

Novel interactions between mitochondrial superoxide dismutases and the electron transport chain

Wichit Suthammarak,^{1,3*} Benjamin H. Somerlot,^{2*†}
Elyce Opheim,¹ Margaret Sedensky^{1,2‡} and
Philip G. Morgan^{1,2‡}

¹Department of Anesthesiology and Pain Medicine, Center for Developmental Therapeutics, University of Washington and Seattle Children's Research Institute, Seattle, WA, USA

²Department of Genetics, Case Western Reserve University, Cleveland, OH, USA

Summary

The processes that control aging remain poorly understood. We have exploited mutants in the nematode, *Caenorhabditis elegans*, that compromise mitochondrial function and scavenging of reactive oxygen species (ROS) to understand their relation to lifespan. We discovered unanticipated roles and interactions of the mitochondrial superoxide dismutases (mtSODs): SOD-2 and SOD-3. Both SODs localize to mitochondrial supercomplex I:III:IV. Loss of SOD-2 specifically (i) decreases the activities of complexes I and II, complexes III and IV remain normal; (ii) increases the lifespan of animals with a complex I defect, but not the lifespan of animals with a complex II defect, and kills an animal with a complex III defect; (iii) induces a presumed pro-inflammatory response. Knockdown of a molecule that may be a pro-inflammatory mediator very markedly extends lifespan and health of certain mitochondrial mutants. The relationship between the electron transport chain, ROS, and lifespan is complex, and defects in mitochondrial function have specific interactions with ROS scavenging mechanisms. We conclude that mtSODs are embedded within the supercomplex I:III:IV and stabilize or locally protect it from reactive oxygen species (ROS) damage. The results call for a change in the usual paradigm for the interaction of electron transport chain function, ROS release, scavenging, and compensatory responses.

Key words: lifespan; superoxide dismutase; reactive oxygen species; electron transport chain; supercomplexes; heat shock protein.

Abbreviations

HNE 4-hydroxynonenal

Correspondence

Philip G Morgan, Seattle Children's Research Institute, 1900 9th Ave, Seattle, WA 98101, USA. Tel.: +1 206-884-1101; fax: +1 206-987-7661; e-mail: pgm4@uw.edu

*These authors contributed equally as primary authors to this article.

†Deceased.

‡These authors contributed equally as senior authors to this article.

³Present address: Department of Biochemistry, Faculty of Medicine Siriraj Hospital, Aroon-amarin Road, Srisavarindira Building 10th Floor, Office #1008, Bangkok-noi, Bangkok 10700, Thailand

Accepted for publication 20 July 2013

IGA in-gel activity
mtSOD mitochondrial superoxide dismutase
ETC electron transport chain
MRC mitochondrial respiratory chain

Introduction

In 1956, Denham Harman first proposed that the accumulation of oxidative damage from reactive oxygen species (ROS), by-products of normal metabolism, is responsible for aging (Harman, 1956). Multiple studies have brought this theory into question (Yang *et al.*, 2007; Doonan *et al.*, 2008; Gems & Doonan, 2009; Yang & Hekimi, 2010a,b), such that the roles of ROS in aging are not yet fully resolved. The main site of production of ROS in the cell is the electron transport chain (ETC) within the mitochondrial inner membrane (Koopman *et al.*, 2010). Studies in the model organism *Caenorhabditis elegans* have tested the role of oxidative damage in the process of aging by chemical manipulation of ROS levels (paraquat or juglone), or manipulation of ROS production by inhibiting electron transport (Senoo-Matsuda *et al.*, 2001; Hartwig *et al.*, 2009; Heidler *et al.*, 2010). More recently, the power of this organism as a genetic model has been applied to alter the expression of components of the ETC (Ishii *et al.*, 1998; Kayser *et al.*, 2004a,b; Yang & Hekimi, 2010b) or of scavengers of ROS (Yang *et al.*, 2007; Doonan *et al.*, 2008; Van Raamsdonk & Hekimi, 2009; Cabreiro *et al.*, 2011). In particular, scavengers both within the mitochondrion (e.g., mitochondrial superoxide dismutase (mtSOD) and glutathione reductase) and within the cytosol (e.g., cytoplasmic SOD and catalase) have been studied in detail (Sampayo *et al.*, 2003; McElwee *et al.*, 2004; Yang *et al.*, 2007; Doonan *et al.*, 2008).

Defects in the ETC are linked to ROS generation, can decrease respiration rates, alter oxidative damage to cells, delay development, and impair fertility (Ishii *et al.*, 1998; Grad & Lemire, 2004; Kayser *et al.*, 2004a). Initial theories linked the amount of oxidative damage and lifespan in several mitochondrial mutants. For example, several short-lived mutants (e.g., *gas-1*, a complex I mutant; *mev-1*, a complex II mutant) have increased oxidative damage to mitochondrial proteins (Hartman *et al.*, 2001; Yang *et al.*, 2009), while the long-lived mutant *clk-1* (*clk-1* codes for an enzyme required for ubiquinone synthesis) displays significantly less oxidative damage to mitochondrial proteins than wild type (Yang *et al.*, 2009).

If ROS play a major role in aging, one would expect that defects in ROS scavenging should adversely affect lifespan. *C. elegans* has five different genes that encode different superoxide dismutases (*sod-1-5*), which catalyse the enzymatic conversion of the toxic superoxide molecule to the less reactive species, hydrogen peroxide (Van Raamsdonk & Hekimi, 2009). *sod-1* encodes the major cytosolic superoxide dismutase (SOD), while *sod-2* encodes the primary SOD found in the mitochondrion (Yang *et al.*, 2007). Expression of *sod-1* or *sod-2* was increased in *C. elegans* under conditions that increased lifespan (Honda *et al.*, 2008).

Keaney *et al.* (2004) showed that when *C. elegans* was exposed to superoxide generators, the addition of superoxide dismutase mimetics lengthened lifespans, though not back to normal. Under normal

conditions, these mimetics did not affect lifespans. However, the role of ROS in aging is disputed (Doonan et al., 2008; Gems & Doonan, 2009; Van Raamsdonk & Hekimi, 2009; Cabreiro et al., 2011). Hekimi reported that a deletion of *sod-2* lengthened lifespan and that *clk-1*; *sod-2* lived longer than the long-lived *clk-1*, despite increased oxidative damage in mitochondrial protein (Yang et al., 2007). In fact, Van Raamsdonk eliminated all of the superoxide dismutases in *C. elegans* without shortening lifespan (Van Raamsdonk & Hekimi, 2012). The overall conclusion was that superoxide dismutase had little relevance to normal lifespan in *C. elegans* (Yang et al., 2007; Doonan et al., 2008; Gems & Doonan, 2009; Van Raamsdonk & Hekimi, 2009, 2012).

Several authors have attempted to clarify the role of SODs in determining lifespan by the use of other longevity mutants. The well-studied and long-lived mutant *daf-2* initiates a longevity pathway involving the insulin/IGF-I signaling pathway that ultimately causes nuclear localization of DAF-16, a FOXO transcription factor. The activation of *daf-16* increased the expression of *sod-3*, an mtSOD normally expressed in very low levels in wild-type worms (Honda et al., 2008). However, elimination of both mitochondrial *sod-2* and *sod-3* failed to suppress the long lifespan of *daf-2* (Honda & Honda, 2002; Yang et al., 2007). These studies suggested that the upregulation of *sod-3* (and presumably decreased superoxide damage to mitochondria) did not significantly contribute to the long lifespan of *daf-2*. However, others have shown that superoxide levels may be decreased by non-SOD mechanisms involving the activation of the insulin/IGF-I signaling pathway, implying that superoxide may still play a role in aging in *daf-2* (Yanase & Ishii, 2008; Yazaki et al., 2011). The protein product of *gst-10* scavenges the toxic ROS-generated product, 4-hydroxynonenal (HNE) (Cohn et al., 1996); its overexpression extends the lifespan of N2, while the *gst-10* null reduces lifespan (Ayyadevara et al., 2005, 2007), in support of a role for ROS damage in aging.

In the majority of studies that implicate ROS damage or mitochondrial function in the animal's phenotype, these parameters are generally inferred and seldom measured. Difficulties intrinsic to measuring ROS production/damage or mitochondrial function have limited the rigorous interpretation of data. Currently, the data concerning ROS and aging are complicated, even in a simple animal model. In this study, our goal was to determine the relationship between mitochondrial function, oxidative

damage, and lifespan in *C. elegans* by studying the effect of mtSOD mutants on the ETC, as well as determining the effect of eliminating mtSOD activity in mitochondrial mutants. To date, there have been few reports of the effects of the mitochondrial *sods* on mitochondrial function (Melov et al., 1999), their role in minimizing ROS damage to the mitochondrial machinery, or their relative importance, if any, in the face of mitochondrial dysfunction. Our results show that the mitochondrial SODs are bound to components of the ETC and that effects of loss of mtSODs can counteract each other, thereby complicating earlier simple interpretations of the roles of mtSODs in health and aging.

