A mitochondrial ROS signal activates mitochondrial turnover and represses TOR during stress

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DECLARATION

I certify that this thesis is my own work and I have correctly acknowledged the work of collaborators. I also declare that this thesis has not been previously submitted for a degree or any other qualification at this or any other university.

Ashwin Sriram
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ABSTRACT

Ageing and age-related diseases are multidimensional processes characterised by the accumulation of damaged mitochondria and a reduced capacity to respond to stress. While mitochondria play a central role in cellular signalling and stress adaptation, how damaged mitochondria accumulate and whether this affects healthy lifespan and onset of age-related diseases is unclear. In the following chapters, I demonstrate how mitochondrial turnover is involved in the process of stress adaptation, mediated by mitochondrial reactive oxygen species (mtROS), using the power of *Drosophila* genetics.

I focus on the role of mitochondria, specifically on how Reactive oxygen species (ROS) are involved in the process of temperature adaptation. Additionally, I establish that ROS acts as a signalling molecule in communicating with different downstream processes. Combining different approaches to measure ROS, I have dissected the nature of this signal.

I show that an increase in mtROS associated with thermal stress is produced as a consequence of over-reduction of the electron transport chain and identify the molecular intermediate as H_2O_2. Moreover, I study in detail the downstream targets and demonstrate that the levels of mtROS regulate levels of Pink1. Furthermore, I provide evidence that mitochondrial H_2O_2 (mtH_2O_2) act as a mitochondrial signal to activate mitophagy, and to repress Target of rapamycin (TOR). When this signal is supressed, mitochondrial respiration is markedly diminished, canonical Pink1-Parkin mediated mitophagy is obstructed and TOR signalling is hyper-activated. This in turn causes the accumulation of damaged proteins and organelles and a drastic reduction in fly lifespan. I show that restoring mitophagy in a low mtH_2O_2 background restores mitochondrial respiration, TOR signalling and rescues lifespan.

In the following chapters, I describe in detail a novel mitochondrial H_2O_2-Pink1/Parkin-TOR signalling axis that regulates cellular quality control and how it can be modulated by pharmacological and genetic interventions. My results also reveal the existence of a novel ROS signalling pathway that regulates mitochondrial density, which in turn determines the activation of TOR signalling and lifespan.

In summary, I show that (1) mtH_2O_2 is instrumental for the process of canonical Pink1-Parkin mediated mitophagy; (2) mtH_2O_2 production can be precisely regulated *in vivo*; (3) Mitochondrial turnover determines the activation of TOR signalling; and (4) Mitochondrial turnover is important in maintaining homeostasis of an organism.

KEYWORDS: Mitochondria, *Drosophila,* ROS, ageing.

AWARDS AND PUBLICATIONS

Awards

- Newcastle University Travel Award for the Molecular Basis of Ageing and Disease conference, Suzhou, China. 2015.
- Travel award for Coimbra Healthy Ageing Symposium, Coimbra, Portugal. 2015.
- Travel award for 6th Annual Alliance for Healthy Ageing Conference, Newcastle upon Tyne, United Kingdom. 2015.
- Won the best oral presentation award in the Postgraduate meeting in Newcastle University, Newcastle upon Tyne, United Kingdom. 2015.
- Won the best poster award in Institute for Cell and Molecular Biosciences away day, Newcastle University, Newcastle upon Tyne, United Kingdom. 2015.

Peer-reviewed Publications

1. Scialo, F., **Sriram, A (co-first author)**., Fernandez-Ayala, D., Gubina, N., Lõhmus, M., Nelson, G., Logan, A., Cooper, H.M., Navas, P., Enriquez, J.M., Murphy, M.P. and Sanz, A. Mitochondrial ROS produced via reverse electron transport extends animal lifespan. *Cell Metabolism*. 2016 Apr 12; 23(4):725-34.

2. Stefanatos, R., **Sriram, A (co-first author).**, Kiviranta, E., Mohan, A., Ayala, V., Jacobs, HT., Pamplona, R. and Sanz, A. DJ-1β Regulates Oxidative Stress, Insulin-Like Signalling, and Development in *Drosophila melanogaster. Cell Cycle*. 2012 Oct 15; 11(20):3876-86.

3. Scialo, F., **Sriram, A (co-first author)**, Naudí, A., Ayala,V., Jové, M., Pamplona, R. and Sanz, A. Target of rapamycin activation predicts lifespan in fruit flies. *Cell Cycle*. 2015 Sep 17; 14(18):2949-58.

4. Kemppainen, K., Rinne, J., **Sriram, A.**, Lakanmaa, M., Zeb, A., Tuomela, T., Popplestone, A., Singh, S., Sanz, A., Rustin, P. and Jacobs, HT. Expression of the *Ciona intestinalis* alternative oxidase in *Drosophila* ameliorates diverse phenotypes due to cytochrome oxidase deficiency. *Human Molecular Genetics*. 2014 Apr 15; 23(8):2078-93.

5. Mallikarjun, V., **Sriram, A.**, Scialo, F. and Sanz, A. The interplay between mitochondrial protein and iron homeostasis and the role this plays in ageing. *Experimental Gerontology*. 2014 Aug; 56:123-34.

6. Syrjänen, L., Valannea, S., Kuuslahtia, M., Tuomelaa, T., **Sriram, A**., Sanz, A., Jacobs, HT., Rämet, M. and Parkkilaa, S. β carbonic anhydrase is required for female fertility in *Drosophila melanogaster. Frontiers in Zoology*. 2015 Aug 22; 12:19.

ABBREVIATIONS LIST

ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BNE	Blue Native Electrophoresis
BSA	Bovine Serum Albumin
C.elegans	*Caenoharbditis elegans*
CAFE	Capillary Feeding
cDNA	Complementary Deoxyribonucleic Acid
CO_2	Carbon-di-Oxide
CR	Caloric Restriction
CYT C	Cytochrome c
DAH	Dahomey
DEPC	Diethylpirocarbonate
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethyldiaminetetraacetic Acid
EGTA	Ethyl Glycol Tetraacetic Acid
ETC	Electron Transport Chain
FAD	Flavin Adenine Dinucleotide
$FADH_2$	Reduced Flavin Adenine Dinucleotide
FeS	Ferrous-Sulphate cluster
FMN	Flavin Mononucleotide
$FMNH_2$	Reduced Flavin Mononucleotide
G3P	Glycerol-3-Phosphate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H_2O_2	Hydrogen Peroxide
HCl	Hydrogen Chloride
$^{\cdot}OH$	Hydroxyl Radical
HRP	Horseradish Peroxidase
KCl	Potassium Cyanide
KDa	Kilo Dalton units
KH_2PO_4	Monopotassium phosphate
Mb	Mega base

MDa	Mega Dalton units
MELAS	Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes
MFRTA	Mitochondrial Free Radical Theory of Ageing
$MgCl_2$	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
QH_2	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.cerevisiae	*Saccharomyces cerevisiae*
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SMT	Somatic Mutation theory
Sod1	Superoxide Dismutase 1
Sod2	Superoxide Dismutase 2
TMPD	Tetramethyl-1,4-benzenediamine dihydrochloride
UAS	Upstream Activating Sequence

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Chapter 1. Introduction

1.1 An overview of mitochondria

Mitochondria are distinctive organelles found in the cytoplasm of nearly all eukaryotic cells. They are thought to have originated from an ancient symbiosis event that occurred when a nucleated cell engulfed a *Rickettsia*- like ancestor prokaryote (Emelyanov, 2001). As a result of this event, the host and the endosymbiont gradually co-evolved becoming dependent on one another. Conversely, the host cell relied on the engulfed prokaryote to live in a nutrient-rich environment free from predators. Over time, the engulfed prokaryote developed into the mitochondria and energy produced due to endosymbiosis became critical to eukaryotic evolution (Roger, 1999). Mitochondria have their own circular DNA (mtDNA), consisting of 37 genes, which encode 13 polypeptides and are exclusively used in the electron transport chain (ETC) for generating adenosine triphosphate (ATP) (Lightowlers *et al.*, 2015). The remaining genes encode for tRNAs and rRNAs (Wallace, 1999). The number of mitochondria per cell varies between cell-types and species. For example, in mammals, red blood cells have none while more metabolically active cell-types, such as skeletal muscle, can have thousands (Zhang *et al.*, 2011).

Mitochondria are double-membrane organelles consisting of an outer membrane surrounding an inner membrane. The space between the two membranes is termed as the intermembrane space. The inner membrane has a greater surface area and hence folds back on itself, creating compartments known as cristae that protrudes into the innermost space termed the mitochondrial matrix (Palade, 1952). Figure 1.1 shows the mitochondrial structure. The respiratory chain complexes are concentrated in the cristae membranes. The matrix mainly consists of the mtDNA and its protein scaffold, collectively known as the nucleoid. mtDNA is typically held in concatenated rings with a single mitochondrion containing 2-5 copies of its genome (Pohjoismaki *et al.*, 2009). Within the matrix are also present the nuclear-encoded ribosomes that translate the 13 mitochondrial transcripts into proteins. The matrix also contains enzymes that are responsible for the tricarboxylic acid cycle (TCAC) reactions that act as entry of electrons into the inner membrane, where they are transferred from one protein complex to another (Balaban, 1990). The inner mitochondrial membrane is the site of ETC, which contains protein Complexes I-V. Protons build up in the spaces between the inner and outer membrane creating a proton gradient for ATP synthesis. The inner membrane is largely impermeable to all molecules except those ferried through a pore such as the TIM (translocase

of the inner membrane) complex. Alternatively, there are also other pores like Porins or Calcium uniporters that help in the exchange of a large array of molecules through passive diffusion or membrane potential respectively. Shuttles for ADP, ATP, Aspartate and Malate have also been described (Klingenberg, 2008). The intermembrane space is a key component in the process of mitochondrial oxidative phosphorylation (Campbell, 2002). Protons translocated from the matrix by Complex I, Complex III and Complex IV into the intermembrane space generate an electro-chemical gradient across the inner membrane providing energy that is used by Complex V to synthesize ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i) (Cooper, 2000). The mitochondrial inner membrane also contains various proteases such as HTRA2/OMI (Kang *et al.*, 2013) and the iAAA proteases involved in mitochondrial quality control. The outer membrane acts as a gateway to the mitochondrion, containing a series of channel proteins called PORINS that allow the free movement of small proteins and ions into the intermembrane space (Alberts, 2002). The outer membrane also contains complex protein channels called TOM (translocase of the outer membrane), and the TOM/TIM complex allows only nascent mitochondrial proteins to permeate through the membrane (Pfanner and Geissler, 2001). This process is carried out by a mitochondrial isoform of heat shock protein 70 (HSP70) in an ATP-dependent fashion whereupon they are folded and processed by other mitochondrial HSPs and proteases (Kang *et al.*, 1990).

Mitochondria are integral to many cellular functions, including maintaining energy homeostasis, synthesis of iron-sulphur clusters (ISC) and heme cofactors, storage and regulation of calcium, thermogenesis, initiating apoptosis and numerous other roles in signalling processes that vary depending on tissue type (Lackner and Nunnari, 2009). Hence dysfunctional mitochondria are implicated in many diseases. Inherited mitochondrial diseases typically result in progressive, thus far incurable neuropathies and myopathies (tissues most dependent on mitochondrial function). However, many sporadic and age-related diseases are also thought to have a mitochondrial component (Lane *et al.*, 2015).

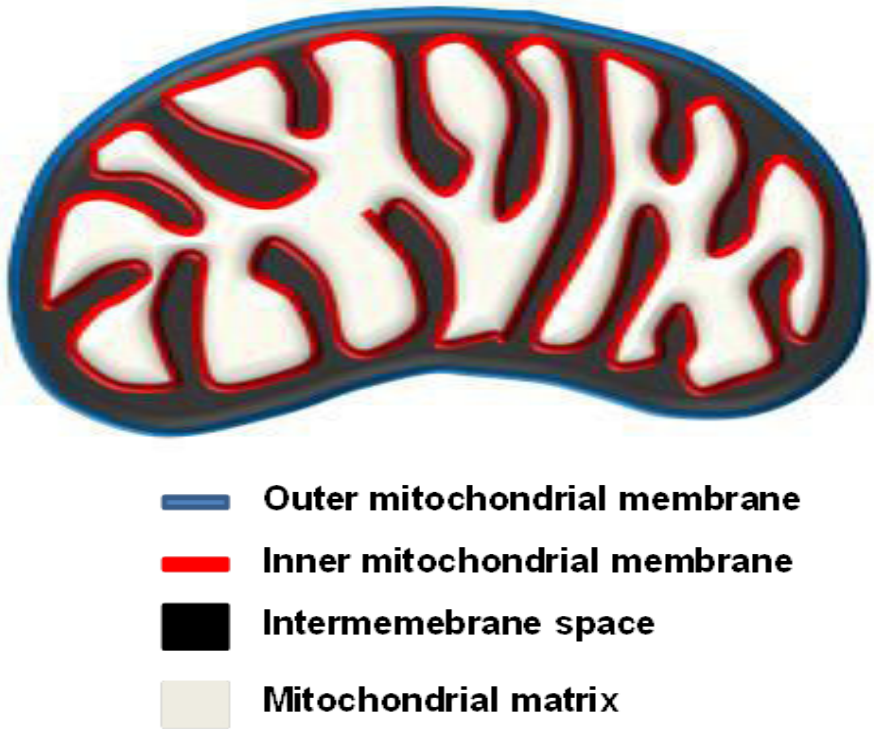

Figure 1.1 Mitochondrial structure

Different parts of the mitochondria are denoted with different colours in the figure.

1.2 The Electron Transport Chain (ETC)

ETC is the key component of mitochondria and is absolutely essential 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 and its malfunction can cause severe damage to the cell. Alternatively, glycolysis is used to produce energy albeit in a less efficient way, considering the amount of ATP generated per molecule of glucose oxidized to form pyruvate and the 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 coenzyme Q co-exist. The initiation of ETC takes place by transferring the electrons to carrier molecules NADH (Nicotinamide adenine dinucleotide) and $FADH_2$ (Flavin adenine dinucleotide). Electrons are also contributed to the ETC by glycerol-3-phosphate dehydrogenase, by transferring two electrons from $FADH_2$ to the ETC. There are also few other proteins like flavoproteins (Flavoprotein Fp1 and Flavoprotein Fp2) that can transfer electrons as well. Overall, these electron carriers are produced by the Krebs' cycle inside the mitochondrion, the only exception being glycerol 3 phosphate dehydrogenase (G3PDH). The transfer of electrons generates a proton motive force (Dimroth *et al.*, 2000) which consequentially generates the energy used for oxidative phosphorylation. The electrons are transferred in a stepwise manner, from the $FMNH_2$ (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 mobile 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. It 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 (SDH). Complex II also transfers electrons to ubiquinone thereby reducing the ubiquinone pool (Cecchini, 2003). In this process, an electrochemical gradient resulting in a potential of 150-180 mV is produced which is known as the proton motive force. It is generated as a result of 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 (P_i) at Complex V (Boyer, 1997). Figure 1.2 illustrates the function of different complexes in the ETC and complete working of the OXPHOS (Oxidative Phosphorylation) system.

In this process, electrons can escape from Complexes I, II or III and reduce oxygen to superoxide (or other types of ROS) (Porter and Brand, 1995; Finkel and Holbrook, 2000). This can result in oxidative stress which is thought to be responsible for the decline in mitochondrial function associated with ageing (Rattan, 2006; Valko *et al.*, 2007). However, there is no electron leak at Complex IV, where always four electrons (and two protons) are used to reduce oxygen to water.

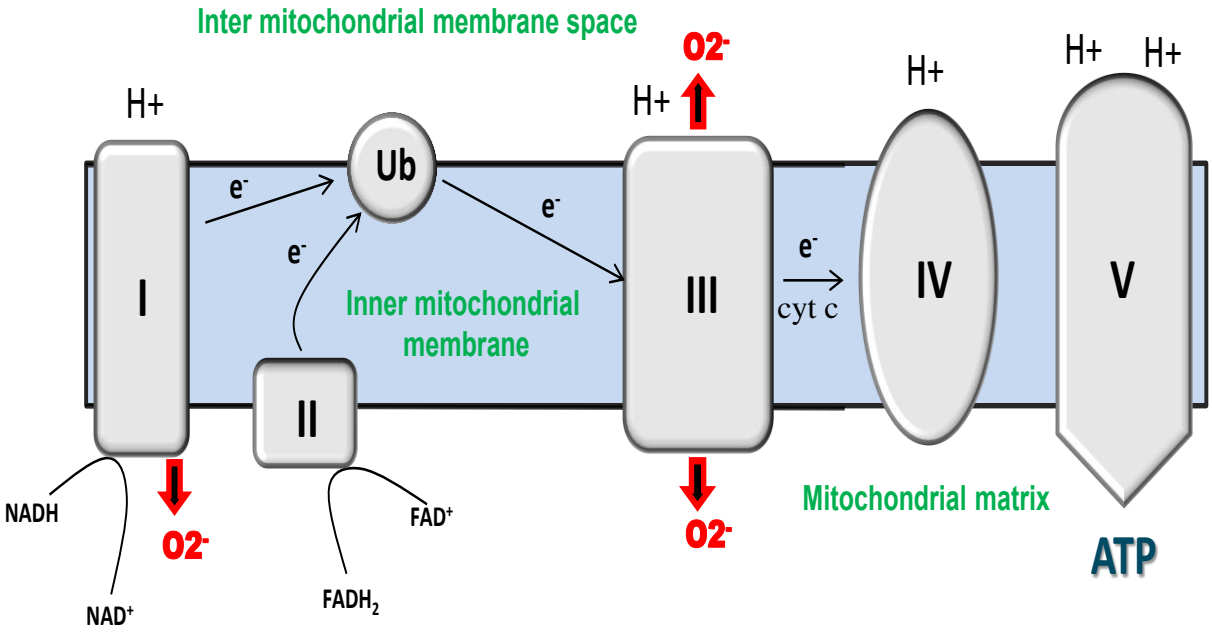

Figure 1.2 Production of adenosine triphosphate by the flow of electrons through different multiprotein complexes located in the inner mitochondrial membrane

The multiprotein complexes are connected to each other by the electron carrier's cytochrome c and coenzyme Q. This figure also points out the most important sites of ROS production.

1.3 Mitochondrial biogenesis

Over the years, it has been proved that physical activity increases the amount of mitochondria in order to compensate for the energy expenditure during exercise (Melanson *et al.*, 2009). However, the molecular mechanisms of this process are being elucidated only very recently. Mitochondrial biogenesis is also considered as a major tool that is responsible for maintaining mitochondrial number within the cell. It is not only a change in number of mitochondria but it is also responsible for maintaining mitochondrial size and mass (Dominy and Puigserver, 2013). Mitochondria have a bacterial origin and hence have its own genome and can auto replicate. The mitochondrial genome is very small and encodes only a few very important proteins and their function is mainly supported by proteins that are encoded in the nucleus. Proteins that are made in the nucleus are recognised by mitochondria through a mitochondrial targeting sequence in the protein itself (Alberts, 2002). This targeting sequence is detected by the translocases of inner and outer mitochondrial membrane (TIM and/or TOM) (Neupert and Herrmann, 2007) and the strict regulation of protein translocation has been shown to play a vital role in mitochondrial biogenesis. Moreover, mitochondrial biogenesis is a process where mitochondria are formed *de novo* from pre-existing mitochondria, hence stringent conditions are absolutely essential. This important regulation of mitochondria is the reason behind changes in activity of the mitochondria in different tissues and increase in their number with respect to ATP requirement (Jornayvaz and Shulman, 2010).

In recent years, studies in many model organism has made it evident that Peroxisome Proliferator activated receptor co-activator 1 γ or PGC1α is the factor responsible for control of mitochondrial biogenesis (Fernandez-Marcos and Auwerx, 2011) (Lin *et al.*, 2002). Also, activation of PGC1α in turn increases the activation of Nuclear respiratory factor (NRF1 and/or 2) (Wu *et al.*, 1999). NRF2 is responsible for the transcription of many mitochondrial genes. It activates mitochondrial transcription factor A or TFAM which is responsible for increase in mtDNA content. This signalling cascade was described in several model organisms by utilizing genetic and pharmacological interventions. Additionally, novel results emerging from this field have also described the interaction of PGC1α with many other proteins, one of them being UCP1 (Uncoupling Protein 1). PGC1α induced the activity of UCP1 which also led to an increase in mitochondrial biogenesis (Puigserver *et al.*, 1998). Thus PGC1α is responsible for linking several stimuli and cues from the environment. This denotes the importance of mitochondrial biogenesis, as it can be induced by different stimulus so that the cell can respond to different requirements (Jornayvaz and Shulman, 2010).

Several studies have demonstrated that induction of PGC1α activity is pivotal in activation of mitochondrial biogenesis. After this discovery, there have been numerous studies demonstrating the activation of PGC1α by investigating many upstream events that lead to its induction. One of the most prominent studies showed that the activation of AMPK (Adenosine Mono-Phosphate Kinase) is responsible for the induction of PGC1α (Wan *et al.*, 2014). AMPK is a major energy sensor for the cell and it is activated when the ATP/AMP ratio inside the cell is low (Wang *et al.*, 2003), which in turn activates PGC1α and NRF1 and/or 2 leading to increased mitochondrial biogenesis. Experiments performed with mice expressing the catalytically inactive version of AMPK showed a decrease in activity of PGC1α and less mitochondria per volume of the cell (Kobilo *et al.*, 2014). On the contrary, overexpression of AMPK or expressing a constitutively active version of AMPK, increased PGC1α induction and mitochondrial content (Liang *et al.*, 2014). AMPK overexpression in mice also protected the observed decrease in mitochondrial content with age (Canto *et al.*, 2009). On the other hand, expression of phospho-mimetic active version of AMPK in *C.elegans* and *D.melanogaster* has been demonstrated to extend lifespan and maintain mitochondrial content by increasing expression of PGC1α (Mair *et al*., 2011; Burkewitz *et al*., 2015; Ulgherait *et al.,* 2014). It has also been demonstrated that the loss of function mutation in AMPK decreases lifespan of mice (Viollet *et al*., 2003). In addition to this, overexpression of AMPK has been demonstrated to increase lifespan non-cell autonomously as well (Ulgherait *et al.,* 2014).

Over the last few years, it has been shown that Nitric Oxide (NO) increases mitochondrial content (Nisoli and Carruba, 2006). Increase in Nitric Oxide Synthase (NOS) within the cell increases the content of nitric oxide, leading to an increase in guanylate cyclase. This in turn increases cyclic guanidine mono-phosphate or cGMP, which leads to PGC1α induction and mitochondrial biogenesis (Nisoli *et al.*, 2004).

One of the major regulators of mitochondrial biogenesis is the silent mating type information regulation 2 homologue or SIRT1 (Lagouge *et al.*, 2006). It has been demonstrated that fasting in many model organisms activates nutrient sensing signalling conducted by SIRT1 (Li, 2013). This activation of nutrient signalling increases the activity of SIRT1, which activates PGC1α by deacetylation. Studies have demonstrated that resveratrol, a drug that induces the expression of SIRT1, increases the induction of PGC1α thereby increasing mitochondrial biogenesis (Lagouge *et al.*, 2006). Moreover overexpression of SIRT1 has also

produced similar results. However, the effect of resveratrol in inducing SIRT1 has not been convincingly demonstrated (Higashida *et al.*, 2013).

Mitochondrial biogenesis is controlled differently in different tissues. One of the factors that regulates this mechanism is the p38 mitogen activated protein kinase or p38 MAPK (Ihsan *et al.*, 2015). It has been demonstrated that muscle activation or contraction increases the expression of p38 MAPK, which leads to an increase in PGC1α and mitochondrial biogenesis (Knutti *et al.*, 2001). This has also been demonstrated by experiments on p38 MAPK overexpressing mice (Nishida *et al.*, 2004). Figure 1.3 is a schematic representation of all the upstream elements discovered that converge onto PGC1α.

All the above mentioned mechanism have also been linked to caloric restriction (Canto and Auwerx, 2009). Although it is not very clear how this happens and the mechanism is unknown, it can be hypothesized that it is an evolutionary adaptation linking the mitochondria to environmental cues. As energy efficiency of the whole organism is regulated by mitochondria, it is clearly evident that there is an increase in less damaged mitochondria in the organism. These mitochondria utilize less oxygen from the environment, thereby producing less ROS. However, more work on this hypothesis is necessary in order to better understand the basic biology of the system. Understanding this mechanism can lead to the development or discovery of novel therapeutics that can potentially be a cure for mitochondrial diseases.

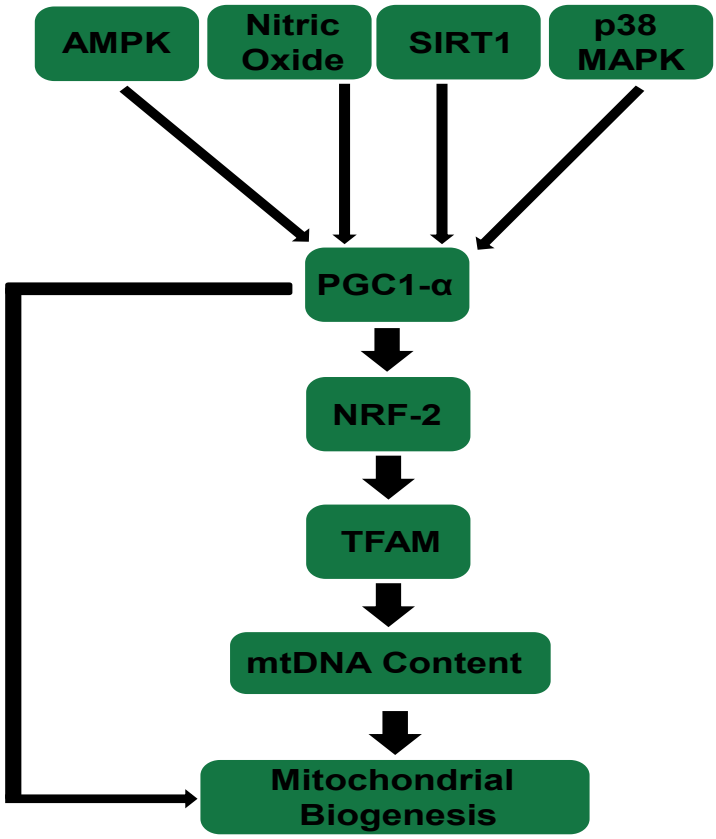

Figure 1.3 The mitochondrial biogenesis pathway

Several upstream elements like AMPK, Nitric Oxide, SIRT1 and p38 MAPK converge on to PGC1-α which regulates NRF-2 and TFAM, which in turn regulates mtDNA content and mitochondrial biogenesis.

1.4 Mitochondrial diseases

The entire organism requires functioning of mitochondria for ATP production. This is essential because the mitochondria are one of the only sites in the cell where consumed food is converted to ATP by the process of cellular respiration (Alberts, 2002). ATP can be made through glycolysis (glucose metabolism) as well, but contributing to only less than 10% of all the ATP produced in most tissues. Mitochondrial diseases are caused by mutations in mitochondrial (Taylor and Turnbull, 2005) and nuclear DNA (Angelini *et al.*, 2009). It can be acquired and inherited from both parents containing mutations in their mitochondrial as well as the nuclear genome. The nuclear DNA is in two pairs of equivalent chromosomes which are inherited in a Mendelian fashion, one from the male and one from the female. Whereas, the mitochondrial DNA is only inherited from the female parent (Taylor and Turnbull, 2005). Genome variants are another complication, where the same mutation manifests as different disease in different individuals (Stankiewicz and Lupski, 2010). For example, the same mutation can manifest as a liver syndrome in one patient and a neuronal disease in a different patient. They also cause changes in severity of the disease making it extremely difficult to predict the cause. Moreover, the manifestation of diseases also depends on the mitochondrial mutation specific to the tissue. For example, a mitochondrial mutation in the brain or the heart, where the energy demands are high, can cause a severe phenotype (Stankiewicz and Lupski, 2010); whereas, the same mutation in the blood or the liver may not cause the same severe phenotype. A few different diseases caused due to the mitochondrial dysfunction are described below.

Mitochondrial myopathies are the most common diseases caused due to mitochondrial dysfunction. This is a maternally inherited disease and is caused due to an inherited mutation in the mitochondrial DNA. Mitochondrial myopathies cause ragged red fibres in muscle and causes muscular dysfunction. It is manifested with accumulation of glycogen and neutral lipids (Fernandez-Sola *et al.*, 1992).

Diabetes mellitus and sensorineural hearing loss is another case of mutation in mitochondrial DNA that is inherited maternally. It has been found that this is caused by a point mutation in the 3243rd base pair in the circular human mitochondrial DNA. However, not all patients have a causal inherited mutation. The patients inheriting this mutation generally show symptoms of diabetes and have severe hearing loss (Kitabchi *et al.*, 2009).

Mitochondrial encephalomyopathy, lactic acidosis and stroke like episodes (MELAS) is the most studied mitochondrial disease. This disease is maternally inherited, but in some cases it is caused by certain mutations in the nuclear genome as well. The patients inheriting one or more of either mitochondrial mutations or nuclear mutations generally show symptoms of muscle weakness, migraine, nervous disorders and seizures. Accumulation of lactic acid is generally found in all these cases, which is hypothesized to be the primal cause of all these phenotypes (Tzoulis *et al.*, 2012). Patients with MELAS are found with a mutation in the mitochondrial DNA region encoding subunits of the OXPHOS Complex I like *ND1* or *ND5* or a mutation in tRNA encoding region of the mitochondrial DNA (Pavlakis *et al.*, 1984). However mutations in the tRNA encoding region has been shown to cause more severe phenotypes.

Leigh syndrome is one the most predominant mitochondrial diseases found around the world (Baertling *et al.*, 2013). Patients with these acquired mutations show severe symptoms of neurometabolic disorders and severely damaged nervous system. The muscular system is also affected with symptoms of ataxia. Respiratory failure manifests in the final stages of this disease. Mutation in the *SURF1* gene encoded in the nucleus or *mt-ATP6* gene encoded in the mitochondria has been found responsible for this mitochondrial disease. SURF1 is responsible for the assembly of OXPHOS Complex IV and mt-ATP6 is a part of the Complex V in the ETC that aids in producing ATP in the mitochondria (Baertling *et al.*, 2014). Figure 1.4 summarizes few of the many mitochondrial diseases caused by mitochondrial and nuclear DNA mutations in different tissues.

Our knowledge on mitochondria has improved significantly over the years; however there are only limited opportunities to treat mitochondrial diseases. Many vitamins like vitamin K and vitamin E have been prescribed for the improvement of the mitochondrial disease phenotypes. (Parikh *et al.*, 2009). However, the efficiency of neither vitamin E nor vitamin K intervention has yet been thoroughly established. On the other hand, membrane penetrating antioxidants have been developed and used to improve the phenotypes caused by mitochondrial diseases. Some examples of the same are pyruvate and N-acetylcysteine. These antioxidants have been shown to decrease the oxidative stress caused by mitochondrial dysfunction (Enns, 2014). Recently a technique known as spindle transfer has been developed, which involves the transfer of nuclear DNA into another healthy egg, leaving the mutated mitochondrial DNA behind. This process has been tested in primates and has been very successful using a pro-nuclear transfer technique (Tachibana *et al.*, 2009). Moreover, research conducted in the

Wellcome Trust Centre for Mitochondrial Research at Newcastle University has demonstrated that dysfunctional mitochondria of a mother with mitochondrial DNA mutations can be replaced with the healthy mitochondria of another woman by using a new IVF-based technique. This process enables the nuclear DNA, which encodes 99.9 % of the genetic information from the mother and father, to remain unchanged (Cravel *et al.*, 2010).

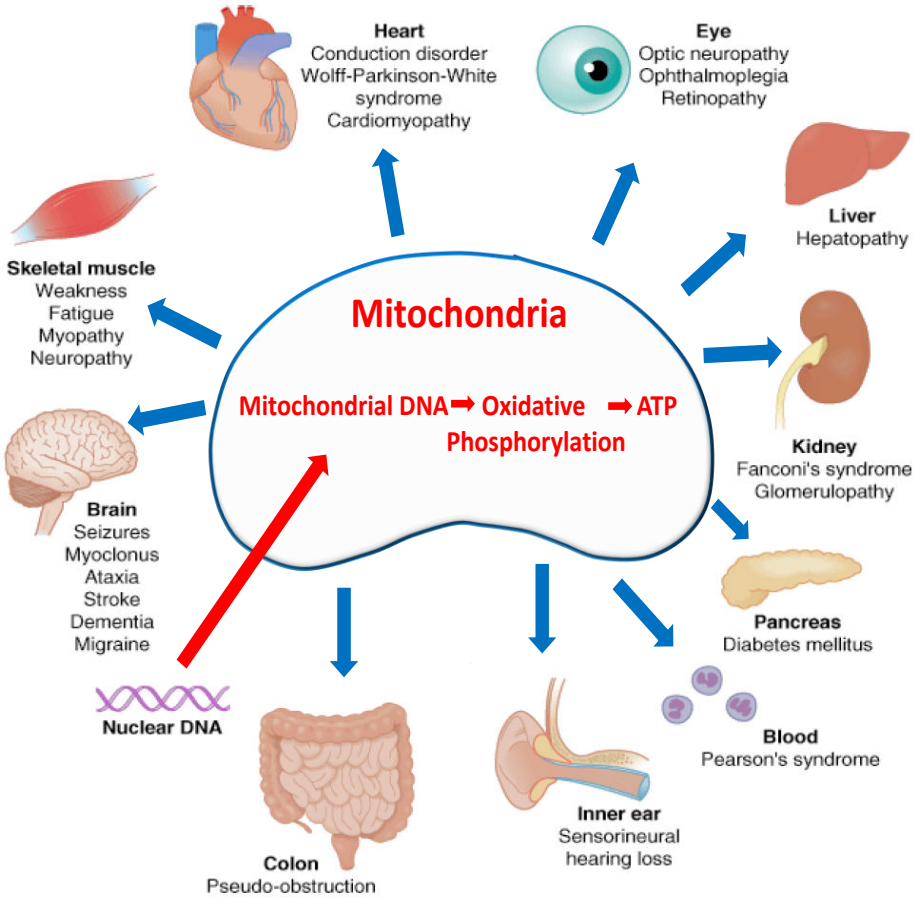

Figure 1.4 Mitochondrial diseases in humans

A summary of few of the many mitochondrial diseases caused by mitochondrial and nuclear DNA mutations in different tissues (Adapted from Fauci et al, 2011).

1.5 Ageing

Ageing is a multi-faceted process which leads to the loss of molecular fidelity and an increased vulnerability to cellular stress. There is a very complicated etiology provided by recent studies, proving that ageing has a strong genetic component, and that genetic modifications can alter lifespan in many model organisms such as *Drosophila melanogaster* (flies), *Caenorhabditis elegans* (worms) and *Mus musculus* (mice) (Longo and Fontana, 2011). Notable attempts to combat ageing have been made and among these are the experimental trials using "anti-ageing drugs" on nematodes, flies and vertebrates like mice and rats (Anisimov *et al.*, 2011). One example of such drug is rapamycin, which increases both mean and maximum lifespan even when administrated to old mice (Wang *et al.*, 2011). Additionally, some genetic strategies like knockout of *daf-2* (Insulin Receptor) and overexpression of *daf-16* (FOXO) have been demonstrated to extend the lifespan of nematodes (around 70-80%) (Kenyon *et al.*, 1993; Kenyon, 2011). However, a much smaller effect was observed in other model organisms, such as flies or mice (20-40%) (Clancy *et al.*, 2001; Bluher *et al.*, 2003; Dillin *et al.*, 2002; Giannakou *et al.*, 2004). Genetics has been demonstrated to determine only a part of human longevity with the rest being determined by other factors such as diet, exercise and other environmental factors (Rattan, 2012). Additionally, this is only true for mean lifespan; whereas, maximum lifespan of an organism is genetically pre-programmed (Prinzinger *et al.*, 2005).

1.6 Molecular theories of ageing

The molecular theories of ageing focuses on damage or loss of functionality of biological molecules. This includes all kinds of molecules like proteins, DNA and/or lipids, which might affect the protein synthesis or gene regulation of the organism (Weinert and Timiras, 2003b). There are two basic theories found under this category. They are i) genetic and ii) non-genetic. As the name suggests, the genetic theories of ageing emphasizes the role of specific genes in relation to ageing (Kanungo, 1975). One prominent genetic theory is the codon restriction theory. Codon restriction theory states that ageing 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 demonstrated to be related to telomerase which an enzyme that protects it from continuous shortening (Olovnikov, 1996). However most of these theories have been challenged over the years.

The non-genetic theories of ageing have their foundation on the physiological aspects of ageing. The accumulative waste theory of ageing (Hirsch *et al.*, 1989) proposes that, accumulation of cellular debris causes ageing. This theory is supported by the fact that, accumulation of the pigment lipofuscin is the most reliable ageing biomarker. Lipofuscin are fine granular pigmented molecules, which are debris products of lysosomal digestion. Essentially, lipofuscin is the by-product of incomplete oxidation of unsaturated fatty acids, glycoxidation of proteins and partial digestion of cellular organelles (Gaugler, 1997). When these by-products accumulate they can interfere with normal cellular function (Weinert and Timiras, 2003).

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 mechanism. The redox hypothesis states that, disruption of these redox sensitive thiol elements by, i.e. accumulation of oxidative stress, is the cause of ageing (Jones, 2008). On the other hand, cellular theories are often closely related to the functions at molecular levels as well. One example of this theory is the process of apoptosis, a form of programmed cell death. Apoptosis has been demonstrated to play a predominant role in ageing (Warner, 1997). This view on cellular theories is compatible with the oxidative stress theories of ageing where these phenomena also lead to cell death (Weinert and Timiras, 2003a).

1.7 Mitochondrial Free Radical Theory of Ageing

Mitochondrial Free Radical Theory of Ageing (MFRTA) is one of the most prominent theories in the era of ageing research and it can be integrated into almost all the theories mentioned earlier. MFRTA was first proposed by Denham Harman in 1956, as the “Free radical theory of ageing”. Harman stated ageing as a consequence of accumulation of damage caused by radicals, generated during normal metabolic processes (Harman, 1956). Harman's free radical theory of ageing was further revised and published in the year 1972, as the “Mitochondrial Free Radical Theory of Ageing” (Harman, 1972; Harman, 1983) which states that the respiratory complexes of the ETC produces reactive oxygen species (ROS) as a by-product 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 ROS with ageing: increased ROS production, accumulation of mutations in mitochondrial DNA (mtDNA) and progressive respiratory chain dysfunction (Kirkinezos and Moraes, 2001). In proof of this

concept, caloric restriction extended 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) and not with an increase in amount of antioxidant defences or repair mechanisms (Sanz *et al.*, 2006).

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. The manipulation of endogenous antioxidant levels does not produce the expected change in lifespan, either. For example, the knock-out of superoxide dismutase 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 SOD2 are long-lived in spite of having much higher levels of oxidative damage (Zhang *et al.*, 2009). 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 (Parkes *et al.*, 1998; Sohal, 2002; Magwere *et al.*, 2006). These contradictory results can be explained by hypothesising that ROS acts more as signalling molecule than as an agent for causing damage.

1.8 *Drosophila* as a model system

Drosophila is one of the most widely used model systems for studies on genetics and developmental biology (Reeve, 2001). It was first used in the early 1900's by Thomas H. Morgan, Jeff Bridges and Alfred Sturtevant for the study of sex linkage and genetic recombination. Since then, *Drosophila* has been widely used in genetics and molecular biology. It's numerous advantages, including; short generation time, low cost, ease of culture, well-characterised genetics and high number of offsprings, make it ideal to study ageing (Rubin and Lewis, 2000). Furthermore, it has been used as the model organism as 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. *Drosophila* has a genome of just 13,600 protein-coding genes, when compared to the 40,000 genes in human genome (Halligan and Keightley, 2006), and it is less redundant. It was completely sequenced in the year 2000 (Adams *et al.*, 2000). The fact that the genetic information in flies are distributed on four chromosomes, 3 pairs of autosomal chromosomes, and one pair of sex chromosomes, make it easier for genetic interventions.

Genetics has been a valuable tool in research, and numerous approaches have been used to gain insight into the function of those genes that have orthologues in humans. To generate these detectable phenotypes there has been two main approaches: i) by using the gain of function, which involves conferring new or additional function of the protein by the process of target specific mutation (gain of function mutation) or adding additional DNA segments to increase the translation of the protein (transgenic overexpression); and ii) by using a loss of function, which involves mutations that confers to the loss of protein function or knocking-down or knocking-out of genes by targeted RNAi or deletion events respectively. The double stranded RNA mediated RNA interference (RNAi) strategy has been recently introduced as a powerful tool. RNAi involves the use of double stranded RNA or small interfering RNA, to knockdown expression of a specific gene by causing degradation of the target RNA (Mello and Conte, 2004). The transgenic approach has been used in this thesis and involves the over expression of a gene in the fly or for expressing a protein from another organism (*Ciona intestinalis)* in the fruit flies, to study its molecular functions in the whole organism.

1.9 The GAL4/UAS and the Geneswitch/UAS system in *Drosophila*

The GAL4/UAS system is one of the most powerful systems used for increasing or reducing gene expression in *Drosophila*. The GAL4/UAS adapted from yeast is a system made up of two elements, namely the yeast GAL4 activator protein and the UAS (Upstream Activating Sequence). The gene of interest is cloned into a vector, which is primarily coupled with the UAS sequence. The GAL4 binds very specifically to the promoter of the desired gene and drives its expression via interaction with the UAS (Brand and Perrimon, 1993; Duffy, 2002). However, neither the GAL4 protein (derived from yeast) nor the UAS exist in wild type flies, making the system very specific.

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 lines are crossed, the binding of the driver (GAL4) to the UAS site takes place, leading to the expression of the desired gene in resulting offsprings (Jones, 2009). Figure 1.5 is a schematic representation of how the GAL4/UAS system works.

In the GeneSwitch/UAS system, the elements are similar; however this is a conditional expression system which is a modified version of the yeast GAL4 protein. It is activated only in presence of the inducer drug RU-486. Moreover, expression of the construct in GeneSwitch/UAS system depends on dosage of the drug. Thus increasing or decreasing the

dosage of Mifepristone or RU-486 (inducer drug) in the culture vial can increase or decrease the efficiency of expression. These are the systems that allow spatial and temporal expression or knockdown of genes.

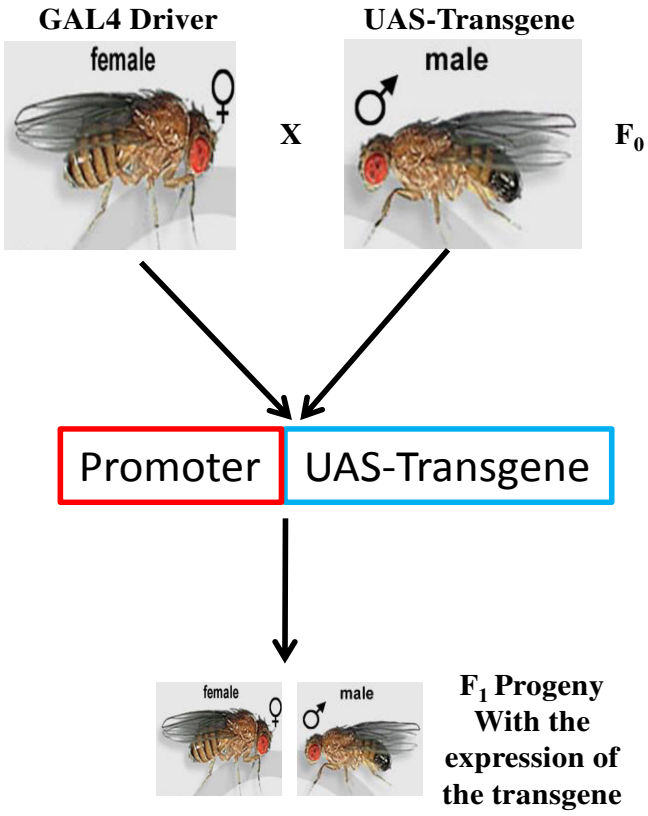

Figure 1.5 The GAL4/UAS system

Virgin females with the GAL4 driver are crossed to males with the UAS-Transgene (F_0). This results in binding of the GAL4 promoter to the UAS-Transgene, resulting in F_1 progeny with expression of the transgene. Fly images taken from Roote and Prokop, 2013.

1.10 *Drosophila melanogaster* as a model system to study ageing

The development of the fruit fly undergoes a four stage life cycle; egg, larva, pupa and imago or adult over a span of 10 days. Once fertilized, the embryo develops in the egg for around one day at 25^{o}C before hatching as a larva. The larva progresses through three stages of development over four days, until it pupates. The tiny larva that emerges is called the first instar larvae. It feeds on the substrate that the eggs were laid in and after a day it molts into a larger worm-like form known as the second instar larvae. This again feeds and molts into the third instar larvae in the next 2 days. The larvae would have significantly grown at this stage and will start to move and climb the surface of its container where it undergoes pupation. During pupation, metamorphosis takes place and the adult fly emerges from an eclosion (pupal case) over the course of 4 days. Most of the embryonic and larval tissue is destroyed during metamorphosis. The adult tissues are developed from a group of undifferentiated, mitotic cells known as imaginal discs and histoblasts formed since early-embryonic development. Figure 1.6 shows the life cycle of *Drosophila*.

Drosophila is poikilothermic and does not thermoregulate as mammals do. The metabolic rate and lifespan is determined by their environmental temperature. Thus, ambient temperature affects the development time, fecundity and fertility in fruit flies. When in lower temperatures, they slow the development process; whereas, higher temperatures have the opposite effect. Especially at higher temperature it has been shown that they accumulate damage faster and they have a very short lifespan. This short lifespan and their limited complexity for the control of lifespan make it a very valuable tool for ageing studies. This has been used to our advantage in this work.

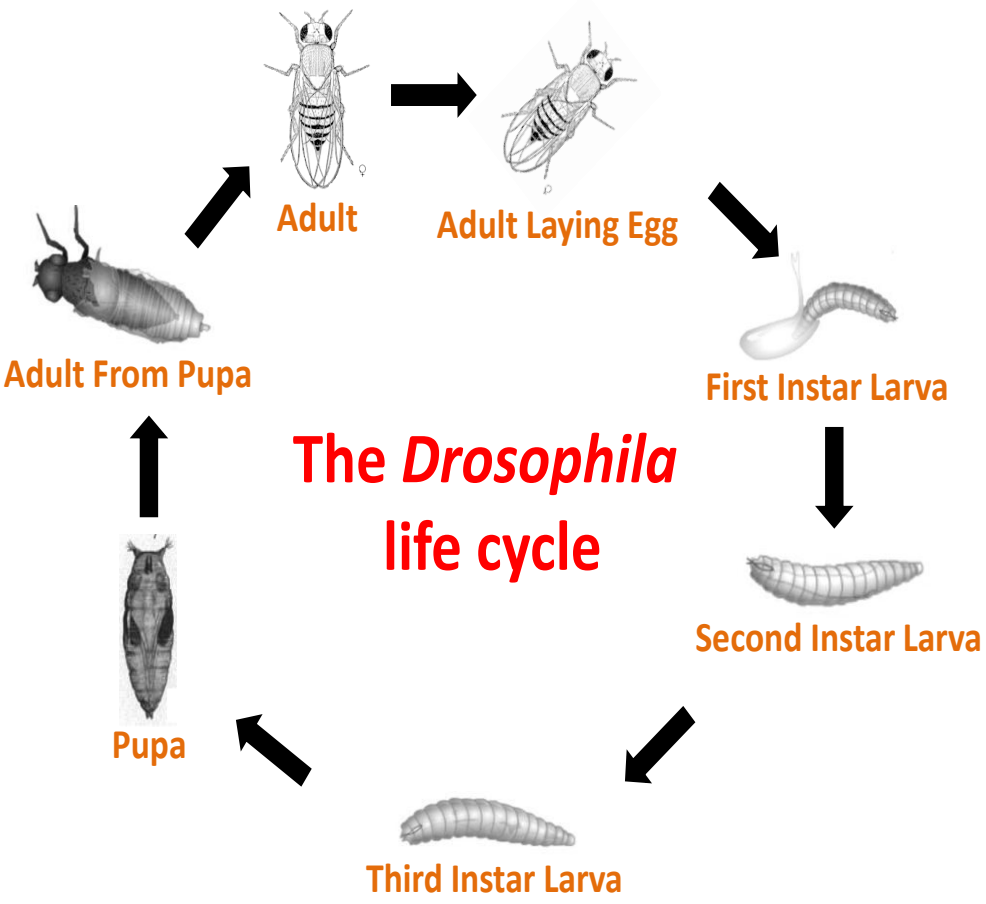

Figure 1.6 Drosophila life cycle

Drosophila lay their eggs, the eggs hatch into first instar larva which develops through different stages until it pupates. *Drosophila* then hatch from the pupae, where the larvae have been metamorphosing to form adults (Adapted from Elland, 2006).

