TAMPEREEN TEKNILLINEN YLIOPISTO
TAMPERE UNIVERSITY OF TECHNOLOGY

ASHWIN SRIRAM

EFFECT OF THE REDUCTION OF RESPIRATORY COMPLEX I LEVELS ON *DROSOPHILA MELANOGASTER* LIFESPAN

Master of Science Thesis

Examiners: Dr. Alberto Sanz &
Professor Matti Karp
Examiners and topic approved in faculty council meeting on 6th May,2012

ABSTRACT

TAMPERE UNIVERSITY OF TECHNOLOGY
Master's Degree Programme in Science and Bioengineering
SRIRAM, ASHWIN: Effect of the reduction of Respiratory Complex I levels on *Drosophila melanogaster* lifespan
Master of Science Thesis, 71 pages
September 2012
Major: Biotechnology
Supervisor: Dr. Alberto Sanz
Reviewers: Dr. Alberto Sanz, Professor Matti Karp
Keywords: Complex I, ROS (Reactive Oxygen Species), Aging, Drosophila

Respiratory Complex I is the main generator of Reactive Oxygen Species (ROS) inside the cell. ROS are generated during aerobic respiration, which occurs inside the mitochondria and generates most of the energy used by aerobic organisms. In this study we have provided evidence supporting the Mitochondrial Free Radical Theory of Aging (MFRTA), which states that ROS, produced inside the mitochondria cause aging. To test this we have utilised the power of Drosophila genetics to study the effects knocking-down one of the subunits of Complex I has on the Complex I assembly, energy homeostasis, and lifespan in *Drosophila melanogaster*.

A series of crosses were performed, in which an RNAi construct for the gene CG6020 (encoding the 39kDa subunit of complex I) was combined with ectopic expression of the yeast Ndi1 protein to compensate the redox disequilibria caused by a lack of NADH re-oxidation in flies expressing only the knock-down. Both the RNAi construct and the NDI1 transgene were expressed using the binary UAS/GAL4 expression system. The resulting progeny were used to evaluate the knockdown of Complex I and to perform lifespan studies and complementary experiments.

The knockdown of the nuclear encoded subunit of complex I (CG6020) was verified at the RNA level, protein level and at the whole-complex level by qPCR, western blotting and Blue Native Gel electrophoresis, respectively. The decrease in complex I respiration was also quantified by polarographic measurements using an Oroboros instrument (Oxygraph 2k), and the change in hydrogen peroxide production was studied in isolated mitochondria. Moreover, other physiological parameters –fertility, activity, food intake- were also studied to understand how longevity was affected in flies expressing the complex I knockdown.

The results from shown here illustrate that, the knockdown of the 39kDa subunit (encoded by CG6020) specifically decreases the concentration of complex I. This specifically affects complex I-linked respiration, and at the same time reduces the generation of hydrogen peroxide in isolated mitochondria. Moreover, knock-down of

complex I increases life-span without affecting fly mobility or food intake. Despite a few shortcomings, substantial evidence has been provided to show that, the increase in life-span of the flies is only due to reduction in the concentration of complex I.

ACKNOWLEDGEMENTS

This research work was carried out at the Institute of Biomedical Technology, University of Tampere, Finland under the supervision of Dr. Alberto Sanz.

I owe my deepest of gratitude to Dr. Alberto Sanz for giving me an opportunity to carry out this research project in his lab and under his esteemed guidance. I am deeply indebted to him especially for the confidence that he always placed in me, his encouragement and support throughout the work. I am grateful to Professor Matti Karp for reading the thesis as an external examiner and for his constructive comments.

I am also very grateful to Essi Kiviranta and Milja Luukonen for their laboratory technical support during the work. Many thanks to Venkatesh Mallikarjun for his help with the English language of the thesis.

Finally, it is my great pleasure to express my sincere and heartfelt thanks to all my love one(s) and all my lab mates, whose friendship and support kept me going, during the work. Last, but not the least, I remember my family and fondly at this time for their endless love and always believing in me. To those who have indirectly contributed to this research, you kindness means a lot to me.

Thank you, one and all.

Tampere, June 20th, 2012

Ashwin Sriram

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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*
CAFÉ	Capillary Feeding
cDNA	complementary Deoxyribonucleic Acid
CO2	Carbon-di-Oxide
CPEO	Chronic progressive external ophthalmoplegia
CR	Caloric Restriction
CyO	Curly winged fly (Genetic Marker)
CYT C	Cytochrome c
DAB	3.3′-Diamidobenzidine tetra hydrochloride
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
MELAS	Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes
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
PCD	Programmed Cell Death
PD	Parkinson's Disease
PDHα	Pyruvate Dehydrogenase
PVDF	Polyvinylidene Diflouride
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 Electrophoresis

SMT	Somatic Mutation theory
SOD 1	Superoxide Dismutase 1
SOD 2	Superoxide Dismutase 2
TMPD	Tetramethyl-1,4-benzenediamine dihydrochloride
UAS	Upstream Activating Sequence

Aims of the research

Respiratory complex I is the main generator of Reactive Oxygen Species (ROS) inside the cell. The Mitochondrial Free Radical Theory of Aging (MFRTA) proposes that damage caused by Reactive Oxygen Species during normal metabolism is responsible for aging. Hence, diminishing the amount of complex I should decrease the production of ROS, and consequently, increase lifespan - if MFRTA is correct. The study presented here was conducted to elucidate the role of Complex I in aging and age related diseases. We have reduced the level of complex I using an RNAi construct designed to knockdown the nuclear-encoded CG6020 gene, whose protein product is the 39 KDa subunit of Complex I.

The specific aims of this study were as follows:

(1) To elucidate the involvement of Complex I in the production of ROS

(2) To investigate how Complex I levels affect lifespan.

(3) To study the role of ROS in regulating lifespan.

1. Introduction

Aging is considered to be one of the most intriguing physiological processes as it is the root cause of many diseases. We believe that, if we study aging we will eventually be able to cure or delay most of these age-related diseases. For this purpose, we have manipulated the activity of mitochondrial complex I, which is considered to be a key regulator of the aging process. *Drosophila melanogaster* was used for all experiments due to its extremely compatible genetic make-up.

The respiratory complex I or NADH: ubiquinone oxidoreductase present in the mitochondria is the largest of the protein complexes found in the electron transfer chain. It is composed of around 46 subunits with a total size of about 1 MDa. Along with complexes III and IV, complex I couples proton translocation to electron transfer and establishes the proton gradient used to generate most of the energy used by the cell. Along with this, complex I is the main generator of ROS in the mitochondrion. Its implication in ROS production is one of the main motivations behind our study. By utilizing RNA interference to knockdown specific genes, we have obtained several Drosophila lines expressing a complex I knockdown.

Mutations or knockdown of the genes encoding these complex I subunits, can lead to electron transfer chain dysfunction, which is often lethal in fruit flies. To compensate for this loss, we have ectopically expressed yeast NDi1, which is a non-proton translocating enzyme that bypasses complex I in *Saccharomyces cerevisiae*. Previous studies in our lab have shown that NDi1 can restore part of the function of complex I (Sanz et al., 2010b). It has been shown that, by the transfer of electrons through NDi1, there is a reduction in ROS generated by complex I, and by knocking down complex I; we hoped to further reduce the production of ROS, in turn increasing lifespan if MFRTA is correct.

The aim of this thesis was to elucidate the effect of knocking-down a specific subunit of complex I, and thus reduce its assembly. By doing this, we sought to understand role of complex I in aging, longevity and ROS production. A series of crosses was performed throughout the project, creating Drosophila lines expressing both the RNAi knockdown construct for the gene CG6020 of complex I and Ndi1, with both under control of a binary UAS/GAL4 system. The resulting progeny was used for all experiments and to analyze the ability of knockdown of complex I to increase lifespan.

2. Review of the Literature

2.1 Aging

Aging can be defined as a multi-faceted process which results in the loss of molecular fidelity and an increased vulnerability to cellular stress. There is a very complicated etiology provided by recent studies proving that aging has a strong genetic component and that genetic modulation can alter lifespan in many model organisms, such as *Drosophila melanogaster* and *Caeonoharbditis elegans* (Longo and Fontana, 2011). Attempts to combat aging have been made. Notable among these the experimental trials using "anti-aging drugs" on nematodes flies and vertebrates like mice and rats (Anisimov et al., 2011). One example of such drugs is rapamycin, which increases both mean and maximum lifespan, even when administrated to old mice (Wang et al., 2011). Nonetheless, there have been no successes in preventing human aging. Equally, some genetic strategies that have been shown to extend the lifespan of nematodes have a much smaller effect on other model animals, such as flies or mice. However, these results should be taken with caution in relation to human beings. Genetics has been shown to determine only 25% of human longevity, with the rest being determined by other factors such as diet, exercise or other environmental factors (Rattan, 2012).

To study both aging and longevity, we must be able to distinguish between mean and maximum life span (MLSP). MLSP is defined as the maximum number of year that an organism can live because of its characteristic genetic make-up (Leonid and Natalia, 1991). On the other hand, Mean Lifespan pertains to the number of years an individual can live, provided a very specific ecosystem (Millar and Zammuto, 1983). For example an individual born and brought up in Africa, would live only up to the age of around 30-40 but an individual born and brought up in Europe, will live up to the average age of around 70. Most of the economically stable countries have been very successful in establishing a suitable environment which favors the increase of Mean Lifespan. However, Maximum Life Span Potential has not been modified during human history (Wilmoth et al., 2000).

2.2 Theories of Aging

There are numerous theories of aging, which can be divided according to their level of specificity and hierarchically, depending on which level (molecular, cellular, organism) they refer to.

2.2.1 Molecular theories of Aging

Molecular theories of aging have been described in different contexts and at different levels. The molecular theories of aging focus on damage or loss of functionality of biological molecules. This includes all kinds of biological molecules like proteins, DNA and/or lipids, which might affect the protein synthesis or gene regulation of the organism (Weinert and Timiras, 2003).

There are two basic theories found under this category. They are i) Genetic and ii) Non-genetic. And, there are numerous theories that fall under both of these categories. As the name suggests, the genetic theories of aging emphasize the role of specific genes in relation to aging (Kanungo, 1975). The latter explains aging postulates at the protein level. One prominent genetic theory is the codon restriction theory. Codon restriction theory states that aging is caused by a decrease in the accuracy of protein translation; thereby leading to a decrease in efficiency of protein synthesis (Strehler et al., 1971). One of the other genetic theories is the Hayflick's Limit theory. This theory states that, there is a limit to the number of times a cell can divide (Hayflick and Moorhead, 1961; Shay and Wright, 2000). This phenomenon has since been shown to be related to telomerase (Olovnikov, 1996). The somatic mutation theory, on the other hand, describes that mutations can take place within the cell, and that they accumulate over time, causing aging (Gavrilov and Gavrilova, 2002).

The non-genetic theories of aging lay its foundation on physiological aspects. The accumulation of waste theory of aging (Hirsch et al., 1989) proposes that, accumulation of cellular debris causes aging. According to accumulative waste theory, the accumulation of the pigment lipofuscin, is the most reliable aging biomarker. Lipofuscin are finely granular pigmented molecules, which are debris products of lysosomal digestion. Essentially, lipofuscin is the byproduct of the oxidation of unsaturated fatty acids, glycoxidation of proteins and incomplete digestion of cellular organelles (Gaugler, 1997). When these byproducts accumulate, they can interfere with normal cellular function (Weinert and Timiras, 2003). A more specific version of this theory is the glycation theory. Glycation theory suggests that incomplete oxidation of glucose causes the accumulation of reactive species, such as methylglyoxal that can go on to damage protein, lipids and DNA, causing the accumulation of cellular waste (Gavrilov and Gavrilova, 2002).

A more recent theory is the thiol redox hypothesis. All biological systems contain redox elements and the organization of these elements occurs through a redox circuit. These redox-sensitive elements are insulated, and can be activated only with the help of specific catalytic mechanisms. The redox hypothesis states that disruption of these redox sensitive thiol elements by, i.e. accumulation of oxidative stress is causative of aging (Jones, 2008).

2.2.2 Cellular theories of Aging

The cellular theories of aging concentrate on aging at the cellular level, although these theories are often closely related to those at the molecular level. One example of these theories is that apoptosis, a form of programmed cell death, has a central role in aging (Warner, 1997). Other example is the telomeric theory of aging, which proposes that cell senescence caused by a reduction in the size of telomeres (after each cell division) is responsible for aging (Olovnikov, 1996; Weinert and Timiras, 2003). Cellular senescence is defined as the biological aging of an organism, after it attains its maturity, which is it inability to participate in the normal functions of the cell. Related to both of these theories, there is the aging pacemaker theory that postulates that one type of cell or tissue interferes with cell proliferation or cell differentiation, therefore initiating the process of senescence (aging of cells) throughout the body. This view of cellular senescence is compatible with the oxidative stress theories of aging where these phenomena also lead to cellular senescence or cell death (Weinert and Timiras, 2003).

2.2.3 System theories of Aging

Systems theories of aging state that aging is a consequence of the malfunction of different organ systems. Systems theories include the rate of living theory, autoimmune theory and the neuroendocrine control theory. The rate of living theory proposes that, the difference in maximum lifespan between different species is due to the variation in metabolic rate associated with changes in mass. Consequently, life expectancy of an individual or an organism is inversely proportional to the metabolic rate (Speakman et al., 2002). However, contrary to the rate of living theory there are a has been a lot of experimental evidence stating that metabolism and life span are not necessarily correlated (Khazaeli et al., 2005a; Lin et al., 1998; Marden et al., 2003; Tatar et al., 2001). Originally, this theory formed the basis of the oxidative stress theory of aging, but nowadays they are clearly differentiated (Hulbert et al., 2007). The neuroendocrine control theory suggests that with age there is a loss of functionality of the receptors of the hormones rather than a decline in the function of the endocrine hormone itself (Weinert and Timiras, 2003). This leads to organ malfunction. Finally, the autoimmune theory suggests that, as the organism ages the immune system losses its effective capability to fight against foreign organisms, cancer cells and other aging related factors, leading to diseases related to aging.

2.2.4 Evolutionary theories of aging

The evolutionary theories of aging explain in brief, the concepts of how evolutionary changes in an organism, can play a vital role in longevity. There are two major lines of research in the aspects of evolutionary theories of aging: the accumulation of mutations theory and the antagonistic pleiotropy theory. The accumulation of mutations theory suggests that, gene mutation is unavoidable in individuals. These mutations over successive generations transform themselves to deleterious mutations, accumulating over time, and thereby increasing the mortality rate (Medawar, 1952). On the other hand, the antagonistic pleiotropy theory states that, the late-onset deleterious phenotypes are favored by natural selection because the genes responsible for them confer a selective advantage before reproductive maturity is reached (Williams, 1957). The major difference between the two theories is that in the former, the negative effects of mutation accumulate only at old age, but in the latter the deleterious mutations can be found in the gene pool itself.

2.2.5 The Network theory of aging

The network theory of aging combines postulates from the evolutionary theory, the molecular theory and the cellular theory of aging. It hypothesizes that; the process of aging is indirectly controlled by a vast network of cellular and molecular defense mechanisms. Cells are continuously exposed to a variety of stress factors, and they have developed a number of mechanisms in the course of evolution, to cope with a huge variety of stressors. All these mechanisms are interconnected and form a network of cellular defense systems. When one of these systems fails to act, senescence takes place, paving the path for the process of aging to occur (Franceschi et al., 2000). The network theory of aging is considered to be one of the most elaborate theories of aging, as there has been a genuine effort to combine all the aspects of different theories and postulates.

2.2.6 Mitochondrial Free Radical Theory of Aging

The Mitochondrial Free Radical Theory of Aging (MFRTA) is one of the most prominent theories in the recent era of aging studies, and it can be integrated into almost all the theories formerly mentioned. MFRTA was first proposed by Denham Harman in 1956, as the “Free radical theory of aging”. Harman stated aging as a consequence of accumulation of damage caused by the free radicals, generated during the normal metabolic processes (Harman, 1956).

