Regulation of Pro-Longevity ROS by ROS-Handling Enzymes in *C. elegans*

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February 2020

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy.

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Abstract

The relationship between reactive oxygen species (ROS) and aging is multifaceted and complex. The free radical theory of aging oversimplifies this relationship in predicting that increased mitochondrial ROS production is the cause of aging. Many findings contradict the core concepts of this theory. Notable among these, is a previous finding in our laboratory that shows increased levels of mitochondrial ROS, via mutations such as *isp-1* and *nuo-6* or treatment with very low doses of the pro-oxidant Paraquat (PQ) can increase the lifespan of the wild-type *Caenorhabditis elegans* in a process that links elevated mitochondrial superoxide generation to the activation of the intrinsic apoptosis pathway. This finding focuses on the role of ROS as signaling molecules in the process of aging. In the studies presented in this thesis, we use *C. elegans* as a model organism to study the ROS-dependent regulation of longevity using a systematic genetic dissection approach which utilizes mutants of the worm's ROS-handling enzymes such as superoxide dismutases (SODs) and catalases (CTLs), as well as mutations that increase mitochondrial ROS production such as *isp-1* and *nuo-6*. In chapter 2, we analyze the regulation of lifespan in *C. elegans* by SOD-2 and SOD-3, the worm's two mitochondrial SODs that are highly similar to each other. We show that despite their similarity, their deletions have opposite effects on the lifespan of the worm, which suggests that the two enzymes have specific roles with regards to the regulation of longevity. We also show that SOD-2 and SOD-3, whose expression is tissue-specific, interact with each other in an age-dependent epistatic manner, in the wild-type, to regulate the lifespan of the worm. Furthermore, in chapter 2, we describe our findings which suggest the involvement of ROS signaling in a PQ-induced developmental arrest phenotype of *sod-2* knock-out mutants. In chapter 3, we describe our systematic genetic analysis of the epistatic role of SODs and CTLs in the regulation of lifespan in *C. elegans*. The primary finding of this comprehensive analysis shows that the pro-longevity effect of mitochondrial superoxide signals relies mainly on an increase in the cytoplasmic levels of hydrogen peroxide produced by SOD-1, the worm's primary cytoplasmic SOD. Overall, our findings show that ROS are indeed involved in aging, but their involvement is in a regulatory capacity and not as lifespan limiting, damaging molecules.

Résumé

La relation entre les espèces réactives de l'oxygène (ERO) et le vieillissement est complexe et comporte plusieurs facettes. Une des théories du vieillissement simplifie cette relation en prédisant qu'une augmentation mitochondriale des ERO est la cause du vieillissement. Mais plusieurs découvertes contredisent le concept central de cette théorie. Parmi celles-ci, une étude de notre laboratoire a démontré que des niveaux augmentés des ERO mitochondriaux par des mutations telles que *isp-1* et *nuo-6,* ou par traitement avec de faibles doses de paraquat (PQ), une molécule pro-oxydante, pouvaient augmenter la durée de vie du nématode *Caenorhabditis elegans* par un processus qui lierait la production élevée de superoxide mitochondrial à l'activation de la voie de signalisation de l'apoptose. Cette étude antérieure se concentre principalement sur le rôle des ERO comme molécules de signalisation au cours du processus de vieillissement. Pour les études présentées dans cette thèse, nous avons utilisé *C. elegans* comme organisme modèle afin d'étudier la régulation de la durée de vie par les EROs. Pour ce faire, nous avons utilisé une approche de dissection génétique systématique qui utilise des mutants sans une ou plusieurs des enzymes qui contrôlent les niveaux de ERO, tel que les superoxides dismutases (SODs) et les catalases (CTLs). Nous avons aussi utilisé des mutations qui augmentent la production d'ERO, telles qu'*isp-1* et *nuo-6*. Dans le chapitre 2, nous analysons l'influence sur la durée de vie de *C. elegans* de deux superoxide dismutases très semblables (SOD-2 et SOD-3). Nous avons démontré que malgré leur similarité, les deux enzymes ont des rôles bien distincts sur la régulation de la longévité. De plus, nous avons montré que les deux enzymes, dont le patron d'expression tissulaire est different,, interagissent l'une avec l'autre de façon épistatique pour régler la durée de vie du vers. Dans ce même chapitre, nous présentons des études qui suggèrent que des ERO agissent aussi comme molécules de signalisation qui règlent le développement du ver. Dans le chapitre 3, nous décrivons notre analyse génétique systématique du rôle des SODs et des CTLs sur la régulation de la durée de vie de *C. elegans*. La découverte principale de cette analyse démontre que l'effet pro-longévité du superoxide mitochondrial dépend principalement de l'augmentation des niveaux de peroxyde d'hydrogène produit par SOD-1, la superoxide dismutase principale du cytoplasme. Globalement, nos découvertes démontrent que les ERO sont bien impliquées dans le processus de vieillissement, mais leur rôle et principalement un rôle régulateur et non pas un rôle de limitation de la durée de vie par la toxicité.

Acknowledgements

There are many to thank for their help during my graduate studies at McGill. First, I would like to thank Dr. Siegfried Hekimi, my supervisor, for his patience and guidance throughout my studies. Working on these projects under Dr. Hekimi's supervision has been a privilege and an opportunity of a lifetime. I would also like to thank my supervisory committee members Dr. Michael Hendricks and Dr. Tamara Western for their guidance and help throughout my studies. Very special thanks to Dr. Robyn Branicky for her continuous help and advice during the course of my PhD studies. I would also like to thank all members of the Hekimi lab, past and present, especially Maximilian Kramer-Drauberg, Ying Wang, Eve Bigras and David Desjardins for their friendship and sharing their knowledge. Lastly, many thanks to my family and friends. Their moral support and encouragement have been invaluable and kept me going all this time.

Contribution to original knowledge

The original work in this thesis is presented in two chapters (two and three) and investigates different aspects of the ROS-dependent regulation of lifespan and development using a genetic approach. In chapter 2, we show that the two mitochondrial superoxide dismutase enzymes of *C. elegans* (SOD-2 and SOD-3), despite their overwhelming structural similarity, perform different roles with regards to the longevity of the worms and have an epistatic relationship. Furthermore by tagging the genomic loci of *sod-2* and *sod-3,* we also show that they are expressed in different tissues, which in combination with our other findings suggests that cells that express *sod-2* and those that express *sod-3* could communicate with each other using ROS signals. Finally, we show that the developmental arrest of *sod-2* mutants in response to low dose PQ treatment likely occurs as a result of ROS signaling.

Chapter 3, describes our systematic genetic dissection of a part of the ROS network of *C. elegans,* focusing on the regulation of ROS-dependent longevity by the synergistic action of superoxide dismutase and catalase enzymes. The data presented in this chapter show, for the first time, that changes in mitochondrial superoxide production need to be translated by cytoplasmic SOD-1 into a hydrogen peroxide signal in order to induce longevity in the worms.

Preface and contribution of authors

This thesis is comprised of 4 chapters, in accordance with the Graduate and Postdoctoral Studies Thesis Guidelines. Chapter 1 is an introduction and literature review, chapters 2 and 3 are presentations of the research that I carried out during my PhD studies, and chapter 4 is a general discussion and summary of the material presented in this thesis. All data presented in chapters 2 and 3 was collected and analyzed by me. A few mutant strains that were used in the projects described in this thesis were constructed previously by other members of our laboratory. These are properly marked in the relevant material and methods sections of the thesis. Furthermore, Dr. Robyn Branicky examined the expression of *sod-2* and *sod-3* lines in N2 and in *daf-2*, and acquired the images shown in section 2.3.7. The thesis was written by me with editorial help from my supervisor Dr. Siegfried Hekimi.

List of common acronyms

CTL	Catalase
ETC	Electron transport chain
FUDR	5'-fluoro-2'-deoxyuridine
GPX	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
•OH	Hydroxyl radical
NAC	N-acetyl cysteine
NGM	Nematode growth medium
$O_2^{\bullet-}$	Superoxide
PQ	Paraquat
PRDX	Peroxiredoxin
RNAi	RNA interference
ROS	Reactive oxygen species
SOD	Superoxide dismutase

Chapter 1:
An overview of the research on the aging process, the biology of reactive oxygen species (ROS), and of ROS-dependent longevity

1.1. An overview of the modern theories of aging

The age-old questions of why and how all living beings age and whether eternal longevity is possible have been a central theme of scientific, philosophical and mythical literature since the dawn of history and yet the answer eludes us. One of the first surviving literary works that makes an attempt at dealing with the concept of aging and longevity is a treatise written by the Greek philosopher Aristotle in third century BCE. In his *Longevity and Shortness of Life,* Aristotle organizes his observations of the lifespans of various organisms known to him based on their physiological characteristics (warm-blooded or cold-blooded), the properties of their habitats (warm, cold, aquatic or terrestrial) and their body sizes. He then goes on to conclude that long-lived animals are those that are terrestrial, large and live in warmer climates. He posits that these animals have more "humidity" and "warmth" in them which enable them to live long. Short-lived and aging species, however, are "dry" and "cold" and losing moisture and warmth over time is the cause of their aging (Barnes 1984). He comes to this conclusion by observing how fast animal bodies dry up after death and how the wrinkled skin of aging people seems to have lost its moisture. Aristotle's erroneous deduction is, of course, the result of an incorrect view of the cause and effect relationships in the process of aging. While Aristotle's logical fallacy in his ancient theory of aging may be evident to the modern scientist, as we will discuss in the following sections, some modern theories of aging have also fallen into similar pitfalls.

At present, there is not a well-defined explanation for the aging of organisms and the process of aging at the cellular level is extremely intricate (Bengtson and Settersten Jr 2016). There are biochemical pathways and even compounds that significantly affect the speed of aging and the overall lifespan of organisms; however, an "aging switch" that can turn the whole aging process on or off does not seem to exist. In this section, we will focus on some of the most influential theories of aging acknowledged by the scientific community in the past few decades. As we discuss each theory and mechanism of aging, it becomes more evident that at the molecular level there is an underlying connection between all these mechanisms and aging is a multifaceted process.

1.1.1 Aging from an evolutionary point of view

From an evolutionary perspective, aging is a progressive decline in reproductive success and an increase in mortality with advancing age (Flatt and Partridge 2018). Evolutionary theories of aging

focus on the fertility of the individual species and evolutionary factors such as natural selection to explain why organisms age (Kirkwood 2011). The general predictions of the evolutionary theories of aging are summarized in the following two principles:

(1) It is not likely that specific genes are selected by evolutionary mechanisms whose function is to promote aging (Kirkwood and Austad 2000). Gene-expression profiling of aging cells in different tissues of a variety of organisms shows that the expression of certain groups of genes are altered with age (Frenk and Houseley 2018). Also, interventions such as caloric restriction (Lee, Klopp et al. 1999, Al-Regaiey 2016) and pro-longevity ROS treatment (Yee, Yang et al. 2014) are shown to alter gene expression patterns, and these changes must be what promotes longevity. It is possible that such studies might also reveal late-acting deleterious genes. However, evolutionary theories maintain that although these gene expression patterns change with age, it is more likely that these effects are secondary consequences and not the main causes of the ageing process. This is because, in populations, the strength of selection for survival and fecundity often declines with age whereas alleles with positive impacts on fitness components are selected for early in life even though they may have negative effects in late-life (Figure 1.1) (Flatt and Partridge 2018).

(2) Aging is not programmed. Early evolutionary biologists believed that senescence is programmed to limit generation size and accelerate the turnover of generations in order to facilitate adaptation of the organisms to the changing environment (Weismann, Poulton et al. 1891). One essential problem with this view is that, for most species, senescence is not the main reason of death. Natural mortality, in contrast with what is seen in protected populations, is mainly caused by external factors such as predation, infections or exposure to elements and is mainly seen in young individuals. Simply, wild animals do not survive long enough to become old (Kowald and Kirkwood 2016). Therefore natural selection has limited opportunity to influence the process of senescence and even in organisms whose senescence does contribute to mortality rate in the wild (e.g. some large mammals) a hypothetical “accelerated aging gene” would not benefit the individual in any way. Also, it is hard to imagine how an equilibrium can be maintained for these accelerated aging genes when individuals that have inactivated alleles of those would enjoy a huge selection advantage (Kirkwood and Austad 2000). Also, empirical evidence from the thousands of mutation screenings that lead to increased lifespan, has identified no gene that can completely abolish aging. These genes, if they existed, would be prone to inactivation by mutation (Kirkwood and Melov 2011). Additionally, at older ages, there may be adverse gene actions from deleterious

genes that escaped forces of natural selection or form pleiotropic genes that exchange beneficial effects at an early age with harmful effects at older ages (Kirkwood 2011). However, selection forces act at the level of reproduction and selective pressure after reproductive phase does not exert any effect. Therefore, genes that offer longevity may be selected against those that offer benefits at an early age to gain competitive advantage in reproduction and survival (Bengtson and Settersten Jr 2016). As an example, this has been shown in a study using long-lived *C. elegans age-1* mutants and wild-type as a model (Walker, McColl et al. 2000). Under normal laboratory conditions (protected environment), in mixed worm cultures of both the long-lived *gas-1* mutants and wild-type there was no consistent change in allele frequency and there is no evidence of a trade-off between longevity and other traits. However, when availability of food was used as a selective pressure, after a few generations, a large reduction in *gas-1* allele frequency indicated a substantial difference in relative fitness compared with the wild-type: the long-lived mutants lost the race.

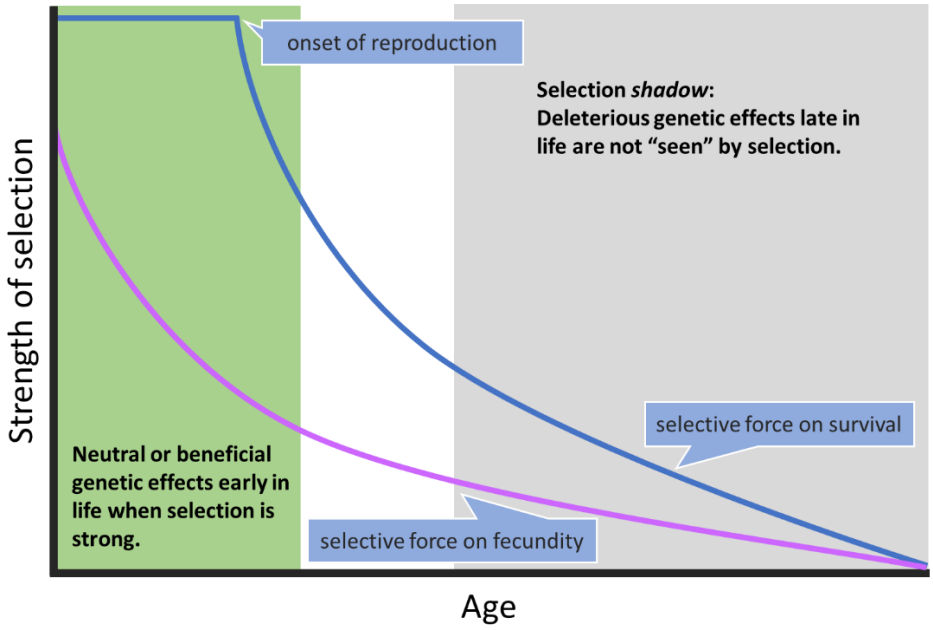

Figure 1.1. The strength of selection declines with age. Often the force of selection on survival and fecundity declines with age. In this case, alleles that have neutral or beneficial effects on survival and fecundity in early stages of life but have deleterious effects in later stages of life (antagonistic pleiotropy) can accumulate in a population. Therefore, these late-life negative effects in the "selection shadow" cannot be effectively removed by selective forces and lead to senescence. Figure modifed after (Flatt and Partridge 2018).

Although these principle predictions of the evolutionary theories of aging are backed up by empirical data and mathematical models (Kowald and Kirkwood 2016) some argue against them on the grounds that programmed longevity actually benefits populations by providing more resources for the young individuals (Goldsmith and Azinet 2016). Some also argue that programmed longevity and not programmed senescence is a more suitable title for the process (Longo 2019). Whether programmed aging is a myth or not, the idea of programmed longevity appears to have benefited the medical and pharmaceutical industries the most (Goldsmith 2016).

1.1.2 The telomere theory of aging

Physical ends of the linear chromosome structure are called telomeres. The human telomere DNA consists of two parts: a double-stranded DNA several thousand nucleotides long with the repeating nucleotide sequence TTAGGG and a single stranded part of 5 – 400 nucleotides overhanging at the 3' end of the DNA (Monaghan, Eisenberg et al. 2018). The specific nucleotide sequence and structure of telomere prevent recombination through side by side crossover which causes a change in the DNA sequence that should remain unaltered in dividing cells. Also, telomeres protect against sticking, fusion and enzymatic degradation of the chromosome endings (Hockemeyer and Collins 2015). After each round of DNA replication and cell division, telomeres become shorter by about 100 nucleotides causing cells to enter senescence after a finite number of cell divisions. This replicative senescence occurs as a result of DNA shortening at the chromosomal ends (Hayashi, Cesare et al. 2015). The loss of DNA is the consequence of the site-directed action of DNA polymerase. This enzyme simply cannot perform at the telomere and each time replication happens, the chromosome loses a short piece of the telomere. When, after numerous replication rounds, the length of the chromosome falls below a critical length, this damage to the DNA is recognized by intrinsic control mechanisms and further cell divisions are inhibited (Greider 2016). In 1961, Hayflick proposed that the ability of human cells to divide is limited to approximately fifty times (Hayflick limit) and after this limit is reached cells will stop dividing (Hayflick and Moorhead 1961). This formed the basis of the *telomere theory of aging* which assumes that the gradual shortening of chromosomal DNA and the ensuing replicative senescence is a determining factor in aging and lifespan (Bengtson and Settersten Jr 2016).

However, not all cell types suffer from replicative senescence. For example, stem cells and germ cells that need to undergo continuous division use an enzyme called telomerase to overcome

the end-replication problem of DNA polymerase (Schmidt and Cech 2015). This enzyme contains a segment of RNA which complements the TTAGGG repeats at the telomeres. With its own template, telomerase binds to the 3' end of the DNA strand and elongates it for exactly six nucleotides and repeats the process several times. Afterwards, other enzymes including DNA polymerase complete the lagging strand and produce a double-stranded chromosomal DNA end (Wu, Upton et al. 2017). This process is shown in Figure 1.2.

The discovery of telomerase in the early 1980's (Greider and Blackburn 1985), created the hope that replicative senescence could be prevented by targeted activation of telomerase. Since then, the role of telomerase reactivation in the propagation of cancer cells has also been extensively studied (Akincilar, Unal et al. 2016). In the context of aging, however, a comparison of telomere length in different species yields interesting results. For example, telomere length in humans is about 10kb while mice have longer telomeres of 20 – 50 kb. Furthermore, while telomerase is expressed in most mouse tissues during adulthood, in adult humans, telomerase expression and activity in most tissues is almost completely nonexistent. One explanation is that due to their short lifespan, mice do not require heavy DNA maintenance that long-lived organisms such as humans require. That is to say before wild-type mice can develop cancer, they could end up as prey to other animals. Therefore, only in species like humans that live long enough for cancer to be a threat, tumor control systems such as telomerase inactivity are developed to prevent cancer (Shay 2016). A connection between telomerase activity and aging and, of course, the hazardous nature of manipulating telomerase activity at an organismal level has been shown in transgenic mice. Transgenic over-expression of a subunit of telomerase in transgenic mice extended their maximum lifespan by 10 percent but it also increased their mortality rate in the first years of life (Gonzalez-Suarez, Geserick et al. 2005). However, in cancer-resistant mice over-expressing tumor-suppressor genes such as p53 this early mortality rate is reduced and lifespan in increased (Tomas-Loba, Flores et al. 2008). Additionally, links between telomerase activity and aging has been reported in humans, too. A special haplotype of telomerase is present in a population of Ashkenazi centenarians which leads to longer telomeres and longer life (Atzmon, Cho et al. 2010). Furthermore, human syndromes that cause progressive and enhanced aging known as progeria such as Werner syndrome and the Hutchinson-Gilford syndrome are known to show greater telomere shortening (Shay 2018).

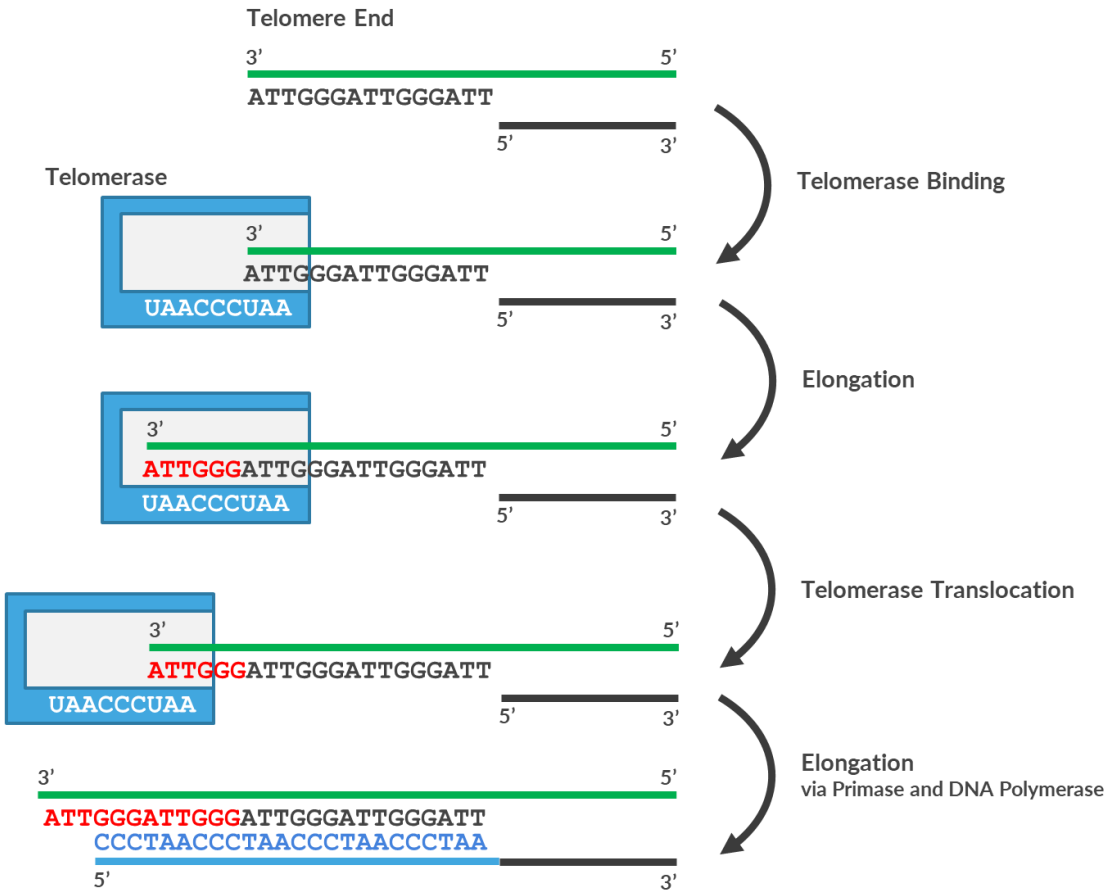

Figure 1.2. Action of telomerase to prevent loss of DNA at telomeres. Telomerase contains a short RNA which is complementary to the sequence at the overhanging 3' end of telomeres. After DNA is constructed from the template RNA, the telomerase translocates and repeats this process several times. The missing complementary DNA strand is then synthesized in 5' → 3' direction by a host of other enzymes including DNA polymerase. Modified after (Müller-Esterl 2017).

In summary, although telomerase activity has been linked with aging, telomere length is not indicative of lifespan as mice have much longer telomers than humans (Calado and Dumitriu 2013). Also, despite the fact that telomerase activity protects cells against replicative senescence, it does not prevent senescence caused by other pathways such as cell death resulting from DNA damage, ROS toxicity or the activation of oncogenes (Shay 2018). Additionally, the telomere theory of aging does not explain the aging process in post-mitotic organisms such as *C. elegans* (Riddle, Blumenthal et al. 1997). In the next section, we will review a group of aging theories that consider the accumulation of cellular components or specific cell types with altered metabolic processes as the cause of aging.

1.1.3. The DNA damage accumulation theory of Aging

Throughout the lifespan of organisms, DNA mutations and damage accumulate. For example, mutation of genes that are responsible for maintaining the integrity of the nuclear DNA lead to human syndromes connected to accelerated aging (Vermeij, Hoeijmakers et al. 2016, Gordon, Brown et al. 2019). The *DNA damage accumulation theory of aging* proposes that aging is essentially caused by the functional changes that result from the accumulation of DNA damage over the lifetime of an organism. These functional changes, in turn, impair cellular homeostasis and cause cellular death and will eventually lead to the death of the organism (Szilard 1959). In fact, throughout the life of a cell, all biomolecules, not just DNA, experience damage that causes structural and functional damage. For example, oxidation of amino acid side chains in proteins causes structural changes but powerful cellular machinery of protein degradation recycles the damaged proteins while new molecules are produced to replace them (Alber and Suter 2019). Similarly, the constant synthesis and degradation of lipids during metabolic and cellular repair processes ensure that damaged lipids are also recycled (Huang and Freter 2015). The nuclear DNA, on the other hand, needs to remain unchanged through the life cycle of the cell and when DNA damage is extensive apoptosis occurs and damaged cells are removed. However, very efficient cellular DNA repair mechanisms are constantly at work to ensure most DNA damage is not permanent and the integrity of genome is maintained (Cadet and Davies 2017). Therefore, it means that lethal accumulation of DNA damage is caused by failing DNA repair and defense mechanisms in old individuals.

In order to test the DNA Damage Theory of Aging, Chevanne and colleagues compared the repair of DNA strand breaks in lymphocytes from young people, old individuals and centenarians. They showed that DNA repair in lymphocytes from centenarians is as effective as in those obtained from young individuals (Chevanne, Calia et al. 2007). Another similar study analyzed DNA breaks in lymphocytes from subjects of different age groups (20–35, 63–70, 75–82 years) and the resistance of lymphocyte DNA to oxidative stress-induced damage and the repair activity were measured. The authors reported an increase in oxidative base damage in old age which was not caused by the failure of either antioxidant defense or DNA repair. These data suggest that in old age either DNA repair and the antioxidant defense systems are induced to compensate for an increasing age-associated challenge or older people, as survivors, were genetically predisposed to have relatively high levels of antioxidant defenses and DNA repair

earlier in their lives, compared to those who did not survive to such an age (Humphreys, Martin et al. 2007). Also, genome-wide association studies for age-related disorders resulted in a wide range of genes that are possible disease susceptibility factors and suggest that when genes that can be connected to longevity are studied, many different factors and conditions can potentially contribute to aging (Jeck, Siebold et al. 2012). Therefore, many genes, each with modest effects likely contribute to aging and negatively affect longevity by increasing susceptibility to age-associated disease and early death, whereas other genes may slow down the aging process leading to a long life. However, the precise genetic factors of aging and their interaction with behavioral and environmental factors that cause longevity are not understood (Murabito, Yuan et al. 2012).

In summary, according to the DNA damage accumulation theory, aging is caused by naturally occurring DNA damage that are not repaired and accumulate over time. This DNA damage can contribute directly to aging by increasing cell and organ dysfunction or indirectly by inducing apoptosis or senescence (Figure 1.3).

1.1.4. Accumulation theories of aging

According to this category of aging theories the built-up of cellular components or specific cell types whose properties have changed, and their function is compromised, results in tissue dysfunction and loss of homeostasis which in turn will cause aging. *Clinker theories of aging* focus on metabolic waste products whose accumulation in cells over time hampers normal cellular function (Bengtson and Settersten Jr 2016). For example, lipofuscin, an oxidized lipid-containing molecule produced during catabolic reactions, builds up in the lysosomes of post-mitotic cells such as neurons and cardiac myocytes. If lipofuscin is not degraded or ejected via exocytosis, lysosomal functions such as macromolecular catabolism, autophagy and cytoplasmic trafficking are negatively affected (Gray and Woulfe 2005).

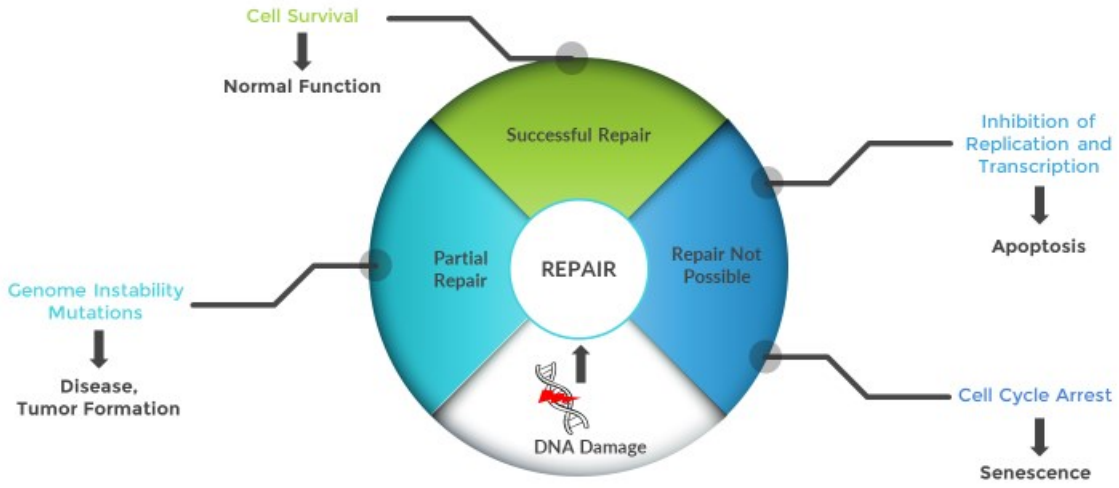

Figure 1.3. Possible outcomes of DNA damage in cells. DNA replication and repair is a highly regulated and complex process. However, accidental DNA damage may occur as a result of metabolic changes, irradiation or environmental toxins. If these damages are successfully repaired by DNA repair mechanisms, the cell will continue functioning normally. But insufficient DNA repair can cause disease or may lead to the formation of tumors, apoptosis or senescence.

Another example is the increase in collagen cross-links in the skin and bone tissue over time. Cross-linking alters the properties of collagen which results in stiff joints and bone and loss of dermal elasticity (Robins 2007). Accumulation of advanced glycation end-products (AGEs) in multiple organ systems is the third example of metabolic waste that is linked to aging. Glycation is the non-enzymatic reaction between reducing sugars, such as glucose, and proteins, lipids or nucleic acids (Ahmed 2005). AGEs are a heterogeneous group of molecules that include compounds such as carboxymethyl-lysine, glucosepane and fructoselysine which target collagen in epidermis and aged and diabetic dermis hampering the normal function of these tissues (Gkogkolou and Böhm 2012, Gautieri, Passini et al. 2017). Furthermore, AGEs can also bind to specific receptors and cause a pro-inflammatory immune response (Hu, Jiang et al. 2015). Finally, misfolded protein aggregates are believed to contribute to aging and various pathologies by forming crystallin accumulation in the eye, neurofibrillary tangles in the brain and amyloid deposition in the heart (Klaips, Jayaraj et al. 2017). It is not clear, however, that accumulation of

molecules such as the ones described above is a consequence of aging or the cause of it (Chaudhuri, Bains et al. 2018). While initially it might seem natural that accumulation of insoluble material is the cause of cellular or tissue dysfunction (Bjorksten 1968), it is also possible that physiological responses such as misfolded protein aggregation are protective responses (Derham and Harding 1997, Kaushik and Cuervo 2015).

Cellular organelles such as peroxisomes, lysosomes, mitochondria and cell nuclear membranes that have reduced functionality as a result of age-related alterations in their structure or metabolism are the focus of *clunker theories of aging* (Bengtson and Settersten Jr 2016). Mitochondrial DNA damage theory of aging, initially focused on the damage to mitochondrial DNA which lead to increased reactive oxygen species (ROS) production which was theorized to cause even more damage and contribute to aging (Harman 1972). The original theory has been invalidated by a series of observations which indicated antioxidant treatment or over- or under-expression of enzymatic regulators of ROS had ambiguous effects on lifespan (Lapointe and Hekimi 2010) and in fact ROS are able to contribute to longevity (Yang and Hekimi 2010a, Yang and Hekimi 2010b). Therefore, a new mitochondrial theory of aging was proposed where the focus shifted to other aspects of mitochondrial physiology such as mitochondrial biogenesis and turnover (Knuppertz and Osiewacz 2016), apoptosis (TeSlaa, Setoguchi et al. 2016), cellular senescence (Wiley and Campisi 2016), calcium mobilization (Granatiero, De Stefani et al. 2017) and epigenetic changes in mitochondrial DNA (D'Aquila, Bellizzi et al. 2015). In this new version of the mitochondrial theory of aging, aberrant mitochondrial function alters important homeostatic pathways such as apoptosis, senescence and energy metabolism. These changes will lead to altered phenotypic expression and energy metabolism which are associated with an aging phenotype (da Costa, Vitorino et al. 2016).

According to the peroxisome theory of aging, accumulative damage to peroxisomes hampers their different functions and leads to cell death and aging (Bengtson and Settersten Jr 2016). Similar to mitochondria, peroxisomes can also multiply by fission. They play an important role in the metabolism of ROS and in antiviral innate immunity for the detection of cytosolic viruses (Dixit, Boulant et al. 2010, Erdmann 2016). Other activities of peroxisomes include production of ether phospholipids and bile acids, catabolizing long-chain fatty acids through beta-oxidation and providing mitochondrial tricarboxylic acid cycle with metabolic intermediates (Demarquoy and Le Borgne 2015, Erdmann 2016). There is some evidence that support the

connection of peroxisomes to cellular aging. The protective role of peroxisomal enzymes in excessive levels of ROS has been linked to aging (Beach, Burstein et al. 2012). Some treatments which increase longevity such as caloric restriction and some mild stressors have been shown to activate NAD^+ salvage pathways in yeast peroxisome (Ghislain, Talla et al. 2002). Additionally, peroxisomes act as modulators of diacylglycerol levels which sensitizes cells to age-related stresses (Feng, Ren et al. 2007, Goldberg, Bourque et al. 2009). Finally, peroxisomes also regulate the levels of non-esterified fatty acids which accelerate age-related necrotic and apoptotic cell death (Jungwirth, Ring et al. 2008).

The lysosome theory of aging states that loss of functional capacity of lysosomes is the cause of the aging phenotype (Carmona-Gutierrez, Hughes et al. 2016). Lysosomes are major degradation facilities of the cell. Cellular components are disassembled for recycling in lysosomes. Also, organelles like mitochondria, when damaged, undergo component disassembly at lysosome (Deretic and Klionsky 2018). Additionally, lysosomes are involved in cellular processes such as nutrient sensing, stress resistance, cellular development, differentiation and apoptosis (Perera and Zoncu 2016). Some findings support this view of the role of lysosomes in aging. During temporary starvation, lysosomes facilitate survival by performing autophagy and catabolizing macromolecules (Takagi, Kume et al. 2016). As part of their routine cellular maintenance procedures, lysosomes also remove damaged proteins and organelles to enhance longevity (Knuppertz and Osiewacz 2016). However, lysosomes are also a potential location for the formation of lipofuscin (Gray and Woulfe 2005) which leads to their own malfunction and causes damaged and dysfunctional organelles and macromolecules to accumulate and hamper cellular functions (Zhang 2013). Therefore, loss of function in lysosomes is a potential mechanism for cumulative loss of function in cells and tissues with age.

In summary, the accumulation theories of aging focus on the potential impact of amassed cellular waste (e.g. misfolded proteins, AGEs, lipofuscin, dysfunctional organelles) over time. Clearly, overloading a living system with non-degradable material takes its toll, however, it is very difficult to untangle the cause and effect relationship in all these scenarios. Do organisms age because all this waste builds up in their cells over time and modifies the normal function of cells and tissues, or this loss of functional capacity is simply a byproduct of advancing age?

1.1.5. Free radical theory of aging

The free radical theory of aging was originally proposed by Harman in 1956 (Harman 1956). Roughly two decades later, he proposed a modified version of that theory, highlighting the role of mitochondrial reactive oxygen species (ROS) in aging (Harman 1972). The premise of free radical theory of aging is that ROS (e.g. $O_2^{\bullet-}$, H_2O_2 and •OH reviewed in section 1.2.1) can oxidize and damage molecular components such as DNA, proteins and lipids when ROS detoxification mechanisms are not sufficient to maintain ROS levels at optimal levels for normal physiology. The principles that the free radical theory of aging was based on revolve around the idea that mitochondria are the main source of cellular ROS production and they act as some kind of "biological clock" which modulates lifespan. Some observations that corroborate this theory included the following:

(1) Loss of function in aging mitochondria inevitably leads to aberrant cell and tissue properties and contribute to the process of aging (Lee and Wei 2012).

(2) ROS generation and oxidative damage increases with age (Barja 2004). For example, it was demonstrated that in isolated mitochondria from older animals ROS generation is increased (Sohal and Sohal 1991) while their ratio of reduced to oxidized glutathione is decreased (Asensi, Sastre et al. 1999).

(3) ROS production is enhanced when mitochondrial function is inhibited (Li, Ragheb et al. 2003) and oxidatively damage macromolecules increase with age (Stadtman 2006).

(4) Sever increase in oxidative stress and damage is seen in many age-dependent diseases such as neurodegenerative and vascular diseases (Mariani, Polidori et al. 2005, Stefanatos and Sanz 2018).

Some other evidence also appeared to support the free radical theory of aging. For example, lack of the mitochondrial superoxide dismutase (SOD2) is lethal in mice and *Sod2*$^{-/-}$ animals die within a few weeks after birth and exhibit such severe phenotypes as enlarged myocardium (Li, Huang et al. 1995, Huang, Carlson et al. 2001). Also, mice that lack the cytoplasmic superoxide dismutase (SOD1) have very high levels of oxidative damage, mainly die from hepatocellular carcinoma and have a very short lifespan. However, the association of such deleterious phenotypes and pathologies with aging is questionable (Hekimi, Lapointe et al. 2011).

In *Drosophila melanogaster* (the fruit fly), over expression of the cytoplasmic and mitochondrial superoxide dismutase increased the lifespan of both *Sod1* and *Sod2* null flies that were short-lived (Phillips, Campbell et al. 1989, Sun and Tower 1999, Duttaroy, Paul et al. 2003)

but overexpression of superoxide dismutases and other antioxidant enzymes in other studies did not increase lifespan at all (Mockett, Sohal et al. 2010). However, by overexpressing SOD2 or human uncoupling protein 2 in adult fly neurons, researchers were able to reduce mitochondrial ROS production and increase lifespan which can be a supporting evidence of the free radical theory of aging (Cho, Hur et al. 2011).

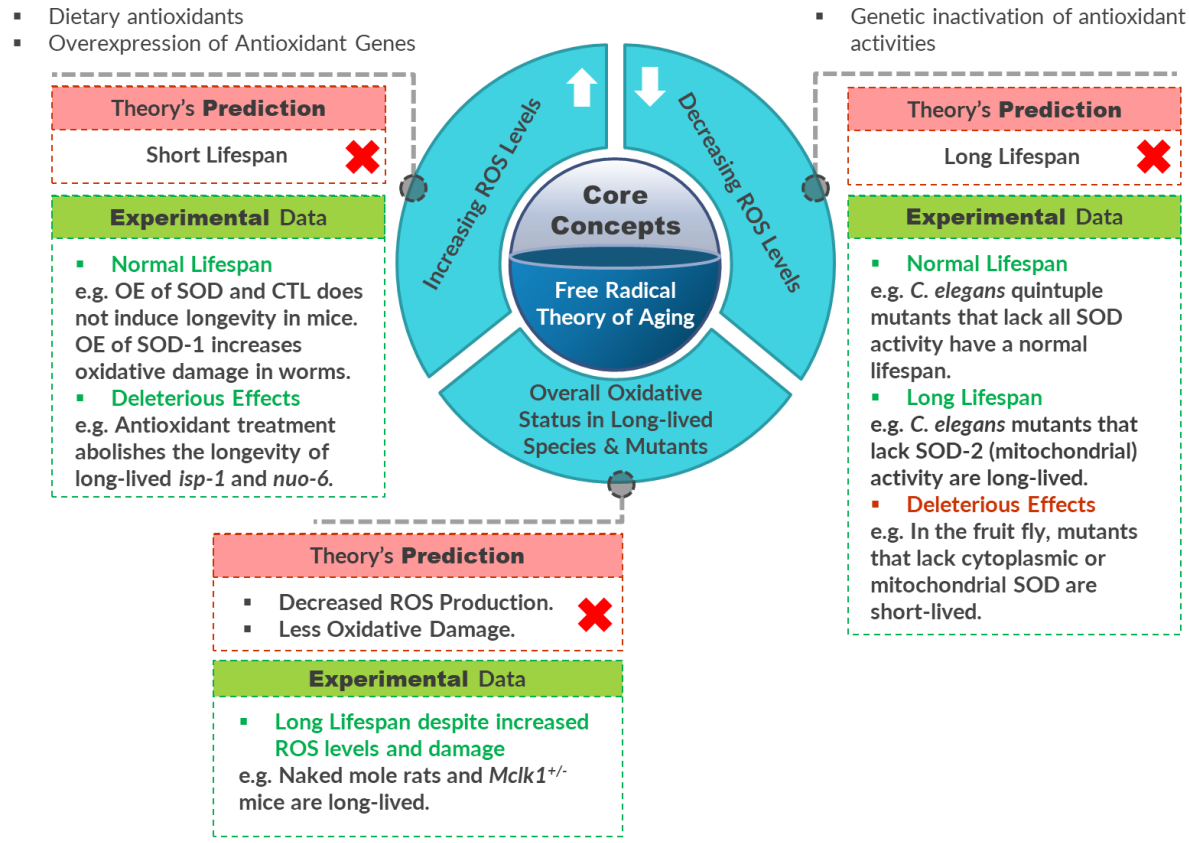

Figure 1.4. The core concepts of the free radical theory of aging are contradicted by experimental data. According to the free radical theory of aging, increasing ROS levels will shorten lifespan while decreasing ROS levels lengthen lifespan. Experimental data from various species and mutants do not support this. (ROS = Reactive oxygen species, OE = Overexpression, SOD = Superoxide dismutase, CTL = catalase)

In *C. elegans,* initially researchers found that overexpression of SOD-1 (cytoplasmic superoxide dismutase) increases the lifespan of the worm (Doonan, McElwee et al. 2008). Further studies showed that lifespan extension by SOD-1 overexpression is not connected to oxidative damage or $O_2^{\bullet-}$ antioxidant defense mechanisms and in fact it increases levels of cellular ROS and

molecular damage (Cabreiro, Ackerman et al. 2011). Figure 1.4 summarizes the findings that are in direct contrast with the core concepts of the free radical theory of aging.

Despite other revisions to the free radical theory of aging (Beckman and Ames 1998), there are a wide variety of incompatible results that the theory fails to explain:

(1) The overexpression of some antioxidant enzymes in fruit flies only manage to extend the lifespan of those animals that lack them in the first place and the overexpression of superoxide dismutases, catalases or a combination of both does not extend the lifespan of genetically engineered mice (Muller, Lustgarten et al. 2007, Perez, Van Remmen et al. 2009). In *C. elegans,* although overexpression of the cytoplasmic superoxide dismutase (SOD-1) increases lifespan, not only is this longevity effect independent of both ROS damage and the role of SOD-1 in $O_2^{\bullet-}$ detoxification, the overexpression of SOD-1 also increases ROS levels and damage despite the longevity of the worms (Cabreiro, Ackerman et al. 2011).

(2) Treatments that involved the use of antioxidants in various organisms including humans did not demonstrate beneficial effects on longevity and in some cases were shown to have deleterious outcomes (Howes 2006).

(3) The level of ROS production and longevity in different species do not show a direct correlation (Chen, Hales et al. 2007, Csiszar, Podlutsky et al. 2012).

(4) There are several mutants and species that exhibit high levels of ROS production and oxidative damage but are long-lived. For example, the naked mole-rat, which is the longest living rodent, shows high oxidative damage levels (Andziak, O'Connor et al. 2006) (Figure 1.5). Also, these animals, which live up to 30 years exhibit a significant increase in ROS levels (Labinskyy, Csiszar et al. 2006). Also, while *Mclk1*$^{+/-}$ mice have a significant increase in ROS levels and oxidative stress, they are long-lived (Lapointe and Hekimi 2008). In *C. elegans,* mutations in a mitochondrial complex III subunit (ISP-1) or a mitochondrial complex I subunit (NUO-6) leads to a massive increase in lifespan despite increased mitochondrial ROS generation (Yang and Hekimi 2010b). Furthermore, a reduction in superoxide detoxification in long-lived mitochondrial mutants of the worm via RNA interference does not shorten their lifespan despite a measurable increase in oxidative damage (Yang, Li et al. 2007). Interestingly, mutants of *C. elegans* that completely lack all superoxide dismutase activity are not short-lived (Van Raamsdonk and Hekimi 2012) and the

mutant worms that lack the mitochondrial superoxide dismutase are even long-lived (Van Raamsdonk and Hekimi 2009).

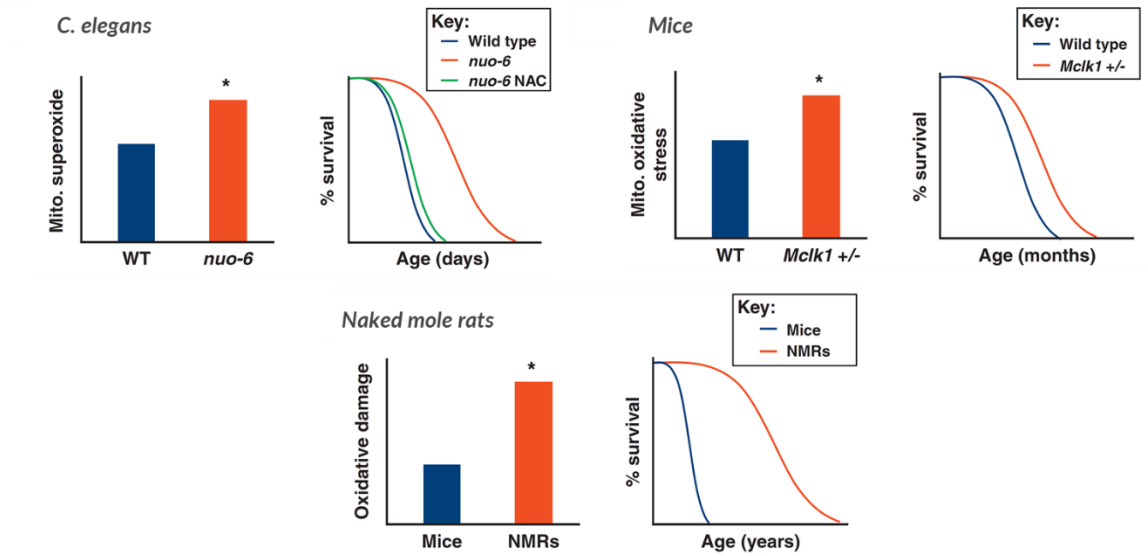

Figure 1.5. Schematic figures showing organisms with high ROS phenotypes that are long-lived. Mitochondrial mutants of *C. elegans* such as *nuo-6* have high levels of mitochondrial superoxide but they are long-lived. Treatment of these long-lived mutants with antioxidants abolishes their lifespan. *Mclk1*$^{+/-}$ mice have higher levels of mitochondrial oxidative stress but are long-lived compared to the wild-type. Naked mole rats are the longest living rodents, but they have high levels of oxidative damage in their tissues. The bars and aging do not represent specific data but summarize and illustrate the findings of several studies. The asterisks (*) indicate that statistically significant differences have been found in the studies that are summarized by the graphs. Figure adapted from (Hekimi, Lapointe et al. 2011).

These observations are in direct contrast with the core concepts of the free radical theory of aging. Moreover, even though ROS-induced pathologies are associated with age-associated diseases (Davalli, Mitic et al. 2016), there is no clear cut evidence that directly points to ROS as the cause of aging and it is more likely that increased ROS production in aging cells is a consequence of aging rather than its cause where increased ROS generation in aging represents a signal that activates protective mechanisms which are aimed at enhancing longevity (Hekimi, Lapointe et al. 2011). Indeed, recent advances in ROS biology (discussed in section 1.3) have confirmed their crucial role as an important mechanism in cellular signaling (Wang, Branicky et al. 2018) and activation of protective mechanisms that enhance longevity (Yee, Yang et al. 2014).

As any other single-cause theory of aging, the free radical theory of aging has fallen short of delivering on its promise. Therefore, our laboratory and others have proposed alternative interpretations of the data that had led to the formation of the free radical theory of aging (Blagosklonny 2008, Lapointe and Hekimi 2010). For example, it has been proposed that the correlation between ROS damage and aged phenotype is because of the role of ROS in modulating signal transduction pathways that respond to the type of cellular stresses which are a result of aging (Hekimi, Lapointe et al. 2011). So, ROS increase with age, not because they cause aging but because they are part of signaling mechanisms that exist to combat aging.

Perhaps a good lesson to take away from all the proposed aging theories since the time of Aristotle is that "aging is unquestionably complex" (Kirkwood 2011) and most likely a result of a the interaction of a plethora of genetic, behavioral and environmental factors. Oversimplification of such a complicated process in a single-cause theory is simply not possible. It is more likely that an interconnected matrix of individual and population traits and their interactions with the environment is responsible for aging and in the case of humans even socioeconomic and psychological elements play a role (Bengtson and Settersten Jr 2016).

In this section we reviewed some of the important theories of aging and how experimental evidence using model organisms such as mouse, the fruit fly and *C. elegans* were used to examine them. Among these theories, we discussed how the free radical theory of aging failed to explain the fundamental discrepancies between its core concepts and experimental data. In the following section, we will discuss the biology of ROS and elements of the cellular ROS network such as ROS-handling enzymes. After that we will revisit the connection between ROS and aging in the context of the findings on the long-lived mitochondrial mutants of *C. elegans isp-1* and *nuo-6* which were introduced in this section.

1.2. An overview of reactive oxygen species (ROS)

1.2.1. Properties and reactivity of ROS

Reactive oxygen species (ROS) is a collective term used to describe partially reduced or excited forms of O_2. These include the free radicals superoxide ($O_2^{\bullet-}$) and hydroxyl (•OH), which contain unpaired electrons, as well as hydrogen peroxide (H_2O_2) (Halliwell and Cross 1994). Among other mechanisms, the most common source of $O_2^{\bullet-}$ production within the cell is the electron flow in the mitochondrial electron transport chain (ETC) leaking onto O_2 to form $O_2^{\bullet-}$ (Murphy 2009).

H_2O_2 can be produced by spontaneous dismutation of $O_2^{\bullet-}$ but superoxide dismutases (SODs) catalyze this reaction at a constant rate which is about 20,000 times faster than the spontaneous mutation of $O_2^{\bullet-}$ (Gray and Carmichael 1992). Two important subcellular compartments of H_2O_2 production are mitochondria and peroxisomes (Lennicke, Rahn et al. 2015, Winterbourn 2017). Both of these organelles use the produced ROS as a means of signal transduction and communication with each other and other subcellular compartments (Schrader, Costello et al. 2015, Shadel and Horvath 2015, Fransen, Lismont et al. 2017). H_2O_2 is then reduced to water by catalase or glutathione-dependent peroxidases at different subcellular compartments (see section 1.4). The highly reactive •OH is either formed by H_2O_2 via the Fenton reaction catalyzed by iron ions, or by $O_2^{\bullet-}$ converted to H_2O_2 and further to •OH in a process called the Haber-Weiss reaction (Figure 1.6) (Koppenol 1993, Kehrer 2000).

$$Fe^{3+} + O_2^{\bullet-} \longrightarrow Fe^{2+} + O_2 \qquad \text{Step 1}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \qquad \text{Step 2}$$

$$O_2^{\bullet-} + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^- + O_2 \qquad \text{Net Reaction}$$

Figure 1.6. The Haber-Weiss Reaction. •OH is generated from H_2O_2 and $O_2^{\bullet-}$ via the Haber-Weiss reaction. This reaction can occur under physiological conditions. Iron catalyzes this reaction.

The distinct chemical properties of different ROS such as their reactivity, half-life and lipid solubility set them apart (D'Autreaux and Toledano 2007). •OH is the most reactive and highly toxic form of ROS. Its extremely short half-life and indiscriminate reactivity with biological molecules makes •OH an unlikely candidate to function as a messenger since it reacts with most molecules at or close to its production site (Schieber and Chandel 2014). $O_2^{\bullet-}$ and H_2O_2 each show preference towards specific biological targets. $O_2^{\bullet-}$ is electrically charged and therefore cell membrane impermeable and targets iron-sulfur clusters (D'Autreaux and Toledano 2007). $O_2^{\bullet-}$ is also a precursor for H_2O_2. Chemical properties of H_2O_2, its target specificity and its concentration in the picomolar-nanomolar make it suitable to act as an intra- and inter-cellular messenger. (Droge 2002, D'Autreaux and Toledano 2007, Forman, Maiorino et al. 2010, Schieber and Chandel 2014). The properties and reactivity of different ROS are summarized in Table 1.1.

Table 1.1. Properties and reactivity of ROS. Modified after (Mittler 2017).

ROS	**$t_{1/2}$**	**Migration Distance**	**Reactivity**	**Production Site**
Superoxide ($O_2^{\bullet-}$)	1-4 µs	30 nm	Reaction with Fe-S proteins Dismutation to H_2O_2	Mitochondria, peroxisomes
Hydrogen Peroxide (H_2O_2)	>1 ms	>1 µm	Reaction with proteins by targeting cysteine/methionine residues Reaction with heme proteins Reaction with DNA	Peroxisomes, mitochondria, cytosol
Hydroxyl Radical (•OH)	1 ns	1nm	Extremely reactive with all biomolecules: DNA, RNA, lipids, proteins	Iron and H_2O_2 (Fenton reaction)

1.2.2. Sources of cellular ROS production

There are many different intracellular sources of ROS, but from a quantitative standpoint, mitochondria are considered the most significant ROS producers (Liu, Fiskum et al. 2002). During the flow of electrons down the respiratory chain, a small fraction of them can prematurely leak to molecular oxygen to produce $O_2^{\bullet-}$ (Murphy 2009). In isolated mitochondria, complex I and complex III of the ETC are the major sites of $O_2^{\bullet-}$ production (Brand 2010). This $O_2^{\bullet-}$ can be directly produced into or relocate to the intermembrane space through anion channels (Han,

Williams et al. 2001, Han, Antunes et al. 2003, Muller, Liu et al. 2004). Additionally, at least in some cell types, mitochondrial $O_2^{\bullet-}$ can exit the cell (Lynch and Fridovich 1978, Li, Zhu et al. 2016). Mitochondrial $O_2^{\bullet-}$ can also be dismutated to H_2O_2 by mitochondrial or cytoplasmic SODs (Figure 1.7) (G., A. et al. 1973, Adam-Vizi 2005).

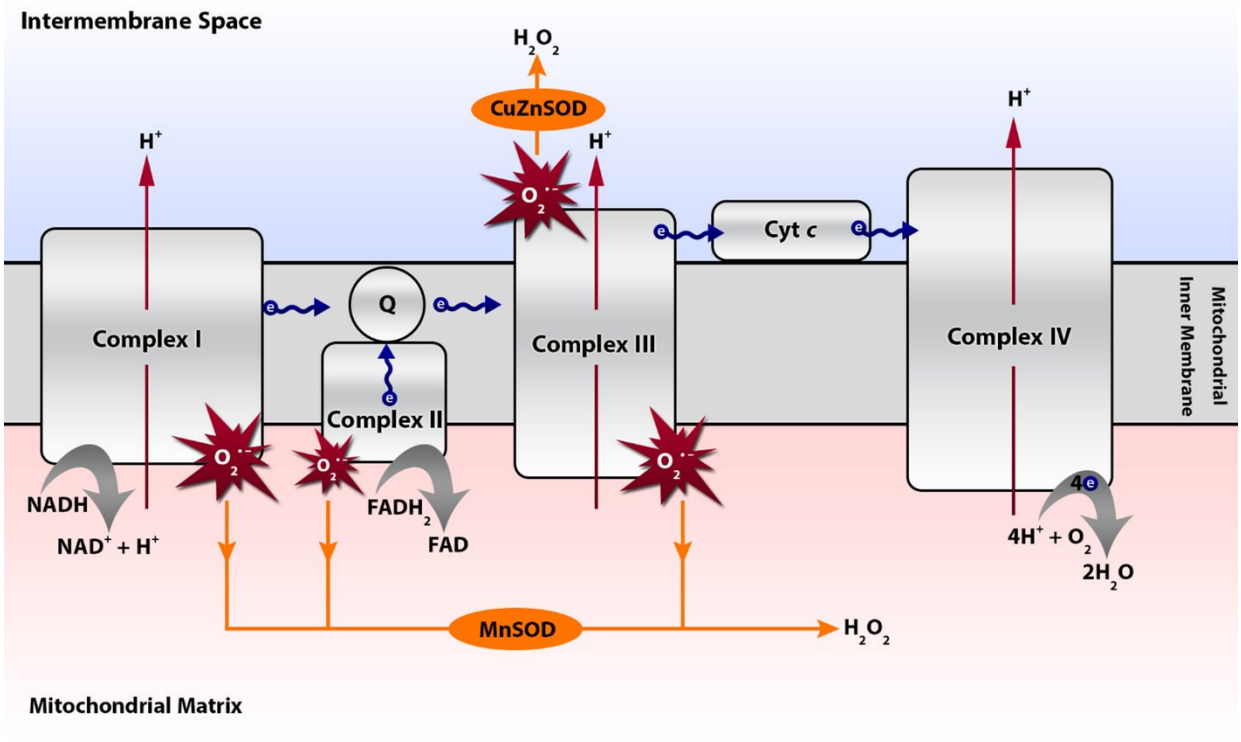

Figure 1.7. Sites of ROS production at the mitochondrial electron transport chain (ETC). While electrons move down the ETC, they may prematurely leak to molecular oxygen and produce $O_2^{\bullet-}$ mostly at the sites of complexes I, II and III. In mitochondrial matrix, manganese superoxide dismutase (MnSOD) enzymes use this $O_2^{\bullet-}$ to produce H_2O_2. This H_2O_2 can relocate to the intermembrane space. Also, H_2O_2 is directly produced in the intermembrane space by copper/zinc superoxide dismutase (CuZnSOD) enzymes. See section 1.4 for an overview of ROS-handeling enzymes.

Another important intracellular source of ROS is NADPH oxidase (NOX) family of enzymes. These membrane-associated enzymes use nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor to produce a local ROS micro-environments (Bedard and Krause 2007). The seven mammalian NADPH oxidases have been found in most tissues and are at cellular membranes and within intracellular compartments, such as endosomes and endoplasmic reticulum (ER) (Bedard and Krause 2007, Krause 2007). *C. elegans* has a homolog of NOX family named

Duox1/BLI-3. Among performing other roles, the redox activity of Duox1/BLI-3 has been shown to promote oxidative resistance and longevity in the worm (Ewald, Hourihan et al. 2017).

Peroxisomes are unique in that they display mechanisms to maintain the equilibrium between production and scavenging of ROS, and in particular H_2O_2. A variety of enzymes that reside within peroxisomes use long-chain fatty acids, hydroxy acids, amino acids, purines, pyrimidines and uric acid as substrate to produce ROS. These enzymes include acyl-CoA oxidases, urate oxidase, d-amino acid oxidase, d-aspartate oxidase, l-pipecolic acid oxidase, l-α-hydroxyacid oxidase, polyamine oxidase, and xanthine oxidase. This enzymatic activity mostly results in the production of H_2O_2 but also some $O_2^{\bullet-}$ and •OH are produced (Antonenkov, Grunau et al. 2010).

To counteract the deleterious effects of uncontrolled ROS production, mammalian peroxisomes also possess some antioxidant enzymes such as catalase, CuZnSOD, as well as glutathione peroxidase and members of the peroxiredoxin family of enzymes (Fujii and Ikeda 2002, Fransen, Nordgren et al. 2012, Morita and Imanaka 2019). We will discuss these enzymes and their regulatory roles in the cellular ROS networks in more detail in section 1.4.

1.2.3. Oxidative stress and harmful effects of excessive ROS

Uncontrolled increases in ROS levels can lead to free radical mediated chain reactions that indiscriminately targets biological molecules. Proteins are the major targets of damage induced by oxidative stress because they are the most abundant molecules in the cell and also because they conduct most of the cellular processes (Dalle-Donne, Rossi et al. 2006). Exposure to toxic levels of ROS may change every level of protein structure from primary to quaternary which in turn leads to physical changes in protein structure and function. ROS-induced damage to proteins can cause peptide backbone cleavage, cross-linking and modification of amino acid side chains (Davies 2005). Protein carbonylation is a very common form of ROS-induced damage to proteins and is considered a hallmark of oxidative stress. It leads to protein malfunctions such as inhibition of enzymatic and binding activities (Yao, Rahman et al. 2011, Curtis, Hahn et al. 2012). Additionally, some functionally inactive proteins are not efficiently degraded by proteasomal and lysosomal pathways and form protein aggregates that accumulate in intracellular compartments or at the extracellular environment (Grune, Merker et al. 2003). The accumulation of damaged proteins can inhibit the proteasome (Grune, Jung et al. 2004). As a consequence of the decreased capacity of

the cell to remove damaged proteins, the accumulation of damaged and misfolded proteins is accelerated and continues up to the point that the protein aggregates cause metabolic dysfunctions or the initiation of apoptosis or necrosis (Dalle-Donne, Aldini et al. 2006, Butterfield and Dalle-Donne 2014).

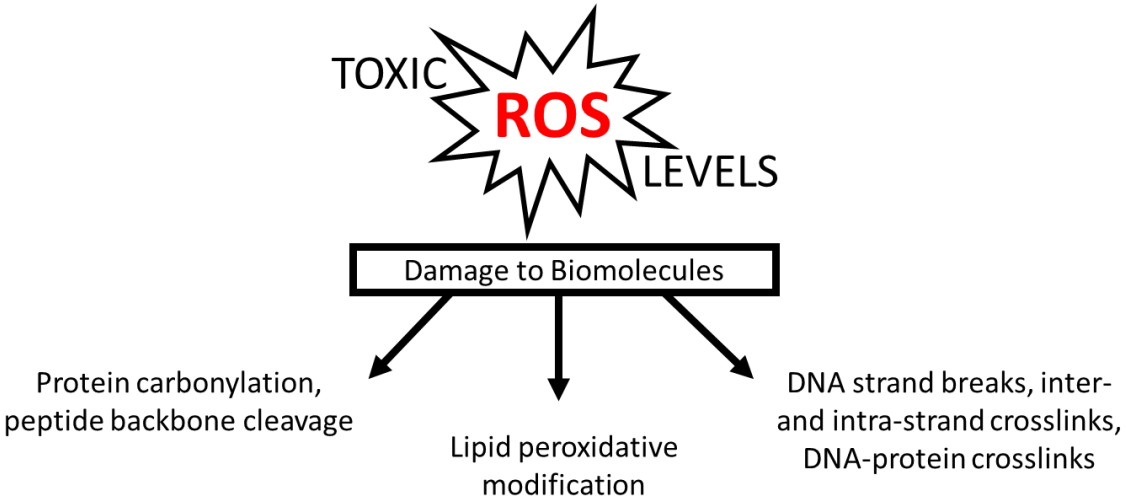

Figure 1.8. Toxic levels of ROS cause damage to biological molecules. Although homeostatic ROS levels are needed for normal cellular processes uncontrolled increase in ROS levels causes damage to biological molecules and hampers cellular functions. Protein carbonylation is a common form of ROS-induced damage to proteins and is a hallmark of oxidative stress. It leads to protein malfunctions such as inhibition of enzymatic activities. ROS-induced peroxidation of membrane lipids causes changes in biological properties of the membrane such as the degree of fluidity which can lead to inactivation of membrane-bound receptors or enzymes and can increase tissue permeability. Also, the structure and function of DNA can be compromised by exposure to high levels of $O_2^{\bullet-}$ and •OH. This can result in oxidative modification of bases which ultimately leads to DNA strand breaks, inter- and intra-strand crosslinks and DNA-protein crosslinks.

Hydroxyl radicals (•OH) cause damaging and potentially lethal peroxidative modification to lipids (Ayala, Mu et al. 2014). ROS-induced peroxidation of membrane lipids causes changes in biological properties of the membrane such as the degree of fluidity which in turn can lead to inactivation of membrane-bound receptors or enzymes and increase tissue permeability (Farmer and Mueller 2013). To make matters worse, lipid peroxidation contributes to and exacerbates cellular damage by producing oxidized products. Some of these are chemically reactive and can irreversibly change the structure of important biomolecules (Barrera 2012). Additionally, the structure and function of DNA can be compromised by exposure to high concentrations of $O_2^{\bullet-}$ and •OH, resulting in oxidative modification of bases which ultimately leads to DNA strand breaks, inter- and intra-strand crosslinks and DNA-protein crosslinks (Figure 1.8) (Jena 2012).

1.3. ROS as signaling molecules

In the past few years some evidence has emerged that points at the role of ROS in intracellular signaling. The data covers functions that are involved in the regulation of a wide range of functions such as signaling pathways related to inflammation (Daiber 2010, Dikalov and Nazarewicz 2013, Schulz, Wenzel et al. 2014), regulation of insulin signaling (Bashan, Kovsan et al. 2009) and regulation of germline development and lifespan (Shibata, Branicky et al. 2003, Yang and Hekimi 2010a, Yang and Hekimi 2010b, Yee, Yang et al. 2014, Schaar, Dues et al. 2015). In the following sections we will discuss some advantages of using ROS as signaling molecules and review the roles of specific types of ROS that act in intracellular signaling.

1.3.1. Advantages of using ROS as signaling molecules

Although ROS were previously viewed as predominantly harmful due to their role in oxidative stress, major scientific breakthroughs in the past two decades have firmly established their role as important signaling molecules in living systems (Moloney and Cotter 2018, Wang, Branicky et al. 2018).

There are several advantages to using ROS as signaling molecules. First, cells have the ability to produce and remove different forms of ROS at the same time (e.g. through the action of ROS-handling enzymes by changing the rates of ROS production and scavenging), which leads to rapid and dynamic changes in ROS concentrations. For example, in mouse and human airway basal stem cells (ABSCs) a ROS-activated mechanism controls the dynamic intracellular ROS flux from low to moderate levels to regulate airway stem cell proliferation cycle. For the correct operation of self-renewal and proliferation machinery in airway basal stem cells, intracellular flux from low to moderate ROS levels is required. ABSCs that are continuously exposed to high ROS levels are proliferation deficient. On the other hand, under normal circumstances, a flux change that causes increased ROS levels to moderate levels leads to the activation of a specific pathway (Nrf2-Notch1) which promotes self-renewal and proliferation. Nrf2 also triggers enzymatic ROS scavenging mechanisms that return overall ROS levels to a low state which brings the ABSC back to the quiescent state. Tightly-regulated inhibition of this pathway results in increased ROS levels until they reach a high enough level again to activate the pathway for the repeat of this cycle (Figure 1.9) (Paul, Bisht et al. 2014).

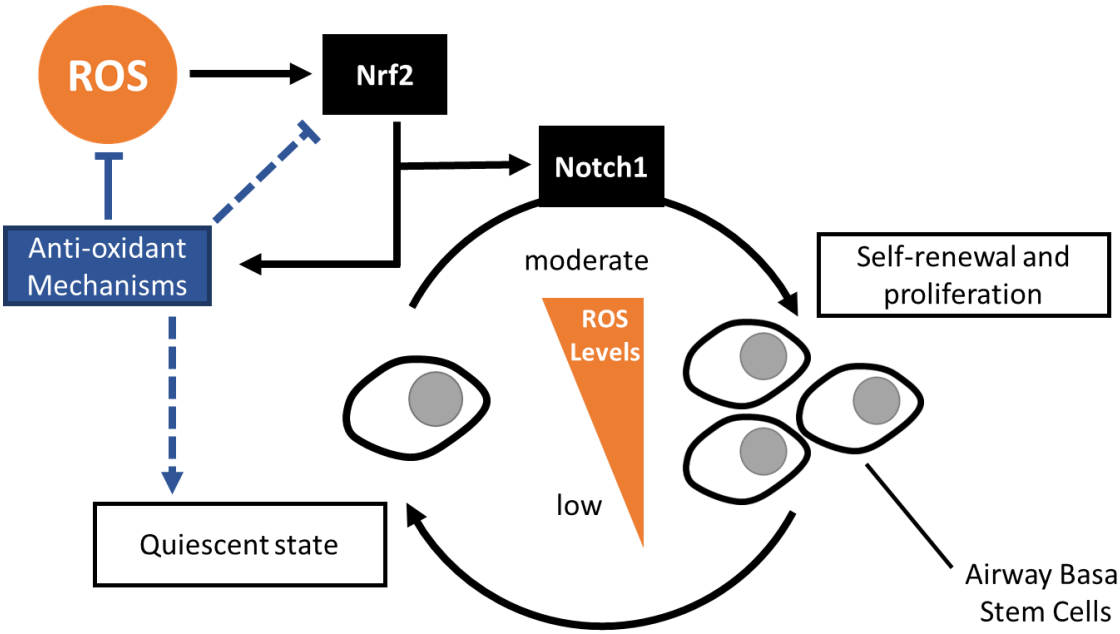

Figure 1.9. ROS-mediated regulation of self-renewal and proliferation in mouse and human airway basal stem cells (ABSCs). A ROS-activated mechanism controls the dynamic intracellular ROS flux from low to moderate levels to regulate airway stem cell proliferation cycle. For self-renewal and proliferation to take place in airway basal stem cells, intracellular flux from low to moderate ROS levels is required. ABSCs that are continuously exposed to high ROS levels are proliferation deficient. Under normal conditions, a flux change that causes increased ROS levels to moderate levels lead to activation of a specific pathway (Nrf2-Notch1) which promotes self-renewal and proliferation. Nrf2 also triggers enzymatic ROS scavenging mechanisms that return overall ROS levels to a low state which brings the ABSC back to the quiescent state. Tightly-regulated inhibition of this pathway results in increased ROS levels until ROS reach a high enough level again to activate the pathway for the repeat of this cycle. Diagram based on (Paul, Bisht et al. 2014).

Another advantage of using ROS for signaling purposes arises from the inherent ability of the cells for subcellular localization of ROS signals, which can be tightly controlled. In this regard, increases in local ROS production can be confined to an organelle to spatially control ROS accumulation and turn it into a highly specific signal. As an example, in a previous study in our laboratory, we have shown how increased mitochondrial ROS can activate signaling pathways that can lead to a wide range of effects including lifespan extension in *Caenorhabditis elegans.* Yee *et al.* have shown that increased mitochondrial ROS production in *C. elegans* triggers protective mechanisms via the mitochondria-associated intrinsic apoptosis pathway. In this organism, the intrinsic apoptosis pathway can be activated by two different BH3-only proteins but only one of

them (CED-13), upon activation by increased mitochondrial ROS production, induces a unique pattern of gene expression that promotes survival instead of apoptosis (Yee, Yang et al. 2014). (see section 1.5.3 and Figure 1.17 for a more detailed discussion of the role of the intrinsic apoptosis signaling pathway in the longevity of *C. elegans*)

Additionally, ROS can be involved in auto-propagating long-range signal transfers throughout the organism. In this way, each cell along the route of the signal activates its own ROS production machinery autonomously in order to rapidly carry a signal over long distances. An example of this can be found in *Arabidopsis thaliana* where a rapid systemic ROS signal travels at a rate of 8.4 centimeters per minute to relay a distress message in response to wounds, heat, cold, high-intensity light, and salinity stresses. This distress signal is initiated and maintained by $O_2^{\bullet-}$ and its derivative ROS produced by the plant NADPH Oxidase (NOX): respiratory burst oxidase homolog D (RBOHD). Accumulation of ROS in the extracellular spaces between cells allows for the high-speed travel of this systemic signal (Figure 1.10) (Miller, Schlauch et al. 2009) in a process that utilizes the mechanism of ROS induced ROS release (see section 1.3.6) (Mittler, Vanderauwera et al. 2011, Evans, Choi et al. 2016).

Also, as discussed in section 1.2.1, distinct biochemical properties of different ROS have enabled them to act as versatile signaling molecules that can be conveniently integrated into numerous signaling pathways. For example, ROS and calcium (Ca^{2+}) signaling are connected in a bidirectional manner (Görlach, Bertram et al. 2015). Ca^{2+} signaling regulates a range of cellular functions including metabolism, contraction, secretion, gene expression, cell survival and cell death (Berridge 2012). In addition to the Ca^{2+} storage and release by the endoplasmic reticulum (ER), specialized calcium transport systems are also localized in other cellular organelles such as mitochondria and mitochondrial Ca^{2+} uptake regulates the rate of energy production, controls the magnitude and spatiotemporal patterns of intracellular Ca^{2+} signals, and plays a major role in cell survival and death (Orrenius, Zhivotovsky et al. 2003). Ca^{2+} from ER cisternae mainly moves through Ca^{2+} release channels such as inositol 1,4,5-trisphosphate receptors (IP_3R) and ryanodine receptors (RyR) which exist in mitochondrial associated membranes (MAMs), as well. On the other hand, Ca^{2+} from the cytoplasm can move into the mitochondria through voltage dependent anion channels (VDAC), too (Kaufman and Malhotra 2014). High levels of Ca^{2+} stimulate ETC activity leading to increased ROS production. Increased ROS levels can in turn target ER-based calcium channels leading to increased release of Ca^{2+} and even more increased

ROS levels (Adam-Vizi and Starkov 2010, Kaufman and Malhotra 2014). Uncontrolled elevation of ROS levels and high Ca^{2+} load can open the mitochondrial permeability transition pore (mPTP) permanently and result in the release of pro-apoptotic factors and cell death (Bonora, Wieckowski et al. 2015). (Also, see section 1.3.4 on ER-mitochondrial interface and redox nanodomains.)