1.11 Alternative respiratory enzymes

One of the major causes of mitochondrial dysfunction is the failure of the ETC (Koenig, 2008). Hence therapeutics is mainly aimed to decrease mitochondrial dysfunction in mitochondrial diseases. However, it has been debated whether this is because of the loss of ATP or the excess ROS generated by ETC (Adam-Vizi *et al.*, 2005; Brookes *et al.*, 2004). The ETC contains five respiratory complexes; hence the damage to one of these components can cause the failure of the whole system. An idea to prevent or improve the failure of ETC complexes is to bypass this damage. Many eukaryotes and some prokaryotes contain alternative respiratory enzymes in their mitochondria to bypass this damage in their ETC components. This can be explained as an evolutionary mechanism to protect themselves against toxins that were present in the environment at early stages of evolution. These toxins were able to block the respiration of an organism by blocking its ETC. However, as toxins in the environment started reducing, higher organisms arrested the production of these alternative respiratory enzymes (McDonald *et al.*, 2004), and was eventually removed from the genome of higher organisms.

The alternative respiratory enzymes are non-proton pumping but help in electron transfer. Due to this function they can compensate for the loss of ATP only partially, but can be more effective to bypass damage to the ETC components. These alternative respiratory enzymes are smaller than the ETC components; hence are easy to assemble by the organism and are less prone to damage dependent loss of function (Unden and Bongaerts, 1997).

Alternative Oxidase (AOX) is one example for this alternative enzyme. It is present in the ETC of the mitochondria in all plants and fungi and also in some higher organisms like *Ciona intestinallis*. It is a comparatively small protein and has no proton pumping properties. The protein accepts electrons from ubiquinol and reduces oxygen directly to water, bypassing the function of Complex III and IV. AOX is also resistant to cyanide and this can be explained as an evolutionary adaptation (Veiga *et al.*, 2003). Figure 1.7 shows how AOX works in ETC.

Few other examples of alternative respiratory enzymes are NADH dehydrogenase internal 1 (NDi1), NADH Dehydrogenase external 1 (NDe1) and NADH Dehydrogenase external 2 (NDe2). Budding yeast does not have the ETC Complex I, but has NDi1 inside the mitochondrial membrane facing the mitochondrial matrix. They also contain NDe1 and NDe2, which are present in the intermembrane space. The alternative respiratory enzyme NDi1 has been demonstrated to bypass the dysfunction of Complex I (Sanz *et al.*,

2010;Cannino *et al.*, 2012a). Moreover, plants contain all of the above mentioned alternative respiratory enzymes, making their ETC extremely branched; therefore less prone to damage (Affourtit *et al.*, 2001). Figure 1.7 shows how NDi1 works in ETC.

These alternative respiratory enzymes are potentially a good method to identify diseases in lower model organisms, which can thereby lead to inventing and discovering potential cure in humans. However, adding to this potential benefit, expression of an exogenous protein can cause opposing immune reactions. These enzymes have shown to produce many beneficial effects and some have been discussed below. The expression of AOX has been successfully achieved in mice and flies. AOX expression in flies rescued several phenotypes caused by the knockdown of Complex IV subunit CoxVb, *Surf1* which is a Complex IV assembly factor and *Cyclope* (Complex IV subunit COXVIc (Fernandez-Ayala et al, 2009) (Kemppainen *et al.*, 2014). AOX expression has also been shown to rescue the phenotypic effects caused by the mutation in *Dj-1β*, which is involved in Parkinson's disease (Fernandez-Ayala *et al.*, 2009). AOX expression was also used to rescue the loss in survival caused by cyanide poisoning in mice (El-Khoury *et al.*, 2013). In this study, we have used the expression of AOX to analyse the role of ROS signalling in flies (Figure 1.7). AOX also prevents over-reduction of the ubiquinone pool, which we hypothesize as the signal responsible for several cellular signalling mechanisms.

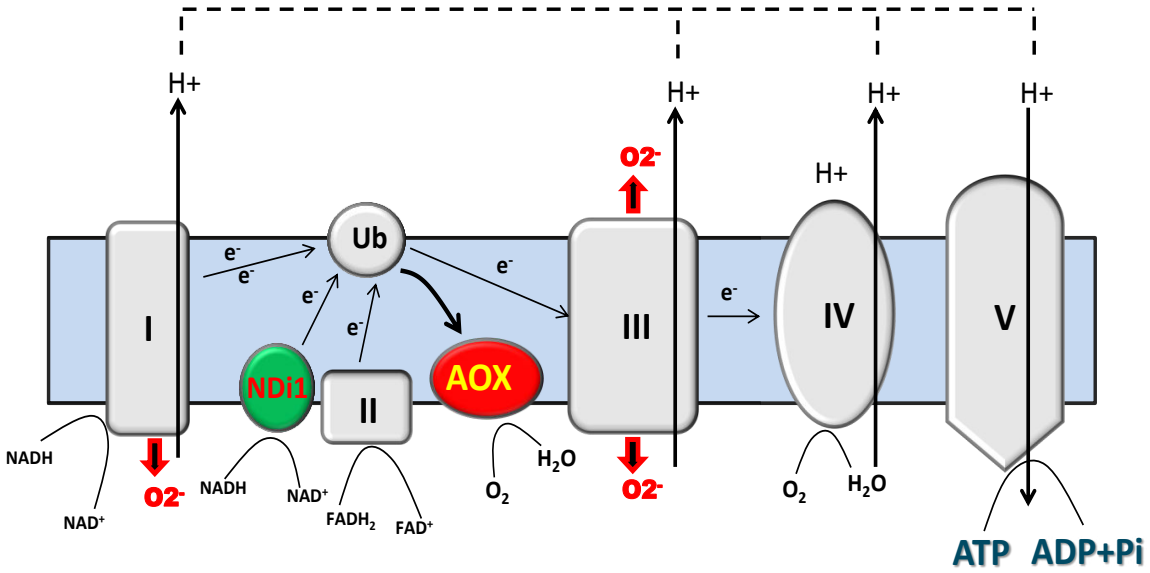

Figure 1.7 Schematic diagram illustrating the effects on electron transport after the expression of alternative enzymes; AOX and NDi1

NDi1 and AOX bypass Complex I and Complex III of the ETC respectively, thereby reducing the leak of electrons; thereby production of ROS. This aids in rescuing disease phenotypes caused by Complex I and Complex III. Additionally AOX and NDi1 oxidise and reduce the ubiquinone pool respectively.

1.12 Reactive oxygen species (ROS)

Free radicals are defined as an atom or group of atoms with one or more unpaired electrons. They are typically very reactive and therefore they have a very short half-life. They are generally unavoidable consequences of aerobic metabolism and are produced by stepwise reduction of oxygen due to electron transfer. ROS is a collective term, which includes many molecules containing oxygen centred radical. However, ROS is also subcategorized to free radicals which are molecules with unpaired electrons. A few examples of these kind of molecules are, the hydroxyl radical (OH^-) and Superoxide (O_2^-). Additionally, Hydrogen peroxide (H_2O_2) is not a free radical but is ROS.

Accumulation of ROS inside the cell is the major cause of oxidative damage, which is termed as oxidative stress. One of the major implications of the free radical theory of ageing is that, ROS causes cellular damage. Energy production by the ETC leads to the formation of ROS, which causes accumulation of damage in mitochondria (Sanz *et al.*, 2008;Luo *et al.*, 2013). For a good part of 20th century existence of ROS *in vivo* was not acknowledged or were usually considered as deleterious entities, but the dual nature of ROS in both damage and signalling have been extensively studied. Many studies have been emerging showing ROS as signalling molecules (D'Autreaux and Toledano, 2007).

There are two types of oxygen molecules that are highly reactive (i) Singlet oxygen or $_1O^2$ is produced by the absorption of sufficient energy to reverse the spin of an oxygen electron (ii) Superoxide or O_2^- is produced by stepwise monovalent reduction of the oxygen atom (Valentine *et al.*, 1984). This is a very harmful by-product and can react with an array of molecules. However $_1O^2$ is studied mostly in plants thorough generation of hydroxyl radicals. It oxidizes protein, unsaturated fatty acids and DNA in plants (Triantaphylides and Havaux, 2009).

Superoxide is very well studied in many higher organisms. However, its reactivity is still not completely understood. It is the primary free radical in the cascade to generate other components of ROS. Since it is the primary step in this reaction, it is one of the most highly reactive ROS species and its reactive half-life is approximately 1µs (Fridovich *et al.*, 1997). Due to its considerably short half-life, superoxide can be involved in a lot of redox reactions and is generally dismutated to H_2O_2 either non-enzymatically by auto dismutation, or enzymatically by Superoxide Dismutase (SOD), which catalyses this reaction (McCord *et al.*, 1969).

$$2O_2^- + 2H+ \xrightarrow{SOD} H_2O_2+O_2$$

Hydrogen peroxide is generated under both normal and stress conditions. It is produced by several sources like mitochondria, endoplasmic reticulum and in cytoplasm (Boveris *et al.*, 1972). Its reactivity with other molecules is more predominant than superoxide because of it half-life of around 1ms (Gough e*t al.*, 2011). Hydrogen peroxide is a better studied molecule in the process of signalling, as H_2O_2 does not contain any charge and can permeate through biological membranes (Veal *et al.*, 2007). Due to this property, combined with considerably high half-life, it can activate signalling cascades and cause damage far away from the site of production. H_2O_2 generally activates or deactivates an enzyme by targeting its methionine or cysteine residues. This can change the conformational state or folding of the protein and this determines its activity (Chung *et al.*, 2013).

Hydroxyl radicals or peroxinitrite are the most reactive oxygen species. Hydroxyl radicals are produced in a two-step process involving H_2O_2 and O_2^-, which involves a metal catalysis part as well (Cohen and Heikkila, 1974). These are called as Fenton reactions.

$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^-OH + {}^{\cdot}OH$$
$$O_2^{\cdot -} + H_2O_2 \rightarrow {}^{\cdot}OH + {}^-OH + O_2$$

Nitric oxide is formed by the oxidation of nitrogen, which is catalysed by an enzyme called nitric oxide synthase. Since nitric oxide contains an unpaired electron it is extremely reactive. However peroxynitrite a by-product of the nitric oxide, formed by the reaction between superoxide and nitric oxide is the much more harmful than nitric oxide itself (Hou *et al.*, 1999). Nitric oxide also acts an important messenger acting in vasodilation and neurotransmission. It also acts as an anti-tumor and anti-pathogenic agent. A sufficient level of nitric oxide is also very important in protection of organs like liver from ischemic damage. On the other hand, sustained levels of nitric oxide can cause tissue toxicity and also vascular collapse. Moreover an increase in nitric oxide has been discovered to be a causal agent for diseases like Juvenile Diabetes and Multiple Sclerosis (Heinrich et al., 2013).

$$N_2 + O_2 \rightarrow 2NO$$

1.13 The role of ROS in signalling and oxidative damage

Role of ROS in signalling is considered to be an evolutionary adaptation that prevents excessive damage caused by different ROS entities (Dowling *et al.*, 2009). Its role in activation of an array of transcription factors is already a very well-studied mechanism. Redox signalling, another well-studied concept, can be facilitated by ROS molecules; where the amino acid residues of proteins are oxidized by ROS (Ray *et al.*, 2012). Due to their abundance in the cell, proteins are one of the main targets of oxidation by ROS. Oxidation causes changes like polarity or size in the amino acids leading to a change in the polypeptide; thereby changing the structure, stability and activity of the protein.

Accumulation of O_2^- is generally connected more to stress than signalling as it cannot diffuse through biological membranes. Moreover, O_2^- is extremely reactive compared to its counterparts and it reacts with many molecules close to its site of production. Superoxide reacts mainly with iron-sulphur clusters and releases free iron, toxic to the cellular environment. However, the role of superoxide in signalling has been shown to activate antioxidant response and cell death in many organisms (Kim and Park, 2003; Kadowaki *et al.*, 2005). There are also studies on the presence of a superoxide channel present in mitochondria that can transport superoxide from the inside to the outside which is used for signalling (Han *et al.*, 2003).

Hydrogen peroxide is studied more extensively than superoxide as a signalling molecule due to its very high reactive half-life of 1ms; hence proteins in vicinity are more prone to reduction or oxidation (Schaar *et al.*, 2015). As mitochondria are dynamic organelles they can also be transported to a certain site, for H_2O_2 to diffuse through the mitochondrial membrane and act in signalling (Bienert *et al.*, 2006). Some extensively studied processes in which H_2O_2 is involved are (i) activation of Hypoxia inducible factor (HIF), which is responsible for maintaining metabolism within the cell (Jung *et al.*, 2008) (ii) activation of Phophoinositol 3 Kinase or PI3K that induces growth (Sadidi *et al.*, 2009) (iii) activation of nuclear factor kappa-light-chain-enhancer of activated B cells or NF-kappaB responsible for controlling several processes like survival, production of antioxidants, activation of transcription factors and also in prevention of cancer (Oliveira-Marques *et al.*, 2009) (iv) activation of Mitogen activated protein kinase or MAPK which is responsible for proliferation of cell (Bhat and Zhang, 1999). These signalling cascades consequently control important systems like increase

in stem cell renewal, differentiation and proliferation, immune response and longevity. Figure 1.8 is a flowchart showing the role of ROS in activating and deactivating different signalling mechanisms.

Although recent studies have significantly improved our understanding of the role of ROS in signalling, more studies and more precise techniques to study ROS *in vivo* will help us discover novel mechanisms which are absolutely essential for the cell. Improvement in these techniques will also aid us to delineate the pros and cons of ROS.

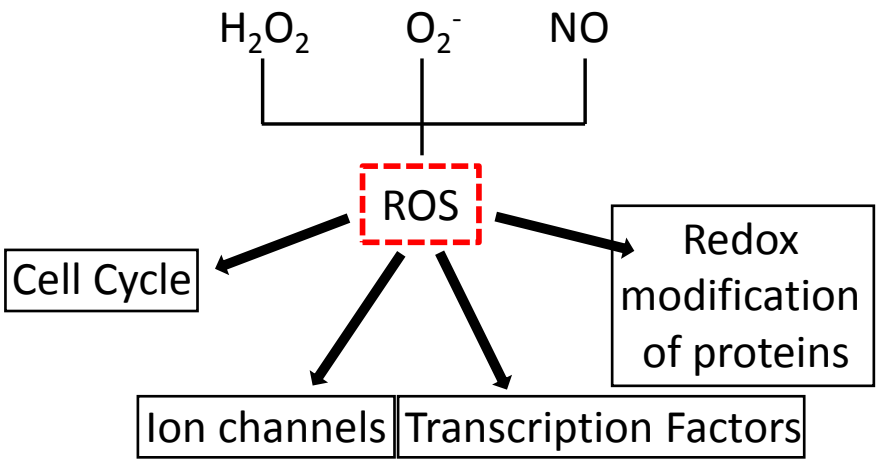

Figure 1.8 A flow chart showing different ROS entities acting in several signalling pathways

Different ROS entities like H_2O_2, O_2^- and NO play a role in activating and deactiving many signalling mechanisms.

1.14 Role of ROS in diseases

The role of ROS has been studied in many diseases like Alzheimer's, Parkinson's and other neurodegenerative disorders (Alfadda and Sallam, 2012). However, it is still unclear whether ROS is a cause or consequence of these disease phenotypes. The only clear evidence is the studies that show the role of ROS in cardiovascular disease. It has been demonstrated that ROS causes oxidation of low density lipoprotein (LDL) which leads to atherogenesis, consequentially leading to atherosclerosis and then cardiovascular diseases (Vogiatzi *et al.*, 2009).

It is hypothesized that ROS plays a role in many cases of cancer (Liou and Storz, 2010). For example, it has been shown that ROS can cause oxidative DNA damage, thereby increasing the risk of cancer. However, whether this is a cause of cancer progression or a consequence of cancer cell metabolism is still unclear. In mice, the only study that has surfaced is the knockout of antioxidants, which increases DNA polymorphisms, in turn increasing the risk of cancer (Van Remmen *et al.*, 2003; Tony *et al.*, 2009). Another concept is the role of ROS in ageing (Loeb *et al.*, 2005), where it is demonstrated in many organisms that consuming a low calorie diet increases lifespan significantly. It is hypothesized that this increase in lifespan is due to the decrease in oxidative stress, which is facilitated by decrease in ROS (Ungvari *et al.*, 2008).

1.15 Antioxidants

Superoxide dismutase (SOD) is the primary line of defence against ROS. It helps the cell in breaking down superoxide to hydrogen peroxide, with the help of metal ion cofactors. Three superoxide dismutases have been discovered across different model organism namely; copper/zinc, manganese, ferrous and nickel superoxide dismutase. In humans, copper/zinc cofactor based superoxide dismutase is present in the cytosol and in the mitochondrial inner membrane space and the manganese version is present in mitochondria. The version of superoxide dismutase present in mitochondria is proven to be very important as mitochondria are the source of abundant ROS production (Li *et al.*, 2011). It has been demonstrated that mice lacking the mitochondrial version of superoxide dismutase die as soon as they are born. However, the mice lacking other types of superoxide dismutases do not have a very well defined pathology (Hashizume *et al.*, 2008). It is hypothesized that the progression of damage is much more prominent than the homeostatic state. Although there have been so many studies with the same enzyme, it is still not completely clear how the enzyme functions. For example, in worms, it has been demonstrated that loss of all superoxide dismutase genes does

not have any effect on lifespan (Van Raamsdonk and Hekimi, 2012). Overexpression of these genes does not cause any beneficial effects either (Perez *et al.*, 2009). Additionally, in flies it has been demonstrated that overexpression of *Sod2* increases lifespan (Sun *et al.*, 1999); however overexpression of *SOD2* in mice does not (Huang *et al.*, 2000).

The catalase enzyme catalyses the conversion of H_2O_2 to H_2O and O_2. An iron or manganese cofactor is used in this process. In humans, the catalase enzyme is generally found localizing to peroxisome (Walton and Pizzitelli, 2012). The cofactor of catalase enzyme is oxidized by one molecule of H_2O_2 and then is regenerated by transferring the bound oxygen to the second molecule of the substrate. The role of catalase is very well studied and is an important step in making the ROS molecule inert. However knockout of catalase does not cause any deleterious effects in mice (Hamilton *et al.*, 2012).

Peroxiredoxins are also used in the conversion of H_2O_2 to H_2O. Moreover, it is also used in the conversion of organic hydroperoxides and peroxynitrates. Peroxiredoxins are enzymes that require a cysteine oxidization to be active (Rhee *et al.*, 2005) and due to its extremely high importance in an organism's array of processes, knockout of this enzyme in mice led to a shortened lifespan (Lee *et al.*, 2003).

Thioredoxins and glutathione peroxidases are one of the most abundant antioxidants present in any system across species. Thioredoxins are highly reactive reducing agents that are responsible for keeping the enzymes in a reduced state. They facilitate the reduction of proteins by cysteine thiol-disulfide exchange. On the other hand glutathione peroxidases are present in three forms (i) reductases; that catalyses the conversion of H_2O_2 to organic hydroperoxide; (ii) peroxidases; that catalyses the conversion of H_2O_2 to H_2O and O_2 and; (iii) Transferases; that are responsible for lipid peroxidation (Harris *et al.*, 2015). Peroxidases are the one of the most abundant antioxidant in the cell. Experiments have demonstrated that knockout of glutathione peroxidase significantly reduces lifespan in mice (de Haan *et al.*, 1998).

1.16 Role of autophagy in cellular maintenance

Autophagy is a process that is used by the cell for intracellular degradation. It involves the formation of an isolation membrane in one portion of the cytoplasm. A double membrane structure is formed called autophagosome, which is a vacuole like structure that engulfs all the organelles and intracellular debris. It then fuses with the lysosome; an organelle responsible

for digesting the debris by utilizing its acidic environment. As damaged proteins or organelles are degraded by the lysosome, amino acids and nutrient components of those structures are released back to the cellular environment as building blocks of components that were digested (Glick *et al.*, 2010).

Autophagy has been studied for a long time as a bulk or non-specific process that just engulfs a part of the cytoplasm, but recent evidence shows that autophagy is a more specific process. It has been demonstrated to be targeted specifically to damaged parts of the cell; like organelles or even proteins (Li *et al.*, 2012). Further investigation has led to the discovery of diseases like Carney complex syndrome, Crohn's disease, Vici syndrome, Hereditary spastic paraparesis; that are caused by disruption of these specific autophagy pathways (Jiang and Mizushima, 2014) (Kaushik *et al.*, 2010).

1.17 Autophagy: process and function

TOR or mTOR (mammalian/mechanistic Target of Rapamycin), is a serine/threonine kinase that regulates a wide array of functions like cell growth, cell survival, protein synthesis and translation (described in detail in Chapter 1.22). Adding to these functions, TOR is also a major regulator of autophagy. The activation of TOR is responsible for deactivation or destabilization of the Unc-51 like Autophagy Activating Kinase 1 (ULK1) complex that consists of Autophagy related 13 (ATG13), Family Kinase-Interacting Protein of 200 kDa (FIP200) and ATG101 proteins. In absence of TOR activation (i.e. under conditions of starvation, where nutrients are much more necessary to survive than to promote growth), phosphorylation of ULK1 is halted stabilizing the complex. It is then translocated to the endoplasmic reticulum that provides a membrane source for the autophagosome (Jung *et al.*, 2010). In parallel, PI3K activation also activates several autophagy genes. The recruiting of ATG's is responsible for the formation and expansion of the isolation membrane and eventually an omegasome (Heras-Sandoval *et al.*, 2014). More specifically, LC3 or ATG8 is responsible for a very important step in autophagy which is the maturation of autophagosome. In the absence of lipidation of LC3, the autophagosome formation is disrupted and autophagy is terminated. This step has been proven to be absolutely essential, as rescuing the phenotype caused by a mutation in LC3 has not been demonstrated (Tanida *et al.*, 2008). Following this step, SNARE (soluble NSF attachment protein receptor protein) and HOPS (homotypic fusion and protein sorting protein) tether and form a complex. This is the final step in the process

where the fusion of autophagosome and lysosome occur (Jiang *et al.*, 2014). Figure 1.9 illustrates the process of autophagy.

Although the signalling cascade was discovered in budding yeast, in the past two decades it has been studied in many other organisms like worms, flies, mice and primates. Discovery of these processes has led to the development of many knockout animals. Most of the knockouts in the autophagy signalling cascade produced similar phenotype which is evident that autophagy is a linear signalling cascade (Mizushima and Levine, 2010). It is still unclear how damage to organelles or proteins is detected by the autophagic machinery. Pharmacological targets are being discovered and developed alongside the discovery of new genes in the autophagy pathway (Rubinsztein *et al.*, 2012).

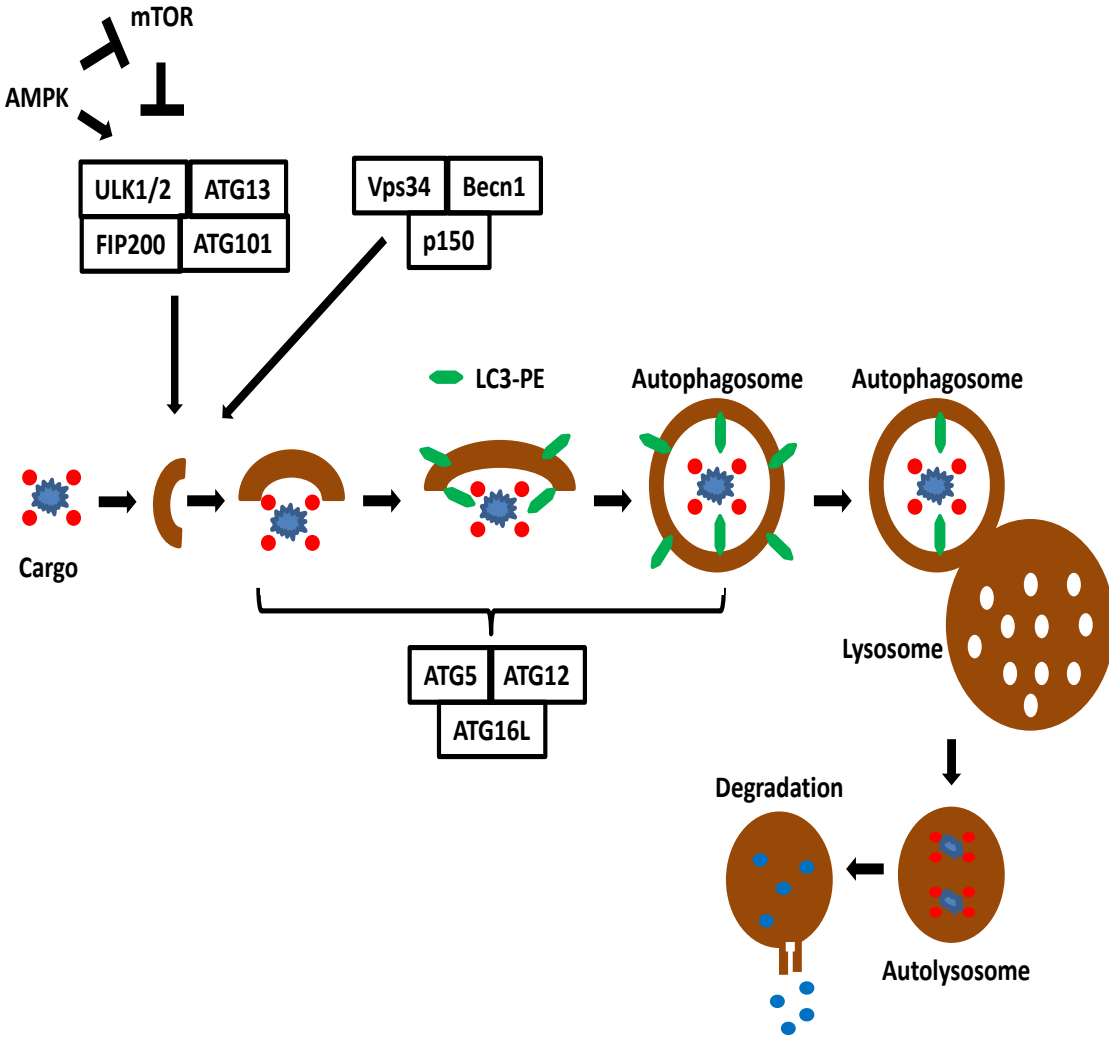

Figure 1.9 The process of autophagy

Autophagy is a complex process involving many proteins for activation and sustenance (Adapated from Kimmelman, 2011).

1.18 Various kinds of autophagy that occur within the cell

Autophagy is mainly subdivided into macro-autophagy, micro-autophagy and chaperone mediated autophagy. Macro-autophagy is bulk degradation or non-specific of debris within the cell, but in recent years more specific macro autophagic processes have been discovered (Feng *et al.*, 2014). Some examples of specific macro-autophagy are; (i) Mitophagy or the degradation of mitochondria (described in detail in Chapter 1.19); (ii) Pexophagy or the degradation of peroxisomes; that are detected by PEX proteins which translocate to the surface of peroxisomes. This recruits the ATG protein, ATG30, which flows into the canonical autophagy pathway for degradation of the whole organelle; (iii) Reticulophagy or the degradation of the Endoplasmic Reticulum; LC3 has been demonstrated to localize to ER in order to detect and activate downstream elements to sequester ER to the lysosome and; (iv) Aggrephagy or recycling of damaged and aggregated proteins is a more specific process. Disruption of aggrephagy has also been shown to cause many diseases (Reggiori *et al.*, 2012). Aggrephagy is usually an aggregation of damaged proteins that cannot be recycled. These aggregated proteins are usually targeted by p62, which recruits and activates LC3 for further autophagic degradation (Reggiori *et al.*, 2012).

Microautophagy is a more unspecific process which does not involve any recruitment or specific activation. It is generally a direct lysosomal degradation process which is just bulk turnover of many or most of the cytosolic components. However, recently there are reports showing more specific microautophagy. Three types of microautophagy have been discussed extensively; (1) Micropexophagy, the process of degradation of damaged or superfluous peroxisomes. In this process of autophagy, instead of the peroxisomes being targeted or sequestered to the lysosome, the lysosome fuses with the peroxisome directly (Mijaljica *et al.*, 2011); (2) Micromitophagy, the process of unspecific recycling of damaged mitochondria. It is split in two; (i) Involves engulfment of mitochondria by the lysosome and; (ii) Mitochondria fuse with lysosome for its degradation. Among other kinds of microautophagy, micromitophagy is least characterised (Mijaljica *et al.*, 2011) and; (3) Microautophagy of the nucleus, is the process of degradation of a damaged part of the nucleus. In this process the damaged nucleus is engulfed by endoplasmic reticulum membrane and the lysosome then engulfs this structure for degradation (Mijaljica *et al.*, 2011).

Another major kind of autophagy is chaperone mediated autophagy (Kaushik and Cuervo, 2012). Chaperone mediated autophagy is a specific target recognition process. This type of autophagy is mainly for damaged proteins that are targeted by specific chaperones like HSC70 or HSP90. The proteins targeted by these chaperones are recognized by LAMP2 (Lysosome associated membrane protein 2), which sequesters them directly to the lysosomes.

1.19 Mitochondrial specific autophagy or mitophagy

Mutation in PTEN-induced putative kinase (*PINK1*) and/or *PARKIN* genes in humans have been associated with familial and sporadic forms of Parkinson's disease. In 2008, Richard Youle and his colleagues established that PARKIN and PINK1 are responsible for degradation of damaged mitochondria (Narendra *et al.*, 2008). They described the mechanism as a canonical PINK1-PARKIN pathway that is responsible to detect and recycle damaged mitochondria. PINK1 is a protein that is encoded in the nucleus and transported to the mitochondria. Under normal circumstances, PINK1 is usually cleaved in the mitochondria by different proteases. This cleaved version of PINK1 is completely inactive. When cells are subjected to CCCP, depolarization of the mitochondria is answered by stabilization of PINK1 (Narendra *et al.*, 2008). Under these circumstances, PINK1 is not processed by proteases; however, the mechanism of how this happens is still unclear. The stabilized version of PINK1 is responsible for recruiting and activating PARKIN. PARKIN then ubiquitinates several proteins on the outer membrane of mitochondria like Mfn2 or Drp1, which are responsible for recruiting several ATG proteins. Consequentially damaged mitochondria as a whole organelle is transported to the lysosome for complete degradation (Narendra *et al.*, 2008; Jin *et al.*, 2010). It is becoming more evident that the activation of PINK1 and PARKIN can occur only through the detection of damaged mitochondria. It has also been demonstrated that PINK1 auto phosphorylates itself when mitochondria depolarize, which is responsible for the activation of other downstream processes (Okatsu K *et al.*, 2012). Few other studies have also demonstrated that p62 and LC3 can orchestrate the sequestering of the mitochondria to the autophagosome; however, these studies are not very clear (Park *et al.*, 2014; Strappazzon *et al.*, 2015). More importantly, *Pink1* and *Parkin* knockout mice show a very mild phenotype of Parkinson's when compared to human cohorts (Chesselet and Richter, 2011). Figure 1.10 is a schematic representation of canonical mitophagy.

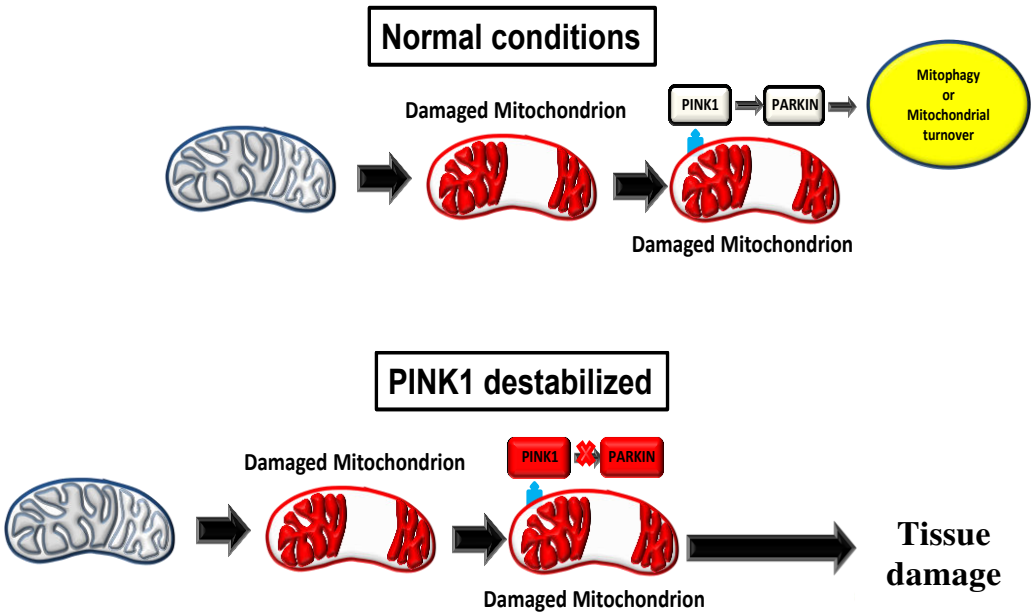

Figure 1.10 Schematic representation of canonical mitophagy

When mitochondria are depolarized or damaged, PINK1 is stabilized on the outer mitochondrial membrane. This in turn recruits PARKIN to sequester mitochondria to the autophagosome (Top panel). However when there is a deficiency in Pink1 these damaged mitochondria are not recognized and start accumulating (Bottom panel).

1.20 Proteasomal degradation of damaged proteins

Protein turnover is generally the balance between protein synthesis and protein degradation. When this balance is lost, the cell is under stress which causes many disease phenotypes. In general, protein synthesis happens when in an anabolic or a more fed state; and protein breakdown happens when in a catabolic or a starved state. Decrease in breakdown of proteins have also been found to be directly proportional to increase in age (Razanov *et al.*, 2002).

Proteosomal degradation of ubiquitinated protein is a process that recognizes independent proteins and breaks down the peptide bonds in an energy dependent manner. This process is also mainly used in the breakdown of misfolded proteins and broken down proteins are generally 7-8 base pairs long. They are further degraded into separate amino acid components. These amino acids can be used for manufacture of new proteins. Proteasomal assembly and components are different in different model organisms, but the number or subunits that are necessary to be assembled are always very complex. In most organisms, the proteasome consists of a 20S, 16S, 19S and a 11S subunits (Murata *et al.*, 2009). The whole complex is responsible for breaking down proteins by breaking peptide bonds between amino acids using ATP, produced by the cell. This is the final step of protein degradation. Before this process the proteins are tagged with a marker or a flag for degradation. This tagging is undertaken by ubiquitin, a very small protein of around 8kDa, that is responsible for tagging damaged proteins and is present in all organisms. These ubiquitin tagged proteins are then poly-ubiquitinated, a process by which the ubiquitin protein binds to itself to form a long poly-ubiquitin chain. Formation of this chain is the final stage in targeting damaged proteins to the proteasome (Li and Ye, 2008).

1.21 Role of dysfunctional autophagy and protein turnover in disease

Dysfunctional autophagy is involved in many disease phenotypes. One of the first ever described diseases involving dysfunctional autophagy is Static encephalopathy of childhood with Neuro-degeneration in Adulthood (SENDA). In patients, a mutation in *WIPI4* gene was discovered. With the help of more functional studies in other model organisms like worms and flies (Grimmel *et al.*, 2015), this gene was found to be involved in the formation of the autophagosome. This was the first ever study showing the link between autophagy and neurodegenerative disease (Saitsu *et al.*, 2013). The most important characteristic of this disease is accumulation of iron in the brain. Patients suffering from the disease present with symptoms like spastic paraplegia, mental retardation, Parkinsonism and dystonia. Even

though studies have been very thorough, it has proven to be extremely difficult to find a mechanism of why accumulation of iron in the brain occurs. Following this, finding a cure for the same has been a daunting task for researchers in the field (Ebrahimi-Fakhari, 2013).

Parkinson's disease is the second most common form of progressive neurodegeneration. There are two kinds of this disease; the autosomal recessive and the sporadic form of Parkinson's. Even though extensively studied, neither a proper mechanism nor a cure to Parkinson's has yet been found. Patients with familial Parkinson's are found to have mutations in *PARKIN*, *PINK1*, *SNCA* (α-Synuclein), *LRKK2* (Leucine-rich repeat kinase 2) or *GBA* (Glucocerebrosidase) (Nalls *et al.*, 2001). Moreover as *PARKIN* and *PINK1* have been recently discovered to be involved in mitophagy, patients with Parkinson's are found to have an accumulation of dysfunctional mitochondria and symptoms such as tremors, muscle rigidity, gait and flexed posture are seen among many other phenotypes. It has been especially difficult for researchers to find a cure because of the dual or multiple functions of the same protein (Duty and Jenner, 2011). For example, PARKIN is involved in other processes like ER protein translocation along with protein degradation (Takahashi *et al.*, 2003), and a defect in autophagy is not the only phenotype caused by PINK1 or PARKIN mutation.

Studies showing involvement of autophagy in cancer has been very complicated, since autophagy in cancer is like a double-edged sword. In primary stages of cancer, autophagy is inhibited to prevent cancer, as the cells lose their recycling ability. After the development of cancer, autophagy is necessary for the maintenance involving cyto protection (Mathew *et al.*, 2007). Mutation in the *BECLIN1* gene has been found in many cancers like breast, ovarian and prostate cancer. BECLIN is involved in recruiting ATG14, which progresses into autophagy. Other than autophagy, BECLIN is also very important for its anti-apoptotic effect, which is necessary to prevent cancer in the early stages (Qu *et al.*, 2003). Several other proteins, apart from BECLIN, like ATG5 and UVRAG have been found mutated in many cancer types (Park *et al.,* 2014). Cancer is the most extensively studied disease; therefore, prevention or cure of cancer is not far-fetched.

Defective proteasomal degradation of damaged proteins is also involved in many diseases. One of the most extensively studied diseases among this is Alzheimer's. It is the cause of 60-70% of dementia cases and is a chronic neurodegenerative disease. Patients present with symptoms like short-term memory loss, problems with language, disorientation and behavioural issues. Even though extensive research for Alzheimer's is happening for the past

century, no treatment to improve the symptoms have been discovered yet due to the complexity of molecular mechanisms involved (Ubhi and Masliah, 2013).

Examples of few other diseases caused by dysfunctional autophagy or protein turnover are; (i) Crohn's disease, which is caused due to a mutation in the *ATG16* gene (Baumgart and Sandborn, 2012). These patients present with symptoms like weight loss and severe vomiting; (ii) Hereditary spastic paraplegia is another disease that is caused due to a dysfunctional SPASTIN (SPG4) protein. SPASTINS are generally involved in membrane trafficking and proteolysis. Patients are found to have many conditions of neurological disorders (Depienne *et al.*, 2007) and; (iii) Another very rare disease is Vici Syndrome, which is caused due to a mutation in the *EPG5* gene, which is involved in autophagosome maturation. Patients with the Vici syndrome present with a multisystem disorder (del Campo *et al.*, 1999). However, with the emergence of exome sequencing and whole genome sequencing, there have been many new genes discovered involved in these processes. Due to the discovery, pharmacological interventions have been more plausible.

1.22 The TOR signalling pathway

Most of the organisms have evolved specific mechanisms to efficiently switch between catabolic and anabolic states, which allowed them to survive and respond to stress under the conditions where nutrient availability is scarce. One such pathway is the mTOR or the mammalian Target of Rapamycin pathway. The mTOR (TOR hereafter) pathway responds to several environmental changes and it is apparent that TOR controls many of the cellular processes. Its most significant role has been related to growth and proliferation, as nutrient sensing is a very important signal for the growth of an organism. mTOR is a serine/threonine protein kinase and it belongs to the phophoinositide3-kinase (PI3K) family. This protein kinase interacts with several proteins in order to form two protein complexes namely, TOR Complex 1 and TOR Complex 2. Both the TOR complexes are large and contain six and seven very well studied protein components respectively. These proteins bind to their specific complexes in order to inhibit or activate them; sensing the conditions which are dictated to them by upstream regulating elements (Laplante and Sabatini, 2012).

TOR was first discovered in the model organism *Saccharomyces cerevisiae,* where genetic screens identified TOR Complex 1 and TOR Complex 2 as targets of the molecule rapamycin. Rapamycin is a macrolide produced by *Streptomyces hygroscopicus* bacteria that gained

attention because of its anti-proliferative and anti-inflammatory properties (Li *et al.*, 2014). Shortly after the discovery of TOR and rapamycin, many different approaches were used to identify the physical binding of rapamycin to TOR to inhibit TOR Complex 1. Although the inhibitory effects of rapamycin on TOR signalling is clear, the mechanism of action has proven to be much more complex than originally anticipated. However the process by which rapamycin forms a complex with FK506-binding protein and directly interacts and inhibits TOR Complex 1 but not TOR Complex 2 is still in the dark. The hypothesis that rapamycin may interfere with the structural integrity of TOR Complex 1 or with the specific activity of its kinase domain has still not been proven 20 years after the discovery of TOR signalling.

TOR has been proven to regulate many cellular mechanisms downstream and it is one of the major nutrient sensors in all organisms. Because of the complex signalling networks linked with TOR, it is a major cause for numerous diseases. TOR deregulation, mostly hyper-activation, has been linked to human diseases like cancer, obesity, type 2 diabetes and neurodegeneration. Furthermore, it has been demonstrated in several model organisms, ranging from yeast to mice, that activation or repression of TOR both genetically and pharmacologically can improve diseases phenotypes. Repression of TOR pharmacologically by rapamycin has also been shown to extend lifespan. This has led to pharmacologically activation or repression of the pathways for treatment of diseases. Moreover, as rapamycin is already a FDA approved drug, discovering its mode of action will be an important step forward in the future (Dazert and Hall, 2011).

1.23 Upstream regulators of TOR

TOR complexes are very well characterised and it has been demonstrated that both these complexes converge from two very different and distinct upstream regulators. TOR Complex 1 integrates several inputs like growth factors, oxygen levels of the cell, amount of amino acids and it also senses the energy status of the cell. Since the number of upstream elements that are sensed by the TOR Complex 1 is numerous, downstream cellular mechanism like protein synthesis, autophagy and lipid synthesis, are also varied (Laplante and Sabatini, 2012). Most of these process and signalling cascades have been described in detail in the Chapter 1.24.

One of the most important signalling responses that are mediated by TOR is oxygen sensing (Filomeni *et al.*, 2014). Oxygen sensing pathway also determines the energy status of the cell and this converges on to TOR signalling pathway (Filomeni *et al.*, 2014). This response is

mainly mediated by Adenosine monophosphate-activated protein kinase or AMPK, which is the major fuel gauge of the cell that detects the balance between three forms of nucleotides namely; Adenosine monophosphate (AMP), Adenosine diphosphate (ADP) and Adenosine triphosphate (ATP). In conditions of low energy state or low oxygen state, AMPK phosphorylates the heterodimer Tuberous Sclerosis Complex 1 and 2 (TSC 1 and 2). TSC 1 and 2 complex is a GTPase activation protein responsible for the activation of Ras homolog enriched in brain protein or RHEB. This activation activates RHEB, which in turn binds to the TOR complex. Overall in case of oxygen sensing mechanism, AMPK is responsible for deactivation or repression of TOR in conditions of low energy or low oxygen. This leads to improvement in glycolysis and energy production by better recycling of the available nutrients. On the other hand, it also might be responsible for halting development or growth of the organism which requires a constant supply of energy.

In recent years, TOR has also been demonstrated to respond to DNA damage, which is also a cause of oxygen stress or low energy stress (Xu *et al.*, 2012). It has been shown that DNA damage increases the expression of Phosphatase and Tensin homolog deleted on chromosome 10 or PTEN. Increase in PTEN has been shown to downregulate TOR signalling axis severely, which in turn is shown to increase DNA damage response. A feedback loop involving the activation of AMPK has also been demonstrated to be involved in response to DNA damage (Shen *et al.*, 2007).

Insulin signalling is one of the processes that is extensively controlled by TOR. Most of the signalling pathways that control insulin signalling converge on to the TOR signalling pathway, which then controls downstream cellular processes. Insulin signalling is initiated by insulin receptors which are present on the surface of cellular membranes (Laplante and Sabatini, 2012). These insulin receptors (INR's) are ubiquitous and are also present in almost all organisms. The insulin signalling pathway then branches into two distinct pathways, the PI3K and RAS pathways (Oldham and Hafen, 2003). Phophotidyl inositol 3-kinase or PI3K activation controls AKT or the Protein Kinase B signalling (PKB), and RAS activation controls the extracellular regulated signalling regulated kinase or ERK1 pathway (Guardiola-Diaz *et al.*, 2012). Both these pathways are studied for their involvement in metastasis. AKT pathway follows the canonical route of TSC 1 and 2 inhibition, which activates TOR signalling. It has also been demonstrated that AKT, upon sensing nutrients or insulin, can directly bind and inhibit RAPTOR which is a negative regulator of TOR, again leading to the hyper-activation of TOR.

Tumor Necrosis Factor α or TNF α, another widely studied molecule for its mechanistic action in metastasis, is also involved in activation of TOR by an inhibitory phosphorylation of TSC 1 and 2. This leads to a persistent activation of TOR. Nutrient and insulin signalling are also major regulators of growth and proliferation. Canonical WNT pathway controls TOR signalling which is very important for maintaining homeostasis of the organism. WNT pathway controls cell growth, proliferation, polarity differentiation and development. WNT activation inhibits glycogen synthase kinase 3 β (GSK3), which in turn activates the TSC 1 and 2 network in order to inhibit TOR signalling (Kikuchi *et al.*, 2011).

Another major function of TOR signalling is the detection of amino acids, especially Leucine and Arginine. Recently it has also been demonstrated that arginine controls mTOR activity in a TSC2-RHEB dependent manner (Carroll et al., 2016). The canonical pathway commences with specific amino acids which are sensed by the mammalian RAG GTPases (Laplante and Sabatini, 2012). RAG GTPases in mammals comprises of four proteins namely RAG A, B, C and D. RAG A and RAG B proteins contain a GTP domain and is activated in the presence of GTP. Whereas, RAG C and RAG D proteins are activated in the presence of GDP. RAG A/B heterodimer interacts with RAPTOR, inactivating the protein by phosphorylation, which in turn activates TOR. This activated version of TOR is transported from the cytosol to the lysosome by an unknown mechanism. The translocation and activation of TOR on the lysosomal surface are controlled by the interaction between the RAG A/B heterodimer and RAGULATOR. It is coupled to the activation of GTP loaded RHEB, but this cascade is activated only if the RAGULATOR protein chaperones TOR to the lysosomal surface. Nonetheless, other proteins have also been demonstrated to sense amino acids to respond to the status of the cell. Proteins like Mitogen-Activated protein kinase or MAPK3, Mammalian vacuolar protein sorting 34 homolog or hVPS34 and Inositol polyphosphate monokinase (IPMK), have been demonstrated to activate TOR by the RAS-RAGULATOR system (Lynch *et al.*, 2000).

Finally, in order to control lipid biogenesis, TOR signalling also acts in response to lipids to produce the right amount of lipids necessary for homeostasis. It has been demonstrated that Phosphatidic acid (PA) activates mTOR, but there are no studies that show the mechanism of action. On the contrary, the process of activation has been studied intensively and it has been shown that overexpression of Phosphatidic acid synthase (PAS) and exogenous addition of Phosphatidic acid (PA) activates TOR persistently. Molecular basis of this activation have

been emerging and preliminary studies have demonstrated that this activation might be due to the stabilization of TOR complex by PA (Foster, 2013).

All these upstream regulators of TOR converge on several cellular processes which control many activities of the cell. The de-regulation of many of these pathways have been demonstrated to cause distinctive diseases like cancer, obesity, diabetes, hepatic steatosis, etc. However, it is still not completely clear how upstream elements regulate TOR. Moreover, it is also unknown how these cascades are prioritized by the cell. Pharmacological inhibition or activation of these pathways, which then converge on to TOR, will aid in discovering new small molecules that need not necessarily act specifically on TOR for its beneficial effects (Laplante and Sabatini, 2012).

1.24 Cellular mechanisms controlled by TOR signalling

The TOR signalling pathway is involved in activation of many signalling cascades; however, autophagy is one of the very few processes that TOR de-activates (i.e. inhibition of TOR increases autophagy). This is regulated by inhibitory phosphorylation of UNC 51 like kinase (ULK1) or the mammalian autophagy related gene 13 (ATG13). The phosphorylation of ATG13 or ULK1 is responsible for inhibition of autophagy and this machinery is initiated by TOR. TOR controls autophagy by inhibitory phosphorylation of DAP1 (death-associated protein 1) that in turn suppresses autophagy. Moreover, the activation of ATG18 by TOR has been extensively studied to show they increase autophagy. Although TOR signalling has been shown to regulate autophagy, it has also been demonstrated that TOR directly regulates lysosomal biogenesis. TOR phosphorylates Transcription factor EB or TFEB that prevents the protein from entering into the nucleus, where it activates an array of autophagy initiating genes (Roczniak-Ferguson *et al.*, 2012). Nutrient sensing has a major role to play in autophagy. When the organism is low on nutrients, the nutrients that are already present recycle more efficiently. This is an evolutionary mechanism that is controlled by TOR, as the presence of nutrients has a major role in activation or repression of TOR signalling. In case of a low nutrient environment the cell represses TOR, which in turn increases autophagy for more efficient recycling of the available nutrients to support other cellular processes.