Harman's free radical theory of aging was further revised and published in the year 1972, as the “Mitochondrial Free Radical Theory of Aging”. MFRTA (Harman, 1972; Harman, 1983) states that the respiratory complexes of the electron transport chain produce Reactive Oxygen Species as by-products of normal oxygen metabolism. Furthermore, MFRTA states that these ROS molecules go on to damage nucleic acid, proteins and lipids. This in-turn would lead to age-related disorders and diminished longevity. Many facts relate mitochondrial Reactive Oxygen Species with aging: increased ROS production, accumulation of mutations in mitochondrial DNA (mtDNA) and progressive respiratory chain dysfunction.

MFRTA also makes many other predications. One of which is that the damage accumulated by longer living species is lower than species with shorter lifespan (Perez-Campo et al., 1998). In fact, long-lived species produce fewer ROS than short-lived species (Muller et al., 2007), and have biological molecules more resistant to oxidation (Hulbert et al., 2007; Pamplona and Barja, 2007). Since they generate less damage, they also have less antioxidants and repair mechanisms (Sanz et al., 2006). From an evolutionary point of view, this is the most adaptive strategy since resources are scarce, and reduced damage generation is energetically more efficient (Page and Stuart, 2011). Calorie restriction extends lifespan in most animal species (Guarente, 2005; Partridge et al., 2005; Sinclair, 2005). Such extension is related with a reduction in the generation of damage (including mtROS), not with an increase in the amount of antioxidant defenses or repair mechanisms (Sacher, 1977). The figure 2.1 explains the different predications of the Mitochondrial Free Radical theory of aging schematically. It is shows the different researches conducted to prove or falsify the predications.

However, some evidence contradicts MFRTA. For example, the administration of antioxidants does not extend lifespan (Sanz et al., 2006); although it could be argued that these antioxidants are not targeted to the appropriate place or administered at the appropriate time. However, the manipulation of endogenous antioxidant levels does not produce the expected increase in lifespan, either. For example, the knock-out of superoxide dismutases 1 or 2 dramatically reduces the lifespan of flies and mammals (Mackay and Bewley, 1989), but this phenomenon does not occur in C. *elegans* (Van

Raamsdonk and Hekimi, 2009). Moreover, heterozygous knock-out mice for SOD 2 are long-lived in spite of having much higher levels of oxidative damage.

On the other hand, over-expression of SOD2 in flies has no effect on lifespan (Mockett et al., 1999). Although over expression of SOD1 increases its lifespan by 30% (Orr and Sohal, 1994), this is paradoxically related with more oxidative stress rather than less (Magwere et al., 2006; Parkes et al., 1998; Sohal, 2002). These contradictory results can be explained if ROS relevant for aging are produced in a very specific place, and they target specific molecules that cannot be protected by antioxidants. As we shall see this can be the case for ROS generated by respiratory complex I.

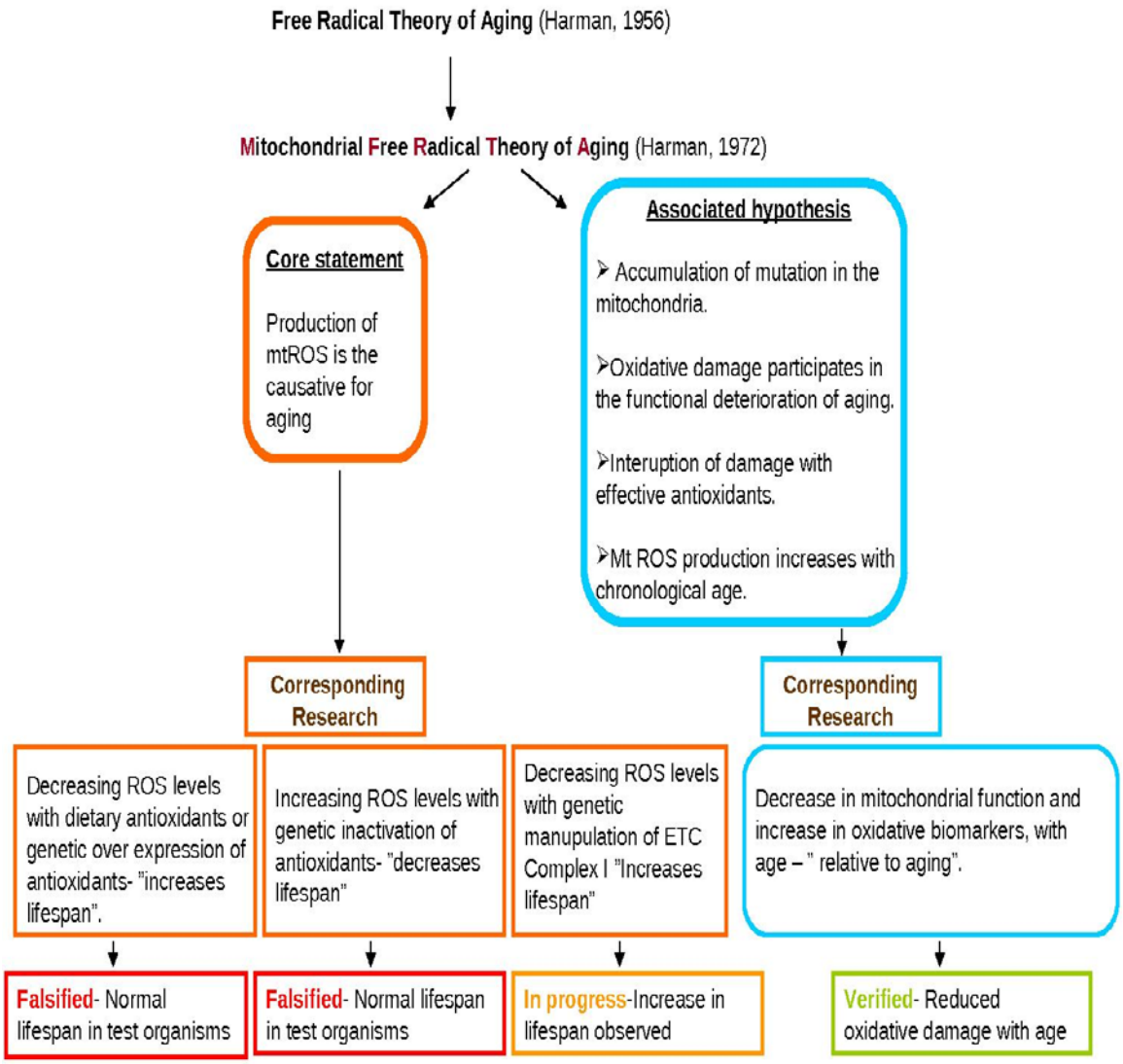


Figure 2.1*: The above figure schematically explains the hypothesis made by the Mitochondrial Free Radical Theory of Aging, and also the associated hypotheses. The research conducted to provide evidence for these hypotheses are also clearly explained.*

2.3 Electron Transport Chain

The electron transport chain is the key component of mitochondria and is absolutely necessary to maintain metabolism through the synthesis of ATP, and the assembly of iron-sulphur clusters (Karp, 2008). Oxidative phosphorylation (OXPHOS) is considered to be the most efficient way of generating energy in aerobic cells. Malfunction of OXPHOS can cause severe damage to the cell. An alternative is the use of glycolysis, which also produces energy, albeit in a less efficient way, considering amount of ATP generated per molecule of glucose oxidized, and amount of waste produced.

Oxidative phosphorylation takes place as a step-wise process which requires five multiprotein enzyme Complexes (Complex I, II, III, IV and V). All these components are embedded in the cristae of the mitochondria where the two electron carriers, cytochrome C and coenzymes Q co-exist. The initiation of the electron transport chain takes place by transferring the electrons to the carrier molecules NADH (Nicotinamide adenine dinucleotide) and FADH2+ (flavin adenine dinucleotide). These electron carriers are produced by the Krebs' cycle, inside the mitochondrion. The transfer of electrons is used to generate a proton motive force (Dimroth et al., 2000), which generates the energy used for oxidative phosphorylation. The electrons are transferred in a stepwise manner, from the FMNH2 (flavin mononucleotide, reduced) through the iron-sulphur clusters of complex I to the ubiquinone pool, in a two-step transfer process (Mitchell, 1979). In this process, 4 protons are translocated from the mitochondrial matrix to the intermembrane space (Hirst, 2005). Ubiquinol is oxidized by complex III (cytochrome bc1 complex), where another proton translocation event occurs (Trumpower, 1990). Four protons are pumped in this process, by complex III to the intermembrane space (Schultz and Chan, 2001). Complex III, transfers two electrons to the other electron carrier of the ETC, cytochrome c (Hunte et al., 2003). Complex IV, also known as cytochrome c oxidase, re-oxidizes cytochrome c and translocates four protons across the inner membrane. Complex IV then transfers four electrons and two protons to oxygen, which is the terminal electron acceptor (Yoshikawa et al., 2006).

Complex II (succinate dehydrogenase) is involved in a different pathway to the one followed by NADH. The stepwise electron transfer is similar, but unlike the NADH pathway, there is no proton translocation by the succinate dehydrogenase (Cecchini, 2003). In this whole process, an electrochemical gradient resulting in a potential of 150-180 mV is produced, known as the proton motive force. The proton motive force is generated as a result of the accumulation of protons in the inter-membrane space and is used to produce ATP, when protons diffuse back into the matrix through Complex V (also known as ATP synthase). The electrochemical gradient drives the formation of ATP from ADP and free phosphate at complex V (Boyer, 1997). Figure 2.2 illustrates the function of the different complexes in the Electron Transport Chain and total working of the OXPHOS (Oxidative Phosphorylation) system.

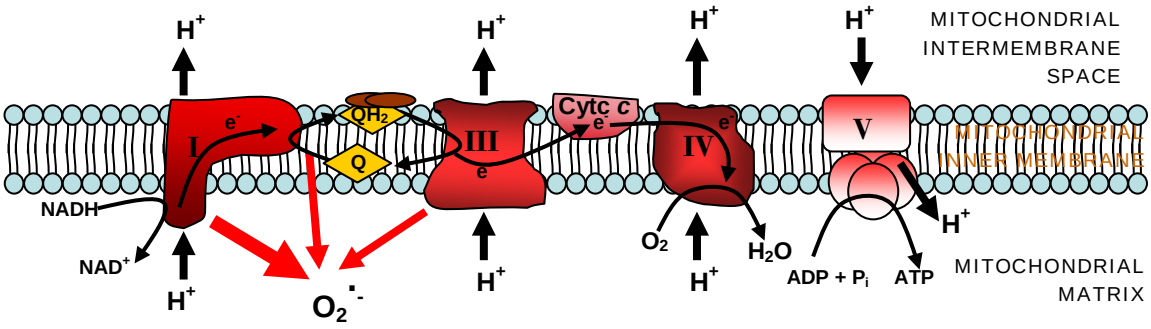


Figure 2.2: *This figure illustrates the production of adenosine tri phosphate by the flow of electrons through different multiprotein complexes located in the inner mitochondrial membrane. The different complexes are connected to each other by the electron carriers cytochrome c and coenzynme Q. The figure also points out the most important sites of ROS production.*

There is no electron leak at complex IV, where always four electrons (and two protons) are used to reduce oxygen to water. However, at complexes I, II or III electrons can escape and incompletely reduce oxygen to superoxide (or other ROS) (Finkel and Holbrook, 2000; Porter and Brand, 1995). This can result in oxidative stress which is thought to be are responsible for the decline in the mitochondrial function associated with aging (Rattan, 2006; Valko et al., 2007).

2.4 Reactive Oxygen Species (ROS)

Free radicals are defined as an atom or group of atoms with one or more unpaired electrons. Free radicals are typically very volatile and can damage cellular components. Free radicals are hypothesized to accelerate the progression of age-related diseases and in turn, reduce longevity. ROS is a collective term, which includes many molecules containing oxygen centered radical. A few examples of these kind of molecules are, the hydroxyl radical (HO^-) and the Hydrogen peroxide (H_2O_2). Accumulation of ROS inside the cell is the major cause of oxidative damage to the cell, which is termed as oxidative stress. To highlight a predominant fact, one of the major implications of free radical theory of aging is that, ROS causes cellular damage. Energy production by the electron transport chain leads to the formation of ROS, which causes accumulation of damage in mitochondria.

2.5 Complex I

The respiratory complex I is a multiprotein complex located in the inner mitochondrial membrane. The complex I, is also referred to as the NADH: ubiquinone reductase or NADH dehydrogenase, named according to its function. This protein complex is one of the entry routes of oxidative phophorylation, in the mitochondria (Nakamaru-Ogiso et al., 2010).

Complex I has a molecular mass of about 1 MDa in eukaryotes and 550 kDa in prokaryotes (Clason et al., 2010). It is by far the largest enzyme complex among all the other complexes and is composed of at least 45 different subunits, of which the primary structures have been determined (Carroll et al., 2006; Voet Judith and Donald, 2004). These subunits are split into two types of subunits, namely, one array of subunits encoded by the nuclear DNA (36 subunits) and other array of subunits encoded by the mitochondrial DNA (7 subunits). Among this large number of subunits, 14 subunits are designated as core subunits and the rest are designated as supernumery subunits, which are similar in both eukaryotes and prokaryotes. Of these 14 core subunits, 7 are encoded by the mt DNA, and are named as ND1, -2, -3, -4, -4L, -5 and -6. The 7 other core subunits are encoded by the nuclear DNA (Yano, 2002).

The 3D structure of complex I shows that it is composed of two major segments, the peripheral segment and the membrane segment. When fully assembled these two segments adopt an L-shaped structure consisting of two arms perpendicular to each other (Grigoreiff, 1998). The peripheral segment is a catalytic domain and comprises of 12 nuclear encoded subunits and the membrane segment consists of hydrophobic subunits, comprising all of the mtDNA encoded subunits (Sazanov and Hinchliffe, 2006).

The passage of electrons starts from Complex I. Its main function is to serve as an entry point for electrons. In this process, complex I translocates four protons per molecule of NADH oxidized (Brandt, 2006; Voet et al., 2008). The overall reaction taking place at complex I is as follows.

$$NADH + H^+ + Q + {}_nH_{in}{}^+ \longleftrightarrow NAD^+ + QH_2 + {}_nH_{out}{}^+ \ (n=2\text{-}4)$$

Complex I is not only involved in electron transfer. Complex I is also a major generator of ROS (Murphy, 2009; Turrens and Boveris, 1980). In general, the rate of ROS production of mitochondria from post-mitotic tissues is lower in long-lived species than in short-lived species (Barja, 2004). Interestingly, differences in ROS production between short and long-lived animals are exclusively due to differences in ROS produced by complex I (Herrero and Barja, 1997). Caloric Restriction (CR) is a normal experimental method to extend lifespan and study aging. CR decreases ROS generation by isolated mitochondria in different tissues, for example, brain, skeletal muscle, heart, liver or kidney (Lambert et al., 2007). Once again, the decrease in ROS is observed only at complex I (Esterhazy et al., 2008).

Another function that is regulated by complex I is the maintenance of the equilibrium between NAD+ and NADH. The equilibrium between NAD+ and NADH determines three basic cellular functions that are related to aging: (1) glycolysis, (2) unsaturation of the membranes and (3) activity of enzymes involved in DNA maintenance such as sirtuins or PARP. Due to that, it is possible that complex I is regulating longevity by ROS independent mechanisms (Stefanatos and Sanz, 2011). Moreover, an appropriate equilibrium between NAD+/NADH also determines the production of superoxide by complex I (Kussmaul and Hirst, 2006).

2.6 Medical relevance of complex I

The electron transport chain plays a pivotal role in the cell cycle. Hence it is very clear that any defects in the OXPHOS system can affect either the efficiency of ATP production or the assembly of iron-sulphur clusters - both of which are fundamental for cell survival. Even with the proper functioning of the electron transport chain, there is leak of electrons, which lead to the production of ROS. This in turn causes oxidative stress, which can also leading to many fatal diseases. Around 50 % of all cases of complex I deficiencies have been reported to generate fatal mitochondrial diseases (Smeitink et al., 2001). These diseases are produced by mutations in the genes encoding complex I subunits.