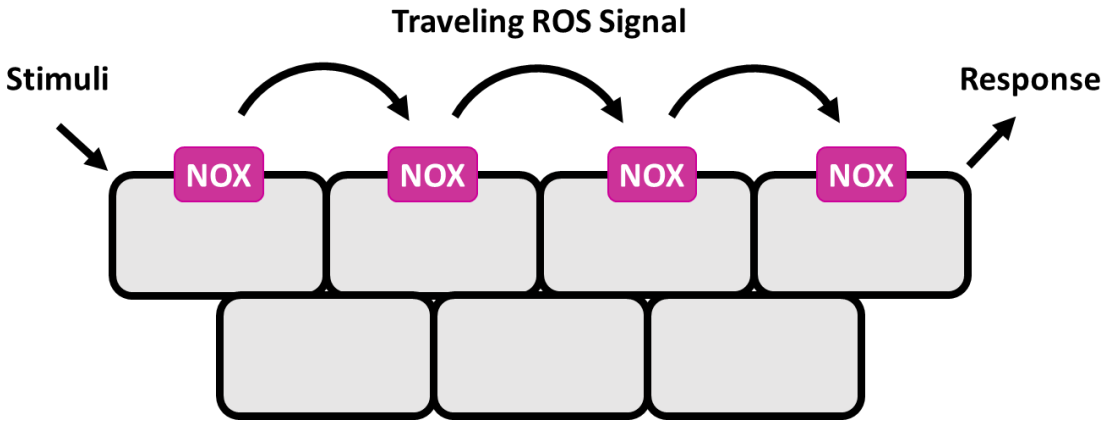

Figure 1.10. Auto-propagating long-range signal transfer over the span of several cells in in *Arabidopsis thaliana*. A rapid systemic ROS signal travels at a rate of 8.4 centimeters per minute to relay a distress message in response to wounds, heat, cold, high-intensity light, and salinity stresses. This distress signal is initiated and maintained by $O_2^{\bullet-}$ and its derivative ROS produced by the plant NADPH Oxidase (NOX). Accumulation of ROS in the extracellular spaces between cells allows for the high-speed travel of this systemic signal. Diagram modified after (Mittler, Vanderauwera et al. 2011).

Finally, as most changes in cellular homeostasis could lead to a change in the steady state of cellular ROS levels, it is conceivable how a link between metabolism and ROS levels can turn ROS into an excellent signaling mechanism for monitoring the changes in cellular metabolism. In fact this relationship between ROS and metabolism is so tightly regulated that disturbed redox homeostasis is considered a hallmark of cancer cells (Panieri and Santoro 2016).

1.3.2. Superoxide radical and ROS signaling

As mentioned before, in animal cells, $O_2^{\bullet-}$ is produced mainly by mitochondrial complexes I and III (Murphy 2009). Some studies that suggest $O_2^{\bullet-}$ functions as part of intracellular signaling machinery are briefly discussed below.

In a study conducted on the activation of inflammasomes, it was shown that inhibition of mitochondrial complexes I and III, the major sources of ROS in mitochondria, significantly hampered the activation of NLRP3 inflammasome which suggested the involvement of $O_2^{\bullet-}$ in this signaling pathway (Zhou, Yazdi et al. 2011). Furthermore, the release of cytokines, which also happens during an inflammation response, is shown to be significantly reduced as a result of treatment with a mitochondrial targeted radical scavenger. As this effect correlates with increased levels of mitochondrial $O_2^{\bullet-}$, it suggests a key role for $O_2^{\bullet-}$ in this signaling pathway (Bulua, Simon et al. 2011). In these two studies, pharmacological changes to the mitochondrial function (especially those that target complexes I and III) or use of antioxidants or radical scavengers show an effect on signaling pathways.

In another study, the connection between $O_2^{\bullet-}$ and signaling was shown by using chemical inhibitors and genetic manipulation of mitochondrial SOD and investigating their effects on the activity of a certain pathway. The authors demonstrated that inhibition of the mitochondrial permeability transition pore, which facilitates the transfer of $O_2^{\bullet-}$ from mitochondria to cytoplasm, prevented the activation of NADPH-oxidase while deficiency of mitochondrial SOD increased NADPH-oxidase activation, suggesting that the signaling agent in this pathway is $O_2^{\bullet-}$ and not H_2O_2 (Swenja, Sebastian et al. 2014).

The notion that $O_2^{\bullet-}$, due to its polarity, cannot participate in signaling since it cannot exit mitochondria, has faded away in recent years due to the emerging evidence. Recent studies demonstrate that, indeed, $O_2^{\bullet-}$ can be released from mitochondria through the mitochondrial permeability transition pore and anion channels (Lustgarten, Bhattacharya et al. 2012, Hou, Ghosh et al. 2014). Additionally, the release of $O_2^{\bullet-}$ from mitochondria has been shown through using $O_2^{\bullet-}$-sensitive spin probes and electron spin resonance spectroscopy (Piskernik, Haindl et al. 2008). Taken together, these data suggest the direct involvement of $O_2^{\bullet-}$ in intracellular signal transduction pathways.

1.3.3. Hydrogen peroxide as a signaling molecule

In contrast to $O_2^{\bullet-}$, H_2O_2 is more stable and its unique chemical properties (see section 1.2.1) and interaction with specific active sites of target proteins make H_2O_2 more suited to act in ROS-dependent signaling pathways (Chen, Thomas et al. 2003). In this section we briefly discuss some evidence that suggest a signaling role for H_2O_2 in vascular endothelial cells.

H_2O_2 has been shown to regulate different aspects of vascular endothelial cell function including growth, proliferation, survival and inflammatory responses (Cai 2005). While a slight increase and strict control over H_2O_2 levels is necessary for vascular homeostasis, excessive ROS production or abnormally low levels of H_2O_2 can negatively impact vascular function (Ramachandran, Levonen et al. 2002). Studies show that in endothelial cells, the growth regulating p90RSK protein (Colavitti, Pani et al. 2002) and early growth factor 1 (Egf1) (Wung, Cheng et al. 1999) are activated by redox-dependent activation of Erk1/2 MAPK by H_2O_2. Additionally, a tight regulation between H_2O_2 and the key angiogenic growth factor VEGF control a variety of angiogenic effects including tube formation, migration and proliferation (Ushio-Fukai, Alexander et al. 2004) and H_2O_2 upregulates VEGF mRNA and protein expression (Chua, Hamdy et al. 1998, Colavitti, Pani et al. 2002). However, VEGF is also capable of activating NADPH oxidase, as an important source of ROS production, in vascular endothelial cells (Griendling Kathy, Sorescu et al. 2000, Abid, Tsai et al. 2001). Furthermore, in a study that investigated the regulation of cell migration, overexpression of a mitochondria-targeted catalase construct suppressed vascular endothelial growth factor (VEGF)-induced cell migration which suggested a role for H_2O_2 in the process (Wang, Zang et al. 2011). Overall, these studies point to the involvement of H_2O_2 as the mediator of ROS signaling.

1.3.4. Spatiotemporal concentration gradients of H_2O_2 in signaling

The local distribution of H_2O_2 concentration differs in tissues, cells and even at the level of different subcellular compartments (Antunes and Cadenas 2000, Marinho, Cyrne et al. 2013, Huang and Sikes 2014). For example, the concentration of H_2O_2 in blood plasma is approximately 1-5 μM which is about 100 times higher than the estimates for the concentration of H_2O_2 in the cells. Most of this H_2O_2 in blood plasma comes from plasma membrane NOXs of phagocytes, endothelial cells and platelets and also from the activity xanthine oxidase released from hepatocytes into the circulation (Forman, Bernardo et al. 2016).

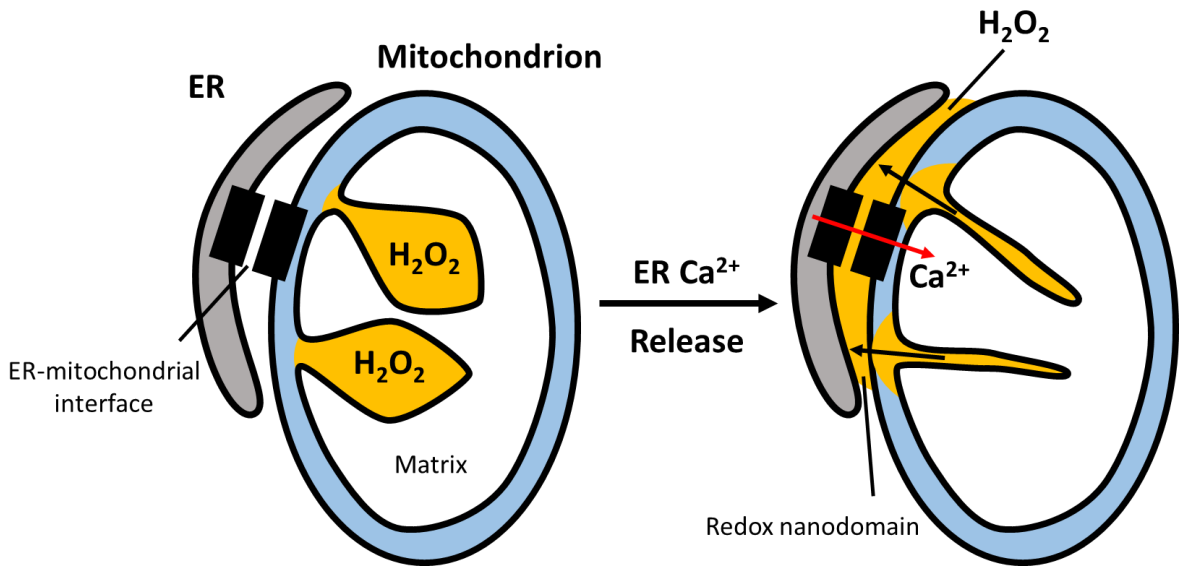

Figure 1.11. Calcium-dependent creation of redox nanodomains at the site of ER-mitochondrial interface. To create redox nanodomains, mitochondrial respiration generates H_2O_2 in the intermembrane/cristae space. Then, Ca^{2+} uptake at the ER-mitochondrial interface induces compression of the dilated cristae to force their volume of H_2O_2 (shoed in yellow) through aligned cristae junctions to the ER-mitochondrial interface, causing a transient elevation of H_2O_2. Diagram modified after (Booth, Enyedi et al. 2016).

There is also a concentration gradient for H_2O_2 within organelles such as mitochondria. While the H_2O_2 in mitochondrial matrix originates from complexes I and II, the origin of most of the H_2O_2 in the intermembrane space is believed to originate from complex III (Bleier, Wittig et al. 2015). This difference in the source of H_2O_2 creates a dynamic H_2O_2 nanodomain at the ER-mitochondrial interface that is induced by and influences Ca^{2+} signals known as a "redox nanodomain". To create these redox nanodomains, mitochondrial respiration generates H_2O_2 in the intermembrane/cristae space. Then, Ca^{2+} uptake at the ER-mitochondrial interface induces compression of the dilated cristae to force their volume of H_2O_2 through aligned cristae junctions to the ER-mitochondrial interface, causing a transient elevation of H_2O_2 (Figure 1.11) (Booth, Enyedi et al. 2016). Additionally, it has been shown that different concentrations of H_2O_2 are maintained in the nucleus and cytosol to allow for specificity in redox signaling (Hansen, Moriarty-Craige et al. 2007).

Although the chemical nature of H_2O_2 allows it to travel freely across biological membranes, in order to create and maintain H_2O_2 concentration gradients, aquaporins have also

been shown to facilitate H_2O_2 translocation (Bienert, Schjoerring et al. 2006, Bienert, Møller et al. 2007).

H_2O_2 levels also demonstrate controlled fluctuations at different stages of an organism's life. For example, studies using a genetically encoded fluorescent sensor (HyPer) and GFP fusion proteins in *C. elegans* show that H_2O_2 levels are generally higher during larval development but decrease during the reproductive stage of the animals and increase again when the worm begins to age (Back, De Vos et al. 2012, Knoefler, Thamsen et al. 2012).

1.3.5. Molecular mechanisms of ROS-dependent signaling

Chemical reactivity of ROS differentiates them from other signaling molecules. While •OH, because of its indiscriminate reactivity towards biological molecules, is not a suitable candidate for a signaling molecule, both superoxide and H_2O_2 have preferred biological targets which sets their target specificity (Halliwell and Gutteridge 2015). Iron-sulfur clusters are the main cellular targets of superoxide due to their high electrostatic attraction, but they are not targeted by the uncharged H_2O_2. As a result of the interaction between superoxide and iron-sulfur clusters, the cluster is oxidized, and iron is released (D'Autreaux and Toledano 2007, Halliwell and Gutteridge 2015). This mechanism of targeting is important in interactions of superoxide with iron-sulfur clusters in proteins such as in the inactivation of mitochondrial aconitase (Figure 1.12) (Gardner, Raineri et al. 1995).

In addition to its direct interactions with biological molecules, superoxide is the precursor to H_2O_2 (produced by SODs) which acts as a major signaling molecule among ROS due to its stable chemistry and high specificity for reaction with active sites of enzymes and proteins that have a cysteine in the thiolate form, and additional features to break an O-O bond (Toppo, Flohe et al. 2009, Forman, Maiorino et al. 2010). A major mechanism of H_2O_2 signaling involves the reversible oxidation and reduction of reactive cysteine residues (known as "redox-sensitive cysteine switches") of certain proteins by H_2O_2. The thiol (sulfur) group of the amino acid cysteine is easily oxidized. Once thiols are oxidized, disulfides form between two thiols in close proximity to one another. Formation of disulfides plays an important role in protein folding and maintaining the structure of proteins, and some thiols act as redox sensitive switches (Figure 1.12) (Klatt and Lamas 2000, Fratelli, Gianazza et al. 2004, Hisabori, Hara et al. 2005). Although thiols such as cysteine show high affinity for reaction with H_2O_2, their reaction with $O_2^{\bullet-}$ is relatively slow and

cannot compete with SODs. Therefore, cysteine oxidation by $O_2^{\bullet-}$ might be physiologically significant only where SODs are not present such as in compartments like phagosomes or endosomes (Cardey and Enescu 2009, Winterbourn 2016).

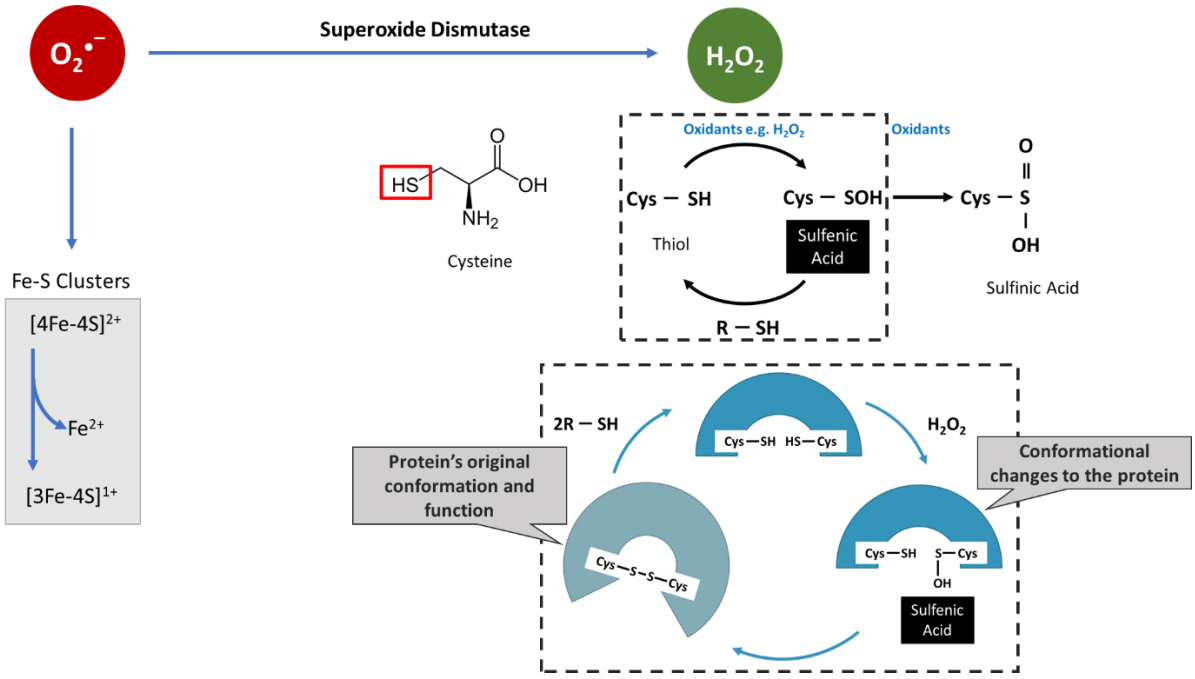

Figure 1.12. Molecular mechanisms of ROS signaling. (Left) Iron-sulfur clusters are the main cellular targets of superoxide due to their high electrostatic attraction. As a result of the interaction between superoxide and iron-sulfur clusters, the cluster is oxidized, and iron is released. (Right) A major mechanism of H_2O_2 signaling involves the reversible oxidation and reduction of reactive cysteine residues (known as "redox-sensitive cysteine switches") of certain proteins by H_2O_2. Oxidants and in particular H_2O_2 modify specific reactive cysteine residues within proteins to sulfenic acids (R-SOH), this is followed by a corresponding change in protein function. This modification, which has distinct effects on functional properties of proteins (e.g.enzymes), is used by cells to activate or inactivate protein functions due to changes in redox states.

When redox-sensitive switches change from a thiol state to disulfide, the shape and function of the protein also changes. The redox state of the cellular compartment where the protein resides (or the redox system that the protein belongs to) determines the tendency of the redox-sensitive switch to be in one state or another (thiol or disulfide). Therefore, changes in the redox state of the immediate environment of a large number of proteins can change their form and function (Jones 2008). This effect is exploited by the living organisms to activate or inactivate

protein functions due to changes in redox states e.g. in different cellular compartments or after their transport from one organelle to another (Figure 1.12) (Hansen, Watson et al. 2004).

Maintaining a narrow range for the redox state of an organelle or cellular compartment is necessary to establish a meaningful threshold. In this way, a change in the global redox state will signal environmental and metabolic changes or stress and can provoke an appropriate response. However, production of H_2O_2 in a cell or cellular compartment can induce conformational changes in proteins in close proximity to the site of generation to indicate local changes and/or initiate a signaling cascade, as well (Dwivedi and Kemp 2012). A good example of this is the oxidation of the catalytic cysteine residue of protein tyrosine phosphatases (PTPs) by H_2O_2 which inhibits the activity of this family of enzymes (Chiarugi and Cirri 2003, Holmstrom and Finkel 2014).

1.3.6. ROS-induced ROS release

The phenomenon where the production or release of ROS by one cellular compartment or organelle triggers generation or release of ROS by another compartment or organelle is referred to as ROS-induced ROS release (RIRR) (Zorov, Filburn et al. 2000, Lee, Bae et al. 2006, Zorov, Juhaszova et al. 2014, Daiber, Di Lisa et al. 2017, Kim, Kim et al. 2017). The concept of RIRR first emerged through studies conducted on cardiomyocytes where thousands of electrically independent mitochondria were found to function in a "grid" and changes in $\Delta\Psi m$, calcium and ROS at a subset of mitochondria within the grid was able to alter ROS production in neighboring or remote mitochondria within that grid (Zorov, Filburn et al. 2000). These early studies and subsequent research showed that in addition to producing low levels of ROS, mitochondria are able to respond to exogenous and endogenous ROS levels by increasing their own ROS generation. It was theorized that due to accumulation of endogenous ROS via normal respiration or perhaps because of certain dysfunctional processes, a collapse of mitochondrial $\Delta\Psi m$ is followed by opening a mitochondrial pore that would release ROS from the mitochondria into the cytosol and therefore act as a natural "safety valve" to prevent ROS from reaching dangerously high levels inside mitochondria. This process occurs naturally and results in a "flickering" phenotype in which mitochondria occasionally open their pores and release ROS and calcium into the cytosol. However, the researchers later found that through stress or pathology, this natural safety measure could irreversibly turn into a hazardous mechanism that increased cytosolic ROS and calcium levels so high as to trigger autophagy or apoptosis (Figure 1.13) (Aon, Cortassa et al. 2003, Brady,

Elmore et al. 2004, Cortassa, Aon et al. 2004, Brady, Hamacher-Brady et al. 2006, Nivala, Korge et al. 2011, Zorov, Juhaszova et al. 2014).

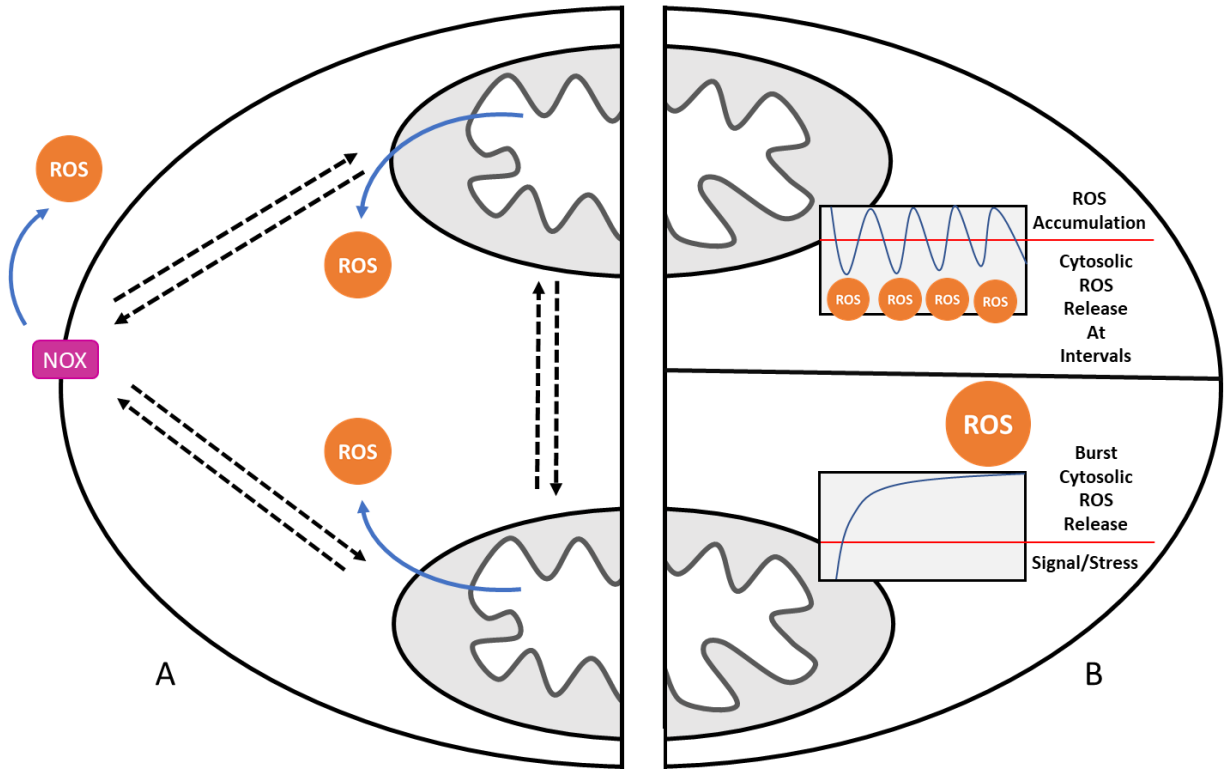

Figure 1.13. ROS induced ROS release (RIRR). (A) Potential communication between mitochondria and NOXs using RIRR machinery. Mitochondrial ROS release can induce ROS production by NOXs and vice versa. (B) ROS release from mitochondria during normal metabolism (Top) and in response to stress or signal (Bottom). As ROS accumulates within mitochondria, it is released to the cytoplasm at regular intervals through the mitochondrial permeability transition pore (mPTP). However, during stress or in response to various signals the mPTP may irreversibly open and release huge amounts of ROS into the cytosol. This burst of cytosolic ROS might signal apoptosis or other physiological events. Diagram based on (Zandalinas and Mittler 2017).

Nevertheless, a new paradigm has emerged from the discovery of RIRR that demonstrates the importance of this process in modulating signaling pathways in addition to its potentially hazardous impact in a range of pathologies (Kimura, Zhang et al. 2005, Zinkevich and Gutterman 2011). One important aspect of RIRR signaling is redox communication between NADPH-oxidases (NOXs) and mitochondria (Zinkevich and Gutterman 2011). In animal cells, different members of the NOX family of enzymes can be found at the plasma membrane, ER, mitochondrial membrane and nuclear membrane (Sirokmány, Donkó et al. 2016). In fact, NOXs act as a link

between various signal transduction pathways and the process of RIRR in a way that various signaling events could trigger mitochondrial ROS release and also mitochondrial ROS release can initiate a wide range of other processes. Therefore, the current concept of RIRR is supported by evidence that encompasses mitochondria-to-mitochondria, mitochondria-to-NOX, NOX-to-NOX and mitochondria-to-NOX communication (Figure 1.13) (Graham, Kulawiec et al. 2010, Brandes, Weissmann et al. 2014, Sahoo, Meijles et al. 2016, Singel and Segal 2016, Wenzel, Kossmann et al. 2017).

An example of the action of RIRR in animal cells is shown in a study by Kim *et al*. This study shows that in human umbilical vein endothelial cells, Nox2 senses Nox4-drived H_2O_2 (both NOXs localize to ER) to induce mitochondrial ROS production via p66Shc, a key mitochondrial ROS regulator. Increased mitochondrial ROS production, in turn, promotes sustained activation of vascular endothelial growth receptor type 2 (VEGFR2) activation enhancing angiogenesis responses. This study represents a ROS induced ROS release mechanism where ROS-mediated NOX-to-NOX and NOX-to-mitochondria communication allows for ER-mitochondria communication and activation of a specific pathway (Kim, Kim et al. 2017).

1.3.7. Maintaining the balance of the ROS network by ROS-handling enzymes

Organisms have a number of enzymes that mediate ROS homeostasis and redox signaling such as SODs for conversion of $O_2^{\bullet-}$ into H_2O_2, and catalases (CTLs), peroxiredoxins (PRDXs) and glutathione peroxidases (GPXs) for catabolizing H_2O_2 (see section 1.4 for an overview of these enzymes with a focus on the ROS network of *C. elegans*). Therefore, in the ROS network, the fine balance that exists between ROS production for signaling and baseline physiological ROS levels through ROS removal and preservation is accomplished by these enzymatic components (Mittler, Vanderauwera et al. 2004).

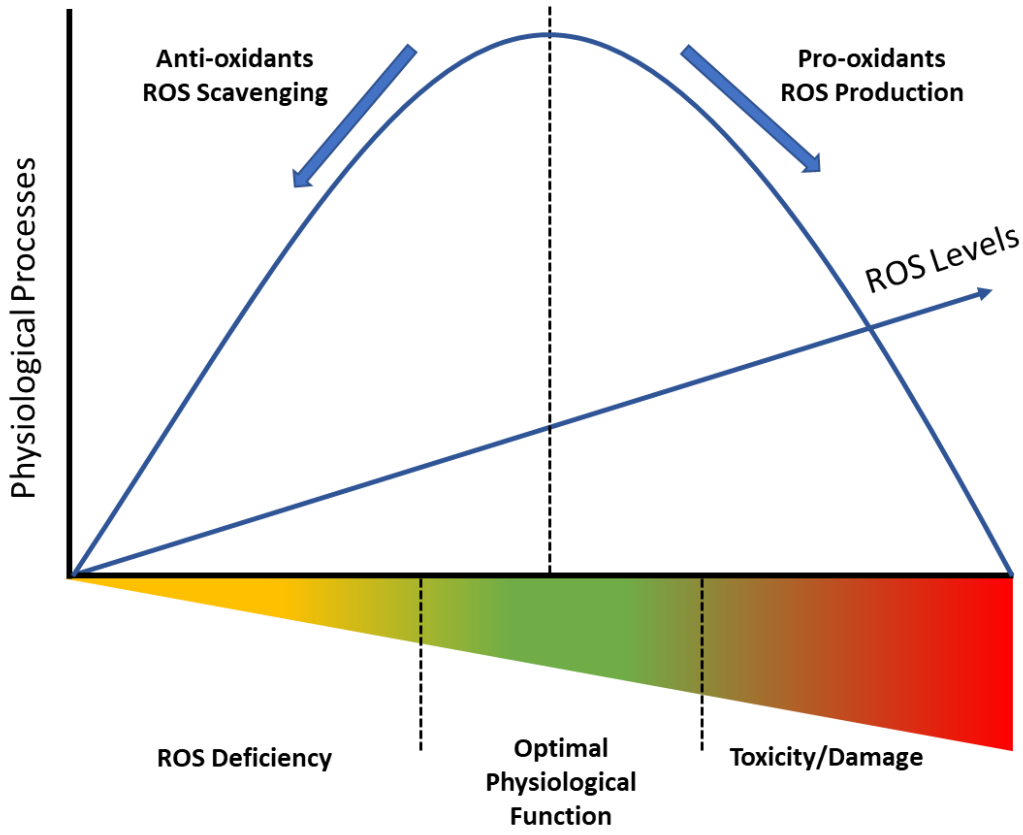

Figure 1.14. Maintaining the balance of ROS network for optimal physiological function of the organism. Fine-tuning and maintenance of optimal ROS levels by a set of redox active enzymes and other means of manipulating ROS levels is necessary for normal cellular processes; not because ROS have a toxic effect on life, but because they, in fact, promote the natural health and function of the cells. Diagram modified after (Desjardins, Cacho-Valadez et al. 2017, Mittler 2017).

The drastic effects of manipulating the ROS-handling enzymes are particularly well-demonstrated when in the genetic studies that involve knockouts of redox active enzymes where important aspects of the organism such as its lifespan is altered. For example, in long-lived *daf-2* mutants of *C. elegans*, loss of SOD-4 (the animal's extracellular SOD) enhances average lifespan by 12% (Doonan, McElwee et al. 2008). Also, in *C. elegans* wild-type animals removal of a major mitochondrial SOD (SOD-2) results in a dramatic lifespan increase, enhancing average lifespan by 35% and maximum lifespan by 58% (Van Raamsdonk and Hekimi 2009) while removal of a peroxiredoxin (PRDX-2) reduces average lifespan by 19% (Oláhová, Taylor et al. 2008).

Another interesting example that demonstrates how ROS homeostasis can affect normal functions of the cells is through the studies which show that cancer cells need to constantly maintain higher than normal levels of ROS to continue proliferation (Diebold and Chandel 2016).

It is shown that these cancer cells maintain ROS levels above cytostatic levels but below cytotoxic levels. This is achieved through sustaining a delicate balance between ROS production by mitochondria and NADPH oxidases and ROS scavenging by SODs, GPXs and PRDXs alongside active repair mechanisms (Poillet-Perez, Despouy et al. 2015).

Overall, studies in various areas support the view that ROS-dependent fine-tuning and maintenance by this set of redox active enzymes is necessary for normal cellular processes; not because ROS have a toxic effect on life, but because they, in fact, promote the natural health and function of the cells. Even views that once considered cell death to be a product of oxidative damage are now replaced by models indicating that ROS trigger a signaling machinery that ends in cell death (Berghe, Linkermann et al. 2014, Conrad, Angeli et al. 2016, Xie, Hou et al. 2016).

Moreover, the studies which indicate that decreasing ROS levels below a certain threshold can negatively affect different aspects of cellular proliferation, differentiation and immunity (Owusu-Ansah and Banerjee 2009, Tormos, Anso et al. 2011, Maryanovich, Oberkovitz et al. 2012, Sart, Song et al. 2015) make a strong case that a "basal level" of ROS below cytotoxic levels is required to allow normal redox biology reactions and the related essential processes for life. This dependency of normal cellular functions on maintaining ROS levels, made possible by ROS-handling enzymes, can be depicted as an inverted U-shaped curve response in which the optimum ROS levels depend on the cell types, environmental conditions, developmental stage and other factors that affect the organism (Figure 1.14) (Van Raamsdonk and Hekimi 2012, Desjardins, Cacho-Valadez et al. 2017, Mittler 2017).

In the next section (1.4), we will discuss the important components of the ROS network of *C. elegans* such as the ROS-handling enzymes of the worm, their sites of action and the movements of ROS within cells. We will also review the phenotypes associated with the loss of these enzymes in the worm such as changes in lifespan. However, more detailed information about lifespan in connection with ROS and mitochondrial long-lived mutants of *C. elegans* is reviewed in section 1.5.

1.4. ROS-handling enzymes of *C. elegans*

C. elegans produces several families of ROS handling enzymes that participate in the detoxification of excessive ROS and/or mediating ROS signals. One group of these enzymes is the SODs. They are responsible for converting superoxide to oxygen and H_2O_2 (McCord and

Fridovich 1969). In *C. elegans*, there are five distinct *sod* genes that code for different isoforms of SODs which reside in different subcellular compartments. There are two cytosolic Cu/ZnSODs (SOD-1 and SOD-5), two mitochondrial MnSODs (SOD-2 and SOD-3) and one extracellular Cu/ZnSOD (SOD-4) (Larsen 1993, Giglio, Hunter et al. 1994, Giglio, Hunter et al. 1994, Suzuki, Inokuma et al. 1996, Fujii, Ishii et al. 1998).

The major sinks for H_2O_2 in *C. elegans* are catalases (CTLs), peroxiredoxins (PRDXs) and glutathione peroxidases (GPXs). Three catalase enzymes have been identified in the worm. *ctl-1*, *ctl-2* and *ctl-3* have very similar sequences. *ctl-2* encodes the peroxisomal catalase (CTL-2). *ctl-1* expresses a cytosolic catalase and *ctl-3* encodes another cytosolic member of the family which appears to be expressed only in the pharyngeal muscle cells and cell bodies of neurons but it is not well-characterized (Togo, Maebuchi et al. 2000, Kamath, Fraser et al. 2003, Petriv and Rachubinski 2004, Gems and Doonan 2009).

C. elegans encodes three PRDXs: *prdx-2, prdx-3* and *prdx-6*. Of these, only *prdx-2* is moderately characterized as a typical 2-Cys peroxiredoxin which contains two catalytic cysteine residues (Oláhová, Taylor et al. 2008).

The nematode has eight known genes that encode for GPX enzymes. Studying the expression patterns of *gpx-1, gpx-2, gpx-6* and *gpx-7* through GPX::GFP fusion proteins under the control of their endogenous promoters has shown that they are primarily expressed in the intestine (Sakamoto, Maebayashi et al. 2014). A summary biological functions, phenotypes and subcellular/tissue specific expression of some of the worm's ROS-handling enzymes is presented in Table 1.2 and Figure 1.15 presents the important elements of ROS network in *C. elegans*.

1.4.1. Superoxide dismutases

1.4.1.1. Biology and localization of SODs

Of the five SOD enzymes produced by *C. elegans*, SOD-1 accounts for approximately 80% of total SOD activity. It is ubiquitously expressed throughout the tissues and is considered the major cytosolic Cu/ZnSOD of the worm. Although loss of SOD-1 has been found to reduce lifespan, this effect has been determined not to be connected with accelerating the age-dependent increase in molecular damage to protein and lipid (Doonan, McElwee et al. 2008).

SOD-2 is the major mitochondrial MnSOD. It is embedded within the mitochondrial supercomplex I:III:IV (similar to SOD-3 where/when it is expressed) and its loss extends the

lifespan of the wild-type animals and some mutant strains with a complex I defect (Doonan, McElwee et al. 2008, Van Raamsdonk and Hekimi 2009, Suthammarak, Somerlot et al. 2013).

Table 1.2. Important genes encoding redox active enzymes in *C. elegans*.

Gene	Product	Subcellular/Tissue localization	Effect of Knockout on WT Lifespan	Other Information
sod-1	Major Cu/ZnSOD	Cytosolic, Mitochondrial intermembrane space	Decreased/None	Overexpression extends lifespan via IIS
sod-2	Major MnSOD	Mitochondrial supercomplex I:III:IV	Increased	
sod-3	Inducible MnSOD	Mitochondrial supercomplex I:III:IV	None	mRNA levels increased in dauer larvae and *daf-2* background
sod-4	Cu/ZnSOD	Extracellular, Membrane-bound	None	Deletion in *daf-2* background enhances longevity
sod-5	Inducible Cu/ZnSOD	Cytosolic	None	Expression in L3 larvae limited to some neurons
ctl-1	Catalase	Cytosolic	None	
ctl-2	Catalase	Peroxisomal	Decreased	Deletion in *clk-1* background causes accelerated development
prdx-2	2-Cys Proxiredoxin	2 types of pharyngeal neurons	None	Low brood size Slow development
gpx-x	Glutathione peroxidase	Intestine	None	Quadruple mutant short-lived *gpx-6* required for *eat-2* longevity

SOD-3 is another mitochondrial MnSOD isoform of the worm. The amino acid sequence of SOD-2 and SOD-3 is very similar (Hunter, Bannister et al. 1997, Henderson, Bonafè et al. 2006, Doonan, McElwee et al. 2008, Honda, Tanaka et al. 2008). However, despite their structural similarity and colocalization at the site of mitochondrial supercomplex I:III:IV (Suthammarak, Somerlot et al. 2013), SOD-2 and SOD-3 might not be expressed in the same cells, tissues or even in the same mitochondria (Henderson, Bonafè et al. 2006, Doonan, McElwee et al. 2008, Honda, Tanaka et al. 2008).

Although *sod-3* mRNA levels are elevated in dauer larvae and *daf-2* long-lived mutants (Honda and Honda 1999, Dong, Venable et al. 2007), its relative contribution to SOD levels

regarding overall SOD activity and MnSOD protein levels is very small (Doonan, McElwee et al. 2008).

SOD-5, similar to SOD-1, is a Cu/ZnSOD that localizes to cytoplasm. The expression of *sod-5::gfp* in L3 larvae has been found to be limited to the ASI, ASK, and ASG amphid neurons but and loss of SOD-5 alone or in combination with SOD-1 does not appear to affect lifespan or elicit other discernable phenotypes (Jensen and Culotta 2005, Doonan, McElwee et al. 2008).

Under normal laboratory conditions in adult worms, *sod-3* and *sod-5* are not expressed or their expression levels are very low. However, they are mainly expressed in dauer larvae and during larval development (Honda and Honda 1999, Essers, de Vries-Smits et al. 2005, Jensen and Culotta 2005, Doonan, McElwee et al. 2008, Yanase and Ishii 2008, Zhi, Feng et al. 2014). Nevertheless, both *sod-3* and *sod-5* are inducible by a variety of factors. For example, the introduction of mitochondrial mutations that increase mitochondrial ROS production and treatment with the pro-oxidant paraquat have been shown to induce *sod-3* and *sod-5* expression (Feng, Bussière et al. 2001, Wolf, Nunes et al. 2008, Dingley, Polyak et al. 2010, González-Cabo, Bolinches-Amorós et al. 2011, Erkut, Vasilj et al. 2013, Suetomi, Mereiter et al. 2013, Song, Zhang et al. 2014, Oh, Park et al. 2015, Rathor, Akhoon et al. 2015, Wu, Huang et al. 2016).

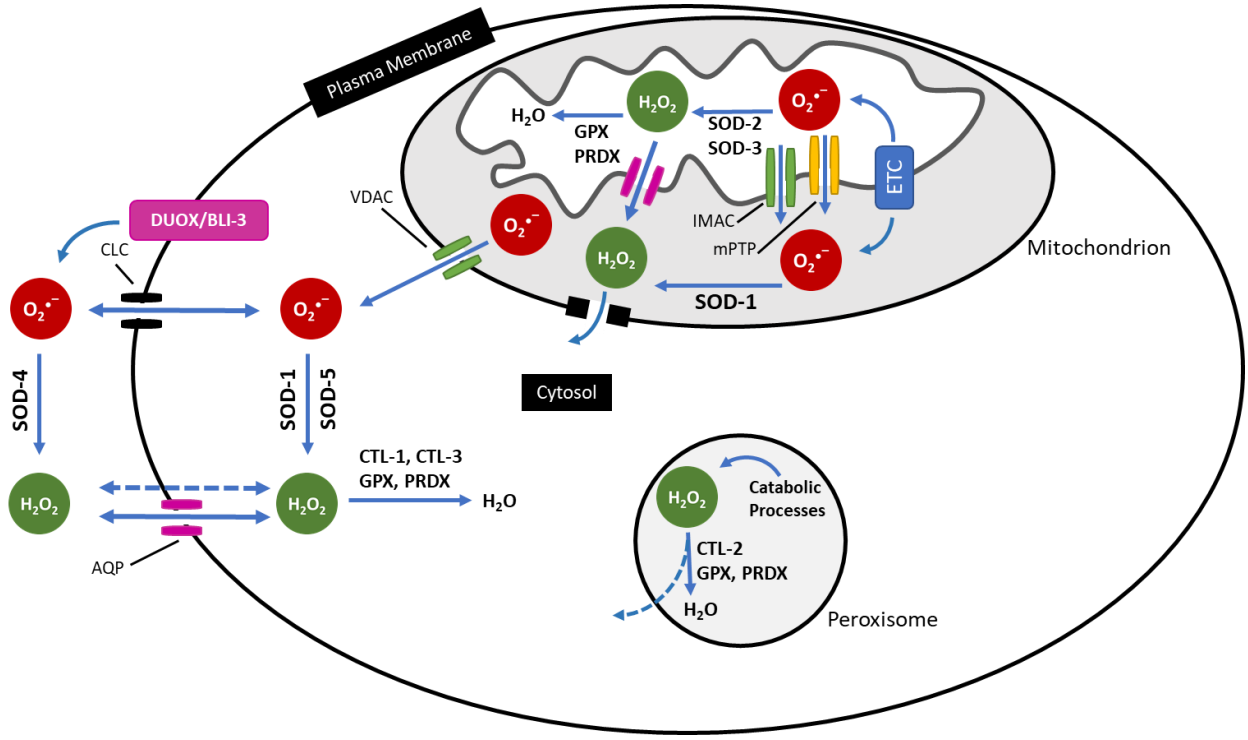

Figure 1.15. An overview of the important elements of ROS network in *C. elegans*. ROS are produced at different sites within the cell and in the extracellular space. Mitochondrial ETC and NADPH Oxidase (DUOX/BLI-3) are notable producers of $O_2^{\bullet-}$. The mitochondrial ETC produces $O_2^{\bullet-}$ inside the mitochondrial matrix and into the mitochondrial intermembrane space. Within the matrix, MnSODs (SOD-2 and SOD-3) convert $O_2^{\bullet-}$ into H_2O_2 which is either further reduced by peroxiredoxins (PRDXs) and glutathione peroxidases (GPXs) or translocates to the intermembrane space. The $O_2^{\bullet-}$ that is directly produced into the intermembrane space or is translocated there from the matrix via inner membrane anion channels (IMAC) and/or mitochondrial permeability transition pores (mPTP) either exits the mitochondria via voltage-dependent anion channels (VDAC) or is used by Cu/ZnSOD (SOD-1) to produce H_2O_2. Part of the H_2O_2 within the intermembrane space escapes to the cytoplasm where it is catabolized by various enzymes including CTL-1 and CTL-3. H_2O_2 is also produced in peroxisomes as a result of the ongoing catabolic processes. The portion of H_2O_2 that does not translocate from peroxisomes into the cytosol is further reduced by a whole host of enzymes including CTL-2, PRDXs and GPXs. Cytosolic Cu/ZnSODs (SOD-1 and SOD-5) convert $O_2^{\bullet-}$ into H_2O_2 within the cytosol. SOD-4, another Cu/ZnSOD isoform, converts extracellular $O_2^{\bullet-}$ into H_2O_2. Both $O_2^{\bullet-}$ and H_2O_2 are able to travel between cytosol and extracellular space using chloride channels (CLC) and aquaporins (AQP) respectively. Diagram modified after (Wang, Branicky et al. 2018).

The *sod-4* gene is predicted to encode two extracellular Cu/ZnSOD isoforms which are the products of alternative splicing of mRNA (Fujii, Ishii et al. 1998). Loss of *sod-4* has been reported to enhance *daf-2* longevity and Daf-c phenotype through possible ROS-signaling activity (Doonan,

McElwee et al. 2008). However, in the wild-type background, removal of *sod-4* has not been reported to affect sensitivity to oxidative stress or lifespan (Doonan, McElwee et al. 2008, Van Raamsdonk and Hekimi 2009, Schaar, Dues et al. 2015).

1.4.1.2. Loss of SODs, ROS sensitivity and lifespan

Because of the proposed connection between ROS and aging put forth by the oxidative theory of aging, many studies have investigated the effects of loss of SODs on ROS sensitivity and lifespan in *C. elegans.* Loss of *sod-1* and *sod*-2, but not the other *sod* genes, leads to significant hypersensitivity to oxidative stress following treatment with pro-oxidants such as paraquat and juglone (Doonan, McElwee et al. 2008, Yanase, Onodera et al. 2009, Van Raamsdonk and Hekimi 2012). However, this effect has been shown to be independent of lifespan (Schaar, Dues et al. 2015, Dues, Schaar et al. 2017). In fact, overexpression of *sod-1* can cause both ROS hypersensitivity and increased longevity at the same time. The increased longevity effect of *sod-1* overexpression, however, is not due to increased $O_2^{\bullet-}$ detoxification or increased H_2O_2 levels. It has been suggested that overexpression of *sod-1* may increase the levels of SOD-1 protein so high as to overwhelm the cellular protein folding machinery and as a consequence inducing an unfolded protein response that enhances longevity (Doonan, McElwee et al. 2008, Cabreiro, Ackerman et al. 2011). Moreover, although *sod-2* mutants are hypersensitive to oxidative stress, they are long lived (Van Raamsdonk and Hekimi 2009, Dingley, Polyak et al. 2010).

Furthermore, Van Raamsdonk *et al.* have demonstrated that SOD is dispensable for normal lifespan in *C. elegans* but a quintuple mutant that lacks SOD activity exhibits slower physiologic rates such as slower defecation cycle and decreased movement. Also, *sod* quintuple mutants are hypersensitive to superoxide mediated oxidative stress during both developmental period and adulthood but their sensitivity to H_2O_2 is not different from that of the wild-type animals which is consistent with the function of SODs in $O_2^{\bullet-}$ detoxification. Furthermore, *sod-12345* worms show increased sensitivity to osmotic stress, cold stress, and heat stress. (Van Raamsdonk and Hekimi 2012).

1.4.1.3. Role of SODs in ROS-dependent signaling

The natural and rather unique role of SODs among ROS-handling enzymes as both a consumer and a producer of different forms of ROS makes them especially suited to function as mediators

in ROS signaling networks. In the context of aging, for example, loss of *sod-4* has been proposed to modulate IIS in *daf-2* background (Doonan, McElwee et al. 2008). Similarly, current evidence points to the role of *sod-3* as another regulator of longevity in the *daf-2(lf)* background (Honda, Tanaka et al. 2008). Furthermore, during the development of *C. elegans,* in a RAS gain-of -function mutant background, down-regulation of *sod-1* or *sod-4* via RNAi treatment is shown to down-regulate the RAS signaling pathway responsible for vulval development via increasing $O_2^{\bullet-}$ levels or diminished H_2O_2 production (Shibata, Branicky et al. 2003). Another example of the involvement of SODs in ROS signaling is the specific role that SOD-1 plays to mediate the worm's avoidance behavior in response to pathogen-induced ROS generation (Horspool and Chang 2017).

1.4.2. Catalases

Catalase was first characterized in bovine liver as a tetrameric enzyme with one heme group per subunit (Sumner and Gralén 1938, Sund, Weber et al. 1967). Heme-containing catalases have been found in a wide range of organisms from bacteria to humans. They convert two molecules of H_2O_2 into two molecules of water and one molecule of oxygen in a two-step catalytic mechanism (Deisseroth and Dounce 1970, von Ossowski, Hausner et al. 1993). Many heme catalases bind NADPH but H_2O_2 is the source of both oxidative and reductive potential during their normal catalytic cycle (Fita and Rossmann 1985); however, low intracellular concentration of NADPH has been shown to cause low catalase activity (Hoffschir, Daya–Grosjean et al. 1998, Kirkman, Rolfo et al. 1999).

1.4.2.1. Biology and localization of CTLs

C. elegans has three catalase genes with very similar sequences. *ctl-2* encodes a peroxisomal catalase (CTL-2). *ctl-1* expresses a cytosolic catalase and *ctl-3* encodes another cytosolic catalase which appears to be expressed only in the pharyngeal muscle cells and cell bodies of neurons. CTL-3 is not well characterized but 2D DIGE analysis of proteomes of L3 and dauer larvae shows that CTL-3 is highly upregulated during the dauer state but is not expressed in L3 larvae (Gems and Doonan 2009, Erkut, Vasilj et al. 2013).

1.4.2.2. Loss of CTLs and mutant phenotypes

In *C. elegans*, deletion of *ctl-1* has not been reported to result in any discernible phenotype and the mutants have a normal lifespan. However, *ctl-2* mutants have been reported to have a shorter lifespan compared to the wild-type (Petriv and Rachubinski 2004). However, in the long-lived *clk-1* mutant background that develop more slowly than the wild-type, while removal of either *ctl-1* or *ctl-2* does not affect longevity, loss of *ctl-2* has been reported to cause accelerated development (Petriv and Rachubinski 2004). Interestingly, both *ctl-1* and *ctl-2* knockout mutants demonstrate decreased rate of oxidative damage accumulation compared to the wild-type as shown by the slower increase in protein carbonyl levels (Petriv and Rachubinski 2004).

1.4.2.3. Effects of increased catalase activity

While overexpression of the entire *C. elegans* gene cluster (i.e. *ctl-1*, *ctl-2*, and *ctl-3*) can shorten the survival of the animals, it has been shown that this effect is not due to an increase in oxidative damage but instead is mainly caused by increased internal hatching of the larvae. However, overexpression of *sod-1* suppresses the internal hatching phenotype, suggesting that maybe it is caused by H_2O_2 deficiency. Also, when internal hatching of the larvae is prevented through the use of the DNA replication inhibitor fluorodeoxyuridine, FUdR, overexpression of catalase gene cluster either alone or in addition to overexpression of *sod-1* still slightly shortens life span of the animals (Doonan, McElwee et al. 2008).

1.4.3. Peroxiredoxins

1.4.3.1. An overview of the biology and general classification of peroxiredoxins

Peroxiredoxins constitute a highly conserved family of peroxidase enzymes that possess a conserved "proxidactic" cysteine residue (C_P) which participates in the reduction of peroxides. Peroxides oxidize this C_p-SH residue to produce cysteine sulfenic acid (C_P-SOH). The C_P-SOH then reacts with a "resolving" cysteine residue (C_R) to form a disulfide which is eventually reduced by an electron donor to conclude a complete catalytic cycle (Hall, Nelson et al. 2011, Rhee, Woo et al. 2012).

All peroxiredoxin enzymes possess the conserved C_P residue. However, based on the location or the absence of the C_R residue, peroxiredoxins are categorized into three subfamilies: 2-

Cys, atypical 2-Cys, and 1-Cys PRDXs (Chae, Robison et al. 1994, Rhee, Kang et al. 2001, Wood, Schröder et al. 2003). The homodimeric 2-Cys PRDXs have two conserved C_P and C_R cysteine residues per subunit. In these PRDXs, the C_P-SOH of one subunit reacts with the C_R-SH of the other subunit resulting in the formation of an inter-subunit disulfide bond. In atypical 2-Cys PRDXs the C_P-SOH of a subunit reacts with the C_R-SH of the same subunit to form an intra-subunit disulfide bond. In contrast to these two types of 2-Cys PRDXs, 1-Cys PRDXs, which lack the C_R residue, form a disulfide bond with the C_R-SH from other proteins or small thiol molecules (Figure 1.16) (Fisher 2011).

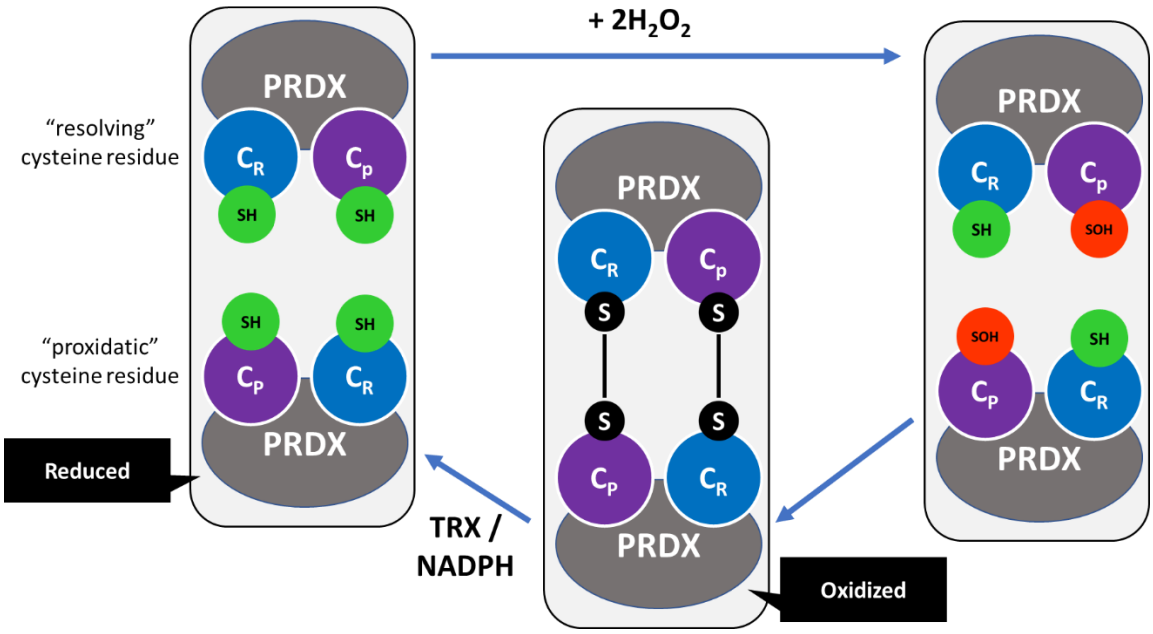

Figure 1.16. The catalytic cycle of a homodimeric 2-cys peroxyredoxin (PRDX). The homodimeric 2-Cys PRDXs have two conserved C_P and C_R cysteine residues per subunit. In these PRDXs, the C_P-SOH of one subunit reacts with the C_R-SH of the other subunit resulting in the formation of an inter-subunit disulfide bond.

1.4.3.2. The role of peroxiredoxins in ROS-dependent signaling in various organisms

As antioxidants, peroxiredoxins indeed play their role in protecting cells against the oxidative damage inflicted by excessive amounts of H_2O_2 and organic hydroperoxides (Fisher and signaling 2011, Knoops, Goemaere et al. 2011). However, the fact that multiple enzymes such as catalases and glutathione peroxidases also exist within a single cell or subcellular compartment to remove peroxides is an indication that PRDXs do not function only in antioxidant defense system of the

organism. Unfortunately, data on the role of PRDXs in ROS signaling in *C. elegans* is not available. Therefore, in order to demonstrate the role of PRDXs in ROS-dependent signaling, we will use examples from other model systems in the following paragraphs.

A good example of the complexity of the functions undertaken by PRDXs is demonstrated in the photosynthesizing chloroplast where multiple peroxiredoxins and glutathione peroxidases use different electron donors and are plugged into different redox networks to mediate various aspects of ROS signaling in these organelles (Dietz and cells 2016).

In mammalian cells, PRDXs function as key regulators of local H_2O_2 concentration. The H_2O_2 produced by the cells activates intracellular signaling by oxidizing thiols in various proteins such as protein tyrosine phosphates (PTPs) (Lee, Kwon et al. 1998, Rhee, Chae et al. 2005). In this context, because the thiol groups of these target proteins react far more slowly with H_2O_2 when compared with the C_P residue of PRDXs (Winterbourn 2013), the PRDX molecules in the vicinity need to be temporarily inactivated to allow for H_2O_2 to react with its target in the neighboring proteins. In this way, highly active PRDXs are temporarily "switched off" to permit higher levels of H_2O_2 in their immediate area so that the ROS signal can be transmitted to target proteins. A good example of this localized regulation of H_2O_2 concentration is shown when a centrosome-associated peroxidase is transiently inactivated through phosphorylation at Thr^{90} during early mitosis (Lim, Lee et al. 2015).

However, PRDXs can also directly mediate H_2O_2-dependent oxidation of redox-regulated proteins. In this case, the C_P of the PRDX in sulfenic (C_p-SOH) or disulfide (C_p-S-S-C_R) states, forms an intermediate inter-molecular bond with the target protein. Following the resolution of this disulfide bond through reaction with another Cys-SH of the target protein, PRDX is reduced and the function of the target protein is altered (Latimer, Veal et al. 2016, Netto, Antunes et al. 2016, Toledano, Huang et al. 2016). Yeast transcription factor PAP (Vivancos, Castillo et al. 2005) and mammalian transcription factors STAT3 (Sobotta, Liou et al. 2015) and ASK1 (Nadeau, Charette et al. 2007) are examples of H_2O_2-targeted proteins whose activation is mediated by PRDXs.

1.4.3.3. The significance of peroxiredoxin hyperoxidation in ROS-dependent signaling

Hyperoxidation of 2-Cys PRDXs occurs when the C_P-SOH is further oxidized to C_P-SO_2H before the disulfide bond is formed. This process inactivates the peroxidase ability of the enzyme (Yang,

Kang et al. 2002). Sulfiredoxin, an ATP-dependent enzyme, can reverse this hyperoxidation and restore the peroxidase ability of the PRDX (Biteau, Labarre et al. 2003, Woo, Kang et al. 2003). One of the proposed functions for this reversible inactivation of PRDXs through hyperoxidation is to allow for H_2O_2 levels to increase significantly under specific conditions to promote ROS signaling (Wood, Poole et al. 2003). For example, Prx III, a mitochondrial PRDX in the cortex of the adrenal gland of mice, is inactivated following its hyperoxidation by H_2O_2 generated in the process of the conversion of cholesterol into corticosterone. This inactivation results in an elevation in H_2O_2 levels which triggers a series of events that lead to the inhibition of steroidogenesis (Kil, Lee et al. 2012).

1.4.3.4. Peroxiredoxins and mutant phenotypes in *C. elegans*

In *C. elegans*, there are three known PRDX genes; *prdx-2, prdx-3* and *prdx-6* (Van Raamsdonk, Hekimi et al. 2010, Back, Braeckman et al. 2012). The mitochondrial peroxiredoxin of the worm is encoded by *prdx-3* and the cytosolic form is encoded by *prdx-2*; both of which are 2-Cys PRDXs. *prdx-6*, the worm's 1-Cys PRDX, is not well-characterized but based on its amino acid sequence and protein domain information, it is predicted to be cytosolic and an ortholog of human PRDX6 (Back, Braeckman et al. 2012).

RNAi knockdown worms of *prdx-3* exhibit decreased total energy availability, lower motility and diminished brood size but do not exhibit either increased levels of oxidized protein or a change in lifespan. Moreover, *prdx*-3-knockdown animals show increased oxygen consumption accompanied by reduced levels of ATP which is a hallmark of mitochondrial uncoupling.(Ranjan, Gruber et al. 2013).

Studies that investigated knockout mutants of the worm's cytosolic PRDX (*prdx-2*) report no change in lifespan. However, *prdx-2* mutants exhibit low brood size and slow development (Isermann, Liebau et al. 2004, Oláhová, Taylor et al. 2008, Kumsta, Thamsen et al. 2011). The expression of *prdx-2* has been shown to be specific to two types of pharyngeal neurons: the single pharyngeal interneuron I4 and the sensory Interneuron I2 where it is likely involved in H_2O_2-signaling (Isermann, Liebau et al. 2004). Finally, it has also been shown that PRDX-2 is required for normal levels of insulin secretion and inhibition of DAF-16 and SKN-1 through insulin/IGF-1-like signaling under nutrient-rich conditions (Oláhová and Veal 2015).

1.4.4. Glutathione peroxidases

Similar to PRDXs, most of our current understanding of the structure and function of glutathione peroxidases (GPXs) comes from model systems other than *C. elegans*. Therefore, in the following sections we will first discuss important points regarding the biology of GPXs based on model systems such as mammalian cells and then we will review the known phenotypes and expression patterns of GPXs in *C. elegans.*

1.4.4.1. An overview of the biology of glutathione peroxidases

One of the important enzymatic defense systems against ROS-induced damage by lipid hydroperoxides and excessive H_2O_2 is the glutathione redox system. Using glutathione as the reductant, GPXs are responsible for the reduction of H_2O_2 to water or organic hydroperoxides to their corresponding alcohols (Arthur and CMLS 2001). Most eukaryotes express GPX family of enzymes. Of the eight GPX enzymes expressed in mammalian cells (GPx1-GPx8), GPx1-GPx4 are selenoproteins which contain a selenocysteine residue in their catalytic center. Selenocysteine is a cysteine analogue with a selenium-containing selenol group in place of the sulfur-containing thiol group. The existence of this selenocysteine residue contributes to the enzyme's efficiency and its removal and replacement with cysteine has been shown to significantly reduce the activity of the enzyme (Rocher, Lalanne et al. 1992, Maiorino, Aumann et al. 1995). In redox-active proteins, the use of selenium instead of sulfur, in addition to increasing the catalytic efficiency of the protein, can have a protective effect because one-electron oxidized product of selenium, the selanyl radical, is not oxidizing enough to modify proteins, but the cysteine-thiyl radical is able to do this (Nauser, Steinmann et al. 2012).

However, except for human GPx6, in other known mammalian systems GPx5-GPx8 host a cysteine residue in their catalytic center instead of a selenocysteine (Kryukov, Castellano et al. 2003). From a phylogenetic point of view, most of these "nonselenium" GPXs are categorized into a cluster known as phospholipid hydroperoxide glutathione peroxidase (PHGPX) (Herbette, Roeckel-Drevet et al. 2007). These PHGPXs specialize in directly removing the hydroperoxides generated in biological membranes (Thomas, Maiorino et al. 1990) as a result of the ROS-induced peroxidation of unsaturated fatty acids esterified in membrane phospholipids (Esterbauer, Schaur et al. 1991, Riley 1994, Palozza, Sgarlata et al. 1996).

1.4.4.2. Glutathione peroxidases in *C. elegans:* known phenotypes and expression patterns

C. elegans produces a whole host of polyunsaturated fatty acids; so much so, that polyunsaturated fatty acids constitute approximately thirty percent of its total fatty acids (Watts 2002, Brock and Watts 2007). Therefore, it is not surprising that all eight identified genes encoding for GPXs in the worm (*gpx-1 – gpx-8*) are nonselenium PHGPXs. Additionally, a phylogenetic analysis of the GPXs expressed by the worm shows a close relationship between GPX-1, GPX-2, GPX-6, GPX-7 and GPX-8 and human and mouse PHGPXs. However, since *C. elegans* GPX-8 lacks Cys and Trp residues in its catalytic center, it is likely that the protein does not act as a glutathione peroxidase (Sakamoto, Maebayashi et al. 2014).

Amino acid sequence analysis of *C. elegans* GPXs suggests that GPX-1, GPX-2, GPX-6 and GPX-7 are homologs of PHGPXs. Transgenic expression of GPX::GFP fusion proteins under the control of their endogenous promoters has shown that GPX-1::GFP and GPX-7::GFP are both expressed in the cytoplasm of intestinal cells with GPX-1::GFP also being expressed in the nuclei of these cells. GPX-2::GFP is mainly expressed in the anterior end of the intestine and in some neurons located at the head and tail of the animal. Finally, GPX-6::GFP expression (but not the expression of other GPX::GFP fusion proteins) is greatly enhanced in the intestinal tissue throughout L2-L4 stages only by induction via starvation treatment. Overall, these data indicate that *gpx-1, gpx-2, gpx-6* and *gpx-7* are mainly expressed in the intestine of the worm (Sakamoto, Maebayashi et al. 2014).

With regards to the phenotypes of *gpx* mutants, all *gpx-1, gpx-2, gpx-6* and *gpx-7* single mutants and their quadruple *phgpx* mutant are viable and reach adulthood without any noticeable abnormality in their physiologic rates or fecundity compared to the wild-type. However, it has been reported that the maximum lifespan of the quadruple mutant animals is shorter than that of the wild-type and they show an age-dependent increase in mortality rate. Furthermore, deletion of *gpx-6* has been found to abolish the lifespan extension of *eat-2* mutants conferred via dietary restriction, suggesting that it plays a key role in dietary restriction-induced longevity in *C. elegans* (Sakamoto, Maebayashi et al. 2014).

In this section, we reviewed the biology of ROS and ROS signaling, examined various aspects of the ROS network, including ROS-handling enzymes with focus on *C. elegans* and discussed the phenotypes (including lifespan) that result from genetic manipulation of the ROS-

handling enzymes. In the next section, we will discuss the connection between ROS and longevity further by exploring the existing data on the mitochondrial mutants of *C. elegans* and discussing how manipulation of ROS levels by chemical and genetic means affects the lifespan of the worm.

1.5. ROS and Aging

As discussed in section 1.1.5, the potential toxicity of ROS, especially ROS that originates from the mitochondria has been the foundation of the free radical theory of aging which considers accumulated oxidative damage the main cause of aging (Harman 1956, Harman 1972, Ku, Brunk et al. 1993). Nevertheless, mounting evidence that questioned the validity of this view has led our laboratory and others to formulate alternative views (Blagosklonny 2008, Lapointe and Hekimi 2010). Essentially, it appears that ROS increase with advancing age is not the cause of aging, but rather ROS increase with age because they are involved in modulating signal transduction pathways that protect against cellular stresses that are a consequent of aging (Hekimi, Lapointe et al. 2011).

Several lines of evidence point to the uncoupling of ROS and aging. These studies were mainly described in section 1.1.5 in connection with the free radical theory of aging. Briefly, studies from our laboratory and others have shown that an increase in oxidative damage due to knocking down or knocking out SODs in *C. elegans* does not lead to a decrease in lifespan (Yang, Li et al. 2007, Doonan, McElwee et al. 2008, Yen, Patel et al. 2009, Van Raamsdonk and Hekimi 2012). Even the quintuple mutants of the worm that lack all of their five SOD enzymes and are hypersensitive to exogenous experimental increases in ROS levels have a normal lifespan (Van Raamsdonk and Hekimi 2012). Interestingly, worms that lack their mitochondrial SOD (SOD-2) are even long-lived (Van Raamsdonk and Hekimi 2009) (See section 1.4.1.1 for more information on SOD-2 and Chapter 3 for the longevity of *sod-2* mutants). On the other hand, using SOD mimetics to increase superoxide dismutase activity does not prolong lifespan, either (Keaney, Matthijssens et al. 2004) and lifespan extension via SOD overexpression is unrelated to antioxidant activity (Cabreiro, Ackerman et al. 2011). Therefore, the causality between ROS and aging has not been established. In the following section, we will review three *C. elegans* mitochondrial mutants that live long despite mutations that result in an increased ROS production in their mitochondria.

1.5.1. Long-lived mitochondrial mutants of *C. elegans: clk-1, isp-1* and *nuo-6*

The *C. elegans clk-1* mutants were one of the first long-lived mutants that were identified twenty-five years ago (Wong, Boutis et al. 1995). The *clk-1* gene encodes a hydroxylase necessary for

ubiquinone (coenzyme Q) biosynthesis (Ewbank, Barnes et al. 1997) and is essential for the normal mitochondrial function (Felkai, Ewbank et al. 1999). For example, despite being long-lived, the mitochondria of *clk-1* mutants lose the ability to accumulate an inner membrane potential-dependent dye at a younger age compared with the wild-type (Felkai, Ewbank et al. 1999). Moreover, purified *clk-1* mitochondria have both increased $O_2^{\bullet -}$ levels and oxidative stress (Yang and Hekimi 2010a). ETC complex activity has been shown to be normal in *clk-1* mutants but electron transfer from complex I to complex III was found to be decreased as measured spectrophotometrically using enzyme assays (Kayser, Sedensky et al. 2004).

Following a screen for *clk-1*-like mutants, in which slow-growing and slow-defecating mutants were examined, *isp-1(qm150)* was identified. This point mutation affects one amino acid in the vicinity of the iron-sulfur center in the Rieske Iron Sulfur Protein of mitochondrial complex III (Feng, Bussière et al. 2001). In addition to the slow rate of development and slow physiological rates such as slow defecation, which are hallmarks of the long-lived mitochondrial mutants, *isp-1(qm150)* worms have decreased whole worm oxygen consumption (Feng, Bussière et al. 2001) and decreased oxidative phosphorylation in isolated mitochondria when using either malate or succinate as an electron donor (Falk, Kayser et al. 2006). *sod-3* (a mitochondrial SOD) expression was also found to be increased in *isp-1(qm150)*; however, deletion of a FOXO gene (*daf*-16) that modules the expression of *sod-3*, does not decrease the longevity of *isp-1(qm150)* mutants suggesting that increased *sod-3* expression levels does not contribute to the longevity of *isp-1(qm150)* (Feng, Bussière et al. 2001). Additionally, using MitoSOX staining to measure ROS levels in the pharynx of *isp-1(qm150)* mutants showed no differences from the wild-type (Dingley, Polyak et al. 2010). Although there is a trend towards decreasing levels of carbonylated proteins in *isp-1(qm150),* the examination of oxidative damage to proteins in mutants shows no significant difference compared to the wild-type (Yang, Li et al. 2007). Yang *et al.* then treated *isp-1(qm150)* mutants with RNAi against *sod-2,* the primary mitochondrial SOD of the worm, to see whether decreased oxidative damage contributed the mutants' long lifespan. However, even though RNAi against *sod-2* significantly increased the levels of carbonylated proteins in *isp-1(qm150)* compared to the wild-type, these *isp-1(qm150)* mutants still lived as long as the RNAi untreated mutants. Therefore, similar to *clk-1* mutants, the longevity of *isp-1(qm150)* is not caused by a decrease in oxidative damage, either.

Like *isp-1(qm150),* the long-lived *nuo-6(qm200)* mutants were also identified in a screen for slow-developing and slow-defecating mutants. *nuo-6* encodes a subunit of complex I of the electron transport chain which causes a decrease in complex I activity and whole worm oxygen consumption in the mutants. Similar to *clk-1*, *nuo-6(qm200)* mutants also have increased ATP levels despite their decreased mitochondrial function (Yang and Hekimi 2010b). RNAi treatment against *sod-2,* the worm's main mitochondrial SOD, does not shorten the lifespan of *nuo-6(qm200)* but even slightly increases their longevity (Yang and Hekimi 2010a). Furthermore, it is shown that although RNAi against *nuo-6(qm200)* and *isp-1(qm150)* also increases the wild-type's lifespan, it does not result in decreased oxygen consumption like the point mutations and RNAi treatment appears to exert its longevity effect by triggering a mitochondrial stress response (Yang and Hekimi 2010b). Also, the *isp-1(qm150);nuo-6(qm200)* double mutants are long-lived but they do not live longer than the single mutants showing that the effects of these mutations on lifespan are not additive and they likely increase longevity through the same mechanism by increasing the mitochondrial ROS generation (Yang and Hekimi 2010b).

The study of the *isp1(qm150)* and *nuo-6(qm200)* mitochondrial mutants of *C. elegans* has led to very strong evidence linking increased mitochondrial ROS generation to increased lifespan: purified mitochondria from both mutants show increased levels of $O_2^{\bullet-}$ generation (Yang and Hekimi 2010a), antioxidant treatment (with *N*-acetyl cysteine or vitamin C) suppresses their longevity (Yang and Hekimi 2010a), and treatment with very low concentrations (0.1 mM) of the pro-oxidant paraquat phenocopies their effects on lifespan (Yang and Hekimi 2010a, Yee, Yang et al. 2014). In chapters 3 and 4, we will review and explore the pro-longevity effects of the pro-oxidant compound paraquat in more detail and discuss how using it as a tool has furthered our understanding of the relationship between ROS and aging.