On the other hand, TOR positively regulates energy balance. It has been demonstrated that TOR directly controls ATP production in the cell by controlling OXPHOS in the mitochondria and glycolysis, which are two major pathways involved in producing ATP. Firstly, there are reports proving that TOR alters OXPHOS activity by directly regulating

mtDNA (Villa-Ceusta *et al*., 2014), which is responsible for encoding major components of OXPHOS system or ETC. This is mediated by TOR by activating PPARγ–Coactivation activator (PGC1α). The activation of PGC1α is responsible for increasing mitochondrial biogenesis (Cunningham *et al.*, 2007). Secondly, TOR is responsible for regulation of glycolysis. This is carried out by the upregulation of Hypoxia inducible factor 1α or HIF1α, which is responsible for the activation of many genes involved in glycolysis (Cheng *et al.*, 2014). These transcriptional regulations are said to have intermediate factors as the presence of TOR in the nucleus is minimal (Sun *et al*., 2011). Moreover, mitochondria also participate in a signalling cascade that lead to inhibition of TOR activity (Schieke and Finkel, 2006). However, the involvement of these mitochondrial signals is still not understood.

TOR also positively regulates lipid biogenesis, which is necessary for forming membranes in many organelles of all organisms. TOR directly activates Sterol regulatory element-binding protein 1 or 2 (SREBP 1 or 2), which is a major transcription factor for genes that improve fatty acid and cholesterol biogenesis. TOR also regulates SREPB by inhibitory phosphorylation of LIPIN1, which is responsible for inactivation of SREBP (Porstmann *et al.*, 2008). It has also been demonstrated that TOR controls adipogenesis by activation of Peroxisome-Proliferator-activated receptor γ (PPARγ) (Kim and Chen, 2004). Positive regulation of lipid biogenesis and TOR are closely linked because of growth regulation, as lipids are major building blocks in the formation of almost all membranes during the development of an organism.

The most important role of TOR is probably the positive regulation of protein synthesis. TOR controls protein synthesis by controlling two different mechanisms. To begin with, TOR phosphorylates the EIF 4E-binding protein or 4EBP. This phosphorylation inhibits 4EBP from binding to the EIF4E protein, which in turn activates the protein and cap dependent translation of proteins. The second mechanism is regulated by phosphorylation of S6 Kinase1 protein (S6K1), which is responsible for the activation of mRNA biogenesis, translation initiation and also elongation in protein translation (Wang and Proud, 2006). It is very well known that S6K1 regulates most parts of the translational machinery, but mechanism by which it coordinates most of these processes is still unclear. On the other hand, TOR regulates protein translation by interacting with promoter regions of RNA polymerase transcribed genes. This is controlled by two mechanisms; (i) TOR activates Tripartite motif containing protein-24 or TIF-1A. This promotes the interaction with RNA polymerase 1 or POL1 which increases translation (Mayer *et al.*, 2004) and; (ii) TOR inhibits MAF1 via inhibitory

phosphorylation, which is a repressor of Polymerase III or POLIII. This increases the presence of tRNA which leads to increase in translation (Shor *et al.*, 2010). Overall, protein synthesis is one of the major processes controlled by TOR as this directly corresponds to the growth of an organism. Figure 1.11 is a flow chart of the upstream elements that control TOR and the downstream signals that TOR orchestrates.

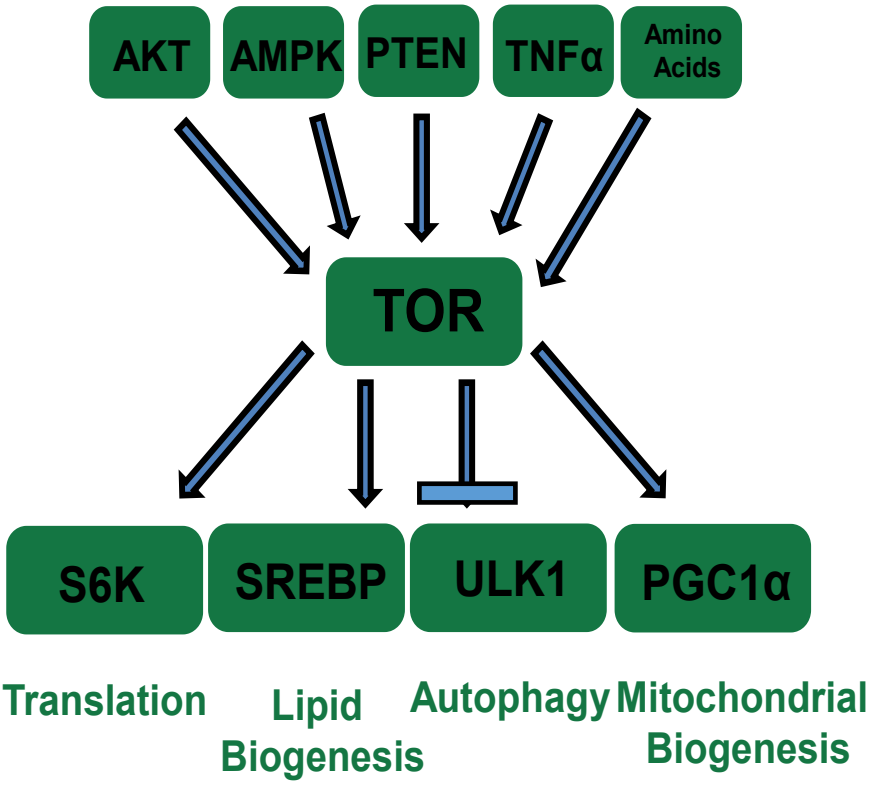

Figure 1.11 The TOR signalling pathway

The diagram summarizes the upstream elements that control and TOR and the downstream signalling mechanisms that TOR orchestrates.

1.25 Role of TOR signalling in diseases

In majority of cancers, it has been observed that TOR signalling is hyper activated. Activation of TOR is responsible for increase in glycolysis and growth of cancer cells. In many cancers, a mutation in *P53*, which is a tumor suppressor or *TSC1/2* heterodimer is found to be the cause of the disease (Hodges *et al.*, 2001). When TSC 1and 2 is not present to inhibit TOR, it is hyper-activated and leads to excessive growth. This TOR hyper-activation can also lead to increase in inhibitory phosphorylation of 4EBP, which in turn leads to an increase in protein translation. This supports the hypothesis that cancer cells need more proteins that are necessary to support its proliferation by cell cycle increase and metastasis. Ribosome biogenesis is also increased in these cases which are likely to promote metastasis by providing machinery for growth. Although ERK1 and S6K1 have not been shown to play a very important role in cancer, more studies have started emerging regarding the same. It has also been shown in many cases that an increase in lipid biogenesis is important for maintenance of cancer, especially an activation in SREBP1 (Guo *et al.*, 2014). Another pathway that leads to cancer through TOR regulation is the autophagy pathway; an absolute balance in autophagy is necessary for maintaining homeostasis of the cell. Pharmacological inhibition of parts of this signalling cascade has been demonstrated to halt metastasis in many cases (Mathew *et al.*, 2007).

Involvement of TOR in many metabolic diseases has been demonstrated, but to isolate a particular mechanism is very difficult as it controls an array of metabolism controlling genes. Fasting and feeding states in mice have been used to study these effects controlled by TOR (Laplante and Sabatini, 2012). They switch between the anabolic and catabolic states that are controlled by TOR, which in turn controls metabolism. This part of TOR signalling cascade has not been thoroughly studied as a full body knockout of *TOR* causes embryonic lethality in mice (Murakami *et al.*, 2004). For overcoming these conditions, tissue specific knockout mice have been used and promising results have been emerging from these studies (Russell *et al.*, 2011).

In the brain, hypothalamus is the part that maintains energy balance and it is a complex network of signals that responds and packages signals to TOR. In response to energy balance, TOR can deactivate a gene called orixigenic neuropeptide or *NPX*, which responds to the food intake by an organism (Wiczer and Thomas, 2010). This deactivation leads to intake of more food leading to obesity which increases the incidence of other diseases such as type 2

diabetes. In many studies it has been shown that conditional knockout of *TOR* in brain leads to lean mice even when fed with a high fat diet (Yang *et al.*, 2012).

One other major disease without a proper cure is type 2 diabetes, which is also said to be a cause of hyper-activation of TOR (Zoncu *et al.*, 2011). Increase in TOR causes an increase in phosphorylation of S6K1, which is responsible for a feedback loop inhibition of insulin signalling leading to insulin resistance, thereby causing type 2 diabetes. As an evidence, it has been shown that knockout of S6K1, reduced this feedback loop and decreased insulin resistance (Leibowitz *et al.*, 2008).

TOR has also been shown to control muscle contraction through synthesis of specific proteins. In many cases, the conditional knockout of *TOR* in mice is said to cause myopathy. This is due to the loss of functionality of OXPHOS, ultimately leading to death. It has also been shown that through the control of PGC1α, TOR increases the accumulation of glycogen in muscles (Risson *et al.*, 2009).

TOR has also been shown to cause many neurodegenerative diseases like Parkinson's, Alzheimer's, Huntington's and Amyotrophic Lateral Sclerosis. The common cause of all or most of these neurodegenerative diseases are protein aggregates or damaged proteins. Hyper-activation of TOR leads to a decrease in autophagy, which causes protein aggregation as proteins are not recycled. Additionally, TOR activation also promotes protein synthesis, which increases the possibility of protein aggregation (Laplante and Sabatini, 2012). Many mouse models have been developed to phenocopy human diseases like Parkinson's and Huntington's which causes neurodegration and increases the presence of aggregate prone proteins. Pharmacological inhibition of TOR has been shown to rescue these phenotypes providing neuroprotection and improving clearance of these proteins.

It is not very surprising that one of the many pathways that play a role in ageing is TOR. Although it has been demonstrated that TOR and dietary restriction produces similar increase in lifespan. However, it has also been demonstrated that there is no additive effect with both interventions; which denotes that they act in the same pathway (Blagosklonny, 2010). Additionally it has been demonstrated that TOR is hyper- activated in aged organisms and rapamycin feeding can extend the lifespan of old mice (Harrison *et al.*, 2009). However, specific molecular mechanisms of these pathways are still unknown. TOR is also shown to be involved in many other lesser studied diseases, one of which is Nonalcoholic fatty liver

disease. When TOR is activated, ketogenesis and lipogenesis are increased, which causes accumulation of fat in the liver leading to the disease (Rinella, 2015).

1.26 Pharmacological inhibitors of the TOR complex

TOR was first discovered in budding yeast when the mechanism of action for rapamycin was investigated. Rapamycin was initially used as an anti-fungal agent and also as an immunosuppressant. The drug was approved by FDA as an immunosuppressant to be used during transplantation of organs. However, it was continually examined for its anti-proliferative action as a valid therapy for cancer (Seto, 2012). It is still under investigation and rapamycin is not used in cancer trials because of its unstable chemical structure and insolubility in water. To overcome these limitations, many other analogs of rapamycin like; Temosirolimus, Everolimus and Deforolimus have been used and have been demonstrated to inhibit TOR. These drugs have been studied for the past decade and research has shown many advantages and disadvantages over rapamycin. These drugs have still not been used in human trials. There have also been some rapalogs discovered like 32-deoxy rapamycin or zotorolimus. These novel drugs have been used instead of rapamycin in many studies due to their chemical stability and water solubility unlike rapamycin, making them more convenient for human trials (Lamming *et al.*, 2013).

Even though it has been almost 20 years since TOR was discovered, there is no clear proof of concept of how TOR is inhibited by rapamycin. Rapamycin has also been shown to inhibit TOR by a completely novel mechanism as it does not bind to the kinase domain of TOR. Pre-clinical trials have demonstrated to be anti-proliferative for some cancers. However most of them are resistant to rapamycin which makes it more difficult to study (Dumont and Su, 1996). As novel inhibitors of TOR are being discovered at a very fast rate and also the mechanisms of action for old inhibitors are surfacing, we can be sure that more precise and effective drugs will be discovered in the near future.

1.27 Aims

The ability of an organism to respond to stress progressively declines with age; therefore studying the process of stress adaptation can aid in understanding the mechanisms of quality control that fail during ageing. It has been already reported that damaged mitochondria accumulate with age (Lopez-Otin *et al*., 2013). Understanding why this happens under a stress condition that mimics the ageing process is beneficial to prevent or delay the onset of age related diseases. It has also been shown that metabolic rate increases with temperature, where the mitochondria play a crucial role (Scialo *et al*., 2015). I aimed to investigate the signalling mechanisms through which mitochondria regulate this adaptation. The specific aims of this study were;

1) To ascertain if a mitochondrial ROS signal is essential for this adaptation (Chapter 3).
2) To understand which ROS entity; either H_2O_2 or O_2^- is involved in this process (Chapter 4).
3) To investigate the role of mitochondrial ROS in controlling TOR signalling (Chapter 5).
4) Can mitochondrial turnover be responsible to maintain homeostasis of an organism (Chapter 6)?

Chapter 2. Materials and methods

2.1 Reagents

All reagents used in this thesis, their providers and catalogue numbers are listed in Table 2.1.

Table 2.1 List of reagents

Reagent	Company	Catalogue number
Acetyl coenzyme A lithium salt	Sigma	A2181-100MG
Active dried yeast	Dutscher Scientific	789093
Adenosine 5′-diphosphate sodium salt (ADP)	Sigma	A2754-5X1G
Antimycin A	Santa Cruz Biotechnology	sc-202467A
Bovine Serum Albumin (BSA)	Sigma	A7906-100G
BSA (fatty acid free)	Sigma	A7030-10G
Bradford Reagent	Sigma	B6916-500ML
Bromophenol Blue	Sigma	B0126-25G
Chroloform	Sigma	288306
Clarity Western ECL substrate kit	Biorad	170-5061
cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets in EASYpacks.	Roche	4693159001
DEPC water	Qiagen	129112
Dichlorofluorescin (H_2DCF)	Sigma	D6883-50MG
5,5′-Dithiobis(2-nitrobenzoic acid)	Sigma	D8130-5G
D-(+)-Glucose	Santa Cruz Biotechnology	sc-211203A
Dithiothreitol (DTT)	Life Technologies	R0862
Digitonin	Santa Cruz Biotechnology	sc-280675
Dimethylsulfoxide (DMSO), sterile filtered	Santa Cruz Biotechnology	sc-359032

DNase I + DNase I Buffer	Thermo Fisher Scientific	89836
dNTP	Thermo Fisher Scientific	18427088
Drosophila Agar Type II (Apex bioresearch products)	Dutscher Scientific	789150
D-(+)-Saccharose	VWR	27480.294
Ethanol (EtOH)	Fisher Scientific UK	E/0650DF/17
Ethylenediaminetetraacetic acid (EDTA)	Sigma	EDS-100G
Ethyleneglycol-bis(2-amino-ethylether)-N,N,N',N'-tetracetic acid (EGTA)	Sigma	03777-10G
Guanidine Hydrochloride	Santa Cruz Biotechnology	sc-202637
sn-Glycerol 3-phosphate bis(cyclohexylammonium) salt	Sigma	G7886-5G
HEPES	Sigma	H3375-25G
Isopropanol	Sigma	190764
Magnesium Chloride, Hexahydrate	Santa Cruz Biotechnology	sc-203126A
Maize meal	TRS white maize meal	4767967
MitoSOX	Life Technologies	M36008
Nipagin M (methyl 4-hydroxybenzoate)	Sigma	H5501-500G
Phosphate Buffered Saline (PBS) tablets	Cambio	MC-09-9400-100
Phospho(enol)pyruvic acid monopotassium salt	Sigma	860077-1G
PMSF (Phenylmethanesulfonyl fluoride)	Sigma	P7626-1G
Potassium cyanide (KCN)	Sigma	60178-25G
Potassium Hydroxide (KOH)	Sigma	484016-1KG
Potassium Phosphate, Monobasic (KH_2PO_4)	Santa Cruz Biotechnology	sc-203211A
L-Proline	Sigma	P5607-25G

Propionic acid	VWR	8.00605.1000
Propyl gallate	Sigma	P3130-100G
ProSeive EX Running Buffer	Lonza	200307
ProSeive EX Transfer Buffer	Lonza	200309
Random Hexamer Primer	Thermo Fisher Scientific	48190011
RevertAid Reverse Transcriptase + RevertAid Reverse Transcriptase Buffer	Thermo Fisher Scientific	EP0441
RNase Inhibitor	Thermo Fisher Scientific	EO0381
Rotenone	Santa Cruz Biotechnology	sc-203242
SensiFAST SYBR Hi-ROX Kit	Bioline	BIO-92020
Sodium dodecyl sulfate (SDS)	Santa Cruz Biotechnology	sc-264510C
Skimmed milk powder	Sigma	70166-500G
Sodium citrate dihydrate	Sigma	W302600-1KG-K
Sodium L-ascorbate	Sigma	A4034-100G
Sodium pyruvate	Sigma	P2256-25G
Sodium succinate dibasic hexahydrate	Sigma	S9637-500G
Soybean flour	Santa Cruz Biotechnology	sc-215897A
TMPD (N,N,N′,N′-Tetramethyl-p-phenylenediamine dihydrochloride)	Sigma	87890-25G
Treacle	Lyle's Black Treacle	-
Tris	Santa Cruz Biotechnology	sc-3715B
Tris-HCl	Promega	H5121
Triton X-100	Promega	H5141
Tri-reagent	Molecular research centre	TR118
Tween 20	Sigma	P9416-100ML
Wheat germ	MP biomedical	0290328805

2.2 Preparation of fly food

The reagents shown in Table 2.2 were measured into a glass beaker and slowly added to the required volume of deionized water (pre-warmed to 37°C, under constant agitation with a magnetic stirrer) in another glass beaker.

Table 2.2 List of ingredients for preparing of fly food

Weights are listed per 1000 ml of fly food		
Final concentration	**Dry weight**	**Ingredient**
1 % (w/v)	10 g	*Drosophila* agar type II
1.5 % (w/v)	15 g	D-(+)-Saccharose
3 % (w/v)	30 g	D-(+)-Glucose
3.5 % (w/v)	35 g	Active dried yeast
1.5 % (w/v)	15 g	Maize meal
1 % (w/v)	10 g	Wheat germ
1 % (w/v)	10 g	Soya flour
3 % (w/v)	30 g	Treacle

The food ingredients and water were mixed thoroughly and the yeast was allowed to ferment for 5 minutes before the temperature was gradually increased to approximately 85°C. The food mix was allowed to boil for 20 minutes before being allowed to cool to below 70°C. When the temperature dropped to below 70°C, propionic acid (final concentration = 0.5 % (w/v)) and nipagin M (made in a 10 % (w/v) stock solution in absolute EtOH; final concentration in food = 0.1 % (w/v)) were added. Additional deionized water was added to the required volume. Food was stirred continuously throughout the whole procedure. It was then dispensed into vials (2 ml per vial) or bottles (30 ml per bottle) and allowed to set over several hours before being plugged and stored at 4°C until use. Before use, food was allowed to warm up to room temperature for 2 hours. Food supplemented with rapamycin was prepared by adding 50mM stock of rapamycin dissolved in EtOH to achieve a final concentration of 200µM in the food. EtOH supplementation was used as a control food. Both rapamycin and EtOH were added after the addition of propionic acid and nipagin.

2.3 Fly stocks and lifespan assessments

Table 2.3 List of fly stocks

Fly Strain	Source	Details
Dahomey	Bjedov *et al*., 2010	Wild type flies incorporated with the *w1118* mutation by repeated backcrossing.
w^{Dah} *; UAS-AOX*	Fernandez-Ayala *et al*., 2009	Dahomey backcrossed UAS-AOX on 2nd Chromosome.
w^{Dah} *; daGAL4*	Bloomington *Drosophila* Stock Center (BDSC)	Dahomey backcrossed daughterless GAL4 driver on 3rd Chromosome (BDSC N°: 55849).
w^{Dah} *; UAS-mito-ORP1-roGFP2*	Albrecht *et al*., 2011	Dahomey backcrossed mitochondrially targeted ROS reporter on 2nd Chromosome.
w^{Dah} *; UAS-mtCatalse*	Mockett *et al*., 2010	Dahomey backcrossed mitochondrially targeted catalase on 2nd Chromosome.
w^{Dah} *; UAS-Sod2*	Bloomington *Drosophila* Stock Center (BDSC)	Dahomey backcrossed Sod2 overexpressors on 2nd Chromosome (BDSC N°: 24494).
w^{Dah} *; TubGS*	Neretti *et al*., 2009	Dahomey backcrossed Tubulin GAL4/GeneSwitch driver on 3rd Chromosome.
w^{Dah} *; UAS-Pink1*	Park *et al*., 2009	Dahomey backcrossed Pink1 overexpressors on 3rd Chromosome.
w^{Dah} *; UAS-Parkin*	Park *et al*., 2009	Dahomey backcrossed Parkin overexpressors on 3rd Chromosome.

All UAS transgenic and GAL4 driver lines were backcrossed for at least 6 generations into a DAH background. UAS-AOX flies have been previously described in (Fernandez-Ayala *et al*., 2009). Detailed descriptions of fly stocks are shown in Table 2.3. The flies were maintained on standard media (1% agar, 1.5% sucrose, 3% glucose, 3.5% dried yeast, 1.5% maize, 1% wheat, 1% soya, 3% treacle, 0.5% propionic acid, 0.1% Nipagin). The transgenes were expressed by crossing virgin females with the GAL4 gene, under the control of the *daughterless* promoter

with males containing the UAS-Transgene construct. In order to induce gene expression using tubulin-Gene Switch-Gal4 an appropriate amount of Mifepristone (RU-486) stock solution (or the same amount of ethanol) was added to produce a final concentration of 200 μM in the standard food. With the help of this system I was able to temporally modulate the expression of the transgene or the knockdown by changing the concentration of the drug in the food. Additionally since this expression system is inducible, I was able to avoid any developmental phenotypes that might be caused by the GAL4 system. We have made use of the ubiquitous daGAL4 expression system or tubulin-Gene Switch-Gal4 for expression in all the flies used in subsequent experiments. Controls were obtained by crossing males containing the transgene constructs, with Dahomey virgin flies for the daughterless promoter crosses. Only female flies were used in this study. 24 hours after eclosion the flies were anesthetized on CO_2 and collected to vials with standard fly food at a density of 20 flies per vial. The flies were then maintained at 29^{o}C in a light: dark cycle controlled incubator for further experiments. For flies used for lifespan experiments, flies were changed to new vials every 2-3 days and the number of dead flies was scored. Lifespan experiments were performed with a minimum of 80 flies and repeated at least twice. For lifespan experiments in hypoxia and hyperoxia, 20 flies per vial were maintained at oxygen levels of 5% and 50%, respectively, at 25°C. Flies in hypoxia and hyperoxia were changed to new vials once in 7 days and 4 days respectively to avoid detrimental effects of rapid reoxygenation. The median and maximum lifespan (the last 10% of surviving flies) were calculated for each experiment. Adult flies, developed at 25^{o}C and aged at 29^{o}C for approximately 10 days were used for all the experiments, unless otherwise stated.

2.4 Quantitative real time PCR

RNA extraction

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 then 500μl of isopropanol was added. RNA was precipitated at room temperature for 10 minutes, and then followed by

centrifugation. The obtained pellet was thoroughly washed by adding 1ml of DEPC water with 75% ethanol. Washing was followed by vortexing the mix, and then centrifuging at 7500g at 4°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 in order to remove any DNA contamination. Further, the samples were incubated at 37°C for approximately 1 hour.

Ethanol precipitation of the DNaseI treated samples was followed after that. The 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. The samples were incubated at -20°C for a minimum of 30 minutes up to 1 hour. Then, the samples were centrifuged at 16000g for 20 minutes at 4°C. The supernatant was removed by using a vacuum needle followed by washing with 1ml of 75% ethanol in DEPC-Water. The step was repeated to ensure proper washing. The pellet was left to dry at room temperature for 5 minutes, and then re-suspended with, 10µl of DEPC water. The RNA concentration was then measured using a Nano-Drop 2000c (Thermo Scientific, Wilmington, USA) apparatus, and adjusted to around 1µg/µl, and then stored at -80°C for cDNA synthesis.

cDNA Synthesis

For each cDNA sample prepared, a triplicate was made, and then pooled together for complete cDNA synthesis. Each synthesis reaction has a final volume of 20 µl, and a total of 2µg of RNA sample. All the following reactions were performed in a 96 well 0.2 ml plate. 2µl of the 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°C for 3 minutes. After incubation, the samples were added with 4µl of 5X reverse transcriptase buffer and 1µl of RNase inhibitor. The samples were then incubated for 10 minutes at 25°C. Finally 2µl of Reverse transcriptase was added to each individual reaction mix.

The samples were then, again incubated in a stepwise manner in a programmed cycle of 25°C for 10 minutes, 37°C for 60 minutes and 70°C for 10 minutes. All the triplicated samples were pooled in a single tube and then, the cDNA was stored at -20°C.

qPCR-Standard curve method

The "primer 3" primer design software was used to design the best primer for each specific gene. The cDNA samples were thawed. 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 nucleic acid free water (FERMENTAS INC., Maryland, USA), and then serial diluted to three other concentrations, namely 1, 1:10, 1:100 and 1:1000, making four standard dilutions in total. 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. Samples were diluted 20 times. All the standards and samples were added in triplicates in the 96 well plates and then stored for further use.

The reaction mix contains 10µl of SENSI FAST enzyme (Bioline, Taunton, USA), 0.4µl of forward and reverse primer and 5.2 µl of Nuclease free water. The total adds upto 16µl of the reaction mix in the well. I have used fast optical 96 well plates (Applied Biosystems, San Francisco, USA) for qPCR. Then 4µl of samples and standards were pipetted into the reaction mix, making the reaction volume to 20µl. The reaction mix and the samples were thoroughly mixed. The plate was then sealed with optical clear cover and subjected for a short spin. The plate was placed in a StepOne, qPCR machine (Applied Biosystems, San Francisco, USA) and then, the cycle involving 95 ^{o}C for 5s, 60^{o}C for 10s and then finally 72^{o}C for 5s was initiated. This cycle is a repeated upto 45 times for the valid quantification. The values were quantified by StepOne v2.1 software (Applied Biosciences, San Mateo, USA). All the samples used for quantifications with different primers were also used for quantification with *Gapdh* primers for *Gapdh* (*CG12055*). These values were then used for normalization from the values obtained from other primers. A list of all primers used in this thesis is given in Table 2.4.

Table 2.4 List of Primers

Gene/Primer Name	**Primer Sequence**
16s Forward	5’ - TTCGTCCAACCATTCATTCC - 3’
16s Reverse	5’ - TCTAACCTGCCCACTGAAAA - 3’
Catalase Forward	5’ - TGATTCCTGTGGGCAAAATG - 3’
Catalase Reverse	5’ - CAGACGACCATGCAGCATCT - 3’
Co1 Forward	5’ - GGAGGATTACCTTTTTTAGG - 3’
Co1 Reverse	5’ - CCTGGAGCATTAATTGGAGATG - 3’
Cytb Forward	5’ - AAATTCCGAGGGATTCAATTTT - 3’
Cytb Reverse	5’ - TCAACTGGTCGAGCTCCAAT - 3’
Delg Forward	5’ - CAGCCAGAAGAGTTCCTCCA - 3’
Delg Reverse	5’ - CTCGGGTATCTTCAGGCGTA - 3’
Dj-1 beta Forward	5’ - GGGAAATCCCTCACCTCGTAT - 3’
Dj-1 beta Reverse	5’ - TTTTGAGGGCGAACTCGTAG - 3’
Gapdh Forward	5’ - GACGAAATCAAGGCTAAGGTCG - 3’
Gapdh Reverse	5’ - AATGGGTGTCGCTGAAGAAGTC - 3’
Glyoxalase Forward	5’ - GTCACCTCAATGGTGGCCTTG - 3’
Glyoxalase Reverse	5’ - GCCAAGTTCTCGCTGTACTTTC - 3’
Hsp22 Forward	5’ - CCTTTCACGCCTTCTTCCAC - 3’
Hsp22 Reverse	5’ - TAGCCATCCTTGTTGACGGT - 3’
Hsp60 Forward	5’ - ATGTTCCGTTTGCCAGTTTC - 3’
Hsp60 Reverse	5’ - GATCACATTGCGACCCTTG - 3’
Hsp70 Forward	5’ - ACTCACACACAATGCCTGCT - 3’
Hsp70 Reverse	5’ - TAGACACCCACGCAGGAGTA - 3’
Impl3 Forward	5’ - CGGGATTGGACACCATAAGC - 3’
Impl3 Reverse	5’ - GCAACACCGACATCCTCAAG - 3’
ND1 Forward	5’ - TTGAGAAAGCTGCCTCCCTA - 3’
ND1 Reverse	5’ - CACCACCAACGACAACAAAG - 3’
Parkin Forward	5’ - GATACGTGTTCTGCCGCAAT - 3’
Parkin Reverse	CTGCATTTTGGACAGGGCTT - 3’
Pink1 Forward	5’ - GCCGCAATATGTCGCTGTAT - 3’
Pink1 Reverse	5’ - ATCGTCCTGCAGCTCGATTA - 3’
PxGPx Forward	5’ - AGGTGTTCGCCAAGGTAAGAC - 3’

PxGPx Reverse	5’ - GGTCTGCTTGGCCTTTAGGTA - 3’
Rpl32 Forward	5’ - AGCATACAGGCCCAAGATCGTGAAGAA - 3’
Rpl32 Reverse	5’ - CACGTTGTGCACCAGGAACTTCTTGAA - 3’
Sdhb Forward	5’ - CATCAACACCTCCAAGTCGC - 3’
Sdhb Reverse	5’ - ACAGCATGCACTCGTACAGG - 3’
Sod Forward	5’ - GAACTCGTGCACGTGGAATC - 3’
Sod Reverse	5’ - GGTGGTTAAAGCTGTCTGCGT - 3’
Sod2 Forward	5’ - GTCTGGTGGTGCTTCTGGTG - 3’
Sod2 Reverse	5’ - GCCCGTAAAATTTCGCAAAC - 3’
Spargel Forward	5’ - AAACTGAGGATCCTGGCACA - 3’
Spargel Reverse	5’ - CGCTTTGGATGCTGGCTATT - 3’

2.5 Western blot analysis

Protein extraction

Around 10 flies were anesthetised with CO_2 and stored at -80^{o}C in a tube. Frozen flies or fresh flies were then grinded using a sterile plastic homogenizer, in a homogenizing buffer (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 10 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 or the total fly extract was transferred to a new tube.

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 (DTT) 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 1X Running buffer diluted from 10 X Running buffer (Lonza, Basel, Switzerland). A readymade gel from Bio-Rad (AnyKD, Criterion TGX, Marnes-la-Coquette, France) was used. The protein ladders were filled with volume of 3µl at one end

and 7µl at the other. 25µl of the samples were added in each well accordingly. The tank was subjected to a voltage of 130 V until the proteins have migrated into the gel. The voltage was then increased to 150 V and run for approximately 40 minutes or until the proteins have migrated till the end of the gel. The gel was then removed from its casing and then soaked in the running buffer until further use.

Blotting

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

Dry blotting device, iBlot Dry Blotting System (Invitrogen, New York, USA) was used for dry blotting. The recommended kit, iBlot gel transfer stacks (Invitrogen, New York, USA) were used. They were placed in the recommended order, and the blot was run at 20 V for a span of 6 minutes. After completion, the membrane was carefully transferred to a bath of 1X PBS. For Wet blotting the tank was filled with 1X blotting buffer diluted from 10 X Western Blot transfer buffer (Lonza, Basel, Switzerland). The sandwich was then packed in the proper order and the blot was started. The blotting was done at 40V at room temperature for approximately 1 hour. After the run was complete, the sandwich was unpacked and then, the membrane was carefully transferred to a bath containing 1 X PBS solution.

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 to remove the stain, with water or 1X PBS. The mebranes were then subjected to blocking in 5% Milk, dissolved in 1X PBS-Tween for 1 hour, with continous 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 conditions and manufacturer information for all the anitbodies used are described in detail in the Table 2.5 below.

The membranes were incubated in the primary antibody in 5% Milk, dissolved in 1X PBS-Tween overnight at 4^{o}C. After the incubation, the membranes were washed again with 1X PBS-Tween and incubated in the appropriate secondary antibody complementary to the primary for 1 hour 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) in the ratio 1:1. 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. The images were quantified using ImageJ (La Jola, California).

Table 2.5 List of Antibodies

Antibody	**Company**	**Catalogue #**	**Dilution**
4E-BP	Cell Signaling	9452	1:250
AKT	Cell Signalling	9272	1:1000
AMPK	Cell Signalling	2757	1:1000
AOX	Prof Howy Jacobs, Finland	Fernandez-Ayala *et al.*, 2009	1:100,000
FOXO	Cell Signalling	12829	1:1000
GAPDH	Everest Biotech	EB07069	1:30000
p4E-BP (Thr 37/46)	Cell Signaling	3929	1:250
pAKT (Ser 473)	Cell Signalling	4060	1:1000
pAMPK (Thr 172)	Cell Signalling	2535	1:1000
Parkin	Sigma-Aldrich	SAB1300355	1:2000
pFOXO (Ser 253)	Cell Signalling	9466	1:100
PINK1/PARK6	Sigma-Aldrich	SAB1300802	1:500
pS6K (Thr 389)	Cell Signalling	9206	1:1000
Ref(2)P	Abcam	ab178440	1:100
S6K	Santa Cruz	sc-230	1:250
Sestrin	Dr. Michael Karin lab, San Diego	Lee *et al.*, 2010	1:2500
Ubiquitin	Cell Signaling	3936	1:1000
Lamp1	Abcam	Ab24170	1:1000
Anti-Rabbit	Vector Laboratories	PI-1000	1:10000
Anti-Mouse	Vector Laboratories	PI-2000	1:10000

2.6 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 the 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 crushed using a pestle very carefully. The homogenate was filtered using a 200µm polyamide mesh. Another 1ml of the mitochondria isolation medium with BSA was added on top of the mesh. Homogenate was transferred to a 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 sample was calculated by Bradford's Assay. After which the mitochondria were stored at -80^{o}C for a few experiments. For other experiments fresh mitochondria were used.

2.7 Mitochondrial oxygen consumption measurements

Around 20 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. This was done 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 ETC were considered for measurements. To begin with, Complex I linked substrates, namely; 5µl of 2M pyruvate+5µl of 2M proline and 4µl of 0.5M ADP were added. In order to study respiration without the participation of respiratory

Complex I, 1µl of 1mM rotenone was added. After which 30 µl of 1.3M Glycerol-3-Phosphate was added to analyse Complex III+IV activities and then Complex III was 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 rates were measured by taking into account the stable rates produced after the addition of substrates i.e. Pyruvate+Proline+ADP, G3P and Ascorbate+TMPD. Respiration rates after the addition of KCN was also tabulated. Pruvate+Proline+ADP dependent respiration was tabulated as CI-linked respiration, G3P dependent respiration was tabulated as CIII-linked respiration and the Ascorbate+TMPD respiration minus the respiration after the addition of KCN was tabulated as CIV-linked respiration. 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. The concentrations obtained, were used to normalize the previously obtained respiration rates, and the data were tabulated. The data were then further normalized to the amount of mitochondria calculated by the citrate synthase assay described in Chapter 2.13.

2.8 Mitochondrial ROS production

Around 40 flies 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. The volumes to be added were calculated so that it was made upto 0.5 mg/ml concentration in 100µl final volume of the wells.

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 upto 10 ml by filling up the rest of the volume by 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). 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 2.5) were added in the wells just before the measurements, to validate the immediate reaction response.

Table 2.6 Concentration and final volume of substrates and inhibitors

Reaction Solutions	**Final Concentration**	**Volume Used**
Substrates		
Pyruvate+Proline	5mM	2µl
Inhibitors		
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; (2) mitochondria with oligomycin (used as a blank for the experimental samples); (3) mitochondria + pyruvate+proline (experimental samples).

All of the above mentioned reactions were conducted in separate wells and considered as individual experiments. Mitochondrial concentration was added in equal concentrations for all reactions, according to the calcuations initially made. The susbstrates and the inhibitors volumes used, are mentioned in the Table 2.5 . The reaction plate, containing all the reactions, was measured in kinetic mode in a Chameleon V plate reader (Hidex, Turku, Finland) for a span of 30 minutes. During the whole measurement, the temperature was maintained at 25^{o}C, and the wavelength was set to 530nm excitation and 595nm emission. The reaction was stopped after 30 minutes approximately, and the calculations for ROS production were done using the slope of the increase in fluorescence.

Appropriate experiments were also performed to obtain standard curves, to measure the concentration of ROS subsequentially. 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 the assay buffer were added to each wells to make it up to 100µM final reaction volume in each well, with different volumes of glucose

oxidase namely; 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. Then, the reaction was started by adding 5µl of 280mM glucose to complete the reaction volume. The flourescence was then measured using the same conditions as used for the ROS measurements for the standard curve. The standard curve values were then plotted and the ROS measurements for the samples were plotted against the standard curve to obtain relative flourescence units.

2.9 CAFE assay

The flies were aged for 7 days at 29°C, before using them for the experiments. To begin with, around 10 tubes per fly group were taken. The tubes were then punched with holes on the top and the sides, sufficient for the capillary to fit and for air circulation. 10 tubes with 1 fly each was considered as one experiment and experiments were repeated at least 4 times. 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. After which, the capillaries were 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 29°C for the required measurements.

2.10 Activity of flies

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 allow the inlet of air. 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 at 29°C and a 12 hour alternative light and dark cycle was set. 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. 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.

2.11 Weighing of the flies

Around 10 flies 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 experiments were performed 10 times.

2.12 AMP, ADP and ATP measurements

20 female flies were put to sleep in a tube on ice. Abdomens were removed then the heads and thoraxes were transferred to a tube and placed on ice. The samples were then washed by pipetting in sterile PBS to remove any remaining eggs or food particulates. PBS was removed and replaced with 100 µl of homogenization buffer (6M Guanidine-HCl, 100 mM Tris pH 7.8, 4 mM EDTA). Flies were homogenized using a motor pestle and homogenates were cleared by centrifugation (4^{o}C, 16,000 x g, 20 minutes). 80 µl of supernatant was taken and protein concentration was determined via Bradford assay. 25 µl of undiluted sample was used for AMP determination with the AMP-Glo assay kit (#V5011; Promega, USA), according to the manufacturer's protocol, with slight modifications. Briefly, 25 µl sample or AMP standard was pipetted into a well of a white 96-well plate and 25 µl AMP-Glo reagent was added, mixed by pipetting and allowed to incubate at room temperature for 1 hour. Following which, 50 µl of AMP detection solution was added and the plate was incubated at room temperature for 10 minutes before measurement. Following AMP determination, the samples were used for ADP detection with the ADP-Glo Kinase assay kit (#V6930; Promega, USA), according to the manufacturer's protocol, with slight modifications. Briefly, 25 µl sample or ADP standard was pipetted into a well of a white 96-well plate and 25 µl ADP-Glo reagent was added, mixed by pipetting and allowed to incubate at room temperature for 1 hour. Following which 50 µl of Kinase detection reagent was added and the plate was incubated at room temperature for 1hour before measurement. Following ADP determination, the remaining sample volume was diluted 10 times in dH_2O for ATP determination using the luciferase-based the ATP determination kit (#A22066; Invitrogen, UK) as per the manufacturer's instructions. All measurements were done using a FLOUstar OMEGA microplate reader (BMG Labtech, Germany). Both assays and standards were measured in duplicate and nucleotide concentration was determined through interpolation with a standard curve.

2.13 Mitochondrial density measurements via the citrate synthase assay

Approximately 40-60 flies were immobilised on ice and then transferred to a chilled mortar. Alternatively samples prepared to measure respiration were used. The flies were homogenised in 500 µl of ice-cold mitochondria isolation medium (250 mM sucrose, 5 mM Tris-HCl, 2 mM EGTA), and the homogenate was filtered through cheesecloth. Then, an additional 500 µl of the mitochondria isolation medium containing 1 mM DTT was added, and the samples were frozen at -80°C overnight. Next, the samples were defrosted, and 50 µl of the whole homogenate sample was diluted 1:5 in mitochondria isolation medium containing 1 mM PMSF. The remainder of the whole homogenate sample was used to isolate mitochondria as described above. The isolated mitochondria were then diluted 1:4 in mitochondria isolation buffer containing 1 mM PMSF. Measurements were performed in a 96-well plate, in which 182 µl of reaction buffer (100 mM Tris-HCl (pH 7.5) and 2.5 mM EDTA), 2 µl of 30 mM acetyl-CoA and 2 µl of 10 mM DTNB were added to each well. Finally, the samples (either the whole homogenate or isolated mitochondria) were added. The reaction was initiated by adding 10 µl of 10 mM oxaloacetate (OAA), and the linear increase in absorbance at 412 nm was followed for 20 minutes using a PerkinElmer EnVision 2104 plate reader (PerkinElmer, Waltham, USA). Blanks were made from the same samples without the addition of OAA and then measured. Triplicates of all samples were used. Mitochondrial density was calculated by dividing the specific citrate synthase activity measured in the whole-fly homogenates by the specific citrate synthase activity measured in isolated mitochondria.

2.14 Analysis of protein oxidative damage markers by mass spectrometry

The following experiment was done in the laboratory of Prof Reinland Pamplona, Universitat de Lleida, Lleida, Spain. The levels of markers of protein oxidation [the protein carbonyl aminoadipic semialdehyde (AASA)], glycoxidation carboxymethyl-lysine (CML)], and lipoxidation [malondialdehydelysine (MDAL)] were determined by gas chromatography/mass spectrometry (GC/MS). The trifluoroacetic acid methyl ester derivatives of these markers were measured in acid-hydrolyzed, delipidated and reduced protein samples using an isotope dilution method as previously described (Sanz *et al.*, 2010) with an HP6890 Series II gas chromatograph (Agilent), a MSD5973A Series and a 7683 Series automatic injector, an HP-5MS column (30-m × 0.25-mm × 0.25-µm), and the described temperature program (Sanz *et al.*, 2010). Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and nondeuterated standards. Analyses were carried

out by selected ion-monitoring GC/MS (SIM-GC/MS). The ions used were: lysine and [2H8] lysine, m⁄z 180 and 187, respectively; 6-hydroxy-2-aminocaproic acid and [2H4]6-hydroxy-2-aminocaproic acid (stable derivatives of AASA), m⁄z 294 and 298, respectively; CML and [2H4] CML, m⁄z 392 and 396, respectively; and MDAL and [2H8] MDAL, m⁄z 474 and 482, respectively. The amounts of product were expressed as the μ molar ratio per mol of lysine.

2.15 Measurement of ROS in *Drosophila* brains

Approximately 20-40 adult flies were immobilized on ice. The immobilized flies were then transferred to a piece of aluminium foil placed on ice to keep them immobilized. The flies of the same genotype were used one by one for dissection. Using fine forceps the flies were lifted by its wings to avoid any damage. The flies were then dipped in 70% EtOH for 15 seconds to remove any lipids that might interfere with the dissections. The cleaned flies were then transferred to a glass dissecting well filled with 1X PBS. The brain of the fly was carefully dissected without causing any physical damage to avoid improper measurements. All the dissections were done under a light microscope. Following dissection, fly brains were transferred to a tube containing either in MitoSOX or H_2DCF using a Pasteur pipette. Experiments were done earlier to optimize the concentrations of MitoSOX or H_2DCF. 30 μM final concentration of either MitoSOX or H_2DCF was used to detect superoxide levels in the mitochondrial matrix or total cellular peroxide levels respectively. The fly brains were incubated for 10 minutes and then MitoSOX or H_2DCF was removed and replaced with 1X PBS. The washing with 1X PBS was repeated 3 times. The fly brains were then immediately mounted on a Poly-Lysine coated dish and used for imaging. Images were acquired using an LSM510 confocal microscope (Zeiss, Jena, Germany) equipped with a 10x 0.3 NA objective as Z stacks throughout the sample using either a 543 nm or 488 nm line of an Argon laser to excite MitoSOX or H_2DCF, respectively. For *in vivo* ROS imaging using mtORP1-roGFP reporter lines (Albrecht *et al.*, 2011), whole flies containing the reporter and driver, along with any other indicated constructs were put to sleep on ice before being dissected and imaged under Ex. 488 (reduced) or 405 (oxidized) nm/Em. 510 nm. The total intensity of each individual brain imaged was quantified using ImageJ (La Jola, California).

2.16 Lysotracker staining of *Drosophila* brains

Adult brains were dissected as described in the previous section. An optimized concentration of 30 µM was used for the Lysotracker Red staining (Molecular Probes, Eugene, USA). Incubations and washings were similar to the previous section. Images were acquired using an LSM510 confocal microscope (Zeiss, Jena, Germany) equipped with a 10x 0.3 NA objective as Z stacks throughout the sample using 400nm line of an Argon laser to excite Lysotracker Red. The total intensity of each individual brain imaged was quantified using ImageJ (La Jola, California).

2.17 Proteasome assay

Around 5 flies for 20S Proteasome and 15 flies for 26S proteasome were homogenized in the respective homogenizing buffers containing, 0.5% Triton X-100, 250mM NaCl, 3mM EDTA, 3mM EGTA and 20mM Tris pH 7,55 for 20S and 0.2% IGEPAL, 5mM ATP, 10% Glycerol, 20mM KCl, 1mM EDTA, 1mM DTT and 20mM Tris pH 7,55 for 26S. After homogenization the samples were cleared by centrifugation at maximum speed for 15 minutes at 4^{o}C. The samples were filtered and the supernatant was collected. The protein concentration of the samples was measured by Bradford assay. 180 µl and 170µl of assay buffer for each reaction containing 25mM Tris pH 7.55 and 25µM Suc-LLVY-AMC, 40µM Boc-LRR-AMC and 100µM Z-LLE-AMC were used for measuring Chymotrypsin-like, Trypsin-Like and Caspase-like activity respectively. 20µl and 30µl of the samples were used for 20S and 26S respectively. The assays were done in a black 96 well plate and measured at wavelength, excitation 380nm and emission 460nm for 30 minutes. The kinetics was stopped and 2µl of 2mM z-leu-leu-leu-al was added to each reaction to inhibit the proteasome and then measured again at the same wavelength, as mentioned above for another 30 minutes. Calculations were done by subtracting the values obtained after the addition of z-leu-leu-leu-al from the values obtained before its addition. Finally the values were normalized to protein concentration determined by Bradford assay.

2.18 PCR arrays

Around 10 flies were used for mRNA isolation as described above. cDNA was synthesised from RNA using the RT^2 first-strand cDNA synthesis kit (Qiagen Technologies, Hilden, Germany). cDNA was added to wells containing RT^2 SYBR Green master mix (Qiagen

Technologies, Hilden, Germany) in an array format of 4x96 wells, with each plate containing 384 wells. Each of these 96-well formats contained 84 signal transduction pathway genes, 5 housekeeping genes and positive and negative controls. The PCR array plate was carefully sealed with an optical adhesive film provided by the manufacturer. A total of 41 cycles were run. The first cycle consisted of 10 minutes at 95°C, and the other 40 cycles were 2-step cycles (15 seconds at 95°C and 1 minute at 60°C). A Bio-Rad CFX384 q-PCR machine was used for all of the above-mentioned protocols (Bio-Rad, Hercules, USA). The average threshold cycle (C_T) value for each well was calculated using Bio-Rad software (Bio-Rad, Hercules, USA). The corresponding (C_T) values were normalised to those of the housekeeping genes and tabulated. A gene was considered to be significantly up or downregulated when the difference between the (C_T) values was greater than or equal to 10%, respectively.

2.19 Statistical analysis

All data were analysed using Prism 6 (GraphPad, La Jolla, California) using either 1-way ANOVA with Newman-Keul's post-test or using the unpaired Student's T-test where appropriate. Lifespan data were analysed using the log-rank Mantel Cox Test. $p<0.05$ was taken as statistically significant. * denotes statistically significant differences between experimental groups. Data are shown as mean $\pm$ SEM.

Chapter 3. A mitochondrial ROS signal is required for stress adaptation

3.1 Mechanisms of stress adaptation

The mechanism of stress adaptation in flies can be studied by culturing them at high temperatures, as the increase in culturing temperature from 25°C to 29°C can impose an additional stress (Loeschcke *et al.*, 1997). Stress adaptation is essential to maintain proper functioning of an organism. This adaptation has shown to fail at older ages, and flies cultured at higher temperatures are an important tool for the study. For instance, *Drosophila* being a poikilothermic organism accumulates damage faster at higher temperatures (Sorensen *et al.*, 2013). The increase in rearing temperature from 25°C to 29°C also causes activation of heat shock response (HSR) and interestingly, induction of HSR extends lifespan at low temperatures (Morrow *et al.*, 2004). In conclusion, identifying the mechanisms controlling stress adaptation and then inducing them in absence of stress could be a valid strategy to extend healthy lifespan.