As it has been previously described, complex I plays a vital role in the production of ROS. One of the major involvements of complex I in medicine is its implication in Parkinson's disease (Chou et al., 2010). Recently, there has been some evidence relating complex I and Parkinson's because of the reactive oxygen species produced. This is because it has been proved that, a perturbation in the mitochondrial function, which is because of the reactive oxygen species produced, can be a causative for Parkinson's disease (Esteves et al., 2010).

There have also been studies relating complex I with the function of the human brain. It has been found that, in patients with extreme bi-polar disorder, the level of activity of complex I was significantly lower, when compared to the normal patients (Andreazza et al., 2010). Related to these studies, it has also been proved that deficiency of complex I leads to slower growth rates in individuals (Moran et al., 2010). However, it has been discovered that mutations in different genes, encoding different subunits of the complex, lead to different disease phenotypes. This explains the variety of pathophysiological manifestations of diseases involving complex I deficiency (Binukumar et al., 2010).

Complex I deficiency or mutation leads to several other diseases, such as Leigh syndrome, mtDNA depletion syndrome, Kearns-Sayre syndrome, Pearson syndrome, mitochondrial encephalopathy, lactic acidosis, MELAS and chronic progressive external opthalmoplegia (CPEO), among others (McFarland et al., 2010; Tucker et al., 2010). Clinical manifestations of these diseases can occur in the early stages of life or in the late stages, making them very difficult to analyze or treat. Recent studies have shown promising results, suggesting therapeutic strategies to extend healthy lifespan, by two common routes. Firstly, by decreasing the amount of ROS produced from complex I and secondly, by overcoming the deficiency of complex I (Sanz et al., 2010c).

Complex I has been also related with diabetes. Diabetes is considered to be one of the major risk factors for developing cardiovascular diseases (Ceriello and Testa, 2009). Recently, Diabetes has been found to be highly related with oxidative stress, which may account for the pathogenesis of most of the diabetic complications (Bonnard et al., 2008; Evans et al., 2002; Maddux et al., 2001). An impairment in complex I can alter mitochondrial metabolism and leads to the development of an impaired glucose-induced insulin secretion, leadings to severe anomalies (Jitrapakdee et al., 2010). A single mitochondrial DNA deletion upto 7 kb is sufficient to reduce the activity of complex I, which has been demonstrated in patients with a unique syndrome of transient diabetes (Poulton, 1992).

The relationship between complex I and many of these diseases maybe mediated by ROS. ROS produced by complex I are negatively correlated with longevity. Therefore, the less ROS produced by complex I, the slower an individual ages. However, as mentioned earlier, antioxidant supplementation or over expression does not prolong lifespan. Moreover, knock-down of most antioxidants does not reduce longevity. These results contradict MFRTA. A possible explanation is that only ROS generated by complex I play a role in aging. In fact, only ROS generated by this complex are correlated with longevity (Stefanatos and Sanz, 2011).

Thus, it may be that these ROS target specific molecules that cannot be protected by antioxidants i.e. unsaturated lipids of the membrane, DNA, or iron-sulphur clusters of complex I. If complex I is the main generator and the main target of ROS, changes in antioxidant levels would not affect longevity, and this is exactly the case. Theoretically, a decrease in the concentration of complex I should decrease the leak of electrons and reduce superoxide concentration, which, if our hypothesis is correct, should extend lifespan. To test this we have reduced the level of complex I in *Drosophila melanogaster*.

2.7 Drosophila as a model system

Drosophila is one of the most widely used model system for studies on genetics and developmental biology (Reeve, 2001). It was first used in the early 1900's by Thomas Morgan, Jeff Bridges and Sturtevant for the study of sex linkage and genetic recombination. Since then, *Drosophila* has been widely used in genetics and molecular biology. As a model organism, *D. melanogaster* has very few disadvantages. The flies cannot be frozen and stored live, as is the case with *Saccharomyces cerevisiase* or *Caenorhabidits elegans*. However, it's numerous advantages, including: short generation time, low cost, ease of culture, well-characterized genetics and high number of offspring make it ideal to study aging (Rubin and Lewis, 2000).

2.7.1 Drosophila genetics

Drosophila has been used as the model organism in the current study. There is a high (around 70%) sequence similarity (Reiter et al., 2001) between *Drosophila* and humans. Thus, results gained in Drosophila can be used to highlight possible novel genetic pathways in humans.

The fruit fly has a very small genome of just 13,600 protein-coding genes, when compared to the 40,000 genes in human genome (Halligan and Keightley, 2006). The total size of the gene is estimated around a size of 180 Mb (Aleksic et al., 2009), and was completely sequenced in the year 2000 (Adams et al., 2000). All the genes in the fruit fly are distributed on four chromosomes, 3 pairs of autosomal chromosomes, and one pair of sex chromosome.

Drosophila genetics has been a valuable tool in research, and numerous approaches have been used in *Drosophila* to gain insight into the function of those genes that have orthologs in humans. To generate these detectable phenotypes there has been two main approaches: i) the Mutational Approach; and ii) the transgenic approach. The mutational approach was invented in the year 1950, when it was discovered that *Drosophila* mutants can be created by X-ray mutagenesis which causes chromosomal rearrangement (Ives, 1959). The transgenic approach involves the expressing of a human disease gene in *Drosophila* to study the role of a disease gene in pathogenesis (Tower, 2000).

The double stranded RNA mediated RNA interference (RNAi) strategy has recently introduced as a powerful tool for determining genes. RNAi involves the use of double stranded RNA or small interfering RNA, to knockdown expression of a specific gene by causing the degradation of the target RNA (Mello and Conte, 2004).

2.7.2 Life cycle of *Drosophila*

Drosophila, like many other insects, undergoes complete metamorphosis. The total life cycle of *Drosophila* includes an egg, larval stage, pupal stage, followed by eclosion and the emergence of an adult fruit fly. The first step of embryogenesis takes place in the oviduct of the female, where the fertilization takes place. The fertilized eggs pass through several stages of development before hatching, they are: i) cleavage takes place for around 2 hours after fertilization; ii) the blastoderm is formed within 30 minutes to 1 hour after cleavage; iii) gastrulation takes place for around 20 minutes; iv) germ band elongation follows for approximately 3-4 hours; v) germ band retraction then takes place, lasting 2-3 hours; vi) head involution follows, leading on to dorsal closure and formation of the trachea; vii) the final step is the differentiation. Generally, embryos take around one day to develop, after which they hatch out as larvae.

The life cycle of the fly begins at this larval stage, by the formation of three stages of larvae, namely, first, second and third instars larvae. The first and the second instar larvae stage lasts around one day, whereas the third instar larvae can last up to two days. This difference between the two is easily noticed as the third instar larval stage is the stage where the larvae become mobile. This is characteristic of a larvae nearing pupa formation. The larvae at this stage climb up the culture tube in order to pupate. The different stages of the *Drosophila* development are shown in figure 2.3. Generally, eclosion of flies takes place during 9-10 days after mating at 25^{o}C. The pupal stage is where the complete metamorphosis of the larvae to fly takes place. However, this is highly dependent on temperature. The higher the temperature, the faster the life cycle of the flies (Ashburner and Thompson, 1978).

2.7.3 The GAL4/UAS system in *Drosophila*

The GAL4/UAS system is one of the most powerful systems used for gene expression in *Drosophila*. It plays a vital part in the transgenic approach adopted for genetic studies in *Drosophila*. The GAL4/UAS system is made up of two elements, namely the GAL4 activator protein and the UAS (upstream activating sequence). The GAL4 binds very specifically to the promoter of the desired gene and drives its expression via interaction with the UAS. To venture into more detail, the gene of interest is basically cloned into a vector, which is primarily coupled with, the UAS sequence. The GAL4 activator specifically binds to the UAS, resulting in expression of the gene of interest (Brand and Perrimon, 1993; Duffy, 2002). The mechanism above is explained in a diagrammatic representation in fig 2.3.

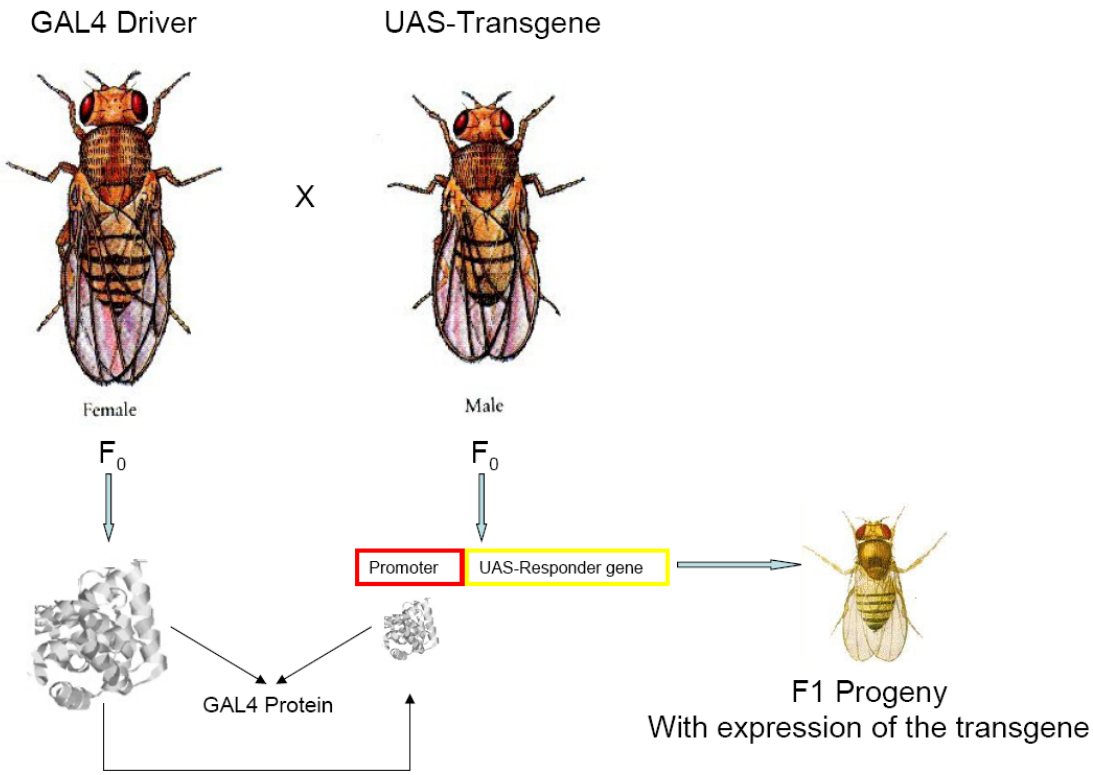


Figure 2.3: *A diagram showing the expression of the required gene in Drosophila, by a GAL 4 /UAS expression system.*

In *Drosophila*, the system is split into two parts. The GAL4 driver is maintained in a separate line, and the gene of interest, is kept under the control of the UAS in another line (Busson and Pret, 2007). When the two different flies are crossed, the specific binding of the driver (GAL4) to the UAS site, takes place, leading to the expression of the desired gene in the resulting offspring (Jones, 2009).

3. Materials and Methods

3.1 Fly stocks

A UAS-RNAi line knocking-down (Identifier number: 13131) the gene CG6020 (39 Kd subunit of complex I) was obtained from Vienna Drosophila RNAi Center. The *Drosophila* line expressing the UAS-NDI1 (NADH dehydrogenase internal 1), from *Saccharomyces cerevisiae*, was made as described in (Sanz et al., 2010b). The daughterless-GAL4 (daGAL4) gene line was used drive expression of both the NDI1 transgene, and the CG6020 knock-down construct. All lines were back-crossed into wild type DAHOMEY (DAH) background for 11 generations.

Both the knockdown construct and the transgene were expressed by crossing virgin females with the GAL4 gene, under the control of the daughterless promoter with males having both the RNAi construct, and/or the Ndi1 transgene. We have made use of the ubiquitous daGAL4 expression system for expression in all the flies used in subsequent experiments. Controls were obtained by crossing males containing, both the RNAi construct and the Ndi1 transgene, with Dahomey virgin flies. An additional control was made by crossing virgin females containing the daughterless-GAL4 construct with DAH males.

The following crosses, represented in the Table 3.1 were performed. The F1 offspring were used for all the experiments. All the female flies, used in the controlled genetic crosses were virgins. To perform controlled mating, all the adults were removed from the bottle, after they have laid eggs in the bottles for around 7 days in 18^{o}C.

The **X** in the table below, is used to indicate that two kinds flies have been mated. In the crosses, that we have performed, the female flies having the daGAL4 promoter sequence, and the desired transgene or knockdown construct was present in the male fly. Where, the daGAL4 promoter aids in the expression of the RNAi mediated knockdown and the NDi1 gene construct.

Table 3.1: *Crosses made to obtain all flies for subsequent experiments. Crossing of RNAi flies with the daGAL4 line . Creation of control lines involved crossing RNAi flies with the Dahomey line, crossing NDi1 flies with the daGAL4 line, and crossing the Dahomey line with the daGAL4 line.*

Parental Generation			**Progeny**
Female (P1)		**Male (P1)**	**F1 Offspring**
2>2 ; 3>3	X	RNAi CG6020>CyO ; NDi1>NDi1	RNAi CG6020/2 ; NDi 1/3
2>2; daGAL4>daGAL4	X	RNAi CG6020> CyO ; NDi1>NDi1	RNAi CG6020>2 ; NDi1>daGAL4
2>2; daGAL4>daGAL4	X	2>2 ; NDi1>NDi1	2>2 ; NDi 1>daGAL4
2>2; daGAL4>daGAL4	X	2>2 ; 3>3	2>2; daGAL4>3

All the progeny flies were developed and maintained on a 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.

3.2 Lifespan studies and Fly Maintenance

Crosses were done in bottles at 18ºC using 30 females and 15 males. Female flies were collected using CO2 anaesthesia within 48 hours of eclosion, and then transferred to a vial containing standard media. As the crosses were performed at 18^{o}C, collection was done every 48 hours to ensure that the flies collected were not virgins. 20 flies per vial were maintained in all the experiments. The temperature of 18^{o}C was maintained in a controlled 12 hour light and dark cycle for respective lifespan studies. Flies were transferred to new vials every 2-3 days, and the number of dead flies was recorded. At least 80 flies per group were used for lifespan studies. For all the other experiments the flies were aged for a minimum of 5 days.

3.3 Quantitative Real time PCR

3.3.1 RNA extraction

Adult flies, developed and aged for 5 days at 18^{o}C were used for this experiment. For complete RNA extraction, a total of 10 flies were anesthetized on ice in a tube and immediately frozen at -80^{o}C. The frozen flies were ground completely using a plastic homogenizer in 50µl of Tri-Reagent (Molecular Research Center Inc, Ohio, USA), and then after grinding another 450µl of Tri-Reagent was added to the Vial. The homogenates were then incubated at room temperature, for 5 minutes. After which, 100 µl of pure chloroform was added and the samples were vortexed thoroughly, followed by incubation at room temperature for 2-3 minutes, before centrifuging for 15 minutes at 12000g at 4^{o}C.

The upper aqueous phase obtained was transferred to a new tube, and mixed with 500µl of isopropanol. RNA was precipitated at room temperature for 10 minutes, and then pelleted by centrifugation. The pellet was thoroughly washed in 1ml of DEPC water with 75% ethanol. Washing was followed by vortexing the mix, and then centrifuging at 7500g at 4^{o}C for 5 minutes. After centrifugation, the ethanol mix was completely removed by using 0.5mm needle for suction.

The pellet was then air dried for 5-10 minutes at room temperature. After this, the RNA pellet was re-suspended with 89µl of DEPC treated water, and then subsequently treated with DNaseI (FERMENTAS INC., Maryland, USA) and DNaseI buffer (FERMENTAS INC., Maryland,USA) 1µl and 10µl respectively in a total volume of 100µl to remove any DNA contamination. Samples were then incubated at 37^{o}C for 1 hour.