1.5.2. Distinguishing ROS-dependent longevity from hormesis

It has been suggested that the beneficial effects of a small increase in ROS levels could be caused by an increase in ROS defenses against ROS-dependent damage. This mechanism is sometimes referred to as mitohormesis (Ristow 2014, Yun and Finkel 2014). We have tested this hypothesis in our laboratory by examining patterns of gene expression linked to mitochondrial ROS longevity. Because the longevity effects of *isp-1(qm150)* and *nuo-6(qm200)* are not additive (Yang and Hekimi 2010b) and the pro-longevity effect of exogenous ROS (0.1 mM paraquat treatment) is

also not additive to the longevity effect of either mutation (Yang and Hekimi 2010a), we reasoned that *isp-1(qm150)*, *nuo-6(qm200),* and the paraquat-treated wild-type worms should show common changes in gene expression for implementing ROS-dependent lifespan extension. Indeed, using Affimetrix gene arrays, we observed a large overlap between the effect of each of these three conditions (*isp-1(qm150)*, *nuo-6(qm200),* and paraquat) compared with the untreated wild type (Yee, Yang et al. 2014). However, the list of altered genes did not indicate a particular mechanism of longevity, nor an upregulation of ROS detoxification or repair of oxidative damage. This was true whether we considered the expression of genes common to all three conditions or when gene expression patterns in each of the three conditions were considered. The notion that hormesis is the mechanism for ROS-dependent longevity, paradoxically implies that ROS cause aging and therefore increased protection against ROS increases lifespan. On the other hand, based on our observations, we believe that constitutively increased mtROS generation increases lifespan because in the absence of actual damage or stress, general mechanisms of stress resistance and damage repair are triggered and amplified (Yee, Yang et al. 2014, Hekimi, Wang et al. 2016).

1.5.3. The intrinsic apoptosis pathway connecting ROS and longevity

The mitochondrial intrinsic apoptosis signaling pathway uses ROS sensors in the process of signal transduction (Circu and Aw 2010). This pathway is different from the extrinsic apoptotic pathway which incorporates receptors at the plasma membrane and interacts with other cells. In vertebrates, apoptosis is tightly controlled and one of the elements that affects it is the mitochondrial ROS through the intrinsic pathway (Wang and Youle 2009). Programed apoptotic cell deaths in *C. elegans* happen during development. In the worm, the apoptotic machinery has several components: EGL-1 (a BH3-only protein, BH3 stands for Bcl2 homology domain 3), CED-9 (Bcl2-homolog), CED-4 (Apaf1-homolog) and CED-3 (Casp9-homolog). CED-4 binds to CED-9 which is tethered to the outer mitochondrial membrane. In the event of programed apoptotic cell death, EGL-1 interacts with CED-9 which leads to the release of CED-4. CED-4 then relocates to the perinuclear membranes and assembles into active apoptosomes which in turn activate the caspase CED-3. However, these proteins are able to performs functions outside the context of apoptosis. For example, CED-4 is involved in S-phase checkpoint regulation (Zermati, Mouhamad et al. 2007) and hypoxic pre-conditioning (Dasgupta, Patel et al. 2007), both CED-4 and CED-3 promote neuronal regeneration (Pinan-Lucarre, Gabel et al. 2012), and CED-9 and EGL-1 can

influence mitochondrial dynamics (Lu, Rolland et al. 2011). These examples suggest a signal transduction role for the individual protein components of the intrinsic apoptosis pathway in other processes. In our laboratory, we have uncovered one of the mitochondrial ROS signals which acts via the intrinsic apoptosis pathway to promote longevity (Yee, Yang et al. 2014) (Figure 1.17).

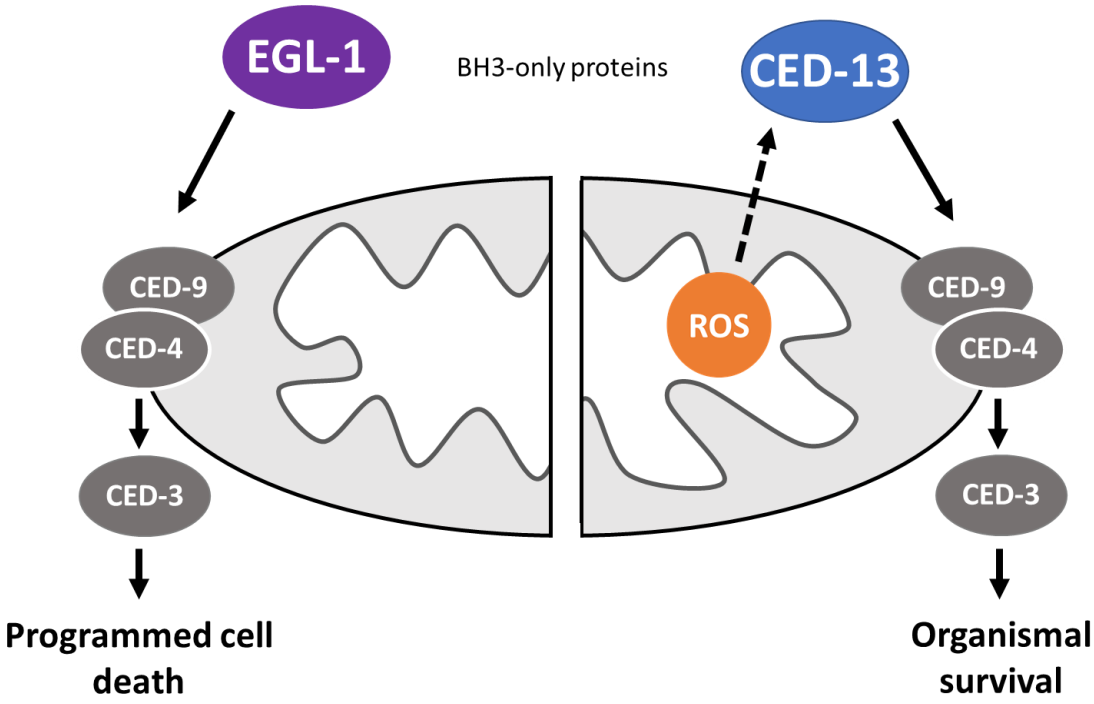

Figure 1.17. Increased mitochondrial ROS production can activate a unique signaling pathway in *C. elegans* that leads to lifespan extension. In *C. elegans*, the intrinsic apoptosis pathway can be activated by two different BH3-only proteins but only one of them (CED-13), upon activation by increased mitochondrial ROS production, induces a unique pattern of gene expression that promotes survival instead of apoptosis. Diagram modified after (Yee, Yang et al. 2014).

This study by Yee *et al.* shows that mutations in the core components of the apoptosis pathway abolish apoptosis in the worm and suppress the longevity of *isp-1(qm150)* and *nuo-6(qm200)*. However, this lifespan suppression is independent from EGL-1 which is required for apoptotic cell death in the worm (Nehme and Conradt 2008). On the other hand, the loss of the only other *C. elegans* BH3-only protein called CED-13, which is not required for apoptosis (Schumacher, Schertel et al. 2005) suppresses the longevity of *isp-1(qm150)* and *nuo-6(qm200)*. The function of BH-3 only proteins in vertebrates is to sense cellular dysfunctions that trigger apoptosis (Galluzzi, Bravo-San Pedro et al. 2014). We have hypothesized that, in a similar way,

CED-13 can also sense the dysfunctional cellular states to initiate protective responses that lead to the longevity of *isp-1(qm150)* and *nuo-6(qm200)* mitochondrial mutants (Yee, Yang et al. 2014, Hekimi, Wang et al. 2016). Interestingly, treatment with very low doses of the pro-oxidant paraquat can bypass this need for CED-13 and *isp-1;ced-13* double mutants can live as long as *isp-1* single mutants when treated with paraquat. This suggest that mitochondrial ROS activates the CED-13-dependent pathway directly, possibly by acting on CED-9 which is physically tethered to the mitochondria.

As mentioned before, long-lived mitochondrial mutants are also slow in processes that consume energy such as slower development and behavior. These phenotypes are suppressed with the loss of apoptotic signaling, as well. But other primary phenotypes of these mutants such as low oxygen consumption and ATP levels are not suppressed. This suggests that, in the long-lived mitochondrial mutants, ROS signaling is inhibiting ATP-consuming processes, perhaps to protect from mitochondrial dysfunction or to divert ATP to protective processes (Yee, Yang et al. 2014, Hekimi, Wang et al. 2016).

In this chapter, we reviewed some of the important theories of aging. We also reviewed important aspects of ROS biology such as ROS signaling and the enzymatic components of the ROS network with a focus on *C. elegans*. We also discussed how recent advances in ROS biology, our understanding of ROS signaling and studying the long-lived mitochondrial mutants *isp-1(qm150)* and *nuo-6(qm200)* are in contrast with the core concepts of the free radical theory of aging. In chapter 2, we will present studies that further explore the effects of the mitochondrial superoxide dismutases on the longevity of *C. elegans* and in chapter 3 we will present a systematic genetic study that analyzes the role of two important classes of ROS-handling enzymes, superoxide dismutases and catalases, in the regulation of lifespan in the worm.

1.6. References

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Chapter 2:
Mitochondrial superoxide dismutases and longevity

2.1. Introduction

In chapter 1, we reviewed the research on the process of aging, the biology of ROS and our previous findings and those of others on ROS-dependent longevity. We saw that the free radical of aging focuses on ROS, and more specifically mitochondrial ROS, as the cause of aging. Based on our previous findings on ROS-induced longevity and other supporting evidence from other research groups we showed that the core concepts of the free radical theory of aging are in stark contrast with the reality of the relationship between ROS and aging. We believe, on the other hand, that the evidence supports the view that ROS do not cause aging. The relationship between ROS and aging is far more complex. It is more likely that when increased ROS levels are observed in aging organisms, this elevation acts as a signal that promotes defensive mechanisms which end up inducing longevity. Previously, we have shown one such way that increased mitochondrial ROS can promote longevity, which is by activating the intrinsic apoptosis pathway (see section 1.5.3). This chapter describes our findings regarding the regulation of longevity by the worm's two mitochondrial MnSODs. In the following sections, we will review the previous research, specifically on the topic of the mitochondrial MnSODs of *C. elegans.*

2.1.1. Unique worm isoforms of SODs in the world of eukaryotes

A fraction of the molecular oxygen in respiring cells is reduced to superoxide radicals ($O_2^{\bullet-}$). Unless effectively managed, uncontrolled increase in the levels of $O_2^{\bullet-}$ and other reactive oxygen species (ROS) derived from it can cause damage to various biological molecules (see section 1.2). The superoxide dismutase (SOD) family of enzymes exist for this purpose. SODs catalyze the dismutation of $O_2^{\bullet-}$ into H_2O_2 and oxygen:

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{\text{SODs}} H_2O_2 + O_2$$

Although the above reaction can occur spontaneously at a very slow rate, which depends on very high concentrations of $O_2^{\bullet-}$, under physiological conditions, SODs can perform this dismutation 20,000 times faster (Forman and Fridovich 1973, McCord, Crapo et al. 1977, Sheng, Abreu et al. 2014), making them one of the most efficient enzymes.

The three distinct isoforms of SODs present in eukaryotic cells differ in the prosthetic metal ion in their active site and their subcellular localization. Copper/zinc SODs (Cu/ZnSODs) mainly localize to the cytosol (Fridovich 1975). For example, Western blot analysis shows that SOD-1, which is the worm's main Cu/ZnSOD, is largely present in the cytosolic fraction (Doonan, McElwee et al. 2008). Additionally, the worm has another cytosolic isoform of Cu/ZnSOD named SOD-5. As we discussed in section 1.4.1, SOD-5 is inducible and its expression in the wild-type adults is not detected or is limited to a couple of neurons. Another isoform of Cu/ZnSODs localizes to the extracellular space (Marklund 1984, Nozik-Grayck, Suliman et al. 2005). In *C. elegans,* this is SOD-4. By itself, SOD-4 is proposed to have two isoforms that result from alternative splicing. Uniquely among eukaryotes, one of these extracellular isoforms appears to be membrane-bound (Doonan, McElwee et al. 2008). Finally, there are the manganese SODs (MnSODs) which are mitochondrial (Weisiger and Fridovich 1973). MnSODs are encoded by nuclear genes as precursor proteins targeted to the mitochondrial matrix. MnSODs are processed to their mature forms by cleavage of their transit peptide (Wispe, Clark et al. 1989). Most eukaryotes have one isoform of MnSOD in their mitochondria, but *C. elegans* uniquely has two of them: SOD-2 and SOD-3. Since the studies that we describe in this chapter are directly related to these two, in the following sections, we will take a closer look at both of them and review the phenotypes associated with their loss in the worm. Note that the unique diversity of the SOD isoforms in *C. elegans* in terms of their function and their subcellular localization offers a powerful tool for studying the role of SODs in ROS dependent phenomena in the context of a complex multi-cellular model organism.

2.1.2. Two isoforms of MnSODs in *C. elegans*: SOD-2 and SOD-3

With the exception of *Zea mays*, which has four isoforms of MnSODs, multiple isoforms of MnSODs are rare in organisms (Zhu and Scandalios 1993). However, *C. elegans* expresses two distinct isoforms of MnSODs. These two isoforms are expressed by *sod-2* (chromosome I) and *sod-3* (chromosome X). These two genes have very similar sequences overall (86% homologous) including in their N-terminal mitochondrial targeting signals (Suzuki, Inokuma et al. 1996, Hunter, Bannister et al. 1997) and they also show striking sequence identity to MnSOD isoforms in other organisms (Figure 2.1). Both SOD-2 and SOD-3 are predicted to be mitochondrial (Hunter, Bannister et al. 1997, Henderson, Bonafè et al. 2006, Doonan, McElwee et al. 2008, Honda,

Tanaka et al. 2008) based on their mitochondrial targeting signals, punctuated expression patterns in GFP translational fusions and colocalization with MitoTracker staining in the worm.

Tissue-specific expression patterns of SOD-2 and SOD-3

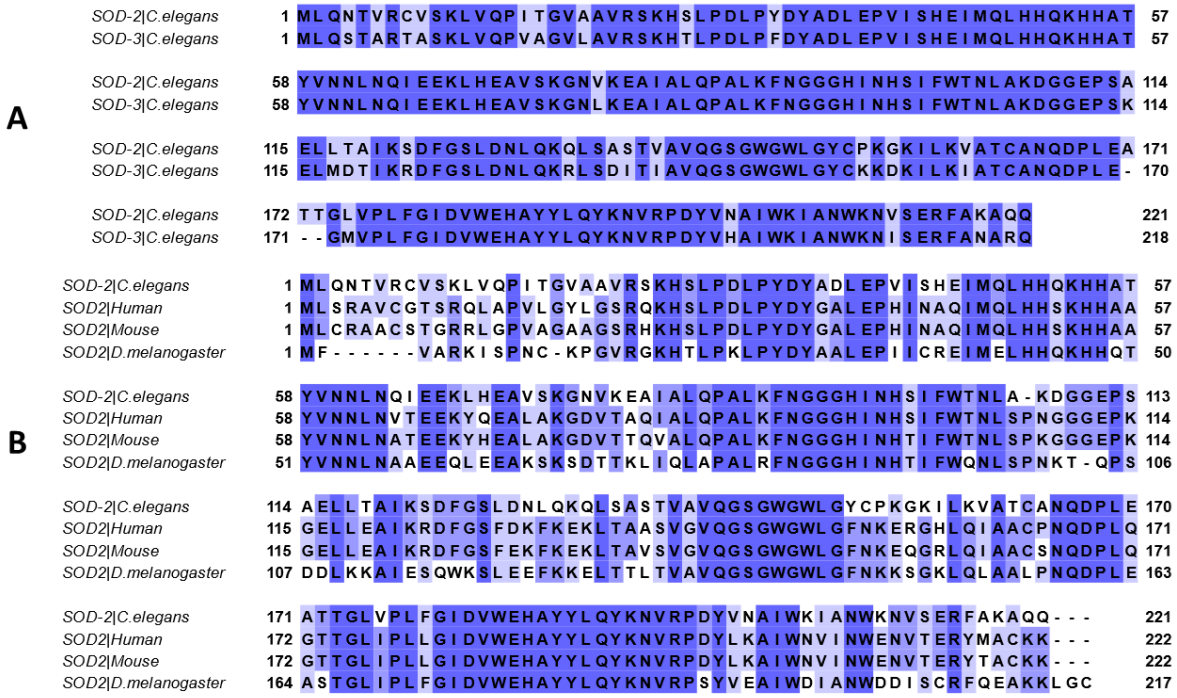
Previous data on tissue-specific expression of SOD-2 and SOD-3 are scarce and all come from studies that use extrachromosomal translational fusions. In one study, in the wild-type background, transgenic *sod-3::gfp* expression has been observed constitutively and weakly in the pharyngeal and intestinal cells (Henderson, Bonafè et al. 2006). The study acknowledges the possibility that the presence of the GFP might be interfering with the mitochondrial targeting of the protein.

In another study, the authors used *sod-3::gfp* and *sod-2::rfp* transgenes to study their expression patterns in the long-lived *daf-2(e1370lf)* background (referred to as *daf-2(lf)* for short) (see section 2.1.6) In *daf-2(lf)* background, both *sod-2::rfp* and *sod-3::gfp* are expressed in the head and the tail of the worms. Additionally, in the head of the worms, expression of *sod-2::rfp* was prominent posterior to the pharyngeal corpus while *sod-3::gfp* expression is more marked at the tip of the head, anterior to the pharyngeal corpus, separating the *sod-2::rfp* expressing cells from *sod-3::gfp* expressing cells (Honda, Tanaka et al. 2008).

Doonan *et al* have looked into the expression patterns of *sod-2* and *sod-3* using extrachromosomal *sod-2::gfp* and *sod-3::gfp* translational fusions, but in the wild-type background (Doonan, McElwee et al. 2008). This study reported marked expression of both *sod-2::gfp* and *sod-3::gfp* in the pharynx where *sod-2::gfp* showed significant expression in procorpus, the terminal bulb region of the pharynx, intestinal cells and various hypodermal cells. Faint expression of *sod-2::gfp* was also observed in some neurons anterior to the pharynx and in the posterior intestine. Additionally, *sod-3::gfp* expression was also seen at the tip of the head, anterior to the pharyngeal corpus similar to the study by Honda *et al* for the expression of *sod-3* in the *daf-2(lf)* background. They also reported that in the wild-type L3 larvae, *sod-3::gfp* is expressed in the pharyngeal corpus and the posterior bulb and *sod-2::gfp* is expressed predominantly in the pharyngeal terminal bulb and weakly in the pharyngeal corpus.

Overall, these three studies suggest that the transgenic expression of *sod-2* and *sod-3* is confined mostly to the pharyngeal region although the transgene expression pattern may not correspond with that of the endogenous genes. However, it is not yet clear whether SOD-2 and SOD-3 are colocalized to the same mitochondria even if they colocalize to the same tissue but the

punctuated expression pattern suggests that they are indeed mitochondrial. These data also show that *sod-2* and *sod-3* translational fusions are expressed in separate tissues. Interestingly, in vitro studies suggest that SOD-2 and SOD-3, localize to the mitochondrial supercomplex I:III:IV to facilitate $O_2^{\bullet-}$ conversion at the site of its production (Suthammarak, Somerlot et al. 2013).



MnSOD Protein	SOD-3 (*C. elegans*)	SOD2 (Human)	SOD2 (Mouse)	SOD2 (*D. melanogaster*)
% Identity vs. *C. elegans* SOD-2	86.43	62.61	63.51	64.18

Figure 2.1. SOD-2 and SOD-3, the two mitochondrial MnSODs of the worm have highly identical sequences with each other and with MnSODs of other species. (A) The sequence of SOD-2 and SOD-3 is approximately 86% identical (dark blue). (B) The sequence of the *C. elegans* SOD-2 shows 62% to 64% similarity to that of the mitochondrial MnSOD of other organisms such as humans, mice and the fruit fly (*D. melanogaster*). Sequence alignments and percentage identity were calculated using NCBI COBALT (www.ncbi.nlm.nih.gov/tools/cobalt/) and alignment visualization was made using Jalview version 2.11.0 for Windows (www.jalview.org). MnSOD protein sequences were obtained form WormBase.org for *C. elegans* and from UniProt.org for other organisms.

2.1.3. Induction of *sod-3* expression by a variety of physiological and environmental factors

It appears that *sod-3* expression in the wild-type background, without induction, is limited. One study, which measured the MnSOD protein activity, reported that in *sod-2* deletion mutants, MnSOD protein activity is not detected, which suggests that the SOD-3 protein may not be expressed largely in the wild-type adults without induction (Doonan, McElwee et al. 2008). This observation also implies that the loss of $O_2^{\bullet-}$ detoxification by SOD-2 does not induce the expression of the SOD-3 protein in any significant manner. This is consistent with Doonan *et al*'s finding that *sod-2* mRNA accounts for 18% of the total *sod* mRNA in the wild-type adults but *sod-3* mRNA contributes to only 1% of the total *sod* mRNA in the wild-type adults without stimulation (Doonan, McElwee et al. 2008). However, a variety of stimuli can induce *sod-3* mRNA expression.

In the long-lived mutants of the insulin signaling pathway of *C. elegans* such as *daf-2(e1370lf)* and *age-1,* the *sod-3* transcript is highly upregulated (Honda and Honda 1999, Honda and Honda 2002, Yanase, Yasuda et al. 2002). Similarly, in other long-lived mutants such as *isp-1* (Feng, Bussière et al. 2001, Dingley, Polyak et al. 2010) and *clk-1* (Yang, Li et al. 2007) (see section 1.5.1) *sod-3* mRNA expression is also induced. Similarly, *sod-3* mRNA expression is induced in the short-lived electron transport chain mutants *gas-1* and *mev-1* (Doonan, McElwee et al. 2008).

Oxidative stress mildly upregulates both *sod-2* and *sod-3* mRNA expression, especially in young worms (Tawe, Eschbach et al. 1998, Yang, Li et al. 2007, Meng, Lv et al. 2017) and loss of SOD-2 induces *sod-3* mRNA expression (Suthammarak, Somerlot et al. 2013).

Other stressors that have been shown to induce *sod-3* mRNA expression include desiccation (Erkut, Vasilj et al. 2013), $CuSO_4$ (Song, Zhang et al. 2014) and treatment with Paraquat (a compound used for stimulating $O_2^{\bullet-}$ generation, PQ) (Tawe, Eschbach et al. 1998) (see section 2.1.8), starvation (Henderson, Bonafe et al. 2006), heat (Wolf, Nunes et al. 2008), folic acid (Rathor, Akhoon et al. 2015), pathogenic bacteria (Chavez, Mohri-Shiomi et al. 2007), knockdown of the iron-sulfur cluster transporter *abtm-1* (Gonzalez-Cabo, Bolinches-Amoros et al. 2011), treatment with pyrroloquinoline quinone (Wu, Huang et al. 2016) and the anti-oxidant N-acetyl-cysteine (NAC) (Oh, Park et al. 2015). One interesting finding that may indicate a signaling role for SOD-3 is that in one study it has been shown that up-regulation of the SOD-3 protein is

required for resistance to *Enterococcus faecalis,* a pathogenic bacterial strain (Chavez, Mohri-Shiomi et al. 2007). In most cases, induction of *sod-3* is primarily dependent on the fork-head transcription factor DAF-16 (Honda and Honda 1999, Henderson, Bonafè et al. 2006).

While we are discussing the phenotypes resulting from the loss of SOD-2 and SOD-3 in the following sections, it is important to bear in mind that the available data on the MnSOD protein activity analysis, *sod-2* and *sod-3* mRNA expression levels and the expression patterns of the two proteins using extrachromosomal translational fusions, reinforce the view that SOD-2 is the worm's primary mitochondrial MnSOD, while SOD-3 is the inducible MnSOD isoform. Also note that the two previous studies by Honda *et al* and Doonan *et al* show that SOD-2-expressing cells and SOD-3-expressing cells are largely separate (Doonan, McElwee et al. 2008, Honda, Tanaka et al. 2008). Additionally, the study by Doonan *et al* shows that *sod-3* expression is more prominent during larval stages and not in the adult wild-type worms without induction.

2.1.4. Loss of SOD-2 and SOD-3 and hypersensitivity to oxidative stress

The loss of SOD-2 leads to hypersensitivity to oxidative stress. We had previously reported that RNAi knockdown of *sod-2* leads to hypersensitivity to 4mM PQ (a high concentration of PQ used to induce oxidative stress) (Yang, Li et al. 2007). Also, *sod-2* knockout worms are sensitive to 40mM PQ (Doonan, McElwee et al. 2008) and 4mM PQ and 240 μM juglone (Van Raamsdonk and Hekimi 2009). On the contrary, we have observed previously that loss of SOD-3 causes only very mild hypersensitivity to PQ treatment (Van Raamsdonk and Hekimi 2009). It is important to note that in the studies mentioned above very high concentrations of PQ (up to 40mM) or juglone are used to induce oxidative stress. This is quite distinct from the pro-longevity effects of very low concentrations of PQ (0.1mM or lower) that we use to induce longevity in *C. elegans* (Van Raamsdonk and Hekimi 2012).

2.1.5. The effects of the loss of SOD-2 and SOD-3 on lifespan in previous studies

Previous studies on the effects of SOD-2 and SOD-3 deletions on the lifespan of the worm report various findings that are in some cases different from what we described in this chapter. In this section we review these previous studies from our laboratory and others.

We have shown previously that the loss of SOD-2 leads to a dramatic increase in the lifespan of the wild-type worms (Van Raamsdonk and Hekimi 2009, Van Raamsdonk and Hekimi

2012). We have also shown that this longevity effect of the loss of SOD-2 is abolished by treatment with N-Acetyl Cysteine (NAC), a H_2O_2 scavenging enhancer (Yang and Hekimi 2010a). Heteroallelic *sod-2(gk256)/sod-2(ok1030)* (a mutant that carries both deletion alleles of *sod-2*) is also long-lived (Van Raamsdonk and Hekimi 2009). Using this heteroallelic mutant ensures that the longevity effect of the loss of SOD-2 (also seen with each allele separately) is indeed the result of the deletion of the *sod-2* gene and is not caused by the presence of some other mutation that could technically be linked to one of these alleles.

However, there are other studies that show a very small increase in lifespan (Dingley, Polyak et al. 2010) or even no change in lifespan (Doonan, McElwee et al. 2008, Honda, Tanaka et al. 2008) in *sod-2* knockout mutants. But, as we discussed in chapter 1, long-lived mitochondrial mutants have a set of typical characteristics such as lengthened defecation cycle, reduced self-brood size and slow development and defecation. We and others have shown that loss of *sod-2* induces these phenotypes in addition to longevity (Doonan, McElwee et al. 2008, Van Raamsdonk and Hekimi 2009).

Previous studies on the effects of the loss of SOD-3 on lifespan also report various data. Previously in studies on the lifespan of *sod-3* mutants we and others have reported no change in lifespan (Honda, Tanaka et al. 2008, Van Raamsdonk and Hekimi 2012, Yee, Yang et al. 2014) or even a slight increase in lifespan (Yen, Patel et al. 2009, Dingley, Polyak et al. 2010). Also, we had previously observed that the loss of SOD-3 slightly shortens the longevity of *sod-2* mutants (Van Raamsdonk and Hekimi 2009). On the contrary, in studies where *sod-2* is not long-lived, loss of SOD-3 does not appear to affect the lifespan of the worms in the *sod-2* background (Doonan, McElwee et al. 2008, Honda, Tanaka et al. 2008).

We will discuss our view on the reason for different data on the effects of the loss of SOD-2 and SOD-3 on the lifespan of *C. elegans* further in the results and discussion sections of this chapter. However, briefly, we believe that varied results in this area are likely due to varied experimental conditions including various growth temperatures (Honda, Tanaka et al. 2008), different methods of age synchronization of the larvae (Dingley, Polyak et al. 2010) or different food sources (e.g. using UV-killed or heat-killed bacteria or different strains of *E. coli*) (Honda, Tanaka et al. 2008, Dingley, Polyak et al. 2010). Additionally, some studies use FUDR (5-fluoro-2′-deoxyuridine) as a means of inducing parental sterility to circumvent the labor-intensive process of separating the offspring from adults during the reproductive period. We and others have shown

that FUDR is known to affect the lifespan in mutant worms (Aitlhadj, Stürzenbaum et al. 2010, Van Raamsdonk and Hekimi 2011, Rooney, Luz et al. 2014, Anderson, Corkins et al. 2016), which is the primary reason we avoided using it for the studies that we describe in this chapter. Also, various studies use different knockout alleles of *sod-2* or *sod-3* (Honda, Tanaka et al. 2008) that are not used in other studies and make the resulting data hard to compare with similar studies due to possible variations caused by allelic differences. Sometimes the studies even use too small sample sizes (Dingley, Polyak et al. 2010) which causes the resulting data to be more prone to error and showing false positives.

2.1.6. A suggested signaling role for SOD-2 and SOD-3 in the regulation of lifespan in *daf-2(lf)* background

Loss-of-function mutants of *daf-2(e1370lf)* (referred to as *daf-2(lf)* for short) are long-lived worms that lack the functionality of an insulin receptor-like protein which regulates various aspects of the worm's physiology and longevity (Kenyon, Chang et al. 1993). A part of the studies that we will describe in this chapter is related to exploring the effects of the loss of SOD-2 and SOD-3 on the lifespan of the *daf-2(lf)* mutants and the possible signaling role and the interaction of the two MnSODs with regards to the lifespan of the worms. In this section, we will review a similar study in this area and summarize its findings.

In a study by Honda, Tanaka *et al.* 2008, the authors investigated the effects of the loss of SOD-2 and SOD-3 on the longevity *daf-2(e1370lf)* mutants. They created *sod-2(sj173)* and *sod-3(sj134)* alleles by mutagenesis with UV-trimethylpsoralen for the purpose of this study. Using a liquid medium solution consisting of 50 mM paraquat they observed that while *sod-2* or *sod-3* single deletions do not affect the oxidative stress resistance phenotype of *daf-2(lf), sod-2* and *sod-3* double deletion eliminates the oxidative stress resistance of *daf-2(lf)* but does not affect the longevity of the triple mutants. This finding uncouples oxidative stress from longevity in the *daf-2(lf)* background.

Additionally, Honda *et al* found that *daf-2;sod-3* double-mutants lived longer than *daf-2*, *daf-2;sod-2* lived shorter than *daf-2,* and the lifespan of *daf-2;sod-2;sod-3* triple mutants was similar to that of *daf-2;sod-3* demonstrating that *sod-3* is epistatic to *sod-2* for *daf-2* longevity. Also, transgenic expression of wild-type *sod-2* and *sod-3* in *daf-2;sod-2* and *daf-2;sod-3*

respectively extends the lifespan of *daf-2;sod-2* and shortens the lifespan of *daf-2;sod-3* suggesting that SOD-2 and SOD-3 may function as lifespan modulators in the *daf-2(lf)* mutant background.

As we discussed before, this study also showed that transgenes of SOD-2::RFP and SOD-3::GFP translational fusions are expressed in the mitochondria of different cells. For example, the showed that while both SOD-2::RFP and SOD-3::GFP are present in the head and tail, their expression patterns in the head indicates a distinctive pattern: *sod-3::gfp* is expressed in the tip of the head, anterior to the pharyngeal corpus while *sod-2::rfp* is expressed in the cells posterior to the pharyngeal corpus. Note that using transgenes as such has inherent limitations. For example, the transgene expression pattern may not correspond with that of the endogenous gene (as acknowledged by other previous studies), transgene expression can be different among siblings of a single strain and arrays are sometimes able to induce RNAi-like effects that suppress endogenous gene function (Evans 2006). Also, Honda *et al* studied the expression patterns of *sod-2::rfp* and *sod-3::gfp* only in the *daf-2(lf)* background where the pattern might be different from that in the wild type. For example, as we discussed in section 2.1.3, *daf-2(lf)* background is indeed one of the genetic conditions that induces the expression of *sod-3.*

So, what does the study conclude from these data? Based on the epistatic relationship of *sod-2* and *sod-3* in the longevity of *daf-2(lf)* and the observed expression patterns of the SOD-2::RFP and SOD-3::GFP transgenes the authors conclude that under specific redox conditions such as the physiological conditions in the *daf-2(lf)* background and in response to specific cellular redox conditions, SOD-2 modulates redox-signal transduction in the SOD-2-expressing cells to transmit a signal to the SOD-3-expressing cells. This, they suggested, could enable SOD-3 to modulate redox-signal transduction in *daf-2(lf)* to regulate longevity. Whether this happens in the wild type background is not explored in this study. In this chapter, we have described our results on the effects of the loss of SOD-2 and SOD-3 on lifespan in both *daf-2(lf)* and the wild-type backgrounds. However, besides this study by Honda *et al,* there are other findings, as well, that the induction of *sod-3* is connected to signaling pathways rather than a need for $O_2^{\bullet-}$ detoxification. In the next section, we will discuss such findings.

2.1.7. A link between induction of *sod-3* and signaling pathways

In a recent review (Wang, Branicky et al. 2018), members of our laboratory have proposed that the following reasons support the view that *sod-3* is more important in signal transduction pathways in the worm rather than merely being only involved as a means for $O_2^{\bullet-}$ detoxification:

(1) As discussed in section 2.1.3, a wide variety of factors cause upregulation of *sod-3* expression; however, it is unclear whether all such conditions in fact cause oxidative stress (as in such high levels of ROS that they can negatively affect the physiology of the worm) to induce *sod-3* upregulation.

(2) There is little evidence to support that the SOD-3 protein levels in the nematode are consistent with its role in $O_2^{\bullet-}$ detoxification. For example, as mentioned before, the *sod-3* transcript is upregulated in *daf-2* mutants, but a corresponding increase in SOD-3 protein levels is not detected (Doonan, McElwee et al. 2008, Dingley, Polyak et al. 2010). Also, in the wild-type worms, *sod-3* mRNA only accounts for 1% of total mRNA expression. Even if all this mRNA is translated into SOD-3, this amount does not appear to be sufficient for defending against oxidative stress.

(3) Honda, Tanaka *et al.* 2008 found that the loss of SOD-3 lengthens the lifespan of the insulin-signaling mutant *daf-2(lf)* and suppresses the shortened lifespan of *daf-2;sod-2* double mutants (see section 2.1.6). They also found that, at least in *daf-2(lf)* mutants, *sod-2* and *sod-3* are expressed in distinct subsets of cells. They conclude that SOD-3 and SOD-2 are involved in a ROS signaling pathway which acts in intercellular communication to regulate longevity.

(4) Despite the fact that neither decreasing (Doonan, McElwee et al. 2008, Honda, Tanaka et al. 2008, Van Raamsdonk and Hekimi 2009) nor increasing (Henderson, Bonafe et al. 2006) *sod-3* expression has been reported to have any effect on lifespan, the expression of a *sod-3::gfp* reporter is suggested to be the best predictor of remaining lifespan from a set of genes whose expression is age-dependent (Sanchez-Blanco and Kim 2011).

The findings that we describe later in this chapter are also consistent with the view that SOD-3 is more likely functioning in a ROS-dependent signaling role in the regulation of longevity, rather than being there solely to defend the worm against deleterious effects of ROS.

2.1.8. Paraquat (PQ) treatment and longevity

Paraquat (PQ) is a redox-cycling compound that undergoes reduction-oxidation reactions to produce $O_2^{\bullet-}$ mainly at the mitochondrial electron transport chain, mostly at the site of complex I (as shown in isolated mitochondria) (Figure 2.2 A) (Castello, Drechsel et al. 2007, Cocheme and Murphy 2008). In high doses, PQ is widely used in ROS biology to induce cellular and especially mitochondrial oxidative stress (Bus, Aust et al. 1976, Cocheme and Murphy 2008, Robb, Gawel et al. 2015) in particular in studies involving *C. elegans* (Senchuk, Dues et al. 2017) where doses as high as 200 mM PQ are used to induce oxidative stress in the worm. However, to induce ROS-dependent longevity in the wild-type worms, we use very low concentrations of PQ (typically 0.1 mM PQ). Previously, we have shown that in wild-type worms, very low dose PQ treatment increases lifespan in a dose-dependent manner until a peak lifespan is reached. However, further increase in ROS levels induced by PQ will result in a shortening of lifespan due the possible deleterious effects of ROS outweighing its beneficial pro-longevity effects. We have proposed that this relationship between the levels of ROS and lifespan in *C. elegans* follows the pattern of an inverted U-shaped curve model (Van Raamsdonk and Hekimi 2009) (Figure 2.2 B). We have also shown that this inverted U-shaped curve model can be expanded to include the effects of anti-oxidants (Desjardins, Cacho-Valadez et al. 2017). Later in this chapter we also present original data from the studies described in this thesis that clearly demonstrate this inverted U-shaped curve relationship between ROS and lifespan.

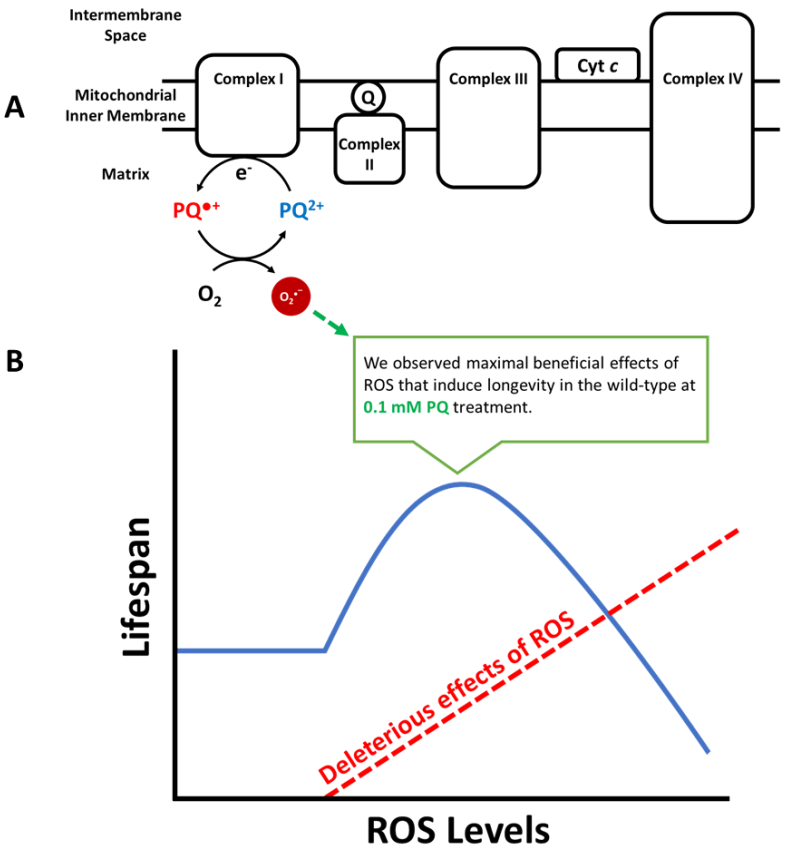


Figure 2.2. PQ-stimulated production of $O_2^{\bullet-}$ at the ETC causes a dose-dependent increase in lifespan that can be represented in an inverted U-shaped curve. (A) PQ is a redox-cycling compound that stimulates the production of $O_2^{\bullet-}$ at the ETC. In isolated mitochondria, this is shown to occur mostly at the site of complex I. (B) Treating the wild-type worms with different doses of PQ increases their lifespan in a dose-dependent manner. This pro-longevity effect of PQ treatment is maximal at 0.1 mM for the wild-type. When PQ treatment doses are increased further, the lifespan of the worms decrease suggesting that at this point perhaps the deleterious effects of increased ROS outweigh its beneficial prolongevity effects (Van Raamsdonk and Hekimi 2009).

2.1.9. Previous studies on the effects of paraquat treatment on *sod-2* and *sod-3* knock-out backgrounds

A part of the studies that are described in this chapter involve exploring the effects of PQ treatment on the lifespan and development of *sod-2* and *sod-3* mutants. Some of the previous studies in our laboratory have explored the effects of PQ on *sod-2* and *sod-3* mutants in various contexts. In this section, we briefly review these previous results.

We have previously shown that the longevity of *sod-2* mutants is due to increased mitochondrial ROS output (Van Raamsdonk and Hekimi 2009) and that is why treatment with the antioxidant N-acetyl-cysteine (NAC) abolishes *sod-2*'s longevity (Yang and Hekimi 2010a). However, contrary to the pro-longevity effects of low-dose PQ treatment on the wild-type, we had observed that 0.2 mM PQ treatment causes developmental arrest in *sod-2* mutants (Van Raamsdonk and Hekimi 2009). This 0.2 mM dose of PQ in that particular study was used to test the resistance of the *sod-2* mutants against oxidative stress and it was not intended as a pro-longevity treatment. On the other hand, we and others had previously observed that the lifespan of the *sod-3* mutants was similar to that of the wild-type (Honda, Tanaka et al. 2008, Van Raamsdonk and Hekimi 2012) but in one of our previous studies we found that the lifespan of *sod-3* mutants was not extended by PQ treatment (Yee, Yang et al. 2014). We will discuss these findings further in more detail in connection to the data that we present in this chapter.

2.1.10. The goals of the studies described in this chapter

As we mentioned earlier, few studies that had previously looked into different aspects of SOD-2 and SOD-3 and the effects of their single and double deletions on lifespan had used various experimental conditions (e.g. different mutant strains, food sources, temperatures, etc.) and as a result they have reported results that are sometimes not consistent with each other. In the following sections of this chapter of the thesis we will describe studies that we conducted using our standard conditions (described in section 2.2) and canonical alleles of *sod-2(ok1030), sod-2(gk257), sod-3(tm760)* and *sod-3(gk235)* which are well characterized and used in many studies in our laboratory and others. The goal is to clarify the roles of mitochondrial MnSODs in the regulation of lifespan in *C. elegans* and their epistatic relationship both in the wild-type and in the *daf-2(e1370lf)* mutant backgrounds. We will also present an insight into cell/tissue specific expression patterns of the worm's MnSODs using endogenously tagged *sod-2* and *sod-3* alleles. Finally, we will explore the PQ-induced developmental arrest of *sod-2* mutants and its possible connection to mitochondrial ROS signaling.

2.2. Materials and Methods

2.2.1. Strains and genetics

All strains were maintained at 20°C, on solid nematode growth medium (NGM), and were fed *E. coli* OP50. Bristol N2 was used as the wild-type. The following mutant strains were used: LGI: *sod-2(ok1030), sod-2(gk257), sod-2::mKate2, nuo-6(qm200)*; LGII: *ctl-1(ok1242), ctl-2(ok1137), ctl-3(ok2042)*; LGIII: *daf-2(e1370lf), let-60(n1046gf), let-60(n1046gf)*-C118S; LGIV: *isp-1(qm150)*; LGX: *sod-3(tm760), sod-3(gk235), sod-3::egfp, gas-1(fc21)*. All the double- and triple-mutant strains were generated using standard genetic methods and were verified using polymerase chain reaction and restriction enzyme digest where applicable.

The *let-60(n1046gf)*-C118S strain is also designated as *let-60 (qm225gf)* and was created using CRISPR by Maximillian-Kramer Drauberg, a member of our laboratory. The endogenously tagged alleles of *sod-2* and *sod-3* namely *sod-2::mKate2* and *sod-3::egfp* were provided by Dr. Robyn Branicky (another member of our laboratory). The methods for the construction of these strains are described below. Also, the method for the construction of *nuo-6 sod-2,* which was a particularly difficult strain to make, is described in detail in section 3.2, since this strain was more extensively used in the studies described in chapter 3.

Construction of *let-60(n1046gf)-c118s*

The LET-60 amino acid Cys118 (C118) was modified using the CRISPR/Cas9 gene editing protocol as described previously (Paix, Folkmann et al. 2015). Briefly, a tool at http://crispr.mit.edu was used to select crRNA sequences targeting *let-60*.
The *let-60* gene-specific crRNA was 5'-AGGTTCCTATGGTCTTGGTAGUUUUAGAGCUA UGCUGUUUUG-3' the co-CRISPR *dpy-10* crRNA and the tracRNA (Dharmacon) were re-suspended in 10mM Tris pH 7.4 to 8 µg/µl. The purified Cas9 protein (PNA Bio. Inc.) was reconstituted in water to 2 mg/ml.
The repair templates for let-60-C118S was 5'-TGAAATTATCAGTCAATGGTTGAATAT TTGTATTTCTTCTAGGTTCCTATGGTGTTGGTAGGCAATAAATCTGATTTGTCATCTC GATCAGTCGACTTCCGAACAGTCAGTGAGACA-3'. The repair template was resuspended in water to 1 µg/µl and 500 ng/µl for *dpy-10*. The injection mix contained 6.25 µl Cas9 protein,

1.25 µl tracrRNA, 0.2 µl crRNA *dpy-10*, 0.275 µl ssODN *dpy-10*, 0.5 µl crRNA *let-60*, 1.1 µl ssODN *let-60-C118S*, 0.25 µl KCl (1M) and 0.375 µl Hepes pH 7.4 (200 mM). The mix was activated for 15 min at 37°C and injected into the distal gonads of young adult worms. F1 roller worms were singled out and after they laid eggs, they were genotyped by single worm lysis and PCR using the primers 5'-GTGAGACATGCCTCCTCGAC-3'and 5'-GGTGTCGTATTTTTGG CGCGA-3'for the *let-60* modification. The *let-60* modification was confirmed by sequencing the PCR product. The non-roller F2 worms were sequenced to verify successful editing.

Studying expression patterns of *sod-2::mKate2* and *sod-3::egfp* endogenously tagged transgene strains

The strategy for producing *sod-2* and *sod-3* tagged knock-ins by CRISPR was conceived of by Dr. Ju-Ling Liu in our laboratory. Constructs were made, injected for homologous recombination and screened for by the company Knudra. Cre-Recombinase was injected by Dr. Robyn Branicky to remove the transformation marker cassette (*unc-119*) which was flanked by loxP sites. Dr. Branicky also crossed the CRISPR knock-in lines into *daf-2(e1370)* mutants and examined the expression of *sod-2* and *sod-3* lines in N2 and in *daf-2*, and acquired the images shown in section 2.3.7. Fluorescent images were captured on an Olympus BX63 microscope at 60× magnification using an EXi Blue^{TM} camera and the CellSens Dimension software.

2.2.2. Measuring adult lifespan with and without PQ treatment

We used PQ in most of the studies described in this thesis as a stimulator of mitochondrial $O_2^{\bullet-}$ production. In all these experiments we used PQ treatment as one of the experimental conditions and control conditions were PQ was not present were always used alongside the treated worms. Since the majority of the lifespan experiments described in this thesis are presented as part of chapter 3, a detailed step-by-step process for setting up such experiments with relevant diagrams is presented in section 3.2. In this section, we briefly describe those steps.

Solid nematode growth medium (NGM) was prepared as described previously (Stiernagle 1999). When the experiment involved PQ treatment, Paraquat (Sigma-Aldrich, St. Louis, USA) was also added to the treatment NGM at a final concentration pertinent to each condition in the experiment. *E. coli* OP50 (a strain of *E. coli* that grows slowly) grown on normal NGM petri dishes

(worm plates) was transferred onto the PQ plates using a platinum pick instead of seeding the bacteria directly onto the PQ plates. Transferring bacteria from normal NGM plates to plates containing PQ is crucial because this treatment may not allow for sufficient growth of OP50. Therefore, if OP50 is directly seeded onto the treatment plates, worms that are put onto these plates may run out of food quickly and starve, which affects the results of the lifespan experiments. Control NGM plates containing no PQ received the bacteria in a similar way through transferring grown bacterial cultures with platinum picks from other seeded NGM plates to provide exactly the same conditions as the PQ plates. Worms were transferred to freshly made NGM or PQ plates once every week to maintain the consistency in the experimental conditions (e.g. moisture content of the NGM gel).

All lifespan measurements described in this chapter were performed at 20°C. Between 20 – 50 adult worms with lots of eggs inside them (gravid adults) were put on normal NGM plates or PQ plates that were prepared as we described above. These gravid adults were given 3 hours to lay eggs (limited lay) and were then removed from these plates. The plates with the eggs were incubated at 20°C until the larvae developed into a stage when their reproductive system was fully formed but they did not have eggs yet. This is called the young adult stage. These young adult worms were transferred onto experimental plates. This marked the beginning of the lifespan assay.

An experimental pool of 40 – 50 worms was used for each condition per trial and each lifespan experiment was performed 2 – 4 times in separate independent trials. Worms that died prematurely as a result of internal hatching of the progeny (when eggs hatch inside the mother and kill it) or had crawled out of the plates were replaced from a backup pool. Data from all trials of an experiment were pooled and statistical analysis was performed using GraphPad Prism (v6.01) and ordinary one-way ANOVA with Sidak's correction for multiple comparisons.

2.2.3. Developmental arrest assays

Developmental arrest assays were performed at 20°C following a 1-hour limited lay. PQ plates were made as described in section 2.2.2. Approximately 100-150 eggs were left on PQ and control NGM plates to hatch. The larvae were inspected twice daily at 12-hour intervals to determine the developmental stage. Depending on the genotypes, the arrest condition was scored after 3 days for strains with a developmental rate similar to the wild-type and after 5 days for strains with slower

rates of development. Additionally, to confirm the arrest condition the plates with the developmentally arrested worms were monitored until 7 days after hatching.

2.3. Results

2.3.1. *sod-2* deletion lengthens lifespan while *sod-3* deletion shortens lifespan of the wild-type

As discussed in section 2.1.2, we had shown previously that the loss of *sod-2* lengthens the lifespan of *C. elegans* (Van Raamsdonk and Hekimi 2009). However, the experimental conditions those experiments differed from the experimental conditions of this study (see section 2.2). Briefly, for the studies presented here, we did not use FUDR as we and others have shown that it can affect the lifespan of some mutant strains of the worm (Van Raamsdonk and Hekimi 2011, Anderson, Corkins et al. 2016). Additionally, in the current studies, we measured the adult lifespan of the worms using two different knockout alleles of *sod-2,* similar to our previous study, and the experiment was carried out at 20°C.

We confirmed that under our standard experimental conditions, both mutant alleles of *sod-2(ok1030)* and *sod-2(gk257)* lengthened the adult lifespan of the wild-type (Figure 2.2 A), suggesting that the loss of *sod-2* increases lifespan in the absence of FUDR, as well.

Given that *sod-2* affects the lifespan of the worm, we wondered whether the loss of the other mitochondrial MnSOD of the worm, *sod-3*, also has an effect on lifespan. Therefore, we measured the adult lifespan of two different knockout alleles of *sod-3*. We found that both alleles of *sod*-3, significantly shorten the adult lifespan of the worm. Since both alleles have the same effect, it appears that the decreased lifespan of these strains is indeed due to the loss of *sod-3* (Figure 2.2 B).

The opposite effects of the loss of SOD-2 and SOD-3 on lifespan are interesting since both of these MnSOD proteins are localized to the mitochondrial matrix and are very similar in their amino acid sequences (Hunter, Bannister et al. 1997). Although it is possible that the opposite effects of the SOD-2 and SOD-3 removal on lifespan could be the result of the small variations in the amino acid sequence of the two proteins, this can also be caused by the differences in their temporal and/or tissue-specific expression patterns as we discussed in the previous sections. To explore the different effects of *sod-2* and *sod-3* on the lifespan of the wild-type further, we studied their PQ treatment dose response patterns described in the following two sections.

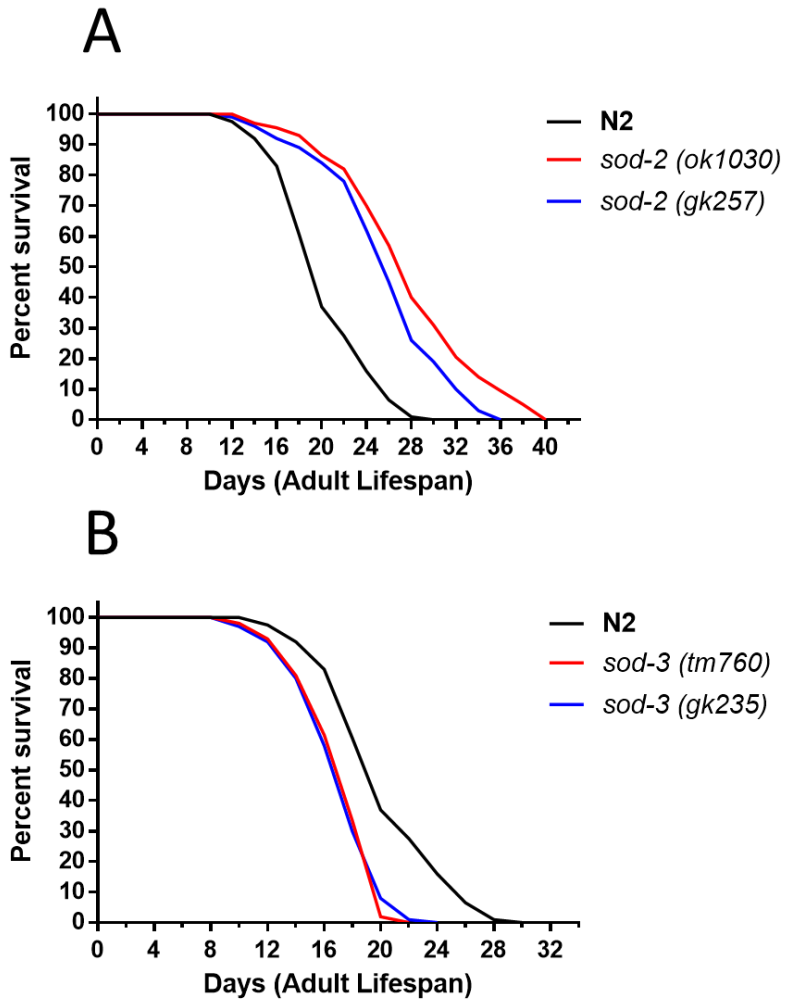


Figure 2.3. The loss of SOD-2 and SOD-3 has opposite effects on lifespan. (A) While both mutant strains of *sod-2* are long-lived compared to the wild-type, (B) both mutant strains of *sod-3* are short-lived. N2, *sod-2 (ok1030)* and *sod-3 (tm760)* n=200, 4 independent trials, all other conditions n=100, 2 independent trials. 50 worms per trial.

2.3.2. The lifespan-shortening effect of loss of SOD-3 is mostly additive to the lifespan-lengthening effects of paraquat (PQ) treatment

As we discussed in section 2.1.8, we had previously established that increasing ROS levels via low-dose PQ treatment has a biphasic effect on the lifespan of wild-type worms which can be represented in an inverted U-shaped dose-response curve (Van Raamsdonk and Hekimi 2012, Desjardins, Cacho-Valadez et al. 2017). Based on this model, the pro-survival effect of increased ROS levels elicits a dose-dependent increase in lifespan; however, at higher ROS levels the

deleterious effects of elevated ROS levels outweigh their pro-survival effects. This imbalance between beneficial and deleterious effects of ROS causes a decrease in lifespan in the second phase of the inverted U-shaped curve after the peak (also see Figure 2.2).

In order to test whether PQ has the same dose-dependent effect on *sod-3* mutants as it does on the wild-type worms, we measured the adult lifespan of *sod-3* knockout worms after treatment with 0.025, 0.05, 0.075, 0.1, 0.125 and 0.175 mM concentrations of PQ. We confirmed that the wild-type exhibits an exquisite PQ dose-sensitivity, responding accordingly to even the smallest changes in PQ doses that we used (Figure 2.4 left graph set). We also found that, similar to the wild-type, low-dose PQ treatment induces a dose dependent lifespan-increase in *sod-3 (tm760)* mutants (Figure 2.4 right graph set), as well.

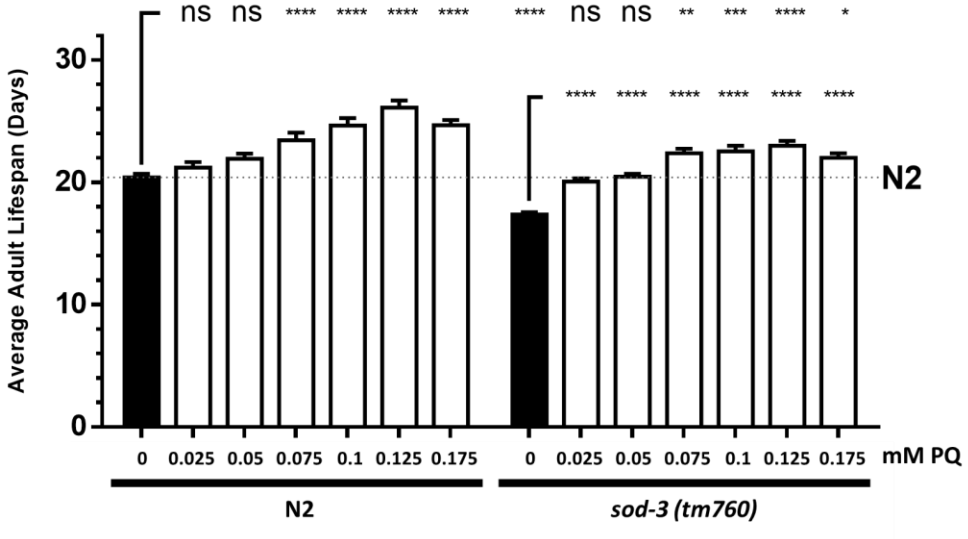


Figure 2.4. The lifespan-shortening effect of the loss of SOD-3 is additive to the lifespan-lengthening effect of paraquat (PQ) treatment. PQ treatment increases lifespan of both the wild-type and *sod-3 (tm760)* in a dose dependent manner. However, the dose-reponse inverted U-shaped curve resulting from the lifespan changes in *sod-3* mutant background is flattened compared to the inverted U-shaped curve of the wild-type (N2) lifespan. N2 (0 PQ) and *sod-3* (0 PQ) n=200, 4 independent trials, all other conditions n=100, 2 independent trials. 50 worms per trial. Comparison of the average adult lifespans: Ordinary one-way ANOVA with Sidak's correction for multiple comparisons. **** | P<0.0001, *** | P<0.001, ** | P<0.01, * | P<0.05

However, despite the apparent PQ-induced increase in the average adult lifespan of *sod-3* mutants, for all the concentrations that we tested, the average adult lifespan of *sod-3* mutants is shorter than that of their wild-type counterparts treated at the same concentration of PQ. As a

result, the inverted U-shaped dose-response curve for the *sod-3* mutants has a flattened form when compared with that of the wild-type. This suggests that first, *sod-3* is not required for the effect of PQ on lifespan extension, and second, the lifespan-shortening effect of *sod-3* mutation is additive to the lifespan-lengthening effect of PQ treatment. It is also possible that the flattened form of the inverted U-shaped curve for the lifespan of *sod-3* implies that SOD-3 could nonetheless play a minor role in PQ-dependent lifespan increase, which might require the production of H_2O_2 by superoxide dismutases. In other words, worms may require the action of SOD-3 at least in the subset of cells/tissues where/when it is expressed in order to fully benefit from the pro-longevity effect of PQ treatment. This view is consistent with the role of SOD-3 as a mitochondrial MnSOD that can participate, albeit to a lesser extent, in the metabolism of PQ-induced $O_2^{\bullet -}$ in the mitochondria. The low impact of the effect of *sod-3* on the effectiveness of the pro-longevity effect of PQ treatment is also consistent with its small contribution to MnSOD activity in the wild-type background that we discussed in section 2.1.3.

In the following two sections, we will explore the effects of *sod-2* deletion on the pro-longevity PQ treatment and we will also explore the effects of PQ treatment on the development of *sod-3* mutant worms and test whether the length of PQ treatment also affects its pro-longevity effect.

2.3.3. PQ treatment shortens the lifespan of *sod-2* mutants at all concentrations tested that allow for viability

As we showed in section 2.3.1, the loss of *sod-2* causes a significant increase in the adult lifespan of the wild-type. However, we had previously found that exposure to 0.2 mM PQ (as a means to test the ROS resistance of the worms) causes developmental arrest in *sod-2* mutants (Van Raamsdonk and Hekimi 2009). We will explore the PQ-induced arrest of *sod-2* further down the road in section 2.3.8. But for the purpose of our discussion in this section, we were able to confirm that under our standard experimental conditions, even exposure to 0.1 mM PQ, which induces longevity in the wild-type, causes developmental arrest in the *sod-2* knockouts. Therefore, in order to find a concentration of PQ that allows for the normal development of *sod-2* mutants we exposed them to 0.05, 0.02, 0.01, 0.005, 0.001 mM PQ at birth. We found that even at 0.05 mM PQ, most of the mutants still go into developmental arrest, but at lower concentrations, *sod-2* worms can develop normally. Nevertheless, even treatment with PQ concentrations as low as 0.01 and 0.02

mM still shortens the long adult lifespan of *sod-2* mutants in such a way that at 0.02 mM, where the wild-type's lifespan is slightly increased, the lifespan of *sod-2* is shortened all the way down to the level of the untreated wild-type worms which marks a dramatic decrease in the average adult lifespan of *sod-2* (Figure 2.5). Therefore, treatment with all tested concentrations of PQ that allow for viability shortens the adult lifespan of *sod-2* mutants. Note that this was not the case with the mutants of *sod-3,* the other MnSOD of the worm. The observation that there is no PQ concentration which can induce pro-longevity in the *sod-2* background suggests that PQ has only deleterious effects on the lifespan of *sod-2* mutants which is consistent with the expected major action of PQ in mitochondria.

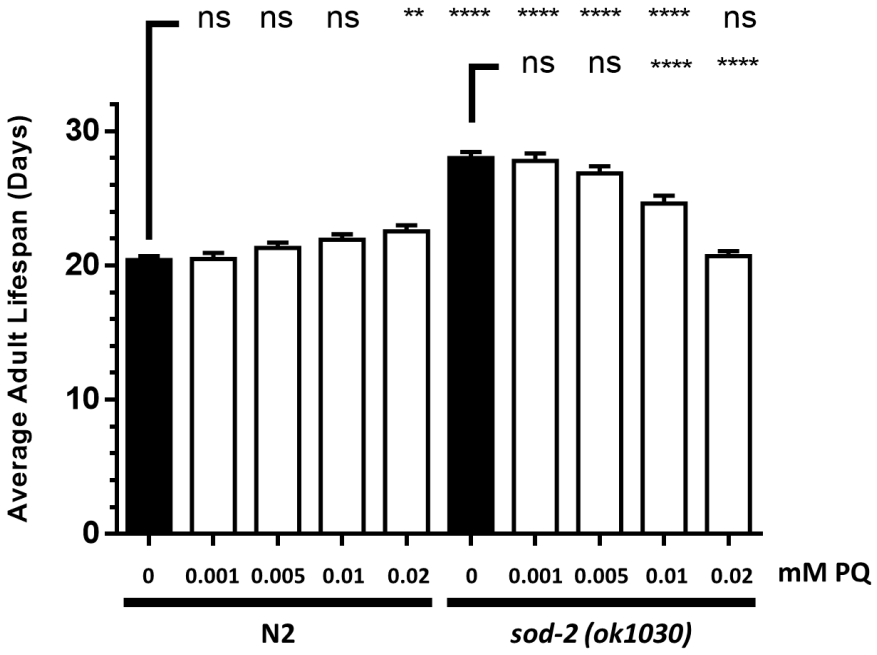


Figure 2.5. At concentrations that allow viability, treatment with PQ shortens the long lifespan of *sod-2* mutants. Even at very low concentrations, PQ treatment is able to extend the lifespan of the wild-type animals. At 0.02 mM (the highest concentration that allows *sod-2* to develop normally), PQ induces a slight increase in lifespan in the wild-type whereas it significantly reduces the lifespan of *sod-2* mutants and brings down their average lifespan to match that of the untreated wild-type animals. N2 (0 PQ) and *sod-2* (0 PQ) n=200, 4 independent trials, all other conditions n=100, 2 independent trials. 50 worms per trial. Comparison of the average adult lifespans: Ordinary one-way ANOVA with Sidak's correction for multiple comparisons. **** | P<0.0001, ** | P<0.01

Additionally, it is possible that the lifespan extension by PQ treatment and the resulting inverted U-shaped longevity represent both increased H_2O_2 generation and the deleterious effects of increases in $O_2^{\bullet-}$ levels. The deleterious effects of increasing $O_2^{\bullet-}$ levels do not necessarily mean damage to biomolecules, although this can be a possibility. Deleterious effects could also mean impairment of the natural signaling roles of $O_2^{\bullet-}$ as a result of increased generation of $O_2^{\bullet-}$. For example, as we discussed in section 1.3.5, $O_2^{\bullet-}$ signal can specifically interact with iron-sulfur clusters in proteins and inactivate them such as the inactivation of mitochondrial aconitase by $O_2^{\bullet-}$ (Gardner, Raineri et al. 1995). This "faulty" signaling can indeed have deleterious effects.

Looking back at the effect of even the smallest doses of PQ treatment on the longevity of *sod-2*, it appears that the PQ-induced increase in $O_2^{\bullet-}$ has deleterious effects when it is not accompanied by a corresponding sufficient increase in H_2O_2 levels that SODs are involved in. To expand on this, let's consider a few of points about *sod-2* mutants:

(1) The loss of SOD-2, as the worm's major mitochondrial MnSOD, causes increased mitochondrial $O_2^{\bullet-}$ production and leads to the longevity of *sod-2* mutants (Van Raamsdonk and Hekimi 2009).

(2) The observation that the longevity of *sod-2* disappears in response to NAC treatment (Yang and Hekimi 2010a), which enhances H_2O_2 scavenging, implicates H_2O_2 alongside $O_2^{\bullet-}$ with regards to the longevity effect of the loss of *sod-2* in the wild-type background.

(3) Given that SOD-2 is the worm's major mitochondrial MnSOD, its removal could simultaneously lead to maximal $O_2^{\bullet-}$ generation (which translates into longevity) and a drop in the H_2O_2 levels sourced by SOD-2 that does not exist anymore. This appears to be the limit for the physiology of the *sod-2* background since increasing $O_2^{\bullet-}$ further with PQ treatment does not lengthen the lifespan of the mutants and shortens it; which brings us back to the inverted U-shaped curve model and its representation of the beneficial and deleterious effects of ROS in longevity.

Considering all these three points, if this maximal $O_2^{\bullet-}$ generation is to be accompanied by a corresponding and sufficiently high level of H_2O_2 generation (for the pro-longevity effect), it may be that SOD-2 itself needs to participate either in the production of this H_2O_2 purely for the sake of increasing H_2O_2 levels directly or to remove excessive $O_2^{\bullet-}$ at the source of its production in the mitochondria to tip the scales in favor of H_2O_2 indirectly. Note that in the case of indirect participation, other players such as other SODs like SOD-1 can be the source of the required H_2O_2, but tipping the scales in favor of H_2O_2 in this $O_2^{\bullet-}$ vs. H_2O_2 balance scenario may require the action

of SOD-2 to remove $O_2^{\bullet-}$ right at the site where it is produced. This is consistent with our previous finding that PQ treatment increased the expression levels of both SOD-1 (cytoplasmic) and SOD-2 as the worm's main SODs (Yang and Hekimi 2010a). We will, of course, explore this further in chapter 3 where we systematically dissect some elements of the worm's ROS network to manipulate $O_2^{\bullet-}$ and H_2O_2 levels at different subcellular compartments.

2.3.4. PQ acts both during development and adulthood including on *sod-3* mutants

Since we had found that the lifespan-shortening effect of *sod-3* mutation is additive to the lifespan-lengthening effect of PQ treatment (see section 2.3.2), and we had previously observed that PQ treatment slows down the developmental rate of the wild-type (Van Raamsdonk and Hekimi 2009), we wondered whether the effects of PQ on the length of the developmental period of the worms could cause this additivity. To further investigate this, we treated one experimental group of *sod-3* and wild-type animals with 0.1 mM PQ from birth (as per our standard experimental conditions) but started the PQ treatment of another experimental group from day 1 of adulthood. We found that regardless of the starting point, 0.1 PQ treatment indeed lengthens the adult lifespan of both the wild-type and *sod-3* mutants albeit to different degrees. In other words, the animals that received the treatment from birth lived longer than those which received the treatment during adulthood only and both treatment groups do live longer than untreated controls (Figure 2.6 A).

In terms of percent change in average adult lifespan, treating the wild-type with 0.1mM PQ from birth lengthens their average lifespan by approx. 25% but when 0.1mM PQ treatment begins at young adulthood the increase in average adult lifespan is only approx. 10%. In a similar way, the average adult lifespan of *sod-3* is lengthened by a staggering approx. 36% compared to untreated *sod-3* when the mutants are treated with 0.1mM PQ from birth. However, when the treatment begins at young adulthood, this increase in average adult lifespan, while still visible, drops to appx. 11%.

Next, in order to investigate the effects of 0.1 mM PQ treatment on the developmental rate of *sod-3* mutants we tested both *sod-3(tm760)* and *sod-3(gk235)*. We found that although 0.1 mM PQ treatment lengthens the developmental period of the wild-type by 1 day, it lengthens the developmental period of both *sod-3* strains by ½ day (Figure 2.6 B).

Taken together these data show that the lifespan extension by PQ treatment is not a purely developmental process and the magnitude of the PQ-induced longevity correlates with the length of the treatment. Also, since PQ treatment lengthens the developmental rate of *sod-3* mutants less than that of the wild-type this suggests that the full effect of PQ treatment on the rate of development of the worms at least partially depends on SOD-3.

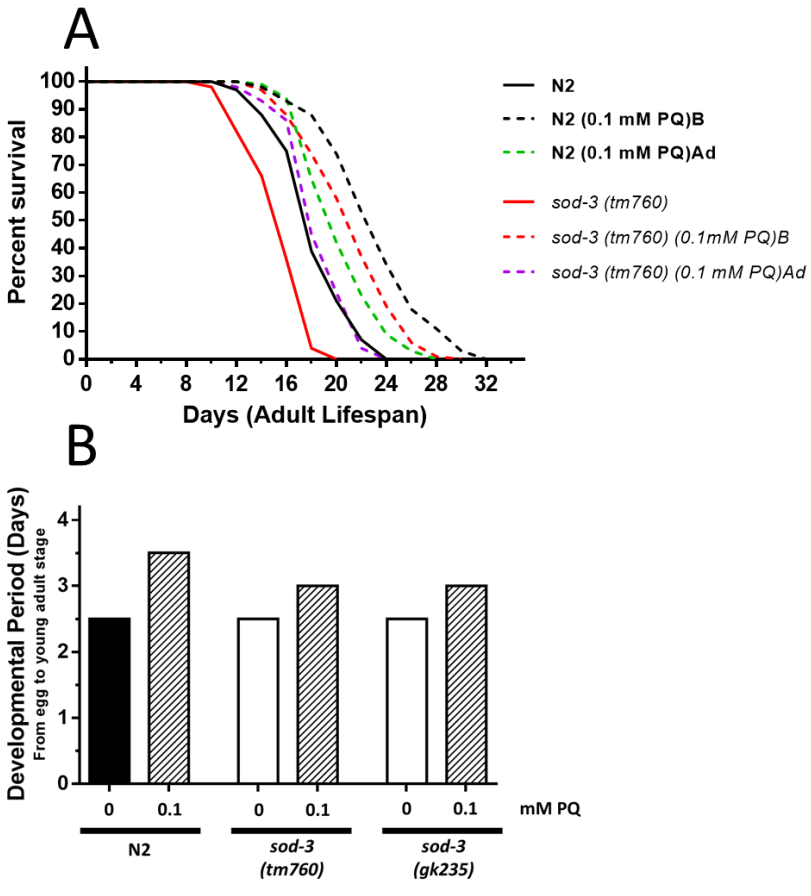


Figure 2.6. PQ acts both during development and adulthood on *sod-3* mutants. (A) 0.1 mM PQ treatment lengthens the lifespan of both the wild-type and *sod-3* mutants whether it is administered from birth (0.1 mM PQ B) or only from day 1 of the adulthood (0.1 mM PQ Ad). (B) 0.1 mM PQ treatment lengthens the developmental period of the wild-type (N2), *sod-3(tm760)* and *sod-3(gk235)*. However, while the developmental period of the wild-type is increased by 1 day, the developmental period of *sod-3* mutants is increased by only half a day. For the lifespan assays in (A) n=100, 2 independent trials, 50 worms per trial. For the developmental rate assays in (B) n=300, 3 independent trials.

2.3.5. *sod-2* and *sod-3* interact on lifespan with an age-dependent epistatic pattern

In section 2.1.5, we reviewed different studies from our laboratory and others that used various experimental conditions and had reported different patterns of interaction between *sod-2* and *sod-3* with regards to lifespan. For example, we had reported previously that *sod-3* mutation slightly shortened the long lifespan of *sod-2* mutants (Van Raamsdonk and Hekimi 2009) while other studies had found that *sod-3* deletion had no effect on lifespan where *sod-2* was not long-lived (Doonan, McElwee et al. 2008, Honda, Tanaka et al. 2008). To investigate the interaction of *sod-2* and *sod-3* on the lifespan of the worms under our standard experimental conditions we used double mutants of the canonical alleles *sod-2(ok1030);sod-3(tm760)*. We found that the adult aging curve for *sod-2*;*sod-3* double mutants has a biphasic pattern. That is, the lifespan curve, at the onset of aging, shows a higher mortality rate for *sod-2;sod-3* double mutants, similar to *sod-3* single mutants but as the worms become older, this mortality rate slows down so that the maximum lifespan of *sod-2;sod-3* is very similar to that of *sod-2* (Figure 2.7).