Results

Specific extension of lifespan by *sod-2* and *sod-3*

As previously reported, *gas-1(fc21)* (a complex I mutant) and *mev-1(kn1)* (a complex II mutant) are short-lived in comparison with wild type (N2) (Fig. 1) (Ishii et al., 1998; Kayser et al., 2004a). *sod-2(gk257)* had a normal lifespan in agreement with Gems and colleagues (Keaney et al., 2004; Doonan et al., 2008). However, the loss of *sod-2* in three mutant backgrounds that share common mitochondrial phenotypes, including sensitivity to oxidative stress, had very different results. *sod-2;gas-1* lived longer than both *gas-1(fc21)* and the wild type, N2. *sod-2;mev-1* was short-lived and not different from *mev-1* (Fig. 1). *sod-2;isp-1* (*isp-1* is a complex III mutant) was a synthetic lethal.

In agreement with previous studies (Doonan et al., 2008; Van Raamsdonk & Hekimi, 2009), the lifespan of *sod-3* was not different from N2 (Fig. S1). However, the mutation *sod-3* differed from *sod-2* in its interactions with *gas-1*, *mev-1*, and *isp-1*. The lifespan of *gas-1sod-3* was not different than that of *gas-1*. *mev-1;sod-3* had a significantly shorter lifespan than did *mev-1* (Fig. S1). The lifespan of *isp-1; sod-3* was significantly longer than either N2, *isp-1*, or *sod-3* alone (Fig. S1). *sod-2; sod-3* had a lifespan similar to N2 as previously reported (Van Raamsdonk & Hekimi, 2009) (Data not shown).

Effects of *sod-2* and *sod-3* on reproductive capacity and postembryonic development

We measured times of postembryonic development (Fig. 2A) and rates of egg-laying (Fig. 2B) in all mutants as measures of health. Compared

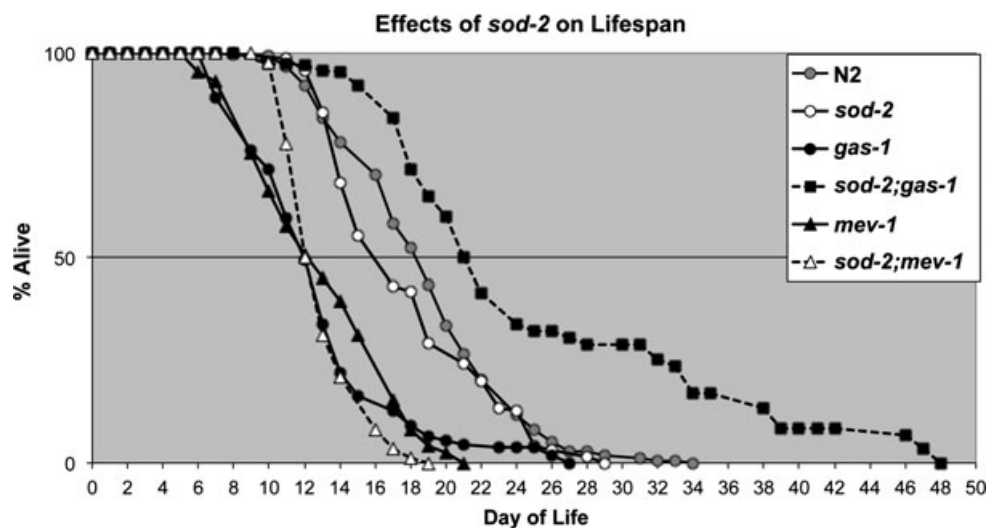


Fig. 1 Lifespans, effects of *sod-2*. Lifespan studies started on day 0 defined as the day of hatching. Lifespans of wild type (N2), *gas-1*, *mev-1*, *sod-2*, *sod-2;mev-1*, and *sod-2;gas-1*. *sod-2;gas-1* lives longer than N2 (median lifespans, N2 19.2 ± 0.5 days, *sod-2*, 18.1 ± 0.8 days, *gas-1*, 11.4 ± 0.4 days, *sod-2;gas-1*, 24.2 ± 0.9 days).

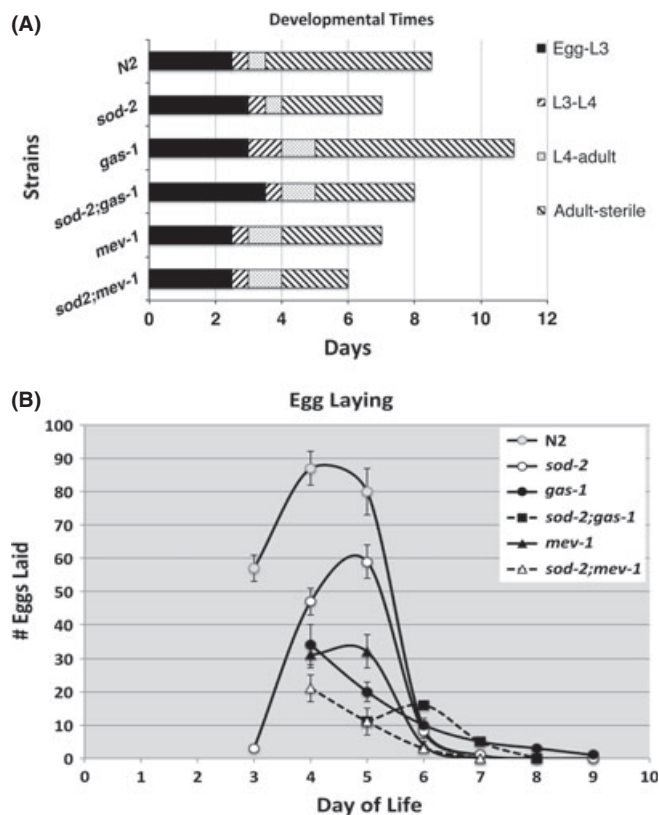


Fig. 2 Developmental Traits. (A) Postembryonic Times. Rates of postembryonic development of the animals were determined from synchronized cultures beginning at day 0 as eggs. Adult animals were scored until cessation of egg laying. Adult sterile refers to the time from first day of adulthood to ceasing to lay eggs. At least 100 eggs were followed in each experiment and repeated three independent times. (B) Egg Laying. Fecundity of the animals was measured by the number of eggs laid per worm per day. Day 0 was the day that parental animals were eggs. Data are represented as mean \pm SEM from three independent worm cultures (totaling 20–30 adult animals for each strain).

with N2, postembryonic times (from egg to adult) of *sod-2*, *gas-1*, and *mev-1* were increased and fecundities were decreased. However, lengths of the reproductive periods (from adult to sterile) were decreased in *sod-2* and *mev-1*, but increased in *gas-1* (Fig. 2A). *sod-2* did not alter the times of postembryonic development of *gas-1* and *mev-1* but did decrease their reproductive capacities (Fig. 2B). *sod-3* did not affect the development or fecundity of *gas-1*, *mev-1*, or *isp-1* (data not shown).

Effects of *sod-2* and *sod-3* on respiration and electron transport chain function

Compared with N2, the complex I-dependent state 3 oxidative phosphorylation capacity was significantly decreased in *sod-2* but not in *sod-3* animals (Fig. 3A,B). No additional effect on respiration was seen in *sod-2;sod-3* compared with *sod-2*. Complex I respiratory capacity was less in *sod-2;gas-1* than in either single mutation alone (Fig. 3A). Complex II-dependent respiration was unchanged in the *sod* mutants (3B). Respiration in other mitochondrial mutants was as previously described (Ishii *et al.*, 1998; Kayser *et al.*, 2001). State 4 respirations were identical to N2 in all mutants (data not shown). Because of the specific decreased complex I-dependent respiratory capacity of *sod-2*,

we next examined specific enzymatic activities of the electron transport chain.