Following DNaseI treatment, ethanol precipitation was used to purify RNA. RNA was precipitated by adding 1/10 volumes of 3M Sodium Acetate pH 5.2 and 2.5 volumes of 95% ethanol in DEPC treated water. RNA was precipitated at -20°C for a minimum of 30 minutes up to 1 hour. Samples were then centrifuged at 16000g for 20 minutes at 4^{o}C. The supernatant was removed by using a vacuum needle followed by two washes with 1ml of 75% ethanol in DEPC-Water. The pellet was left to dry at room temperature for 5 min, and then re-suspended in 10µl of DEPC water. The RNA concentration was then measured using a Nano-Drop 2000c (Thermo Scientific, Wilmington, USA) apparatus, and adjusted to around 1ug/µl, and then stored at -80^{o}C for cDNA synthesis.

3.3.2 cDNA synthesis

For each cDNA sample prepared, triplicates were made, and then pooled together for complete cDNA synthesis. Each synthesis reaction has a final volume of 20 µl, and a total of 2ug of RNA. All of the following reactions were performed in a 96 well 0.2 ml plate. 2µl of the 1ug/ul RNA sample was added to the corresponding reaction well, containing 9.6µl of DEPC water, 1µl of 10mM dNTP and 0.4µl of random hexamers. The mixed samples were then incubated in a thermal cycler at 90^{o}C for 3 minutes. After incubation, 4µl of 5X reverse transcriptase buffer and 1µl of RNase inhibitor as added to each sample. The samples were then incubated for 10 minutes at 25^{o}C. Finally 2µl of Reverse transcriptase was added to each individual reaction mix.

The samples were then incubated in a programmed cycle of 25^{o}C for 10 minutes, 37^{o}C for 60 minutes and 70^{o}C for 10 minutes. All the triplicated samples were pooled in a single tube and then, the cDNA was stored at -20^{o}C. The cDNA samples were checked for contamination via electrophoresis on a 2% agarose gel. For this verification procedure, a PCR was run using primers that span an intronic region, allowing differentiation of cDNA from genomic DNA via differences in amplicon size. 5µl of the PCR product were taken in a tube and mixed with DNA loading dye and the samples were loaded. The gel was also loaded with a positive control, to compare for contamination. The gel was run at around 120V for 30 minutes-1 hour.

3.3.3 qPCR-Standard Curve Method

Primers were designed using the “primer 3” primer design software found at [GIVE WEBSITE ADDRESS]. A stock was prepared by pooling together 5µl of all the prepared cDNA samples. 20 µl of the stock is then diluted in 80µl of nuclease-free water (FERMENTAS INC., Maryland, USA). Serial dilutions from this stock were made, namely 1:10, 1:100 and 1:1000. The stock was prepared in order to determine the standard curve and to be sure that, all the gene amplifications fall inside the standard curve. cDNA samples were diluted 20 times. All the standards and samples were added in triplicates in the 96 well plates and then stored at -20^{o}C.

The reaction mix used for qPCR contained 10µl of SENSI FAST enzyme (Bioline, Taunton, USA), 0.4µl of 20uM forward and reverse primer and 5.2 µl of Nuclease-free water. Fast optical 96 well plates (Applied Biosystems, San Francisco, USA) were used for qPCR. 4µl of samples and standards were pipetted into the reaction mix, making the final reaction volume20µl. The reaction mix and the samples were thoroughly mixed. The plate was then sealed with optical clear cover and spun down. qPCR was performed using a StepOne qPCR machine (Applied Biosystems, San Francisco, USA). The PCR cycle used consisted of 95 ^{o}C for 5s, 60^{o}C for 10s and then finally 72^{o}C for 5s. This cycle was repeated 45 times. The values were quantified by StepOne v2.1 software (Applied

Biosciences, San Mateo, USA). Amplification values of all the genes were normalized to values obtained from the amplification of the GAPDH gene (CG12055), in all the samples.

3.4 Western Blot Analysis

3.4.1 Protein Extraction

Around 10 flies, which were developed for approximately 5 days at 18^{o}C, were anesthetised with CO2 and stored at -80^{o}C in an eppendorf. Fresh flies were used for Ndi1 probing due to technical reasons. Frozen flies or fresh flies were then homogenized using a sterile plastic homogenizer, in homogenizing buffer (0,15gms of 1,5% Triton X-100, 1 tablet of pre-made complete mini EDTA-free protease inhibitor and 1 tablet of available phosphatase inhibitor (Roche Diagnostics, Mannheim, Germany), dissolved in 1 X PBS. After crushing the flies, they were incubated on ice for around 15 minutes, to let the protease inhibitor work. The samples were then subjected to centrifugation for 15 minutes at 13000g at 4^{o}C. Following this, the supernatant (total fly protein extract) was collected.

3.4.2 SDS-PAGE

The protein concentration of the samples was measured using a Bradford Assay. The samples were diluted up to 35 µg of protein in each sample with required amount of sterilized water and Sample buffer (40% v/v of Glycerol, 8% v/v SDS, 25% v/v 1M Tris-HCL pH 6.8 and Bromophenol blue slurry 0.015%). 8ml of sterilized water is added along with 20% v/v of 1M Dithiothreitol just before using. The diluted samples were then heated on a heating block at 100^{o}C for a minimum of 5 minutes and then moved to ice.

The electrophoresis tank was filled with Running buffer (0.25 M Trizma, 1.92 Glycine and 1% SDS). Ready-made gels from Bio-Rad (Criterion TGX, Marnes-la-Coquette, France) were used in all electrophoresis experiments. The protein ladders were filled with volume of 3µl at one end and 7µl at the other. 25µl of sample, containing 35 µg of protein, was loaded. The tank was subjected to a voltage of 80 V until the proteins have migrated into the gel. And then, the voltage was increased to 120 V and run for approximately 1 ½ hours or until the proteins migrated to the end of the gel. The gel was then removed from its casing and left soaking in running buffer until further use.

3.4.3 Blotting

Two techniques were used for transfer of proteins to a nitrocellulose membrane. I) Dry Blotting and II) Wet Blotting.

3.4.3 a) Dry Blotting

An iBlot dry blotting device (Invitrogen, New York, USA) was used for dry blotting. The recommended kit, iBlot gel transfer stacks (Invitrogen, New York, USA) were used. Stacks were assembled as per the manufacturer's instructions and run at 20 V for 6 minutes. After completion, the membrane was carefully transferred to a bath of 1X PBS.

3.4.3 b) Wet Blotting

The tank was filled with blotting buffer (10X stock containing 0.25 M Trizma, 1.92 Glycine and dissolved in water. 1 X working solution contains 10X blotting buffer, methanol and water in the ratio 1:2:7, respectively). The sandwich was then packed in the proper order and the blot was started. The blotting was done at 30mA overnight or 300-400mA for approximately 1-2 hours. The whole blotting procedure is done at 4^{o}C. After the run was complete, the sandwich was unpacked and the membrane was carefully transferred to a bath containing 1 X PBS solution.

3.4.4 Immunodetection

After transfer, the membranes were washed throughly with 1X PBS and then stained with Ponceau S (0.1 % w/v Ponceau S in 5% v/v acetic acid, made upto 1 litre with double distilled water) for a few seconds, to verify the complete transfer of proteins to the membranes. The membranes are washed throughly with water or 1X PBS to remove the stain. The mebranes were then subjected to blocking in 5% Milk, dissolved in 1X PBS-Tween for 2 hours, with continuous shaking. Different dilutions of primary antibodies with the appropriate secondary antibodies were used, which were also diluted in 5% Milk in 1X PBS-Tween.

The concentrations are as follows: anti-Complex I 39 Kda subunit (provided by Prof. Howard T Jacobs, Institute of Biomedical Technology, University of Tampere, Finland) 1:5000; anti-Ndi1 1:15000 (provided by the laboratory of Dr. Takao Yagi, Scripps Research Institute, La Joya, California); anti-Complex V Subunit Alpha (Anti-ATP5A antibody (Mitosciences, Oregon, USA)) 1:200,000; anti-GAPDH (c Terminus) 1:30000 (Everest Biotech Ltd, Oxforshire, United Kingdom), the secondary antibodies used were, horse anti-mouse IgG (Vector Labaratories, Burlingame, USA) used at 1:10000; anti-rabbit (Vector Labaratories, Burlingame, USA) used at 1:10000 and anti-goat (Santa Cruz Biotechnologies, Santa Cruz, USA) used at 1:5000.

The membranes were incubated in the primary antibody in 5% Milk, dissolved in 1X PBS-Tween for 2 hours at room temperature or overnight at 4^{o}C. After the incubation, the membranes were washed again with 1X PBS-Tween and incubated in the appropriate secondary antibody for 2 hours at room temperature. The membranes were then washed repeatedly with 1X PBS-Tween and then, finally, washed with PBS. For exposing the membranes or detection of the antibody, they were treated with substrate solutions Luminol enhancer and Immuno Star HRP peroxide buffer (Bio-Rad, Marnes-la-Coquette, France) at a 1:1 ratio. The membranes were 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.

3.5 Isolation of mitochondria

Around 40 flies were immobilized by placing on ice and then transferred to a chilled mortar. The number of flies used, were similar for all experiments involving the use of isolated mitochondria. 1 ml of ice-cold mitochondria isolation medium with BSA (250mM sucrose, 5mM Tris-HCL, 2mM EGTA, 0.1% w/v of BSA) was added. Then, the flies were gently crushed using a pestle. The homogenate was filtered using a 200µm polyamide mesh. And, another 1ml of the mitochondria isolation medium with BSA was added on top of the mesh. Homogenate was transferred to an eppendorf tube and centrifuged at 200g for 5 minutes at 4^{o}C.

Supernatant was collected and centrifuged at 9000g for 10 minutes at 4^{o}C. The obtained pellet was then resuspended in 50µl of isolation buffer without BSA (250mM sucrose, 5mM Tris-HCL, 2mM EGTA). The protein concentration of isolated mitochondria was calculated by Bradford's Assay. The mitochondria were then stored at -80^{o}C for a few experiments. For some experiments, fresh mitochondria were used. The use of mitochondria in different conditions, are mentioned specifically in the following experiments.

3.6 Separation and analysis of activity of Respiratory Complexes by Blue Native PAGE and in gel assay

Isolated mitochondria were used for this experiment. The procedure for isolation of mitochondria is, as explained previously. Fresh mitochondria from flies which were developed at 18^{o}C and aged for approximately 5 days were prepared for blue native PAGE. The protein concentration of the fresh mitochondria was measured just before the experiment using Bradford's Assay. The samples were then diluted to 100µg of protein per sample using appropriate ammounts of 50 mM Aminocaproic acid in 50 mM BisTris, Dodecylmaltoside and Native PAGE sample buffer (50mM BisTris, 6M HCL, 4% v/v Glycerol, NaCl 1% w/v, Ponceau stain 0,4% w/v, made upto 10 ml with water). The organelle proteins, were then solubilized in 25µl of ice cold 1X Native PAGE sample buffer containing 1% digitonin (Invitrogen, New York, USA) and Protease inhibitors (Roche, San Francisco, USA). The samples were mixed gently before being incubated on ice for 15 minutes. After incubation, the samples were centrifuged at 20000 g for 30 minutes at 4^{o}C. The supernatant was transferred to a new tube and mixed with 1.5µl of Coomassie Brilliant blue stain and 10 µl of 4 X Native PAGE sample buffer.

The tank was set up for the procedure and a readymade NativePAGE Novex 3-12% Bis-Tris gels (Bio-Rad, Marnes-la-Coquette, France) was used. Different buffers were prepared for the experiment, 20X Native PAGE running buffer (21% w/v BisTris, 18% w/v Tricine dissolved in water upto 1000 ml); 10X Anode Buffer (Native PAGE running buffer 50ml dilutted upto 1000 ml in water); 20 X Cathode Buffer additive (Coomassie G-250 1 gram in 250 ml Water); 1 X Dark blue Cathode buffer (5 % v/v 20X Native PAGE running buffer, 5 % v/v 20 X Cathode Buffer additive, made upto 200 ml with water); 1 X Light blue Cathode buffer (5 % v/v 20X Native PAGE running buffer, 0.5 % v/v 20 X Cathode Buffer additive made upto 200 ml with water).

Diluting these above mentioned solutions, to specefic dilutions 1 X Anode buffer, 1 X Light blue Cathode buffer and 1 X Dark blue cathode buffer were made. These three solutions, were used for the experiment. The tank was filled with 1 X Dark blue Cathode buffer, and with 1 X Anode buffer. Protein marker was added in the first well as a loading control before adding the samples. Around 15 µl of sample was loaded in each well, and the gel was run at 120 V for about 1 hour before 1 X Dark blue Cathode buffer was replaced with 1 X light blue cathode buffer. The apparatus was then placed at 4^{o}C overnight and maintained at a voltage of around 25-30V.

After the run was completed, the gel was removed from its plastic casing and stained with colorimetric staining solutions for around 1 hour to visualize all the respiratory complexes and the total protein content. Specific colorimetric activity buffers were also

used for visualising the activity of specific complexes. The gel was incubated in the activity buffers for around 15-120 minutes, the time span of incubation depending on the different complex acitivity buffers used.

For in-gel acttivity of complex I, the gels were incubated in a complex I activity buffer (2 mM Tris, 0,1 mg/ml NADH, 2,5 mg/ml Nitroblue tetrazolium chloride, pH 7,4), followed by fixing with destaining solution (50% Methanol, 10% acetic acid, dissolved in water) by incubation for 20 minutes. For in-gel activity of Complex IV, the gels were incubated in a complex IV activity buffer (5 mg 3.3′-diamidobenzidine tetra hydrochloride [DAB] dissolved in 9 ml phosphate buffer [0.05 M, pH 7,4], 1 mM catalase [20 µg/ml], 10 mg cytochrome *c*, and 750 mg sucrose). The gel was incubated for 2 hours for this specific complex activity buffer, and then was fixed with the destaining solution for 20 minutes. All these procedures were carried out at room temperature. After all the activity incubations were complete, the gels were rinsed thoroughly with water and then scanned.

3.7 Mitochondrial Oxygen consumption Measurements

Around 40 or 60 flies, which were approximately 5 days old and developed at 18^{o}C were collected in a vial and stunned on ice. The flies were transferred to a chilled mortar, and then, 500µl of isolation buffer without BSA (250mM sucrose, 5mM Tris-HCL, 2mM EGTA) was added. The flies were crunched to a certain extent, being extra careful not to break the mitochondrial membrane, and to avoid misleading respiration measurements. The homogenate was transferred on top of a 200µm polyamide net and collected in a beaker and transferred to a tube. Additional 500µl of isolation buffer without BSA was added on top of the net. All the extracts were carefully removed and transferred to a tube. This sample was immediately used for the oxygen consumption measurements.

Mitochondrial oxygen consumption from the homogenates was measured by high resolution respirometry, using an Oxygraph 2-K (Oroborous instruments, Innsbruck, Austria). Prior to the measurements, the chambers were thoroughly cleaned with 70% Ethanol and distilled water to remove any kind of contamination. Exactly 50µl of the fly homogenate was added in each chamber already filled with 1.95ml of assay buffer with BSA (120mM KCL, 5mM KH_2PO_4, 3mM Hepes, 1mM EGTA,1mM $MgCl_2$, 0.2% w/v BSA and calibrated to pH 7.2 at 25^{o}C) and chambers were closed. All complexes in the electron transport chain were considered for measurements. To study respiration through Complex I, 5µl of 2M pyruvate and 5µl of 2M proline and 4µl of 0.5M ADP were added. To study respiration without complex I, 1µl of 1mM rotenone was added. After which, 30 µl of 1.3M Glycerol-3-Phosphate was added to analyse respiration through Complexes III and IV. Complex III was then inhibited by 1µl of 5mM Antimycin A. After the reaction was completely blocked, Complex IV substrates Ascorbate (0.8M) and TMPD (0.2M)

were added in the respective volumes of, 5µl and 4µl. The whole reaction was inhibited by 2µl of 1M Potassium Cyanide.

