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THE EFFECT OF MITOCHONDRIAL SUPEROXIDE ON DROSOPHILA LIFESPAN

Master's thesis

Examiner: Dr. Alberto Sanz and Professor Matti Karp.
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ABSTRACT

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One of the aims of this research is to create a system with high mitochondrial oxidative stress. This was done by knocking down Superoxide dismutase 2 using RNA interference technology in *Drosophila melanogaster*. This knocking down would result in a decreased efficiency of the flies to dismutate superoxide and thus resulting in high superoxide levels. The consequences of such a system whose conditions are similar to that seen in late-life stages were studied. According to the Mitochondrial Free Radical Theory of Aging (MFRTA) the damage caused by ROS produced during normal metabolism is determinant of aging. The validity of MFRTA was checked in this system. Also from previous work, it is known that alternative enzymes NDI1 and AOX reduce ROS production and that NDI1 extends lifespan in flies. The mechanism by which NDI1 extends lifespan was not clear. We set out to investigate how NDI extends lifespan by co-expressing NDI1 and AOX in the flies expressing SOD2 RNAi using a binary system GAL4/UAS. NDI1 was previously found to decrease ROS production as well as increase complex I specific substrate oxidation. Here we try to find if NDI1 functions by a ROS dependent or independent method to extend lifespan. The alternative enzyme AOX was expressed in parallel to be used as a control due to its location in the electron transport chain (ETC) and its complementation of complex III as opposed to NDI1 which compliments complex I of the ETC.

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Aravind Kumar Mohan.

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ABBREVIATIONS

ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BNE	Blue Native Electrophoresis
BSA	Bovine Serum Albumin
C.elegans	*Caenoharbditis elegans*
cDNA	complementary Deoxyribonucleic Acid
CR	Caloric Restriction
CyO	Curly winged fly (Genetic Marker)
CYT C	Cytochrome c
DAH	Dahomey
DEPC	Diethylpirocarbonate
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethyldiaminetetraacetic Acid
EGTA	Chelating agent Ethyl Glycol Tetraacetic Acid
ETC	Electron Transport Chain
FAD	Flavin Adenine Dinucleotide
FADH2	reduced Flavin Adenine Dinucleotide
FeS	Ferrous-Sulphate cluster
FMN	Flavin Mononucleotide

FMNH2	reduced Flavin Mononucleotide
G3P	Glycerol-3-Phosphate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H2O2	Hydrogen Peroxide
HCL	Hydrogen Chloride
HO	Hydroxyl Radical
HRP	Horseradish Peroxidase
KCL	Potassium Cyanide
KDa	Kilo Dalton units
KH2PO4	Monopotassium phosphate
Mb	Mega base
MDa	Mega Dalton units
MFRTA	Mitochondrial Free Radical Theory of Aging
MgCl2	Magnesium Chloride
MLSP	Maximum Lifespan
mtDNA	mitochondrial Deoxyribonucleic Acid
mtROS	mitochondrial Reactive Oxygen Species
NAD+	Nicotinamide Adenine dinucleotide
NADH	reduced Nicotinamide adenine dinucleotide
NDi1	NADH dehydrogenase internal 1
nDNA	nuclear Deoxyribonucleic Acid
OXPHOS	Oxidative Phosphorylation
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PBS-T	Phosphate Buffer Saline-Tween

PD	Parkinson's Disease
PDHα	Pyruvate Dehydrogenase
Q	Ubiquinone
QH2	Ubiquinol
QPCR	Quantitative Polymerase Chain Reaction
REDOX	Reduction-Oxidation
RNA	Ribo-Nucleic Acid
RNAi	Ribonucleic Acid interference
ROS	Reactive Oxygen Species
RT-PCR	Real Time Polymerase Chain Reaction
S.cerviciae	*Saccharomyces cerevisiae*
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis9
SMT	Somatic Mutation theory
SOD 1	Superoxide Dismutase 1
SOD 2	Superoxide Dismutase 2
TMPD	Tetramethyl-1,4-benzenediamine dihydrochloride
UAS	Upstream Activating Sequence

1. INTRODUCTION

Aging is a highly complex process which must be investigated from different perspectives. In order to understand aging, it is required to define aging first. Aging is the result of progressive accumulation of deleterious changes that reduce an organism's ability to resist stress causing a decrease in survival possibilities. Aging is of particular importance now because almost all countries will face population aging in the near future. Population aging is the process by which the population of older individuals becomes a proportionally larger share in the whole population. As the aging populations increase, the number of people susceptible to age related diseases like cardiovascular disease, cancer, diabetes also increases. According to the report by United Nations Department of Economic and social affairs, population division in 2002, Europe had the highest proportion of older individuals and was projected to remain so until 2050 with approximately 37% of its population 60 years or over this age. Therefore it is important not only study about the age related diseases, but the basic mechanism of aging in order to pave the way for healthy lifespan.

Various studies were aimed at increasing the maximum lifespan of different model organisms. In our previous works, we expressed alternative enzymes AOX (*Ciona intestinalis* Alternative Oxidase) and NDI1 (Yeast NADH dehydrogenase internal 1) in *Drosophila melanogaster* and found that they reduce the mitochondrial ROS production. NDI1 was found to increase complex I substrate oxidation and reduce ROS at complex I. But the mechanism as to how NDI1 increases lifespan was still not solved. It is not clear if NDI1 increases lifespan in a ROS dependent or independent mechanism. To understand the mechanism by which NDI1 extends lifespan and thereby understanding the implications of complex I in aging was the main motivation of this work.

2. REVIEW OF LITERATURE

2.1 Theories of aging

There are many theories of aging which are focused on three different levels of hierarchy- molecular, cellular and systemic levels.

2.1.1 Molecular theories of aging

The molecular theories of aging emphasize on the damage and/or loss of functionality of biological molecules like proteins or DNA which may affect the gene regulation or protein synthesis. The codon restriction theory states that during aging, the accuracy of translation is impaired due to the decrease in efficiency of some synthetases. The gene regulation theory and dysdifferentiation theory both emphasize on the changes in gene regulation as the main reason for aging. While the gene regulation theory hypothesizes that senescence results from the changes in gene expression after reproductive maturity is reached, the dysdifferentiation theory states that mistakes in protein synthesis are due to the gradual accumulation of molecular damage which causes an aberrant expression of genes. The error catastrophe theory postulates that the error in information transfer happens in the synthetic and enzymatic machinery of the cell rather than in DNA and that the summation of these errors leads to a point beyond which the cell cannot survive. Somatic mutation and DNA damage theories state that the integrity of the DNA is the controlling factor of aging process and that the integrity is affected either by somatic mutations that occur during day-to-day cell replication or by environmental insults to DNA (Weinert BT and Timiras PS. 2003).

2.1.2 Cellular aging

Theories about cellular aging include wear-and-tear, mitotic clock and apoptosis theory. The wear and tear theory states that injuries of daily living accumulate and lead to the organism's efficiency and thus death. The mitotic clock or telomere hypothesis of aging provides a molecular mechanism for counting the number of cell divisions by the shortening of telomere. This theory also explains the widely believed notion that senescence evolved to limit the number of cell divisions thereby preventing the accumulation of multiple mutations which might result in the cell becoming malignant. The apoptosis theory predicts that the main function of apoptosis or programmed cell death is to be a defensive mechanism against immortal or dysfunctional cells and also that it helps in regulating the cell number. The free radical theory states that aging is a result of accumulation of damages caused by free radicals (superoxides, ROS and RNS) which are produced during the normal metabolism (Weinert BT and Timiras PS. 2003).

2.1.3 System theories of aging

This includes theories like the rate of living, neuroendocrine control theory and immunological theory. The rate of living theory states that the life expectancy of a species is inversely proportional to its metabolic rate. The neuroendocrine control theory of aging is based on the importance of synchronization of signals from the endocrine and neurological systems for normal functioning. It hypothesizes that the effective homeostatic adjustments declines with age which leads to aging and death. The immunological theory of aging stresses on the importance of immune response from the innate and adaptive immune systems when a foreign body is encountered. This theory is based on the findings that there is a progressive decline in the functioning of immune systems with age, the ability to induce certain T-cells and the involution of thymus due to these changes.

2.1.4 Evolutionary theories of aging

There are two major evolutionary theories which try to explain aging and the limited longevity of biological species. The accumulation of mutation theory states that aging is the result of declining force of natural selection with age. According to this theory, late-acting deleterious mutations will be accumulated (passively) during aging and thus leading to mortality late in life. The antagonistic pleiotropy theory states that the late-acting mutations will be actively selected if they have a beneficial effect early in life (Gavrilov LA and Gavrilova NS. 2002).

2.1.5 Reliability theory of aging

It is a general theory about systems failure. The theory predicts the age-related failure kinetics of a system based on its given architecture and reliability of its components. Among other things this theory provides explanations to some fundamental questions about aging like 1) why biological species deteriorate with age and 2) why mortality rates increase with age in many species. The reliability theory also states that aging is a direct consequence of systems redundancy (Weinert BT and Timiras PS. 2003).

2.2 Mitochondrial Free Radical Theory of Aging (MFRTA)

The Mitochondrial Free Radical Theory of Aging (MFRTA) was originally proposed by Denham Harman in 1956 and 1972, previously named as the “Free Radical Theory of Aging”.

MFRTA obtained a greater support after the discovery of the Anti-Free radical enzyme superoxide dismutase (SOD) by McCord and Fridovich in 1969. After its discovery

many other anti-oxidant systems have also been found. Later, the production of highly reactive and unstable molecules as a result of incomplete reduction of oxygen by isolated mitochondria during normal metabolism was described by Britton and his colleagues (Alberto Boveris et al, 1972;1973). From in-vitro studies in isolated mitochondria, it is estimated that 0.1 to 4 % of the oxygen consumed by mitochondria leaks and result in the incomplete reduction of oxygen. Even though the cells possess a set of anti-oxidant and repair systems, they are not 100% effective. This can be seen from the formation of various products of oxidative damage to cells, for example, damage to DNA, protein, lipid peroxidation reactions, etc.

It is important to understand the difference between mean and maximum lifespan in order to better understand aging. Mean lifespan is the average or mean number of years an organism can live in a specific environment (for example the life expectancy of a man in Somalia is around 50 years whereas in Finland it is around 80 years) and Maximum lifespan potential (MLSP) is the maximum number of years an individual in a species can live (for example the maximum lifespan for a rat is four years whereas for humans it is 122 years).

The mitochondrial free radical theory of aging states that aging is the result of accumulating oxidative damage caused by reactive oxygen species generated during normal metabolism. Previously it was believed that longevity can be increased with the help of antioxidants, but this strategy proved to be wrong as they failed to increase the MLSP in mammals (Huang TT et al. 2000; Schriner SE et al. 2000; Chen X et al. 2004 and Sanz et al. 2006.). Changes in anti-oxidant capacity and oxidative damage during aging have been reported in various tissues and species. This failure to increase lifespan can be explained by comparative studies between animals. It was found that long-lived species had lower concentration of anti-oxidant systems compared to short-lived species. This can be explained by the fact that long-lived animals produce less oxidative damage than short-lived animals by producing less ROS and thus they need less antioxidants. There may be two different possibilities to increase lifespan, they are 1. to increase the defensive and repair systems and 2. to decrease damage. From an evolutionary point of view, it seems logical that the latter would be favored as it is the less energy demanding mechanism. According to this concept, mitochondrial ROS production negatively correlates with the MLSP in different comparisons including different species of mammals, birds, bats, snakes and insects (Barja G. 2007.). Especially interesting, there are comparisons between birds and mammals. They show that long-lived birds produce fewer ROS than short-lived mammals in spite of having similar rates of oxygen consumption. This indicates that longevity is related to the number of electrons that leak out of the mitochondrial electron transport chain (to become ROS) and not to the amount of oxygen that is consumed (Schriner SE et al. 2000; Barja G. 2007.). Also in support of the MFRTA, some experimental results show that there is a direct correlation between mitochondrial ROS production and oxidative damage to mitochondrial DNA (mtDNA).

Levels of oxidative damage to mtDNA also seem to correlate with the mtROS produced and its negative correlation with MLSP. Thus the free radicals produced during normal metabolism of mitochondria oxidatively damage mtDNA and the accumulation of alterations in the mtDNA would be partly responsible for the aging process.

2.3 Mitochondria

Mitochondria apart from their function in oxidative phosphorylation to produce ATP (Lehninger AL, 1962) also function in the regulation of metabolism, cell cycle, cell death (McBride HM et al. 2006) and have a central role in maintaining a static cell ionic composition (Darley-Usmar V. 2004). They are surrounded by two membranes namely the outer mitochondrial membrane which largely contains porins or VDAC (voltage dependent anion channels which allow the passage of hydrophilic molecules like sugars, amino acids) and the inner mitochondrial membrane which has numerous folds called cristae. The inner mitochondrial membrane is impermeable to most solutes and it encloses the mitochondrial matrix. The impermeability of inner membrane to H^+ provides the basis for mitochondrial energy transduction. Oxidative phosphorylation is the final step in the biochemical pathway involved in the production of ATP. The oxidative phosphorylation system (OXPHOS) consists of five multi-protein complexes (I-V) and two electron carriers which are embedded in the lipid bilayer of the mitochondrial inner membrane. Assembly of the OXPHOS system depends on the coordinated expression of nuclear and mitochondrial genes. It is made up of at least 89 protein subunits, a majority of which are encoded by the nuclear genome (Pagliarini DJ et al. 2008) and the rest by the mitochondrial genome. The mitochondrial genome encodes 13 essential polypeptides which are part of different complexes (except complex II which is also a TCA cycle enzyme). It also encodes two rRNAs and 22 tRNAs required for the translation of these proteins (Anderson S et al. 1981). This is true in the case of most metazoans including humans and *Drosophila.*

The human mitochondrial DNA (mt DNA) is intron-less, multi-copy, circular and made up of 16,569 bp. The two strands of mt DNA are divided into Heavy and Light strands depending on pyrimidine and purine content that result in different buoyant densities. The non-coding region contains the triplex D-loop which is also the site of origin of replication of mt DNA. Each strand has a transcription promoter (HSP, LSP).

2.4 OXPHOS complexes

Five complexes and two electron carriers are involved in the process of oxidative phosphorylation. Complex I also called as NADH dehydrogenase is composed of 42-43 different polypeptides including FMN containing flavo proteins and 8 Fe-S clusters (Hinchliffe et al. 2005). It is L-shaped with a long arm and the short arm extends in to the matrix. Complex I is specifically inhibited by rotenone. Complex II also called as

succinate dehydrogenase is membrane bound and is also a component of the citric acid cycle. It also has FAD and iron-sulfur centers bound to it. Ubiquinone is an electron carrier which transfers electrons from complex I and II to complex III. It is a lipid soluble benzoquinone and is diffusible in both layers of the phospholipid bilayer of the inner membrane. Complex III or cytochrome bc1 complex is composed of 9-10 polypeptides including a [2Fe-2S] cluster. Cytochrome C is a peripheral protein which is facing the intermembrane space and functions by transferring electrons from complex III to complex IV. Complex IV or cytochrome C oxidase reduces O_2 to H_2O with 4 e^- obtained from reduced cytochrome c in a reaction which also consumes 4 H^+ from the matrix (Hatefi. 1985). ATP synthase or complex V is an F-type ATPase which has two components, an integral protein F_0 and a peripheral protein F_1 (Boyer PD. 1998). The F_0 which is in contact with the central stalk of the complex can be rotated and is fuelled by H^+ (Walker JE et al. 1995) which results in the rotational catalysis of ADP phosphorylation by F_1 component.