The respiratory chain mutants had ETC enzymatic rates as previously described (Fig. 3C–E) (Ishii *et al.*, 1998; Kayser *et al.*, 2001, 2004a; Senoo-Matsuda *et al.*, 2001). Compared with N2, *sod-2* significantly reduced complex I-related activities; the change in complex II rate did not reach significance ($P = 0.01$) (Fig. 3C–E). NADH ferricyanide reductase (NFR), which measures activity through the very proximal part of complex I and is taken as a measure of the amount of the complex (Suthammarak *et al.*, 2010), was decreased in *sod-2*. Complex I activity, which measures flow of electrons through the entire complex, was decreased to a greater extent in *sod-2* than was NFR (Fig. 3C,D). Similar to *gas-1*, *sod-2;gas-1* exhibited decreased activities of complex I (Fig. 3C,D) and I-III (not shown). *sod-2;mev-1* exhibited decreased complex I and complex II functions (Fig. 3C,E). Complex II function of *sod-2;mev-1* was not significantly different than that of *mev-1* (Fig. 3E). *sod-3* showed no differences from N2 in complex I or II activities (data not shown). Complex III and IV activities of all the mutants remained normal (data not shown).

Changes in the formation of mitochondrial supercomplexes

As *sod-2* exhibited complex I respiratory dysfunction (Fig. 3A), we determined whether the formation of mitochondrial I:III:IV supercomplexes was affected in *sod-2(0)* animals. BNGs revealed a 28% decrease in the formation of supercomplexes I:III and I:III:IV_n in *sod-2(0)* mitochondria by complex I in-gel activity (CI-IGA) (Fig. 4A,B). The amount of complex I in the supercomplexes was decreased in all other mutants except *mev-1* as also determined by CI-IGA (Fig. 4B). *sod-2;gas-1* had less complex I-containing supercomplexes than either *sod-2* or *gas-1* (30% of that in *gas-1* and 10% of that in *sod-2*). The loss of complex I in *sod-2* was further shown by a decrease in the complex I protein NDUF52 in the supercomplexes (Fig. 4C). *sod-3* BNGs were normal (Fig. 5E).

We then studied the location of SOD-2 by BNG analysis. Western blots of BNGs indicated that SOD-2 co-localized with the I:III:IV supercomplex (Fig. 4D). We have confirmed this finding in BNGs from mouse mitochondria as well (data not shown). The residual binding of anti-SOD-2 in the *sod-2* mutant was much decreased in the *sod-2;sod-3* mutant, indicating that there was cross-specificity between SOD-2 and SOD-3 for this antibody (Fig. 52). In addition, the results show that SOD-3 is also localized to the I:III:IV supercomplex.

HNE damage in mitochondrial protein and total worm protein

The loss of *sod-2* increased HNE damage to mitochondrial protein in *gas-1* and *mev-1*, but not total worm protein (Fig. 5A–D). The extent of oxidative damage to supercomplex protein was assessed by normalizing HNE staining to total Coomassie staining. BNGs of mitochondria showed an increase in HNE staining in the *sod-2;sod-3* double mutant compared with either mutant alone or N2 (Fig. 5E). In those mutants containing complex I mutations (*gas-1* or *nuo-6*), HNE staining more than doubled compared with N2 (quantification shown in Fig. 5F) in Western blots of native gels (Fig. 53).

The response of antioxidant defense mechanisms

Our earlier studies comparing gene expression of *gas-1* and *mev-1* showed that *sod-3*, *hsp-6* (involved in the mitochondrial unfolded protein response; mtUPR), and glutathione-S-transferase-14 (*gst-14*) were significantly increased in *gas-1*, but not in *mev-1* (Falk *et al.*, 2008).

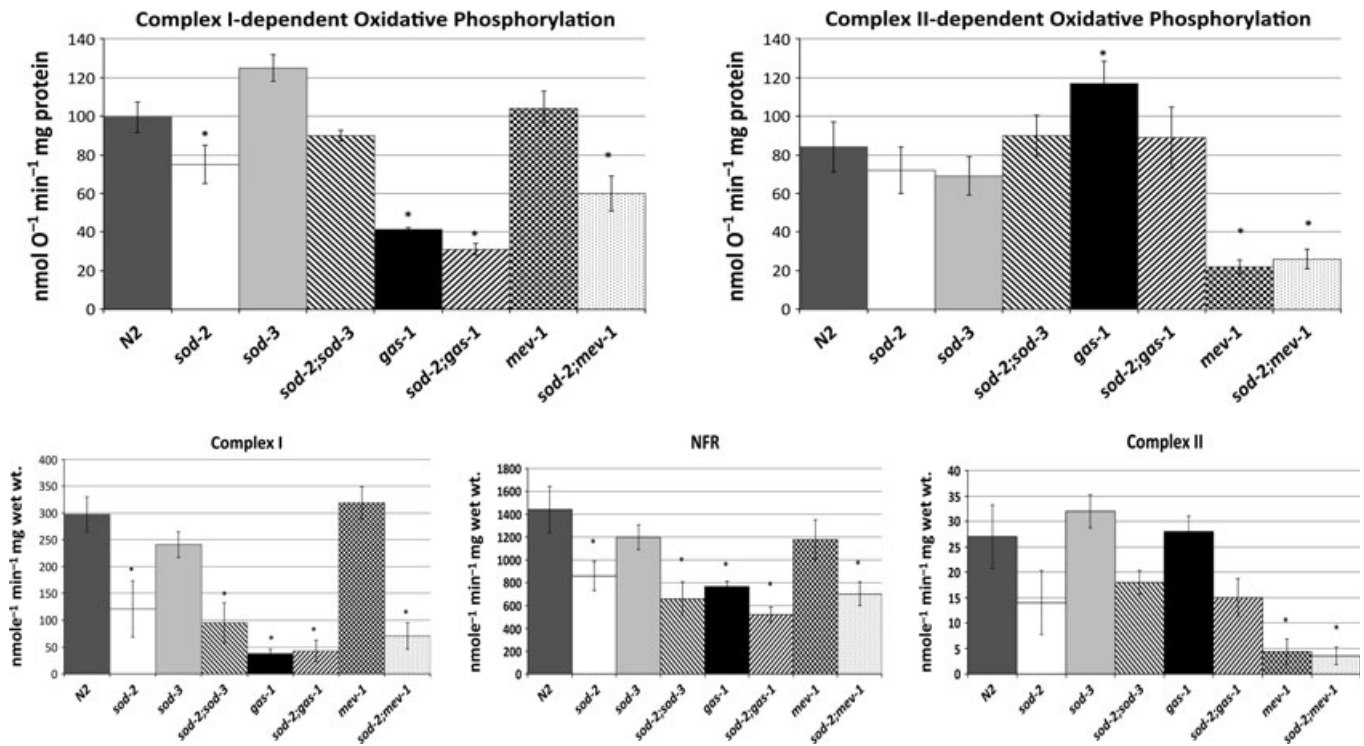


Fig. 3 Oxidative Phosphorylation Studies. (A) Complex I. State 3 rates of oxidative phosphorylation of wild type, *gas-1*, *mev-1*, *sod-2*, *sod-3*, *sod-2;sod-3*, *sod-2;gas-1*, and *sod-2;mev-1* using malate as a substrate. *gas-1*, *sod-2*, *sod-2;gas-1*, and *sod-2;mev-1* each have decreased complex I-dependent respiratory capacities. (B) Complex II. State 3 complex II-dependent rates using succinate as a substrate. No change in complex II-dependent rates was noted in *sod-2* or *sod-3*. (C–E) Electron transport chain function. Respiratory chain enzymatic activities of wild type, *gas-1*, *mev-1*, *sod-2*, *sod-3*, *sod-2;sod-3*, *sod-2;gas-1*, and *sod-2;mev-1*. Enzymatic activity of: (C) CI (D) NFR and (E) CII are shown. Complex I rates were decreased in *sod-2* and *sod-2* containing double mutants. CIII and CIV rates were normal in all mutants and are not shown. Data in all panels are represented as mean \pm SEM from three to eight independent worm cultures. * indicates statistical significance as $P < 0.05$ in comparison with wild type (with a Bonferroni correction for $n = 8$ resulting in $P < 0.00625$ for significance as described in Statistical analysis).