Mitochondria are important organelles that act as one of the central regulators of stress adaptation in many organisms. For example, muscle mitochondrial stress has shown to activate AMPK signalling and consequentially muscle mitohormesis in mice (Ost *et al.*, 2015). Their activity is strongly linked to temperature changes (Guderley and St-Pierre, 2002). However, it is not clear yet how mitochondria adjust their own function in response to stress. There have been several studies suggesting that both ROS-dependent and ROS-independent mitochondrial mechanisms can contribute to ageing and stress adaptation (Scialo *et al.*, 2013). ROS are intriguing molecules as they cause oxidative damage and also act as signal messengers (Ristow and Schmeisser, 2011). This duality leads to a complicated relationship between ROS and stress adaptation. However, it is unknown how these adaptations are initiated at the molecular level.

Accumulation of damaged mitochondria is one of the major hallmarks of ageing and many neurodegenerative diseases (Osellame and Duchen, 2013). Unfortunately, we are still lacking the knowledge on what causes the decline in the mitochondrial function that is observed. Understanding these mechanisms will aid us to prevent many neurodegenerative diseases such as Parkinson's or Alzheimer's.

Mitochondrial turnover is the result of the balance between mitochondrial biogenesis and mitochondrial elimination. Mitochondrial biogenesis is regulated by the convergence of signals on the peroxisome proliferator-activated receptor gamma (PPARγ) family that promote transcription of mitochondrial genes, activating nuclear respiratory factor 1 and 2 (NRF 1 or 2)

(Scarpulla, 2011). On the other hand, the elimination of the damaged mitochondria is done by a process called mitophagy, which is a selective macro-autophagic process. Canonical mitophagy is mediated by the PTEN-induced putative kinase 1 (PINK1) and PARKIN (Clark *et al.*, 2006). The cleavage of PINK1 by mitochondrial proteases is inhibited and the full length PINK1 protein stabilizes on the mitochondrial outer membrane upon mitochondrial depolarization (Narendra *et al.*, 2008). This stabilization of PINK1 recruits PARKIN and activates it through ubiquitination (Matsuda *et al.*, 2010). Ubiquitination of PARKIN is responsible for ubiquitination dependent activation or deactivation of several cytosolic proteins, one of them being p62 or ref (2) p in flies, which is responsible for recruiting the autophagic machinery (Song *et al.*, 2016). Mitochondria are eventually sequestered to the lysosome to be digested. Alternatively, other pathways independent of PARKIN have also been shown. NDP52 and OPTINUERIN, two proteins which were previously linked to xenophagy, have been shown to be recruited by PINK1 upon stabilization to recycle damaged mitochondria (Lazarou *et al.*, 2015). Unfortunately, it is not very clear how mitophagy occurs *in vivo*, as the process has been demonstrated *in vitro* where the conditions are non-physiological. It has been shown that mitochondria that use OXPHOS (cells cultured in galactose) inhibit mitophagy by avoiding the crucial recruitment and activation of DRP1 for undergoing fission, thereby avoiding the recruitment of PARKIN (MacVicar and Lane, 2014).

In this study, I aimed to analyse the role of mitochondria in stress adaptation focusing on the role of mtROS and its downstream effects. In this chapter, *Drosophila melanogaster* was used as the experimental model. I took advantage of the alternative oxidase (AOX), which is not a part of the ETC of humans or fruit flies, but is present in plants and fungi (McDonald *et al.*, 2009). AOX reduces the generation of mtROS, preventing the over-reduction of ETC (Scialo *et al.*, 2016). Figure 3.1 illustrates the function of AOX in the ETC. Overall; these changes in the mitochondria of the fruit flies decrease the ability of the organism to respond to stress.

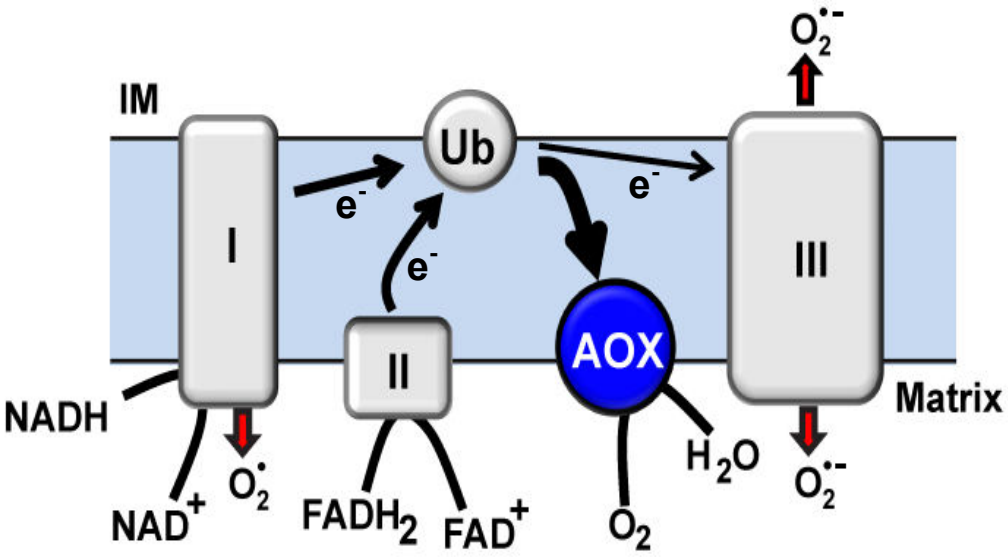

Figure 3.1 Ectopical expression of AOX in fruit flies and a schematic of how AOX interacts with the ETC

The expression of AOX bypasses the flow of electrons through Complex III and Complex IV, by directly receiving electrons from the ubiquinone pool and converting oxygen to water. AOX expression thereby also reduces generation of mtROS from Complex III and prevents over-reduction of the ETC.

3.2 AOX expression reduces ROS production

To begin with, I verified the expression of AOX in flies by western blotting (Figure 3.2A). Next, in order to verify that AOX expression reduces ROS production, I took an *in vitro* approach. Using the Peroxidase/Amplex Red ROS detection system I verified that AOX expression significantly decreased ROS *in vitro* (Figure 3.2 B). To then determine if AOX reduces ROS *ex vivo*, I dissected fly brains from AOX expressing flies (AOX flies hereafter) and their respective controls. Fly brains were then stained with MitoSOX, a dye that accumulates in the matrix of the mitochondria due to its positive charge, and fluoresces upon its reaction with superoxide. AOX flies showed a significant decrease in the MitoSOX fluorescence (Figure 3.2 C and D). In order to re-confirm the results with MitoSOX, I also stained fly brains with H_2DCF, a dye that is specific to peroxides (i.e. emits fluorescence when oxidized by H_2O_2 or other peroxides). AOX flies showed significantly less fluorescence with H_2DCF as well, confirming that superoxide and peroxide levels were reduced (Figure 3.2 C and D).

Finally, in order to verify if mitochondrial ROS levels were reduced *in vivo*, I used a mitoORP1-roGFP reporter line. roGFP is an engineered GFP sensor with a modified cysteine sensitive for oxidation (Albrecht *et al.*, 2011). The probe is also engineered to be sensitive for H_2O_2 oxidation as it is fused to ORP1 which is a H_2O_2 sensor. Flies containing the reporter and da-GAL4 driver with the AOX construct were used. The mitoORP1-roGFP reporter lines with the da-GAL4 driver were used as controls. The flies expressing AOX along with the reporter constructs showed significantly less peroxide levels (Figure 3.3 A and B). These results confirmed that AOX reduces ROS *in vivo* as well.

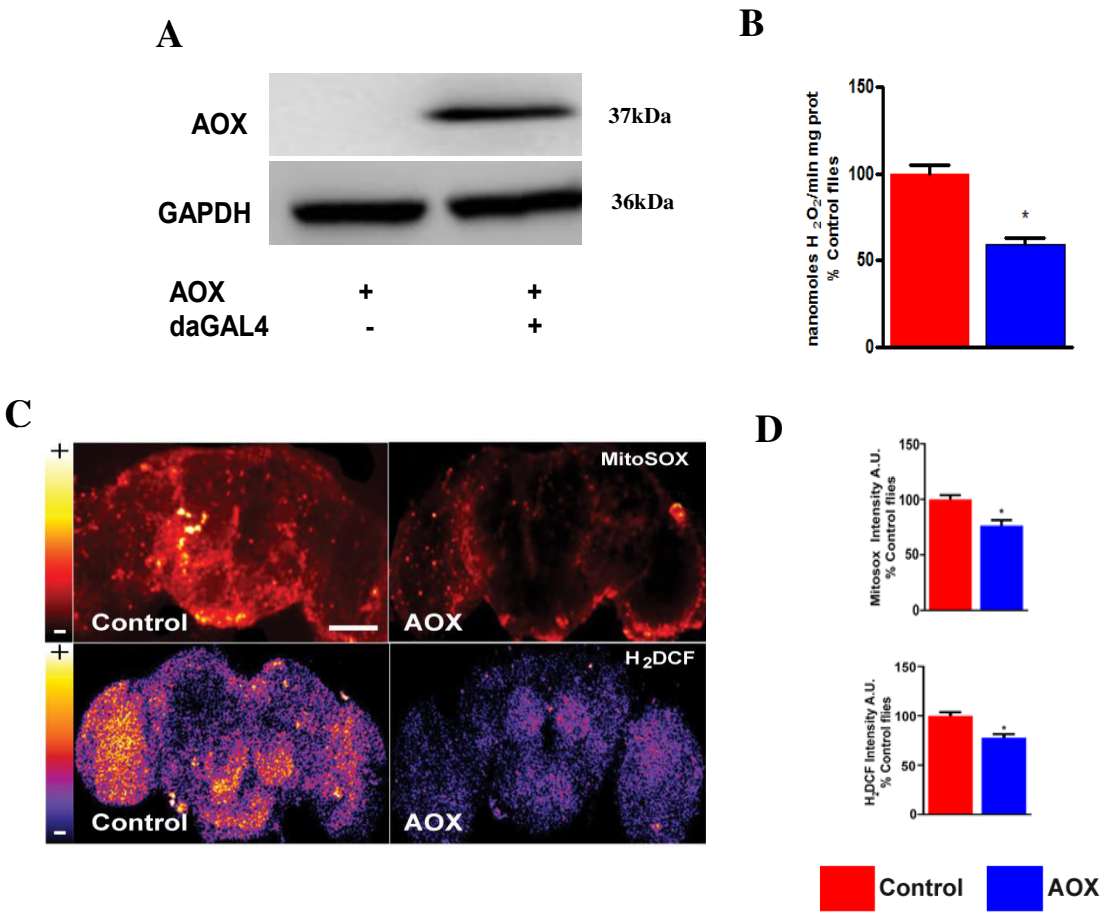

Figure 3.2 AOX expression reduces ROS levels in vitro and ex vivo.

(A) Representative western blot membrane showing AOX protein levels of indicated genotypes. GAPDH is used for normalization purpose. **(B)** Quantification of *in vitro* ROS production by Amplex Red assay of indicated genotypes (n=5) (Control = 0.1940 ± 0.009 nanomoles H_2O_2 min^{-1} mg $protein^{-1}$). **(C)** Representative images of dissected fly brains stained with MitoSOX and H_2DCF of indicated genotypes. **(D)** Quantification of **C** (n=5). Non-expressing flies (*2>UAS-AOX*) (Control in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using student's t-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. Scale bars are 100µm. This work was done in collaboration with Dr. Filippo Scialo. The molecular weight of proteins detected by western blots are given as kiloDalton (kDa) units.

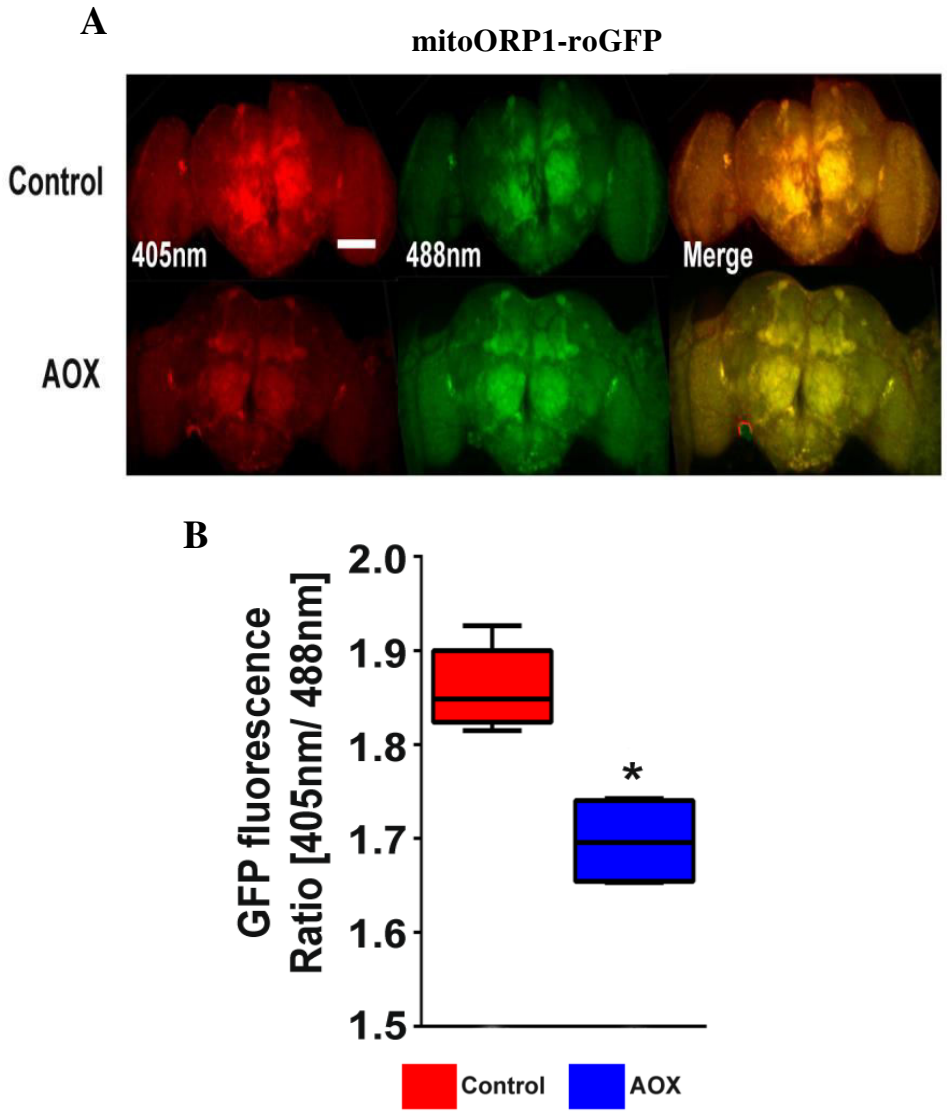

Figure 3.3 ROS production in vivo is reduced in AOX flies.

(A) *In vivo* ROS measurements in brains dissected from flies expressing a mitochondrially-localized redox-active GFP-based reporter (mitoORP1-roGFP) alone (top, n=5) or with AOX expression (+AOX; bottom, n=5). A daughterless GAL4 promoter was used for the expression of the transgene. **(B)** Quantification of non-expressing (Control in the figure) and *UAS-AOX*; *UAS-mtORP1-roGFP* expressing (AOX in the figure) flies **(A).** P values were calculated using student's t-test. Data are shown as whisker box plots minimum-maximum. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. Scale bars are 100µm. This work was done in collaboration with Dr. Filippo Scialo.

3.3 AMP, ADP, ATP levels and behaviour are not affected in AOX flies

As AOX is ectopically expressed in the mitochondrion of the fly (Fernandez-Ayala *et al.*, 2009), I performed experiments to check if the expression of AOX perturbs energy levels. I measured the steady state levels of AMP, ADP and ATP in AOX flies. An AMP, ADP or ATP detection kit was used to measure nucleotide levels. I did not detect any changes in the AMP, ADP or ATP levels (Figure 3.4 A). Additionally, as the steady state levels of these nucleotides were unchanged, the ratios were also not altered (Figure 3.4 B).

I then analysed if AOX expression affects any of the fly behaviours. I was able to confirm that the expression of AOX does not cause any alteration in the activity of the fly, by using Trikinetics Locomotion detection software (Figure 3.5 A). AOX expression also did not change the feeding behaviour of the flies, measured with a capillary feeding assay (CAFÉ assay); over a period of 5 days (Figure 3.5 B). However, the weight of the flies was modestly increased (Figure 3.5 C).

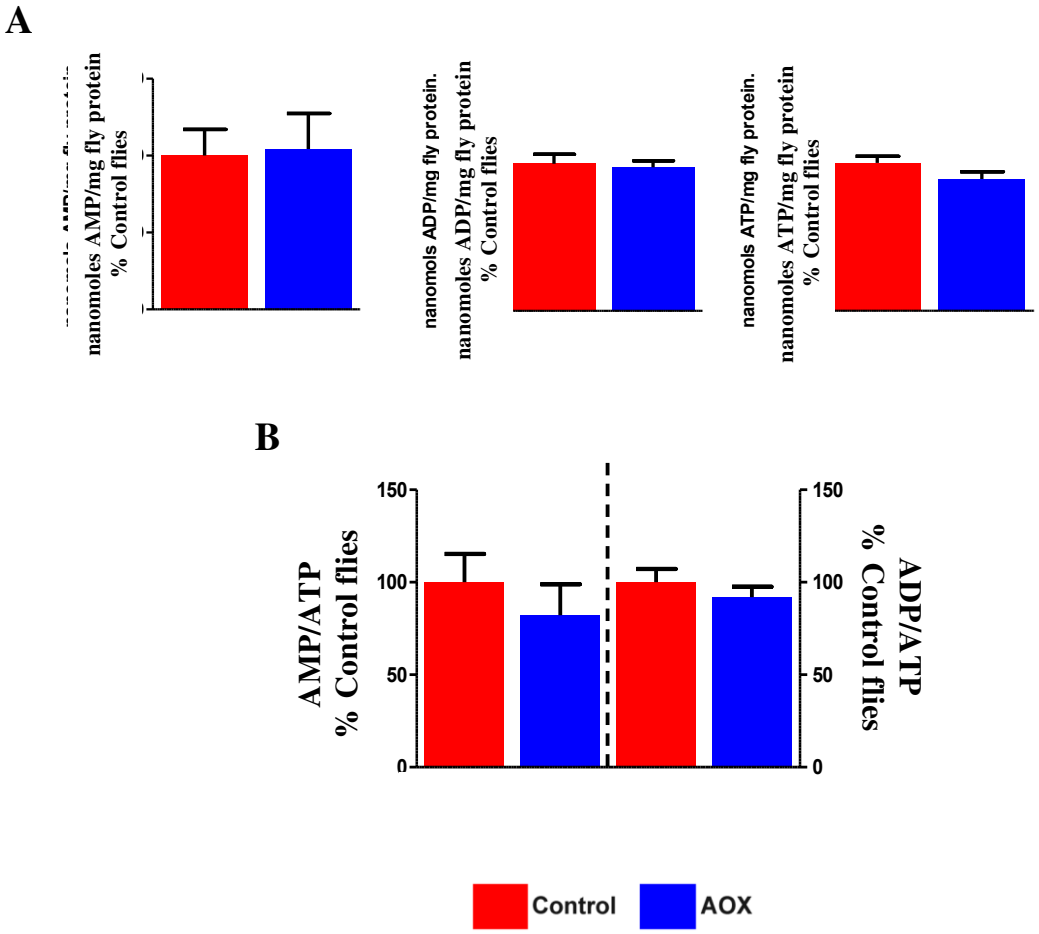

Figure 3.4 Nucleotide levels are unaltered in AOX flies.

(**A**) AMP, ADP and ATP levels of indicated genotypes (n=8) (Control AMP = 101.5±17.14 nanomoles mg protein^{-1}; Control ADP = 754.9±49.57 nanomoles mg protein^{-1}; Control ATP = 1236±69.06 nanomoles mg protein^{-1}) **(B)** Ratio of the nucleotides AMP: ATP and ADP: ATP of indicated genotypes (n=8). Non-expressing flies (*2>UAS-AOX*) (Control in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using student's t-test. Data are shown as mean ± SEM.

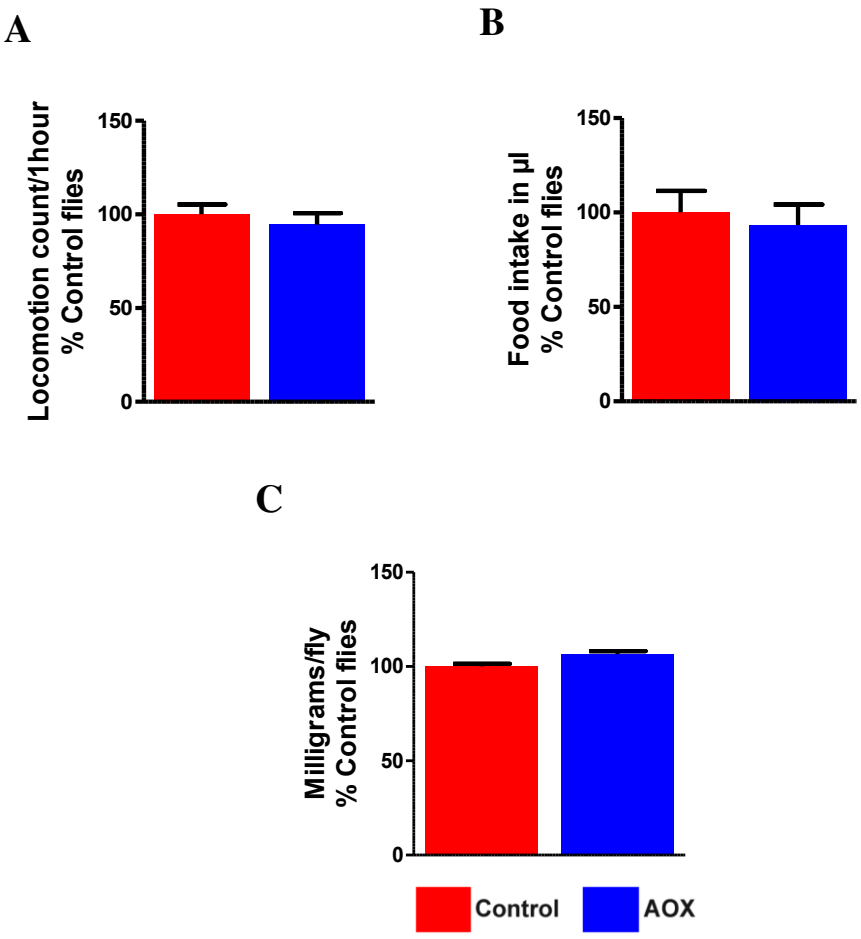

Figure 3.5 AOX expression does not affect fly behaviour.

(A) Locomotion count per hour of indicated genotypes (n=40) (Control = 82.08±4.378 Counts Hour^{-1}). **(B)** Food intake measured by CAFÉ assay of indicated genotypes (n=20) (Control = 1.155±0.132 µl). **(C)** Weight in milligrams of indicated genotypes (n=10) (Control = 1.103 milligrams per fly). Non-expressing flies (*2>UAS-AOX*) (Control in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using student's t-test. Data are shown as mean ± SEM.

3.4 Oxidative stress is not reduced, but antioxidant levels are downregulated in AOX flies

I analysed if the total oxidative stress in the AOX flies were reduced, since I observed a decrease in ROS levels in AOX flies (Figure 3.2). I analysed the levels of specific markers of oxidative damage (aminoadipic semi aldehyde (AASA) and glutamic semi aldehyde (GSA) for detection of oxidation, carboxymethyl-lysine (CML) was used for detection of glycoxidation and carboxyethyl-lysine (CEL) was used for detection of glycoxidation and MDA-lys was used as a marker for lipoxidation) by mass spectrometry. However, I did not observe any overall differences in oxidative, glycoxidative or lipoxidative damage, except for an increase in lipoxidation and glycoxidation in AOX flies (Figure 3.6).

I then measured mRNA levels of different antioxidants like *Superoxide dismutase (Sod), Superoxide dismutase 2 (Sod2), Catalase, Glutathion Peroxidase (PXGPx), Glyoxalase 1 and dj-1beta (Park7*) by quantitative PCR. Even though the expression of AOX did not reduce oxidative protein damage, it caused a significant decrease in levels of most of the antioxidants measured, except for *Sod2* mRNA levels which was increased; supporting the fact that the lower ROS levels in AOX flies causes a feed forward response to reduce the levels of antioxidants (Figure 3.7).

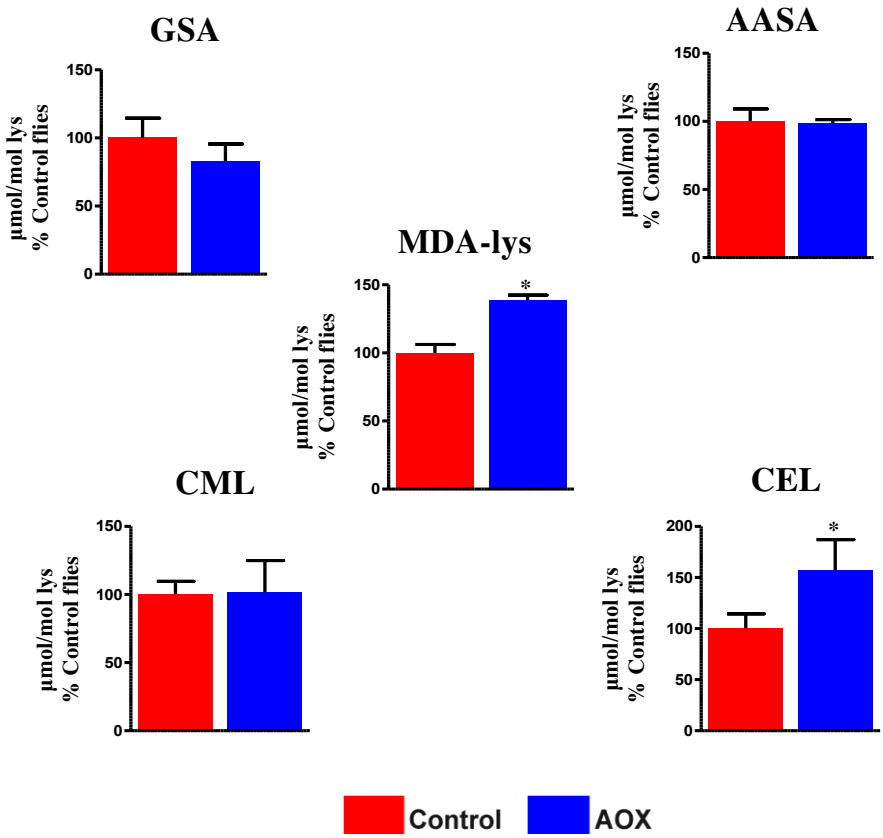

Figure 3.6 Markers of oxidative damage are not reduced in AOX flies.

Measurements for markers of oxidative (GSA, AASA), glycoxidative (CML, CEL) damage to proteins and lipoxidative (MDA-lys) damage to proteins of indicated genotypes (n=4) (Control GSA = 9910±1578 µmoles moles lys^{-1}; Control AASA = 178±52 µmoles moles lys^{-1}; Control MDA-lys = 307±116 µmoles moles lys^{-1}; Control CML = 121±20 µmoles moles lys^{-1}; Control CEL = 767±73 µmoles moles lys^{-1}). Non-expressing flies (*2>UAS-AOX*) (Control in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using student's t-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. This work was done in collaboration with Dr. Reinald Pamplona, Lleida, Spain.

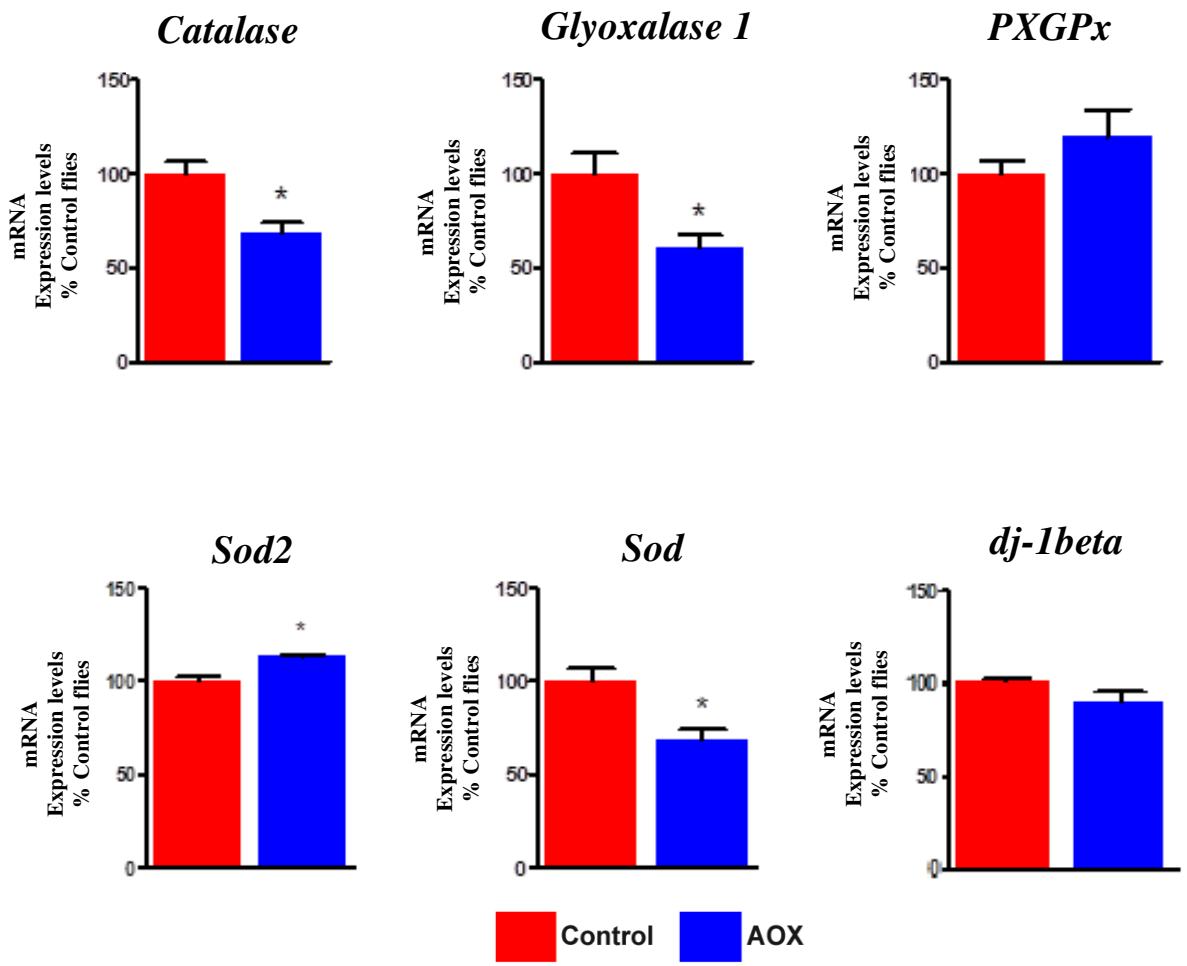

Figure 3.7 Antioxidant levels are decreased in AOX flies.

Quantification of mRNA levels of *Sod, Sod2, Catalase, PXGPx, Glyoxalase 1 and dj-1beta* by quantitative PCR of indicated genotypes (n=4). Non-expressing flies (*2>UAS-AOX*) (Control in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using student's t-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes.

3.5 Mitochondrial density is increased in AOX flies

I sought to investigate if mitochondrial turnover was disrupted. I used the Citrate synthase assay as a proxy to measure mitochondrial density. I found that AOX flies had significantly more mitochondrial density (Figure 3.8 A). I confirmed the citrate synthase results by measuring mtDNA copy number of one mitochondrial and one nuclear gene by using quantitative PCR (described in detail in Chapter 2.4). The ratio clearly showed that there was a significant increase in mtDNA copy number in AOX flies (Figure 3.8 B). This led me to believe that AOX expression may have disrupted the ROS signalling mechanisms that are essential for stress response.

In order to ascertain that changes in mitochondrial number was caused by AOX expression, and therefore by reduced mtROS levels, I used the inducible GeneSwitch system to temporarily regulate the expression of AOX. AOX was induced by collecting and ageing the flies on the food with RU-486 (explained in materials and methods). To cease AOX expression appropriately aged flies were removed from the RU-486 food and transferred to food with just EtOH. I analysed the protein levels of AOX to verify induction and removal of the protein (Figure 3.9 A) and observed that upon AOX induction, mitochondrial density increased; whereas, the opposite was observed when expression of AOX was ceased (Figure 3.9 B). This suggested that mitochondrial density is modified in response to changes in AOX/mtROS levels.

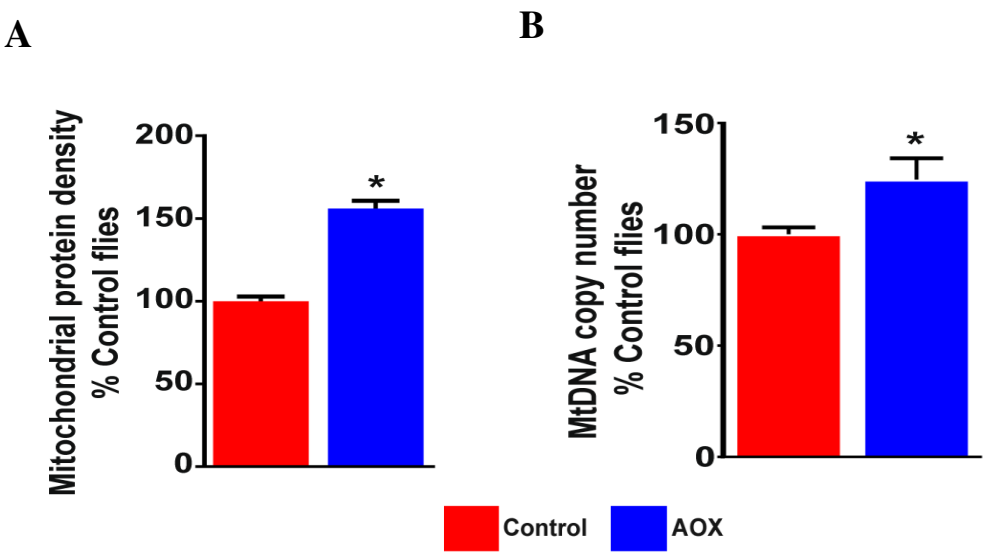

Figure 3.8 Mitochondrial density is increased in AOX flies.

(A) Citrate synthase activity measuring mitochondrial density of indicated genotypes (n=18) (Control = 2.481±0.071 Absorbance Units mg protein^{-1}). **(B)** mtDNA copy number of indicated genotypes (n=8). Non-expressing flies (*2>UAS-AOX*) (Control in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using student's t-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes.

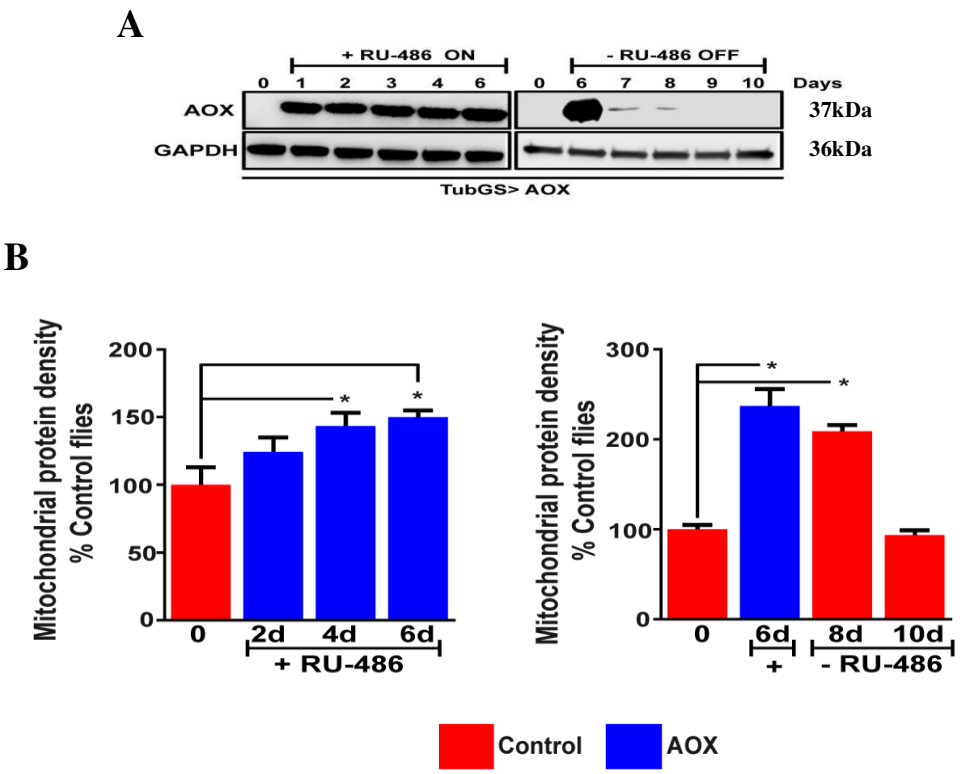

Figure 3.9 Induction and removal of AOX expression increases and decreases mitochondrial density respectively.

(A) Representative western blot membranes showing AOX protein levels. Left panel representing AOX protein levels in non-expressing flies (0) and *UAS-AOX* expressing flies (*TubGS>UAS-AOX*) (+RU-486 ON) for 1,2,3,4 and 6 days were used. Right panel representing AOX protein levels in non-expressing flies (0), *UAS-AOX* expressing flies (*TubGS>UAS-AOX*) un-induced after expression for 5 days earlier (-RU-486 OFF) for 6,7,8,9 and 10 days were used. GAPDH is used for normalization purpose. **(B)** Left panel representing citrate synthase measurements in non-expressing flies (0) and *UAS-AOX* expressing flies (*TubGS>UAS-AOX*) (+RU-486 ON) for 2, 4 and 6 days (n=4) (Control = 0.5157±0.067 Absorbance Units mg protein^{-1}). Right panel representing citrate synthase measurements in non-expressing flies (0), *UAS-AOX* expressing flies (*TubGS>UAS-AOX*) un-induced after expression for 5 days earlier (-RU-486 OFF) for 6, 8 and 10 days (n=4) (Control = 0.250±0.011 Absorbance Units mg protein^{-1}). An inducible Tubulin GeneSwitch GAL4 promoter was used for the expression of the transgene. P values were calculated using 1-way ANOVA with Newman-Keul's post-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. The art lines in the figure denote the statistically significant difference between the marked genotypes. The molecular weight of proteins detected by western blots are given as kiloDalton (kDa) units.

3.6 Mitochondrial biogenesis is unaltered in AOX flies

As I have established in Chapter 3.4 that there is an accumulation of mitochondria, I wanted to check if mitochondrial biogenesis was altered. To check if mitochondrial biogenesis was increased in response to the presence of AOX in ETC, I measured levels of expression of S*pargel* (homologue of mammalian Pgc-1α) and *Ets at 97D* (*Delg,* homologue of the α subunit of mammalian transcription factor NRF-2/GABP) using quantitative PCR. Only a modest increase in mRNA levels of *Spargel* was observed, while *Delg* mRNA levels were unchanged (Figure 3.10).

To further confirm if the increase in mitochondrial density was caused by the modest increase in mRNA levels of *Spargel*, I checked mRNA levels of several mitochondrial genes by quantitative PCR. Although mRNA levels of mitochondrial Cytochrome b (*Cytb*; CIII subunit) was increased, mRNA levels of other ETC components like mitochondrial NADH-ubiquinone oxidoreductase chain 1 (*ND1*; CI subunit), Succinate dehydrogenase, subunit B (*Sdhb*; CII subunit), mitochondrial Cytochrome c oxidase subunit I (*CoI*; CIV subunit) were either unchanged or decreased (Figure 3.11). Expression of mitochondrial genes was not consistently induced in AOX flies, making it unlikely that the increase in mitochondrial number was caused by an increase in mitochondrial biogenesis. This led me to suspect an alteration in mitochondrial turnover caused by AOX expression.

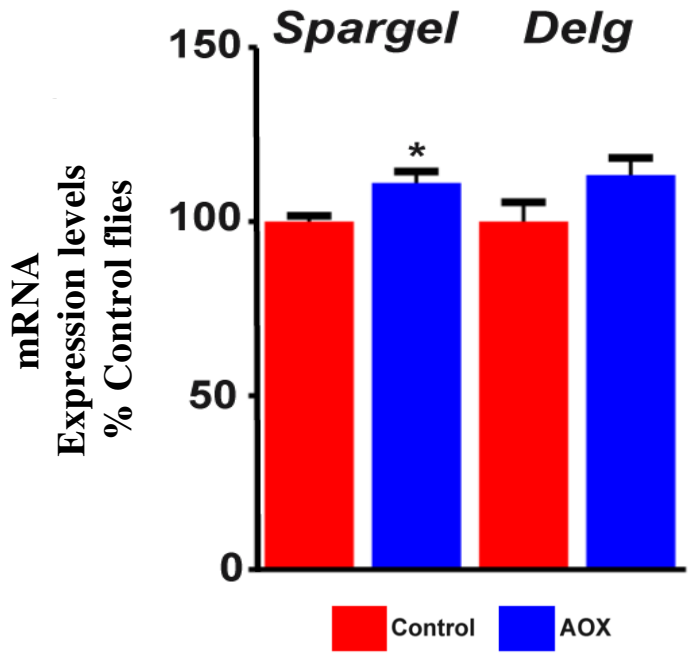

Figure 3.10 Spargel and Delg levels are unaltered in AOX flies.

Quantification of mRNA levels of *Spargel* and *Delg* by quantitative PCR of indicated genotypes (n=4). Non-expressing flies (*2>UAS-AOX*) (Control in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using student's t-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes.

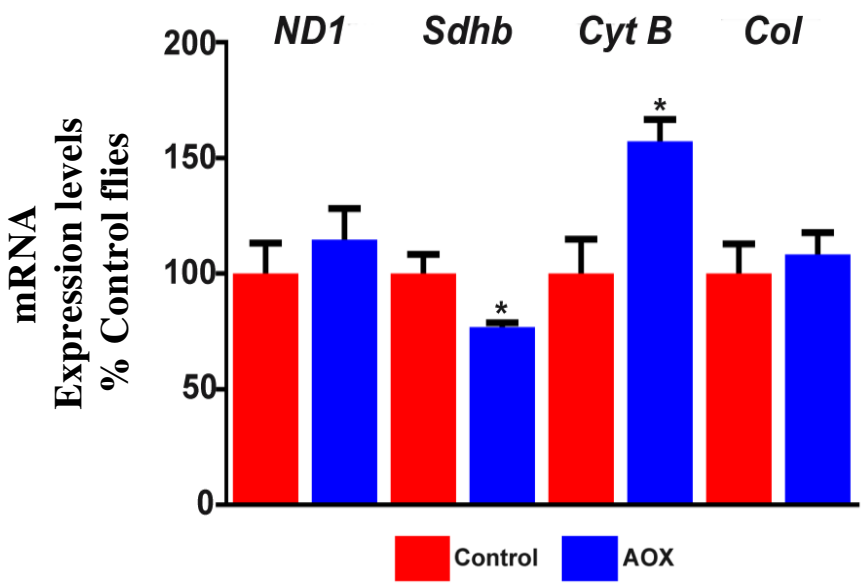

Figure 3.11 mRNA levels of several mitochondrial genes were unaltered in AOX flies.
Quantification of mRNA by quantitative PCR of indicated genotypes (n=4). Non-expressing flies (*2>UAS-AOX*) (Control in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using student's t-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes.

3.7 Mitochondrial functionality is compromised in AOX flies

To understand the mitochondrial function of these flies, I performed high-resolution respirometry. I hypothesized that the mitochondrial accumulation phenotype caused in the AOX flies might be due to the improper recycling of damaged mitochondria. Pyruvate, Proline and ADP stimulated CI-linked respiration, G3P stimulated CIII- linked respiration and Ascorbate and TMPD stimulated CIV-linked respiration was unchanged when normalized to total protein content of the samples (Figure 3.12 A). However, when I normalized these respiration measurements to the total mitochondrial density (measured using citrate synthase assay as described earlier), I observed a significant decrease in respiration of AOX flies across the panel (Figure 3.12 B). The fact that mitochondrial respiration was significantly reduced in AOX flies even though mitochondrial density was increased; denote that the accumulated mitochondria are severely damaged.

I also observed an increase in the mRNA levels in markers of mitochondrial unfolded protein response (Heat shock protein 22 (*Hsp22*) and Heat shock protein 60 (*Hsp60*)) and also in the levels of markers of cytosolic stress (Heat shock protein 70 (*Hsp70*)), indicating an accumulation of dysfunctional mitochondria (Figure 3.13 A) (Deocaris *et al.*, 2006). In order to confirm this observation, I measured expression levels of *Ecdysone-inducible gene L3* (*ImpL3*) (the Drosophila homologue of lactate dehydrogenase A) and found a strong up-regulation of mRNA levels (Figure 3.13 B). Upregulation of *Impl3* levels (used as a proxy for mitochondrial damage) also indicates that the cells have switched to a more glycolytic metabolism.

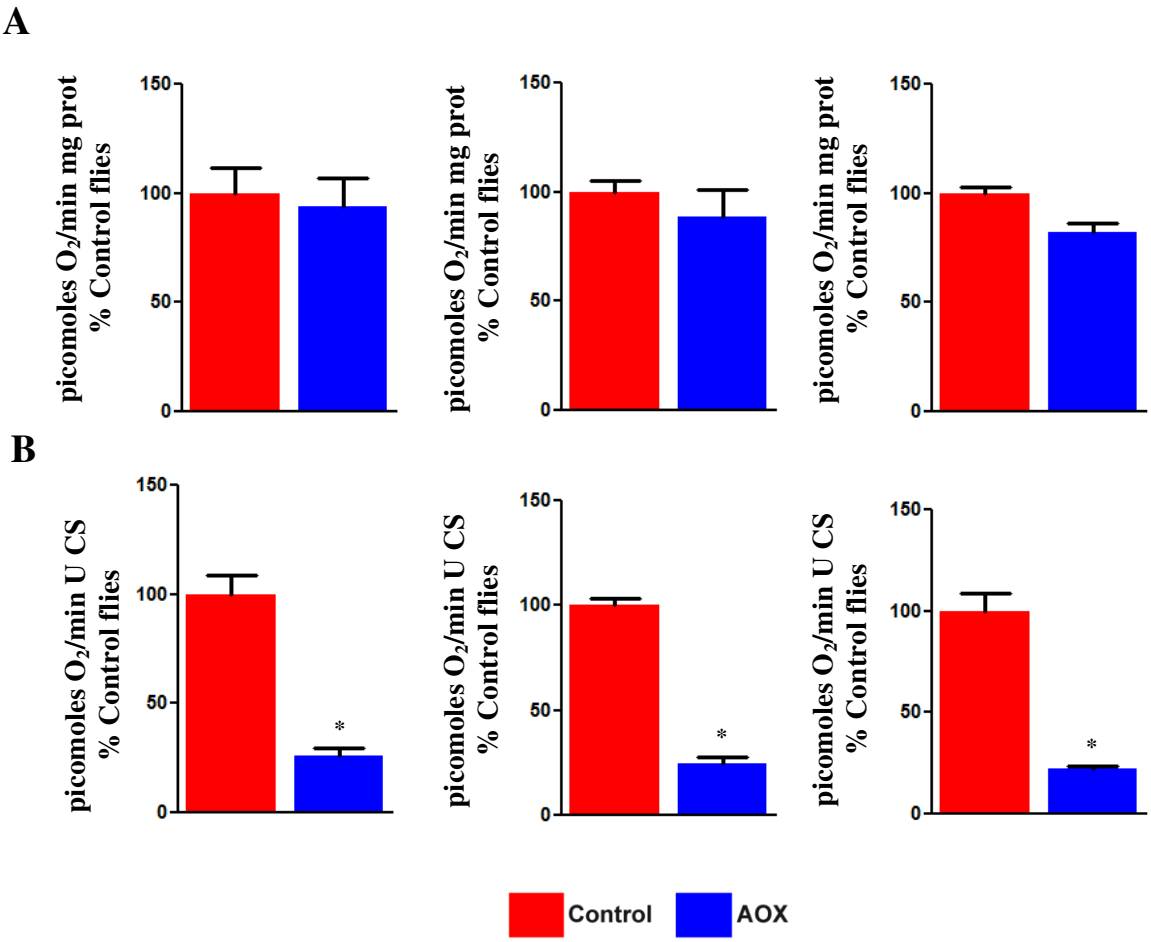

Figure 3.12 Mitochondrial respiration is decreased in AOX flies.

(A) Comparison of oxygen consumption of indicated genotypes (n=6). CI-linked (Control = 654.3±76.70 picomoles O_2 min^{-1} mg protein $^{-1}$), CIII-linked (Control = 486±25.73 picomoles O_2 min^{-1} mg protein $^{-1}$) and CIV-linked (Control = 2360±25.73 picomoles O_2 min^{-1} mg protein $^{-1}$) oxygen consumption measured. **(B)** Representing respiration data normalized to respiration per mitochondrion in indicated genotypes (n=6). CI- CIII- and CIV-linked respiration was measured. Non-expressing flies (*2>UAS-AOX*) (Control in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using student's t-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes.