During the mitochondrial energy conversion reaction, the TCA cycle enzymes produce high energy electron through oxidation of carbon or organic-acid oxidation and transfer them to the electron carriers nicotinamide adenine dinucleotide (NADH(reduced), NAD+ (oxidized)) or flavin adenine dinucleotide ($FADH_2$(reduced), FAD(oxidized)) respectively. These electrons are transferred through the different complexes up until complex IV where they are used to convert O_2 to H_2O. This transport of electrons across the electron transport chain is coupled with oxidative phosphorylation to produce ATP. Complex I, III and IV also function as H^+ pumps which are driven by the free energy of the coupled oxidation reactions (Navarro A et al. 2007). The H^+ ions are pumped from the matrix to the intermembrane space. This proton motive force generated across the membrane is made use of by complex V to produce ATP (Figure 2.1).

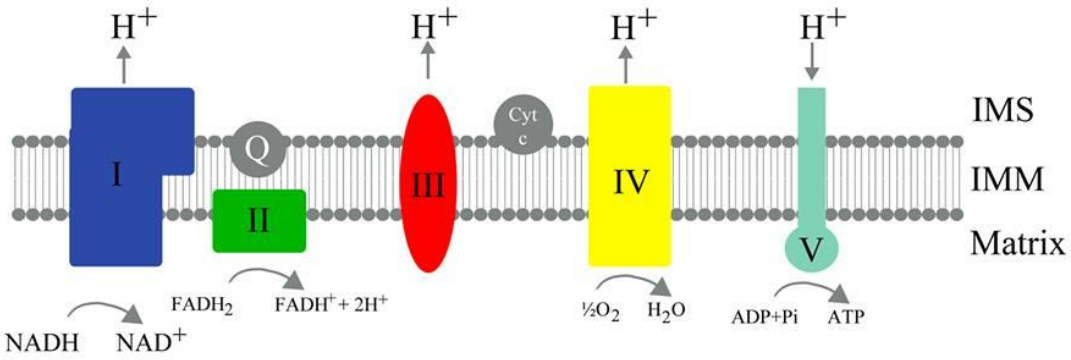


Figure 2.1 *Figure representing the OXPHOS system in mitochondria. I-V represent respiratory complexes I-V.The diagram indicate the several processes during the generation of ATP which includes transfer of electron through different complexes and coupled transfer of H^+ ions across the intermembrane space (adopted from Smeitink J et al. 2001)*

The rate of respiration and the rate of ATP synthesis depend on the cellular demand for energy. When more ATP is being used by the cell, it is broken down into ADP and when ADP availability increases, the rate of respiration and of ATP synthesis increases. This can referred as state 4 and state 3 as defined by Chance and Williams in 1956. State 4 is defined as the state at which there is availability of respiratory substrates but not ADP. It is also called as resting state respiration. State 3 is the state at which there is availability of respiratory substrates as well as ADP and is the state of maximum O_2 consumption and rate of ATP synthesis. It is also called active respiration (Chance B, Williams GR. 1956).

2.5 ROS

Free radicals are atoms, molecules or ions that have unpaired valence electrons. Reactive oxygen species is a collective term which includes molecules containing oxygen-centered radicals such as the superoxide radical anion ($O_2^{\bullet-}$), the hydroxyl radical ($^{\bullet}OH$) and peroxyl radical (ROO) and also non-radical derivatives molecular oxygen like hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and singlet oxygen ($^1O_2$).

The major pathway by which superoxide is formed in most organisms is by incomplete reduction of oxygen with only one electron. This happens especially in the electron transport chain since mitochondria is the place where most oxygen is consumed. In the mitochondrial electron transport chain, the electrons are transported through a series of protein complexes (described above) to a terminal electron acceptor (described above) which creates a proton motive force to produce ATP. These electrons sometimes prematurely leak directly to O_2 to form the superoxide radical. Although ROS are well described messengers of cellular processes, at high concentrations they can be harmful to the organism. It is for this reason that the levels of ROS have to be tightly regulated. A cell is said to be in a state of oxidative stress when the amount of ROS exceeds the defense mechanisms. Scavenging or detoxification of excessive ROS is achieved with the help of antioxidant defense system comprising of antioxidant enzymes like superoxide dismutases (SOD1 and SOD2), catalases, Glutathione peroxidases (GPx), glutathione reductase, glucose-6-phosphate dehydrogenase, thioredoxin reductase, peroxiredoxins and non-enzymatic anti-oxidants like Vitamins A,C and E, glutathione, α lipoic acis, mixed carotenoids, antioxidant minerals (Cu, Zn, Mn) and other cofactors (Maritim AC et al. 2002).

2.6 Types of ROS

Superoxide (O_2^-) is the primary ROS formed in the cell in the process involving stepwise reduction of O_2. O_2^- can initiate a series of reactions to generate secondary ROS mainly through enzyme or metals-catalyzed processes (Halliwell B et al. 1984; Valko M et al. 2005). It is formed by the addition of an electron to molecular oxygen. O_2^- has both oxidizing and reducing properties and has been shown to oxidize enzymes containing [4Fe-4S] clusters and also reduce cytochrome C (Imlay JA. 2003). Two O_2^- molecules get dismutated rapidly to form hydrogen peroxide (H_2O_2) and this reaction is accelerated by the enzyme superoxide dismutase (SOD). O_2^- although being a free radical is not highly reactive and is not able to penetrate lipid molecules. Hence O_2^- is usually present in the same cellular compartment where they are generated.

Hydrogen peroxide (H_2O_2) is moderately reactive and is generated in the cell during normal metabolism. H_2O_2 has no unpaired electrons (is not a free radical) and is readily able to cross biological membranes and can consequently cause oxidative damage to sites far from its site of production. Although H_2O_2 performs an important role as signaling molecule in intracellular processes (Rhee SG. 1999), it also plays a role as an intermediate in the formation of more reactive ROS including HOCl and $^{\bullet}OH$ via oxidation of transition metals. The H_2O_2 formed is scavenged by three different enzyme systems namely catalases, glutathione peroxidases and peroxiredoxins (Chae HZ et al. 1999 ; Mates JM et al. 1999). The formation of $^{\bullet}OH$ is dependent on O_2^- and H_2O_2 and is therefore indirectly dependent on the antioxidant enzymes scavenging them. $^{\bullet}OH$ is generated in a reaction called the Haber-Weiss reaction. It consists of the following reactions.

$H2O2 + Cu^{+}/Fe^{2+} \rightarrow {}^{-}OH + {}^{\bullet}OH + Cu^{2+} / Fe^{3+}$ (Fenton reaction) (Reaction 1)

Superoxide helps in recycling the metal ions through the following reaction.

$Cu^{2+} / Fe^{3+} + O_2^{-} \rightarrow Cu^{+}/Fe^{2+} + O_2$ (Reaction 2)

$^{\bullet}OH$ has a single unpaired electron and is the most reactive among all ROS. $^{\bullet}OH$ can react with all biological molecules and cause oxidative cellular damages like lipid peroxidation, protein damage and DNA damage. Transition metals may be released from sources like ferritin, Fe-S clusters in enzymes on interaction with O_2^{-} (Fridovich I. 1997).

Although the exact mechanism of production and regulation of ROS are not fully understood, in the absence of pathology and other external oxidative stress, ROS are mainly produced in the mitochondrial ETC in both complex I and III (Muller FL et al. 2004) and are considered by many as directly responsible or implicated in aging, diseases like cancer, diabetes and other degenerative diseases. ROS can damage cellular macromolecules like lipids, proteins and DNA. Lipid peroxidation is the major oxidative damage process in tissues mainly due to the high sensitivity of polyunsaturated fatty acids (PUFA) to ROS and the sensitivity increases exponentially as a function of the number of double bonds per fatty acid molecule (Barja G. 2004). Final products of lipid peroxidation (eg. malondialdehyde) and other secondary radicals generated also cause damage to tissue proteins and DNA (Pamplona R et al. 2004).

2.7 Enzymatic antioxidants

The presence of enzymes in living organisms which can decompose oxygen radicals led to the consideration of the role and importance of free radicals in biology. There are three major classes of antioxidative enzymes namely Superoxide dismutases, catalases and glutathione (GSH) peroxidases (GPX) and peroxiredoxins. Some of the non-enzymatic antioxidants are ascorbic acid (Vitamin C), α-tocopherol (vitamin E), glutathione, β carotene and vitamin A. Many other enzymes also perform roles as antioxidants by indirect means. They perform by backing up the primary antioxidative enzymes, for example the enzyme glutathione reductase replenishes GSH from glutathione-disulphide (GSSG). Some enzymes also help in the transport of reactive compounds, e.g. the glutathione S-transferases.

2.7.1 Superoxide dismutase

SODs were the first genuine ROS metabolizing enzymes to be discovered (McCord JM et al. 1969) and are the primary source of defense against superoxide. This discovery led to the postulation of the superoxide theory of oxygen toxicity and that free radicals are important metabolic products. They catalyze the reaction in which two superoxide mol-

ecules are dismutated to hydrogen peroxide and molecular oxygen (Reaction 3) and is thereby a source of hydrogen peroxide in the cell. H2O2 formed from this reaction can be detoxified by catalase or GPx system.

$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ (Reaction 3)

The enzyme is present in most of the subcellular compartments where superoxides are generated. Four classes of SODs were found to contain either a dinuclear Cu/Zn or mononuclear Fe, Mn or Ni at their active sites. Three isozymes of SODs found in humans are Copper/Zinc SOD (Cu/Zn SOD), Mangenese SOD (Mn SOD or SOD2) and an extracellular SOD (SOD3). These enzymes catalyze the dismutation by successive oxidation and reduction of the transition metals ions at its active site with a high reaction rate (Hsieh Y et al. 1998). Mn SOD or SOD2 is a homotetramer with a molecular weight of 96 kDa containing of Mn atom per subunit which cycles from Mn(III) to Mn(II) and back to Mn(III) in a two-step dismutation reaction of superoxide. As the electron transport chain is the major producer of oxygen radical, the nuclear encoded SOD2 which is localized mainly in the mitochondria is the primary antioxidant enzyme that functions to remove this superoxide radical. Cu/Zn SOD or SOD1 is made up of two identical subunits of molecular weight 32kDa each containing a metal cluster and the active site is constituted by Cu and Zn atoms bridged by a common His 61 ligand (Banci L et al. 1998). Extracellular SODs (EC-SOD) are tetrameric, Cu/Zn containing mainly found in interstitial spaces of tissues and extracellular fluids (Marklund S. 1980).

2.7.2 Catalase

They are tetrameric enzymes containing four identical subunits which are tetrahedrally arranged and each subunit containing a ferriprotoporphyrin group. It has a molecular mass of 240 kDa. It functions efficiently in detoxifying H2O2 to water and oxygen as seen from the Reaction 4.

$2\ H_2O_2 \rightarrow H_2O + O_2$ (Reaction 4)

Catalase is predominantly located in peroxisomes in mammalian cells. It also reduces the risk of hydroxyl radical formation through the Fenton reaction. In animals hydrogen peroxide is detoxified by catalase and GPX. Catalase protects the cell from hydrogen peroxide produced within them. Even though catalase is not essential for the survival, it plays an important role in acquisition of tolerance to oxidative stress (Hunt C et al. 1998).

2.7.3 Glutathione peroxidase and glutathione reductase

The selenium containing glutathione peroxidase is comprised of four identical subunits each containing a selenocysteine (Sec) residue which is essential for its functioning. The

80kDa enzyme catalyzes the reduction of hydrogen peroxide to water by using reduced glutathione (GSH) as the source of hydrogen (Reaction 5).

ROOH + 2 GSH → ROH + GSSH + H2O (Reaction 5)

According to Yan and Harding, 1997 the glutathione redox system is the major source of protection against low levels of oxidative stress under normal physiological conditions whereas the catalase has a significant role in protection against severe oxidative stress (Figure 2.2). The glutathione disulphide that is formed during the detoxification of hydroperoxides or hydrogenperoxide is recycled back to glutathione by the enzyme glutathione reductase using the cofactor NADPH generated by glucose 6-phosphate dehydrogenase (Maritim AC et al. 2003).

2.7.4 Peroxiredoxins (Prxs)

Peroxiredoxin is a family of peroxidases that are present in organisms from all kingdoms. It exists in multiples isoforms. It was shown that *Drosophila Melanogaster* and Humans have five and six isoforms of this protein respectively (Rhee SG et al. 2001). Prxs contain a conserved cysteine residue in the N-H_2-terminal portion of the molecule and mostly also containing a conserved Cysteine residue at the COOH-terminal region. Prxs use redox-active cysteins to reduce peroxides by a two-step reaction. On reaction with H_2O_2, the cysteine residue is oxidized to Cys-SOH which then reacts with Cys-SH of the other subunit to form an intermolecular disulfide .This disulfide is reduced specifically by thioredoxin and the reduced thioredoxin is regenerated by thioredoxin reduxtase (Trx) using NADPH (Rhee SG et al. 2001; Seo MS et al. 2000).

2.8 Non-enzymtic antioxidants

Endogenous non-enzymatic molecules also play a role in maintaining oxidative stress homeostasis. These molecules get oxidized after reacting with ROS and have to be reduced back to regain their antioxidant capacity. Glutathione (GSH) and ascorbate are the main non-enzymatic molecules that operate in the hydrophilic compartment. The reduced thiol group of cysteine residue of GSH is important for its antioxidant activity. It can directly react with ROS and also act as a co-substrate of GPX enzymes. Ascorbate is the next most abundant non-enzymatic antioxidant next to GSH. After its reaction with ROS, oxidized ascorbate has to be reduced back through the NADPH-GSH or NADH-dependent reductases. The protein thioredoxin plays a protective role against oxidative stress by scavenging ROS in co-operation with peroxiredoxin/thioredoxin-dependent peroxidase (Mitsui A et al. 2002). Tocopherols and carotenoids act inside lipophilic environments. Tocopherol vitamin E inhibits the propagation of lipid peroxidation by reducing lipid peroxyl groups to hydroperoxides. Coenzyme Q is an important carotenoid which is present in all cellular membranes and functions by scavenging lipid

radicals. The coenzyme Q is regenerated by reductase enzymes using NADPH/NADH as cofactors (Sanz A et al. 2006).

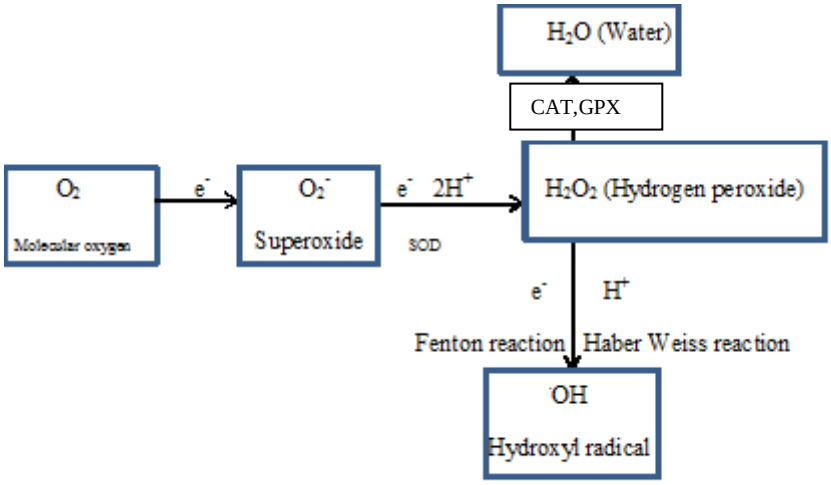


Figure 2.2: *Schematic representation of generation of reactive oxygen species (ROS) by stepwise monovalent reduction of O_2 leading to the formation of O_2^-, H_2O_2 and $^{\cdot}OH$. O_2^- is dismutated to H_2O_2 and H_2O_2 is converted to H_2O by catalase and GPX (adopted from Scialo F et al. 2012).*

2.9 Sites and topology of ROS production

Although free radicals can be generated from different cellular sites, in healthy tissues the main source of free radicals is the mitochondrial electron transport chain. To understand the role of Mitochondrial ROS in ageing and related diseases it is important to understand the mechanism of its production. The primary product of electron leakage is superoxide which then gives rise to Hydrogen peroxide, Hydroxyl radicals etc.