Individual HAMILTON GASTIGHT syringes (Hamilton Bonaduz AG, Bonaduz, Switzerland) were used for all substrates and inhibitors. Respiration was measured as the stable rate produced, after the addition of substrate and the addition of specific inhibitors.

The protein concentrations of the previously extracted samples were measured by Bradford's Assay. O2 consumption values were normalized to protein concentration.

3.8 Fertility Assay

Experiments were also performed to check whether the knockdown of Complex I affects fertility. Around 100 female flies of all the groups were collected separately by CO_2 anesthesia and crossed with approximately 50 males flies (2>CyO:Ndi1>daGal4)) in bottles at 18^{o}C for over 48 hours. After this, the flies were again anesthetized on CO_2 and females alone were collected to new vials and stored at 18^{o}C to observe development. After 1 week, the parental female flies were discarded. Subsequently, the number of flies eclosing from each vial was counted for a span of 10 days, collecting every alternate day.

3.9 Mitochondrial ROS production

Around 40 flies, aged for approximately 5 days at 18^{o}C, were stunned on ice and used for the isolation of mitochondria as described before. The mitochondria were immediately placed on ice after the extraction. The protein concentration of the samples was measured by Bradford assay. Final protein concentration used in the following assay was 0,5 mg/ml concentration in 100µl final reaction volume.

The buffer was prepared containing 50µl of 10mM of commercially available Amplex red (Invitrogen, Oregon , USA) stock solution, 100µl of 10 mM Horse Radish Peroxidase (Invitrogen, Oregon , USA) stock solution and 80µl of 6250 U/ml SOD stock solution (Sigma-Aldrich, Buchs, Switzerland). The whole mix was made up to 10 ml with assay buffer containing BSA (120mM KCL, 5mM KH_2PO_4, 3mM Hepes, 1mM EGTA,1mM $MgCl_2$, 0.2% w/v BSA and calibrated to pH 7.2 at 25^{o}C). The prepared 100 µl volume of buffer was always stored on ice. In each well appropriate amount of buffer was added, considering the amount of substrates, inhibitors and mitochondria to be added. Initially, the mitochondria were added in all the required wells, and then, all the inhibitors were added according to specific final concentrations. The appropriate

substrates (Table 3.2) were added in the wells just before the measurements, to validate the immediate reaction response.

Table 3.2*: The substrates and inhibitors used and their concentration and final volume are described in the table below*.

Reaction Solutions	Final Concentration	Volume Used
Substrates		
Pyruvate+Proline	5mM	2µl
ADP	1mM	1µl
Glycerol-3-Phosphate	20mM	2µl
Inhibitors		
Rotenone	0.5µM	1µl
Antimycin A	0.5µM	1µl
Oligomycin	1µg/µl	2 µl

A total of 9 experiments were performed. A blank without mitochondria but with all the other components of the reaction was used as negative control. The experiments are as follows: (1) mitochondria alone (first control); (2) the mitochondria with oligomycin (second control, that was used as a blank for the experimental samples); (3) mitochondria + pyruvate+proline substrate was added to initiate the reaction; (4) G3P instead of pyruvate+proline susbstrate; (5) G3P + rotenone; (6) pyruvate+proline and G3P; (7) pyruvate+proline + ADP; (8). pyruvate+proline+ rotenone; and (9) G3P+ antimycin A. Additionally, two other reactions with mitochondria + inhibitors were also used as specific blanks for those reactions involving inhibitors.

All of the above mentioned reactions were conducted in separate wells and considered as individual experiments. Mitochondrial concentration was added to equal concentration for all reactions, susbstrate and inhibitor volumes used are mention in table 3.2. The reaction plate, containing all the reactions, was measured in kinetic mode in a Chameleon V o plate reader (Hidex, Turku, Finland) for a span of 30 mins (530nm excitation and 595nm emission). The temperature was maintained at 25^{o}C over the course of the experiment. The reaction was stopped after approximately 30 mins, and the calculations for ROS production were done using the slope of the increase in fluorescence.

Appropriate experiments were also performed to obtain standard curvesused to measure ROS concnetrations. The standard curve for calculating the units of flourescence produced was prepared using glucose oxidase enzyme. A glucose oxidase stock was prepared by adding 0.04 U/ml in water. A solution of glucose (280mM) was also prepared for each reaction. Appropriate volumes of assay buffer were added to each

well to a 100µM final reaction volume, with different volumes of glucose oxidase (0.5, 10, 15, 20, 25 µl). The stock of the glucose oxidase was diluted upto 1:1000 times in water before adding it to the wells. The reaction was started by adding 5µl of 280mM glucose. The flourescence was then measured at the same temperature, and excitation-emission wavelength as used for ROS measurements for a duration of 45 minutes, for both the standard curves. The average of the subsequent standard curve values were used for the calculation of units of flourescence produced.

3.10 CAFE (Capillary Feeding) Assay

The flies were aged for 2 days at 18^{o}Cprior to collection for experiments. To begin with, around 10 tubes per fly group were taken, and punched with holes on the top and the sides, sufficient for the capillary to fit and for ventilation. 10 tubes with 1 fly each was considered as one experiment. The flies were then anesthetized on CO_2 and then transferred to a 1.5 ml tube. The capillaries were then filled with CAFE assay food containing 5% sucrose and 5% yeast extract. The capillaries were then fit inside the holes in the tube with the flies. The whole container, in which all the tubes were placed, was also filled with a small amount of water to maintain equilibrium in humidity. The quantification of actual ingestion of food by the flies was done by putting a known amount of food in the capillaries, and then measuring the variations every 24 hours. The evaporation of the food was controlled by measuring the capillaries in the tubes without flies. The analysis was carried out for around 120 hours. The whole set up was placed at 18^{o}C for the required measurements.

3.11 Activity of Flies

Flies that were aged for 3 days o at 18^{o}C were used for the following experiment. Around 40 flies were anesthethised on CO_2 and put in separate capillaries with 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).

The capilarries were plugged at one end with rubber cork, and other end with a sponge to let air in. The locomotion activity of the flies was measured using a Digitherm Circ Kinetics monitoring incubator (Tritech Research, Los Angeles, USA). The analysis temperature was maintained 18^{o}C, with a 12 hour alternative light and dark cycle. The capillaries with fly food and the flies were connected to a monitoring device. The number of times the fly crossed the center of the capillary was monitored by an in-built sensor and the readings were integrated by using TriKinetics software. The average locomotor activity of 40 flies every 30 minutes were combined and calculated for statistical analysis. The total analysis was done over a time span of 96 hours.

3.12 Weighing of the flies

Around 10 flies, which were collected immediately after eclosion, were anesthetized by CO_2 and then transfered into a tube. The weight of the tube was normalized and the weight of the flies was calculated. The procedure was repeated for flies aged for 5 days in a tube with standard food. Both the experiments, using flies immediately after eclosion and 5 days old flies, were performed 10 times to obtain statistically significant data.

3.13 Statistical Analysis

All data were analyzed using using GraphPad Prism 4. The level of significance of all the data were evaluated by one way ANOVA, with $p<0.05$ being the accepted level for statistical significance. Newman-Keuls Multiple Comparison Test was chosen as Post-Anova test to compare different experimental groups.

4. Results

We verified the knockdown of the CG6020 gene at the RNA, protein and whole complex assembly levels by several procedures. In parallel to this, we also analyzed how knockdown of complex I affects mitochondrial respiration and ROS generation.

4.1 Verification of knock-down of the mRNA, of the CG6020 gene (Subunit 39Kd of complex I).

Flies expressing both the RNAi construct for CG6020 and the ectopic protein Ndi1 (RNAi CG 6020>2; Ndi1>daGAL4) were used as the target groups; whereas three others lines were used as control: (1) non-expressing flies (RNAi CG 6020>2; NDI1>3); (2) a control for the Ndi1 gene in the target lines, namely flies expressing only the yeast Ndi1 protein (2>2; Ndi1>daGAL4); and (3) flies with the daughterless-GAL4 construct, that drives ubiquitously the expression of the genes with an UAS promoter (2>2; daGAL4>3). Expression levels of each of the selected genes were analyzed, using q-PCR.

The resulting data shows that, the expression levels of the CG6020 gene have been significantly down-regulated. There was an approximately 2 fold decrease, after normalization to the house-keeping gene GAPDH, in the mRNA levels of CG6020 in the line where the knockdown was induced. There was no significant difference in the levels of the same gene in any of the control lines, proving efficiency and specificity of the RNAi construct (fig 4.1).

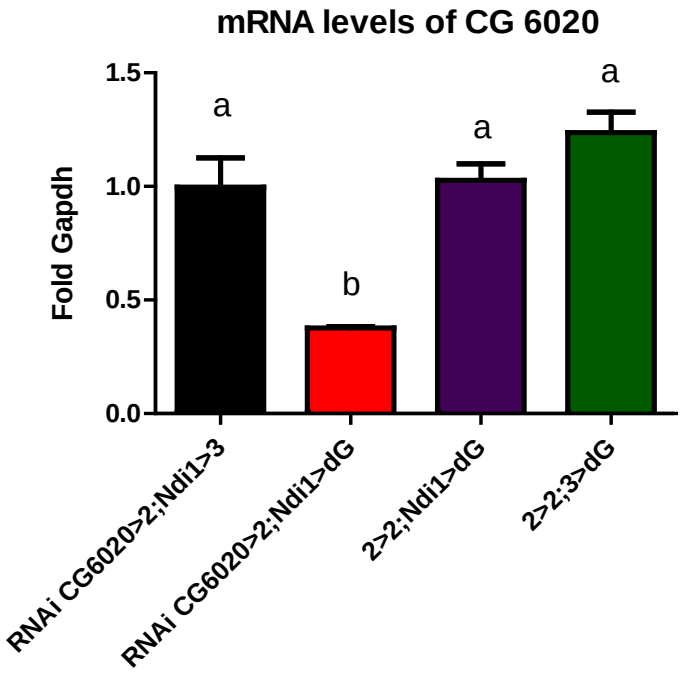


Figure 4.1: *Relative expression levels of mRNA of the CG 6020 gene measured by q-PCR. Data are shown as the average ± SEM of 3 independent samples per group. Colored bars represent the RNAi non-expressing line, the RNAi expressing line, the Ndi1 construct expressing line and the daughterless-GAL4 construct control line, respectively. The RNAi CG 6020 indicates the presence of the RNAi construct for the CG 6020 gene, Ndi1 indicates the presence of the gene that codes for Ndi1 protein, and dG indicates the presence of the daughterless-GAL4 construct. Y-Axis denotes the times fold increase in amplification of that specific gene. Different letters, a or b indicate a significant statistical differences between the groups. (ANOVA, P<0,05).*

The data obtained also shows a very significant amplification in the Ndi1 gene in with the Ndi1 gene and the daGAL4 construct (fig 4.2). The amplification in both target lines was noted to be almost equal, as they have one copy of the Ndi1 construct.

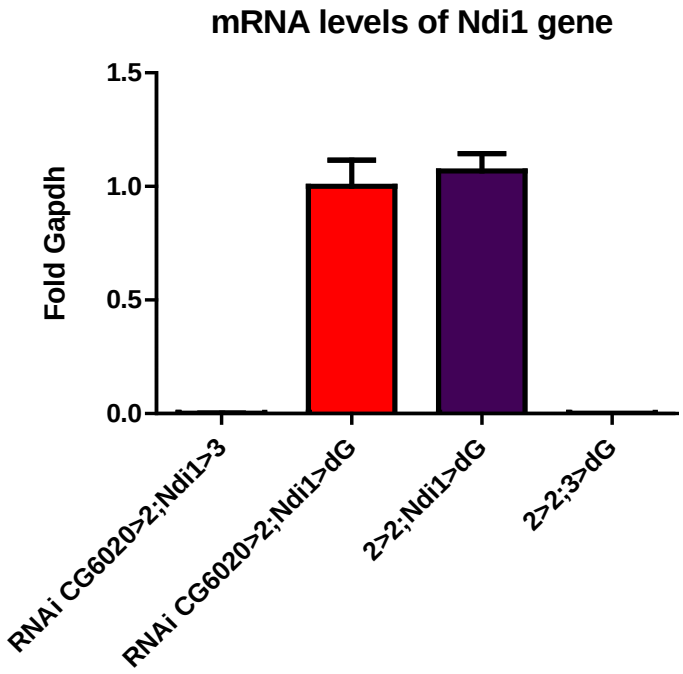


Figure 4.2*: Relative expression levels of mRNA of Ndi1 gene measured by q-PCR. Data are shown as the average ± SEM of 3 independent samples per group. Colored bars represent the RNAi non-expressing line, the RNAi expressing line, the Ndi1 construct expressing line and daughterless-GAL4 construct control line, respectively. The RNAi CG 6020 indicates the presence of the RNAi construct for the CG 6020 gene, Ndi1 indicates the presence of the Ndi1 gene, and dG indicates the presence of the daughterless-GAL4 construct. Y-Axis denotes the times fold increase in amplification of that specific gene.*

4.2 Confirmation of the knock-down at protein level by western blot

Western blots were performed on four lines (target and control lines, as described in the previous experiments) using specific antibodies for the 39kDa subunit. No signal was detected in the group where 39kDa subunit was knocked down using RNAi (fig 4.3A), whereas a clear band of similar intensity was present in all the control groups. No differences were observed in the levels of complex V subunit alpha or complex IV subunit COX IVa, demonstrating the specificity of the knock-down (fig 4.3B and 4.3C). A similar probing of Ndi1 on the same target lines and control lines also proved the expression of Ndi1 (fig 4.3D), only when the gene was expressed using the daughterless-GAL4 construct. GAPDH, a cytosolic protein, which is unaltered, was used as a loading control for all the experiments performed (fig 4.3E).

RNAi CG6020	+	+	-	-
NDI1 ^{A46}	+	+	+	-
daGAL4	-	+	+	+

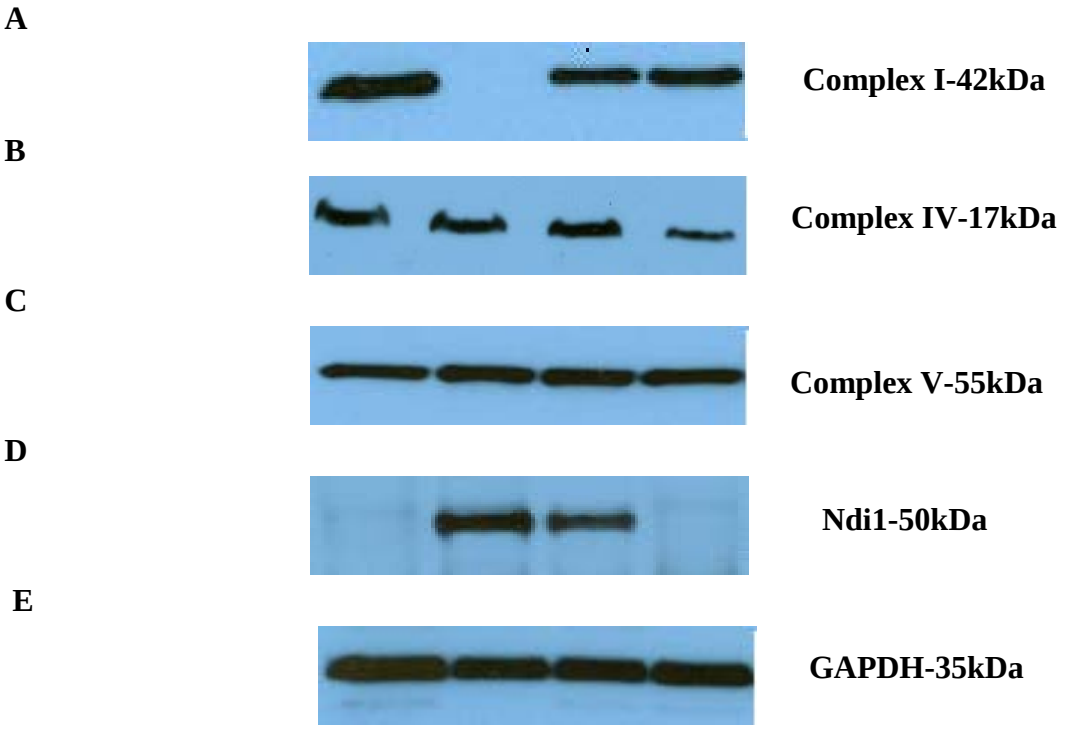


Figure 4.3: *Western blot using protein extracted from whole flies homogenates of the indicated genotypes, namely the RNAi expressing line, the RNAi non-expressing lines, the Ndi1 construct expressing line and the daughterless-GAL4 construct, control line. The RNAi CG6020 indicates the presence of the RNAi construct for the CG6020 gene, Ndi1 indicates the presence of the gene that codes for Ndi1 protein, and daGAL4 indicates the presence of the daughterless-GAL4 construct. Probed with A) Complex I subunit 39Kda and B) Complex IV, subunit COX IV a C) ATP synthase Complex V, subunit alpha D) Ndi1 E) GAPDH. Symbol + denotes the presence of the gene or the expression system and -, the absence of the gene or the expression system.*

4.3 Measurement of complex I assembly and activity by BN-PAGE and in gel activity

Respiratory complexes were separated by BN-PAGE to test how the knock-down of 39kDa subunit affects the assembly of Complex I. A general staining of the gel was made to study the assembly of all respiratory complexes. Levels of complexes II, III, IV and V were unchanged in all the groups, but complex I concentration was reduced in the lines where the knock-down of the gene CG6020 was induced (fig 4.4). In parallel, the activity of Complex I and IV was studied using specific staining buffers. .