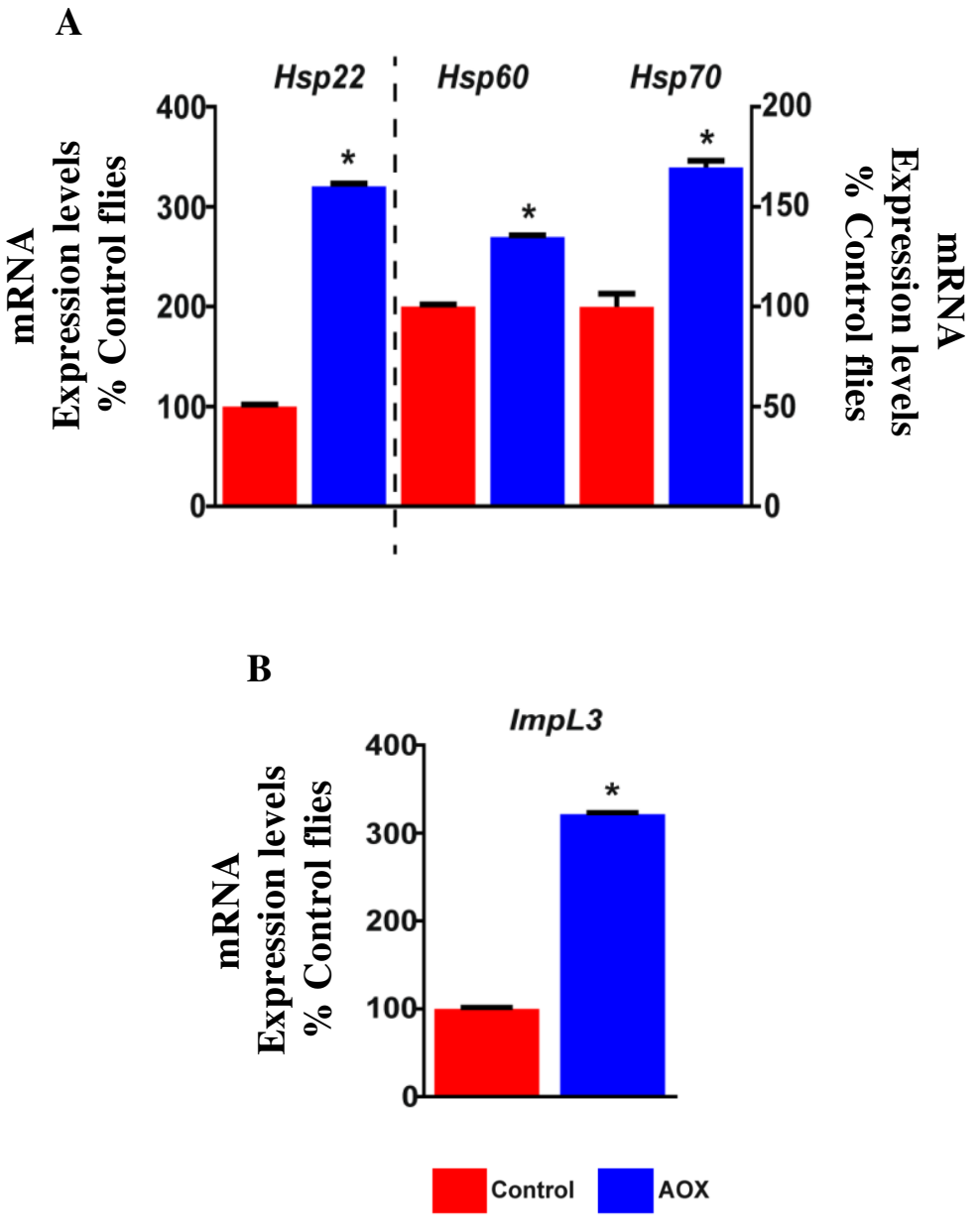

Figure 3.13 mRNA levels of mtHSP's and ImpL3 are upregulated in AOX flies.

(A) Quantification of mRNA levels of *Hsp22*, *Hsp60* and *Hsp70* by quantitative PCR of indicated genotypes (n=4). **(B)** Quantification of mRNA levels of *ImpL3* by quantitative PCR of indicated genotypes (n=4). Non-expressing flies (*2>UAS-AOX*) (Control in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using student's t-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes.

3.8 Pink1 protein levels are decreased in AOX flies

In fruit flies, mitochondria are recycled by the canonical Pink1-Parkin mitophagy pathway (Clark *et al.*, 2006) (discussed in detail in Chapter 1.19). I found a significant decrease in protein levels of Pink1 measured by Western Blotting, but the protein levels of Parkin were not changed (Figure 3.14 A and B). These data indicated that recycling of damaged mitochondria was disrupted by reduction of mtROS levels.

I also measured mRNA levels of *Pink1* and *Parkin* and found a significant decrease in mRNA levels of *Pink1* but not *Parkin* (Figure 3.15). This indicates that Pink1 is regulated at post-transcriptional level as well.

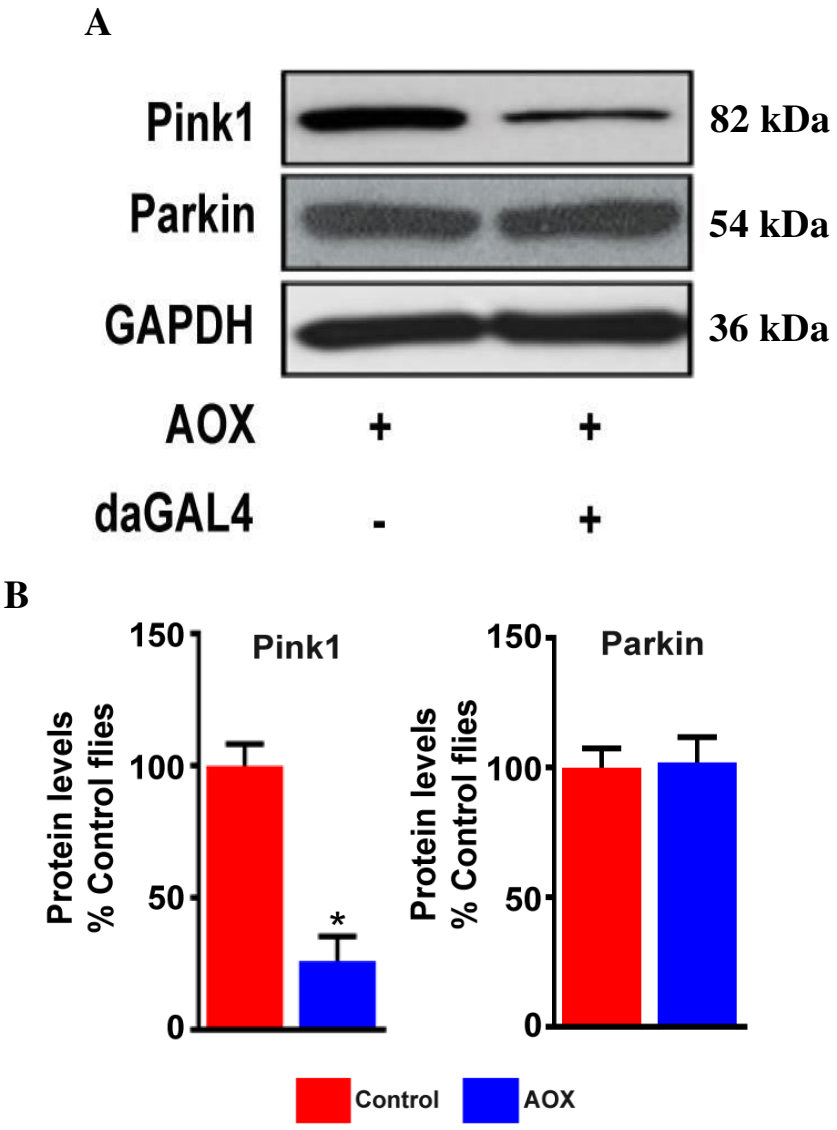

Figure 3.14 Pink1 but not Parkin protein levels are decreased in AOX flies.

(A) Representative western blot membranes showing Pink1 and Parkin protein levels of indicated genotypes. GAPDH is used for normalization and quantification purposes. **(B)** Quantification of **(A)** (n=3). Non-expressing flies (*2>UAS-AOX*) (Control in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using student's t-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. The molecular weight of proteins detected by western blots are given as kiloDalton (kDa) units.

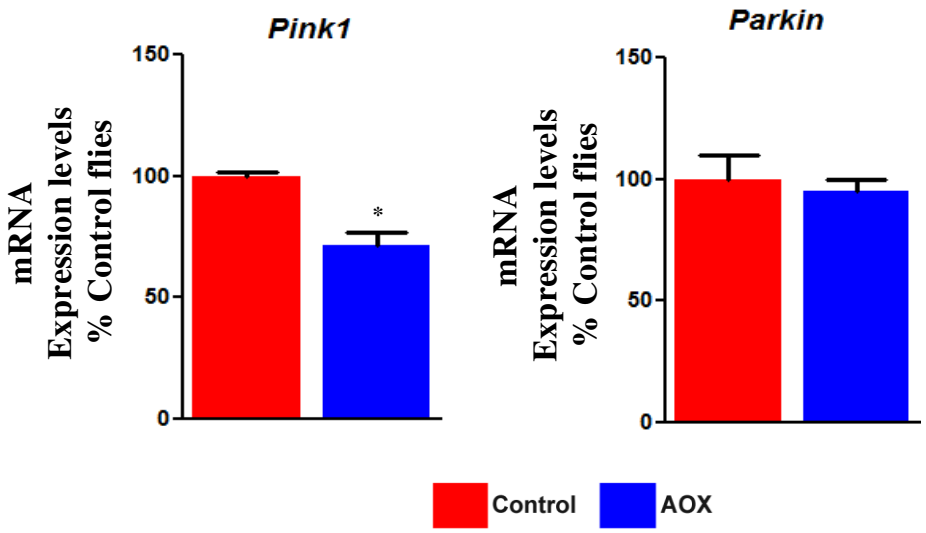

Figure 3.15 Pink1, but not Parkin mRNA levels are reduced in AOX flies.

Quantification of mRNA levels of *Pink1* and *Parkin* by quantitative PCR of indicated genotypes (n=4). Non-expressing flies (*2>UAS-AOX*) (Control in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using student's t-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes.

3.9 Protein turnover mechanisms are disrupted in AOX flies

Mechanism of protein turnover is well established and well conserved in almost all organisms (discussed in detail in Chapter 1.20). To begin with, I checked the functionality of protein turnover mechanisms were functioning properly. I used a previously described assay to measure the activity of 20S and 26S proteasome (Rivett *et al*., 2002). I detected a decrease in Chymotrypsin-like, Trypsin-Like and Caspase-like activity in both 20S and 26S proteasome in AOX flies (Figure 3.16 A and B).

Loss of proteostasis is also considered as a hallmark of ageing (Ryazanov *et al*., 2002). Protein levels of Ubiquitin and p62 (Ref (2) P) were significantly increased (Figure 3.17 A and B), which also indicated dysfunctional protein turnover. However, defective protein turnover may also be caused by the loss in autophagy (described in the following chapters).

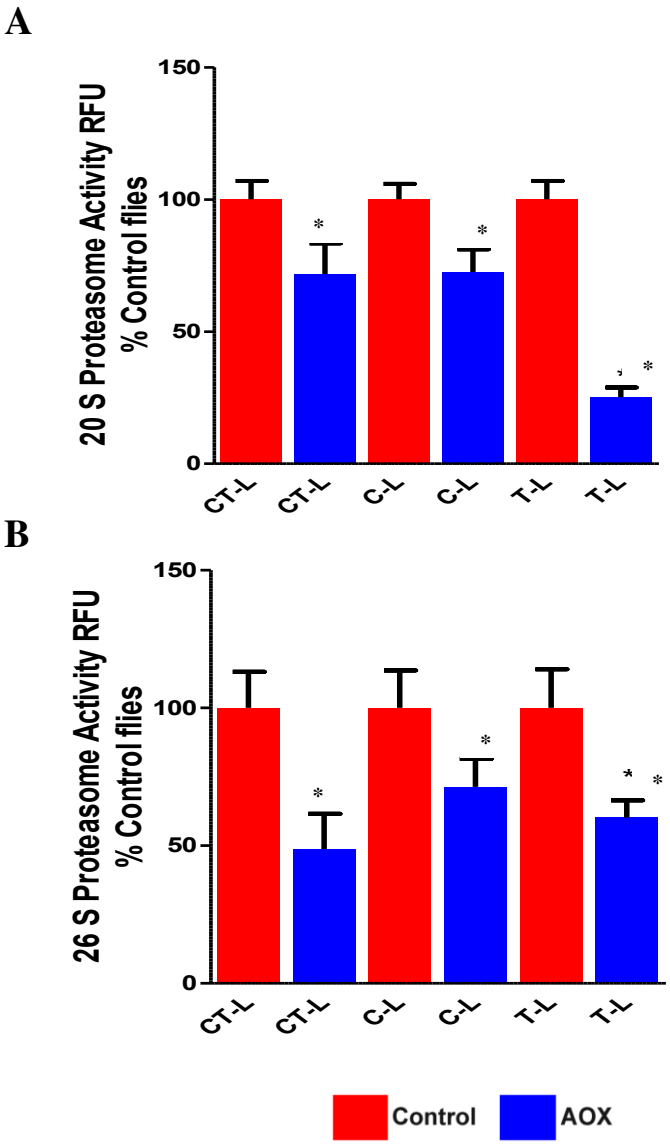

Figure 3.16 Proteasome activity is decreased in AOX flies.

(A) Measurements for Chymotrypsin like (CT-L), Caspase like (C-L) and Trypsin like (T-L) 20S proteasome activity of indicated genotypes (n=6). **(B)** Measurements for Chymotrypsin-like (CT-L), Caspase-like (C-L) and Trypsin-like (T-L) 26S proteasome activity of indicated genotypes (n=6). Non-expressing flies (*2>UAS-AOX*) (Control in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using student's t-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes.

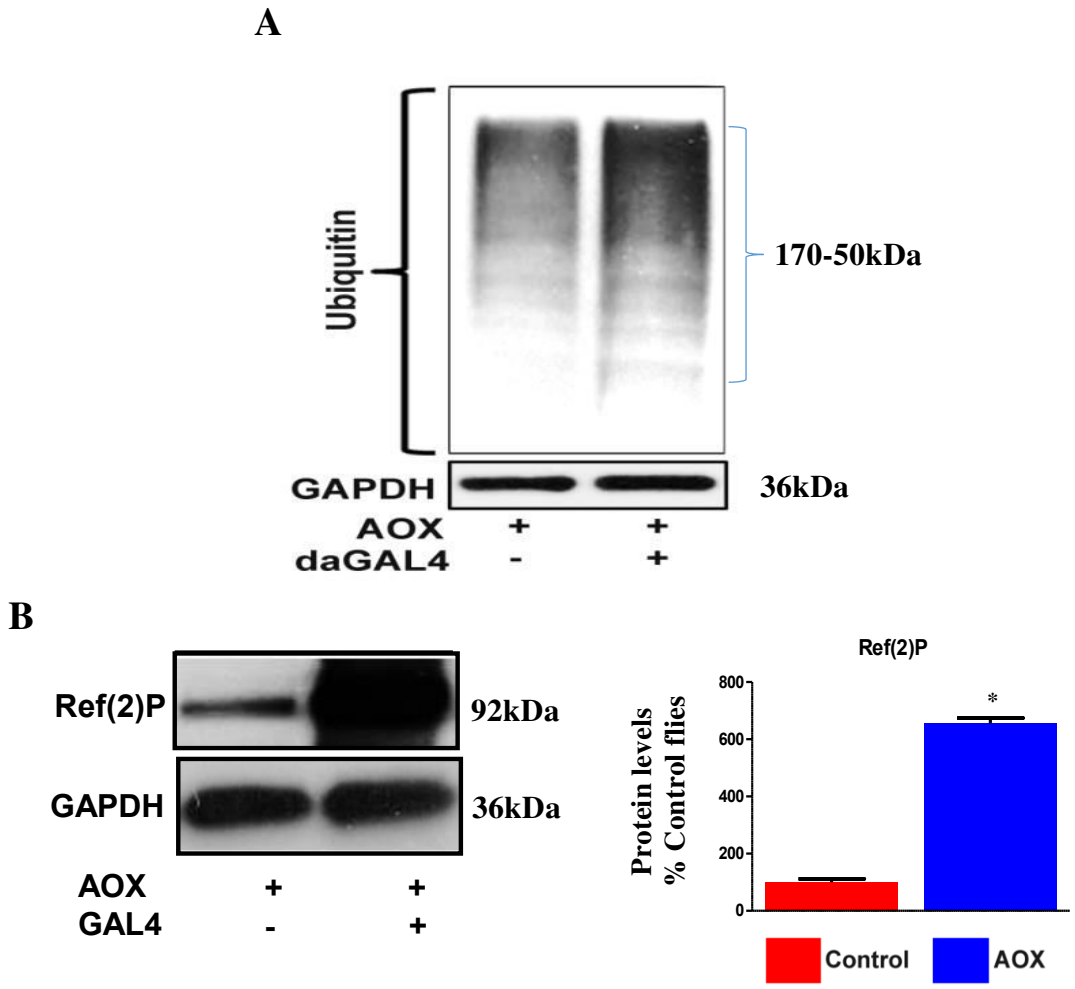

Figure 3.17 Ubiquitinated proteins and Ref (2) P protein levels are increased in AOX flies.

(A) Representative western blot membrane showing ubiquitinated protein levels of indicated genotypes. GAPDH is used for normalization and quantification purposes. **(B)** Left panel showing representative western blot membrane showing Ref (2) P protein levels of indicated genotypes. GAPDH is used for normalization and quantification purposes. Right panel showing quantification of left panel (n=3). Non-expressing flies (*2>UAS-AOX*) (Control in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using student's t-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. The molecular weight of proteins detected by western blots are given as kiloDalton (kDa) units.

3.10 Inactivation of proper quality control mechanisms compromises the survival of AOX flies

I then observed that expression of AOX drastically reduced survival of the flies (Figure 3.18) at high temperatures; whereas, normal culture temperature does not alter AOX lifespan (Fernandez-Ayala *et al.*, 2009). It can be hypothesized that with the loss of mtROS signal caused by AOX expression, the flies were not able to activate the necessary signals and overcome persistent damage.

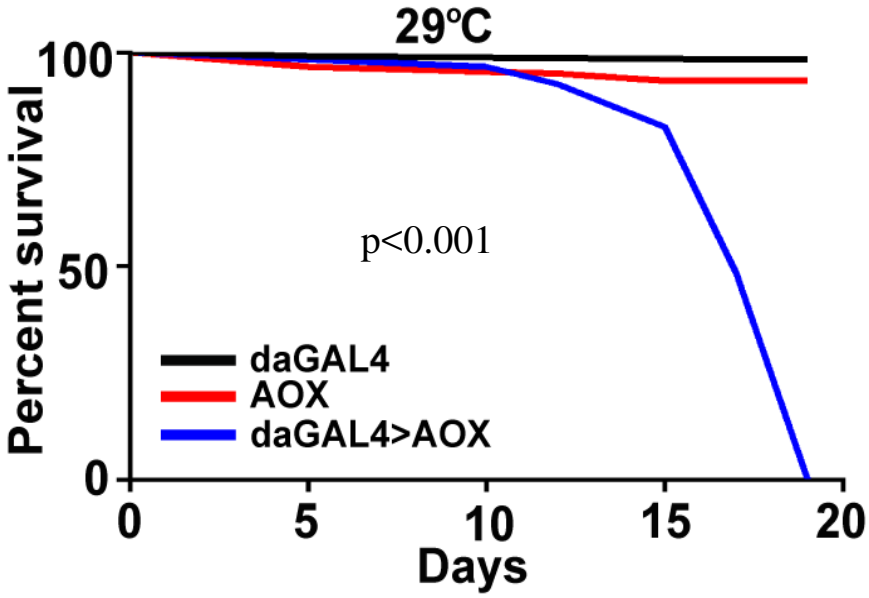

Figure 3.18 AOX expression decreases fly lifespan.

Survival curve of indicated genotypes (n=200). A table with statistics for the lifespan is presented in Appendix A.

Chapter 4. Determining the nature of the ROS signal generated under stress

4.1 A background on mitochondrial ROS signalling mechanisms

Mitochondrial ROS were initially not considered as signalling molecules since they were said to cause oxidative damage. This dual function of ROS has made it very complicated to establish how or why ROS causes ageing. However, carefully controlling levels of oxygen and maintaining very similar physiological levels of ROS in cell culture; it has been evident that this mtROS signal might be responsible for initiating major signalling pathways essential for healthy ageing (Hamanaka and Chandel, 2010). Furthermore, extensive studies are required in order to elucidate these ROS signalling mechanism *in vivo*.

ROS are often considered as a unique entity, but over the decade it has been reported that different ROS have different properties and are independent molecules (Scialo *et al.*, 2013). For instance, extreme increase of O_2^- is detrimental for *Drosophila*, but increasing cellular levels of H_2O_2 extends lifespan (Sohal, 1988; Broughton *et al.*, 2005; Stefanatos *et al.*, 2012). Similarly, O_2^- and H_2O_2 may play different roles in mitochondrial turnover and activation of other downstream mechanisms. Therefore, using strategies to delineate the effect of different ROS molecules is necessary to understand how they operate *in vivo*. On the other hand, mild mitochondrial stress caused by the increase in ROS, increases or activates signalling pathways that are needed to protect the organism long term. This activation has been demonstrated to prolong the organism's lifespan. This mechanism known as mitohormesis is gaining attention and is being explored extensively owing to the work across different model organisms from yeast, mice and rats (Yun and Finkel, 2014).

Results presented in Chapter 3 indicated that the loss of mtROS signalling (AOX flies) compromised many of the survival mechanisms, thereby compromising fly lifespan. However, as expression of AOX modifies the generation of mtROS by controlling the redox state of Coenzyme Q (CoQ), I was not able to differentiate the ROS entity responsible for this signalling (H_2O_2 or O_2^-). AOX is also upstream of the antioxidant response to H_2O_2 or O_2^- (El-Khoury *et al.*, 2014) and I have already demonstrated that AOX reduces both ROS species.

To understand which of the ROS species (H_2O_2 or O_2^-) is involved in this signalling, I took advantage of the powerful genetics of *Drosophila melanogaster*. I ectopically expressed Catalase in the mitochondrial matrix. Catalase converts H_2O_2 to H_2O, making the ROS molecule completely inert. I also overexpressed Sod2, which is in the mitochondrial matrix and converts O_2^- to H_2O_2. Figure 4.1 represents the function of Sod2 and mitochondrial Catalase in the mitochondria. In this chapter, I specifically manipulate the levels of both superoxide and Hydrogen peroxide to understand which ROS entity is responsible for the changes in the mitochondrial functionality observed in Chapter 3.

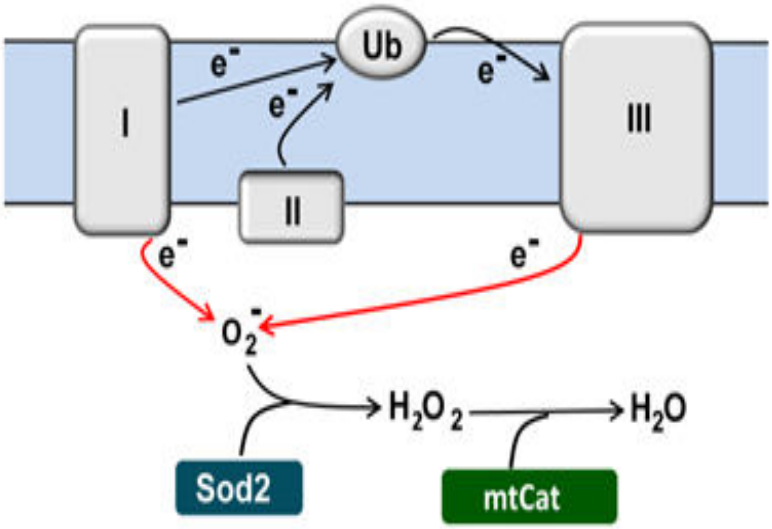

Figure 4.1 Superoxide dismutase 2 (Sod2) and mitochondrial Catalase (mtCAT) interact with O_2^- and H_2O_2 respectively.

Sod2 and mtCAT are important antioxidants in the mitochondria converting O_2^- to H_2O_2 and H_2O_2 to H_2O, respectively.

4.2 Ectopical expression of Catalase and overexpression of Sod2 in the mitochondria reduces H_2O_2 and O_2^- respectively

To validate the fly models, I analysed the mRNA levels of *Catalase* and *Sod2* in the mitochondrial Catalase expressing flies (mtCAT flies hereafter) and the Sod2 overexpressing flies (Sod2 flies hereafter), respectively. I detected a significant increase in mRNA levels of both *Catalase* and *Sod2* in the respective flies (Figure 4.2 A). To confirm that mitochondrial expression of Catalase reduces H_2O_2 production, I dissected fly brains from mtCAT flies and their respective controls. When the fly brains were stained with MitoSOX, mtCAT flies showed no decrease in MitoSOX fluorescence, confirming that mtCAT does not affect superoxide levels (Figure 4.2 B). I also stained fly brains with H_2DCF, a dye specific to peroxides that fluoresces in reaction with H_2O_2 and other peroxides. mtCAT flies showed significantly less fluorescence with H_2DCF, confirming that mtCAT reduces H_2O_2 and functions as expected (Figure 4.2 C). On the other hand, Sod2 flies showed a significant decrease in MitoSOX fluorescence confirming that overexpression of Sod2 reduces O_2^- levels (Figure 4.2 B). I also found that overexpression of Sod2 increases fluorescence of brains stained with H_2DCF, indicating that Sod2 overexpression increases H_2O_2 levels (Figure 4.2 C).

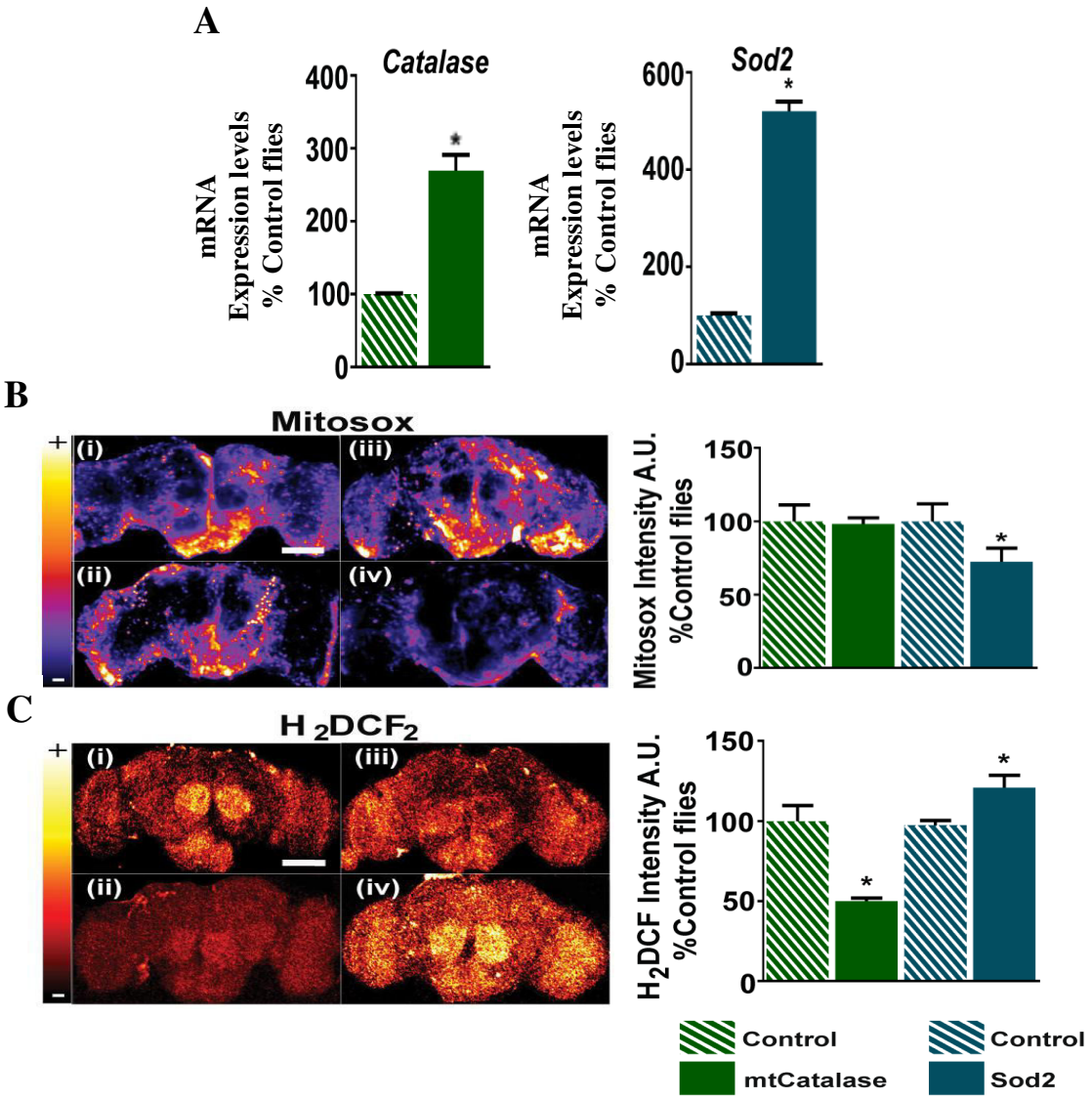

Figure 4.2 Reduction in H_2O_2 and O_2^- is observed in mtCAT and Sod2 flies respectively.

(A) Quantification of mRNA levels of *Catalase* and *Sod2* by quantitative PCR of indicated genotypes (n=4) **(B)** Left panel showing representative images of dissected fly brains stained with MitoSOX of indicated genotypes. Right panel showing quantification of left panel (n=5). **(C)** Left panel showing representative images of dissected fly brains stained with H_2DCF of indicated genotypes. Right panel showing quantification of left panel (n=5). (i) Non-expressing flies (*2>UAS-mtCAT*) (Control in the figure), (ii) *UAS-mtCAT* expressing flies (*daGAL4>UAS-mtCAT*) (mtCatalase in the figure), (iii) non-expressing flies (*2>UAS-Sod2*) (Control in the figure) and (iv) *Sod2* overexpressing flies (*daGAL4>UAS-Sod2*) (Sod2 in the figure) were used. A daughterless GAL4 promoter was used for the expression of transgenes. P values were calculated using student's t-test. Experimental groups were compared to their respective controls. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. Scale bars are 100µm. This work was done in collaboration with Dr. Filippo Scialo.

4.3 Oxidative stress is not reduced and antioxidant levels are unaltered in mtCAT and Sod2 flies

I analysed the levels of specific markers of oxidative damage as described in the Chapter 3.4, since ROS levels were significantly altered in these flies. However, I did not see any significant difference in oxidative, glycoxidative or lipoxidative damage between the groups, except for a moderate increase in AASA (marker for protein oxidation) in mtCAT flies (Figure 4.3 A).

I then wanted to see if there was any retrograde response to other antioxidants (Whelan and Zuckerbraun, 2013). I detected only moderate changes in the mRNA levels of *Glutathion Peroxidase and dj-1β* were down regulated; whereas, mRNA levels of *Glyoxalase 1* was unregulated in Sod2 flies. However, in mtCAT flies only *Glutathion Peroxidase* mRNA levels were downregulated. Other antioxidants like *Sod, Sod2, and Catalase* were unaltered (Figure 4.3 B). These results demonstrate that the feedback mechanisms are not active in these fly models.

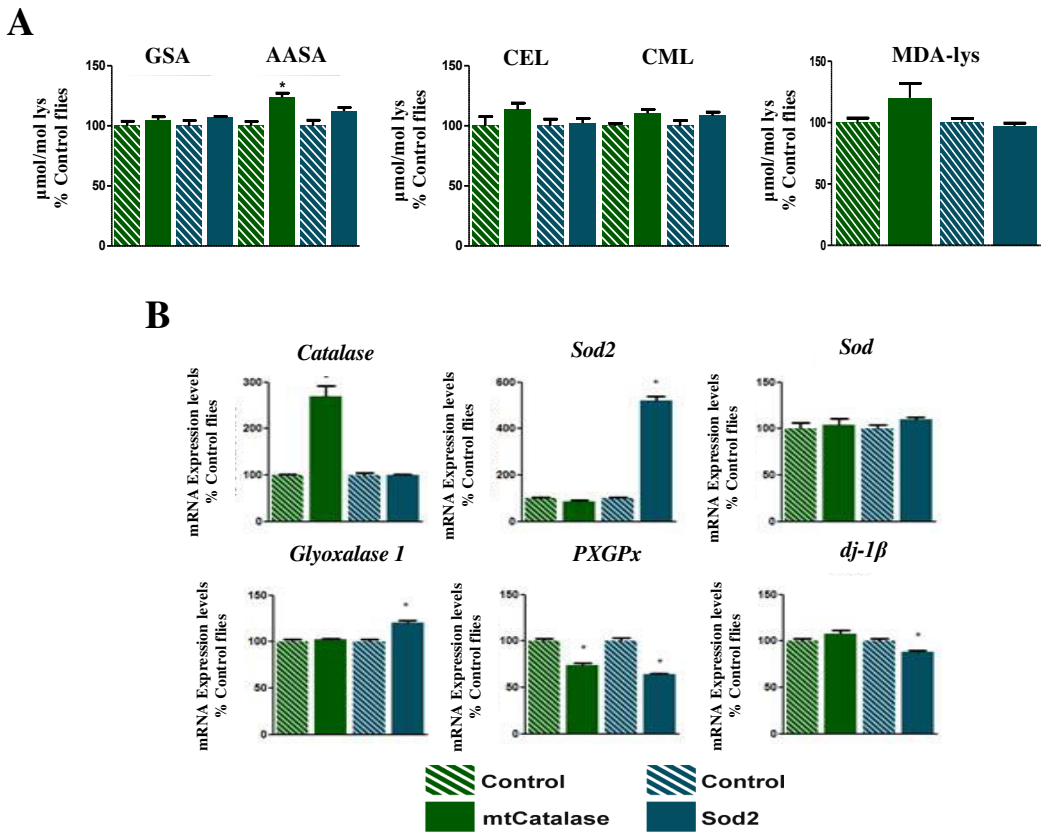

Figure 4.3 Oxidative stress is not reduced and antioxidant levels are unaltered.

(A) Measurements for markers of oxidative (GSA, AASA), glycoxidative (CML, CEL) and lipoxidative (MDA-lys) damage in flies of indicated genotypes (n=6) (Control (mtCAT) GSA = 6890±268.9 µmoles moles lys^{-1}, Control (Sod2) GSA = 7153±319.1 µmoles moles lys^{-1}) (Control (mtCAT) AASA = 1404±522 µmoles moles lys^{-1} and Control (Sod2) AASA = 597.8±16.16 µmoles moles lys^{-1}) (Control (mtCAT) CML = 227.4±17.4 µmoles moles lys^{-1} and Control (Sod2) CML = 512.5±27.55 µmoles moles lys^{-1}) (Control (mtCAT) CEL = 1329±27.13 µmoles moles lys^{-1} and Control (Sod2) CEL = 1113±49.83 µmoles moles lys^{-1}) (Control (mtCAT) MDA-lys = 353±23.45 µmoles moles lys^{-1} and Control (Sod2) MDA-lys = 319±22 µmoles moles lys^{-1}). This work was done in collaboration with Dr. Reinald Pamplona. **(B)** Quantification of mRNA levels of *Sod, Sod2, Catalase, Glutathion Peroxidase, Glyoxalase 1* and *dj-1β* by quantitative PCR of indicated genotypes (n=4). *Catalase* and *Sod2* qPCR measurements are same as Figure 4.2 A, but with addition of other experimental groups. Non-expressing flies (*2>UAS-mtCAT*) (Control in the figure), *UAS-mtCAT* expressing flies (*daGAL4>UAS-mtCAT*) (mtCatalase in the figure), non-expressing flies (*2>UAS-Sod2*) (Control in the figure) and *Sod2* overexpressing flies (*daGAL4>UAS-Sod2*) (Sod2 in the figure) were used. A daughterless GAL4 promoter was used for the expression of transgenes. P values were calculated using student's t-test. Experimental groups were compared to their respective controls. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes.

4.4 Mitochondrial density is increased in mtCAT but not in Sod2 flies

In order to test whether O_2^- or H_2O_2 had a leading role in the phenotypes observed in AOX flies, I measured the mitochondrial density in mtCAT and Sod2 flies. I detected a significant increase in mitochondrial density in mtCAT flies; whereas, mitochondrial density was significantly reduced in Sod2 flies (Figure 4.4 A).

I then measured mtDNA copy number to confirm the increase in mitochondrial density. The ratio between the nuclear and the mitochondrial DNA clearly showed a significant increase in mtCAT flies and a significant decrease in Sod2 flies (Figure 4.4 B). These results demonstrate that O_2^- and H_2O_2 may have different effects on downstream signalling cascades.

Corroborating my results from this chapter, I have established that mtCAT flies phenocopy AOX flies, denoting that H_2O_2 may be involved in maintaining mitochondrial functionality. Moreover, mitochondrial recycling was improved in Sod2 flies, which as described earlier produce less O_2^- and more H_2O_2. These findings also ascertain the fact that an increase in mtH_2O_2 is not harmful and can function as a ROS messenger.

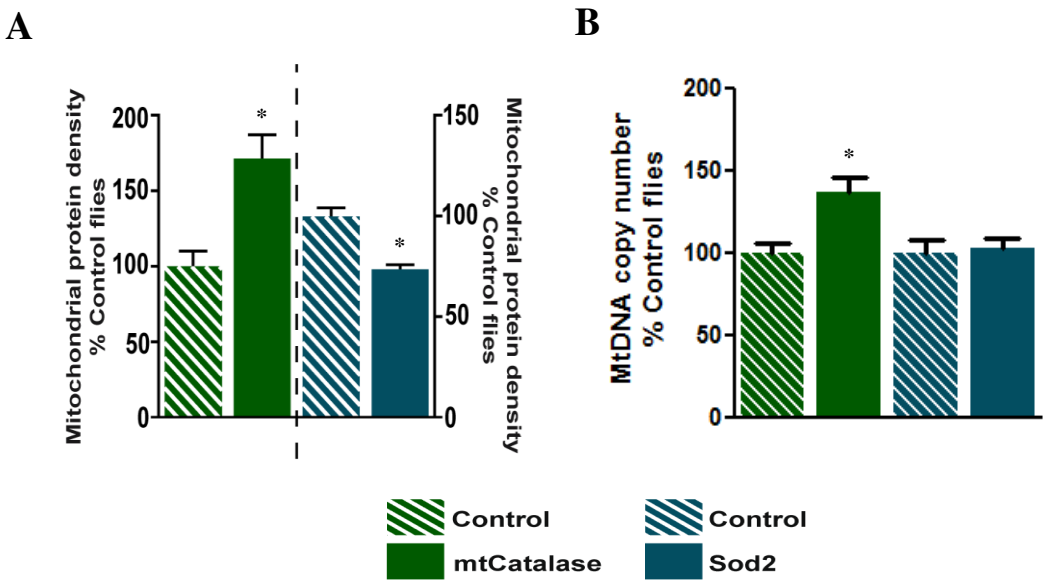

Figure 4.4 Mitochondrial density is increased in mtCAT but not in Sod2 flies.

(A) Citrate synthase activity measuring mitochondrial density of indicated genotypes (Control (mtCAT) = 1.652±0.16 Absorbance Units mg protein^{-1} and Control (Sod2) = 1.882±0.079 Absorbance Units mg protein^{-1}) (n=9). **(B)** mtDNA copy number of indicated genotypes (n=4). Non-expressing flies (*2>UAS-mtCAT*) (Control in the figure), *UAS-mtCAT* expressing flies (*daGAL4>UAS-mtCAT*) (mtCatalase in the figure), non-expressing flies (*2>UAS-Sod2*) (Control in the figure) and *Sod2* overexpressing flies (*daGAL4>UAS-Sod2*) (Sod2 in the figure) were used. A daughterless GAL4 promoter was used for the expression of transgenes. P values were calculated using student's t-test. Experimental groups were compared to their respective controls. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes.

4.5 *Spargel* and *Delg* mRNA levels are unaltered in both mtCAT and Sod2 flies

In order to discern whether the increase in mitochondrial density is not because of an increase in mitochondrial biogenesis, I measured the mRNA levels of *Spargel* and using quantitative PCR. I confirmed that mitochondrial biogenesis was not altered in response to changes in mtH_2O_2, as both mtCAT and Sod2 did not alter mRNA levels of *Spargel* and *Delg* (Figure 4.5).

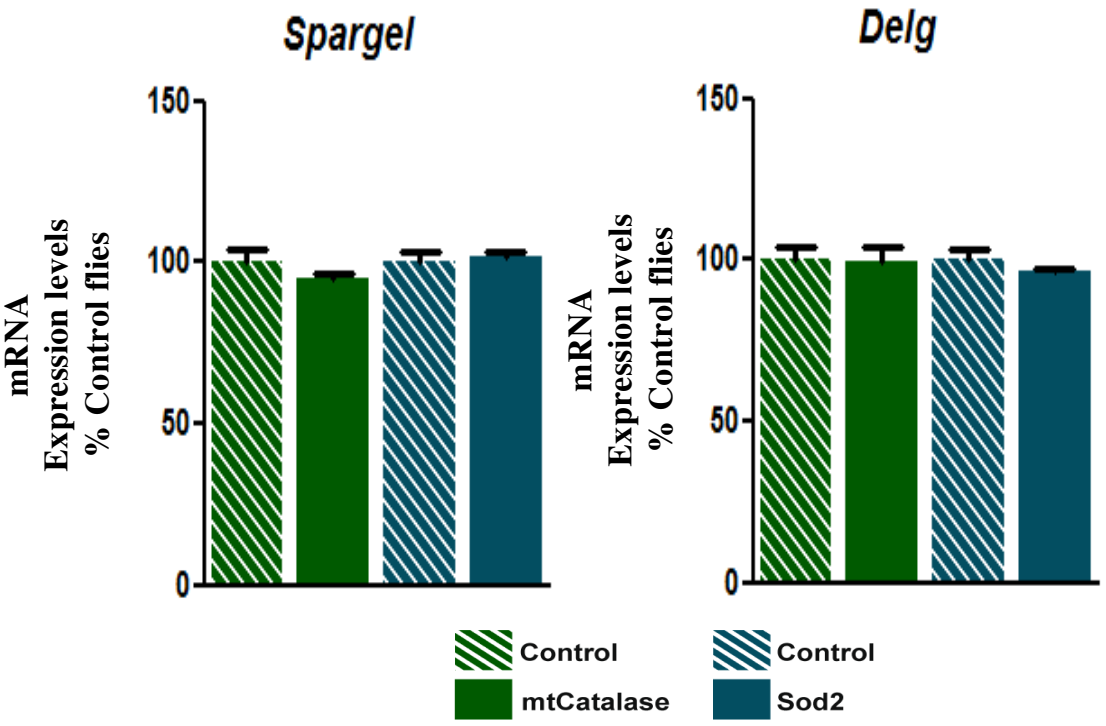

Figure 4.5 Spargel and Delg mRNA levels are unaltered in both mtCAT and Sod2 flies.

Quantification of mRNA levels of *Spargel* and *Delg* by quantitative PCR of indicated genotypes (n=4). Non-expressing flies (*2>UAS-mtCAT*) (Control in the figure), *UAS-mtCAT* expressing flies (*daGAL4>UAS-mtCAT*) (mtCatalase in the figure), non-expressing flies (*2>UAS-Sod2*) (Control in the figure) and *Sod2* overexpressing flies (*daGAL4>UAS-Sod2*) (Sod2 in the figure) were used. A daughterless GAL4 promoter was used for the expression of transgenes. P values were calculated using student's t-test. Experimental groups were compared to their respective controls. Data are shown as mean ± SEM.

4.6 Mitochondrial functionality is compromised in mtCAT flies but improved in Sod2 flies

I then used the mtCAT and Sod2 flies to analyse mitochondrial respiration. Similar to the AOX flies, I did not observe any difference between the samples when the respiration measured was normalized to total protein content of the samples (Figure 4.6 A). However, mtCAT expression decreased CI, CIII and CIV-linked respiration, when the measurements were normalized to their respective mitochondrial densities. On the other hand, Sod2 expression increased mitochondrial respiration. These experiments demonstrate that mtCAT flies phenocopy AOX flies, denoting a leading role of mtH_2O_2 in maintaining mitochondrial functionality (Figure 4.6 B).

In order to ascertain that mitochondria are damaged, I analysed the mRNA levels of different heat shock proteins, as they are used as markers for mitochondrial damage. I observed severe induction in Heat shock protein 22 (*Hsp22*) and Heat shock protein 70 (*Hsp70*), indicating an accumulation of dysfunctional mitochondria in mtCAT flies. However, we only observed a modest but not significant increase in Heat shock protein 60 (*Hsp60*). Sod2 flies on the other hand, decreased the induction of *Hsp70* and did not affect other markers (Figure 4.7). In order to confirm this observation, I measured mRNA levels of *ImpL3* (used as a marker for mitochondrial damage) and found a strong up-regulation in mtCAT flies; whereas, the mRNA levels remained unaltered in Sod2 flies (Figure 4.7). This indicated that loss of mtH_2O_2 signalling and loss in recycling of damaged mitochondria caused the fly to switch to a more glycolytic metabolism.

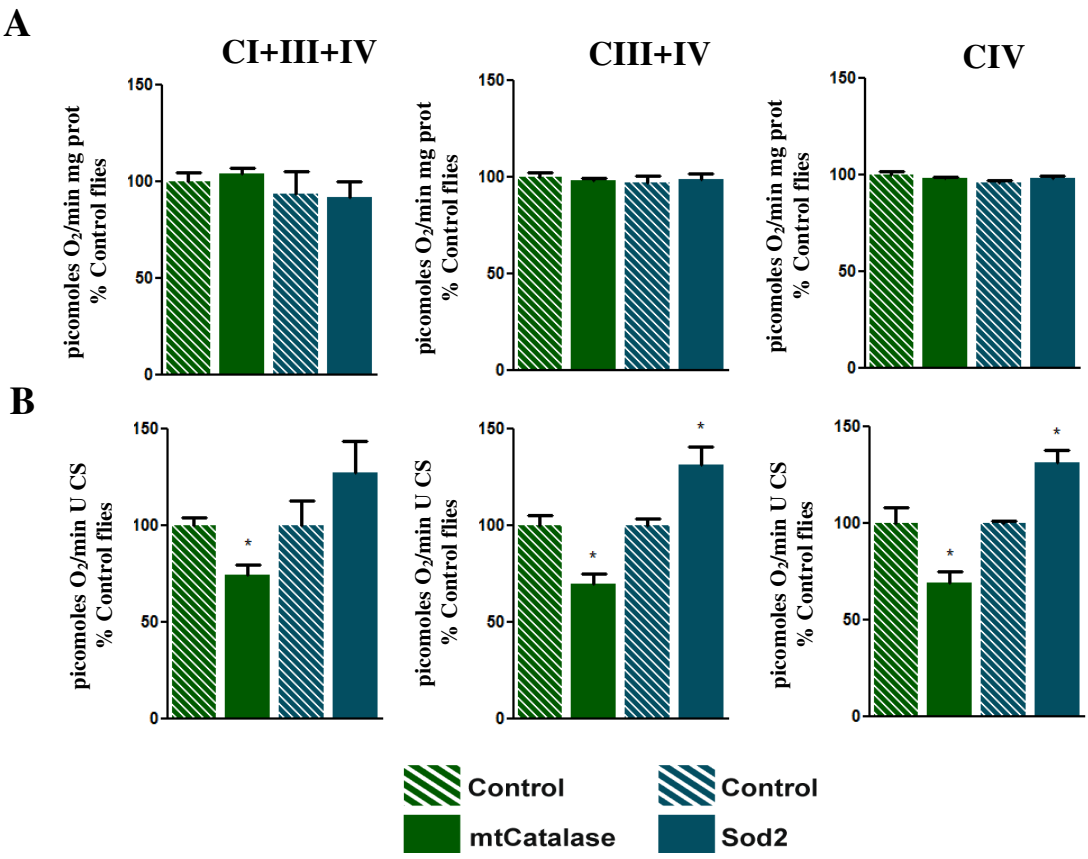

Figure 4.6 Mitochondrial respiration is decreased in mtCAT flies and improved in Sod2 flies.