In 2003 a study by Satomi Miwa and others in flies reported that the leakage usually occurs at complex I where the superoxide formed gets passed to the inner matrix, at complex III where the superoxide formed gets dissipated to both side of the mitochondrial inner membrane and by the enzyme *sn*-glycerol 3-phosphate dehydrogenase which produces superoxide mainly in the cytosolic side of the membrane (Miwa S et al.2003). In this study, in isolated mitochondria, the capacities of Complex I and Complex III of the Electron Transport chain (ETC) to generate superoxide was determined by using pyruvate+proline and glycerol 3 phosphate respectively as substrates. Inhibitors of complex I (rotenone), and complex III (antimycin A and myxothiazol for center i and o respectively) were used in combination to study the direction (forward or reverse electron flow) and specific sites of ROS production. The amount of superoxide radical produced from the electron transport chain depends on various factors like the protonmotive force, local oxygen concentration and the reduced state of ETC. It is also sug-

gested that 0.1- 4 % of electrons that are passed down the electron transport chain leak to form superoxide *in vivo*.

2.10 Targets of ROS

The ROS produced can attack many cellular macromolecules like proteins, lipids and DNA. Damaging these cellular molecules can result in changes in membrane properties, ion transport, enzyme activity, protein cross-linking, inhibition of protein synthesis and DNA damage which could result in many consequences leading to diseases and ultimately cell death (Figure 2.3).

2.10.1 Lipids

Increase in ROS levels can lead to enhanced lipid peroxidation thus affecting the cell's normal functioning. Lipid peroxide aggravates the oxidative stress through the production of lipid-derived radicals which can in turn react with proteins and DNA and cause damage. The polyunsaturated fatty acids (PUFAs) are particularly sensitive to ROS damage. The lipid peroxidation reaction can be started by a hydroxyl radical or singlet oxygen. The superoxide and hydrogen peroxide do not have enough reactivity to cause oxidative damage to lipid membranes but they can react with transition metals producing hydroxyl radicals which can cause lipid peroxidation, protein carbonylation and oxidation of DNA and RNA. The lipid peroxidation reaction involves three steps namely initiation, propagation and finalization. Initiation starts with the removal of an electron adjacent to a double bond which results in an alkyl radical. A conjugated diene is formed when the alkyl radical formed in stabilized by rearrangement of it electrons. The alkyl radicals produced cause inter and intramolecular cross linking disrupting the lipid membranes. The second stage of lipid peroxidation has the form of a chain reaction where the alkyl radical frequently reacts with oxygen generating lipid hydroperoxides. The electrons needed for this reaction are obtained from adjacent fatty acids resulting in the formation of further alkyl radicals. The third stage or finalization involves fragmentation of hydroperoxides into products like malondialdehyde (MDA) or 4-hydroxynonenal which can react with other molecules resulting in secondary damage to the cell. It has been shown that the degree of fatty acid unsaturation is negatively correlated with maximum longevity (Pamplona R et al. 2002). Also in agreement with this, long-lived mammals and birds have low levels of lipid peroxidation products in mitochondria and tissues (Pamplona R et al. 1999, 2000).

2.10.2 Proteins

Proteins can get modified in a variety of ways during its reaction with ROS. Protein's activity can be modulated by nitrosylation, carbonylation, glutathionylation and disulphide bond formation. They can also undergo conjugation with products of fatty acid peroxidation. Tissues injured due to high levels of ROS contain increased concen-

trations of carbonylated proteins. Thiol groups and sulphur containing groups are susceptible sites of attack by ROS. Proteins which have a transition metal in its catalytic core (for example aconitase) are particularly sensitive to oxidative damage. Oxidation of iron-sulphur centers by O_2^- is irreversible and leads to inactivation of the enzyme. During aging there is an increased amount of oxidized proteins which can also explain the loss in activity of respiratory enzymes such as cytochrome c and ATP synthase. Although all amino acids in proteins can be oxidatively modified, the most common are cysteine, tyrosine and methionine (Dean RT et al, 1997) while methionine being particularly susceptible to oxidation by various reactive intermediates (Stadtman ER, 2003). Lipid peroxidation products can modify amino acids in proteins resulting in the formation of a variety of adducts collectively called advanced lipoxidation end products (ALEs). Reactive carbonyl species are formed during oxidation of carbohydrates and were identified as intermediates in advanced glycoxidation end products in proteins (AGEs) (Thorpe SR and Baynes JW, 2003). These protein-adducts and cross links can contribute to structural and functional deterioration of protein function. In accordance with these findings different studies suggest that the longevity of a species increases with decrease in methionine content in the proteins and glyco and lipoxidation derived protein damage (PamplonaR and Barja G. 2007).

2.10.3 DNA

Although superoxide and hydrogen peroxide do not have enough reactivity to attack the DNA, the hydroxyl radical which is formed when hydrogen peroxide reacts with a transition metal is capable of damaging the DNA. DNA damage could result in changes in the encoded proteins thus resulting in possible malfunctions or even inactivation of the protein. Oxidative attack on DNA can result in oxidation of deoxyribose, strand breakage, removal of nucleotides and a variety of modifications to the bases of nucleotides. DNA bases are oxidatively attacked by addition of $^{\bullet}OH$ to double bonds whereas the sugar is attacked by hydrogen abstraction from deoxyribose. Various products of DNA damage are formed, for example 8-oxo-7,8 dehydro-2'-deoxy guanosine, hydroxymethyl urea, urea, thymine glycol and saturated products of which 8-hydroxyguanine is the most commonly observed product and used in various studies as the biomarker of oxidative damage to DNA. Of all the macromolecular damage caused by ROS, the damage to DNA must be the most important as it may lead to permanent alteration or loss of coded information. The damage to mtDNA is of particular importance due to its close proximity to the source of mtROS production. The steady state level of mtDNA damage depends on the rate of mtROS production (Pamplona R and Barja G. 2007), long lived species having low rates than the short lived species. Also oxidative damage to mtDNA was found to be 5 to 9 times greater than to nDNA in the heart and brain of six mammalian species studied and the MLSP was found to be inversely proportional to 8-oxodG levels of mtDNA (Barja G and Herrero, 2000).

Accumulation of mtDNA mutations like deletions and point mutations occurs during aging. A common mutation which removes a 5 kb region between the ATPase and ND5 genes was described in patients with mitochondrial diseases and was also shown to be present in various types of aged tissues. MtDNA point mutations in aged individuals associated with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke (MELAS) and Myoclonic Epilepsy with Ragged Red Fibres (MERRF) were described and shown to be non-detectable in young individuals (Cortopassi GA and Arnheim N. 1990). These mutations could result in respiratory chain deficiencies. Various mutator mouse models were created to prove the causal role of mitochondria in aging. A mouse model which had the proof reading deficient version of mtDNA polymerase gamma (POLG) was developed and it was found that mice had more mtDNA point mutations and deletions. Interestingly no proof regarding increased oxidative damage was found suggesting that mutations in mtDNA do not increase ROS production (Greaves LC, Turnbull DM. 2009).

The role of mtDNA in the aging process is controversial. It is believed that the oxidative lesions do not result in reduction in lifespan (Van Remmen H et al. 2003) or alteration of mitochondrial function (Stuart JA et al. 2005) due to the fact that these lesions can repaired easily and do not lead to accumulation of mutations (Krishnan KJ et al. 2008). It has been suggested that the pattern of mutation found in aging relates to errors made by mt-polymerase gamma during replication and also because levels of mutations in mutator mice are never reached in wild type mice, care has to be taken while interpreting the results. The proportion of deletions in mtDNA are said to be more abundant than point mutations and may have an important role in aging. Although the role of mtDNA mutations needs to be investigated more an alternative explanation for their role in aging was proposed by Richter (Richter C. 1988) where he proposed that the mitochondrial fragment produced by ROS damage or replication errors would escape mitochondria and accumulate in nDNA and therefore inducing aging.

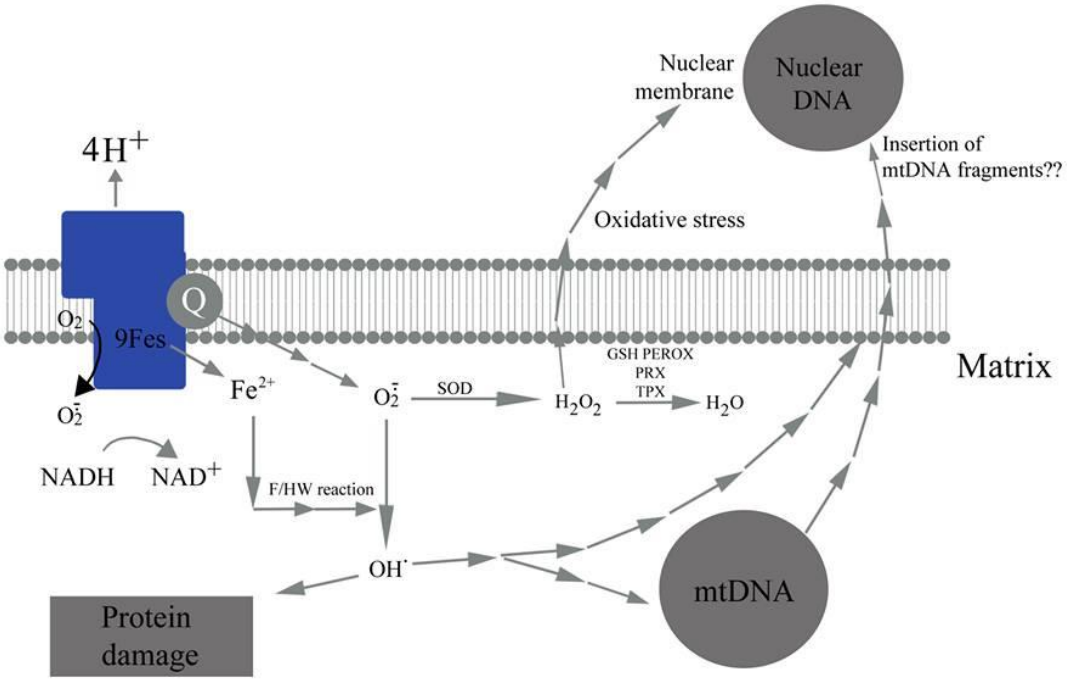


Figure 2.3: *Figure showing superoxide production from complex I dismutated to H_2O_2 and then to H_2O by antioxidant enzymes. The superoxide still manages to attack the Fe-S cluster in complex I releasing iron which inturn can react with superoxide and H_2O_2 to form highly reactive hydroxyl radical. The hydroxyl radical can attack the biomolecules meanwhile H_2O_2 can diffuse far away from mitochondria and potentially cause damage to nuclear DNA on reaction with free iron or copper. F. reaction: Fenton reaction, HW.Reaction: Haber-Weiss reaction (adopted from Scialo F et al. 2012).*

2.11 Validity of MFRTA

The most important prediction of MFRTA is that the individuals producing low levels of mitochondrial free radicals should live longer. It has been found in mammals that the mitochondrial free radical generation is negatively correlated with longevity (Ku HH et al. 1993), the same relationship was reported in different species of flies (Sohal RS et al. 1995). Dietary restriction (DR) have been found to decrease mtROS production (Gredilla R and Barja G. 2005), increase MLS and delay most age related dieases like Alzeimer's, cancer and sarcopenia (Sohal RS and Weindruch R. 1996). Since other parameters like insulin signaling, cellular autophagy are also altered during DR it cannot be established that the increase in lifespan is exclusively due to decrease in mtROS production. The solution to this problem would be to analyze models which have a longer lifespan. There can be three possible ways to extend lifespan 1) by decreasing damage accumulation 2) increase defense and/or 3) increase repair. From an evolutionary point of view, the most effective way to increase lifespan would be by decreasing damage accumulation. In fact it has been found that long lived individuals have biological molecules which are more resistant to molecular damage along with their

mitochondria producing fewer ROS. The susceptibility to get oxidized is mainly associated with chemical composition of the biomolecules and studies have found that methionine residues of proteins (Stadtman ER et al. 2003), highly unsaturated fatty acids (Hulbert AJ et al. 2006) and guanine content in mtDNA (Bjelland S and Seeberg E. 2003) are most susceptible to oxidation by free radicals. In accordance with this, the long lived individuals (mammals and birds) were found to have fewer fatty acid unsaturation, protein methionine and guanine content in mtDNA (Pamplona R and Barja G. 2007). A good way to check MFRTA would be to specifically reduce ROS production. Decrease in mtROS production during DR occurs specifically at complex I (Sanz A et al. 2006) although other studies also support the role of complex III (Chen Y et al. 2012). Due to difficulties with manipulating antioxidant levels, it is necessary to employ a different strategy which requires targeting damage generation and not damage repair.

2.12 Alternative enzymes (Ndi1 and AOX)

Studies in Fungi showed that the expression of an alternative enzyme, the alternative oxidase (AOX) resulted in a reduction of ROS production and an increase in lifespan. Our group along with other groups managed to express the alternative enzymes NADH dehydrogenase internal 1 (NDI1) and alternative oxidase (AOX) in *Drosophila melanogaster* (Sanz A et al. 2010(a, b), Bahadorani S et al. 2010, Fernandez-Ayala DJ et al. 2009). It was believed that these alternative enzymes played a role in maintaining mitochondrial redox balance and sustain mitochondrial metabolism via the TCA cycle and its associated pathways when the OXPHOS system is inhibited (Rustin P and Queiroz C.1985). The expression of AOX resulted in reduced production of ROS in isolated mitochondria but interestingly failed to increase the lifespan in *Drosophila melanogaster* (Sanz A et al. 2010a). Expression of NDI1 in Drosophila melanogaster on the other hand increases lifespan and also mtROS production was reduced in old flies but not in young flies (Figure2.6) (Sanz A et al. 2010b). The enzyme AOX is located downstream of ubiquinone, passes the electrons directly to molecular oxygen, reducing it to water and thus bypassing complexes III and IV . It was found that flies expressing AOX were resistant to antimycin and cyanide which are inhibitors of complex III and IV respectively. NDI1 is located upstream of ubiquinone and serves as a non-proton translocating alternative enzyme which can replace complex I (Figure2.4). It was found to increase substrate oxidation when provided with complex I substrates (Bahadorani S et al. 2010) , rescue lethality caused by complex I knockdown and have resistance to complex I toxin rotenone (Sanz et al. 2010b). The location of these alternative enzymes suggest that ROS production by complex I maybe be mechanistically linked to aging (Stefanatos R and Sanz A. 2011).