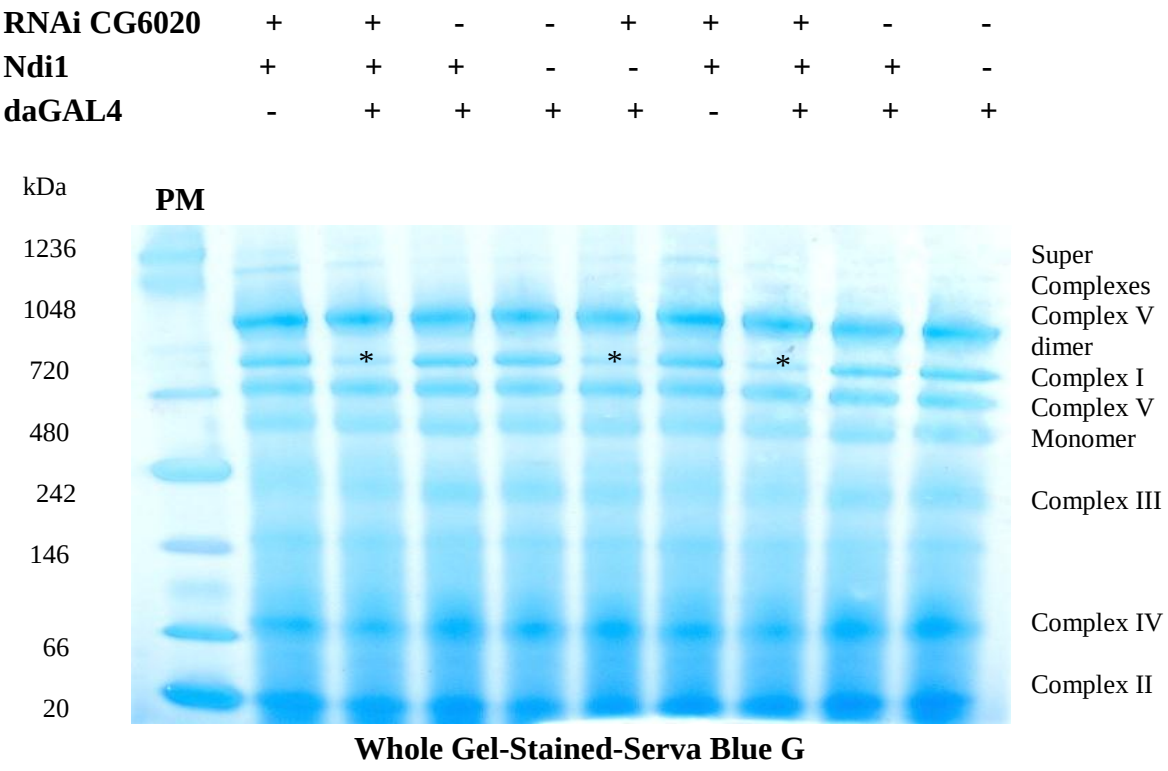
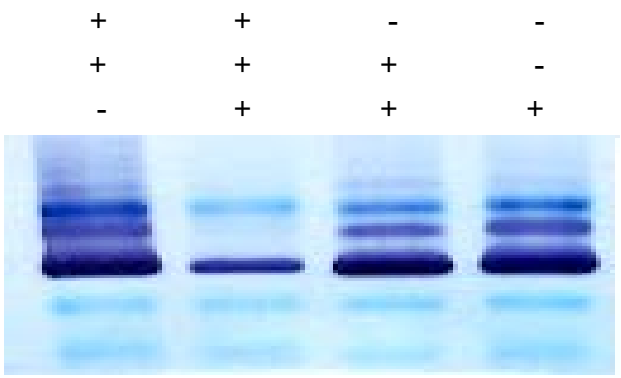


Figure 4.4*: BNE gel of fresh mitochondrial protein extract of female flies from the RNAi expressing line, the RNAi non-expressing line, the Ndi1 construct expressing line and the daughterless-GAL4 construct control line, displaying the separation of different complexes determined by size. The RNAi CG 6020 indicates the presence of the RNAi construct for the CG 6020 gene, NDI1A46 indicates the presence of the gene that codes for NDI1 protein, and daGAL4 indicates the presence of the daughterless-GAL4 construct. '+' denotes the presence of the gene or the expression system and '-' denotes the absence of the gene or the expression system. The first lane was loaded with a protein marker. The diagram clearly presents the separation of different Complexes (1-V) and supercomplexes with respect to size in kDa (kilo Daltons), which are mentioned in the sides of the diagram. An asterisk in the diagram denotes the missing Complex I in the RNAi expressing samples.*

Staining of complexes I and IV, shows a specific decrease in complex I activity in the lines where CG6020 was knocked-down (fig 4.5A), whereas complex IV activity was similar in all the groups (fig 4.5B). These results indicate that complex I activity is specifically reduced, and other complexes of ETC were not affected by the knockdown. This is also complemented by results from experiments in the following sections.

A



B

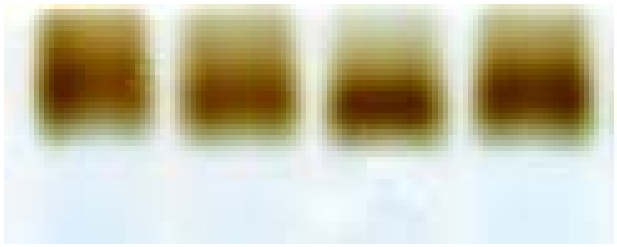


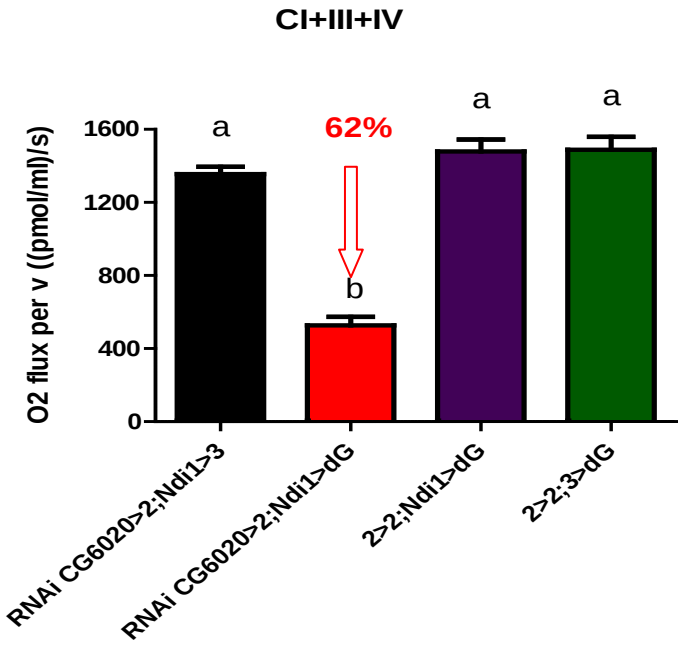
Figure 4.5*: In gel activites of complex I and IV of mitochondrial extracts of the RNAi expressing line, the RNAi non-expressing lines, the Ndi1 construct expressing line and the daughterless-GAL4 construct control line. The RNAi CG 6020 indicates the presence of the RNAi construct for the CG 6020 gene, Ndi1 indicates the presence of the gene that codes for Ndi1 protein, and daGAL4 indicates the presence of the daughterless-GAL4 construct. Activity staining of mitochondrial complexes. A) Complex I and B) Complex IV. Knockdown expressing lines cleary shown a specific decrease in the activity of Complex I activity when compared to the control lines.*

4.4 Study of mitochondrial respiration using high-resolution respirometry

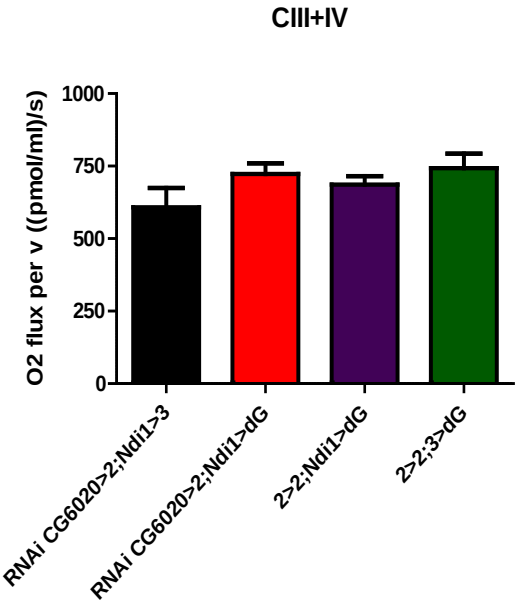
We have also verified the knockdown of complex I by measuring mitochondrial respiration using high resolution respirometry.

Mitochondrial respiration was measured by adding complex I-linked substrates, pyruvate+proline, followed by ADP (Adenosine diphosphate) to initiate oxidative phosphorylation (state 3). Target lines and control lines the same as in previous experiments, namely the RNAi expressing line, the RNAi non-expressing lines, the NDi1 construct expressing line and the wild type line. State 3 respiration -normalised to protein concentration, showed similar respiration in all control lines. However, the line where complex I was knocked-down showed a significant reduction in respiration (One way ANOVA, ($P<0,05$)). The decrease in respiration was around 62% (fig 4.6A). Then, G3P was added as a complex III-linked substrate. Under these circumstances, no differences in respiration were observed between flies with complex I-knockdown and controls (fig 4.6B). Specific complex IV substrates- ascorbate and Tetramethyl-p-Phenylenediamine (TMPD), produced similar results, with no statistical significance between the different groups (fig 4.6C). This indicates a specific knock-down of complex I, and no interference with other complexes.

A



B



C

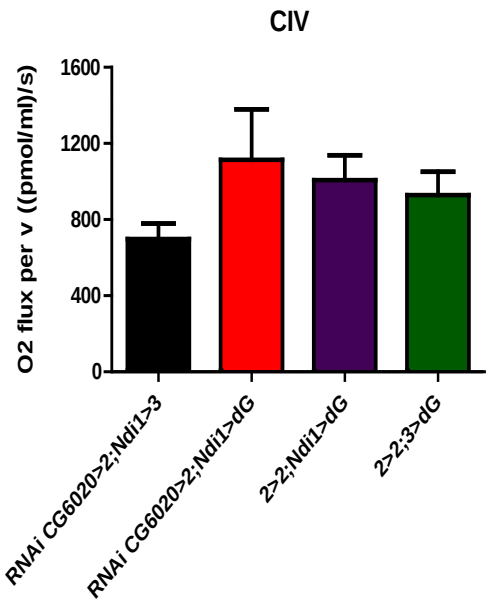


Figure 4.6*: Mitochondrial respiratory flux rates per mg of protein of whole fly homogenates. Colored bars represent the RNAi non-expressing line, the RNAi expressing lines, the Ndi1 construct expressing line and the daughterless-GAL4 construct control line, respectively. The RNAi CG6020 indicates the presence of the RNAi construct for the CG 6020 gene, Ndi1 indicates the presence of the gene that codes for Ndi1 protein, and dG indicates the presence of the daughterless-GAL4 construct. Data are shown as the average ± SEM of 6 independent samples per group. A) Complex I+III+IV dependent respiration with pyruvate+proline as substrate. B) Complex III+IV dependent respiration with G3P as a substrate C) Complex IV dependent respiration with TMPD+Ascorbate as substrates. All the measurements were done in presence of ADP. The percentage in Figure A denotes the intensity of the knockdown. Different letters a or b indicate a significant statistical differences between the groups. (ANOVA, P<0,05).*

4.5 mtROS production

The amount of Hydrogen peroxide (H_2O_2) produced from isolated mitochondria of different flies was also analyzed. A total of 40 flies were used for the extraction of mitochondria, and was considered as an independent experiment.

The production of H_2O_2 was significantly lowered in the complex I knockdown line compared to the control lines when Complex I-linked substrates, Pyruvate and Proline were used (fig 4.7B). Under these experimental conditions H_2O_2 is produced from both complexes I and III. An approximately 55% reduction in H_2O_2 production was visualized under these conditions. Maximal production of ROS by complex I (using pyruvate + proline + Rotenone) was also 67% lower in flies possessing reduced complex I levels (fig 4.7A). A similar trend was also seen in flies expressing the Ndi1 gene alone, although the difference was not statistically significant.