(A) Comparison of oxygen consumption of indicated genotypes (n=3). CI-linked (Control (mtCAT) = 2063±92.68 picomoles O_2 min^{-1} mg $protein^{-1}$ and Control (Sod2) = 1993±242.7 picomoles O_2 min^{-1} mg $protein^{-1}$), CIII-linked (Control (mtCAT) = 1308±32.32 picomoles O_2 min^{-1} mg $protein^{-1}$ and Control (Sod2) = 1270±45.17 picomoles O_2 min^{-1} mg $protein^{-1}$) and CIV-linked (Control (mtCAT) = 9947±154.8 picomoles O_2 min^{-1} mg $protein^{-1}$ and Control (Sod2) = 9555±86.12 picomoles O_2 min^{-1} mg $protein^{-1}$) oxygen consumption measured in indicated genotypes. **(B)** Representing respiration data normalized to respiration per mitochondrion in indicated genotypes (n=3). CI- CIII- and CIV-linked respiration was measured. Non-expressing flies (*2>UAS-mtCAT*) (Control in the figure), *UAS-mtCAT* expressing flies (*daGAL4>UAS-mtCAT*) (mtCatalase in the figure), non-expressing flies (*2>UAS-Sod2*) (Control in the figure) and *Sod2* overexpressing flies (*daGAL4>UAS-Sod2*) (Sod2 in the figure) were used. A daughterless GAL4 promoter was used for the expression of transgenes. P values were calculated using student's t-test. Experimental groups were compared to their respective controls. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes.

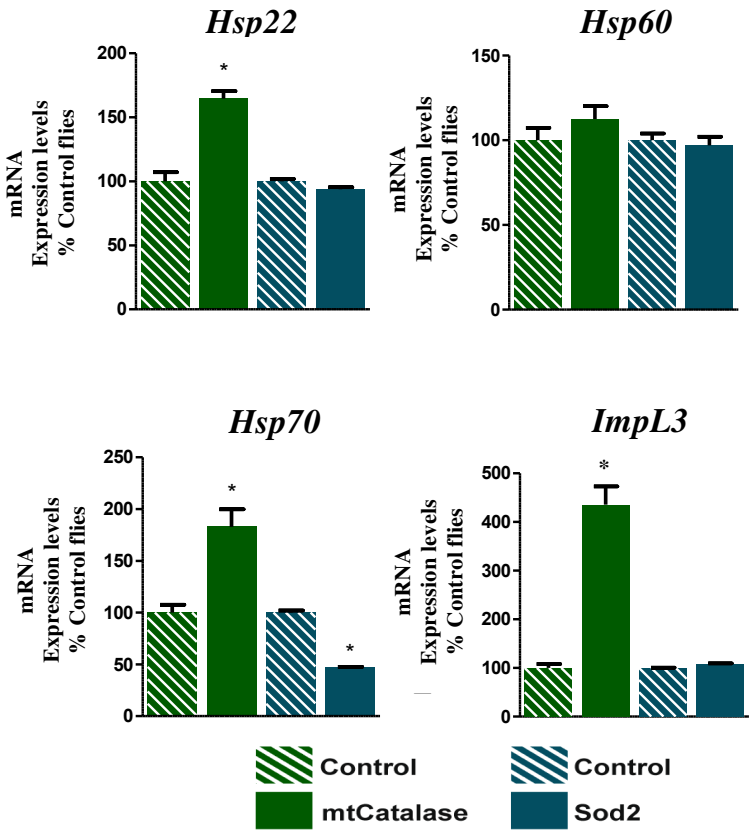

Figure 4.7 mRNA levels of mtHSP's and ImpL3 are upregulated in mtCAT flies.

Quantification of mRNA levels of *Hsp22, Hsp60, Hsp70 and ImpL3* by quantitative PCR of indicated genotypes (n=4). Non-expressing flies (*2>UAS-mtCAT*) (Control in the figure), *UAS-mtCAT* expressing flies (*daGAL4>UAS-mtCAT*) (mtCatalase in the figure), non-expressing flies (*2>UAS-Sod2*) (Control in the figure) and *Sod2* overexpressing flies (*daGAL4>UAS-Sod2*) (Sod2 in the figure) were used. A daughterless GAL4 promoter was used for the expression of transgenes. P values were calculated using student's t-test. Experimental groups were compared to their respective controls. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes.

4.7 Pink1 protein levels are decreased in mtCAT flies

Canonical Pink1-Parkin mitophagy is generally considered to be active when the mitochondrial membrane potential is low, which in turn is generally associated with damaged mitochondria (discussed in detail in Chapter 1.19). In this chapter, I show that an active mtH_2O_2 signal regulates the levels of Pink1, probably affecting Parkin recruitment to the mitochondrion. By Western Blotting, I demonstrate that Pink1 protein levels are significantly reduced in mtCAT flies; whereas, Pink1 protein levels are increased in Sod2 flies. The protein levels of Parkin are however unaltered (Figure 4.8 A and B). Taking into account the loss in Pink1 protein levels in AOX and mtCAT flies, and also a modest increase in Sod2 flies, I can hypothesize that a mtH_2O_2 signal regulates Pink1 levels *in vivo*.

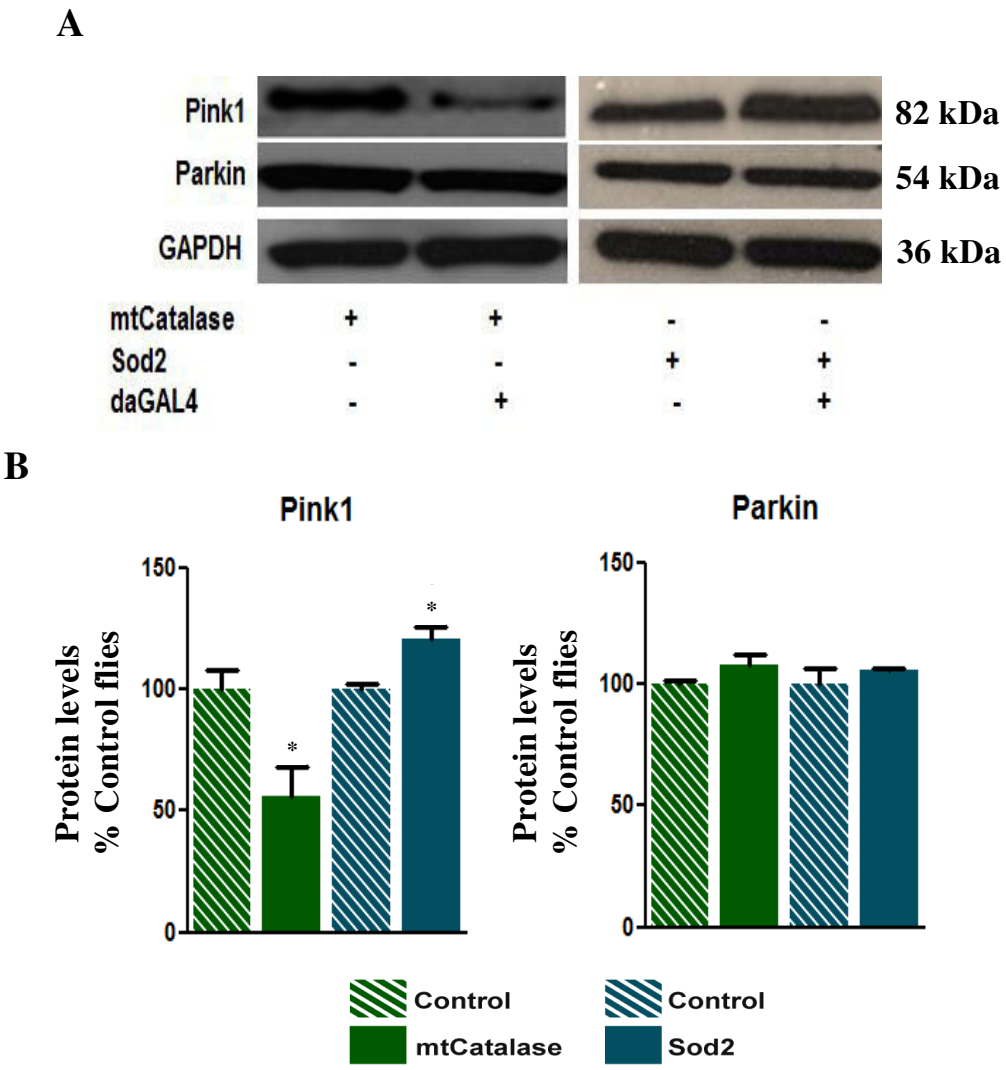

Figure 4.8 Pink1 protein levels are decreased in mtCAT flies.

(A) Representative western blot membranes showing Pink1 and Parkin protein levels. GAPDH is used for normalization and quantification purposes. **(B)** Quantification of **(A)** (n=3). Non-expressing flies (*2>UAS-mtCAT*) (Control in the figure), *UAS-mtCAT* expressing flies (*daGAL4>UAS-mtCAT*) (mtCatalase in the figure), non-expressing flies (*2>UAS-Sod2*) (Control in the figure) and *Sod2* overexpressing flies (*daGAL4>UAS-Sod2*) (Sod2 in the figure) were used. A daughterless GAL4 promoter was used for the expression of transgenes. P values were calculated using student's t-test. Experimental groups were compared to their respective controls. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. The molecular weight of proteins detected by western blots are given as kiloDalton (kDa) units.

4.8 Protein turnover is disrupted in mtCAT flies

I then used mtCAT and Sod2 flies, to examine whether O_2^- or H_2O_2 is responsible for the modulation of protein turnover. Although the effect was not as severe as in AOX flies, mtCAT flies reduced Caspase-Like and Trypsin-Like activity of the 20S proteasome and Chymotrypsin-Like and Caspase-Like activity of the 26S proteasome. Whereas, Sod2 overexpressing flies increased the activities of all the parameters studied in the 20S proteasome, but activity of the 26S proteasome remained largely unchanged (Figure 4.9 A and B). It can be hypothesized that the 20S proteasome is the primary target for the ROS signal. It is a more efficient way of recycling proteins that does not require the proteins to be ubiquitin tagged for recycling. However, when the mtH_2O_2 signal is reduced, proteasomal activity is always reduced; and when the mtH_2O_2 signal is increased the activity is always improved. This further supports the hypothesis that mtH_2O_2 signal is not only responsible for mitochondrial turnover but also for mechanisms involving protein turnover.

I also found a significant increase in Ubiquitin tagged and p62 (Ref (2) P in flies) protein levels in mtCAT flies; whereas, protein levels remained unaltered in Sod2 flies (Figure 4.10 A and B). This confirms the results obtained earlier in this chapter, and that protein turnover mechanisms are altered by mtH_2O_2.

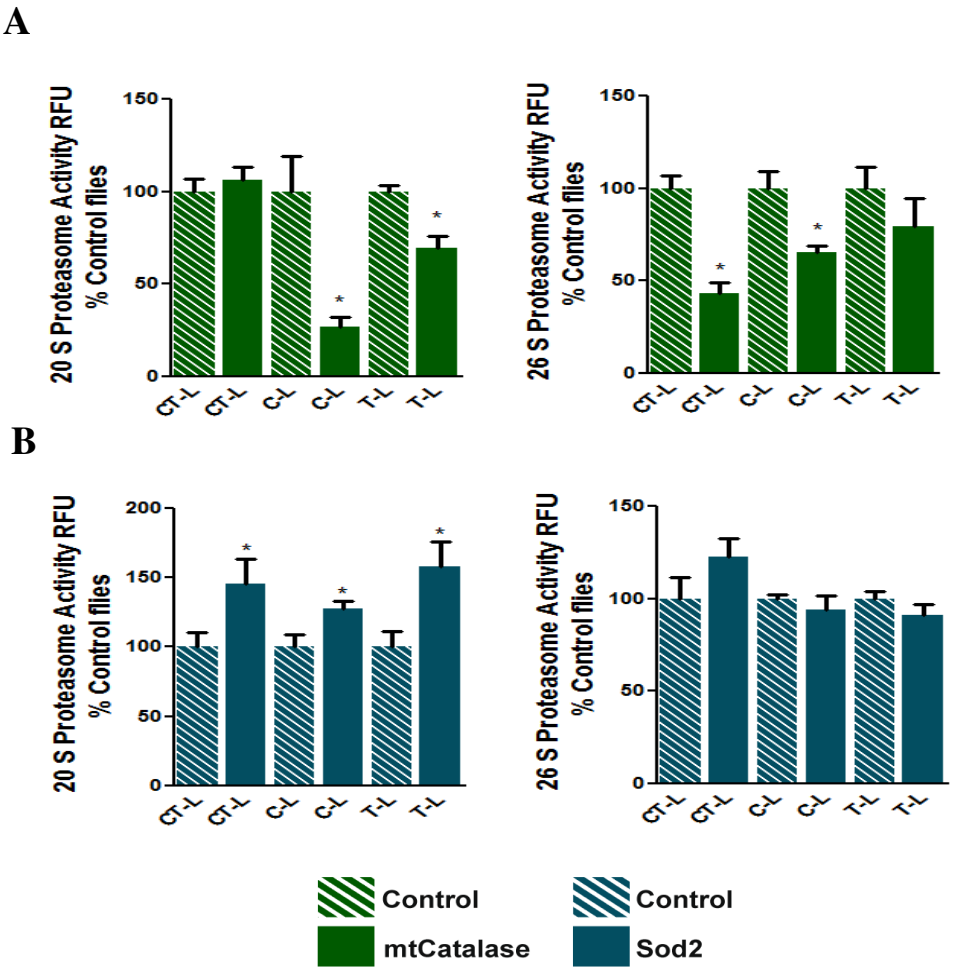

Figure 4.9 Proteasomal activity is decreased in mtCAT flies and increased in Sod2 flies.

(A) Measurements for Chymotrypsin-like (CT-L), Caspase-like (C-L) and Trypsin-like (T-L) 20S and 26S proteasome activity of indicated genotypes (n=6) **B)** Measurements for Chymotrypsin-like (CT-L), Caspase-like (C-L) and Trypsin-like (T-L) 20S and 26S proteasome activity of indicated genotypes (n=6). Non-expressing flies (*2>UAS-mtCAT*) (Control in the figure), *UAS-mtCAT* expressing flies (*daGAL4>UAS-mtCAT*) (mtCatalase in the figure), non-expressing flies (*2>UAS-Sod2*) (Control in the figure) and *Sod2* overexpressing flies (*daGAL4>UAS-Sod2*) (Sod2 in the figure) were used. A daughterless GAL4 promoter was used for the expression of transgenes. P values were calculated using student's t-test. Experimental groups were compared to their respective controls. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes.

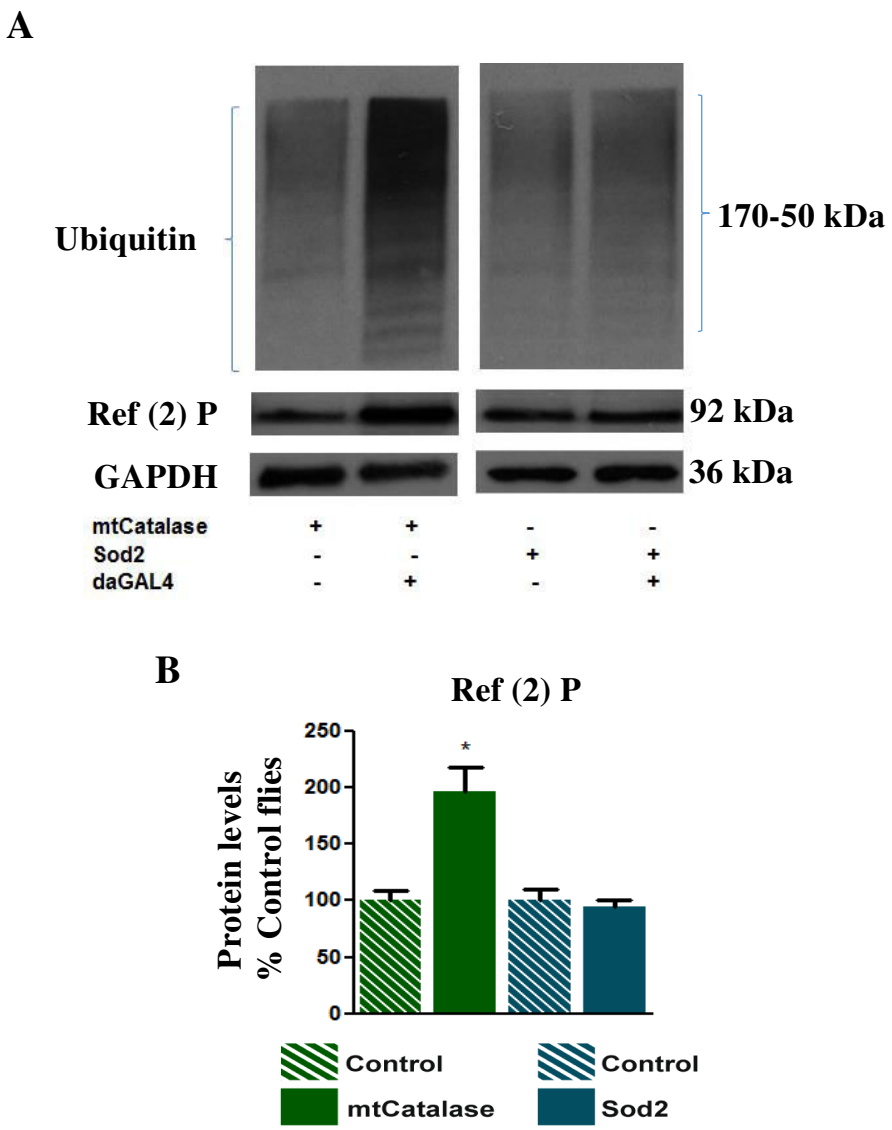

Figure 4.10 Ubiquitinated proteins and Ref (2) P protein levels are increased in mtCAT flies.

(A) Representative western blot membranes showing Ubiquitin and Ref (2) P protein levels of indicated genotypes. GAPDH is used for normalization and quantification purposes. **(B)** Quantification of bottom panel of **(A)** (n=3). Non-expressing flies (*2>UAS-mtCAT*) (Control in the figure), *UAS-mtCAT* expressing flies (*daGAL4>UAS-mtCAT*) (mtCatalase in the figure), non-expressing flies (*2>UAS-Sod2*) (Control in the figure) and *Sod2* overexpressing flies (*daGAL4>UAS-Sod2*) (Sod2 in the figure) were used. A daughterless GAL4 promoter was used for the expression of transgenes. P values were calculated using student's t-test. Experimental groups were compared to their respective controls. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. The molecular weight of proteins detected by western blots are given as kiloDalton (kDa) units.

4.9 mtH_2O_2 activates a transcriptional program for stress adaptation

To further understand the mechanisms involved in the control of adaptation by mtH_2O_2, I analysed the expression of 87 genes involved in signal transduction by using quantitative PCR. AOX expression altered the expression of 44% of these genes, while mtCAT expression and Sod2 overexpression had a weaker effect altering the expression of 20% and 27% of the genes, respectively. Such differences can be explained because AOX directly affects the leak of electrons from the ETC and suppresses ROS production from both sides of the inner membrane; whereas, mtCAT and Sod2 can only intercept a portion of ROS released into the matrix, specifically acting on H_2O_2 and superoxide, respectively. Decreased mtH_2O_2 signal caused a down-regulation of genes, while increased mtH_2O_2 levels were associated with up-regulation of genes. Two pathways appeared to be most affected: P53 signalling (*cathD, ef1alpha48d, mus209, tsp66e*) and adaptation to hypoxia (*CG10899, tango, and reptin*). More importantly, the genes up-regulated in AOX or mtCAT flies were never up-regulated in Sod2 overexpressing flies, while the genes down-regulated in AOX or mtCAT flies were never down-regulated in Sod2 flies (Figure 4.11). This observation pertains to the fact that an extremely specific transcriptional programme is associated with the levels of mtH_2O_2. Moreover, reducing O_2^- and reducing H_2O_2 in two different fly models produced distinctly different transcriptional profiles. These results clearly demonstrate that O_2^- and H_2O_2 should not be considered as a single ROS entity for studies related to activation and/or deactivation of signal mechanisms. They should be explored as two distinctly different ROS molecules and should be modulated independent to one another in order to determine more robust mechanisms. Table 4.1 shows the list of all the genes analysed in the qPCR array for AOX, mtCAT and Sod2 flies.

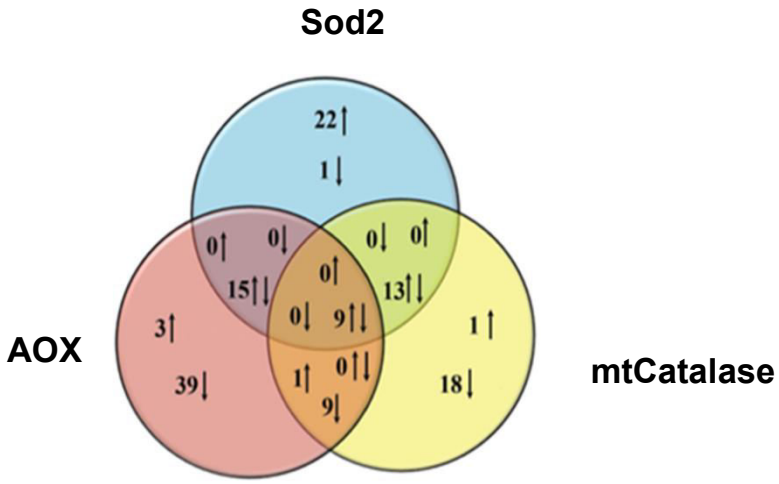

Figure 4.11 Distinctly different trancription profiles are observed with a decrease and increase in mtH_2O_2.

Venn diagram showing number of genes upregulated (↑) and number of genes downregulated (↓) in response to changes in mtH_2O_2 levels. *daGAL4>AOX* (AOX in the figure), *daGAL4>UAS-mtCAT* (mtCatalase in the figure) and *daGAL4>UAS-Sod2* (Sod2 in the figure) flies were used, compared to respective controls. A daughterless GAL4 promoter was used for the expression of transgenes. P values were calculated using student's t-test. $p<0.05$; denotes statistically significant fold change between mentioned genotypes.

Table 4.1 Transcription profile for all genes in conditions of decreased and increased mtH_2O_2.

All Genes in the qPCR array that are downregulated, upregulated or unchanged, when mtH_2O_2 is decreased or increased. *daGAL4>AOX* (AOX in the table), *daGAL4>UAS-mtCAT* (mtCatalase in the table) and *daGAL4>UAS-Sod2* (Sod2 in the table) flies were used, compared to their respective controls. A daughterless GAL4 promoter was used for expression of the transgenes. P values were calculated using student's t-test. $p<0.05$; denotes statistically significant fold change between mentioned genotypes.

Pathways and Genes involved	**AOX**	**mtCatalase**	**Sod2**
Hedgehog Pathway	**Fold Change**	**Fold Change**	**Fold Change**
Atonal (ato) CG 7508	0.81	NC	NC
Interference Hedgehog (iHog) CG 9211	NC	NC	NC
Mirror (mirr) CG10601	0.84	NC	NC
Patched (ptc) CG2411	0.83	NC	NC
Supernumery limb (smlb) CG3412	0.86	0.85	1.34
Smoothened (smo) CG11561	NC	0.81	NC
Wingless (wg) CG4889	0.89	NC	1.38
Hippo Pathway	**Fold Change**	**Fold Change**	**Fold Change**
Branchless (Bnl) CG4608	0.79	NC	NC
Cyclin E (cycE)CG3938	NC	0.85	NC
Diminutive (dm) CG10798	NC	NC	NC
Expanded (ex) CG4114	0.83	NC	NC
Four Jointed (fj) CG10917	0.86	NC	NC
Kibra Ortholog (Kibra) CG33967	NC	NC	NC
Outstretched (os) CG5993	0.88	NC	NC
Spitz (Spi) CG10334	NC	NC	NC
Thread (th) CG12284	NC	0.84	1.33
Unpaired 2 (upd2) CG5988	0.77	NC	NC
Unpaired 3 (upd3) CG33542	NC	NC	NC
Vein (vn) CG10491	NC	NC	NC
Hypoxia	**Fold Change**	**Fold Change**	**Fold Change**
CG10899	0.86	NC	1.33
Glucose Transporter 1 (glut1) CG43946	0.85	NC	NC
Ecdysone-inducible gene L3 (Impl3) CG10160	1.43	NC	NC
Tango (tgo) CG11987	0.85	0.75	1.29
Cysteine string protein (csp) CG6395	NC	NC	1.34
Cyclin G (cycG) CG11525	0.74	NC	1.33
Ornithin Decarboxylase 1 (odc1) CG8721	NC	0.78	NC
Ornithin Decarboxylase 2 (odc2) CG8719	0.81	NC	NC
Reptin (rept) CG9750	0.79	0.84	1.29

JAK/STAT Pathway			
	Fold Change	**Fold Change**	**Fold Change**
Cytochrome P451-4e1 (Cyp4e1) CG2062	NC	NC	NC
G-Protein α subunit (Galpha73b) CG12232	0.88	NC	NC
Grain (grn) CG9656	NC	NC	NC
Heat shock protein 27 (Hsp27) CG4466	0.79	0.87	1.33
Net (net) CG11450	NC	NC	NC
Suppressor of cytokine signaling 36E (socs36e) CG15154	0.81	NC	NC
Notch Pathway			
	Fold Change	**Fold Change**	**Fold Change**
Delta (di_delta) CG3619	0.84	NC	NC
Fringe (fng) CG10580	0.79	NC	NC
Hairy (h) CG6496	0.76	NC	NC
Hairy (hey) CG11194	↓0.84	NC	NC
Notch (n) CG3936	NC	NC	1.40
Oxidative Stress			
	Fold Change	**Fold Change**	**Fold Change**
Glutamate Cysteine Ligase modifier (gclm) CG4919	NC	NC	1.33
Glutathione S transferase D1 (gstd1) CG10045	NC	0.79	1.28
Keap1 (keap1) CG3962	NC	NC	NC
Refractory to sigma 1 (ref(2)p)	0.81	0.85	NC
Thioredoxin-2 (trx-2) CG31884	NC	NC	1.31
p53 Pathway			
	Fold Change	**Fold Change**	**Fold Change**
Cathepsin D (cathD) CG1548	0.87	0.81	1.34
CG8630	NC	NC	NC
Elaongation Factor 1α48D (ef1alpha48d) CG8280	NC	0.88	1.29
Heat shock protein cognate 1 (hsc70-1) CG8937	1.35	NC	NC
Mutagen sensitive 209 (mus209) CG9193	0.81	0.84	1.39
p53 CG33336	NC	0.79	1.40
Tetraspanin 66E (tsp66e) CG4999	0.80	0.80	1.37
PPAR Pathway			
	Fold Change	**Fold Change**	**Fold Change**
CG10924	NC	NC	NC
CG2107	NC	NC	NC
CG3961	NC	NC	NC
Fatty Acid transport protein (fatp) CG7400	NC	NC	NC
Midway (mdy) CG31991	NC	NC	1.34

Phosphoinositide dependent kinase (pk61c) CG1210	NC	0.80	NC
TGF beta Pathway	**Fold Change**	**Fold Change**	**Fold Change**
Brinker (brk) CG9653	NC	NC	NC
Division abnormally delayed (dally) CG4974	NC	NC	NC
Decapentaplegic (dpp) CG9885	NC	NC	NC
Follistatin (fs) CG33466	NC	NC	NC
Ionotrophic receptor 76a (ir76a) CG42584	0.84	NC	NC
Toll/IMD/NfkB Pathway	**Fold Change**	**Fold Change**	**Fold Change**
Attacin-A (atta) CG10146	0.81	NC	NC
Attacin-B (attb) CG18372	NC	NC	NC
Attacin-C (attc) CG4740	NC	NC	NC
Attacin-D (attd) CG7629	NC	NC	NC
Cactus (cact) CG5848	0.77	0.79	1.41
Defensin (def) CG1385	NC	NC	NC
Drosomycin (drs) CG10810	NC	NC	NC
Metchnikowin (mtk) CG8175	NC	NC	NC
Torso Pathway	**Fold Change**	**Fold Change**	**Fold Change**
Brachyentron (byn) CG7260	0.81	NC	NC
Fork head (fkh) CG10002	0.79	NC	NC
Hunchback (hb) CG9786	0.84	NC	1.33
Huckebein (hkb) CG9768	0.77	NC	1.37
Tailless (tll) CG1378	0.80	NC	1.67
Wnt Pathway	**Fold Change**	**Fold Change**	**Fold Change**
Arrow (arr) CG5912	0.84	NC	NC
Axin (axn) CG7926	NC	NC	NC
CyclinD (cycD) CG9096	0.77	0.74	1.38
Frizzled (fz) CG17697	NC	NC	NC
Nemo (nm) CG7892	0.76	NC	NC
Pangolin (pan) CG34403	NC	0.84	NC
Ultrabithorax (ubx) CG10388	0.84	NC	NC
Wnt Oncogene Analog 4 (wnt4) CG4698	0.84	NC	NC
Wnt Oncogene Analog 5 (wnt5) CG6407	NC	NC	NC

4.10 Survival of flies with low levels of mtH_2O_2 is compromised; whereas, increasing mtH_2O_2 improves fly lifespan

In this chapter I have demonstrated that several pathways are altered due to the loss of mtH_2O_2 signal. I wanted to verify if these alterations affect the survival of fruit flies. Interestingly, when survival analyses were performed, mtCAT expressing flies lived significantly shorter under a mild thermal stress (Figure 4.12). It can be hypothesized that, a balance in mtH_2O_2 production is necessary. When this balance is lost by reducing mtH_2O_2, signalling cascades like mitochondrial turnover and/or protein turnover are disrupted, reducing fly survival. On the other hand, when mtH_2O_2 was increased by overexpressing Sod2, fly survival was significantly increased (Figure 4.12).

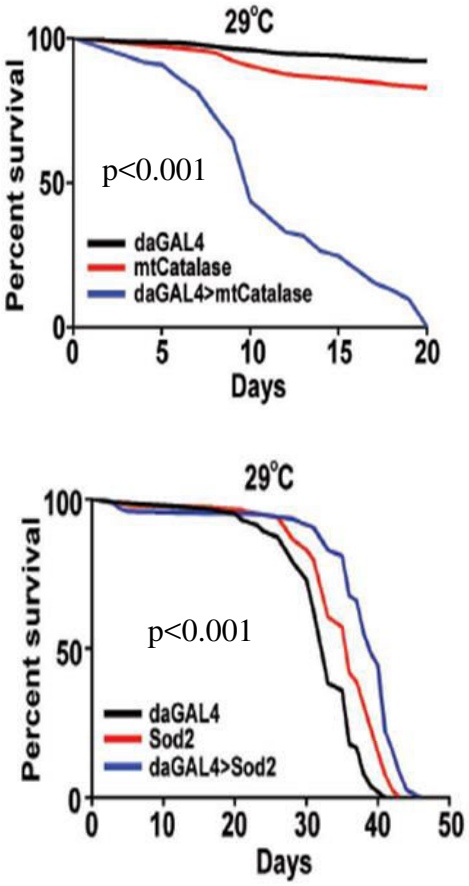

Figure 4.12 Decreasing mtH_2O_2 (mtCAT flies) reduces fly lifespan; whereas, increasing mtH_2O_2 (Sod2 flies) increases fly lifespan.

Survival curves of indicated genotypes (n=200). A table with statistics for the lifespan is presented in Appendix A.

4.11 mtH_2O_2 is necessary to modulate a hyperoxic and hypoxic stress response signal

To discern if the observed decrease in survival was specific to thermal stress, I exposed the AOX, mtCAT and Sod2 flies to hypoxia (5% Oxygen) and hyperoxia (50% Oxygen). Interestingly, I found that H_2O_2 signal is necessary in order to adapt to changes in oxygen concentration, as the flies with decreased levels of H_2O_2 (AOX and mtCAT flies) lived shorter in both conditions (Figure 4.13). However, when the H_2O_2 levels were increased (Sod2 flies), there was a modest increase in lifespan in both conditions (Figure 4.13). It can also be argued that the decrease in lifespan of AOX flies in hypoxic conditions is caused by its interaction with the ETC. As AOX reduces the ROS produced by CIII, it has been demonstrated that this ROS plays a major role in hypoxic adaptation. However, I observed a decrease in lifespan of AOX flies in hyperoxic conditions, which demonstrates the role of mtH_2O_2 signal for adaptation under oxygen stress. Furthermore, I also detected a decrease and increase in lifespan in mtCAT and Sod2 flies, respectively, in both conditions. In summary, it can be ascertained that mtH_2O_2 signal is necessary to orchestrate a response to changes in oxygen concentration. Further experiments are necessary in order to elaborate this signalling cascade.

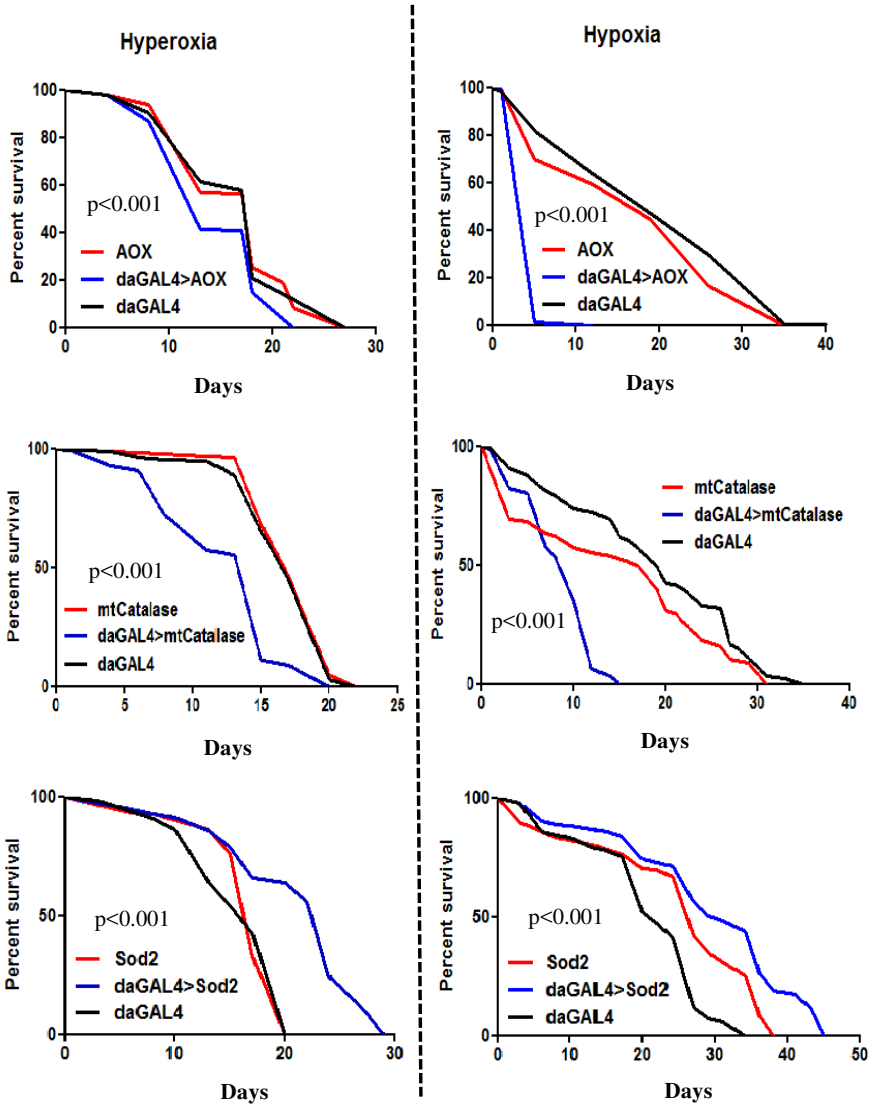

Figure 4.13 mtH$_2$O$_2$ is necessary for adaptation under oxygen stress.

Survival curves of indicated genotype in 50% Oxygen (Hyperoxia) (n=200) and survival curves of indicated genotype in 5% Oxygen (Hypoxia) (n=200). A table with statistics for the lifespan is presented in Appendix A.

Chapter 5. mtH_2O_2 regulates TOR signalling

5.1 A brief overview of signalling mechanisms involved in aging

An appropriate signalling following a stress response is necessary to activate pathways that detect and respond to stress. These pathways (e.g. nutrient sensing or energy sensing pathways) are responsible to make adjustments in case of energy demands or nutrient requirements (Jewell and Guan, 2013), including the necessary modifications to mitochondrial function. It has been previously hypothesized that de-regulation of the nutrient sensing pathways or a reduction in mitochondrial function is due to the damage caused by the excessive ROS produced, and was considered a hallmark of ageing (Harman, 1972). This hypothesis however was challenged in the following years. Moreover, in recent years several studies have demonstrated that ROS can be a consequence of ageing rather than a cause (Copeland *et al.*, 2009).

One of the major pathways responsible for these alterations is the TOR pathway (discussed in detail in Chapter 1.22). It has been shown that pharmacological inhibition of TOR Complex I by rapamycin increases autophagy. This preserves cellular homeostasis by improving the recycling of nutrients and damaged proteins (Bjedov *et al.*, 2010). Even though there are several downstream targets of TOR, increase in autophagy has been the most prominent one studied, related to lifespan extension. On the other hand, mild down-regulation of ETC genes has also proven to be beneficial as it causes a mild mitochondrial dysfunction. It then interferes with an array of pathways like, activation of mitochondrial UPR (Unfolded protein response) or repression of insulin signalling that extends lifespan (Copeland *et al.*, 2009 ; Owushu-Ansah *et al.*, 2013). Both phenomena seem related and there have been several reports showing that TOR can directly regulate mitochondrial function (Schieke and Finkel, 2006). However, it is unclear if TOR stimulates or represses mitochondrial function.

In this chapter, I demonstrate how ROS, or more specifically mtH_2O_2, affects TOR signalling, since this interaction has not been demonstrated earlier. I hypothesized that there might be alterations in the TOR pathway, as I observed significant changes in the transcription profile of flies with low mtH_2O_2. Moreover, protein and organelle turnover, two major mechanisms controlled by TOR, were also impaired in these conditions. To further confirm this hypothesis, I used AOX flies cultured in a mild heat stress; 29^{o}C, which reduced both mtH_2O_2 levels and mitochondrial turnover. I also inhibited TOR by feeding rapamycin to the flies to analyse the impact of TOR inhibition on downstream signalling pathways. Figure 5.1 is a schematic illustration of the TOR signalling pathway.

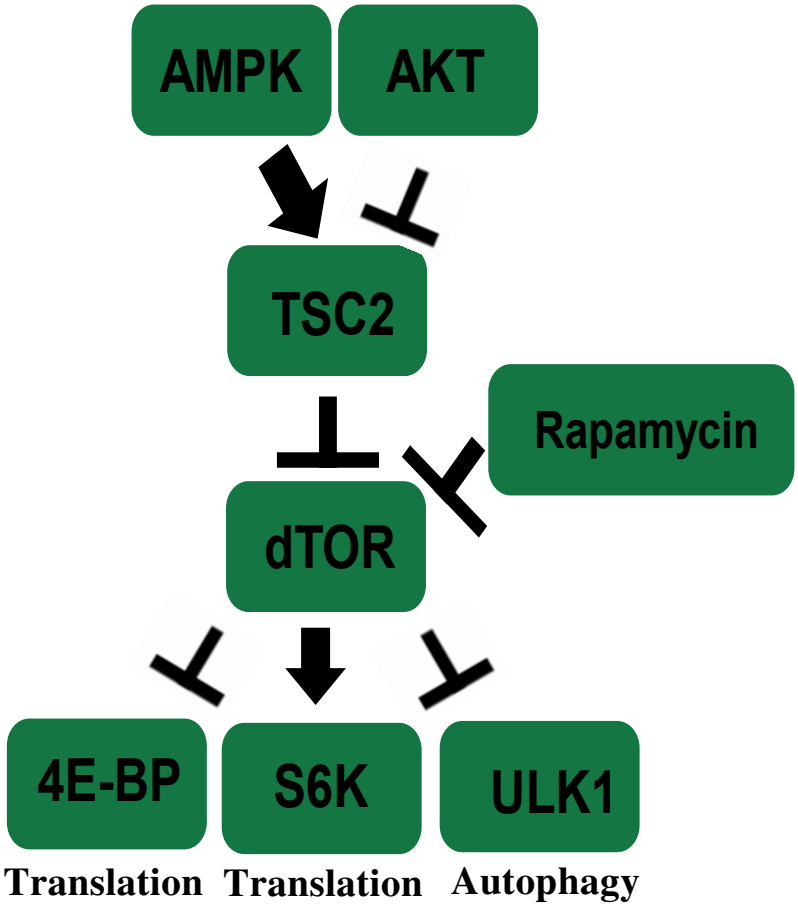

Figure 5.1 Schematic diagram illustrating in brief the upstream and downstream elements of TOR signalling.

TOR integrates several signalling cascades. It is one the major pathways that controls homeostasis of an organism by mediating cellular nutrient and energy sensing.

5.2 Lysosomal number is decreased when the mtH_2O_2 signal is disrupted

I have established in Chapter 3 and 4, that mtH_2O_2 signal is required to initiate mitochondrial turnover by acting as a signal to flag damaged mitochondria. Since mitochondrial turnover was disrupted in flies with low mtH_2O_2 (AOX and mtCAT); and was increased in flies with high mtH_2O_2 (Sod2), I wanted to understand whether general autophagy or macro-autophagy was altered by a mtH_2O_2 signal. First, I stained whole fly brains with Lysotracker Red, a fluorescent dye that labels acidic organelles. It is used as a general indicator for the amount of lysosomes. I observed a significant decrease in the fluorescence by Lysotracker Red in AOX flies and mtCAT flies, but not in Sod2 flies (Figure 5.2 A and B). I also previously observed an accumulation of p62 (Ref (2) P in flies) and decreased proteasome activity in the AOX and mtCAT flies (described in Chapter 3.8 and 4.8). Altogether these results indicate dysfunctional autophagy (Korolchuk *et al.*, 2010; Glick *et al.*, 2010); whereas, none of these changes were observed in Sod2 flies. This supports the hypothesis that mtH_2O_2 signal is involved in maintaining a proper autophagic flux.

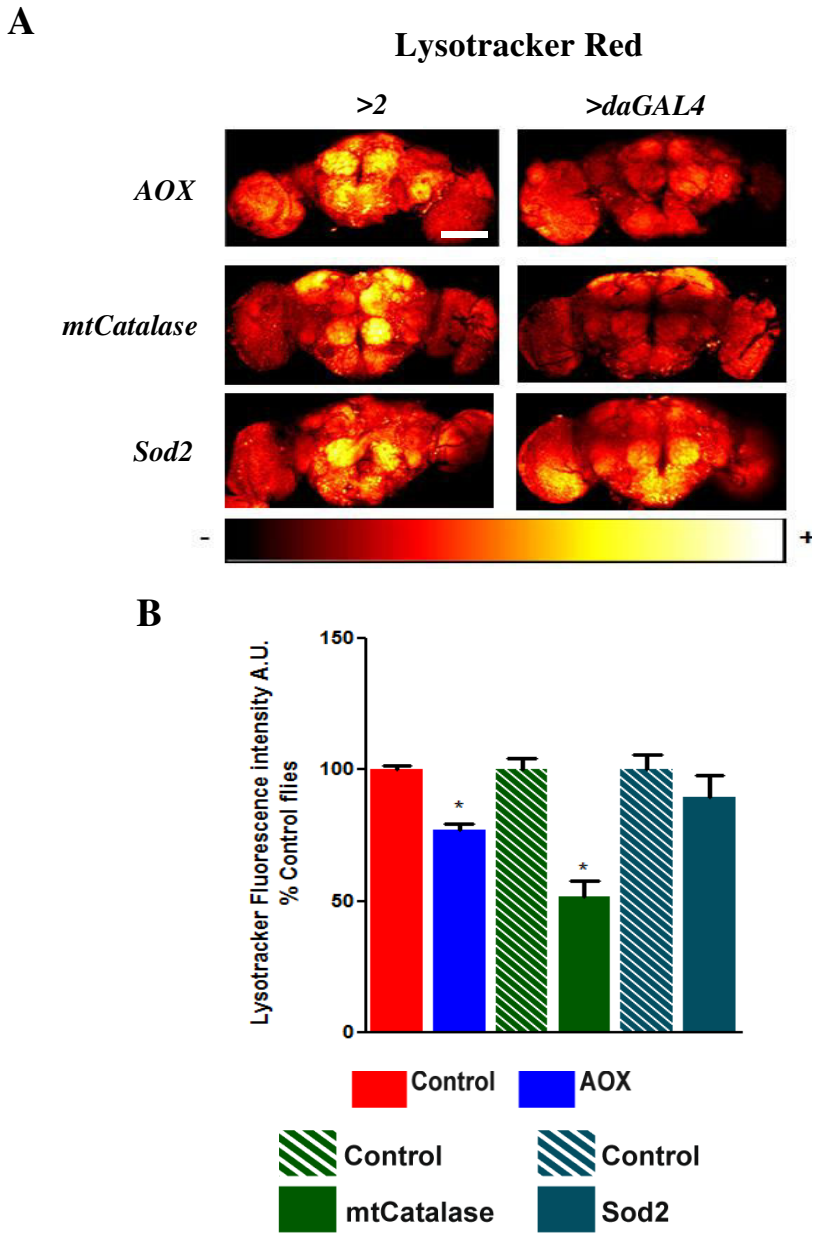

Figure 5.2 Lysosomal number is decreased when the mtH2O2 signal is disrupted.

(A) Representative images of dissected fly brains stained with Lysotracker Red of indicated genotypes. **(B)** Quantification of **(A)** (n=4). Non-expressing flies (*2>UAS-AOX*) (Control in the figure), *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure), non-expressing flies (*2>UAS-mtCAT*) (Control in the figure), *UAS-mtCAT* expressing flies (*daGAL4>UAS-mtCAT*) (mtCatalase in the figure), non-expressing flies (*2>UAS-Sod2*) (Control in the figure) and *Sod2* overexpressing flies (*daGAL4>UAS-Sod2*) (Sod2 in the figure) were used. A daughterless GAL4 promoter was used for the expression of transgenes. P values were calculated using student's t-test. Experimental groups were compared to their respective controls. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. Scale bars are 100µm. This work was done in collaboration with Dr. Filippo Scialo.

5.3 TOR signalling is hyper-activated in AOX flies

Autophagy is negatively regulated by TOR through inhibitory phosphorylation of ULK1 (Nazio *et al.*, 2013). As I observed a decrease in autophagy in the previous chapter, I decided to investigate if TOR signalling was altered in AOX flies. I detected a significant increase in phosphorylated state of RPS6-p70-protein kinase (S6K) (proxy for TOR activity), which is a direct phosphorylation target of TOR. The non-phosphorylated state of the protein was unchanged, which denoted that the increase in phosphorylation was not because of the abundance of the protein but because of TOR hyper-activation. This was further confirmed by measuring the phosphorylation state of another downstream target of TOR, Thor (4E-BP) (eukaryotic translation initiation factor 4E binding protein), which was significantly increased as well (Figure 5.3 A and B).

In order to further confirm that the hyper-activation of TOR signalling was caused by AOX expression; I used the "GeneSwitch" inducible expression system to modulate the expression of AOX (described in detail in Chapter 1.9). I observed that activation of TOR signalling (phosphorylation of S6K as a proxy) occurred in parallel to the increase in mitochondrial density. (Figure 3.9 B). I observed that when AOX expression was induced, TOR signalling was hyper-activated, and when AOX expression was halted, TOR signalling was reverted back to normal (Figure 5.4 A and B).

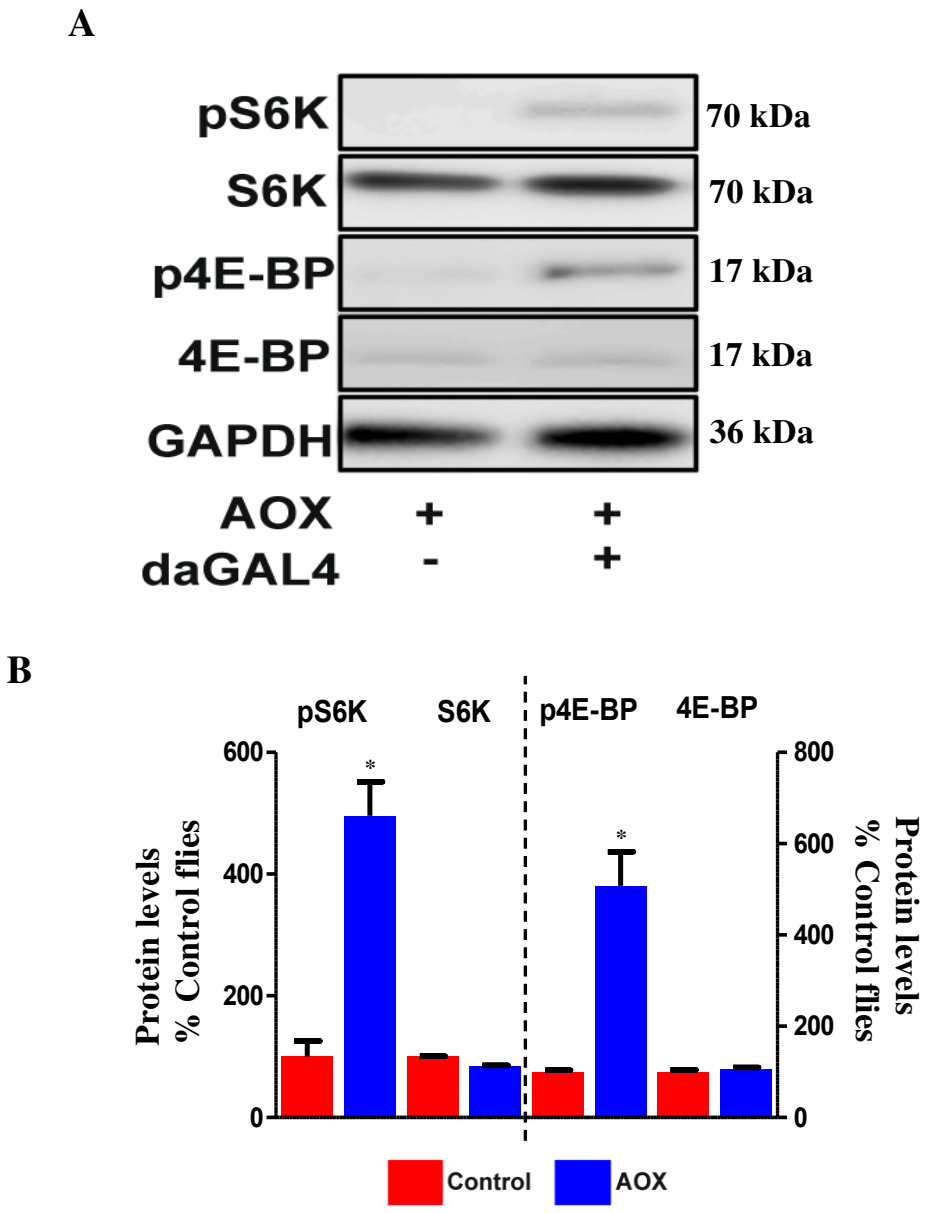

Figure 5.3 TOR is hyper-activated in AOX flies.