Others have shown that the mtUPR plays an important role in determining lifespan (Durieux et al., 2011; Baker et al., 2012). However, changes in the mtUPR did not explain the effects of *sod-2(0)* on *gas-1* and *mev-1* (Figs S4, S5). To further understand the role of antioxidant defense mechanisms in the lifespans of mitochondrial mutants, we studied the expression of genes encoding antioxidant proteins by real-time PCR (Table 1). Expression of *sod-1*, a cytoplasmic superoxide dismutase, remained unchanged in all mutants in comparison with wild type. *gas-1* and *mev-1* did not alter the expression of *sod-2* compared with wild type. Expression of *sod-3* and *gst-14* was increased in all strains except those containing *mev-1*. The highest levels of both were found in *sod-2;gas-1*. We also found that a triglyceride lipase (K04A8.5) (providing a potential alternate substrate source in a complex I mutant) was upregulated in *gas-1* and *sod-2;gas-1* ($2.9(\pm 1.3)$ and $7.06(\pm 1.8)$ fold compared with N2). As both *sod-3* and K04A8.5 are known to be downstream from *daf-16* (the FOXO transcription factor in the *daf-2* pathway), we hypothesized that the lifespan extension in *sod-2;gas-1* might be *daf-16* dependent. As reported earlier by Kondo et al. (2005), we found that DAF-16 was localized to the nucleus in *gas-1* and *mev-1* (not shown). However, we found that exposure of *gas-1* or *sod-2;gas-1* to *daf-16* RNAi extended lifespan [mean lifespan *gas-1*:10 days vs. 19 days (grown on *daf-16* RNAi); *sod-2;gas-1*: 17 days vs. 24 days (grown on *daf-16* RNAi)]. Thus, the extended lifespan of *sod-2;gas-1* is not *daf-16* dependent.

If increased expression of *sod-3* or *gst-14* causes the prolonged lifespan of *sod-2;gas-1*, then interfering with their expression should shorten the lifespan of *sod-2;gas-1*. We found that the triple mutant

sod-2;gas-1;sod-3 was lethal, consistent with this hypothesis. In contrast, and contrary to this hypothesis, RNAi knockdown of *gst-14* in *sod-2;gas-1* animals more than doubled their lifespan on the same food source (HT115) (Fig. 6). Development of those animals is accelerated compared with *sod-2;gas-1* animals grown in the absence of *gst-14* RNAi (data not shown). We obtained similar results for the effect of *gst-14* RNAi on *gas-1* animals (prolonged lifespan) (Fig. 6). In both cases, the lifespan curves were biphasic, in that a subset of animals died very early in life (app. day 9), while others were long-lived. Exposure to *gst-14* RNAi did not prolong the lifespan of *mev-1* (Fig. 6), nor the wild-type N2 (data not shown).

Discussion

We expected that combining the loss of SOD-2 with mitochondrial mutants known to increase oxidative stress (Kayser et al., 2004a; Kondo et al., 2005; Yang & Hekimi, 2010a) would be detrimental to lifespan. Loss of SOD-2 did in fact slow development and decrease fecundity compared with N2 and significantly worsened those phenotypes when coupled with *gas-1* and *mev-1*. Surprisingly though, loss of SOD-2 specifically increased the lifespan of *gas-1*, but not that of *mev-1*. We could not attribute the lengthened lifespan of *sod-2;gas-1* to any improvement in respiratory function of *gas-1* or change in levels of ROS damage. In fact, no single component of mitochondrial physiology that we studied correlates simply with lifespan (Fig. 7). However, loss of *sod-2* directly decreased mitochondrial respiration, supercomplex formation, and ETC function. Therefore, the scavenging functions of the

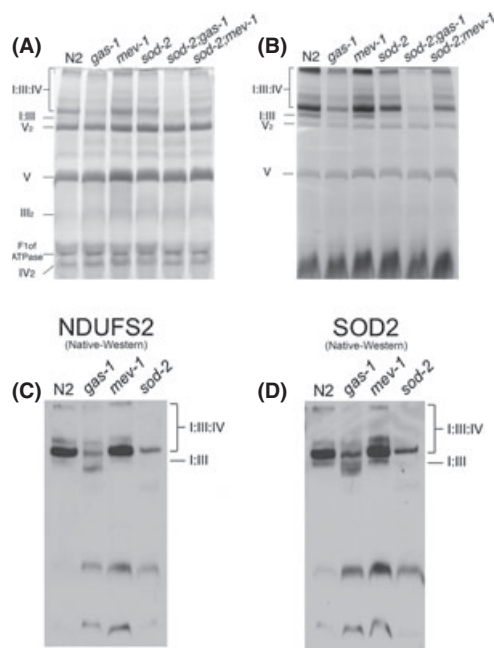


Fig. 4 Supercomplex formation. Digitonin-based blue native gels (BNGs) from frozen mitochondrial pellets of N2 and the mutants as indicated. (A) Coomassie staining of BNGs. Identification of supercomplex I:III (I:III), supercomplex I:III:IV (I:III:IV), dimeric complex V (V_2), monomeric complex V (V), dimeric complex III (III_2), unbound form of the F1 part of complex V (F1 of ATPase), and dimeric complex IV (IV_2) are inferred from previously published proteomic data and molecular weights (Suthammarak et al., 2009). (B) Complex I in-gel activity (CI-GA) staining from the same samples as in A in a parallel gel. (C) A Western blot of a BNG probed with anti-NDUFS2, a complex I subunit. NDUFS2 is the mammalian homologue of GAS-1. This blot confirms the decrease in the amount of complex I in supercomplexes of *gas-1* and *sod-2*. (D) Western blot from BNGs of mitochondrial mutants probed with anti-SOD-2. Note that SOD-2 is localized to the I:III:IV supercomplex. All gels were repeated three independent times. Figures are representative gels or blots.

mtSODs must be considered in conjunction with their effects on respiration itself. Others have previously reported decreases in complex I- and II-dependent function in SOD-2-deficient mice (Melov et al., 1999; Van Remmen et al., 2001). This dependence of complex I function on SOD-2 extends to *C. elegans* as well, and as such implies an ancient conserved dependence of the ETC on this ROS scavenger.

The surprising presence of SOD-2 in I:III:IV supercomplexes (Fig. 4D) indicates that SOD-2 plays a pivotal role in local scavenging of superoxide at the site of its production. However, the lack of a large increase in ROS damage to supercomplexes in a *sod-2* animal may indicate that SOD-3 is also capable of performing this function, especially when the amount of supercomplex I:III:IV is reduced. This possibility is strengthened by two observations: 1) ROS damage to the supercomplexes is relatively increased if both SOD-2 and SOD-3 are absent; and 2) in an animal that is sensitive to oxidative stress (*gas-1*), loss of both mtSODs is lethal. Our findings are consistent with the hypothesis that the mtSODs, which are associated with the I:III:IV supercomplex, protect components of the supercomplex from ROS damage; their loss is accompanied by damage to specific proteins in the mitochondrial ETC (Fig. 5E,F).

A dependence of the amount of supercomplex I:III:IV on SOD-2 in supercomplexes has not been previously noted. Maranzana et al. (2013) did report that the stability of the I:III:IV supercomplex was linked to superoxide production in bovine heart mitochondria. In contrast to our results, they found increased ROS when I:III:IV deteriorated. However, they measured ROS production from an intact complex I that had been

acutely dissociated from the supercomplex with detergent. We measured accumulated ROS damage generated *in vivo* from animals that had lifelong complex I dysfunction due to the loss of SOD-2. These animals also upregulated the expression of inducible mtSOD in worms, *sod-3*. Lifelong decreased mitochondrial respiration, with increased SOD-3, partially accounts for the lack of increased ROS damage in *gas-1* and *sod-2;gas-1*. We further postulate that the loss of SOD-2 (in *sod-2;gas-1*) locally increases ROS damage to the key components of the ETC causing a dissolution of supercomplex I:III:IV, which, in a negative feedback loop, decreases complex I-dependent respiration and limits superoxide production from complex I. However, as complex I function is decreased in *sod-2* mitochondria out of proportion to the amount of ROS damage, it is also possible that the mtSODs may directly serve as stabilizing factors in the I:III:IV supercomplex.

The loss of *sod-2* did not result in global cellular ROS damage to proteins (Fig. 5A–D), nor in a large increase in ROS damage to mitochondrial proteins. This is perhaps not surprising because loss of superoxide dismutase, by itself, will not increase ROS production nor decrease the ultimate conversion of ROS to nonharmful molecules. The increases in HNE staining seen in total mitochondrial proteins in *sod-2;gas-1* and *sod-2;mev-1* (Fig. 5A) are in proteins not maintained in MRC complexes in native gels (Fig. 5F). These proteins may be soluble matrix proteins, which normally dissociate from the MRC complexes under conditions used for BNGs, or damaged proteins that are eliminated from the MRC complexes. We favor the third possibility, although further study is necessary to confirm the model.

We also found that *sod-3* expression was significantly upregulated in *sod-2* animals (Table 1), in contrast to Back et al. (2010). However, their study did find a 1.5-fold increase in *sod-3* expression that did not reach statistical significance. Our nematodes were grown on OP50, not K12 as in the Back study. It is possible that the decreased bacterial lawn of the uracil-requiring bacteria (OP50) introduced an extra level of stress, resulting in more upregulation of *sod-3*.

