# Analysis of mitochondrial quality control using a *Drosophila* model of Parkinson's disease

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# Abstract

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. Despite recent advances, the cause for most PD cases remains unclear. The discovery of mutations in *PINK1* (*PTEN-induced putative kinase 1*) reinforced the importance of mitochondrial impairment in PD. Mitochondria are essential organelles for energy generation in eukaryotic cells, whose compromise can eventually cause cell death. Multicellular organisms have evolved quality control mechanisms to ensure the viability of mitochondria and ultimately the cell. Molecular quality control through the mitochondrial chaperones and proteases acts to promote the proper folding of polypeptides and the degradation of misfolded or damaged proteins. When molecular quality control is overwhelmed, organellar quality control ensures mitochondrial recycling through a selective form of autophagy called mitophagy. *PINK1* has been proposed to act in both molecular and organellar quality control, by modulating the activity of chaperones, namely HtrA2 and TRAP1, and acting on mitophagy through Parkin recruitment to damaged mitochondria.

The work in this thesis provides evidence of a genetic interaction between *Trap1*, *Pink1* and *parkin* in *Drosophila melanogaster*. *Trap1* is essential to maintain mitochondrial and dopaminergic neuronal functions and is associated with resistance to stress. Importantly, neuronal expression of *Trap1* is sufficient to rescue the *Pink1* mutants. Moreover, the expression of *Trap1* ameliorates *parkin*-mutant phenotypes and *parkin* expression suppresses *Trap1*-mutant phenotypes, suggesting that molecular and organellar quality control pathways act in parallel downstream from *Pink1*.
p62 is an autophagy adaptor that acts in the PINK1/Parkin pathway, facilitating the aggregation and elimination of depolarised mitochondria through mitophagy. In this work it is shown that loss-of-function mutations in the *Drosophila* orthologue of p62, *ref(2)P*, result in a reduction in lifespan and age-dependent neurodegeneration. *ref(2)P* expression rescues the *Pink1*-mutant phenotypes and its presence is essential for the *parkin*-mediated rescue of *Pink1* mutant flies.

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# Abbreviations

| | |
|---|---|
| 6-OHDA | 6-hydroxydopamine |
| A | Adenine |
| AD | Alzheimer's disease |
| ALFY | Autophagy-linked FYVE |
| AMP | Adenosine monophosphate |
| AMPK | AMP-activated protein kinase |
| APP | Amyloid precursor protein |
| AR-JP | Autosomal recessive juvenile Parkinsonism |
| Atg | Autophagy-related protein |
| ATP | Adenosine triphosphate |
| BBB | Blood brain barrier |
| BiP | Binding immunoglobulin protein |
| bp | Base pairs |
| BSA | Bovine serum albumin |
| C | Cytosine |
| CCCP | Protonophore m-chlorophenylhydrazone |
| CNS | Central nervous system |
| COMT | Cathecol-O-methyl transferase |
| DA | Dopamine |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |
| ECL | Enhanced chemiluminescence |

| | |
|---|---|
| EDTA | Ethylenediaminetetraacetic acid |
| ER | Endoplasmic reticulum |
| ETC | Electron transport chain |
| G | Guanine |
| GBA | Glucocerebrosidase |
| GFP | Green fluorescent protein |
| GWAS | Genome-wide association study |
| HA | Hemagglutinin |
| HD | Huntington's disease |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HPLC | High performance liquid chromatography |
| HRP | Horseradish peroxidase |
| Hsp | Heat shock protein |
| HtrA2 | High temperature requirement protein A2 |
| IMM | Inner mitochondrial membrane |
| IMS | Inter-membrane space |
| IF | Immunofluorescence |
| kDa | Kilo Dalton |
| Keap1 | Kelch-like ECH-associated protein 1 |
| KO | Knockout |
| LBs | Lewy bodies |
| LB | Lysogeny broth |
| LC3 | Microtubule-associated protein light chain 3 |
| L-dopa | Levodopa |
| LDS | Lithium dodecyl sulfate |

| | |
|---|---|
| LIR | LC3 interacting region |
| LRRK2 | Leucine-rich repeat kinase 2 |
| MAO | Monoamine oxidase |
| MAPT | Microtubule associated protein tau |
| Mfn | Mitofusin |
| mitoGFP | Mitochondria-targeted green fluorescent protein |
| MPTP | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| mPTP | Mitochondrial permeability transition pore |
| mRNA | Messenger ribonucleic acid |
| mL | Millilitre |
| mg | Milligram |
| mM | Millimolar |
| min | Minutes |
| N- | Amino |
| NAC | *N*-acetylcysteine |
| ng | Nanogram |
| nM | Nanomolar |
| NMJ | Neuromuscular junction |
| NRF1 | Nuclear respiratory factor 1 |
| Nrf2 | Nuclear factor erythroid 2-related factor 2 |
| OD | Optical density |
| OMM | Outer mitochondrial membrane |
| OTC | Ornithine transcarbamylase |
| OXPHOS | Oxidative phosphorylation |
| PARIS | Parkin interacting substrate |

| | |
|---|---|
| PARL | Presenilin-associated rhomboid-like protein |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PD | Parkinson's disease |
| PE | Phosphatidylethanolamine |
| PGC-1α | PPARγ coactivator-1α |
| PINK1 | PTEN-induced putative kinase 1 |
| PPARγ | Peroxisome proliferator-activated receptor gamma |
| PTEN | Phosphatase and tensin homologue |
| PVDF | Polyvinylidene difluoride |
| ref(2)P | refractory to sigma P |
| RING | Really interesting new gene |
| RNA | Ribonucleic acid |
| RNAi | Ribonucleic acid interference |
| ROS | Reactive oxygen species |
| rpm | Rotations per minute |
| RT | Room temperature |
| SD | Standard deviation |
| siRNA | Small interfering ribonucleic acid |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| sec | Seconds |
| SNCA | α-synuclein gene |
| SQSTM1 | Sequestosome 1 |
| Su(P) | Suppressor of ref(2)P sterility |

| | |
|---|---|
| T | Thymine |
| TBS | Tris-buffered saline |
| TE | Tris-EDTA |
| TEMED | N,N,N,N,-tetramethylethylenediamine |
| TH | Tyrosine hydroxylase |
| TNF | Tumour necrosis factor |
| TRAP1 | TNF receptor-associated protein 1 |
| Tris | Tris(hydroxymethyl)aminomethane |
| UAS | Upstream activation sequence |
| UBA | Ubiquitin associated domain |
| UBL | Ubiquitin-like domain |
| UBL-5 | Ubiquitin-like protein 5 |
| UPR | Unfolded protein response |
| $UPR^{mt}$ | Mitochondrial unfolded protein response |
| UPS | Ubiquitin-proteasome system |
| UV | Ultraviolet |
| VDAC1 | Voltage-dependent anion-selective channel protein 1 |
| VPS35 | Vacuolar protein sorting-associated protein 35 |
| v/v | Volume per volume ratio |
| *w* | *white* gene |
| WT | Wild type |
| w/v | Weight per volume ratio |
| ΔOTC | Truncated ornithine transcarbamylase |
| ΔΨm | Mitochondrial membrane potential |
| µg | Microgram |

| | |
|---|---|
| µl | Microlitre |
| µM | Micromolar |

# Chapter 1

# Introduction

# Chapter 1: Introduction

## 1.1 Parkinson's disease: an overview

In 1817 James Parkinson presented his observations of six individuals with *Paralysis Agitans*, in a monograph entitled "An essay on the shaking palsy" (Parkinson, 2002). He considered that this disease should be properly nominated and described. Due to the nature of the disease, it was easily mistaken with other diseases, as mentioned in the preface of the publication: "some have regarded its characteristic symptoms as distinct and different diseases, and others have given its name to diseases different essentially from it; whilst the unhappy sufferer has considered it as an evil, from the domination of which he had no prospect to escape". Even nowadays the "evil" cannot be dominated, since no cure has yet been discovered for this "shaking palsy". In James Parkinson's publication, the "shaking palsy" is defined as "involuntary tremolous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured" (Parkinson, 2002).

Jean-Martin Charcot later implemented the designation Parkinson's disease (PD), and also highlighted its fundamental features: resting tremor, bradykinesia (slowness or absence of voluntary movement), rigidity (increased resistance to passive movement of the limbs) and postural impairment (Figure 1.1). Subsequently, in 1912, Friedrich Lewy described another hallmark of PD: the neuropathologic lesions, consisting of cytoplasmatic proteinaceous inclusions, known as Lewy bodies (LBs) (Shulman et al., 2011).

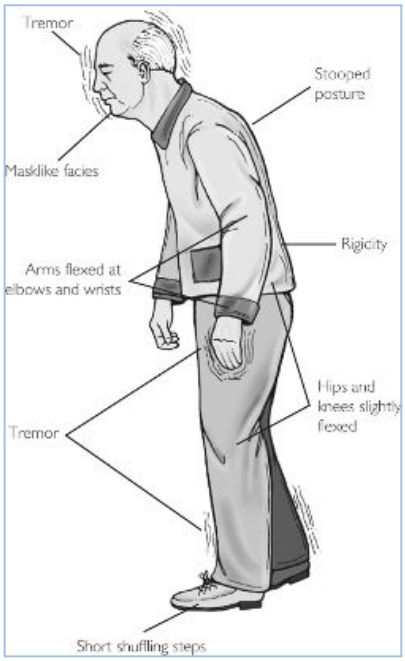



**Figure 1.1: Schematic representation of a Parkinson's disease patient and its characteristic symptoms.** Figure adapted from *http://schoolworkhelper.net/. St. Rosemary Educational Institution.*

The selective injury of dopaminergic neurons of the substantia nigra was first described by Tretiakoff in 1919 and later perceived by Hassler (1937 and 1938) and also confirmed by Greenfield and Bosanquet in 1953 (Holdorff, 2002). Remarkable progress has been achieved from several studies of PD, and yet, the definition of this disorder is still not completely clear. It is a rather complex syndrome, and a better knowledge about its aetiology and pathogenesis is fundamental for the development of a diagnostic test or marker, as well as therapeutic strategies and a possible cure for this disease.

The loss of dopaminergic neurons in the substantia nigra, and the consequent loss of dopamine (DA) in their striatal projections, causes the motor-related symptoms of PD, which can be ameliorated using the DA precursor levodopa (L-dopa) or dopaminergic agonists. However, PD patients have additional symptoms that are L-dopa resistant, which suggests that non-dopaminergic neurons are also affected. Indeed,

several areas of the brain show pathology at different stages of the disease. The neuronal loss in PD begins much before the manifestation of motor symptoms, which usually means that at the moment of the diagnosis about 60% of the dopaminergic neurons have already been lost (Braak et al., 2003). There are several symptoms that can be detected at earlier stages, such as the loss of the sense of smell, constipation, sleep disturbances and cardiovascular dysfunctions (Sun et al., 2007).

The progression of PD is usually slow and always irreversible. There are no available therapies to alter the course of the disease, meaning no agents are available that could either slow, arrest or reverse the neurodegeneration. The available therapies are primarily symptomatic. Both pharmacological and surgical interventions can ameliorate some of the symptoms, but these approaches are also associated with considerable side effects. The treatment of Parkinsonism aims to replenish the DA levels with the use of L-dopa, usually associated with inhibitors of the enzymes responsible for DA degradation, monoamine oxidase (MAO) and cathecol-O-methyl transferase (COMT).

PD is the second most common neurodegenerative disease, after Alzheimer's disease (AD), and the most common neurodegenerative movement disorder. It's prevalence of about 1% at age 60, increases to 5% at age 85, and it seems to affect more the masculine gender compared to the feminine (Van Den Eeden et al., 2003). Due to the gradual increase in life expectancy in the world population (Lutz et al., 2008), it is estimated that the prevalence of PD is also going to increase. It is, therefore, important to gain further insights into the disease mechanisms in order to improve the quality of life for PD sufferers.

### 1.1.1 Aetiology of PD

Most cases of PD are sporadic, with no associated inheritance pattern and of unknown cause. These cases are also known as idiopathic PD. Recently, familial forms of PD, which are caused primarily by monogenic mutations in different *PARK* loci have also been identified (discussed in section 1.2). There are three major factors in the aetiology of PD: age, environment and genetics. The role played by each of these factors has not been entirely unravelled (Figure 1.2).

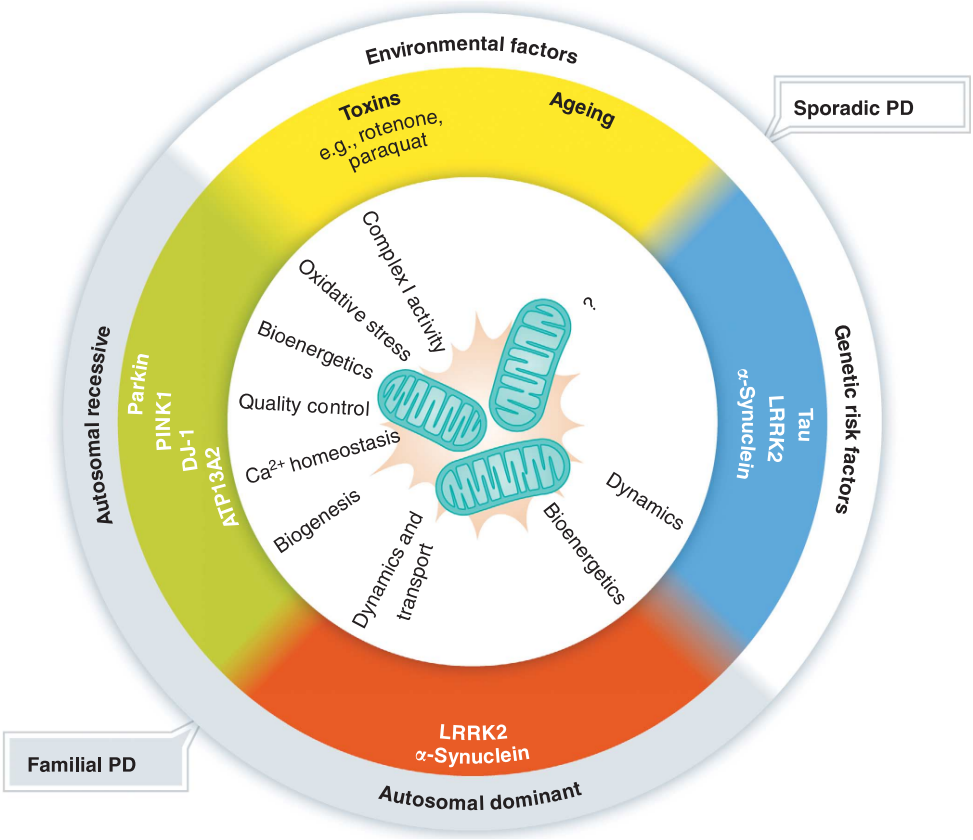



**Figure 1.2: Aetiology of Parkinson's disease (PD) and emerging links to mitochondria.** Heritable forms of PD can be caused by mutations in various genes: *LRRK2*, *α-synuclein*, *PINK1*, *Parkin*, *DJ-1*, *ATP13A2*. The sporadic cases of PD are considered to result from a variety of factors, from genetic susceptibility to the environment. Both genetics and environment can impact on mitochondrial function, through the regulation of bioenergetics, dynamics, transport and quality control. Figure from Exner et al., 2012.

Important insights into the aetiopathogenesis of PD were obtained by the manifestation of Parkinsonism in individuals accidentally exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP was a contaminant of a "designer" drug, a synthetic opiate, and several drug users exposed to MPTP developed a PD-like syndrome. MPTP is the precursor of the neurotoxin $MPP^+$ that blocks mitochondrial Complex I, consequently decreasing the ATP production and substantially increasing reactive oxygen species (ROS) generation and oxidative stress. MPTP is converted to its metabolite $MPP^+$ by MAO-B in glia cells, and is then actively taken-up by dopaminergic neurons via DA transporters, being primarily toxic to the substantia nigra. The MPTP-induced Parkinsonism in humans mimics PD, with similar clinical features, including symptoms, and responses and complications to therapy (Langston, 1987).

The concept that the environmental exposure to toxins causes PD is particularly supported by the MPTP studies. Moreover, these studies were also crucial for linking mitochondrial dysfunction and oxidative stress as key events in the pathogenesis of PD. In addition, post-mortem analysis of PD brains have revealed increased oxidative stress in the substantia nigra; linked to decrease complex I activity, increased levels of Fe (II) and decreased levels of reduced glutathione. There is also evidence of oxidative damage to proteins, lipids and DNA (Olanow et al., 2004).

The search for non-genetic risk factors for the development of PD has been intense. However, not only the methodologies applied, but also the experimental designs were often not appropriate to obtain definite conclusions from those studies. The improvement of the studies is needed, and especially the inclusion of a higher number of individuals in those studies in order to increase their statistical power.

To date, the only non-genetic risk factors with consistent results from different studies are the age and smoking behaviour. Age increases the risk of PD, whereas

cigarette smoking decreases this risk, although the exact mechanism that confers this protection has not been established. The nicotine present in the cigarettes has been proposed to act as a neuroprotective agent. Nicotine may increase the release of DA at synapses and it may also act as an antioxidant or modulate the activity of the enzyme MAO-B (de Lau and Breteler, 2006). Examples of other factors that may decrease the susceptibility to develop PD are the ingestion of coffee and non-steroidal anti-inflammatory drugs (NSAIDs). By contrast, an increased risk has been associated with exposure to pesticides, herbicides, solvents, farming and rural living (Di Monte et al., 2002). The effect of several of these factors in the aetiopathology of PD is currently under investigation.

The organochloride pesticide dieldrin has been detected in brains of PD patients, but not in those of the controls. Dieldrin was also found to be a risk factor in epidemiological studies, along with other pesticides, such as paraquat (Hancock et al., 2008). The discovery of heritable cases of PD highlights the contribution of genetic factors for developing this disease. Twin and family studies suggest that genetic factors may play an important role in sporadic PD. A positive family history is associated with a higher risk for PD (Warner and Schapira, 2003).

The search for susceptibility genes has been made essentially by linkage and association analysis. One of the major challenges is the fact that Parkinsonism is a feature of other neurodegenerative and non-degenerative diseases, and it might be difficult to distinguish them from PD without the confirmation by autopsy. An additional challenge comes from the fact that the motor symptoms manifest only when the disease is well advanced, meaning that pre-symptomatic patients can be considered as not having the disease.

Nevertheless, despite all the potential difficulties, genome-wide association studies (GWASs) have been a useful tool to identify common genetic variants associated with PD. Some of the loci associated with PD from GWASs correspond to genes identified in familial cases of PD. This is particularly interesting, because it supports the idea that common mechanisms are involved in both genetic and idiopathic PD. Some of the genes identified from GWASs are: *Glucocerebrosidase* (*GBA*), *Leucine-rich repeat kinase 2* (*LRRK2*), *SNCA*, *microtubule associated protein tau* (*MAPT*) (Keller et al., 2012).

## 1.2 Monogenic forms of PD

The identification of gene mutations that cause PD was an invaluable discovery, since it allowed an enormous improvement in our understanding of the mechanistic features underlying the pathogenesis of this disease. The monogenic forms of PD are rare when compared to the sporadic form, which account for 90-95% of the cases. The identification of *PARK* genes made possible the generation of genetic animal models, which are of great value for scientific research. Mutations in the genes *SNCA*, *LRRK2*, *Parkin*, *PINK1*, *DJ-1*, and *ATP13A2* are well validated to cause monogenic forms of PD (Table 1.1). The first two genes are related to autosomal dominant PD, whereas the last four have been linked to a form of autosomal recessive Parkinsonism. Most of the dominant mutations act through a gain-of-function mechanism. Conversely, most of the recessive mutations result in the absence of the encoded protein or in an inactive protein, and thus act through a loss-of-function mechanism (Corti et al., 2011). This seems to be case of the PD-linked gene mutations as well. Some of the genetic forms present atypical clinical features, such as young onset, onset with dystonia, and early

onset of dementia. Furthermore, Lewy body pathology does not seem to be present in some of the cases.

Whether the PD-related gene mutations converge on the same cellular pathway is not known. However, a better knowledge about the pathogenic mechanism for each one of the genes/mutations would certainly provide valuable information about the dopaminergic-neuronal loss, also in the common forms of PD.

**Table 1.1: Parkinson's disease-associated loci and genes with conclusive evidence (adapted from Corti et al., 2011 and Martin et al., 2011).**

| PARK locus | Gene | Inheritance | Clinical phenotype |
|---|---|---|---|
| PARK1/4 | *SNCA* | Dominant; rare sporadic | Early-onset; Parkinsonism with common dementia |
| PARK8 | *LRRK2* | Dominant; sporadic | Late-onset Parkinsonism |
| PARK2 | *Parkin* | Recessive; sporadic | Early-onset, slow progression Parkinsonism |
| PARK6 | *PINK1* | Recessive | Early-onset, slow progression Parkinsonism |
| PARK7 | *DJ-1* | Recessive | Early-onset Parkinsonism |
| PARK9 | *ATP13A2* | Recessive | Early-onset Parkinsonism with Kufor-Rakeb syndrome |

More recently, other gene mutations have been identified and linked to familial cases of PD, such as mutations in the *VPS35* gene (Ando et al., 2012, Sheerin et al., 2012, Vilarino-Guell et al., 2011). *VPS35* encodes for the vacuolar protein sorting-

associated protein 35 and the function and pathogenic mechanism for these mutations are currently being investigated.

### 1.2.1 α-synuclein

The identification of a mutation in the *SNCA* gene in a family of PD patients in the late nineties (Polymeropoulos et al., 1997), together with the finding that α−synuclein is the major component of LBs (Spillantini et al., 1997), caused a dramatic change on the view of genetic contribution in the development of PD. Since then, distinct mutations in the α-synuclein gene, *SNCA*, have been found in familial cases of PD. In addiction to the point mutations (Kruger et al., 1998, Polymeropoulos et al., 1997, Zarranz et al., 2004), gene duplications and triplications have also been reported (Chartier-Harlin et al., 2004, Ibanez et al., 2004, Singleton et al., 2003). Interestingly, a gene triplication of *SNCA* causes a form of PD that has earlier onset and rapid progression when compared to the duplication, suggesting that the expression levels of α-synuclein are related to its toxicity.

α-synuclein is a protein of 140 amino acids, with a physiological function that is not clearly unravelled. Nonetheless, it seems to be involved in synaptic plasticity and vesicular regulation of DA (Bellani et al., 2010). The synuclein-protein family comprises the α, β and γ members and do not seem to be present in invertebrates or bacteria, since no homologues have been identified. α-synuclein is highly expressed in the central nervous system (CNS) of vertebrates, found primarily localized at presynaptic terminals, associated with vesicles and the plasma membrane. This protein appears to have a naturally unfolded nature *in vitro*, but it has been suggested that within the cell it associates with phospholipid membranes and acquires a α-helical rich conformation that promotes its stabilization (Eliezer et al., 2001).

In order to have a better understanding of the function of α-synuclein, knockout (KO) mice were generated. Nevertheless these animals do not show neurodegeneration (Chandra et al., 2004). The triple KO mice (absence of the three members of the synuclein family) show a decrease in lifespan and age-dependent neuronal dysfunction (Greten-Harrison et al., 2010). As opposed to the small effect of suppressing α-synuclein expression, enhanced expression of α-synuclein, in a range that is well matched with gene multiplication, clearly inhibits synaptic transmission by decreasing the release of neurotransmitters (Nemani et al., 2010).

From the fact that α-synuclein is prone to unfold and aggregate *in vitro*, as well as the presence of α-synuclein aggregates in LBs, originated the concept that α-synuclein toxicity may be a result of its propensity to form aggregates. The observation that expression of the *SNCA* mutant variants and multiplications increases the formation of oligomers and fibrils in cultured cells, transgenic animals and human brain further supported this hypothesis (Martin et al., 2011). Additionally, the fairly selective vulnerability of dopaminergic neurons to α-synuclein pathology may be due to a stabilizing effect of DA on protofibrillar α-synuclein aggregates that cause toxicity (Conway et al., 2001). Furthermore, aggregation of the α-synuclein is enhanced with paraquat and rotenone treatments. α-synuclein aggregation as a toxic mechanism in PD could also explain the age-dependent increase in the incidence of the disease. ROS and ROS-induced damage to proteins, lipids and DNA increase with age and oxidation and nitrosylation of α-synuclein also promote its aggregation (Martin et al., 2011).

From the majority of studies focusing on the biological properties of α-synuclein, it appears that protein misfolding and aggregation plays a crucial role in the pathogenesis of PD. However, it is not clear which species are more toxic, oligomers or

fibrils. It is possible that the formation of aggregates constitutes a protective mechanism to decrease toxicity. Nevertheless, the presence of aggregates in cell and animal models seems to correlate with increased toxicity (Wan and Chung, 2012). This controversy should be elucidated by further studies employing distinct model systems of neurodegeneration caused by α-synuclein.

### 1.2.2 LRRK2

The *PARK8* locus encodes for the Leucine-rich repeat kinase 2 (LRRK2), also known as dardarin, a large protein of 2527 amino acids, with multiple domains. As a member of the ROCO family of proteins, LRRK2 is characterised by the presence of a Ras-of-Complex (ROC) GTPase domain beside a C-terminal-of-ROC (COR) linker region. Additionally, LRRK2 contains a serine/threonine kinase domain. The central ROC-COR-kinase catalytic region is flanked by putative protein-protein interaction domains: leucine-rich repeat motifs, ankyrin and WD40 domain. LRRK2 dimerisation is essential for its kinase activity.

LRRK2 is expressed in many tissues, and within the brain its expression is lower in the substantia nigra, when compared to the high levels of expression in the striatum, cerebral cortex, cerebellum and hippocampus (Gandhi et al., 2009). Despite a large body of evidence for LRRK2 kinase activity and the identification of several substrates *in vitro*, it is still not clear whether these are authentic substrates *in vivo*, in mammals (Trancikova et al., 2012). However it is known that LRRK2 undergoes autophosphorylation and this process seems to regulate its kinase activity.

Mutations in the *LRRK2* gene are the most frequent cause of heritable PD. Most of the *LRRK2* mutations affect the ROC-COR-kinase region. To date, it has not been possible to define a common mechanism of action for all the mutations reported. Some of the

mutant forms of the protein result in increased kinase activity *in vitro*, whereas other mutations do not. The fact that the mutations are dominantly inherited, together with the absence of deletions or truncation mutations support the idea that the toxic mechanism of LRRK2 is due to a gain-of-function (Corti et al., 2011). As mentioned above, *LRRK2* mutations not only cause autosomal dominant familial PD, but can also increase the risk of developing sporadic PD (Shulman et al., 2011).

Animal models have been used to elucidate the function of LRRK2, as well as the perturbations caused by the PD-linked mutations. Several LRRK2 transgenic mice show defects in dopaminergic neurotransmission (Gandhi et al., 2009). LRRK2 has been implicated in a diversity of pathways involving several cellular functions: vesicular trafficking, neurite outgrowth, cytoskeletal regulation, autophagy, mitochondrial function, and translational control. LRRK2 may regulate mitochondrial function through the regulation of mitochondrial morphology and turnover (Tsika and Moore, 2012). In spite of the intense scientific investigation focusing on LRRK2, further studies are required to elucidate the physiological function and mechanism that causes PD in the presence of pathologic mutations. The identification of specific substrates for this kinase could be of great value to address its function *in vivo*.

### 1.2.3 Parkin

Mutations in the *Parkin* gene are the most common cause of early-onset Parkinsonism. Actually, the onset of the disease is often before 20 years of age, and was given a distinct designation: autosomal recessive juvenile Parkinsonism (AR-JP). *Parkin* mutations are associated with a marked loss of dopaminergic neurons of the substantia nigra. Several mutations in *Parkin* have been reported, from missense

mutations to gene rearrangements (Abou-Sleiman et al., 2006) and Parkin is generally found in LBs from either sporadic or familial PD patients.

Parkin is a protein of 465 amino acids, with an ubiquitin-like (UBL) domain at the amino (N) terminus, two RING (Really Interesting New Gene) fingers domain at the carboxyl (C) terminus and an in-between RING (IBR) domain separating the UBL from the RING domains. Parkin functions as an E3 ubiquitin ligase (Shimura et al., 2000, Zhang et al., 2000), and it has been hypothesized that the pathological mechanism of *Parkin* mutations is the loss of its E3 ligase activity. However, this hypothesis was not supported by *in vitro* ubiquitination assays, whereby single amino acid substitutions only rarely affect Parkin ligase activity (Corti et al., 2011).

Expression of wild-type (WT) Parkin protects cultured neuronal cells against several insults, such as serum withdrawal and treatment with agents, namely hydrogen peroxide and $MPP^+$. On the contrary, expression of PD-linked mutant forms of Parkin enhances oxidative damage and apoptotic cell death (Hyun et al., 2005). Interestingly, Parkin itself can be modified by oxidative and nitrosative stress, which alters its E3 ligase function and can cause its inactivation (Chung et al., 2004, Yao et al., 2004). Moreover, nitrosylated Parkin has been detected in brains of PD patients and also those from animals treated with MPTP and rotenone. Furthermore, DA can compromise the E3 ligase activity of Parkin. Together, these observations suggest that inactivation of Parkin might be of great relevance for PD pathogenesis (Abou-Sleiman et al., 2006). Moreover, a large variety of stressors, including paraquat, rotenone, $MPP^+$, nitric oxide, 6-hydroxydopamine (6-OHDA) and iron, as well as DA, seem to have the ability to cause Parkin insolubility and aggregation. These findings suggest that Parkin aggregation could also be a possible mechanism of PD pathogenesis in sporadic cases of the disease (Wang et al., 2005).

Several putative Parkin substrates have been identified, including Cell division control related protein 1 (CDCrel-1), Parkin-associated endothelin receptor-like receptor (Pael-R) and Synphilin-1, among others. CDCrel-1 belongs to a family of GTPases, the septins. It is highly expressed in the nervous system and has been found to associate with synaptic vesicles. Expression of CDCrel-1 can cause neurodegeneration (Dawson and Dawson, 2010). Pael-R is expressed in dopaminergic neurons, although its expression is more profound in oligodendrocytes. In cell culture experiments, Pael-R expression has been shown to activate the unfolded protein response (UPR), and Pael-R itself becomes insoluble. This phenomenon can be suppressed by Parkin expression (Dawson and Dawson, 2010). Synphilin-1, a α-synuclein-interacting protein, has been shown to interact with and be ubiquitinated by Parkin.

None of these three substrates fulfil the criteria of a "true" substrate, as none of these proteins has been consistently shown to accumulate in the absence of Parkin *in vivo*. More recently, a new substrate was identified that accumulates in the absence of Parkin activity in PD. Parkin interacting substrate (PARIS) is up-regulated in the brain of sporadic PD patients, as well as in the brain of *Parkin* conditional KO mice, particularly in the striatum and substantia nigra. PARIS is a transcriptional repressor of peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1α (PGC-1α) expression. The overexpression of PARIS causes loss of dopaminergic neurons in the substantia nigra, which can be rescued by co-expression of either Parkin or PGC1-α (Shin et al., 2011). Additional substrates are likely to be identified in the future, and this will shed light into mechanisms relevant for the dopaminergic neuronal loss due to *Parkin* mutations.

Important information about the physiological role of Parkin has been achieved using animal models, which have revealed that Parkin is important for mitochondrial

function, although the mechanism is currently being elucidated and it will be further discussed below.

### 1.2.4 PINK1

PTEN-induced putative kinase 1 (PINK1) is a 581-amino-acids protein. It is ubiquitously expressed, contains an N-terminal mitochondrial targeting sequence, a predicted transmembrane domain, a highly conserved serine/threonine kinase domain and a C-terminal regulatory domain. PINK1 was first shown to be up-regulated in cancer cells by the tumour suppressor phosphatase and tensin homologue (PTEN) (Unoki and Nakamura, 2001). PINK1 is expressed throughout the human brain and in all cell types. The immunostaining of endogenous PINK1 consists of a cytoplasmic punctate pattern, consistent with mitochondrial localization (Gandhi et al., 2006). PINK1 seems to be a mitochondrial membrane integral protein with a cytosol-facing kinase domain (Zhou et al., 2008). The transmembrane domain is essential for anchoring PINK1 to the mitochondrial membrane. PINK1 appears to be a rapidly turned-over protein, under normal conditions, and its expression levels are normally low (Kawajiri et al., 2011). PINK1 has been detected in LBs from sporadic PD brains (Gandhi et al., 2006).

Mutations in *PINK1* are the second most common cause of autosomal recessive early-onset Parkinsonism. Numerous point mutations and truncations affecting different domains of the protein have been identified. These mutations can reduce or impair the kinase activity and enhance PINK1 degradation or misfolding (Dagda et al., 2009). The precise functions of this mitochondrial kinase are currently the subject of intense scrutiny with many functional insights resulting from work done in animal models, especially *Drosophila melanogaster*. The mitochondrial localization of PINK1, as well

as the discovery of its impact in mitochondrial function have supported the view that mitochondrial dysfunction might be a central mechanism in PD pathogenesis.

### 1.2.5 DJ-1

Mutations in *PARK7*, which encodes the chaperone DJ-1, have been found in a small number of autosomal recessively inherited cases of PD. DJ-1 is a small protein of 189 amino acids (20 kDa) that belongs to the highly conserved ThiJ/Pfp1 family of molecular chaperones, which are up-regulated by oxidative stress. DJ-1 protein is expressed in the nervous system as well as in other tissues. It can be found in different subcellular localizations: cytoplasm, nucleus and mitochondrion. DJ-1 exists as a homodimer and it has been suggested that the oxidative state of its cysteine residue at position 106 is important for the chaperone activity of the protein. In the presence of oxidative stress DJ-1 translocates from the cytoplasm to the outer mitochondrial membrane (OMM), which seems to account for a neuroprotective effect (Corti et al., 2011).

DJ-1 was first associated with oncogenesis and infertility in mice. DJ-1 has been reported to interact with several proteins and has been ascribed several functions, such as transcription regulation, oncogenic transformation, mRNA stabilization, redox-sensitive molecular chaperone activity and antioxidant (Cookson, 2003). One of the PD-linked *DJ-1* mutations was found to increase the degradation of DJ-1 by the proteasome (Alvarez-Castelao et al., 2012). The localization of DJ-1 to the mitochondria seems to protect cells from some insults and this reinforces the importance of mitochondria for PD pathogenesis.

### 1.2.6 ATP13A2

The ATP13A2 protein localizes to the lysosomal membranes and is a large transmembrane protein with predicted ATPase activity. It has been shown that truncated forms of the protein can be retained in the endoplasmic reticulum (ER) and be degraded by the proteasome. The function of this protein is unknown, although its knockdown or the expression of the PD-linked mutated forms cause general lysosomal deficiency and cell death (Dehay et al., 2012b). Interestingly, a significant increase in the ATP13A2 mRNA levels has been found in the substantia nigra of PD patients (Dehay et al., 2012a).

## 1.3 Animal models of PD

An ideal animal model of PD should faithfully reproduce the clinical manifestations (behavioural defects), pathological components and molecular abnormalities characterizing the disease. The reality is usually somehow far from the ideal, as the animal models do not perfectly mimic the aetiology, progression, or pathology of PD. Despite this difficulty, studies using animal models of PD have given a significant contribution to the current knowledge about this complex and multifactorial disorder.

### 1.3.1 Toxin-induced animal models

Since the incident of human contamination with MPTP and appearance of PD-like symptoms in some of the individuals, several efforts have been made to develop animal models based on MPTP treatment. As mentioned above, the MPTP metabolite $MPP^+$ can accumulate in mitochondria from dopaminergic neurons and cause Complex I

inhibition, resulting in decreased efficiency of oxidative phosphorylation (OXPHOS) and increased production of ROS. MPTP-based animal models have been very useful in PD research and it has been noticed that the phenotypes can vary in some extent depending on the animal species and on the nature of the treatment, either acute or chronic. Non-human primates are more vulnerable than rodents to MPTP treatment. In general, MPTP-based animal models show parkinsonian features that resemble the human disease, loss of dopaminergic neurons and DA content in nigrostriatal regions, α-synuclein-positive inclusions and a good response to L-dopa and DA agonists (Hisahara and Shimohama, 2010).

6-OHDA is another neurotoxin that has been used to develop animal models of PD. 6-OHDA is unable to cross the blood brain barrier (BBB), meaning that systemic administration is not suitable to obtain the neurotoxic effect in the brain. Intracerebral injections of the toxin cause loss of tyrosine hydroxylase (TH)-positive neurons and loss of DA content in nigrostriatal regions, without formation of LBs. Animals have good response to L-dopa and DA agonists treatments. The 6-OHDA mechanism of action is also in the mitochondria, causing inhibition of Complex I.

The pesticide and insecticide rotenone is still currently used worldwide, although there has been a voluntary reduction of its usage due to its association with PD. Rotenone is a naturally occurring compound from the roots of *Lonchocarpus* species. It is a lipophilic substance, which can permeate cellular membranes easily and thus crosses the BBB. Rotenone is a potent mitochondrial Complex I inhibitor, and animal models based on treatment with this toxin show loss of dopaminergic neurons, associated with reduced DA content in nigrostriatal regions of the brain. Animals treated with rotenone display parkinsonian features that show a good response to L-dopa and DA agonists treatments. LBs can also be present (Beal, 2010). However, there

is variability in the results achieved by treating animals with rotenone. Moreover, rotenone treatment is associated with high animal mortality, which makes this model not advantageous when compared to other toxin-induced animal models for studying PD (Blesa et al., 2012).

The herbicide paraquat has been linked to PD by epidemiologic studies, and its administration to animal models causes a parkinsonian phenotype similar to that of MPTP treatment. Decrease DA content can be detected in nigrostriatal regions and chronic treatment with paraquat leads to the appearance of LBs neuropathology (Hisahara and Shimohama, 2010).

The toxin-based animal models have been very useful for testing new potential therapeutic compounds; however, many of these compounds did not achieve good results in clinical trials. It is still controversial whether these animal models can mimic the human pathology effectively and reproducibly.

### 1.3.2 Genetic animal models

The modelling of PD in several genetic animal models has provided powerful information about the molecular and cellular mechanisms of neurodegeneration caused by mutations in the *PARK* genes. Mouse models are generally preferred in the studies of human diseases, in part because they possess homologues for many human disease-related genes, and they also have a complex neuronal network somehow similar to humans. The availability of genetic manipulation techniques, along with well-known cell biology and specific behavioural tests constitute other advantages of using mouse model systems. Despite these advantages, the use of mouse models in the PD research has faced difficulties, since no real recapitulation of the PD features has been easily achieved.

Based on the presumed gain-of-function mechanism of α-synuclein toxicity, the overexpression of the WT and PD-linked mutant forms of the protein has been used to create genetic animal models for studying PD. In mice, overexpression of WT human α-synuclein in neurons causes a reduction in the striatal dopaminergic nerve terminals, a decline in the mice motor performance, and the formation of proteinaceous inclusions, immunoreactive for both synuclein and ubiquitin, in both the cytoplasm and nucleus (Masliah et al., 2000). In spite of the formation of α-synuclein inclusions in the substantia nigra and other areas of the brain, no dopaminergic neuronal death has been reported for the mice expressing either the human WT or mutant form of α-synuclein. To increase the specificity of this model for the studies of PD, strategies, such as the use of adenoviral vectors for the expression of WT or mutant human α-synuclein, were employed. These approaches led to the development of mice, rats and non-human primates models that show a substantial loss of dopaminergic neurons. The formation of α-synuclein-rich aggregates was observed, and, interestingly, in a rat model, this phenomenon could be prevented by co-expression of Parkin (Shulman et al., 2011).

Other model organisms were used to study α-synuclein pathology, and even in yeast, *C. elegans* and *Drosophila*, expression of human α-synuclein causes toxicity. These model organisms have been very useful for large-scale screens for modifiers (genetic or pharmacological) of α-synuclein toxicity. Interestingly, it has been reported that the expression of the chaperone heat shock protein (Hsp) 70 ameliorates the toxicity induced by human α-synuclein in *Drosophila*, indicating that abnormal protein folding is likely to be an important factor in PD pathogenesis (Auluck et al., 2002).

Little has been reported for animal models based on genetic manipulation of *LRRK2*. Nevertheless, experimental evidences from *LRRK2* transgenic mice implicate a

gain-of-function mechanism in the neurotoxicity induced by LRRK2 (MacLeod et al., 2006). Of great interest is the finding that α-synuclein aggregation and neurotoxicity is enhanced in the presence of high levels of LRRK2, while, conversely, the α-synuclein neurotoxicity is diminished in the absence of LRRK2.

The mouse models generated for studying the effects of the absence (KO mice) of Parkin, PINK1 or DJ-1 have a very subtle phenotype, with no apparent loss of dopaminergic neurons (Blesa et al., 2012). Nevertheless, it has been reported that a transgenic mice model expressing a mutant form of Parkin present age-dependent locomotor defects and dopaminergic neuronal degeneration (Lu et al., 2009).

The fruit fly *Drosophila melanogaster* has been shown to be a very good model system for studying the effects of KO either *parkin*, *Pink1* or *DJ-1*. Important insights to the PD field came from studies using *Drosophila* as a model system. Numerous benefits can be found in the use of this invertebrate to model human disorders, including neurodegenerative diseases, such as PD.

## 1.4 *Drosophila melanogaster*: a powerful model for PD studies

*Drosophila* has emerged has a powerful tool for studying PD pathogenesis. The use of this invertebrate in many research fields is well established. The whole genome of the fruit fly has been sequenced and there are plenty of genetic technologies available for using this model organism.

There are many advantages of working with *Drosophila*: it is a small animal, which makes it easy and cheap to maintain large number in the laboratory; it has a compact genome size (approximately 13 000 genes), with limited genetic redundancy; it has a short generation time and lifespan; there are mutants available for many of the

genes; and it presents a complex nervous system. Additionally, 77% of the human disease genes are conserved in the fruit fly (Guo, 2012). Phenotypic analysis can be achieved in the fly when gene expression is reduced or eliminated (KO) or by overexpression of a gene product. In case whereby a disease-related human gene does not have an orthologue in *Drosophila*, a gain-of-function approach to express the human protein in the fly can be used, and the consequences of such manipulation are observed. This approach is still very useful and informative in exploring the cellular and molecular mechanisms involved. The *Drosophila* genome encodes orthologues of all the PD-related genes that have been identified, with the exception of PARK1/4 (α-synuclein). Heterologous expression of either WT or mutant forms of human α-synuclein leads to the development of a PD-like features in the fruit fly (Auluck et al., 2002).

There are several genetic tools of an enormous value in using *Drosophila* as an animal model. Many transposon insertion lines have been generated and the insertion sites have been identified. Importantly, these lines are widely available from *Drosophila* stock centres. The transposon insertions can affect gene expression when inserted in the coding region or the regulatory regions of a gene. Alternatively, null mutations can be generated by imprecise excision of the P element, creating a gene deletion in the coding region.

The tissue-specific overexpression or down-regulation of a gene can be easily achieved in *Drosophila* using the UAS/GAL4 system (Brand and Perrimon, 1993). This powerful system makes use of the yeast transcription activator GAL4, capable of stimulating transcription of reporter genes under the control of Upstream Activating Sequences (UAS). Characterised *Drosophila* promoters can thus be used to drive GAL4 transcription, which allows specific, temporal and spatial expression of GAL4. These

GAL4 expressing lines are commonly known as drivers (Brand and Perrimon, 1993). In the UAS/GAL4 system, the expression of the gene of interest, the responder, is controlled by the presence of the UAS element. Since the transcription of the responder requires the presence of GAL4, its absence in the GAL4-responsive lines maintains them in a transcriptionally silent state. To achieve expression of a gene, a GAL4-responsive line must be crossed with a GAL4-expressing line. The resulting progeny will express the responder in a spatial and temporal manner reflected by the GAL4 driver (Figure 1.3) (Duffy, 2002).