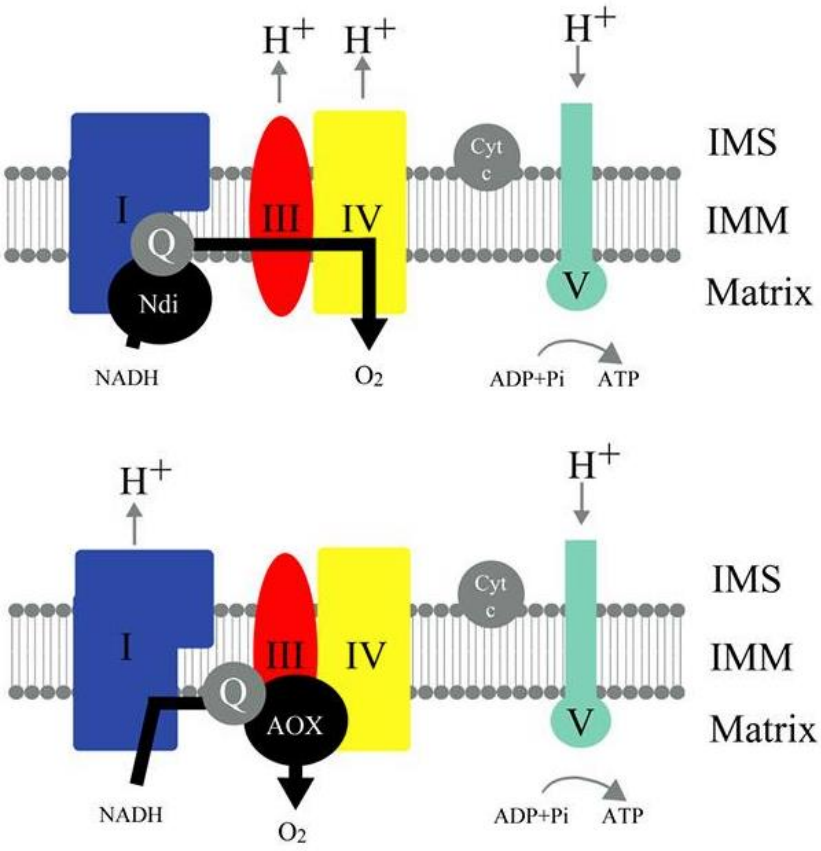


Figure 2.4. *Figure showing the respiratory chain along with the alternative enzymes which were expressed. The enzymes NDI1 and AOX are located upstream and downstream of Ubiquinone respectively. Continuous and dotted lines represent electron and proton flow respectively. Abbreviations: I-V, Complexes I-V ; AOX, Alternative oxidase; NDI1, NADH dehydrogenase internal 1; Q, Ubiquinone (adopted from Rustin P, Jacobs HT. 2009).*

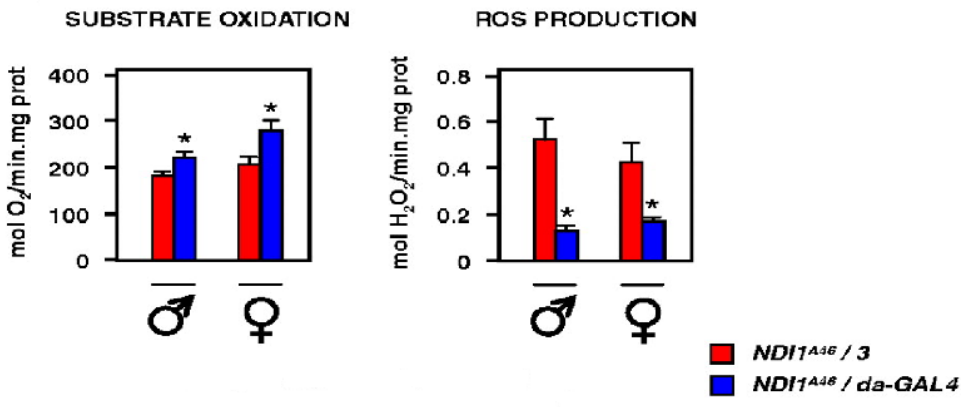


Figure 2.5. *NDI1 expression reduces ROS production (pyruvate + proline substrate mix) and increases respiration (pyruvate + proline + sn-glycerol-3-phosphate, state 3). * indicates a statistically significantly difference between different experimental groups (reproduced from Sanz A et al. 2010b).*

2.13 SOD2 knockdown

SOD2 is vital for life in an oxygen rich environment. As the mitochondrial respiration is the main source of ROS in a cell, the mitochondrially localized SOD (SOD2) was thought to have an important role in cell's defense against oxidative stress. Knockout models of this enzyme have been shown to produce organisms which have cardiac and neurological defects leading to a shortened lifespan in both *Drosophila* and mouse (Duttaroy A et al. 2003; Li Y et al. 1995). A study by Li Y et al. showed that knockout mice developed dilated cardiomyopathy, metabolic acidosis, lipid accumulation in skeletal muscle and liver and a shortened lifespan of 10 days. Knockdown of SOD2 in Drosophila results in oxidative damage in brain and increased neuronal death (Paul A et al. 2007) and also indicate that a loss of SOD2 activity results in early adult-onset mortality (Kirby K et al. 2002). Also *Drosophila* SOD2 null mutants were reported to have functional deterioration in an accelerated manner and proposed that this model could be of particular importance to methodologies that aim at reversing the damages caused by oxidative stress (Nicole Piazza et al. 2009). These findings suggest a role of oxidative stress in determining the lifespan and that reducing the expression of SOD2 would be a good method to study the mechanisms of aging and test other methods that could delay or interfere with aging.

In this study we used *Drosophila melanogaster* as the model organism in which we knocked down mitochondrial superoxide dismutase (SOD2) by RNA interference thereby increasing superoxide levels to study if according to the mitochondrial free radical theory of aging, it would mimic the late-life stages in the organism. As the expression of the alternative enzyme NDI1 (Yeast dehydrogenase) was previously shown to decrease ROS production (Figure 2.5) as well as increase lifespan in Drosophila and AOX (*Ciona intestinalis* Alternative Oxidase) expression was also shown to reduce ROS production but had no effect on lifespan, We co-expressed Ndi1 and AOX (as a control) in our superoxide knockdown model to study the mechanism by which Ndi1 extends lifespan.

2.14 Drosophila as a model organism

In order to understand aspects of human aging simpler and shorter lived organisms can be used as laboratory model organisms. In this study *Drosophila* is used as the model organism. The genome is made up of four chromosomes, 3 sets of autosomes and a pair of sex chromosomes. It has a high sequence similarity with humans and was completely sequenced (Adams MD et al. 2000). Drosophila has a well-differentiated brain, complex behaviors, a heart and Malphigian tubules which are homologues of kidneys and dioecy (males and females). The various genetic tools and methodologies and powerful systems for gene-knockout and targeted mutagenesis makes it an excellent model for this study and many models for human age related diseases have been developed.

The lifecycle of *Drosophila* comprises of different stages namely egg, larval stage and pupal stage which is followed by eclosion and emergence of adult flies. Fertilization of the egg takes place in the oviduct of female fly and undergoes several stages before hatching as larvae. The larvae have three stages of development namely first, second and third instar larvae, the first and second lasting for a day each and the third lasting up to two days. During the third instar larval stage, the larva become mobile and climb up the culture bottle to pupate. The complete metamorphosis of the larva to fly takes place in the pupal stage and the eclosion usually takes place in 9-10 days at 25°C (Ashburner M and Thompson JN. 1978).

The GAL4/ upstream activating sequence (UAS) which is based on the yeast GAL4 transcription factor is a powerful tool for targeted gene expression. It works by activating the transcription of its target genes by binding to UAS cis-regulatory sites (see figure 2.6 for an example). In *Drosophila* the two components of the GAL4/UAS system are maintained in different lines thereby allowing many combinatorial possibilities. The driver line provides tissue specific expression of GAL4 and the responder line carries the gene of interest under the control of UAS (Busson D and Pret AM. 2007).

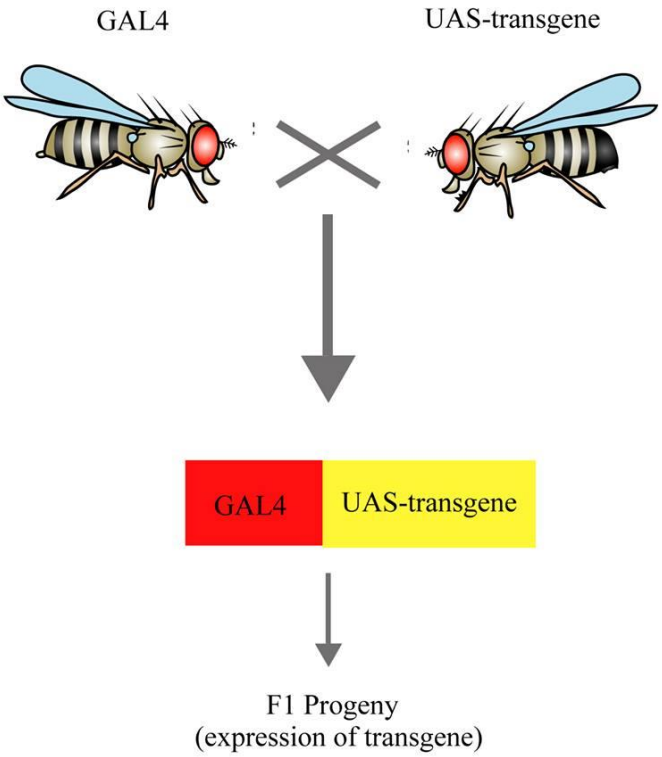


Figure 2.6: *Figure showing an example of how the GAL4/UAS system is used for expression of a transgene. Two transgenic lines are created. The first contains the transgene placed downstream of the UAS activating domain which contains the GAL4 binding sites. Transgenic flies that express GAL4 are created which when crossed with the other line results in a progeny expressing the transgene. In the absence of GAL4 the transgene remains inactive (adopted from Miratul MK et al. 2002).*

3 MATERIALS AND METHODS

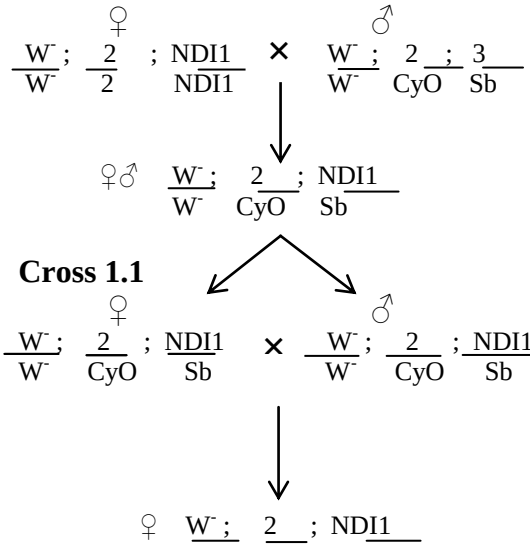
3.1 Fly stocks

A UAS-RNAi line (ID 42162) knocking down the protein coding gene (CG8905) for superoxide dismutase 2 (Mn) was obtained from Vienna Drosophila RNAi Center. The *Drosophila* lines expressing UAS-AOX from *Ciona intestinalis* and UAS-NDI1 from *Saccharomyces cerevisiae* were made as described in (Fernandez-Ayala DJ et al. 2009) and (Sanz A et al. 2010b) respectively. The daughterless-GAL4 (daGAL4 or GAL4) line obtained from Bloomington Fly Stock Center was used to drive the expression of both the SOD2 RNAi knockdown as well as the alternative enzymes (NDI1 or AOX).

The different groups of flies used in subsequent experiments are as follows. 1) SOD2 RNAi line, 2) SOD2 RNAi line + NDI1 , 3) SOD2 RNAi line + AOX expressing flies were obtained by crossing males of the above mentioned groups with virgin females with GAL4. An example scheme of how the group SOD2 RNAi line+NDI1 were obtained is showed in the Figure1. Other groups of experimental flies were obtained using similar schemes. A control group was obtained by crossing males of SOD2 RNAi line with Dahomey virgin flies.

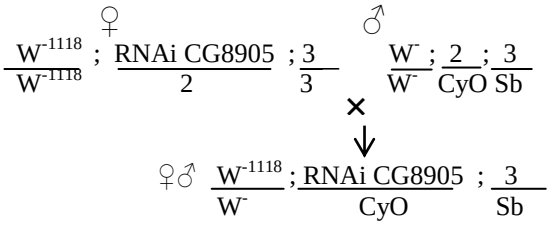
All the crosses made in Figure 1 used virgin females. The progeny from cross 5 was used for subsequent experiments. The flies to be collected in each cross were identified on the basis of eye color and bristle morphology phenotypes. CyO and Sb are markers on balancing chromosomes 2 and 3 respectively. To perform a controlled mating all the flies used in the crosses were removed from the bottles after around 7 days of laying eggs at 18°C. All the flies were raised and maintained in standard media: 1% agar, 1,5% sucrose, 3% glucose, dried yeast, 1,5% maize, 1% wheat, 1% soya, 3% treacle, 0,5% propionic acid, 0,1% Nipagin.

Cross 1.

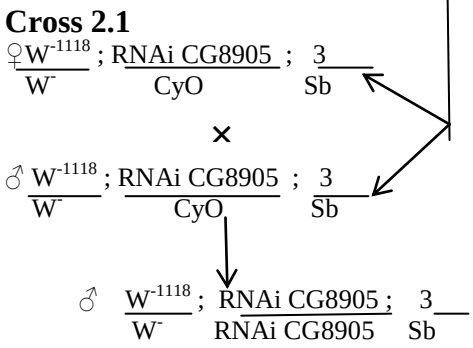


Cross 1.1

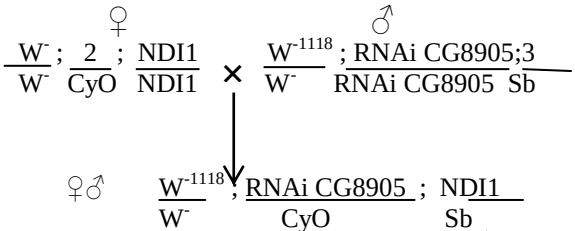
Cross 2.



Cross 2.1



Cross 3.



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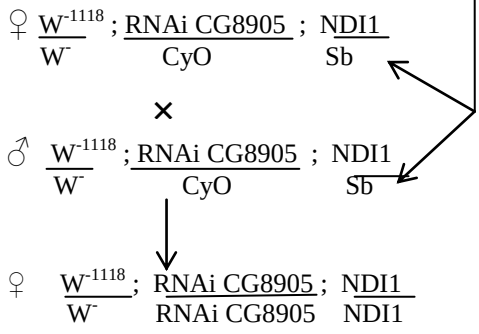


Cross 4.

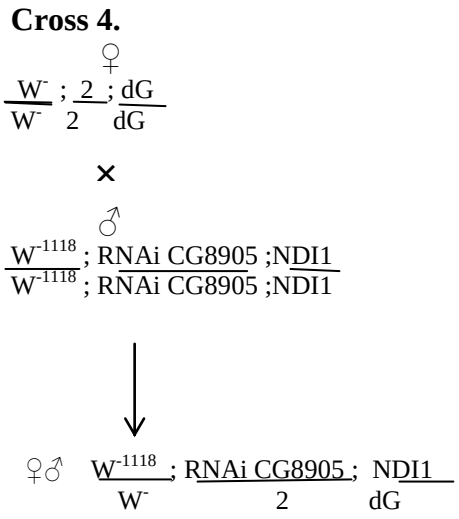


Figure 3.1. *Crossing scheme made to obtain flies expressing SOD2(CG8905) RNAi and NDI1, the expression of which is driven by daughterless GAL4. All the females collected and used for crosses were virgins. 2and 3 denotes wild-type chromosomes 2 and 3 respectively. CyO and Sb are markers on balancing chromosomes 2 and 3 respectively. The flies to be collected in each cross were identified on the basis of eye color and bristle morphology phenotypes. RNAi (CG8905) represents the presence of transgene responsible for RNAi of mitochondrial SOD (SOD2) and NDI1 represents the presence of alternative enzyme NADH dehydrogenase internal1.Similar crosses were made with flies having AOX insert and crossed with RNAi CG8905 flies. RNAi CG8905 expressing flies were made by crossing the product of cross 2.1 with 2>2 ; dG>dG.*

From this point in this report, the different experimental groups will be represented with the following names.

SOD2>W: contains transgene for SOD2 RNAi but no GAL4 driver.

SOD2>GAL4: contains transgene for SOD2 RNAi and GAL4 driver.

SOD2>NDI1: contains transgene for SOD2 RNAi and NDI1 transgene both driven by -GAL4.

SOD2>AOX: contains transgene for SOD2 RNAi and AOX transgene both driven by -daGAL4.