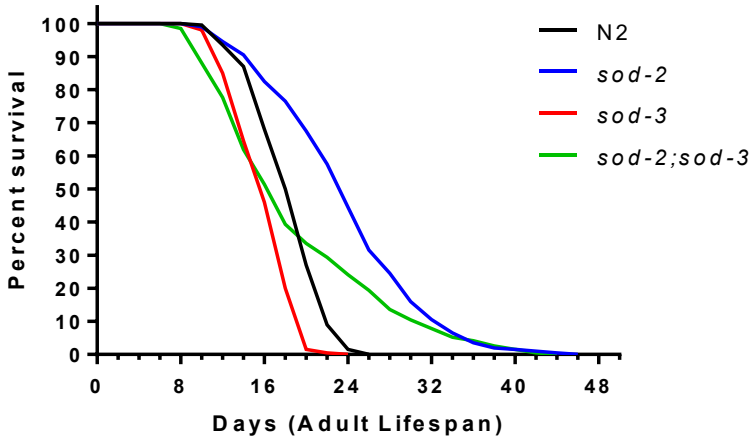


Figure 2.7. *sod-2* and *sod-3* interact on lifespan with an age-dependent epistatic pattern. While *sod-3* mutants live shorter than the wild-type (N2) and *sod-2* mutants live longer, *sod-2;sod-3* double mutants exhibit a biphasic pattern of aging where the mortality rate is higher at first but as the worms become older the mortality rate decreases so that the maximum lifespan of *sod-2;sod-3* is similar to that of *sod-2. sod-2;sod-3* n=191, all other conditions n=200, 4 independent trials, 40-50 worms per trial.

This complex epistatic pattern in the adult aging curve of *sod-2;sod-3* double mutants suggests that either the requirement for the pro-longevity ROS generation changes over time in aging worms or it might hint at a more complex signaling interaction as suggested by the previous work of Honda *et al* (see section 2.1.6). In the following section, we will describe another part of our studies that further suggests the existence of an age-dependent epistasis between *sod-2* and *sod-3*.

2.3.6. Loss of both SOD-2 and SOD-3 extends lifespan non-additively in a *daf-2(lf)* background

As one of the first studies that investigated the effects of superoxide dismutases on lifespan regulation, Honda *et al* found that *sod-3* is epistatic to *sod-2* in modulating lifespan in a *daf-2* loss-of-function background: *daf-2(e1370lf)* (Honda, Tanaka et al. 2008). We presented an overview of this study and its findings in section 2.1.6. Briefly, for this study, Honda *et al* had created their own knockout alleles of *sod-2(sj173)* and *sod-3(sj134),* which have not been used in other studies since then. Also, their experimental conditions differed from our standard conditions. For example, hypochlorite was used to synchronize worms for the experiments (we use the limited lay technique to avoid stressing the worms), UV-inactivated OP50 was used as the food source (we use live OP50) and worms were grown at 15°C until the last larval developmental stage and then they were shifted to 20°C (we conduct all experimental stages at 20°C).

Therefore, in order to further investigate the epistatic relationship between SOD-2 and SOD-3 in *daf-2(lf)* background, we revisited this issue using our standard experimental conditions and the canonical knockout alleles of *sod-2(ok1030)* and *sod-3(tm760),* which have been used in many studies. We found that the loss of both SOD-2 and SOD-3 lengthens the adult lifespan in the *daf-2(lf)* background and the adult lifespan lengthening by the loss of SOD-3 is indeed greater than the adult lifespan extension by the loss of SOD-2; however, the lifespan of the triple mutant *daf-2;sod-2;sod-3* is similar to that of *daf-2;sod-3* (Figure 2.8). This is consistent with *sod-2* and *sod-3* having an epistatic relationship. However, it appears that *daf-2(lf)* reverses the epistatic relationship of *sod-2* and *sod-3* compared to what is observed in the wild-type (see section 2.3.5). That is, while in the wild-type, loss of SOD-3, shortens the lifespan of *sod-2* mutants in the wild-type background, in the *daf-2(lf)* background, loss of SOD-3 lengthens the lifespan of *daf-2;sod-2*. Note that the lifespan of *daf-2* mutants is already very long, *sod*-2 and *sod*-3 both lengthen this

long lifespan even further with *sod-3* having a larger effect. But unlike the wild-type, in the *daf-2(lf)* background the loss of *sod-3* does not shorten the *sod-2*-induced longevity but lengthens it. The pattern and magnitude of the change of the effects produced by the loss of *sod-2* and *sod-3* in the *daf-2(lf)* background militates for a signaling interaction between the two superoxide dismutases. Honda *et al,* also suggested that in the *daf-2(lf)* background, separation of the SOD-2-expressing cells and SOD-3-expressing cells, added an element of intercellular communication to the epistasis of SOD-2 and SOD-3 in the regulation of lifespan. In the next section, we will describe our findings regarding the cell/tissue-specific patterns of *sod-2* and *sod-3* expression using their endogenously tagged chromosomal genes.

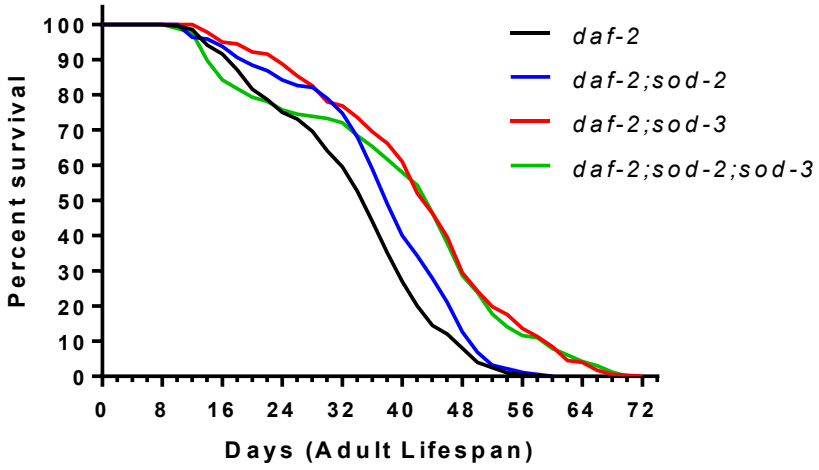


Figure 2.8. The loss of both SOD-2 and SOD-3 extends the adult lifespan non-additively in a *daf-2(lf)* background. Removal of SOD-2 causes an increase in the adult lifespan in *daf-2(lf)* backgorund and the loss of SOD-3 causes an even greater increase in the adult lifespan of *daf-2(lf)*. However, the lifespan of *daf-2;sod-3* is similar to that of *daf-2;sod-2;sod-3*. *daf-2* n=200, *daf-2;sod-2* n=190, *daf-2;sod-3* n=177 *daf-2;sod-2;sod-3* n=164. 4 independent trials, 40-50 worms per trial.

2.3.7. *sod-2* and *sod-3* are expressed largely in different tissues

2.3.7.1. A comparison of the expression patterns of *sod-2* and *sod-3* in the wild-type and *daf-2(e1370lf)* backgrounds

As we discussed in section 2.1.2, a couple of studies had previously investigated the expression patterns of *sod-2* and *sod-3* in the worm. In these studies, the researchers had used extrachromosomal GFP or RFP translational fusions to investigate the expression patterns of *sod-2* and *sod-3* in the wild-type (Doonan, McElwee et al. 2008) and in the *daf-2(e1370lf)* (here referred to as *daf-2(lf)* for short) (Honda, Tanaka et al. 2008) backgrounds. These studies reported that *sod-2* and *sod-3* translational fusions are expressed in separate tissues. Here, for the first time, we have the tools to see endogenous expression of *sod-2* and *sod-3* by tagging the genomic loci of each gene with a fluorescent tag (*sod-2::mKate2* and *sod-3::egfp*) using CRISPR. The schematic structure of *sod-2* and *sod-3* genomic loci after modification by CRISPR is shown in Figure 2.9 A and Figure 2.9 B shows the expression of *sod-2* and *sod-3* in the head region of both the wild-type (N2) and *daf-2(lf)* adults. These fluorescent images, which are taken using the same settings for the wild-type (N2) and *daf-2(lf),* show that both *sod-2* and *sod-3* expressions are higher in *daf-2(lf)* background in comparison with the wild-type. We found that it is not clear whether the expression of *sod-3* can be detected at all in the wild-type (N2) background since it cannot be distinguished from the background fluorescence observed in non-fluorescent worms when imaged with the same settings. Because of this, all other images in the following sections are from *daf-2(lf)* background. In Figure 2.9 B, we see that *sod-2* is strongly expressed in head neurons. Also, in some neurons weak *sod-3* expression can also be detected. Additionally, both *sod-2* and *sod-3* may be expressed in the region of the posterior bulb of the pharynx, although it is unclear in which tissue. The fact that the endogenous expression of *sod-3::egfp* is not detected in the wild-type (N2) background is indeed consistent with the previous findings by Doonan *et al* that reported *sod-3* mRNA contributes to only 1% of the total *sod* mRNA in the wild-type adults without stimulation (Doonan, McElwee et al. 2008), therefore we might expect that endogenous SOD-3 protein expression would be difficult to detect in the unstimulated wild-type (N2) background. However, in the *daf-2(lf)* background, which *sod-3* expression is mildly induced, we see some expression in neurons. In the following section, we will look at the expression patterns of *sod-2* and *sod-3* in different tissues in the *daf-2(lf)* background.

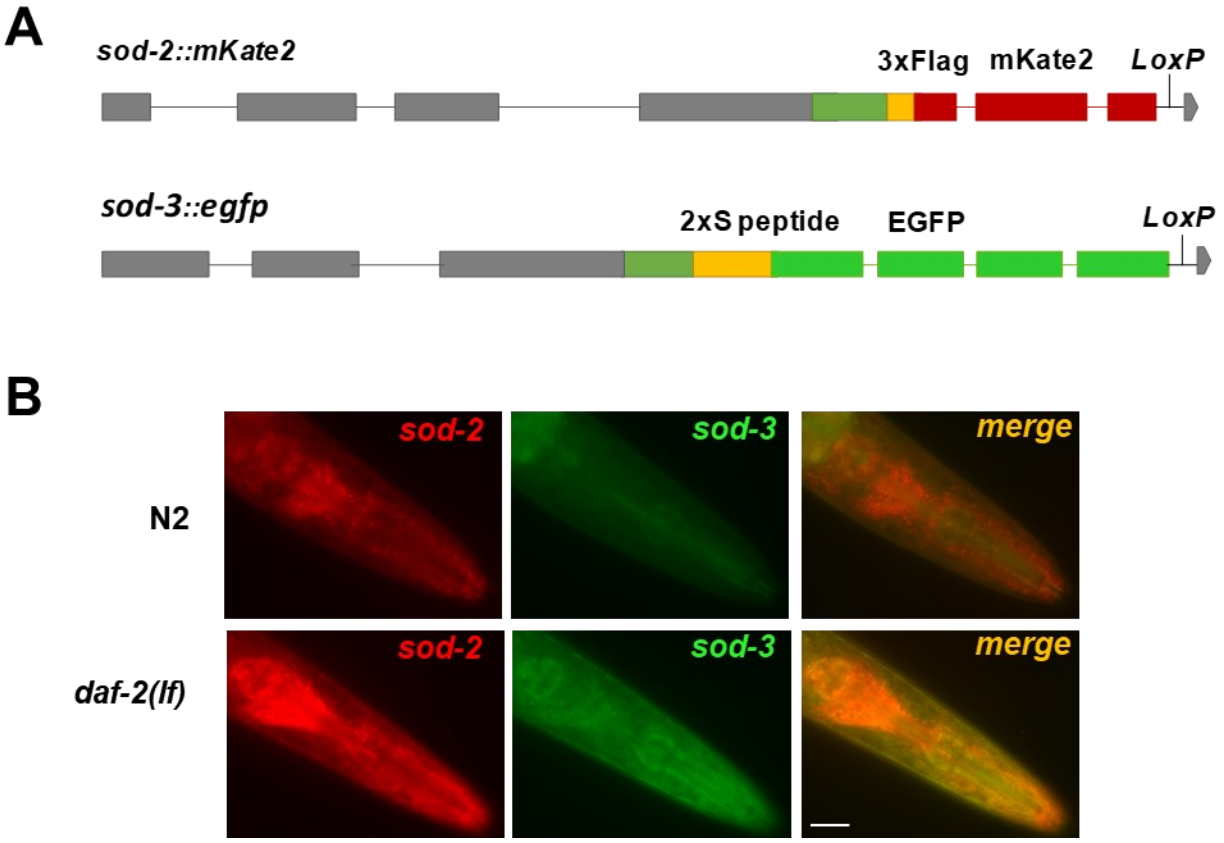


Figure 2.9. Structure of the genomic loci of *sod-2* and *sod-3* after CRISPR modification to include a fluorescent tag and expression patterns of *sod-2* and *sod-3* in the head of the wild-type (N2) and *daf-2(lf)* backgrounds. (A) Schematic of *sod-2* and *sod-3* genomic loci after modification by CRISPR. Both modifications include a fluorescent tag (mKate2 or egfp), a tag for protein purification shown in yellow (Flag tag or S peptide) and some synthetic codons shown in green (which code for the same amino acids as the endogenous protein, but help to distinguish the endogenous from the CRISPR loci alleles by sequencing). As mKate2 and egfp are the reporters for *sod-2* and *sod-3* expression, respectively, images of *sod-2* and *sod-3* expression are pseudo-colored red and green, respectively. **(B)** Expression of *sod-2* and *sod-3* in the head region of the wild-type (N2) and *daf-2(lf)* adults. Fluorescent images are taken with the same settings for N2 and *daf-2(lf)*. *sod-2* expression is higher in the *daf-2* background. *sod-3* expression is also higher in the *daf-2* background; it is not clear in fact whether *sod-3* expression can be detected at all in the wild-type (N2) mutants as it cannot be distinguished from the background fluorescence observed in non-fluorescent worms when imaged with the same settings. Because of this, all subsequent images shown are from *daf-2(lf)* mutants. *sod-2* is strongly expressed in head neurons; in some neurons weak *sod-3* expression can also be detected. Both *sod-2* and *sod-3* may be expressed in the region of the posterior bulb of the pharynx, although it is unclear in which tissue. Scale bar represents 20µm.

2.3.7.2. Expression of *sod-2* and *sod-3* in the adults of the *daf-2(lf)* background

To further investigate the expression patterns of *sod-2* and *sod-3* in the *daf-2(lf)* background we looked at the midbody region Figures 2.10 and 2.11 and the tail region Figure 2.12. We found that in the midbody region, *sod-3* expression can be detected in the body wall muscle in a pattern which is consistent with the expression of *sod-3* in the mitochondrial network. Also, *sod-2* may be expressed in the body wall muscle but only weakly (Figure 2.10). Additionally, *sod-2* expression can be detected in germ cells in a pattern that is consistent with expression in the mitochondrial network. Furthermore, *sod-2* expression can be detected in the vulval region and strong *sod-2* expression can be observed in sperm and oocytes (Figure 2.11). In the tail region, *sod-2* expression can be detected in the hypodermis in a pattern consistent with expression in the mitochondrial network and in a tail neuron. However, *sod-3* expression appears diffuse and is therefore likely a background signal (Figure 2.12). All the regions where *sod-2* and *sod-3* expressions were detected are summarized in Figure 2.13.

Overall, our findings show that *sod-2* and *sod-3* are expressed largely in different tissues with some overlap in neurons. Honda *et al* also showed that in the *daf-2(lf)* background in the head region, expression of *sod-2::rfp* and *sod-3::gfp* were distinct, separating the *sod-2::rfp* expressing cells from *sod-3::gfp* expressing cells with very small overlaps (Honda, Tanaka et al. 2008). Also, we found that *sod-3* expression is much weaker than *sod-2* expression (note that the illumination settings for *sod-2* and *sod-3* are not the same). In fact, given how weak it is, *sod-3* expression could no be reliably detected in the wild-type (N2) under these growth conditions. Nevertheless, *sod-3* expression could be seen in *daf-2(lf)* background. This is consistent with *sod-3* being an inducible *sod* which is known to be induced in *daf-2(lf)* mutants. Therefore, an expansion on the current study could examine how the expression of the *sod-2* and *sod-3* reporters is altered by inducers of ROS (like PQ) and other mutant backgrounds known to affect ROS levels such as *isp-1* and *nuo-6* mutations or other mutants of *sod* and catalase genes.

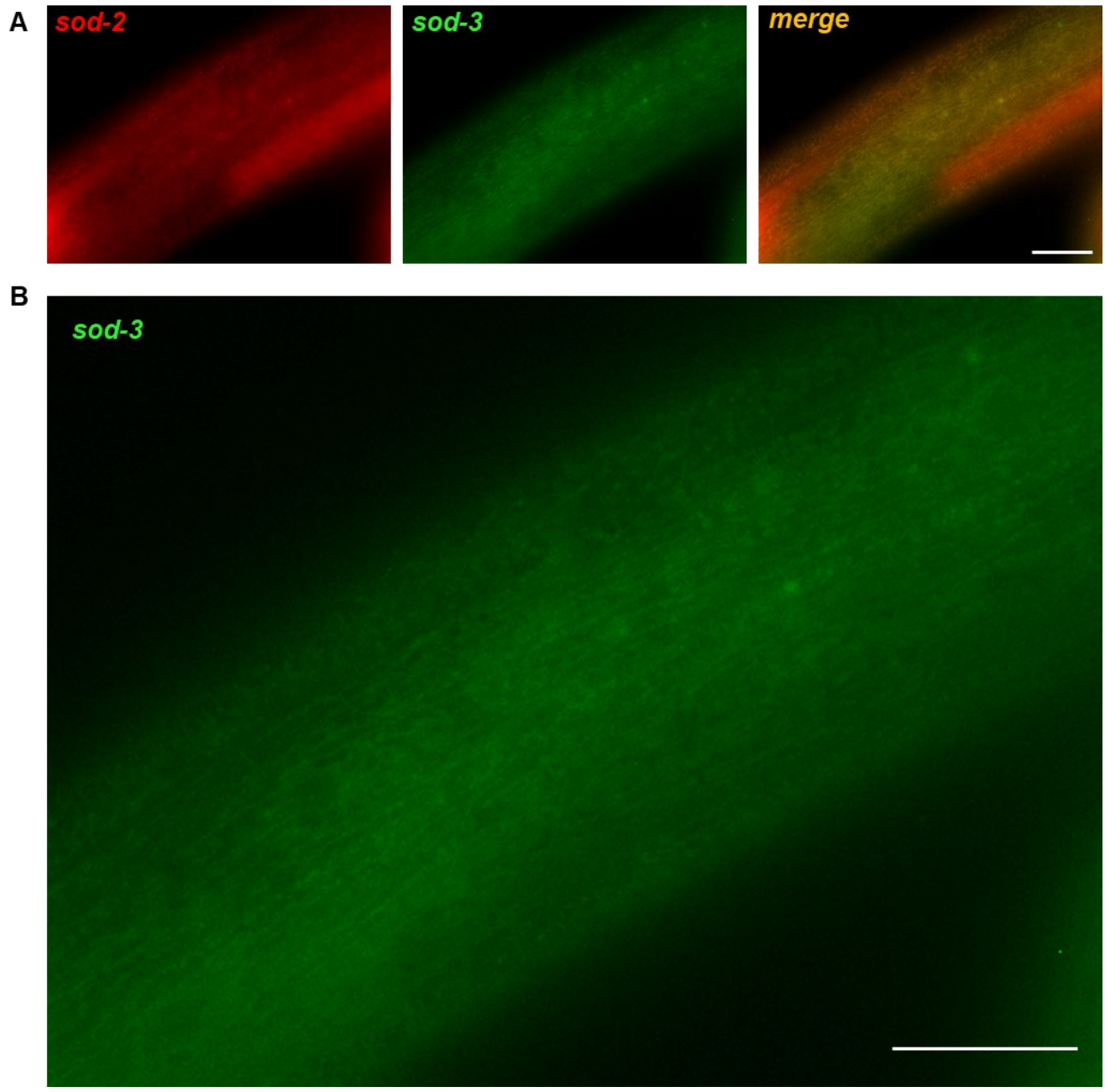


Figure 2.10. Expression of *sod-2* and *sod-3* in the midbody region of a *daf-2(lf)* adult. **(A)** In this plane of focus, *sod-3* expression can be detected in the body wall muscle in a pattern consistent with expression in the mitochondrial network. *sod-2* may also be expressed in the body wall muscle, but if so, only weakly. **(B)** Enlarged version of *sod-3* panel from A**.** Scale bars represent 20µm.

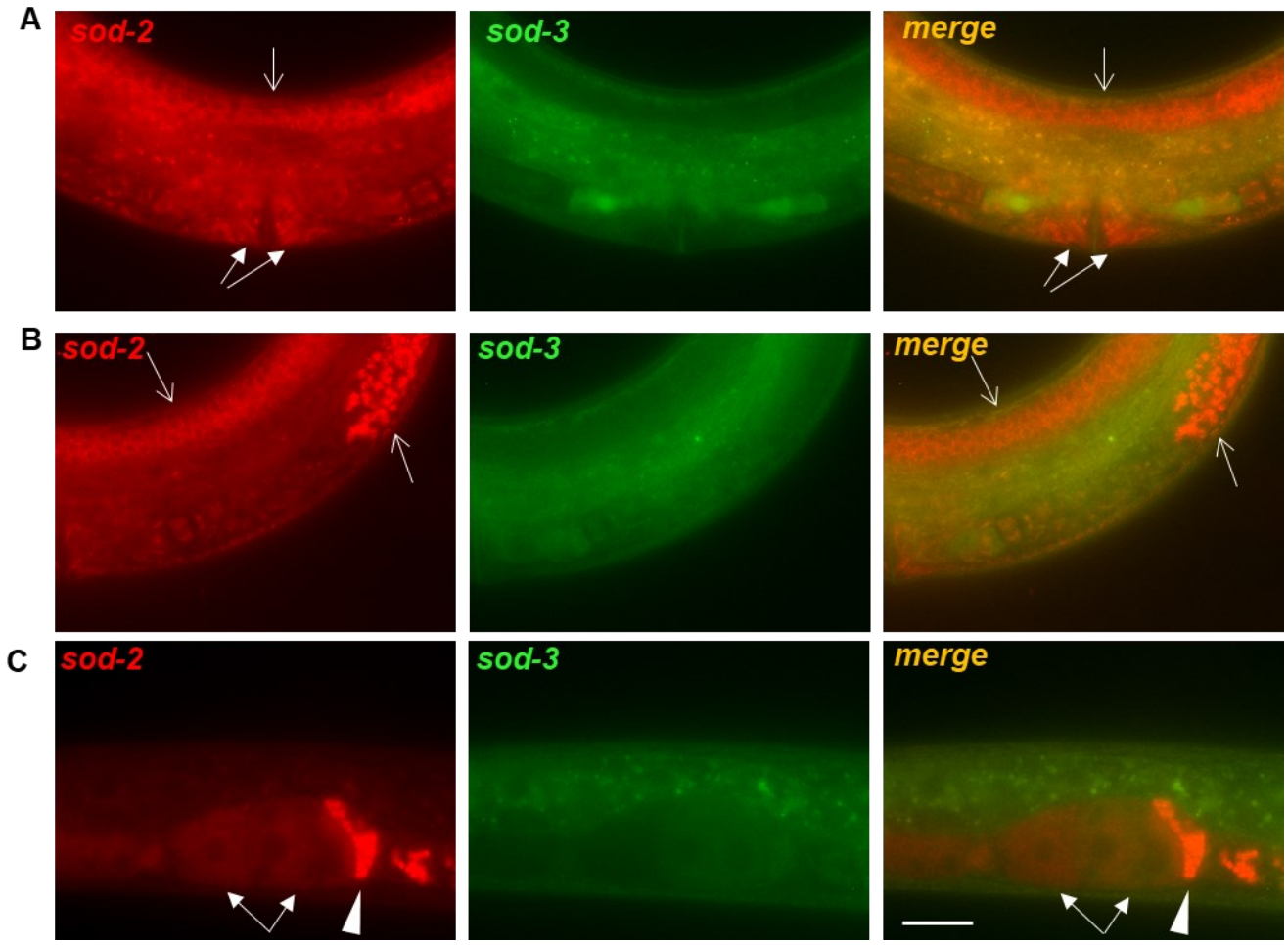


Figure 2.11. Expression of *sod-2* and *sod-3* in the midbody region of a *daf-2(lf)* adult. (A) In this plane of focus, *sod-2* expression can be detected in germ cells in a pattern consistent with expression in the mitochondrial network (open arrow). *sod-2* expression can also be detected in the vulval region (closed arrows), in what appears to be hypodermis, although it may also be vulval muscle. *sod-3* expression is not detected in any of these tissues. Note that *sod-2* and *sod-3* both appear to be expressed in the intestine but we cannot distinguish whether this is actually expression of the reporters or due to background and/or autoflourescence. **(B)** Expression of *sod-2* and *sod-3* in the midbody region of another *daf-2(lf)* adult. In this plane of focus, *sod-2* expression can be detected in germ cells in a pattern consistent with expression in the mitochondrial network (open arrow). As in (A), *sod-3* expression is not detected. **(C)** Expression of *sod-2* and *sod-3* in the midbody region of an older *daf-2(lf)* adult. In this plane of focus, strong *sod-2* expression can be detected in sperm (arrowheads) and in oocytes (closed arrows). Scale bar represents 20µm.

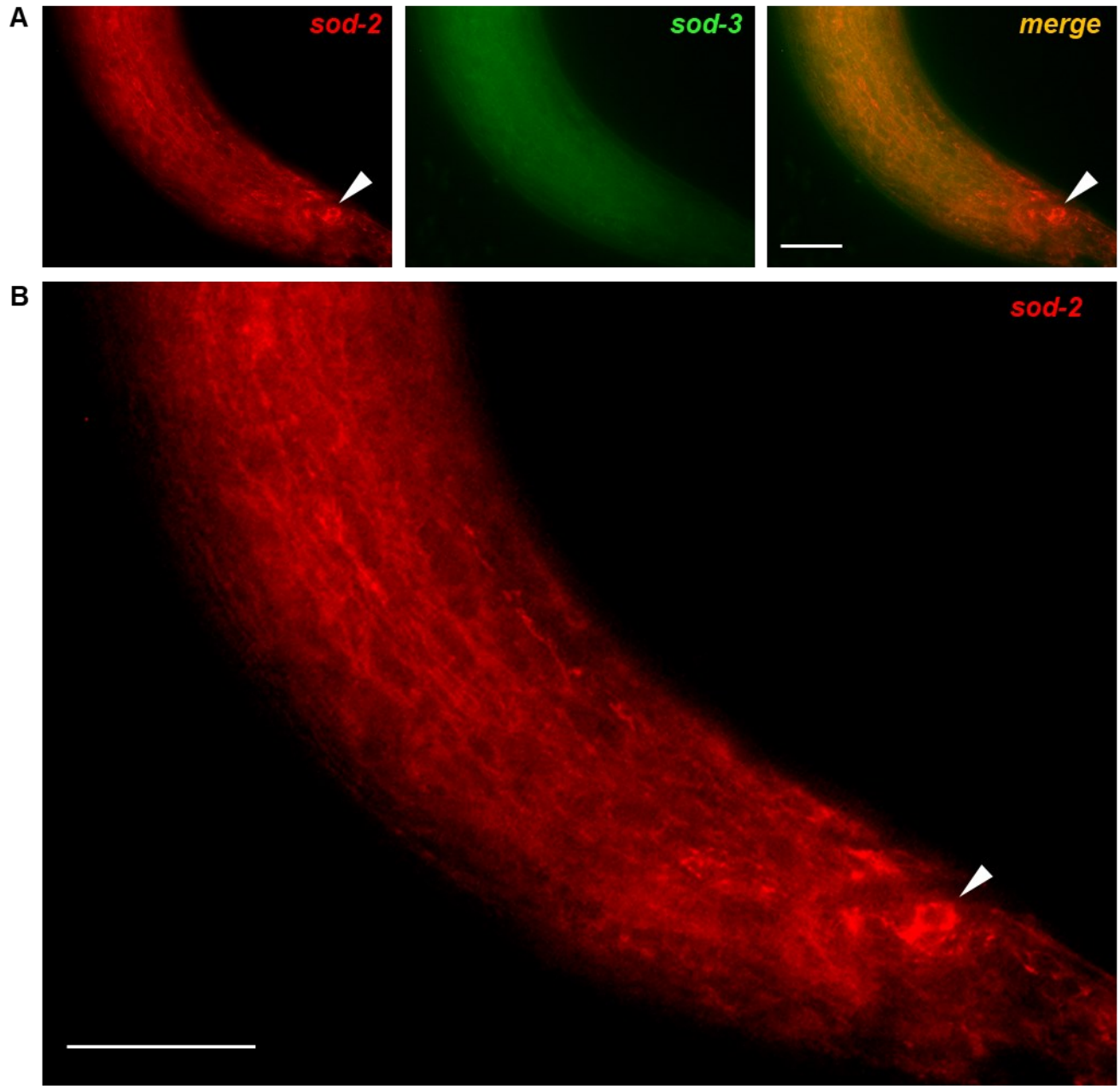


Figure 2.12. Expression of *sod-2* and *sod-3* in the posterior region of a *daf-2(lf)* adult. (A) In this plane of focus, *sod-2* expression can be detected in the hypodermis in a pattern consistent with expression in the mitochondrial network. *sod-3* expression appears diffuse and is therefore likely to be background signal. *sod-2* expression can be detected in a tail neuron (arrowhead) where *sod-3* expression cannot be detected. **(B)** Enlarged version of *sod-2* panel from A**.** Scale bars represents 20μm.

Tissue	*sod-2*	*sod-3*
neurons	strong	weak, some
muscle	weak or none	moderate
hypodermis	moderate	weak or none
germline	strong	weak or none

Figure 2.13. Summary of tissues in which expression of *sod-2* and *sod-3* have been observed. Although all images shown are in *daf-2* background, *sod-2* expression has also been examined in the wild-type (N2) background and appears to be the same as what is observed in the *daf-2(lf)* background.

2.3.8. PQ treatment of *sod-2* knockout mutants leads to developmental arrest

As mentioned before, we had previously established that 0.1mM PQ treatment dramatically extends wild-type's lifespan due to increased mitochondrial $O_2^{\bullet-}$ production (Yang and Hekimi 2010a). We had also found that the deletion of SOD-2 extends the lifespan of the worm (Van Raamsdonk and Hekimi 2009) as a result of increased mitochondrial $O_2^{\bullet-}$ levels (Van Raamsdonk and Hekimi 2012). *C. elegans* goes through 4 larval developmental stages (L1 – L4) before becoming fertile adults. In a previous study that tested oxidative stress resistance of *sod* mutants we had found that 0.2mM PQ treatment causes the *sod-2* mutants to go into developmental arrest at L1 stage (Van Raamsdonk and Hekimi 2009). However, during the course of the current studies we found that even 0.1mM PQ treatment, the optimal concentration of PQ that increases the lifespan of the wild-type, also causes the *sod-2* mutants to go into developmental arrest at L2 stage. Therefore, in addition to the canonical allele of *sod-2(ok1030),* we also tested another allele of *sod-2(gk257)* and obtained similar results (Figure 2.14).

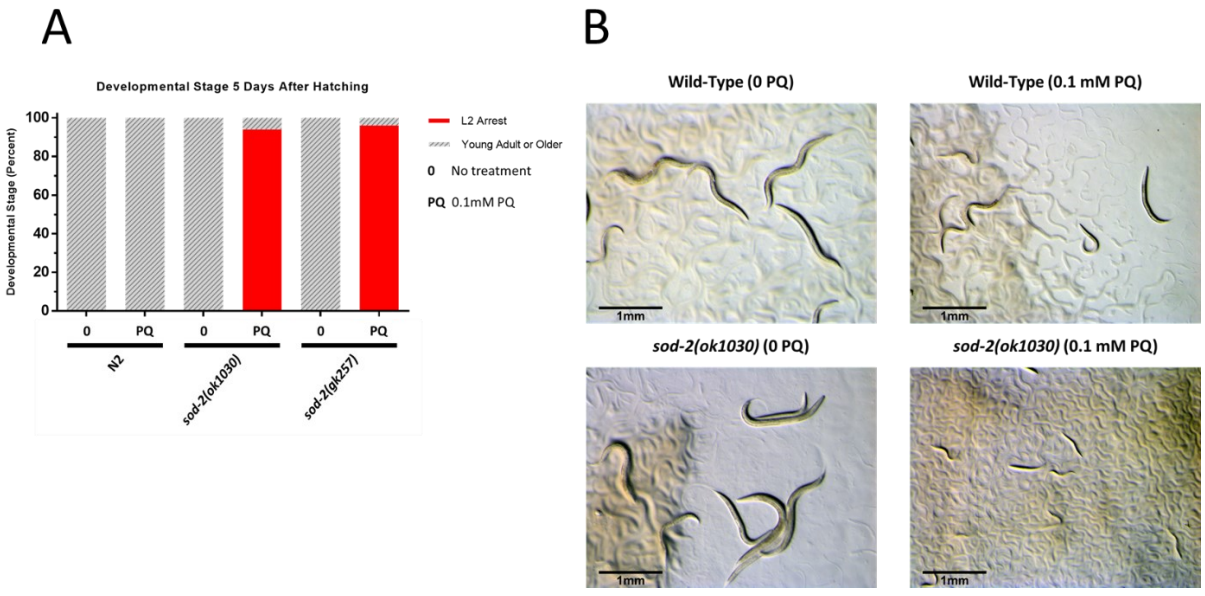


Figure 2.14. PQ treatment of *sod-2* knockout mutants leads to developmental arrest. (A) Both the canonical allele of *sod-2(ok1030)* and an alternative strain of *sod-2(gk257)* go into developmental arrest at L2 stage in reponse to 0.1mM PQ treatment. (B) Shows the development of the wild-type and *sod-2(ok1030)* 3 days after birth. While 0.1 mM PQ treatment slightly slows down the development of the wild-type, it causes developmental arrest in *sod-2* mutants. All conditions n=300 – 400. 3 independent trials. 100 - 150 worms per trial.

The developmental arrest of *sod-2* mutants is not reversible after five days after birth. Among all *sod* mutants of *C. elegans*, the PQ-induced developmental arrest is specific to *sod-2.* This is not surprising, given the function of SOD-2 as the worm's principal mitochondrial MnSOD and considering the function of PQ as $O_2^{\bullet-}$-generating agent within the mitochondria. However, further investigation described in the following sections suggest that the developmental arrest might not be merely a result of ROS toxicity and may be caused by the function of ROS in signaling.

2.3.9. Attempted manipulation of superoxide and peroxide levels with *sod-1*, *sod-3, ctl-1, 2* or *3*, or NAC produce only minor effects on the developmental arrest of *sod-2*

In the previous sections we discussed that while both SOD-2 and SOD-3 are the worm's mitochondrial MnSODs and their amino acid sequences are very similar, we have found that *sod-3* deletion shortens the lifespan of the wild-type and *sod-2* deletion lengthens it. Also, *sod-3* is epistatic to *sod-2* on lifespan (see section 2.3.5). Because of *sod-2* and *sod-3's* epistasis on lifespan, we tested whether removal of SOD-3 would affect the PQ-induced developmental arrest of *sod-2* mutants. We found that removing SOD-3 does not affect the developmental arrest in any way (Figure 2.15 A).

Next, we tested whether decreasing H_2O_2 levels can affect the PQ-induced arrest of *sod-2* mutants. We also found that removing SOD-1, the worm's main cytoplasmic SOD, does not affect the arrest; however, concurrent removal of SOD-1 and NAC treatment (which enhances H_2O_2 scavenging) exacerbates the arrest causing the *sod-1;sod-2* double-mutants to go into arrest earlier through the development at L1 rather than L2. On the contrary, treatment with NAC alone does not affect the arrest phenotype in either *sod-2(ok1030)* or *sod-2(gk257)* (Figure 2.15 A).

To further investigate the effects of H_2O_2 levels on the arrest phenotype, we sought to increase H_2O_2 levels by removing each of the three CTL enzymes in the *sod-2* mutant background. We found that removal of CTL enzymes also exacerbates the PQ-induced developmental arrest of *sod-2* so that the *sod-2;ctl-1, sod-2;ctl-2* and *sod-3;ctl-3* double mutants go into developmental arrest at the L1 stage instead of at L2 (Figure 2.15 B). Overall, all these responses are not consistent with the known function of NAC or the enzymes. Taken together these data show that neither increasing nor decreasing H_2O_2 levels can affect the PQ-induced developmental arrest in a

significant way. The most parsimonious explanation at this time is that excessive PQ-dependent generation of $O_2^{\bullet-}$ in mitochondria harboring SOD-2 produces an excessive signal that slows down the development of the *sod*-2 mutants to the point of arrest.

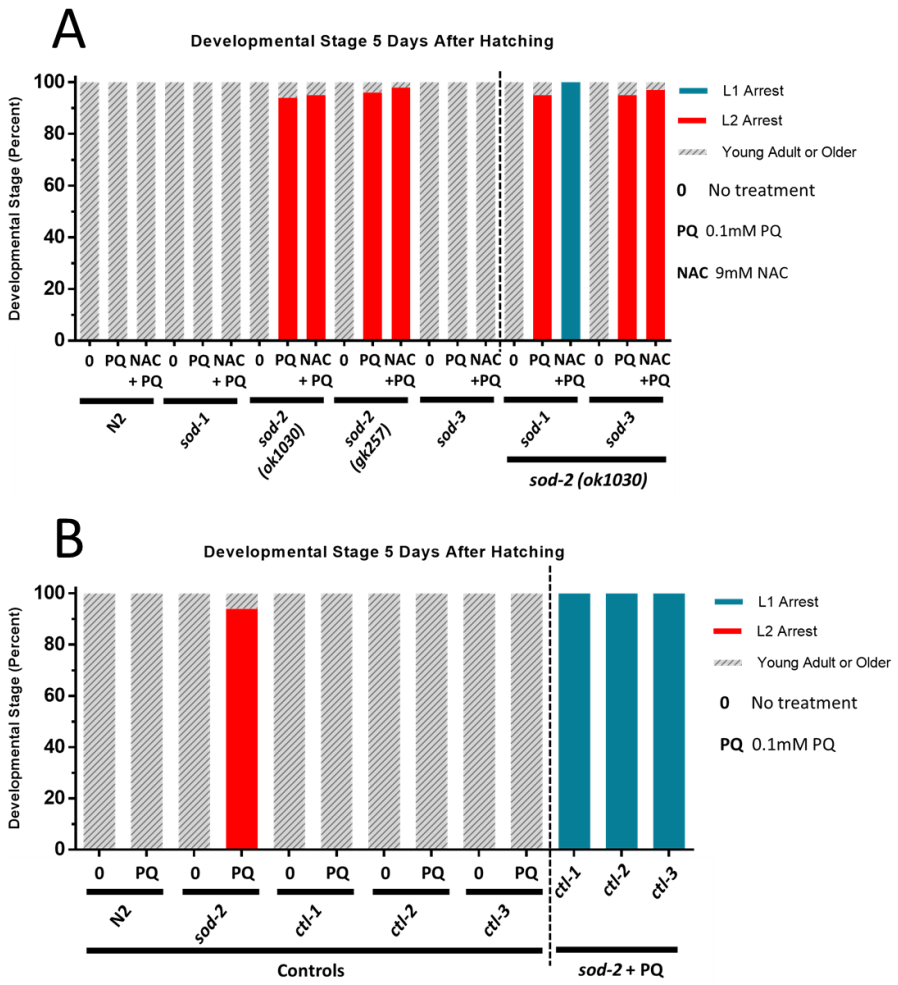


Figure 2.15. Attempted manipulation of superoxide and peroxide levels produce only minor effects with regards to the PQ-induced developmental arrest of *sod-2* mutants. (A) NAC treatment of *sod-2(ok1030)* or *sod-2(gk257)* does not affect the PQ-induced arrest phenoype of *sod-2*. Removal of SOD-1 (the worm's principal cytoplasmic SOD) or SOD-3 (the worm's inducible mitochondrial SOD) does not affect the PQ-induced arrest of *sod-2.* However, simultaneous removal of SOD-1 and NAC treatment exacerbates the arrest, causing the *sod-1;sod-2* double mutants to arrest at L1 stage. (B) Removal of each of the three CTL enzymes from *sod-2* background also exacerbates the arrest phenotype causing the *sod-2;ctl-x* double-mutants to go into arrest at L1 instead of L2, when PQ normally induces arrest in *sod-2* mutants. All conditions n=300 – 400. 3 independent trials. 100 - 150 worms per trial.

2.3.10. A *let-60rasgf* mutation partially prevents the PQ-induced arrest of *sod-2*

Ras proteins are molecular switches that are turned on and off through a regulated GDP/GTP cycle. These proteins are important regulators of cell proliferation and differentiation. All Ras protein family members act as intermediates that mediate the signal from upstream tyrosine kinases to a downstream targets in order to activate nuclear factors that control gene expression and protein synthesis (Scheffzek and Shivalingaiah 2019).

In *C. elegans,* Ras is encoded by the *let-60* gene which is responsible for a variety of developmental processes, including vulva development (Sternberg and Han 1998). A gain-of-function mutation of *let-60* (shown as *let-60rasgf* for short) leads to the multi-vulva phenotype in the worm (Figure 2.16 A).

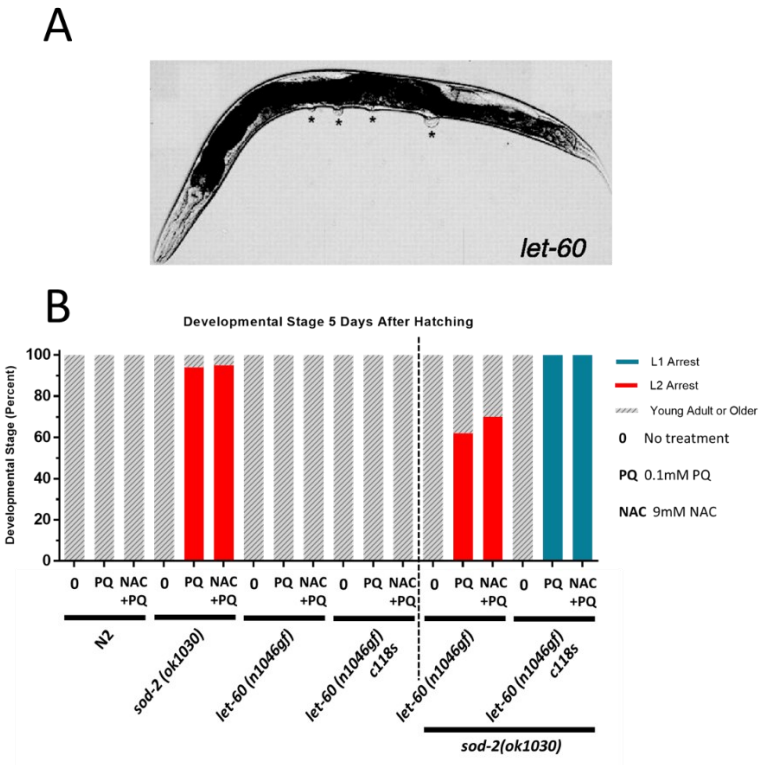


Figure 2.16. A *let-60ras* gain-of-function mutation partially prevents the PQ-induced developmental arrest of *sod-2.* (A) A *let-60rasgf* mutation causes a multi-vulva phenotype in *C. elegans.* Photo from (Shibata, Branicky et al. 2003) (B) *let-60rasgf* partially suppresses the PQ-induced developmental arrest of *sod-2*; however, substituting an active cysteine residue (C118) by serine in *sod-2;let-60rasgf* background and thus eliminating the H_2O_2-dependent inhibition of LET-60rasgf, compeletely restores the PQ-induced arrest phenotype of *sod-2* and *sod-2;let-60rasgf-c118s* go into developmental arrest at L1 stage. All conditions n=300 – 400. 3 independent trials. 100 - 150 worms per trial.

We have shown previously, that the activity of *let-60ras* is redox regulated (Shibata, Branicky et al. 2003). In order to investigate whether the PQ-induced arrest of *sod-2* involves a redox signaling element during the development, we tested if *sod-2;let-60rasgf* also goes into developmental arrest when exposed 0.1mM PQ. We found that indeed a *let-60gf* mutation is able to partially suppress the arrest phenotype (Figure 2.16 B). The partial rescue of the PQ-induced *sod-2* arrest by a *let-60rasgf* mutation argues for the involvement of a developmental signaling process that at least in part goes through the Ras signaling pathway. In the following section, we describe experiments that attempt to investigate the role of Ras signaling in the arrest by manipulating a ROS-sensitive cysteine switch in the LET-60 protein.

2.3.11. Removing H_2O_2-dependent inhibition of LET-60ras gain-of-function restores full developmental arrest

In studies conducted in our laboratory in connection with the role of *let-60ras* in vulva development and lifespan, we found that a cysteine residue (C118) in LET-60ras is a redox sensitive cysteine switch (see section 1.3.5 for more details on redox sensitive cysteine residues) which can be oxidized by H_2O_2. This redox active cysteine residue enables LET-60 to be controlled through redox signaling (unpublished ongoing work by Maximillian Kramer-Drauberg and Dr. Robyn Branicky). In order to test whether removing the H_2O_2-dependent inhibition of LET-60rasgf can alter its effect on the arrest phenotype we introduced a mutation in *sod-2;let-60rasgf* background that substituted the redox active C118 with serine (designated as *let-60rasgf*-C118S). This substitution eliminates the redox sensitivity of LET-60rasgf. We found that 0.1mM while the arrest phenotype is partially suppressed in *sod-2;let60rasgf,* treatment of *sod-2;let-60gf*-C118S with 0.1 mM PQ causes the double mutants to fully go into developmental arrest (Figure 2.16 B). In other words, the partial rescue of the arrest by *let-60rasgf* and is suppressed by inactivating the redox sensitivity of the protein. Furthermore, as a result of the treatment, *sod-2;let-60rasgf*-C118S double mutants arrest earlier at the L1 stage rather than the L2 stage. Additionally, treatment with NAC does not affect the arrest in either *sod-2;let-60rasgf* or *sod-2;let-60rasgf*-C118S. Although no explicit model is yet available, the findings with *let-60rasgf* reinforce the likelihood that the PQ-induced arrest phenotype of *sod-2* is the result of faulty signaling and not a less specific response to damage. Next, since manipulating H_2O_2 levels does not affect the arrest phenotype (see section 2.3.9) we wondered whether manipulating $O_2^{\bullet-}$ levels can affect the arrest phenotype.

Our findings regarding the effects of increased $O_2^{\bullet-}$ on the PQ-induced arrest of *sod-2* are described in the following section.

2.3.12. Associating the loss of SOD-2 with electron transport chain mutations (*isp-1*, *nuo-6* and *gas-1*) fully suppresses the PQ-induced developmental arrest

As we discussed in section 1.5.1, *isp-1* and *nuo-6* mutants are long-lived mitochondrial mutants of *C. elegans* whose longevity is ROS-dependent. These ETC mutants have increased mitochondrial $O_2^{\bullet-}$ production and lower metabolic rates (Yang and Hekimi 2010a, Yang and Hekimi 2010b). *isp-1* encodes a Rieske iron sulfur protein in mitochondrial ETC complex III (Feng, Bussière et al. 2001) and *nuo-6* encodes a subunit of complex I (Yang and Hekimi 2010b).

gas-1 is another ETC mutant of complex I which encodes for a 49 kDa subunit of complex I (Morgan and Sedensky 1994, Kayser, Morgan et al. 1999). In contrast to the long-lived *isp-1* and *nuo-6* ETC mutants, *gas-1* are short-lived compared with the wild-type (Hartman, Ishii et al. 2001). However, *gas-1* mutants have also been reported to have increased $O_2^{\bullet-}$ levels in sub-mitochondrial particles despite a decrease in $O_2^{\bullet-}$ in intact mitochondria likely due to increased mitochondrial SOD expression that effectively removes $O_2^{\bullet-}$ from their mitochondria (Kondo, Senoo-Matsuda et al. 2005).

In order to investigate the effects of high mitochondrial $O_2^{\bullet-}$ production on the PQ-induced arrest of *sod-2*, we treated the *isp-1;sod-2, nuo-6 sod-2* and *gas-1;sod-2* with 0.1mM PQ from birth. Surprisingly, we found that all three ETC mutations (*isp-1, nuo-6* and *gas-1*) completely suppress the arrest phenotype (Figure 2.17 A).

Then we wondered whether $O_2^{\bullet-}$ resistance may enable these mutations to suppress the PQ-induced arrest of *sod-2*. To find out whether this is the case, we treated all the double mutants that partially suppressed (*sod-2;let-60gf* see section 2.3.10) or completely suppressed (*isp-1;sod-2, nuo-6 sod-2* and *gas-1;sod-2*) the arrest phenotype with 1 mM PQ. We found that all the strains that are exposed to high concentration of PQ go into developmental arrest at L1 stage, including the wild-type (Figure 2.17 B). Therefore, the finding that *isp-1, nuo-6* and *gas-1* completely rescue the PQ-induced *sod-2* arrest cannot be due to PQ-resistance by these mitochondrial ETC mutations.

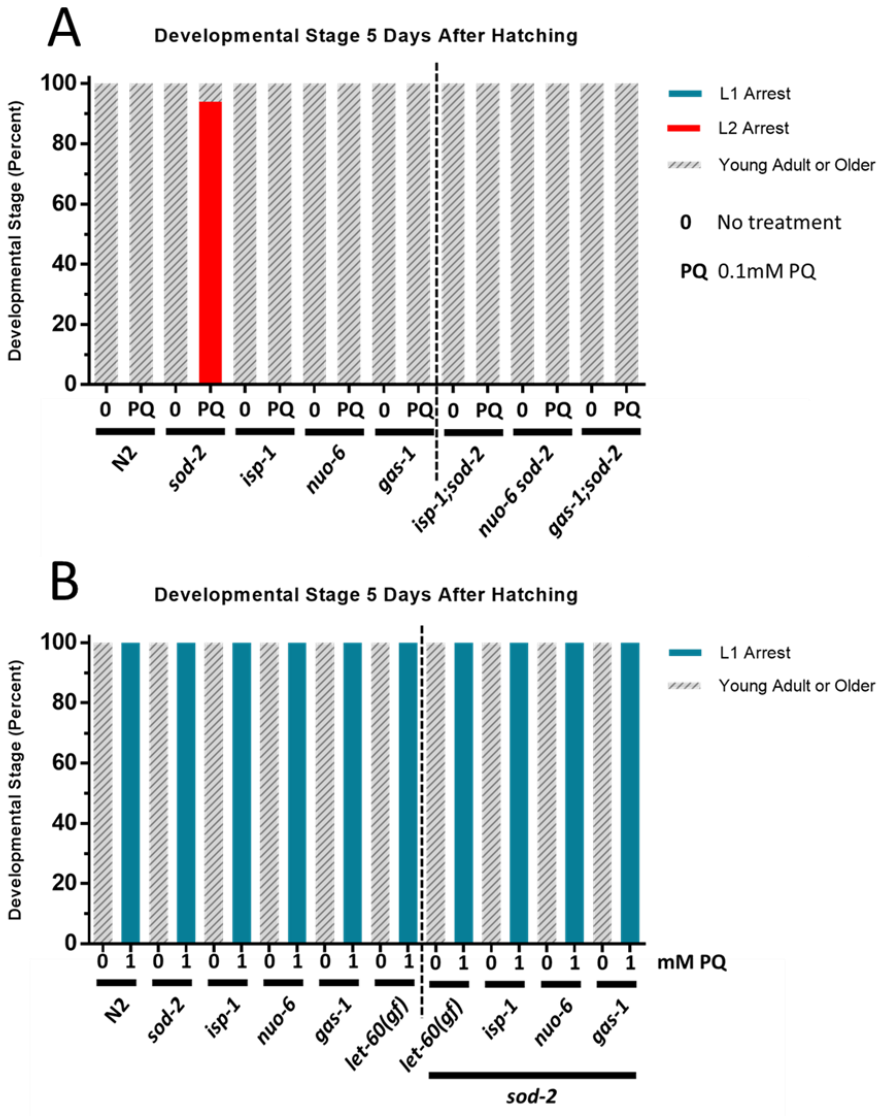


Figure 2.17. Mitochondrial electron transport chain mutations *isp-1, nuo-6* and *gas-1* suppress the PQ-induced arrest of *sod-2* mutants. (A) Mitochondrial ETC mutations that affect $O_2^{\bullet-}$ metabolism, completely suppress the arrest phenotype. (B) All the tested strains including those that partially or completely suppress the PQ-induced arrest of *sod-2*, go into developmental arrest at L1 stage when treated with 1 mM PQ. All conditions n=300 – 400. 3 independent trials. 100 - 150 worms per trial.

The finding that the mitochondrial ETC mutations that cause increased $O_2^{\bullet-}$ production can suppress the PQ-induced arrest of *sod-2* mutants is unexpected. First, these findings reaffirm the view that simple damage is not the cause of the arrest phenotype as the mutations that are able to suppress the arrest at very low dose of PQ (0.1 mM) are not able to do so at a higher concentration (1 mM) and altered mitochondrial $O_2^{\bullet-}$ metabolism plays a role in preventing the arrest. Second,

the data shows that the relationship between ETC mutations, mitochondrial $O_2^{\bullet -}$ and H_2O_2 generation are not understood. Understanding this relationship was part of the motivation that led to the studies that are described in chapter 3 of this thesis where we explore these questions in detail in connection with lifespan.

2.4. Discussion

2.4.1. Opposite effects of removing SOD-2 and SOD-3

As we saw in section 2.1.2, *sod-2* and *sod-3* encode the worm's two mitochondrial SODs and exhibit an 86% homology (Suzuki, Inokuma et al. 1996, Hunter, Bannister et al. 1997) in their amino acid sequences (also see Figure 2.1). Despite this similarity in sequences, we have observed that deletion of these genes has opposite effects on lifespan. The loss of *sod-2* lengthens the lifespan of the worms while the loss of *sod-3* shortens it. In both cases, different data has been reported in previous studies. For example, while some studies, similar to our results, report a dramatic increase in lifespan after deletion of *sod-2* (Van Raamsdonk and Hekimi 2009, Van Raamsdonk and Hekimi 2012), others report a very small increase in lifespan (Dingley, Polyak et al. 2010) or even no change in lifespan (Doonan, McElwee et al. 2008, Honda, Tanaka et al. 2008) in *sod-2* knockout mutants compared to the wild-type. Previous studies on the effects of *sod-3* deletion on lifespan also report conflicting results. For example, some studies indicate no change in lifespan compared to the wild-type in the absence of SOD-3 (Honda, Tanaka et al. 2008, Van Raamsdonk and Hekimi 2012, Yee, Yang et al. 2014) while others show even a slight increase in the lifespan compared to the wild-type (Yen, Patel et al. 2009, Dingley, Polyak et al. 2010). Our data, however, consistently shows that deletion of *sod-3* results in the shortening of the adult lifespan in *sod-3* mutants. Two questions arise from the comparison of all these studies. First, why there is so much variety in the data obtained in different studies regarding these two MnSOD enzymes? Second, why the removal of two proteins that are both localized to the mitochondria and have similar sequences has opposite effects on lifespan?

To address the inconsistency between the data from different studies, we believe it is primarily due to the difference in experimental conditions. For example, the compound FUDR, a commonly utilized chemical that induces parental sterility in worms, is used in one of our previous studies that reports no change in the lifespan of *sod-3* mutants compared to the wild-type (Van Raamsdonk and Hekimi 2009). Although FUDR does not seem to affect the lifespan of the wild-type, we and others have shown that it can affect the lifespan of several *C. elegans* mutants (Aitlhadj, Stürzenbaum et al. 2010, Van Raamsdonk and Hekimi 2011, Rooney, Luz et al. 2014, Anderson, Corkins et al. 2016). During the course of our studies described in chapter 3, we have also shown that FUDR lengthens the lifespan of *sod-1* and *sod-1;sod-2* mutants (see section

3.3.2.4). Nevertheless, FUDR is widely used in lifespan studies that involve *C. elegans* because it considerably eases the labor-intensive nature of these studies by preventing the eggs from hatching and therefore eliminating the need for transferring the worms to new plates every day or every other day in order to prevent the mixing of adults with their progeny. In the studies that we describe in this thesis we did not use FUDR (other than as a treatment in one experiment) precisely to avoid its influence on the lifespan data. Therefore, it is reasonable to believe one of the factors that could account for different results among all these studies is the use of FUDR.

Another plausible reason for varying results between different studies is the different alleles used in the experiments. In our studies we used the canonical alleles for *sod-2* and *sod-3*: *sod-2(ok1030), sod-2(gk257)*, *sod-3(tm760), sod-3(gk235)* and confirmed the lifespan data using both alleles to show that *sod-2* lengthens the adult lifespan and *sod-*3 shortens it. However, in Honda *et al* study that reports no change in the lifespan for both *sod-2* and *sod-3* compared to the wild-type, the authors had used a pair of deletion alleles of *sod-2(sj173)* and *sod-3(sj134)* that they had constructed and were never used again in any other lifespan studies (Honda, Tanaka et al. 2008). This study also uses experimental conditions that differed from ours. For example, they used UV-killed bacteria (Honda, Tanaka et al. 2008), while our standard protocol for lifespan experiments uses live bacterial cultures as the food source for the worms (also see section 2.2). Additionally, in one study that reported a slight lengthening of lifespan for *sod-3(gk235)* the total sample size was too small (n=56) (Dingley, Polyak et al. 2010) which could increase the chance of erroneous results. One more factor to consider is that we measured the adult lifespan of the worms because different genetic backgrounds such as *sod-2* or PQ treatment affect the length of the developmental period in the worms. By measuring the adult lifespan, we made sure these variations in the developmental rate of the worms do not interfere with lifespan data. Sometimes other studies do not make it clear whether adult lifespan is measured, or the developmental period of the worms is also included (total lifespan). Therefore, we believe not using FUDR which is known to affect lifespan (but alleviate the labor-intensive nature of lifespan experiments), using canonical, well-characterized alleles of the genes, large sample sizes in our experiments and measuring the adult lifespan instead of total lifespan have greatly contributed to obtaining consistent results throughout the course of our studies presented in this thesis.

This brings us to our second question. Why deletion of *sod-2* lengthens the lifespan of the wild-type but deletion of *sod-3* shortens the lifespan of the wild-type when both have such similar

sequences? To answer this question, we need to consider several factors. First, the expression levels of these two proteins need to be considered. While *sod-2* is the main MnSOD of the worm and its contribution to the total *sod* mRNA in the wild-type is about 18%, *sod-3* is the inducible MnSOD and *sod-3* mRNA account for only 1% of the total *sod* mRNA in the wild-type. Additionally, in the absence of SOD-2, MnSOD activity in the wild-type could not be detected, which shows very low levels of SOD-3 activity in the absence of SOD-2 (Doonan, McElwee et al. 2008). Also, in long-lived mutants of the insulin signaling pathway such as *daf-2(lf)* and *age-1,* although the *sod-3* transcript is upregulated (Honda and Honda 1999, Honda and Honda 2002, Yanase, Yasuda et al. 2002), and *sod-3* mRNA levels are elevated in dauer larvae and *daf-2(lf)* long-lived mutants (Honda and Honda 1999, Dong, Venable et al. 2007), the relative contribution of SOD-3 to both the overall SOD activity and MnSOD protein levels is very small (Doonan, McElwee et al. 2008). Additionally, in these studies neither decreasing (Doonan, McElwee et al. 2008, Honda, Tanaka et al. 2008, Van Raamsdonk and Hekimi 2009) nor increasing (Henderson, Bonafe et al. 2006) *sod-3* expression has had any effect on lifespan. But the expression of a *sod-3::gfp* reporter is proposed to be the best predictor of remaining lifespan from a set of genes whose expression is age-dependent (Sanchez-Blanco and Kim 2011). These data indicate that while SOD-2 functions as the worms main mitochondrial MnSOD, SOD-3 may not be purely function as a $O_2^{\bullet-}$ detoxifying enzyme and its role in ROS signaling may be more pronounced.

Second, there is evidence of epistasis between *sod-2* and *sod-3*. The study by Honda *et al* (also see section 2.1.6) and our study on the same subject (also see section 2.3.6) show that there is an epistatic relationship between *sod-2* and *sod-3* with regards to lifespan in *daf-2(lf)* long-lived background. Our findings regarding the epistasis between *sod-2* and *sod-3* in lifespan in the wild-type background reaffirm this connection. Therefore, taken together, the inducible nature of *sod-3,* its small contribution to overall antioxidant SOD activity and the epistasis between *sod-2* and *sod-3* in lifespan suggest that despite their sequence homology, they may perform more specialized duties in the absence of which lifespan is lengthened for *sod*-2 mutants or shortened in *sod-3* mutants.

Third, the opposite reactions of *sod-2* and *sod-3* mutants to mild increase in ROS levels strongly argue for this view that SOD-2 and SOD-3 functionally perform different roles with regards to lifespan. That SOD-2 and SOD-3 occupy different functional niches is further demonstrated by our data that shows increasing ROS levels via PQ treatment lengthens the lifespan

of *sod*-3 mutants in the same way that it increases the lifespan of the wild-type, however, there is one distinction: the lifespan shortening effect of the loss of SOD-3 is additive to the lifespan lengthening effect of PQ treatment. On the contrary, as we discussed in section 2.3.3, there is no concentration of PQ that can lengthen the lifespan of *sod-2* mutants and the lowest concentration of PQ that allows for the normal development of *sod-2* mutants, suppresses their longevity. The opposite reaction of *sod-2* and *sod-3* mutants to increased $O_2^{\bullet-}$ levels is another indication of their functional diversity.

Another factor that needs to be considered when addressing the different effects of *sod-2* or *sod-3* deletion on lifespan is the spatial and possibly temporal differences in their expression. Data on tissue specific expression patterns for SOD-2 and SOD-3 is scarce and in previous studies relies on extrachromosomal expression of fluorescent-tagged proteins. Based on these early studies, SOD-2 and SOD-3 expression has been observed in the pharynx, intestinal cells and hypodermal cells of the worm (Henderson, Bonafè et al. 2006, Doonan, McElwee et al. 2008). It is not clear, however, whether SOD-2 and SOD-3 are colocalized to the same tissues or mitochondria (Honda, Tanaka et al. 2008). Our studies described in section 2.3.7 using endogenously tagged SOD-2 and SOD-3 also demonstrate that these two MnSODs are expressed in different tissues. Therefore, it stands to reason that another factor which can contribute to the opposite effects of *sod-2* or *sod-3* deletion on lifespan is the tissue specificity of their expression. Finally, our findings show that *sod-2* and *sod-3* interact on lifespan with an age-dependent epistatic pattern (described in section 2.3.5). This is represented in a biphasic aging curve where the younger *sod-2;sod-3* double mutants have a high mortality rate (similar to *sod-3* mutants). However, in the older *sod-2;sod-3* mutants the mortality rate decreases and they are able to live as long as *sod-2* mutants. In other words, younger adults in the wild-type background need SOD-3 to fully benefit from the longevity effect of the loss of SOD-2. But older worms (the second phase of the aging curve) do not need SOD-3 to benefit from the pro-longevity effect of the loss of SOD-2. This implies that there might be a time-specific aspect to the responsibilities of SOD-3, as well.

2.4.2. Age-dependent interaction between SOD-2 and SOD-3 to modulate lifespan

As we discussed before, in previous studies on lifespan of *sod-2;sod-3* double mutants loss of SOD-3 has either slightly shortened the longevity of long-lived *sod-2* mutants (Van Raamsdonk

and Hekimi 2009) or has not affected the lifespan of *sod-2* in studies where *sod-2* is not long-lived at all (Doonan, McElwee et al. 2008, Honda, Tanaka et al. 2008). In our studies however, we observe an age-dependent biphasic pattern in the lifespan of *sod-2;sod-3* which suggests that in the *sod*-2 background, young worms need SOD-3 in order to benefit from the pro-longevity effect of loss of SOD-2. However, as the worms become older, their reliance on SOD-3 for benefiting from the longevity effect of the loss of SOD-2 disappears and the maximum lifespan of *sod-2;sod-3* is similar to that of *sod-2*. Is this age-dependency on SOD-3 for longevity due to the role of SOD-3 in ROS metabolism during the early stages of adulthood? Let's explore the answers to this question.

We showed that the loss of SOD-3 results shortens the adult lifespan in the wild-type background (also see section 2.3.1) but this lifespan shortening effect is additive to the lifespan-lengthening effect of the pro-longevity ROS induced by PQ-treatment (also see section 2.3.2). This additivity remains unchanged in PQ dose response experiments and in experiments where PQ is administered only after the worms reach young adulthood (also see section 2.3.4). Furthermore, when *sod-3* mutants are treated with PQ from birth their average adult lifespan is lengthened by about 36% while the wild type's average adult lifespan is lengthened by appx. 25% when treated with the same dosage of PQ from birth. On the other hand, when PQ treatment starts at young adulthood for both *sod-3* mutants and the wild-type, the percent change increase in average adult lifespan for both backgrounds is very similar: an 11% increase for *sod-3* and a 10% increase for the wild-type. This has three implications. First, both the wild-type and *sod-3* benefit from the pro-longevity effect of PQ treatment in the same way. Second, the intensity of the pro-longevity effect of PQ treatment depends on the duration of the treatment. And third, the average lifespan of *sod-3* increases more dramatically than the wild-type (36% vs 25%) when they are exposed to elevated ROS levels from birth but the increase in the average lifespan of both *sod-3* and the wild-type is similar when elevated ROS levels are introduced at the young adult stage (11% vs 10%). This last point is interesting because it suggests that at certain points during development, SOD-3 is required to produce ROS (perhaps in a tissue specific manner). When it is not present, PQ treatment partially compensates for the ROS deficiency, hence the 36% increase in average lifespan for *sod-3* versus the 25% for the wild-type. However, when worms are past young adulthood, the lack of SOD-3 hardly has any effect on the percent change in their average adult lifespan. Therefore, both *sod-3* and the wild-type benefit from pro-longevity ROS equally when PQ treatment begins after young

adulthood. This implies the age-dependency of SOD-3 requirement. In the *sod-2;sod-3*'s biphasic lifespan curve a similar pattern of age-dependency on SOD-3 is also evident. Younger worms need SOD-3 to benefit from the pro-longevity effect of SOD-2, however, as they become older this dependency on SOD-3 disappears and those double-mutants that survive later into the adulthood live just as long as *sod-2* mutants.

In *daf-2(lf)* background, however, the effects of the loss of SOD-2 and SOD-3 are reversed compared to the wild-type background. In our studies, in the wild-type background, the loss of SOD-3 shortens the lifespan of *sod-2*. However, in *daf-2(lf)* background, the loss of SOD-3 lengthens the lifespan of *sod-2*. This reversal could be due to the altered physiology of mitochondrial $O_2^{\bullet-}$ metabolism in *daf-2(lf)* background compared to the wild-type background. In *daf-2(lf)* background, there is a detectable increase in mitochondrial $O_2^{\bullet-}$ (Yang and Hekimi 2010a). It could also be related to the increased expression of *sod-3* in the *daf-2(lf)* mutants. In both cases, however, the results are consistent with epistasis between *sod-2* and *sod-3* and the complexity and the magnitude of the effects suggest a signaling interaction between the two MnSODs to regulate lifespan.

2.4.3. PQ-induced arrest of *sod-2*: ROS damage or signaling?

Given the function of SOD-2 as the worm's major mitochondrial MnSOD (Doonan, McElwee et al. 2008, Gems and Doonan 2009) it is not surprising that the mutants are sensitive to $O_2^{\bullet-}$ produced by PQ mainly in the mitochondrial matrix (Castello, Drechsel et al. 2007, Cocheme and Murphy 2008). Our current and previous studies (Van Raamsdonk and Hekimi 2009) show that among all five mutants of *sod* genes, *sod-2* mutants are the only ones that go into developmental arrest by very low dose PQ treatment. However, what is surprising is that mitochondrial mutants such as *isp-1, nuo-6* and *gas-1* that alter $O_2^{\bullet-}$ metabolism in mitochondria are able to completely rescue the PQ-induced arrest of *sod-2* (also see section 2.3.12). Another mutation that can partially rescue the arrest is a *let-60rasgf* mutation (also see section 2.3.10) but removing the H_2O_2-dependent inhibition of LET-60rasgf restores the complete PQ-induced arrest of *sod-2* (also see section 2.3.11). However, we have shown that manipulating H_2O_2 levels via introduction of *ctl* mutations, *sod-1* or *sod-3* mutations or NAC treatment does not result in major changes regarding the arrest. Taken together, for the following reasons we are believe it is more likely that the PQ-induced *sod-*

2 developmental arrest is due to faulty $O_2^{\bullet-}$ signaling rather than being caused purely by damage of excessive ROS:

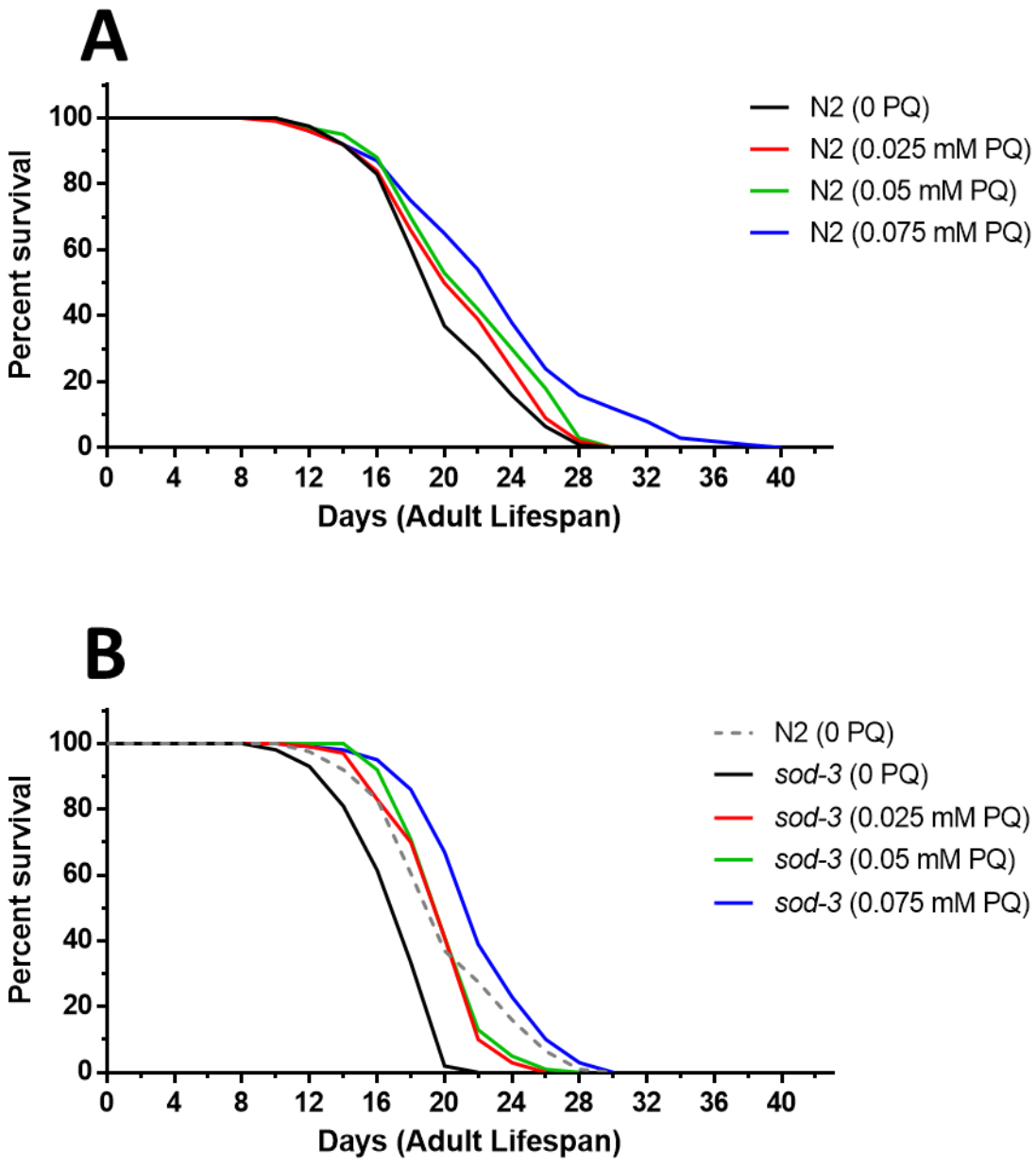
1. Increasing or decreasing H_2O_2 levels via genetic and/or chemical means does not cause any major changes in the arrest phenotype which leads us to believe that $O_2^{\bullet-}$ must be the cause of the arrest.
2. Mutations like *isp-1, nuo-6* and *gas-1,* which specifically alter the mitochondrial $O_2^{\bullet-}$ metabolism, are able to completely rescue the arrest phenotype.
3. A *let-60rasgf* mutation that is connected to ROS signaling (Shibata, Branicky et al. 2003) can partially rescue the developmental arrest; however, upon losing its ROS-sensitive cysteine switch, the protein loses its ability to suppress the arrest phenotype.
4. All strains that we tested, including the wild-type, go into developmental arrest when treated with high doses of PQ and none of the mutations mentioned above can rescue this condition. This demonstrates the damaging effects of excessive ROS, which cannot be rescued.