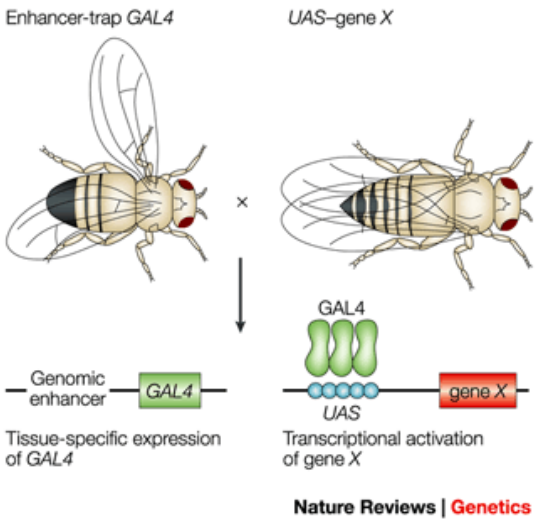



**Figure 1.3: Directed gene expression in *Drosophila*.** To generate transgenic lines expressing GAL4 in numerous cell- and tissue-specific patterns, the GAL4 gene is inserted randomly into the genome, driving GAL4 expression from numerous different genomic enhancers. A GAL4-dependent target gene can then be constructed by sub-cloning any sequence behind GAL4 binding sites (UAS). The target gene is silent in the absence of GAL4. To activate the target gene in a cell- or tissue -specific pattern, flies carrying the *UAS-Gene X* are crossed to flies expressing GAL4. In the progeny of this cross, it will be possible to activate *UAS-Gene X* in cells where GAL4 is expressed. Figure adapted from Muqit and Feany, 2002.

An additional advantage for the use of the fruit fly as a model system is the easiness of conducting large-scale screenings. Having a disease-associated phenotype, one can look for genetic enhancers or suppressors of the phenotype. In addition, it is

also possible to search for chemical compounds that suppress the phenotype. Importantly, the studies in *Drosophila* can be performed in any of the developmental stages of this animal. The life cycle of the fruit fly consists of four stages: egg, larvae, pupae, and adult. The larval stage is divided into three subdivisions, termed the first, second and third instar larvae (Figure 1.4).

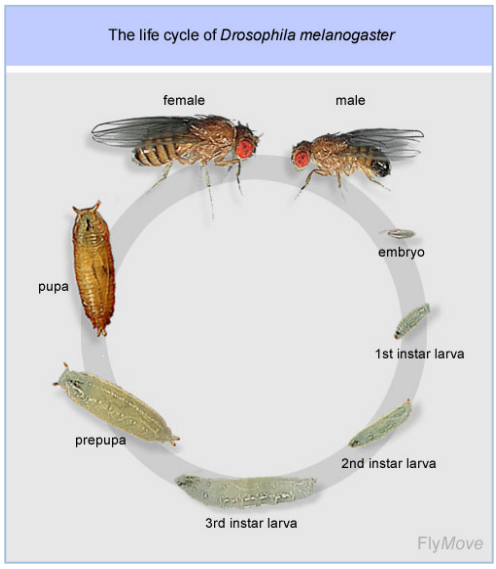



**Figure 1.4: The life cycle of *Drosophila melanogaster*.** Several stages of development in the fruit fly life cycle: the egg gives rise to an embryo, followed by the larval stage (first, second and third instar). The larvae will transform into pupae from which the adult fly emerges. Figure adapted from http://www.anatomy.unimelb.edu.au/researchlabs/whitington/index.html

Great enlightenment about PD pathogenesis was obtained from work employing *Drosophila* as an experimental model. While mutations of the PD recessive genes, *PINK1*, *Parkin* and *DJ-1* did not originate strong phenotypes in vertebrate animal models, the *Drosophila* models showed strong phenotypes. Studies in the flies were the main source for most of the current knowledge of *Pink1* and *parkin*.

## 1.5 The PINK1/Parkin pathway

As mentioned above, mitochondrial involvement in PD pathogenesis has been highlighted in the past, following the discovery of the toxic effects of $MPP^+$ and mitochondrial toxins, such as rotenone and paraquat (Abou-Sleiman et al., 2006). The generation of null mutant flies for *parkin* and *Pink1* strongly reinforced the importance of mitochondria for PD pathogenesis.

*Drosophila parkin* mutants have been characterised, showing complete male sterility, severe mitochondrial pathology, apoptotic muscle cell death, and behavioural abnormalities (flight and climbing defects) (Greene et al., 2003, Pesah et al., 2004). Importantly these mutants show a loss of dopaminergic neurons (Whitworth et al., 2005). Interestingly, when *Pink1* mutant flies were generated, a clear resemblance of their phenotype to that of *parkin* mutants was noticed. *Pink1* mutants show male sterility, apoptotic muscle degeneration, mitochondrial morphological defects (swollen mitochondria with disrupted cristae), decreased levels of ATP and mitochondrial DNA (mtDNA) and behavioural abnormalities (flight and climbing defects) (Clark et al., 2006, Park et al., 2006). Additionally, dopaminergic neuronal loss has also been reported (Park et al., 2006).

Genetic-interaction studies have demonstrated that *parkin* expression can compensate for the loss of *Pink1*, since the *Pink1* mutant phenotype could be rescued by parkin. However, the *parkin* mutant phenotype could not be rescued by *Pink1* expression. Moreover, double mutants of *Pink1* and *parkin* do not show an enhancement of the phenotype compared to either of the mutants alone. All these observations suggested that *Pink1* and *parkin* act in a common linear genetic pathway,

with *Pink1* acting upstream of *parkin* to promote mitochondrial integrity (Clark et al., 2006, Park et al., 2006, Yang et al., 2006).

Following the identification of the genetic interaction between *Pink1* and *parkin* in *Drosophila*, further studies focused on the molecular basis of such interaction. PINK1 is a mitochondrial protein, whose kinase domain seems to face the cytosol (Zhou et al., 2008), making it possible that putative PINK1 substrates reside in this sub-cellular compartment. It has been reported that PINK1 directly phosphorylates Parkin, thus controlling its E3 ligase activity. Moreover, the PINK1 mutations related to PD seem to impair this phosphorylation (Sha et al., 2010). More recently, the phosphorylation of Parkin by PINK1 was confirmed by another group, which proposed that the phosphorylation occurs at serine 65 in the UBL domain of Parkin, enhancing its ligase activity (Kondapalli et al., 2012).

The *Drosophila* models were also pivotal in showing a genetic interaction between *Pink1*, *parkin* and the mitochondrial fusion and fission machineries. Both *Pink1* and *parkin* null male mutants are infertile. During spermatogenesis in *Drosophila*, mitochondria undergo significant morphological changes that require fusion to form a large spherical nebenkern and subsequent fission when the nebenkern unfurls to yield two mitochondrial derivatives (Fuller, 1993). It was noticed that *Pink1* and *parkin* mutants did not have these two characteristic mitochondrial derivatives, as if the fission of the nebenkerns was impaired. Further investigation showed that either overexpressing mitochondrial fission factors or down-regulating fusion factors ameliorated the severity of the *Pink1* and *parkin* mutant phenotypes (Deng et al., 2008). However, Pink1 and parkin do not seem to act in parallel with the fusion and fission machineries. The knockout of key regulators of mitochondrial dynamics (*dMfn*, *opa1*, *drp1*) causes lethality in flies. Additionally, the loss-of-function of *Pink1* or *parkin*

causes a different phenotype from the *drp1* loss of function. All these genetic studies suggest that Pink1 and parkin play a regulatory role in mitochondria dynamics (Deng et al., 2008).

## 1.6 Mitochondria: essential organelles of eukaryotic cells

Mitochondria are cellular organelles responsible for the production of the majority of the adenosine triphosphate (ATP). Additionally they act by buffering the concentration of calcium, and participate in different pathways that influence cellular homeostasis and fate, including cell death cascades. Mitochondria are two-membrane organelles with an outer membrane facing the cytosol and an inner membrane facing the matrix; the space between the membranes is referred to as the inter-membrane space (IMS). Both the outer and the inner membranes are composed of phospholipids and proteins; however, they have different properties. The outer membrane is highly permeable to small molecules due to the presence of porins within its structure, which allows the IMS to maintain approximately the same concentrations of ions and sugars as the cytosol. On the contrary, the inner membrane does not exhibit such permeability, thus the passage of molecules to the matrix is highly selective. Most IMS proteins have well-characterised roles in apoptosis following the release from the mitochondria into the cytosol, as it is the case of cytochrome c, Smac/DIABLO, HtrA2/Omi and endonuclease G (Endo G) (Radke et al., 2008).

The inner mitochondrial membrane (IMM) exhibits several invaginations that form the mitochondrial cristae. This increases its surface area and enables a higher efficiency of energy production. It is in the IMM that the electron transport chain (ETC) is located (Figure 1.5). The ETC is comprised of five protein complexes and is

responsible for the OXPHOS that results in the production of energy in the form of ATP, which is accompanied by consumption of oxygen and production of water. Complexes I, III and IV pump protons ($H^+$) into the IMS and create a gradient essential for the generation of ATP by Complex V (ATP synthase). This proton gradient is crucial for the normal functioning of mitochondria and is known as the mitochondrial membrane potential ($\Delta\Psi m$). ROS are also generated as a by-product of OXPHOS. ROS production can be partially modulated by two cofactors, coenzyme $Q_{10}$ at Complex III and cytochrome c at Complex IV (Mattson et al., 2008).

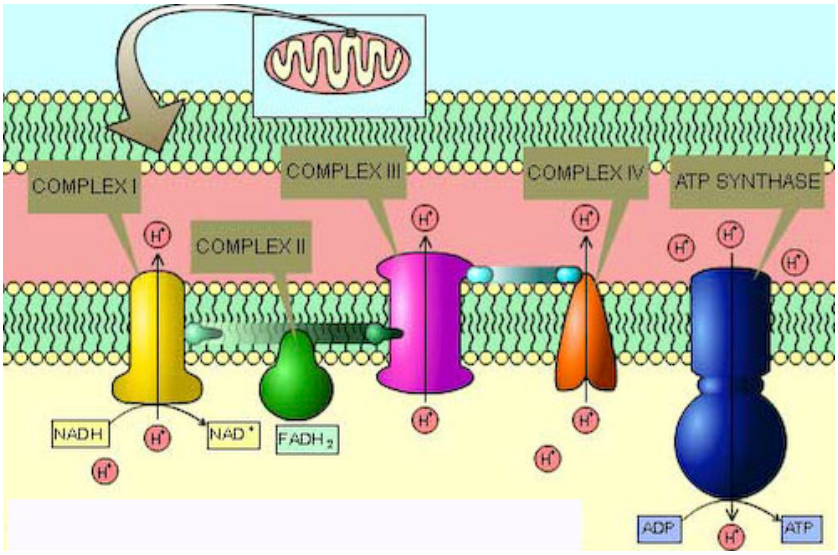



**Figure 1.5: Electron transport chain (ETC) at the inner mitochondrial membrane (IMM).** Electrons flow from one protein complex to the next, and during this process protons pass across the IMM to the inter-membrane space (IMS) at Complexes I, III and IV. An electrochemical gradient is generated through the increased proton content in the IMS compared to the matrix, known as the mitochondrial membrane potential ((ΔΨm). The proton gradient is used by the ATP synthase for the phosphorylation of ADP to ATP. The final acceptor of electrons at the ETC is molecular oxygen ($O_2$), which is reduced to water at Complex IV. Figure from http://schoolworkhelper.net/electron-transport-and-chemiosmosis/.

The mitochondrial matrix is a sub-compartment with high protein content, when compared to the cytosol. It accommodates the enzymes required for the Krebs cycle, the fatty acid oxidation and the haeme synthesis, as well as several chaperones and proteases involved in the folding and degradation of proteins. Additionally, the matrix

contains mtDNA, together with the proteins required for its transcription and translation. Nonetheless most of mitochondrial proteins (~1500) are encoded by nuclear DNA, thirteen proteins involved in the ETC are mitochondrially encoded (Ryan and Hoogenraad, 2007). This constitutes a challenge for the cell, since both nuclear and mitochondrial transcription must be tightly coordinated. Due to its localisation near the ETC, mtDNA is susceptible to oxidative damage, which may result in mutations.

The number of mitochondria is variable according to the tissue and cell type, and it reflects the energetic needs of the cell. For example, neuronal and muscle cells have high mitochondrial content when compared to other cell types. The number of mitochondria can be modulated, but these organelles cannot be generated *de novo*. Mitochondria biogenesis involves an increase in the translation of mitochondrial proteins (both nuclearly and mitochondrially encoded); recruitment of these proteins to the mitochondria, which results in mitochondria enlargement; and finally, division and generation of additional mitochondrion units (Ryan and Hoogenraad, 2007). Within a cell, a mitochondrion cannot therefore be considered as an individual and independent organelle. Mitochondria behave as a dynamic network, exhibiting successive cycles of fusion (mitochondrion units combining with each other) and fission (separation of mitochondrion units). Mitochondrial dynamics increases the ability of the cell to respond to different demands. The proteins responsible for mitochondrial dynamics are relatively well characterised, although new proteins are continually being identified that play roles on this active process. In mammals, at least three proteins are required for mitochondrial fusion: mitofusins Mfn1 and Mfn2 (for fusion of the OMM), and optic atrophy 1 (OPA1) (fusion of the IMM). Mutations in Mfn2 have been associated with Charcot-Marie-Tooth Disease type 2 (Braathen et al., 2010, Kijima et al., 2005), whereas mutations in OPA1 can cause dominant optic atrophy in humans (Hudson et

al., 2008). *Drosophila melanogaster* presents one sole orthologue of mitofusins, *dMfn* or *Marf*.

The opposing process of mitochondrial fission requires the dynamin-related protein Drp1 and Fis1. Most of the Drp1 protein resides in the cytosol, and it seems to be recruited to discrete spots on mitochondria to participate in the fission process (Chan, 2006). Due to the importance of mitochondria for cellular homeostasis, it is undeniable that the dysfunction of these organelles may result in severe consequences. Depending on the extent of the mitochondrial damage, distinct signalling pathways will be activated. If the extent of the damage is too severe, it might cause the dissipation of the $\Delta\Psi m$ and the release of pro-apoptotic proteins (such as cytochrome c and HtrA2), resulting in cell death.

Mitochondrial dysfunction has long been associated with the natural chronic process of ageing, as well as with neurodegenerative diseases, such as Huntington's disease (HD), AD and PD, and with metabolic diseases, such as diabetes. Neurons are particularly sensitive to mitochondrial dysfunction, which most likely reflects their high energetic needs and their absolute dependence on mitochondria to obtain most of the ATP required for neuronal function (de Castro et al., 2010). The presence of protein aggregates is also a characteristic of neurodegenerative diseases, not only PD (as mentioned above) but also AD. The importance of a proper protein folding has long been recognised, and the effects of un-/misfolding and aggregation have been linked to many pathological conditions. These aggregates may be cytosolic, such as LBs in PD and fibrillary tangles in AD, or extracellular, such as amyloid plaques in AD. However, little is known about the effects of unfolded proteins in mitochondria.

Protein homeostasis is essential to maintain the cells in a healthy state; therefore, it is crucial to prevent the accumulation of protein in a non-native form. A recent study

in the nematode *C. elegans* has shown that in this multicellular animal, a considerable number of proteins aggregate with age (David et al., 2010). These aggregation-prone proteins may also be related to the deregulation of proteostasis in some neurodegenerative diseases. A better understanding of the mechanisms involved in protein misfolding and aggregation will certainly be of a major interest to explain the causes and/or progression of certain diseases.

## 1.7 Mitochondrial quality control

Oxidative damage is likely to occur in the mitochondria (the main ROS source), presumably affecting protein, lipids and mtDNA. Multiple quality control systems exist to protect mitochondria, and ultimately the cell, against damage (Figure 1.6).

First, as part of the molecular quality control system within the organelle, mitochondrial chaperones and proteases are essential for the protein folding, the assembly of protein complexes and the degradation of misfolded and damaged proteins. Second, at the organellar level, there is a mechanism of quality control that depends on the dynamic nature of mitochondrial fusion and fission events (Tatsuta and Langer, 2008). When fusing with healthy mitochondria, damaged mitochondria can recover by sharing the essential solutes, metabolites and other components from the healthy partner. However, if the damage is too severe, mitochondria will no longer undergo fusion, but will, instead, lose their $\Delta\Psi m$ and be eliminated by autophagy, a process known as mitophagy. Recently, another potential mechanism of organellar quality control has been proposed. It consists in the formation of mitochondria-derived vesicles that seem to carry selected cargo directly to the lysosomes (Soubannier et al., 2012). This process does not require the loss of $\Delta\Psi m$ and is independent of ATG5 and microtubule-

associated protein light chain 3 (LC3), suggestive that this may complement the mitophagy process. Finally, when the damage to mitochondria is too severe, cellular quality control mechanisms can be activated. Upon the opening of the mitochondrial permeability transition pore (mPTP) and release of pro-apoptotic proteins from the IMS, the cell may undergo apoptosis.

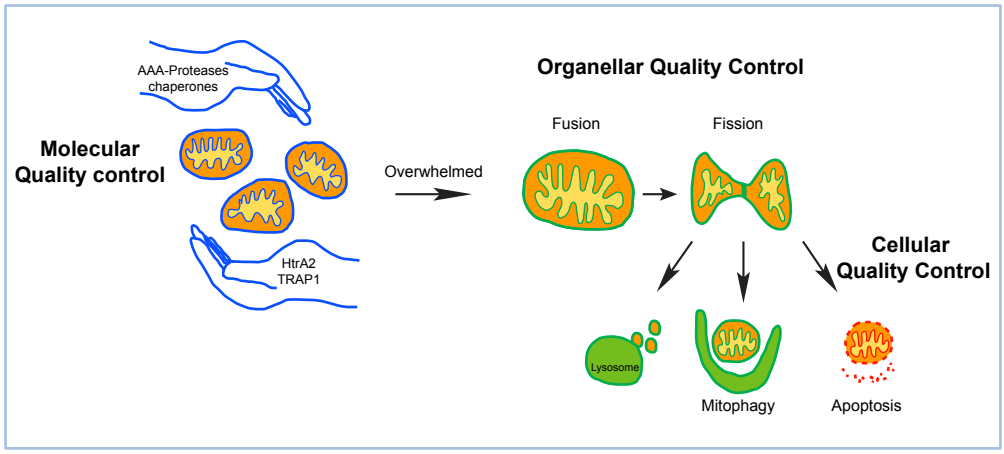



**Figure 1.6: Various mechanisms and effectors ensure mitochondrial quality control.** At the molecular level, it is assured through the protective action of chaperones and proteases, such as molecular chaperones in the mitochondrial matrix, AAA-proteases in the inner membrane, and HtrA2 and TRAP1 in the IMS (drawing highlighted in blue). When this mechanism of defence is not sufficient to maintain fully functional mitochondria, a second level of quality control exists at the organellar level. Fusion with healthy mitochondria might be sufficient to restore the functionality of a mitochondrion. Moreover, the formation of vesicles with mitochondrial content may be part of the organellar quality control system, by transporting potentially damaged products directly to lysosomes. In the presence of a more severe damage, fission might occur, producing small mitochondria that will not fuse, but will be degraded by autophagy (mitophagy) (highlighted in green). If the damage to the mitochondria is too severe, it might lead to the opening of the mitochondrial permeability transition pore (mPTP), releasing pro-apoptotic proteins into the cytosol, resulting in the activation of apoptosis (drawing highlighted in red). Figure adapted from Costa et al., 2012.

### 1.7.1 Effectors of molecular quality control: chaperones and proteases

Molecular chaperones play critical roles in the maintenance of protein homeostasis by aiding in the folding of newly synthesised and/or imported proteins, the assembly of multimeric protein complexes, protein translocation across membranes and protein degradation. In addition, molecular chaperones are essential for cellular

signalling. In eukaryotes, chaperones are segregated to the cytosol or to specific cellular compartments, such as the ER and mitochondria. The mechanisms through which the levels of the chaperones are modulated have not been well studied. Nevertheless, it is known that signalling pathways activating chaperone-encoding genes are repressed by free chaperones that are not engaged by client proteins (Yoneda et al., 2004). In contrast, if the levels of client proteins are too high, the availability of free chaperones can be dramatically decreased, and the repressor effect will not occur, possibly resulting in increased transcription of chaperones.

The mitochondrial chaperones and proteases are fundamental components of the mitochondrial quality control system, since they selectively remove unfolded or misfolded proteins, thus avoiding their deleterious accumulation. A failure in molecular quality control is emerging as a feature of neurodegenerative diseases, such as PD.

#### 1.7.1.1 AAA proteases

ATP-dependent proteases are present in the mitochondrial matrix as well as in the IMM. The $AAA^+$ (ATPases associated with various cellular activities) superfamily of peptidases is able to eliminate misfolded proteins from the mitochondria, and plays a regulatory role during mitochondrial biogenesis by processing proteins. Mutations in some of these proteases have been implicated in neurodegenerative conditions, such as the m-AAA protease subunit paraplegin in hereditary spastic paraplegia (Lee et al., 2011). Furthermore, a mutation in the mitochondrial chaperone Hsp60 has been identified in hereditary spastic paraplegia. The mitochondrial matrix chaperone Hsp60 mediates ATP-dependent folding of a wide variety of proteins, making it an important player in the molecular quality control mechanism.

### 1.7.1.2 PINK1, HtrA2 and TRAP1

The mitochondrial protein PINK1 plays important roles in mitochondrial quality control. It participates in molecular quality control through interactions with high-temperature-requirement protein A2 (HtrA2) and possibly the Tumour Necrosis Factor (TNF) receptor-associated protein 1 (TRAP1) and at the organellar level via its activity in signalling pathways involving Parkin and mitophagy. Mutations in the *PARK13* gene, encoding for HtrA2, have been found in sporadic PD patients, and HtrA2 protein has been detected in LBs (Bogaerts et al., 2008, Strauss et al., 2005).Furthermore, an increase in phosphorylated HtrA2 in brain tissues of sporadic PD patients and, conversely, reduced phosphorylation in brain tissue of PD patients with *PINK1* mutations, have also been reported (Plun-Favreau et al., 2007).

HtrA2 (also known as Omi) was first identified as a mammalian homologue of the bacterial endoprotease HtrA. In bacteria, HtrA acts as a chaperone at normal temperatures and as a protease at high temperatures and removes damaged or denatured proteins from the periplasm (Faccio et al., 2000). HtrA2 is a serine protease with a N-terminal inhibitor of apoptosis (IAP) domain identical to those in the *Drosophila* protein Reaper. It is localised in the IMS, and following certain stimuli, it is released to the cytosol, where it binds to IAPs, inhibiting their effects and promoting apoptosis. In addition to its caspase-dependent action, HtrA2 promotes cell death in a caspase-independent manner through increased proteolytic activity (Martins et al., 2003). HtrA2 was initially thought to be a pro-apoptotic protein homologous to Reaper. However, the observation that HtrA2-mutant mice present an extremely shortened lifespan (~ 30 days) and a neurological phenotype with parkinsonian features has promoted a different view of the role of this protein (Martins et al., 2004).

The phosphorylation state of HtrA2 influences its proteolytic activity and is dependent on PINK1 (Plun-Favreau et al., 2007). PINK1 and HtrA2 interact with each other, although it is not clear whether PINK1 phosphorylates HtrA2. Nevertheless, the phosphorylation of HtrA2 occurs upon p38 Mitogen Activated Protein Kinase (MAPK) activation and requires PINK1. It appears that p38, PINK1 and HtrA2 are components of a stress-sensing pathway and that phosphorylation of HtrA2 increases its proteolytic activity and enhances its cell-protective effects (Figure 1.7). The observation that higher levels of phosphorylated HtrA2 are present in the brains of sporadic PD patients has led to the hypothesis that this stress-sensing pathway is activated in PD. However, the precise stimulus that originates the stress is not known. Interestingly, in brains from PD patients with *PINK1* mutations, the levels of phosphorylated HtrA2 are reduced, emphasising the importance of PINK1 for HtrA2 phosphorylation (Plun-Favreau et al., 2007). This reduced HtrA2 phosphorylation may account for the pathogenic effects of the PD-linked *PINK1* mutations. Thus, HtrA2 appears to be part of a quality control pathway, acting downstream of PINK1.

HtrA2 KO mice show accumulation of unfolded proteins in the mitochondria, as well as defects in respiration. Loss of HtrA2 augments ROS levels, and treatment with antioxidants is neuroprotective in this condition. The up-regulation of the transcription factor C/EBP homology protein (CHOP) specifically in the brain of the Htra2 KO mice indicates the activation of the integrated stress response. Enhanced levels of CHOP have also been detected in brain tissue of idiopathic PD patients (Moisoi et al., 2009). This increase in the levels of CHOP specifically in the brain may account for the neuronal loss observed in this mouse model. However, the observation that CHOP is also up-regulated in regions of the brain that do not undergo neuronal death indicates that CHOP up-regulation is an early event that precedes neuronal loss.

HtrA2 may also play a role in the pathogenesis of AD. HtrA2 interacts with both intracellular amyloid β (Aβ) and the C-terminal tail of presenilins, although the functional significance of these interactions is obscure (Gupta et al., 2004, Park et al., 2004). More recently, HtrA2 has been linked to the amyloid precursor protein (APP) metabolism (Huttunen et al., 2007). A small percentage of HtrA2 localises alongside ER membranes and binds to immature APP *in vitro*. Finally, in HtrA2$^{-/-}$ cells, APP is stabilised, and Aβ production is increased. These observations further suggest that HtrA2 has a protective effect and functions in protein homeostasis.

Another piece of evidence relating PINK1 to mitochondrial molecular quality control comes from an *in vitro* study that revealed a physical interaction between PINK1 and the mitochondrial chaperone TRAP1 (Hsp75) (Pridgeon et al., 2007). PINK1 phosphorylates TRAP1 (also located in the IMS), which protects cells against apoptosis induced by hydrogen peroxide treatment. Notably, the PINK1-dependent phosphorylation of TRAP1 inhibits the release of cytochrome c from mitochondria and thereby increases cell survival under certain stress conditions (Pridgeon et al., 2007) (Figure 1.7). TRAP1 may therefore be another downstream effector of PINK1 involved in the molecular quality control pathway. TRAP1 is structurally related to the HSP90 family, possesses an ATP-binding domain and shows ATPase activity *in vitro*. TRAP1 activity can be inhibited by the Hsp90 inhibitors radicicol and geldanamycin. Nevertheless, TRAP1 does not form complexes with the classic co-chaperones of Hsp90 and might have distinct functions from the other members of the HSP90 family (Felts et al., 2000). In addition, TRAP1 exerts a protective effect against apoptosis when cells are subjected to various apoptotic stimuli (Hua et al., 2007, Masuda et al., 2004). Moreover, high levels of the TRAP1 protein may be involved in drug resistance in certain cancer treatments (Costantino et al., 2009, Landriscina et al., 2010). Further

investigation is required to unravel the role of TRAP1 in mitochondrial quality control and possibly other signalling pathways.

It is clear that maintenance of proteostasis is crucial for the health of the cell, and it is logical to hypothesise that regulation of quality control mechanisms must be of a great importance when an insult occurs. As mentioned above, the various sub-compartments of the cell exhibit different subsets of chaperones and proteases that have location-specific functions. Accordingly, depending on the insult and the organelle affected, different stress responses might be activated.

### 1.7.2 A mitochondrial unfolded protein response ($UPR^{mt}$) with its origin in the mitochondrial matrix

The concept of anterograde communication between the nucleus and mitochondria is well established; however, the communication process from the mitochondria to the cytosol and nucleus, the so-called retrograde signalling, remains unclear. The main problems in understanding this process lie in the identification of the molecules responsible for the initiation of the signal in the mitochondria and how this signalling is transmitted to the nucleus to alter nuclear gene expression. The mitochondrial retrograde signalling pathway is associated with both physiology and pathology. This signalling pathway exists in simple organisms, such as yeast, where it has primarily been studied, and in multicellular organisms. Despite the fact that the retrograde signalling pathway is conserved, the proteins and molecular mechanisms involved do not seem to be.

Compartment specificity in signalling by chaperones has been demonstrated for the ER, the cytosol and the mitochondrial matrix. These compartment-specific signals are responses to the presence of unfolded proteins in each one of these compartments.

Following accumulation of unfolded proteins in the cytosol, heat shock-sensitive transcription factors (HSFs) are activated and increase the transcription of proteins involved in adaptation to this stress, a process known as the heat-shock response (HSR). The signal derived from an excess of unfolded proteins in the ER, on the other hand, is sensed by the luminal domains of transmembrane proteins and is propagated through their cytoplasmic domains, ultimately leading to the transcription of nuclear genes encoding for proteins that alleviate the stress in the ER (e.g., the ER chaperone Binding immunoglobulin protein, BiP). This process is known as the unfolded protein response of the ER ($UPR^{ER}$) (Benedetti et al., 2006). The stress responses can also be induced by drug treatments. For example, treatment of cells with arsenite, which results in the accumulation of unfolded proteins in the cytoplasm, selectively activates cytoplasmic-chaperone gene expression. On the other hand, treatment of cells with tunicamycin, which blocks ER-specific N-linked glycosylation, activates the $UPR^{ER}$, resulting in increase expression of ER-localised chaperones (Yoneda et al., 2004).

Remarkably, little is known about the specific stress response resulting from the accumulation of unfolded proteins in the mitochondria, despite the fact that many different model systems have been used to investigate this. Nevertheless there are several important recent findings from studies in *Caenorhabditis elegans*, *Drosophila* and mammalian models systems.

The nematode *C. elegans* has been extensively used to study the $UPR^{mt}$. The ability to use sensitive reporter genes, as well as the ease of genetically manipulating this worm, makes it a convenient animal model to address this issue. The existence of an $UPR^{mt}$ in *C. elegans* became evident after the observation that provoking stress in the mitochondrial matrix, either by reduction of mtDNA through treatment with ethidium bromide or down-regulation of mitochondrial chaperones or proteases (*spg-7*), resulted

in transcriptional up-regulation of the mitochondrial chaperones *hsp-6* (*C. elegans* orthologue of the human mtHsp70) and *hsp-60* (Yoneda et al., 2004). Up-regulation is a specific response for these stress-modulator molecules, as the cytosolic or ER chaperones are not up-regulated nor are other mitochondrial enzymes. Further studies using an RNA interference (RNAi) library screening led to the discovery of several genes required for the UPR$^{mt}$ signalling (Figure 1.7).

The ubiquitin-like protein 5 (UBL-5) is essential for the UPR$^{mt}$ activation, although its down-regulation does not result in the accumulation of misfolded proteins in the mitochondria, suggesting that it must act downstream of this event. Moreover, upon mitochondrial stress, *ubl-5* is transcriptionally up-regulated, and its protein levels are increased in the nucleus (Benedetti et al., 2006). This might correspond to a feed-forward mechanism to amplify the stress response. It is of note that UBL-5 mRNA levels are reasonably high in mitochondrion-rich human tissues, such as the heart, skeletal muscle, liver, and kidney, and that polymorphisms in UBL-5 have been linked to obesity and diabetes (Benedetti et al., 2006).

*dve-1* is another gene involved in the UPR$^{mt}$ (Haynes et al., 2007). The DVE-1 protein is a putative transcription factor that is localised to the nucleus and possesses a predicted homeobox-related DNA-binding domain. Under mitochondrial stress, DVE-1 exhibits an altered nuclear distribution (punctate) and binds to the promoters of the mitochondrial chaperone genes *hsp-6* and *hsp-60*. Furthermore, in stressed worms, DVE-1 forms a complex with UBL-5. Interestingly, UBL-5 is also able to form a complex with the mammalian orthologue of DVE-1, Special AT-rich sequence-binding protein 2 (SATB2), but the relevance of the formation of this complex in the UPR$^{mt}$ in mammalian cells is unknown (Haynes et al., 2007).

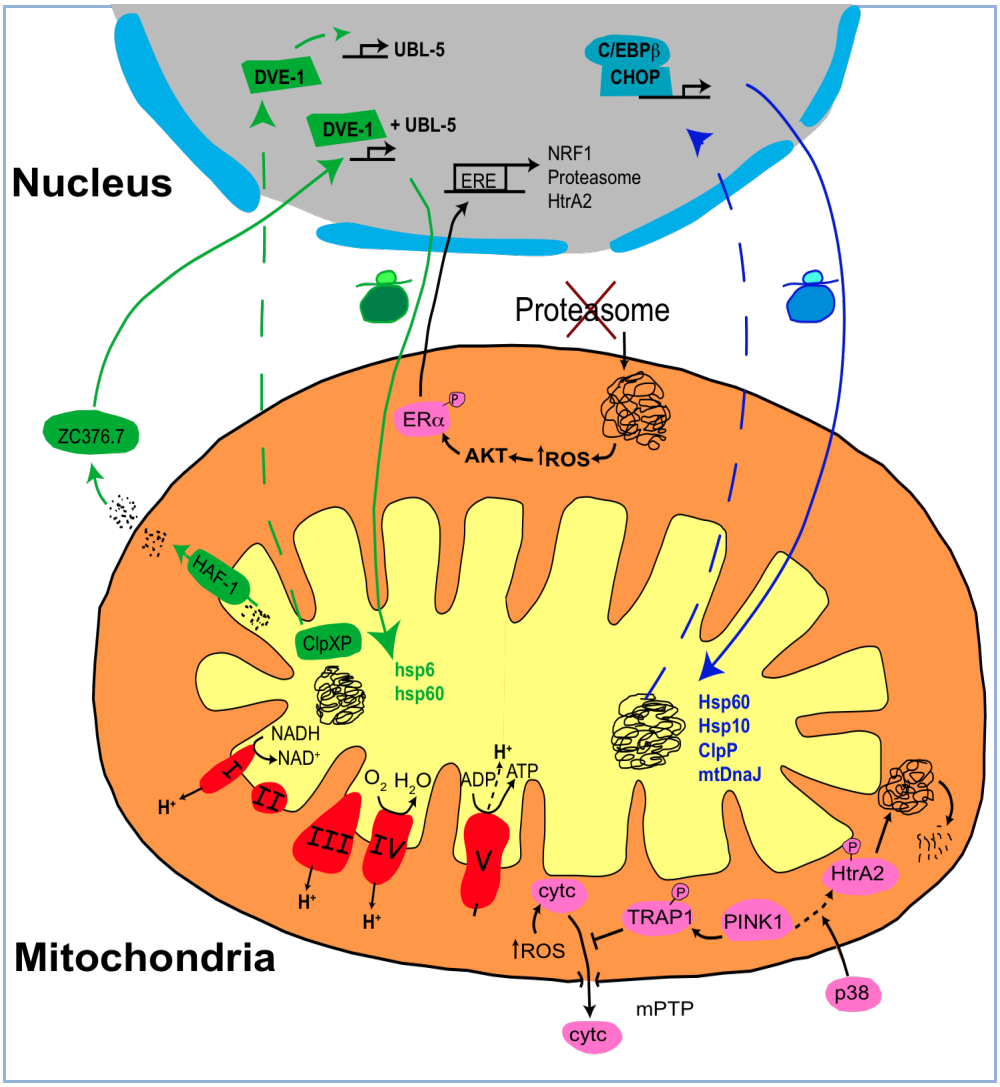



Figure 1.7: Mitochondrial stress signalling. Key components of the molecular quality control machinery and mitochondrial unfolded protein response (UPR$^{mt}$) are represented. Retrograde signalling transmits information from the mitochondria to the nucleus (dashed lines), whereas anterograde signalling transmits information in the opposite direction (solid lines). The mechanism of UPR$^{mt}$ activation *C. elegans* is highlighted in green on the left. In *C. elegans*, the accumulation of unfolded proteins in the mitochondrial matrix may be sensed by the ClpXP complex, which might generate a signal leading to certain nuclear events. The nature of this signal remains elusive, although ClpXP activity is known to result in the formation of small peptides that are extruded to the IMS through the peptide transporter HAF-1. Once in the IMS, the peptides can be released onto the cytosol and possibly activate a receptor or other effector molecules. The bZIP transcription factor ZC376.7 is activated by this peptide efflux from mitochondria and translocates to the nucleus, where it up-regulates UPR$^{mt}$-responsive genes. The nuclear events include the transcriptional up-regulation of *ubl-5* and the redistribution of DVE-1 in the nucleus. While both events are downstream of ClpXP, only *ubl-5* transcriptional activation is downstream of HAF-1. This UPR$^{mt}$ activation results in increased levels of both hsp60 and hsp6 mitochondrial chaperones. The mechanism of UPR$^{mt}$ activation in mammalian cells is highlighted in blue on the right side of the figure. The presence of an excess of unfolded proteins in the mitochondrial matrix promotes the transcriptional up-regulation of *Chop* and the formation of a complex between the transcription factor CHOP and C/EBPβ in the nucleus, resulting in the transcriptional activation of mitochondrial stress-responsive genes, such as *Hsp60*, *Hsp10*, *ClpP* and *mtDnaJ* and increased levels of their encoded proteins in the mitochondria. HtrA2 is located in the IMS and is part of the protein quality control machinery. The proteolytic activity of HtrA2 is phosphorylation-dependent, requires the presence of PINK1 and is mediated through p38 pathway activation. The molecular chaperone TRAP1 is also located in the IMS

and might be responsible for the proper folding and assembly of proteins and complexes. TRAP1 has also a cytoprotective effect in inhibiting the release of cytochrome c upon an increase in the levels of ROS in the mitochondria. The UPR of the IMS is represented in the upper middle part of the mitochondrion in the figure. This stress response can be activated on accumulation of aggregates of misfolded proteins in the IMS, which can be a result of failure of the proteasome, or a failure of the molecular quality control in this compartment. The presence of protein aggregates leads to the overproduction of ROS, activation of Akt and phosphorylation of the Oestrogen Receptor α (ERα). Activation of the ERα results in transcriptional activation of NRF1, the proteasome and HtrA2. Figure adapted from Costa et al., 2012.

CLPP-1 is also essential for UPR$^{mt}$ signalling. CLPP-1, the *C. elegans* orthologue of the bacterial ClpP protease, is localised to the mitochondrial matrix. ClpP is the ATP-dependent proteolytic sub-unit of a heteromeric complex formed with a partner AAA+ ATPase (Ortega *et al*., 2004). Bacterial ClpP functions in conjunction with two partner chaperones, ClpX and ClpA, associated with a wide range of substrates. Moreover, an increase in the levels of misfolded proteins enhances the degradation of these substrates. ClpX is the only ClpP partner known in eukaryotes, and to date, the substrates of mitochondrial ClpP remain unknown. In *C. elegans*, CLPP-1 is necessary for DVE-1 activation. Silencing of CLPP-1 by RNAi prevents the redistribution of DVE-1 in the nucleus and the transcriptional up-regulation of UBL-5, suggesting an upstream action of CLPP-1 in activation of the UPR$^{mt}$. Once CLPP-1 is localised to the mitochondria, it is likely that it senses the mitochondrial perturbation and mediates the proteolysis signal that is sent to the nucleus. Interestingly, CLPP-1–dependent proteolysis is required for the UPR$^{mt}$, as an inhibitor of CLPP-1 proteolytic activity abolishes its effect in activating the stress response (Haynes *et al*., 2007). ClpX is also relevant for UPR$^{mt}$ signalling in *C. elegans*. There are two isoforms of *ClpX* genes in the worm, both of which encode proteins homologous to bacterial ClpX, and when both genes are silenced, the UPR$^{mt}$ is attenuated. It appears that the function of ClpX in the worm is comparable to that in bacteria and mammals (Haynes *et al*., 2010). In bacteria, ClpP breaks down proteins into small peptides. Furthermore, studies in

yeast have shown that mitochondrial peptides can be extruded to the IMS and subsequently to the cytosol.

The mitochondrial transporter responsible for the extrusion of peptides from the mitochondrial matrix in yeast is the Mitochondrial inner membrane half-type ATP-binding cassette (ABC) transporter (Mdl1p). Interestingly, there are multiple orthologues of Mdl1p in *C. elegans*, and the most evolutionarily conserved of these, HAF-1, plays a role in the UPR$^{mt}$ signalling, as deletion mutations in the *haf-1* gene cause impairment of this stress response. Additionally, HAF-1 seems to be located in the IMM, which is consistent with its function in transporting cargo from the matrix to the IMS (Haynes et al., 2010). Epistasis analysis has placed HAF-1 upstream of *ubl-5* transcriptional induction, but not of DVE-1 redistribution in the nucleus, which suggests the existence of other transcription factors involved in the UPR$^{mt}$.

Subsequently, the protein ZC376.7, which includes a C-terminal leucine zipper domain, a predicted nuclear localisation sequence, and a nuclear export sequence, was identified. In unstressed worms, ZC476.7 is localised diffusely in the cytosol, but following mitochondrial stress it translocates to the nucleus. ZC376.7 appears to act downstream of HAF-1 and CLPP-1, because the nuclear localisation of this transcription factor in stressed worms is attenuated by down-regulation of either HAF-1 or CLPP-1 (Haynes et al., 2010). Further studies are required to better understand this signalling mechanism. It is still unclear how small peptides can activate the UPR$^{mt}$ and influence the subcellular distribution of ZC376.7. It is also of a particular interest to determine the downstream effectors that influence the function of DVE-1.

Interestingly, a recent work using *C. elegans* as a model system has demonstrated that the UPR$^{mt}$ can be activated in a cell-non-autonomous manner (Durieux et al., 2011). The mitochondrial stress caused by down-regulation of a

component of the ETC in the nervous system results in the activation of the UPR$^{mt}$ in the intestine of the animal (Figure 1.8). However, it seems that UBL-5 is not required for the UPR$^{mt}$ activation in this cell-non-autonomous manner. It is still not known which signal is transmitted from the nervous system to the periphery, and additional research is required to address this point.

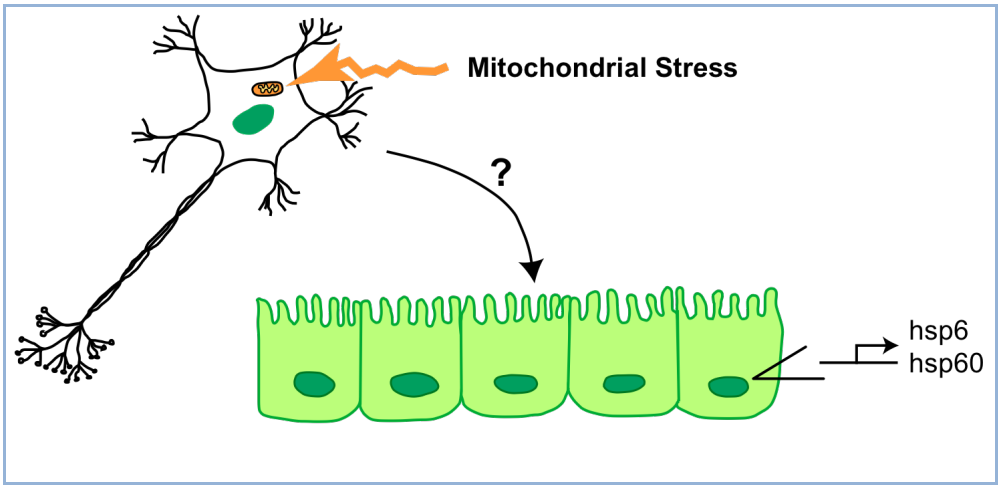



**Figure 1.8: Cell non-autonomous activation of the UPR$^{mt}$ in *C.elegans*.** Generation of mitochondrial stress in the central nervous system by depletion of an ETC protein sub-unit results in the activation of the UPR$^{mt}$ in distant tissues, such as the worm intestine (transcriptional up-regulation of *hsp6* and *hsp60* in the intestinal cells). The nature of the signal transmitted from the brain to the intestine is currently unknown. Figure adapted from Costa et al., 2012.

The existence of the UPR$^{mt}$ was first identified in mammalian cells by a work of Zhao and colleagues (Zhao et al., 2002). The expression of a truncated form of the mitochondrial matrix protein ornithine transcarbamylase (ΔOTC), which accumulates in an insoluble state in the matrix, results in the activation of nuclear genes encoding for mitochondrial stress proteins. Upon the accumulation of ΔOTC in mitochondria, the mitochondrial chaperones Hsp60, Hsp10, and mtDnaJ, as well as the protease ClpP, are up-regulated. In mammalian cells, mtHsp70 is not up-regulated by ΔOTC accumulation, which is consistent with its function as a protein transporter across membranes, rather

than a stress-sensing molecule. The transcription factor CHOP and CCAAT/enhancer-binding protein β (C/EBPβ) form a hetero-dimer and participate in this mitochondrial stress response by binding to the promoters of the UPR$^{mt}$-responsive genes. Furthermore, CHOP protein levels are increased in this model, indicating that *Chop* itself is an UPR$^{mt}$-responsive gene.

Six novel UPR$^{mt}$-responsive genes, encoding for the mitochondrial proteases YME1L1 and MPPβ, the import component Tim17A, and the enzymes NDUFB2, Endo G and thioredoxin 2, have been identified (Aldridge et al., 2007). In addition to the CHOP recognition element, these genes contain two other conserved elements in their promoters: mitochondrial unfolded protein response element (MURE) 1 and 2, which are present in nine out of the ten genes found to be up-regulated specifically in the UPR$^{mt}$, with the exception of the Hsp60/10 bidirectional promoter. The transcription factors that bind to the CHOP element are known (for example, CHOP and C/EBPβ), but the transcription factors that bind to MURE elements remain elusive.