3.2 Western blotting

A total protein extract was first prepared with around 10 flies which were either fresh and stunned on ice or flies which were frozen at -80°C. the flies were homogenized using a sterile plastic homogenizer in homogenizing buffer (0,15 gm of 1,5% Triton X-100, 1 tablet of pre-made complete mini EDTA-free protease inhibitor and 1 tablet of phosphatase inhibitor(Roche Diagnostics, Mannheim, Germany)) dissolved in 1X PBS. The homogenate was incubated on ice for 15 minutes and then centrifuged at 13000g for 15 minutes at 4°C. The total fly extract (supernatant) was then collected in a separate tube.

3.2.1 SDS PAGE

Following are the different buffers that were used

Table3.1. *Table showing the constituents and their concentration of the different buffers used.*

Sample loading buffer 4X	**Running buffer 10X**	**Blotting buffer** (pH around 8.3)
40% glycerol	0,25M Trizma	0,25M Trizma
8% SDS	1,92M Glycine	1,92M Glycine
1M Tris-HCl (pH6.8)	1% SDS	
0.004% Bromophenol blue slurry		
20% Dithiothreitol (DTT)		

1X working solution of running buffer was prepared by diluting it with sterile water and 1X working solution of blotting buffer was prepared by making a solution of 10X blotting buffer, methanol and sterile in the ratio 1:2:7.

The protein concentration of the total protein extract was measured using Bradford assay. The samples were diluted with sterilized water and sample loading buffer so as to get 35 µg of protein in 25 µl of sample which will be loaded to the gel during electrophoresis. The diluted samples were then heated at 100°C using a heat block for 5 minutes and immediately placed on ice.

The electrophoresis tank was filled with running buffer and the ready-made gels from Bio-Rad (Criterion TGX, Marnes-la-Coquette, France) were placed inside the tank. Protein ladder was filled in 3µl and 7µl to each end with samples in between to identify the order of the samples during blotting. The gel was run on a voltage of 80V until the protein from the wells have migrated into the gel and then the voltage was increased to 120V, allowed to run for around 2-3 hours or until the proteins have migrated to the bottom of the gel. The gel was then removed from the electrophoresis chamber and kept soaking in the 1X running buffer.

3.2.2 Blotting

Both dry blotting and wet blotting were used to transfer proteins to a nitrocellulose membrane.

Dry blotting was done using an iBlot dry blotting device (Invitrogen, New York, USA). Recommended kit, iBlot gel transfer stacks were used. The stacks were assembled and blotting was done according to the manufacturer's instructions. After the transfer of proteins, the membrane was transferred to a container with 1X PBS.

Wet blotting was done at 4°C. The blotting buffer working solution was added to the tank and the sandwich for the blotting was assembled and placed. Blotting was done at 40/50mA overnight or 400mA for 1-2 hours. An ice pad was used to cool the blotting buffer by placing it inside the blotting chamber. The sandwich was then unpacked and the membrane was moved to a container with 1X PBS.

3.2.3 Immunodetection

The membrane was then washed with PBS-Tween for few seconds and then stained with Ponceau S (0.1% w/v Ponceau S in 5% v/v acetic acid made up to 1 liter with double distilled water) for few seconds to verify the proper transfer of protein from gel to the membrane. The membrane was then washed with milli-Q water to remove the stain and blocked using 5% milk in PBS-Tween for 2-3 hours in a shaker. Following blocking, primary antibody diluted in 5% Milk in PBS-Tween was applied to the membrane and incubated at 4°C overnight or room temperature for 2 hours. The membrane was then washed with PBS-Tween three times for 10 minutes each and a secondary antibody diluted in 5% Milk in PBS-Tween was applied and incubated at room temperature for 1 hour. The membrane was again washed three times with PBS-Tween for 10 minutes and the membrane was now ready for exposing. It was first treated with substrate solutions Luminol enhancer and Immuno Star HRP peroxide buffer (Bio-Rad, Marnes-la-Coquette, France) in 1:1 ratio for 3-5 minutes. The membrane then was exposed using a Kodak Biomax hypercasette and developed using a AGFA developer (AGFA, Mortel, Belgium). Fuji Medical X-Ray films (FUJIFILM, Tokyo, Japan) were used for developing.

The primary antibodies and their concentrations used for immunodetection are as follows: anti-SOD2, anti-complex I NDUFS3 used at 1:10,000; anti-Complex IV used at 1:10,000 (Anti-COX IV Antibody, Abcam, USA); anti-Complex V Subunit Alpha (Anti-ATP5A antibody (Mitosciences, Oregon, USA)) used at 1:200,000; anti-GAPDH (c Terminus) used at 1:30,000 (Everest Biotech Ltd, Oxforshire, United Kingdom), anti-pyruvate dehydrogenase (PDH) E1α subunit (MitoSciences, Eugene, Oregon, USA) used at 1:10,000.
The secondary antibodies concentrations used for immunodetection were, horse anti-mouse IgG (Vector Labaratories, Burlingame, USA) used at 1:10,000; anti-rabbit (Vector Labaratories, Burlingame, USA) used at 1:10,000 and anti-goat (Santa Cruz Bio-

technologies, Santa Cruz, USA) used at 1:5000. All the dilutions were made with 5% Milk in PBS-Tween.

3.3 Quantitative real time PCR

3.3.1 RNA extraction

A total of 10 flies were anesthetized on ice, placed in a tube and frozen at 80°C. 50 µl of Tri-Reagent (Molecular Research Center Inc, Ohio, USA) was added to the tube and the frozen flies were ground using a plastic homogenizer. Another 450 µl of Tri-Reagent was then added to the tube and the homogenate was incubated at room temperature for 5 minutes. Then 100 µl of pure chloroform was added, samples were vortexed thoroughly and incubated at room temperature for 2-3 minutes. The samples were then centrifuged at 12000g for 15 minutes at 4°C.

The upper aqueous phase obtained after centrifugation was transferred to a new tube and and mixed with 500 µl of isopropanol. RNA was precipitated at room temperature for 10 minutes and pelleted by centrifugation. The pellet was then washed thoroughly in 1 ml of DEPC water with 75% ethanol. Following washing, the mix was vortexed and centrifuged at 7500g for 5 minutes at 4°C. The ethanol mix was then removed by using a 0.5mm needle using suction.

The pellet was air dried for 5-10 minutes at room temperature, re-suspended with 89 µl DEPC treated water and treated with DNaseI and DnaseI buffer (FERMENTAS INC.,Maryland, USA), 1 µl and 10 µl respectively to remove DNA contamination. The samples were then incubated at 37°C for 1 hour.

The RNA was then purified by ethanol precipitation. 1/10 volumes of 3M Sodium Acetate with pH 5.2 and 2.5 volumes of 95% ethanol in DEPC water were added to the samples and precipitated at -20°C for 30 minutes to 1 hour. The samples were then centrifuges at 16000g for 20 minutes at 4°C. The supernatant was removed using a vacuum needle and the pellet was washed twice with 1ml of DEPC-water containing 75% ethanol. The pellet was air dried at room temperature for 5 minutes and re-suspended in 10µl DEPC-water. The concentration of RNA was then measured using Nano-Drop 2000c (Thermo Scientific, Wilmington, USA) and adjusted to 1µg/µl and stored at -80°C for cDNA synthesis.

3.3.2 cDNA synthesis

Triplicates were prepared for each of the cDNA samples prepared and at the end pooled together for complete cDNA synthesis. The final reaction volume for each synthesis reaction was 20µl containing 2µg of RNA and all reactions were performed in a 96 well 0.2ml plate. 2 µl of 1µg/µl RNA samples were added to the corresponding wells con-

taining 9.6µl DEPC water, 1µl of 10mM dNTP and 0.4µl of random hexamers. The mix was then incubated for 3 minutes at 90°C. 4µl of 5X reverse transcriptase buffer and 1 µl of RNase inhibitor were then added to each of the sample wells and then incubated at 25°C for 10 minutes. 2µl of reverse transcriptase was then added to all the sample wells.

The samples were then incubated in a thermocycler in a programmed cycle of 25°C for 10 minutes, 37°C for 60 minutes and 70°C for 10 minutes. The triplicated samples were then pooled together in a single tube and stored at -20°C.

3.3.3 QPCR standard curve method

The primers for the experiment were made using the "primer3" primer design software. A stock was first prepared by pooling 5µl of all the cDNA samples together in order to determine the standard curve and so that the gene amplifications will fall inside the standard curve. 20 µl of the stock was then diluted with 80 µl of nuclease free water (FERMENTAS INC.,Maryland, USA). Serial dilution of 1:10, 1:100 and 1:1000 were then made from this dilution. cDNA samples were diluted 20 times separately and all the standards and samples were then added to the 96 well plates and stored.

Q-RT-PCR was performed by using StepOnePlus Real-time PCR instrument (Applied Biosystems, San Francisco, USA) and the manufacturer's Fast SYBR Green reagents. The reaction volume was 20µl and contained 10µl Fast SYBR green Master mix, 0.4µl of 20µM forward and reverse primers and 5.2µl nuclease free water. Fast Optical 96 well plates were used for this experiment. 4µl of standards and samples were added to the reaction mix in respective wells and mixed thoroughly. The plate was then covered with optical clear cover and spun down. The PCR cycle used was 95°C for 5s, 60°C for10s, 72°C for 5s and the cycle was repeated for 45 times. The amplification values of each gene were normalized with amplification values of the gene GAPDH (CG 12055) for all the experimental samples.

3.4 DNA extraction and Quantification of mtDNA copy number

Total DNA was extracted from 30 females or 40 males as described in Sullivan et al. 2000. DNA pelletes were resuspended in 100 µl of sterilized Milli-Q H_2O and stored at 4 °C.

Quantitative PCR was carried out using QuantiTect SYBR Green PCR Kit (Qiagen). Specific primers were used to amplify a fragment of the 16S rRNA (FBgn0013686) gene of mtDNA and the nuclear single copy gene for ribosomal protein L32 (FBgn0002626). PCR reaction conditions were performed according to standard conditions for SYBR Green PCR (Qiagen) in total volume 20 ul: QuantiTect SYBR Green PCR Master Mix (2x), each of primers 10uM, DNA template 3 ul. PCR assays were

performed in triplicate for each DNA sample. Mitochondrial DNA copy number relative to nuclear DNA was determined by comparing mitochondrial and nuclear gene amplifications.

3.5 Lifespan studies

All the crosses were done with 40 females and 20 males of each group in bottles at 18°C. Flies were collected from the bottles after eclosion using CO_2 anaesthesia and transferred to smaller vials with standard media in counts of 20 flies per vial. Flies were collected every 48 hours to ensure that the flies collected were not virgins and maintained in a controlled 12h light-dark cycle at 25°C. The vials were changed every 2-3 days and the number of dead flies was counted for lifespan studies. Prism GraphPad software was used to construct lifespan curves. At least 100 flies of each group were used for the lifespan experiment.

3.6 Activity of flies

The locomotor activity of flies was measured in a Digitherm CircKinetics monitoring incubator (Tritech Research, Los Angeles, USA) at 25ºC in a controlled 12 hour dark/light cycle. Flies of different age were anesthetized using CO_2, and then put into separate capillaries which had standard fly food (1% agar, 1,5% sucrose, 3% glucose, dried yeast, 1,5% maize, 1% wheat, 1% Soya, 3% treacle, 0,5% propionic acid, 0,1% Nipagin). Only female flies were used for this experiment. The capillary was sealed on one side with a rubber cork and on other side with a sponge so that air can pass through. The activity was monitored using TriKinetics Activity Monitors (Trikinetics Inc., Waltham, USA) wherein the number of times the fly crosses the center of the tube per hour is counted and integrated using Trikinetics software.

3.7 Isolation of Mitochondria

Around 80 flies were used for isolation of mitochondria. The flies were immobilized by placing them on ice and then transferred to a chilled mortar. 1 ml of ice cold mitochondrial isolation medium with BSA (250mM sucrose, 5mM Tris-HCL, 2mM EGTA, 0.1% w/v of BSA) was added and the flies were gently crushed using a pestle. The homogenate was then filtered using a 200 µm polyamide mesh and then another 1 ml of the mitochondrial isolation medium was added through the filter. The homogenate was then transferred to an eppendorf tube and centrifuged at 200g for 5 minutes at 4°C. The supernatant was collected and centrifuged at 9000g for 10 minutes at 4°C. The pellet obtained was resuspended in 50µl of isolation medium without BSA. The protein concentration of the samples was measured using Bradford assay. The isolated mitochondria were either immediately used or stored at -80°C depending on the necessity of the experiments conducted.

3.8 Mitochondrial O_2 consumption

Mitochondria were isolated as described in this section and used immediately for measuring oxygen consumption. High resolution respirometry was used to measure mitochondrial oxygen consumption using oxygrapgh 2-K (Oroboros, Innsbruck, Austria). The chambers were thoroughly cleaned with 70% ethanol and distilled water to remove any contaminants. 0.25-0.5 mg of mitochondria were used in each experiment and incubated in assay buffer (120 mM KCl, 5 mM KH_2PO_4, 3 mM Hepes, 1 mM EGTA, 1 mM $MgCl_2$, 0.2% bovine serum albumin, pH 7.2 at 25 °C). All the complexes in the ETC were considered as canditates for measurement. Complex I respiration was initiated by adding 5µl of 2M pyruvate and 5µl of 2M proline. 30 µl of 1.3M Glycerol-3-Phosphate was added to study the respiration of complex III and IV. 1µl of 1mM rotenone and 1µl of 5mM Antimycin A were used to inhibit complex I and III respectively. 4µl of 0.5M ADP was added to measure the state3 repiration.5µl of Ascorbate (0.8M) and 4µl of TMPD (0.2M) were used as substrate for complex IV and 2µl of 1M Potassium Cyanide was used to inhibit complex IV. Separate HAMILTON GASTIGHT syringes (Hamilton Bonaduz AG, Bonaduz, Switzerland) were used for addition of all substrates and inhibitors to the reaction chamber. Respiration was measured as the stable rate, after the addition of specific substrates and inhibitors.

3.9 Blue Native PAGE and in-gel activity

For performing Blue Native PAGE, mitochondria from 5-7days old flies were isolated by following the previously mentioned protocol. The protein concentration was measured using Bradford assay. The sample was then diluted to contain 100µg of protein in a volume of 40µl using appropriate amounts of 750mM aminocaproic acid in 50mM Bis-Tris, dodecylmaltoside. Then 2.5µl of dodecyl maltoside (10%) was added, mixed well and incubated for 10-15 minutes. After incubation the samples were centrifuged at 12000g for 15 minutes at 4°C. The supernatant was transferred to a different tube and just before adding the samples to the gel 2.5µl of Native PAGE sample buffer (500 mM Aminocaproic acid, 2,5% Serva Blue G made up to 10 ml with 50mM BisTris) was added, mixed well and kept on ice for 5 minutes.

The cathode buffer comprised of 50mM Tricine and 15mM BisTris and the anode buffer comprised of 50mM BisTris. The tank was set up for the procedure and 15 µl or more of the sample and 10µl of standards was added to the wells of readymade NativePAGE Novex 3-12% Bis-Tris gels (Bio-Rad, Marnes-la-Coquette, France). Electrophoresis was performed at 4°C using a limited current of 70V for 1 hour. The blue cathode buffer (0.01% Serva BlueG in cathode buffer) was changed to cathode buffer to visualize the bands in the gel and when the samples have reached the separation gel the voltage was increased to 100V and run for 5 hours.

The staining solution comprised of 50% Methanol, 10% Acetic Acid, 0,05% Serva Blue G and water and the de-staining solution comprised of 50% methanol, 10% acetic acid and water. After the run was completed, the gel was stained for 1 hour to visualize the total protein content and calorimetric activity buffers specific for each complex were used to visualize the activity of the complex. The gel was incubated with activity buffers for a time span which differs for different complex activity buffers used.