2.4.4. Statement connecting chapter 2 to chapter 3

Chapter 2 was dedicated to the analysis of the regulation of lifespan in *C. elegans* by its two mitochondrial MnSODs and the PQ-induced arrest phenotype of *sod-2* mutants. In the next chapter, we will explore the regulation of lifespan by ROS-handling enzymes further by systematically dissecting parts of the ROS network of the worm on a quest to find the subcellular compartments and ROS-handling enzymes that are involved in the transduction of the mitochondrial $O_2^{\bullet-}$ signal that causes longevity in mutants like *sod-2, isp-1* and *nuo-6.*

2.5. Supplementary data for chapter 2

2.5.1. Lifespan curves for dose response experiments described in sections 2.3.2 and 2.3.3.



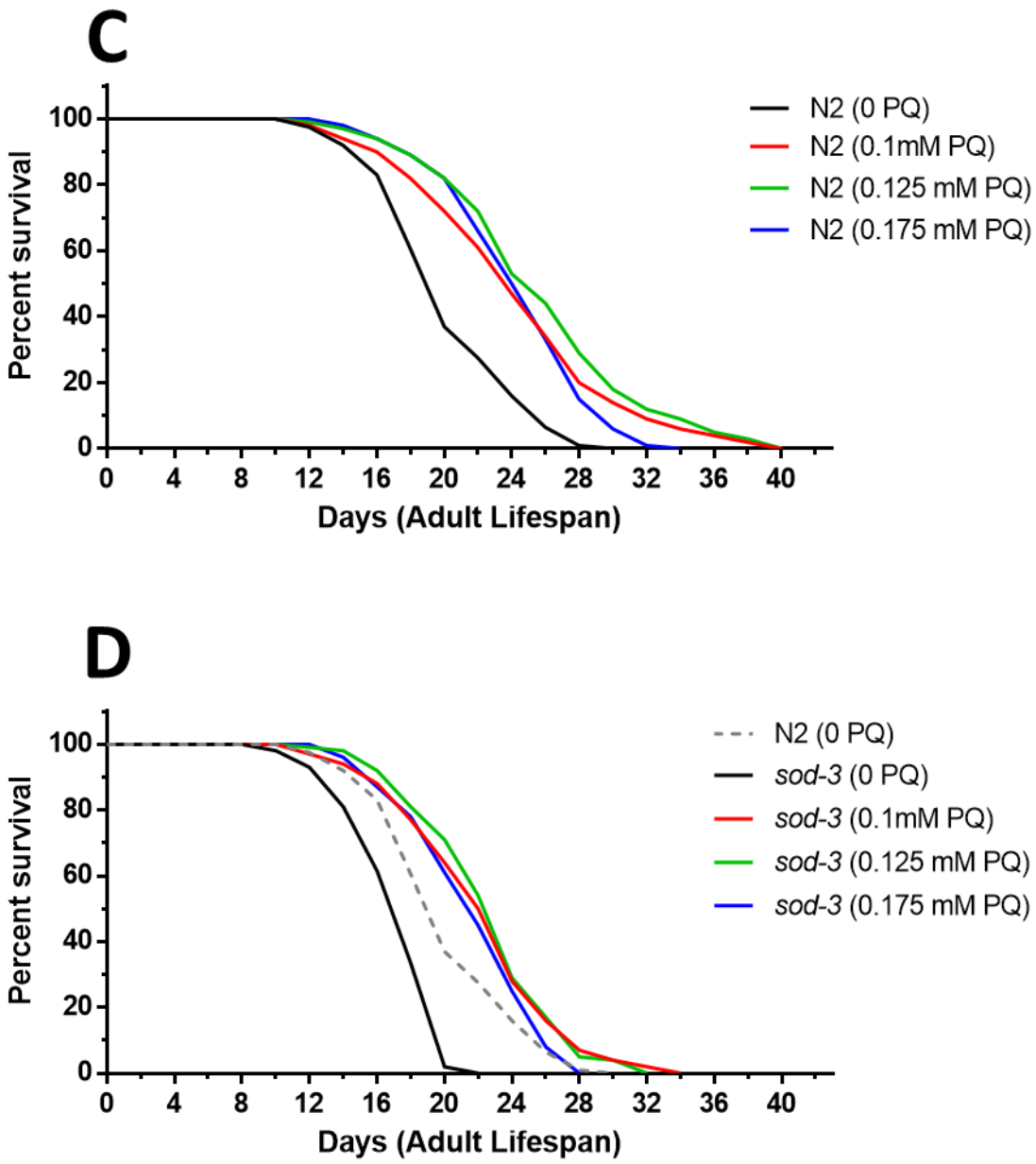


Figure S2.1. Lifespan curves for paraquat (PQ) dose response experiments for (A, C) the wild-type (N2) and (B, D) *sod-3 (tm760)* mutant worms. Average adult lifespans are the worms are shown in Figure 2.4. Similar doses for the wild-type and *sod-3* mutant worms are organized into two separate panels for clarity. In panels that show *sod-3* dose-response curves (B and D), the wild-type lifespan (N2 0 PQ) is shown for reference. Sample sizes and other data points are shown in Table S2.1.

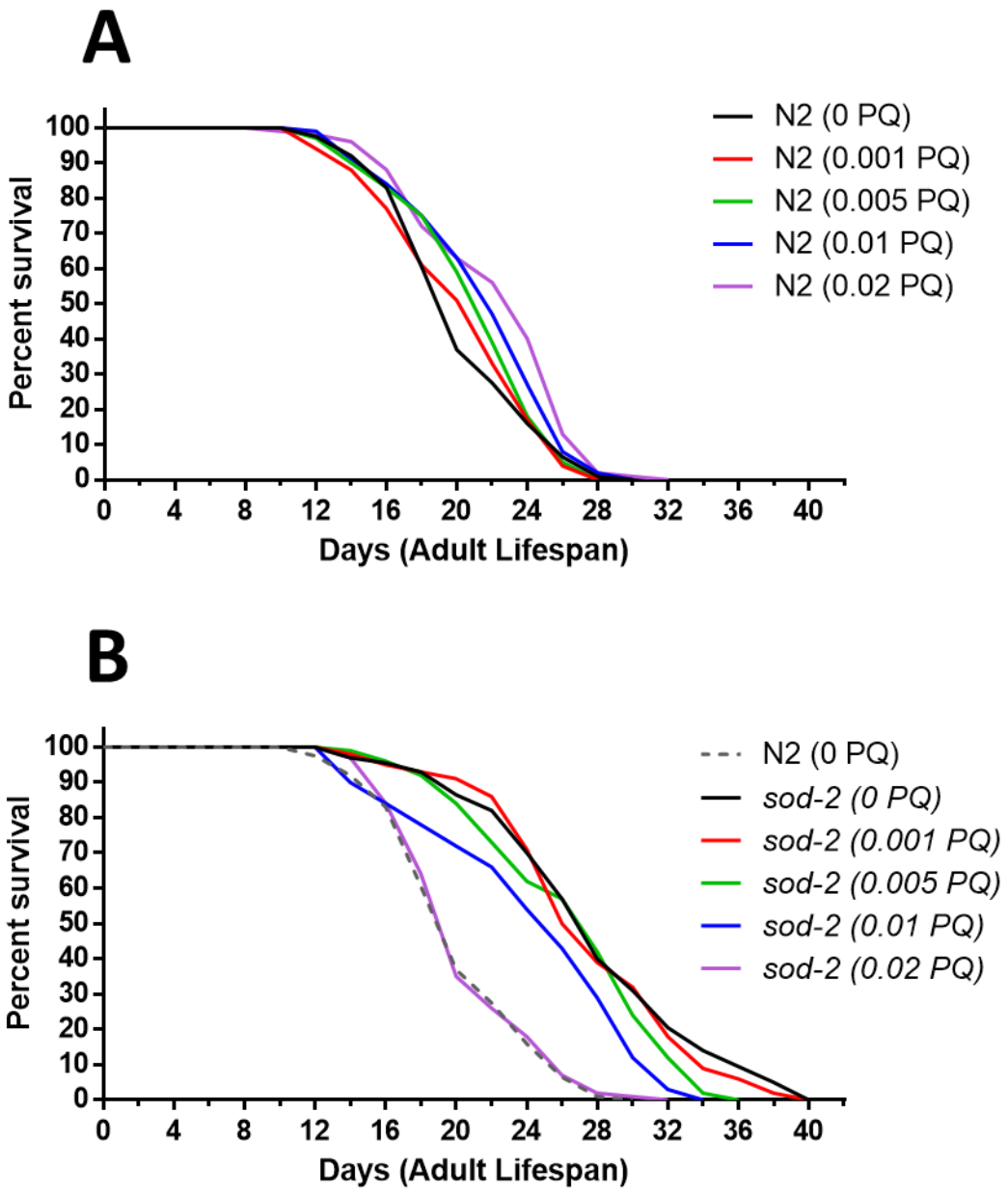


Figure S2.2. Lifespan curves for paraquat (PQ) dose response experiments for (A) the wild-type (N2) and (B) *sod-2 (ok1030)* mutant worms. Average adult lifespans are the worms are shown in Figure 2.5. In panel B, which shows *sod-2* dose-response curves, the wild-type lifespan (N2 0 PQ) is shown for reference. Sample sizes and other data points are shown in Table S2.1.

2.5.2. Summary of the lifespan data

Table S2.1. Summary of the data from the lifespan experiments described in chapter 2.

Condition	Trials	N	Average Adult Lifespan ± SEM	Maximum Adult Lifespan
Figures 2.3, 2.4, 2.5				
N2	4	200	20.42 ± 0.28	30
N2 + 0.001 mM PQ	2	100	20.5 ± 0.43	28
N2 + 0.005 mM PQ	2	100	21.32 ± 0.40	28
N2 + 0.01 mM PQ	2	100	21.92 ± 0.42	30
N2 + 0.02 mM PQ	2	100	22.56 ± 0.45	32
N2 + 0.025 mM PQ	2	100	21.22 ± 0.44	30
N2 + 0.05 mM PQ	2	100	21.92 ± 0.45	30
N2 + 0.075 mM PQ	2	100	23.46 ± 0.60	40
N2 + 0.1 mM PQ	2	100	24.66 ± 0.61	40
N2 + 0.125 mM PQ	2	100	26.12 ± 0.60	40
N2 + 0.175 mM PQ	2	100	24.68 ± 0.43	34
sod-2 (ok1030)	4	200	28.02 ± 0.44	40
sod-2 (ok1030) + 0.001 mM PQ	2	100	27.8 ± 0.56	40
sod-2 (ok1030) + 0.005 mM PQ	2	100	26.86 ± 0.53	36
sod-2 (ok1030) + 0.01 mM PQ	2	100	24.62 ± 0.59	34
sod-2 (ok1030) + 0.02 mM PQ	2	100	20.7 ± 0.39	32
sod-3 (tm760)	4	200	17.38 ± 0.19	22
sod-3 (tm760) + 0.025 mM PQ	2	100	20.06 ± 0.28	26
sod-3 (tm760) + 0.05 mM PQ	2	100	20.46 ± 0.25	28
sod-3 (tm760) + 0.075 mM PQ	2	100	22.4 ± 0.35	30
sod-3 (tm760) + 0.1 mM PQ	2	100	22.54 ± 0.47	34
sod-3 (tm760) + 0.125 mM PQ	2	100	23 ± 0.41	32
sod-3 (tm760) + 0.175 mM PQ	2	100	22 ± 0.38	28
sod-2 (gk257)	2	100	26.06 ± 0.53	36
sod-3 (gk235)	2	100	17.32 ± 0.29	24
Figure 2.6				
N2	2	100	18.54 ± 0.29	24
N2 + 0.1 mM PQ B	2	100	23.44 ± 0.41	32
N2 + 0.1 mM PQ Ad	2	100	20.7 ± 0.3	28
sod-3 (tm760)	2	100	15.72 ± 0.24	20
sod-3 (tm760) + 0.1 mM PQ B	2	100	21.6 ± 0.36	30
sod-3 (tm760) + 0.1 mM PQ Ad	2	100	19 ± 0.25	24

Condition	Trials	N	Average Adult Lifespan ± SEM	Maximum Adult Lifespan
Figure 2.7				
N2	4	200	18.71 ± 0.24	26
sod-2 (ok1030)	4	200	24.2 ± 0.49	46
sod-3 (tm760)	4	200	16.31 ± 0.2	24
sod-2 (ok1030);sod-3 (tm760)	4	191	19.37 ± 0.59	44
Figure 2.8				
daf-2 (e1370)	4	200	33.86 ± 0.78	60
daf-2 (e1370);sod-2 (ok1030)	4	190	37.59 ± 0.79	60
daf-2 (e1370);sod-3 (tm760)	4	177	42.51 ± 0.99	72
daf-2;sod-2;sod-3	4	164	39.97 ± 1.24	70

2.6. References

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Chapter 3:
Modulation of longevity by superoxide dismutases and catalases

3.1. Introduction

As we discussed in chapter 1, ROS have been a major focus in aging studies. The free radical theory of aging, which was first proposed in the 1950's, actually regarded ROS as the cause of aging (Harman 1956). This theory assumes that the chemical reactivity of ROS makes them toxic by default and powerful ROS-handling enzymes such as superoxide dismutases (SODs) and catalases (CTLs) exist solely to protect the organisms against the resulting damage. This ROS-induced damage, according to the free radical theory of aging, accumulates over time and causes aging. However, in section 1.1.5 we reviewed evidence from various organisms, including *C. elegans,* which demonstrate ROS generation and detoxification can indeed be uncoupled from aging. For example, in our laboratory, we have shown that mitochondrial mutants such as *isp-1* and *nuo-6* (discussed below and in section 1.5) live long precisely because of increased mitochondrial ROS production (Yang and Hekimi 2010b) via activating the intrinsic apoptosis pathway (Yee, Yang et al. 2014) (see section 1.5.3). Therefore, our laboratory and other researchers have proposed alternative interpretations for the role of ROS in aging (Blagosklonny 2008, Lapointe and Hekimi 2010). For example, we have proposed that the correlation between ROS damage and aged phenotype is because ROS modulate signal transduction pathways that respond to the type of cellular stresses which are a result of aging (Hekimi, Lapointe et al. 2011). In other words, ROS increase with age, not because they cause aging but because they are part of signaling mechanisms that exist to protect against aging. In this chapter, we will present further evidence from our systematic genetic studies of two major ROS-handling enzyme families in *C. elegans* (SODs and CTLs) which shows how these enzymes are involved in modulating ROS-induced longevity in the worm.

3.1.1. The mitochondrial ETC mutants: *isp-1* and *nuo-6*

The *isp-1* mutation affects an amino acid near the iron-sulfur center in the Rieske Iron Sulfur Protein of mitochondrial complex III (Feng, Bussière et al. 2001). The mutant worms exhibit slow rate of development and slow physiology such as slow defecation, decreased whole worm oxygen consumption (Feng, Bussière et al. 2001) and decreased oxidative phosphorylation in isolated mitochondria when using either malate or succinate as an electron donor (Falk, Kayser et al. 2006). So, is the longevity of *isp-1* caused by decreased ROS damage? ROS levels in the pharynx of *isp-*

1 mutants is not different from the wild-type as measured by MitoSOX staining (Dingley, Polyak et al. 2010). Also, the examination of oxidative damage to proteins in *isp-1* mutants shows no significant difference compared to the wild-type, although there is a trend towards decreasing levels of carbonylated proteins. However, increasing levels of carbonylated proteins by treatment of *isp-1* mutants with RNAi against *sod-2* (the primary mitochondrial SOD) does not affect the longevity of the worms (Yang, Li et al. 2007). Therefore, the long lifespan of *isp-1* is not caused by a decrease in oxidative damage.

In *nuo-6* mutants a point mutation affects a subunit of complex I of the electron transport chain which causes a decrease in complex I activity and whole worm oxygen consumption. *nuo-6* mutants also have increased ATP levels despite their decreased mitochondrial function (Yang and Hekimi 2010b). Similar to *isp-1,* RNAi treatment against *sod-2* does not shorten the lifespan of *nuo-6* but in this case the *sod-2* RNAi treatment even slightly increases *nuo-6's* longevity (Yang and Hekimi 2010a).

RNAi treatment against *nuo-6* and *isp-1* also increases the wild-type's lifespan but it does not result in decreased oxygen consumption like the point mutations do and the RNAi treatment appears to exert its longevity effect by triggering a mitochondrial stress response (Yang and Hekimi 2010b). Additionally, the *isp-1;nuo-6* double mutants are long-lived but they do not live longer than the single mutants showing that the effects of these mutations on lifespan are not additive and they likely increase longevity through the same mechanism by increasing the mitochondrial ROS generation (Yang and Hekimi 2010b).

The study of the *isp-1* and *nuo-6* has led to strong evidence that links increased mitochondrial ROS generation to increased lifespan. For example, purified mitochondria from both mutants show increased levels of $O_2^{\bullet-}$ generation (Yang and Hekimi 2010a). However, increasing ROS scavenging activity in the mutants through antioxidant treatment with N-acetyl cysteine (NAC) or vitamin C suppresses their longevity (Yang and Hekimi 2010a) (Figure 3.1). Finally, Affimetrix gene arrays show that treatment with very low concentrations (0.1 mM) of the pro-oxidant paraquat (PQ) phenocopies the effects of *isp-1* and *nuo-6* point mutations on lifespan (Yee, Yang et al. 2014). As we discussed in chapter 2, PQ is widely used as a pro-oxidant in ROS biology. like for the data presented in chapter 2, in this chapter we have also used PQ, in addition to *isp-1* and *nuo-6* mutations, to further study the regulation of ROS-dependent longevity in *C.*

elegans. In the next section, we will review how use of PQ in longevity studies using *C. elegans* as a model organism has provided us valuable insights.

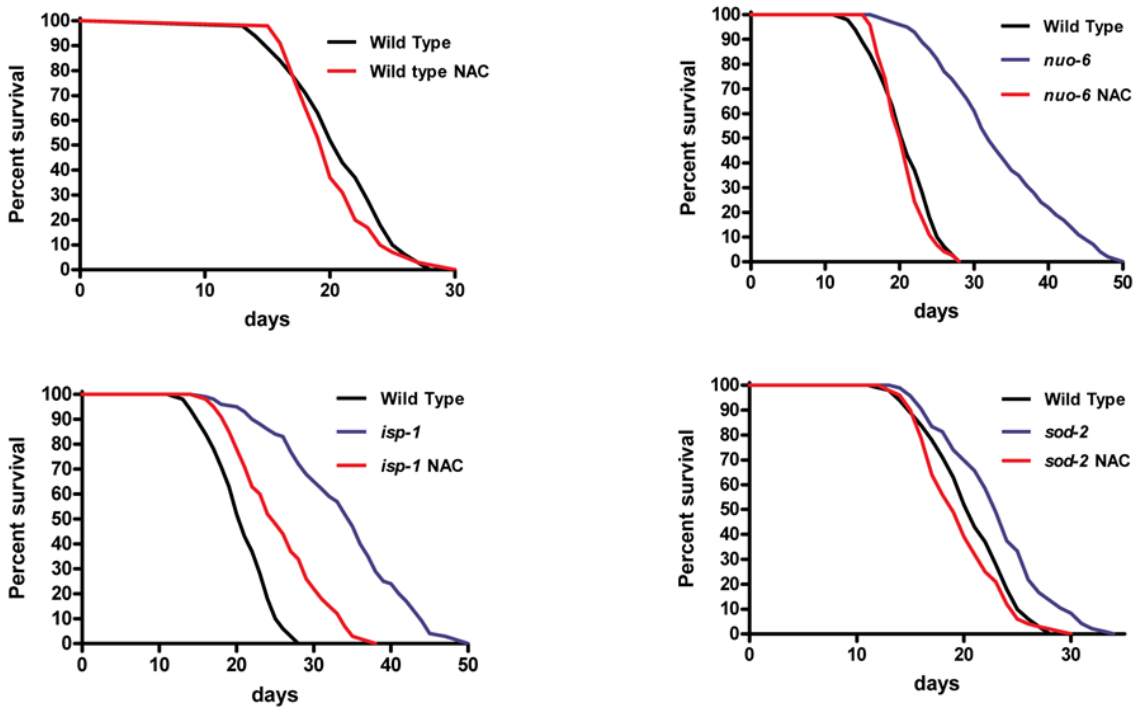


Figure 3.1. Treatment with N-acetyl cysteine (NAC) suppresses ROS-dependent longevity. NAC is antioxidant that enhances H_2O_2 scavenging. While NAC treatment does not affect the normal lifespan of the wild-type worms, the longevity of mitochondrial mutants *isp-1* and *nuo-6*, whose long lifespan is believed to be due to increased mitochondrial $O_2^{\bullet-}$ production, is suppressed by NAC treatment. Loss of *sod-2* also lengthens the lifepsan of the worms (see chapter 2). Due to the role of SOD-2 as the main mitochondrial MnSOD, the longevity of *sod-2* mutants is also believed to be caused by increased mitochondrial $O_2^{\bullet-}$ production. NAC treatment suppresses the longevity of *sod-2,* as well. Taken together, these observations suggest that H_2O_2 is likely the species that is responsible for the longevity of these mutants. Figure adapted from (Yang and Hekimi 2010a).

3.1.2. PQ and longevity in *C. elegans*

PQ (1,1′-dimethyl-4,4′-bipyridinium dichloride) is a redox-cycling compound. It can accept electrons from an electron donor (e.g the ETC) and transfer them to molecular oxygen to generate $O_2^{\bullet-}$. In high doses, PQ is widely used in ROS biology to induce cellular and especially mitochondrial oxidative stress (Bus, Aust et al. 1976, Cocheme and Murphy 2008, Robb, Gawel

et al. 2015) in particular in studies involving *C. elegans* (Senchuk, Dues et al. 2017) where 200 mM PQ is used to induce oxidative stress in the worm. Within mitochondria, the main site of $O_2^{\bullet-}$ generation by PQ appears to be complex I where electrons are transferred to PQ^{2+} to form a radical cation ($PQ^{\bullet+}$) which further reacts with molecular oxygen to produce $O_2^{\bullet-}$ (Figure 3.2) (Cocheme and Murphy 2008).

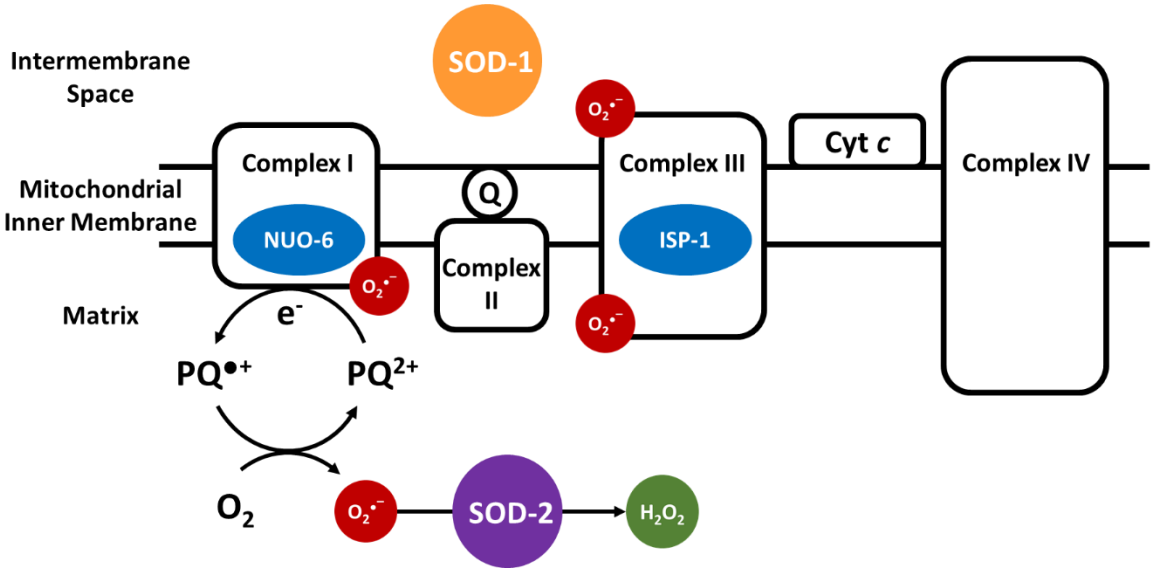


Figure 3.2. Schematic figure showing the paraquat (PQ)-induced production of $O_2^{\bullet-}$ in the ETC and the location of *isp-1* and *nuo-6* mutations. PQ can accept electrons from the ETS, likely at the site of complex I, and transfer them to O_2 to produce $O_2^{\bullet-}$. Within mitochondrial matrix, SOD-2, the primary mitochondrial MnSOD, converts H_2O_2 to $O_2^{\bullet-}$. The $O_2^{\bullet-}$ which is produced directly into the intermembrane space or relocates there from the mitochondrial matrix, becomes a target for SOD-1 either in the mitochondrial inner membrane or in the cytoplasm when it escapes the mitochindria. In this figure we have also shown the location of the affected proteins by the *isp-1* and *nuo-6* mutations. This depiction is only for reference and is not intended to show any kind of mechanism.

In contrast to the large doses of PQ used for worm studies involving PQ, we have determined that very low concentrations of PQ can increase the lifespan of the wild-type worms in a dose dependent manner where the optimum concentration for maximizing lifespan under our experimental conditions is 0.1 mM (Van Raamsdonk and Hekimi 2012) (Also see Figure 2.3). The correlation between the different PQ doses and lifespan of the worms reveals an inverted U-shaped curve (see Figure 2.1) which shows a decrease in lifespan as the concentration of PQ increases beyond the peak of the curve at 0.1 mM (Van Raamsdonk and Hekimi 2012). This suggests that

although increased mitochondrial ROS generation extends lifespan, increasing ROS generation further can weaken the beneficial pro-longevity effects of ROS as a result of the toxicity of abnormally high ROS generation. Of course, intrinsic factors such as cell/tissue type and genetic composition determine the optimum levels of beneficial ROS (Desjardins, Cacho-Valadez et al. 2017) (also see Figure 1.14). For example, the wild-type worms fully benefit from 0.1 mM PQ treatment and their lifespan is extended by approximately 67%. In contrast, the quintuple mutants that lack all of their SOD enzymes (*sod-12345*) but their lifespan is similar to that of the wild-type experience, show a 56% decrease in their lifespan and no concentration of PQ can increase their lifespan (Van Raamsdonk and Hekimi 2012). This can be explained in two ways. First, the *sod-12345* mutants completely lack a mechanism to detoxify $O_2^{\bullet-}$. Therefore, toxic levels of $O_2^{\bullet-}$ as a result of PQ treatment can completely mask the beneficial pro-longevity effects of the treatment. Another possibility is that the quintuple mutants does not benefit from the pro-longevity PQ treatment because it does not have any SOD enzymes and subsequently it does not have sufficient H_2O_2 which is produced by the SODs (Hekimi, Wang et al. 2016). H_2O_2 is believed to be the main cellular ROS messenger due to its greater stability and ability to cross membranes (even without the help of specialized structures such as aquaporins) (Holmstrom and Finkel 2014). Therefore, the lack of sufficient H_2O_2 production in the absence of SODs could shorten lifespan by preventing normal, beneficial, ROS signaling in *sod-12345.* The idea an inverted U-shaped curve can demonstrate the relationship between lifespan and ROS levels has enabled us to conceptualize the data from ROS-dependent longevity experiments to illustrate the comparative ROS profiling of the experimental conditions. Some examples are shown in (Van Raamsdonk and Hekimi 2012) and (Hekimi, Wang et al. 2016). In section 3.4.4, we have also discussed how the data presented in this chapter fits into the inverted U-shaped curve for the relationship between ROS and lifespan.

The pro-longevity effect of PQ treatment is additive to the longevity of some previously identified long-lived mutants. For example, *eat-2* mutants (which live long because of calorie restriction) and *daf-2* mutants (which live long because of reduced insulin-like signaling) both live even longer when treated with 0.1 mM PQ but the same PQ treatment is not additive to the longevity of *isp-1* and *nuo-6* mutants (Yang and Hekimi 2010a). Additionally, Affimetrix gene array data show a considerable overlap in gene expression for *isp-1, nuo-6* and wild-type treated with 0.1 mM PQ (Yee, Yang et al. 2014). Also, these three conditions (i.e. *isp-1, nuo-6* and wild-type + 0.1 mM PQ) share the key phenotypes of the long-lived mitochondrial mutants such as slow

development, slow physiological rates (e.g. defecation and swimming) and smaller brood size in comparison to the wild-type (Yee, Yang et al. 2014). Taken together, these observations show that low-dose PQ treatment phenocopies the longevity of *isp-1* and *nuo-6* mutations.

However, there appears to be a small difference between the mechanism through which PQ induces longevity and the one that *isp-1* or *nuo-6* use. In section 1.5.3 we discussed a study from our laboratory which shows that one of the ways *isp-1* and *nuo-6* mutations transmit the pro-longevity ROS signal is through the intrinsic apoptosis signaling pathway (Yee, Yang et al. 2014). Briefly, this pathway includes EGL-1 (a BH3-only protein), CED-9 (Bcl2-homolog), CED-4 (Apaf1-homolog) and CED-3 (Casp9-homolog). CED-4 binds to CED-9 which is tethered to the outer mitochondrial membrane. Upon activation of programed apoptotic cell death, EGL-1 interacts with CED-9 which leads to the release of CED-4. CED-4 then relocates to the perinuclear membranes and assembles into active apoptosomes which in turn activate the caspase CED-3. However, for the transduction of the *isp-1* and *nuo-6's* longevity signal, the intrinsic apoptotic pathway needs to be activated by the only other BH3-only protein that the worm possesses: CED-13. That is why, for example, *isp-1;ced-13* double mutants have a shortened lifespan. However, 0.1 mM PQ treatment does not require CED-13 for its pro-longevity effect. That is why when *isp-1;ced-13* double mutants are treated with 0.1 mM PQ, their lifespan is lengthened back to the longevity of *isp-1* while the pro-longevity effect of PQ is not additive to that of the *isp-1.* This suggests that PQ can bypass the need for CED-13 and activate the intrinsic apoptosis pathway directly, possibly by affecting CED-9 which is physically associated with mitochondria.

3.1.3. Using SODs and CTLs ROS-dependent mechanisms in *C. elegans*

In chapter 1 section 1.4, we reviewed SODs and CTLs and their dual roles as protective enzymes against ROS toxicity and the part they possibly play in the ROS network as regulators of ROS-related phenomena such as signaling. In the analysis of the regulation of ROS-dependent longevity presented in this chapter, we have used combinations of *sod* and *ctl* mutants in *isp-1, nuo-6* and wild-type backgrounds along with PQ treatment. Therefore, in this section, we will revisit these two classes of enzymes to review the points that are most pertinent to the specific contents of this chapter.

3.1.3.1. SOD isoforms and their localization

C. elegans has five genes that encode SODs. SOD-1, SOD-4 and SOD-5 are Cu/ZnSODs and SOD-2 and SOD-3 are MnSODs (Jensen and Culotta 2005, Yang, Li et al. 2007, Doonan, McElwee et al. 2008) (Figure 3.3). SOD-1 is responsible for nearly 80% of total SOD activity in the worm and also 80% of the total *sod* mRNA expression belongs to *sod-1* (Doonan, McElwee et al. 2008) (see Table 1.2 for an overview). Their job is to convert $O_2^{\bullet-}$ to H_2O_2 and they are the only family of enzymes that does this in the worm. Of course, $O_2^{\bullet-}$ can react with itself and spontaneously produce H_2O_2, but SODs do this conversion 20,000 times faster (McCord 1999). Unlike mammals that have only three isoforms of SOD, the worm has five. The fact that the control of $O_2^{\bullet-}$ and H_2O_2 levels in *C. elegans* is divided among five different isoforms that have different properties and are expressed in different subcellular compartments and possibly tissues makes the worm an exceptionally good model organism for studying the diversity of the biological roles of SODs.

The five SOD isoforms of the worm are compartmentalized. SOD-1 and SOD-5 are predicted to be intracellular cytoplasmic Cu/Zn SODs (Larsen 1993, Jensen and Culotta 2005). SOD-4, on the other hand, is predicted to be extracellular Cu/Zn SOD. Alternative splicing may produce two different forms of SOD-4. Both of these forms have signal peptides but one of them also has a putative transmembrane domain, which makes it the first example of a membrane-bound SOD (Fujii, Ishii et al. 1998). However, it is not known in which cells SOD-4 is expressed (Wang, Branicky et al. 2018). SOD-1 localizes to the cytoplasm of most cells in *C. elegans.* This has been confirmed through transgenic expression and western blotting (Doonan, McElwee et al. 2008, Yanase, Onodera et al. 2009). SOD-5 is another cytosolic SOD of the worm. Although SOD-5 expression has been detected in the cytoplasm of a small subset of neurons, this may not be an indicative of the true expression pattern of *sod-5* because it is an inducible *sod* (see below) (Doonan, McElwee et al. 2008).

SOD-2 and SOD-3 are very similar and they are both predicted to be mitochondrial MnSODs (Hunter, Bannister et al. 1997, Henderson, Bonafe et al. 2006, Doonan, McElwee et al. 2008, Honda, Tanaka et al. 2008). Previous transgenic studies show SOD-2 and SOD-3 expression are in intestinal cells, pharynx, and hypodermal cells (Henderson, Bonafe et al. 2006, Doonan, McElwee et al. 2008). But as we discussed in chapter 2, it is not clear whether the two proteins are co-localized to the same tissue or mitochondria (Honda, Tanaka et al. 2008). In the mitochondria,

SOD-2 and SOD-3 have been reported to localize to the supercomplex I:III:IV of the ETC. This suggests that these SODs can interact with $O_2^{\bullet-}$ right at the site of its production (Suthammarak, Somerlot et al. 2013). The localization of the worm's SOD isoforms is shown in Figure 3.3.

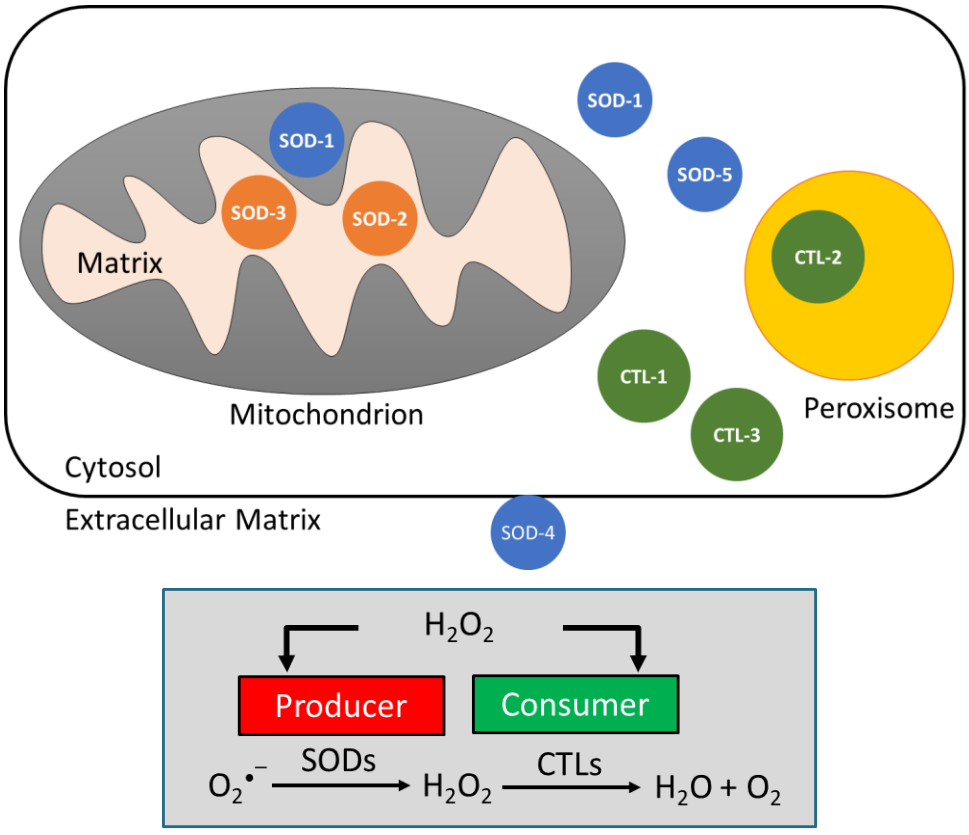


Figure 3.3. Schematic figure showing the subcellular localization of different isoforms of SODs and CTLs in *C. elegans* and their synergistic action. (Top Panel) *C. elegans* has five genes that encode SODs. SOD-1, SOD-4 and SOD-5 are Cu/ZnSODs and SOD-2 and SOD-3 are MnSODs. SOD-1 is the worm's primary cytoplasmic SOD and likely acts in the mitochondrial intermembrane, too. SOD-5 is the inducible cytoplasmic SOD. SOD-4 has two isoforms that result from alternative splicing. One of these isoforms has a putative transmembrane domain and is likely membrane-bound. SOD-2 is the worm's primary MnSOD and SOD-3 is inducible. Both SOD-2 and SOD-3 localize to the mitochondrial matrix. Like other eukaryotes, the worm has a proxisomal CTL (CTL-2), but *C. elegans* uniquely has two cytoplasmic CTLs, which is unusual for eukaryotes. (Bottom Panel) SODs and CTLs act in synergy with each other to regulate levels of $O_2^{\bullet-}$ and H_2O_2. SODs use $O_2^{\bullet-}$ to produce H_2O_2 and CTLs consume this H_2O_2 and convert it to water and oxygen. The unique diversity of SODs and CTLs, their synergy for controling the levels of $O_2^{\bullet-}$ and H_2O_2 in *C. elegans* and the fact that these enzymes are expressed in different subcellular compartments and possibly tissues makes the worm an exceptionally good model organism for ROS studies. Also, see Figure 1.15 for a more detailed overview of the worm's ROS network.

3.1.3.2. Induction of *sod* genes

The expression of *sod-1* and *sod-2* as the worm's major *sod* genes are found to be mildly upregulated by oxidative stress (Tawe, Eschbach et al. 1998, Yang, Li et al. 2007, Meng, Lv et al. 2017), and possibly as a result of the loss of other *sod* genes (Van Raamsdonk and Hekimi 2009, Yanase, Onodera et al. 2009, Back, Matthijssens et al. 2010). However, these findings are often conflicting. Perhaps one reason is that the experimental conditions for these studies vary greatly. For example, PQ concentrations ranging from 0.1 mM to 100 mM were used to induce oxidative stress. However, there is a one report on cell-specific upregulation of *sod-1* in *C. elegans* (Horspool and Chang 2017). This study shows that in response to pathogenic bacteria, *sod-1* expression is specifically induced in a unique neuron (the ASER neuron) where its upregulation is required for the worm's pathogen avoidance behavior. The authors believe these findings suggest that pathogen-induced ROS activates a SOD-1-dependent pathway which is responsible for mediating the avoidance behavior in the worm.

Both *sod-3* and *sod-5* are strictly inducible and in the wild-type, under normal conditions, there is little to no expression of either of them (Honda and Honda 1999, Doonan, McElwee et al. 2008). The induction of *sod-3* is mostly dependent on the forkhead transcription factor DAF-16 (Honda and Honda 1999). A wide range of environmental conditions and genetic mutations have been found that can induce *sod-3* transcription. For example, in long-lived mutants of the insulin signaling pathway such as *daf-2* (Honda and Honda 1999) and the long-lived mutants such as *isp-1* (Feng, Bussiere et al. 2001, Dingley, Polyak et al. 2010), *clk-1* (Yang, Li et al. 2007) the *sod-3* transcript is highly upregulated. Loss of *sod-2* (Suthammarak, Somerlot et al. 2013), and stressors such as desiccation (Erkut, Vasilj et al. 2013), $CuSO_4$ (Song, Zhang et al. 2014) and treatment with PQ (Tawe, Eschbach et al. 1998) can also upregulate *sod-3* expression. By using transcriptional reporters, studies have also reported *sod-3* expression upregulation by stresses such as heat (Wolf, Nunes et al. 2008), starvation (Henderson, Bonafe et al. 2006), and pathogenic bacteria (Chavez, Mohri-Shiomi et al. 2007). Even treatment with the antioxidant N-acetyl-cysteine (NAC) has been reported to upregulate *sod-3* expression (Oh, Park et al. 2015). In most of these studies the specificity of *sod-3* upregulation is not clear with one interesting exception. It is shown that up-regulation of SOD-3 in the intestine of the worm is required for resistance to *Enterococcus faecalis* (Chavez, Mohri-Shiomi et al. 2007).

The findings on the induction of *sod-5* are much more limited. Some of the conditions that induce the expression of *sod-3,* appear to induce *sod-5* expression as well. For example, *sod-5* upregulation has been reported in long-lived insulin signaling pathway mutants (Doonan, McElwee et al. 2008, Song, Zhang et al. 2014). Desiccation (Erkut, Vasilj et al. 2013) and exposure to $CuSO_4$ (Song, Zhang et al. 2014) have also been found to upregulate *sod-5*. However, the importance and specificity of *sod-5* upregulation in these cases is also unknown.

3.1.3.3. The localization of CTLs and their associated phenotypes

The worm has three catalase genes with very similar sequences that are arranged in a tandem array (Figure 3.4). *ctl-1* and *ctl-3* both encode two forms of cytosolic CTL. *ctl-2* encodes a peroxisomal catalase (Gems and Doonan 2009, Erkut, Vasilj et al. 2013). The subcellular localization of the worm's three CLTs are shown in Figure 3.3.

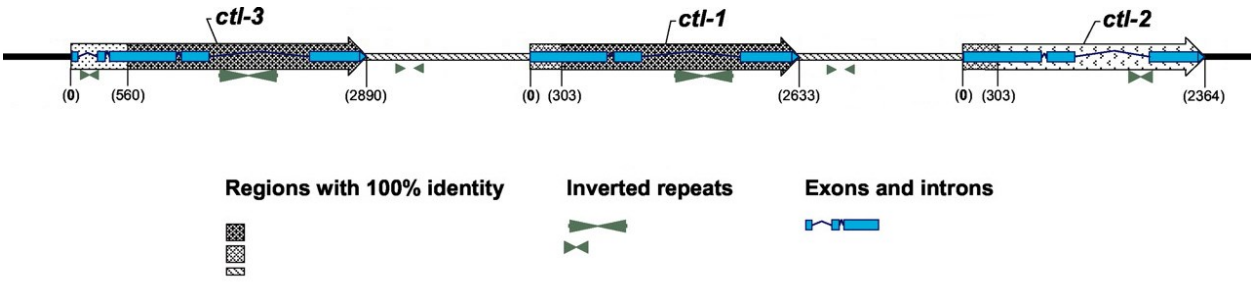


Figure 3.4. The structure of *ctl* gene cluster in the *C. elegans* genome. This figure shows a span of 11,931 bp of the genomic DNA of the worm at the locus of the three catalase genes of *C. elegans* is presented. The *ctl* genes have very similar sequences and the region contains a large number of inverted DNA repeats. The adenyl residue of the initiating codon of each catalase gene is designated "0" to function as the point of reference for numbering the nucleotide sequences of a particular catalase gene. Figure modified from (Petriv and Rachubinski 2004).

Loss of *ctl-1* has not been associated with any discernible phenotypes and the mutants have a normal lifespan as the wild-type animals. However, one study reports that *ctl-2* mutants have a shorter lifespan compared to the wild-type (Petriv and Rachubinski 2004). In long-lived *clk-1* mutant background (see section 1.5.1) that develop more slowly than the wild-type, while removal of either *ctl-1* or *ctl-2* does not affect longevity, loss of *ctl-2* (the peroxisomal CTL) has been reported to cause accelerated development (Petriv and Rachubinski 2004). Interestingly, both *ctl-1* and *ctl-2* knockout mutants demonstrate decreased rate of oxidative damage accumulation

compared to the wild-type as shown by the slower increase in protein carbonyl levels (Petriv and Rachubinski 2004) which suggests that the shortened lifespan of *ctl-2* mutants in this study by Petriv *et al.* may not due to increased oxidative damage due to lack of H_2O_2 detoxification.

In one study, the overexpression of the entire *C. elegans* gene cluster (i.e. *ctl-1*, *ctl-2*, and *ctl-3*) decreased the survival of the transgenic animals (Doonan, McElwee et al. 2008). However, in the same study the authors determined that this effect is not due to an increase in oxidative damage but instead it is caused by increased internal hatching of the larvae (i.e. when larvae hatch inside the mother and kill it). Interestingly, the authors showed that overexpression of *sod-1* suppresses the internal hatching phenotype in these transgenic worms. This suggests that likely the internal hatching was caused by H_2O_2 deficiency not oxidative stress.

As mentioned before, in the studies described in this chapter, we have used deletion mutations of the worm's *ctl* genes in combination with *sod* mutants to study the role of ROS in lifespan regulation. In addition to CTLs, other enzymatic systems such as peroxiredoxins (see section 1.4.3) and glutathione peroxidases (section 1.4.4) also have the responsibility of handling H_2O_2. However, these other systems are much less studied and described in *C. elegans*. On the other hand, CTLs are relatively better studied in the worm and they have a synergy with SODs in that they use the product of SODs (H_2O_2) as substrate to produce H_2O and O_2 (Figure 3.3) and their subcellular localization also partially overlaps with that of SODs. A good example of this synergy is the study by Doonan *et al* (described above) where they show how *sod-1* overexpression can suppress the internal hatching phenotype caused by *ctl* overexpression. Also, there are three *ctl* genes in *C. elegans* which makes them more convenient for our systematic genetic studies with *sod* combinations than, for example, the eight glutathione peroxidase genes (*gpx-1* – *gpx-8*). Finally, while CTL only localizes to the matrix of peroxisomes in mammals (and most eukaryotes) (Zhou and Kang 2000), the worm, curiously, has two cytosolic forms of CTL, as well. This makes *C. elegans* CTLs a unique candidate for studying the regulatory roles of ROS in cell.

3.1.3.4. Genetic dissection of SODs and CTLs in *C. elegans* as a tool to study ROS-Dependent longevity and other phenotypes

C. elegans provides an excellent model organism for investigating ROS-dependent longevity and other phenotypes that rely on ROS for signal transduction. In addition to its simple anatomy, the worm offers a complex molecular biology with localized subcellular distribution of

several different major families of ROS-handling enzymes. For example, the studied expression patterns of different SOD isoforms and lack of their compensatory upregulation as a result of different *sod* mutations (Back, Matthijssens et al. 2010) supports the idea that the five *C. elegans sod* genes are not functionally redundant (Braeckman, Smolders et al. 2016) and are responsible for a precise and purposeful regulation of ROS levels in their respective subcellular compartments where and/or when they are active. In a similar way, even the study that reports the upregulation of *sod-5* as a result of *sod-1* deletion (Yanase, Onodera et al. 2009) might be indicating a general stress response (mediated via IGF-1/insulin-like signaling) to the knockout of a major SOD (Doonan, McElwee et al. 2008, Yanase, Onodera et al. 2009, Cabreiro, Ackerman et al. 2011).

A similar degree of complexity is observed in other redox-active enzyme families of *C. elegans,* such as CTLs, although they are not as much studied as SODs. For example, despite their sequence similarity, *ctl* gene expression shows a specific subcellular expression pattern where CTL-1 is cytosolic but CTL-2 is peroxisomal and is responsible for eighty percent of total catalase activity in the cell (Togo, Maebuchi et al. 2000, Petriv and Rachubinski 2004).

Additionally, phenotypes such as lifespan extension in electron transport chain (ETC) mutants (*isp-1* and *nuo-6*) (Yang and Hekimi 2010a, Yang and Hekimi 2010b) point to the significance of precise redox control in *C. elegans* and the magnitude of the outcomes resulting from the manipulation of the worm's redox systems. As we discussed in section 1.5.3 this increase in lifespan of ETC mutants is linked to the intrinsic apoptosis pathway where the ROS-dependent activation of a specific set of proteins activates a cell protective program rather than the cell death machinery (Yee, Yang et al. 2014). Moreover, it has been shown that in mitochondrial mutants the subcellular site of ROS production is also particularly important in ROS-dependent regulation of lifespan (Schaar, Dues et al. 2015) where it is shown that deletion cytoplasmic and mitochondrial *sods* has opposite effects on lifespan.

Given the wide range of influence and specificity of ROS action in *C. elegans* and the subcellular and spatiotemporal specificity of redox active enzymes in this organism, the systematic dissection of various ROS-handling enzymes in our present study provides a valuable tool regarding the subcellular and/or tissue-specific origins, route of travel, destination and nature of a ROS signal that elicits a certain phenotype or triggers a specific physiological event that is ROS dependent.

3.1.4. Rationale and design of the study

3.1.4.1. Hypothesis

As discussed in section 3.1.1, in our laboratory, we had previously characterized two long-lived ETC mutants: *isp-1* (Feng, Bussière et al. 2001) and *nuo-6* (Yang and Hekimi 2010b) whose longevity is due to increased mitochondrial $O_2^{\bullet-}$ production (Yang and Hekimi 2010a). We had also found that loss of *sod-2,* but not other *sod* genes, leads to the long lifespan of *sod-2* mutants (Van Raamsdonk and Hekimi 2009). The role of SOD-2, as the worm's primary MnSOD, in dealing with the $O_2^{\bullet-}$ that originates from the ETC, suggests that the loss of *sod-2* leads to increased lifespan as a result of increased mitochondrial $O_2^{\bullet-}$ production, too. Furthermore, we found that treating the wild-type worms with very low doses of PQ, a $O_2^{\bullet-}$ generator (see section 1.4.2) also increases their lifespan in a dose dependent manner (Van Raamsdonk and Hekimi 2012) and this PQ treatment phenocopies the longevity effects of *isp-1* and *nuo-6* ETC mutations (Yee, Yang et al. 2014). However, we also found that treatment with N-acetyl cysteine (NAC), which enhances H_2O_2 scavenging, suppresses the longevity of *isp-1, nuo-6* and *sod-2* (Yang and Hekimi 2010a).

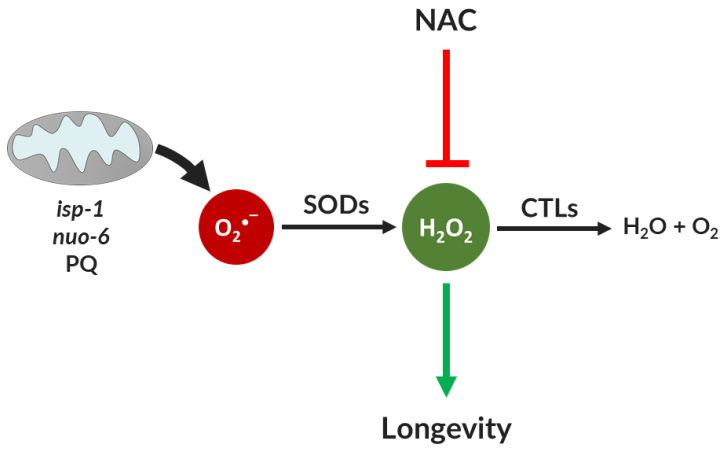


Figure 3.5. Our hypothesis for the involvement of SODs in coveying the mitochondrial $O_2^{\bullet-}$ signal that leads to the longevity of *isp-1* and *nuo-6* mutants and wild-type worms that are treated with PQ. *isp-1* and *nuo-6* mutations increase mitochondrial $O_2^{\bullet-}$ production. Treatment with PQ, a $O_2^{\bullet-}$ generator, phenocopies both mutations. All three conditions lead to ROS-dependent longevity. However, NAC, which enhances H_2O_2 scavenging, suppresses the longevity of *isp-1* and *nuo-6*. This implies that SODs are involved in converting the mitochondrial $O_2^{\bullet-}$ to the H_2O_2 which may be responsible for the longevity effect of *isp-1, nuo-6* and PQ treatment. Also, CTLs are able to remove this H_2O_2 from the intracellular enviroment and countereffect the SODs.

The suppression of a mitochondrial $O_2^{\bullet-}$ signal through H_2O_2 scavenging implies that SODs and CTLs are likely involved in this process as mediators of the pro-longevity ROS. We, therefore, hypothesized that some of the worm's SODs must be involved in this process and the action of the CTLs can counter that effect. This hypothesis, which is outlined in Figure 3.5, marked the beginning of a large study that involved constructing a large number of mutants and scoring their lifespans multiple times with and without the use of PQ treatment.

3.1.4.2. Design and goals of this study

Based on our hypothesis, we decided to construct all the double- and triple-mutant conditions in the wild-type and *isp-1* background and score their lifespans with and without the use of PQ. The lifespan experiments were repeated multiple times. When findings appeared to be significant, we tested them in the equivalent double- or triple-mutant compositions in *nuo-6* background to further explore the findings. For this purpose, we constructed some 59 double- and triple-mutants, 54 of which were specifically made for this study (see section 3.2 for details of the strains and lifespan experiments). This valuable collection of multiple mutants can be used in future studies of ROS biology, as well. Throughout this study, we aimed to answer these questions:

1. Is H_2O_2 the pro-longevity signal?
2. What are the sources and sinks of the pro-longevity signal?

In the following sections, we present the data that answers these questions.

3.2. Materials and methods

3.2.1. Strains and genetics

All strains were maintained at 20°C, on solid nematode growth medium (NGM), and were fed *E. coli* OP50. Bristol N2 was used as the wild-type.

The following mutant strains were used: LGI: *sod-2(ok1030), nuo-6(qm200)*; LGII: *sod-1(tm783), sod-5(tm1146), ctl-1(ok1242), ctl-2(ok1137), ctl-3(ok2042)*; LGIII: *sod-4(gk101)*; LGIV: *isp-1(qm150)*; LGX: *sod-3(tm760)*.

Construction of the multiple mutant strains

This study required the construction of complete set of multiple mutants. Since we were looking to trace the path of pro-longevity ROS from mitochondria to its destination, we needed all the possible combination of SODs as producers of H_2O_2 in different subcellular compartments and CTLs as sinks of H_2O_2 that are also compartmentalized. In this way, we took advantage of the unique diversity of the worm's SOD and CTL enzymes to track the longevity signal by means of genetic dissection.

We constructed all the double- and triple-mutant strains using standard genetic methods and verified them using polymerase chain reaction (PCR) and restriction enzyme digest where there were point mutations such as *isp-1* and *nuo-6*. A complete list of all 54 strains that we used for this study is provided in Table 4.1. Most of these were constructed during the course of this study and only a few (marked with an asterisk) were made beforehand in our laboratory. Sometimes a particular combination required multiple attempts to construct due to the fact that they dramatically altered the ROS physiology of the worm and many of the embryos or larvae died before reaching adulthood and being able to lay eggs, which generally reduced our chances of isolating them. This was a common problem especially in triple mutation combinations that included *isp-1* or *nuo-6* and major SOD and CTL enzymes like *sod-1, sod-2* and *ctl-1*. Nevertheless, when these particular strains were finally constructed, they did not appear to be in poor health. In the case of four of the triple mutants (marked in red in Table 4.1), the combination of the mutations appeared to be embryonic lethal since after numerous attempts and even with the aid of pro-oxidants and anti-oxidants during the construction process, we were not able to obtain them. We discuss these in section 3.4.

Table 3.1. List of all the double- and triple-mutants used in the studies described in this chapter. The multiple mutant strains marked with an asterisk (*) were created previously in our laboratory. All other strains were constructed during the course of this study. Triple mutations marked in red were embryonic lethal (see section 3.4.2 for discussion).

Single Mutants	Double Mutants	Triple Mutants (*isp-1*)	Triple Mutants (*nuo-6*)
isp-1	*sod-1 ctl-1*	*isp-1;ctl-2 sod-1*	*nuo-6;ctl-2 sod-1*
nuo-6	*sod-2;ctl-1*	*isp-1;ctl-2;sod-2*	*nuo-6;ctl-2;sod-2*
	sod-3;ctl-1	*isp-1;ctl-2;sod-3*	*nuo-6;ctl-2;sod-3*
	sod-4;ctl-1	*isp-1;ctl-2;sod-4*	*nuo-6;ctl-2;sod-4*
	sod-5 ctl-1	*isp-1;ctl-2 sod-5*	*nuo-6;ctl-2 sod-5*
sod-1	*nuo-6;sod-1*	*isp-1;ctl-3 sod-1*	*nuo-6;ctl-3 sod-1*
sod-2	***nuo-6 sod-2***	*isp-1;ctl-3;sod-2*	*nuo-6;ctl-3;sod-2*
sod-3	*nuo-6;sod-3*	*isp-1;ctl-3;sod-3*	*nuo-6;ctl-3;sod-3*
sod-4	*nuo-6;sod-4*	*isp-1;ctl-3;sod-4*	*nuo-6;ctl-3;sod-4*
sod-5	*nuo-6;sod-5*	*isp-1;ctl-3 sod-5*	*nuo-6;ctl-3 sod-5*
ctl-1	*isp-1;sod-1*	*isp-1;ctl-1 sod-1*	*nuo-6;ctl-1 sod-1*
ctl-2	*isp-1;sod-2**	*isp-1;ctl-1;sod-2*	***nuo-6;ctl-1;sod-2***
ctl-3	*isp-1;sod-3*	*isp-1;ctl-1;sod-3*	*nuo-6;ctl-1;sod-3*
	isp-1;sod-4	*isp-1;ctl-1;sod-4*	*nuo-6;ctl-1;sod-4*
	*isp-1;sod-5**	*isp-1;ctl-1 sod-5*	*nuo-6;ctl-1 sod-5*
	*sod-2;ctl-1**		
	*sod-2;ctl-2**		
	*sod-2;ctl-3**		
	nuo-6;ctl-1		
	nuo-6;ctl-2		
	nuo-6;ctl-3		
	isp-1;ctl-1		
	isp-1;ctl-2		
	isp-1;ctl-3		

Additionally, some mutations existed on loci on the same chromosome. Which made it more difficult to obtain them. This was particularly true in the case of *nuo-6* and *sod-2*. The genomic locus of *nuo-6* is at 1.70 cM on chromosome I and that of *sod-2* is at 2.83 cM (based on the information available at wormbase.org) on the same chromosome. This puts them about 1 cM

apart, which means there is only a 1% chance for homologous recombination to occur per cross to obtain a heterozygous animal that carries both *sod-2* and *nuo-6.* Therefore, to construct this double mutant we had to link each gene to a genetic marker which has a visible phenotype and does not require molecular genotyping to find. We chose *dpy-5(e61),* a classical allele that makes the worms look short and fat to link to *nuo-6.* Which is about 2 cM upstream of *nuo-6.* For *sod-2,* we chose *unc-29(e1072)* which is about 2cM downstream of *sod-2*'s locus. *unc* mutations hamper the movement of the worms so that they move in a distinctive circle and have a particular posture that makes them easy top spot among hundreds of worms on a plate.

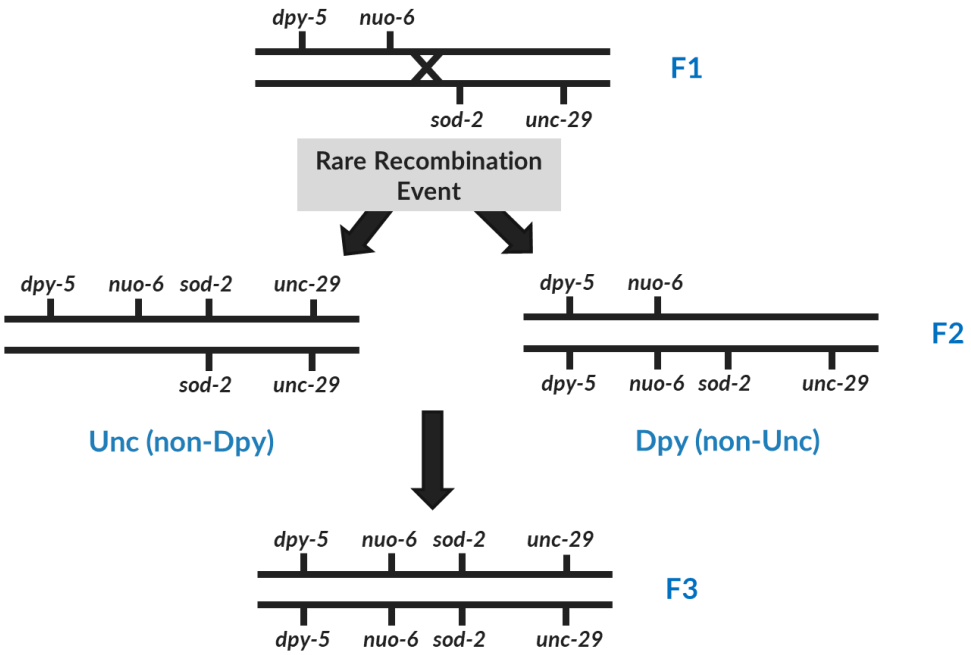


Figure 3.6. Construction of *nuo-6 sod-2* using genetic markers. *nuo-6* and *sod-2* are both located on chromosome I with an approximate distance 1 cM from each other. We linked *dpy-5* and *unc-29* to *nuo-6* and *sod-2* respectively. Then *nuo-6 dpy-5* males were crossed with *sod-2 unc-29* hermaphrodites. A rare recombination event (1% chance) would result in our target chromosome which carried both *nuo-6* and *sod-2.* To find this chromosome, we isolated Unc (non-Dpy) and Dpy (non-Unc) worms from F2 progeny and looked for Unc-Dpy worms in F3. Molecular genotyping (PCR) was used to confirm the genotype of *nuo-6 sod-2* in Unc-Dpy background and at various points during the process of outcrossing *dpy-5* and *unc-29*.

We constructed *nuo-6 dpy-5* and *sod-2 unc-29* by crossing *nuo-6* males and *dpy-5* hermaphrodites and *sod-2* males with *unc-29* hermaphrodites. Since *dpy-5* and *unc-29* alleles are not dominant, we isolated about 50 F1 progeny that looked wild-type and left them to self-fertilize.

Then we isolated the *dpy* and *unc* F2 progeny and genotyped them using PCR to make sure they carried *nuo-6* and *sod-2* respectively. To clean out any background mutations that the markers may have carried, we outcrossed *nuo-6 dpy-5* with *nuo-6* males and *sod-2 unc-29* with *sod-2* males 3 times.

In the next step, we crossed *nuo-6 dpy-5* males with *sod-2 unc-29* hermaphrodites and isolated approximately 300 heterozygous F1 progeny on individual plates. Among F2 progeny, we were looking to isolate Unc (non-Dpy) and Dpy (non-Unc) worms that may carry our target chromosome with both *nuo-6* and *sod-2* on it (Figure 3.6). This is only possible as a result of a rare recombination event at the locus between *nuo-6* and *sod-2* as shown in Figure 3.6. In this case the chance of the rare recombination event was about 1%. Among the F3 progeny of Unc (non-Dpy) and Dpy (non-Unc) worms, we looked for plates were 25% of the progeny were Unc-Dpy. At this step we used PCR to confirm whether our target chromosome existed on a particular plate. When we finally found the chromosome, we isolated the worms and let them self-fertilize to obtain homozygous animals. After that we repeated more or less the same process, to outcross *dpy-5* and *unc-29* using *nuo-6* males. This was a rather long process and unfortunately, we experienced a couple of failed attempts, but it was worth it finally when *nuo-6 sod-2* double mutants were made and were ready for the experiments!

We carried out all the crosses in *isp-1* and *nuo-6* backgrounds in 16°C. This caused the worms to grow more slowly than they would normally do in 20°C, however, in our experience, this allowed for better survivability of the multiple mutants. Once the genotype of a strain was confirmed using PCR, the strain would be maintained in 20°C and was kept well-fed for at least 3 – 5 generations before it was used for lifespan experiments. This ensured that the worms were healthy and other factors such as starvation did not skew the data of the experiments.

3.2.2. Measuring adult lifespan with and without PQ treatment

C. elegans goes through 4 larval stages (L1 – L4). Following L4 stage, before the worms develop eggs inside their ovaries, they are called young adults. Worms that already have a lot eggs inside their ovaries and are laying eggs are called gravid worms (Corsi, Wightman et al. 2015). Recognizing these stages in *C. elegans* life cycle is an important part of setting up lifespan experiments.

For the lifespan studies described in this chapter (and throughout this thesis) we used the following process as our standard experimental procedure. These steps are also shown in Figure 3.7:

1. Solid nematode growth medium (NGM) is prepared as described previously (Stiernagle 1999). When the experiment involves PQ treatment, Paraquat (Sigma-Aldrich, St. Louis, USA) is also added to the NGM at a final concentration pertinent to each experiment. This final concentration is 0.1 mM for the double- and triple-mutant lifespan experiments described in this chapter. NGM is poured into sterile plastic petri dishes that we normally call "plates". The plates are left in room temperature for about 48 hours for the NGM "gel" to solidify and its moisture content to normalize. The plates should not be too wet.

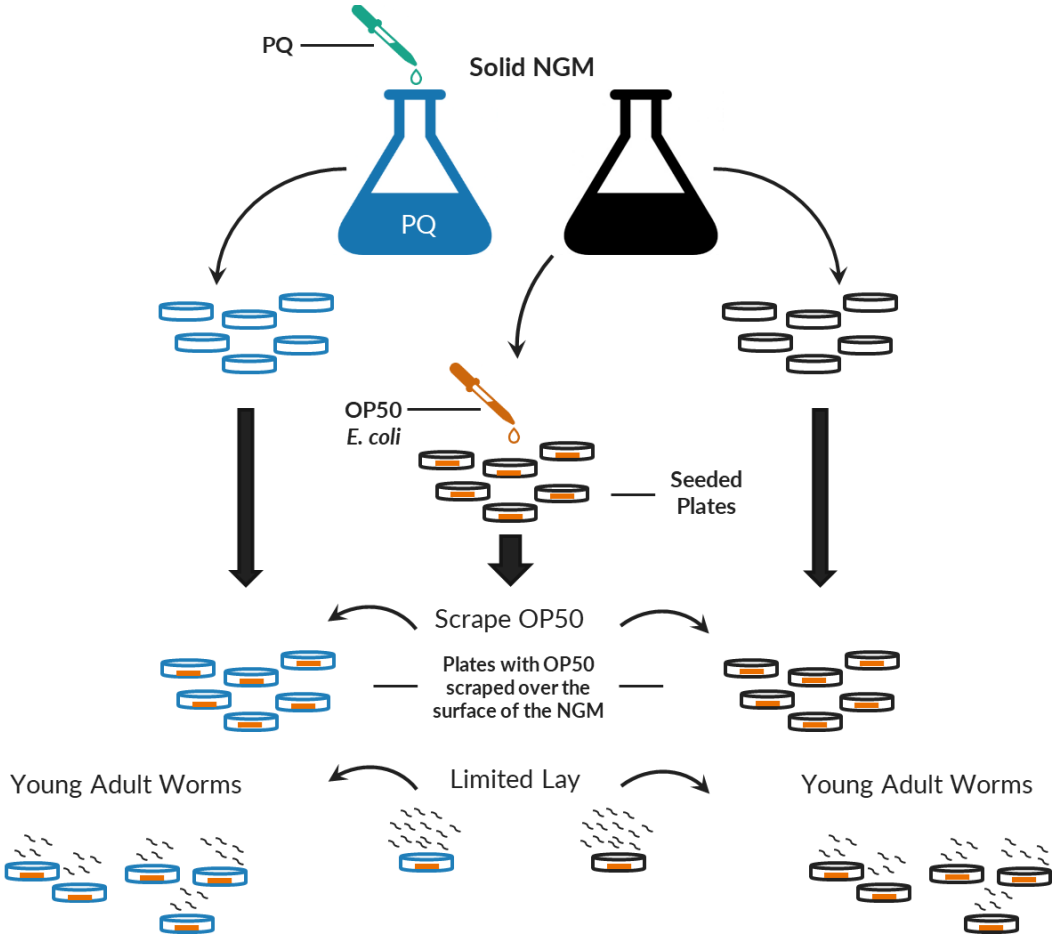


Figure 3.7. Steps for preparing solid NGM plates and setting up a lifespan experiment that involves the use of PQ. After solid NGM (for PQ and non-PQ conditions) is prepared, it is poured into small plastic petri dishes ("plates"). Some plates are seeded with *E. coli* OP50 which is the food source of the worms. OP50 is scraped onto the surface of the plates that we use for experiements that involve the use of PQ. After worms from the limited lay reach the young-adult stage, they are transferred to experimental plates and the lifespan study begins.

2. We use OP50 (a slow growing strain of *E. coli*) as the food source for the worms. A few drops of the bacterial culture are poured onto each plate to seed them. The plates are then left at room temperature for 24 – 48 hours to give the bacteria time to grow. We use these "seeded plates" to keep the strains well-fed at 20°C for at least 3 – 5 generations before using them for lifespan experiments. This ensures the health of the progeny and prevents the undesirable effects of starvation from affecting our lifespan experiments.

3. To prepare the plates that we use for lifespan experiments involving the use of PQ (e.g. experiments described in this chapter), we gather the OP50 from the seeded plates using a sterile platinum wire "pick" and scrape them over the surface of the plates that we intend to use for experiments. Normally, the entire OP50 content of a seeded plate is transferred to an experimental plate. Scraping OP50 is done for both PQ plates and plates which do not have PQ to maintain consistency between the two conditions. This adds to the labor-intensive nature of lifespan experiments, but we find it necessary as we have realized that the effect of PQ on lifespan is better demonstrated when we scrape OP50 onto the plates. PQ appears to prevent the proper growth of bacteria if OP50 is seeded directly onto PQ plates and the worms will starve during the experiment if they do not have enough food.

4. We leave gravid worms (between 20 – 50 depending on the genotype as some strains have smaller brood size than the wild-type) on PQ and non-PQ plates for 3 hours to give them time to lay eggs. This is called a "limited lay". In this way, we ensure that all the embryos on that plate are synchronized and will hatch at the same time. The gravid worms are then picked off the limited lay plates and the embryos are maintained at 20°C (like all the other stages of the experiments) until they develop into young adults (i.e. hermaphrodites that do not have eggs inside their ovaries). Note that the limited lay for the worms that are going to be used for PQ studies is done on PQ plates and similarly the limited lay for the worms that are going to be used in non-PQ studies is done on non-PQ plates.

5. Once the worms reach the young-adult stage on limited lay plates, they are transferred to experimental plates 25 worms per plate. This marks the beginning of the lifespan experiment. For each condition, we use 5 plates. The status of the worms (dead or alive) is recorded every other day for the duration of the experiment on two of these plates (lifespan plates). The 2 lifespan plates

are selected randomly after all five plates are populated with young adults. The other 3 serve as back up. If during the course of the experiment, one of the worms on lifespan plates crawls off the plate or dies as a result of internal hatching of the embryos (when eggs hatch inside the mother and kill it) we replace that worm with a randomly selected one from the back up plates.

6. During the period when the worms are laying eggs, we transfer the worms from each of the 5 experimental plates to fresh scraped plates every other day (Appx. 125 worms per condition including backup worms) to prevent mixing of the progeny with the mothers. After the worms stop laying eggs, we transfer them to fresh scraped plated every six days to maintain optimal living conditions for the worms. Also, if fungal contamination is seen on a plate, the worms are transferred immediately to a fresh, clean plate. If bacterial contamination occurs and persists after transferring the worms to a fresh clean plate, the affected lifespan plates will be discarded and the trial with the affected strain needs to be repeated if the total sample size of the pooled data is below 100.

7. To score lifespan, we record the number of dead and alive worms on a lifespan plate every other day. To score a worm as dead, first we gently "poke" the area around the worm on the NGM. The distortion of the surrounding gel area causes the worm to move if it is not dead. If this does not work, we gently pick up the worm and move it to place on the plate that does not have food on it. If a worm is not dead, it will move its head slightly to look for the food or inspect the change in the surrounding environment. This requires patience, so we leave the worm while checking other plates. When we get back to it, if it is not moving or has not moved from that position, we poke the worm with the platinum wire pick gently on its tail. If no movement is detected at this stage, the worm is scored dead and is picked off the lifespan plate.

For each lifespan experiment we conducted 2 – 3 independent trials. The data for each trial was recorded separately and once all these trials were complete, the data was pooled, and statistical analysis was performed using GraphPad Prism (v6.01) and ordinary one-way ANOVA with Sidak's correction for multiple comparisons.

In order to understand and present the large data set of this study we have used data matrices and meaningful sub-sets organized in graphs and tables. In section 3.3.1, we have discussed the analysis, organization and presentation of the lifespan data for the studies presented in this chapter.

3.3. Results

3.3.1. Presentation and analysis of the lifespan data sets for the double- and triple-mutants

As described previously throughout this chapter, in order to investigate whether H_2O_2 is the pro-longevity signal and to find the sources and sinks of such a signal, we constructed a set of double- and triple-mutant knockouts of different SODs (logically one of the main sources of H_2O_2) and CTLs (one of the main sinks of H_2O_2) in *isp-1, nuo-6* and the wild-type (N2) backgrounds. The high mitochondrial $O_2^{\bullet-}$ production of *isp-1* and *nuo-6* leads to their longevity but treatment with NAC which enhances H_2O_2 scavenging suppresses their longevity (Yang and Hekimi 2010a). Therefore, SODs appeared to be the missing link between the mitochondrial $O_2^{\bullet-}$ signal and the H_2O_2 which confers the effects of this signal. We also used PQ as a $O_2^{\bullet-}$ generator which phenocopies *isp-1* and *nuo-6* (Yee, Yang et al. 2014) to further investigate the pro-longevity effect of increased ROS in the wild-type and *isp-1* and *nuo-6* backgrounds which are already sensitized to this effect and have altered mitochondrial $O_2^{\bullet-}$ metabolism. To investigate the roles of SODs and CTLs, we scored the lifespan of the double- and triple-mutants shown in Table 4.1 in the wild-type background and where we observed significant effects, we investigated further using equivalent double- or triple-mutant combinations in the *nuo-6* background. For each experiment, the lifespans of all conditions were scored in multiple trials (each of which lasted between 1 to 2 months) and the data from all trials were pooled. We organized these lifespan data of *sod* and *ctl* multiple mutant combinations in 21 graph sets and data tables based on the double- or triple-mutants used and their background (i.e. *isp-1, nuo-6* or the wild-type). For comparisons we used average adult lifespan and did not include larval stages in our lifespan analysis because the duration of development varies between different backgrounds and will cause interference with data interpretation if included in lifespan analysis. For example, *isp-1* and *nuo-6* mutations as well as 0.1 mM PQ treatment, all cause the developmental stages to last longer compared to the untreated wild-type. Therefore, not including the developmental period in the lifespan data is the logical choice to avoid masking of true effects or false positives. An example of these 21 graphical data sets with the key to its statistical analysis is provided in Figure 3.8. Note that this figure is not intended to present the data, it is rather intended to focus on the key to the process of data analysis

and discovery that we have used. All 21 such graph sets along with the corresponding data table are presented in section 3.5.