A connection between PD and the UPR$^{mt}$ has recently been established. Brain tissue from PD patients (sporadic or associated with *PINK1* mutations) has been shown to have higher levels of unfolded mitochondrial respiratory complexes, associated with higher levels of Hsp60 (Pimenta de Castro et al., 2012). Moreover, *Drosophila Pink1* and *parkin* mutants show an accumulation of unfolded mitochondrial respiratory complexes, as well as up-regulation of the mitochondrial chaperones Hsp60 and Hsc70-5 (the fly orthologue of the mammalian mtHsp70). The subsequent generation of a fly model of protein misfolding in the mitochondria (by expressing ΔOTC) show similar results to the PD fly models. More importantly, the ΔOTC flies show a phenotype that mimics some of the features presented by *Pink1* and *parkin* mutants, such as respiratory defects and reduced ATP levels, reduced mtDNA content and mitochondrial respiratory

complexes, impairment of the climbing performance, mitochondrial morphological defects in the thoracic muscles, and a reduced lifespan (Pimenta de Castro et al., 2012). The expression of ΔOTC in flies results in mitochondrial fragmentation and activation of autophagy, possibly as a means to eliminate these dysfunctional mitochondria. Of great interest, the expression of *parkin* in the ΔOTC flies was able to rescue their defective phenotype in an autophagy-dependent manner, since this rescue was abolished in an *Autophagy-specific gene 1* (*Atg1*)-mutant background (Pimenta de Castro et al., 2012). This work has strengthened the concept that protein homeostasis in the mitochondria is of great importance and its unbalance can be associated with the pathogenesis of various diseases, including PD.

### 1.7.3 A distant signalling pathway originating in the mitochondrial IMS

The ubiquitin-proteasome pathway probably represents the most common process of protein quality control within the cell. Conjugation of a substrate with the ubiquitin chains is important for various physiological functions, from cell signalling to target substrates for degradation. The proteasome exists in both the nucleus and the cytoplasm and is responsible for the protein turnover in these cellular compartments. Interestingly, the proteasome is also responsible for the degradation of ER proteins, because a fraction of the proteasome is associated with ER membranes (Kalies et al., 2005). In the ER, misfolded or damaged proteins are recognised by chaperones, such as BiP, and transported to the cytosol, where are degraded by the proteasome. Inhibition of the proteasome activity results in the accumulation of misfolded proteins in the ER. Similarly, inhibition of the proteasome also results in the accumulation of unfolded proteins in the IMS (Radke et al., 2008). Inhibition of either Hsp90 or the proteasome causes proteins to accumulate in the mitochondria (Margineantu et al., 2007).

Endo G is a protein that accumulates in the IMS following proteasome inhibition (Radke et al., 2008). Overexpression experiments using either WT or a mutant form of Endo G have shown that both forms are substrates for ubiquitination, however the mutant form is ubiquitinated at a much higher level. The mitochondrial targeting signal is not required for the conjugation with ubiquitin. These observations are consistent with the existence of a protein quality control system in the cytoplasm prior to the import of proteins to the mitochondria. Further analyses have shown that upon proteasome inhibition, WT Endo G is cleaved by HtrA2, while the mutant forms of the protein are not. Elimination of mutant Endo G appears to depend on the proteasome pathway.

A defect in proteasome activity may cause the accumulation of mitochondrial proteins and deregulate their activity. HtrA2 seems to be part of a second checkpoint following the action of the proteasome in the cytosol (Radke et al., 2008). Consistent with this, mutations in HtrA2 may impair its function and lead to unwanted accumulation of its putative substrates. Furthermore, mutant forms of the IMS proteins are resistant to HtrA2 degradation, and this could augment their deleterious effect and result in mitochondrial collapse.

The consequences of unfolded-proteins accumulation in the IMS have not been explored, although a recent study showed that upon IMS stress a distinct protective pathway is activated (Papa and Germain, 2011) (Figure 1.6). The expression of a mutant form of Endo G results in the formation of aggregates in the IMS and mitochondrial stress. Under these conditions, HtrA2 and proteasome components increase in an oestrogen receptor α (ERα)-dependent manner. Accumulation of mutant Endo G does not result in the up-regulation of CHOP or Endo G, in contrast to what is observed during the $UPR^{mt}$. Endo G accumulation also does not cause increased expression of

BiP or BIM, which is a characteristic feature of the $UPR^{ER}$. Using a luciferase reporter with oestrogen-responsive elements (EREs), it has been observed that the accumulation of various IMS proteins activates ERE-containing genes. Additionally, the activation of EREs depends on ERα phosphorylation and not ERβ. ERα appears to be phosphorylated by Akt, which in turn is activated by ROS. ROS overproduction has been reported following mutant Endo G overexpression and is inhibited by the antioxidant *N*-acetylcysteine (NAC), suppressing the activation of the ERE reporter. Nuclear respiratory factor 1 (NRF1) is a transcription factor that activates the expression of genes involved in mitochondrial respiration and can be induced by ER activation. IMS stress results in enhanced *NRF1* transcription. Furthermore, increased levels of HtrA2 have been detected following IMS stress induction, as have increases in proteasome activity.

### 1.7.4 Organellar quality control

Although the mitochondrial molecular quality control machinery can respond efficiently to some insults, it can also be overwhelmed. In this situation, the organellar quality control must take over. This second line of defence relies on the dynamic nature of mitochondria and its ability to undergo consecutive cycles of fusion and fission. As mentioned above, it is reasonable to consider that a little damage in a mitochondrion unit can be diluted if it fuses with healthy mitochondria. However, if there is significant damage, mitochondrion units may become depolarised and be segregated, being removed by mitophagy. Mitophagy is the selective engulfment of mitochondria by autophagosomes, followed by its fusion to lysosomes, forming the autophagolysosome, in which the mitochondria are digested. The term mitophagy has been proposed to

distinguish this selective form of autophagy from the more general process (Lemasters, 2005).

Generally, autophagy is an adaptive process that is activated under certain conditions, such as starvation, to maintain cellular homeostasis. During autophagy, intact organelles and portions of the cytosol are sequestered into the autophagosomes (a double-membrane vesicle). After the fusion with lysosomes, the contents can then be digested, allowing the cells to eliminate damaged or harmful components and, through catabolism and recycling, can also allow the cell to maintain nutrient and energy homeostasis (He and Klionsky, 2009). An interesting experimental approach has been used to show the selective removal of damaged mitochondria by mitophagy. The photo-irradiation of mitochondria causes their damage by increased levels of ROS, resulting in depolarisation and permeabilization of these organelles and their subsequent sequestration by autophagosomes (Kim and Lemasters, 2011).

The importance of mitophagy as a quality control mechanism was also supported by the *Atg7* conditional knockout mouse model, in which the autophagy impairment results in the accumulation of damaged mitochondria in certain tissues (Komatsu et al., 2005, Wu et al., 2009).

The *PINK1/Parkin* pathway has been suggested to play a central role in the mechanism of disposal of damaged mitochondrial through mitophagy. Several findings are consistent with this role, although the mechanism is still not entirely understood (Jin and Youle, 2012). First, it has been established that under mitochondrial depolarizing conditions, by treating cultured cells with the uncoupler of OXPHOS calbonyl cyanide m-chlorophenylhydrazone (CCCP), Parkin is recruited to mitochondria and results in their elimination from the cells through mitophagy (Narendra et al., 2008). Further studies have also revealed that the recruitment of Parkin to depolarized mitochondria is

dependent on the presence, as well as the kinase activity, of PINK1 (Geisler et al., 2010, Matsuda et al., 2010, Narendra et al., 2010, Vives-Bauza et al., 2010). Under normal conditions, PINK1 seems to be rapidly cleaved by the presenilin-associated rhomboid-like protein (PARL) (Deas et al., 2011a), and the cleaved form is then degraded by the proteasome (Muqit et al., 2006). However, upon CCCP treatment, PINK1 seems to be stabilized at the OMM and this resulted in the translocation of Parkin to the mitochondrial surface (Narendra et al., 2010). Once recruited to mitochondria, Parkin ubiquitinates various OMM proteins that recruit other proteins and initiate mitophagy (reviewed by (Jin and Youle, 2012)). Examples of substrates for Parkin ubiquitination are Mfn1 and Mfn2, and the *Drosophila* dMfn is also ubiquitinated by parkin. Upon ubiquitination, these proteins are targeted for degradation by the proteasome, in a p97-dependent manner. This observation suggests that Parkin-mediated degradation of MFNs impedes mitochondrion units to undergo fusion, and therefore segregate the depolarized mitochondria for mitophagy (Poole et al., 2010, Tanaka et al., 2010, Ziviani et al., 2010). Mitochondrial dynamics seems to play an important role in mitophagy. For example, knockdown of dynamin-related protein 1 (Drp1) results in elongated mitochondria and defective mitophagy (Chen and Chan, 2009). It is reasonable to anticipate that smaller units are more easily engulfed by an autophagosome than the large structures.

Interestingly, accumulation of dMfn has been detected in *Pink1* and *parkin* mutant flies (Ziviani et al., 2010). It had been previously shown that these mutants have a defect in mitochondrial fission or an enhancement of fusion (Deng et al., 2008, Poole et al., 2008). In the absence of Pink1 or parkin, dMfn does not seem to be ubiquitinated by parkin, but accumulates in mitochondria, which fail to undergo fission, indicating that mitophagy is impaired in this situation (Ziviani et al., 2010). This could represent a

pathogenic mechanism in PD, since this failure of organellar quality control may result in the accumulation of damaged mitochondria, which can be particularly harmful for neuronal cells. Results from studies in mammals show variability in the predominant mitochondrial morphology (either more fused or fragmented) depending on the type of tissue or cells tested. It is clear that PINK1 and Parkin interfere with mitochondrial dynamics and must play a role in the mitochondrial function, although exactly how PINK1 and Parkin exert their function is not currently known.

The voltage-dependent anion-selective channel protein 1 (VDAC1) is another mitochondrial substrate ubiquitinated by Parkin and it seems to be required for the recruitment of p62 to mitochondria (Geisler et al., 2010). The ubiquitin-binding adaptor p62, also known as sequestosome 1 (SQSTM1), is involved in the process of aggregation of ubiquitinated proteins, and it seems to target these aggregates for autophagosomes by binding to microtubule-associated protein light chain 3 (LC3). Additionally, p62 accumulates on mitochondria after the Parkin-mediated ubiquitination of mitochondrial substrates (Ding et al., 2010, Geisler et al., 2010). Thus, it is possible that p62 acts as an adaptor molecule to dispose ubiquitin-labelled mitochondria through mitophagy. Although it is clear that p62 binds to the ubiquitinated substrates and facilitates aggregation, it is not entirely clear if it is essential for mitophagy, as contradicting results have been reported (Ding et al., 2010, Geisler et al., 2010, Narendra et al., 2010, Okatsu et al., 2010).

A model of PINK1/Parkin pathway mediating mitophagy has been proposed (Figure 1.9). Nonetheless, further investigation is needed, especially because the conditions in which some of the results were obtained are far from the normal physiologic condition, such as the use of CCCP to depolarize mitochondria. It would be of great interest to have a better knowledge of the physiological mitochondrial turnover

that is necessary to maintain a healthy pool of mitochondria, and thus maintain homeostasis in the cells. It is also essential to make sure that the PINK1/Parkin pathway operates in a physiologic condition to promote the degradation of damaged mitochondria. Additionally, the identification of the downstream targets of this process is of great importance.

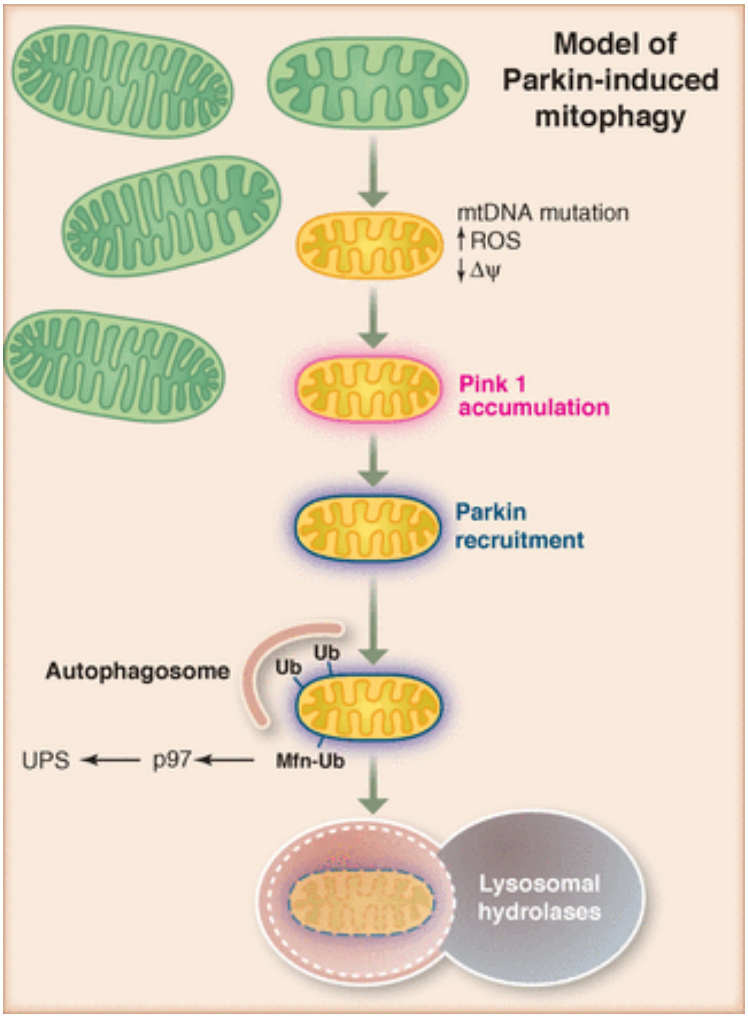



**Figure 1.9: Model of the PINK1/Parkin-mediated mitophagy.** Under conditions where the mitochondrial membrane potential (ΔΨm) is maintained, PINK1 is imported into mitochondria and rapidly cleaved by the proteases and degraded, being present in mitochondria at low levels. Under depolarizing conditions, such as treatment with the uncoupler CCCP, the full-length PINK1 accumulates in the outer mitochondrial membrane (OMM), inducing the translocation of Parkin to the mitochondrial surface. Parkin ubiquitinates mitochondrial proteins, such as Mitofusins (MFNs) and VDAC1. MFNs are then degraded by the proteasome, which requires the protein p97. The protein adaptor p62 is also recruited to mitochondria and these organelles are selectively degraded by mitophagy. Figure from Youle and van der Bliek, 2012.

## 1.8 Aims of this project

Mitochondrial dysfunction and oxidative stress have consistently been implicated in PD pathogenesis. Much information about the PD-linked genes *PINK1* and *Parkin* came from studies using *Drosophila* as a model system. This thesis aims to further investigate the molecular mechanisms implicated in the mitochondrial pathology of *Pink1* and *parkin* mutants, by using *Drosophila melanogaster* as a model system. Particularly, aspects of mitochondrial quality control pathways, both molecular (through the involvement of chaperones and proteases) and organellar (mitophagy) will be emphasized.

The role of TRAP1, a proposed *in vitro* cellular substrate of PINK1 will be characterised *in vivo*. Evidence supporting Trap1 as a putative effector of molecular quality control in the mitochondria will be presented in Chapter 3. Further epistasis analysis of *Trap1* with *Pink1* and *parkin* will be detailed in Chapter 4.

Furthermore, p62 has been proposed to act as an adaptor molecule for targeting damaged mitochondria for mitophagy. This thesis also aims to characterise the *Drosophila* orthologue of p62, *ref(2)P*, as an effector of mitochondrial quality control, as well as its role in the Pink1/parkin pathway *in vivo* (Chapter 5).

This work essentially uses genetic approaches to perform epistasis analysis of the PD-linked genes *Pink1* and *parkin* with the putative modulators of mitochondrial quality control *Trap1* and *ref(2)P*.

# Chapter 2

# General materials and methods

# Chapter 2: General materials and methods

## 2.1 Reagents

### 2.1.1 Chemicals suppliers

All chemicals were of analytical grade purity or higher and supplied by Sigma-Aldrich Company UK Limited, unless otherwise stated.

| Reagent | Supplier |
|---|---|
| Precision Plus dual color protein standards | Bio-Rad (Hemel Hempstead, UK) |
| Hoechst 33258 | Calbiochem (Nottingham, UK) |
| EDTA, NaCl, KCl | Fisher Scientific (Loughborough, UK) |
| Ethanol | |
| Methanol | |
| Glacial acetic acid | Merck Millipore (Nottingham, UK) |
| PVDF | |
| Proteinase K | New England Biolabs Ltd (Hitchin, UK) |
| 2X *Taq* master mix | |
| 1 kb DNA ladder Quick-load | |
| Vectashield | Vector Laboratories (Burlingame, CA, USA) |
| Rapamycin | Santa Cruz (Santa Cruz, CA, USA) |

### 2.1.2 Antibodies

| Primary antibodies | Dilution | Supplier |
|---|---|---|
| Anti-α-tubulin B-5-1-2 (mouse) | 1:2000 | Sigma-Aldrich (St. Louis, MO, USA) |
| Anti-NDUFS3 (mouse) | 1:1000 | Abcam-Mitosciences (Eugene, OR, USA) |
| Anti-Tyrosine Hydroxylase (rabbit) | 1:1000 | Millipore (Billerica, MA, USA) |
| Anti-HA High Affinity (3F10) (rabbit) | 1:1000 | Roche (Welwyn Garden City, UK) |
| Anti-TRAP1 (clone 30) (mouse) | 1:1000 WB<br>1:50 IF | BD Transduction Laboratories (Franklin Lakes, NJ, USA) |
| Anti-dMfn (rabbit) | 1:2000 | Gift from A. Whitworth (University of Sheffield, UK) |

| Secondary antibodies | Dilution | Supplier |
|---|---|---|
| Goat anti-mouse Alexa 564 | 1:200 | Invitrogen Molecular Probes (Paisley, UK) |
| Sheep anti-mouse IgG, HRP | 1:50 000 | GE Healthcare (Little Chalfonf, UK) |
| Donkey anti-rabbit, HRP | 1:10 000 | |

## 2.2 Fly stocks

All experimental procedures were performed using *Drosophila melanogaster*. The fly stocks were maintained on standard cornmeal agar media. All stocks, crosses and flies used in the experiments were maintained at 25˚C under a cycle of 12 hours of

light and dark. Most of the strains used are described below, although some others are described in detail in the relevant chapters.

***Drosophila* strains:**

***w*$^{1118}$**: carry a deletion in the sex-linked *white* (*w*) gene, resulting in white eyes on this strain. It is used for the production of transgenic flies, because it allows the selection of transformants based on the changes of eye colour. It was used as a control for *Trap1*$^{4}$ and *ref(2)P* mutants.

***w*$^{1118}$; *Sco/CyO*; *MKRS/TM6B***: carry chromosome balancers and markers for second and third chromosomes. These were used for balancing stocks.

***w*$^{1118}$; *Sco/CyO***: carry chromosome balancers and markers for the second chromosome.

***FM7a***: chromosome balancer for the X chromosome, associated with the *Bar* eye marker.

**GAL4 lines:**

***w; +; daGAL4*** and ***yw; Act5cGAL4/CyO***: ubiquitous GAL4 drivers.

***elavGAL4/CyO***: pan-neural GAL4 driver.

***w; enGAL4/CyOGFP***: drives expression of GAL4 in the posterior compartment of the imaginal disc, in an *en* pattern.

***w; UASmitoGFP/CyO***: expressing mitochondria-targeted green fluorescent protein (mitoGFP) under the control of a GAL4 driver.

All the fly strains listed above were obtained from the Bloomington Stock Center (Indiana University, Bloomington, IN, USA), except for *da*GAL4 driver.

*da*GAL4, *pink1*$^{B9}$, UAS-*park*$^{C2}$ , UAS-*parkin*$^{62B}$ and *park*$^{25}$ strains were a gift from A. Whitworth (MRC, Centre for Developmental and Biomedical Genetics, University of Sheffield, Sheffield, UK).

In this work, *Pink1*$^{B9}$ and *park*$^{25}$ mutant stocks were maintained with GAL4 drivers in the following genotypes.

***Pink1*$^{B9}$*/FM6; +; daGAL4***

***Pink1*$^{B9}$*/FM7a; elavGAL4/CyO; +***

***w; Act5CGAL4/CyO; park*$^{25}$*/TM6B***

*Trap1*$^{4}$ mutants were a kind gift from J.E. Treisman (New York University School of Medicine, NY, USA).

***w; Trap1*$^{4}$*/CyO; +***

*Atg1*$^{\Delta 3d}$ strain was kindly provided by Dr. T.P. Neufeld (University of Minnesota, Minneapolis, MN, USA).

***w; +; Atg1*$^{\Delta 3D}$*/TM6B***

*ref(2)P*$^{od2}$ and *ref(2)P*$^{od3}$ were a gift from Dr. I. Nezis (Institute for Cancer Research, Oslo, Norway).

***w; ref(2)P*$^{od2}$*/CyO***

***w; ref(2)P*$^{od3}$*/CyO***

## 2.3 Genotyping *Drosophila melanogaster*

### 2.3.1 Genomic DNA extraction

An individual fly was placed in a 1.5 mL microfuge tube and subjected to fast freezing in dry ice. The single fly was then homogenized in 400 μL of Tail Lysis Buffer (TLB) (100 mM Tris:Cl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) with freshly

added 100 μg/mL of Proteinase K. The samples were incubated at 55˚C overnight in a thermal mixer with an agitation speed of 800 rpm. After a centrifugation at 14 000 g for 5 min, the supernatant was transferred to a fresh tube containing 400 μL of isopropanol and vortexed for a few seconds. The samples were centrifuged again at 14 000 g for 5 min. The majority of the supernatant was removed, leaving a small volume in the tube, and this was centrifuged for 5 min at 14 000 g. The remaining supernatant was removed, carefully, to not disturb the pellet at the bottom of the tube. The samples were left for 5 min with the tubes open to allow them to air dry, after which the pellet was resuspended in 50 μL of nuclease-free water. The DNA suspension was incubated in a thermal mixer at 37˚C for 30 min with agitation (1200 rpm). For genotyping, 2 μL of the DNA was used for each Polymerase Chain Reaction (PCR).

### 2.3.2 Polymerase Chain Reaction

For fly genotyping, the 25 μL of reaction mixture contained 2 μL of DNA, 0.5 μL forward primer (25 μM stock), 0.5 μL reverse primer (25 μM stock), 12.5 μL *Taq* 2X master mix and 9.5 μL nuclease-free water. The *Taq* 2X master mix (New England Biolabs) consists of *Taq* DNA polymerase, dNTPs, standard *Taq* reaction buffer and stabilizers. The standard cycling conditions for the routine PCR are listed in Table 2.1.

**Table 2.1: Standard reaction conditions for PCR**

| PCR step | Temperature | Time (min:sec) |
|---|---|---|
| **1. Initial denaturation** | 94˚C | 2:00 |
| **2. Denaturation** | 94˚C | 0:45 |
| **3. Annealing** | 55˚C | 0:45 |
| **4. Extension** | 68˚C | 1:00 - 2:00, depending on the size of PCR product |
| Repeat step 2 to 4, 30 times | | |
| **Final extension** | 68˚C | 10:00 |

#### 2.3.2.1 Oligonucleotide design

Oligonucleotides designed for PCR were between 20 and 22 bp in length with an AT:GC ratio of approximately 50% to ensure an annealing temperature of around 50-60ºC.

### 2.3.3 Agarose gel electrophoresis

Loading buffer (10 mM Tris-HCl (pH 7.6), 60% v/v glycerol, 60 mM EDTA, 0.15% v/v orange G) was added to the DNA in a 1:5 ratio and resolved by electrophoresis on an agarose gel, with concentration varying between 0.7 and 2%, depending on the size of the DNA fragments being analysed. The agarose gel was obtained by dissolving agarose in 1X TAE (40 mM Tris acetate, 1 mM EDTA pH8.0) supplemented with ethidium bromide (0.5 μg/mL). 2 μL of 1 kb DNA ladder were added to a separate well and electrophoresis was carried out at 100 V for 1 hour. The DNA bands were visualised with ultraviolet (UV) light and images were captured using

a Gene Genius CDC gel documentation system (Syngene, Cambridge, UK). The length of the fragments was determined by comparison to the DNA ladder bands.

### 2.3.4 Recovery of DNA from an agarose gel

Standard QIAquick Gel Extraction Kit (QIAGEN) was used to purify the DNA from the agarose gel according to the manufacturer's instruction.

### 2.3.5 DNA sequencing

DNA samples were analysed by Protein and Nucleic Acid Chemistry Laboratory (PNACL, University of Leicester) to be run on an ABI 377 sequencer.

## 2.4 RNA extraction from *Drosophila*

Three male flies, 3 days old, were used per sample. The flies were collected in RNase-free 1.5 mL microfuge tubes and subjected to fast freezing in dry ice. The isolation of total RNA was performed using the RNeasy Mini System Kit (QIAGEN), according to the manufacturer's instruction. The flies were homogenised in 200 μL of RLT (RNeasy Lysis Buffer) with freshly added β-mercaptoethanol (1:100) using a motorised pestle. After centrifugation at 16 000 g for 3 min, the supernatant was transferred to QIAshredder columns (QIAGEN) and centrifuged for 2 min at 16 000 g, to filter out insoluble debris. 200 μL of RNase-free 70% ethanol was added to the cleared lysate, mixed immediately by pipetting and transferred to RNeasy mini column (QIAGEN) placed in a 2 mL collection tube. After centrifugation at 8000 g for 30 sec, the flow-through was discarded. 700 μL of Buffer RW1 were added to the column and centrifuged for 30 sec at 8000 g. The columns were transferred to new collection tubes and 500 μL of Buffer RPE were added to the column and centrifuged for 30 sec at 8000

g. The flow-through was discarded and another 500 μL of Buffer RPE were added to the column and centrifuged for 2 min at 8000 g. The flow-through was discarded and the column was centrifuged again for 1 min at full speed to eliminate any remaining Buffer RPE. To elute, the RNeasy column was transferred to a new 1.5 mL collection tube and 30 μL of RNase-free water were added directly onto the RNeasy silica-gel membrane. Columns were centrifuged for 1 min at 8000 g to elute. The RNA concentration was measured using a NanoDrop 2000 Micro-Volume UV-Vis Spectrophotometer (Thermo Scientific), by measuring the optical density (OD) at 260 nm. The samples were normalized to the same RNA concentrations, typically 50 ng/μL.

## 2.5 Real-time Quantitative Reverse Transcription PCR (qRT-PCR)

To perform real-time one-step qRT-PCR, QuantiTect® SYBR® Green Kit (QIAGEN) was used. The description of the components of the kit can be found in Table 2.2 (as described in the handbook provided by QIAGEN). Using this product, both reverse transcription and PCR take place in a single tube, with all the reagents being added at the beginning of the procedure.

As recommended by the manufacturer, QuantiTect Primer Assays, a ready-to-use primer sets that were guaranteed to provide specific and sensitive quantification when use in combination with QuantiTect SYBR Green Kits, were used. A reaction volume of 30 μL was used per sample. In general, 100 ng of RNA were used per reaction, normally corresponding at a volume of 2 μL.

**Table 2.2: Description of QuantiTect SYBR Green RT-PCR Kit components**

| | **Component** | **Description** |
|---|---|---|
| Master Mix | HotStarTaq DNA polymerase | Modified form of a recombinant 94-kDa DNA polymerase, originally isolated from *Thermus aquaticus*, cloned into *E. coli* |
| | QuantiTect SYBR Green RT-PCR buffer | Contains Tris.Cl, KCl, $(NH_4)_2SO_4$, 5mM $MgCl_2$, pH 8.7 |
| | dNTP mix | Contains dATP, dCTP, dGTP and dTTP/dUTP of ultrapure quality |
| | Fluorescent dyes | SYBR Green I and ROX |
| | QuantiTect RT Mix | Contains an optimized mixture of the QIAGEN products Omniscript Reverse Transcriptase and Sensiscript Reverse Transcriptase, both of which are recombinant heterodimeric enzymes expressed in *E. coli* |
| | RNase-free water | Ultrapure quality, PCR grade |

The reaction mixture contained: 15 μL of master mix, 0.3 μL of QuantiTect RT Mix, 3 μL of the QuantiTect Primer Assay (0.5 μM of forward primer and 0.5 μM of reverse primer), and RNase-free water to a total volume of 30 μL. The mixture was distributed to PCR tubes according to the number of samples to be tested and RNA templates were then added directly on to the reaction mixture. The reaction was carried on a real-time cycler Mx4000 from Stratagene (La Jolla, CA, USA). The conditions of the reaction are described in Table 2.3.

**Table 2.3: Real-time cycler conditions**

| Step | Time (min:sec) | Temperature |
|---|---|---|
| **1. Reverse transcription** | 30:00 | 50°C |
| **2. PCR initial activation** | 15:00 | 95°C |
| **3. Denaturation** | 00:15 | 94°C |
| **4. Annealing** | 00:30 | 55°C |
| **5. Extension** | 00:30 | 72°C |
| Repeat steps 3-5 for 40 cycles | | |

## 2.6 Immunofluorescence and confocal microscopy

To perform protein expression analysis in the wing discs, third instar larvae were inverted in ice-cold PBS and fixed in 3% (v/v) paraformaldehyde, washed in PBS + 0.2% (v/v) Triton (PBT) 3 times for 10 min and blocked for 1 hour with PBT + 1% (w/v) BSA, with gentle rotation. The incubation with the primary antibody, anti-TRAP1 clone 30 (BD Biosciences), 1:100 in PBT + 1% BSA was carried out overnight at 4˚C. After 4 washes of 10 min in PBT + 1% BSA with gentle rotation, the samples were incubated with the secondary antibody (1:200), α-mouse Alexa 564 (Molecular Probe Inc.), and Hoechst 33342 (1:500) (Molecular Probe Inc.) in PBT + 1% BSA, for 3 to 4 hours at room temperature (RT) with very gentle rotation. The samples were washed 4 times 10 min in PBT. The wing discs were dissected, mounted on slides with Vectashield (Vector Laboratories Inc.) and imaged using a Carl Zeiss LSM510 confocal microscopy system (Jena, Germany).

## 2.7 Western Blotting

### 2.7.1 Protein extraction

Protein extraction, from either the whole flies or heads only, was carried out depending on the objective of a particular experiment. The number of flies/heads used for protein extraction also varied depending on the proteins being analysed. Flies were anesthetized and collected in 1.5 mL microfuge tubes and subjected to fast freezing in dry ice. For the collection of fly heads, these were separated from the fly body using a scalpel. The samples were homogenized in 100 μL of ice-cold extraction buffer (0.1% Triton X100, 10 mM EDTA, 1mM DTT, 100 mM KCl, 20 mM HEPES (pH 7.5), 5% glycerol, 1 μg/mL chymostatin, 1 μg/mL leupeptin, 1 μg/mL antipain and 1 μg/mL pepstatin A) using a motorised plastic pestle. The homogenates were centrifuged at 4˚C for 10 min at 16 000 g. The supernatant was recovered (~ 80 μL) and the protein concentration was measured using Bradford assay. For this purpose, 1 μL of lysate was added to 1 mL of 1X Bradford reagent and the OD at 595 nm was measured using a spectrophotometer (Eppendorf). The samples were normalized to the lower concentration by addition of extraction buffer. 80 μL of protein samples were transferred to fresh 1.5 mL microfuge tubes, to which 26.6 μL of NuPAGE 4X LDS sample buffer (Invitrogen) that contains glycerol and lithium dodecyl sulfate (LDS) and supplemented with 1% β-mercaptoethanol were added. The samples were then boiled at 100˚C for 5 min and subjected to Sodium dodecyl sulphate (SDS)-Polyacrylamide gel electrophoresis (PAGE) analysis immediately or stored at -20˚C.

### 2.7.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

#### 2.7.2.1 SDS-PAGE gel preparation

Polyacrylamide gels were prepared using a Bio-Rad mini-PAGE system. There are two parts in the polyacrylamide gel: the stacking gel and the resolving gel. The resolving gel buffer contained 1.5 M Tris:HCl and 0.1% (w/v) SDS and the stacking gel buffer contained 0.5 M Tris:HCl and 0.1% (w/v) SDS, pH 6.8. Both buffers were filtered using polystyrene vacuum filter with a cellulose acetate membrane, 0.22 micron, and kept in storage bottles of 500 mL at RT.

The components of the stacking gel are listed in Table 2.4.

**Table 2.4 Components of the stacking gel of polyacrylamide gel**

| Component (stock) | Volume (mL) |
|---|---|
| Stacking gel buffer | 2.5 |
| Acrylogel (40%) | 0.975 |
| 10% APS | 0.1 |
| TEMED | 0.01 |
| Water | 6.3 |

The percentage of acrylamide in the resolving gel varied depending on the molecular weight of the proteins being analysed. For proteins with higher molecular weights, gels with lower concentration of acrylamide were used. The components for the resolving gel of 10% and 12.5% acrylamide are listed in the Table 2.5.

Combs were inserted into the stacking gel to form wells for sample loading.

**Table 2.5: Components of the resolving gel of a polyacrylamide gel**

| Component (stock) | Volume (mL) for 10% gel | Volume (mL) for 12.5% gel |
|---|---|---|
| Resolving gel buffer | 5 | 5 |
| Acrylogel (40%) | 5 | 6.2 |
| 10% APS | 0.2 | 0.2 |
| TEMED | 0.02 | 0.02 |
| Water | 9.8 | 8.6 |

### 2.7.2.2 SDS-PAGE

Samples were loaded onto the polyacrylamide gels, along with a coloured protein standard (Bio-Rad). Running buffer (2.5 mM Tris, 0.25 M glycine, 0.1% (v/v) SDS) was used for the electrophoresis at 100 V for 10 min, followed by 1 hour at 200 V. The progress of the electrophoresis was followed with the dual colour protein standard, and the running time could be adjusted based on the molecular weight of the proteins being analysed.

### 2.7.2.3 Transfer of proteins onto PVDF membranes

Following electrophoresis, proteins were transferred onto a polyvynilidene difluoride (PVDF) membrane (Millipore) using a BioRad mini Trans Blot kit, also known as wet transfer, at 300 mA for 1 hour and 30 min. The efficiency of transfer and equality of loading were checked by ponceau red (0.1% (w/v) Ponceau S, 5% (v/v) acetic acid) staining, after which the proteins were fixed in the membrane by washing it with methanol for 1-2 min. The membranes were allowed to air dry before being used for immunobloting.

#### 2.7.2.4 Protein immunobloting

Membranes were incubated in 5%(w/v) skimmed milk in Tris Buffered Saline (TBS)-Tween (TBST) (50 mM Tris, 150 mM NaCl, pH 8.1, 0.1% (v/v) TWEEN 20) for 1 hour at RT to block non-specific sites of antibody binding. Incubation with the primary antibodies, diluted in TBST with 5% skimmed milk, was performed overnight at 4°C or 1 hour at RT (for anti-tubulin antibody). The membranes were washed for 15 min followed by two washes of 5 min in TBST and subsequently incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at RT. The membranes were washed for 20 min followed by four washes of 5 min in TBST. Membranes were exposed to enhanced chemiluminescence (ECL) solution (Pierce) for 5 min. Antibody complexes were visualized on X-ray film (Hyperfilm).

## 2.8 Respirometry analysis

Mitochondrial respiration was assayed at 37°C by high-resolution respirometry using an OROBOROS Oxygraph-2k (OROBOROS, Innsbruck, Austria). To assess state-3 respiration, flies were anesthetized, two flies were transferred to 1.5 mL tube and homogenised in MiR05 respiration buffer (20 mM HEPES, 1m mM $KH_2PO_4$, 110 mM sucrose, 20 mM taurine, 60 mM K-lactobionate, 0.5 mM EGTA, 3 mM MgCl, 1g/L fatty-acid-free BSA). The samples were immediately analyzed. To assess Complex I respiration samples were supplemented with 2 mM malate, 10 mM glutamate and 5 mM ADP. To assess Complex II respiration, the Complex I inhibitor rotenone (1 μM) was added to the samples, followed by the addition of the Complex II substrate succinate (10 mM). To measure uncoupled mitochondrial respiration, flies were frozen in dry ice prior to homogenization in MiR05. Uncoupled Complex II respiration was determined

in the presence of rotenone and succinate. The DatLab software package (OROBOROS, Innsbruck, Austria) was used for data acquisition (2 sec time intervals) and analysis, including calculation of the time derivative of oxygen concentration, signal deconvolution (dependent on the response time of the oxygen sensor) and correction for instrumental background oxygen flux.

## 2.9 ATP assay

For ATP assay, the CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used. It is a luminescence-based technique, and the luminescent signal is proportional to the amount of ATP present. Five male flies were homogenized in 100 μL of Tris-EDTA (TE) buffer (100 mM Tris, 4 mM EDTA, pH 7.8) with 6 M guanidine-HCl, to inhibit ATPases. The homogenates were subjected to fast freezing in liquid nitrogen, boiled at 100°C for 5 min and cleared by centrifugation at 16 000 g for 10 min. 80 μL of supernatant were recovered and a part of it was diluted (1:50) in TE buffer. 100 μL of the diluted samples was added per well of a white 96-well-plate. After a 30 min equilibration time, 100 μL of the luminescent solution was added to each sample. After 10 min of incubation, the luminescence was measured on a GENios Multifunction Reader (TECAN, Switzerland). The protein concentration of the samples was determined by the Bradford method by adding 1 μL of the sample (not diluted) to 1 mL of 1X Bradford, and the absorbance was measured at 595 nm using a Spectrophotometer (Eppendorf). ATP levels of the samples were determined by normalising the luminescence values to the protein concentration (RFU/mg of protein).

## 2.10 Electrochemical detection of neurotransmitters in fly heads

For sample preparation, fifteen male fly heads (20 days old) were dissected out under mild $CO_2$ anaesthesia, and subjected to fast freezing in dry ice. The heads were then homogenized in 50 μL chilled 0.1 M perchloric acid using a motorized, hand-held tissue homogenizer. The chilled homogenates of fly heads were centrifuged for 2 min at 16000 g, and the supernatant was filtered through a low-binding Durapore (0.22μm) PVDF membrane using Ultrafree-MC centrifugal devices. The DA and serotonin measurements were performed immediately after sample preparation. Supernatant fluid (30 μL) was eluted at a flow rate of 50 μL/min through a 150 × 1.0 mm C18 column. The mobile phase contained 50 mM phosphoric acid, 8 mM NaCl, 0.1 mM EDTA, 12.5% (v/v) methanol, 500 mg/L octanesulfonic acid (OSA) (pH 6.0). Analysis was performed using an Alexys LC-EC system equipped with a DECADE II electrochemical detector (Range, 10 nA; Digital noise filter, 0.05 Hz; Cell potential, 0.7 V) (ANTEC). Standards of DA and serotonin were analysed for the absolute estimation of the concentration of DA and serotonin (Appendix 2).

## 2.11 Behavioural tests

### 2.11.1 Climbing assay

A countercurrent apparatus was used to perform the climbing assay (Figure 2.1). Twenty male flies were placed into the first chamber, taped to the bottom, and given 20 sec to climb a distance of 10 cm. Flies that successfully climbed 10 cm or beyond proceed to the second chamber, and another 20 sec were given to the flies to climb the 10-cm distance. This procedure was repeated a total of five times and at the end the

number of flies in each chamber was counted to calculate the climbing index. At least 80 flies were used for each genotype.

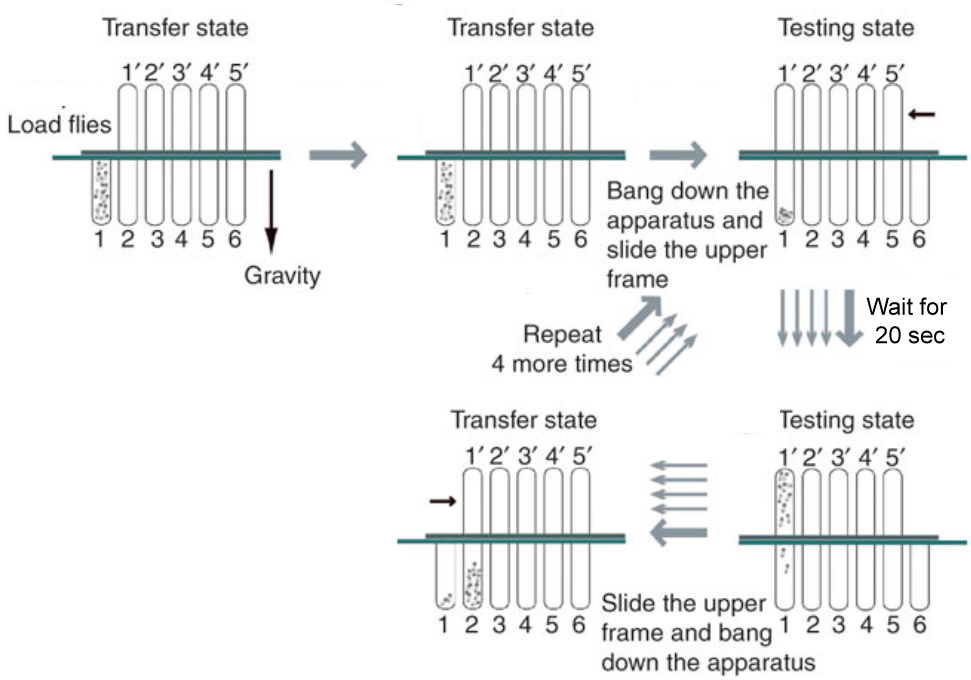



**Figure 2.1: Schematic representation of the climbing assay using a countercurrent apparatus.** Figure adapted from Inagaki et al., 2010.

## 2.12 Lifespan analysis

100 to 200 newly eclosed male flies of each genotype were divided in groups of 10 and placed in vials containing fresh fly food. The flies were maintained at 25°C and transferred to new vials every 3 to 4 days and the number of dead flies was recorded. The experiment was finalized when all the flies were dead. Data were presented as Kaplan-Meier survival distributions and the significance determined by log-rank tests.

## 2.13 Stress treatments

### 2.13.1 Heat stress

For heat sensitivity, 4-day-old male flies were divided into groups of 15 and placed in empty vials (no food or water). The flies were kept for 30 min at 25°C, after which the heat stress was applied by placing the vials in a water bath at 37°C for 1 hour. This was followed by a 30-min recovery at 25°C and a new heat stress for 1 hour at 37°C. Flies were then transferred for vials containing fresh food and maintained at 25°C. The number of flies alive was recorded 24 hours later. 100 flies of each genotype were tested for heat stress.

### 2.13.2 Drug treatments

Boiled fly food was supplemented with the drugs at 40-50°C. The following drugs were used: Paraquat (1,1'-Dimethyl-4,4'-bipyridinium dichloride) (5 mM), antimycin A (50 mM) and rotenone (0.6 mM).

The newly eclosed males were placed in normal fly food for 1 day prior to their transfer to vials containing the drug. Groups of 10 males per vial were transferred to new vials every 2 to 3 days and the number of dead flies was recorded. The assay was completed after all the flies have died. 100 flies of each genotype were analysed and the results are presented as Kaplan-Meier survival distributions and the significance determined by log-rank tests.

## 2.14 Statistical analysis

The data are presented as mean values, and the error bars indicate ± Standard Deviation (SD). Inferential statistical analysis was performed using the Prism and

StatMate software packages (www.graphpad.com). The significance level is indicated as **** for $P < 0.0001$, *** for $P < 0.001$, ** for $P < 0.01$, * for $P < 0.05$ and ns for $P > 0.05$.

# Chapter 3

# Trap1 as a putative effector of molecular quality control in mitochondria

# Chapter 3: Trap1 as a putative effector of molecular quality control in mitochondria

## 3.1 Introduction

TRAP1 was first identified in a yeast two hybrid screen to be interacting with the intracellular domain of the TNF receptor type I (TNFR-1IC) (Song et al., 1995). TRAP1, also known as Hsp75, is the only known member of the HSP90 family that localises to the mitochondria. The mitochondrial localization is evident from many different studies; however, different mitochondrial sub-compartment localisations were proposed, such as the matrix (Cechetto and Gupta, 2000) and the IMS (Pridgeon et al., 2007). TRAP1 has also been detected in extra-mitochondrial localizations, in particular in zymogen granules, cardiac sarcomeres and cell surface of endothelial cells from blood vessels (Cechetto and Gupta, 2000). TRAP1 is expressed in various tissues, such as the liver, kidneys, brain, pancreas, lungs, skeletal muscle and placenta and it is also expressed at high levels in transformed cell lines (Song et al., 1995).