(A) Representative western blot membranes showing pS6K, S6K, p4E-BP and 4E-BP protein levels of indicated genotypes. GAPDH is used for normalization and quantification purposes. **(B)** Quantification of **(A)** (n=3). Non-expressing flies (*2>UAS-AOX*) (Control in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using student's t-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. The molecular weight of proteins detected by western blots are given as kiloDalton (kDa) units.

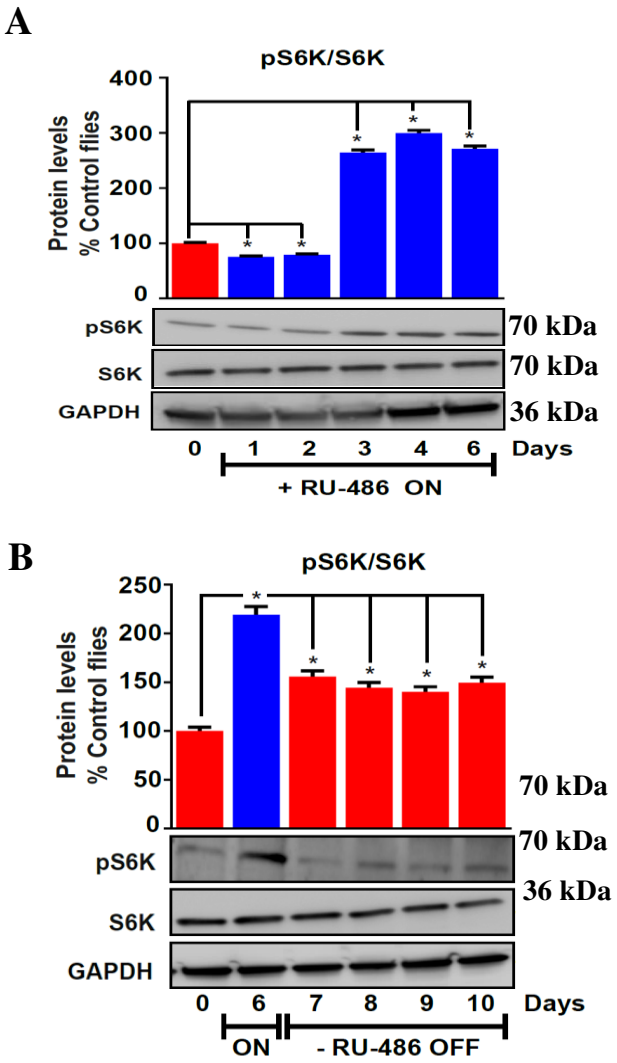

Figure 5.4 TOR is hyper-activated and restored parallel to induction and removal of AOX expression respectively.

(A) Representative western blot membranes and their respective quantification showing pS6K and S6K protein levels in indicated genotypes (n=3). Non-expressing flies (0) and *UAS-AOX* expressing flies (+RU-486 ON) for 1,2,3,4 and 6 days were used. **(B)** Representative western blot membranes and their respective quantification showing pS6K and S6K protein levels in indicated genotypes (n=3). Non-expressing flies (0) and *UAS-AOX* expressing flies un-induced after expression for 5 days earlier (-RU-486 OFF) for 6,7,8,9 and 10 days were used. GAPDH is used for normalization and quantification purposes. An inducible Tubulin GeneSwitch GAL4 promoter was used for the expression of the transgene. P values were calculated using 1-way ANOVA with Newman-Keul's post-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. The art lines in the figure denote the statistically significant difference between the marked genotypes. The molecular weight of proteins detected by western blots are given as kiloDalton (kDa) units.

5.4 Insulin and insulin-like growth factor signalling is modified in AOX flies

In order to investigate whether the upstream regulators of TOR were altered when the mtH_2O_2 levels were decreased, I used AOX flies to check the levels of several proteins that are positive and negative regulators of TOR. Firstly, I measured the phosphorylated protein levels of Akt1 (AKT). Akt1 is a downstream target of Insulin/insulin-like growth factor signalling (IIS) and Chico (insulin receptor (INR)) in flies, which are key regulators of energy metabolism and growth (Clancy *et al.*, 2001). The phosphorylated state of AKT is a positive regulator of TOR. In order to prevent over activation of TOR, the phosphorylation of AKT is also regulated through a negative feedback loop by a downstream target of TOR; S6K1 (Wan *et al.*, 2007). Interestingly, I observed a reduction in the phosphorylation of AKT in AOX flies, suggesting that the negative feedback loop is active (Figure 5.5 A and B). To further confirm this activation, I measured protein levels of forkhead box, sub group O (FOXO), which is a direct target of AKT. In accordance to the activation state of AKT, I detected a decrease and increase in the phosphorylated and non-phosphorylated form of FOXO respectively, indicating its activation and translocation to the nucleus (Figure 5.5 A and C) (Tzivion *et al.*, 2011). These results confirmed that the decrease in the phosphorylation state of AKT is not an artefact and the feedback loop is active.

Additionally, as FOXO was activated in AOX flies, I analysed the levels of one of its transcription targets; Sestrin, which is responsible for the activation of AMP-activated protein kinase α subunit (AMPKα) (Lee *et al.*, 2010). I detected an increase in protein levels of Sestrin that correlated with an increase in the presence of phosphorylated-AMPK, which is responsible for deactivation of TOR (described in detail in Chapter 1.22) (Figure 5.6 A,B and C) (Serfontein *et al.*, 2010). These data indicate that the upstream negative regulators of TOR were active, however, were not sufficient to supress TOR in conditions of low mtH_2O_2.

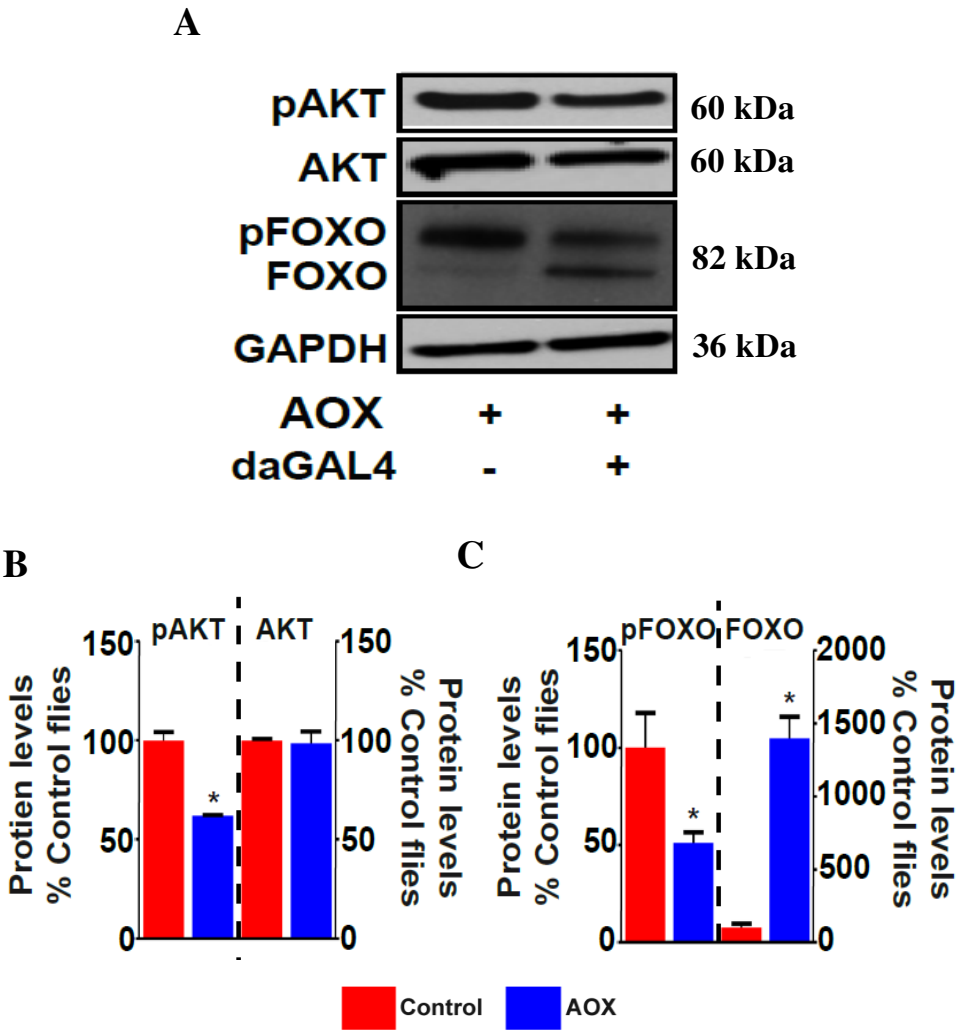

Figure 5.5 Insulin and insulin-like growth factor signalling (IIS) are downregulated in AOX flies.

(A) Representative western blot membranes showing pAKT, AKT, pFOXO and FOXO protein levels of indicated genotypes. GAPDH is used for normalization and quantification purposes. **(B)** Quantification of pAKT and AKT from **(A)** (n=3). **(C)** Quantification of pFOXO and FOXO from **(A)** (n=3). Non-expressing flies (*2>UAS-AOX*) (Control in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using student's t-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. The molecular weight of proteins detected by western blots are given as kiloDalton (kDa) units.

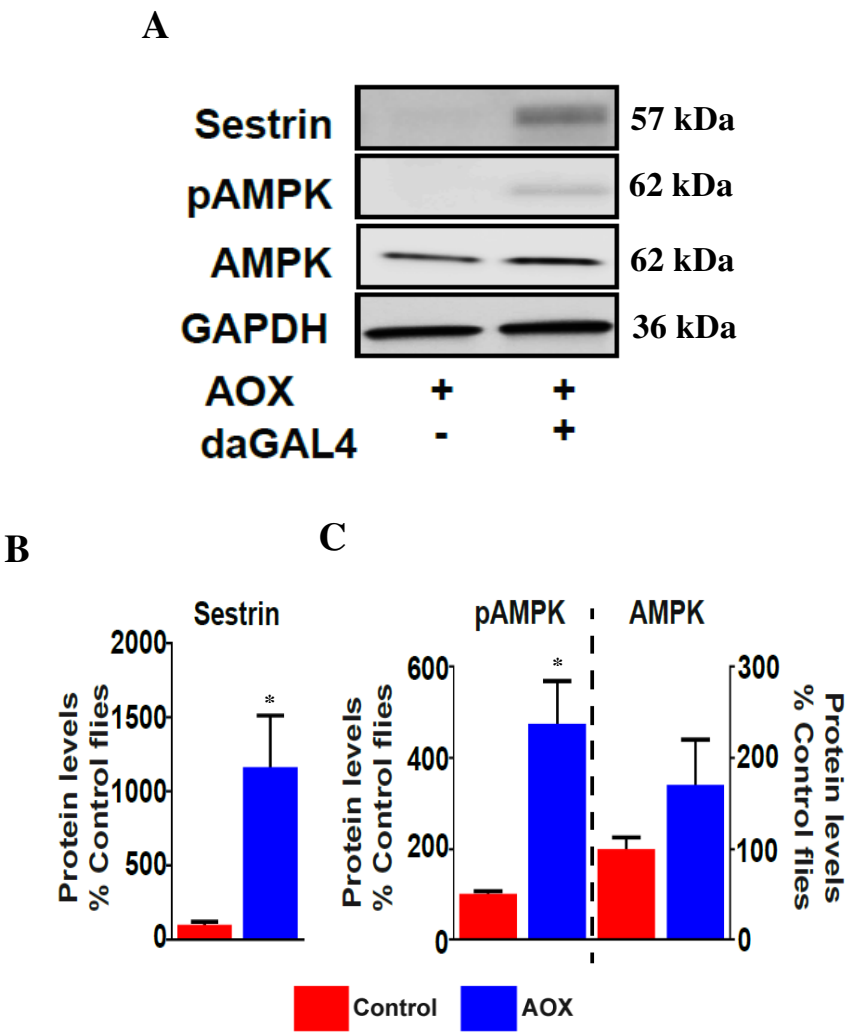

Figure 5.6 The feedback loop necessary to inhibit TOR hyper-activation is activated in AOX flies.

(A) Representative western blot membranes showing Sestrin, pAMPK and AMPK protein levels of indicated genotypes. GAPDH is used for normalization and quantification purposes. **(B)** Quantification of Sestrin from (**A**) (n=3). **(C)** Quantification of pAMPK and AMPK from (**A**) (n=3). Non-expressing flies (*2>UAS-AOX*) (Control in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using student's t-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. The molecular weight of proteins detected by western blots are given as kiloDalton (kDa) units.

5.5 Decrease in lysosomal content in AOX flies is rescued by pharmacological inhibition of TOR.

I have observed in Chapter 5.3 that TOR was hyper-activated in AOX flies and it was linked to reduced autophagy, reduced mitochondrial turnover and severely shortened lifespan (Chapter 5.2, 3.5 and 3.9). I then wanted to investigate whether the pharmacological inhibition of TOR by rapamycin (described in detail in Chapter 1.26) would reverse or at least partially rescue the phenotype conferred by AOX expression.

I was able to successfully inhibit TOR signalling in AOX flies by feeding them with rapamycin, as verified by the decrease in phosphorylation of S6K (Figure 5.7 A and B). This clearly shows that the rapamycin doses used for the inhibition of TOR was effective. I then wanted to analyse if the rapamycin treatment improved lysosomal number in AOX flies. Lysotracker staining demonstrated that rapamycin treatment restored lysosomal content in AOX flies (Figure 5.8 A and B). I reconfirmed these results by checking the protein levels of Lamp1 which was also restored upon rapamycin treatment (Figure 5.8 C and D).

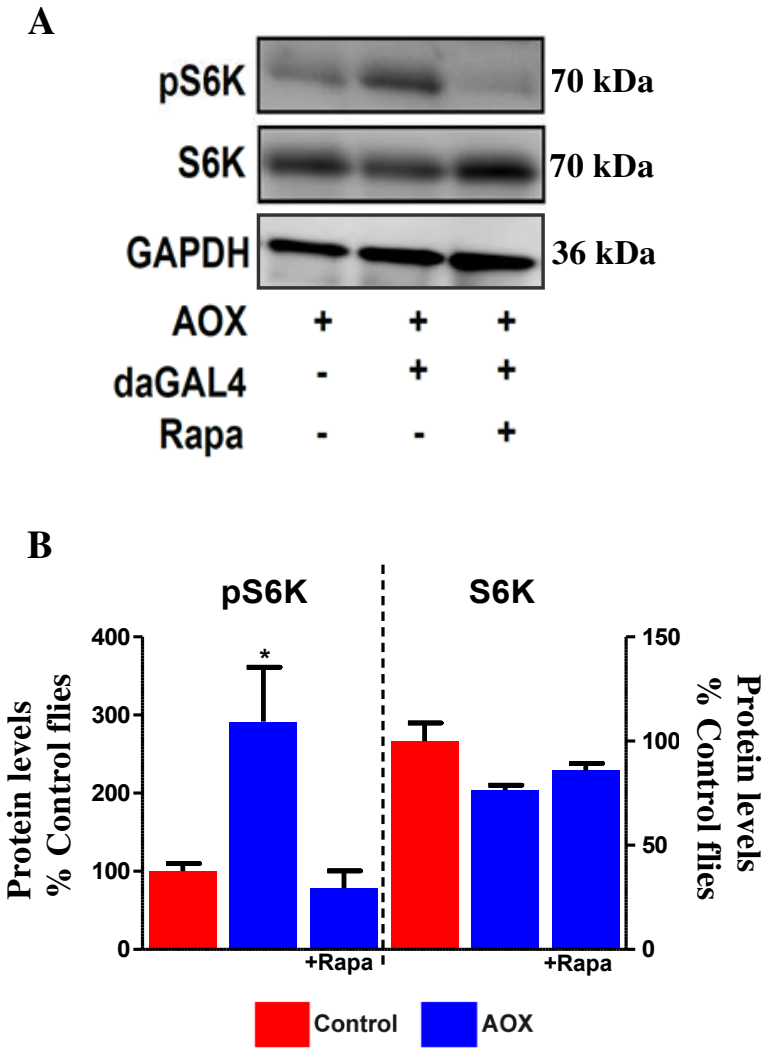

Figure 5.7 TOR signalling is restored when AOX flies are fed with rapamycin.

(A) Representative western blot membranes showing pS6K and S6K protein levels of indicated genotypes. GAPDH is used for normalization and quantification purposes. **(B)** Quantification of (**A**) (n=3). Non-expressing flies (*2>UAS-AOX*) (Control in the figure), *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) fed with rapamycin (AOX + Rapa in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using 1-way ANOVA with Newman-Keul's post-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. The molecular weight of proteins detected by western blots are given as kiloDalton (kDa) units.

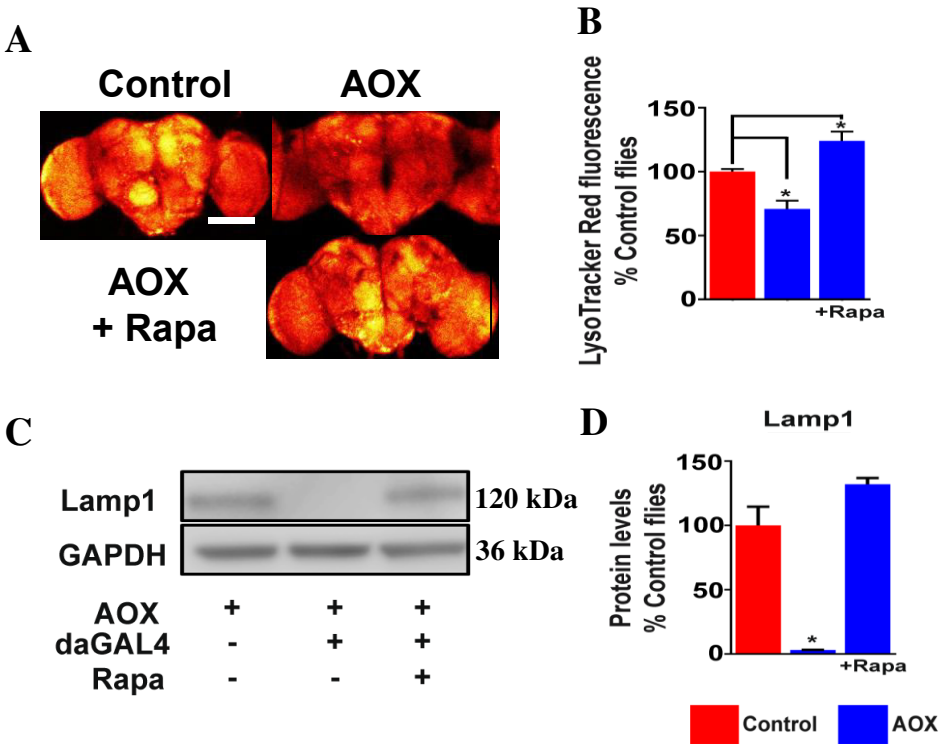

Figure 5.8 Lysosomal content in AOX flies is rescued when the flies are fed with rapamycin.

(A) Representative images of dissected fly brains stained with Lysotracker Red of indicated genotypes. **(B)** Quantification of **(A)** (n=4). This work was done in collaboration with Dr. Filippo Scialo. **(C)** Representative western blot membrane showing Lamp1 protein levels of indicated genotypes. GAPDH is used for normalization and quantification purposes. **(D)** Quantification of **(C)** (n=3). Non-expressing flies (*2>UAS-AOX*) (Control in the figure), *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) fed with rapamycin (AOX + Rapa in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using 1-way ANOVA with Newman-Keul's post-test or using student t-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. The art lines in the figure denote the statistically significant difference between the marked genotypes. Scale bars are 100µm. The molecular weight of proteins detected by western blots are given as kiloDalton (kDa) units.

5.6 Mitochondrial accumulation is rescued by rapamycin feeding in AOX flies

We have previously demonstrated that a mtH_2O_2 signal is necessary; in order to initiate turnover of damaged mitochondria (Chapter 3 and Chapter 4). However, I hypothesized that when the mtH_2O_2 signal responsible to flag damaged mitochondria or to stabilize Pink1 is absent; improving autophagy may not be necessarily advantageous. This may be because the autophagy machinery is not able to successfully detect damaged mitochondria, specifically for sequestering them to the autophagosome. This phenomenon might lead to improper recycling of mitochondria by the autophagic machinery, by using alternate mitochondrial markers other than Pink1, which do not flag damaged mitochondria.

I then checked if the increase in mitochondrial density in AOX flies was rescued by rapamycin treatment. Rapamycin treatment, as expected, reduced the mitochondrial density in AOX flies (Figure 5.9).

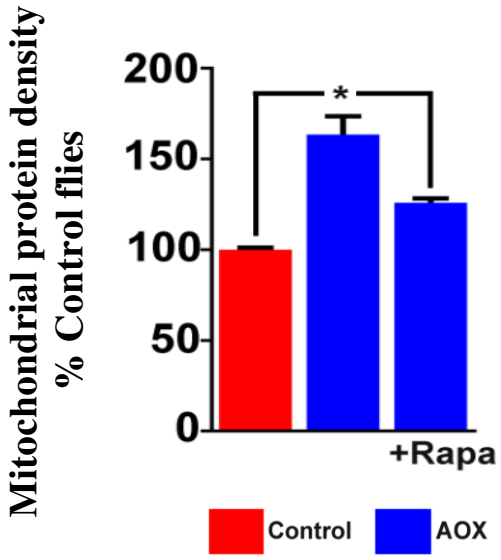

Figure 5.9 Mitochondrial accumulation is rescued in AOX flies fed with rapamycin.
Citrate synthase activity measuring mitochondrial density of indicated genotypes (Control = 1.159±0.013 Absorbance Units mg protein^{-1}) (n=9). Non-expressing flies (*2>UAS-AOX*) (Control in the figure), *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) fed with rapamycin (AOX + Rapa in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using 1-way ANOVA with Newman-Keul's post-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. The art lines in the figure denote the statistically significant difference between the marked genotypes.

5.7 Mitochondrial dysfunction is not rescued by rapamycin feeding in AOX flies

Interestingly, reduction in mitochondrial respiration was not rescued by rapamycin feeding in AOX flies. As described earlier (Chapter 3.6), I did not see any difference between AOX flies and controls when respiration rates were normalized to total protein content; however, AOX flies fed with rapamycin had a modest but significant decrease in respiration (Figure 5.10 A). Additionally, even though AOX flies fed with rapamycin, rescued mitochondrial accumulation, the respiration rates per mitochondrion were not increased as expected, when normalized to mitochondrial density (Figure 5.10 B). This confirms the hypothesis that a mtH_2O_2 signal is necessary to flag damaged mitochondria for recycling.

To reconfirm that mitochondrial dysfunction was persistent after rapamycin feeding; I measured mRNA levels of *Hsp22* and *Hsp60* whose levels are increased when mitochondria are damaged (Chapter 3.6). I detected a significant increase in mRNA levels of both *Hsp's* in the rapamycin fed AOX flies, surprisingly, even more than AOX flies without rapamycin feeding. I detected a similar increase with mRNA levels of *Impl3* as well, indicating that the flies are switching to a more glycolytic metabolism, due to the severely dysfunctional mitochondria (Figure 5.11). In summary, these results support the hypothesis that mitochondrial recycling upon rapamycin feeding is not specific to damaged mitochondria. All or most of the damaged mitochondria are hence not recycled exacerbating the phenotype of the fly. These results also indicate that hyper-activation of TOR in AOX flies can be a consequence and not the cause of accumulation of damaged mitochondria. It can be further hypothesized that the accumulation of mitochondria or the increase in mitochondrial number is interpreted as a signal to grow by the cell; thus consequently activating TOR to increase cell size and protein synthesis. Further investigation however is required in order to dissect and demonstrate this hypothesis.

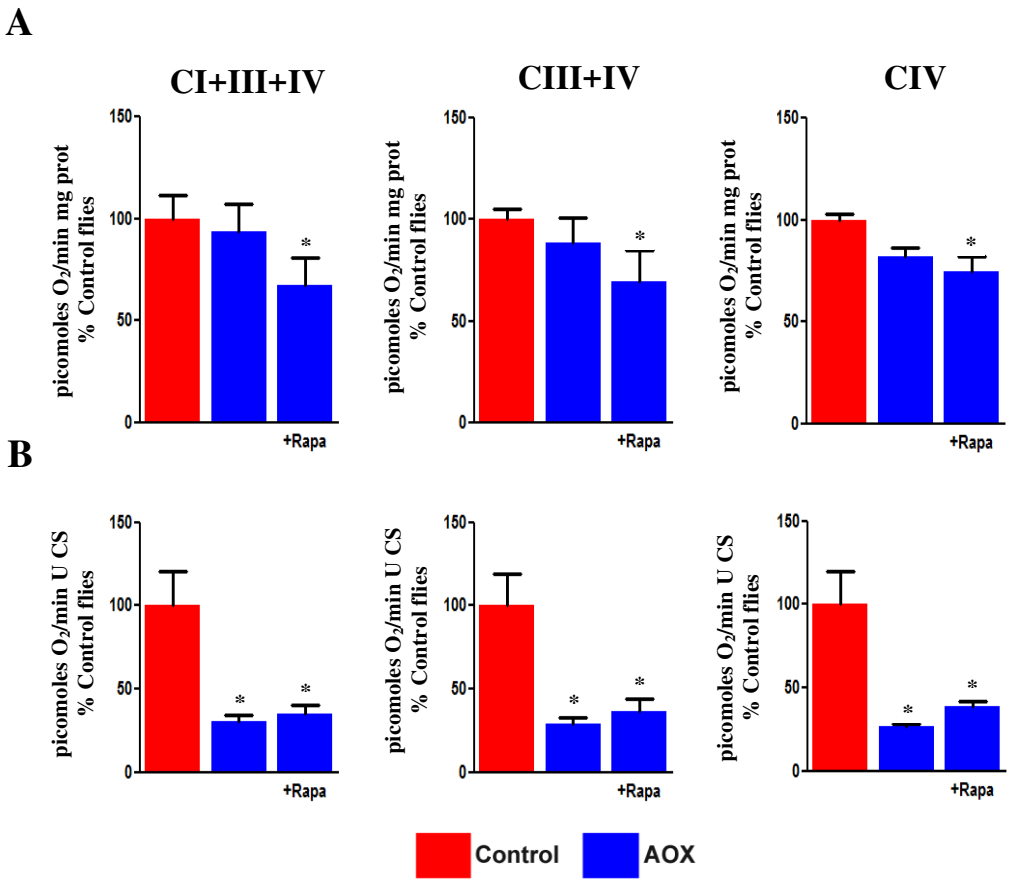

Figure 5.10 Mitochondrial respiration is not rescued in AOX flies fed with rapamycin.

(A) Comparison of oxygen consumption of indicated genotypes (n=3). CI-linked (Control = 544±25.98 picomoles O_2 min^{-1} mg $protein^{-1}$), CIII-linked (Control = 328.58±35.98 picomoles O_2 min^{-1} mg $protein^{-1}$) and CIV-linked (Control = 1243±40 picomoles O_2 min^{-1} mg $protein^{-1}$) oxygen consumption measured. **(B)** Representing respiration data normalized to respiration per mitochondrion of indicated genotypes (n=3). CI-linked, CIII-linked and CIV-linked oxygen consumption measured. Non-expressing flies (*2>UAS-AOX*) (Control in the figure), *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) fed with rapamycin (AOX + Rapa in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using 1-way ANOVA with Newman-Keul's post-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes.

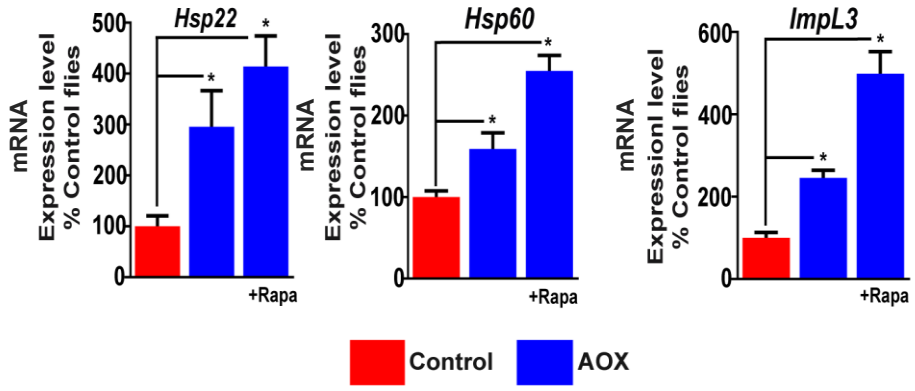

Figure 5.11 Mitochondrial damage is exacerbated in AOX flies fed with rapamycin.

Quantification of mRNA levels of *Hsp22, Hsp60* and *ImpL3* by quantitative PCR of indicated genotypes (n=4). Non-expressing flies (*2>UAS-AOX*) (Control in the figure), *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) (AOX in the figure) and *UAS-AOX* expressing flies (*daGAL4>UAS-AOX*) fed with rapamycin (AOX + Rapa in the figure) were used. A daughterless GAL4 promoter was used for the expression of the transgene. P values were calculated using 1-way ANOVA with Newman-Keul's post-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. The art lines in the figure denote the statistical significance between all three genotypes.

5.8 Survival of AOX flies is not rescued by rapamycin feeding

Rapamycin has been shown to increase lifespan in several model organisms like yeast, worms, flies and mice. A dose dependent increase in lifespan has also been demonstrated in many model organisms (Bjedov *et al.*, 2010). As I previously detected a hyper-activation of TOR and a severely compromised lifespan in AOX flies, I wanted to investigate if feeding rapamycin to AOX flies might improve their survival.

To begin with, I fed AOX flies with rapamycin every day and analysed their survival. Interestingly, AOX flies fed with rapamycin had an even shorter lifespan than AOX flies without the feeding (Figure 5.12 A). This can be explained by the fact that mitochondrial damaged was exacerbated in AOX flies fed with rapamycin when compared to AOX flies feeding on normal food (Figure 5.10 and 5.11). I then hypothesized that the continuous feeding of rapamycin can cause defects in translation by inhibiting TOR Complex II, leading to a severe reduction in lifespan. This can be an additional burden for AOX flies, thereby reducing their lifespan even more drastically. To test the hypothesis, I fed AOX flies with rapamycin for only 2 days a week. Transient feeding did not reduce the lifespan of AOX flies, but neither did it rescue their lifespan (Figure 5.12 B). In summary, these results demonstrate that a mtH_2O_2 signal might be necessary in order to keep TOR signalling in check and maintain homeostasis.

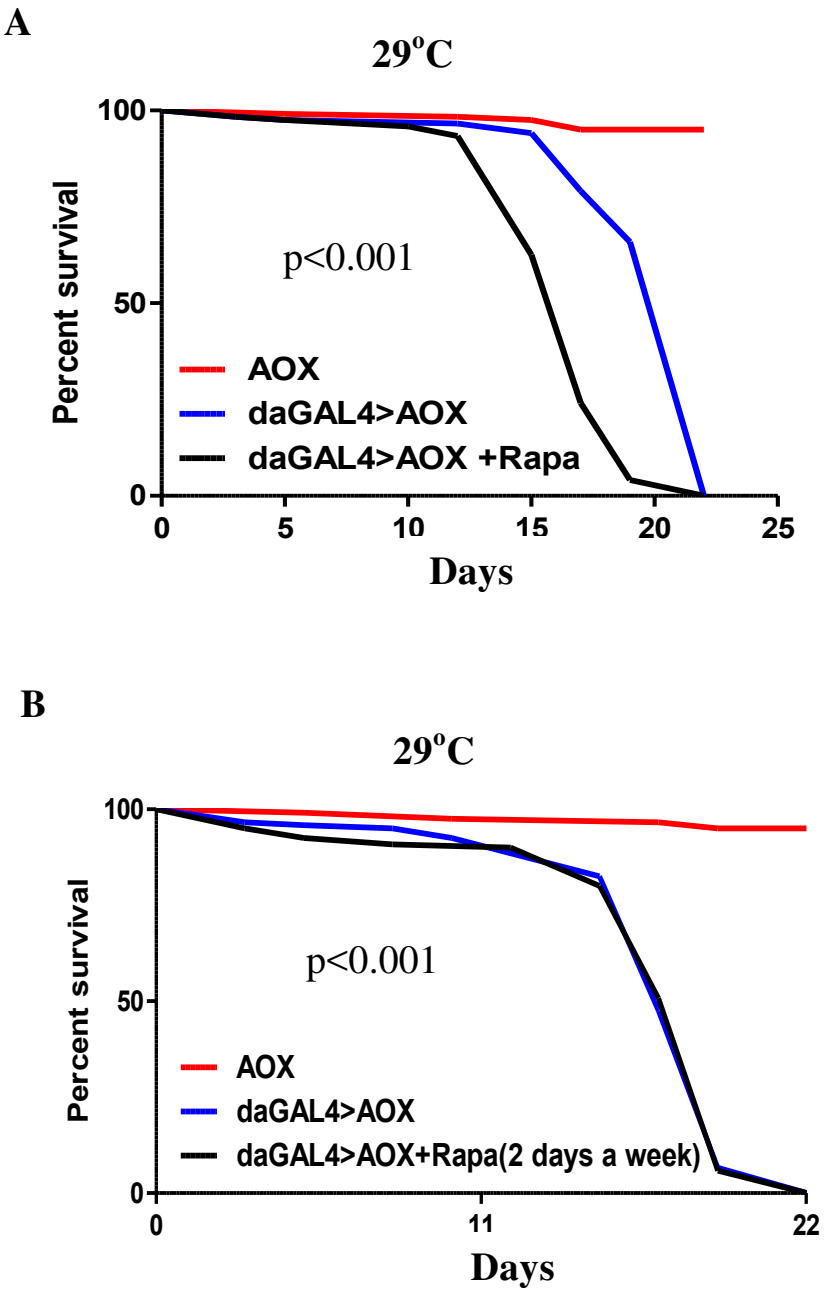

Figure 5.12 Continuous feeding of rapamycin shortened the lifespan of AOX flies; whereas, lifespan was unaltered upon transient feeding.

Survival curves of indicated genotype. (n=200). A table with statistics for the lifespan is presented in Appendix A.

Chapter 6. Restoring mitophagy rescues the physiological phenotype triggered by low mtH_2O_2 levels

6.1 Overview of canonical Pink1-Parkin mediated mitophagy

Canonical mitophagy is mediated by the PINK1-PARKIN axis, but how Pink1 is activated upon mitochondrial damage and Parkin is recruited by Pink1 is still a highly debated topic. It has been suggested that PINK1 needs to auto-phosphorylate itself upon mitochondrial depolarization in order to recruit PARKIN. However, there have also been some reports supporting the hypothesis that PARKIN is responsible for the activation of PINK1 (Okatusu *et al.*, 2012;Narendra *et al.*, 2008). On the other hand, numerous other studies in flies and worms have also demonstrated that Parkin overexpression rescues Pink1 mutant phenotype, denoting the fact that Pink1 can also be completely dispensable for mitochondrial turnover (Clark *et al.*, 2006).

I have demonstrated in Chapter 3 and 4, that mitophagy is disrupted when mtH_2O_2 levels are decreased (AOX and mtCAT flies). In this chapter, I restored mitophagy in a low mtH_2O_2 background (AOX flies) to see if restoration of mitophagy can rescue TOR signalling and lifespan of AOX flies. This will help us to differentiate the upstream and downstream elements of this network. Figure 6.1illustrates the canonical PINK1-PARKIN mitophagy pathway.

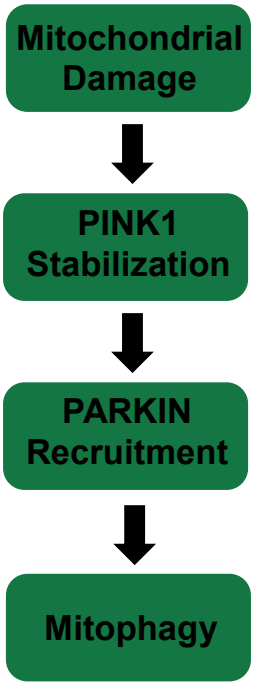

Figure 6.1 Schematic diagram illustrating in brief the canonical PINK1-PARKIN mitophagy pathway.

It has been demonstrated that PINK1 and PARKIN are essential for detection and turnover of damaged mitochondria.

6.2 Verification of overexpression of Parkin and Pink1 in *Drosophila*

Parkin and Pink1 overexpression was achieved by using a *UAS-Parkin* and *UAS-Pink1* constructs and an inducible Tubulin GeneSwitch GAL4 promoter. I verified the overexpression by measuring protein levels of Parkin and Pink1 in the respective overexpressing fly lines. I detected a significant increase in protein levels for both Parkin and Pink1, confirming that the overexpression constructs are functional (Figure 6.2).

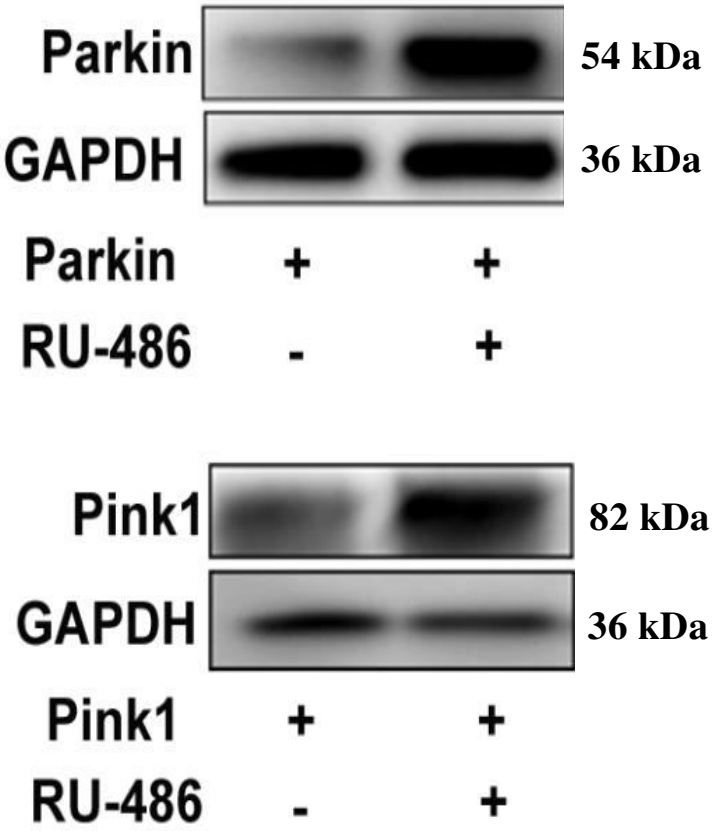

Figure 6.2 Validation of Pink1 and Parkin overexpression.

Top panel showing representative western blot membrane of Parkin protein levels in non-expressing flies (*TubGS>UAS-Parkin*; –RU-486 OFF) (Parkin in the figure) and *Parkin* overexpressing flies (*TubGS>UAS-Parkin*; +RU-486 ON) (Parkin in the figure). Bottom panel showing representative Western blot membrane of Pink1 protein levels in non-expressing flies (*TubGS>UAS-Pink1*; –RU-486 OFF) (Pink1 in the figure) and *Pink1* overexpressing flies (*TubGS>UAS-Pink1*; –RU-486 ON) (Pink1 in the figure) flies. An inducible Tubulin GeneSwitch GAL4 promoter was used for the expression of the transgene. GAPDH was used for normalization purpose. The molecular weight of proteins detected by western blots are given as kiloDalton (kDa) units.

6.3 Parkin, but not Pink1 overexpression restores mitochondrial turnover in AOX flies

Results presented in Chapter 3 and 4 indicated that mitochondrial turnover was regulated by mtH_2O_2, and reduction of mtH_2O_2 compromised stress adaptation and survival. I then sought to understand whether increasing mitochondrial turnover by activating the canonical Pink1-Parkin mitophagy pathway would rescue the AOX phenotype. In agreement with the hypothesis, Parkin overexpression in AOX flies reduced the accumulation of mitochondria (Figure 6.3). Surprisingly, Pink1 overexpression in AOX flies did not rescue the mitochondrial accumulation (Figure 6.3). These results partially validated the hypothesis that the Pink1 protein needs to undergo an oxidative modification to be activated. When the mtH_2O_2 necessary for redox regulation of the protein is absent, overexpression of the protein is not beneficial. Moreover, these results also indicate that overexpression of Parkin is a bypass for this H_2O_2 signal.

To further understand the process of Parkin dependent mitophagy, I stained live fly brains with H_2DCF. Parkin overexpression reduced the ROS levels modestly, but not significantly, in the AOX flies. This might be due to the decrease in damaged mitochondria; however, Pink1 overexpression did not change the ROS levels (Figure 6.4 A and B). This experiment demonstrates that Parkin is located downstream of the ROS signal.

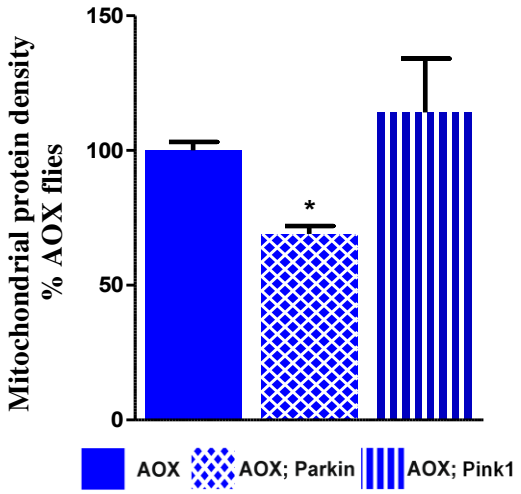

Figure 6.3 Parkin, but not Pink1 overexpression rescues the mitochondrial accumulation caused by AOX expression.

Citrate synthase activity measuring mitochondrial density of indicated genotypes (Control = 0.7783±0.024 Absorbance Units mg protein^{-1}) (n=9). AOX expressing flies (*TubGS>UAS-AOX*; +RU-486 ON) (AOX in the figure), UAS-AOX; *Parkin* overexpressing flies (*TubGS>UAS-AOX; UAS-Parkin*; +RU-486 ON) (AOX; Parkin in the figure) and UAS-AOX; *Pink1* overexpressing flies (*TubGS>UAS-AOX; UAS-Pink1*; +RU-486 ON) (AOX; Pink1 in the figure). An inducible Tubulin GeneSwitch GAL4 promoter was used for the expression of the transgene. P values were calculated using 1-way ANOVA with Newman-Keul's post-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes.

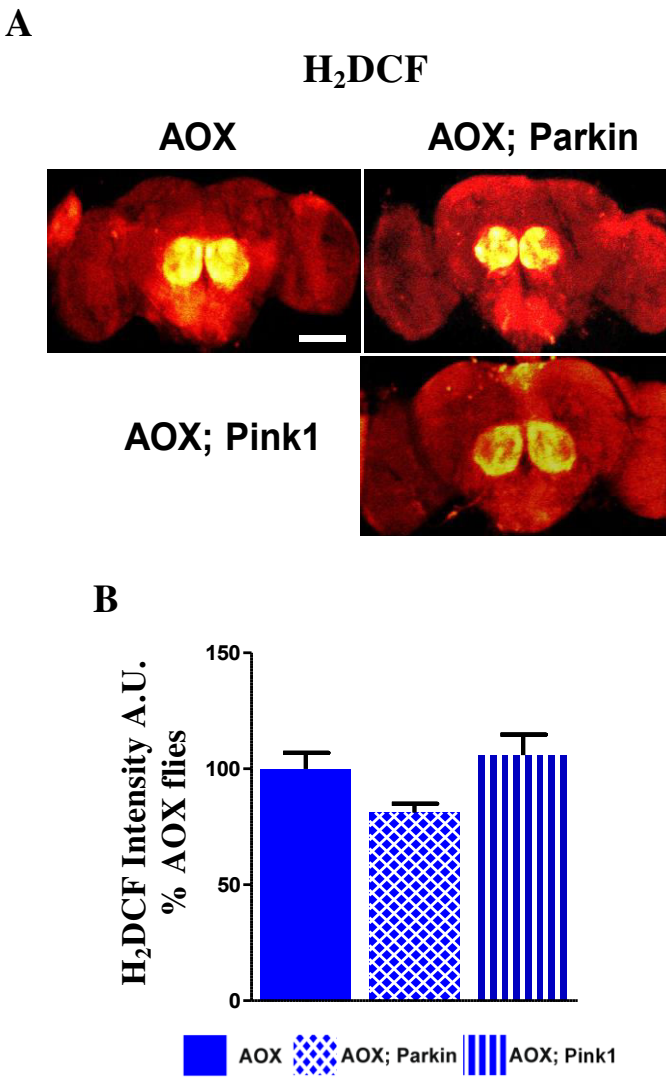

Figure 6.4 Neither Parkin nor Pink1 overexpression alters ROS levels in AOX flies.

(A) Representative images of dissected fly brains stained with H_2DCF of indicated genotypes. **(B)** Quantification of **A** (n=5). AOX expressing flies (*TubGS>UAS-AOX*; +RU-486 ON) (AOX in the figure), UAS-AOX; *Parkin* overexpressing flies (*TubGS>UAS-AOX; UAS-Parkin*; +RU-486 ON) (AOX; Parkin in the figure) and UAS-AOX; *Pink1* overexpressing flies (*TubGS>UAS-AOX; UAS-Pink1*; +RU-486 ON) (AOX; Pink1 in the figure). An inducible Tubulin GeneSwitch GAL4 promoter was used for the expression of the transgene. P values were calculated using 1-way ANOVA with Newman-Keul's post-test. Data are shown as mean ± SEM. This work was done in collaboration with Dr. Filippo Scialo.

6.4 Parkin overexpression improves the mitochondrial functionality of AOX flies

In order to verify the recycling of damaged mitochondria, I checked if the mitochondrial functionality was restored after restoring mitophagy. The respiration normalized to total protein content was unaltered as observed earlier (Figure 6.5 A). However, when normalized to their respective mitochondrial densities, mitochondrial respiration was improved by Parkin overexpression in AOX flies but not by Pink1 overexpression (Figure 6.5 B). Combined, these experiments confirm that Parkin overexpression restores recycling of damaged mitochondria, hence improving its function.

I also detected a modest rescue in mRNA levels of *Hsp22*; which demonstrated that mitochondrial function is restored after restoring mitophagy. However, I did not detect any changes in the *Hsp60* mRNA levels. This can be explained by the fact that induction in *Hsp60* is not as extreme as other *Hsp's* in the AOX flies. Additionally, there was also no rescue in mRNA levels of *Hsp70*, but there was a significant increase in induction when Pink1 was overexpressed along with AOX. The mRNA levels of *ImpL3* were also rescued by Parkin overexpression; whereas, Pink1 overexpression did not rescue the induction (Figure 6.6). This experiment demonstrates that Parkin overexpression, but not Pink1 overexpression, can restore different mitochondrial phenotypes caused by low mtH_2O_2 levels.