How does *sod-2* extend the lifespans of *gas-1* and *clk-1* (Yang et al., 2007), but not *mev-1* or *isp-1*? Both *gas-1* and *clk-1* have decreased complex I function as well as increased ROS production (Hartman et al., 2001; Kayser et al., 2001, 2004a,b; Yang et al., 2009). The extended lifespans of *sod-2;gas-1* and *sod-2;clk-1* (Yang et al., 2007) likely result from decreased metabolism coupled with an increase in the expression of *sod-3* (and perhaps other ROS scavengers). The slowing of complex I-dependent respiration can be viewed as a negative feedback mechanism to limit the production of ROS from the I:III:IV supercomplex in the face of defective superoxide scavenging. In addition, it appears that *sod-2* function is necessary for the upregulation of complex II activity normally seen in *gas-1*. It is unclear why this is so, but this change may further decrease the metabolic rate in *sod-2;gas-1* and also limit the production of superoxide. Although enzymatic activity of complex II is decreased in *sod-2;mev-1*, overall integrated function of the mitochondrion, as reflected in mitochondrial respiratory capacity, is not affected.

Loss of *sod-3*, which is upregulated in *gas-1*, does not change the lifespan of *gas-1*, yet it increases the lifespan of *isp-1* and decreases the lifespan of *mev-1*. We do see a small increase in complex I activity staining in BNGs of *sod-3* animals; perhaps, this partially compensates for the loss of complex III in *isp-1* (Suthammarak et al., 2010) and can extend its lifespan. If so, one might presume that this same phenomenon would operate in *mev-1*, yet loss of *sod-3* in *mev-1* decreases its lifespan. Studies are now being undertaken to characterize the interaction of *sod-3* with supercomplex I:III:IV formation.

The role of *gst-14* as a response to complex I defects is also not clear. Several forms of GST proteins are known to scavenge HNE-damage

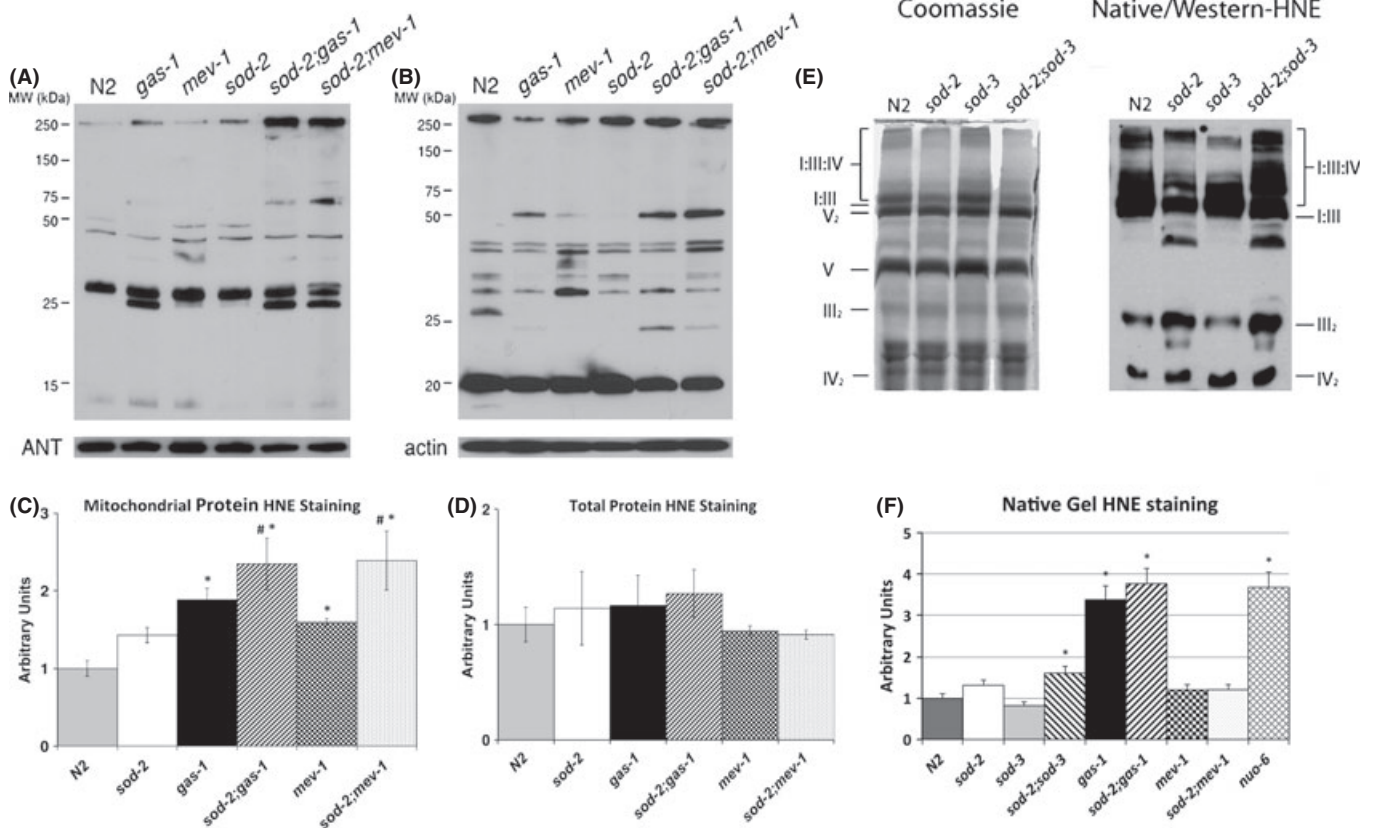


Fig. 5 Oxidative damage to proteins. Western blots of (A) mitochondrial protein and (B) total worm protein probed with anti-HNE antibody. Adenine nucleotide translocase (ANT) and actin were used as loading controls as shown. (C, D) Quantification of HNE-modified protein from (A) and (B) (C and D, respectively). Legend for strains applies to both (C) and (D). Histogram fill patterns for strains are the same as for Figs 3 and 5F. Data shown in arbitrary units are normalized to wild-type (N2) levels as mean \pm SEM from three independent samples. * indicates different than amount in N2, $P < 0.05$ in comparison with wild type (with a Bonferroni correction for $n = 6$ resulting in $P < 0.0083$ for significance as described in Statistical analysis). # indicates the amount of staining in *sod-2;gas-1* and *sod-2;mev-1* is similarly increased compared with *gas-1* and *mev-1*, respectively. (E) BNG and Western blot from BNGs of *sod* mutants (probed with anti-HNE). (F) Ratios of HNE oxidative damage to Coomassie staining in a sister BNG normalized to N2 (taken as unity) in multiple mitochondrial mutants. The BNG and Western blot are shown in Fig. S4, Supporting Information. The values are the averages from three gels. Strains containing complex I mutations (*gas-1* and *nuo-6*) show the most increase in relative HNE staining. *sod-2*; *sod-3* also showed a significant increase in HNE staining, compared with N2. Each gel was repeated at least three times. * indicates statistical significance as $P < 0.05$ in comparison with wild type (with a Bonferroni correction for $n = 9$ resulting in $P < 0.0056$ for significance as described in Statistical analysis).

Table 1 Relative quantification of mRNA level of antioxidant genes. Levels of expression of 4 antioxidant genes (*sod-1*, *sod-2*, *sod-3*, and *gst-14*) in the mutants were normalized to that of the wild type

Strains\Genes	<i>sod-1</i>	<i>sod-2</i>	<i>sod-3</i>	<i>gst-14</i>
N2	1 \pm 0.1	1 \pm 0.2	1 \pm 0.1	1 \pm 0.3
<i>gas-1</i>	1.4 \pm 0.3	0.8 \pm 0.2	7.8 \pm 1.3*	70.9 \pm 1.2*
<i>mev-1</i>	0.9 \pm 0.2	0.8 \pm 0.1	3.5 \pm 0.8*	0.04 \pm 0.02*
<i>sod-2</i>	0.9 \pm 0.1	0	5.4 \pm 0.8*	19.7 \pm 1.8*
<i>sod-2;gas-1</i>	1.4 \pm 0.2	0	36.2 \pm 2.1*	305.9 \pm 124.1*
<i>sod-2;mev-1</i>	1.4 \pm 0.0	0	6.8 \pm 0.7*	2.3 \pm 1.7

Data are represented as mean \pm SEM from 3 to 6 day 1 adult cultures.