The gel was cut to suitable sections and incubated with Complex II activity buffer (4.5 mM EDTA, 10 mM KCN, 0.2 mM phenazine methasulfate, 84 mM succinic acid and 50 mM NTB in 1.5 mM phosphate buffer (pH 7.4)) and Complex IV activity buffer (5 mg 3:30-Diamidobenzidine tetrahydrochloride (DAB) dissolved in 9 ml phosphate buffer (0.05 M, pH 7.4), 1 ml catalase (20 µg/ml), 10 mg cytochrome c, and 750 mg sucrose). The gels were then fixed with de-staining solution for 20 minutes, washed with distilled water and scanned.

3.10 Statistics analysis

All data were analyzed using GraphPad Prism 4. The level of significance of all the data were evaluated by one way ANOVA, with $p<0.05$ as the accepted level for statistical significance. Lifespan data were analyzed using the Kaplan Meier Log-Rank Test. Newman-Keuls Multiple Comparison Test was used to compare different experimental groups.

4 RESULTS

We verified the expression of SOD2 RNAi in the different groups of flies at mRNA and protein level. The expression of the alternative enzymes AOX and NDi1 was shown previously (Sanz A et al. 2010a,b). Then the lifespan and activity of the flies were monitored. Simultaneously the mitochondrial respiration with different substrate mix, the mitochondrial respiratory complex assembly and its functioning were also analysed. Finally we also checked if the increase in oxidative stress affects mtDNA copy number and mRNA levels of specific subunits of respiratory complexes (including mitochondrial and nuclear subunits).

4.1 Verification of SOD2 knockdown at protein and mRNA level

Verification of the knockdown at mRNA level was done by Q-RT-PCR using the four experimental groups as described in the section fly stocks. The expression levels of SOD2 for the different experimental groups were normalised with the house-keeping gene GAPDH.

The results (Figure 4.1A) show that there is a clear down regulation of the enzyme SOD2 in the groups expressing RNA interference. A significant difference is seen between the non-expressing group and the groups expressing the RNAi, construct alone or with NDI1/AOX.

Verification of knockdown at protein level was done by western blotting.The different groups used here are SOD2 RNAi non-expressing group, SOD2 RNAi expressing group, SOD2 expressing + Ndi1/ AOX as described in the section Fly stocks. Another control group of flies expressing only GAL4 line was used in this experiment. Protein extract of both sexes of flies after eclosion was probed with a specific SOD2 antibody and the result as seen in the Figure 4.1B) shows that the groups not expressing SOD2RNAi and containing only daGAL4 shows a strong band as opposed to the rest of the groups expressing SOD2RNAi which only shows a faint band or no band.

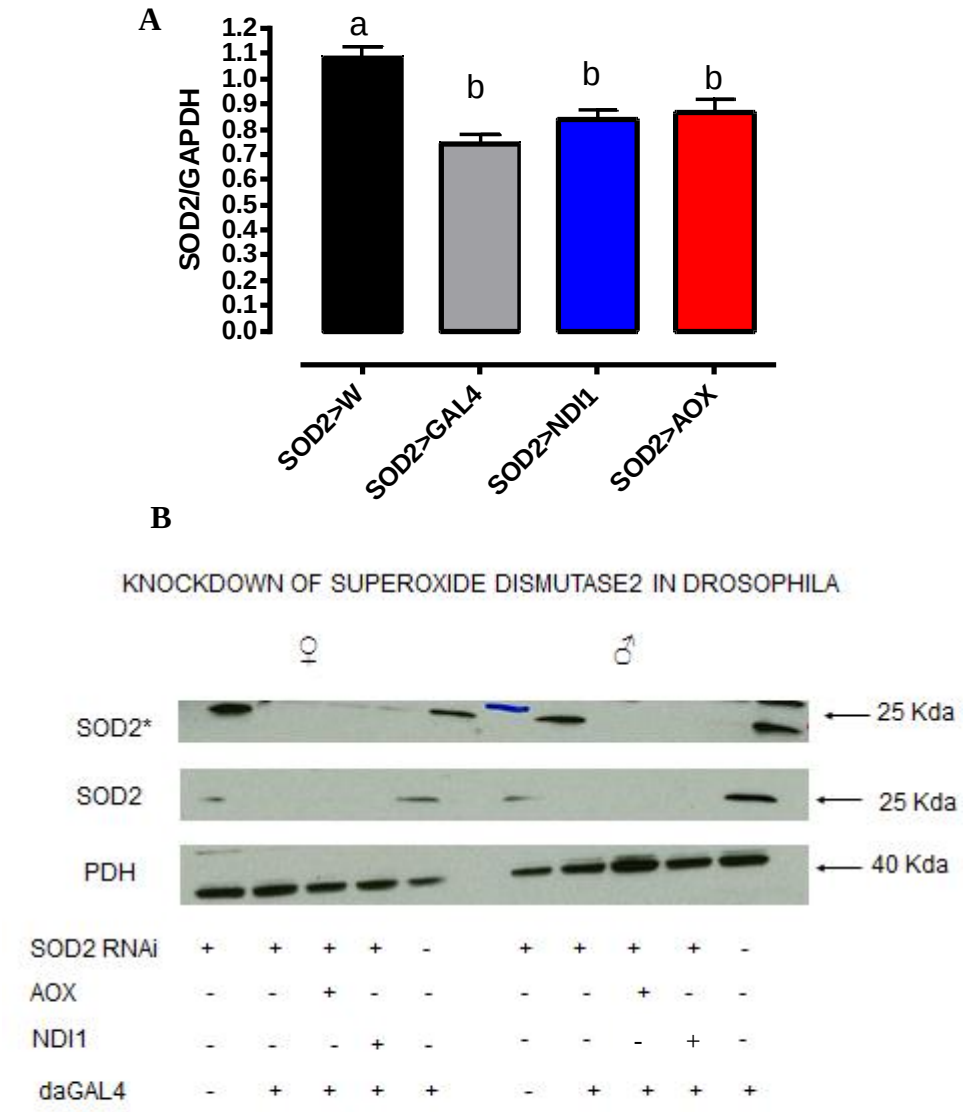
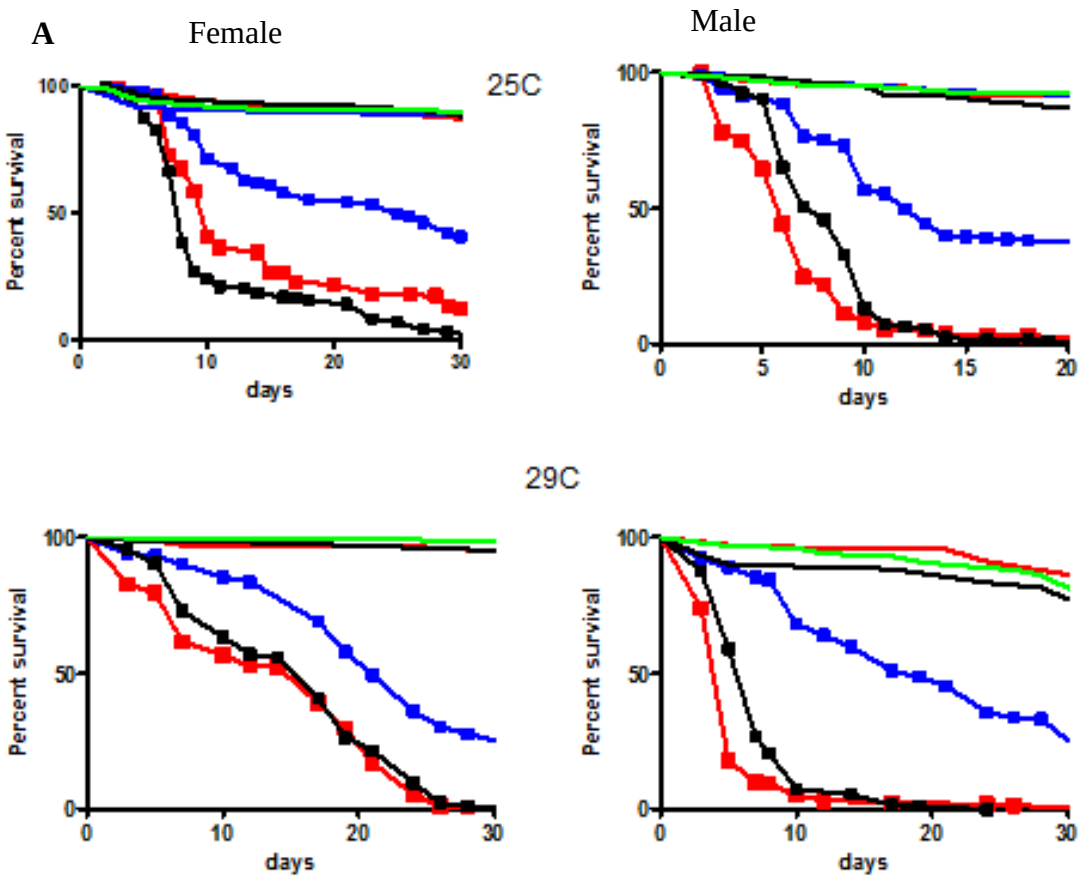


Figure 4.1: *Verification of Knockdown of SOD2. (A) Verification of Knockdown of SOD2 at RNA level for different groups in the following order, SOD2RNAi non-expressing group (SOD2>W), SOD2 RNAi expressing group (SOD2>GAL4), SOD2 RNAi+NDI1 expressing group (SOD2>NDI1) and SOD2 RNAi+AOX expressing group (SOD2>AOX) by Q-RT-PCR normalized with control RNA, GAPDH. a and b represent statistical difference between groups (mean±SEM, p< 0.001, n=3-4 for each group). (B) Western blot with different groups probed with antibodies against SOD2 and PDH. + and – represent the presence or absence of SOD2 RNAi, AOX, NDI1 transgene and GAL4 driver in the flies. The knockdown can be seen from the presence of a faint or no bands in the SOD2 RNAi expressing groups*

4.2 Ndl 1 partially rescues lifespan and activity of the flies

Lifespan studies were carried out for both sexes of flies at 25°C and 29°C. The four groups used in the previous experiments along with flies with only GAL4 and flies containing SOD2>AOX with GAL4 absent was used as additional control. The Prism Graphpad software was used to construct the survival curves. It can be seen from the Figure 4.2A that the groups expressing the RNAi have a significantly reduced lifespan compared to the control (non-expressing) groups. It can also be seen that NDI1 expression extends the lifespan of both males and females at both temperatures.

Measurement of locomotor activity of the flies was performed at different ages. No significant difference was seen at day 3 of lifespan. At day 6 a significant decrease in activity of the group expressing the SOD2 RNAi without any of the alternative enzymes was observed. The groups expressing SOD2 RNAi and alternative enzymes NDI1 and AOX show a significantly increased activity at day 6. At day 8 all groups expressing the RNAi and RNAi+ NDI1/AOX show a significantly decreased locomotor activity as compared to the control group SOD2>W (Figure4.2B).



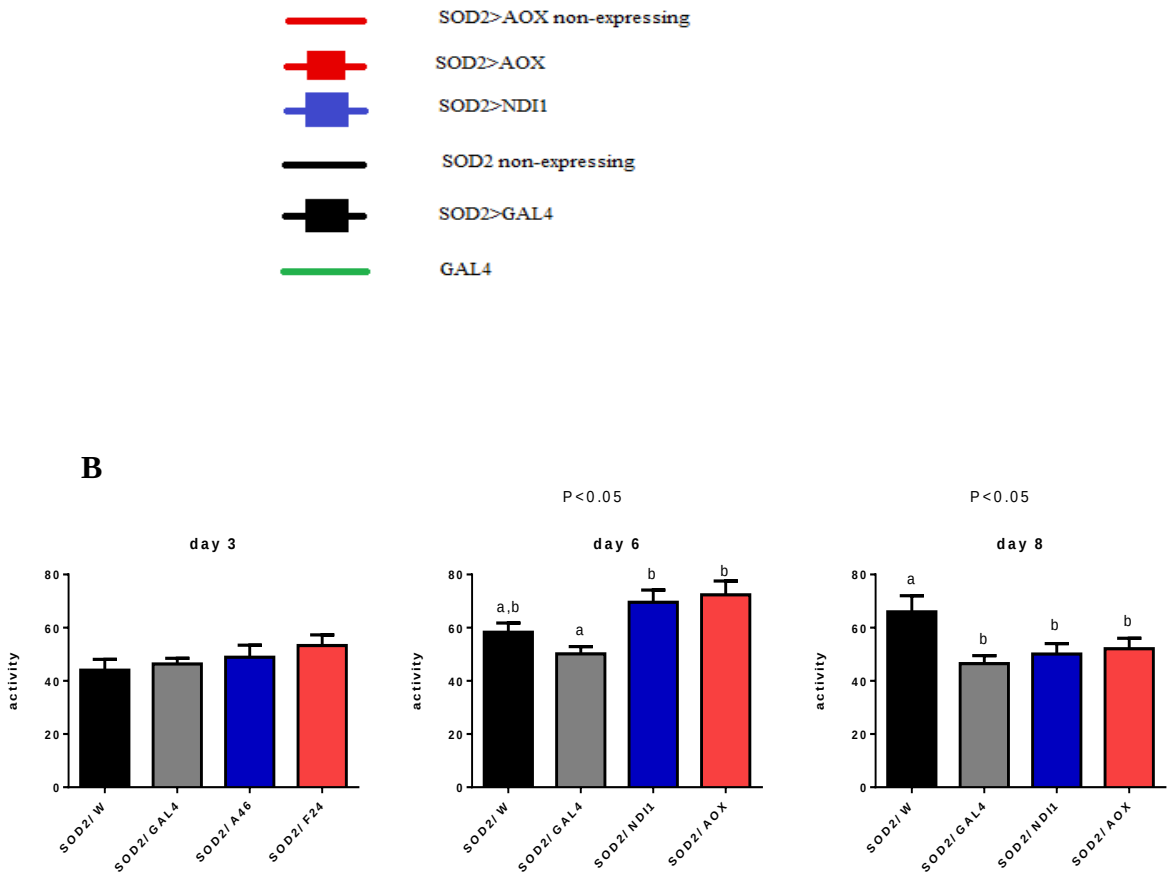


Figure 4.2: *NdI 1 partially rescues lifespan and activity of the flies. (A) Survival curves of different groups, flies with only the GAL4 construct, SOD2RNAi non-expressing group, SOD2 RNAi expressing group, SOD2 RNAi+NDI1 expressing group, SOD2 RNAi+AOX expressing group, SOD2 RNAi+AOX non-expressing group. f,m represent female and male flies respectively. Flies expressing RNAi have a shortened lifespan and NDI1 co-expression partially rescues it. (B) Activity of different groups mentioned measured using a 24 hours activity monitor at 25 ºC in a controlled 12h dark/light cycle. NDI1 co-expression partially increases the activity of the flies compared to the SOD2RNAi expressing flies. a and b represent statistical difference between groups(mean±SEM, p< 0.05, n= at least 25 for each group).*

4.3 Increase in superoxide levels decreases complex I-linked respiration. By-pass of complex I by NDI1 partially rescues this phenotype.

Mitochondrial respiration was measured using isolated mitochondria for the different groups as described. After the addition of assay medium and mitochondria, the following substrates and inhibitors were added in the following order and measurements were taken after each substrate or inhibitor was added. First pyruvate+ proline was added followed by ADP. Rotenone was added followed by Glycerol3-phosphate and then ADP. Antimycin was added next. TMPD was added next followed by ADP.

The measurement for all groups after the addition of each substrate is shown in Table 4.1. From the figure 4.3A, it can be observed that mitochondrial respiration using complex I substrates during state 3 is significantly decreased in all the groups expressing SOD2RNAi. By-pass of respiratory complex I using NDI partially rescues or improves the respiration when complex I substrates were used. No difference in mitochondrial respiration was seen between the groups when complex III substrates (G3P+ADP) were added (Figure 4.3B). Also when TMPD is added, no difference in state 3 respiration was seen between the different groups.

