cadoret A *et al.* Insulin anti-apoptotic signaling involves insulin activation of the nuclear factor κB-dependent survival genes encoding tumor necrosis factor receptor-associated factor 2 and manganese-superoxide dismutase. *J Biol Chem* 1999; **274**: 30596–30602.

58 Fernandez V, Tapia G, Varela P *et al.* Redox up-regulated expression of rat liver manganese superoxide dismutase and Bcl-2 by thyroid hormone is associated with inhibitor of ?B-a phosphorylation and nuclear factor κB activation. *J Endocrinol* 2005; **186**: 539–547.

59 Visner GA, Dougall WC, Wilson JM *et al.* Regulation of manganese superoxide dismutase by lipopolysaccharide, interleukin-1, and tumor necrosis factor. Role in the acute inflammatory response. *J Biol Chem* 1990; **265**: 2856–2864.

60 Ishii N, Fujii M, Hartman PS *et al.* A mutation in succinate dehydrogenase cytochrome b causes oxidative stress and ageing in nematodes. *Nature* 1998; **394**: 694–697.

61 Harman D. Aging: a theory based on free radical and radiation chemistry. *J Gerontol* 1956; **11**: 298–300.

62 Harman D. The free radical theory of aging. *Antioxid Redox Signal* 2003; **5**: 557–561.

63 Clancy DJ, Gems D, Harshman LG *et al.* Extension of life-span by loss of CHICO, a *Drosophila* insulin receptor substrate protein. *Science* 2001; **292**: 104–106.

64 Holzenberger M, Dupont J, Ducos B *et al.* IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature* 2003; **421**: 182–187.

65 Keaney M, Matthijssens F, Sharpe M *et al.* Superoxide dismutase mimetics elevate superoxide dismutase activity *in vivo* but do not retard aging in the nematode *Caenorhabditis elegans*. *Free Radic Biol Medical* 2004; **37**: 239–250.

66 Magwere T, West M, Riyahi K *et al.* The effects of exogenous anti-oxidants on lifespan and oxidative stress resistance in *Drosophila melanogaster*. *Mech Ageing Dev* 2006; **127**: 356–370.

67 Hayflick L, Moorhead PS. The limited *in vitro* lifetime human diploid cell strains. *Exp Cell Res* 1961; **25**: 595–621.

68 Noda A, Ning Y, Venable SF, Pereira-Smith OM, Smith JR. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp Cell Res* 1994; **211**: 90–98.

69 Smith JR, Pereira-Smith OM. Replicative senescence: implications for *in vivo* aging and tumor suppression. *Science* 1996; **273**: 63–67.

70 Stein GH, Beeson M, Gordon L. Failure to phosphorylate the retinoblastoma gene product in senescent human fibroblasts. *Science* 1990; **249**: 666–669.

71 Packer L, Fuehr K. Low oxygen concentration extends the lifespan of cultured human diploid cells. *Nature (London)* 1977; **267**: 423–425.

72 Parrinello S, Samper E, Krtolica A *et al.* Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nat Cell Biol* 2003; **5**: 741–747.

73 Balin AK, Goodman DB, Rasmussen H *et al.* The effect of oxygen and vitamin E on the lifespan of human diploid cells *in vitro*. *J Cell Biol* 1977; **74**: 58–67.

74 Honda S, Matsuo M. Shortening of the *in vitro* lifespan of human diploid fibroblasts exposed to hyperbaric oxygen. *Exp Gerontol* 1983; **18**: 339–345.

75 Chen Q, Ames BN. Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells. *Proc Natl Acad Sci USA* 1994; **91**: 4130–4134.

76 von Zglinicki T, Saretzki G, Docke W *et al.* Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence? *Exp Cell Res* 1995; **220**: 186–193.

77 Gorbunova V, Seluanov A, Pereira-Smith OM. Expression of human telomerase (hTERT) does not prevent stress-induced senescence in normal human fibroblasts but protects the cells from stress-induced apoptosis and necrosis. *J Biol Chem* 2002; **277**: 38540–38549.

78 Honda S, Matsuo M. Relationships between the cellular glutathione level and *in vitro* life span of human diploid fibroblasts. *Exp Gerontol* 1988; **23**: 81–86.

79 Blander G, de Oliveira RM, Conboy CM *et al.* SOD1 knock down induces senescence in human fibroblasts. *J Biol Chem* 2003; **278**: 38966–38969.

80 Serra V, von Zglinicki T, Lorenz M *et al.* Extracellular superoxide dismutase is a major anti-oxidant in human fibroblasts and slows telomere shortening. *J Biol Chem* 2003; **278**: 6824–6830.

81 Lee AC, Fenster BE, Ito H *et al.* Ras proteins induce senescence by altering the intracellular levels of reactive oxygen species. *J Biol Chem* 1999; **274**: 7936–7940.