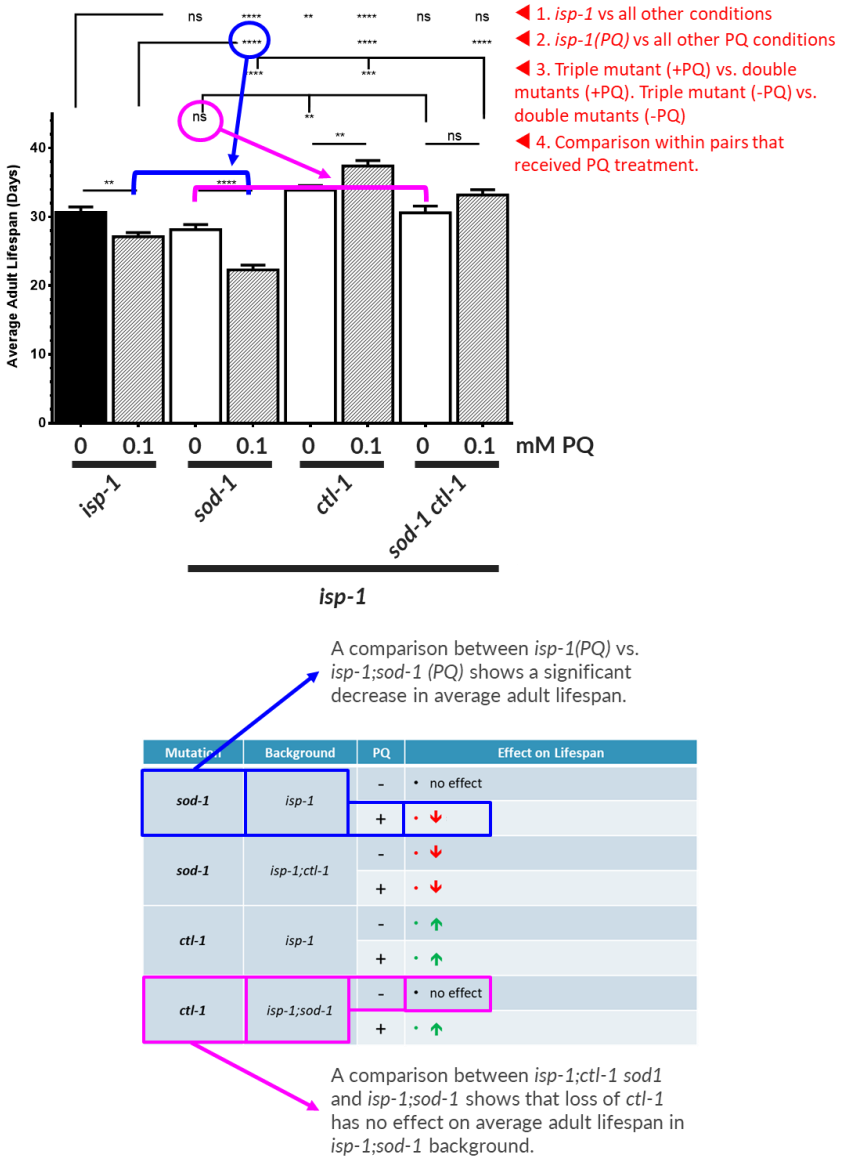


Figure 3.8. An example demonstrating the systematic statistical analysis of a sample data set of the studies presented in this chapter. This figure is not intended to present the results. Its purpose is to indicate the statistical analysis performed on this sample data set which is one of the 21 data sets from this study.(Left Panel) The average adult lifespan data of pooled multiple trials for each strain is presented in a bar graph. The comparisons are performed among 4 families (indicated in red next to the bar graphs). (Right Panel) A summary of the data from the bar graph is shown in a table. Two examples are highlited in blue an purple in both panels to show the corresponding portions of the data that they refer to. For each of the four families of comparisons, statistical analysis is performed using One-Way Anova with Sidak's correction for multiple comparisons. Sample sizes for experiments are between 100 – 200 based on 2 – 3 independent trials (for more details see section 3.2 on materials and methods).

To see the "big picture" in the vast data sets of this study, we also organized the pooled lifespan data from these 21 data sets into six data matrices. These data matrices included comparisons of average adult lifespan based on the background of the experimental conditions. These backgrounds are:

1. *isp-1* background
2. *isp-1* + 0.1 mM PQ background
3. *nuo-6* background
4. *nuo-6* + 0.1 mM PQ background
5. The wild-type (N2) background
6. The wild-type (N2) + 0.1 mM PQ background

These six data matrices are shown in section 3.5. Organizing the data in this way enabled us to view and analyze general trends in lifespan changes as well as spotting details and their relationship with other pieces of the puzzle. However, presenting these data in the forms discussed above in this section is not feasible since the important points are not immediately visible. Instead, for the purpose of presenting the results in this section, we have broken down the data in graph sets that are meaningful to the context of each section. Note that because of the way we have opted to present the data, one or some particular data points (for example the control) may be repeated in several graphs. Our intention is not to show duplicate data. We present the data in this way because all the data points in a particular graph belong to the same study and become meaningful when they are presented together rather than in separate graphs. In any given case, the controls were also present in all pertinent trials and their data were pooled.

Another important note is that in some cases we have shown the adult lifespan as lifespan curves and in other cases as average adult lifespan in bar graphs. Our reasons for the method of presenting the data are twofold. First, presenting lifespan data in the form of bar graphs that show average adult lifespan makes comparisons and statistical analysis more practical. For all these data sets that are presented in bar graph format, the corresponding lifespan curves are shown in section 3.5. Second, in some cases showing bar graphs instead of the lifespan curves omits certain features of the data. For example, if a lifespan curve is biphasic, this feature cannot be shown in a bar graph that only includes the average lifespan. All the pooled data from multiple trials for the lifespan experiments are shown in tables in section 3.5.

3.3.2. The pro-longevity signal induced by mitochondrial $O_2^{\bullet-}$ is an increase of SOD-1-dependent cytoplasmic H_2O_2

Interestingly, through analyzing the lifespan data sets of all these double- and triple-mutants, we found that although the initial ROS change is in mitochondria, this ultimately produces a change in cytoplasmic H_2O_2 levels, and this change is necessary for lengthening the lifespan as it is the pro-longevity signal. In the following sections, we present the data that serve as evidence for this conclusion.

3.3.2.1. The short lifespan of the *sod-1* mutants is rescued by the loss of cytoplasmic *ctl*

Loss of *sod-1* has been associated with no change (Van Raamsdonk and Hekimi 2009) or a shortening (Doonan, McElwee et al. 2008, Yanase, Onodera et al. 2009) of lifespan in the worm. We also found that *sod-1* mutants are short-lived compared to the wild-type (N2). So, we wondered whether their short lifespan is the result of insufficient cytoplasmic H_2O_2. CTLs are very efficient H_2O_2 sinks and loss of any of the worm's *ctl* genes (*ctl-1, ctl-2, ctl-3*) does not affect their lifespan. However, while loss of *ctl-2* (peroxisomal) partially rescues the lifespan shortening effect of *sod-1* deletion, removal of *ctl-1* (cytoplasmic) fully rescues the short lifespan of *sod-1* (Figure 3.9). In the absence of SOD-1, much less H_2O_2 is made in the cytoplasm but when we remove the CTLs, we are actually removing one of the sinks of this H_2O_2 which rescues the deficiency of H_2O_2 in the cytoplasm. In the wild-type, we do not have the stimulation of the pro-longevity effect caused by $O_2^{\bullet-}$ metabolism in the mitochondria, so the magnitude of the effect is small. Therefore, for the next step we tried 0.1 mM PQ treatment to increase mitochondrial stimulation and $O_2^{\bullet-}$ production.

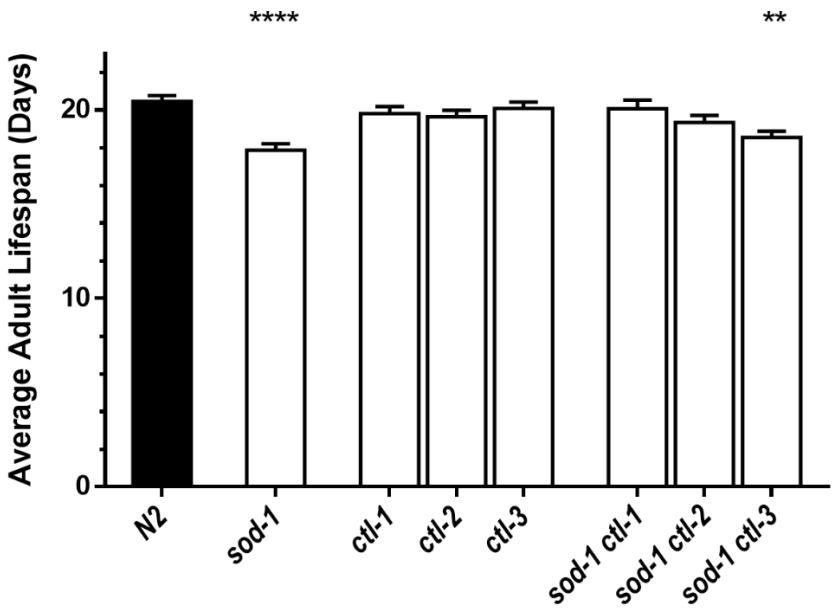


Figure 3.9. The shortening effect of *sod-1* deletion is rescued by the loss of cytoplasmic *ctl.* The loss of SOD-1, the worm's primary cytoplasmic SOD, shortens the lifespan of the wild-type (N2). However, this lifespan shortening effect is the result of cytoplasmic H_2O_2 deficiency and not increased ROS damage caused by the loss of a major SOD. That is why removing the worm's main cytoplasmic H_2O_2 sink, CTL-1, rescues the short lifespan of *sod-1* mutants by increasing the levels of cytoplasmic H_2O_2. N2, *sod-1, ctl-1, ctl-2, ctl-3* n=150, 3 independent trials, all other conditions n=100, 2 independent trials. 50 worms per trial. Comparison of the average adult lifespans vs. N2: Ordinary one-way ANOVA with Sidak's correction for multiple comparisons. **** | P<0.0001, ** | P<0.01, Error bars represent SEM

3.3.2.2. *sod-1* is required for the pro-longevity effect of PQ treatment

In section 3.1.2, we discussed that 0.1 mM PQ treatment increases the lifespan of the wild-type worms by stimulating mitochondrial $O_2^{\bullet-}$ production. In Figure 3.10, average adult lifespan of *sod* and *ctl* mutants is shown with and without 0.1 mM PQ treatment. Similar to the wild-type, PQ treatment also increases the lifespan of *sod-3, sod-4, sod-5* and all *ctl* mutants. *sod-2,* of course, is a special case; PQ treatment, shortens the lifespan of *sod-2* (see chapter 2). Here we found that loss of SOD-1, the worm's primary cytoplasmic SOD, completely suppresses the pro-longevity effect of PQ. Additionally, the magnitude of the lifespan-shortening effect of loss of SOD-1 has amplified due to increased stimulation by PQ. Taken together, these observations suggest that SOD-1 is required for the pro-longevity effect of PQ. Next, we increased stimulation further by adding the *isp-1* mutation to the mix in addition to PQ treatment.

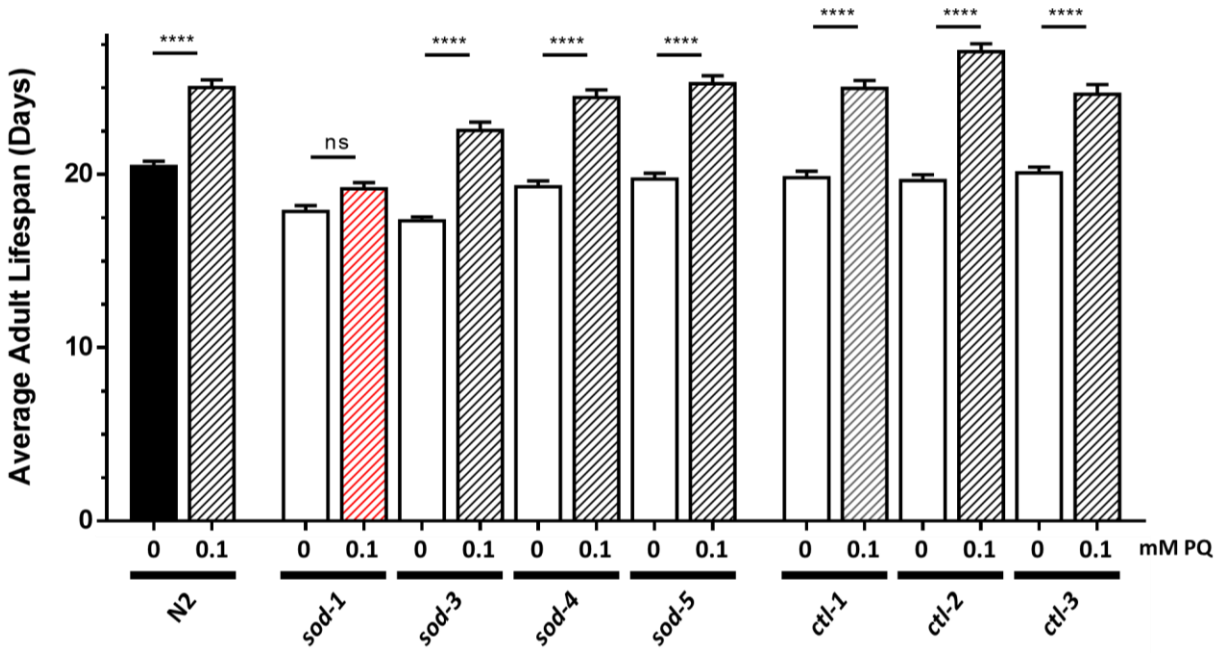


Figure 3.10. *sod-1* is required for the pro-longevity effect of PQ treatment. In this graph, we have compared the average adult lifespan of the wild-type (N2), *sod-1, sod-3, sod-4, sod-5* and *ctl* mutants with and without 0.1 mM PQ treatment. *sod-2* is not included because there is no concentration of PQ that increases its lifespan and we discussed it in chapter 2. Except for *sod-1,* PQ treatment increases the average adult lifespan of all other conditons. Under the stimulation of PQ, which mainly increases mitochondrial $O_2^{\bullet-}$ generation, the lifespan shortening effect of loss of *sod-1* (Red bargraph) is even more pronounced compared with the wild-type whose lifespan greatly increases with PQ treatment. All conditions n=150, 3 independent trials. 50 worms per trial. Comparison of the average adult lifespans in pairs: Ordinary one-way ANOVA with Sidak's correction for multiple comparisons. **** | P<0.0001, Error bars represent SEM

3.3.2.3. The effects of *sod-1* and PQ on *isp-1* and *nuo-6* are suppressed by the loss of cytoplasmic CTL-1

As we discussed before, *isp-1* and *nuo-6* mutations and PQ act in the mitochondria. Worms treated with PQ or *isp-1* and *nuo-6* mutants live long because the metabolism of $O_2^{\bullet-}$ in their mitochondria has changed. By combining *isp-1* and PQ or *nuo-6* and PQ we looked to increase the stimulation to see the effect of *sod-1* deletion. In the previous sections we saw that *sod-1* mutations shortens the lifespan of the wild-type.

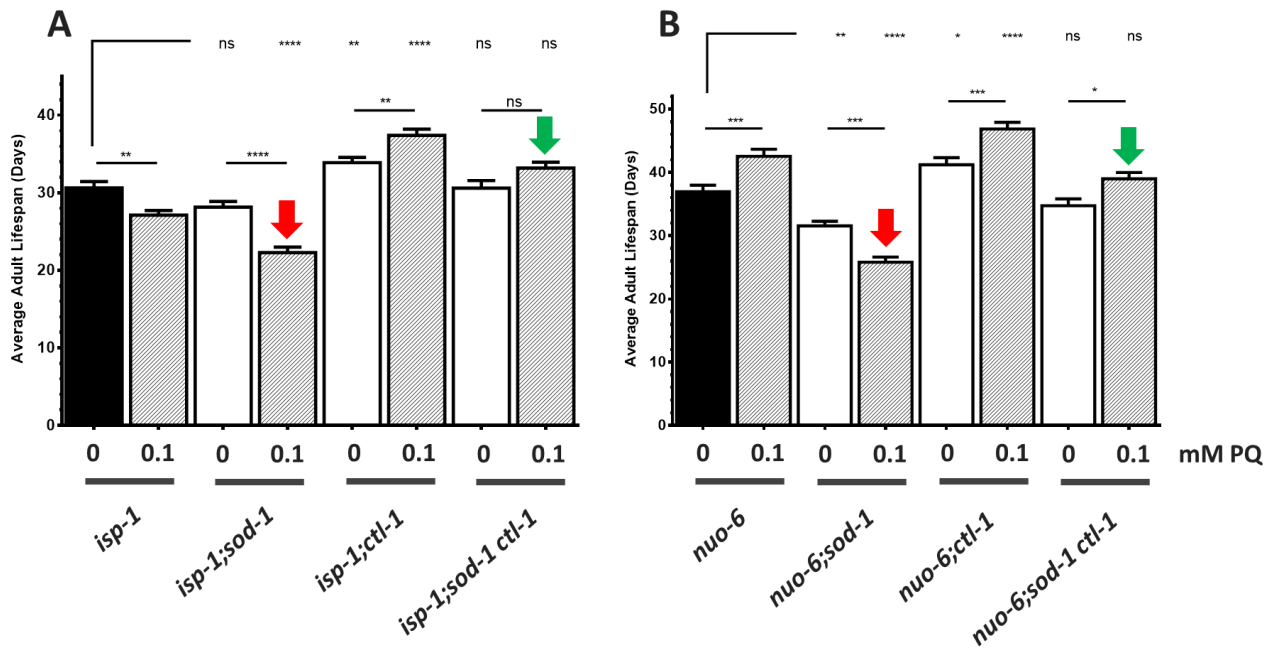


Figure 3.11. The effects of *sod-1* and PQ on *isp-1* and *nuo-6* are suppressed by the loss of cytoplasmic CTL-1. The average adult lifespan of *isp-1* (A) and *nuo-6* (B) of *sod-1* and *ctl-1* mutants are shown with and without 0.1 mM PQ treatment. The combined effects of the loss of *sod-1* and PQ stimulation shortens the lifespan of *isp-1* and *nuo-6* (Red Arrows). However, loss of CTL-1, as a major cytoplasmic sink of H_2O_2, restores the lifespan of both *isp-1* and *nuo-6* (Green Arrows), suggesting that the lifespan shortening effect of *sod-1* deletion and PQ treatment is due to cytoplasmic H_2O_2 deficiency. *isp-1* and *isp-1;ctl-1* (0 and 0.1 mM PQ), n=150, 3 independent trials, all other conditions n=100, 2 independent trials. 50 worms per trial. Comparison of the average adult lifespans in pairs and vs. control: Ordinary one-way ANOVA with Sidak's correction for multiple comparisons. **** | P<0.0001, *** | P<0.001, ** | P<0.01, * | P<0.05, Error bars represent SEM

In Figure 3.11 A, we see that in a background that includes both *isp-1* and PQ, the worms live even shorter. But what causes the large lifespan shortening effect by *sod-1* deletion in the *isp-1* background in the presence of PQ? We see that the removal of *ctl-1,* the main cytoplasmic CTL, fully restores the lifespan of *isp-1;ctl-1 sod-1* to the level of *isp-1*. This shows that both *isp-1* and PQ need SOD-1 for increasing cytoplasmic H_2O_2. Therefore, by removing CTL-1, as a major H_2O_2 sink, we help increase cytoplasmic H_2O_2 and restore *isp-1*'s longevity. In other words, this suggests that in the absence of SOD-1 (and even simply in *isp-1*) PQ is lifespan-shortening because an increase in the $O_2^{\bullet-}$ levels in the mitochondria of *isp-1* (and/or elsewhere in the case of PQ), can have deleterious lifespan-shortening effects when it is uncoupled from or insufficiently accompanied by (e.g. in the case of *isp-1* itself) a protective, lifespan-lengthening effect of elevated

cytoplasmic H_2O_2 . We have demonstrated this concept, in more detail, in the *isp-1* data set from Figure 3.11 A in section 3.4.4. So far, we have seen that SOD-1 is required for the longevity effect of both *isp-1* and PQ. It is also true about *nuo-6* (Figure 3.11 B). However, an additional feature in *nuo-6* lifespan data is that *sod-1* deletion shortens the lifespan of *nuo-6,* just like the way it affects the lifespan of the wild-type. This suggests that *nuo-6;sod-1* mutants, also rely on the action of SOD-1 to maintain the required H_2O_2 levels for their longevity.

Next, we wondered if *sod-1* is required for the longevity of *sod-2* as well, since as we discussed in section 3.1.1, NAC (a H_2O_2 scavenger) treatment suppresses the lifespan of all three conditions (*isp-1, nuo-6* and *sod-2*).

3.3.2.4. SOD-1 is required for the pro-longevity effect of the loss of SOD-2

In chapter 2, we discussed that loss of SOD-2, the primary mitochondrial SOD, increases the lifespan of *C. elegans*. Given the role of SOD-2 in the metabolism of $O_2^{\bullet-}$ within mitochondria, we expect that this longevity effect would also result from increased mitochondrial ROS generation. Also, like *isp-1* and *nuo-6,* the longevity of *sod-2* mutants is also suppressed by NAC treatment which enhances H_2O_2 scavenging. Since we have shown that SOD-1 is required for the pro-longevity effect of *isp-1, nuo-6* and PQ (which phenocopies those two mutations), we wondered whether SOD-1 is also required for the pro-longevity effect of loss of SOD-2. We found that loss of SOD-1, indeed, suppresses the longevity of *sod-2* mutants. Figure 3.12 A shows the adult lifespan curves of *sod-1;sod-2* double mutants in comparison with the single mutants and the wild-type (N2) under our normal experimental conditions.

However, previous results from our laboratory indicated that *sod-1;sod-2* double mutants also live long (Van Raamsdonk and Hekimi 2009) under different experimental conditions. In that study, *sod-1* had a normal lifespan, too. In the study by Van Raamsdonk *et al,* 100 µM FUDR (5-Fluoro-2′-deoxyuridine) was used to prevent extensive internal hatching (larvae hatching inside of the mother) of the *sod* double-mutants. FUDR is widely used in *C. elegans* lifespan studies to induce maternal sterility to prevent internal hatching and somewhat alleviate the labor-intensive nature of lifespan studies.

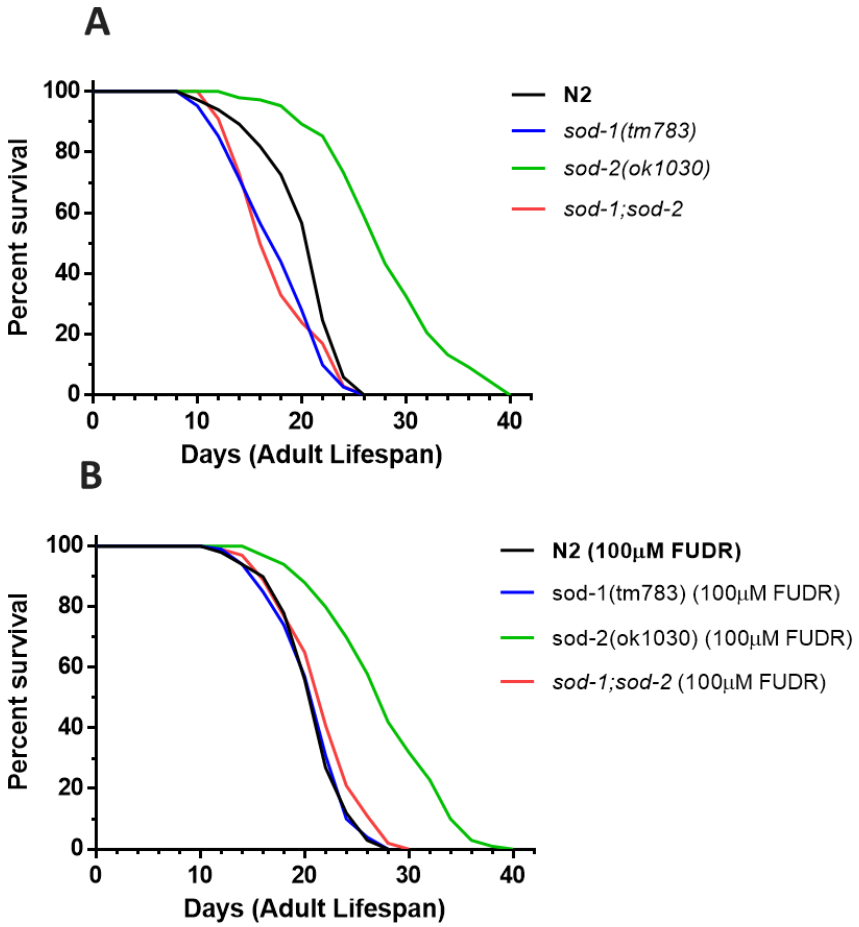


Figure 3.12. SOD-1 is required for the pro-longevity effect of the loss of SOD-2. The adult lifespan curves of the wild-type (N2), *sod-1, sod-2* and *sod-1;sod-2* double mutants under our standard experimental conditions (A) and when using 100 μM FUDR to induce maternal strility (B). Deletion of *sod-2* leads to longevity in the wild-type background. However, loss of SOD-1, suppresses this longevity by eliminating the cytoplasmic source of H_2O_2. Treatment with 100 μM FUDR is known for inducing longevity in some mutan backgrounds; howver, while this treatment does not affect the lifespan of the wild-type, it restors the shortened lifespan of *sod-1* mutants but even with FUDR treatment, the longevity effect of *sod-2* deletion is still suppressed by the loss of *sod-1*. N2, *sod-1, sod-2* n=150, 3 independent trials, all other conditions n=100, 2 independent trials. 50 worms per trial.

FUDR does not affect the lifespan of the wild-type, but it is known to induce longevity in some mutant backgrounds (Aitlhadj, Stürzenbaum et al. 2010, Van Raamsdonk and Hekimi 2011, Rooney, Luz et al. 2014, Anderson, Corkins et al. 2016), which was the primary reason why we did not use it for this study. Therefore, to further investigate the difference between our data in this study and the study by Van Raamsdonk *et al,* we repeated the *sod-1;sod-2* lifespan experiment with 100 μM FUDR. We confirmed that treatment with FUDR does not affect the adult lifespan

of the wild-type and it also suppresses the lifespan shortening effect of the loss of SOD-1. However, even with the use of 100 μM FUDR, loss of SOD-1 still suppresses the long lifespan of *sod-2* mutants (Figure 3.12 B). This suggests that, like *nuo-6, isp-1* and PQ, the pro-longevity effect of *sod-2* deletion is also dependent on the action of SOD-1 to produce H_2O_2 in the cytoplasm.

3.3.2.5. Loss of cytoplasmic CTLs lengthens lifespan with the greatest changes in mitochondrial $O_2^{\bullet-}$

So far, we have shown that an increase in the cytoplasmic H_2O_2 is necessary for the pro-longevity effect of increased mitochondrial $O_2^{\bullet-}$ which PQ treatment and *isp-1, nuo-6* and *sod-2* rely on. We also presented that SOD-1, which acts in the cytoplasm, modulates this effect. To investigate the role of cytoplasmic H_2O_2 further, we looked at the data from our set of multiple mutants to see what the general trends in the lifespan of different backgrounds indicate. We found that indeed in both maximally stimulated backgrounds of *isp-1* and *nuo-6* with PQ, removing the CTLs increases the lifespan of *isp-1*+PQ and *nuo-6*+PQ even further (Figure 3.13). This demonstrate that in both cases the pro-longevity effect of ROS is not maximal, and that is why increasing H_2O_2 levels by removing the CTLs is able to maximize the pro-longevity effect of ROS. In fact, a feature immediately visible in Figure 3.13 in this regard is that the longevity effect of *sod-2* deletion is additive to both *isp-1*+PQ and *nuo-6*+PQ. This is not surprising since we determined that *sod-2* longevity also works through the action cytoplasmic SOD-1; although SOD-2 itself localizes to the mitochondria.

However, another outstanding feature, is the pro-longevity effect of the loss of SOD-4 (the worm's extracellular/membrane bound SOD isoforms) in *nuo-6*+PQ background (Figure 3.13 A) and not in *isp-1*+PQ (Figure 3.13 B). Based on our current working model of cytoplasmic H_2O_2 as the pro-longevity signal and the general visible patterns in the lifespan data, one possibility is that lack of SOD-4 may cause an increase in the influx of extracellular $O_2^{\bullet-}$ into the cytoplasm where it is converted into H_2O_2 by SOD-1 and promotes longevity (see Figure 1.15 for an overview of the worm's ROS network). Of course, loss of SOD-4 does not have such the same effect in *isp-1* background even when it is stimulated by PQ. This is not unusual. Although, both *isp-1* and *nuo-6* share numerous characteristics that are hallmarks of long-lived ETC mutants, such as high mitochondrial $O_2^{\bullet-}$ production, low ATP levels and slow development and behavior (Yee, Yang et al. 2014), they are not completely similar. No two mutants are.

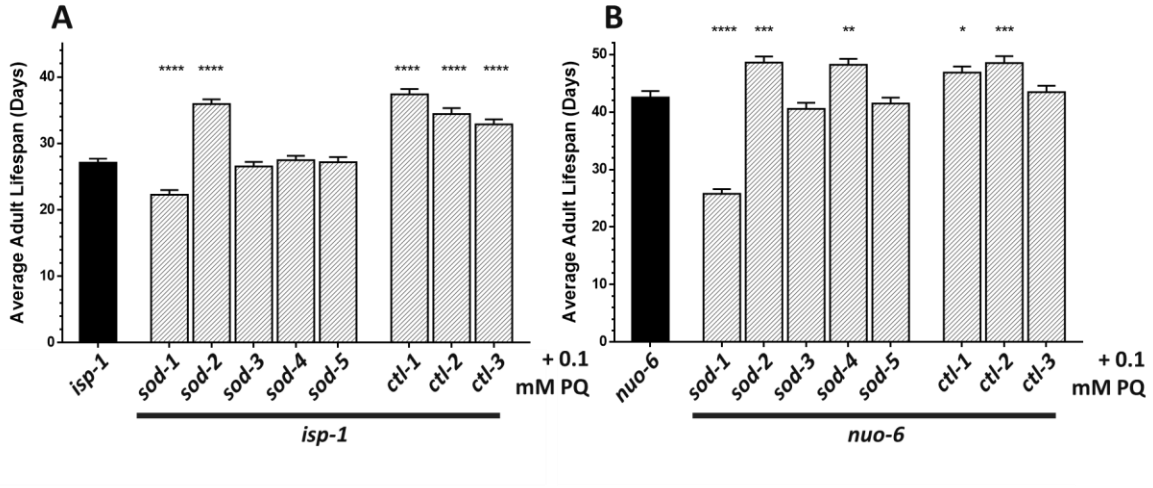


Figure 3.13. Loss of cytoplasmic CTLs lengthens lifespan with the greatest changes in mitochondrial $O_2^{\bullet-}$. Graphs show the effects of loss of all *sods* and *ctls* under the maximally stimulated conditions of 0.1 mM PQ treatment and *isp-1* (A) and *nuo-6* (B) mutations. In both backgrounds loss of *sod-1* causes a decrease in the average adult lifespan of the worms due to decreasing levels of cytoplasmic H_2O_2. However, in all other strains (that have *sod-1*) we do not see a decrease in lifespan compared to the control. On the contrary, the loss of all three *ctls* in *isp-1* background and *ctl-1* and *ctl-2* in *nuo-6* background result to an increase in lifespan since removing them as sinks of H_2O_2 in the cytoplasm makes H_2O_2 more available to induce longevity. In a similar way, the SOD-1-dependent longevity of the loss of *sod-2,* also increases the lifespan in both backgrounds. A unique feature of *nuo-6* is that the loss of *sod-4* (extracellualr/membrane-bound SOD) also increases in longevity. One exaplanatio for this effect is that the loss of SOD-4 may cause an increase in the influx of extracellular $O_2^{\bullet-}$ into the cytoplasm, where SOD-1 is present to convert this $O_2^{\bullet-}$ into H_2O_2 which in turn acts as a pro-longevity signal (see text for more details). *isp-1, isp-1;sod-2,* and *isp-1;ctl-1* n=150, 3 independent trials, all other conditions n=100, 2 independent trials. 50 worms per trial. Comparison of the average adult lifespans vs. control: Ordinary one-way ANOVA with Sidak's correction for multiple comparisons. **** | P<0.0001, *** | P<0.001, ** | P<0.01, * | P<0.05, Error bars represent SEM

In fact, the two panels in Figure 3.13 showcase some other differences of *isp-1* and *nuo-6,* too. For example, we can see that under these maximally stimulated conditions the average adult lifespan of *isp-1* is shorter than that of the *nuo-6.* We can also see that the loss the three CTLs has relatively larger impact on the average adult lifespan of *isp-1*. However, in both cases increasing ROS by removing either *sod-2* or *ctls* extends their lifespan even further, which goes to show that in their unstimulated state even the long-lived *isp-1* and *nuo-6* still have room for a little more pro-longevity ROS. We will discuss this relationship between ROS and longevity further in the context

of our inverted U-shaped model in section 3.4. We will also present our model for the path of the pro-longevity effect of the mitochondrial $O_2^{\bullet -}$.

3.4. Discussion

3.4.1. Using SODs and CTLs to systematically study the regulation of ROS-dependent longevity in *C. elegans*

In this chapter we reviewed two long-lived ETC mutants of *C. elegans* (*isp-1* and *nuo-6*) that single-handedly have challenged the core concepts of the free radical theory of aging (see section 1.1.5). These mutants have dysfunctional mitochondria that generates more $O_2^{\bullet-}$ than the mitochondria of the wild-type. This increase in mitochondrial $O_2^{\bullet-}$ transmits a signal that promotes longevity in these mutants (Yang and Hekimi 2010a). In our laboratory, we have previously shown that the mitochondrial intrinsic apoptosis pathway is partially responsible for translating the mitochondrial $O_2^{\bullet-}$ signal to longevity in the worms (Yee, Yang et al. 2014). The intrinsic apoptosis pathway can be activated by either of the worm's BH3-only proteins: EGL-1 and CED-13 (see section 1.5.3). Upon activation by EGL-1, this conserved pathway promotes apoptotic cell death that occurs during the development of *C. elegans*. However, when *isp-1* and *nuo-6* activate this pathway through CED-13, it promotes protective mechanisms that lead to longevity. The intrinsic apoptosis pathway has three other protein components: CED-9 (Bcl2-homolog), CED-4 (Apaf1-homolog) and CED-3 (Casp9-homolog). The BH3-only protein interacts with CED-9 which leads to the release of CED-4 and ultimately the activation of CED-3. Interestingly, PQ treatment, which phenocopies the ROS-dependent longevity of the *isp-1* and *nuo-6* mutations, can bypass the need for CED-13 and activate the intrinsic apoptosis pathway directly, possibly by affecting CED-9 which is physically associated with mitochondria.

Loss of SOD-2, also leads to longevity in *C. elegans* (Van Raamsdonk and Hekimi 2009). SOD-2 is the worm's primary mitochondrial MnSOD and is believed to localize to the supercomplex I:III:IV of the ETC where it can interact with $O_2^{\bullet-}$ right at the site of its production (Suthammarak, Somerlot et al. 2013). Therefore, the natural function of SOD-2 implies that the longevity of the *sod-2* mutants may be due to increased mitochondrial $O_2^{\bullet-}$ generation, too.

However, we also observed that treatment with NAC suppresses the longevity of *isp-1, nuo-6* and *sod-2* which, of course, confirmed the involvement of ROS in their longevity but also highlighted the role of H_2O_2 in this regard. NAC enhances H_2O_2 scavenging by boosting the glutathione-dependent antioxidant systems such as peroxiredoxins (see section 1.4.3) and

glutathione peroxidases (see section 1.4.4) (Bavarsad Shahripour, Harrigan et al. 2014). NAC and in general thiol-containing compounds, do not target $O_2^{\bullet-}$ irrespective of their concentration (Aldini, Altomare et al. 2018). This is partially due to the fact that the reaction rate of these compounds is far slower for interactions with $O_2^{\bullet-}$ than the reaction rate of SODs (Winterbourn 2016). Another reason that makes the involvement of H_2O_2 in the longevity of *isp-1, nuo-6* and *sod-2* more likely, is that the biochemical properties of H_2O_2 and its specificity for targeting mechanisms such as cysteine switches (see section 1.3.5) enables it to act as a messenger to carry a redox signal from the its site of production to a target site. Therefore, among all ROS, H_2O_2 is considered most suitable for redox signaling (Sies 2017). So, as the SODs hold a monopoly over the conversions of $O_2^{\bullet-}$ to H_2O_2, it appeared that their involvement in the mitochondrial $O_2^{\bullet-}$ induced longevity was evident. Fortunately, the worm has a rather research-friendly collection of SODs with subcellular localizations that can help us identify the path of the pro-longevity ROS in addition to the specific kind of ROS that is involved. Worms are unusual in that way. Most eukaryotes have three SOD isoforms (cytoplasmic, mitochondrial, extracellular) while *C. elegans* has five. This diversity is not simply a redundancy. The different isoforms of SODs in the worm indeed appear to have specific functions to perform. For example, in chapter 2 we discussed how the two virtually identical mitochondrial MnSODs in *C. elegans* show tissue-specific expression patterns and even their deletion has opposite effects on lifespan. Therefore, the study of the role of the different SOD isoforms of the worm can offer valuable insights into the redox systems of the long-lived mutants (*isp-1, nuo-6* and *sod-2*) that we are interested in.

For this study, we also needed to be able to manipulate the sinks of H_2O_2 to be able to detect the effects of increasing or decreasing H_2O_2 levels by manipulating the SODs in different subcellular compartments. We chose CTLs for this purpose. Like SODs, the CTL enzymes of *C. elegans* are also unusual in that the worm has three of them. Most eukaryotes have one peroxisomal CTL but *C. elegans* has two cytoplasmic ones (CTL-1 and CTL-3) in addition to the peroxisomal CTL-2. The fact that worms have evolved two cytoplasmic CTLs implies that a very tight control over the regulation of cytoplasmic H_2O_2 is very important to the different aspects of the worm's physiology. Therefore, to find the path and nature of the pro-longevity ROS in the ETC mutants *isp-1* and *nuo-6*, we decided to conduct a genetic dissection of SODs and CTLs in these backgrounds using deletion alleles (knock-out mutants). The alternative was using RNA interreference (RNAi) by feeding to knock down the expression of SODs and CTLs. This method,

of course, has the advantage of offering a faster option compared to the generation of dozens of multiple-mutant strains, but there are caveats. First, the presence of some molecular features in *ctl* gene cluster of *C. elegans,* such as a high level of sequence identity (Figure 3.4), make it next to impossible to use the results of methods such as RNAi as definitive answers to the roles of individual CTLs in the worm's process of aging (Petriv and Rachubinski 2004). Second RNAi is known to induce unexpected phenotypes (De-Souza, Camara et al. 2019) and even mimic a phenotype through activating a completely different mechanism than the actual mutation. For example, it appears that RNAi against *isp-1* and *nuo-6* extends the lifespan of the worms not via increasing $O_2^{\bullet-}$ production but through the induction of mitochondrial unfolded protein response (Yang and Hekimi 2010b, Durieux, Wolff et al. 2011). To avoid these problems, we set out to create a set of *sod* and *ctl* multiple mutant combinations in *isp-1* and *nuo-6* backgrounds that offer a valuable tool for any study that involves investigating the redox systems of the worm.

3.4.2. Lessons from the lethality of some combinations of mutations

Some of these multiple mutants were difficult to construct due to the fact that the two genes of interest were located on the same chromosome very close to each other. This was a technical issue and was particularly true about *nuo-6 sod-2* (see section 3.2.1). However, for some triple-mutants we had to try generating the strains several times due to the problems caused by the altered ROS physiology of the combined effects of the multiple mutations. For example, *nuo-6;ctl-2;sod-2* and *isp-1;ctl-2;sod-2* were particularly hard to generate. We used *nuo-6;ctl-2* or *isp-1;ctl-2* males and *nuo-6 sod-2* and *isp-1;sod-2* hermaphrodites respectively to generate these. This simplified the crosses and increased our chances (1 in 16) of easily obtaining the triple mutants. Due to the large sample of F2 worms (Appx. 60 worms) that were used for molecular genotyping (PCR) and after three attempts for *nuo-6;ctl-2;sod-2* and two attempts for *isp-1;ctl-2;sod-2,* we were still not able to construct them, and it was starting to look like the mutant combination was lethal. However, on the next attempt they were made. We initially thought this was due to the effects of increased ROS toxicity because we had removed a major mitochondrial SOD, and the proxisomal CTL. However, both strains looked healthy and apart from the reduced brood size, slow development and slow behavior which are typical of *nuo-6* and *isp-1* they had no other phenotypes that were immediately discernable. But they had a surprise in store for us when we used them in the lifespan studies. The average adult lifespans of both triple mutants were shorter than that of *nuo-6* (Figure

S3.17) and *isp-1* (Figure S3.5). However, increasing ROS levels through PQ stimulation restored the triple-mutant's lifespan to *nuo-6* and *isp-1.* A possible explanation is that based on our current findings, by increasing cytoplasmic H_2O_2 the longevity effect of *sod-2* under PQ stimulation is additive to lifespan shortening effect of the combined loss of *ctl-2* and *sod-2* in both cases. However, this also means it is possible that these two strains were probably hard to obtain not because they were affected by the negative effects of ROS toxicity because they had too much ROS, but maybe they were hard to obtain because most of the embryos that carried the triple-mutations did not have enough ROS which they needed during their development and failed to develop as a result. Some of the developmental features of the worm such as vulval development are known to be regulated by ROS (Shibata, Branicky et al. 2003). We will revisit the topic of optimum levels of ROS in the following sections.

However, *isp-1;ctl-1;sod-2, isp-1;ctl-1;sod-4, nuo-6;ctl-1;sod-2* and *nuo-6;ctl-1;sod-4* turned out to be impossible to generate, perhaps due to the embryonic lethality of the combined mutations. In addition to trying to construct them several times, we also used PQ and NAC separately in the plates for increasing ROS levels or decreasing ROS levels. We thought this might help the larvae to develop if they were suffering from ROS toxicity or ROS insufficiency. Neither of these conditions helped, of course. But a closer inspection of the mutation combinations reveals a pattern. As we discussed before, by removing CTL-1, we are eliminating an important cytoplasmic sink of H_2O_2. This leads to increased cytoplasmic levels of H_2O_2. We can see in Figure 3.13, that the loss of *sod-2* in both *isp-1* and *nuo-6* backgrounds under the stimulation of PQ also induces longevity through the SOD-1-dependent increase of cytoplasmic H_2O_2. One possibility is that uncontrolled increase of cytoplasmic H_2O_2 in *isp-1;ctl-1;sod-2* and *nuo-6;ctl-1;sod-2* results in ROS toxicity. Alternatively, it is also possible that during the development, the worm needs to be able to increase and decrease H_2O_2 levels in demand (likely for signaling purposes) and in case of these combinations of mutations it cannot do so because it needs CTL-1 for this purpose. After all the worm is unique in having a cytoplasmic CTL. Using a genetically encoded fluorescent sensor (HyPer) and GFP fusion proteins in *C. elegans* some studies have reported that H_2O_2 levels are generally higher during different stages of the worm's life cycle (Back, De Vos et al. 2012, Knoefler, Thamsen et al. 2012). In Figure 3.13, we can see that loss of SOD-4 in the maximally stimulated background of *nuo-6* + PQ also leads to increased longevity although this effect is specific to *nuo-6* and is not seen in *isp-1* background. It is possible that in the absence of SOD-4

in the extracellular space, more $O_2^{\bullet-}$ finds its way into the cytoplasm where it is converted to H_2O_2 by SOD-1. This is also one possible explanation for the lethality of *isp-1;ctl-1;sod-4* and *nuo-6;ctl-1;sod-4*.

3.4.3. The path of the pro-longevity ROS

SOD-1 is the worm's primary cytoplasmic Cu/ZnSOD. Nearly 80% of total SOD activity in the worm belongs to SOD-1 and *sod-1* accounts for 80% of the total *sod* mRNA in *C. elegans* (Doonan, McElwee et al. 2008). The SOD-1 protein appears to be ubiquitously expressed and localizes to the cytosol and mitochondrial intermembrane space (Doonan, McElwee et al. 2008). Some studies have shown that knocking out SOD-1 in the wild-type background shortens the lifespan of the worm (Yang, Li et al. 2007, Doonan, McElwee et al. 2008, Yen, Patel et al. 2009) and some others have reported no change (Van Raamsdonk and Hekimi 2009, Schaar, Dues et al. 2015). In both studies that reported no change in the lifespan of *sod-1* mutants, 100 µM FUDR was used to induce maternal sterility. In section, 3.3.2.4 we showed that 100 µM FUDR treatment can indeed suppress the lifespan shortening effect of the loss of SOD-1 in the wild-type and our laboratory and other groups have also shown that FUDR extends the lifespan of several mutant backgrounds (Aitlhadj, Stürzenbaum et al. 2010, Van Raamsdonk and Hekimi 2011, Rooney, Luz et al. 2014, Anderson, Corkins et al. 2016). That is the primary reason that discouraged us from using FUDR for lifespan studies despite the fact that it reduces the labor-intensiveness of such experiments. In the study by Doonan *et al,* they created *sod-1::gfp* fusions to monitor the expression patterns of *sod-1.* They realized that fusion of GFP to SOD-1 reduced specific activity of the enzyme. Nevertheless, they were able to rescue the shortened lifespan of their *sod-1* mutants by using this transgenic expression of *sod-1::gfp* (Doonan, McElwee et al. 2008). We also found that under our standard conditions (i.e. 20°C, no FUDR, measuring adult lifespan) deletion of *sod-1* shortens the lifespan of the wild-type. If this is due to increased ROS toxicity, then increasing ROS levels via enzymatic and chemical means would enhance the toxicity effect. But this is clearly not the case. Treatment with 0.1 mM PQ does not further shorten the lifespan of *sod-1* mutants but it does not lengthen their lifespan, either (Figure 3.10). Furthermore, loss of CTL-1, the worm's main cytoplasmic CTL, rescues the lifespan shortening effect of the loss of SOD-1 (Figure 3.9). This happens because CTL-1 is a cytoplasmic sink for H_2O_2 and removing it increases the H_2O_2 levels in the cytoplasm. This means that although SOD-1 is believed to localize to the

mitochondrial intermembrane space in addition to the cytoplasm, the pro-longevity H_2O_2 produced by SOD-1 needs to travel to the cytoplasm to exert it effect. We see this in other backgrounds, too.

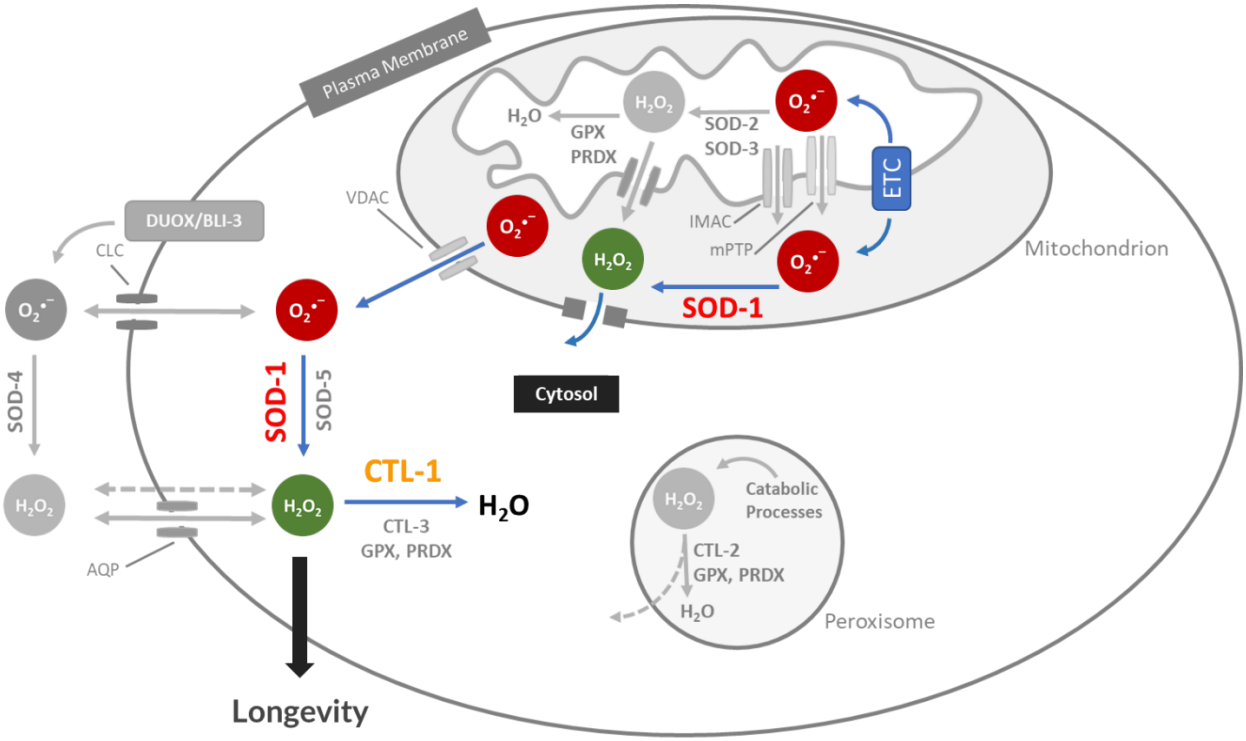


Figure 3.14. The path of the pro-longevity ROS in the ROS network of *C. elegans*. This diagram shows the path of the pro-longevity ROS and important elements of the ROS network of *C. elegans*. The mitochondrial $O_2^{\bullet-}$ signal is converted to H_2O_2 by SOD-1 in the cytoplasm. This SOD-1-dependent increase in cytoplasmic H_2O_2 promotes longevity. Also, see Figure 1.15 for an overview of the important elements of the worm's ROS network.

In *isp-1* and *nuo-6* backgrounds only removal of CTL-1 suppresses the lifespan shortening effect of the loss of SOD-1 (Figure 3.11). Loss of the other two CTLs (CTL-2 and CTL-3) does not have this effect in *isp-1* and *nuo-6* backgrounds either with PQ stimulation or without it (see Figures S3.4 and S3.9 for *isp-1* and S3.15 and S3.16 for *nuo-6*). Besides CTL-1, the worm has another cytoplasmic CTL (CTL-3) that is poorly studied and appears to have limited expression. In one report, the expression of a GFP reporter under the control of a promoter fragment extending 657 bp upstream from the *ctl-3* initiating codon was localized to pharyngeal muscle cells and neurons (Petriv and Rachubinski 2004). Our observations of the smaller effects of the loss of CTL-3 on lifespan of the worms compared to the loss of CTL-1 are also consistent with the limited

action of CTL-3. Using Two-Dimensional Difference Gel Electrophoresis (2D DIGE), one study found that *ctl-3* is particularly active during dauer formation in the worms. Dauer formation occurs when the nematode is developmentally arrested due to harsh environmental conditions such as lack of food. Taken together our findings suggest that the pro-longevity signal induced by the mitochondrial $O_2^{\bullet-}$ is an increase of SOD-1-dependent cytoplasmic H_2O_2. This model is shown in Figure 3.14.

3.4.4. Maximum beneficial ROS effects occur with the greatest changes in cytoplasmic H_2O_2 levels

We had previously proposed that the relationship between ROS levels and longevity follows the pattern of an inverted U-shaped curve (Van Raamsdonk and Hekimi 2012). This represents the beneficial pro-longevity effects of ROS and its deleterious effects. For example, when the wild-type is treated with PQ at different doses, its lifespan increases in a dose dependent manner (see Figure 2.3). However, there is a peak for how much we can increase longevity via increasing ROS levels. When this peak is reached, increasing ROS levels further actually reduces lifespan. Our new data set from this study confirms this model and adds a flavor of H_2O_2 to it.

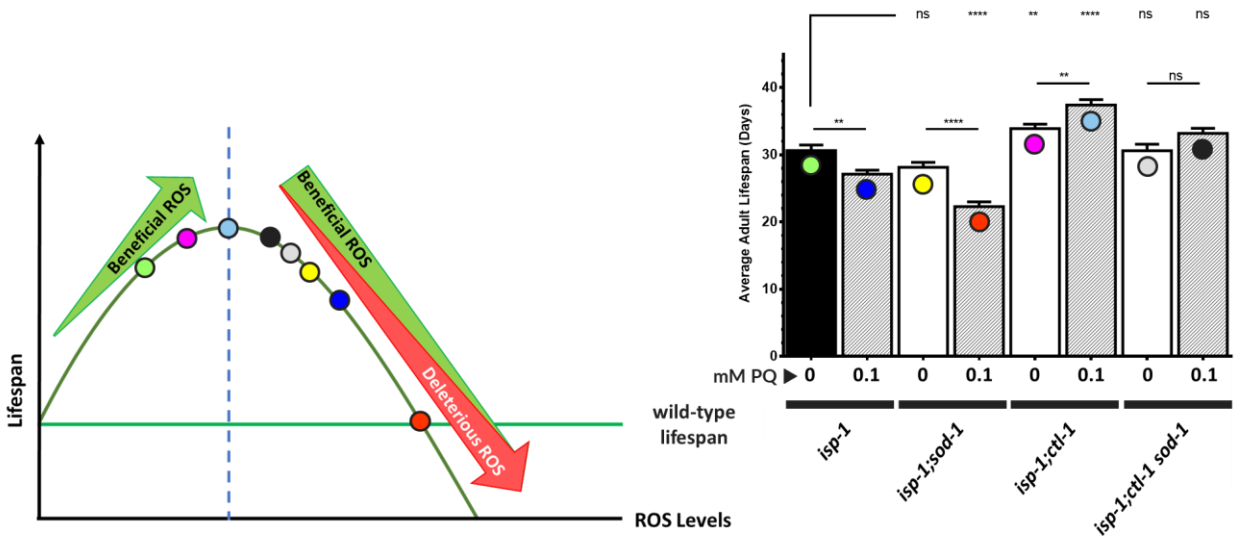


Figure 3.15. An inverted U-shaped curve represents the relationship between ROS levels and longevity. An increase in ROS levels increases longevity, however, by increasing ROS levels further, the deleterious effects of ROS outweigh the beneficial effects and cause a decrease in lifespan. (See text for description)

In Figure 3.15, on the left panel we have shown the inverted U-shaped diagram that represents the relationship between ROS levels and longevity. The right panel shows the average adult lifespan of *sod-1* and *ctl-1* single- and double-deletions in *isp-1* background with and without the stimulation of PQ. We presented this data in section 3.3.2.3 and it is shown here for reference. What is immediately visible in the diagram is that *isp-1;ctl-1*+PQ stands at the peak of the curve (light blue). Mitochondrial $O_2^{\bullet-}$ production is maximally stimulated in this genetic composition via the combined effects of *isp-1* mutation and 0.1 mM PQ treatment. Increasing cytoplasmic levels of H_2O_2 by removing CTL-1 has maximally optimized the longevity of this condition. Removing PQ stimulation lowers mitochondrial $O_2^{\bullet-}$ and SOD-1-dependent levels of cytoplasmic H_2O_2 and decreases longevity (purple). Putting CTL-1 (the H_2O_2 sink) back in the mix, decreases the longevity further by lowering cytoplasmic H_2O_2 levels (*isp-1* light green). By manipulating the levels of cytoplasmic H_2O_2 in this way, we are increasing or decreasing the longevity of the worm in the beneficial pro-longevity range of ROS.

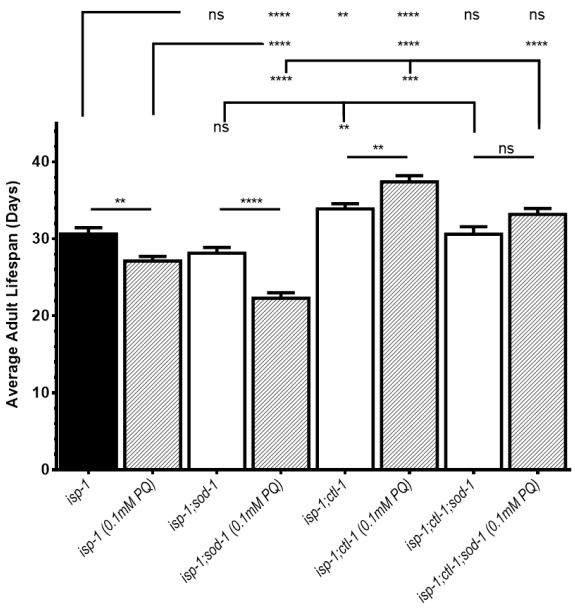
Now let's look at this from another direction. *isp-1;sod-1*+PQ has experienced the strongest lifespan shortening effect in the group (red) which represents the deleterious effects of ROS. In fact, The average adult lifespan of *isp-1;sod-1*+PQ is not significantly different than that of the wild-type (see Figure S3.23). In *isp-1;sod-1*+PQ we have a maximally stimulated background for mitochondrial $O_2^{\bullet-}$ production, however, the absence of SOD-1 means low levels of cytoplasmic H_2O_2 and decreased longevity. Let's leave the maximum stimulation in place for now but increase cytoplasmic H_2O_2 at the same time by removing its sink (CTL-1). We immediately see a marked increase in lifespan (Black). However, the lifespan of *isp-1;ctl-1 sod-1*+PQ is not that different from *isp-1;ctl-1 sod-1* (gray), because the pro-longevity effect of PQ requires the SOD-1-dependent production of cytoplasmic H_2O_2 and this triple mutant does not have SOD-1, to begin with. Also, lack of SOD-1 in *isp-1;ctl-1 sod-1* (gray) means that removing the sink of H_2O_2 (CTL-1) does not increase the lifespan either (yellow).

Finally, let's go back to *isp-1;sod-1*+PQ (red) which has maximum mitochondrial $O_2^{\bullet-}$ stimulation but not SOD-1 to convert it to cytoplasmic H_2O_2 (pro-longevity signal). If we put SOD-1 back in, cytoplasmic H_2O_2 increases and so does lifespan (dark blue).

3.5. Supplementary data for chapter 3

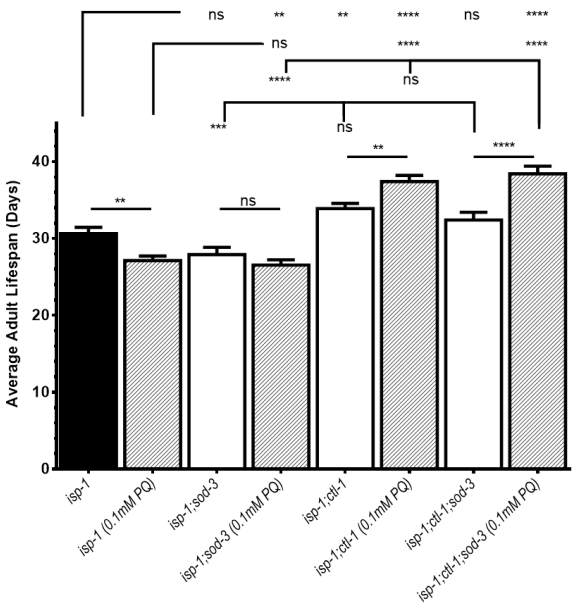
3.5.1. Analysis of lifespan in multiple-mutant sets in *isp-1* background

Note: All the data presented in the graph sets throughout this section are based on 2 or 3 independent trials with a total sample size of 100 – 200. Statistical analysis is performed using One-Way Anova with Sidak's correction for multiple comparisons. Comparisons are preformed among 4 data sets (families) indicated in the sample graph presented in section 3.3.1. **** | P<0.0001, *** | P<0.001, ** | P<0.01, * | P<0.05, Error bars represent SEM



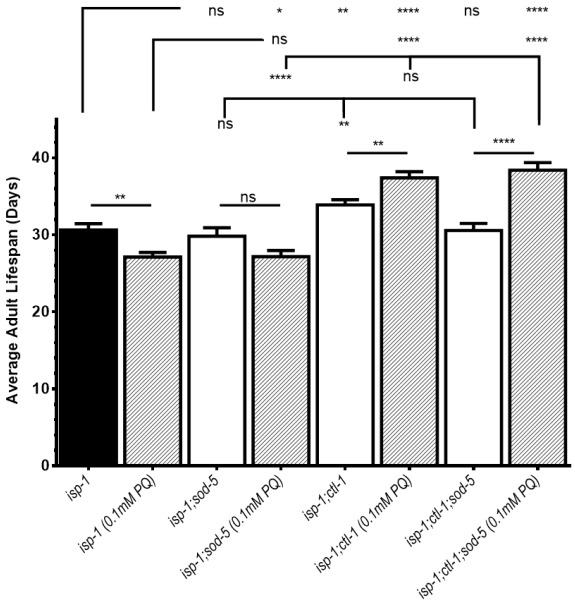
Mutation	Background	PQ	Effect on Lifespan
sod-1	*isp-1*	-	• no effect
		+	• ↓
sod-1	*isp-1;ctl-1*	-	• ↓
		+	• ↓
ctl-1	*isp-1*	-	• ↑
		+	• ↑
ctl-1	*isp-1;sod-1*	-	• no effect
		+	• ↑

Figure S3.1. Analysis of the average adult lifespan of *isp-1;ctl-1 sod-1* with (+) and without (-) 0.1 mM PQ treatment.



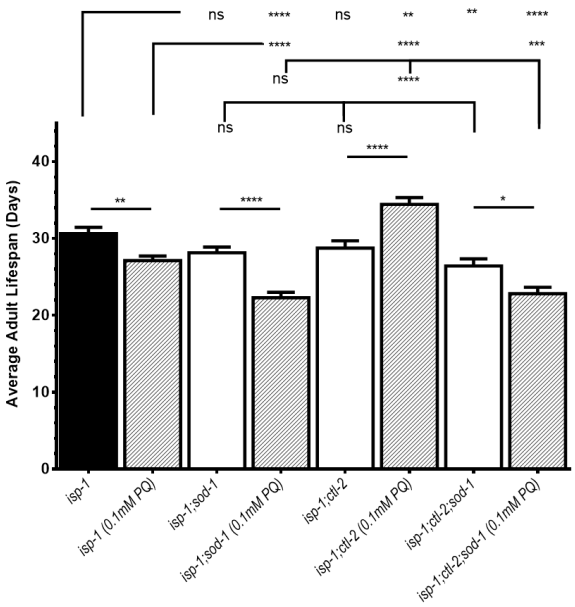
Mutation	Background	PQ	Effect on Lifespan
sod-3	*isp-1*	-	• no effect
		+	• no effect
sod-3	*isp-1;ctl-1*	-	• no effect
		+	• no effect
ctl-1	*isp-1*	-	• ↑
		+	• ↑
ctl-1	*isp-1;sod-3*	-	• ↑
		+	• ↑

Figure S3.2. Analysis of the average adult lifespan of *isp-1;ctl-1;sod-3* with (+) and (-) 0.1 mM PQ treatment.



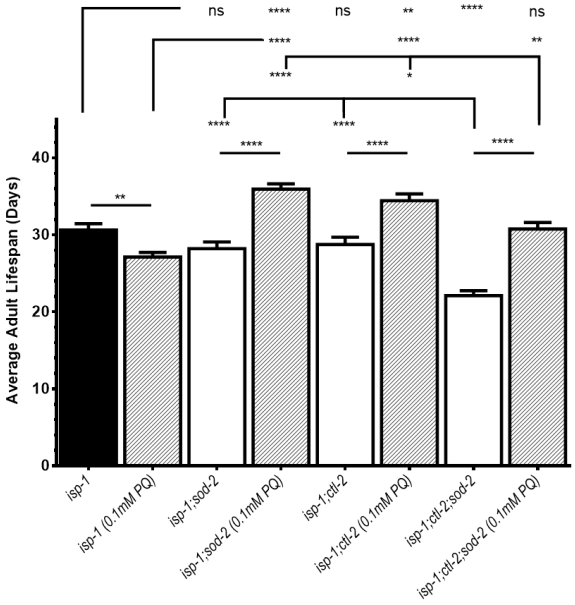
Mutation	Background	PQ	Effect on Lifespan
sod-5	*isp-1*	-	• no effect
		+	• no effect
sod-5	*isp-1;ctl-1*	-	• ↓
		+	• no effect
ctl-1	*isp-1*	-	• ↑
		+	• ↑
ctl-1	*isp-1;sod-5*	-	• no effect
		+	• ↑

Figure S3.3. Analysis of the average adult lifespan of *isp-1;ctl-1 sod-5* with (+) and without (-) 0.1 mM PQ treatment.



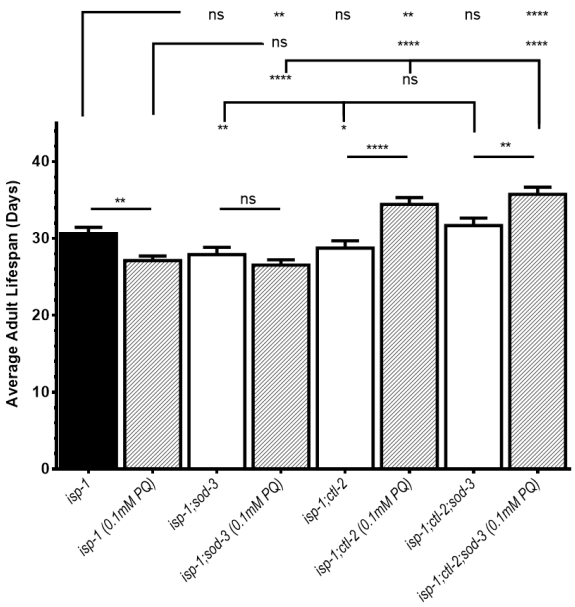
Mutation	Background	PQ	Effect on Lifespan
sod-1	*isp-1*	-	• no effect
		+	• ↓
sod-1	*isp-1;ctl-2*	-	• no effect
		+	• ↓
ctl-2	*isp-1*	-	• no effect
		+	• ↑
ctl-2	*isp-1;sod-1*	-	• no effect
		+	• no effect

Figure S3.4. Analysis of the average adult lifespan of *isp-1;ctl-2 sod-1* with (+) and without (-) 0.1 mM PQ treatment.



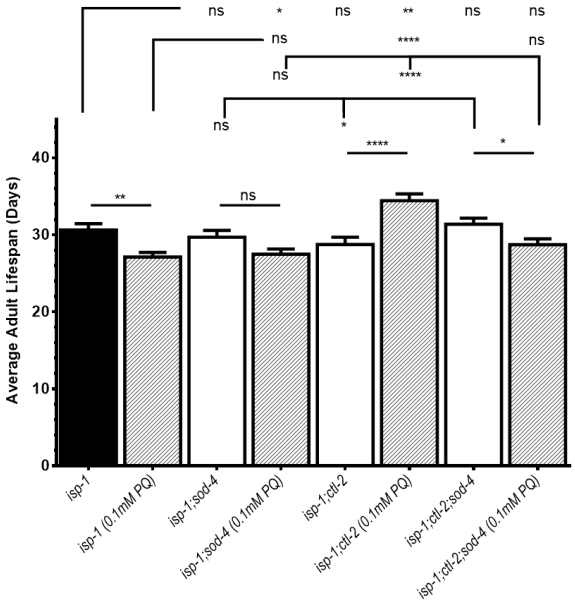
Mutation	Background	PQ	Effect on Lifespan
sod-2	*isp-1*	-	• no effect
		+	• ↑
sod-2	*isp-1;ctl-2*	-	• ↓ = N2
		+	• ↓
ctl-2	*isp-1*	-	• no effect
		+	• ↑
ctl-2	*isp-1;sod-2*	-	• ↓ = N2
		+	• ↓

Figure S3.5. Analysis of the average adult lifespan of *isp-1;ctl-2;sod-2* with (+) and without (-) 0.1 mM PQ treatment.



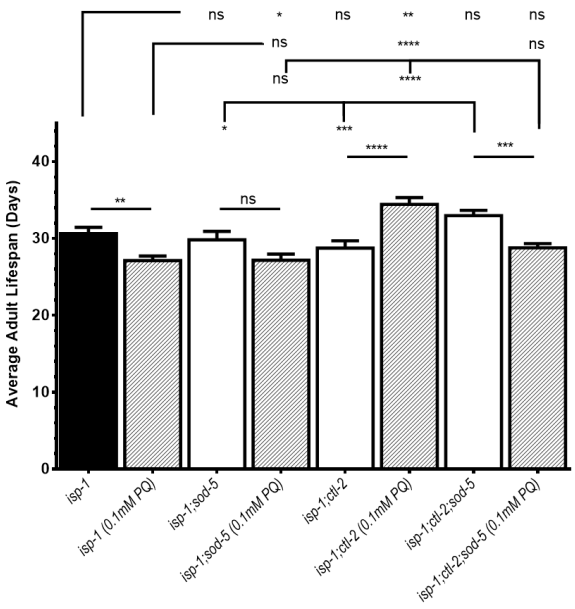
Mutation	Background	PQ	Effect on Lifespan
sod-3	*isp-1*	-	• no effect
		+	• no effect
sod-3	*isp-1;ctl-2*	-	• ↑
		+	• no effect
ctl-2	*isp-1*	-	• no effect
		+	• ↑
ctl-2	*isp-1;sod-3*	-	• ↑
		+	• ↑

Figure S3.6. Analysis of the average adult lifespan of *isp-1;ctl-2;sod-3* with (+) and without (-) 0.1 mM PQ treatment.



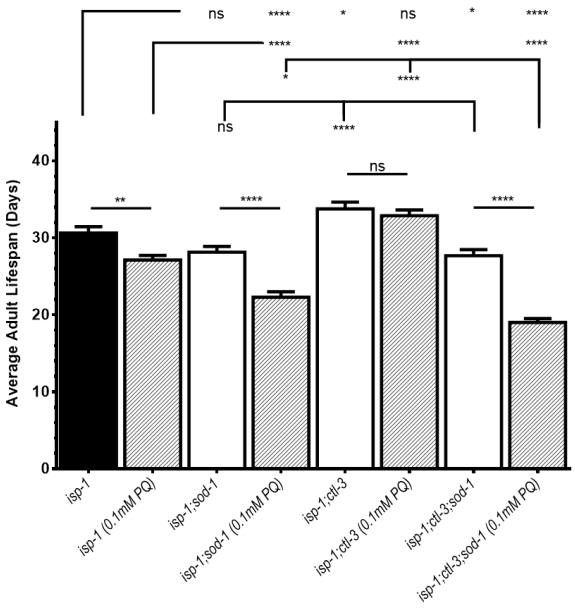
Mutation	Background	PQ	Effect on Lifespan
sod-4	*isp-1*	-	• no effect
		+	• no effect
sod-4	*isp-1;ctl-2*	-	• ↑
		+	• ↓ = *isp-1*
ctl-2	*isp-1*	-	• no effect
		+	• ↑
ctl-2	*isp-1;sod-4*	-	• no effect
		+	• no effect

Figure S3.7. Analysis of the average adult lifespan of *isp-1;ctl-2;sod-4* with (+) and without (-) 0.1 mM PQ treatment.



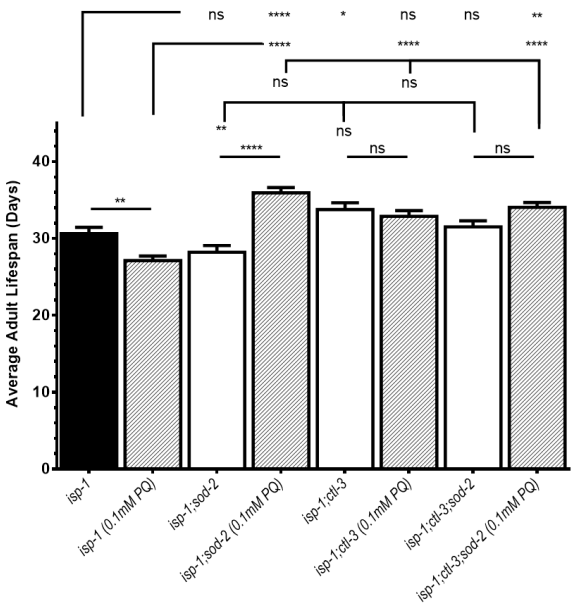
Mutation	Background	PQ	Effect on Lifespan
sod-5	*isp-1*	-	• no effect
		+	• no effect
sod-5	*isp-1;ctl-2*	-	• ↑
		+	• ↓ = *isp-1*
ctl-2	*isp-1*	-	• no effect
		+	• ↑
ctl-2	*isp-1;sod-5*	-	• ↑
		+	• no effect

Figure S3.8. Analysis of the average adult lifespan of *isp-1;ctl-2 sod-5* with (+) and without (-) 0.1 mM PQ treatment.