Table 4.1: Mitochondrial oxygen consumption (pico moles of O2/ mg mt protein)

Substrate **A**	**SOD2/ W-**	**SOD2/ GAL4**	**B** **SOD2/ NDI1**	**SOD2/ AOX**
Pyruvate+Proline (PP)	614 ± 94	466 ± 110	509 ± 69	582 ± 139
Pyruvate+Proline (PP)+ ADP	19195 ± 1793	9127 ± 1560	14196 ± 1481	10166 ± 1592
RCR	32.5 ± 2.2	27.5 ± 3.0	28.4 ± 2.1	19.3 ± 2.9
Substrate	**SOD2/ W-**	**SOD2/ GAL4**	**SOD2/ NDI1**	**SOD2/ AOX**
Glycerol 3 phosphate (Sn3gp)	2237 ± 372	2262 ± 183	2103 ± 386	3877 ± 494
Glycerol 3 phosphate (Sn3gp)+ADP	9557 ± 1705	8815 ± 917	8571 ± 1583	9005 ± 1069
RCR	4.3 ± 0.2	3.9 ± 0.3	4.3 ± 0.5	2.4 ± 0.2
TMPD	11396 ± 831	10529 ± 515	11334 ± 1085	10741 ± 962
TMPD+ADP	18729 ± 1480	16003 ± 887	19985 ± 1334	17280 ± 2013

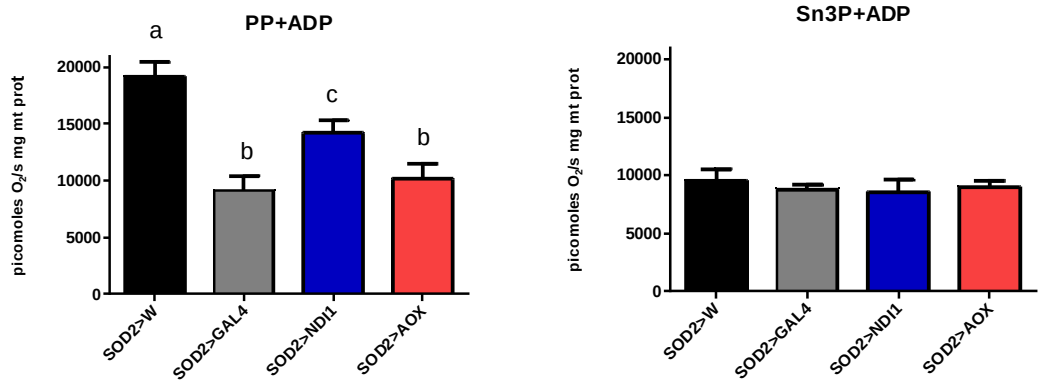


Figure 4.3: *NDI1 partially rescues respiration. Oxygen consumption using isolated mitochondria at state3 measured in different groups in the following order. SOD2RNAi non-expressing group (SOD2>W), SOD2 RNAi expressing group (SOD2>GAL4), SOD2 RNAi+NDI1 expressing group (SOD2>NDI1) and SOD2 RNAi+AOX expressing group (SOD2>AOX) using A) complex I and B) complex III linked substrates. The level of Oxygen consumption at complex I of all RNAi expressing groups are significantly reduced and NDI1 co-expression partially improves the respiration at complex I. Co-expression of AOX does not improve the oxygen consumption. Letters a and b represent significant difference between the groups(mean±SEM, p< 0.05).*

4.4 Increased mitochondrial oxidative stress does not affect the mitochondrial copy number and mRNA levels.

To measure the mtDNA copy number, whole DNA of the four groups of flies was extracted and copy number was measured by real time qPCR using primers for complex I subunit ND1 and GAPDH. The values obtained were normalized to the values of the control group SOD2/W (non-expressing group). It can be seen from the figure 4.4A that the mitochondrial copy number of the test samples SOD2/GAL4 and SOD2/NDI1 were not altered as compared to the control group SOD2/W. But the mtDNA copy number of the group SOD2/AOX was significantly reduced.

The expression levels of different genes were measured using Q-RT PCR. The mRNA levels of nuclear encoded genes (Succinate dehydrogenase (Succ dH) and complex I subunit (39kd)) and mitochondrial encoded genes (cyt B, COX1, ATP6 and 16S) were measured relative to the house-keeping gene GAPDH. The values obtained were then normalized to the values of the control group SOD2/W. It can be seen from the figure 4.4 B that the mRNA levels were not affected or reduced in any of the groups mentioned.

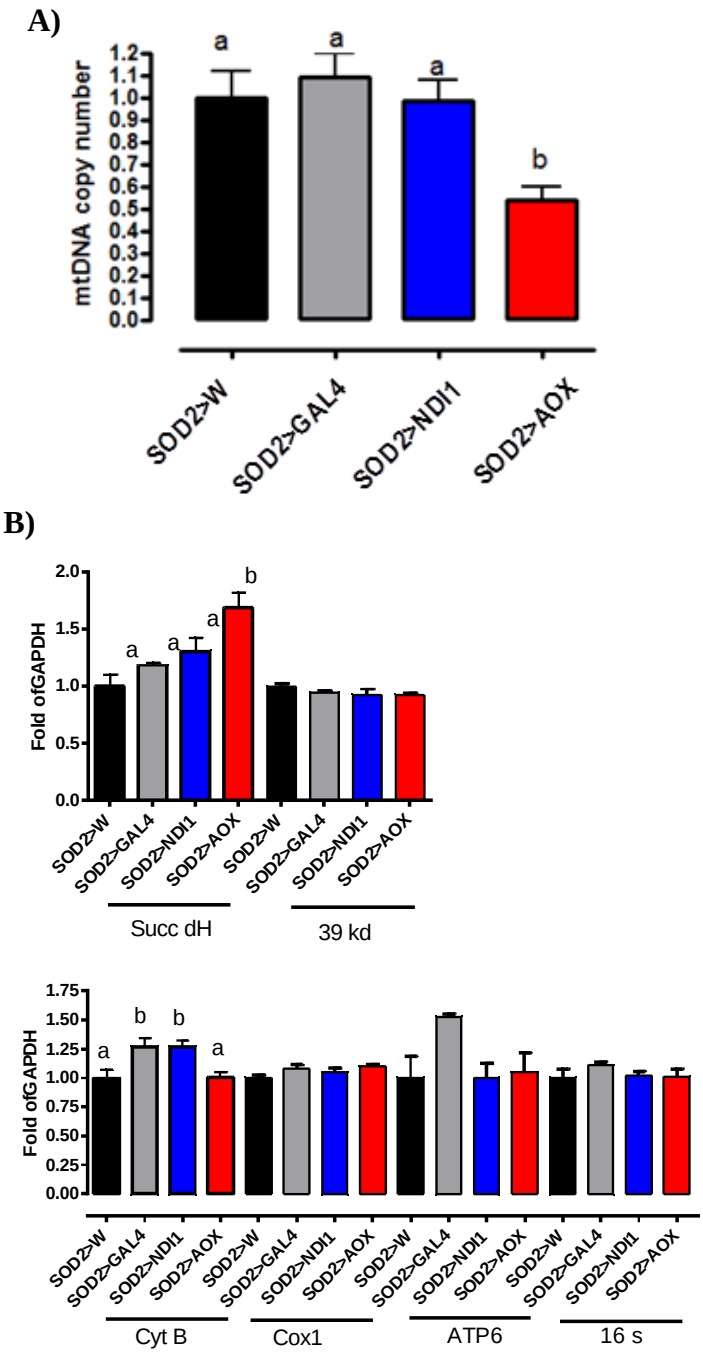
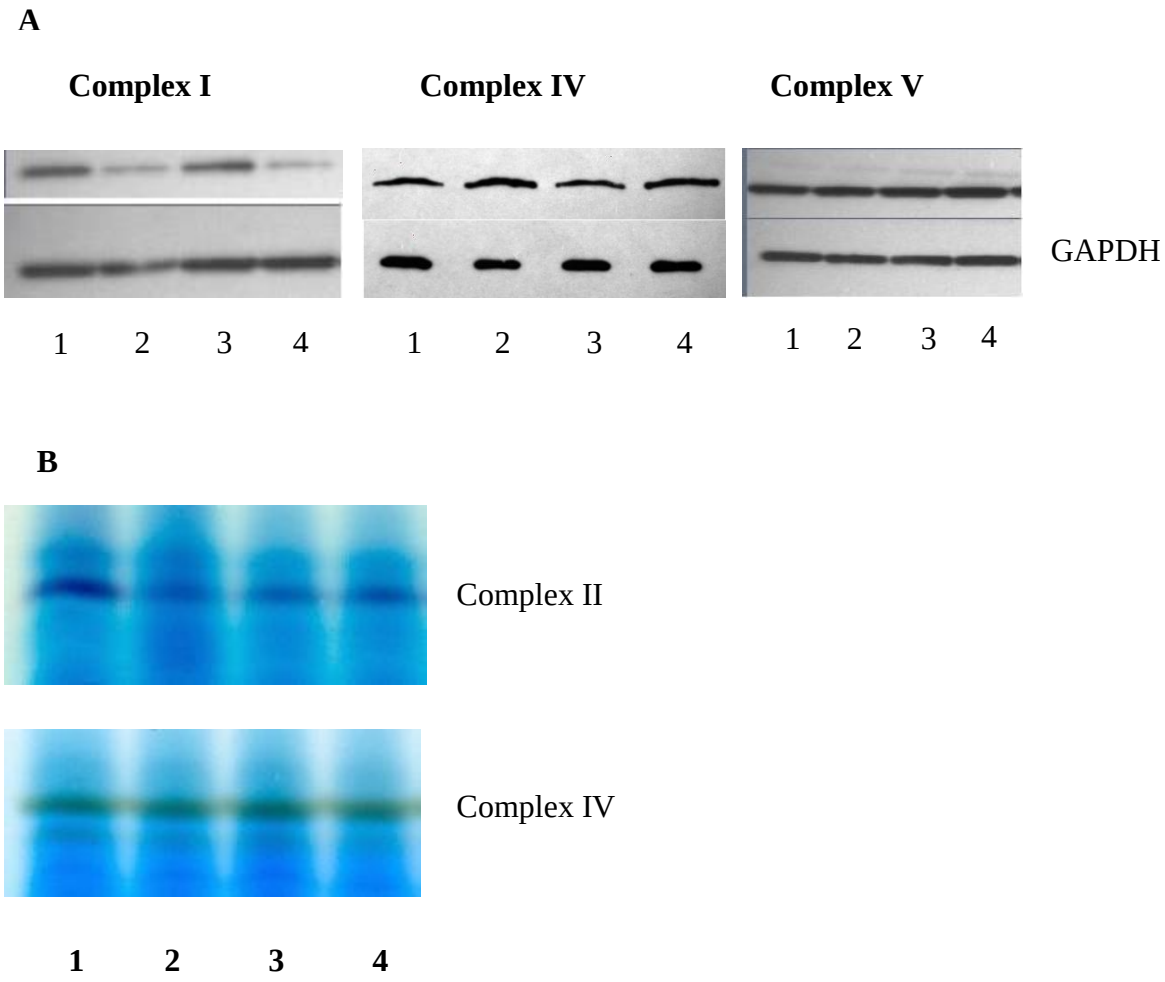


Figure 4.4 ***A)*** *The mitochondrial copy number was measured using real time qPCR using isolated whole DNA of samples represented in the following order in the figure. SOD2RNAi non-expressing group (SOD2>W), SOD2 RNAi expressing group (SOD2>GAL4), SOD2 RNAi+NDI1 expressing group (SOD2>NDI1) and SOD2 RNAi+AOX expressing group (SOD2>AOX). The mtDNA copy number was measured using primers for complex I subunit ND1 and GAPDH and the values obtained was normalized to values of the control group SOD2/W.* ***B)*** *mRNA levels (represented as folds of GAPDH)of nuclear subunits (Succ dH, 39kd) and mitochondrial subunits (cyt B, Cox1, ATP6, 16s) measured by Q-RT-PCR in the different groups mentioned. The values obtained were normalized to values of the control group SOD2/W. Letters a and b represent significant difference between groups(mean±SEM, $p < 0.05$. n=3-4, triplicates were used for each sample).*

4.5 Western blots and Blue Native PAGE to study the assembly and activity of OXPHOS complexes.

Western blotting was done with the same groups of flies as mentioned and probed with antibodies specific for each complex to study if the increased oxidative stress affects the protein assembly. Atleast three different samples (considered as different experiments) and three replicates (in each experiment) were used to perform this experiment. The bands obtained after each experiment were scanned and quantified using ImageLab to determine the intensity (Figure 4.6). The Prism GraphPad software was used to combine and analyze the results. The bands which closely represent the average intensity of the bands are presented in the figure 4.5A. It can be seen that the complex I assembly is affected in all the groups expressing SOD2RNAi compared to SOD2/W (non-expressing group) and the group expressing SOD2RNAi+Ndi1 partially improves complex I assembly. No decrease in complex IV and complex V assembly was observed.

Mitochondrial respiratory complexes were separated using BN-PAGE and section of gels containing specific complexes were cut and used for determining their activity. From the figure it can be clearly seen that the activity of complex II is decreased in all the groups expressing SOD2RNAi with or without NDI1/AOX compared to the control group SOD2/W (Figure 4.5B). No difference in the activity of complex IV was observed between the different groups.



1 - SOD2>W; **2** - SOD2>GAL4; **3** - SOD2>NDI1; **4** - SOD2>AOX.