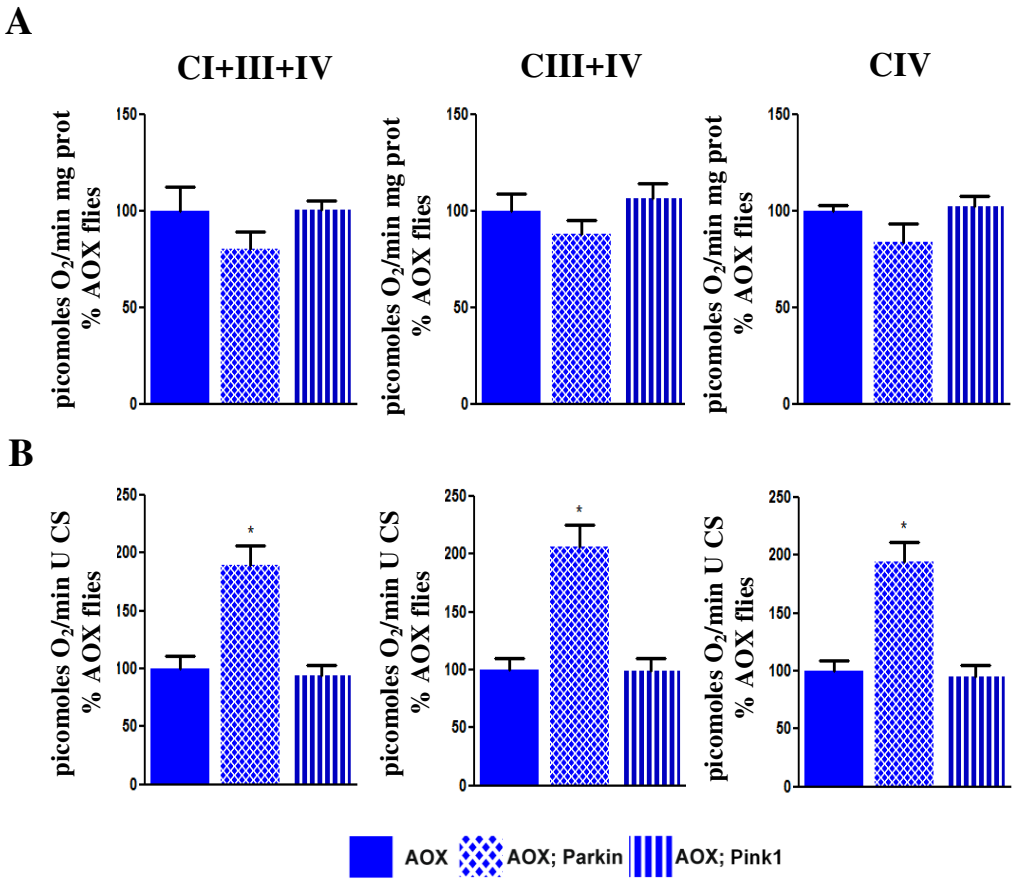

Figure 6.5 Parkin overexpression rescues mitochondrial respiration of AOX flies.

(A) Comparison of oxygen consumption of indicated genotypes (n=4). CI-linked (Control = 1065±133.4 picomoles O_2 min^{-1} mg $protein^{-1}$), CIII-linked (Control = 590.3±52.3 picomoles O_2 min^{-1} mg $protein^{-1}$) and CIV-linked (Control = 3155±86.36 picomoles O_2 min^{-1} mg $protein^{-1}$) oxygen consumption **(B)** CI-linked, CIII-linked and CIV-linked oxygen consumption measurements normalized to respective mitochondrial density (n=4). AOX expressing flies (*TubGS>UAS-AOX*; +RU-486 ON) (AOX in the figure), UAS-AOX; *Parkin* overexpressing flies (*TubGS>UAS-AOX; UAS-Parkin*; +RU-486 ON) (AOX; Parkin in the figure) and UAS-AOX; *Pink1* overexpressing flies (*TubGS>UAS-AOX; UAS-Pink1*; +RU-486 ON) (AOX; Pink1 in the figure). An inducible Tubulin GeneSwitch GAL4 promoter was used for the expression of the transgene. P values were calculated using 1-way ANOVA with Newman-Keul's post-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes.

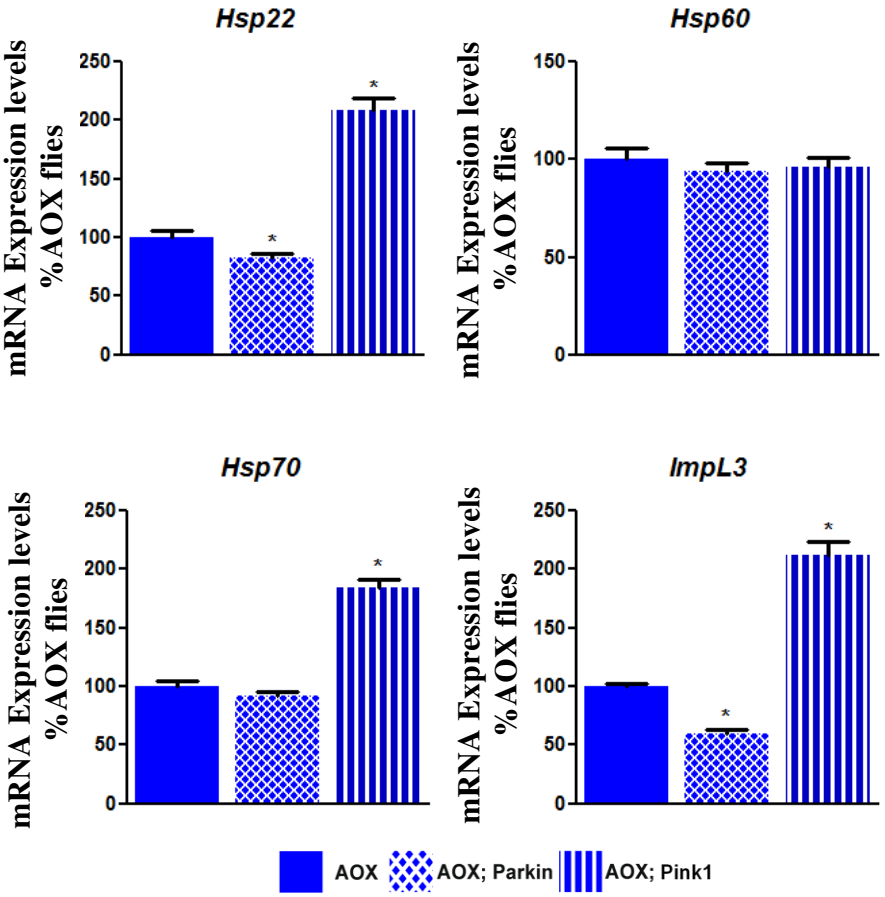

Figure 6.6 Mitochondrial damage is improved by Parkin overexpression in AOX flies.

Quantification of mRNA levels of *Hsp22, Hsp60, Hsp70 and ImpL3* by quantitative PCR in indicated genotypes (n=6). AOX expressing flies (*TubGS>UAS-AOX*; +RU-486 ON) (AOX in the figure), UAS-AOX; *Parkin* overexpressing flies (*TubGS>UAS-AOX; UAS-Parkin*; +RU-486 ON) (AOX; Parkin in the figure) and UAS-AOX; *Pink1* overexpressing flies (*TubGS>UAS-AOX; UAS-Pink1*; +RU-486 ON) (AOX; Pink1 in the figure). An inducible Tubulin GeneSwitch GAL4 promoter was used for the expression of the transgene. P values were calculated using 1-way ANOVA with Newman-Keul's post-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes.

6.5 Parkin overexpression restores TOR signalling in AOX flies

As I have observed earlier that AOX expression causes the hyper-activation of TOR (Chapter 5.3), I wanted to understand if TOR signalling directly controls mitochondrial function or vice-versa. I measured phosphorylation of S6K as a proxy for TOR activation. In accordance to the results seen in mitochondrial respiration and mitochondrial density, Parkin but not Pink1 overexpression restored TOR signalling in AOX flies (Figure 6.7 A and B). This suggests a direct control of TOR signalling by mitochondrial activity. Combined, these results prove that restoring mitophagy is enough to restore TOR signalling, indicating that an appropriate mitochondrial turnover is essential.

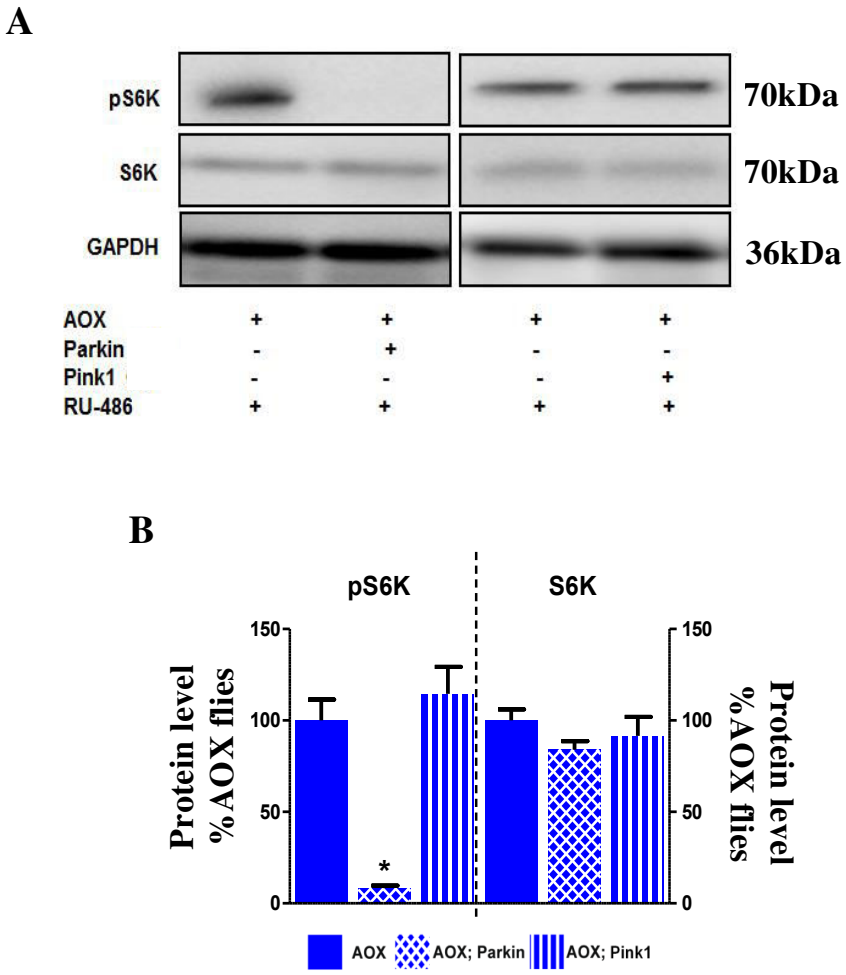

Figure 6.7 Hyper-activation of TOR in AOX flies is restored by Parkin overexpression.

(A) Representative western blot membranes showing pS6K and S6K levels in indicated genotypes. GAPDH is used for normalization and quantification purposes. **(B)** Quantification of **(A)** (n=3). AOX expressing flies (*TubGS>UAS-AOX*; +RU-486 ON) (AOX in the figure), UAS-AOX; *Parkin* overexpressing flies (*TubGS>UAS-AOX; UAS-Parkin*; +RU-486 ON) (AOX; Parkin in the figure) and UAS-AOX; *Pink1* overexpressing flies (*TubGS>UAS-AOX; UAS-Pink1*; +RU-486 ON) (AOX; Pink1 in the figure). An inducible Tubulin GeneSwitch GAL4 promoter was used for the expression of the transgene. P values were calculated using 1-way ANOVA with Newman-Keul's post-test. Data are shown as mean ± SEM. * = $p<0.05$; denotes statistically significant difference between mentioned genotypes. The molecular weight of proteins detected by western blots are given as kiloDalton (kDa) units.

6.6 Parkin overexpression rescues the survival of AOX flies

As demonstrated in Chapter 3.9, AOX expression caused a significant decrease in lifespan. Since I have established that accumulation of damaged mitochondria activates TOR signalling and reduces lifespan, I hypothesized that activation of mitochondrial turnover in these conditions will rescue the short lifespan of AOX flies.

I performed lifespan analysis in both Parkin overexpressing flies and Pink1 overexpressing flies, co-expressing AOX. I observed a significant extension in the survival, when Parkin was overexpressed in AOX flies; whereas, Pink1 overexpression did not alter AOX lifespan (Figure 6.8). This was in accordance to the lack of effect on other phenotypes studied. In summary, this chapter shows that mitochondrial turnover is indispensable for a healthy and long survival.

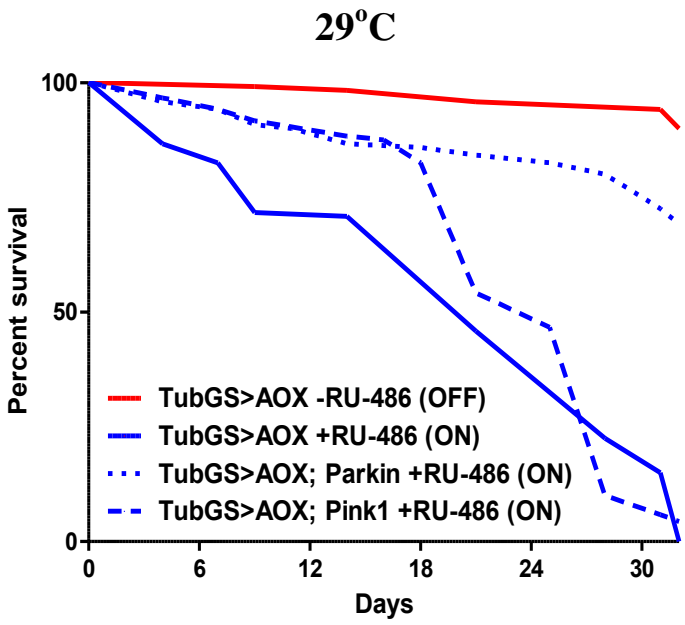

Figure 6.8 Parkin overexpression rescues the lifespan of AOX flies.
Survival curves of indicated genotypes (n=200). A table with statistics for the lifespan is presented in Appendix A.

Chapter 7. Discussion

7.1 Introduction

Accumulation of damaged mitochondria is a common characteristic for ageing and neurodegenerative diseases (Bratic and Larsson, 2013), but how and why this happens is still a mystery. Although ROS can be hypothesized as a proximal cause for this phenotype, studies have not been successful in demonstrating this effect definitively. ROS often functions as a dual entity, in damage as well as in signalling. The function of ROS, that causes damage to proteins and molecules have been very well studied and linked to many diseases, particularly that of ageing (Alfadda and Sallam, 2012). Even though many studies have emerged making it evident that ROS also functions in signalling pathways like hypoxia and apoptosis (Deng *et al.*, 2012) (Qutub and Popel, 2008), it is still unclear if or how ROS are responsible for activating or deactivating many other signalling pathways like mitophagy and/or insulin signalling. Most of the studies on ROS signalling are also demonstrated *in vitro*; either in cell culture or isolated mitochondria. Cell culture has many advantages, but when considering studies on ROS signalling it might be less efficient. Culturing cells in a dish expose cells to high levels of oxygen than the ones observed *in vivo*. This can be responsible for the production of excessive ROS, as cells can induce a Hyperoxia like response, increasing ROS consequentially. This response can also lead to activation or deactivation of many signal transduction pathways (Kwak *et al.*, 2006). Furthermore, cells that are used for experiments are also cultured on glucose instead of galactose. Glucose makes the cell highly glycolytic, making the cell completely dependent on glycolysis for their energy demands; therefore making the mitochondria dormant or with very low functionality. This hypothesis has also already been tested and it has been observed that PARKIN dependent mitophagy is altered according to the media in which cells are cultured. In these studies, it was observed that PARKIN dependent mitophagy in cells occur only when cultured in glucose. Under these conditions, PARKIN is overexpressed and FCCP is added to the culture media. However, when cells are cultured in galactose, mitochondrial OXPHOS is active and PARKIN dependent mitophagy is inhibited. Even upon FCCP addition, PARKIN dependent mitophagy is minimal (MacVicar and Lane, 2014). In summary, as ROS signalling mainly depends on mitochondrial function, signalling studies in cell cultures may lose their credibility.

Activation or deactivation of TOR signalling has already been studied in many diseases and in ageing (Kaeberlein, 2013). TOR signalling plays a major role in cellular mechanisms like cell growth, proliferation, protein translation, autophagy and many more. It integrates many upstream elements, thus making it one of the most important signal transduction pathways. There have been numerous studies emerging from the TOR signalling cascade since its discovery (Heitman *et al.*, 1991), but there is no solid proof of how TOR signalling performs as a unit. Many studies have demonstrated that inhibiting TOR improves many disease phenotypes like cancer and has also been shown to extend healthy lifespan (Seufferlein and Rozengurt, 1996; Neff *et al.*, 2013). On the other hand, activation of TOR has been shown to improve homeostasis by promoting the translation of proteins. Even though many pharmacological and genetic interventions to activate and deactivate TOR have been used, the mechanism of activation or inhibition is still not very clear. However, with the discovery of new and more specific inhibitors and/or activators, further studies will improve our understanding on the molecular basis of the signalling cascade.

TOR signalling modulates mitochondrial bioenergetics in many ways. It activates PGC1 α, which is a major modulator for mitochondrial biogenesis (Diop *et al.*, 2015). It has also been shown to improve availability of substrates for glycolysis, which is utilized by the mitochondria as well (Sun *et al.*, 2011). On the other hand, not many studies have demonstrated the role of mitochondria in controlling TOR signalling. It has been shown that mitochondrial ROS regulates many downstream mechanisms of TOR like autophagy or apoptosis (Gibson, 2013) (Simon *et al.*, 2000), but there are no studies showing a direct regulation of TOR by mitochondrial ROS. In many model organisms mitochondrial damage and TOR signalling are increased in parallel during ageing (Bratic and Larsson, 2013), but whether this is a cause or a consequence of ageing is still not determined.

In summary, I provide evidence that mitochondrial H_2O_2 signal is necessary for stress adaptation. When this signal is blocked by two different genetic interventions, mitochondrial turnover is halted. This causes an accumulation of damaged mitochondria which in turn causes persistent hyper-activation of TOR. I have further demonstrated that increasing mitochondrial turnover in a low mitochondrial H_2O_2 (AOX flies) background reduces mitochondrial density and restores TOR signalling. However, reverting the hyper-activation of TOR in a low mitochondrial H_2O_2 background does not improve this phenotype. This demonstrates that activation of TOR signalling can be a homeostatic response, instead of a consequence, in order to improve glycolysis for energy balance. I aim to elucidate this redox

signalling pathway in detail which will be a valuable clinical tool to prevent ageing and onset of age-related diseases.

7.2 A mtH_2O_2 rather than a O_2^- signal is responsible for cellular stress response

As discussed earlier, ROS are considered as a double entity; causing damage and acting as signalling molecules. Many studies which describe ROS as a damaging agent have been performed which is due to the fact that damage can be easily studied. Whereas, it is extremely difficult to perform experiments to study ROS as a signalling molecule due to its very short half-life of around 1µs to 1ms. ROS has been shown to act as signals by modifying cysteine residues in proteins, changing their conformation and therefore their activity (Miki and Funato, 2012). This phenomenon has been described in signalling cascades like apoptosis, hypoxia and few other (Ray *et al.*, 2012). To overcome the hurdles of studying ROS as a signalling molecule in cell culture, I have taken an *in vivo* approach altering ROS by genetic interventions. I ectopically expressed AOX in the fruit fly (Figure 3.2 A). AOX accepts electrons from the ubiquinone pool, thus preventing the over-reduction of the ubiquinone pool, the leak of electrons and the generation of ROS (Fernandez-Ayala *et al.*, 2009). I hypothesize that this prevention in reduction of the ubiquinone pool caused by AOX is the block in the signal that is responsible for cellular stress response.

Currently, there is a huge controversy in the field of ROS regarding the most appropriate method to measure ROS *in vivo* (Halliwell and Whiteman, 2004). To ascertain that AOX reduces the leak of electrons from the ETC and thereby reduces ROS, I measured ROS using four different techniques to demonstrate the change in different ROS molecules. AOX flies significantly reduced O_2^- and H_2O_2 levels both *in vitro* and *ex vivo* (Figure 3.2 B, C and D). Furthermore, I also confirmed the decrease in ROS *in vivo* (Figure 3.3 A and B). However, this reduction in ROS did not improve the damage caused to proteins (Figure 3.6). This led me to hypothesize that ROS might act as a signal instead of a damaging molecule. It can also be hypothesized that a certain level of ROS is necessary to facilitate the cellular signalling processes.

As AOX has no proton pumping properties and can reduce proton motive force, it is possible that it affects ATP production. However, I measured the levels of ATP, ADP, and AMP in the fly and observed no changes (Figure 3.4 A and B). This can also occur because of the upregulation of glycolysis, which will be discussed in the following pages. Moreover,

feeding, locomotion or the weight of the fly were unchanged denoting that AOX expression did not cause any phenotypic changes (Figure 3.5 A, B and C). These results demonstrate that the effect of AOX predominantly alters ROS levels.

AOX is upstream of the dismutation of O_2^- or H_2O_2; hence it is difficult to determine the ROS species involved in the signalling process. O_2^- is rapidly dismutated to H_2O_2 by Superoxide dismutases, like SOD1, in the intermembrane space and cytosolic compartments and SOD2, in the mitochondrial matrix. On the other hand, H_2O_2 is converted to water by many enzymes like CATALASE, GLUTATHIONE PEROXIDASES and PEROXIREDOXINS making the molecule inert. H_2O_2, however, when compared to O_2^- is less reactive and due to its neutral charge can diffuse through biological membranes and act in signalling far from its site of production. To differentiate these ROS signals, I used two different fly models. (i) I ectopically expressed Catalase in the mitochondria of the fly (Figure 4.2 A) and (ii) I overexpressed a version of superoxide dismutase which is present in the mitochondria (Figure 4.2 A). I demonstrated that ectopic expression of Catalase reduces H_2O_2 but does not alter levels of O_2^- (Figure 4.2 B and C). Sod2 overexpression on the other hand reduced levels of $O_2^-$and increased H_2O_2 levels marginally (Figure 4.2 B and C), therefore being a very valuable tool in studying ROS signalling. In addition to studying ROS signalling, these flies have been used in several labs to study their effect on lifespan. Over the last decade or two, several labs have demonstrated contradictory lifespan results. Overexpression of Sod2, in some studies did not affect the lifespan of flies and in some studies extended fly lifespan (Mockett *et al.*, 1999; Mockett *et al.*, 2010; Mockett *et al.*, 2001). On the other hand, mitochondrial Catalase expression has never been demonstrated to extend lifespan of flies (Mockett *et al.*, 2003). However, in mice, mitochondrial Catalase expression extended lifespan (Schriner *et al.*, 2005). It has to be taken into consideration the different experimental conditions used for these lifespan analysis e.g. diet or background that can significantly affect these results. More stringent conditions have to be maintained in order to demonstrate these results more convincingly.

I observed that a reduction in mitochondrial H_2O_2 in flies (AOX and mitochondrial Catalase), caused an increase in mitochondrial density. Whereas, the increase in mitochondrial H_2O_2 (Sod2) reduced mitochondrial density (Figure 3.8 A and Figure 4.4 A). Mitochondrial density is also a balance between mitochondrial turnover and mitochondrial biogenesis. In order to confirm whether there was any alteration in biogenesis, I measured *Spargel* and *Delg* levels, which regulate mitochondrial biogenesis in flies. I detected only a modest increase in mRNA

levels of *Spargel* in AOX flies (Figure 3.10). Furthermore, *Spargel* mRNA levels were unchanged in the mitochondrial Catalase expressing flies (Figure 4.5). I also detected a significant increase in mtDNA copy number, without any changes in mtDNA encoded ETC subunits (Figure 3.8 B, Figure 3.11 and Figure 4.4 B). These results indicate that the increase in mitochondrial density is caused due to accumulation of damaged mitochondria rather than an increase in mitochondrial biogenesis. To confirm that there is accumulation of damaged or respiratory deficient mitochondria, I analysed mitochondrial respiration. To begin with, I used whole homogenates of the AOX flies, which showed no difference in respiration when normalized to total protein content (Figure 3.12 A). Since I detected an increase in mitochondrial density, independent of mitochondrial biogenesis, it is unlikely that the mitochondrial bioenergetics is preserved. I then measured mitochondrial density in these homogenates used for respiration. When the respiration values from the homogenates were normalized to the mitochondrial density values instead of the total protein content, I saw a significant decrease in respiration of AOX flies (Figure 3.12 B). Interestingly, I observed a similar decrease in respiration in mtCAT flies; whereas, in Sod2 flies, mitochondrial respiration was increased upon normalization (Figure 4.6 A and B). This analysis of respiration was used, as it gave an estimation of respiration per mitochondrion. I also measured mRNA levels of UPR genes (*Hsp's*) and glycolysis regulator (lactate dehydrogenase (*ImpL3*)), which were upregulated in response to reduction in mitochondrial H_2O_2 (Figure 3.13 A and Figure 4.7). These results denote the increase in presence of damaged mitochondria. Overall, these experiments support the hypothesis that an active mitochondrial H_2O_2 signal is required to alter mitochondrial bioenergetics in response to stress.

Results from Figures 3.8, 4.4, 3.12 and 4.6, have demonstrated that damaged mitochondria accumulate when mitochondrial H_2O_2 is reduced; therefore, I wanted to understand the mechanism behind this accumulation. Since Pink1-Parkin dependent mitophagy is one of the major regulators of mitochondrial turnover, I analysed protein levels of Pink1 and Parkin. I observed that canonical Pink1-Parkin mitophagy was reduced with loss of the H_2O_2 signal (Figure 3.14 A, B and Figure 4.8 A, B). These results show that ROS; more specifically mtH_2O_2, is required to initiate mitochondrial turnover independent of the generation of oxidative damage. In relation to this, I demonstrated that mitochondrial H_2O_2 levels regulate the levels of Pink1. When H_2O_2 signalling is interrupted, Pink1 levels rapidly decay which may halt mitophagy, explaining the accumulation of respiratory deficient mitochondria that I observed. However, I did not detect any changes in the Parkin protein levels (Figure 3.14 A, B and Figure 4.8 A, B). It can be hypothesized that the protein levels of Parkin do not explain the activity of the protein,

since the phosphorylated or the redox state of the protein is not measured. Additionally, as Parkin is a cytosolic protein it is most likely controlled by a mediator protein that relays the ROS signal over to Parkin. I hypothesize that a mitochondrial H_2O_2 signal is essential to stabilize and activate Pink1. This can be achieved by an interaction between the cysteine residues in the Pink1 protein and H_2O_2. On the other hand, it is also possible that Pink1 activity is regulated by kinases or phosphatases that are redox regulated. More experiments dissecting the hypothesis are however required.

Through simultaneous expression analysis of 87 genes, I have demonstrated that changes in mitochondrial H_2O_2 have a consistent effect on gene expression, supporting the existence of different transcriptomic signatures for high and low levels of mtH_2O_2 (Figure 4.11 and Table 4.1). The most significant changes in gene expression were in hypoxia and P53 signalling. These results are not unexpected, as hypoxia adaptation is regulated by ROS produced at Complex III (Bleier and Drose, 2013). Preventing over-reduction of the ubiquinone pool via expression of an alternative oxidase (AOX) was expected to, and did in fact, reduced mitochondrial ROS and lifespan of flies exposed to hypoxia. A high proportion of the transcripts up-regulated by H_2O_2 in response to high temperatures (e.g. *Tsp66E*, *CathD* or *Ref (2) P*) were involved in autophagy. Accordingly, in transgenic flies with reduced mtH_2O_2 levels, the autophagic response was attenuated (Figure 5.2). The failure of these cellular mechanism caused the flies with low mitochondrial H_2O_2 (AOX and mtCAT) to live shorter. On the other hand, flies with increased mitochondrial H_2O_2 (Sod2) lived significantly longer (Figure 3.18 and Figure 4.12).

In summary, I have established several novel genetic approaches to modify the ROS levels *in vivo* in the fruit fly. Using these techniques, I have revealed alternative effects of different ROS molecules. I demonstrated that these ROS molecules have distinctly different roles in redox signalling. Moreover, I have provided evidence for the existence of a mitochondrial H_2O_2 signal in the process of canonical mitophagy. Furthermore, I have also demonstrated that interruption of this mtH_2O_2 signal disrupts the response for stress adaptation.

7.3 Interaction between mitochondria and TOR

TOR signalling controls many signalling cascades and has been studied for several years. However, a distinct list of signalling cascades that TOR controls still remains unknown. It is very complex as it integrates many upstream and downstream elements. Since TOR controls many signalling cascades, it is safe to assume it also plays an important role in mitochondrial

bioenergetics. It has been demonstrated that TOR signalling controls mitochondrial functionality, however, the vice-versa has not been very well studied. Mitochondrial ROS has been shown to control many downstream elements that are also controlled by TOR (Schieke and Finkel, 2006), but a direct control of TOR by mitochondria or mitochondrial ROS has not been shown. Mitochondria has also been shown to control insulin signalling and nutrient signalling that are upstream of TOR, but these studies have been contested since. Mitochondrial biogenesis is another pathway governed by TOR. TOR signalling controls the production of new mitochondria and mitochondrial DNA by controlling the transcription of *PGC1α* and *NRF1* (described in detail in Chapter 1.24). Mitochondrial recycling is also one of the pathways that interact with TOR. It controls the autophagic machinery, which plays a major role in mitophagy (Jung *et al.*, 2010). Even though TOR signalling has been shown to interact with mitochondrial functionality and vice-versa, it is still not evident how this happens. In summary, I have established a link between mitochondrial turnover and TOR activation. I have also demonstrated that mitochondria are upstream regulators of TOR. Additionally, I have demonstrated that mitochondrial ROS is directly linked to TOR rather than interacting with the downstream effectors of TOR.

First, I observed transcriptional changes mainly in the insulin signalling pathway and the autophagy pathway when the levels of mitochondrial H_2O_2 was reduced (Figure 4.11). Both these pathways are major signalling cascades that are controlled by TOR. This was the primary reason for me to delve into studying the TOR signalling cascade. Secondly, I also observed a decrease in mitophagy, when the mitochondrial H_2O_2 was reduced. I hypothesized that TOR signalling can be disrupted, causing an autophagy dependent accumulation of damaged mitochondria. I observed hyper-activation of TOR signalling, confirmed by the increase in phosphorylation of S6K and 4E-BP (Figure 5.3 A and B). I also observed a decrease in autophagy, which can be linked to hyper-activation of TOR signalling (Figure 5.2 A and B). Additionally, in order to verify that TOR signalling is modified in response to AOX expression, in the sense; decrease in mitochondrial ROS and mitochondrial accumulation, I used the inducible system as described earlier to induce or terminate AOX expression. I have also previously demonstrated that mitochondria accumulate in parallel to the expression of AOX and are reverted when the AOX expression is halted. I observed a similar activation of TOR signalling (Figure 5.4 A and B). However, it is still unclear if the activation of TOR is because of the decrease in the ROS signal or because of the increase in mitochondrial accumulation.

In order to verify if the activation of TOR was a cause or consequence of the mitochondrial accumulation, I checked if the upstream inhibitors of TOR were active. Initially, I verified if the well-described S6K feedback loop was active (Efeyan and Sabatini, 2010). This feedback loop is responsible for keeping the activation of TOR in check as TOR hyper-activation can cause cancer. I have demonstrated earlier that TOR was hyper-activated when mitochondrial density was increased. However, I also detected an activation of the feedback loop, as mentioned earlier, demonstrated by the decrease in phosphorylation of AKT, mediated by S6K (Figure 5.5 A and B). The decrease in phosphorylation of AKT leads to FOXO activation (Figure 5.5 A and C) and consequentially TOR inactivation in an AMPK-TSC2 dependent manner (Figure 5.6 A, B and C). The failure of the feedback loop to deactivate TOR denotes that the activation of TOR is persistent due to the reduction of mtH_2O_2 or increase in mitochondrial density. Figure 7.1 is a schematic representation of the TOR signalling pathway.

I then wanted to verify if the hyper-activation of TOR is an adaptive response. In order to understand this, I used rapamycin to inhibit TOR in AOX flies. Rapamycin is a well-known drug used for the inhibition of TOR and has been shown to extend lifespan robustly in many organisms (Ehninger *et al.*, 2014). As described earlier, TOR signalling was hyper-activated in AOX flies, which caused a reduction in lifespan (Figure 3.18 and 5.3). I fed AOX flies with rapamycin in order to determine if deactivation of TOR will be beneficial for these flies. Rapamycin efficiently inhibited TOR, improved autophagy and increased mitochondrial turnover (Figure 5.7, 5.8 and 5.9). However, the functionality of mitochondria was not rescued. Mitochondrial respiration was disrupted in AOX flies fed with rapamycin, and respiration was even lower than the flies that were not fed with rapamycin. When normalized to mitochondrial density, the difference was more evident (Figure 5.10). I hypothesize that this might be because of improper signalling to recycle damaged mitochondria. It is possible that damaged mitochondria might need a functional signal in order to be recycled and this signal is H_2O_2. In AOX flies, this signal is lost causing accumulation of mitochondria. In support of this hypothesis it has been demonstrated that overexpression of ATG18 (increasing autophagy), significantly reduced mitochondrial density in mice, independent of PINK1 or PARKIN. It is very unlikely that damaged mitochondria are flagged and specifically recycled in this process (Chang *et al.*, 2013). Moreover, when AOX flies are fed with rapamycin, the increase in autophagy can cause un-specific recycling of mitochondria, leaving behind a very low number of functional mitochondria. Finally, the flies fed with rapamycin lived even shorter than AOX flies, which demonstrated that maybe TOR activation is a necessary

adaptation (Figure 5.12). I also fed the AOX expressing flies with rapamycin two days a week. However, I did not see any improvement in the fly lifespan, and the flies had similar lifespan as the AOX flies without rapamycin (Figure 5.12). I hypothesize that the increase in TOR is a homeostatic response; improving glycolysis to compensate the presence of dysfunctional mitochondria and sustain energy production (Sun *et al.*, 2011; Cheng *et al.*, 2014).

In summary, I show evidence of persistent activation of TOR due to loss of ROS signalling and mitochondrial accumulation. I demonstrate that an active S6K feed-back loop is not enough to repress TOR signalling. I also show evidence of a mitochondrial signal that is absolutely essential to recycle mitochondria, without which the increase of downstream pathways is nullified. I have thus demonstrated a novel existing link between mitochondria and TOR, and that activation of TOR is determined by the mitochondria.

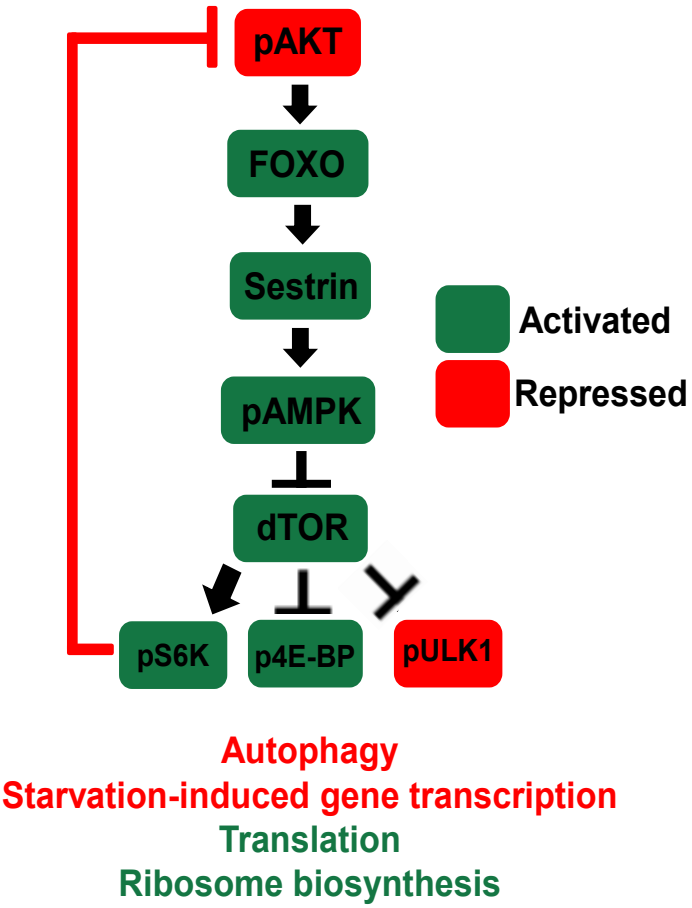

Figure 7.1 A schematic of upstream elements that control TOR and downstream elements that TOR controls.

Phosphorylation dependent activation or deactivation of several proteins in the cascade, flow into TOR, which inturn (i) inhibits (red text in figure) mechanisms like autophagy and starvation-induced gene transcription and (ii) activates (green text in figure) mechanisms like translation and ribosome biosynthesis among many others.

7.4 Mitochondrial turnover controls TOR signalling

From experiments performed earlier, I was unable to understand how mitochondria and mTOR interact, i.e. I was unable to discern if mitochondrial H_2O_2 directly controls TOR or if H_2O_2 controls TOR by controlling mitochondrial turnover. It will indeed be very interesting to understand if mitochondrial accumulation is a pseudo signal that activates TOR; wherein the cell interprets mitochondrial accumulation as signal to promote proliferation. To test this hypothesis, I overexpressed two of the proteins involved in canonical mitophagy; Pink1 and Parkin, to restore mitophagy (Figure 6.2).

Overexpression of Parkin in AOX flies restored mitochondrial turnover and reduced mitochondrial accumulation. However, Pink1 overexpression did not restore mitochondrial respiration nor did it reduce the amount of mitochondria (Figure 6.3 and 6.5). Pink1 protein was clearly overexpressed in the flies, confirmed by Western blot; however, since Western blotting technique only measured the levels of total protein, I was unable to check the activity of the protein. I hypothesize that overexpression of Pink1 is not enough to initiate mitophagy. It has been shown that auto-phosphorylation of Pink1 after mitochondrial membrane potential dissipation is absolutely essential for the process of mitophagy (Kondapalli *et al.*, 2012). PINK1 activation after its auto phosphorylation is responsible to recruit PARKIN (Okatsu *et al.*, 2012; Kondapalli *et al.*, 2012). However, a redox dependent cysteine activation of PINK1 has not been shown yet. I hypothesize that a mitochondrial H_2O_2 signal is responsible for the activation of Pink1 and this activation is responsible for conformational changes in the Pink1 protein avoiding degradation of the protein. This was also confirmed by checking the Pink1 proteins levels in the flies that have more H_2O_2 (Sod2). Sod2 flies had more Pink1 protein (Figure 4.8), which partially supports the hypothesis that the protein avoids degradation. However as mentioned earlier, activation of the protein was still not verified. Additionally, it has been shown that PARKIN needs to be recruited in order to be involved in mitophagy. This does not explain why Parkin overexpression increases mitochondrial turnover in AOX flies. However, Parkin has been shown to rescue Pink1 mutant fly phenotypes (Park *et al.*, 2006). It can be hypothesized that, Parkin, when overexpressed, does not need to be recruited and activated by Pink1; and it can be involved in mitochondrial turnover independent of Pink1. It is not surprising that neither Pink1 nor Parkin overexpression in the AOX flies improved the mitochondrial ROS signal (Figure 6.4). These experiments also demonstrate that Parkin is located downstream of the mitochondrial H_2O_2 signal. More experiments are required in order to determine the intermediate protein that connects the mitochondrial H_2O_2 signal and mitochondrial turnover.

In order to ascertain that accumulation of defective mitochondria is directly responsible for the hyper-activation of TOR, I checked TOR signalling in flies overexpressing Pink1 or Parkin, in an AOX flies. When the turnover of mitochondria was improved with Parkin overexpression in AOX flies, TOR signalling was reverted back to basal levels; whereas, Pink1 overexpression, as described earlier, neither improved the mitochondrial turnover nor rescued the hyper-activation of TOR (Figure 6.7). This supports the hypothesis that mitochondrial turnover or accumulation is directly responsible for activation of TOR. Additionally, Parkin overexpression significantly improved the decrease in lifespan caused by AOX expression; whereas, Pink1 overexpression did not (Figure 6.8). This shows that a proper mitochondrial turnover is absolutely essential for survival, which in turn controls TOR signalling.

These results demonstrate how mtH_2O_2 regulates mitochondrial turnover and through it the activation of TOR. When this pathway is disrupted, mitophagy is halted and causes an accumulation of damaged mitochondria. This clearly shows that mitochondrial turnover is instrumental for an appropriate fine-tuning of TOR signalling. Moreover, I also describe a mechanism of how autophagy is disrupted causing the dysfunction of the proteasome and consequentially the accumulation of damaged proteins. As a consequence of the reduction in cellular quality control, the lifespan of flies under stress is severely reduced.

In summary, I have uncovered a novel mtH_2O_2-Pink1/Parkin-TOR pathway that provides us with new targets for pharmacological or dietary interventions. I have established that targeting this pathway is a valid therapeutic strategy that should be considered for the treatment of age-related and mitochondrial disorders. In the near future, I aim to elucidate this redox regulated pathway in detail. Figure 7.2 is a summary of my research findings.

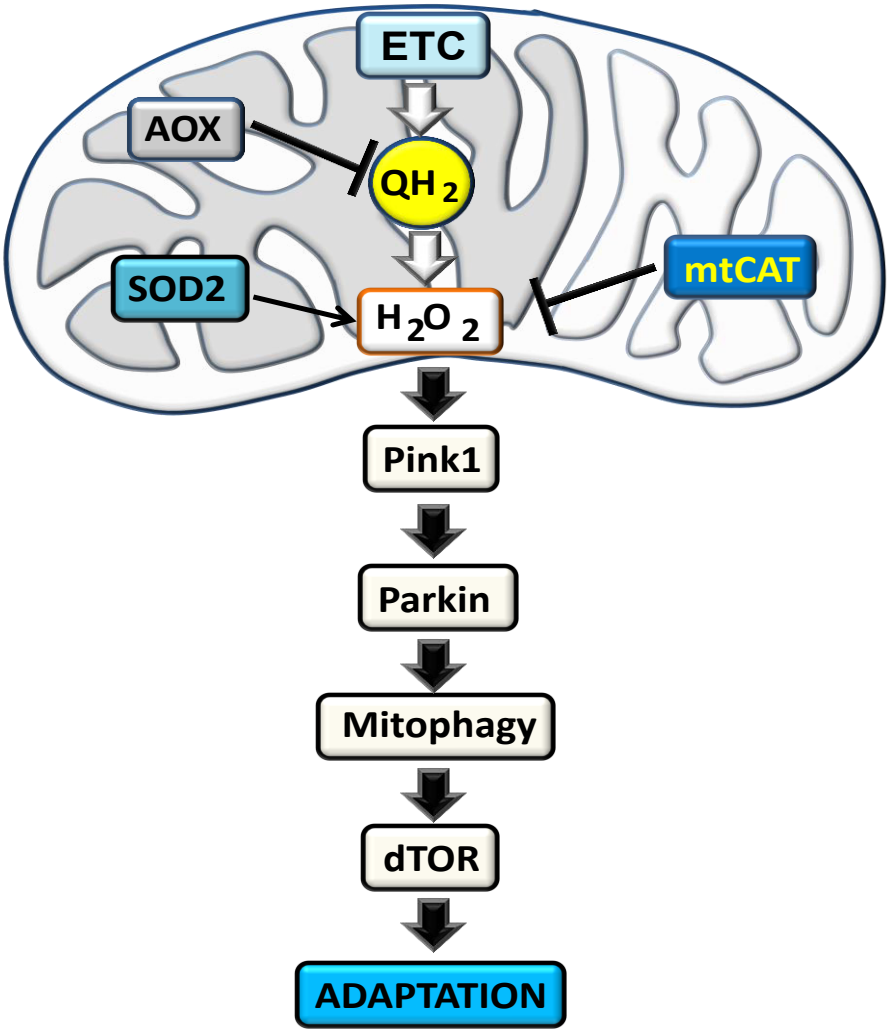

Figure 7.2 Summary of research findings.

I hypothesize that a mitochondrial H_2O_2 signal initiated by the over reduction of ubiquinone pool is responsible for the activation of Pink1. This activation is responsible for the recruitment of Parkin and sequestration of mitochondria to the autophagosome. Mitochondrial density therefore controls the activation of TOR which is responsible for specific adaptations of the organism.

7.5 Future work

Bringing together my findings, I have identified a central role of mitochondrial H_2O_2 in many cellular signalling processes. I have used several genetic approaches to block the ROS signal; like ectopic expression of AOX or ectopic expression of Catalase in the mitochondrion. Even though these techniques are very efficient and the results obtained are reproducible, they are non-physiological conditions in the fruit fly as both AOX and mitochondrial Catalase are non-endogenous proteins. Hence it will be very interesting to see what happens if we block this signal physiologically, either by; (i) overexpressing any of the native antioxidants to block the H_2O_2 signal or; (ii) feed the flies with known antioxidants to supress this ROS signal. However, both these techniques have shortcomings; (i) Overexpressing antioxidants might lead to prevention of damage rather than reducing the ROS signal. Moreover, the affinity towards ROS for different antioxidants is different and might be very difficult or improbable to control. I also hypothesize that either a balance between O_2^- and H_2O_2 or a specific ratio between the two is absolutely essential; hence it will be extremely difficult to maintain this balance with transgenic overexpression; (ii) The feeding experiments are very complicated as I will not be able to control the amount of food that the fly has consumed, hence will be unable to control the concentration of the antioxidant.

I have also mainly used the fly's brain for my ROS measurement experiments; whereas, in other molecular experiments I have used the whole flies, which mostly contain mitochondria from the muscle. Hence it will be good to differentiate the tissue in my experiments. I will also be able to see a much more significant effect by using just the post-mitotic tissue, most extensively found in the thorax and head. This will be advantageous to my work as the repair mechanisms and the damage will be much more pronounced in these tissues and will be advantageous to modulate. Whereas, the abdomen of the fly contains eggs which are constantly undergoing cell division, therefore diluting the effect observed when whole flies are used. It will also be interesting to determine the cell non-autonomous signal, by altering the ROS signal in the brain, which may lead to a phenotypic change in the muscle of the organism. By using tissue specific drivers, I can express AOX or mitochondrial Catalase in the brain or the muscle to see if it alters mitochondrial functionality or decreases the lifespan of the fly, just as much as ubiquitous expression. This will be very useful as I will be able to determine if this ROS signal is existent in different tissues.

It would be interesting to determine the protein or a set of proteins that orchestrate this ROS signal generating from the mitochondria, through oxidative modifications. I will be able to use mass spectrometry and site-specific proteomic mapping to assay a set of mitochondrial proteins to determine selectively modified cysteine residues. This will enable to ascertain the fact that there is indeed a ROS signal that regulates mitochondrial functionality. Additionally, once the redox modification of this protein or these set of proteins are identified, I will be able to modify the protein *in vivo* in different model organisms. This will aid in understanding the physiological relevance and the basic biology of different signalling mechanisms.

Finally, it will be essential to determine how Pink1 is activated. This can be done by mass spectrometry by determining if Pink1 protein has a cysteine dependent on/off switch. If this site is determined, Pink1 can be constitutively activated irrespective of the signal. This model can be used to investigate several steps involved in the signalling process. It will also be essential to determine how Pink1 is cleaved after deactivation. Moreover, it will be essential to determine the process by which TOR signalling is activated detecting the accumulation of damaged mitochondria. By discovering the protein or set of proteins involved in this process, I will be able understand in detail the mechanism by which TOR and mitochondria interact, opening new avenues for pharmacological interventions.

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APPENDIX A: Summary of lifespan experiments performed in this study presented as mean and (maximum) lifespan in number of days.

		29°C	
Figure 3.18	daGAL4	>20(>20)	
	AOX	>20(>20)	
	daGAL4>AOX	17(19)	
P value		<0.001	
Figure 4.12	daGAL4	>20(>20)	
	mtCatalase	>20(>20)	
	daGAL4>mtCatalase	10 (20)	
P value		<0.001	
	daGAL4	33(39)	
	Sod2	36(42)	
	daGAL4>Sod2	39(46)	
P value		<0.001	
		25°C	
		Hypoxia	**Hyperoxia**
Figure 4.13	daGAL4	19 (35)	18 (27)
	AOX	19 (35)	18 (27)
	daGAL4>AOX	5 (12)	13(22)
P value		<0.001	<0.001
	daGAL4	19 (35)	17 (22)
	mtCatalase	18 (35)	17 (22)
	daGAL4>mtCatalase	10 (15)	15 (20)
P value		<0.001	<0.001
	daGAL4	22 (38)	17 (20)
	Sod2	27 (34)	17 (20)
	daGAL4>Sod2	31 (45)	24 (29)
P value		<0.001	<0.001
Figure 5.13		**29°C**	
	AOX	>22(>22)	
	daGAL4>AOX	22(22)	
	daGAL4>AOX+Rapamycin	17(22)	
P value		<0.001	
	AOX	>22(>22)	
	daGAL4>AOX	17(22)	
	daGAL4>AOX+Rapamycin 2 days/week	19(22)	
P value		<0.001	
Figure 6.8		**29°C**	
	TubGS>AOX–RU-486 (OFF)	>44(>44)	
	TubGS>AOX–RU-486 (ON)	21 (31)	
	TubGS>AOX; Parkin +RU-486 (ON)	26 (28)	
	TubGS>AOX; Pink1 +RU-486 (ON)	>44(>44)	
P value		<0.001	