*Indicated statistical significance as $P < 0.05$ in comparison with wild type (with a Bonferroni correction for $n = 5$ resulting in $P < 0.01$ for significance as described in Statistical analysis).

proteins. The HSP-6 family of proteins [also upregulated in *gas-1* (Falk *et al.*, 2008)] has been shown to modify GST proteins to cause mitochondrial targeting (Raza, 2011). We anticipated, given the

tremendous upregulation of this molecule in *sod-2;gas-1*, that *gst-14* overexpression was linked to the extension of lifespan of *sod-2;gas-1* compared with *gas-1*. However, reduction in *gst-14* expression lengthened the lifespan of both *gas-1* and *sod-2;gas-1*. The protein GST-14 is most homologous to the mammalian pro-inflammatory protein, hematopoietic prostaglandin D2 synthase (HPGDS) (Kanaoka & Urade, 2003; Mohri *et al.*, 2009). Given this homology, we hypothesize that GST-14 actually is responsible for an inflammatory response in nematodes, rather than ROS scavenging, and are testing this possibility. It should be noted that there are several other *gst* genes in *C. elegans* that code for homologues of HPGDS; it is not known whether *gst-14* is unique in its role in governing lifespan. Regardless of the specificity of *gst-14*, the lifespan of mitochondrial mutants is governed by an interaction of metabolic rate, ROS damage, and an inflammatory response.

There are some differences in the lifespans in our laboratory compared with reports of others. First, we consistently find little to no increase in the lifespan of *isp-1* compared with N2. This may result partially from our reluctance to remove worms from our lifespan measurements that contain one internal hatched offspring (a 'bagged' worm) in adults exhibiting almost no mobility on the prior day (10–15%

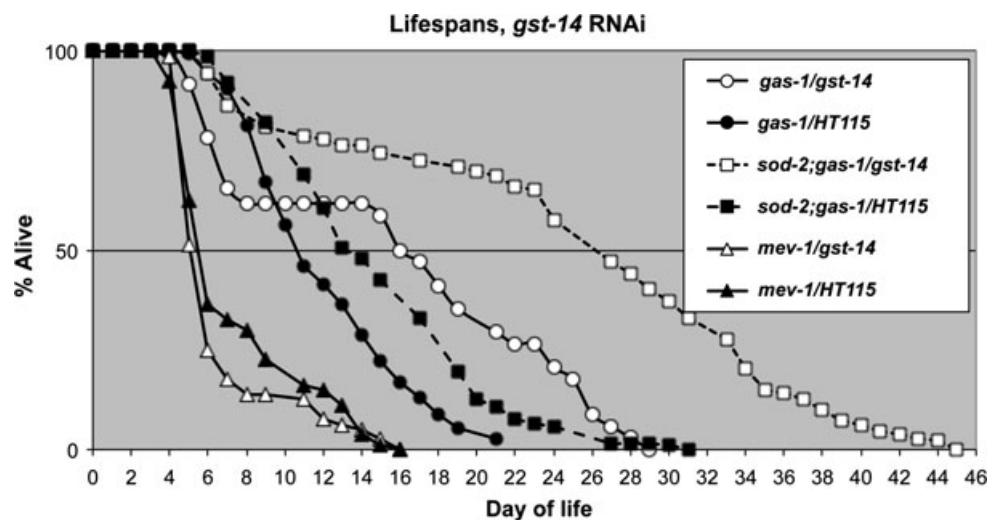


Fig. 6 Compensatory responses of *sod-2*. Effects of *gst-14* RNAi on lifespans of mitochondrial mutants. *gst-14* RNAi caused a significant increase in the lifespan of *gas-1* (mean lifespan 15.7 days with RNAi vs. 10.1 days without RNAi) and *sod-2;gas-1* (mean lifespan 28.4 days with RNAi vs. 13.4 days without RNAi).

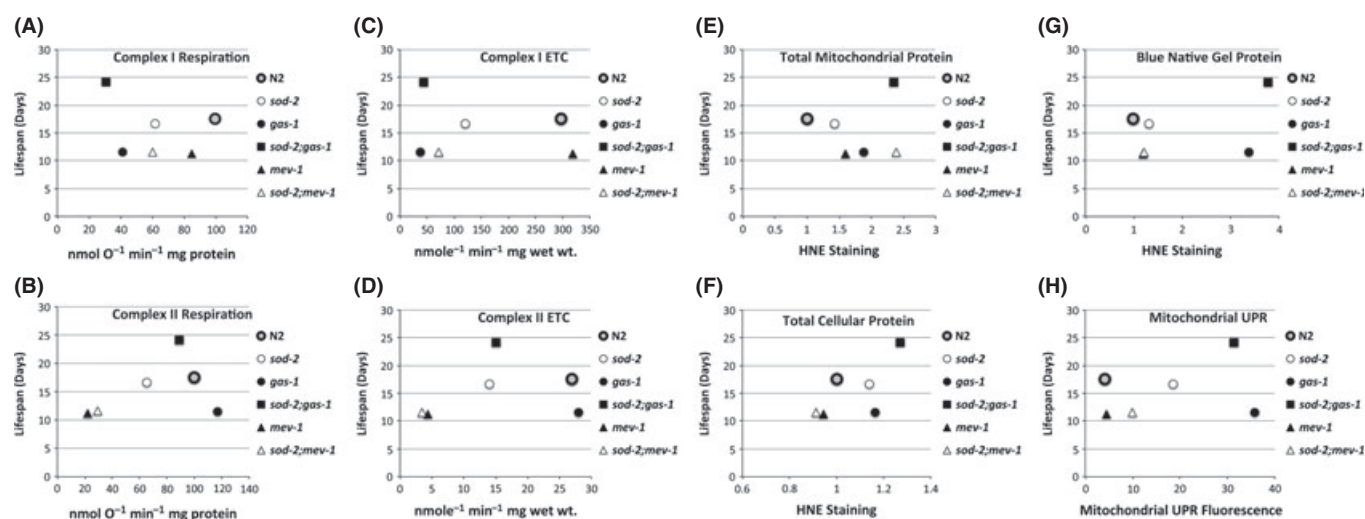


Fig. 7 Comparison of multiple aspects of mitochondrial physiology with lifespan in mitochondrial mutants. Correlation of several different aspects of mitochondrial physiology with lifespan of six strains of *C. elegans*. Lifespan values are from Fig. 1; Respiration and ETC values are from Fig. 3; HNE staining values are from Fig. 5; Mitochondrial UPR fluorescence is from Fig. S4. Symbols for the strains are the same as in Fig. 1. The symbol for N2 is enlarged in all graphs for comparison with other strains. (A) Complex I-dependent oxygen consumption. (B) Complex II-dependent oxygen consumption. (C) Complex I ETC activity. (D) Complex II ETC activity. (E) 4-hydroxynonenal (HNE) antibody staining of total worm protein. (F) HNE antibody staining of total mitochondrial protein. (G) HNE staining of complexes from blue native gels (BNGs). (H) Mitochondrial UPR-dependent fluorescence as measured by HSP-6::GFP expression normalized to that of N2 expressed in arbitrary units. No significant correlation was found with any measurement when compared with lifespan.

of the adults in our studies). In our opinion, these animals represent dead adults that have retained one hatched egg. However, this does not fully explain the differences between our results and those of others. Second, Van Raamsdonk reported a decrease in lifespan for the *sod-2;isp-1* double mutant (Van Raamsdonk & Hekimi, 2009). We were unable to obtain viable *sod-2;isp-1* offspring and so report this as a lethal. We do not know the cause for our differences in *isp-1*-related results, although we suspect feeding differences may play a role. Finally, the finding that loss of DAF-16 increases the lifespan on *gas-1* is extremely surprising. We suspect either that *gst-14* expression is DAF-16 dependent or that the metabolic changes dependent on DAF-16 (Robida-Stubbs *et al.*, 2012) interact with *gas-1*.

The effects of *sod-2* and *sod-3* mutants on the viability and lifespan of *gas-1*, *mev-1*, and *isp-1* support the findings of others that there is not a simple correlation between mitochondrial respiration, superoxide scavenging, ROS damage, and lifespan (Yang *et al.*, 2007, 2009; Doonan

et al., 2008; Gems & Doonan, 2009; Van Raamsdonk & Hekimi, 2009, 2012) (Fig. 7). Clearly the notion that loss of mtSODs simply increases ROS levels and damage is too simplistic. Superoxide dismutase does not actually remove potentially damaging ROS; rather it only speeds up the conversion from one form (superoxide) to another (hydrogen peroxide).

In summary, we have found specific interactions between mitochondrial mutants and the mitochondrial superoxide dismutases. It is clear that losses of mitochondrial SODs have unanticipated effects on lifespan and mitochondrial function. As they are associated with mitochondrial supercomplexes, they may regulate electron transport or supercomplex stability in a negative feedback loop and protect specific proteins within the ETC from ROS damage. Paradoxically, loss of *sod-2* may actually serve as a control point that limits maximal ROS production and keeps the levels of ROS damage relatively constant as found by Yang (Yang & Hekimi, 2010a). Our data would indicate that this decrease is mediated by dissolution of supercomplexes, which are stabilized by the mtSODs,

which in turn decreases mitochondrial respiration. Evolution would likely favor the linkage of presence of an ROS scavenger to the physical entity which generates the ROS.