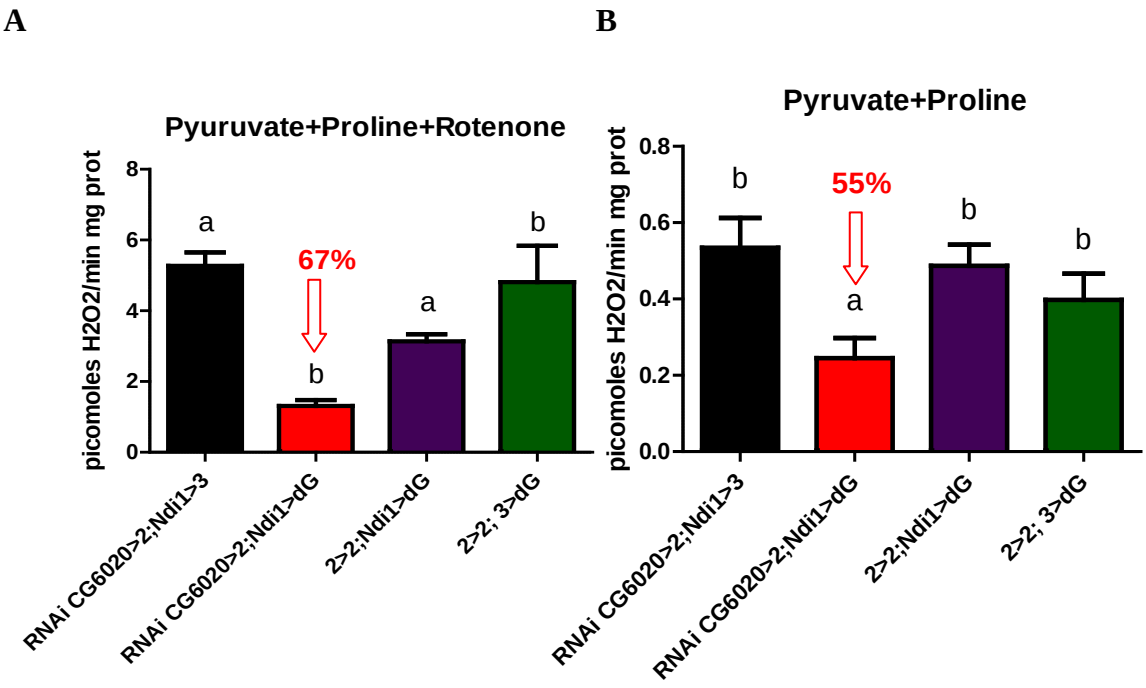


Figure 4.7*: Mitochondrial free radical production using Complex I-linked substrates. Colored bars represent the RNAi non-expressing line, the RNAi expressing line, the Ndi1 construct expressing line and the daughterless-GAL4 construct, control lines, respectively. The RNAi CG 6020 indicates the presence of the RNAi construct for the CG6020 gene, Ndi1 indicates the presence of the gene that codes for Ndi1 protein, and dG indicates the presence of the daughterless-GAL4 construct. A) H_2O_2 levels from mitochondria using complex I-linked substrates (pyruvate+ proline as substrates). B) To study maximal ROS production by complex I rotenone was added to the buffer. Data are shown as the average ± SEM of 8 independent samples per group. Different letters a or b indicate a significant statistical differences between the groups. (ANOVA, P<0,05).*

Experiments with complex III linked substrate (G3P+Rotenone) were also performed to analyze the production of H_2O_2 downstream of complex I (mainly by complex III). Under these conditions, no significant difference between the groups was detected (fig 4.8A). This confirms that the main decrease in ROS detected in experiments described above is located at complex I. Further confirming these results, we measured maximal production by complex III, using G3P + antymicyn A. Under these experimental conditions, complex III is the main generator of ROS; no significant differences were observed between the groups either (fig 4.8B).

A **B**

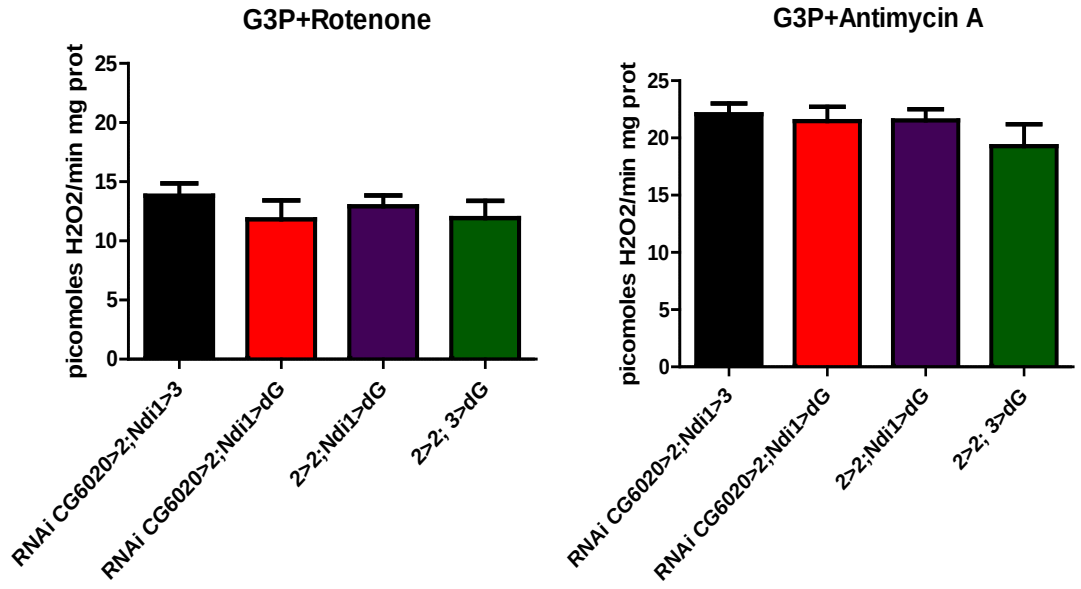


Figure 4.8*: Mitochondrial free radical production using complex III-linked substrate G3P. Colored bars represent the RNAi non-expressing line, the RNAi expressing lines, the Ndi1 expressing line and the daughterless-GAL4 construct, control line, respectively. The RNAi CG 6020 indicates the presence of the RNAi construct for the CG 6020 gene, Ndi1 indicates the presence of the gene that codes for Ndi1 protein, and dG indicates the presence of the daughterless-GAL4 construct. A) H_2O_2 levels from mitochondria using complex III-linked substrate (G3P + rotenone). B) To study maximal ROS production by complex III antymcyin A was added to the buffer. Data are shown as the average ± SEM of 8 independent samples per group. One way ANOVA analysis used to determine the significance, $P<0.05$.*

4.6 Lifespan

To test our main hypothesis: complex I is a master regulator of longevity, we have measured the lifespan in a fly line where complex I assembly was significantly reduced. Flies used in the previous experiments were used again here; the lifespan experiments were conducted at 18^{o}C.

Knockdown of complex I has a significant effect on lifespan in Drosophila. We were able to clearly visualize a significant increase in mean, median and maximum lifespan of the RNAi expressing flies when compared to the other lines (fig 4.9). As previously described (Sanz et al., 2010), Ndi1 extends median lifespan by 19% and maximum lifespan by 7%, whereas the knockdown of complex I with co-expression of Ndi1 increases median lifespan by 22%, and more importantly, maximum lifespan by 14%. This indicates that the less electrons flow through complex I, the more lifespan is extended.

The Prism GraphPad software was used to construct survival curves for calculating the lifespan results. The survival curves were further statistically analyzed using the Log-rank Martel-Cox test. Those analyses showed a statistically significant ($p<0.05$) increase in lifespan of the line with knock-down of complex I compared to the other lines.

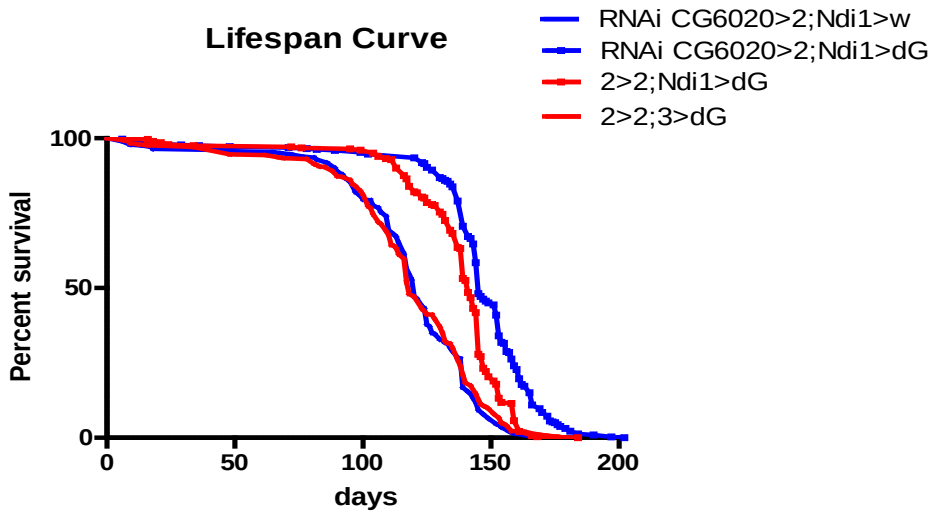


Figure 4.9: *Knockdown of complex I increases lifespan of Drosophila melanogaster. The figure shows the lifespan curves of female flies in Dahomey background. The blue dotted line represents the RNAi expressing flies and also shows a significant increase in median and maximum lifespan. The red dotted lines are the Ndi1 expressors, the blue line denotes the RNAi non-expressing lines and the red line denotes the life span of the flies with daughterless-GAL4 construct. X-Axis represents the number of days and Y-Axis denotes the percentage of survival of the flies.*

4.7 Fertility assay

The levels of fertility of the different groups of flies were measured to check, how reductions in complex I affect this parameter. Crosses for all the four groups were made (crosses explained in the Materials and Methods section). The flies were crossed in vials and the number of flies' eclosing on subsequent days was assessed.

The results obtained show that flies with low levels of Complex I are not sterile, although a small yet statistically significant decrease in the number of flies eclosing was observed. The RNAi expressing lines seemed to be less fertile than the other groups as the number of flies eclosing was less than the number of flies' eclosing in other groups. ANOVA analysis indicate that there is significant differences between the groups ($P<0.05$). However, differences were small and there were none detected by the post-test analysis. Nevertheless, the number of eclosing flies was clearly lower in the RNAi expressing group (fig 4.10).

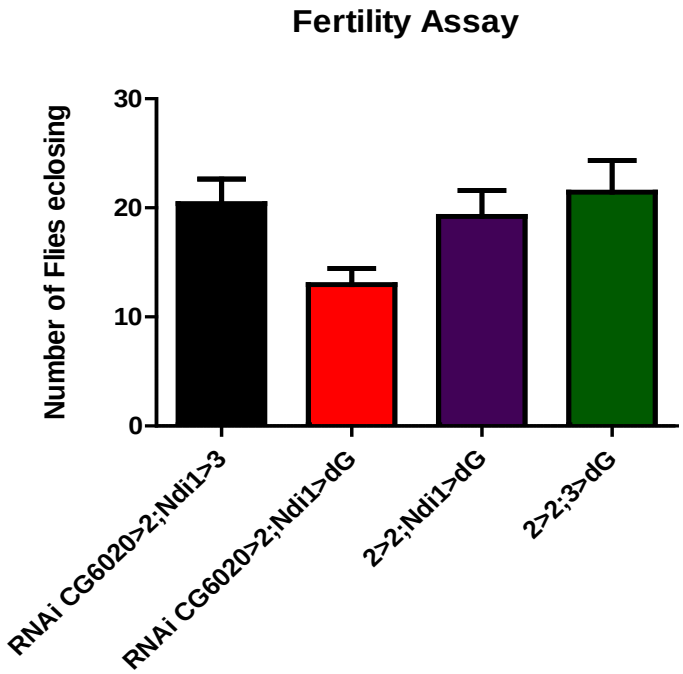


Figure 4.10*: Number of flies eclosing each day were recorded and analyzed. Colored bars represent similar groups, the RNAi non-expressing line, the RNAi expressing line, the Ndi1 construct expressing line and the daughterless-GAL4 construct, control line, respectively. The RNAi CG6020 indicates the presence of the RNAi construct for the CG6020 gene, Ndi1 indicates the presence of the gene that codes for Ndi1 protein, and dG indicates the presence of the daughterless-GAL4 construct. Data are shown as the average ± SEM of 25 independent samples per group. Statistical significance analyzed by 1 way ANOVA, P<0,05.*

4.8 CAFE (Capillary Feeding) assay

We have also assayed the actual intake of liquid food by housing individual female flies in 1.5 ml tubes. A special liquid food containing 5% sucrose and 5% yeast extract was used. The readings were measured every 24 hours. The statistical analysis of the data we have obtained showed a significant increase of food intake by the flies with reduced levels of complex I, although differences were only statistically significant with respect to one of the control groups (fig 4.11).

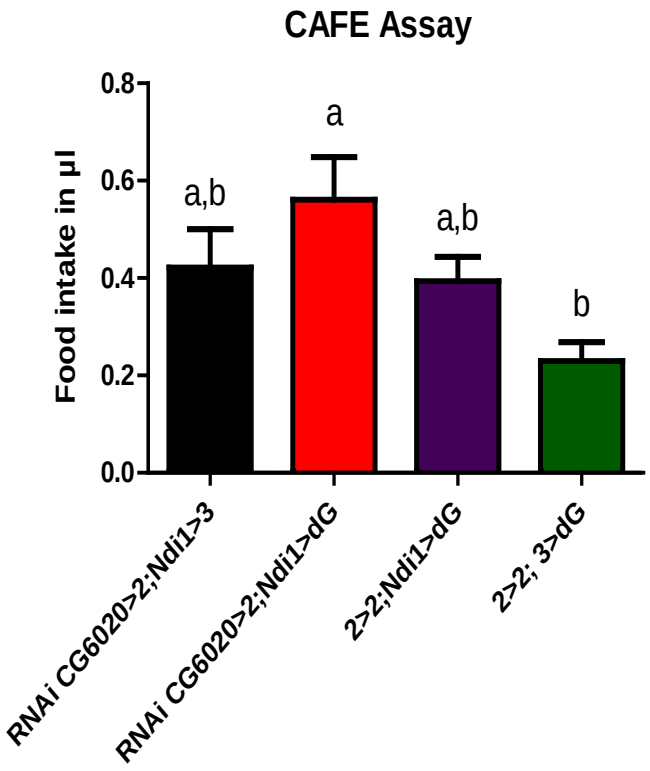


Figure 4.11*: Ingestion of food analyzed by CAFE assay (Capillary Feeding). The volume of consumption of the liquid food is measured every 24 hours, calculated and used for statistical analysis. Colored bars represent the RNAi non-expressing line, the RNAi expressing lines, the Ndi1 construct expressing line and the daughterless-GAL4 construct, control line, respectively. The RNAi CG 6020 indicates the presence of the RNAi construct for the CG 6020 gene, Ndi1 indicates the presence of the gene that codes for Ndi1 protein, and dG indicates the presence of the daughterless-GAL4 construct. Data are shown as the average ± SEM of 40 independent samples per group. Different letters a or b indicate a significant statistical differences between the groups. (ANOVA, P<0,05).*

4.9 Locomotor activity of the flies

There was a significant (one way ANOVA, $p<0.05$), increase in the locomotion of flies expressing the NDI1 protein (fig 4.12). However, no significant difference was detected between the group expressing only Ndi1 compared to the one co-expressing the knock-down construct (ANOVA, $p>0.05$).

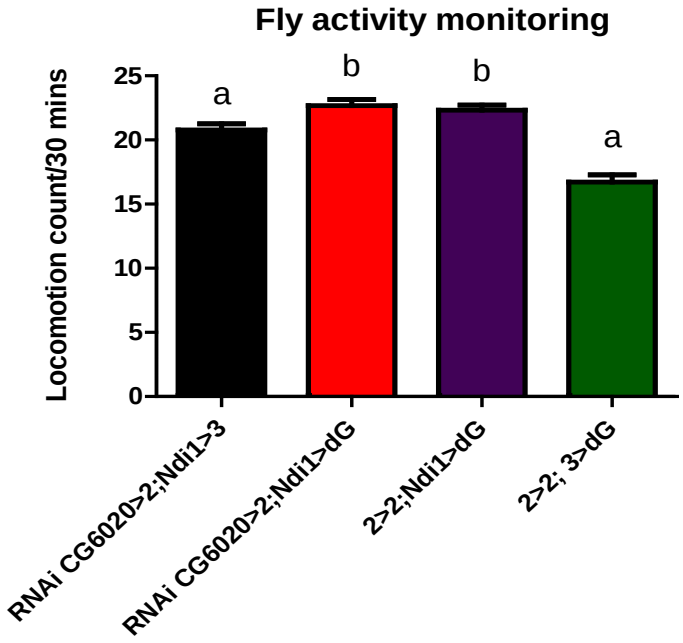


Figure 4.12*: Locomotor activity of flies at 18°C. As many as 32 flies used per experiment and these 32 flies were considered as individual experiments for calculation. The averages locomotor activity of the flies during every 30 minutes was calculate and plotted. Bar chart showing averages of locomotion data for four different groups of flies. Colored bars represent the RNAi non-expressing line, the RNAi expressing line, the Ndi1 construct expressing line and the daughterless-GAL4 construct, control line, respectively. The RNAi CG 6020 indicates the presence of the RNAi construct for the CG 6020 gene, Ndi1 indicates the presence of the gene that codes for Ndi1 protein, and dG indicates the presence of the daughterless-GAL4 construct. Data are shown as the average ± SEM of 32 independent samples per group. Different letters a or b indicate a significant statistical differences between the groups. (ANOVA, P<0,05).*

4.10 Weight of Flies

The weight of the flies measured immediately after eclosion did not show any significant difference between the groups (fig 4.13A). There was a significant difference in weight of flies after they were aged for 5 days. However, the differences were not consistently associated with any specific characteristic of the experimental groups since flies expressing the knockdown of complex I and flies possessing the daughertless-GAL4 construct alone were lighter (fig 4.13B).