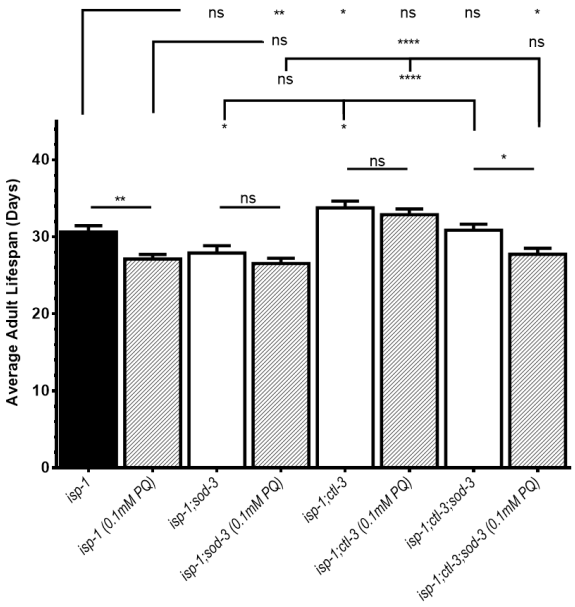
Mutation	Background	PQ	Effect on Lifespan
sod-1	*isp-1*	-	• no effect
		+	• ↓ = N2
sod-1	*isp-1;ctl-3*	-	• ↓
		+	• ↓ = N2
ctl-3	*isp-1*	-	• ↑
		+	• no effect
ctl-3	*isp-1;sod-1*	-	• no effect
		+	• ↓

Figure S3.9. Analysis of the average adult lifespan of *isp-1;ctl-3 sod-1* with (+) and without (-) 0.1 mM PQ treatment.



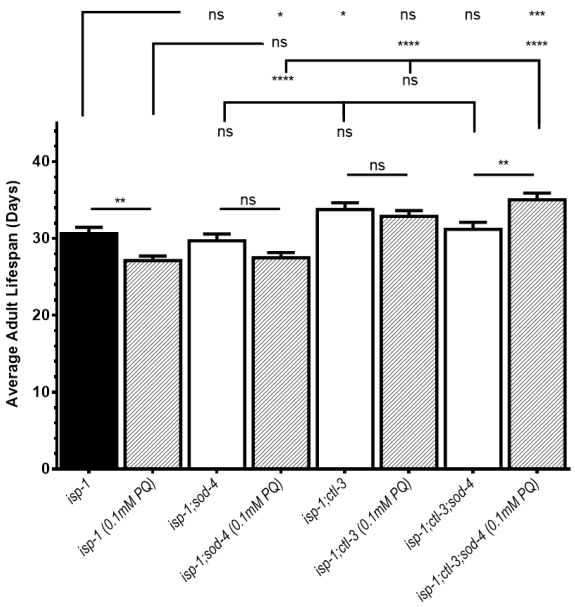
Mutation	Background	PQ	Effect on Lifespan
sod-2	*isp-1*	-	• no effect
		+	• ↑
sod-2	*isp-1;ctl-3*	-	• no effect
		+	• no effect
ctl-3	*isp-1*	-	• ↑
		+	• ↑
ctl-3	*isp-1;sod-2*	-	• ↑
		+	• no effect

Figure S3.10. Analysis of the average adult lifespan of *isp-1;ctl-3;sod-2* with (+) and without (-) 0.1 mM PQ treatment.



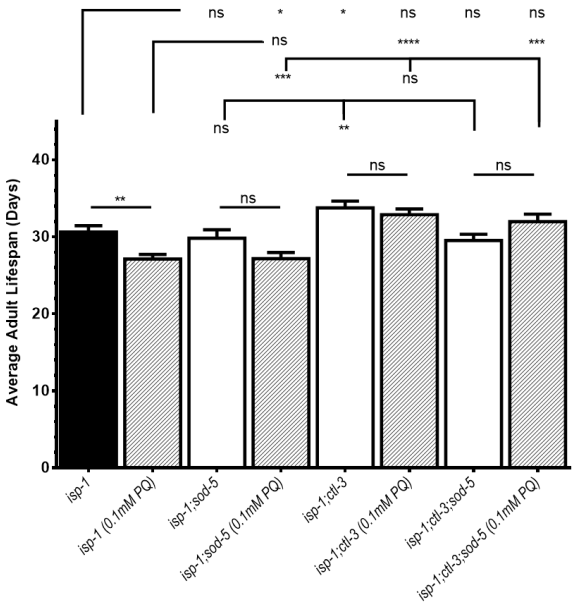
Mutation	Background	PQ	Effect on Lifespan
sod-3	*isp-1*	-	• no effect
		+	• no effect
sod-3	*isp-1;ctl-3*	-	• ↓
		+	• ↓
ctl-3	*isp-1*	-	• ↑
		+	• ↑
ctl-3	*isp-1;sod-3*	-	• ↑
		+	• no effect

Figure S3.11. Analysis of the average adult lifespan of *isp-1;ctl-3;sod-3* with (+) and without (-) 0.1 mM PQ treatment.



Mutation	Background	PQ	Effect on Lifespan
sod-4	*isp-1*	-	• no effect
		+	• no effect
sod-4	*isp-1;ctl-3*	-	• no effect
		+	• no effect
ctl-3	*isp-1*	-	• ↑
		+	• ↑
ctl-3	*isp-1;sod-4*	-	• no effect
		+	• ↑

Figure S3.12. Analysis of the average adult lifespan of *isp-1;ctl-3;sod-4* with (+) and without (-) 0.1 mM PQ treatment.

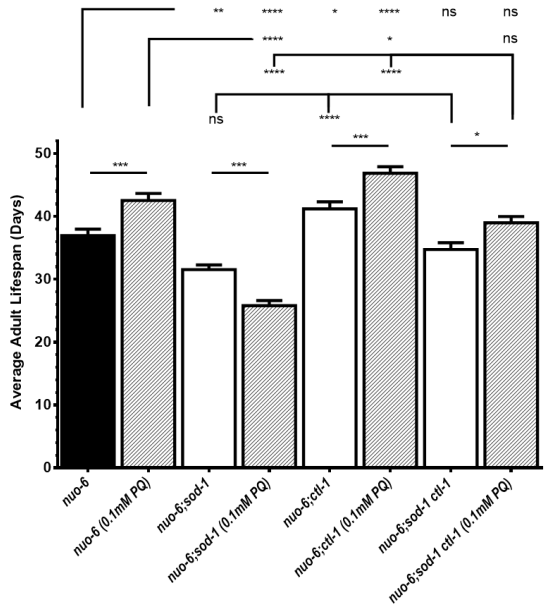


Mutation	Background	PQ	Effect on Lifespan
sod-5	*isp-1*	-	• no effect
		+	• no effect
sod-5	*isp-1;ctl-3*	-	• ↓
		+	• no effect
ctl-3	*isp-1*	-	• ↑
		+	• ↑
ctl-3	*isp-1;sod-5*	-	• no effect
		+	• ↑

Figure S3.13. Analysis of the average adult lifespan of *isp-1;ctl-3 sod-5* with (+) and without (-) 0.1 mM PQ treatment.

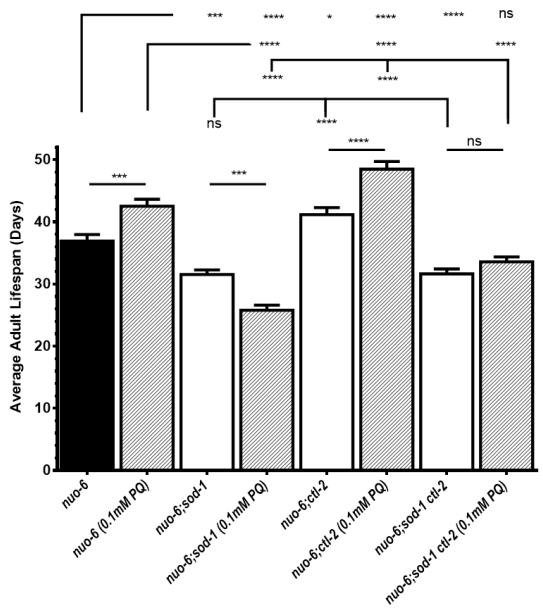
3.5.2. Analysis of lifespan in multiple-mutant sets in *nuo-6* background

Note: All the data presented in the graph sets throughout this section are based on 2 or 3 independent trials with a total sample size of 100 – 200. Statistical analysis is performed using One-Way Anova with Sidak's correction for multiple comparisons. Comparisons are preformed among 4 data sets (families) indicated in the sample graph presented in section 3.3.1. **** | P<0.0001, *** | P<0.001, ** | P<0.01, * | P<0.05, Error bars represent SEM



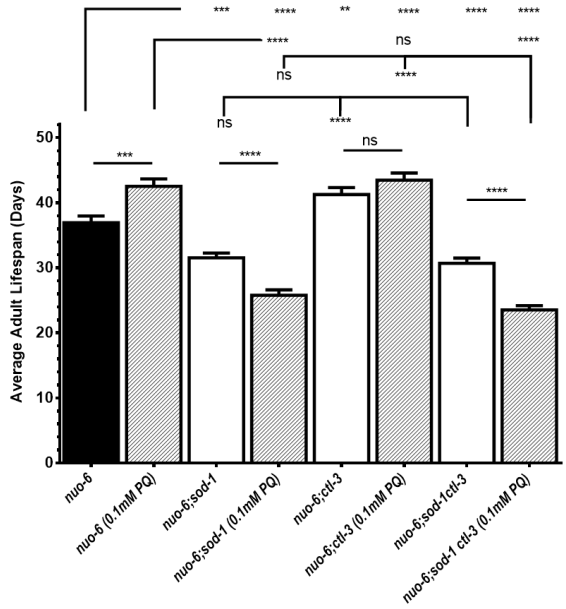
Mutation	Background	PQ	Effect on Lifespan
sod-1	*nuo-6*	-	↓
		+	↓
sod-1	*nuo-6;ctl-1*	-	↓
		+	↓
ctl-1	*nuo-6*	-	↑
		+	↑
ctl-1	*nuo-6;sod-1*	-	no effect
		+	↓

Figure S3.14. Analysis of the average adult lifespan of *nuo-6;ctl-1 sod-1* with (+) and without (-) 0.1 mM PQ treatment.



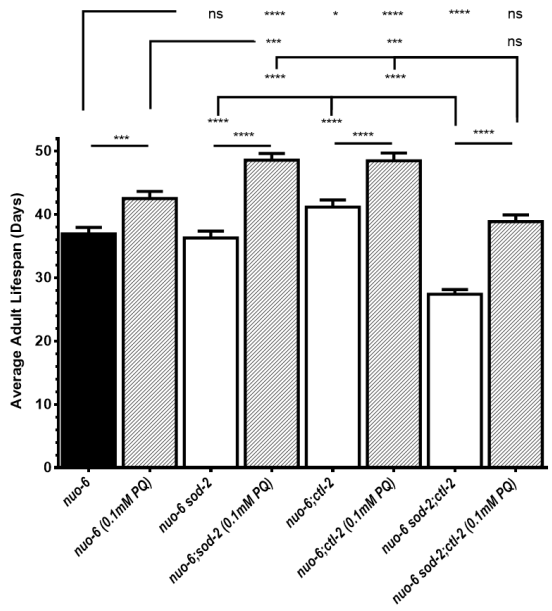
Mutation	Background	PQ	Effect on Lifespan
sod-1	*nuo-6*	-	↓
		+	↓
sod-1	*nuo-6;ctl-2*	-	↓
		+	↓
ctl-2	*nuo-6*	-	↑
		+	↑
ctl-2	*nuo-6;sod-1*	-	no effect
		+	↓

Figure S3.15. Analysis of the average adult lifespan of *nuo-6;ctl-2 sod-1* with (+) and without (-) 0.1 mM PQ treatment.



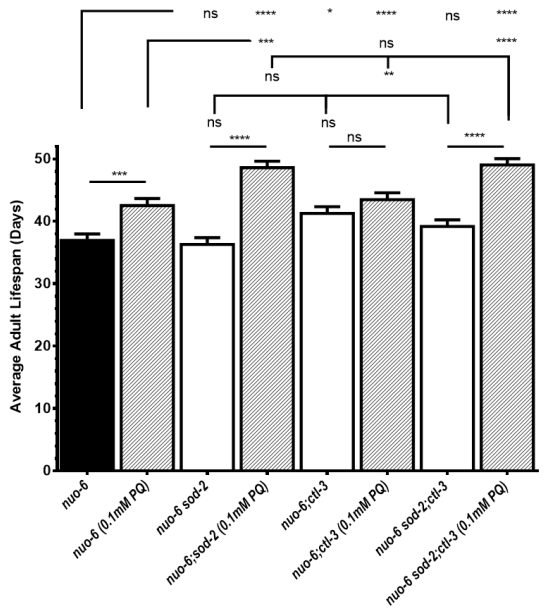
Mutation	Background	PQ	Effect on Lifespan
sod-1	*nuo-6*	-	↓
		+	↓
sod-1	*nuo-6;ctl-3*	-	↓
		+	↓
ctl-3	*nuo-6*	-	↑
		+	no effect
ctl-3	*nuo-6;sod-1*	-	no effect
		+	no effect

Figure S3.16. Analysis of the average adult lifespan of *nuo-6;ctl-3 sod-1* with (+) and without (-) 0.1 mM PQ treatment.



Mutation	Background	PQ	Effect on Lifespan
sod-2	*nuo-6*	-	• no effect
		+	• ↑
sod-2	*nuo-6;ctl-2*	-	• ↓
		+	• ↓
ctl-2	*nuo-6*	-	• ↑
		+	• ↑
ctl-2	*nuo-6 sod-2*	-	• ↓
		+	• ↓

Figure S3.17. Analysis of the average adult lifespan of *nuo-6;ctl-2;sod-2* with (+) and without (-) 0.1 mM PQ treatment.

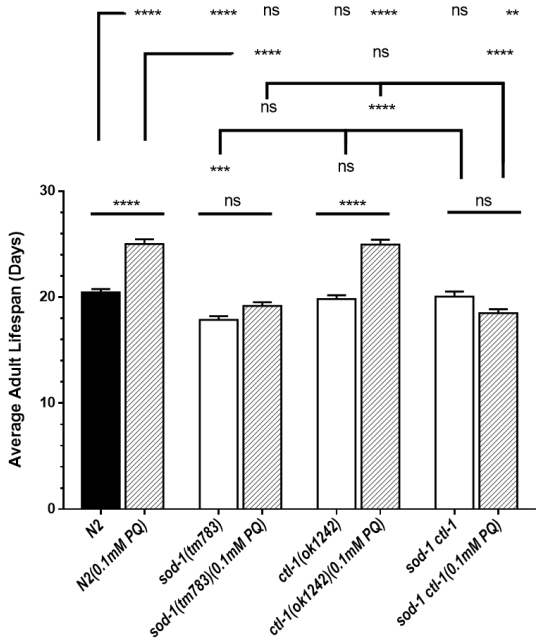


Mutation	Background	PQ	Effect on Lifespan
sod-2	*nuo-6*	-	• no effect
		+	• ↑
sod-2	*nuo-6;ctl-3*	-	• no effect
		+	• no effect
ctl-3	*nuo-6*	-	• ↑
		+	• no effect
ctl-3	*nuo-6 sod-2*	-	• no effect
		+	• no effect

Figure S3.18. Analysis of the average adult lifespan of *nuo-6;ctl-3;sod-2* with (+) and without (-) 0.1 mM PQ treatment.

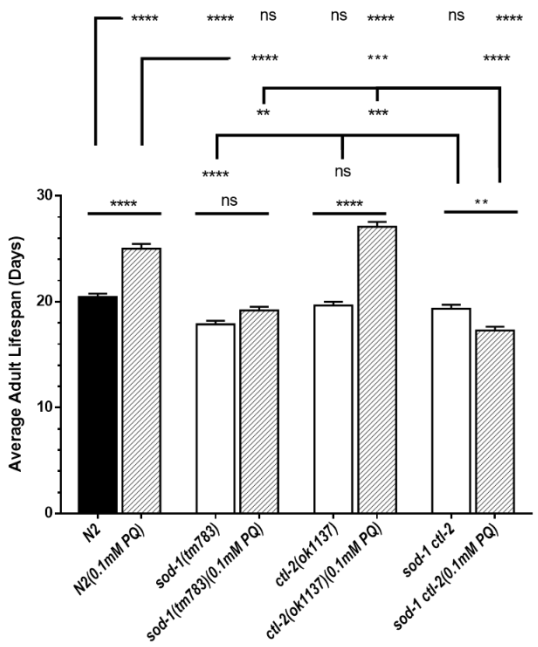
3.5.3. Analysis of lifespan in multiple-mutant sets in the wild-type (N2) background

Note: All the data presented in the graph sets throughout this section are based on 2 or 3 independent trials with a total sample size of 100 – 200. Statistical analysis is performed using One-Way Anova with Sidak's correction for multiple comparisons. Comparisons are preformed among 4 data sets (families) indicated in the sample graph presented in section 3.3.1.



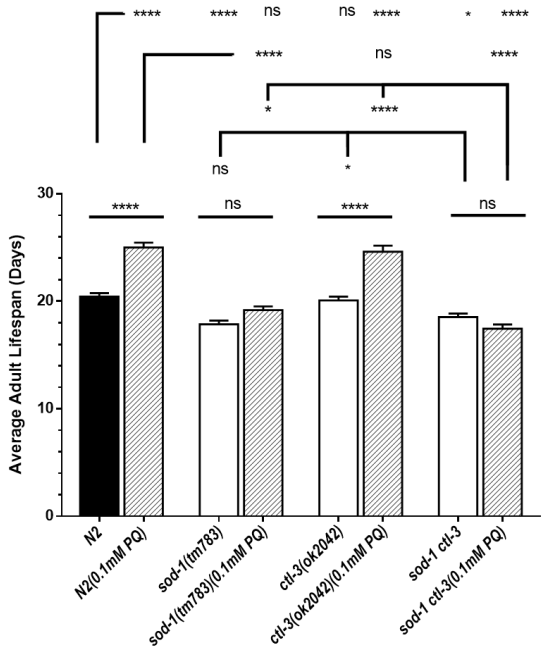
Mutation	Background	PQ	Effect on Lifespan
sod-1	N2	-	• ↓
		+	• ↓
sod-1	*ctl-1*	-	• no effect
		+	• ↓
ctl-1	N2	-	• no effect
		+	• no effect
ctl-1	*sod-1*	-	• ↑
		+	• no effect

Figure S3.19. Analysis of the average adult lifespan of *sod-1 ctl-1* with (+) and without (-) 0.1 mM PQ treatment.



Mutation	Background	PQ	Effect on Lifespan
sod-1	N2	-	• ↓
		+	• ↓
sod-1	*ctl-2*	-	• no effect
		+	• ↓
ctl-2	N2	-	• no effect
		+	• ↑
ctl-2	*sod-1*	-	• ↑
		+	• ↓

Figure S3.20. Analysis of the average adult lifespan of *sod-1 ctl-2* with (+) and without (-) 0.1 mM PQ treatment.



Mutation	Background	PQ	Effect on Lifespan
sod-1	N2	-	• ↓
		+	• ↓
sod-1	*ctl-3*	-	• ↓
		+	• ↓
ctl-3	N2	-	• no effect
		+	• no effect
ctl-3	*sod-1*	-	• no effect
		+	• ↓

Figure S3.21. Analysis of the average adult lifespan of *sod-1 ctl-3* with (+) and without (-) 0.1 mM PQ treatment.

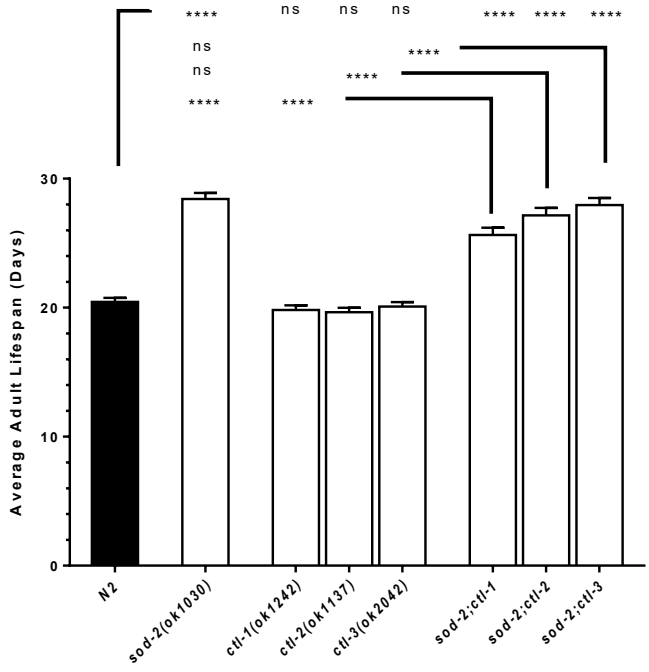


Figure S3.22. Analysis of the average adult lifespan of *sod-2;ctl-x.* Note that lifespan analysis of *sod-2;ctl-x* double-mutants with PQ treatment was not possible. *sod-2* mutants go into developmental arrest when they are treated with PQ. We have studied phenotype is chapter 2.

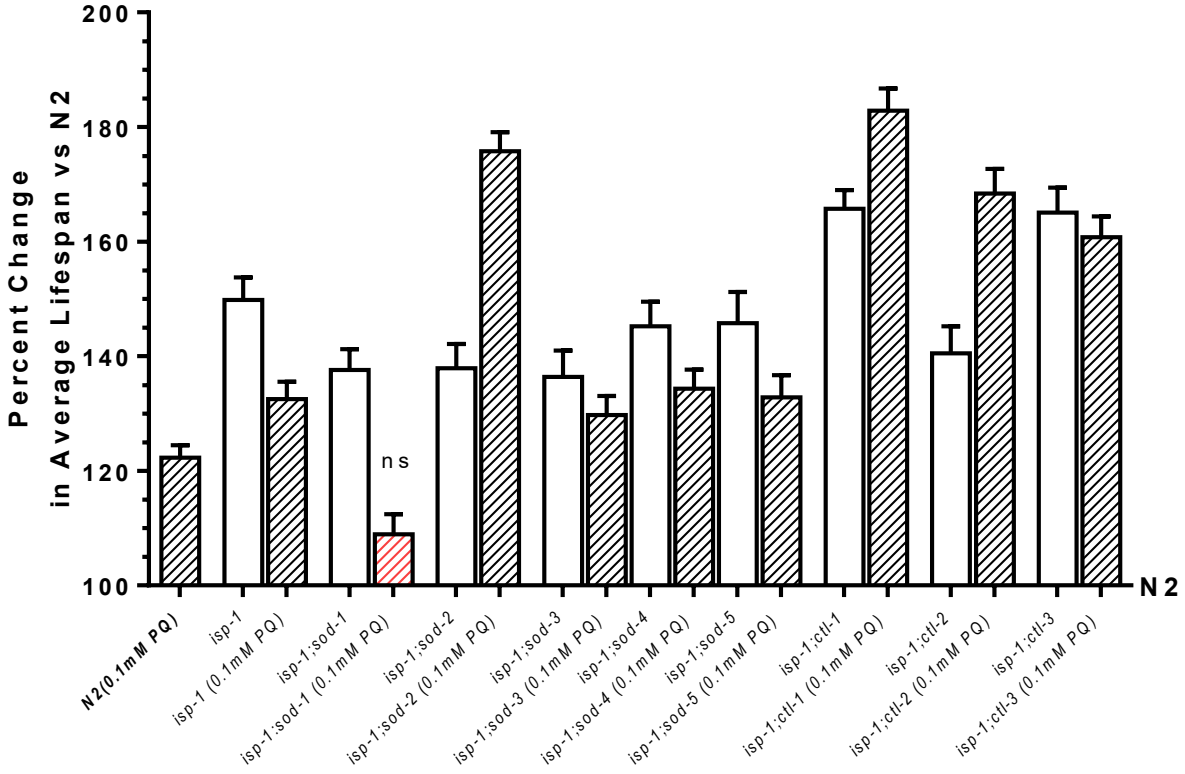


Figure S3.23. An overview of the percent change in the average adult lifespan of *sod-x* and *ctl-x* double-mutants in *isp-1* background with and without PQ treatment compared with the wild-type (N2). Note that the x axis in the graph represents the average adult lifespan of the wild-type, normalized to 100%.

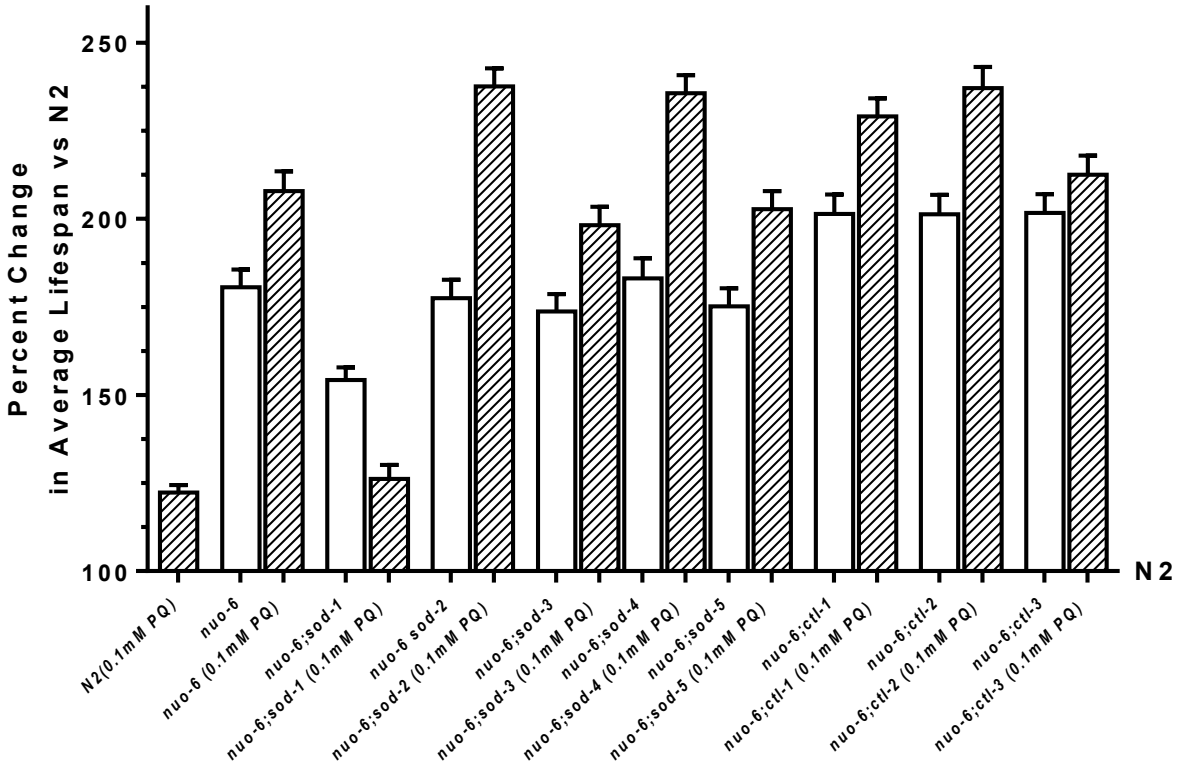


Figure S3.24. An overview of the percent change in the average adult lifespan of *sod-x* and *ctl-x* double-mutants in *nuo-6* background with and without PQ treatment compared with the wild-type (N2). Note that the x axis in the graph represents the average adult lifespan of the wild-type, normalized to 100%.

3.5.4. Data summary tables of the lifespan experiments

Note: These tables summarize the data sets presented in sections 3.5.1 through 3.5.3.

Table S3.1. Summary of the adult lifespan data in *isp-1* background.

BG	BG	*ctl* mutations in the background			
	isp-1	**✚**	***ctl-1***	***ctl-2***	***ctl-3***
***sod* mutations in the background**	**✚**	**30.6 (Control)**	33.9 ⬆	28.7 ■	33.8 ⬆
	sod-1	28.1 ■	30.6 ■ ⬇ ■	26.4 ⬇ ■ ■	27.7 ⬇ ⬇ ■
	sod-2	28.2 ■	☠	22.1 ⬇ ⬇ ⬇	31.5 ■ ■ ⬆
	sod-3	27.9 ■	32.4 ■ ■ ⬆	31.7 ■ ⬆ ⬆	30.9 ■ ⬇ ⬆
	sod-4	29.7 ■	☠	31.4 ■ ⬆ ■	31.2 ■ ■ ■
	sod-5	29.8 ■	30.6 ■ ⬇ ■	34 ■ ⬆ ⬆	29.5 ■ ⬇ ■

Numbers in the cells show the average adult lifespan.

The symbol on the right of the number indicates comparison vs. control *(isp-1)*.

For triple mutants there are a pair of cells below the numbers. For each pair of cells, the symbol on the left indicates comparison vs. *isp-1;ctl-x* and the one on the right indicates comparison vs. *isp-1;sod-x*.

Symbols:

■ No significant change in average adult lifespan

⬆ Significant increase in average adult lifespan

⬇ Significant decrease in average adult lifespan

☠ Embryonic lethality of the condition

Table S3.2. Summary of the adult lifespan data in *isp-1* + 0.1 mM PQ background.

BG	BG	*ctl* mutations in the background			
	isp-1+PQ	✚	*ctl-1*	*ctl-2*	*ctl-3*
sod mutations in the background	✚	**27.1 (Control)**	37.4 ↑	34.4 ↑	32.9 ↑
	sod-1	22.3 ↓	33.18 ↑ ↓ ↑	22.8 ↓ ↓ ■	19 ↓ ↓ ↓
	sod-2	35.9 ↑	☠	30.8 ↑ ↓ ↓	34 ↑ ■ ■
	sod-3	26.5 ■	38.4 ↑ ■ ↑	35.7 ↑ ■ ↑	27.7 ■ ↓ ■
	sod-4	27.5 ■	☠	28.7 ■ ↓ ■	35 ↑ ■ ↑
	sod-5	27.1 ■	38.4 ↑ ■ ↑	28.8 ■ ↓ ■	32 ↑ ■ ↑

Numbers in the cells show the average adult lifespan.

The symbol on the right of the number indicates comparison vs. control (*isp-1* + 0.1mM PQ)

For triple mutants there are a pair of cells below the numbers. For each pair of cells, the symbol on the left indicates comparison vs. *isp-1;ctl-x* + PQ and the one on the right indicates comparison vs. *isp-1;sod-x* + PQ.

Symbols:

■ No significant change in average adult lifespan

↑ Significant increase in average adult lifespan

↓ Significant decrease in average adult lifespan

☠ Embryonic lethality of the condition

Table S3.3. Summary of the adult lifespan data in *nuo-6* background.

BG	BG	*ctl* mutations in the background			
	nuo-6	✚	***ctl-1***	***ctl-2***	***ctl-3***
sod mutations in the background	✚	**36.9 (Control)**	41.2 ↑	41.1 ↑	41.3 ↑
	sod-1	31.5 ↓	34.7 ■ ↓ ■	31.6 ↓ ↓ ■	30.7 ↓ ↓ ■
	sod-2	36.3 ■	☠	27.4 ↓ ↓ ↓	39.2 ■ ■ ■
	sod-3	35.52 ■			
	sod-4	37.4 ■	☠		
	sod-5	35.8 ■			

Numbers in the cells show the average adult lifespan.

The symbol on the right of the number indicates comparison vs. control *(nuo-6)*.

For triple mutants there are a pair of cells below the numbers. For each pair of cells, the symbol on the left indicates comparison vs. *nuo-6;ctl-x* and the one on the right indicates comparison vs. *nuo-6;sod-x*.

Symbols:

■ No significant change in average adult lifespan

↑ Significant increase in average adult lifespan

↓ Significant decrease in average adult lifespan

☠ Embryonic lethality of the condition

Table S3.4. Summary of the adult lifespan data in *nuo-6* + 0.1 mM PQ background.

BG	BG	*ctl* mutations in the background			
	nuo-6+PQ	**✚**	***ctl-1***	***ctl-2***	***ctl-3***
***sod* mutations in the background**	**✚**	**42.5 (Control)**	46.86 ↑	48.5 ↑	43.5 ■
	sod-1	25.8 ↓	38.98 ■ ↓ ↑	33.6 ↓ ↓ ↑	23.52 ↓ ↓ ■
	sod-2	48.6 ↑	☠	38.9 ■ ↓ ↓	49.0 ↑ ↑ ■
	sod-3	40.5 ■			
	sod-4	48.2 ■	☠		
	sod-5	41.5 ■			

Numbers in the cells show the average adult lifespan.

The symbol on the right of the number indicates comparison vs. control (*nuo-6* + 0.1mM PQ)

For triple mutants there are a pair of cells below the numbers. For each pair of cells, the symbol on the left indicates comparison vs. *nuo-6;ctl-x* + PQ and the one on the right indicates comparison vs. *nuo-6;sod-x* + PQ.

Symbols:

■ No significant change in average adult lifespan

↑ Significant increase in average adult lifespan

↓ Significant decrease in average adult lifespan

☠ Embryonic lethality of the condition

Table S3.5. Summary of the adult lifespan data in the wild-type (N2) background.

BG	BG	*ctl* mutations in the background			
	N2	✚	*ctl-1*	*ctl-2*	*ctl-3*
sod mutations in the background	✚	**20.4 (Control)**	19.8 ■	19.6 ■	20.1 ■
	sod-1	17.9 ↓	20.1 ■ ■ ↑	19.3 ■ ■ ↑	18.5 ↓ ↓ ■
	sod-2	28.4 ↑	25.6 ↑ ↑ ↓	27.2 ↑ ↑ ■	27.9 ↑ ↑ ■
	sod-3	17.3 ↓			
	sod-4	19.3 ■			
	sod-5	19.7 ■			

Numbers in the cells show the average adult lifespan.

The symbol on the right of the number indicates comparison vs. control (N2).

For double mutants there are a pair of cells below the numbers. For each pair of cells, the symbol on the left indicates comparison vs. *ctl-x* and the one on the right indicates comparison vs. *sod-x*.

Symbols:

■ No significant change in average adult lifespan

↑ Significant increase in average adult lifespan

↓ Significant decrease in average adult lifespan

Table S3.6. Summary of the adult lifespan data in the wild-type (N2) + PQ background.

BG	BG	*ctl* mutations in the background			
	N2+PQ	✚	*ctl-1*	*ctl-2*	*ctl-3*
sod mutations in the background	✚	**25 (Control)**	24.9 ■	27.1 ⬆	24.6 ■
	sod-1	19.2 ⬇	18.5 ⬇ ⬇ ■	17.3 ⬇ ⬇ ⬇	17.5 ⬇ ⬇ ⬇
	sod-2	Developmental Arrest	Developmental Arrest	Developmental Arrest	Developmental Arrest
	sod-3	22.5 ⬇			
	sod-4	24.4 ■			
	sod-5	25.2 ■			

Numbers in the cells show the average adult lifespan.

The symbol on the right of the number indicates comparison vs. control (N2 + 0.1mM PQ).

For double mutants there are a pair of cells below the numbers. For each pair of cells, the symbol on the left indicates comparison vs. *ctl-x* + PQ and the one on the right indicates comparison vs. *sod-x* + PQ.

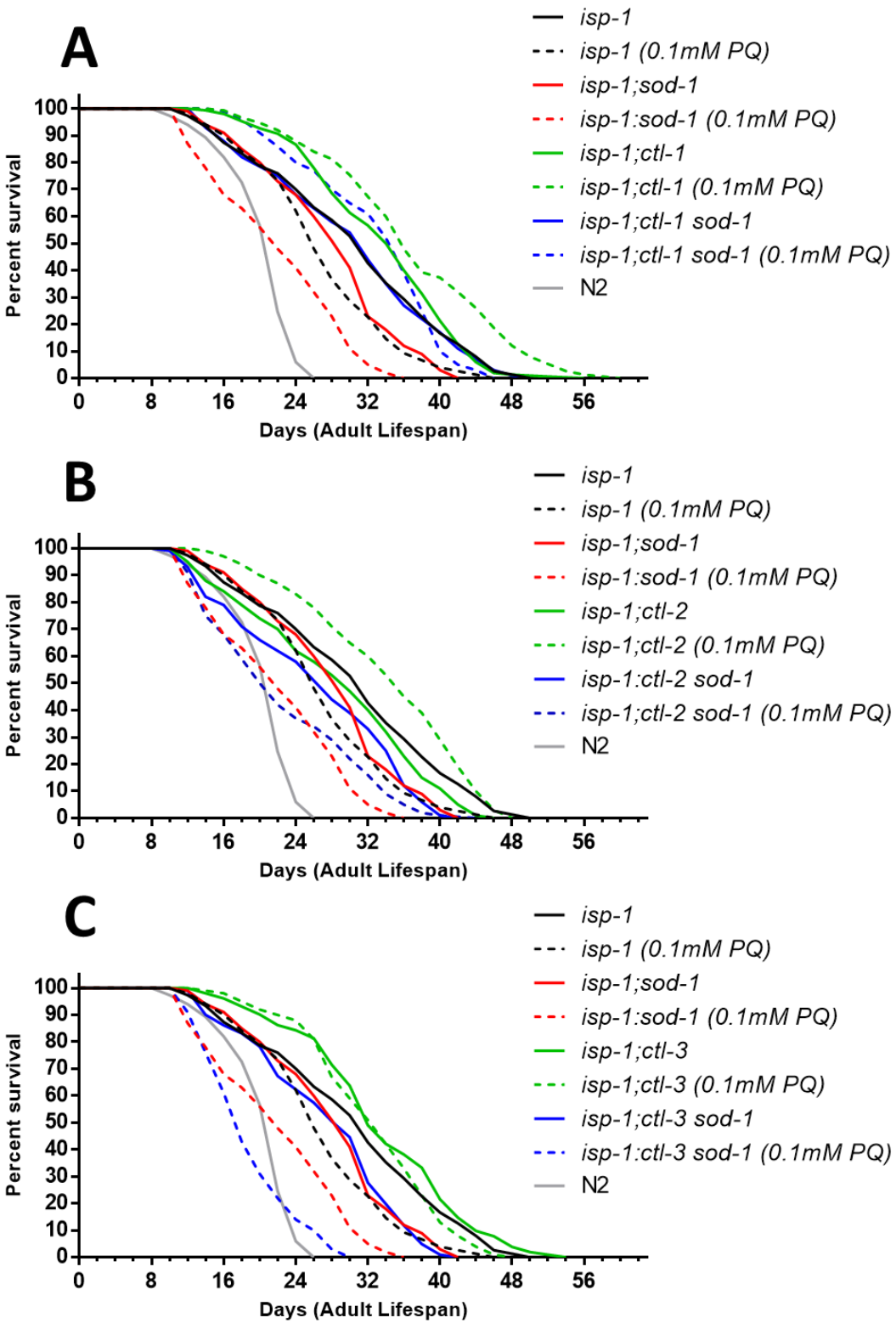
Symbols:

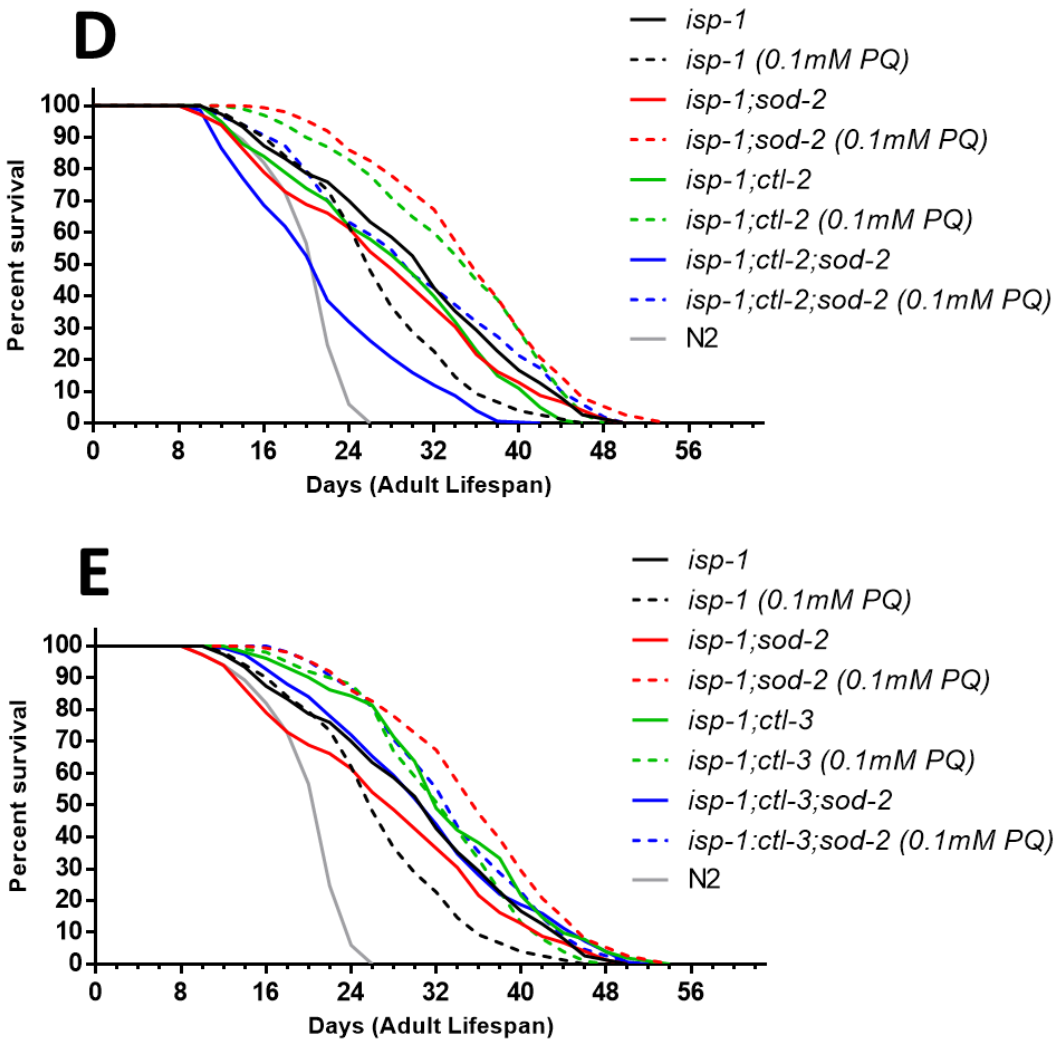
■ No significant change in average adult lifespan

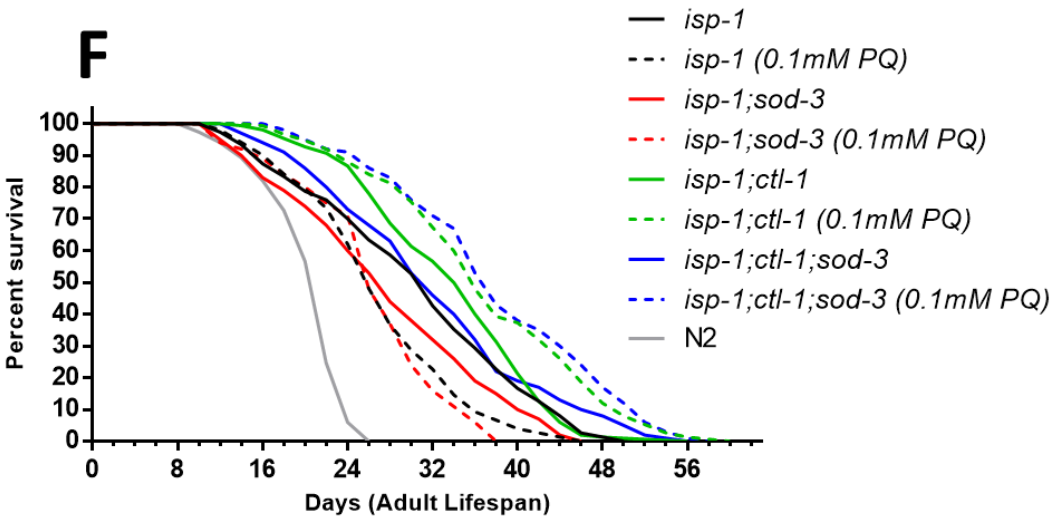
⬆ Significant increase in average adult lifespan

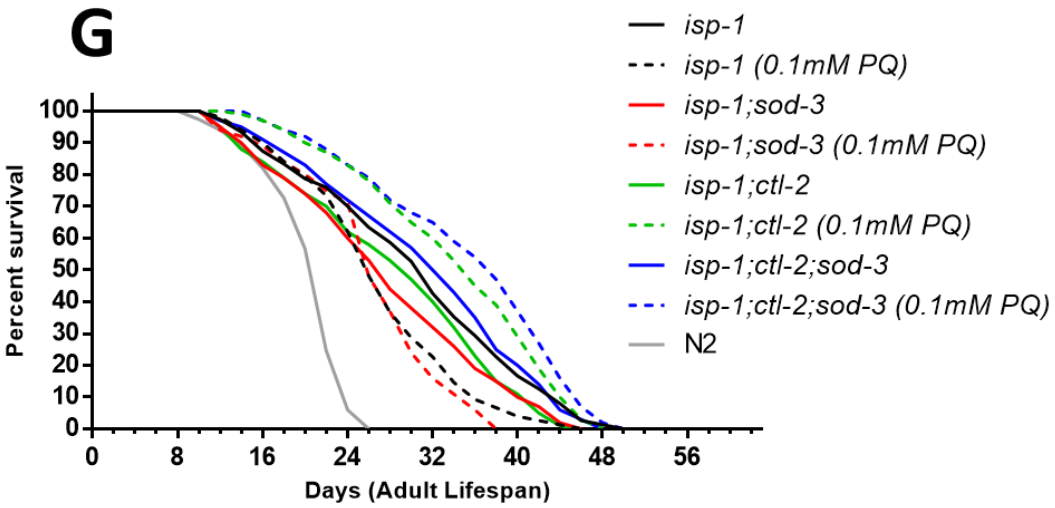
⬇ Significant decrease in average adult lifespan

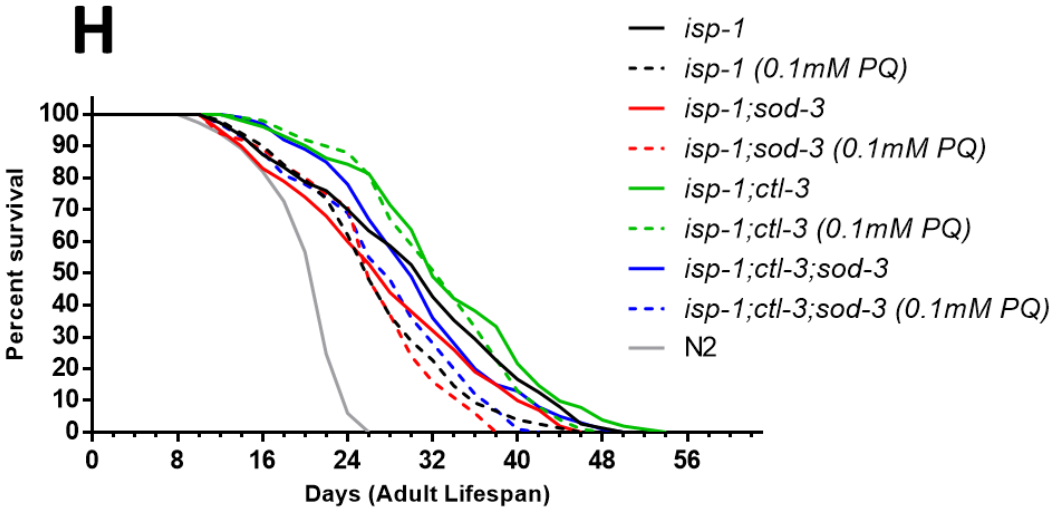
3.5.5. Lifespan curves

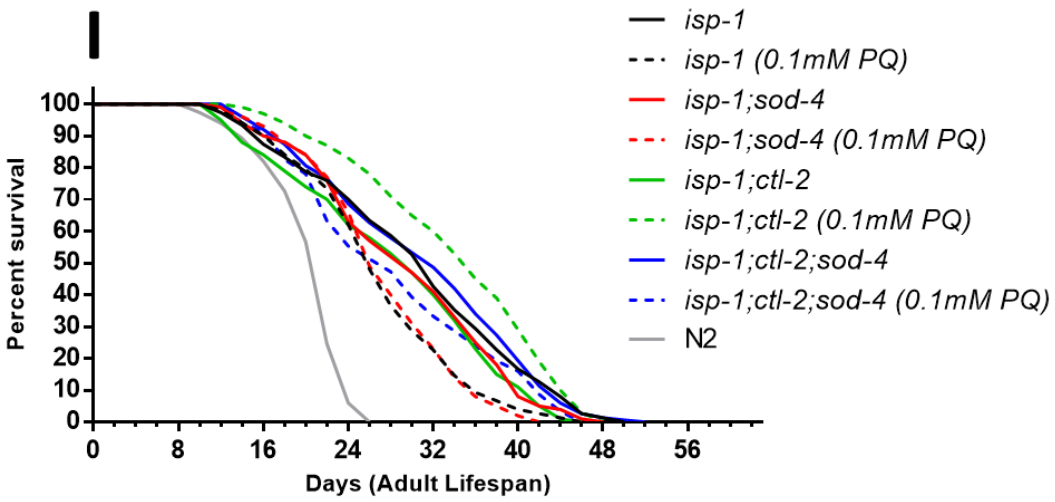


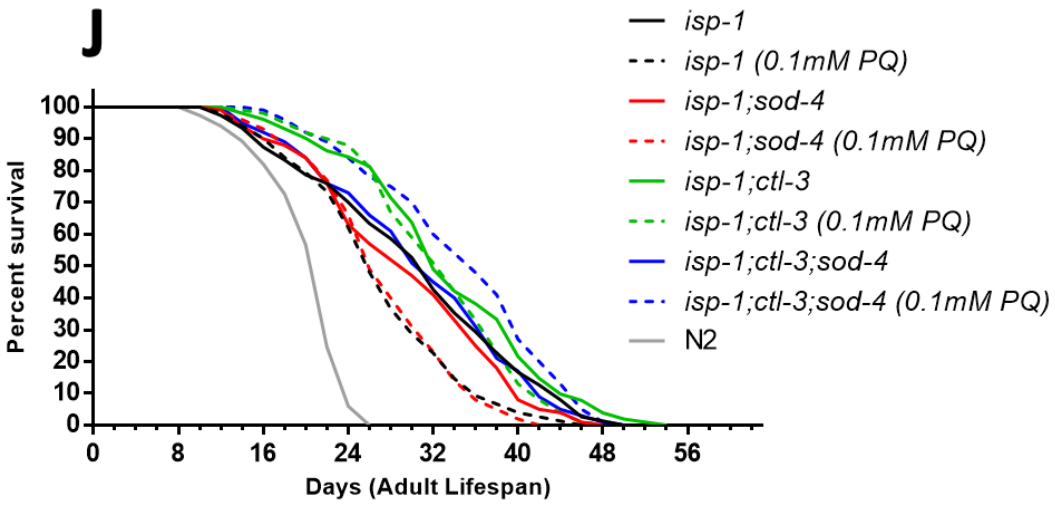

D
Percent survival
Days (Adult Lifespan)
isp-1
isp-1 (0.1mM PQ)
isp-1;sod-2
isp-1;sod-2 (0.1mM PQ)
isp-1;ctl-2
isp-1;ctl-2 (0.1mM PQ)
isp-1;ctl-2;sod-2
isp-1;ctl-2;sod-2 (0.1mM PQ)
N2
E
Percent survival
Days (Adult Lifespan)
isp-1
isp-1 (0.1mM PQ)
isp-1;sod-2
isp-1;sod-2 (0.1mM PQ)
isp-1;ctl-3
isp-1;ctl-3 (0.1mM PQ)
isp-1;ctl-3;sod-2
isp-1:ctl-3;sod-2 (0.1mM PQ)
N2

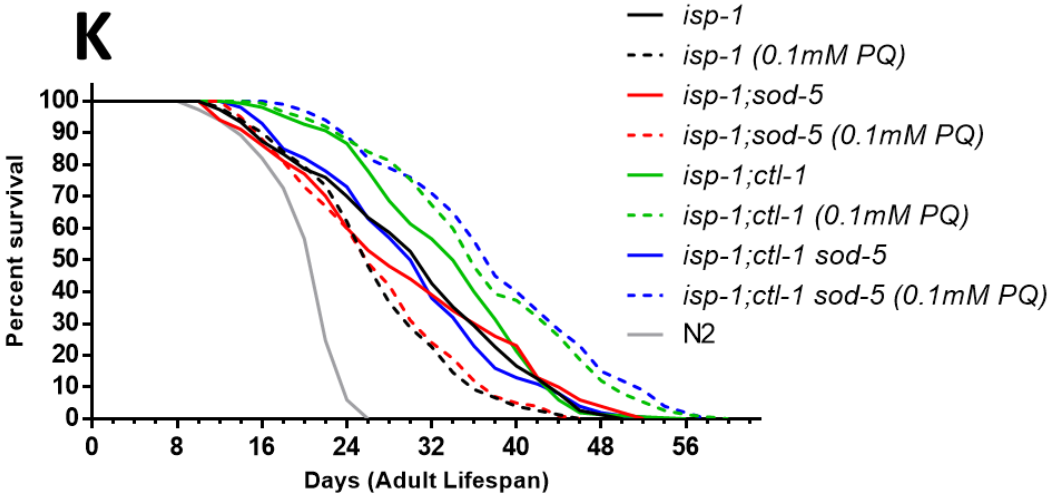

F
Percent survival
Days (Adult Lifespan)
isp-1
isp-1 (0.1mM PQ)
isp-1;sod-3
isp-1;sod-3 (0.1mM PQ)
isp-1;ctl-1
isp-1;ctl-1 (0.1mM PQ)
isp-1;ctl-1;sod-3
isp-1;ctl-1;sod-3 (0.1mM PQ)
N2

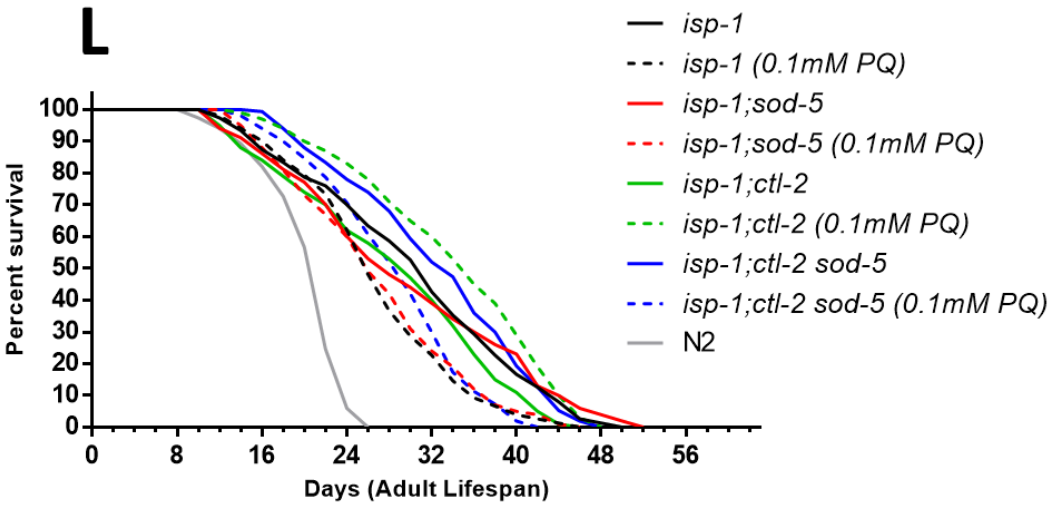

G
Percent survival
Days (Adult Lifespan)
isp-1
isp-1 (0.1mM PQ)
isp-1;sod-3
isp-1;sod-3 (0.1mM PQ)
isp-1;ctl-2
isp-1;ctl-2 (0.1mM PQ)
isp-1;ctl-2;sod-3
isp-1;ctl-2;sod-3 (0.1mM PQ)
N2


H
Percent survival
Days (Adult Lifespan)
isp-1
isp-1 (0.1mM PQ)
isp-1;sod-3
isp-1;sod-3 (0.1mM PQ)
isp-1;ctl-3
isp-1;ctl-3 (0.1mM PQ)
isp-1;ctl-3;sod-3
isp-1;ctl-3;sod-3 (0.1mM PQ)
N2


I
Percent survival
Days (Adult Lifespan)
isp-1
isp-1 (0.1mM PQ)
isp-1;sod-4
isp-1;sod-4 (0.1mM PQ)
isp-1;ctl-2
isp-1;ctl-2 (0.1mM PQ)
isp-1;ctl-2;sod-4
isp-1;ctl-2;sod-4 (0.1mM PQ)
N2


J
Percent survival
Days (Adult Lifespan)
isp-1
isp-1 (0.1mM PQ)
isp-1;sod-4
isp-1;sod-4 (0.1mM PQ)
isp-1;ctl-3
isp-1;ctl-3 (0.1mM PQ)
isp-1;ctl-3;sod-4
isp-1;ctl-3;sod-4 (0.1mM PQ)
N2


K
Percent survival
Days (Adult Lifespan)
isp-1
isp-1 (0.1mM PQ)
isp-1;sod-5
isp-1;sod-5 (0.1mM PQ)
isp-1;ctl-1
isp-1;ctl-1 (0.1mM PQ)
isp-1;ctl-1 sod-5
isp-1;ctl-1 sod-5 (0.1mM PQ)
N2


L
Percent survival
Days (Adult Lifespan)
isp-1
isp-1 (0.1mM PQ)
isp-1;sod-5
isp-1;sod-5 (0.1mM PQ)
isp-1;ctl-2
isp-1;ctl-2 (0.1mM PQ)
isp-1;ctl-2 sod-5
isp-1;ctl-2 sod-5 (0.1mM PQ)
N2

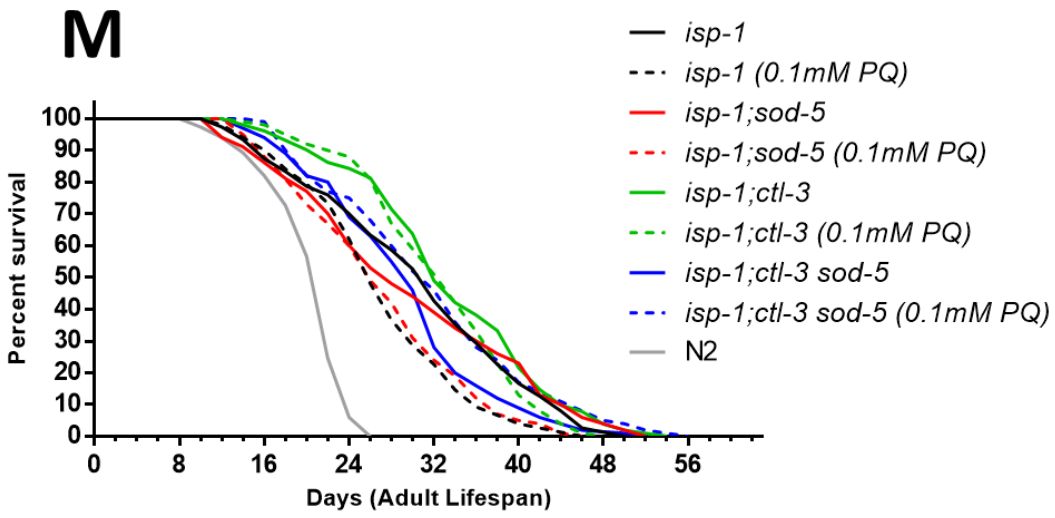
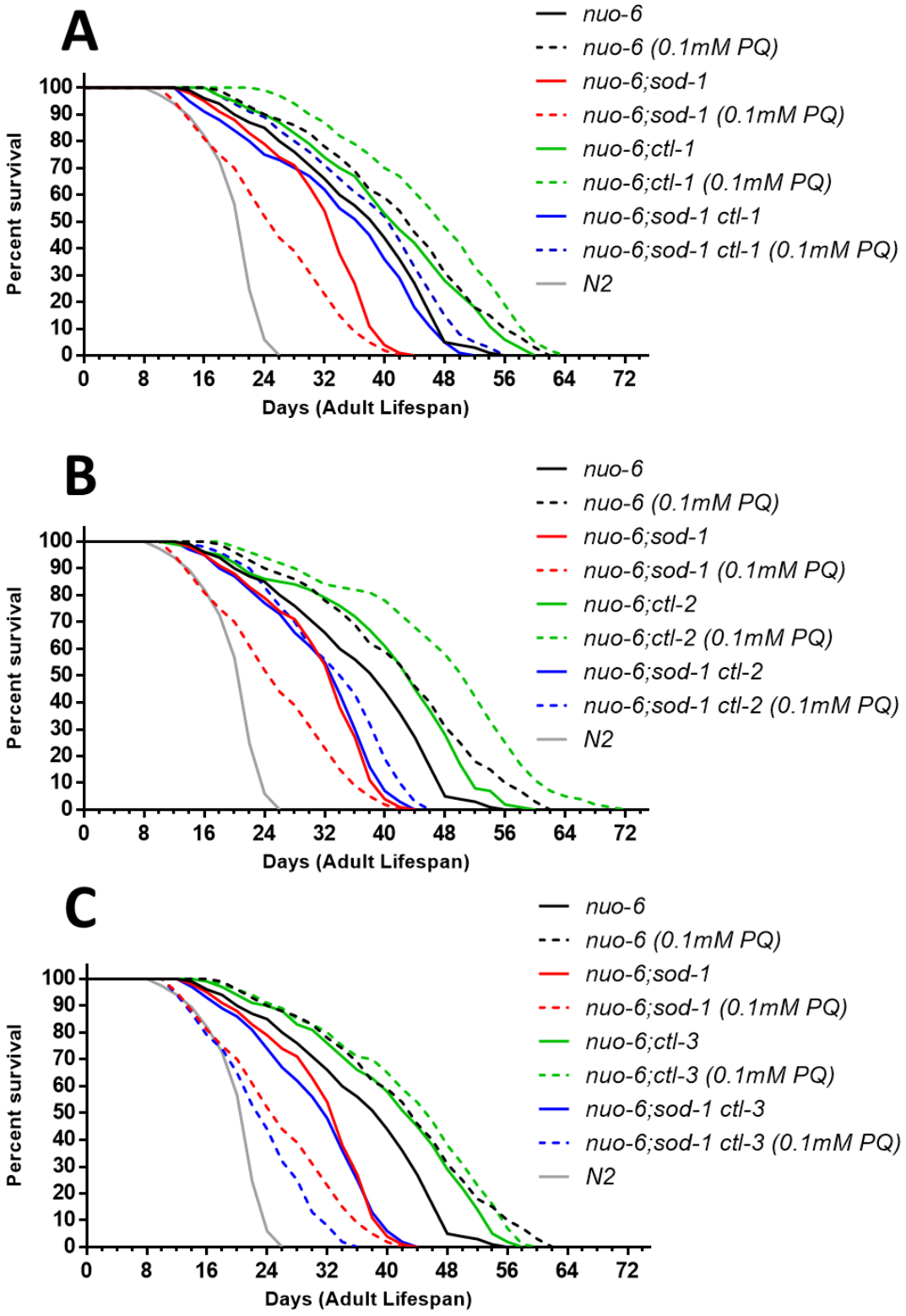
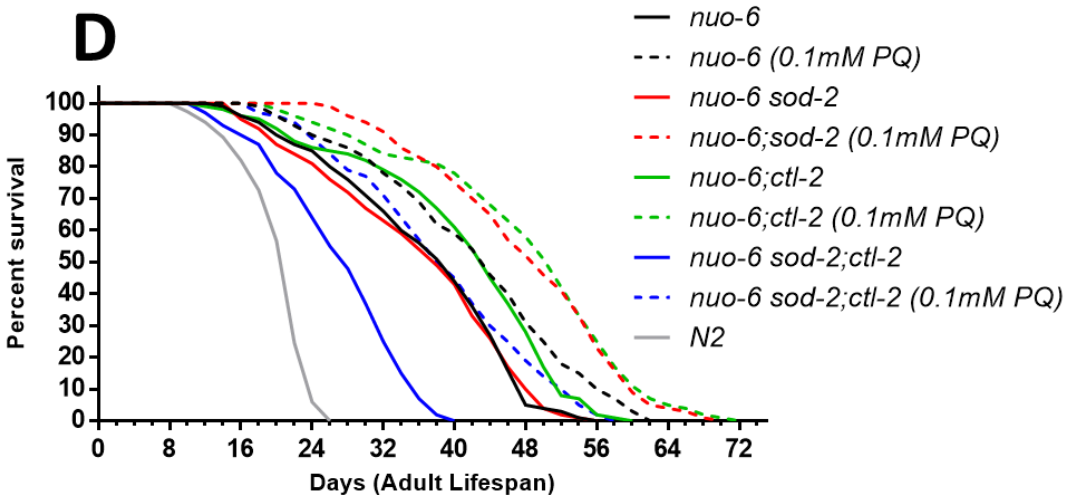
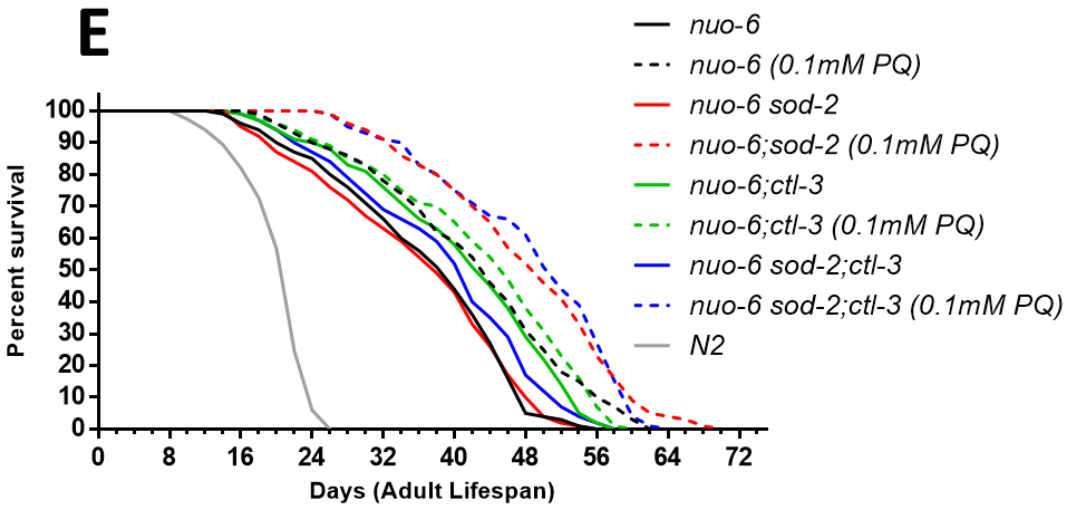
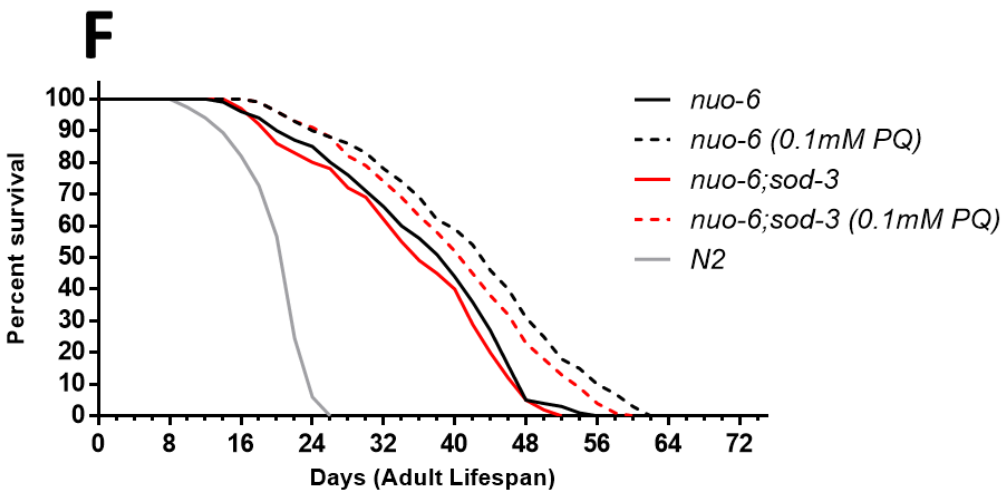

M
Percent survival
Days (Adult Lifespan)
isp-1
isp-1 (0.1mM PQ)
isp-1;sod-5
isp-1;sod-5 (0.1mM PQ)
isp-1;ctl-3
isp-1;ctl-3 (0.1mM PQ)
isp-1;ctl-3 sod-5
isp-1;ctl-3 sod-5 (0.1mM PQ)
N2

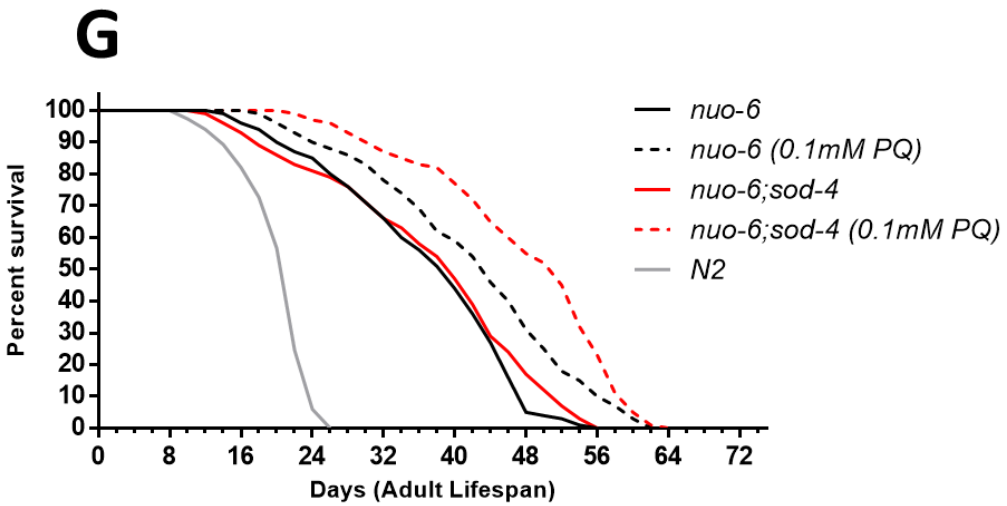
Figure S3.25. Lifespan curves showing the adult lifespan of *isp-1;ctl-x;sod-x* triple mutants with and without paraquat (PQ) treatment. The graphs are organized based on the *sod* mutation in the backgrounds. Note that the triple mutant combinations *isp-1;ctl-1;sod-2* and *isp-1;ctl-1;sod-4* were embryonic lethal (refer to the text for discussion). In each graph, the wild-type (N2) lifespan is shown for reference. Sample sizes and additional data points are shown in Table S3.7.