Experimental procedures

C. elegans strains and culture

Worms were maintained on NGM agar plates seeded with OP50 bacteria at 20 °C. Strains obtained from the *Caenorhabditis* Genetics Center include N2 Bristol (wild type), *gas-1(fc21)*, *mev-1(kn1)*, *isp-1(qm150)*, *sod-2(gk257)*, *sod-3(tm760)*, and SJ4100 (*zcls13[hsp-6p::GFP]*). SJ4100 was kindly shared by Matt Kaerberlein (UW) and Andrew Dillin (UCSF). Double mutants were generated by standard methods when visible phenotypes were available. Cross-progeny were identified by PCR if there were no visible phenotypes. All double mutants were sequenced to confirm genotype and outcrossed a minimum of 4 times.

Lifespan analysis

SAll lifespan assays were conducted at 20 °C on fresh OP50 NGM plates or HT115 plates in RNAi studies. Synchronized cultures of *C. elegans* were generated by seeding gravid young adults on plates to lay eggs for 4 h on day 0. Individual plates were maintained with a maximum of 25 worms per plate. Worms were transferred to new plates every 2 days to avoid counting offspring of the original cohort. Worms that were not active were gently prodded by a platinum worm pick; if worms did not move when prodded 3X, they were scored as dead. Nematodes that died abnormally (bagging, trapped on the side of the petri dish) were excluded from counting as previously described (Kayser *et al.*, 2004a). A minimum of 100 animals were followed for each lifespan experiment, and each lifespan study was repeated at least three times.

Egg laying and hatching

Synchronous young adult worms were allowed to lay eggs for 4 h on day 0. On day one, five L1 or L2 worms were transferred to fresh plates. Once these animals began to lay eggs, they were transferred daily until they no longer laid any eggs. The number of days until the first eggs were laid, the total number of eggs laid, and the number that hatched were determined on each plate. All cultures were maintained at 20 °C.

Oxidative phosphorylation and electron transport chain (ETC) assays

Mitochondria were isolated as previously outlined (Kayser *et al.*, 2001, 2004b). Mitochondrial respiratory capacity was measured as previously described using freshly prepared mitochondria (Kayser *et al.*, 2001, 2004b). The mitochondrial samples that were isolated for oxidative phosphorylation assays were also used as fresh samples for the ETC assays. ETC assays were carried out using techniques previously described (Hoppel *et al.*, 1998). All ETC assays were carried out on fresh mitochondrial samples that were kept on ice and repeated on three independent isolates.

Blue native gels (BNGs) and in-gel activity (IGA)

Blue native gels were performed as previously described (Suthammarak *et al.*, 2009). Western blots and in-gel activities (IGA) of BNGs were

carried out as described by Suthammarak (Suthammarak *et al.*, 2010). All gels were repeated on three independent samples.

Western blots

For Western blots of SDS-PAGE gels and native gels, mitochondria were isolated as described previously (Suthammarak *et al.*, 2009, 2010). Fifty microgram of mitochondrial protein from each strain was loaded onto a 12% SDS-PAGE gel. The gel was run and transferred as previously described (Suthammarak *et al.*, 2009, 2010). Blots were probed with a primary antibody to either 4-hydroxy-2-nonenal (HNE) fluorophore (Calbiochem, San Diego, CA), NDUFS2 (Santa Cruz Biotechnology, Dallas, TX), or SOD-2 (Santa Cruz Biotechnology, Dallas, TX). The membranes were washed as previously described and incubated with horseradish peroxidase-conjugated secondary antibody (Yang *et al.*, 2009) (Santa Cruz Biotechnology, Dallas, TX). Blots were incubated with SuperSignal® West Pico chemiluminescence substrate. Adenosine nucleoside transporter (ANT) antibody (Mitosciences, Eugene OR) was used as a loading control.

Statistical analysis

Analysis of variance was used to analyze groups of data for significant differences. Unpaired Student's *t*-test was then employed to calculate statistical significance of specific pairs if a difference was noted with analysis of variance. Significance was defined as $P < 0.05$ with a Bonferroni correction for the number of strains evaluated with each test (generally eight strains resulting in a $P < 0.05/8 = 0.00625$). Error bars in the figures represent mean \pm SD for each experiment.

Acknowledgments

The authors thank the technical help of Beatrice Predoi, Judith Preston, and Katrina Elsaesser. This work was supported, in whole or in part, by National Institutes of Health Grants GM58881 and AG026273 (MMS and PGM) and by the Royal Thai Government, Science and Technology Scholarship (WS), and the Northwest Mitochondrial Research Guild (WS, PGM, MMS).

Author contributions

WS designed and performed experiments, evaluated results, and contributed to the writing of the article. BHS designed and performed experiments and evaluated results. EO designed methods, performed experiments, and contributed to the writing of the article. MMS and PGM designed and performed experiments, evaluated results, and primarily wrote the article.

References

- Ayyadevara S, Dandapat A, Singh SP, Benes H, Zimniak L, Shmookler Reis RJ, Zimniak P (2005) Lifespan extension in hypomorphic *daf-2* mutants of *Caenorhabditis elegans* is partially mediated by glutathione transferase Ce-GSTP2-2. *Aging Cell* **4**, 299–307.
- Ayyadevara S, Dandapat A, Singh SP, Siegel ER, Shmookler Reis RJ, Zimniak L, Zimniak P (2007) Life span and stress resistance of *Caenorhabditis elegans* are differentially affected by glutathione transferases metabolizing 4-hydroxy-non-2-enal. *Mech. Ageing Dev.* **128**, 196–205.
- Back P, Matthijssens F, Vlaeminck C, Braeckman BP, Vanfleteren JR (2010) Effects of *sod* gene overexpression and deletion mutation on the expression profiles of reporter genes of major detoxification pathways in *Caenorhabditis elegans*. *Exp. Gerontol.* **45**, 603–610.