TRAP1 protein contains three major domains: a N-terminal mitochondria-targeting sequence, an ATPase domain with four ATP-binding sites and a C-terminal HSP90-like domain (Matassa et al., 2012). The function of TRAP1 is not entirely clear. It has been reported, based in *in vitro* studies, that TRAP1 does not have chaperone-like activity under the conditions in which Hsp90 does (Felts et al., 2000). However, this does not exclude the possibility that TRAP1 might have chaperone activity under different conditions and with distinct co-chaperone partners and substrates.

The programmed cell death apoptosis can be induced by a variety of stimuli, such as hypoxia, growth factor withdrawal, heat shock and DNA damage. Proteins from

the different classes of HSPs have been shown to play important roles in regulating apoptosis (Samali and Cotter, 1996). TRAP1 has been shown to protect cells against apoptosis induced by various agents, such as β-hydroxyisovalerylshikonin (a compound extracted from an oriental medicinal plant) (Masuda et al., 2004), cisplatin (Montesano Gesualdi et al., 2007), hypoxia (Xiang et al., 2010), and granzyme M (Hua et al., 2007). TRAP1 expression levels seem to decrease when ROS levels increase and TRAP1 expression promotes the decrease of ROS production (Hua et al., 2007).

TRAP1 inhibits the release of cytochrome c from mitochondria, which does not seem to involve only the attenuation of ROS production. This mitochondrial chaperone has been shown to interact with cyclophilin D (CypD), a regulator of the mPTP, and antagonizes its pro-apoptotic function. Thus, the cells escape apoptosis and survive (Kang et al., 2007). Moreover, TRAP1 expression prevents hypoxia-induced damage in cardiomyocytes; in contrast, the down-regulation of TRAP1 increases the cell death under both normoxic and hypoxic conditions. The cell death induced by TRAP1 knockdown was prevented by the mPTP inhibitor cyclosporine A. TRAP1 protein seems to play an important role in protecting cardiomyocytes against apoptosis by preventing the opening of the mPTP (Xiang et al., 2010).

As mentioned in Chapter 1, TRAP1 has been identified as a cellular substrate of the PINK1 kinase (Pridgeon et al., 2007). PINK1 was shown to have a protective effect against oxidative stress (treatment of cells with hydrogen peroxide), increasing cell survival. Under this stress condition, the phosphorylation of TRAP1 increases in a PINK1-dependent manner and is necessary for the protection against apoptosis. TRAP1 phosphorylation seems to block the release of cytochrome c from mitochondria, thus preventing cell death (Figure 3.1). PINK1 and TRAP1 were shown to physically interact and expressing PINK1 with PD-linked mutations abolished the phosphorylation

of TRAP1 (Pridgeon et al., 2007). These observations suggest that PINK1 can modulate the activity of the molecular chaperone TRAP1, which might be part of a mitochondrial quality control pathway.

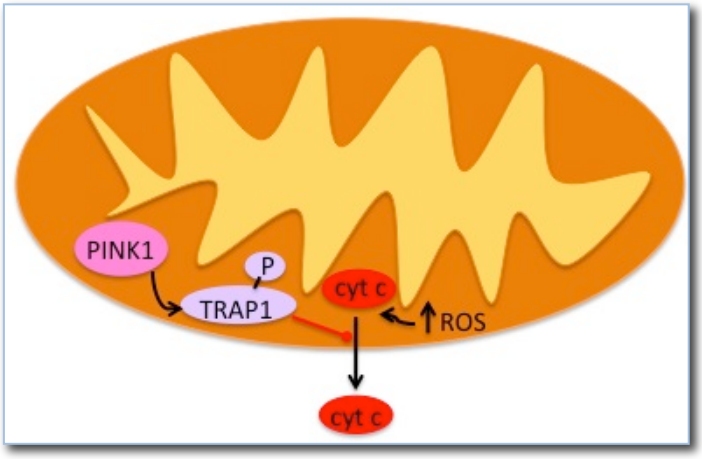



**Figure 3.1: Cytoprotective effect of TRAP1, dependent on its phosphorylation by PINK1.** Under stress conditions, in which the production of ROS is increased, the phosphorylation of the mitochondrial protein TRAP1 is enhanced in a PINK1-dependent manner, and it inhibits the release of cytochrome c from the mitochondria, increasing cell survival.

Recently, TRAP1 has been found to have an impact on the toxicity mediated by the expression of a mutant form of α-synuclein in different model systems, such as *Drosophila* and cultured mammalian cells (Butler et al., 2012). The down-regulation of Trap1 increased the dopaminergic cell loss due to the mutant α-synuclein expression, whereas the overexpression of Trap1 prevented the cell death. Interestingly, the expression of Trap1 does not seem to have a protective effect in all fly models of neurodegeneration, since it failed to rescue the phenotype caused by the expression of Tau with polyglutamine repeats (PolyQ) (Butler et al., 2012).

Amoroso and colleagues described an S6 ATPase protein of the proteasome regulatory subunit, TBP7/ATPase-4/Rpt3, as a new interacting partner for TRAP1 (Amoroso et al., 2011). A small fraction of the TRAP1 protein has been found to localize to the ER membrane in cancer cells, and this seems to be important for a

specific function of TRAP1. The TRAP1/TBP7 complex seems to control the ubiquitination of proteins. The expression levels of two potential TRAP1-interacting proteins, F1ATPase and the 18kDa Sorcin, were analysed following TRAP1 silencing by siRNA, and both proteins were more ubiquitinated and showed reduced levels. The silencing of TRAP1 has also been shown to cause ER stress. The authors propose that the TRAP1/TBP7 complex may constitute a protein quality control system at the ER/mitochondria interface, checking newly formed proteins and allowing them to be repaired from damages and subsequently transported to mitochondria or being targeted for degradation by the proteasome if damages were excessive (Amoroso et al., 2011).

Most of the research work on *TRAP1* was conducted in the cancer field. TRAP1 has been shown to be up-regulated in various types of tumours and seems to be important for cancer cell survival. The expression of TRAP1 increases the resistance of the cells to anti-cancer drugs, such as 5-fluorouracil, oxaliplatin and irinotecan (Costantino et al., 2009). The 18kDa isoform of Sorcin has been identified as a TRAP1-interacting protein, and both of these proteins have been implicated in multidrug resistance in colorectal carcinomas (Landriscina et al., 2010). Indeed, specific TRAP1 inhibitors are in clinical trials for cancer treatment. Therefore, TRAP1 is a potential target for cancer therapy.

The aim of this work is to determine the role of *Trap1 in vivo*, by using *Drosophila* as a model system, with a particular emphasis on the mitochondrial function and the response to stress.

## 3.2 Methods

### 3.2.1 *Drosophila* strains

Three stocks with putative P-element insertions in the *Trap1* gene were obtained from the Bloomington Stock Center: *P{Epgy2}Trap1*$^{EY21851}$, *P{SUPor-P}Trap1*$^{KG06242}$ and *P{Epgy2}Trap1*$^{EY10238}$ and one stock from Exelixis Collection at Harvard Medical School: *PBac{PB}Trap1*$^{c03329}$.

The Trap1 null mutant, *w*$^{1118}$; *Trap1*$^{4}$/*CyO*; +, was a gift from Dr. J. E. Treisman (New York University School of Medicine, NY, USA).

The *Drosophila* strain carrying *piggyBac*-specific transposase: *w*$^{1118}$; *CyO*, *P{Tub-PBac\T}2*/*wgSp-1* and the strain carrying the P-element transposase *ywKi*; +; *Δ2-3/TM3Δ2-3* were obtained from the Department of Genetics, University of Cambridge.

### 3.2.2 Induction of *piggyBac* transposon excision in *Trap1*$^{c03329}$

*piggyBac* transposon excision was induced in *Drosophila* using the crosses scheme shown in Figure 3.2 at 25°C.

Virgin females carrying the *piggyBac* transposon inserted in the *Trap1* gene (*PBac{PB}Trap1*$^{c03329}$) were mated to male flies carrying the *piggyBac*-specific transposase. From the progeny, males carrying both the *piggyBac* transposon and the transposase were selected by a phenotypic selection marker and mated to virgins of the second chromosome balancer (*w*; *Sco*/*CyO*; +). A total of 25 crosses were established, each with three males and six virgin females. Excisions of *piggyBac* transposon were identified by the loss of *w* eye markers (*w*$^{-}$). Single males were mated to six *w*; *Sco*/*CyO*; + virgin females. The progenies *w*; *Trap1*$^{c03329\ excision}$/*CyO* were maintained as a stock for further analysis.

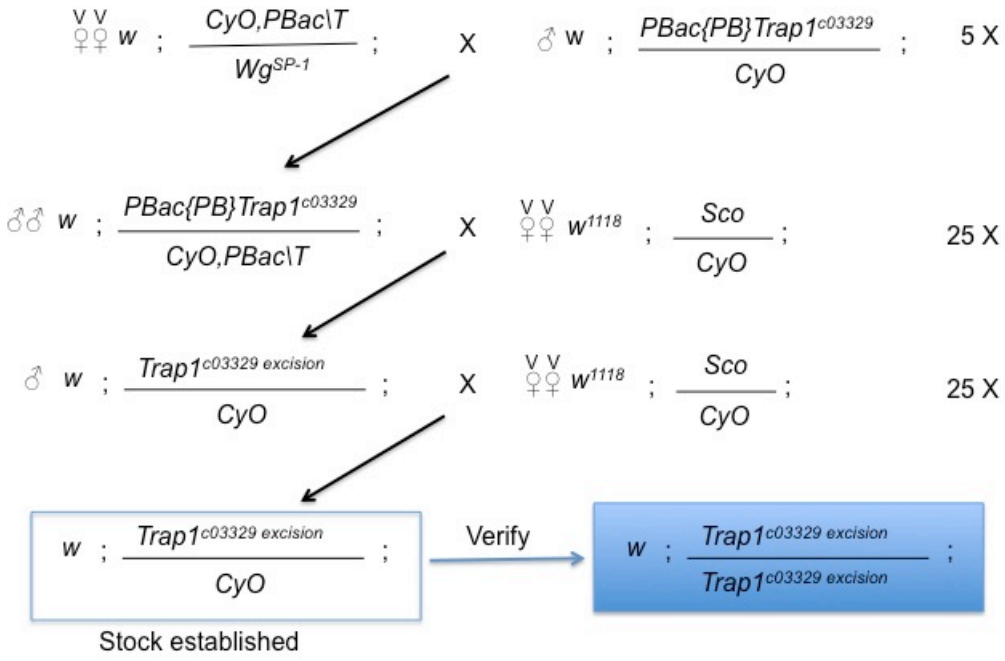



**Figure 3.2: Cross scheme used to induce *piggyBac* transposon excision at the *Trap1* gene.**

### 3.2.3 Analysis of the P-element integration site in *P{Epgy2}Trap1$^{EY21851}$*

The transposable element insertion site and the DNA sequences of the flanking regions were identified from flybase.org. To confirm the integration site is as reported, a PCR screening strategy was designed to allow for the detection of the P element and also for its absence in the control ($w^{1118}$), as well as the P element-excised strains (Figure 3.3). Four primers were designed to conduct the screening by PCR analysis. Two primers were designed to bind to the *Trap1* gene, one forward and one reverse (red and green arrows respectively, in Figures 3.3 and 3.4). The other two primers were designed for the P element (yellow and purple arrows in Figures 3.3 and 3.4). The primers and their sequences are listed in Table 3.1.

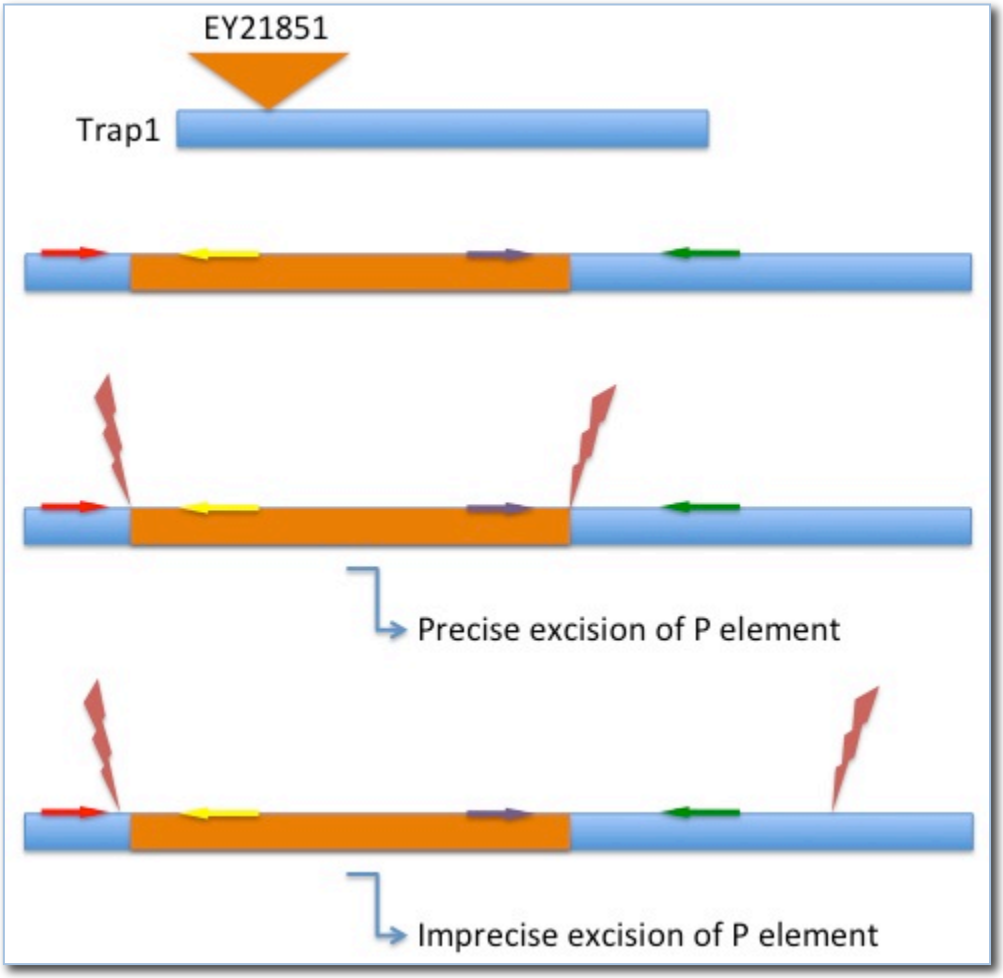



**Figure 3.3: Schematic representation of the P element insertion EY21851 in the *Trap1* gene and the primers used for the PCR screening (arrows).** The scheme also represents the events happening in a precise excision of the P element and a possible imprecise excision of the P element. The blue rectangle represents the TRAP1 gene, whereas the orange rectangle represents the P element EY21851. The figure is not in scale.

**Table 3.1: Primer sequences used for PCR screening of P element EY21851.**

| Primer | Nucleotide sequence | Representation in figures |
|---|---|---|
| **Trap1 F** | CGGCGCACTCTAGTATTTCTC | Red arrow |
| **Trap1 R** | GTGCTCTTCTGCTACGAACC | Green arrow |
| **EY21851 F** | GGTGATAGAGCCTGAACCAGA | Purple arrow |
| **EY21851 R** | CTGCTGCTCTAAACGACGCA | Yellow arrow |

Three different sets of primers were used in the PCR screening strategy to evaluate the presence or absence of the P element (Figure 3.4):

**A**: Trap1 F and EY21851 R

With this set of primers no PCR product could be amplified in the control flies, $w^{1118}$, without the presence of the P element. A PCR product of 715 base pairs (bp) was amplified in strains carrying either one or two alleles of the P element.

**B**: EY21851 F and Trap1 R

This set of primers allowed the formation of a PCR product of 820 bp in flies containing the P element, whereas no PCR product was amplified in flies that do not carry the P element.

**C**: Trap1 F and Trap1 R

With this set of primers the PCR product was a DNA fragment of 611 bp in the control flies. Flies carrying the P element in one of the alleles of *Trap1* (heterozygous) showed a band of 611 bp as well. In strains where the P element is present, no PCR product could be amplified with this set of primers, because the size of the P element is too large (10 908 bp) for amplification.

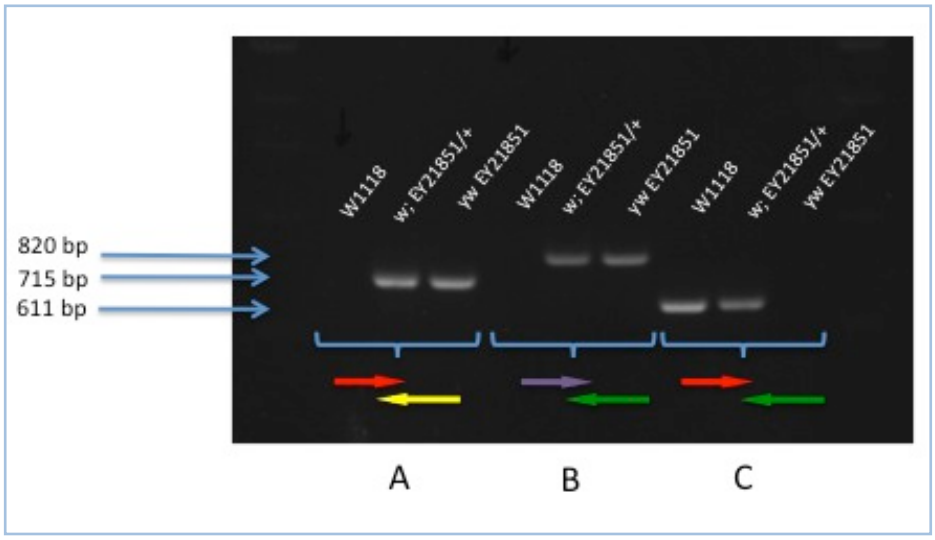



**Figure 3.4: PCR products visualised on an agarose gel.** Strategy A shows a band of 715 bp in flies with the P-element insertion and no band in $w^{1118}$. Strategy B shows a band at 820 bp in flies with P-element insertion and no band in $w^{1118}$. Strategy C shows a band of 611 bp in $w^{1118}$ and heterozygous flies (*EY21851*/+) and no band for the homozygous flies (*EY21851*/*EY21851*) for the P-element insertion. The arrows represent the primers used in each one of the reactions.

### 3.2.4 Induction of the P-element excision in *P{Epgy2}Trap1*$^{EY21851}$

The P-element excision was induced by following the cross scheme in Figure 3.5 at 25°C. Male flies carrying a source of transposase were mated to virgin females carrying the P element transposon inserted in the *Trap1* gene (*P{Epgy2}Trap1*$^{EY21851}$). From the progeny, males carrying both the P element and the transposase were selected by a phenotypic selection marker and mated to virgins of the second chromosome balancer (*w*; *Sco*/*CyO*; +). A total of 80 crosses were established, each with one male and five virgin females. From the cross, P element-excised males (selected by the loss of eye colour) were mated to *w*; *Sco*/*CyO*; + balancer virgin females. Flies were kept as a stock and homozygotes were selected to screen for deletion by PCR.

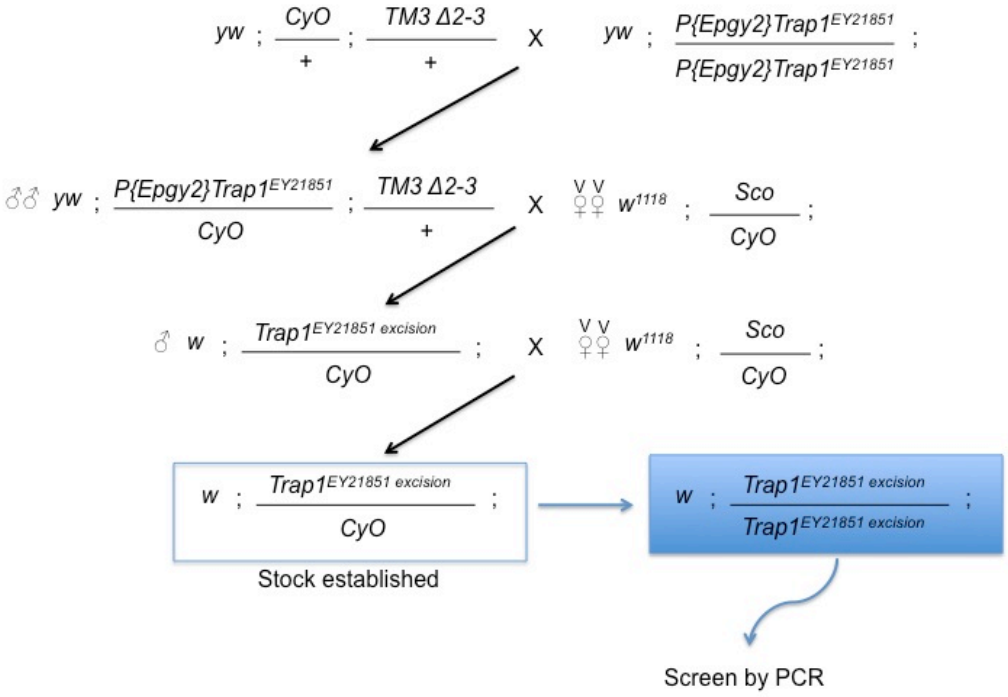



**Figure 3.5: Cross scheme used to induce P-element excision at the *Trap1* gene.**

## 3.3 Results

### 3.3.1 The down-regulation of *Trap1* in *Drosophila* is not sufficient to cause a strong phenotype

In order to address the role of *Trap1* in *Drosophila* it is important to characterise the *Trap1* loss-of-function flies.

The *Trap1* gene is located in the second chromosome, 2R, 42B2-42B2. Various transposon insertions have been identified in the *Trap1* gene region (Figure 3.6), which could potentially affect gene transcription and subsequently the translation of the protein.

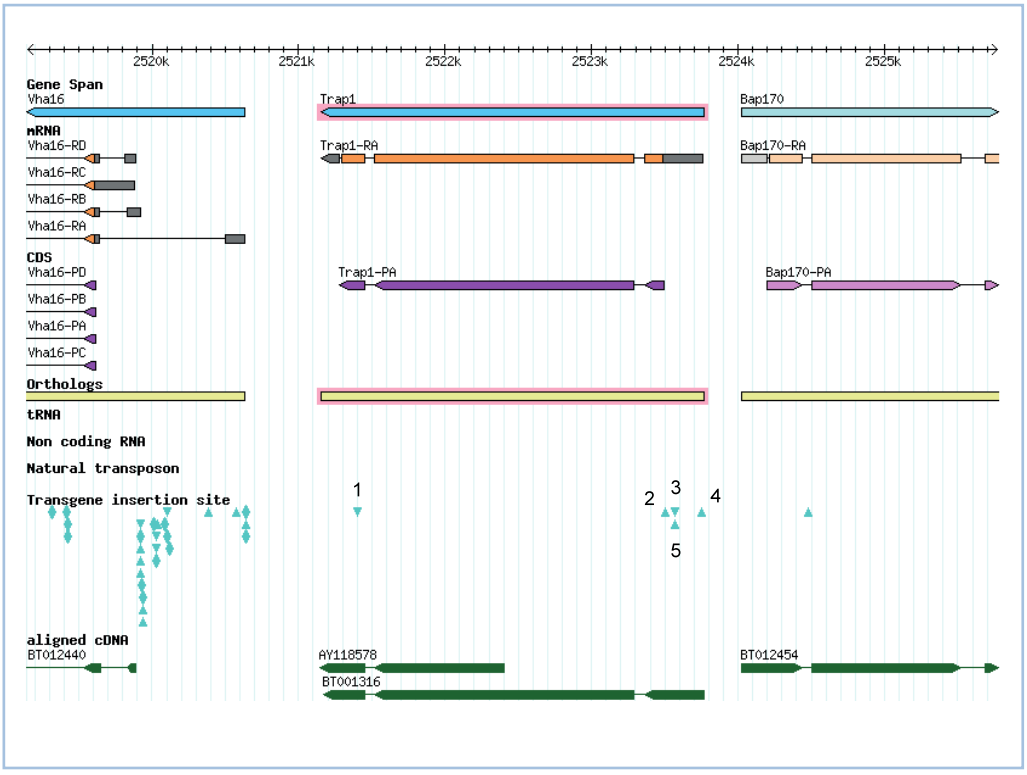



**Figure 3.6: Genomic map of the *Trap1* gene region.** 5 putative P-element insertions in the *Trap1* gene: 1- *P{Epgy2}Trap1*$^{EY21851}$, 2- *PBac{PB} Trap1*$^{c03329}$, 3- *P{SUPor-P}Trap1*$^{KG06242}$, 4- *P{Epgy2}Trap1*$^{EY10238}$ and 5- *P{EP}Trap1*$^{EP2052}$ (not publicly available). Figure adapted from flybase.org.

Transposons are mobile DNA fragments that can insert into and be excised from the genome through the action of the enzyme transposase. The excision may or may not

cause genomic alterations at the original insertion site, depending on whether the excision is precise or imprecise. An imprecise excision usually causes a genomic deletion near the original insertion site. The transposon-mediated imprecise excision is the most extensively used technique for mutagenesis in *Drosophila*. The P-element transposons are commonly used to generate imprecise excisions. On the contrary, the *piggyBac* transposons are considered to always excise precisely by the *piggyBac*-specific transposase and do not create local deletions.

The naturally occurring P elements contain the transposase gene. However, the P elements used in research to achieve mutagenesis in *Drosophila* do not have the coding sequence of the enzyme transposase, which implies that once inserted in the fly genome the P element is immobile, unless the flies are mated with flies carrying a source of transposase. Additionally, the P elements used contain one or more reporter genes that act as markers for the P-element insertion, such as the white gene ($w^+$) to give red eye colour or the yellow gene ($y^+$), which confers brown colour to the fly body (Engels et al., 1990).

Three stocks with putative P-element insertions in the *Trap1* gene were obtained from the Bloomington Stock Center (*P{Epgy2}Trap1*$^{EY21851}$, *P{SUPor-P}Trap1*$^{KG06242}$ and *P{Epgy2}Trap1*$^{EY10238}$) and one stock from Exelixis Collection at Harvard Medical School (*PBac{PB}Trap1*$^{c03329}$).

The line *PBac{PB}Trap1*$^{c03329}$ was analysed intensively, since the insertion is located thirteen bp upstream of the initiation start codon. This line is homozygous lethal at a very early stage of the larval development. The lethality was not rescued by *Trap1* overexpression, since no homozygous flies were obtained. Moreover, the revertant flies of this insertion, obtained by precise excision of the *piggyBac* transposon, were also

homozygous lethal. These observations indicate that the lethality was not due to Trap1 deficiency and there might be other gene or genes affected by this *piggyBac* insertion.

On the other hand, *yw*; *P{EPgy2}Trap1*$^{EY10238}$, *yw*; *P{EPgy2}Trap1*$^{EY21851}$ and *yw*; *P{SUPor-P}Trap1*$^{KG06242}$ were homozygous viable. Real-time qRT-PCR analysis revealed that *P{EPgy2}Trap1*$^{EY21851}$ (hereafter referred to as *EY21851*) had the highest down-regulation of the *Trap1* gene (Figure 3.7), and owing to this result it was used for further analysis.

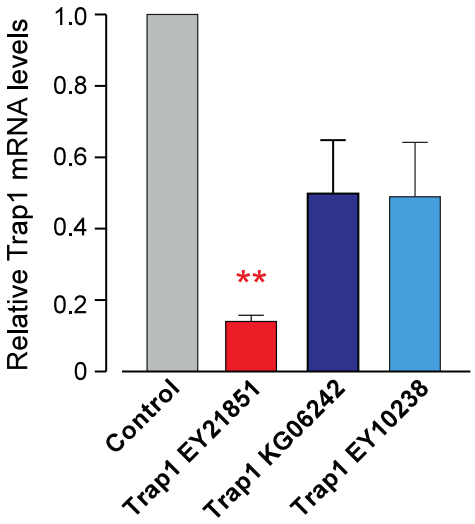



**Figure 3.7: Relative Trap1 mRNA expression in *EY10238*, *EY21851* and *KG06242*.** The three lines have a reduction in the Trap1 mRNA expression compared with the control, and *EY21851* shows the highest down-regulation of the *Trap1* gene. Analysis using the $2^{-\Delta\Delta CT}$ method. Results presented as the means ± S.D. of three biologic replicates. The asterisks indicate significant values, ** $P < 0.01$ (One-way ANOVA with Dunnett's multiple comparison test). Control: *yw*; +; +.

The *EY21851* flies did not have an obvious phenotype, as no morphology alterations or behavioural changes could be detected.

In order to obtain a *Trap1* loss-of-function mutant, experiments were carried out to imprecisely excise the P element in the *EY21851* strain. Ideally, the imprecise excision would create a deletion in the *Trap1* gene, thus generating a null mutation.

The selection of male flies with the P-element excision was first conducted phenotypically, since the loss of the P element resulted in the loss of the orange eye colour (marker present in the transposon). A total of 150 single males were selected and mated with virgin females of the second chromosome balancer to generate fly stocks. A single homozygous male fly was screened for the excision by PCR for each stock (section 3.2.4).

The PCR analysis of more than 100 male flies revealed that the majority of the excisions were precise excisions (Figure 3.8). In 11 cases, a part of the P element together with the intact *Trap1* gene remained in the genome, as revealed by sequencing of the PCR product.

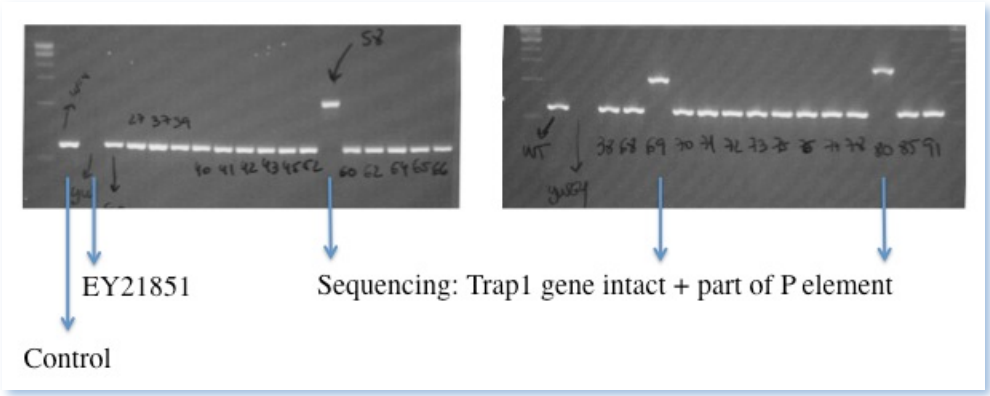



**Figure 3.8: Analysis of the PCR products for screening the P-element excisions in the line *EY21851*.** Pictures of the agarose gels are representative of the overall results (Strategy C in the methods, Section 3.2.4). Primers used were Trap1 F and Trap1 R and the expected PCR product for the control flies ($w^{1118}$) was 611 bp, whereas no PCR product was expected in the flies with the P element EY21851. For the lines showing a band of higher molecular weight than that of the control, the PCR product was sequenced and it revealed that a part of the P element was inserted in the *Trap1* gene.

In the absence of an imprecise excision, experiments were carried out using the *Trap1 EY21851* line, to test whether the down-regulation of the *Trap1* gene could result in a phenotype distinguishable from the controls. The revertant flies of the *EY21851* line

(*Trap1*$^{REV}$), obtained by precise excision of the P element, were used as a control in the experiments.

Analysis of mRNA levels in these flies, showed a reduction of the *Trap1* mRNA level in the *Trap1 EY21851* flies, when compared to the *Trap1*$^{REV}$ (Figure 3.9).

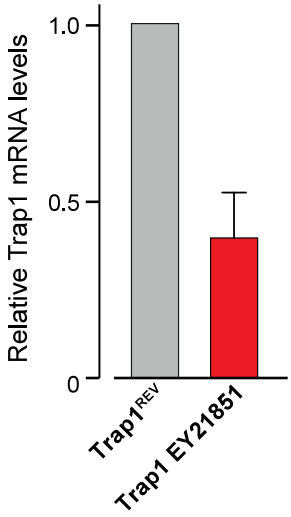



**Figure 3.9: Relative *Trap1* mRNA expression in *EY21851*.** The line *EY21851* shows down-regulation of *Trap1* mRNA when compared to controls. Results are presented as the mean ± SD of three biologic replicates. Trap1$^{REV}$: *w*; *EY21851*$^{REV}$; +, (precise excision of the P element). Trap1 EY21851: *w*; *EY21851/EY21851*; +.

Behavioural analysis, by assessing the climbing performance of the flies, showed no difference between the *EY21851* and the control flies (Figure 3.10). In addition, the analysis of the lifespan also failed to show any difference between the genotypes (Figure 3.11a). However, under oxidative stress treatment, by maintaining the flies in paraquat-containing food, the *EY21851* flies were more sensitive than the controls (Figure 3.11b).

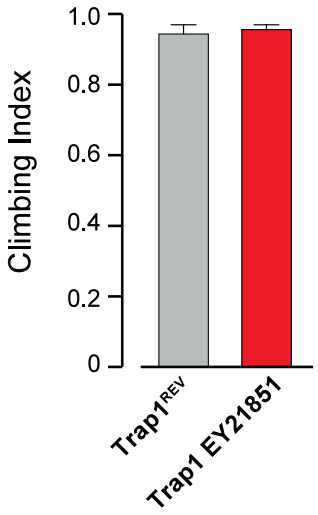



**Figure 3.10: *Trap1 EY21851* flies have a climbing performance similar to that of the controls.** 3-day-old male flies were tested (n > 100 per genotype). Genotypes: Trap1$^{REV}$, *w*; *EY21851$^{REV}$*; +. Trap1 EY21851, *w*; *EY21851/EY21851*; +.

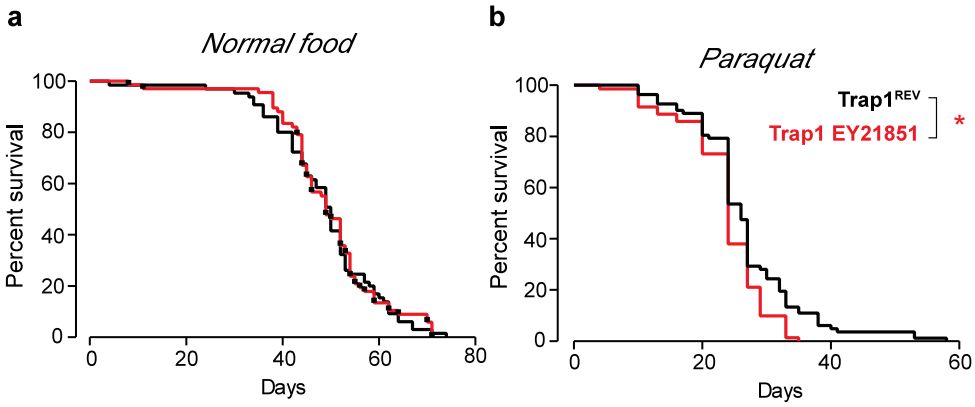


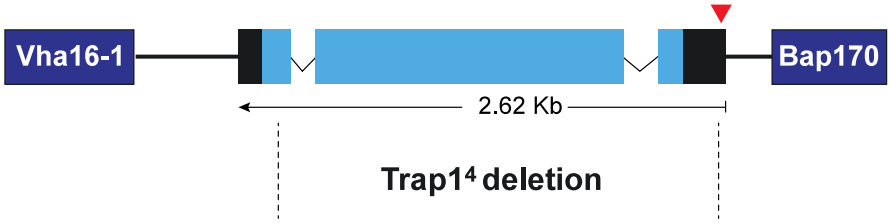

**Figure 3.11: Trap1 knockdown increases sensitivity to stress. (a)** Trap1 knockdown does not alter the lifespan of *Drosophila EY21851* line under normal conditions. Fly viability was scored over a period of 70 days, using a minimum of 100 flies per genotype. **(b)** The *EY21851* flies present a shorter lifespan under paraquat treatment. Fly viability was scored over a period of 60 days, using a minimum of 100 flies per genotype. The asterisks indicate significant values, * P < 0.05 (log-rank, Mantel-Cox test). (a-b) Genotypes: Trap1$^{REV}$, *w*; *EY21851$^{REV}$*; +. Trap1 EY21851, *w*; *EY21851/EY21851*; +.

### 3.3.2 The loss of *Trap1* in *Drosophila* increases the sensitivity to stress and compromises the mitochondrial function of the flies

Work carried out in the laboratory of Dr. J. E. Treisman (New York University School of Medicine, USA), which focused mainly on the gene *Bap170*, located 3' of *Trap1*, had generated imprecise excisions of the P element of *yw; P{EPgy2}Trap1*$^{EY10238}$, which resulted in deletions that affected both *Bap170* and *Trap1* (Carrera et al., 2008). One of these imprecise excision lines, *Trap1*$^{4}$, has a deletion that affected solely the *Trap1* coding region (J. E. Treisman, personal communication), Therefore, *Trap1*$^{4}$ was obtained from Dr. J. E. Treisman for further analysis.

The excision of the P element deleted the majority of the *Trap1* gene (Figure 3.12a). Analysis of mRNA levels by real-time qRT-PCR revealed that *Trap1*$^{4}$ mutants show a total loss of *Trap1* mRNA and that its neighbouring genes were not affected by the imprecise excision of the P element (Figure 3.12b).

**a**




**b**

| | *Amplicons* | | | |
|---|---|---|---|---|
| **Genotype** | **Actin 79B** | **Trap1** | **Vha16-1** | **Bap170** |
| Control | 13.6 ± 0.2 | 19.4 ± 0.1 | 18.0 ± 0.3 | 21.2 ± 0.3 |
| Trap1⁴ | 13.9 ± 0.6 | No $C_T$ | 18.3 ± 1.6 | 21.3 ± 0.1 |

**Figure 3.12: Characterization of the *Trap1*$^4$ mutation. (a)** Genomic map of *Trap1* (cytological location 42B2). Black, untranslated regions; light blue, exons. The P-element insertion (EY10238) is indicated by the red triangle. The neighbouring genes (*Vha16-1* and *Bap170*) are indicated in dark blue. *Trap1*$^4$ deletion, delimited by the dashed lines, removes most of the *Trap1* gene. **(b)** Analysis of the expression levels of *Trap1* and its neighbouring genes. Expression levels were measured by real-time qPCR in 3-day-old flies with the indicated genotypes (mean Ct ± SD, n = 4 per genotype). Expression of actin was used as a control. No Ct for the *Trap1* transcript was detected in *Trap1*$^4$ mutants. Control: *w*$^{1118}$.

The *Trap1*$^4$ mutant flies were viable and developed to adulthood; however, they presented a shortened lifespan compared with the controls (Figure 3.13).

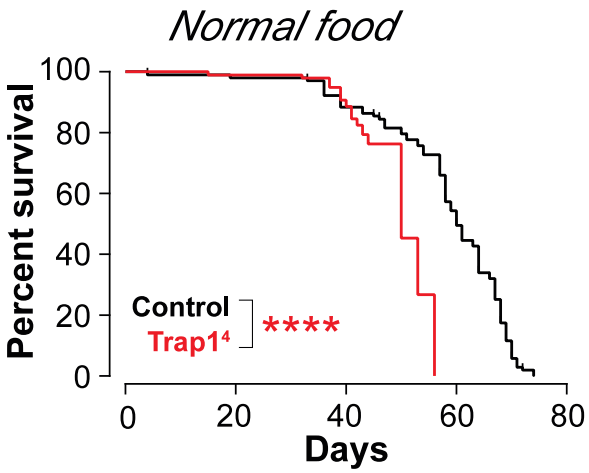



**Figure 3.13: *Trap1⁴* mutants have a decrease in the total lifespan.** *Trap1⁴* mutants (red) have a reduced lifespan compared with the controls (black). Fly viability was scored over a period of 75 days, using a minimum of 100 flies per genotype. The statistical significance is indicated, **** $P < 0.0001$ (log-rank, Mantel-Cox test). Genotypes: Control, $w^{1118}$. Trap1⁴, *w*; *Trap1⁴/Trap1⁴*; +.

Given the proposed role of TRAP1 as a mitochondrial chaperone, the consequences of exposing the *Trap1* mutants to increased levels of stress were investigated. When exposed to a heat stress treatment, the *Trap1⁴* mutants revealed a significant decrease in fly viability when compared to the controls (Figure 3.14a). The *Trap1⁴* mutants were also more sensitive to the treatments with the mitochondrial poisons paraquat, rotenone and antimycin (Figure 3.14 b-d).

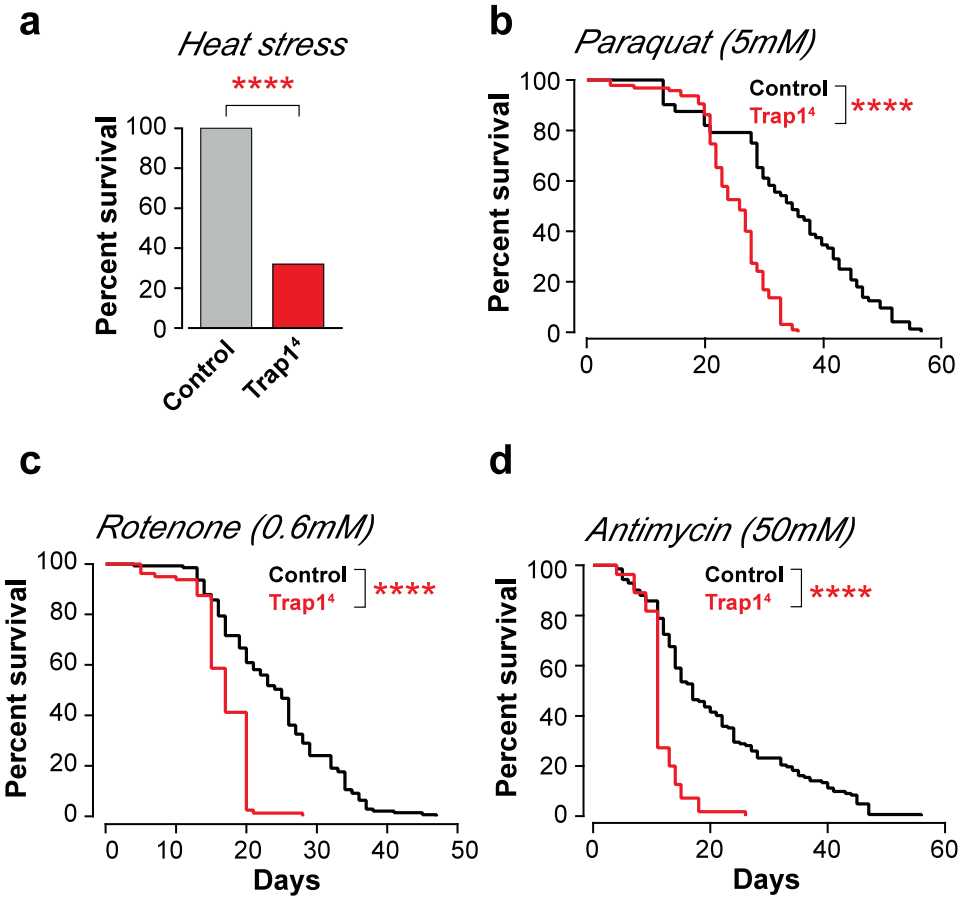



**Figure 3.14: *Trap1*$^{4}$ mutant flies show enhanced sensitivity to stress. (a)** Flies were subjected to heat stress, and viability was assessed after 24 hours. A total of 100 males (4 days old) were assayed for each genotype. The asterisks indicate significant values, **** $P < 0.0001$ (Fisher's exact test, two-sided, alpha < 0.05). **(b-d)** Flies were maintained on food supplemented with the indicated drugs, and the viability was scored over a period of 50 days, using a minimum of 100 flies per genotype. The statistical significance is indicated by the asterisks, **** $P < 0.0001$ (log-rank, Mantel-Cox test). (a-d) Genotypes: Control, $w^{1118}$. Trap1$^{4}$, *w*; *Trap1*$^{4}$/*Trap1*$^{4}$; +.

The *Trap1* mutants showed an age-dependent impairment in the climbing performance, indicative of a locomotor defect (Figure 3.15). In the *Pink1* and *parkin* mutant flies, the locomotor defects result from mitochondrial dysfunction in the skeletal muscles, including the indirect flight muscle (Clark et al., 2006, Park et al., 2006).