Figure 4.5 *A) Western blots of the samples to check the assembly of respiratory complexes. The samples used are in the following order, (1) SOD2RNAi non-expressing group (SOD2>W), (2) SOD2 RNAi expressing group (SOD2>GAL4), (3) SOD2 RNAi+NDI1 expressing group (SOD2>NDI1) and (4) SOD2 RNAi+AOX expressing group (SOD2>AOX). Whole fly homogenates were probed with antibodies specific to each complex. Complex I assembly is affected in flies expressing the RNAi and NDI1 co-expression partially improves the complex I assembly. No change in complex IV and V assembly was observed. B) In-Gel activity of complex II and IV using mitochondrial extracts of the samples in the same order. Activity of complex II is affected in all groups expressing the RNAi and partial improvement is seen when NDI1 is co-expressed.*

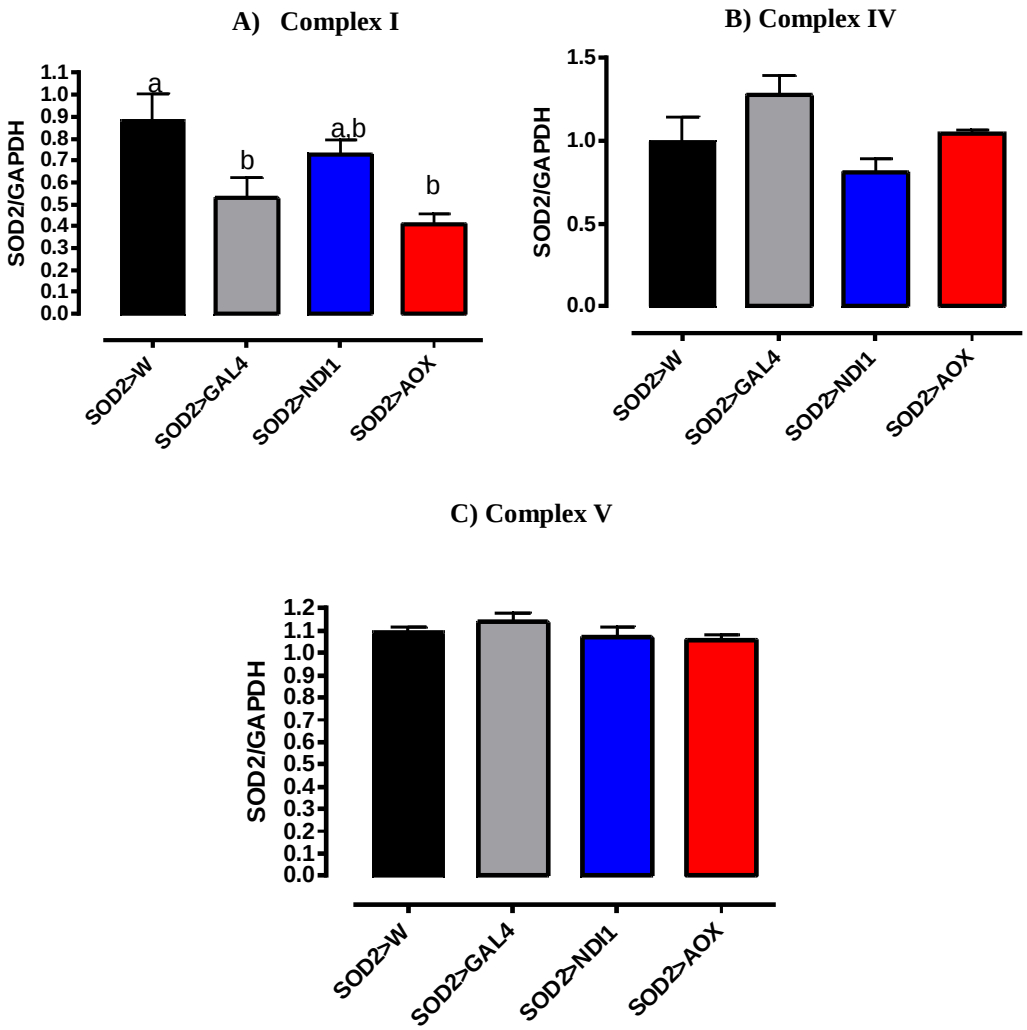


Figure 4.6. *Quantification for intensities of bands obtained for Western Blotting was done by scanning and using ImageLab software. The Prism Graphpad software was used to combine and analyze the results. Intensities of bands obtained by probing with A) Complex I, B) Complex IV, C) Complex V specific antibodies and normalized to GAPDH are presented here. Letters a and b represent significant difference between groups (mean±SEM, p< 0.05. n=3-4.)*

5 DISCUSSION

5.1 SOD2 knockdown decreases lifespan

Oxidative stress has been proposed to play an important role in age related functional declines, both pathological and non-pathological. The activity of SOD enzyme converts superoxide into H_2O_2 and the action of other anti-oxidant enzymes convert H_2O_2 to water. SOD2 or MnSOD which is localized inside mitochondria has been of particular importance because of its ability to detoxify ROS at the site of its production. Thus theoretically knock-out or knock-down of this enzyme should increase the oxidative stress and if the Mitochondrial free radical theory holds true, the increased oxidative stress should result in accelerated aging of the organism in a pattern similar to that of late-life stages. Previous research in knock-out or knock-down of SOD2 enzyme have supported the notion that these models can be used for studies relating age or age-associated diseases and also as models of accelerated aging (Li Y et al. 1995; Nicole Piazza et al. 2009; Paul A et al. 2007).

We have managed to express AOX and NDI1 in *Drosophila* before (Fernandez-Ayala DJ et al. 2009, Sanz A et al. 2010b) and our result (Figure 4.1) shows that the knockdown of SOD2 using RNAi with GAL4 driver in the different experimental groups containing SOD2RNAi without GAL4 driver SOD2RNAi+GAL4, SOD2RNAi+NDI1/AOX+GAL4 was successful. The knockdown was checked in both protein level as well as mRNA level. There is a clear difference in the bands from Western blots between the groups expressing and non-expressing the knock-down, when probed with antibody for SOD2. The mRNA levels of SOD2 normalized to GAPDH shows a clear pattern of down regulation of SOD2 compared to the control group (Figure 4.1).

Results from the lifespan experiment (Figure 4.2 A) make it clear that the lifespan of all groups of flies (both male and female) expressing the SOD2RNAi have a high mortality rate and reduced lifespan. This is consistent with other studies regarding down-regulation of SOD2 (Kirby K et al. 2002; Duttaroy A et al. 2003). This is due to the increased endogenous oxidative stress in mitochondria resulting from SOD2 knock-down and can be interpreted that SOD2 is required for the normal functioning of mitochondria. All the crosses were made and the flies were allowed to develop at 18°C. This is because the numbers of flies that eclose were less and most flies die at an early age. This may be due to the property of the binary expression system GAL4/UAS that the GAL4 protein has a higher affinity towards UAS promoter at higher temperatures,

which induces higher expression of the RNAi construct.Or because the production of ROS is higher at higher temperatures.

5.2 NDI1 expression partially increases lifespan and activity of flies.

We expressed AOX and NDI1 in *Drosophila* primarily to study their effects on OXPHOS disease (dysfunstion) models and to test the prediction of MFRTA. Interestingly NDI1 expression increases the lifespan in *Drosphila* independently of dietary restriction (Sanz A et al. 2010b). As mentioned before both NDI1 and AOX expression reduces ROS production (In old flies in case of NDI1) in mitochondria and only NDI1 expression increases the lifespan. It was proposed that NDI1 could work by a pathway dependent or independent of ROS production (Discussed later). We co-expressed AOX or NDI1 (not together) in a SOD2 knock-down model using the same daughterless GAL4 (daGAL4) driver to study the effect of NDI1 in a model where the oxidative stress is high. Our results (Figure 4.2A) show that NDI1 co-expression with SOD2RNAi partially and yet significantly increases the lifespan compared to the groups which express only SOD2RNAi and SOD2RNAi+AOX. We then measured the activity of flies at different ages (1-10 days) and the results (Figure 4.2B) show that at Day 3 there is no difference in the activity between the groups. It is interesting to notice that at Day 6 the flies expressing SOD2RNAi alone are the least active group. It is important to remember that this group of flies also has one of the highest mortality. Flies expressing SOD2RNAi+NDI1 have an activity higher than SOD2RNAi. Interestingly the flies expressing SOD2RNAi+AOX which also has an increased mortality (almost equal to SOD2RNAi) has an activity equal to the flies co-expressing NDI1. At Day 8 the activity of all groups of flies expressing SOD2RNAi with or without NDI1/AOX co-expression was significantly lower than one of the groups that did not express the RNAi. In fact we predict that the increased mitochondrial oxidative stress results in oxidative damage to various proteins critical to the functioning of the respiratory chain and thus leading to inactivation of these proteins or enzymes. In fact reduced mitochondrial activity resulting in a high energy demand can be the mechanism leading to early death. Interestingly the results obtained from the activity of flies (particularly Day 6) may suggest the same. The group having the highest mortality has the least activity at day 6. Groups expressing NDI1 and AOX have higher activity at this pint maybe due to the alternative enzymes compensating for the dysfunctions in ETC. Alternative enzymes AOX and NDI1 have previously been shown to compensate ETC dysfunctions by maintaining electron transfer (Fernandez-Ayala DJ et al. 2009; Sanz A et al. 2010b). Although AOX expression has been shown to decrease mtROS production when expressed in flies with DAH background, it did not extend lifespan (Sanz A et al. 2010a). The same effect of AOX on lifespan was also observed in our SOD2RNAi model. It can be observed from the results from activity of flies and lifespan that although the expression of alternative en-

zymes (NDI1 and AOX) helps increase the activity of the flies, only NDI1 increases the lifespan.

5.3 NDI1 rescues the decrease in respiration associated with high levels of superoxide.

Mitochondrial respiration at different respiratory chain complexes were measured by adding substrates specific to each complex and state 3 respiration was measured by adding ADP after achieving a steady rate of respiration. The results (Table 4.1 and Figure 4.3) show that increased oxidative stress in mitochondria leads to a decrease in state 3 respiration with complex I substrates in all groups of flies expressing SOD2RNAi compared to the control group but no such decrease was seen when complex III substrate was used.

State 3 respiration using complex I substrates was significantly increased (but not fully compensated) when NDI1 was co-expressed with SOD2RNAi but no difference was seen with AOX co-expression when complex III substrates were used. The higher respiration in complex I of NDI1 co-expressing group compared to the other groups can be explained by the fact that NDI1 oxidizes NADH without the participation of complex I and maybe also because only complex I assembly is affected (shown in figure 4.5A) due to the increased oxidative stress. NDI1 helps avoid the passage of electrons through complex I resulting in lesser leakage of electron from complex I which has been implicated with the process of aging.

Superoxide can attack Fe-S clusters and mitochondrial respiratory complexes I, II, III and aconitase which contain Fe-S clusters are some important targets. Complex I might be particularly sensitive to damage from superoxide due of the presence of 9 Fe-S clusters in its hydrophilic arm. Leakage of electrons from complex I can result in superoxide formation which can attack Fe-S clusters resulting in the release of free iron. The close proximity between the source of damage and its target could mean that the antioxidant enzymes might not have a chance to intervene. The free iron can get involved in Fenton/ Haber-Weiss reactions resulting in the formation of hydroxyl radicals and cause further damage to biomolecules. This would explain the reduction in complex I assembly (Figure 4.5 A) and complex II activity (Figure 4.5B) in the groups expressing SOD2RNAi. From our previous studies, it has been proven that NDI1 reduces mtROS production in vitro (Sanz A et al. 2010b) and by doing so it could reduce potential damage to complex I and complex II by superoxide. This can be supported from the results obtained in this study where NDI1 is shown to partially rescue complex I assembly and complex II activity. This rescue is however not observed with AOX expression. Rescue of respiration (complex I) by NDI1 might have been due to lesser ROS production associated with NDI1 expression thereby resulting in lesser damage to complex I.

Long lived species are characterized by fewer mtROS production and more resistance of their membranes to oxidative stress. AGEs (advanced glycoxidation end products) and ALEs (advanced lipoxidation end products) which are negatively correlated with longevity accumulate with age (Jacobson J et al. 2010; Pamplona R et al. 1999, 2000) and whose concentration is reduced by dietary restriction can be influenced by the efficiency at which complex I functions. In other words, the ratio of NAD+/NADH which is determined by complex I functioning can have an effect on the sensitivity of the membranes to oxidative stress (Stefanatos R et al. 2011). NAD+ is required for the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphogylcerate. A shortage of NAD+ can result in the accumulation of dihydroxyacetone that decomposes to form methylglyoxal, which is the main initiator of glycation reactions. Similarly the enzyme desaturases need electrons and energy from NAD(P)H to introduce double bonds into fatty acids which can result in the formation of ALEs. An efficient complex I would oxidize NADH and thereby maintain only low levels of NADH and thus would ensure that appropriate amounts of NAD+ is available for glycolysis (Scialo F et al. 2012). Therefore maintaining a balance between the oxidized and reduced forms of NADH is important to having membranes that are more resistant to oxidative stress. From the results it is possible to assume that NDI1 extends lifespan by the above mentioned method but it is also possible that ROS production is also reduced at complex I which could also influence lifespan.

5.4 Proteins with Fe-S are affected

O_2^- toxicity can occur by direct oxidation and inactivation of Fe-S proteins and its associated release of iron. The direct attack of O^{2-} on [4Fe-4S] causes the release of an iron ion not directly ligated to the protein. This results in the formation of inactive $[3Fe\text{-}4S]^+$ cluster and simultaneous release of Fe^{2+} and H_2O_2 which can participate in Fenton reaction as mentioned before. It has been shown before that O^{2-} can inactivate other Fe-S proteins like complex I and complex II (Zhang Y et al. 1990). Consistent with these findings and from our results (Figure 4.5 A and 4.5 B) it can be concluded that the complex I assembly and complex II activity are affected due to the increase in mitochondrial oxidative stress. It can also be clearly seen that NDI1 co-expression has at least partially compensated complex I assembly and complex II activity. Consistent with findings from other studies using a SOD2 knockout models, the activity of complex II is also affected due to the increased oxidative stress (Kirby K et al. 2002; Doughlas H et al. 2003).

5.5 mtDNA copy number and mRNA levels are not affected

We checked the effect of increased mitochondrial oxidative stress on mitochondrial copy number and mRNA levels of nuclear and mitochondrial subunits. It could be seen that the levels of mRNA are clearly not affected (Figure 4.4 B). From the results it can be interpreted that the transcription of subunits from different sections of mtDNA and nDNA are not affected by high levels of superoxide. The mitochondrial copy number was decreased in the flies expressing SOD2RNAi+AOX (Figure 4.4 A). Further investigation is needed to analyze it but we have focused only on the mechanism by which NDI1 works.

Although previous studies have shown a correlation between markers of oxidative damage in mtDNA with old age, it is believed that these oxidative damages can be repaired (Stuart JA et al. 2005). Also there have been doubts about the role of ROS in accumulation of mutations in mtDNA. From the results obtained in this study, it was shown that complex I assembly and complex II activity were affected (Figure 4.5 and 4.6) but a corresponding decrease in expression of its subunits is not observed (Figure4.4). This could mean that the decrease in complex I assembly and complex II activity are not as a result of mtDNA damage.

From our results it can be interpreted that complex I has an important role in aging. Due to the increased oxidative stress, the ROS generation at complex I cause a substantial damage to complexes I and II as described earlier. Other studies have reported that the loss of respiratory competence at complex I during aging is accompanied by increased production of mtROS at complex I. From our finding that NDI1 partially extends lifespan and rescues the reduction is respiration at complex I in our model, there can be two possible mechanisms that can be put forward regarding how NDI1 functions, 1) by regulating NAD+/NADH ratio and 2) by its effect on ROS production.

An appropriate balance between NAD+ and NADH is important as it is one of the major sensors for redox state of the cell. During DR, the NADH levels are reduced and during aging the opposite is observed (Sohal RS et al.1990; Scialo F et al. 2012).NAD+/NADH ratio can also control the accumulation of glycoxidative and lipoxidative damage. Interestingly DR also delays the accumulation of AGEs and ALEs which have been negatively correlated with longevity (Pamplona R et al. 2007). It can be inferred that an appropriate balance between NAD+ and NADH helps protect against oxidative damage. Interestingly NAD+/NADH have also been shown to regulate the functioning of complex I (a major contributor of ROS in mitochondria) and other enzymes (Murphy MP. 2009). This could mean that a more efficient complex I would oxidize NADH effectively and thus producing lesser ROS at the same time will have more resistance to oxidative damage. These are the characteristics observed in long lived animals.

NDI1 could function by regulating NAD+/NADH ratio and also by its ability to bypass complex I can also help reduce ROS production. These effects would result in lesser damage generation as well as having more resistance to oxidative damage. The partial extension of lifespan by NDI1 in our model can be the result of an addition of both these effects.

CONCLUSIONS

The aim of the research was to design a system with high mitochondrial oxidative stress and by co-expressing alternative enzymes (NDI1, AOX), we tested if NDI1 still extends lifespan and if so to try and figure out the mechanism by which it is done. A system with high mitochondrial oxidative stress was created by knocking down SOD2 using RNA interference. Both RNAi and alternative enzymes were expressed using a binary system GAL4/UAS.

The knockdown of SOD2 was checked at protein and mRNA levels. It is seen that the knock down results in a reduced lifespan due to early mortality and a partial rescue with NDI1 co-expression is observed.

A reduction in locomotor activity is seen in flies expressing the RNAi.

A partial rescue in complex I specific substrate oxidation is observed in flies co-expressing NDI1.

Complexes with Fe-S clusters are affected. The protein assembly is thus affected resulting in a functional loss leading to energy crisis.

NDI1 co-expression delays the functional deterioration possibly by reducing ROS production at complex I thus protecting it from damage and also by compensating the substrate oxidation at complex I. This compensation might also result in an improved ATP production.

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