- Baker BM, Nargund AM, Sun T, Haynes CM (2012) Protective coupling of mitochondrial function and protein synthesis via the eIF2 α kinase GCN-2. *PLoS Genet.* **8**, e1002760.
- Cabreiro F, Ackerman D, Doonan R, Araiz C, Back P, Papp D, Braeckman BP, Gems D (2011) Increased life span from overexpression of superoxide dismutase in *Caenorhabditis elegans* is not caused by decreased oxidative damage. *Free Radic. Biol. Med.* **51**, 1575–1582.
- Cohn JA, Tsai L, Friguet B, Szewda LI (1996) Chemical characterization of a protein-4-hydroxy-2-nonenal cross-link: immunochemical detection in mitochondria exposed to oxidative stress. *Arch. Biochem. Biophys.* **328**, 158–164.
- Doonan R, McElwee JJ, Matthijssens F, Walker GA, Houthoofd K, Back P, Matscheski A, Vanfleteren JR, Gems D (2008) Against the oxidative damage theory of aging: superoxide dismutases protect against oxidative stress but have little or no effect on life span in *Caenorhabditis elegans*. *Genes Dev.* **22**, 3236–3241.
- Durieux J, Wolff S, Dillin A (2011) The cell-non-autonomous nature of electron transport chain-mediated longevity. *Cell* **144**, 79–91.
- Falk MJ, Zhang Z, Rosenjack JR, Nissim I, Daikhin E, Nissim I, Sedensky MM, Yudkoff M, Morgan PG (2008) Metabolic pathway profiling of mitochondrial respiratory chain mutants in *C. elegans*. *Mol. Genet. Metab.* **93**, 388–397.
- Gems D, Doonan R (2009) Antioxidant defense and aging in *C. elegans*: is the oxidative damage theory of aging wrong? *Cell Cycle* **8**, 1681–1687.
- Grad LI, Lemire BD (2004) Mitochondrial complex I mutations in *Caenorhabditis elegans* produce cytochrome c oxidase deficiency, oxidative stress and vitamin-responsive lactic acidosis. *Hum. Mol. Genet.* **13**, 303–314.
- Harman D (1956) Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.* **11**, 298–300.
- Hartman PS, Ishii N, Kayser EB, Morgan PG, Sedensky MM (2001) Mitochondrial mutations differentially affect aging, mutability and anesthetic sensitivity in *Caenorhabditis elegans*. *Mech. Ageing Dev.* **122**, 1187–1201.
- Hartwig K, Heidler T, Moch J, Daniel H, Wenzel U (2009) Feeding a ROS-generator to *Caenorhabditis elegans* leads to increased expression of small heat shock protein HSP-16.2 and hormesis. *Genes Nutr.* **4**, 59–67.
- Heidler T, Hartwig K, Daniel H, Wenzel U (2010) *Caenorhabditis elegans* lifespan extension caused by treatment with an orally active ROS-generator is dependent on DAF-16 and SIR-2.1. *Biogerontology* **11**, 183–195.
- Honda Y, Honda S (2002) Oxidative stress and life span determination in the nematode *Caenorhabditis elegans*. *Ann. N. Y. Acad. Sci.* **959**, 466–474.
- Honda Y, Tanaka M, Honda S (2008) Modulation of longevity and diapause by redox regulation mechanisms under the insulin-like signaling control in *Caenorhabditis elegans*. *Exp. Gerontol.* **43**, 520–529.
- Hoppel CL, Kerner J, Turkaly P, Turkaly J, Tandler B (1998) The malonyl-CoA-sensitive form of carnitine palmitoyltransferase is not localized exclusively in the outer membrane of rat liver mitochondria. *J. Biol. Chem.* **273**, 23495–23503.
- Ishii N, Fujii M, Hartman PS, Tsuda M, Yasuda K, Senoo-Matsuda N, Yanase S, Ayusawa D, Suzuki K (1998) A mutation in succinate dehydrogenase cytochrome b causes oxidative stress and ageing in nematodes. *Nature* **394**, 694–697.
- Kanaoka Y, Urade Y (2003) Hematopoietic prostaglandin D synthase. *Prostaglandins Leukot. Essent. Fatty Acids* **69**, 163–167.
- Kayser EB, Morgan PG, Hoppel CL, Sedensky MM (2001) Mitochondrial expression and function of GAS-1 in *Caenorhabditis elegans*. *J. Biol. Chem.* **276**, 20551–20558.
- Kayser EB, Sedensky MM, Morgan PG (2004a) The effects of complex I function and oxidative damage on lifespan and anesthetic sensitivity in *Caenorhabditis elegans*. *Mech. Ageing Dev.* **125**, 455–464.
- Kayser EB, Sedensky MM, Morgan PG, Hoppel CL (2004b) Mitochondrial oxidative phosphorylation is defective in the long-lived mutant clk-1. *J. Biol. Chem.* **279**, 54479–54486.
- Keaney M, Matthijssens F, Sharpe M, Vanfleteren J, Gems D (2004) Superoxide dismutase mimetics elevate superoxide dismutase activity *in vivo* but do not retard aging in the nematode *Caenorhabditis elegans*. *Free Radic. Biol. Med.* **37**, 239–250.
- Kondo M, Senoo-Matsuda N, Yanase S, Ishii T, Hartman PS, Ishii N (2005) Effect of oxidative stress on translocation of DAF-16 in oxygen-sensitive mutants, mev-1 and gas-1 of *Caenorhabditis elegans*. *Mech. Ageing Dev.* **126**, 637–641.
- Koopman WJ, Nijtmans LG, Dieteren CE, Roestenberg P, Valsecchi F, Smeitink JA, Willems PH (2010) Mammalian mitochondrial complex I: biogenesis, regulation, and reactive oxygen species generation. *Antioxid. Redox Signal.* **12**, 1431–1470.
- Maranzana E, Barbero G, Falasca AI, Lenaz G, Genova ML (2013) Mitochondrial respiratory supercomplex association limits production of reactive oxygen species from complex I. *Antioxid. Redox Signal.* [Epub ahead of print].
- McElwee JJ, Schuster E, Blanc E, Thomas JH, Gems D (2004) Shared transcriptional signature in *Caenorhabditis elegans* Dauer larvae and long-lived daf-2 mutants implicates detoxification system in longevity assurance. *J. Biol. Chem.* **279**, 44533–44543.
- Melov S, Coskun P, Patel M, Tuinstra R, Cottrell B, Jun AS, Zastawny TH, Dizdaroglu M, Goodman SI, Huang TT, Miziorko H, Epstein CJ, Wallace DC (1999) Mitochondrial disease in superoxide dismutase 2 mutant mice. *Proc. Natl Acad. Sci. U. S. A.* **96**, 846–851.
- Mohri I, Aritake K, Taniguchi H, Sato Y, Kamauchi S, Nagata N, Maruyama T, Taniike M, Urade Y (2009) Inhibition of prostaglandin D synthase suppresses muscular necrosis. *Am. J. Pathol.* **174**, 1735–1744.
- Raza H (2011) Dual localization of glutathione S-transferase in the cytosol and mitochondria: implications in oxidative stress, toxicity and disease. *FEBS J.* **278**, 4243–4251.
- Robida-Stubs S, Glover-Cutter K, Lamming DW, Mizunuma M, Narasimhan SD, Neumann-Haefelin E, Sabatini DM, Blackwell TK (2012) TOR signaling and rapamycin influence longevity by regulating SKN-1/Nrf and DAF-16/FoxO. *Cell Metab.* **15**, 713–724.
- Sampayo JN, Olsen A, Lithgow GJ (2003) Oxidative stress in *Caenorhabditis elegans*: protective effects of superoxide dismutase/catalase mimetics. *Ageing Cell* **2**, 319–326.
- Senoo-Matsuda N, Yasuda K, Tsuda M, Ohkubo T, Yoshimura S, Nakazawa H, Hartman PS, Ishii N (2001) A defect in the cytochrome b large subunit in complex II causes both superoxide anion overproduction and abnormal energy metabolism in *Caenorhabditis elegans*. *J. Biol. Chem.* **276**, 41553–41558.
- Suthammarak W, Yang YY, Morgan PG, Sedensky MM (2009) Complex I function is defective in complex IV-deficient *Caenorhabditis elegans*. *J. Biol. Chem.* **284**, 6425–6435.
- Suthammarak W, Morgan PG, Sedensky MM (2010) Mutations in mitochondrial complex III uniquely affect complex I in *Caenorhabditis elegans*. *J. Biol. Chem.* **285**, 40724–40731.
- Van Raamsdonk JM, Hekimi S (2009) Deletion of the mitochondrial superoxide dismutase sod-2 extends lifespan in *Caenorhabditis elegans*. *PLoS Genet.* **5**, e1000361.
- Van Raamsdonk JM, Hekimi S (2012) Superoxide dismutase is dispensable for normal animal lifespan. *Proc. Natl Acad. Sci. U. S. A.* **109**, 5785–5790.
- Van Remmen H, Williams MD, Guo Z, Estlack L, Yang H, Carlson EJ, Epstein CJ, Huang TT, Richardson A (2001) Knockout mice heterozygous for Sod2 show alterations in cardiac mitochondrial function and apoptosis. *Am. J. Physiol. Heart Circ. Physiol.* **281**, H1422–H1432.
- Yanase S, Ishii N (2008) Hyperoxia exposure induced hormesis decreases mitochondrial superoxide radical levels via Ins/IGF-1 signaling pathway in a long-lived age-1 mutant of *Caenorhabditis elegans*. *J. Radiat. Res.* **49**, 211–218.
- Yang W, Hekimi S (2010a) A mitochondrial superoxide signal triggers increased longevity in *Caenorhabditis elegans*. *PLoS Biol.* **8**, e1000556.
- Yang W, Hekimi S (2010b) Two modes of mitochondrial dysfunction lead independently to lifespan extension in *Caenorhabditis elegans*. *Ageing Cell* **9**, 433–447.
- Yang W, Li J, Hekimi S (2007) A Measurable increase in oxidative damage due to reduction in superoxide detoxification fails to shorten the life span of long-lived mitochondrial mutants of *Caenorhabditis elegans*. *Genetics* **177**, 2063–2074.
- Yang YY, Gangoiti JA, Sedensky MM, Morgan PG (2009) The effect of different ubiquinones on lifespan in *Caenorhabditis elegans*. *Mech. Ageing Dev.* **130**, 370–376.
- Yazaki K, Yoshikoshi C, Oshiro S, Yanase S (2011) Supplemental cellular protection by a carotenoid extends lifespan via Ins/IGF-1 signaling in *Caenorhabditis elegans*. *Oxid. Med. Cell. Longev.* **2011**, 596240.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Fig. S1 Lifespans, *sod-3*.

Fig. S2 Location of SOD-2 and SOD-3 in Supercomplexes.

Fig. S3 Blue Native Gels and HNE staining of Western blots from mitochondrial mutants.

Fig. S4 Expression of *Phsp-6::GFP* in mitochondrial mutants.

Fig. S5 Development on *hsp-6* RNAi.