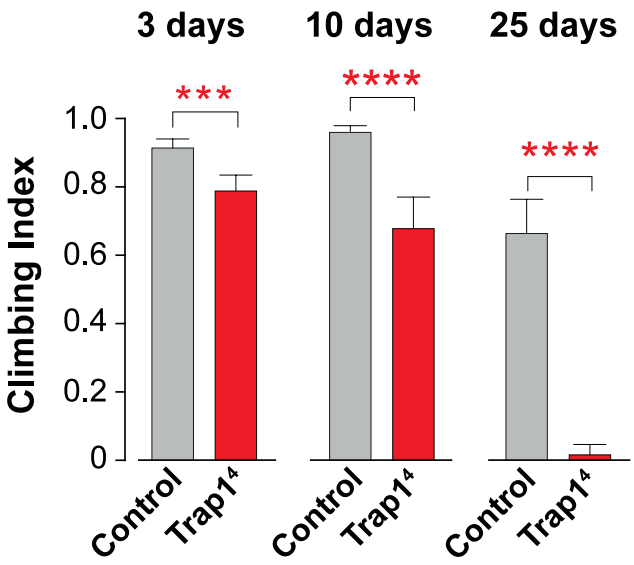



**Figure 3.15: *Trap1*$^{4}$ mutants show a decrease in motor performance.** Flies with the indicated genotypes and ages were tested using a standard climbing assay (mean ± SD, n ≥ 80 flies for each genotype). The asterisks indicate significant values, *** P < 0.001; **** P < 0.0001 (two-way ANOVA with the Bonferroni multiple comparison test). Genotypes: Control, $w^{1118}$. Trap1$^{4}$, *w*; *Trap1*$^{4}$/*Trap1*$^{4}$; +.

The final acceptor of electrons in the ETC is oxygen, which is reduced to water at Complex IV (cytochrome c oxidase). Since this is an essential step prior to the ATP synthesis, the mitochondrial capacity is often assessed from the rates of oxygen consumption (Lanza and Nair, 2010).

To determine whether the loss of *Trap1* leads to mitochondrial impairment, respiration rates were analysed by high-resolution respirometry. Using the twin-chamber instrument manufactured by Oroboros Instruments it is possible to measure oxygen consumption in a small amount of tissue with high sensitivity. Moreover, it is possible to perform sequential measurements involving the titration of substrates, inhibitors and uncouplers (Gnaiger, 2009). The advantages of using this approach include the possibility to assess the respiratory capacity at multiple levels of the ETC and to perform serial measurements in the same sample. The electron transport and the

ATP synthesis are tightly coupled, but some of the energy generated by the ETC is uncoupled from the ATP synthesis (Lanza and Nair, 2010). The experimental uncoupling of mitochondria can be achieved by a freeze-thaw cycle, allowing the measurement of the maximal ETC capacity, since it is not dependent on the production of ATP at Complex V.

The analysis of oxygen consumption rates revealed a significant decrease in the respiratory function of the *Trap1*$^{4}$ mutants (Figure 3.16).

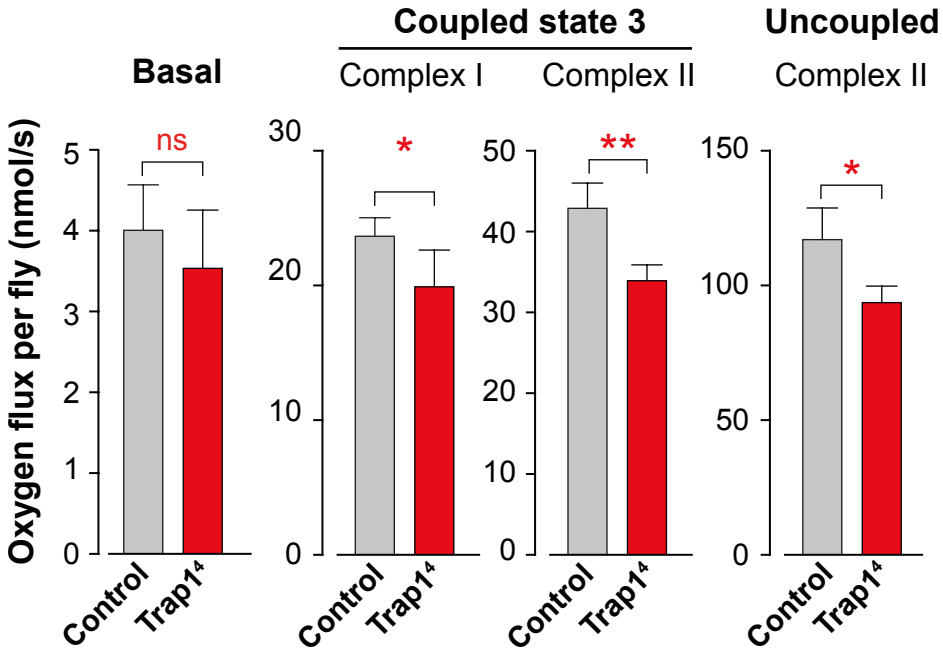



**Figure 3.16: Decreased respiration in *Trap1*$^{4}$ mutant flies.** *Trap1* mutants show decreased oxygen consumption in ADP-stimulated respiration (state 3) in through mitochondria-fed Complex I substrates (coupled state 3 Complex I) and mitochondria-fed Complex II substrates in the presence of the complex I inhibitor rotenone (coupled state 3 Complex II). The *Trap1* mutants also have decreased respiration compared to the control in the uncoupled condition. Oxygen consumption was measured by high-resolution respirometry. Data are shown as the means ± SD (n ≥ 4 in each genotype). The statistical significance is indicated by the asterisks, * $P < 0.05$; ** $P < 0.01$; ns (non significant) $P > 0.05$, (two-tailed unpaired t test). Genotypes: Control, $w^{1118}$. Trap1$^{4}$, *w*; *Trap1*$^{4}$/*Trap1*$^{4}$; +.

A proper mitochondrial function is determined to a larger extent by the expression of mitochondrial proteins, but also by the integrity and functionality of

individual mitochondrial proteins and protein complexes. The analysis of the expression levels of a nuclearly encoded sub-unit of the mitochondrial Complex I by western blotting showed that its levels are reduced in *Trap1*$^{4}$ mutants compared to the controls (Figure 3.17a).

The main function of mitochondria is the generation of ATP through the oxidation of nutrients, such as glucose and fatty acids. In the TCA cycle, energy is released from acetyl groups as reduced coenzymes (NADH, $FADH_2$). Subsequently, the energy generated by electron transport in the ETC is conserved by phosphorylation of ADP to ATP. The capacity for ATP production is frequently used to define the functionality of mitochondria (Lanza and Nair, 2010). The measurement of the ATP levels in *Trap1*$^{4}$ mutants revealed a significant decrease when compared with the controls (Figure 3.17b). Taken together, these results suggest that the loss of Trap1 results in a reduction of the mitochondrial function, associated with a loss of ATP and motor impairment in adult flies.

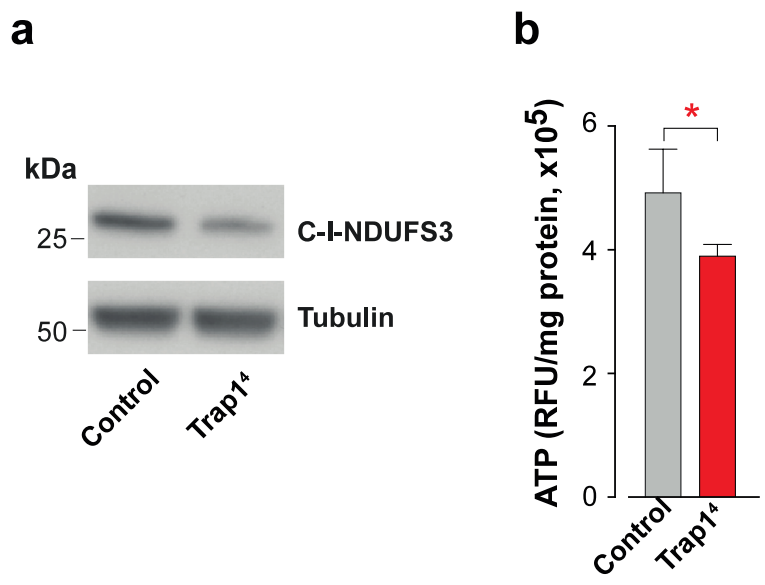



**Figure 3.17: *Trap1*$^4$ mutants show mitochondrial irregularities. (a)** The *Trap1*$^4$ mutants have a reduction in the Complex I protein. Whole-fly protein lysates (20 days old) were used for western blotting analysis with the indicated antibodies. **(b)** The *Trap1*$^4$ mutants have lower levels of ATP when compared to the controls. * P < 0.05 (two-tailed unpaired t test). (a-b) Genotypes: Control, $w^{1118}$. Trap1$^4$, *w*; *Trap1*$^4$/*Trap1*$^4$; +.

The loss of dopaminergic neurons can be indirectly assessed through the analysis of the expression levels of TH, an enzyme expressed in dopaminergic neurons (Wills et al., 2010). There was no detectable difference in the TH levels of *Trap1*$^4$ mutants (Figure 3.18a); however measuring neurotransmitter levels in the heads of *Trap1*$^4$ mutants revealed a significant decrease in the DA content, compared to the controls (Figure 3.18b). Concomitantly, there was an increase in the levels of the neurotransmitter serotonin (Figure 3.18c).

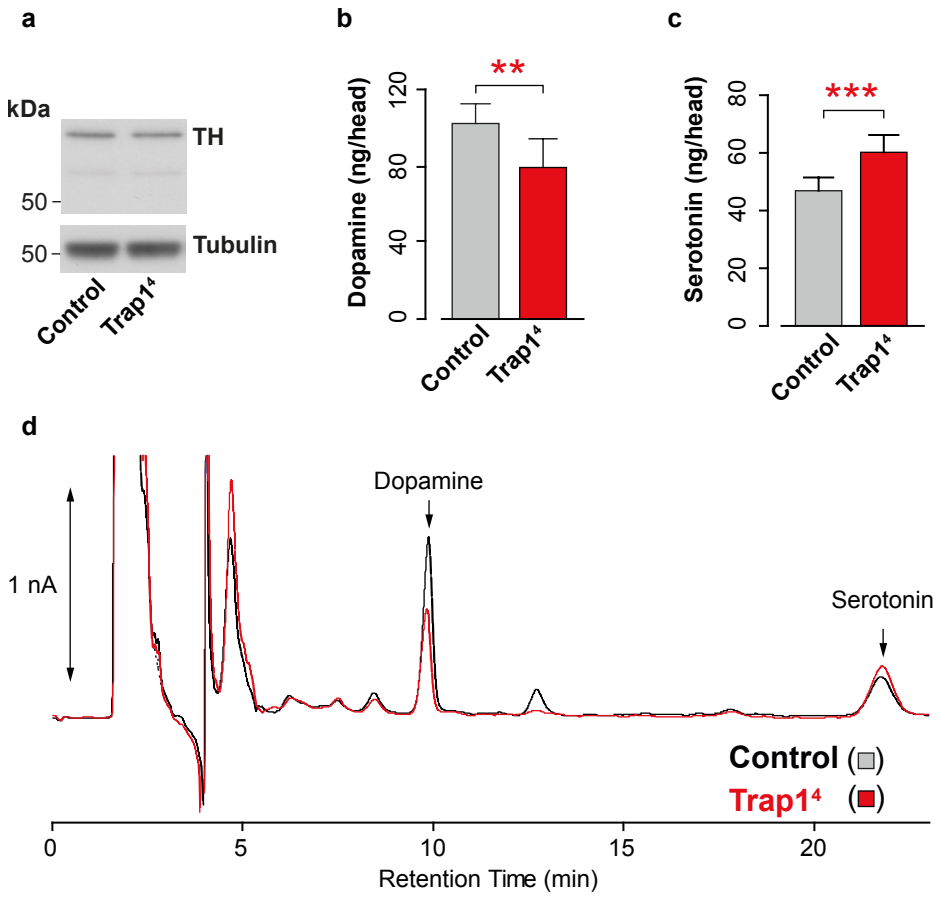



**Figure 3.18: *Trap1*$^{4}$ mutants have a reduction in the brain dopamine levels. (a)** Analysis of tyrosine hydroxylase (TH) levels in *Trap1* mutant flies. Fly-head protein lysates from 20-day-old flies were used for western blotting analysis with the indicated antibodies. **(b)** *Trap1* mutant flies have decreased dopamine levels. **(c)** *Trap1* mutants have increased serotonin levels. (b-c) Neurotransmitter levels were assessed by HPLC with electrochemical detection. Data are shown as the means ± SD (n ≥ 5 for each genotype). The statistical significance is indicated by asterisks, ** P < 0.01, *** P < 0.001 (two-tailed unpaired t-test). **(d)** Representative chromatogram of the HPLC measurements of dopamine and serotonin in *Trap1* mutants and controls. (a-d) Genotypes: Control, $w^{1118}$. Trap1$^{4}$, *w*; *Trap1*$^{4}$/*Trap1*$^{4}$; +.

### 3.3.3 The overexpression of *Trap1* attenuates the age-related decline in motor performance and protects against oxidative stress

In order to gain further insights into the *in vivo* role of the mitochondrial chaperone Trap1, a gain-of-function approach was developed through the generation of *Trap1* transgenic flies. The cloning of the full-length *Drosophila Trap1* into the GAL-responsive pUAST vector was performed previously in the laboratory (unpublished data). The construct was sent to the BestGene Inc. (Chino Hills, CA, USA) for the generation of transgenic flies by P-element mediated transformation. Three independent fly lines were obtained from BestGene (Table 3.2).

**Table 3.2: Transgenic lines of UAS *Trap1*.**

| Transgenic line | Sex of G1 adult | Chromosome of insertion |
|---|---|---|
| 1 | male | third |
| 2 | male | third |
| 3 | male | second |

To confirm the expression of the transgene, UAS *Trap1* male flies were crossed to virgin females of the ubiquitous, *da*GAL4, driver and males carrying both the driver and the transgene were used for the analysis (*da* > *Trap1*, in the Figures). The expression of the transgene was confirmed at the mRNA level by real-time qRT-PCR (Figure 3.19a) and at the protein level by western blotting (Figure 3.19b).

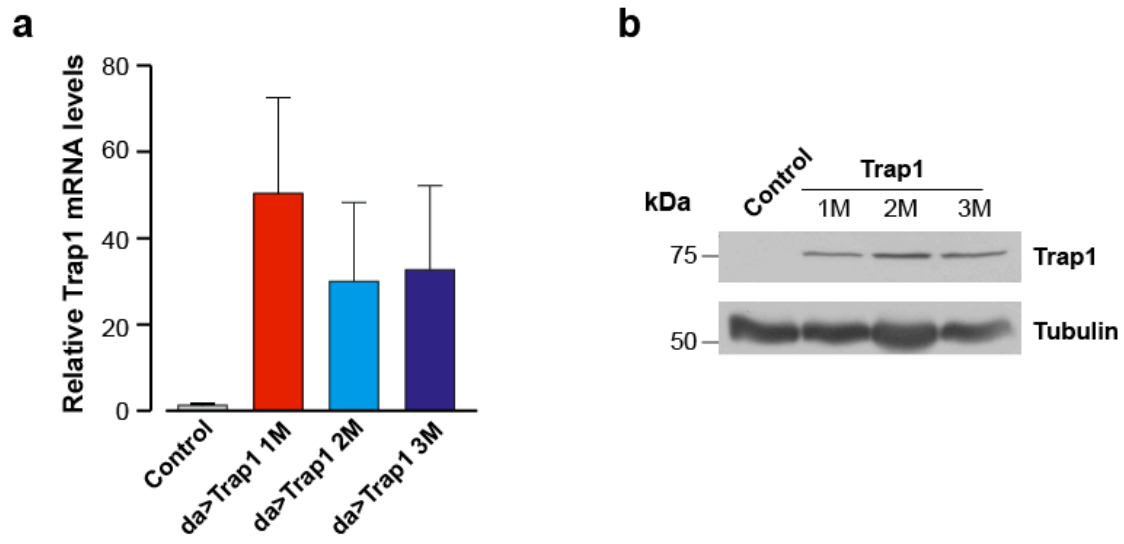



**Figure 3.19: *Trap1* expression in the *Trap1* transgenic lines with the ubiquitous driver *da*GAL4. (a)** Enhanced *Trap1* mRNA levels in the *Trap1* transgenic lines. Results are presented as the mean ± SD of three biologic replicates. **(b)** The expression of Trap1 protein is increased in the transgenic lines. Whole-fly protein lysates were analysed by western blotting with the indicated antibodies. Control, *w*; +; *daGAL4*/+. da>Trap1 1M, *w*; +; *daGAL4*/*UAS Trap1 (1M)*. da>Trap1 2M, *w*; +; *daGAL4*/*UAS Trap1 (2M)*. da>Trap1 3M, *w*; *UAS Trap1 (3M)*/+; *daGAL4*/+.

In addition, the expression of Trap1 was also analysed by immunofluorescence (Figure 3.20). To this purpose, UAS *Trap1* line was crossed to *en*GAL4, UAS*GFP* line. The resulting progenies will have Trap1 and GFP expression in the posterior, but not the anterior, compartment of the larval imaginal discs. This allows the examination of Trap1 expression in GFP positive cells.

The analysis showed that all the transgenic lines expressed Trap1 (Figure 3.20). These experiments also revealed that Trap1 antibody could not detect endogenous Trap1 protein, but could detect the transgenic expression of Trap1 easily.

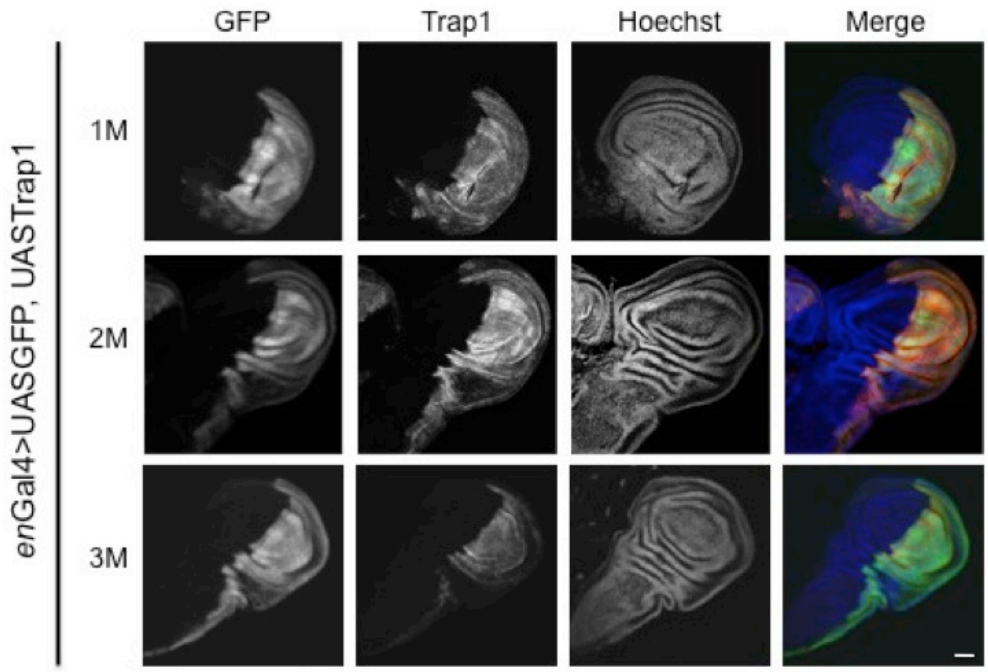



**Figure 3.20: Expression of Trap1 in the larval wing imaginal discs.** The expression of GFP and Trap1 was observed in the posterior compartment of the wing discs (driven by *en*GAL4). The wing discs were also stained with the nuclear dye Hoechst. In the merged colour image, red corresponds to Trap1, blue to nuclei, and green to GFP. Scale bar: 20 μm. Genotype: *w*; *enGAL4,UAS GFP; UAS Trap1*/+.

The mammalian TRAP1 protein was reported to localize to the mitochondria. To investigate the subcellular localization of the *Drosophila* Trap1, crosses were made to express Trap1 and a form of the green fluorescent protein (GFP) targeted to the mitochondria (mitoGFP) under the control of *en*GAL4 driver. The Trap1 protein co-localizes with the mitoGFP (Figure 3.21), indicating that *Drosophila* Trap1 localises to the mitochondria.

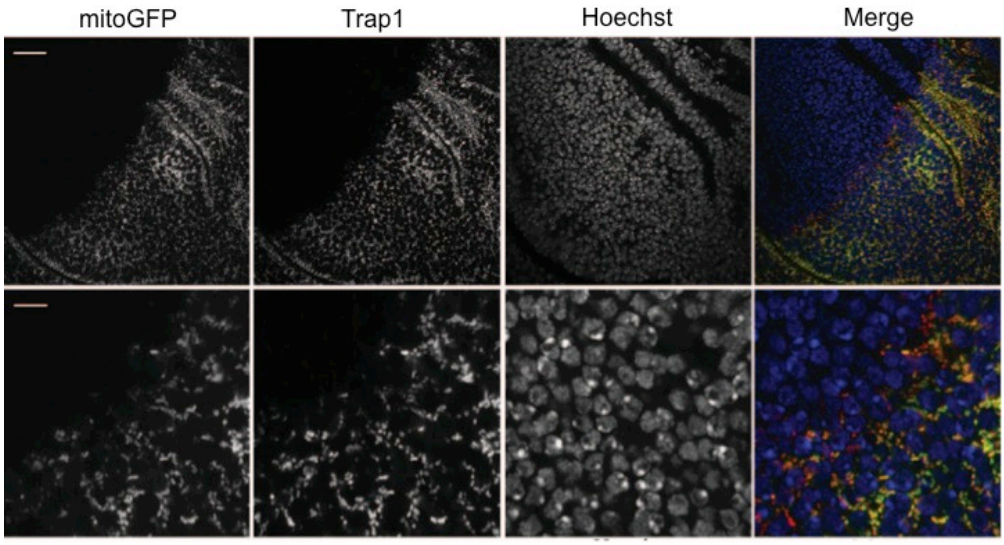



**Figure 3.21: Trap1 localizes to the mitochondria.** Confocal analysis of the posterior compartment of larval wing discs co-expressing Trap1 and a mitochondria-targeted protein (mitoGFP) under the control of the *en*GAL4 driver. In the merged colour image, red corresponds to Trap1, blue to nuclei, and green to mitoGFP. Scale bars: upper 20 μm; lower 5 μm. Genotype: *w*; *enGAL4/UASmitoGFP; UAS Trap1*/+.

To further investigate the phenotypic consequences of the *Trap1* expression, the locomotor performance of these flies was compared to that of the control flies using a climbing assay. Although there was no difference in the climbing ability when the flies were young (3 days old), the *Trap1* expressing flies showed a better climbing performance than the control flies at the age of 25 days (Figure 3.22).

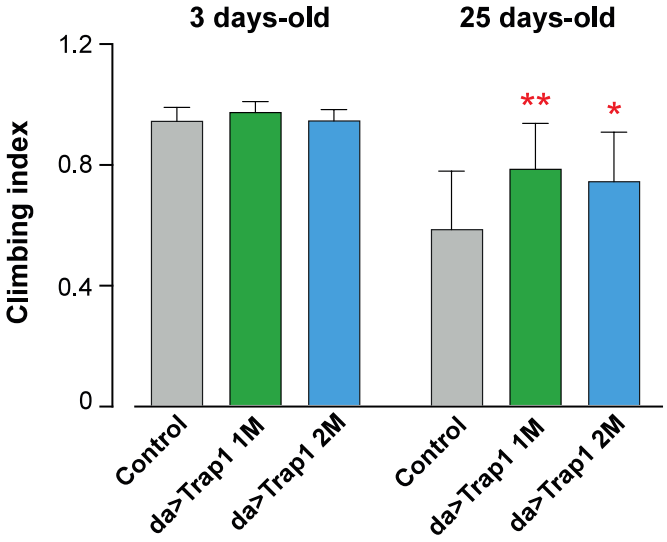



**Figure 3.22: Improved climbing performance in the *Trap1* transgenic flies.** At the age of 3 days old no difference was observed in the climbing ability between the three genotypes tested; however, at the age of 25 days old the Trap1-expressing flies exhibit a better performance in climbing than that of the controls. Flies with the indicated genotypes and ages were tested using a standard climbing assay (mean ± SD, n ≥ 120 flies for each genotype). The asterisks indicate significant values relative to the control, * $P < 0.05$; ** $P < 0.01$ (two-way ANOVA with the Bonferroni multiple comparison test). Control, *w*; +; *daGAL4*/+. da>Trap1 1M, *w*; +; *daGAL4*/*UAS Trap1 (1M)*. da>Trap1 2M, *w*; +; *daGAL4*/*UAS Trap1 (2M)*.

The expression of *Trap1* was also effective in rescuing the climbing defects observed in the $Trap1^4$ mutants (Figure 3.23). Moreover, the expression of *Trap1* in the mutants was also able to restore the levels of the mitochondrial Complex I (Figure 3.24). Taken together these results show that the expression of *Trap1* transgene can compensate for the loss of *Trap1* in the $Trap1^4$ mutants.

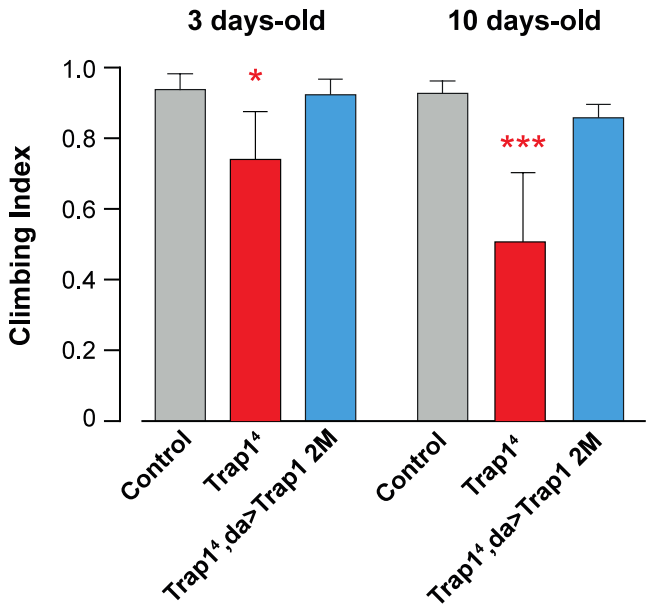



**Figure 3.23: The expression of *Trap1* reversed the decrease in motor performance of *Trap1*$^4$ mutants.** Male flies with the indicated genotypes and ages were tested using a standard climbing assay (mean ± SD, n ≥ 80 flies for each genotype). The asterisks indicate significant values relative to the control, * $P < 0.05$, *** $P < 0.001$ (two-way ANOVA with the Bonferroni multiple comparison test). Genotypes: Control, *w*; *Trap1*$^4$/+; *daGAL4*/+. Trap1$^4$, *w*; *Trap1*$^4$/*Trap1*$^4$; *daGAL4*/+. Trap1$^4$,da>Trap1 2M, *w*; *Trap1*$^4$/*Trap1*$^4$; *daGAL4*/*UAS Trap1 (2M)*.

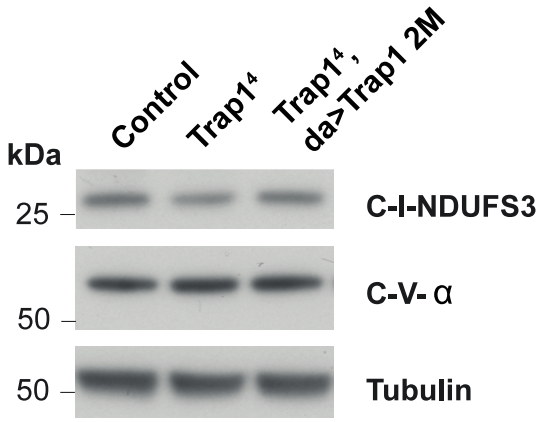



**Figure 3.24: The expression of *Trap1* restored the levels of Complex I in the *Trap1*$^4$ mutants, whereas no differences were detected in the levels of a sub-unit of mitochondrial Complex V.** Whole-fly protein lysates (5 days old) were used for western blotting analysis with the indicated antibodies. Control, *w*; *Trap1*$^4$/+; *daGAL4*/+. Trap1$^4$, *w*; *Trap1*$^4$/*Trap1*$^4$; *daGAL4*/+. Trap1$^4$,da>Trap1 2M, *w*; *Trap1*$^4$/*Trap1*$^4$; *daGAL4*/*UAS Trap1 (2M)*.

It has previously been reported that the treatment of flies with paraquat results in the up-regulation of the mitochondrial chaperones Hsp60 and mtHsp70 as a means to protect mitochondria from the oxidative stress triggered by this pesticide (Pimenta de Castro et al., 2012). Moreover, TRAP1 phosphorylation has been shown being enhanced in response to oxidative stress (Pridgeon et al., 2007). The flies expressing *Trap1* present a normal lifespan compared with the controls (Figure 3.25a). However, *Trap1* expression significantly increased the lifespan of paraquat-treated flies (Figure 3.25b), indicating that the expression of this mitochondrial chaperone protects against oxidative stress *in vivo*.

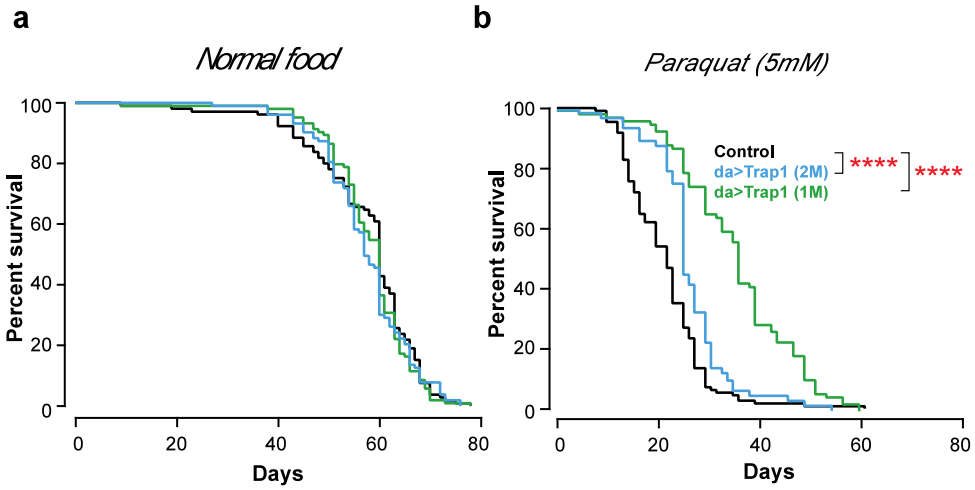



**Figure 3.25: Analysis of the lifespan of the *Trap1* transgenic flies under normal and oxidative stress conditions. (a)** *Trap1* expression does not affect the total lifespan. A total of 100 flies per genotype were maintained on normal food for a period of 80 days. **(b)** *Trap1*-expressing flies (green and blue) show enhanced resistance to paraquat toxicity compared with the controls (black). Fly viability was scored over a period of 80 days using a minimum of 100 flies per genotype. The statistical significance is indicated by the asterisks, **** $P < 0.0001$ (log-rank, Mantel-Cox test). (a-b) Genotypes: Control, *w*; +; *daGAL4*/+. da>Trap1 1M, *w*; +; *daGAL4*/*UAS Trap1 (1M)*. da>Trap1 2M, *w; +; daGAL4*/*UAS Trap1 (2M)*.

## 3.4 Discussion

My work has demonstrated that the *Drosophila* orthologue of the TRAP1 chaperone localizes to the mitochondria, similarly to its mammalian counterpart. Using a loss-of-function approach, through the generation of *Trap1* mutant flies that lack Trap1 expression, I have determined that the loss of Trap1 compromises mitochondrial function. Mutations in either *Pink1* or *parkin* influence mitochondrial dynamics and function, causing an impairment of fission, which affects male fertility by compromising the normal development of mitochondrial derivatives (also known as nebenkern) (Clark et al., 2006, Deng et al., 2008, Park et al., 2006, Riparbelli and Callaini, 2007).

The *Trap1* mutants developed normally to adulthood and were both viable and fertile, suggesting that their mitochondrial defects do not compromise mitochondrial fission and fusion processes required for normal spermatogenesis.

Although *Trap1* mutants show no defects in development, they had a decreased lifespan, progressive locomotor defects and a decrease in the brain DA levels. Taken together, these results suggest that this mitochondrial chaperone is important to minimise the age-dependent decline of mitochondrial function in post-mitotic cells, such as muscle cells and neurons, which rely heavily on mitochondrial function.

The reduction in the DA content in *Trap1* mutants was accompanied by an increase in the serotonin levels. It has been previously reported a decrease in dopaminergic innervation and an increase in the serotonergic innervation in brains of PD patients (Bedard et al., 2011), which may be part of a compensatory mechanism.

The milder phenotype of *Trap1* mutants compared with the *Pink1* mutants (Figure 3.26) suggests that other effectors of molecular quality control, acting downstream of *Pink1*, can compensate for the potential defects caused by the loss of

*Trap1*. In this respect, the serine protease HtrA2, a downstream target of PINK1 (Plun-Favreau et al., 2007, Tain et al., 2009a) involved in mitochondrial molecular quality control (Moisoi et al., 2009), could potentially partially compensate for any disturbances in protein folding in the IMS of mitochondria.

The overexpression of *Trap1* was sufficient to enhance the climbing performance of aged flies and promote resistance to oxidative stress (Figures 3.22 and 3.25). Mitochondria in skeletal muscles are highly specialised towards energy production for contractile activity (Powers et al., 2012), and therefore, the expression of this chaperone might contribute to the maintenance of mitochondrial performance in tissues with high energy demand, such as skeletal muscles, thereby enhancing motor performance.

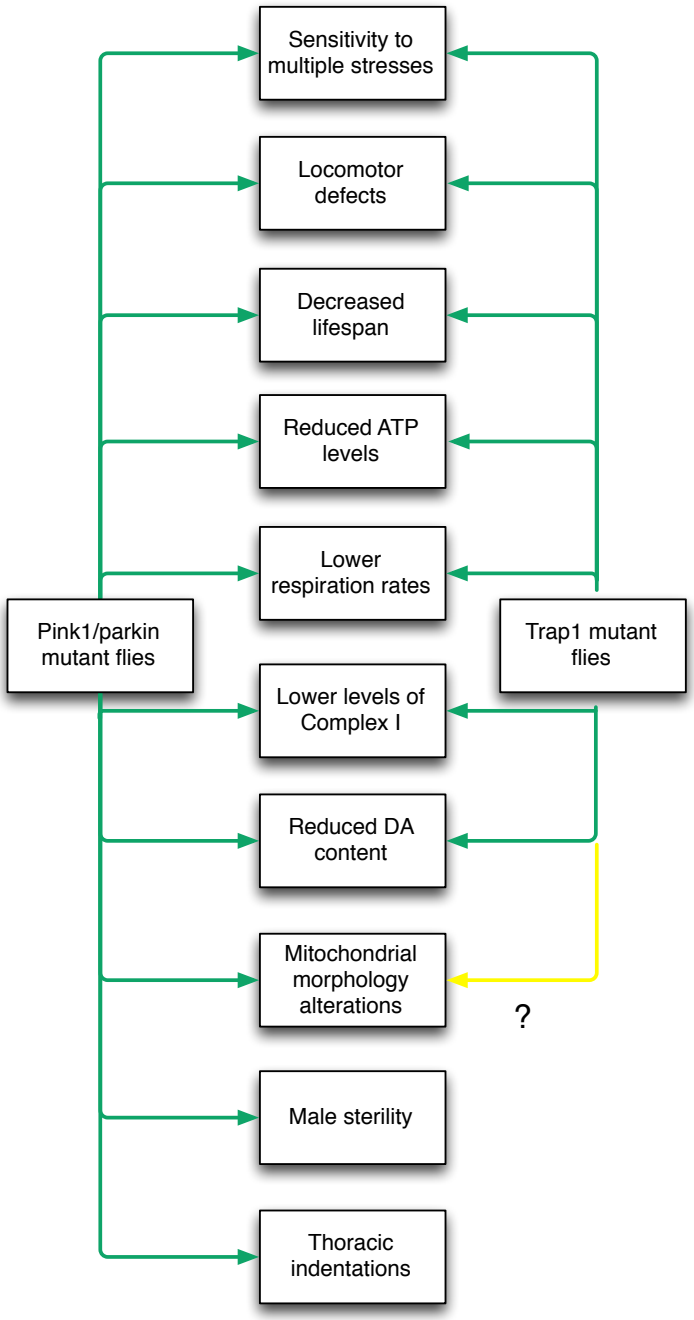



**Figure 3.26: Phenotypic similarities and differences of *Pink1* and *parkin* mutants with *Trap1* mutants.** *Pink*, *parkin* and *Trap1* mutant flies have common phenotypes, such as increased sensitivity to stress, locomotor defects, reduced lifespan, decreased levels of a subunit of Complex I, lower respiration rates and ATP levels. As opposed to Pink1 and parkin mutants, Trap1 mutants do not show thoracic indentations and males are fertile. The mitochondrial morphology of *Trap1* mutants was not analysed in this study.

# Chapter 4

# Epistasis analysis of *Trap1* with *Pink1* and *parkin*

# Chapter 4: Epistasis analysis of *Trap1* with *Pink1* and *parkin*

## 4.1 Introduction

As mentioned in Chapter 1, a large amount of information regarding the PD-linked genes *PINK1* and *Parkin* was obtained from studies using *Drosophila*. Many of the observations in the fly models were subsequently confirmed in studies with mammalian cells.

The *Pink1* and *parkin* mutants show similar phenotypes, essentially characterised by mitochondrial dysfunction, accompained by dopaminergic neuronal degeneration. Both mutants are male sterile, most likely due to the influence of *Pink1* and *parkin* in mitochondrial dynamics and function, causing an impairment of fission, which in turn affects male fertility by compromising the normal development of the nebenkern (Clark et al., 2006, Deng et al., 2008, Park et al., 2006).

Furthermore, both *Pink1* and *parkin* mutants show abnormal wing posture, disorganised muscle fibres with enlarged mitochondria and disrupted cristae, apoptotic muscle degeneration (which causes the appearance of thoracic indentations in the upper part of the thorax), locomotor impairment, reduced lifespan, increased sensitivity to multiple stresses, and dopaminergic neuronal loss (Clark et al., 2006, Greene et al., 2003, Park et al., 2006, Whitworth et al., 2005).

Epistasis analyses have revealed that *Pink1* and *parkin* double mutants do not show increased severity in the phenotype when compared to either of the mutants alone. Moreover, the overexpression of *parkin* can ameliorate the phenotype of *Pink1* mutants, whereas the overexpression of *Pink1* does not affect *parkin* mutant phenotype. These genetic studies suggest that *Pink1* and *parkin* act in a linear genetic pathway that

promotes mitochondrial integrity (Clark et al., 2006, Park et al., 2006, Yang et al., 2006).

TRAP1 was proposed to work downstream of PINK1 in the prevention of mitochondrial dysfunction associated with PD pathogenesis (Pridgeon et al., 2007). The aim of this work is to address whether *Trap1* is part of the *Pink1/parkin* pathway in maintaining mitochondrial integrity. Experimental studies were performed to evaluate the possible genetic interactions between these three genes.

## 4.2 Methods

### 4.2.1 Recombination of UAS *Trap1* (2M) with *park*$^{25}$

In order to recombine the *Trap1* transgene and *park*$^{25}$ mutation in the third chromosome, crosses were carried out as in Figure 4.1. Recombinants where selected for the presence of UAS *Trap1* by eye colour (conferred by *mini-white* in the UAS construct), and the presence of *park*$^{25}$ mutation by PCR screening (Figure 4.2). The primer sequences are the following: Forward (F), `CGTGTCCACGTTTTCCTCCG` and Reverse (R), `TTGTGGCACTGGCGCCCGTC`.

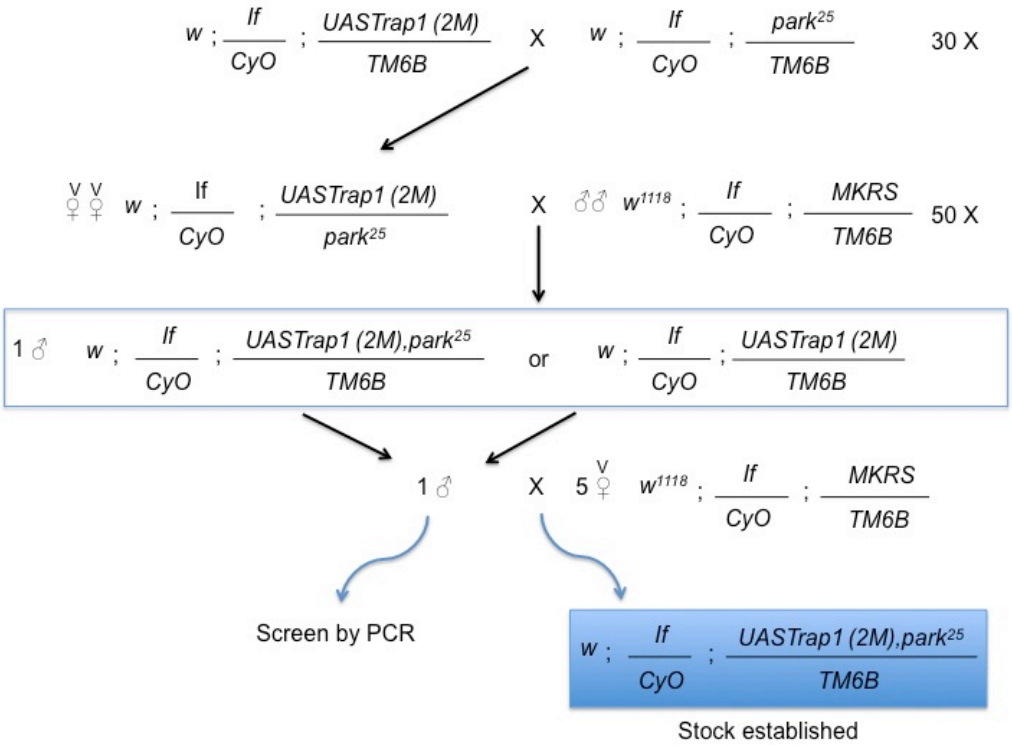



**Figure 4.1: Cross scheme for the recombination of UAS *Trap1* (2M) and *park*$^{25}$.** v♀, virgin female; ♂, male.

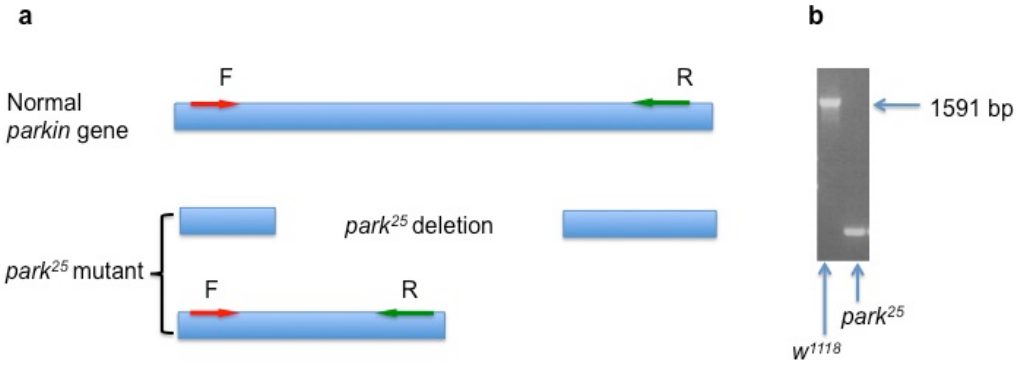



**Figure 4.2: PCR strategy for screening for *park*$^{25}$ mutation in the recombinant flies. (a)** Primers were used that allowed for the distinction between the full length *parkin* gene and the *park*$^{25}$ mutation. **(b)** Confirmation of the validity of the PCR strategy. The expected band of 1591 bp was obtained for the full-length *parkin* gene, whereas a band of a smaller molecular weight was obtained in the *park*$^{25}$ mutants.

## 4.3 Results

### 4.3.1 Trap1 expression rescues the phenotypes associated with the *Pink1* mutants

The expression of TRAP1 in cultured cells was shown to be required for PINK1-dependent inhibition of oxidative stress-induced cytochrome c release and cell death (Pridgeon et al., 2007).