A
Percent survival
Days (Adult Lifespan)
nuo-6
nuo-6 (0.1mM PQ)
nuo-6;sod-1
nuo-6;sod-1 (0.1mM PQ)
nuo-6;ctl-1
nuo-6;ctl-1 (0.1mM PQ)
nuo-6;sod-1 ctl-1
nuo-6;sod-1 ctl-1 (0.1mM PQ)
N2
B
Percent survival
Days (Adult Lifespan)
nuo-6
nuo-6 (0.1mM PQ)
nuo-6;sod-1
nuo-6;sod-1 (0.1mM PQ)
nuo-6;ctl-2
nuo-6;ctl-2 (0.1mM PQ)
nuo-6;sod-1 ctl-2
nuo-6;sod-1 ctl-2 (0.1mM PQ)
N2
C
Percent survival
Days (Adult Lifespan)
nuo-6
nuo-6 (0.1mM PQ)
nuo-6;sod-1
nuo-6;sod-1 (0.1mM PQ)
nuo-6;ctl-3
nuo-6;ctl-3 (0.1mM PQ)
nuo-6;sod-1 ctl-3
nuo-6;sod-1 ctl-3 (0.1mM PQ)
N2


D
Percent survival
100
90
80
70
60
50
40
30
20
10
0
0
8
16
24
32
40
48
56
64
72
Days (Adult Lifespan)
nuo-6
nuo-6 (0.1mM PQ)
nuo-6 sod-2
nuo-6;sod-2 (0.1mM PQ)
nuo-6;ctl-2
nuo-6;ctl-2 (0.1mM PQ)
nuo-6 sod-2;ctl-2
nuo-6 sod-2;ctl-2 (0.1mM PQ)
N2


E
Percent survival
100
90
80
70
60
50
40
30
20
10
0
0
8
16
24
32
40
48
56
64
72
Days (Adult Lifespan)
nuo-6
nuo-6 (0.1mM PQ)
nuo-6 sod-2
nuo-6;sod-2 (0.1mM PQ)
nuo-6;ctl-3
nuo-6;ctl-3 (0.1mM PQ)
nuo-6 sod-2;ctl-3
nuo-6 sod-2;ctl-3 (0.1mM PQ)
N2


F
Percent survival
Days (Adult Lifespan)
nuo-6
nuo-6 (0.1mM PQ)
nuo-6;sod-3
nuo-6;sod-3 (0.1mM PQ)
N2


G
Percent survival
Days (Adult Lifespan)
nuo-6
nuo-6 (0.1mM PQ)
nuo-6;sod-4
nuo-6;sod-4 (0.1mM PQ)
N2

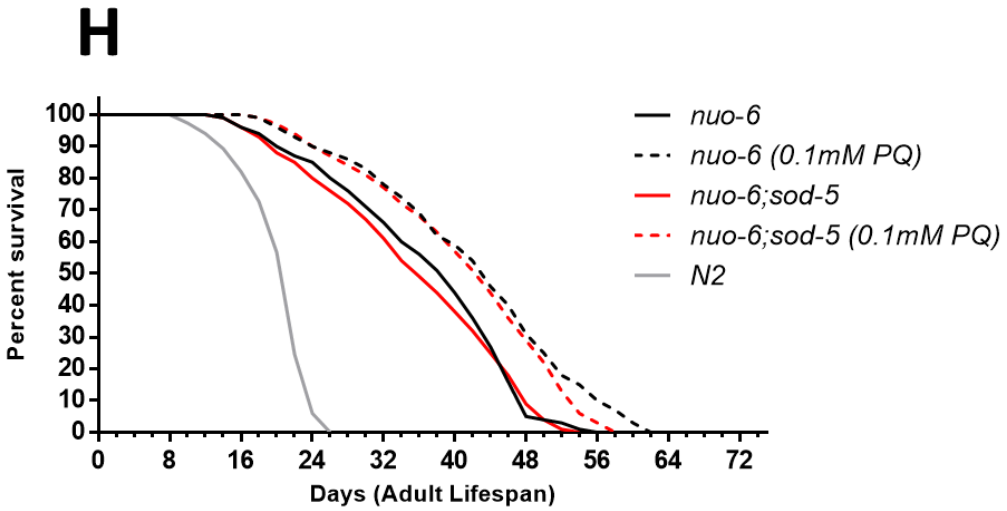
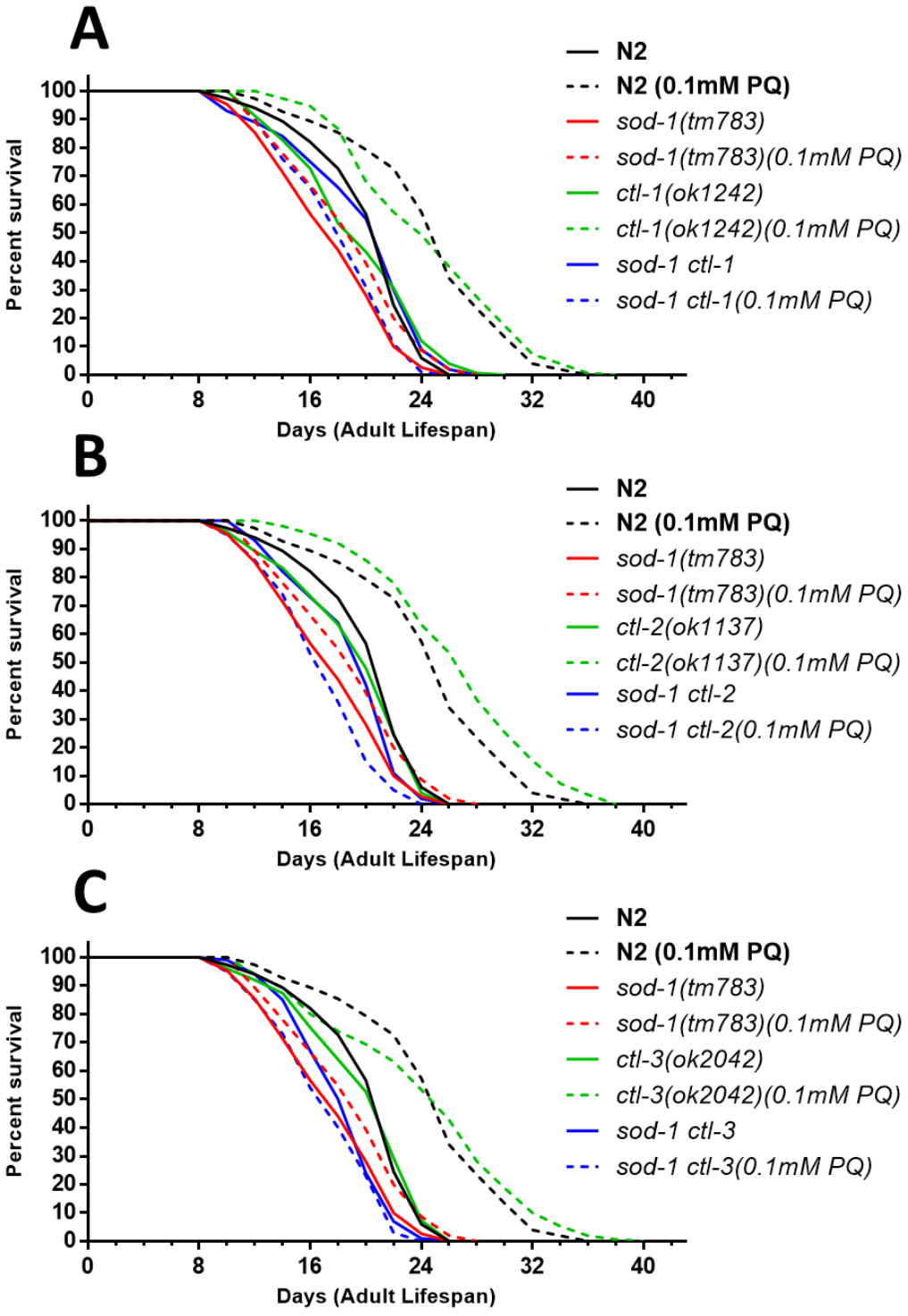
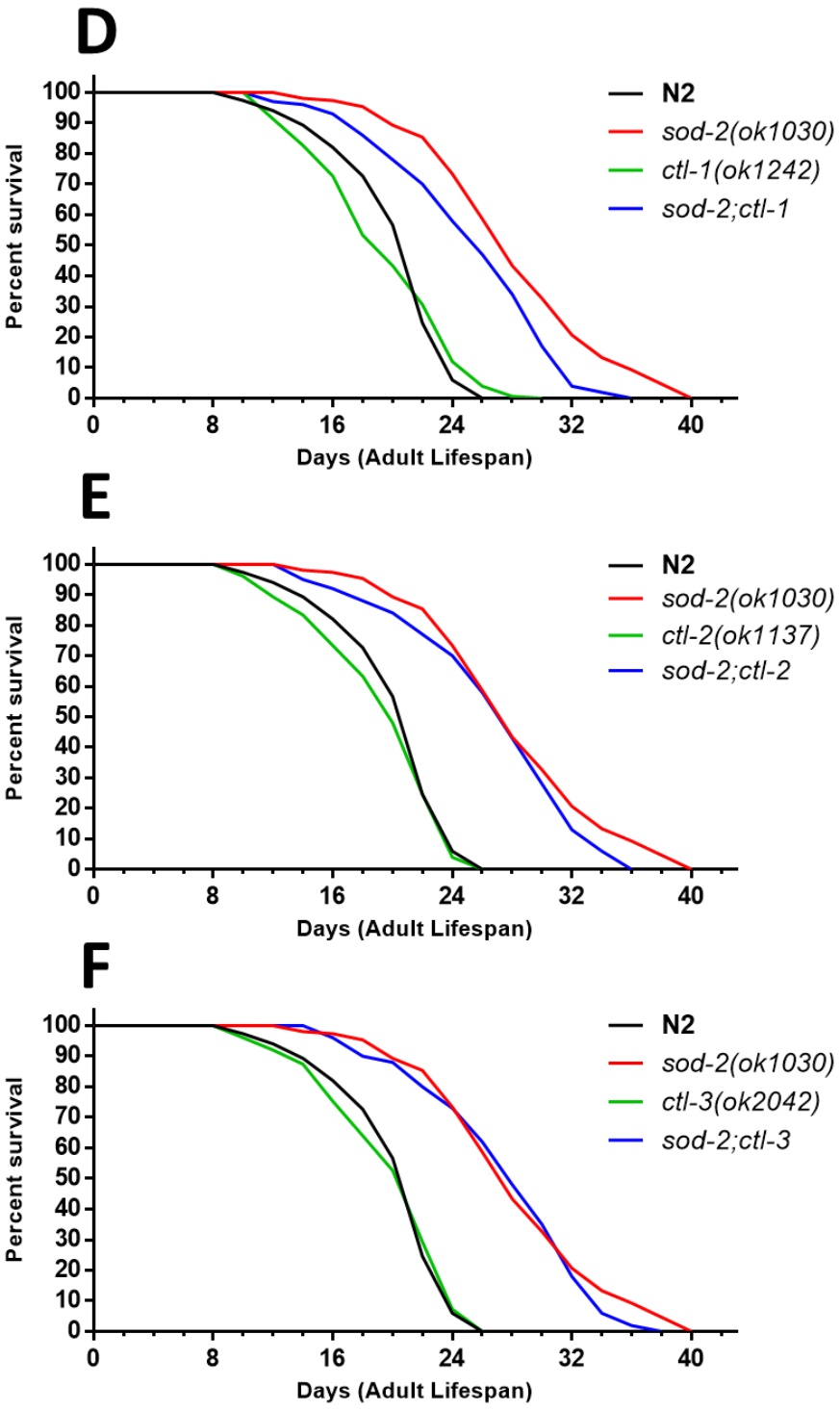

H
Percent survival
Days (Adult Lifespan)
nuo-6
nuo-6 (0.1mM PQ)
nuo-6;sod-5
nuo-6;sod-5 (0.1mM PQ)
N2

Figure S3.26. Lifespan curves showing the adult lifespan of (A – C) *nuo-6;sod-1;ctl-x* (D, E) *nuo-6;sod-2;ctl-x* triple mutants and (F – H) *nuo-6;sod-x* double mutants with and without paraquat (PQ) treatment. The graphs are organized based on the *sod* mutation in the backgrounds. Note that the triple mutant combinations *nuo-6;ctl-1;sod-2* and *nuo-6;ctl-1;sod-4* were embryonic lethal (refer to the text for discussion). In each graph, the wild-type (N2) lifespan is shown for reference. Sample sizes and additional data points are shown in Table S3.7.


A
Percent survival
Days (Adult Lifespan)
N2
N2 (0.1mM PQ)
sod-1(tm783)
sod-1(tm783)(0.1mM PQ)
ctl-1(ok1242)
ctl-1(ok1242)(0.1mM PQ)
sod-1 ctl-1
sod-1 ctl-1(0.1mM PQ)
B
Percent survival
Days (Adult Lifespan)
N2
N2 (0.1mM PQ)
sod-1(tm783)
sod-1(tm783)(0.1mM PQ)
ctl-2(ok1137)
ctl-2(ok1137)(0.1mM PQ)
sod-1 ctl-2
sod-1 ctl-2(0.1mM PQ)
C
Percent survival
Days (Adult Lifespan)
N2
N2 (0.1mM PQ)
sod-1(tm783)
sod-1(tm783)(0.1mM PQ)
ctl-3(ok2042)
ctl-3(ok2042)(0.1mM PQ)
sod-1 ctl-3
sod-1 ctl-3(0.1mM PQ)


D
Percent survival
Days (Adult Lifespan)
N2
sod-2(ok1030)
ctl-1(ok1242)
sod-2;ctl-1
E
Percent survival
Days (Adult Lifespan)
N2
sod-2(ok1030)
ctl-2(ok1137)
sod-2;ctl-2
F
Percent survival
Days (Adult Lifespan)
N2
sod-2(ok1030)
ctl-3(ok2042)
sod-2;ctl-3

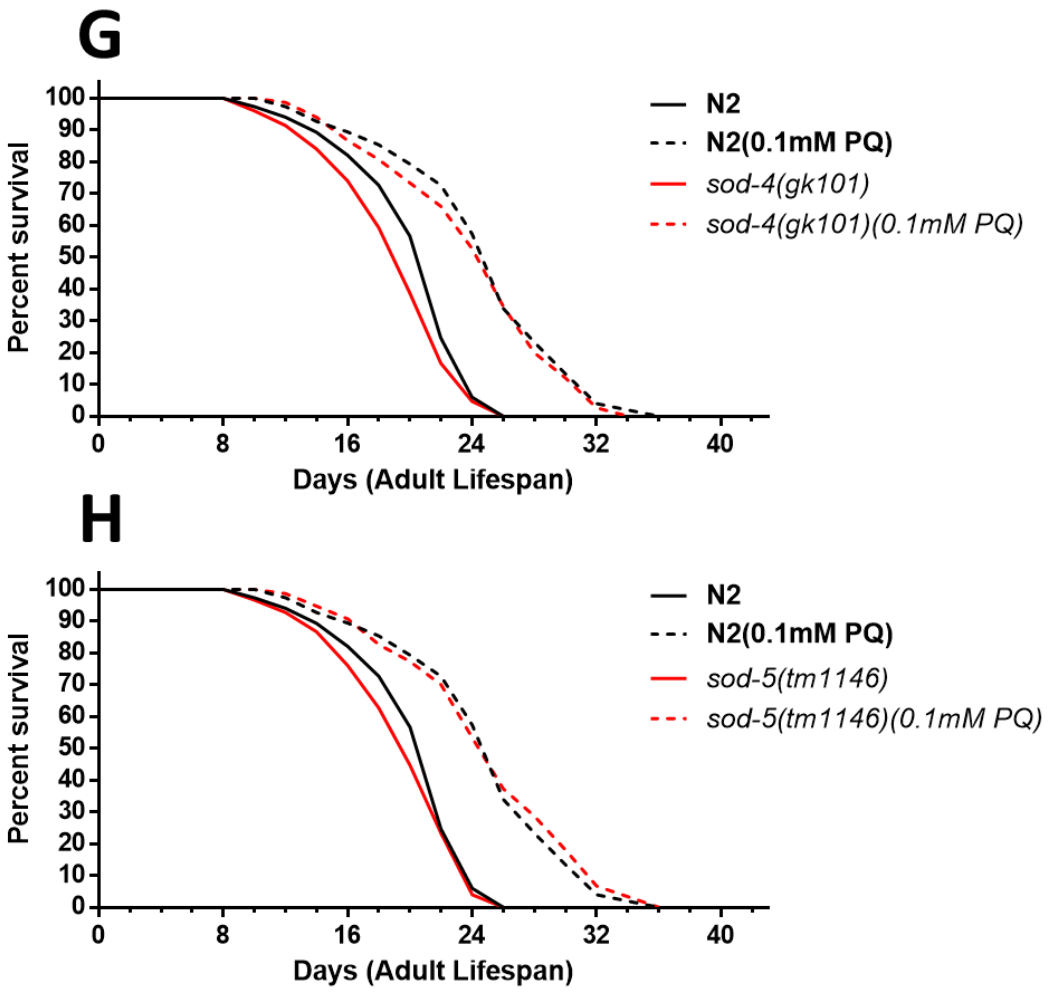


Figure S3.27. Lifespan curves showing the adult lifespan of (A – C) *sod-1;ctl-x* double mutants with and without paraquat (PQ) treatment (D – F) *sod-2;ctl-x* double mutants without PQ treatment, (G) *sod-4* and (H) *sod-5* single mutants with and without PQ treatment. The graphs are organized based on the *sod* mutation in the wild-type (N2) background. Note that the *sod-2* mutation causes the worms to go into developmental arrest as a result of 0.1 mM PQ treatment. Therefore, the graph sets that show the adult lifespan in the *sod-2* mutant background do not include the data on PQ treatment. For discussion about the lifespan of *sod-2* and *sod-3* and their dose response to PQ treatment see chapter 2. Sample sizes and additional data points are shown in Table S3.7.

3.5.6. Summary of the lifespan data

Table S3.7. Summary of the data from the lifespan experiments described in chapter 3.

Condition	Trials	N	Average Adult Lifespan ± SEM	Maximum Adult Lifespan
Wild-Type Background				
N2	3	150	20.45 ± 0.31	26
N2 + 0.1 mM PQ	3	150	25.01 ± 0.44	36
sod-1(tm783)	3	150	17.86 ± 0.34	26
sod-1 + 0.1 mM PQ	3	150	19.17 ± 0.36	28
sod-2(ok1030)	3	150	28.43 ± 0.47	40
sod-3(tm760)	3	150	17.32 ± 0.22	22
sod-3 + 0.1 mM PQ	2	100	22.54 ± 0.47	34
sod-4(gk101)	3	150	19.29 ± 0.33	26
sod-4 + 0.1 mM PQ	3	150	24.43 ± 0.44	34
sod-5(tm1146)	3	150	19.73 ± 0.32	26
sod-5 + 0.1 mM PQ	3	150	25.23 ± 0.47	36
ctl-1(ok1242)	3	150	19.81 ± 0.37	30
ctl-1 + 0.1 mM PQ	3	150	24.96 ± 0.46	38
ctl-2(ok1137)	3	150	19.64 ± 0.35	26
ctl-2 + 0.1 mM PQ	3	150	27.08 ± 0.45	38
ctl-3 (ok2042)	3	150	20.08 ± 0.34	26
ctl-3 + 0.1 mM PQ	3	150	24.61 ± 0.56	40
sod-1 ctl-1	2	100	20.06 ± 0.46	28
sod-1 ctl-1 + 0.1 mM PQ	2	100	18.48 ± 0.38	26
sod-1 ctl-2	2	100	19.34 ± 0.37	26
sod-1 ctl-2 + 0.1 mM PQ	2	100	17.28 ± 0.36	24
sod-1 ctl-3	2	100	18.54 ± 0.33	26
sod-1 ctl-3 + 0.1 mM PQ	2	100	17.46 ± 0.38	24
sod-2;ctl-1	2	100	25.64 ± 0.56	36
sod-2;ctl-2	2	100	27.16 ± 0.57	36
sod-2;ctl-3	2	100	27.96 ± 0.55	38
sod-1;sod-2	2	100	17.82 ± 0.4	26
***isp-1* Background**				
isp-1 (qm150)	3	150	30.64 ± 0.81	50
isp-1 + 0.1 mM PQ	3	150	27.1 ± 0.61	46
isp-1;sod-1	2	100	28.14 ± 0.75	42
isp-1;sod-1 + 0.1 mM PQ	2	100	22.28 ± 0.71	36
isp-1;sod-2	3	148	28.20 ± 0.87	50

Condition	Trials	N	Average Adult Lifespan ± SEM	Maximum Adult Lifespan
isp-1;sod-2 + 0.1 mM PQ	3	150	35.94 ± 0.69	54
isp-1;sod-3	2	100	27.9 ± 0.94	46
isp-1;sod-3 + 0.1 mM PQ	2	100	26.54 ± 0.68	38
isp-1;sod-4	2	100	29.7 ± 0.89	48
isp-1;sod-4 + 0.1 mM PQ	2	100	27.48 ± 0.68	42
isp-1;sod-5	2	100	29.82 ± 1.1	52
isp-1;sod-5 + 0.1 mM PQ	2	100	27.16 ± 0.8	46
isp-1;ctl-1	3	150	33.89 ± 0.67	56
isp-1;ctl-1 + 0.1 mM PQ	3	150	37.4 ± 0.79	60
isp-1;ctl-2	2	100	28.74 ± 0.96	46
isp-1;ctl-2 + 0.1 mM PQ	2	100	34.44 ± 0.88	48
isp-1;ctl-3	2	102	33.76 ± 0.89	54
isp-1;ctl-3 + 0.1 mM PQ	2	100	32.88 ± 0.74	48
isp-1;sod-1 ctl-1	2	100	30.6 ± 0.98	50
isp-1;sod-1 ctl-1 + 0.1 mM PQ	2	100	33.18 ± 0.75	46
isp-1;sod-1 ctl-2	2	100	26.42 ± 0.92	42
isp-1;sod-1 ctl-2 + 0.1 mM PQ	2	100	22.82 ± 0.84	44
isp-1;sod-1 ctl-3	2	101	27.68 ± 0.79	42
isp-1;sod-1 ctl-3 + 0.1 mM PQ	2	100	19.02 ± 0.49	30
isp-1;sod-2;ctl-2	3	150	22.1 ± 0.63	42
isp-1;sod-2;ctl-2 + 0.1 mM PQ	3	150	30.76 ± 0.85	50
isp-1;sod-2;ctl-3	3	150	31.49 ± 0.8	52
isp-1;sod-2;ctl-3 + 0.1 mM PQ	3	150	34.04 ± 0.64	52
isp-1;sod-3;ctl-1	2	100	32.4 ± 1.01	56
isp-1;sod-3;ctl-1 + 0.1 mM PQ	2	100	38.42 ± 0.98	58
isp-1;sod-3;ctl-2	2	100	31.68 ± 0.97	48
isp-1;sod-3;ctl-2 + 0.1 mM PQ	2	100	35.74 ± 0.93	50
isp-1;sod-3;ctl-3	2	100	30.86 ± 0.79	50
isp-1;sod-3;ctl-3 + 0.1 mM PQ	2	100	27.74 ± 0.78	42
isp-1;sod-4;ctl-2	3	150	31.37 ± 0.79	52
isp-1;sod-4;ctl-2 + 0.1 mM PQ	3	150	28.72 ± 0.77	46
isp-1;sod-4;ctl-3	2	100	31.18 ± 0.92	50
isp-1;sod-4;ctl-3 + 0.1 mM PQ	2	100	35.04 ± 0.86	50
isp-1;sod-5;ctl-1	2	100	30.56 ± 0.91	54
isp-1;sod-5;ctl-1 + 0.1 mM PQ	2	100	38.4 ± 0.99	58
isp-1;sod-5;ctl-2	3	150	32.97 ± 0.69	48
isp-1;sod-5;ctl-2 + 0.1 mM PQ	3	150	28.77 ± 0.56	42
isp-1;sod-5;ctl-3	2	100	29.52 ± 0.82	54
isp-1;sod-5;ctl-3 + 0.1 mM PQ	2	100	31.98 ± 0.97	56

Condition	Trials	N	Average Adult Lifespan ± SEM	Maximum Adult Lifespan
***nuo-6* Background**				
nuo-6(qm200)	2	100	36.94 ± 1.02	56
nuo-6 + 0.1 mM PQ	2	100	42.52 ± 1.14	62
nuo-6;sod-1	2	100	31.54 ± 0.73	44
nuo-6;sod-1 + 0.1 mM PQ	2	100	25.8 ± 0.82	42
nuo-6;sod-2	2	100	36.3 ± 1.08	56
nuo-6;sod-2 + 0.1 mM PQ	2	100	48.6 ± 1.05	70
nuo-6;sod-3	2	100	35.52 ± 1.01	52
nuo-6;sod-3 + 0.1 mM PQ	2	100	40.54 ± 1.06	60
nuo-6;sod-4	2	100	37.44 ± 1.19	56
nuo-6;sod-4 + 0.1 mM PQ	2	100	48.2 ± 1.05	64
nuo-6;sod-5	2	100	35.82 ± 1.06	54
nuo-6;sod-5 + 0.1 mM PQ	2	100	41.46 ± 1.05	58
nuo-6;ctl-1	2	100	41.2 ± 1.13	60
nuo-6;ctl-1 + 0.1 mM PQ	2	100	46.86 ± 1.04	64
nuo-6;ctl-2	2	100	41.18 ± 1.12	60
nuo-6;ctl-2 + 0.1 mM PQ	2	100	48.5 ± 1.22	72
nuo-6;ctl-3	2	100	41.26 ± 1.09	58
nuo-6;ctl-3 + 0.1 mM PQ	2	100	43.46 ± 1.12	60
nuo-6;sod-1 ctl-1	2	100	34.72 ± 1.09	52
nuo-6;sod-1 ctl-1 + 0.1 mM PQ	2	100	38.98 ± 1	56
nuo-6;sod-1 ctl-2	2	100	31.64 ± 0.79	44
nuo-6;sod-1 ctl-2 + 0.1 mM PQ	2	100	33.58 ± 0.79	46
nuo-6;sod-1 ctl-3	2	100	30.7 ± 0.8	44
nuo-6;sod-1 ctl-3 + 0.1 mM PQ	2	100	23.52 ± 0.65	36
nuo-6;sod-2;ctl-2	2	100	27.42 ± 0.72	40
nuo-6;sod-2;ctl-2 + 0.1 mM PQ	2	100	38.9 ± 1.03	58
nuo-6;sod-2;ctl-3	2	100	39.18 ± 1.06	58
nuo-6;sod-2;ctl-3 + 0.1 mM PQ	2	100	49.04 ± 1	64
Figure 3.12 B FUDR				
N2 + 100 µM FUDR	2	100	21.16 ± 0.34	28
sod-1(tm783) + 100 µM FUDR	2	100	21.08 ± 0.35	28
sod-2(ok1030) + 100 µM FUDR	2	100	27.96 ± 0.56	40
sod-1;sod-2 + 100 µM FUDR	2	100	22.04 ± 0.38	30

3.6. References

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Chapter 4:
Summary and general discussion

4.1. Summary of background information

In chapter 1, we reviewed the key concepts of aging and ROS biology, their relationship and their relevance to the studies presented throughout chapters 2 and 3. Many theories of aging have been proposed. From an evolutionary perspective, it is not likely that specific genes whose function is to promote aging are actively selected for by evolutionary mechanisms (Kirkwood and Austad 2000) because, in populations, while beneficial alleles with positive impacts on fitness are selected for early in life (even though they may have negative effects in late-life), the strength of selection for survival and fecundity often declines with age (Flatt and Partridge 2018). Additionally, evolutionary biology does not consider aging to be a programmed process. One reason is that a hypothetical "accelerated aging gene" would not benefit the individual in any way. This is because individuals that have inactivated alleles of those accelerated aging genes would enjoy a huge selection advantage over their unlucky counterparts. Also, an equilibrium is next to impossible to maintain for these hypothetical genes as they tend to kill off the individuals that carry them (Kirkwood and Austad 2000).

Another theory of aging is the telomere theory of aging. This was sparked by Hayflick's discovery in 1961. He proposed that the ability of human cells to divide is limited to approximately fifty times (Hayflick limit) and after this limit is reached, cells will stop dividing (Hayflick and Moorhead 1961). This limitation occurs as a result of the mode of function of DNA polymerase. After each round of DNA replication and cell division, telomeres become shorter by about 100 nucleotides which shortens the DNA at the chromosomal ends causing cells to enter senescence after a finite number of cell divisions (Hayashi, Cesare et al. 2015). The discovery of telomerase in the early 1980's (Greider and Blackburn 1985), created the hope that replicative senescence could be prevented by the targeted activation of telomerase. However, although telomerase activity has been linked with aging, telomere length is not indicative of lifespan as, for example, mice have much longer telomers than humans (Calado and Dumitriu 2013). Also, despite the fact that telomerase activity protects cells against replicative senescence, it does not prevent senescence caused by other pathways such as cell death resulting from DNA damage, ROS toxicity or the activation of oncogenes (Shay 2018). Additionally, the telomere theory of aging does not explain the aging process in post-mitotic organisms such as *C. elegans* (Riddle, Blumenthal et al. 1997).

Next, we discussed the DNA damage accumulation theory and other accumulation theories of aging which focus on the piled-up damage-over-time effect to the DNA and other macromolecules and organelles as the cause of aging. However, it is not clear that accumulation of damaged components is a consequence of aging or the cause of it (Chaudhuri, Bains et al. 2018). While initially it might seem natural that the accumulation of insoluble material is the cause of cellular or tissue dysfunction (Bjorksten 1968), it is also possible that physiological responses such as misfolded protein aggregation are protective responses (Derham and Harding 1997, Kaushik and Cuervo 2015).

Finally, there is the free radical theory of aging originally proposed by Harman in 1956 (Harman 1956) and modified later to highlight the role of mitochondrial ROS in aging (Harman 1972). This theory is designed to link ROS and aging and is most relevant to the studies described in this thesis. The premise of the free radical theory of aging is that since ROS (e.g. $O_2^{\bullet -}$, H_2O_2 and •OH) have the ability to oxidize and damage molecular components such as DNA, proteins and lipids, they must be the culprit behind the whole aging enigma. Therefore, the principles of the free radical theory of aging are based on the idea that mitochondria are the main source of cellular ROS production and they act as some kind of "biological clock" which modulates lifespan. The core concepts of this theory have been falsified time and time again by numerous groups including the studies from our laboratory. For example, we have shown in previous studies and the ones presented in this thesis that increasing ROS levels can indeed lead to longevity. Also, increasing ROS detoxification (e.g. by NAC or vitamin C treatment) can suppress longevity. All in all, even though ROS-induced pathologies are associated with age-associated diseases (Davalli, Mitic et al. 2016), there is no clear cut evidence that directly points to ROS as the cause of aging and it is more likely that increased ROS production in aging cells is a consequence of aging rather than its cause where increased ROS generation in aging represents a signal that activates protective mechanisms which are actually aimed at enhancing longevity (Hekimi, Lapointe et al. 2011). However, despite all the empirical evidence against the free radical theory of aging, pharmaceutical conglomerates remain one of its prominent ardent advocates. These days you can buy NAC as an "anti-oxidant supplement that promotes health and longevity" in supermarkets. You can even buy activated charcoal as "an anti-oxidant and detox agent" for those brave souls who want to take charcoal with their morning orange juice!

While it is true that very high levels of ROS (like pretty much any other chemical compound) can indeed cause toxicity and damage to cellular components, not only cells possess multiple complex systems to tightly control ROS metabolism to prevent exactly that, they also take advantage of different aspects of ROS biology and use ROS as signaling molecules. In section 1.3.1, we discussed that apart from the inherent chemical properties of ROS, certain aspects of their biology make them exceptionally suitable for signaling. First, cells are able to produce and remove different forms of ROS at the same time. They accomplish this through the action of ROS-handling enzymes such as SODs and CTLs to change the rates of ROS production and scavenging. This leads to rapid and dynamic changes in ROS concentrations.

Second, the inherent ability of the cells for subcellular localization of tightly controlled ROS signals makes ROS especially suited for signaling. Therefore, increases in local ROS production can be confined to an organelle to spatially control ROS accumulation and turn it into a highly specific signal. For example, we have shown in our laboratory how increased mitochondrial ROS levels can activate the intrinsic apoptosis signaling pathways to induce a wide range of effects including longevity in *C. elegans* (Yee, Yang et al. 2014) (see section 1.5.3 and Figure 1.17 for a more detailed discussion of the role of the intrinsic apoptosis signaling pathway in the longevity of *C. elegans*).

Additionally, ROS can be involved in auto-propagating long-range signal transfers throughout the organism. In this model, each cell along the route of the ROS signal activates its own ROS production machinery autonomously in order to rapidly carry a signal over long distances. An example of this can be found in *Arabidopsis thaliana* (Miller, Schlauch et al. 2009) (also see Figure 1.10) in a process that takes advantage of a mechanism called ROS-induced ROS release (Mittler, Vanderauwera et al. 2011, Evans, Choi et al. 2016) (also see section 1.3.6 for more details).

Finally, because most changes in the cellular homeostasis can lead to a change in the steady state of cellular ROS levels, it is conceivable how a link between metabolism and ROS levels can turn ROS into an excellent signaling mechanism for monitoring the changes in cellular metabolism. This relationship between ROS and metabolism is so tightly regulated that disturbed redox homeostasis is considered to be a hallmark of cancer cells (Panieri and Santoro 2016).

All these unique aspects of ROS biology have enabled cells to use ROS in signaling scenarios. While •OH, because of its indiscriminate reactivity towards biological molecules, is not

a suitable candidate for a signaling molecule, both $O_2^{\bullet-}$ and H_2O_2 have preferred biological targets which sets their target specificity (Halliwell and Gutteridge 2015). Iron-sulfur clusters are the main cellular targets of $O_2^{\bullet-}$ due to their high electrostatic attraction, but they are not targeted by the uncharged H_2O_2. Interaction between $O_2^{\bullet-}$ and the iron-sulfur clusters oxidizes the cluster and iron is released (D'Autreaux and Toledano 2007, Halliwell and Gutteridge 2015). This targeting mechanism is important in the interactions between $O_2^{\bullet-}$ and the iron-sulfur clusters in proteins. For example, $O_2^{\bullet-}$ is involved in the inactivation of the mitochondrial aconitase (Gardner, Raineri et al. 1995).

In addition to its direct interactions with biological molecules, $O_2^{\bullet-}$ is the precursor to H_2O_2 which is produced by SODs. Among ROS, H_2O_2 acts as a major signaling molecule because of its stable chemistry and high specificity for reactions with the active sites of enzymes and proteins that have a reactive cysteine residue in thiolate form, and additional features to break an O-O bond (Toppo, Flohe et al. 2009, Forman, Maiorino et al. 2010). A much-studied mechanism of H_2O_2 signaling involves the reversible oxidation and reduction of these reactive cysteine residues that are known as "redox-sensitive cysteine switches" (Klatt and Lamas 2000, Fratelli, Gianazza et al. 2004, Hisabori, Hara et al. 2005) (also see Figure 1.12).

The activity of SODs, as one of the major producers of cellular H_2O_2, is linked with H_2O_2-dependent signaling. For example, there is a study which shows that in response to pathogenic bacteria, *sod-1* expression is specifically induced in a unique neuron (the ASER neuron) where its upregulation is needed for the worm's pathogen avoidance behavior. The authors believe these findings suggest that pathogen-induced ROS activates a SOD-1-dependent pathway which is responsible for mediating the avoidance behavior in the worm (Horspool and Chang 2017). This brings us to the next item in this summary: SODs.

We reviewed different aspects of SODs such as their subcellular localization and their involvement in the longevity of *C. elegans* throughout chapters 1 – 3. Unlike mammals that have only three isoforms of SOD, the worm has five. The fact that the control of $O_2^{\bullet-}$ and H_2O_2 levels in *C. elegans* is divided among five different SOD isoforms that have different properties and are expressed in different subcellular compartments and possibly tissues makes the worm a well-suited model organism for studying the diversity of the biological roles of SODs. This unique feature also enabled us to trace the path of the pro-longevity ROS signal from the mitochondria to the cytoplasm and was the reason why we chose to incorporate SODs in our studies in the first place.

Briefly, the five SOD isoforms of the worm are compartmentalized. SOD-1 and SOD-5 are predicted to be intracellular cytoplasmic Cu/ZnSODs (Larsen 1993, Jensen and Culotta 2005). SOD-4, on the other hand, is predicted to be extracellular Cu/Zn SOD. Alternative splicing may produce two different forms of SOD-4. Both of these forms have signal peptides but one of them also has a putative transmembrane domain, which makes it the first example of a membrane-bound SOD (Fujii, Ishii et al. 1998). However, it is not known in which cells SOD-4 is expressed (Wang, Branicky et al. 2018). SOD-1 is responsible for nearly 80% of the total SOD activity in the worm and also 80% of the total *sod* mRNA expression belongs to *sod-1* (Doonan, McElwee et al. 2008). It localizes to the cytoplasm of most cells in *C. elegans.* The cytoplasmic localization of SOD-1 has been confirmed through transgenic expression and western blotting (Doonan, McElwee et al. 2008, Yanase, Onodera et al. 2009). SOD-5 is another cytosolic SOD of the worm. Although SOD-5 expression has been detected in the cytoplasm of a small subset of neurons, this may not be an indicative of the true expression pattern of *sod-5* because it is an inducible *sod* (Doonan, McElwee et al. 2008).

SOD-2 and SOD-3 are very similar and they are both predicted to be mitochondrial MnSODs (Hunter, Bannister et al. 1997, Henderson, Bonafe et al. 2006, Doonan, McElwee et al. 2008, Honda, Tanaka et al. 2008). Previous transgenic studies show SOD-2 and SOD-3 expression are in intestinal cells, pharynx, and hypodermal cells (Henderson, Bonafe et al. 2006, Doonan, McElwee et al. 2008). But as we discussed in chapter 2, it is not clear whether the two proteins are co-localized to the same tissue or mitochondria (Honda, Tanaka et al. 2008). In the mitochondria, SOD-2 and SOD-3 have been reported to localize to the supercomplex I:III:IV of the ETC which enables these SODs to interact with $O_2^{\bullet-}$ at the site of its production (Suthammarak, Somerlot et al. 2013).

In addition to SODs, we also used *ctl* knock-out mutants of *C. elegans* in our studies to help us manipulate H_2O_2 levels in different subcellular compartments. CTLs are synergistic with SODs in that they consume the H_2O_2 content which is partially produced by SODs. The worm has three *ctl* genes with very similar sequences that are arranged in a tandem array (also see Figure 3.4). *ctl-1* and *ctl-3* both encode two forms of cytosolic CTL. *ctl-2* encodes a peroxisomal catalase (Gems and Doonan 2009, Erkut, Vasilj et al. 2013). A summary of different aspects of SODs and CTLs such as their subcellular localization and the effects of their deletion on the lifespan of the wild-type worms are presented in Table 1.2. Also, Figure 1.15 summarizes the important features

of the worm's ROS network such as the subcellular localization of SODs and CTLs and the synergistic interaction of these two families of enzymes with regards to ROS metabolism.

Two mutations of the components of the ETC (*isp-1* and *nuo*-6) and PQ were also central to the studies that we presented in this thesis. These provided us with important tools to investigate ROS-dependent longevity in *C. elegans*. The *isp-1* mutation affects an amino acid near the iron-sulfur center in the Rieske Iron Sulfur Protein of mitochondrial complex III (Feng, Bussière et al. 2001). The *isp-1* mutant worms have a slow rate of development. Other aspects of their physiology such as defecation are also slow (Feng, Bussière et al. 2001). They exhibit decreased whole worm oxygen consumption (Feng, Bussière et al. 2001) and decreased oxidative phosphorylation in isolated mitochondria when using either malate or succinate as an electron donor (Falk, Kayser et al. 2006). Previous studies in our laboratory have shown that the longevity of *isp-1* mutants is not induced by decreased ROS damage. ROS levels in the pharynx of *isp-1* mutants is not different from the wild-type as shown by MitoSOX staining (Dingley, Polyak et al. 2010). Also, a study of oxidative damage to proteins in *isp-1* mutants shows no significant difference compared to the wild-type, although there is a trend towards decreasing levels of carbonylated proteins. However, increasing levels of carbonylated proteins by treatment of *isp-1* mutants with RNAi against *sod-2* (the primary mitochondrial SOD) does not affect the longevity of the worms (Yang, Li et al. 2007). Therefore, the long lifespan of *isp-1* is not caused by a decrease in oxidative damage.

nuo-6 mutants have a point mutation that affects a subunit of complex I of the electron transport chain. This causes a decrease in complex I activity and whole worm oxygen consumption. *nuo-6* mutants also have increased ATP levels despite their decreased mitochondrial function (Yang and Hekimi 2010b). RNAi treatment against *sod-2* does not shorten the lifespan of *nuo-6* just like what we had observed for *isp-1.* However, in the case of *nuo-6,* the *sod-2* RNAi treatment even slightly increases *nuo-6's* longevity (Yang and Hekimi 2010a). Considering the findings that we described in chapter 3, this point is interesting, and we will revisit this in the next sections of this chapter. We also, discussed that increasing ROS scavenging activity in *isp-1* and *nuo-6* (and *sod*-2) mutants via antioxidant treatment with N-acetyl cysteine (NAC) or vitamin C suppresses their longevity (Yang and Hekimi 2010a) (also Figure 3.1).

PQ is a redox-cycling compound that we used throughout our studies to stimulate mitochondrial $O_2^{\bullet-}$ production. It can accept electrons from the ETC and transfer them to molecular oxygen to generate $O_2^{\bullet-}$. Within mitochondria, the main site of $O_2^{\bullet-}$ generation by PQ appears to

be complex I where electrons are transferred to PQ^{2+} to form a radical cation ($PQ^{\bullet+}$) which further reacts with molecular oxygen to produce $O_2^{\bullet-}$ (Cocheme and Murphy 2008) (see also Figure 3.2). In high doses, PQ is widely used in ROS biology to induce cellular and especially mitochondrial oxidative stress (Bus, Aust et al. 1976, Cocheme and Murphy 2008, Robb, Gawel et al. 2015) in particular in studies involving *C. elegans* (Senchuk, Dues et al. 2017) where high concentrations such as 200 mM PQ are used to induce oxidative stress in the worm. To stimulate mitochondrial $O_2^{\bullet-}$ production, however, we used 0.1 mM PQ as we had previously established that this concentration maximally induced the pro-longevity effects of PQ in the wild-type and increasing the concentration of PQ further would decrease the lifespan of the worms (Van Raamsdonk and Hekimi 2012). The correlation between the different PQ doses and the lifespan of the worms reveals an inverted U-shaped curve (also see Figure 2.1) which shows a decrease in lifespan as the concentration of PQ increases beyond the peak of the curve at 0.1 mM (Van Raamsdonk and Hekimi 2012). The implication of this finding is that although increased mitochondrial ROS generation extends lifespan, increasing ROS generation further can weaken the beneficial pro-longevity effects of ROS as a result of the deleterious effects of abnormally high ROS generation. Intrinsic factors such as cell/tissue type and genetic composition determine the optimum levels of beneficial ROS, too (Desjardins, Cacho-Valadez et al. 2017). Section 3.3.2.5 demonstrates an example of the effect of genetic composition on the determination of the optimum levels of beneficial ROS. We showed that the same concentration of PQ (0.1 mM) enhances the longevity of *isp-1;sod-2* and *nuo-6 sod-2* beyond that of *isp-1* and *nuo-6,* respectively, suggesting that the double mutants that lack a major mitochondrial MnSOD benefit more from higher mitochondrial ROS levels than their single mutant counterparts.

This concludes our brief review of the highlights from the introductory material from the previous chapters and a few important points from the findings presented in this thesis in relevance to them. In the next sections, we will discuss our findings from chapters 2 and 3, their meanings and their connections.

4.2. Summary and discussion of our findings regarding the mitochondrial MnSODs and their role in regulating longevity and development

4.2.1. The lifespan shortening effect of sod-3 deletion suggests a specificity for the role of SOD-3 in the redox regulation of lifespan in the worm

Most organisms including mammals have one MnSOD isoform that localizes to the mitochondria, but the worms have two: SOD-2 and SOD-3. In chapter 2 we saw that despite the striking similarity between the sequence of SOD-2 and SOD-3, many aspects of their biology are different. For example, one study found that *sod-2* mRNA accounts for 18% of the total *sod* mRNA in the worm while *sod-3* mRNA contributes to only 1% of that. Coupled with the finding that Western blotting detected no MnSOD protein in adult *sod-2* mutants, this suggests that SOD-2 is the worm's main MnSOD (Doonan, McElwee et al. 2008). But does this mean SOD-3 is redundant? Well, no it does not. Two examples confirm this view. First, during the normal life cycle of *C. elegans,* extrachromosomal *sod-3::gfp* is highly expressed in the fully fed larvae in a pattern similar to that of *sod-2::gfp* which suggests that *sod-2* and *sod-3* have common promotor elements and are both specifically needed for the development of the worm. Second, *sod-3* is expressed under the influence of a wide variety of stressors such as lack of food, oxidative stress and exposure to some chemicals (see section 3.1.3.2 for more details). Some genetic backgrounds also induce the expression of *sod-3.* For example, in the long-lived mutants of the insulin signaling pathway such as *daf-2* (Honda and Honda 1999) and the long-lived mutants such as *isp-1* (Feng, Bussiere et al. 2001, Dingley, Polyak et al. 2010) and *clk-1* (Yang, Li et al. 2007) the *sod-3* transcript is highly upregulated. Therefore, there are a variety of situations when the worm specifically needs to upregulate *sod-3* expression although SOD-2 is present. Loss of *sod-2,* however, can also upregulate *sod-3* expression (Suthammarak, Somerlot et al. 2013). But does this mean that SOD-3 is there to take up the duties of SOD-2 in the absence of the latter? This might be true in the context of a general physiological response to the loss of a major mitochondrial ROS-handling enzyme. However, the specific compensatory mechanism of *sod-3* induction in response to these factors is not established (Wang, Branicky et al. 2018). Additionally, our finding that *sod-2* mutants go into developmental arrest (see section 2.3.8) in response to 0.1 mM PQ treatment negates the compensatory role of *sod-3* in the absence of *sod-2*. That is, if *sod-3* specifically took on the job of *sod-2* in its absence, we would not expect to see *sod-2* mutants going into arrest

because they do have SOD-3. Furthermore, our finding that *sod-3* deletion mutants are short-lived and *sod-2* deletion mutants are long-lived (shown by us and others) also strongly argue for the specificity of the action of these two MnSOD isoforms in the context of the worm's longevity.

When a knock-out mutant of a *sod* gene such as *sod-3* is found to be short-lived, naturally one thinks of the deleterious effects of ROS (perhaps a side effect of the free radical theory of aging). However, we had a very simple but effective tool to test this: PQ treatment. If *sod-3* knockouts are short-lived because for some reason they are suffering from ROS deleteriousness, we would expect PQ treatment to make them live even shorter. As we saw in section 2.3.2, this is clearly not the case. In fact, in response to PQ treatment *sod-3* mutants' adult lifespan increases in a dose-dependent manner in a pattern similar to that of the wild-type worms. Therefore, the lifespan-shortening effect of SOD-3 deletion is mostly additive to the lifespan-lengthening effects of PQ treatment. To explain this observation, one possibility is that specifically SOD-3-dependent H_2O_2 production is necessary at a certain point during the worm's lifecycle for the longevity of the worm. In other words, there might be specific cells/tissues that rely on the H_2O_2 that originates from SOD-3 for the longevity of the worm. Even the inverted U-shaped PQ dose response curve of *sod-3* appears to be flattened when we compare it to the inverted U-shaped PQ dose response curve of the wild-type (see Figure 2.4) which implies that SOD-3 could nonetheless play a minor role in PQ-dependent lifespan increase. Several observations support the view that SOD-3 dependent H_2O_2 may play a cell/tissue- and time-specific role in the regulation of longevity in *C. elegans*. First, *sod-3* expression appears to be upregulated during the worm's developmental stages but in the adult worms its expression levels are very low (Honda and Honda 1999, Essers, de Vries-Smits et al. 2005, Jensen and Culotta 2005, Doonan, McElwee et al. 2008, Yanase and Ishii 2008, Zhi, Feng et al. 2014). Second, we showed in section 2.3.6 that *sod-3* and *sod-2* interact on lifespan with an age-dependent epistatic manner. That is, when worms are younger, they need *sod-3* to benefit from the pro-longevity effect of *sod-2* deletion but older worms do not appear to need *sod-3* as much. These two observations suggest that SOD-3 is needed at different times during the worm's lifecycle. Third, we and others (Doonan, McElwee et al. 2008, Honda, Tanaka et al. 2008) have shown that *sod-3* expression pattern is cell/tissue specific and it is indeed involved in the redox regulation of longevity in the *daf-2* background. Taken together, all these observations are consistent with the view that SOD-3 acts in a time-specific and tissue-specific manner which could

make its product (H_2O_2) particularly indispensable for the worm when or where it is needed for longevity.

So, why the lifespan shortening effect of *sod-3* deletion is mostly additive to the longevity effect of PQ treatment, but it is not additive to the longevity effect of *isp-1* and *nuo-6*? While it is true that 0.1 mM PQ treatment phenocopies the *isp-1* and *nuo-6* mutations, there are some differences between the mode of action of PQ and that of the two mitochondrial mutations. For example, in section 3.1.2, we discussed that *isp-1* and *nuo-6's* longevity signal needs to be conveyed to the intrinsic apoptotic pathway through CED-13. But PQ can bypass the need for CED-13 and activate the intrinsic apoptosis pathway directly, possibly by affecting CED-9 which is physically associated with mitochondria. Also, when using tools like PQ (like any other chemical) we cannot guarantee that it reaches all tissues or cell types equally, at the specific time when it is needed or in the amount that it is needed. On the other hand, every single cell in the organism carries mutations like *isp-1* and *nuo-6*. This ensures that, unlike PQ treatment, increased ROS levels caused by these mutations are available where and when they are needed through the lifespan of the worm all throughout the embryonic and larval stages up until its death. It is these differences besides the similarities in the effects of PQ, *isp-1* and *nuo-6* that make their combination such an appealing tool for studying redox biology in the worm. In the next section, we will look at the interaction between SOD-2 and SOD-3 in the regulation of lifespan in *C. elegans.*

4.2.2. The pattern and magnitude of the effects of *sod-2* and *sod-3* on the lifespan of *C. elegans* suggest a signaling interaction between the two

Unlike *sod-3,* loss of *sod-2* lengthens the lifespan of *C. elegans.* In our laboratory, we had found this previously (Van Raamsdonk and Hekimi 2009) and during the course of the studies presented in this thesis we confirmed that deletion of *sod-2* increases the adult lifespan of the worm. However, we were curious as how SOD-2 and SOD-3 would interact in the regulation of lifespan, especially since the results of a previous study by Haonda *et al* had suggested an interaction between the two mitochondrial MnSODs (Honda, Tanaka et al. 2008). In this study, the authors had investigated the interaction and gene expression patterns of *sod-2* and *sod-3* in *daf-2(lf),* a long-lived mutant of the insulin signaling pathway. Using extrachromosomal *sod-2::rfp* and *sod-3::gfp* transgenes, they have shown that in *daf-2(lf)* background, both *sod-2::rfp* and *sod-3::gfp*

are expressed in the head and tail of the worm. Additionally, in the head of the worms, expression of *sod-2::rfp* was prominent posterior to the pharyngeal corpus while *sod-3::gfp* expression is more marked at the tip of the head, anterior to the pharyngeal corpus, separating the *sod-2::rfp* expressing cells from *sod-3::gfp* expressing cells.

A study by Doonan *et al* had also looked into the expression patterns of *sod-2* and *sod-3* using extrachromosomal *sod-2::gfp* and *sod-3::gfp* translational fusions, but in the wild-type background (Doonan, McElwee et al. 2008). This study reported marked expression of both *sod-2::gfp* and *sod-3::gfp* in the pharynx. *sod-2::gfp* also showed significant expression in procorpus, the terminal bulb region of the pharynx, intestinal cells and various hypodermal cells. Faint expression of *sod-2::gfp* was also observed in some neurons anterior to the pharynx and in posterior intestine. Additionally, *sod-3::gfp* expression was also seen at the tip of the head, anterior to the pharyngeal corpus similar to the study by Honda *et al*.

To study gene expression patterns of *sod-2* and *sod-3,* we used CRISPR to tag the *sod-2* and *sod-3* chromosomal genes in the wild-type with mKate2 and egfp, respectively. The results of this study are discussed in section 2.3.7. We found that *sod-2::mKate2* is highly expressed in the nervous system while *sod-3::egfp* has very little expression in the nervous system. On the other hand, *sod-3* expression can be detected in the body wall muscle in a pattern consistent with expression in the mitochondrial network and although *sod-2* may also be expressed in the body wall muscle, its expression is weak in this tissue. We also observed moderate expression of *sod-2* in hypodermis and strong expression of *sod-2* in the germline. Although, there is a very small overlap between the expression of *sod-2::mKate2* and *sod-3::egfp* in the head region*,* we were also able to confirm that cells which express *sod-2::mKate2* are largely separate from those that express *sod-3::egfp*. Our observations of the expression patterns of the two endogenously tagged mitochondrial MnSODs and those by the previous studies that used extrachromosomal transgenes confirm the tissue-specificity of SOD-2 and SOD-3 expression.

Honda *et al* had also looked into the effect of the loss of *sod-2* and *sod-3* on the adult lifespan, both in the wild-type background and in *daf-2(lf)* background. They constructed their own *sod-2* and *sod-3* deletion alleles for these studies and used experimental conditions that were quite different from our standard experimental conditions (see sections 2.1.6 and 2.3.6). They had reported that the adult lifespans of *sod-2* and *sod-3* deletion mutants and *sod-2;sod-3* double-mutants were similar to that of the wild-type. Additionally, in their study, in terms of adult lifespan,

daf-2;sod-3 lives longer than *daf-2* but *daf-2;sod-2* lives shorter than *daf-2.* Also, the lifespan of *daf-2;sod-2;sod-3* triple-mutant is similar to that of *daf-2;sod-3.* This pattern of the effects of *sod-2* and *sod-3* on the lifespan of *daf-2* suggests that *sod-3* is epistatic to *sod-2* for *daf-2* longevity.

So, we decided to investigate the relationship between *sod-2* and *sod-3* in both wild-type and *daf-2* backgrounds using our standard experimental conditions and deletion alleles (see section 2.2). We found that in the wild-type background, while the adult lifespan of *sod-3* was shorter and the adult lifespan of *sod-2* was longer than that of the wild-type, the adult lifespan of *sod-2;sod-3* double-mutants showed a biphasic aging pattern (also see Figure 2.7). The biphasic aging curve of the *sod-2;sod-3* double-mutants initially shows a steep decline indicating a higher mortality rate for younger worms. This is similar to what we see in *sod-3* mutants. However, as the worms get older their mortality rate decreases and the maximum adult lifespan of *sod-2;sod-3* is very similar to that of *sod-2* mutants. This biphasic lifespan curve of *sod-2;sod-3* indicates a complex epistatic relationship between SOD-2 and SOD-3 in the regulation of longevity in the wild-type background. It is possible that the requirement for the pro-longevity ROS generation changes over time in aging worms. That is, younger worms need SOD-3 to maximally benefit from the longevity effect of SOD-2 deletion and as they become older, this reliance on SOD-3 diminishes and they are able to live as long as *sod-2* mutants. Another possibility is that SOD-2 and SOD-3 have a more complex signaling interaction with regards to lifespan as suggested by the previous work of Honda *et al.*

Therefore, we also looked into the interactions of SOD-2 and SOD-3 in the regulation of lifespan in *daf-2(lf)* mutants. We found that that *daf-2(lf)* actually reverses the epistatic relationship of *sod-2* and *sod-3* compared to what we observed in the wild-type. As mentioned previously, *daf-2(lf)* mutants are already long-lived. We showed that both *sod*-2 and *sod*-3 mutations lengthen this long lifespan even further with *sod-3* having a larger effect (also see Figure 2.8). However, unlike what we see in the wild-type, in the *daf-2(lf)* background the loss of *sod-3* does not shorten the *sod-2*-induced longevity but lengthens it. In other words, while in the wild-type background SOD-3 appears to be essential for the full pro-longevity effect of the loss of SOD-2, in *daf-2(lf)* background SOD-3 is suppressing the longevity effect induced by the loss of SOD-2. We believe both the complex epistatic pattern and the magnitude of the change of the effects produced by the loss of *sod-2* and *sod-3* in the *daf-2(lf)* background strongly argue for a signaling interaction between the two MnSODs of *C. elegans* in the regulation of longevity. Some other observations

are consistent with this interpretation. For example, the cell-type/tissue-specificity of the expression pattern of *sod-2* and *sod-3* which indicates the two MnSOD isoforms are expressed in different cells/tissues and even possibly in different mitochondria within the small overlap regions is consistent with the interaction of SOD-2 and SOD-3 in a signaling context. Additionally, upregulation of *sod-3* expression in the *daf-2* background does not seem to be happening as a means to increase the anti-oxidant defences of the worm because first, in *daf-2;sod-2* there are very low detectable levels of MnSOD (presumably SOD-3) and the only SOD whose deletion causes a large and significant decrease in SOD protein activity levels in *daf-2(lf)* is SOD-1, not SOD-2 or SOD-3 (Doonan, McElwee et al. 2008). Taken together, these observations are consistent with a signaling role for the mitochondrial MnSODs in *daf-2(lf)* background between *sod-2*-expressing and *sod-3*-expressing cells/mitochondria and weaken the view that at least in the case of *sod-3* its upregulation is due to a need for increased anti-oxidant defense.

4.2.3. Impaired superoxide signaling could be responsible for inducing developmental arrest in *C. elegans* in the absence of SOD-2

In our laboratory, we had previously observed that *sod-2* deletion mutants go into developmental arrest when treated with 0.2 mM PQ (vs. our pro-longevity PQ treatment at 0.1 mM) (Van Raamsdonk and Hekimi 2009). This 0.2 mM PQ treatment, at the time, was intended to test the mutant's sensitivity to PQ and had served it purpose perfectly. The study reported that *sod-2* deletion mutants go into developmental arrest at L1 in response to 0.2 mM PQ treatment. However, while studying modulation of ROS-dependent longevity by ROS-handling enzymes, we realized that there is no PQ concentration that can induce longevity in the already long-lived *sod-2* mutants. All the concentrations of PQ that we tested only reduced the longevity of *sod-2* even at concentrations as low as 0.01 mM PQ, 10 times lower than the 0.1 mM PQ that induced longevity in the wild-type (also see Figure 2.5). Even the 0.1 mM PQ treatment itself, induced developmental arrest in *sod-2* mutants at L2 stage. Interestingly, the loss of SOD-3, as the other *C. elegans* mitochondrial MnSOD does not induce this PQ arrest phenotype. So, we set out to find what may be the reason. Is the developmental arrest happening because increased mitochondrial $O_2^{\bullet-}$ generation stimulated by 0.1 mM PQ causing ROS toxicity in the absence of SOD-2 (the primary mitochondrial MnSOD of the worm)? Or a change in $O_2^{\bullet-}$ signaling in the mitochondria that lack SOD-2 is causing the developmental arrest?

We know that 0.1 mM PQ treatment slows down the development of the wild-type by at least a day (up to 3.5 days vs. 2.5 day for untreated worms, also see Figure 2.6). We have two ROS players here: H_2O_2 (the product of SOD-2) and mitochondrial $O_2^{\bullet-}$ (since both SOD-2 and PQ act in the mitochondria to alter $O_2^{\bullet-}$ metabolism). To see whether alterations in H_2O_2 levels are responsible for the developmental arrest in *sod-2* mutants we turned to our toolkit of *ctl* deletion mutants, which can increase H_2O_2 levels in different subcellular compartments, *sod-1* deletion mutants, which have reduced cytoplasmic H_2O_2, *sod-3* deletion mutants, which lack the other mitochondrial MnSOD of the worm, and finally NAC, which can enhance H_2O_2 scavenging and thereby reduce its levels. We found that removing each of the three CTLs in 0.1 mM treated *sod-2* background causes the worms to go into arrest at an earlier developmental stage (at L1 rather than L2). As we discussed in chapter 2, *sod-3* mutants develop into adults normally while being treated with 0.1 mM PQ, and so do *sod-1* mutants. We found that removing either *sod-1* or *sod-3* from the 0.1 mM PQ-treated *sod-2* background does not alter the arrest phenotype; however, concurrent removal of *sod-1* and NAC treatment does make the *sod-2;sod-1* double-mutants go into developmental arrest at L1 rather than L2 which makes the arrest phenotype worse. Curiously, removing each of the three *ctl* genes from 0.1 mM treated *sod-2* background has the same effect. It is curious because we the removal of *sod-1* decreases cytoplasmic H_2O_2 levels and treatment with NAC decreases H_2O_2 levels further. But we expect that the removal of CTLs, as sinks of H_2O_2, would increase H_2O_2 levels. Therefore, since neither increasing, nor decreasing H_2O_2 levels affected the arrest phenotype in a positive way, the most parsimonious explanation at this time is that excessive PQ-dependent generation of $O_2^{\bullet-}$ in mitochondria that need SOD-2 but do not have it produces an excessive signal that slows down the development of the *sod*-2 mutants to the point of arrest.

The next step was to investigate whether ROS signaling was affecting the development of these *sod-2* mutants under PQ treatment. *let-60ras* is a signaling pathway that is responsible for vulva formation during the development of the worm. For example, a gain-of-function mutation of *let-60ras* leads to the multi-vulva phenotype in the worm (also see Figure 2.16). In our laboratory, we had previously found that the activity of *let-60ras* is redox regulated (Shibata, Branicky et al. 2003). So, we tested to see how *sod-2;let-60rasgf* would respond to 0.1 mM PQ treatment in terms of developmental arrest. We found that *let-60rasgf* is able to partially rescue the *sod-2* PQ-induced arrest phenotype. That is some 40% of *sod-2;let-60rasgf* double mutants

develop into adults while the rest go into developmental arrest at L2. This partial rescue of the PQ-induced *sod-2* arrest by a *let-60rasgf* mutation suggests that a developmental signaling process that at least in part goes through the Ras signaling pathway is involved in the arrest phenotype. To test the involvement of the Ras signaling pathway in the arrest phenotype we disabled the redox sensitivity of the LET-60rasgf protein.

LET-60 is has a redox sensitive cysteine residue at 118th position (also see section 1.3.5). This 118th cysteine residue (C118) can be oxidized by H_2O_2, which then enables LET-60 to be controlled through redox signaling (unpublished ongoing work by Maximillian Kramer-Drauberg and Dr. Robyn Branicky, members of our laboratory). Using CRISPR, we introduced a mutation in *sod-2;let-60rasgf* background that substituted the redox active C118 with serine (C118S), which eliminates the redox sensitivity of LET-60rasgf. We found that eliminating the redox sensitivity of the LET-60rasgf protein also abolishes the partial rescue of the PQ-induced *sod-2* developmental arrest. Additionally, manipulating H_2O_2 levels with NAC does not affect the arrest in either *sod-2;let-60rasgf* or *sod-2;let-60rasgf*-C118S. The findings with *let-60ras* reinforce the possibility that the PQ-induced arrest phenotype of *sod-2* is caused by impaired signaling and not a less specific response to damage. Therefore, since manipulating H_2O_2 levels does not affect the arrest phenotype, we wondered whether manipulating $O_2^{\bullet-}$ levels can affect the arrest phenotype.

To manipulate mitochondrial $O_2^{\bullet-}$ levels and metabolism we used *isp-1* and *nuo-6* that induce increased mitochondrial $O_2^{\bullet-}$ levels (Yang and Hekimi 2010a, Yang and Hekimi 2010b) and *gas-1* which causes an increase in the $O_2^{\bullet-}$ content of in sub-mitochondrial particles (Kondo, Senoo-Matsuda et al. 2005). As we discussed in section 2.3.1.2, *nuo-6* and *gas-1* mutations affect complex I subunits and *isp-1* affects a subunit of complex III. These mutations alter $O_2^{\bullet-}$ metabolism in the mitochondria. However, unlike *isp-1* and *nuo-6,* which are long-lived, *gas-1* mutants are short-lived compared to the wild-type. To find out whether mitochondrial $O_2^{\bullet-}$ metabolism affects the PQ induced arrest phenotype of *sod-2* we treated *sod-2;isp-1, sod-2 nuo-6* and *sod-2;gas-1* with 0.1 mM PQ. Surprisingly, we found that despite their different effects on lifespan, all three mitochondrial ETC mutations (*isp-1, nuo-6* and *gas-1*) completely suppress the arrest phenotype. This confirms our view of the involvement of $O_2^{\bullet-}$ in the arrest phenotype.

Next, we wondered whether the $O_2^{\bullet-}$ resistance of the ETC mutants was the reason for the complete suppression of the arrest phenotype. So, we treated the double mutants which do not go into PQ-induced developmental arrest at all (*sod-2;isp-1, sod-2 nuo-6* and *sod-2;gas-1*), and *sod-*

2;let-60rasgf, which partially suppresses the PQ-induced developmental arrest, with 1 mM PQ. If they did not go into developmental arrest at 1 mM PQ, it would show that these mutations that completely or partially suppressed the arrest phenotype, did so because they induced PQ resistance in the worms. However, this was clearly not the case. All four double mutants went into developmental arrest at L1 when they were treated with 1 mM PQ.

The finding that the mitochondrial ETC mutations, which cause increased $O_2^{\bullet-}$ production, can suppress the PQ-induced arrest of *sod-2* mutants is unexpected. These findings reconfirm the view that simple damage is not the cause of the arrest phenotype because the mutations that are able to suppress the arrest at a very low dose of PQ (0.1 mM) are not able to do so at higher concentration (1 mM) and strengthen the view that altered mitochondrial $O_2^{\bullet-}$ metabolism plays a role in preventing the arrest.

Considering all these data, we believe that the PQ-induced *sod-2* developmental arrest is more likely due to impaired $O_2^{\bullet-}$ signaling and it is not caused purely by ROS damage. These findings support this view:

1. Manipulating H_2O_2 levels via genetic and/or chemical means does not cause any major changes in the arrest phenotype. This means that $O_2^{\bullet-}$ is more likely to be responsible for the arrest.
2. Mutations like *isp-1, nuo-6* and *gas-1* that specifically alter mitochondrial $O_2^{\bullet-}$ metabolism completely rescue the arrest phenotype. This confirms the view that $O_2^{\bullet-}$ (and not H_2O_2) is involved in the PQ-induced arrest phenotype of *sod-2.*
3. A *let-60rasgf* mutation that is connected to ROS signaling can partially rescue the developmental arrest; however, upon losing its ROS-sensitive cysteine switch, the protein loses its ability to do so. The involvement of a major redox regulated signaling pathway (also active during the development of the worm) in the PQ-induced arrest phenotype of *sod-2,* favors the view that ROS signaling is likely involved in the developmental arrest phenotype.
4. All strains that we tested including the wild-type, go into developmental arrest when treated with high doses of PQ and none of the mutations mentioned that we discussed above can rescue this condition. This shows the damaging effects of excessive ROS, which cannot be rescued.

In this section, we focused on our findings presented in chapter 2 regarding the regulation of lifespan in *C. elegans* by the worm's two MnSODs: SOD-2 and SOD-3. We also discussed our findings using *sod-2* mutants that implicate mitochondrial $O_2^{\bullet-}$ as a signal that can affect the development of the worm. In the next section, we will summarize and discuss our major findings

which, for the first time, show that the pro-longevity signal induced by the mitochondrial $O_2^{\bullet-}$ is an increase of SOD-1-dependent cytoplasmic H_2O_2.

4.3. An increase of SOD-1-dependent cytoplasmic H_2O_2 is the pro-longevity signal induced by the mitochondrial $O_2^{\bullet-}$.

In chapter 3, we discussed our previous findings that showed treatment with N-acetyl cysteine (NAC), which enhances H_2O_2 scavenging, suppresses the longevity of *isp-1, nuo-6* and *sod-2* (Yang and Hekimi 2010a) (also see Figure 3.1). This finding implied the involvement of SODs as one of the major producers of H_2O_2 in the regulation of ROS-dependent longevity induced by increased mitochondrial $O_2^{\bullet-}$ production via *isp-1, nuo-6* and PQ (also see section 3.1.4.1). In the vast project that ensued, we genetically dissected a part of the ROS network of the worm to find the path of the pro-longevity ROS from mitochondria to its target compartment. To do this, we used *isp-1, nuo-6* and PQ as stimulators of mitochondrial $O_2^{\bullet-}$ generation, the five SODs as the consumers of $O_2^{\bullet-}$ and producers of H_2O_2, and the three CTLs as synergistic partners of SODs with regards to H_2O_2 consumption. All the SODs and CTLs in the worm are compartmentalized. Therefore, the *sod* and *ctl* mutations enabled us to genetically manipulate ROS levels at different subcellular compartments. The *isp-1* and *nuo-6* mutations, offered a genetic way of manipulating $O_2^{\bullet-}$ production and PQ provided a chemical means to do so. Also, combining *isp-1* or *nuo-6* with PQ enabled us to change the intensity of $O_2^{\bullet-}$ stimulation. All the multiple-mutant strains that we used for this study are outlined in Table 4.1. We constructed most of these multiple mutants specifically for this project, but they can also serve as a valuable asset for any other study that involves systematic analysis of the redox regulation of a physiological system in the worm. We analyzed the lifespan data from multiple trials of all the relevant strains using data matrices, tables and graph sets that are described in section 3.3.1.

Looking at the data sets as a whole, the first outstanding data point is that among all *sod* and *ctl* deletion mutants in the wild-type background, only *sod-1* mutants are short-lived. One explanation could be that the loss of SOD-1 as the worm's major cytoplasmic SOD increases oxidative damage and accelerates aging. However, previous studies had not been able to detect an age-dependent increase in molecular damage in *sod-1* mutants (Doonan, McElwee et al. 2008). Additionally, if the shortened lifespan of *sod-1* is due to increased ROS deleterious effects, we would expect that enzymatic or chemical increase in ROS levels (e.g. via PQ) would shorten the

lifespan of the *sod-1* mutants even further. This is clearly not the case. Two data points support this view. First, 0.1 mM PQ treatment lengthens the lifespan of the wild-type, *sod-3, sod-4, sod-5* and *ctl* mutants (we discussed *sod-2* in the previous sections) but not *sod-1* mutants. However, 0.1 mM PQ treatment does not shorten the lifespan of *sod-1,* either; it just does not lengthen it. Further shortening in lifespan by PQ treatment is something you would expect to see if increased ROS deleteriousness was responsible for the shortened lifespan of *sod-1* mutants. But we do not see this here. What we see here can be a reduction in the effectiveness of the beneficial effects of the pro-longevity ROS treatment. In other words, lack of SOD-1 seems to have nullified the beneficial effects of PQ. This brings us to our second point. Removing the worm's cytoplasmic CTL-1, which makes more H_2O_2 available in the cytoplasm, suppresses the lifespan shortening effect of the loss of SOD-1. So, the average adult lifespan of *sod-1;ctl-1* double mutants is similar to that of the wild-type. This is because SOD-1 is a major cytoplasmic producer of H_2O_2 and CTL-1 is a major cytoplasmic sink of H_2O_2. If *sod-1* worms live shorter than the wild-type because they do not have enough H_2O_2 in their cytoplasm, removing CTL-1 from the cytoplasm of *sod-1* mutants should restore their lifespan to the wild-type level. This is clearly what we see happening here (also see Figures 3.8 and 3.9). In other words, the lifespan-shortening effect of the loss of SOD-1 is successfully antagonized by sufficient cytoplasmic H_2O_2. These two data points show that SOD-1-dependent H_2O_2 is required for the normal lifespan of the worm and that the pro-longevity effect of the PQ-induced mitochondrial $O_2^{\bullet-}$ signal requires SOD-1-dependent H_2O_2 production.

Next, we looked at the same situation under maximal stimulation for $O_2^{\bullet-}$ production. We achieved this condition through combining each of *isp-1* and *nuo-6* mutations with 0.1 mM PQ treatment (also see Figure 3.11). Here again we see that in the absence of SOD-1, in *isp-1* and *nuo-6* backgrounds 0.1 mM PQ treatment has a lifespan shortening effect. However, similar to what we observed in the wild-type background, in *isp-1;sod-1*+PQ and *nuo-6;sod-1*+PQ backgrounds removing CTL-1, restores the average adult lifespan of both to the level of *isp-1* and *nuo-6,* respectively. That is, under maximum $O_2^{\bullet-}$ stimulation, just like the wild-type background, the lifespan-shortening effect of the loss of SOD-1 is successfully antagonized by sufficient cytoplasmic H_2O_2. This also suggests that an increase in $O_2^{\bullet-}$ levels can have lifespan-shortening effects when it is uncoupled from or insufficiently accompanied by a protective, lifespan-lengthening effect of elevated cytoplasmic H_2O_2. We have presented a detailed analysis of this view in section 3.4.4.

As we discussed previously, the loss of SOD-2, like *isp-1* and *nuo-6* mutations, also lengthens the lifespan of the wild-type. Given that the basic function of SOD-2 is to convert mitochondrial $O_2^{\bullet-}$ into H_2O_2, it is likely that a mitochondrial $O_2^{\bullet-}$ signal is also responsible for the longevity of *sod-2* mutants. So, we were interested to see whether the longevity of *sod-2* also relies on the SOD-1-dependent increase of H_2O_2 levels. We found that indeed this is the case. The longevity of *sod-2* mutants is dependent on SOD-1.

The genetic dissection of the two major families of ROS-handling enzymes (SODs and CTLs) with the help of *isp-1, nuo-6* and PQ, enabled us to trace the path of a pro-longevity $O_2^{\bullet-}$ signal from the mitochondria to the cytoplasm where SOD-1 turns it into H_2O_2 to relay the pro-longevity signal. The path of the pro-longevity ROS signal is summarized in Figure 3.14.

4.4. Conclusion and future studies

The free radical theory of aging holds ROS, and specifically mitochondrial ROS, responsible for the aging of organisms. Our previous findings and the current findings presented in this thesis strongly contradict this view. These findings show that ROS and ROS-handling enzymes are involved in the regulation of lifespan, not through merely causing or preventing damage to the biomolecules, but through signaling mechanisms that regulate lifespan. Previously, we had shown that one way through which the mitochondrial $O_2^{\bullet-}$ signal lengthens the lifespan of *isp-1*, *nuo-6* and PQ-treated worms is by activating the intrinsic apoptosis pathway which in turn may activate the protective mechanisms that lead to longevity in the worm (Yee, Yang et al. 2014). Our current major finding that SOD-1-dependent increase in cytoplasmic H_2O_2 conveys this mitochondrial $O_2^{\bullet-}$ signal could provide a link between the intrinsic apoptosis pathway and mitochondria. Therefore, one way to expand on the research that we presented in this thesis is to further investigate the connection between SOD-1-dependent cytoplasmic H_2O_2 and the intrinsic apoptosis pathway.

4.5. References

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