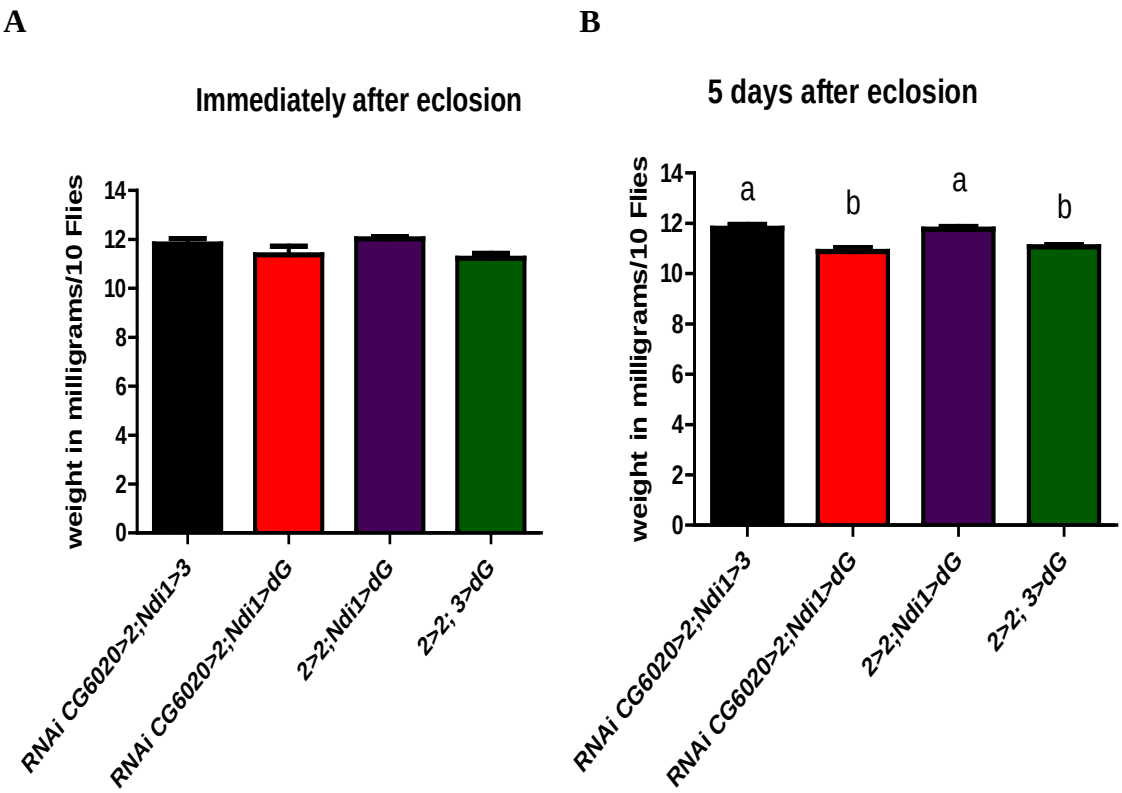


Figure 4.13*: Weight in milligrams of 10 flies per group considered as a single experiment (n=10). Colored bars represent the RNAi non-expressing line, the RNAi expressing line, the Ndi1 construct expressing line and the daughterless-GAL4 construct, control line, respectively. The RNAi CG 6020 indicates the presence of the RNAi construct for the CG 6020 gene, Ndi1 indicates the presence of the gene that codes for Ndi1 protein, and dG indicates the presence of the daughterless-GAL4 construct. Data are shown as the average ± SEM of 10 independent samples per group. A) Flies used for weight measurements immediately after eclosion. B) Flies used for weight measurements 5 days after eclosion. One way ANOVA, P<0,05. The Y-Axis denotes the weight in milligrams of the flies and the X-Axis denotes the different groups of flies used for this experiment. Different letters a or b indicate a significant statistical differences between the groups. (ANOVA, P<0,05).*

5. Discussion

5.1 Knock-down of CG6020 gene prevents complex I assembly

The complex I of the respiratory chain is a multi-subunit structure, made up of about 46 subunits (Yano, 2002). These subunits are encoded by the mitochondrial, and the nuclear genome. Of those subunits, 14 (75, 51, 49, 30 25, PSST, TYKY and the ones coded by mtDNA: ND1-ND6 and ND4L) are the minimum needed for complex I function (oxidation of NADH and pumping of protons). These 14 essential subunits are conserved from bacteria to humans (Yano, 2002), whereas the rest are accessory or supernumerary subunits, whose role in complex I assembly and function is not fully understood yet. A lot of studies have been performed on the catalytic subunits, whereas very little is known about the supernumerary subunits. The catalytic subunits, as mentioned before, have been defined as the major regulators of Complex I activity (Hirst et al., 2003). Loss of any of these catalytic subunits can lead to the loss of Complex I stability and functionality. On the other hand, supernumerary subunits contribute to the correct assembly and stability of complex I. Hence, any defects in the biogenesis of the accessory subunits, or any mutation in the genes encoded by these subunits, may also cause a decrease in complex I functionality (Hirst et al., 2003).

The RNAi-mediated knockdown of those subunits essential for the catalytic activity or assembly of complex I can prove to be lethal in many model organisms (including *Drosophila melanogaster*). Previous studies in our lab have shown that knockdown of these subunits in *D. melanogaster* causes death in the early larval stages (Sanz et al., 2010b). However, ectopic expression of the yeast alternative NADH dehydrogenase internal 1 (NDi1) protein rescues this phenotype. Interestingly, *Saccharomyces cerevisiae* does not possess respiratory complex I, the oxidation of NADH is done by Ndi1 protein (Seo et al., 1998).

Complex I, and specifically ROS produced by this complex, has been shown to be involved in aging. It has been proposed that superoxide produced by complex I goes on to cause oxidative damage in proteins, lipids and DNA, resulting in the accumulation of oxidative damage and cell death (Sanz et al., 2006). If this is true, the reduction of complex I ROS production, or its replacement by a more efficient enzyme (one that produces fewer ROS) should increase lifespan. Interestingly, bypassing complex I, but not complex III or IV, increases Drosophila lifespan (Sanz et al., 2010a). The knock-out or a complete knock-down of complex I is not possible in animals using classical techniques, such as mutagenesis through P elements or RNAi interference. However, a reduction over 50% of complex I is possible by combining RNAi with the expression of NDI1 in *Drosophila melanogaster*. In the present study, we have used this strategy to

assess if reduction of superoxide produced by complex I can extend *Drosophila* lifespan.

Primarily, the knockdown of the complex I was substantially verified at different levels by the use of different techniques. First, we have verified that RNA levels were reduced when the RNAi construct was expressed using a daughterless-GAL4 driver line. The data showed that there was a significant down-regulation in the CG 6020 gene in the flies expressing the knockdown (fig 4.1 and fig 4.2). At the protein level we have observed that the levels of the 39kDa subunit (coded by the gene CG6020) were out of the limit of detection of the western blot (fig 4.2A). Moreover, the knockdown of 39kDa significantly reduces the assembly of the complex I (fig 4.4 and 4.5A), indicating that this subunit is essential for correct assembly of the complex. This demonstrates that this gene encodes a subunit that is involved in complex I assembly. The fact that other complexes were not altered by the knockdown of CG6020 demonstrates its specificity (fig 4.5B).

5.2 Effect of knockdown of CG6020 on mitochondrial respiration

To further verify the knockdown of complex I, high resolution respirometry was utilised. The Oroboros Oxygraph-2k was used to produce quantifiable data, by high resolution measurement of oxygen consumption. Using this, we have measured the functionality of the whole OXPHOS system. So far, this has been the best method for us to verify the knockdown of specific complexes and to establish the effect of the knockdown on the whole respiratory process. Additionally, this method has been demonstrated as the most inexpensive, fastest and easiest to perform, when compared to the other experiments. Importantly, this method yields a precise quantification of the effect of the knockdown on respiration. These results will further help our lab to quantify the effect of knocking-down other subunits of complex I, or even subunits of others respiratory complexes.

The results shown here illustrate how the knockdown of complex I was successfully achieved. The respiration data showed that the flies where complex I was knocked down show a specific decrease in respiration when complex I-linked substrates are used (fig 4.6A), whereas the use of other substrates that, introduce electrons using different respiratory complexes, was not altered (fig 4.6B and fig 4.6C). This support a specific role of CG6020 in complex I function.
Overall, these findings demonstrate an efficient knockdown of CG6020 gene (coding for the 39 Kda), and demonstrate that our model is appropriate for use in testing the Mitochondrial Free Radical Theory of Aging by modulating the generation of damage, which we suspect to be mainly generated by complex I, rather than just providing the

antioxidants for defense, or supplementing any other repair mechanisms. Even though the results produced here are *in vitro*, we suggest that they are extremely relevant to *in vivo* studies as well.

5.3 Knockdown of complex I increases lifespan

Aging, as mentioned before is controlled by various factors. There have been numerous theories proposed to explain it. Here, we have focused on studying the role of free radicals in aging and, in doing so; we sought to test the Mitochondrial Free Radical Theory of Aging (MFRTA).

In preliminary published (Sanz et al., 2010a) and unpublished experiments we did our crosses at 25ºC. However, at this temperature, flies with significantly decreased levels of complex I were extremely weak, and most die within a few days after eclosion (data not shown). Due to the nature of the binary expression system we are using (GAL4), the higher the temperature, the higher is the affinity of the GAL4 protein for the UAS promoter (Brand and Perrimon, 1993; Duffy, 2002). Another possibility is that complex I is essential during development. Thus, performing the crosses at 25ºC prevents the normal development of some essential function (Sanz et al., 2010a). Nevertheless, the development of flies at 18ºC allows flies expressing the knockdown to be viable, whereupon they develop without major physiological alteration (see below).

Interestingly, the knockdown of complex I in combination with Ndi1 extends lifespan beyond the extension gained by the expression of Ndi1 alone. There was an increase in the mean, median and more important, the maximum lifespan of the flies (fig 4.7). This indicates that flies age slower when complex I levels are reduced. This is probably caused by a reduction in the accumulation of irreversible and age-related damage in the flies. *In vitro* measurements of ROS production supported that reduction of complex I levels reduces superoxide leak from the ETC (see below). This can be predicated as the crucial criterion for the increase in lifespan.

In summary, it is clearly proved that the knockdown of the CG6020 gene prolongs lifespan and delays the onset of many age-related diseases. Knock down of a respiratory complex that produces ROS has successfully lowered adult mortality by reducing free radical-mediated damage in flies. Moreover, it would be particularly interesting to see if the same response mechanism takes place in higher organisms or in mammals.

5.4 Production of ROS, effectively reduced by knockdown of complex I

It has been widely accepted that complex I has an extraordinary importance both in the control of oxidative phosphorylation (Yano, 2002) and in the generation of ROS (Turrens and Boveris, 1980). In fact, it has been proposed that complex I is the main generator of free radicals in vivo (Murphy, 2009). MFRTA proposes that ROS generated by ETC contribute to the accumulation of oxidative damage and aging (Lapointe and Hekimi, 2010). Our results support a central role of mitochondrial ROS produced by complex I in aging, and thus support MFRTA. The simultaneous knock-down of a specific subunit of complex I -39kDa, with the co-expression of Ndi1 prevents the assembly of the complex and decreases the production of H_2O_2 in vitro. Our results indicate that only ROS produced by complex I are reduced when complex I is knocked down, since the only reduction we observed was when using complex I-linked substrates (fig 4.9A and fig 4.9B).

The expression of Ndi1 can compensate for the reduction in the oxidation of NADH caused by the knock-down of complex I. On the other hand, it is unknown if Ndi1 produces superoxide, or if it can have other deleterious effect on longevity. Previous studies indicate that expression of Ndi1 decrease age-related ROS production *in vitro* and protein damage, (Sanz et al., 2010b), without reduction in ATP levels (Bahadorani et al., 2010).

Put together, all these data support the association between mtROS and longevity. However, ROS should be measured in vivo, and oxidative damage should be studied before making any definitive conclusions.

5.5 Knockdown of complex I does not reduce physiological activity of *Drosophila melanogaster*

Mitochondrial dysfunction has been shown to be a causative factor for various diseases (Finsterer, 2007), with mutations in complex I subunits responsible for many of them. Hence a mutation in complex I or a reduced activity of complex I, can trigger the onset for various diseases, or in turn lead to various physiological phenotypes.

Mutation or knockdown of specific complex I subunits has been related with sterility in *Drosophila melanogaster*. Sterility has been related to extension of lifespan in *Drosophila* (Barnes et al., 2008). We assessed whether a decrease in fertility could explain the lifespan extension in complex I knockdown expressing flies. The number of flies eclosing was slightly lower in the knock-down flies. However, they were clearly fertile (fig 4.8). Moreover, the period of collection of flies was ten days, due to the

nature of the crosses; some of the flies were slightly delayed during eclosion, or were not viable (e.g. flies where the knockdown construct is expressed without NDI1).

A reduction in body weight and food intake is also related with an extension of fly lifespan (Speakman, 2005). We have studied the body weight and energy metabolism, which is indirectly related to lifespan (Khazaeli et al., 2005b). The idea of forming a relation between aging and energy expenditure, is itself very intuitively attractive (Speakman et al., 2002). This forms the basis of the rate of living theory, which suggesting that the more an organism spends energy, the shorter its lifespan. It is suggested that, food intake, thermoregulation and physical activity all increase the rate of metabolism (Speakman, 2005).This is especially true in poikilothermic organism as fruit flies, where temperature or activity determines lifespan (Lamb, 1968; Speakman, 2005).

As to refute the rate of living theory, we have obtained results that show that elevated metabolism in flies does not cause premature mortality (fig 4.11). We have also provided evidence to support that the extension in lifespan caused by complex I knockdown in is not related with changes in activity, food intake or body weight. Flies with low levels of complex I show no difference in weight when eclosing, compared to control flies (fig 4.13A). Similarly, when the same flies are aged for 5 days, their weight gradually decreases, but differences were not significant when compared to the control flies, with the daughterless-GAL4 control (fig 4.13 B).

The activity or locomotion of a fruit fly can also determine its longevity (Le Bourg et al., 1984). Surprisingly, complex I knock-down flies move more, not less than the controls (fig 4.12). This significantly undermines the idea that the extension of lifespan is caused by a reduction in mobility. Moreover, a reduction in calorie intake, known as CR, it is also associated with extension of lifespan in flies (Piper et al., 2005). We have measured food intake and detected that long-lived flies expressing Ndi1 eat more, not less, than short-lived controls. This discards the hypothesis that a reduction in food intake causes the extended longevity associated with Ndi1 expression, with or without the knockdown of CG6020. All in all, our results support MFRTA.

CONCLUDING REMARKS

The main aim of this thesis was to analyze if an RNAi-mediated reduction in the level of complex I will reduce the amount of ROS produced and extend lifespan. We can conclude the following points from the present study.

1. Using several different approaches, we have validated our strategy to significantly reduce the levels of complex I-linked respiration by knocking down the 39kDa subunit of complex I: (1) qPCR, (2) western blotting, (3) BN PAGE, and (4) high resolution respirometry. Overall, high resolution respirometry was the best method for verification.

2. Complex I linked respiration was specifically reduced after knockdown 39kDa subunit.

3. The amount of ROS produced was significantly reduced in the flies with lower levels of complex I.

4. Knockdown of 39kDa subunit extends *Drosophila* lifespan

5. Complementary results have also been showed that demonstrate that, the extension in lifespan is not caused by sterility, reduction in mobility or reduction in food intake (CR), supporting a role of respiratory complex I in the regulation of lifespan.

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