In order to determine if there is a genetic interaction between *Pink1* and *Trap1* in *Drosophila*, the effect of the expression of *Trap1* in the $Pink1^{B9}$ mutants was investigated.

First, the expression of *Trap1* in the *Pink1* mutant background was confirmed by real-time qRT-PCR (Figure 4.3).

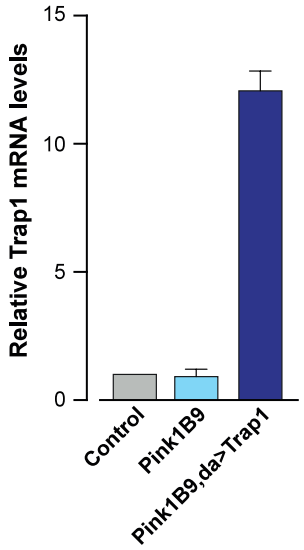



**Figure 4.3: Expression of *Trap1* transgene in *Pink1* mutant using the ubiquitous *da*GAL4 driver.** Expression levels were measured by real-time qPCR in 3-day-old flies. Results are presented as the mean ± SD of three biologic replicates. Genotypes: Control, *w*; +; *daGAL4*. Pink1B9, $Pink1^{B9}$; +; *daGAL4*/+. Pink1B9, da>Trap1, $Pink1^{B9}$; +; *daGAL4*/*UAS Trap1*.

The mitochondrial dysfunction in *Pink1* mutants is associated with muscle pathology, leading to the appearance of thoracic indentations (Appendix 1) and

impaired motor performance. The expression of *Trap1* reduced the appearance of thoracic indentations in these flies (Figure 4.4a) and improved their motor performance (Figure 4.4b).

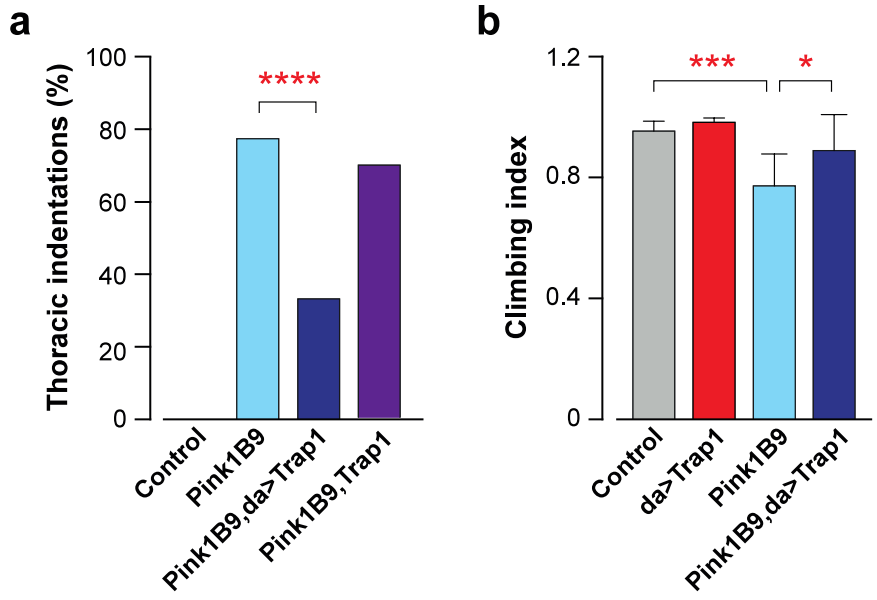



**Figure 4.4: *Trap1* gain-of-function rescues *Pink1* mutant flies. (a)** Expression of *Trap1* rescues the thoracic defects of $Pink1^{B9}$ mutants. The presence of the UAS *Trap1* without the driver (UAS control) had no effect in the degree of thoracic indentations. Thoracic indentations were counted up to 24 hours after eclosion (n > 900 for each genotype). The asterisks indicate significance, **** P < 0.0001 (chi-square, two-sided, alpha < 0.05). **(b)** *Trap1* expression suppresses motor impairment in the $Pink1^{B9}$ mutants. 10-day-old flies with the indicated genotypes were tested using a standard climbing assay (mean ± SD, n = 120 flies per genotype). The statistical significance relative to $Pink1^{B9}$ is indicated by asterisks, * P < 0.05, *** P < 0.001 (one-way ANOVA with Dunnett's multiple comparison test). (a-b) Genotypes: Control, *w*; +; *daGAL4*/+. Pink1B9, $Pink1^{B9}$; +; *daGAL4*/+. Pink1B9, da>Trap1, $Pink1^{B9}$; +; *daGAL4*/*UAS Trap1*. Pink1B9,Trap1, $Pink1^{B9}$; +; *UAS Trap1*/+.

Moreover, the expression of *Trap1* was able to increase the survival of the $Pink1^{B9}$ mutant flies under normal conditions (Figure 4.5a) and also under stress conditions, where flies were maintained in paraquat-containing food (Figure 4.5b). Taken together, these results suggest that the ubiquitous expression of *Trap1* has a beneficial effect in $Pink1^{B9}$ mutant flies.

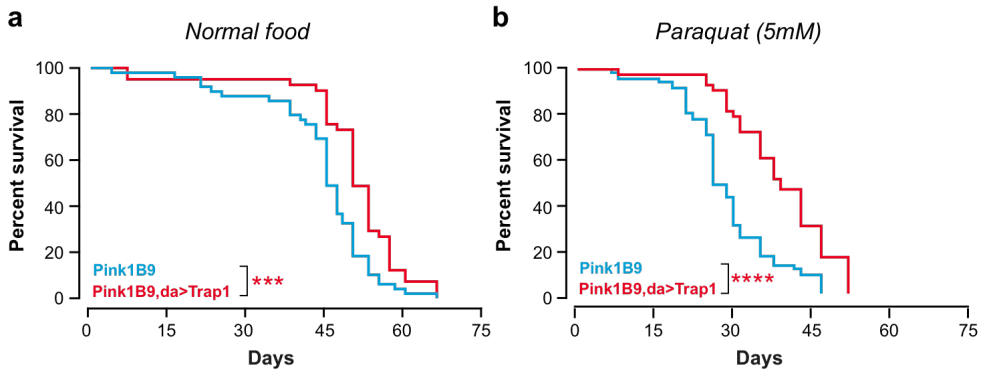



**Figure 4.5: *Trap1* expression enhances the lifespan of *Pink1* mutants.** A minimum of 80 flies per genotype were maintained on either normal (a) or paraquat-containing food (b). Fly viability was scored over a period of 70 days. The statistical significance is indicated by the asterisks, *** $P < 0.001$, **** $P < 0.0001$ (log-rank, Mantel-Cox test). (a-b) Genotypes: Pink1B9, *Pink1*$^{B9}$; +; *daGAL4*/+. Pink1B9, da>Trap1, *Pink1*$^{B9}$; +; *daGAL4*/*UAS Trap1*.

To explore the effect of *Trap1* expression in the mitochondrial phenotype of *Pink1*$^{B9}$ mutant flies, additional assays were carried out. The analysis of mitochondrial respiration by high-resolution respirometry showed that *Pink1*$^{B9}$ mutant flies presented lower respiration rates compared to the controls. A decrease in oxygen consumption in the basal conditions, as well as in through mitochondria-fed Complex I substrates and also mitochondria-fed Complex II substrates was observed (Figure 4.6). The expression of *Trap1* partially recovered the respiration rates in these flies (Figure 4.6). Moreover, the respiration rates in uncoupled conditions were extremely low in the *Pink1*$^{B9}$ mutants compared to the controls, indicating that the ETC is severely damaged in these mutant flies. The expression of *Trap1* also ameliorated the respiration rates of the *Pink1*$^{B9}$ mutants in uncoupled condition (Figure 4.6).

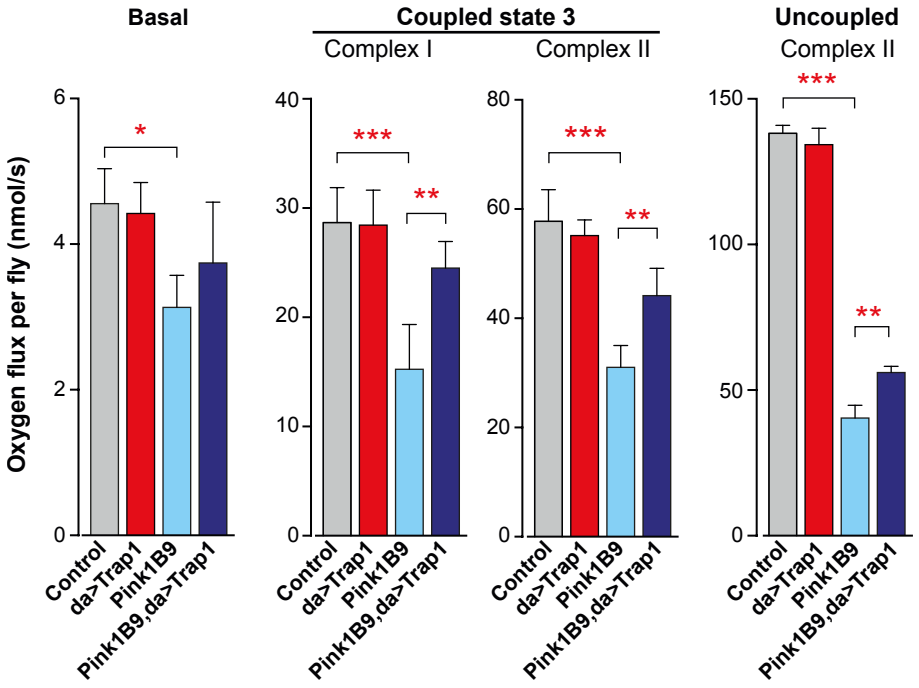



**Figure 4.6: *Trap1* expression partially rescues the mitochondrial respiratory defects in the *Pink1* mutants.** Measurements of state 3 respiration of Complex I and Complex II, and Complex II uncoupled respiration by high-resolution respirometry. The data are shown as the means ± SD (n = 6 for each genotype). The statistical significance is indicated by the asterisks, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA with Dunnett's multiple comparison test). Genotypes: Control, *w*; +; *daGAL4*/+. da>Trap1, *w*; +; *daGAL4*/*UAS Trap1*. Pink1B9, $Pink1^{B9}$; +; *daGAL4*/+. Pink1B9, da>Trap1, $Pink1^{B9}$; +; *daGAL4*/*UAS Trap1*.

*Pink1* mutant flies have also been reported to have depleted levels of mitochondrial Complex I (Liu et al., 2011). The analysis of levels of mitochondrial proteins by western blotting revealed $Pink1^{B9}$ mutants to have reduced levels of Complex I, and this phenotype was suppressed by *Trap1* expression (Figure 4.7a). The analysis of the levels of another mitochondrial respiratory chain complex, Complex V, and of an OMM protein, VDAC, have shown no difference of expression between the genotypes tested (Figure 4.7a). These data indicate that *Pink1* mutants do not have a generalised loss of mitochondria, but present lower levels of Complex I compared to the controls.

In addition, *Pink1* mutants were reported to have a lower level of total ATP (Clark et al., 2006, Park et al., 2006). The expression of *Trap1* was also able to restore, at least partially, the ATP levels of $Pink1^{B9}$ mutants (Figure 4.7b).

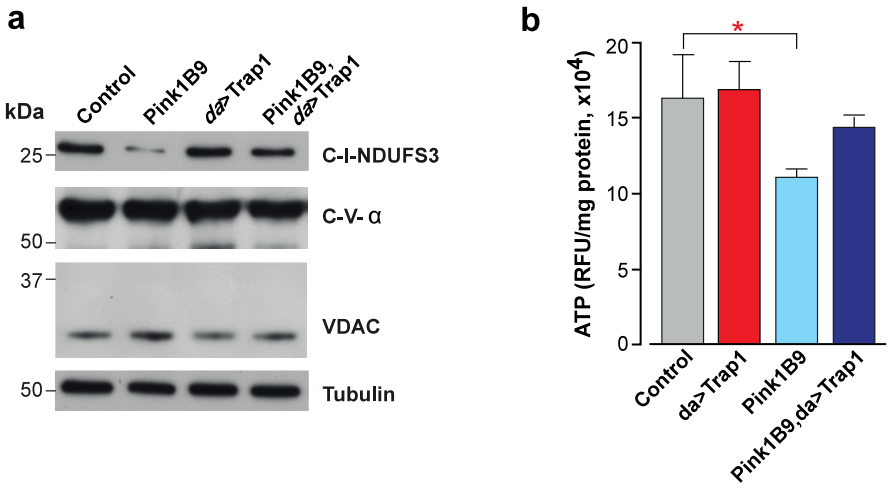



**Figure 4.7: *Drosophila Trap1* reverses mitochondrial dysfunction in *Pink1* mutants. (a)** *Trap1* expression restores the mitochondrial Complex I protein levels in the $Pink1^{B9}$ mutant flies. Whole-fly protein lysates (10 days old) were used for western blotting analysis with the indicated antibodies. **(b)** Expression of *Trap1* increases the ATP levels in the $Pink1^{B9}$ mutants. ATP levels were measured in 10-day-old flies using a bioluminescent assay. Data are shown as the means ± SD (n = 4 for each genotype). The statistical significance, relative to $Pink1^{B9}$, is indicated by the asterisks, * $P < 0.05$ (one-way ANOVA with Dunnett's multiple comparison test relative to the control). (a-b) Genotypes: Control, *w*; +; *daGAL4*/+. da>Trap1, *w*; +; *daGAL4*/*UAS Trap1*. Pink1B9, $Pink1^{B9}$; +; *daGAL4*/+. Pink1B9, da>Trap1, $Pink1^{B9}$; +; *daGAL4*/*UAS Trap1*.

Mitochondria in the *Pink* and *parkin* mutants have been described as enlarged organelles, suggestive of an excess of fusion or a defect in fission (Deng et al., 2008, Poole et al., 2008). The mitochondrial fusion protein dMfn (*Drosophila* mitofusin) was reported to be a downstream target for ubiquitination by parkin, which controls its degradation. In both *Pink1* and *parkin* mutants, higher levels of dMfn were documented (Poole et al., 2010, Ziviani et al., 2010), and this phenomenon was suggested to be an indicator of mitophagy impairment.

To test whether the expression of *Trap1* could impact on the mitophagy pathway as a means to rescue the *Pink1* mutant phenotype, the analysis of the dMfn levels was used as a marker of impaired mitophagy. The expression of *Trap1* had no effect on the accumulation of dMfn seen in the $Pink1^{B9}$ mutants, indicating that mitophagy was not stimulated by *Trap1* expression (Figure 4.8).

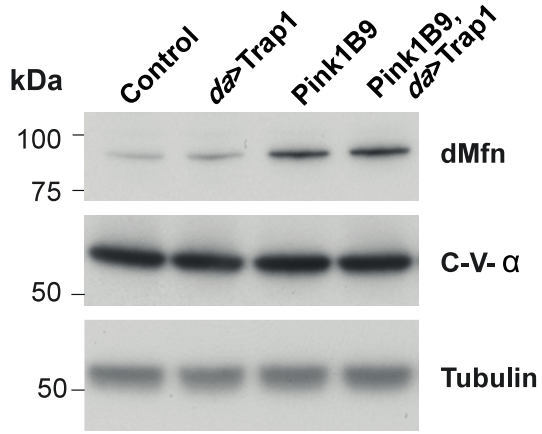



**Figure 4.8: The expression of *Trap1* does not alter the levels of dMfn in *Pink1* mutants.** $Pink1^{B9}$ mutants show an accumulation of dMfn compared to the control flies, which is not reverted by expression of *Trap1*. Complex V-α sub-unit was used as a loading control for mitochondrial content. Whole-fly protein lysates (10-day-old flies) were used for western blot analysis with the indicated antibodies. Genotypes: Control, *w*; +; *daGAL4*/+. da>Trap1, *w*; +; *daGAL4*/*UAS Trap1*. Pink1B9, $Pink1^{B9}$; +; *daGAL4*/+. Pink1B9, da>Trap1, $Pink1^{B9}$; +; *daGAL4*/*UAS Trap1*.

The loss-of-function of *Pink1* in *Drosophila* was associated with dopaminergic neuronal loss. The evaluation of the TH levels in the $Pink1^{B9}$ mutants revealed a significant reduction when compared to the controls, and this reduction could be partially recovered by the expression of *Trap1* (Figure 4.9).

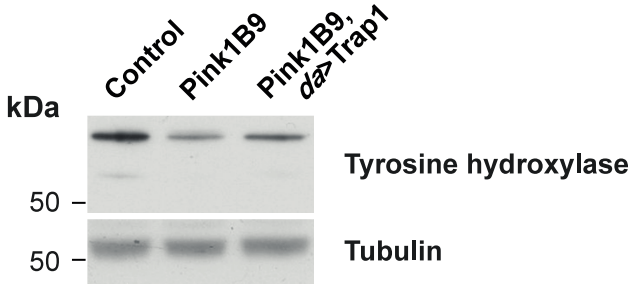



**Figure 4.9: The expression of *Trap1* partially restores the levels of tyrosine hydroxylase in the *Pink1* mutant flies.** Fly-head protein lysates from 20-day-old flies were used for western blotting analysis with the indicated antibodies. Genotypes: Control, *w*; +; *daGAL4*/+. Pink1B9, $Pink1^{B9}$; +; *daGAL4*/+. Pink1B9, da>Trap1, $Pink1^{B9}$; +; *daGAL4*/*UAS Trap1*.

### 4.3.2 Neuronal expression of *Trap1* is sufficient to revert the phenotype of *Pink1* mutants

The genetic removal of the function of *Drosophila* Pink1 results in apoptotic muscle degeneration and a decrease in the brain DA levels as a consequence of the degeneration of dopaminergic neurons (Park et al., 2006).

The loss of mitochondria at neuronal synapses causes defects in neurotransmission of the *Drosophila* neuromuscular junction (NMJ) (Verstreken et al., 2005). Given that one of the most important features of *Pink1* mutant flies is the degeneration of both the indirect flight muscle and dopaminergic neurons, it was tested whether the degeneration of the indirect flight muscle involves a presynaptic component and is therefore preceded by neuronal loss.

The decrease in the TH levels in $Pink1^{B9}$ mutants was reversed upon the expression of *Trap1* using the pan-neural *elav*GAL4 driver (Figure 4.10). This result indicates that the neuronal expression of *Trap1* can suppress neurodegeneration in *Pink1* mutant flies.

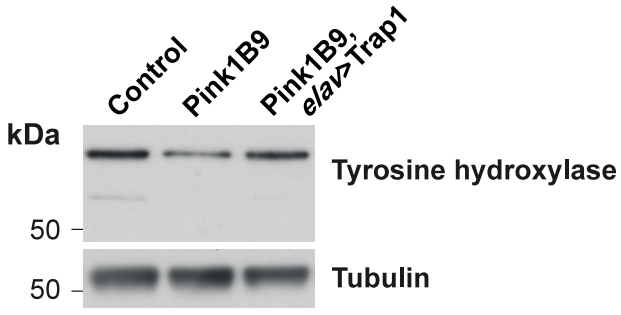



**Figure 4.10: Decreased tyrosine hydroxylase levels observed in the *Pink1* mutants can be partially restored by the expression of *Trap1* in neurons.** Fly-head protein lysates from 20-day-old flies were used for western blotting analysis with the indicated antibodies. Control, *w*; *elavGAL4*/+; +. Pink1B9, $Pink1^{B9}$; *elavGAL4*/+; +. Pink1B9, elav>Trap1, $Pink1^{B9}$; *elavGAL4*/+; *UAS Trap1*/+.

Furthermore, the consequences of the neuronal expression of *Trap1* on phenotypes associated with muscle degeneration were evaluated. The neuronal expression of *Trap1* was sufficient to suppress muscle degeneration in *Pink1*$^{B9}$ mutants, which was reflected in a decrease in the degree of thoracic indentations (Figure 4.11a) and an improved climbing performance (Figure 4.11b). Finally, the neuronal expression of *Trap1* significantly reversed the respiration deficit present in *Pink1* mutant flies (Figure 4.11c).

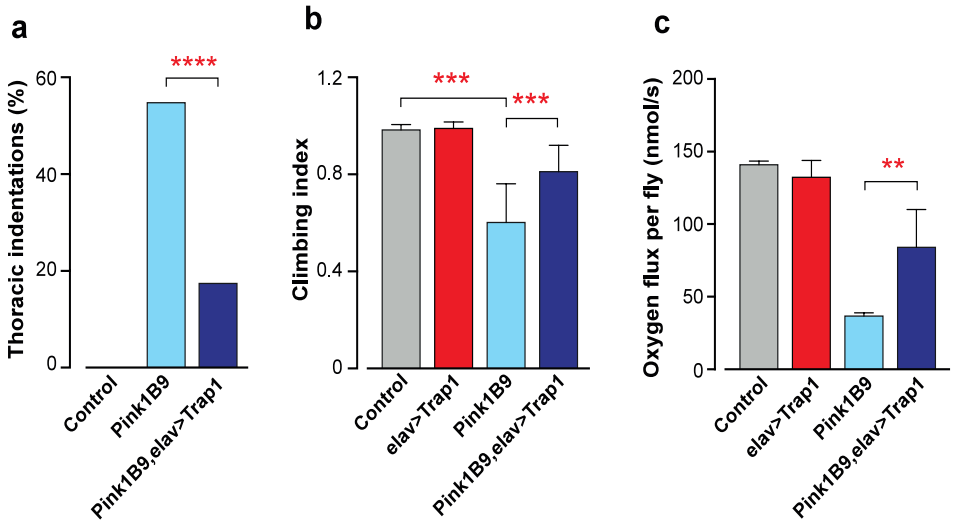



**Figure 4.11: Targeted neuronal expression of *Trap1* rescues mitochondrial dysfunction in *Pink1* mutants. (a)** Neuronal expression of *Trap1* is capable of diminishing the degree of thoracic indentations in the *Pink1*$^{B9}$ mutants. Thoracic indentations were counted in male flies, up to 24 hours after eclosion (n > 300 for each genotype). The asterisks indicate the significance relative to *Pink1*$^{B9}$, **** P < 0.0001 (chi-square, two-sided, alpha < 0.05). **(b)** Targeted neuronal expression of *Trap1* improves the climbing performance of *Pink1*$^{B9}$ mutants. 10-day-old flies with the indicated genotypes were tested using a standard climbing assay (mean ± SD, n = 120 flies per genotype). The statistical significance relative to *Pink1*$^{B9}$ is indicated by the asterisks, *** P < 0.001 (one-way ANOVA with Dunnett's multiple comparison test). **(c)** Expression of *Trap1* specifically in neurons is sufficient to improve mitochondrial respiration in the *Pink1*$^{B9}$ mutants. Complex II-uncoupled respiration was measured by high-resolution respirometry. Data are shown as the means ± SD (n = 6 for each genotype). The statistical significance is indicated by the asterisks, ** P < 0.01 (one-way ANOVA with Dunnett's multiple comparison test relative to the *Pink1*$^{B9}$ mutant). (a-c) Genotypes: Control, *w*; *elavGAL4*/+; +. Pink1B9, *Pink1*$^{B9}$; *elavGAL4*/+; +. Pink1B9, elav>Trap1, *Pink1*$^{B9}$; *elavGAL4*/+; *UAS Trap1*/+.

Taken together, these results indicate that the neuronal rescue of mitochondrial function in *Pink1* mutants by *Trap1* is sufficient to suppress the muscle degeneration and motor impairment in these flies.

### 4.3.3 Trap1 expression compensates for the loss of parkin in *park*$^{25}$ mutants

To address whether *Trap1* interacts genetically with *parkin* in *Drosophila*, the phenotypic consequences of the *Trap1* expression in the *park*$^{25}$ null mutants were investigated.

*parkin* mutants show a very similar phenotype to that of *Pink1* mutant flies, therefore similar assays were performed to evaluate whether the expression of *Trap1* could rescue the *parkin* mutant-associated phenotypes. In order to achieve this, recombination of UAS*Trap1* and *park*$^{25}$ on the third chromosome was carried out. A total of five recombined lines (*w; +; UASTrap1, park*$^{25}$*/TM6B*) were obtained. The ubiquitous expression of *Trap1* using the *Act5C*GAL4 driver was assessed in the *park*$^{25}$ mutant background (Figure 4.12).

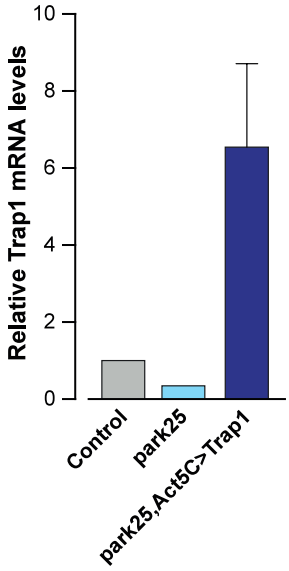



**Figure 4.12: Ubiquitous expression of the *Trap1* transgene in the *park*$^{25}$ mutant background.** Expression levels were measured by real-time qPCR in 3-day-old flies. Results are presented as the mean ± SD of three biologic replicates. Genotypes: Control, *w*; *Act5CGAL4/+; park*$^{25}$*/+*. park25, *w*; *Act5CGAL4/+; park*$^{25}$*/park*$^{25}$. park25, Act5C>Trap1, *w*; *Act5CGAL4/+; UAS Trap1, park*$^{25}$*/park*$^{25}$.

The expression of *Trap1* reduced the number of *parkin* mutant flies with thoracic indentations (Figure 4.13a). However, no improvement in the climbing performance of the *parkin* mutants was achieved by *Trap1* expression (Figure 4.13b).

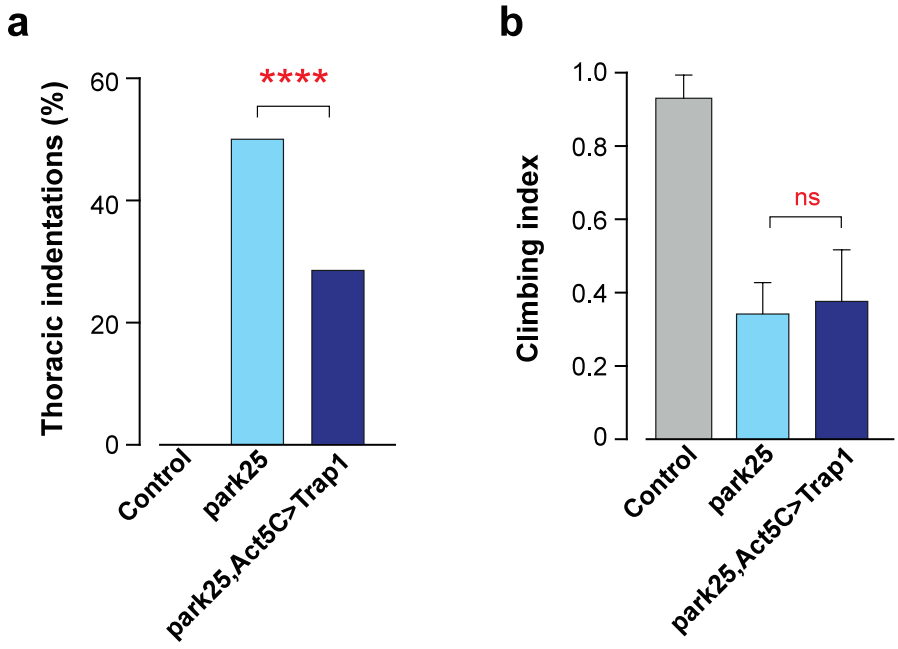



**Figure 4.13: Epistasis analysis of *parkin* and *Trap1*. (a)** *Trap1* expression decreases the degree of thoracic indentations in *parkin* mutant flies, $park^{25}$. Thoracic indentations were counted up to 24 hours after eclosion (n > 250 for each genotype). The asterisks indicate the significance, **** P < 0.0001 (chi-square, two-sided, alpha < 0.05). **(b)** The expression of *Trap1* fails to rescue the climbing defect of the $park^{25}$ mutants. 3-day-old flies with the indicated genotypes were tested using a standard climbing assay (mean ± SD, n > 120 flies per genotype). ns, P > 0.05 (one-way ANOVA with Dunnett's multiple comparison test) (a-b) Genotypes: Control, *w*; *Act5CGAL4/+;* $park^{25}$/+. park25, *w*; *Act5CGAL4/+;* $park^{25}/park^{25}$. park25, Act5C>Trap1, *w*; *Act5CGAL4/+; UAS Trap1,*$park^{25}/park^{25}$.

To evaluate whether the expression of *Trap1* improves the mitochondrial function of $park^{25}$ mutants, the levels of cellular ATP were analysed. This experiment showed a slight increase in the cellular ATP level in $park^{25}$ mutant with *Trap1* expression (Figure 4.14a). The *parkin* mutants also show reduced levels of the mitochondrial Complex I, which could be restored by *Trap1* expression (Figure 4.14b).

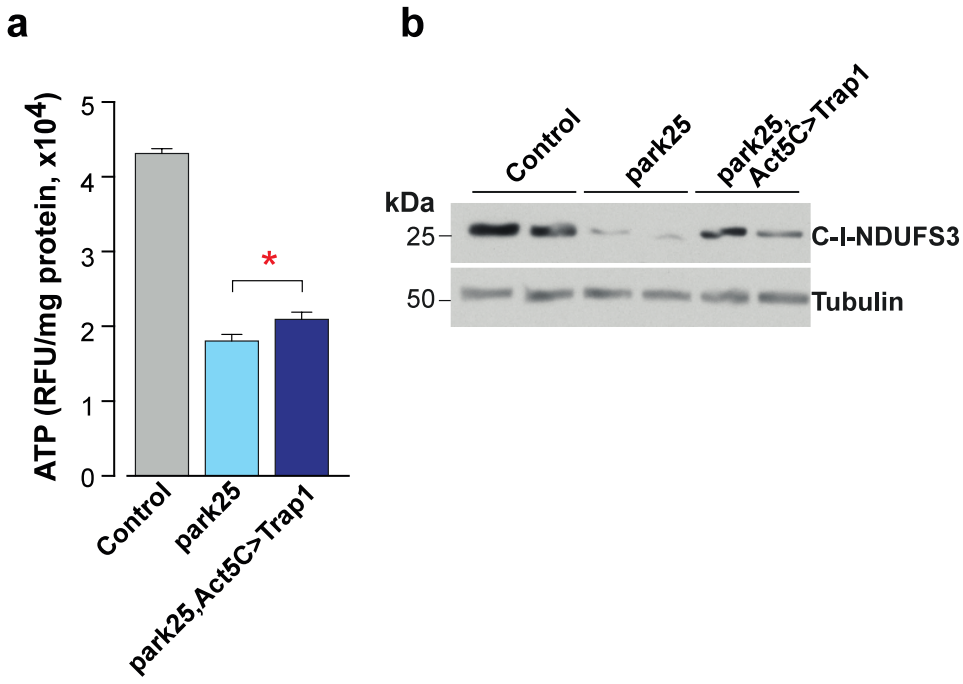



**Figure 4.14: *Trap1* expression ameliorates *parkin*-mutant mitochondrial phenotypes. (a)** *Trap1* expression increases the levels of ATP in the *park*$^{25}$ mutants. The ATP levels were measured using a bioluminescent assay. Data are shown as the means ± SD (n = 4 for each genotype). The statistical significance is indicated by the asterisks, * $P < 0.05$ (one-way ANOVA with Dunnett's multiple comparison test relative to *park*$^{25}$). **(b)** Trap1 expression restores the mitochondrial Complex I protein levels in the *park*$^{25}$ mutant flies. Whole-fly protein lysates from 10-day-old flies were used for western blotting analysis with the indicated antibodies. Control, *w*; *Act5CGAL4/+; park*$^{25}$*/+*. park25, *w*; *Act5CGAL4/+; park*$^{25}$*/park*$^{25}$. park25, Act5C>Trap1, *w*; *Act5CGAL4/+; UAS Trap1,park*$^{25}$*/park*$^{25}$.

Moreover, the expression of *Trap1* increased the survival of the *parkin* mutants under normal conditions (Figure 4.15a) and also paraquat treatment (Figure 4.15b).

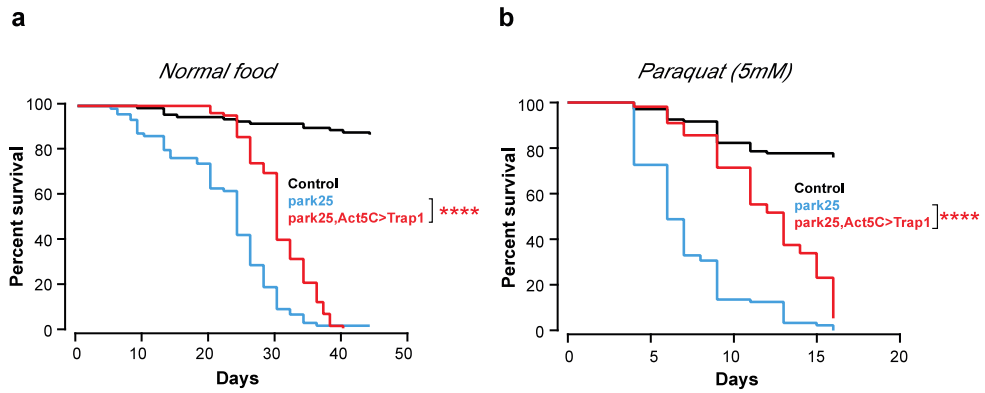



**Figure 4.15: *Trap1* expression enhances the survival of the *parkin* mutants under normal and stress conditions. (a)** Expression of *Trap1* increases the survival of *park*$^{25}$ mutants. Fly viability was scored over a period of 40 days using a minimum of 100 flies per genotype. The statistical significance is indicated by the asterisks, **** $P < 0.0001$ (log-rank, Mantel-Cox test). **(b)** Expression of *Trap1* increases the resistance of *park*$^{25}$ mutants to paraquat-induced stress. Fly viability was scored over a period of 15 days using a minimum of 80 flies per genotype. The statistical significance is indicated by the asterisks, **** $P < 0.0001$ (log-rank, Mantel-Cox test). (a-b) Genotypes: Control, *w*; *Act5CGAL4*/+; *park*$^{25}$/+. park25, *w*; *Act5CGAL4*/+; *park*$^{25}$/*park*$^{25}$. park25, Act5C>Trap1, *w*; *Act5CGAL4*/+; *UAS Trap1*,*park*$^{25}$/*park*$^{25}$.

Taken together, these results indicate that the expression of *Trap1* can partially compensate for the loss of parkin in the *park*$^{25}$ mutants. By expressing *Trap1*, there was an improvement of the majority of the *parkin*-mutant phenotypes analysed, with the exception of the climbing performance of the flies.

### 4.3.4 The expression of *parkin* ameliorates *Trap1* mutant-associated phenotypes

The second approach to investigate the epistasis relationship between *parkin* and *Trap1* was to analyse the effect of *parkin* expression in *Trap1*$^{4}$ mutant flies. The expression of a *parkin* transgene was able to significantly ameliorate the climbing performance of the *Trap1* mutants (Figure 4.16a). Similarly, the *parkin* expression restored the levels of the mitochondrial Complex I, which were reduced in the *Trap1*$^{4}$ mutant flies (Figure 4.16b).

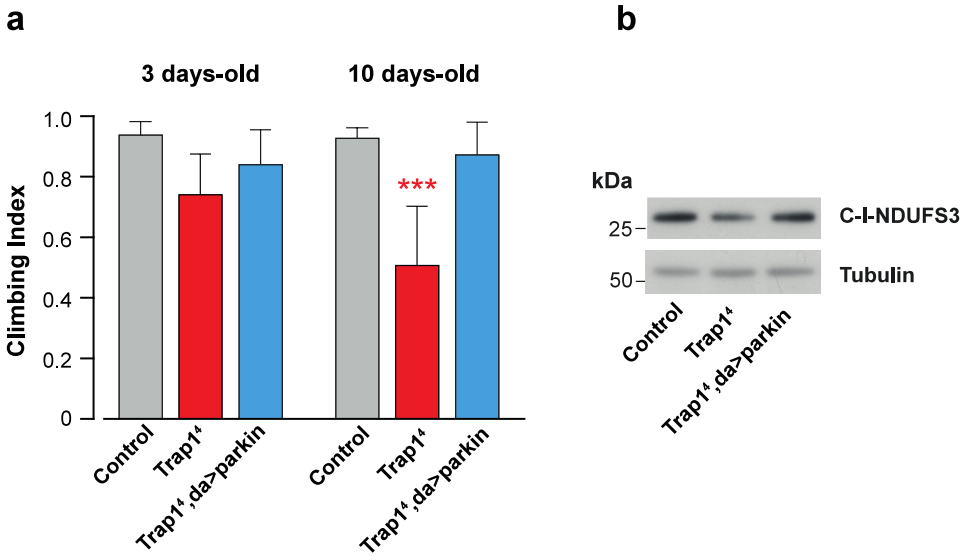



**Figure 4.16**: **The expression of *parkin* rescues *Trap1*$^{4}$-mutant phenotype. (a)** The expression of *parkin* rescues the climbing defects of *Trap1*$^{4}$ mutants. Flies with the indicated genotypes and ages were tested using a standard climbing assay (mean ± SD, n > 60 flies per genotype). The asterisks indicate significant values relative to the control (two-way ANOVA with the Bonferroni multiple comparison test). **(b)** *parkin* expression restores the mitochondrial Complex I protein levels in *Trap1*$^{4}$ mutant flies. Whole-fly protein lysates from 20-day-old flies were used for western blotting analysis with the indicated antibodies. (a-b) Genotypes: Control, *w*; *Trap1*$^{4}$/+; *daGAL4*/+. Trap1$^{4}$, *w*; *Trap1*$^{4}$/*Trap1*$^{4}$; *daGAL4*/+. Trap1$^{4}$, da>parkin, *w*; *Trap1*$^{4}$/*Trap1*$^{4}$; *daGAL4*/*UAS parkin*$^{62B}$.

Taken together these results suggest that *Trap1* and *parkin* interact genetically and they seem to intervene in parallel pathways to promote mitochondrial integrity.

## 4.4 Discussion

The mitochondrial chaperone TRAP1 was identified as a PINK1 substrate and it has been proposed to be part of a molecular quality control pathway (Pogson et al., 2011). In this work, I have shown that the expression of *Trap1* improved the mitochondrial function of *Pink1* mutants and relieved their defective phenotypes, which includes an enhancement of the TH levels. More importantly, it has been observed that the expression of *Trap1* exclusively in neurons was able to rescue the muscle degeneration in the *Pink1* mutants. Mitochondria have been shown to be concentrated in both the presynaptic nerve terminal and the postsynaptic endplates in muscle (Lysakowski et al., 1999), indicating that a decline in mitochondrial bioenergetics may affect synaptic transmission and result in a loss of NMJ innervations. The results of this work suggest that the primary defect in the *Pink1* mutants is neuronal and this seems to precede the muscle degeneration.

Consistent with the hypothesis that Trap1, as a chaperone, acts at the molecular level of mitochondrial quality control, the expression of *Trap1* does not reverse the accumulation of dMfn in the *Pink1* mutants. This result suggests that mitophagy is not being stimulated upon *Trap1* expression.

In *Drosophila*, the expression of *parkin* can rescue the phenotype of *Pink1* mutants, presumably by enhancing the organellar quality control through autophagic disposal of defective mitochondria (de Castro et al., 2011). The results from this work indicate that *Trap1* expression can partially compensate for the mitochondrial defects present in *parkin* mutant flies. Given that Trap1 is likely to act as a molecular chaperone in the clearance of misfolded proteins, its expression may lead to enhanced molecular quality control in mitochondria and therefore decrease the demand for organellar quality control as a clearance mechanism for mitochondria overwhelmed by excessive protein

misfolding. Enhanced expression of Trap1 may therefore decrease the demand on organellar quality control in *parkin* mutants, leading to a decrease in the degree of dysfunctional mitochondria in these insects. The rescue of *parkin* mutants by *Trap1* overexpression was not as remarkable as the *Trap1* rescue of *Pink1* mutants. It remains the possibility that such difference could be due to the fact that the overexpression of *Trap1* was higher when driven by *daGAL4* (driver used for $Pink1^{B9}$ rescue) compared to *act5cGAL4* (driver used for $park^{25}$ rescue) (Figures 4.3 and 4.12). It would be preferable to achieve the same degree of expression of Trap1 to compare the different results.

I also observed that the overexpression of *parkin* was capable of suppressing some of the phenotypic consequences associated with *Trap1* loss-of-function. This suggests that both the molecular and organellar pathways for mitochondrial quality control work in parallel and the enhancement of either one of these can relieve pressure on the other and contribute to a better mitochondrial health.

# Chapter 5

# Mitochondrial quality control mediated by *ref(2)P*

# Chapter 5: Mitochondrial quality control mediated by *ref(2)P*

## 5.1 Introduction

The major cellular pathways used by eukaryotes for the degradation of proteins (either for normal protein turnover or for disposal of damaged proteins) are the ubiquitin-proteasome system (UPS) and autophagy. These processes are essential for maintaining a controlled balance between anabolism and catabolism in order to keep normal cell growth and development. The UPS is the main system for the degradation of short-lived and abnormal or misfolded proteins following labelling with lysine (Lys)-48-linked polyubiquitin chains (Goldberg, 2003). However, misfolded proteins that accumulate and form oligo- and polymeric structures cannot serve as substrates for the UPS, and these aggregates can instead be degraded by autophagy.

There are three forms of autophagy: macroautophagy (usually referred to as autophagy), microautophagy and chaperone-mediated autophagy. Autophagy is thought to be mainly a non-selective process, responsible for the degradation of the majority of long-lived proteins and some organelles (Pankiv et al., 2007). A considerable amount of studies have identified autophagy as a crucial cellular process to avoid accumulation of abnormal proteins in various neurodegenerative diseases.

The autophagic process requires the formation of double-membrane structures called autophagosomes. Two evolutionarily conserved protein conjugation systems are necessary for the autophagosome elongation: Atg12-Atg5- and Atg8-phosphatidylethanolamine (PE). The best-characterised mammalian Atg8 is LC3. During the autophagosomes formation, the cytosolic LC3 (LC3-I) is conjugated with PE (LC3-II), which tightly associates to the membrane of autophagosomes (He and Klionsky, 2009).

The ubiquitin-binding protein p62 is commonly found in inclusion bodies containing polyubiquitinated protein aggregates, also called p62 bodies. p62 is detected in LBs in PD, neurofibrillary tangles in AD, and in Huntingtin aggregates in HD (Kuusisto et al., 2001).

p62 contains an LC3 interacting region (LIR) and can bind to LC3 directly. The LIR motif of p62 is required for the autophagic degradation of p62 bodies. p62 is inducible by stress, and its levels increase upon oxidative stress and inhibition of the proteasome (Pankiv et al., 2007). Moreover, since p62 is normally degraded by autophagy, it accumulates under conditions where autophagy is blocked. The accumulation can be easily detected in autophagy-deficient cells or in tissues from autophagy-deficient animal models. Accumulation of p62 has been used as a marker for inhibition of autophagy or defective autophagic degradation (Bjorkoy et al., 2009). As mentioned in Chapter 1, p62 has been suggested to act as an autophagic adaptor molecule for targeting depolarised and ubiquitin-labelled mitochondria for mitophagy (Geisler et al., 2010).

p62 has been reported to interact with the autophagy-linked FYVE (ALFY) protein and this interaction is important for the formation and autophagic degradation of p62 bodies (Clausen et al., 2010). As opposed to p62, ALFY has not yet been implicated in the degradation of dysfunctional organelles, such as mitochondria. However, that remains an interesting possibility and warrants further investigation.

*refractory to Sigma P*, *ref(2)P*, is the single p62 orthologue of *Drosophila*. This gene has been named refractory (*ref*) owing to the fact that it was identified by the restrictive effect of some of its alleles on sigma virus multiplication (Dezelee et al., 1989). ref(2)P is a protein of 599 amino acids with three domains involved in protein-protein interaction: a Phox and Bem1 (PB1) domain required for multimerization, a ZZ

zinc finger and a ubiquitin-associated domain (UBA) (Nezis et al., 2008). The presence of the UBA domain that enables the protein to bind to ubiquitin suggests that ref(2)P may act as a cargo adapter for the ubiquitinated substrates targeted for autophagic degradation.

ref(2)P has been shown to be required for the formation of protein aggregates in the *Drosophila* adult brain, a process that occurs during the normal ageing of the fly (Nezis et al., 2008). During ageing, proteasome activity and autophagy efficiency decline and the protein aggregates accumulate in the fly brain. Both the PB1 and the UBA domains of *ref(2)P* are necessary for the formation of aggregates, indicating that ref(2)P needs to bind to ubiquitinated proteins and subsequently polymerized by binding to other ref(2)P molecules for protein aggregates formation. Whether this formation of protein aggregates is a protection mechanism, in order to avoid the accumulation of other species that could be more deleterious for the cell is not clear. The genetic removal of p62 in an autophagy-deficient mice model impeded the formation of protein aggregates in different tissues, such as the liver and brain; however it did not prevent the neurodegeneration caused by autophagy deficiency (Komatsu et al., 2007).

In this work, *ref(2)P* loss-of-function mutant flies were characterized in respect to mitochondrial function and resistance to stress. Additionally, I have generated *ref(2)P* transgenic flies for the characterization of the role of *ref(2)P* in the *Pink1/parkin* pathway.

In order to facilitate further studies with the *ref(2)P* transgenic flies, such as detection of protein expression by western blotting analysis, ref(2)P was tagged with hemagglutinin (HA). HA-tag of recombinant proteins has been extensively used, since

HA does not seem to interfere with the activity or distribution of the recombinant protein.

*ref(2)P* was recently shown to play a key role in *parkin*-mediated suppression of mitochondrial dysfunction induced by protein conformational stress (Pimenta de Castro et al., 2012). Since ref(2)P might be an adaptor molecule that targets ubiquitinated substrates for autophagy, I tested in this work whether the expression of *ref(2)P* could rescue the *Pink1* mutant phenotype, perhaps by facilitating the turnover of damaged proteins or mitochondria.

## 5.2 Methods

### 5.2.1 Oligonucleotide design to characterise the gene *Suppressor of ref(2)P sterility, Su(P),* in *ref(2)P* mutants

In order to genotype the flies for the presence or absence of a deletion mutation in the *Su(P)* gene, previously associated with the *ref(2)P* mutants sterility (Bichon et al., 2001), PCR primers were designed so that the 32 bp deletion could be identified. With the combination of primers SuP1 F and SuP1 R, the PCR amplification resulted in a product of 196 bp in the full length *Su(P)* and 164 bp in the presence of the gene deletion. The combination of Primers SuP2 F with SuP2 R amplified a PCR product in the presence of the full-length *Su(P)*, whereas in the presence of the deletion, no PCR product was amplified.

**Table 5.1: Primer sequences used for PCR screening of the *Su(P)* gene.**

| Primer | Nucleotide sequence | Representation in figures |
|---|---|---|
| **SuP1 F** | CACCAGCATCGGTAAATAAGG | Blue arrow |
| **SuP1 R** | TCTTTGGATAAAATAAGGGC | Purple arrow |
| **SuP2 F** | GGGCGAGTCCCTAGAGACTT | Red arrow |
| **SuP2 R** | GCTATAGCGGCCATGTTTTC | Green arrow |

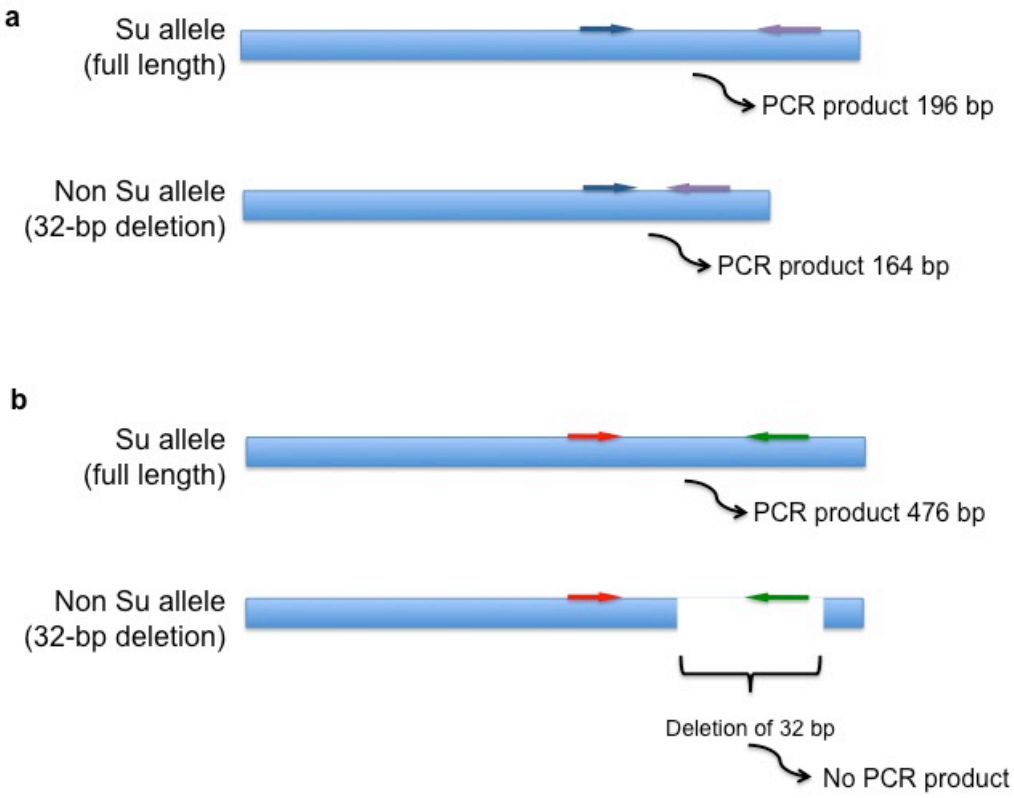



**Figure 5.1: PCR strategy to screen the *Su(P)* gene alleles. a)** In the presence of a Su allele a PCR product of 196 bp is amplified, whereas in the presence of a non Su allele, a smaller PCR product of 164 bp is amplified. **b)** In the presence of the Su allele, a PCR product of 476 bp is amplified. In the absence of a Su allele, the genomic region for the annealing of the reverse primer is not present, thus no amplification of the targeted DNA occurs, leading to the absence of a PCR product.

### 5.2.2 Treatment with drugs inducers of autophagy

Drugs were added to the fly food at lower or higher concentration for larval or adult feeding respectively, as previously described (Tain et al., 2009b). The drugs used included Rapamycin (0.5 μM and 200 μM), metformin (0.25 mM and 100 mM) and trehalose (500 mM, adult feeding only). The newly eclosed males were placed in food containing the drug or ethanol (1% v/v) (vehicle of Rapamycin). Groups of 10 males per vial were transferred to new vials every 2 to 3 days and the number of dead flies was recorded. The assay was completed when all the flies were dead. At least 100 flies

of each genotype were analysed and the results were presented as Kaplan-Meier survival distributions and the significance determined by log-rank tests.

### 5.2.3 Cloning of *ref(2)P* into pUAST

The *ref(2)p* cDNA in pOT2 plasmid vector containing chloramphenicol resistance cassette was obtained from Drosophila Gene Collection of Berkeley Drosophila Genome Project (GH06306). The plasmid was eluted from Whatman disc with 25 μL TE buffer (100 mM Tris, 4 mM EDTA, pH 7.8). For the cloning of *ref(2)P* into pUAST several procedures as described below were performed.

#### 5.2.3.1 Transformation of competent bacteria

NEB10β competent bacteria were used for the transformation. 2 μL of plasmid containing the *ref(2)P* cDNA were added to 50 μL of bacteria and kept for 30 min on ice, followed by a 30 sec heat shock in a 42°C water bath. 200 μL of SOC medium (2% (w/v) Tryptone (pancreatic digest of casein), 0.5% (w/v) yeast extract, 8.6 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$ and 20 mM glucose) were added and incubated at 37°C for 30 min. 100 μL of the resulting culture were spread on LB agar plates containing 30 μg/mL of chloramphenicol and grown overnight at 37°C.

#### 5.2.3.2 Isolation of plasmid DNA by DNA maxiprep

For isolation of large quantities of high quality, pure plasmid DNA, the GENOMEDs JETSTAR 2.0 MAXI kit was used. 250 mL of LB medium containing 100 mg/mL of chloramphenicol was inoculated with a single bacteria colony, which had been grown for 12 hours previously. The culture was maintained at 37°C with agitation at 250 rpm overnight. The bacteria culture was transferred to centrifugation tubes and

centrifuged for 15 min at 5000 g. The supernatant was removed and the bacteria were allowed to dry. The JETSTAR columns were placed on a rack and equilibrated with 30 mL of equilibration buffer E4. In the meantime, bacteria were resuspended in 10 mL of buffer E1 (contained RNase) and 10 mL of buffer E2 was added to lyse the bacteria. After gentle mixing and incubation at RT for 5 min the lysed mix was then neutralized with 10 mL of buffer E3 and centrifuged at 12 000 g for 10 min. The columns were subsequently loaded with the supernatant and this was allowed to run by gravity flow. The column was washed with 60 mL of buffer E6. To elute the plasmid DNA, the column was placed over a clean tube and the DNA was eluted with 15 mL of buffer E6. The DNA was then precipitated with 10.5 mL of isopropanol, followed by centrifugation at 4°C, 12 000 g for 30-40 min. The supernatant was removed and the pellet was allowed to air dry for 10 min, and resuspended in 300 μL of distilled water. Quantity and quality of DNA were assessed by measuring the OD at 280 nm, using a Spectrophotometer (Eppendorf). A small amount of plasmid DNA obtained was run on an agarose gel for confirmation of the expected molecular weight.

#### 5.2.3.3 Vector pUAST

pUAST is a *Drosophila melanogaster* transformation vector (Figure 5.2). It comprises of a fragment of five optimized GAL4 binding sites, followed by the *hsp70* TATA box and transcriptional start site, a polylinker containing restriction sites for EcoRI, BgIII, NotI, Xho, KpnI and XbaI and the SV40 small t intron and polyadenylation site. It also contains the P element ends (P3' and P5'), allowing its incorporation into the genome, and the *mini-white* gene, which acts as a marker for successful incorporation into the genome.

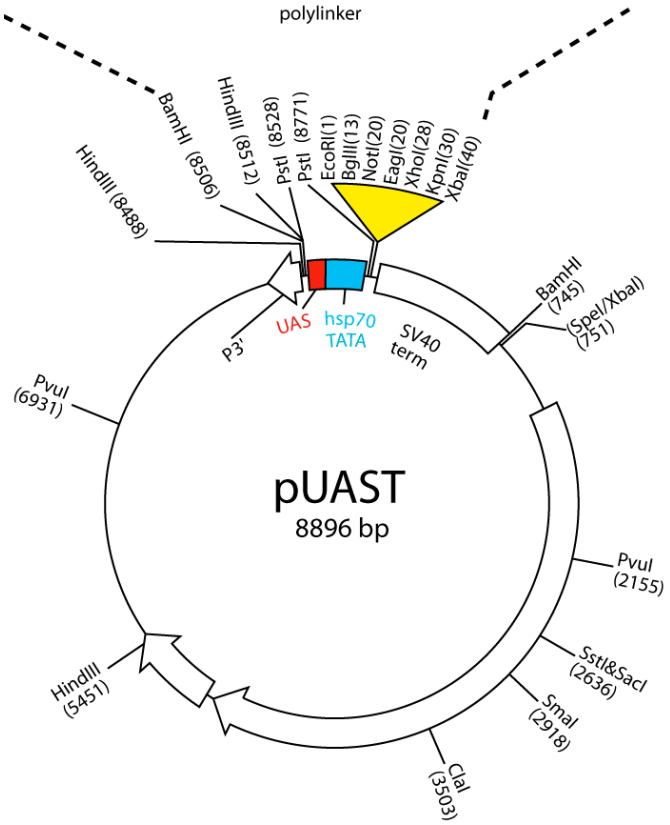



**Figure 5.2: Vector map of pUAST.**
Figure adapted from http://www.gurdon.cam.ac.uk/~brandlab/doc/pUAST_updated.png.

### 5.2.3.4 Restriction enzyme digestion of pUAST

The pUAST vector was digested with restriction enzymes EcoRI and KpnI. BSA was added to the mixture to increase KpnI activity. The components of the digestion mixture are listed in Table 5.1.

The digestion was incubated at 37°C for 3 hours and the product was subjected to an agarose gel electrophoresis to confirm the fragment size and also extraction of DNA fragment.

**Table 5.2: Components of the restriction enzyme digestion of pUAST.**

| Component | Amount |
|---|---|
| DNA (plasmid pUAST) | 5 μg |
| Buffer 1 10X | 5 μL |
| EcoRI | 2 μL |
| KpnI | 2 μL |
| BSA 100X | 0.5 μL |
| Distilled water | 36 μL |

### 5.2.3.5 Purification of the plasmid DNA fragment

Under the UV light, fragments of agarose gel containing the desired DNA were cut with a scalpel and transferred to a 1.5 mL microfuge tube. The Gel Extraction Kit (QIAGEN) was used to purify the DNA. The gel was dissolved in 1.5 mL of Buffer QG and for that was incubated at 50°C for 10 min with occasional vortexing. 750 μL of the gel solution was loaded to the kit column and centrifuged for 1 min at maximum speed. The flow-through was discarded. Another 750 μL of the gel solution were added to the column, centrifuged, and the flow-through was discarded. 750 μL of Buffer PE were added to the column and centrifuged for 1 min at maximum speed. The flow-through was discarded and the column was centrifuged for 1 min at maximum speed. Finally, the column was transferred to a 1.5 mL microfuge tube and DNA was eluted with 50 μL of elution buffer, EB, (10 mM Tris.Cl, pH 8.5). The DNA quantity and quality were assessed by measuring the OD at 280 nm, using a Spetrophotometer (Eppendorf).

#### 5.2.3.6 Oligonucleotide design

To incorporate appropriate restriction enzyme sites for cloning, additional bases were added to the 5' end of the oligonucleotide sequence to ensure maintenance of the correct reading frame. In order to generate transgenic flies with 5' HA tagged version of ref(2)p, the forward primer contained HA sequences after the initiation codon (ATG). The primers sequences used for amplification are listed below, and the blue color represents the restriction enzymes, whereas the green corresponds to the HA sequence.

Forward primer: ATATATAT GAATTC ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT CCG GAG AAG CTG TTG AAA ATC ACC

Reverse primer: ATATATAT GGTACC TTAGTTCGGTTCTGCG

#### 5.2.3.7 Polymerase Chain Reaction

For the PCR, Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen), with 3'→ 5' exonuclease proofreading activity, was used. A High Fidelity 10X PCR Buffer used to increase the efficiency of the PCR reaction was also supplied in the kit. The components and amounts of each component of the PCR reaction were listed in Table 5.2, and the PCR cycling conditions were listed in Table 5.3.

**Table 5.3: Components of the PCR reaction**

| Component | Amount |
|---|---|
| *ref(2)p* DNA | 100 ng |
| Forward primer | 10 μM |
| Reverse primer | 10 μM |
| 10X High Fidelity Buffer | 2.5 μL |
| 50 mM $MgSO_4$ | 1 μL |
| Nuclease-free water | 19.5 μL |
| *Taq*DNA Polymerase High Fidelity | 0.1 μL |

**Table 5.4: PCR cycling conditions**

| PCR step | Temperature | Time (min:sec) |
|---|---|---|
| **1. Initial denaturation** | 94˚C | 2:00 |
| **2. Denaturation** | 94˚C | 0:30 |
| **3. Annealing** | 55˚C | 0:30 |
| **4. Extension** | 68˚C | 2:30 |
| | Repeat steps 2 to 4 30 times | |
| **Final extension** | 68˚C | 10:00 |

The PCR product was digested by the restriction enzymes EcoRI and KpnI for 3 hours at 37˚C, as previously described (section 3.2.1.4) and then run on an agarose gel for further gel extraction and purification of the PCR product, as described in section 3.2.1.5.

#### 5.2.3.8 DNA ligation

Ligation reaction was carried out using a rapid DNA ligation kit (Roche). A vector:insert molar ration of 1:3 was employed in a reaction mix of 50 ng of pUAST vector, 150 ng insert DNA and 5 U DNA ligase diluted in the appropriate volume of ligation buffer. The reaction was incubated for 10 min at RT.

The product of the ligation reaction was used to transform bacteria, as previously described in section 3.2.1.1. The resulting cultures were spread in LB agar plates containing 50 μg/mL ampicillin.

#### 5.2.3.9 Isolation of plasmid DNA by DNA miniprep

Individual bacterial colonies were inoculated into 5 ml LB containing 50 μg/mL ampicillin and incubated at 37ºC, with agitation for 12 hours. Following incubation, cells harvested by centrifugation (5000 g, 10 min) and DNA was isolated using a QIAfilter plasmid miniprep kit (Qiagen) according to the manufacturer's instructions. DNA was eluted in 50 μl of nuclease-free water.

#### 5.2.3.10 DNA sequencing

Automated DNA sequencing was employed to verify the presence of the appropriate insert and the correct reading frame. This was carried out by PNACL (The Protein Nucleic Acid Chemistry Laboratory), University of Leicester.

### 5.2.4 Generation of the *ref(2)P* transgenic flies

The DNA construct (pUAST vector containing the HA-tagged *ref(2)P* cDNA) was sent to BestGene Inc (Chino Hill, CA, USA) for the generation of the transgenic flies by P-element mediated transformation (Figure 5.3).

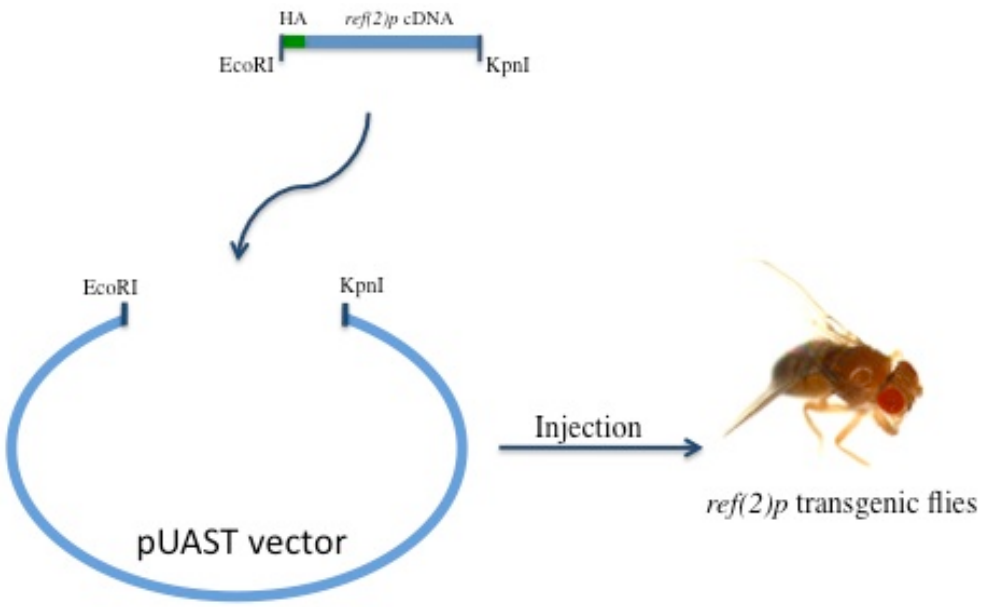



**Figure 5.3: Schematic representation for the generation of *ref(2)P* transgenic flies.**

## 5.3 Results

### 5.3.1 *ref(2)P* mutant flies have reduced lifespan and age-dependent neurodegeneration

*ref(2)P*$^{od2}$ and *ref(2)P*$^{od3}$ mutants were identified and characterised in respect to their involvement with sigma virus multiplication (Gay and Contamine, 1993). *Drosophila ref(2)P*$^{od2}$ and *ref(2)P*$^{od3}$ lack the PB1 and UBA domains, respectively (Figure 5.4). Both mutants are homozygous viable and were reported to be completely male sterile (Wyers et al., 1995). However, it was noticed during the progress of this work that the *ref(2)P*$^{od2}$ strain was male fertile. Indeed, another study also reported that the sterility of the *ref(2)P* mutants was dependent on its interaction with another gene, named *Suppressor of ref(2)P sterility*, *Su(P)* (Bichon et al., 2001). In that work, Bichon et al reported that most of the *Drosophila* strains present a deletion of 32 bp in the *Su(P)* gene, the so-called non-Su alleles, whereas few strains contain the non mutated *Su(P)* gene (Su alleles). *ref(2)P* mutants that contain one Su allele are fertile.

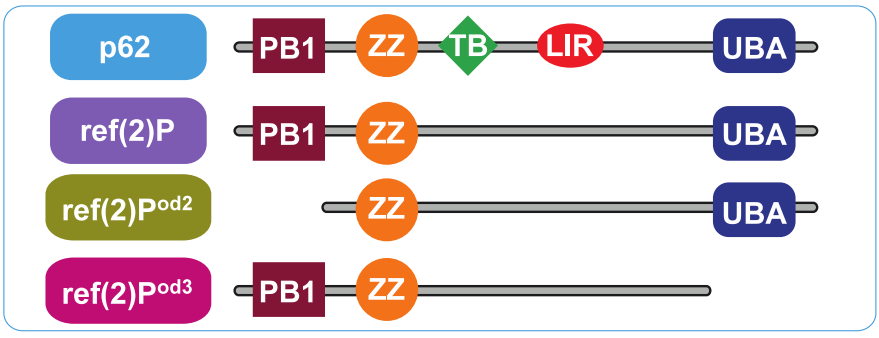



**Figure 5.4: Structural domain organisation of mammalian p62, *Drosophila* ref(2)P, and ref(2)P$^{od2}$ and ref(2)P$^{od3}$ mutations.** p62 has a PB1 domain, a ZZ-type zinc finger domain, a TRAF6-binding (TB) domain, an LC3-interacting region (LIR), and a ubiquitin-associated domain (UBA). ref(2)P has in common with the mammalian p62 the PB1 domain, the ZZ-type finger domain and the UBA domain. ref(2)P$^{od2}$ mutant protein lacks the PB1 domain, and ref(2)P$^{od3}$ mutant protein lacks the UBA domain. Figure adapted from Castro et al, under review by *Human Molecular Genetics*.

My analysis of the *Su(P)* gene in both *ref(2)P* mutant strains revealed that *ref(2)P*$^{od2}$ flies carry mostly the non mutated Su allele, whereas the *ref(2)P*$^{od3}$ mutants present the Su alleles with the deletion (non Su alleles) (Figure 5.5). This result explains the male fertility of *ref(2)P*$^{od2}$ mutants.

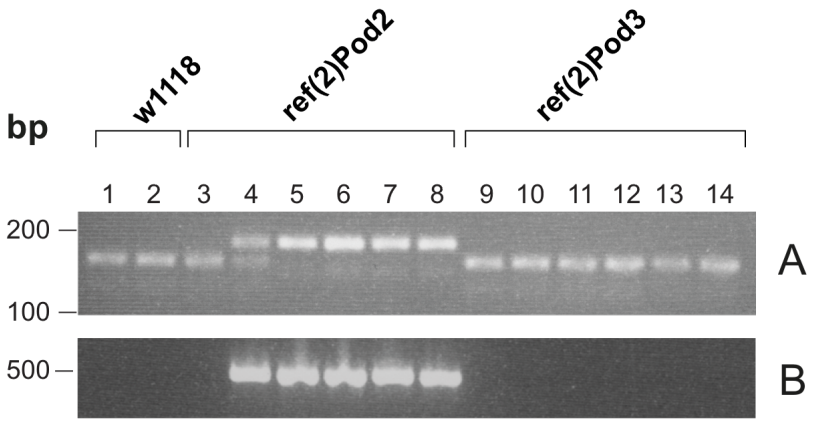



**Figure 5.5: *ref(2)P*$^{od2}$ mutants carry the Su allele (non mutated), whereas *w*$^{1118}$ and *ref(2)P*$^{od3}$ strains carry the non Su allele.** In A, *ref(2)P*$^{od2}$ strain present both the Su allele (band 196 bp) (lanes 4 to 8) and the non Su allele (band 162 bp) (lanes 3 and 4). In B, the absence of PCR amplification (lanes 1 to 3 and 9 to 14) indicates that the flies have a deletion in the *Su(P)* gene.

My main focus on the *ref(2)P* mutants in this work is to characterize the role of *ref(2)P* in terms of their mitochondrial function, lifespan and resistance to stress conditions.

*ref(2)P* mutants show a decline in the climbing ability when compared to the control flies (Figure 5.6). To test whether this defect in climbing was due to an impairment of mitochondrial function, the respiration rates of *ref(2)P* mutants were analysed by high-resolution respirometry. However, no differences were detected in the respiration rates in these mutant flies when compared to the controls (Figure 5.7). Additionally, no differences in the levels of ATP were detected (Figure 5.8).

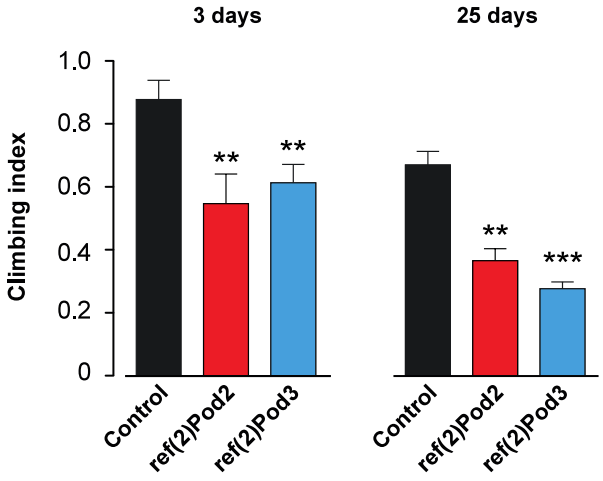



**Figure 5.6: *ref(2)P* mutants show a decrease in the climbing performance compared to the controls**. Flies with the indicated ages and genotypes were tested using a standard climbing assay (mean ±SD, n ≥ 60 flies per genotype). Asterisks indicate statistically significant values relative to the control, ** P < 0.01, *** P < 0.001 (one-way ANOVA with Dunnett's multiple comparison test). Genotypes: Control, $w^{1118}$. ref(2)Pod2, *w; ref(2)*$P^{od2}$*/ref(2)*$P^{od2}$*; +*. ref(2)Pod3, *w; ref(2)*$P^{od3}$*/ref(2)*$P^{od3}$*; +*.

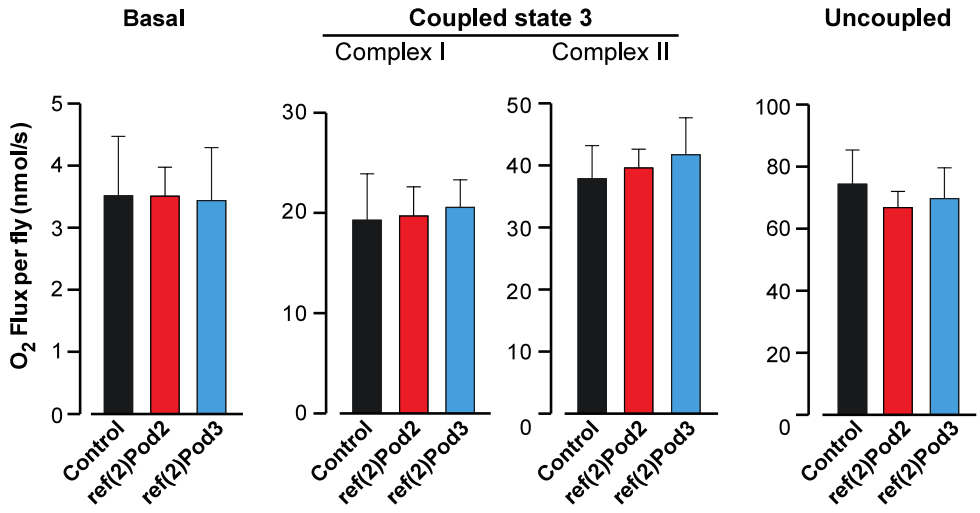



**Figure 5.7: Respiration rates of *ref(2)P* mutants are comparable to that of the controls.** No differences were detected in oxygen consumption either without supplementation of exogenous substrates or in through mitochondria-fed Complex I substrates and mitochondria-fed Complex II substrates, in both coupled and uncoupled conditions. Oxygen consumption was measured by high-resolution respirometry. Data are shown as the means ± SD (n ≥ 6 in each genotype). Genotypes: Control, $w^{1118}$. ref(2)Pod2, *w; ref(2)*$P^{od2}$*/ref(2)*$P^{od2}$*; +*. ref(2)Pod3, *w; ref(2)*$P^{od3}$*/ref(2)*$P^{od3}$*; +*.

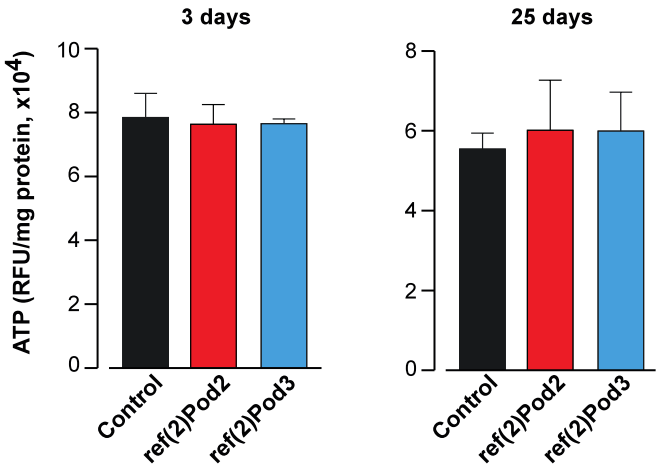



**Figure 5.8: ATP levels of *ref(2)P* mutants are comparable to that of the controls.** ATP levels were measured using a bioluminescent assay. Data are shown as the means ± SD (n = 4 for each genotype). Genotypes: Control, $w^{1118}$. ref(2)Pod2, *w; ref(2)*$P^{od2}$*/ref(2)*$P^{od2}$*; +*. ref(2)Pod3, *w; ref(2)*$P^{od3}$*/ref(2)*$P^{od3}$*; +*.

Contrarily to the *Pink1* mutants, which show a reduction in the levels of mtDNA, *ref(2)P* mutants present an increase in the mtDNA compared to the controls, which is exacerbated with age (experiment performed by a member of the laboratory, Dr. Roberta Tufi) (Figure 5.9).

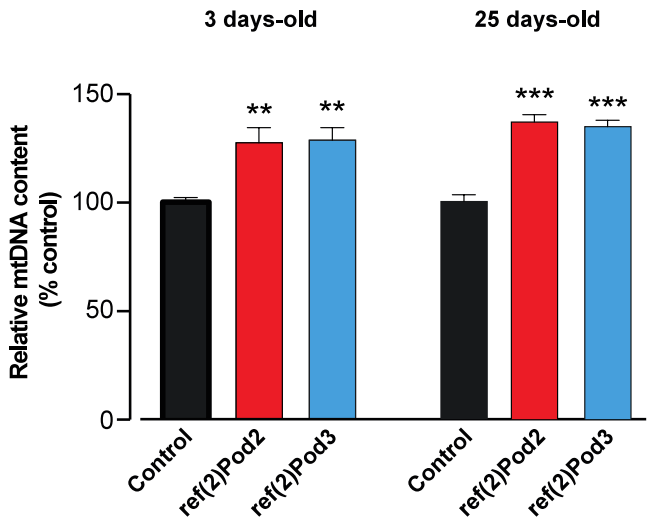



**Figure 5.9: *ref(2)P* mutants have increased levels of mitochondrial DNA (mtDNA).** The ratio of mtDNA to nuclear DNA was measured by real-time qPCR, using flies with the indicated ages (mean ± SD, n = 9 per genotype). Statistically significant values relative to the control are indicated by asterisks (one-way ANOVA with Dunnett's multiple comparison test). Genotypes: Control, $w^{1118}$. ref(2)Pod2, *w; ref(2)*$P^{od2}$*/ref(2)*$P^{od2}$*; +*. ref(2)Pod3, *w; ref(2)*$P^{od3}$*/ref(2)*$P^{od3}$*; +*. Figure adapted from Castro et al, under review by *Human Molecular Genetics*.

Nevertheless, the levels of the mitochondrial transcription factor A (TFAM) are similar to those of the control flies (Figure 5.10), indicating that there is no increase in mitochondrial biogenesis.

*Pink1* and *parkin* mutant flies have been shown to have an accumulation of dMfn (Ziviani et al., 2010). The accumulation of dMfn might be an indicator of an impairment of mitophagy in these flies, hence, the levels of the dMfn were evaluated in the *ref(2)P* mutants. This experiment showed no increase in the levels of dMfn in *ref(2)P* mutants when compared to the control flies (Figure 5.10), while the positive control, *Pink1* mutants, showed increased levels of the dMfn (Figure 5.10).

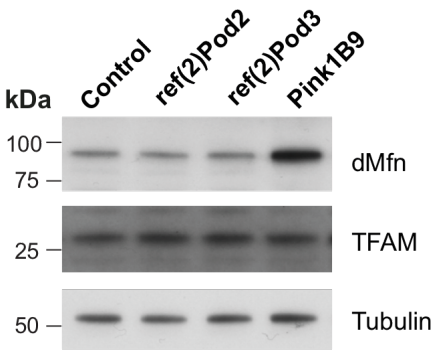



**Figure 5.10: *ref(2)P* mutants have similar levels of TFAM and dMfn to the control flies.** Whole-fly protein lysates (25-day-old flies) were used for western blotting analysis with the indicated antibodies. Genotypes: Control, $w^{1118}$. ref(2)Pod2, *w; ref(2)*$P^{od2}$*/ref(2)*$P^{od2}$*; +*. ref(2)Pod3, *w; ref(2)*$P^{od3}$*/ref(2)*$P^{od3}$*; +*.

*ref(2)P* mutants have a decrease in the lifespan when compared to the controls (Figure 5.11). Furthermore, both mutants show an increased sensitivity to various stress conditions, such as the treatment with the mitochondrial toxins rotenone (Figure 5.12a) and paraquat (Figure 5.12b).

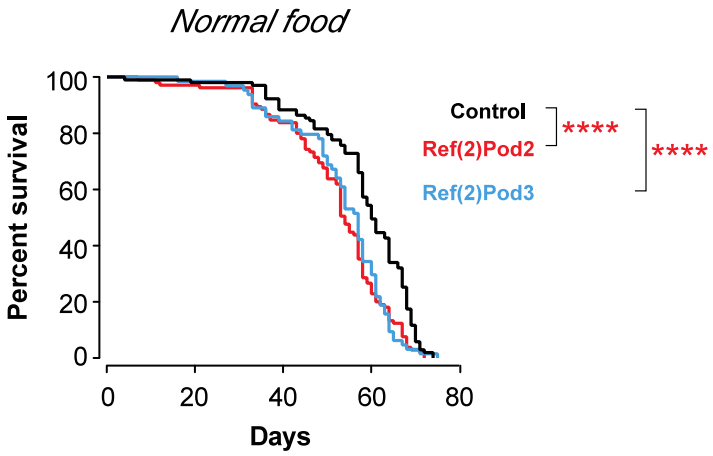



**Figure 5.11: *ref(2)P* mutants have a decreased lifespan.** Fly viability was scored over a period of 75 days. The statistical significance is indicated by the asterisks, **** $P < 0.0001$ (log-rank, Mantel-Cox test). Genotypes: Control, $w^{1118}$. ref(2)Pod2, *w; ref(2)*$P^{od2}$*/ref(2)*$P^{od2}$*; +*. ref(2)Pod3, *w; ref(2)*$P^{od3}$*/ref(2)*$P^{od3}$*; +*.

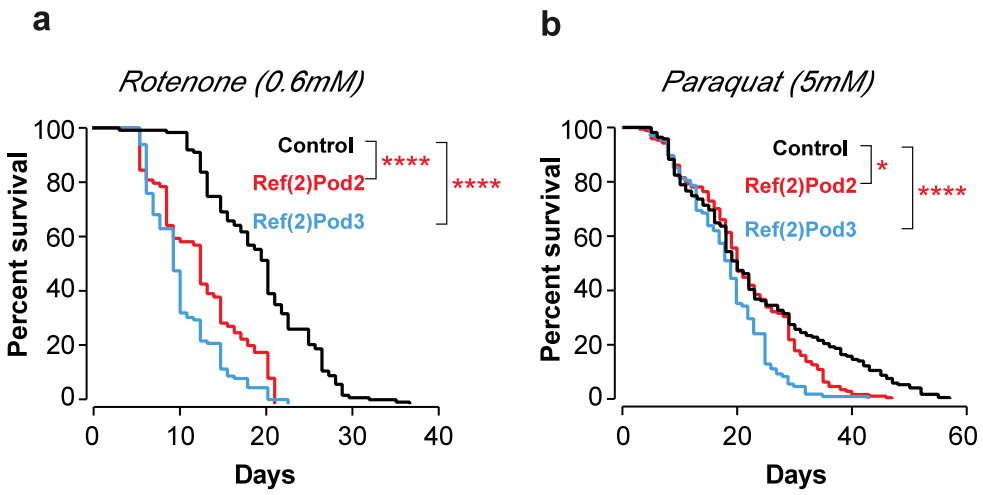



**Figure 5.12: *ref(2)P* mutants are more sensitive to oxidative stress than the control flies. (a-b)** Fly viability was scored over a period of 60 days. The statistical significance is indicated by the asterisks, * P < 0.05, **** P < 0.0001 (log-rank, Mantel-Cox test). Genotypes: Control, $w^{1118}$. ref(2)Pod2, *w; ref(2)*$P^{od2}$/*ref(2)*$P^{od2}$; +. ref(2)Pod3, *w; ref(2)*$P^{od3}$/*ref(2)*$P^{od3}$; +.

Interestingly, the levels of the enzyme TH were found to be significantly reduced in aged *ref(2)P* mutants (Figure 5.13a). The loss of TH correlated with a reduction of DA in the fly heads of *ref(2)P* mutants (Figure 5.13b). These results suggest that *ref(2)P* mutants present an age-dependent dopaminergic neurodegeneration.

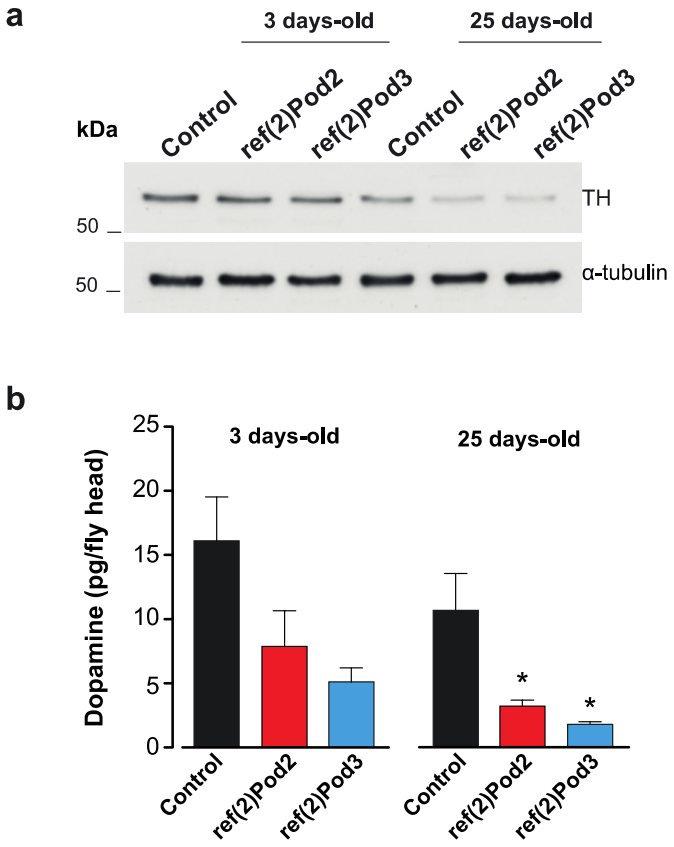



**Figure 5.13: Age-dependent dopaminergic neuronal degeneration in *ref(2)P* mutants. (a)** Decreased tyrosine hydroxylase (TH) levels in *ref(2)P* mutants. Fly-head protein lysates were analysed by western blotting using the indicated antibodies. **(b)** *ref(2)P* mutant flies have decreased dopamine levels. Neurotransmitter levels were assessed by HPLC with electrochemical detection. Data are shown as the means ± SEM (n = 3 for each genotype). The statistical significance is indicated by asterisks, * $P < 0.05$ (one-way ANOVA with Dunnett's multiple comparison test). Experiments performed by past members of the laboratory. Figure adapted from Castro et al, under review by *Human Molecular Genetics*.

#### 5.3.1.1 Pharmacological induction of autophagy causes toxicity in *ref(2)P* mutant flies

Given the proposed role for ref(2)P in the autophagic degradation of defective mitochondria, the effects of the pharmacological induction of autophagy in the lifespan of the *ref(2)P* mutants were tested. Both $ref(2)P^{od2}$ and $ref(2)P^{od3}$ mutants maintained on food supplemented with Rapamycin, an inhibitor of the mammalian target of Rapamycin complex 1 (mTORC1) and inducer of autophagy, showed a dramatic decrease in lifespan (Figure 5.14b and 5.14c), when compared to the vehicle alone (ethanol)-containing food. This result suggests that the loss of ref(2)P function causes lethality upon the induction of autophagy in flies. It is worth noting that the controls were also slightly affected by the Rapamycin treatment; however, the median life of the control flies was not affected (Figure 5.14a). The effect of the Rapamycin treatment in autophagy-deficient flies, $Atg1^{\Delta 3d}$ mutants, was also investigated. This experiment revealed no effect for the drug in the lifespan of $Atg1^{\Delta 3d}$ mutants (Figure 5.14d), suggesting that the autophagy activation is essential for the shortening of the lifespan. Rapamycin has been previously suggested to rescue *Pink1* mutant-associated phenotypes (Tain et al., 2009b), however there is no reference to the lifespan of the flies in that study. The treatment of *Pink* mutants with Rapamycin had no effect on their lifespan (Figure 5.14e).

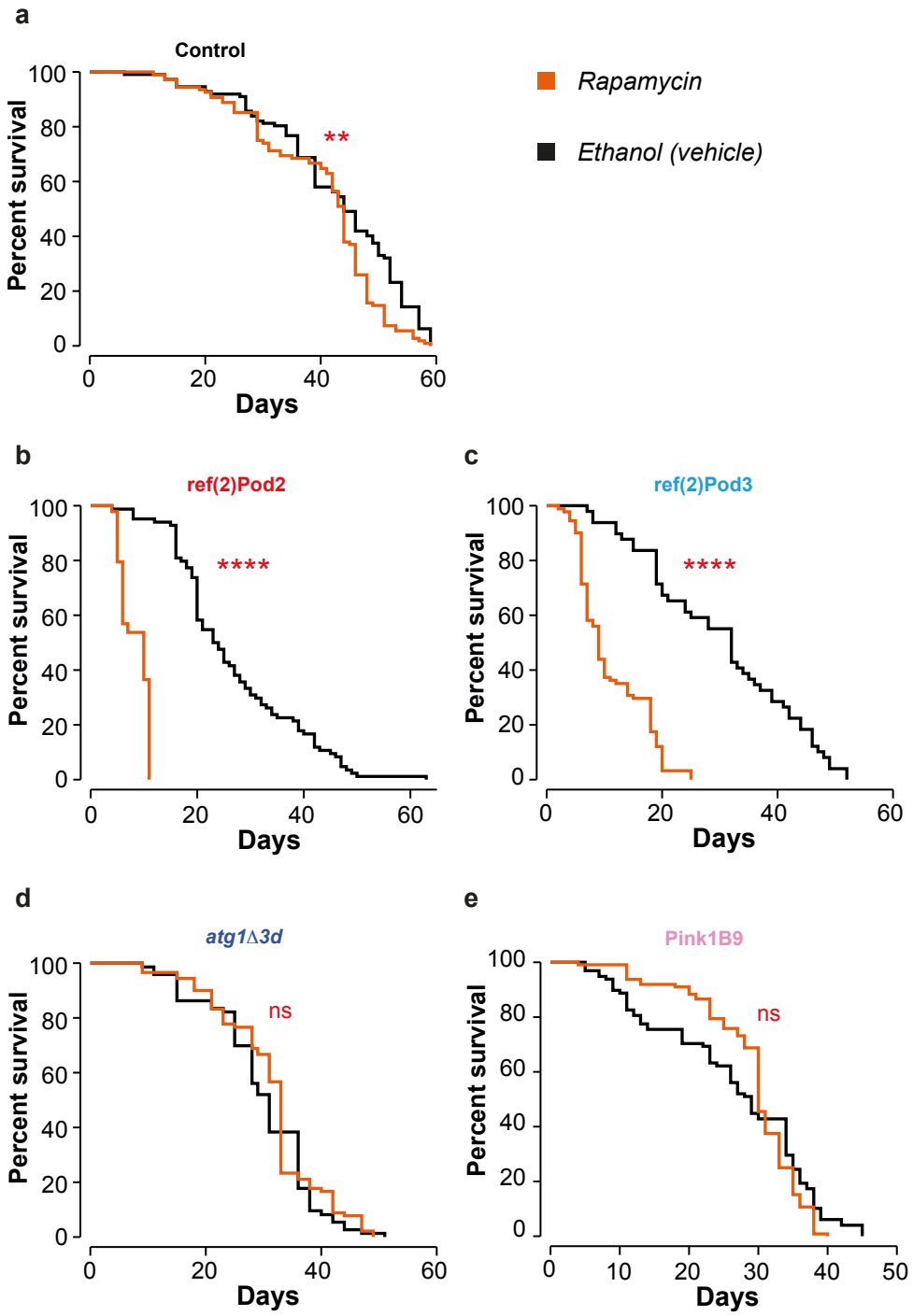



**Figure 5.14: Survival curves under treatment with Rapamycin or vehicle alone. (a)** Rapamycin treatment decreases the lifespan of control flies ($w^{1118}$), but it does not alter their median survival. **(b-c)** *ref(2)P* mutants show a tremendous decrease in lifespan under Rapamycin treatment. **(d-e)** Neither *Pink1* or *Atg1* mutants show an effect in the total lifespan when treated with Rapamycin. (a-e) Fly viability was scored over a period of 60 days using a minimum of 100 flies per genotype. The statistical significance is indicated by the asterisks, ** $P < 0.01$, **** $P < 0.0001$, ns (non significant) (log-rank, Mantel-Cox test). Genotypes: Control, $w^{1118}$. ref(2)Pod2, *w; ref(2)*$P^{od2}$*/ref(2)*$P^{od2}$*; +*. ref(2)Pod3, *w; ref(2)*$P^{od3}$*/ref(2)*$P^{od3}$*; +*. Pink1B9, $Pink1^{B9}$*; +; +*. atg1Δ3d, *w; +;* $atg1^{\Delta 3d}$*/+*.

In order to strengthen the hypothesis that the reduction of the lifespan in the *ref(2)P* mutants was caused by the induction of autophagy, flies were maintained on food supplemented with other drugs known to be autophagy inducers, such as trehalose and metformin. Trehalose is a sugar known to induce autophagy in an mTOR-independent manner and protects cells against the toxic effects of the disease-related proteins huntingtin and α-synuclein (Sarkar et al., 2007). Metformin is a clinically used drug by patients with type-2 diabetes. In hepatocytes, metformin acts to increase the cellular adenosine monophosphate (AMP)/ATP ratio, leading to the activation of AMP-activated protein kinase (AMPK), a key autophagy regulator (Zhou et al., 2001). AMPK activation has been shown to induce autophagy in flies (Pimenta de Castro et al., 2012).

Similarly to the treatment with Rapamycin, *ref(2)P* mutants showed a dramatic decrease in their survival under treatment with either trehalose (Figure 5.15) or metformin (Figure 5.16), confirming the enhanced sensitivity of *ref(2)P* mutants to treatment with autophagy-inducing drugs.

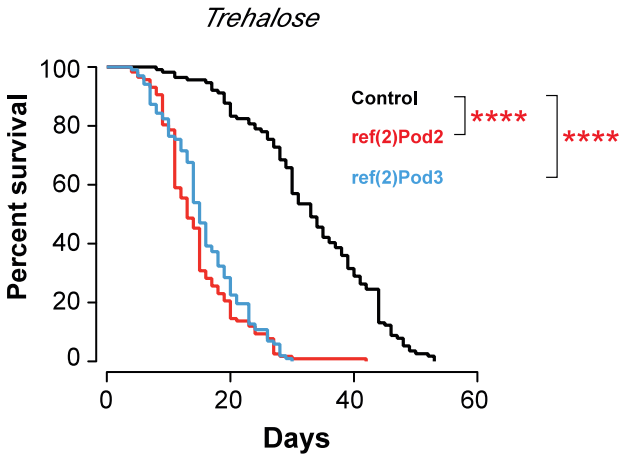



**Figure 5.15: *ref(2)P* mutants show a reduction in lifespan when treated with the autophagy inducer trehalose.** Fly viability was scored over a period of 55 days using a minimum of 100 flies per genotype. The statistical significance is indicated by the asterisks, **** P < 0.0001 (log-rank, Mantel-Cox test). Genotypes: Control, $w^{1118}$. ref(2)Pod2, *w; ref(2)*$P^{od2}$*/ref(2)*$P^{od2}$*; +*. ref(2)Pod3, *w; ref(2)*$P^{od3}$*/ref(2)*$P^{od3}$*; +*.

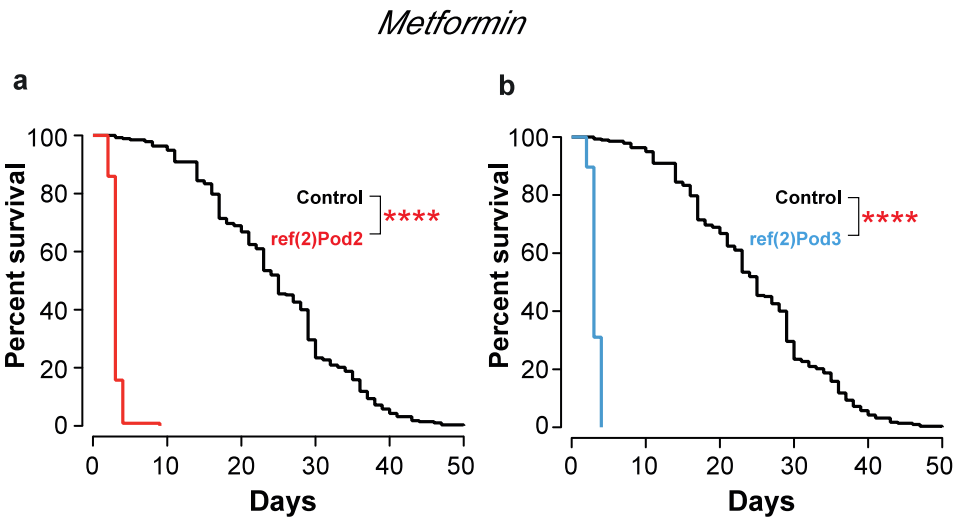



**Figure 5.16: *ref(2)P* mutants have a dramatic decrease in lifespan when treated with metformin. (a-b)** Fly viability was scored over a period of 50 days using a minimum of 100 flies per genotype. The statistical significance is indicated by the asterisks, **** P < 0.0001 (log-rank, Mantel-Cox test). Genotypes: Control, $w^{1118}$. ref(2)Pod2, *w; ref(2)*$P^{od2}$*/ref(2)*$P^{od2}$*; +*. ref(2)Pod3, *w; ref(2)*$P^{od3}$*/ref(2)*$P^{od3}$*; +*.

### 5.3.2 *ref(2)P* is required for *parkin* suppression of *Pink1*$^{B9}$ mutant phenotype

To investigate a possible genetic interaction between the ref(2)P and Pink1, crosses were made to obtain double-mutants of *ref(2)P*$^{od2}$ or *ref(2)P*$^{od3}$ and *Pink1*$^{B9}$. The double mutants do not show an enhanced phenotype when compared to the *Pink1* mutant alone (Figure 5.17).

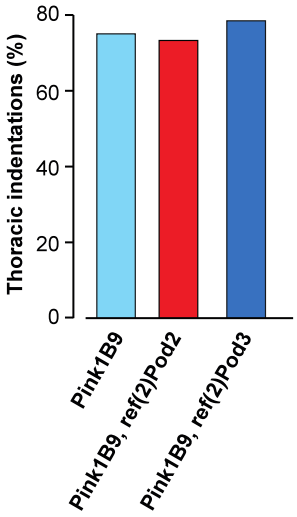



**Figure 5.17: *Pink1* mutants and *Pink1-ref(2)P* double mutants show approximately the same degree of thoracic indentations.** Thoracic indentations were counted in newly eclosed male flies (n > 100 in each genotype). Genotypes, Pink1B9, *Pink1*$^{B9}$*; +; daGAL4*/+. Pink1B9, ref(2)Pod2, *Pink1*$^{B9}$*; ref(2)P*$^{od2}$*/ref(2)P*$^{od2}$*; daGAL4*/+. Pink1B9, ref(2)Pod3, *Pink1*$^{B9}$*; ref(2)P*$^{od3}$*/ref(2)P*$^{od3}$*; daGAL4*/+.

Both *parkin* and *Pink1* are known to genetically interact in *Drosophila*, and parkin and p62 were shown to physically interact in mammalian cells (Pimenta de Castro et al., 2012).

It was demonstrated that the rescue of *Pink1* mutants through expression of *parkin* is dependent on autophagy. In a background of autophagy deficiency, in the *Atg1* and *Pink1* double mutant flies, *parkin* failed to rescue their defective phenotype (Castro et al, under review by *Human Molecular Genetics*). It has also been shown that ref(2)P is required for the parkin-mediated suppression of defects associated with mitochondrial

protein misfolding (Pimenta de Castro et al., 2012), suggesting its importance in clearing damaged mitochondria.

Similarly to the results with the *Atg1* mutant background, the presence of a functional ref(2)P protein seem to be required for the beneficial effect of *parkin* expression in the *Pink1* mutant flies. The *parkin*-mediated rescue of the thoracic indentation was reduced in the double mutants of *Pink1* and *ref(2)P*$^{od2}$ or *ref(2)P*$^{od3}$ (Figure 5.18). The requirement of ref(2)P was also evidenced in *parkin* rescuing the levels of Complex I in *Pink1* mutants (Figure 5.19).

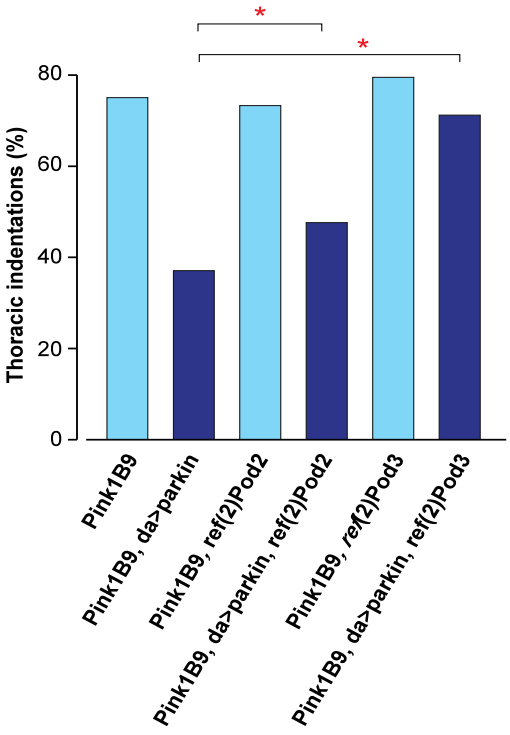



**Figure 5.18: *parkin*-mediated rescue of thoracic indentations in *Pink1* mutants was reduced with *ref(2)P* mutant background.** Thoracic indentations were scored in newly eclosed flies of each indicated genotype. Asterisks indicate the statistical significance relative to Pink1$^{B9}$, da>parkin (chi-square analysis, alpha=0.05, beta=0.20). Genotypes: Pink1B9, *Pink1*$^{B9}$*; +; daGAL4*/+. Pink1B9, da<parkin, *Pink1*$^{B9}$*; UAS park*$^{c2}$/+*; daGAL4*/+. Pink1B9, ref(2)Pod2, *Pink1*$^{B9}$*; ref(2)P*$^{od2}$/*ref(2)P*$^{od2}$*; daGAL4*/+. Pink1B9, da>parkin, ref(2)Pod2, *Pink1*$^{B9}$*; UAS park*$^{c2}$*, ref(2)P*$^{od2}$/*ref(2)P*$^{od2}$*; daGAL4*/+. Pink1B9, ref(2)Pod3, *Pink1*$^{B9}$*; ref(2)P*$^{od3}$/*ref(2)P*$^{od3}$*; daGAL4*/+. Pink1B9, da>parkin, ref(2)Pod3, *Pink1*$^{B9}$*; UAS park*$^{c2}$*, ref(2)P*$^{od3}$/*ref(2)P*$^{od3}$*; daGAL4*/+. Figure adapted from Castro et al, under review by *Human Molecular Genetics*.

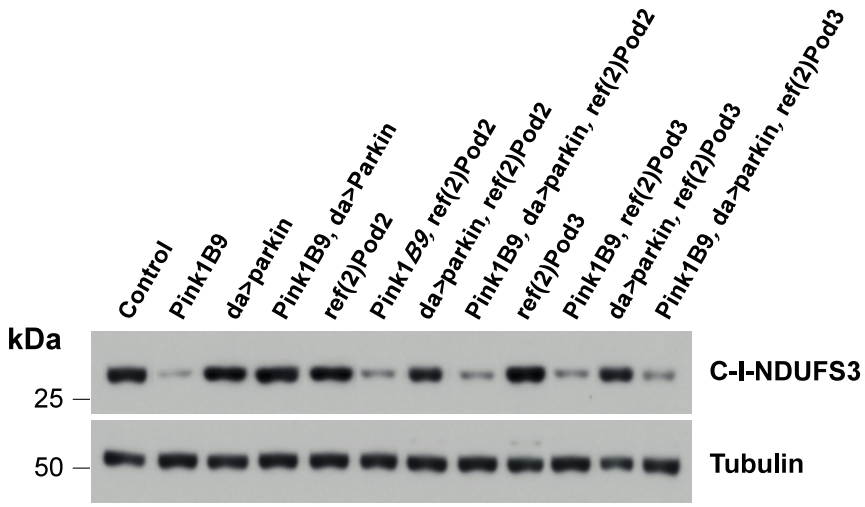



**Figure 5.19: *ref(2)P* mutations blocked the parkin-mediated rescue of Complex I levels in *Pink1* mutant flies.** Whole-fly protein lysates were analysed by western blotting with the indicated antibodies. Genotypes: Control, *w; +; daGAL4*/+. Pink1B9, *Pink1$^{B9}$; +; daGAL4*/+. da<parkin, *w; UAS park$^{c2}$*/+; *daGAL4*/+. Pink1B9, da<parkin, *Pink1$^{B9}$; UAS park$^{c2}$*/+; *daGAL4*/+. ref(2Pod2, *w; ref(2)P$^{od2}$*/*ref(2)P$^{od2}$; daGAL4*/+. Pink1B9, ref(2)Pod2, *Pink1$^{B9}$; ref(2)P$^{od2}$*/*ref(2)P$^{od2}$; daGAL4*/+. da>parkin, ref(2)Pod2, *w; UAS park$^{c2}$, ref(2)P$^{od2}$*/*ref(2)P$^{od2}$; daGAL4*/+. Pink1B9, da>parkin, ref(2)Pod2, *Pink1$^{B9}$; UAS park$^{c2}$, ref(2)P$^{od2}$*/*ref(2)P$^{od2}$; daGAL4*/+. ref(2Pod3, *w; ref(2)P$^{od3}$*/*ref(2)P$^{od3}$; daGAL4*/+. Pink1B9, ref(2)Pod3, *Pink1$^{B9}$; ref(2)P$^{od3}$*/*ref(2)P$^{od3}$; daGAL4*/+. da>parkin, ref(2)Pod3, *w; UAS park$^{c2}$, ref(2)P$^{od3}$*/*ref(2)P$^{od3}$; daGAL4*/+. Pink1B9, da>parkin, ref(2)Pod3, *Pink1$^{B9}$; UAS park$^{c2}$, ref(2)P$^{od3}$*/*ref(2)P$^{od3}$; daGAL4*/+. Figure adapted from Castro et al, under review by *Human Molecular Genetics*.

### 5.3.3 *ref(2)P* expression ameliorates *Pink1* mutant-associated phenotypes

Ten transgenic lines of UAS HA-*ref(2)P* with different insertion sites as listed in Table 5.5 were obtained from BestGene.

**Table 5.5: *ref(2)P* transgenic lines.**

| Transgenic line | Sex of G1 adult | Chromosome of insertion |
|---|---|---|
| 1 | male | third |
| 2 | male | third |
| 3 | male | third |
| 4 | male | third |
| 5 | male | second |
| 6 | male | third |
| 7 | male | third |
| 8 | male | second |
| 9 | male | second |
| 10 | male | third |

To confirm the expression of the transgene, adult males from all the lines were crossed to virgin females of the ubiquitous *da*GAL4 driver and the males progeny containing both the driver and the transgene were selected and tested by western blot analysis. Anti-HA antibody was used to detect the recombinant protein HA-ref(2)P. Based on their protein expression levels, lines 3M and 5M were chosen for further analysis (Figure 5.20).

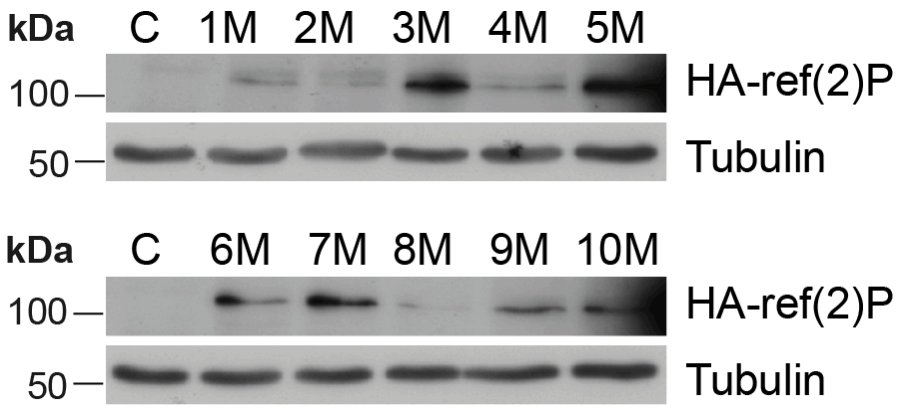



**Figure 5.20: Expression of HA-ref(2)P in all the transgenic lines obtained from BestGene.** Whole-fly protein lysates were used for western blotting analysis with anti-HA and anti-α-tubulin antibodies. Genotypes: C, control, *w; +; daGAL4/+*. 1M, 2M, 3M, 4M, 6M, 7M, and 10 M, *w; +; daGAL4/UAS HA-ref(2)P*. 5M, 8M and 9M, *w; UAS HA-ref(2)P/+; daGAL4/+*.

To gain insight into the role of *ref(2)*P in the *Pink1* pathway, the overexpression of *ref(2)P* in the $Pink1^{B9}$ mutants was tested. The results indicated that the expression of *ref(2)P* improved the thoracic defects of *Pink1* mutants, reducing the number of flies exhibiting thoracic indentations (Figure 5.21a). Additionally, the climbing ability of the *Pink1* mutants was enhanced following *ref(2)P* expression (Figure 5.21b). These results suggest that *ref(2)P* interacts genetically with *Pink1 in vivo*.

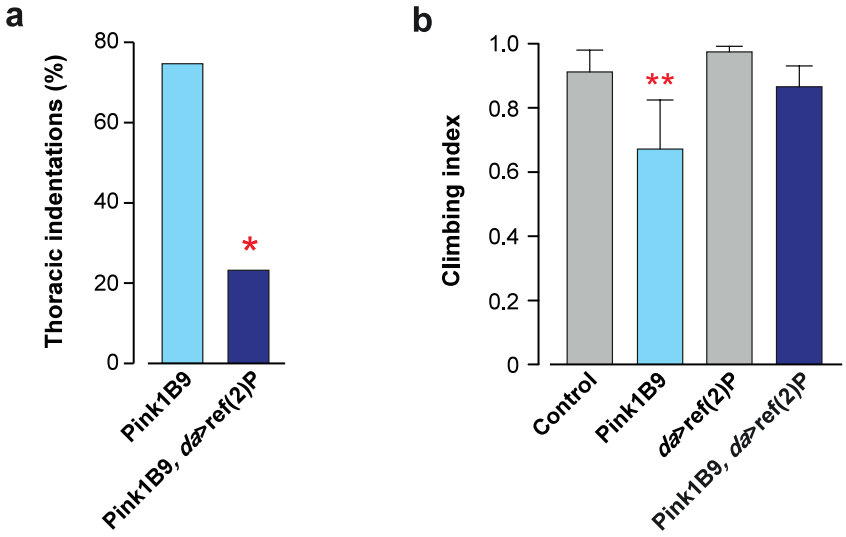



**Figure 5.21: The expression of *ref(2)P* rescues *Pink1* mutant-associated phenotypes. (a)** Expression of *ref(2)P* rescues the thoracic indentations of *Pink1* mutants. Asterisk indicates statistical significance, * $P < 0.05$ (chi-square analysis, alpha=0.01, beta=0.01). **(b)** Expression of *ref(2)P* rescues the climbing defects of *Pink1* flies. Flies were tested using a standard climbing assay (mean ± SD, $n \geq 60$ flies per genotype). Asterisks indicate statistically significant values relative to the control, ** $P < 0.01$ (one-way ANOVA with Dunnett's multiple comparison test). (a-b) Genotypes: Control, *w; +; daGAL4*/+. Pink1B9, *Pink1$^{B9}$; +; daGAL4*/+. da>ref(2)P, *w; UAS ref(2)P*/+; *daGAL4*/+. Pink1B9, da>ref(2)P, *Pink1$^{B9}$; UAS ref(2)P*/+; *daGAL4*/+. Figure adapted from Castro et al, review in *Human Molecular Genetics*.

## 5.4 Discussion

The characterization of *ref(2)P*$^{od2}$ and *ref(2)P*$^{od3}$ mutants revealed that the phenotype associated with the loss of ref(2)P is milder compared to that of the loss of Pink1 and parkin in *Drosophila*. The mitochondrial function of *ref(2)P* mutants seems to be comparable to that of the controls, at least under normal conditions, since no differences were detected in the respiration rates and in ATP levels. However, these flies show a reduction in the total lifespan and enhanced sensitivity to oxidative stress.

Moreover, *ref(2)P* mutants show an increase in mtDNA, but not TFAM protein levels, which suggest an accumulation of mitochondria, presumably because of a defective targeting of damaged mitochondria for mitophagy. Nonetheless, these flies do not show an accumulation of dMfn, consistent with ref(2)P functioning downstream of Pink1 and parkin in targeting mitochondria for mitophagy.

The *ref(2)P* mutants also show an impairment in the climbing performance, which becomes more evident with ageing. Furthermore, it was detected a reduction in the TH and DA levels in aged flies. These results suggest that the effects of the loss of ref(2)P are particularly manifested in old flies, when the accumulation of damaged molecules and organelles increases.

It was previously reported that *ref(2)P* mutant flies fail to accumulate the normal age-related protein aggregates in the brain (Nezis et al., 2008). The formation of these aggregates might be a protective mechanism against misfolded, and otherwise toxic, proteins. Moreover, the formation of aggregates seems to precede their elimination through autophagy (Clausen et al., 2010).

Mammalian p62 has been reported to interact with ALFY and promote the degradation of p62 bodies through autophagy (Clausen et al., 2010). Interestingly, the *Drosophila* orthologue of *ALFY*, *blue cheese*, has been characterised and mutations in

this gene cause a reduction in the lifespan and progressive neural degeneration in fruit flies (Finley et al., 2003). It is worth noting that *blue cheese* mutants seem to have just mild behavioural defects when young. It is only when the flies are old that there was accumulation of ubiquitin aggregates in the fly brain and neurodegeneration (Finley et al., 2003).

The phenotype of the *ref(2)P* mutants seems to be remarkably similar to that of *blue cheese* mutants, which is consistent with the proposed function of ALFY downstream of p62 in mammals. Despite the lack of detailed analysis of neurodegeneration in *ref(2)P* mutants, I have shown that these flies have an age-dependent decrease in TH and DA levels in the brain. Further studies are necessary to characterise the extension of neurodegeneration in *ref(2)P* mutants.

Furthermore, the lethality caused by autophagy inducing drugs requires further investigation, since the mechanism responsible for the toxicity was not explored in this work. It remains to be investigated whether autophagy is indeed being induced in the flies, and particularly in *ref(2)P* mutants, treated with these drugs. It would be interesting to test whether in an autophagic-deficient background the *ref(2)P* mutants would be protected and their lifespan extended.

My work, associated with the work of another member of the laboratory, Ines Castro (Castro et al, under review by *Human Molecular Genetics*), provides evidences that the *Drosophila* orthologue of p62, ref(2)P, is an important component of the Pink1/parkin quality control pathway. The expression of *ref(2)P* rescued the *Pink1* mutant phenotype, indicating that *ref(2)P* acts downstream or in parallel to *Pink1* in *Drosophila*. Moreover, in the presence of *ref(2)P* mutations, the ability of *parkin* overexpression for rescuing *Pink1* mutant flies was decreased, indicating that ref(2)P is required for the parkin-mediated rescue of *Pink1* mutants. These results are in

agreement with the proposed model in which parkin mediates the labelling of dysfunctional mitochondria, by ubiquitinating proteins at the OMM, which in turn recruit p62 that acts as an adaptor molecule to bring the autophagosomes and initiate mitophagy.

Taken together, the analysis of the *ref(2)P* mutants phenotypes, associated with the rescue of *Pink1* mutants by *ref(2)P* overexpression, suggest that ref(2)P acts downstream of *Pink1* in *Drosophila*. The milder phenotype of *ref(2)P* mutants compared to *Pink1* mutants indicates that these two genes are not in a linear genetic pathway. The function of *Pink1* in *Drosophila* seems to go beyond sensing mitochondrial damage and facilitating to target dysfunctional organelles for mitophagy. *Pink1* seems to be important for mitochondrial function and dynamics, which does not seem to be the case of *ref(2)P*. The presence of ref(2)P seems to be particularly important under stress conditions and in aged flies, which is consistent with the fact that in these situations a higher incidence of damage occurs, increasing the need for turnover of proteins and organelles.

It would be interesting to explore further the rescue of *Pink1* mutants by *ref(2)P* overexpression and to investigate the mechanism by which such results are achieved. The proposed mechanism of action of ref(2)P in this work is the enhancement of autophagic clearance of the dysfunctional mitochondria present in *Pink1* mutants, however it has not been tested whether this clearance is increased or not. Additional mechanisms may be involved, such as the activation of the Nrf2/Keap1 pathway, which is involved in stress responses, and whose activation can confer protection, including ameliorating neurodegenerative phenotypes in a fly model of PD (Barone et al., 2011).

# Chapter 6

# General discussion

# Chapter 6: General discussion

Despite the intense scientific work on the molecular mechanisms involved in PD pathogenesis, we still lack a considerable amount of information to achieve a better and more global understanding of the entire process. PD seems to be a rather complex phenomenon, possibly involving various signalling pathways that eventually converge into a single outcome.

Mitochondrial dysfunction, oxidative stress and protein aggregation have all been implicated in the pathogenesis of PD (Schapira, 2011). Toxin-based studies first provided evidence connecting oxidative stress and mitochondrial dysfunction to PD. Further support for the involvement of mitochondria in this disease derived from the discovery of gene mutations in *PINK1*, causing familial autosomal recessive Parkinsonism. Moreover, the elucidation of the role of PINK1 and Parkin in a common pathway that regulates mitochondrial function and dynamics has focused the interest of the scientific community in the role of mitochondria for PD pathogenesis.

An increasing body of evidence indicates that both PINK1 and Parkin are involved in the autophagic degradation of defective mitochondria (mitophagy) that lost their membrane potential and, therefore, need to be selectively targeted for degradation (reviewed by (Deas et al., 2011b)). The mechanisms involved in this process of quality control are not entirely clear. Under conditions that cause loss of $\Delta\Psi m$, the full-length PINK1 accumulates at the OMM and recruits Parkin to the mitochondria. Parkin, in turn, promotes the ubiquitination of mitochondrial proteins at the OMM, such as VDAC1 and mitofusins. Thus, these mitochondria are unable to fuse, and are segregated from the healthy organelles instead. Furthermore, the ubiquitination of VDAC1 recruits p62 to the ubiquitin-labelled mitochondria, promoting their

aggregation and subsequent removal through mitophagy (Geisler et al., 2010, Narendra et al., 2010).

Additional events must be involved in the disposal of dysfunctional organelles. Recently it was proposed that PINK and Parkin regulate mitochondrial trafficking through their connection to the microtubule network (Wang et al., 2011). Upon mitochondrial damage, Miro, a Rho-like GTPase important for mitochondria movement, is phosphorylated and degraded in a PINK1/Parkin-dependent manner. As a consequence, damaged mitochondria are arrested and segregated, prior to their degradation through mitophagy.

Another issue regarding the *PINK1*/*Parkin* pathway of mitophagy that has not yet been elucidated is whether this process occurs in physiological conditions and in living organisms. The experimental limitations of the studies led to the use of uncouplers that cause irreversible loss of $\Delta\Psi m$. It is still unclear if such dramatic loss in $\Delta\Psi m$ replicates more physiological conditions in cells, even in pathological situations. Moreover, it has been difficult to perform experiments without overexpression of PINK1 and Parkin, due to the limited availability of reagents. The majority of these studies were also conducted in mitotic cells, and it would be of great interest to evaluate if these same mechanisms are replicated in post-mitotic cells such as neurons.

The discovery of downstream targets of PINK1 and Parkin has the potential to further elucidate the precise mechanisms of mitochondrial quality control. PINK1 has been proposed to act in mitochondria quality control at the organellar level based on its interaction with Parkin in the mitophagy pathway. Furthermore, it has also been proposed to act at the molecular level of mitochondria quality control, through its interaction with the protease HtrA2 and the chaperone TRAP1.

The work presented in Chapter 4 shows that *Pink1* and *Trap1* interact genetically *in vivo*, in *Drosophila*. The expression of *Trap1* restored mitochondrial function and improved the phenotypic defects presented in *Pink1* mutant flies. More importantly, the expression of *Trap1* in neurons was sufficient to attenuate both neuronal and muscle abnormalities of *Pink1* mutants, which indicates that the primary defect in *Pink1* mutants is neuronal rather than muscular.

Interestingly, *parkin* mutant phenotypes could be partially ameliorated by expression of *Trap1*. It is likely that Trap1, as a mitochondrial chaperone, may promote the stability of mitochondrial proteins and protein complexes, which might partially compensate for the presence of dysfunctional organelles in *parkin* mutants.

Additionally, Trap1 loss-of-function flies presented mitochondrial defects, associated with a decrease in lifespan and increased sensitivity to various stress conditions as described in Chapter 3. The phenotype of *Trap1* mutant flies is to some extent, although not entirely, similar to that of the *Pink1* mutants. The loss of *Trap1* does not affect male fertility, possibly because mitochondrial dynamics is not critically affected in this mutant. Moreover, *Trap1* mutants do not present morphological defects, such as abnormally positioned wings or thoracic indentations, indicating that they do not have muscle degeneration due to increased apoptosis. Similarly to the *Pink1* mutants, a reduction in the Complex I protein level was detected in *Trap1* mutants.

*Pink1* mutants do not have a generalised loss of mitochondria; however, there is a robust reduction in the Complex I levels, assessed by western blot analysis of the NDUFS3 sub-unit (encoded by nuclear DNA). The mitochondrial Complex I is composed of several sub-units, and some of these are encoded by the mitochondrial genome. *Pink1* mutants were shown to have reduced levels of mtDNA (Park et al., 2006), which may result in lower levels of the mtDNA-encoded Complex I sub-units in

these flies. The different sub-units of a multimeric respiratory complex are present in a mitochondrion in a stoichiometric relation. If some of the sub-units are missing, the other sub-units produced by the nuclear genome cannot be assembled at the mitochondrion and are likely to be targeted for degradation (Goldberg, 2003). In this respect it would be interesting to investigate whether *Trap1* mutant flies have a decrease in mtDNA content that could explain the observed loss of Complex I. Additionally, a defect in translation could be excluded through the assessment of Complex I mRNA levels.

The *Trap1*-mutant phenotype was rescued by expression of *Trap1* as well as *parkin* expression. This genetic interaction between *Trap1* and *parkin* might be an indication that in mitochondria the molecular and organellar quality control mechanisms act in parallel and can compensate for defects of one another.

The results presented in Chapters 3 and 4 provided evidences for the importance of *Trap1* in mitochondrial function and its interaction with the *Pink1/parkin* pathway.

In Chapter 5, I have presented evidences for a role of *ref(2)P* in the *Pink1/parkin* pathway by two means: first, I established the requirement of ref(2)P for the *parkin*-mediated rescue of *Pink1*-mutant phenotype; second, demonstrated that the expression of *ref(2)P* rescued the *Pink1*-mutant phenotype.

The analysis of the *ref(2)P*-mutant phenotype revealed no evidence of mitochondrial dysfunction. These flies showed a decreased lifespan and age-dependent dopaminergic neurodegeneration. ref(2)P, as its mammalian orthologue p62, is likely to be involved in targeting the damaged and ubiquitin-labelled mitochondria for mitophagy. Nevertheless, it is not clear whether in physiological conditions this process is critical for cell viability. It is possible that under normal conditions, molecular quality control may be sufficient to ensure mitochondria homeostasis. However, in aged

animals, progressive accumulation of damage that results in mitochondrial dysfunction could increase the requirement for functional mitophagy. Additionally, treatment with oxidative stress inducers is likely to cause damage to mitochondria, and hence increase the requirement for targeting damaged mitochondria for mitophagy. This could explain the mild neurodegenerative phenotype of *ref(2)P* mutants and the absence of more severe phenotypes.

It is worth noting that the dependence on autophagy/mitophagy is variable with the cell type. It is likely that post-mitotic cells, such as neurons, are more vulnerable to defects in autophagy/mitophagy. It was previously reported that knockdown of *Atg* genes in the brain cause neurodegeneration (Hara et al., 2006, Komatsu et al., 2006). It would therefore be important to experimentally address the discrepancies and different susceptibilities to the defects in autophagic pathways between cell populations. The enhancement of autophagy has been linked to neuroprotection, in particular through the clearance of aggregation prone proteins, such as α-synuclein in PD, Aβ in AD and huntingtin in HD (reviewed by (Son et al., 2012)). However, it is also clear that an exacerbated induction of autophagy can result in cell death. The mechanisms underlying autophagy control are only partially unravelled, and considerably less is known about the regulation of mitophagy.

The results presented in Chapter 5 showed that *ref(2)P* mutants are extremely sensitive to autophagy induction. Whether this was due to the high drug concentration was not evaluated yet. However, none of the other strains tested showed such a dramatic reduction in lifespan as *ref(2)P* mutants, indicating that the specificity of this response is due to loss of *ref(2)P*. It would be interesting to pursue these studies further and investigate the mechanism of toxicity involved. Moreover, it would be interesting to test the effect of these autophagy inducers in *Pink1* and *parkin* mutants.

It seems reasonable to consider that an approach consisting exclusively in augmenting mitophagy may not be totally efficient, since it can ultimately result in the total loss of mitochondria within the cell and in cell death. It seems rational that, in addition to enhancing the clearance of defective mitochondria, a parallel stimulation of mitochondria biogenesis would provide further benefits. The expression of *parkin* has been shown to have an impact on mitochondria biogenesis by regulating PGC1α, through its interaction with PARIS (Shin et al., 2011). The combined action of agents that impact on degradation of dysfunctional organelles with agents that stimulate their biogenesis would potentially result in a more effective protection strategy for mitochondria. In Figure 6.1, a schematic representation of the possible sites of action of Trap1 and ref(2)P is shown. The overexpression of *Trap1* protects mitochondria from stress, likely by enhancing the folding and/or degradation of damaged proteins, therefore acting at the mitochondrial molecular quality control level. The overexpression of ref(2)P may facilitate the autophagic degradation of dysfunctional mitochondria. The presence of ref(2)P is important for the parkin-mediated rescue of *Pink1* mutant flies, suggesting that *ref(2)P* play a role in the Pink1/parkin mitochondrial organellar quality control pathway.

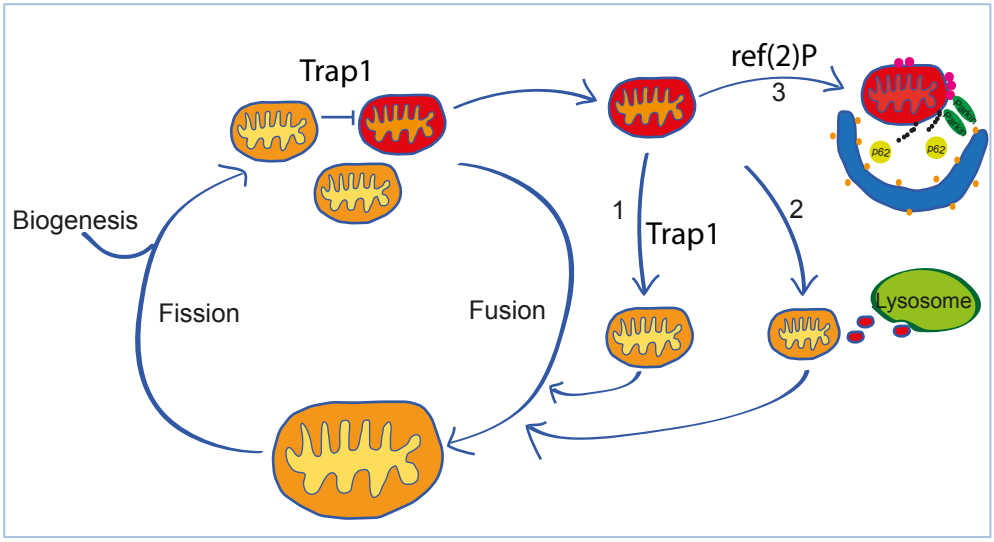



**Figure 6.1: Possible roles of Trap1 and ref(2)P in mitochondrial quality control pathways.** Several lines of defence exist to protect mitochondria and ultimately the cell from damage. 1- Molecular quality control: mitochondria that accumulate damage may recover by the action of molecular chaperones and proteases, which can help in the refolding and/or degradation of un/misfolded proteins. Trap1 is a mitochondrial chaperone that protects from mitochondrial stress *in vivo*, in *Drosophila*. This chaperone may be part of the mitochondrial molecular quality control system and its overexpression may, upon stress, avoid the accumulation of damage in mitochondria. 2- Mitochondria-derived vesicles with cargo targeted to the lysosomes may promote the elimination of damaged components from mitochodria. 3- Elimination of dysfunctional mitochondria through mitophagy. It has been shown that in mammalian systems p62 is involved in the aggregation and possibly autophagic degradation of depolarised mitochondria. The overexpression of *ref(2)P* in *Drosophila* may therefore improve the clearance of defective mitochondria present in *Pink1* mutants and ameliorate their phenotype.

Mammalian p62 has been reported to impact on another important cellular pathway: the Nfr2/Keap1 pathway. p62 binds to Kelch-like ECH-associated protein 1 (Keap1), diminishing its repressor effect on nuclear factor erythroid 2-related factor 2 (Nrf2) and increasing the Nrf2 transcriptional activation (Komatsu et al., 2010). The Nrf2/Keap1 pathway was shown to affect lifespan and resistance to stress in *Drosophila* (Sykiotis and Bohmann, 2008). Moreover, the activation of Nrf2 pathway was also shown to ameliorate the phenotype of a fly model of PD based on α-synuclein expression (Barone et al., 2011). In the work presented in this thesis I did not explore the putative involvement of the Nrf2/Keap1 pathway in the *ref(2)P* studies. However, it is possible that some of the observations, either related to the loss- or gain-of-function of *ref(2)P*, may be due to Nrf2/Keap1 signalling. Thus, it would be interesting to

conduct some studies on that respect. Despite the limitation of reagents, such as antibodies, to perform studies in the fruit fly, genetic studies can be performed easily. In this respect, the generation of *Keap1* and *ref(2)P* double mutants would be interesting, for instance to test the effect of autophagy inducers in these flies. If the lethality of *ref(2)P* mutants was due to its repressor effect on the Nrf2 pathway, the *Keap1* and *ref(2)P* double mutants should be more resistant to the autophagy inducer drug treatments.

In conclusion, the elucidation of the molecular mechanisms involved in all processes that may account for PD pathogenesis can be of great value. Such information would help the understanding of what seems to be a very complex pathology that affects many individuals worldwide. It is essential to understand why the dopaminergic neurons of the substantia nigra are so susceptible to certain toxins and mutations in PD-linked genes. However, it is also important to recognise that other types of neurons and other cell types are also affected. Hopefully, all the efforts to clarify disease-intervenient molecules and mechanisms will improve the development of new therapies. It is absolutely essential to aim for therapies that can alter the course of the disease, rather than the existing symptomatic treatments. With the available information to date, it seems that a multifactorial approach might have better success than the use of single agents, and the combined effect of various agents may significantly ameliorate the quality of life of PD patients.

## 6.1 Future work

The results presented in this thesis lead to some interesting concepts that could be explored further. It would be interesting to investigate the role of Trap1 in the PINK1/Parkin pathway in additional experimental systems, such as mammalian neuronal cell lines. Moreover, it would be interesting to elucidate the molecular mechanisms responsible for the protective effect of *Trap1* expression in *Pink1* and *parkin* mutants. It would also be interesting to determine whether a decrease in ROS production is involved in the protective action of Trap1. Considering the proposed role of TRAP1 in preventing apoptosis through the direct regulation of mPTP opening, it would be interesting to investigate whether TRAP1 blocks apoptotic cell death in the *Pink1* and *parkin* mutants.

Exploring some molecular mechanisms can be challenging in *Drosophila*. It would therefore be advantageous complement the *Drosophila* work with molecular studies in cultured cells. It would also be relevant to identify mitochondrial targets of TRAP1. To date it is not known if TRAP1 interacts with specific proteins or protein complexes, promoting their folding and/or assembly.

*Pink1* and *parkin* mutants were associated with the activation of the UPR$^{mt}$ (Pimenta de Castro et al., 2012). It would be worth investigating whether the loss of *Trap1* results in UPR$^{mt}$ activation, or perhaps an UPR originated in the IMS. To this respect, gene expression analysis could be used to examine the expression of a large amount of genes in a single experiment. Samples could be prepared for *Trap1*$^{4}$ mutants and controls (*Trap1*$^{revertant\ EY10238}$) at two distinct ages, 10 and 25 days old. These samples would include preparations from whole flies and from heads only, to evaluate whether there is any specific alteration in the gene expression profile of *Trap1* mutants in neuronal cells.

Regarding the *ref(2)P* work, it would be interesting to investigate the putative involvement of the Nrf2/Keap1 pathway in the *ref(2)P* phenotype. Due to the lack of available tools some hypotheses may not be easily addressed in *Drosophila*. However, it is possible to perform genetic studies with the available mutants and transgenic flies for *Nrf2* and *Keap1*.

The strong impact of autophagy inducers in the viability of *ref(2)P* mutants is of a particular interest and warrants further investigation. It seems important to obtain a dose response study for the drug treatments. Moreover, it should be tested whether the drugs induce autophagy in the *ref(2)P* mutants. To that purpose, *ref(2)P* mutants could be crossed with a transgenic line that expresses GFP-LC3, and treated with the autophagy inducers. Protein lysates from these flies would then be subjected to western blot analysis to investigate the relative amounts of LC3I and LC3II. It would be also important to do the same type of treatment to *ref(2)P* mutants in an autophagic mutant background. If the activation of autophagy was necessary for the premature dead of *ref(2)P* mutants, the autophagy deficiency should protect these flies from dying so rapidly. Furthermore, a genetic screen of modifiers of the *ref(2)P* viability when treated with metformin (the drug with the stronger effect) could be conducted. To achieve this, *ref(2)P* mutants would be crossed to flies carrying chromosomal deletions (deficiencies), using the Bloomington Deficiency Kit. Progeny would then be screened for changes in lifespan under metformin treatment. Any positive hits would be then subject to further analysis.

# Appendices

# Appendices

## **Appendix 1:** Thoracic indentation in *Pink1*$^{B9}$ mutants.


**Figure appendix 1: Representative pictures of the thoracic indentation in *Pink1* mutants and normal thorax in the rescue with *Trap1* expression and control.** Genotypes: Control, *w; +; daGAL4*/+. Pink1B9, *Pink1*$^{B9}$*; +; daGAL4*/+. Pink1B9,da>Trap1, *Pink1*$^{B9}$*; +; daGAL4*/*UASTrap1*.

**Appendix 2:** HPLC chromatograms of reference standards and respective calibration curves.

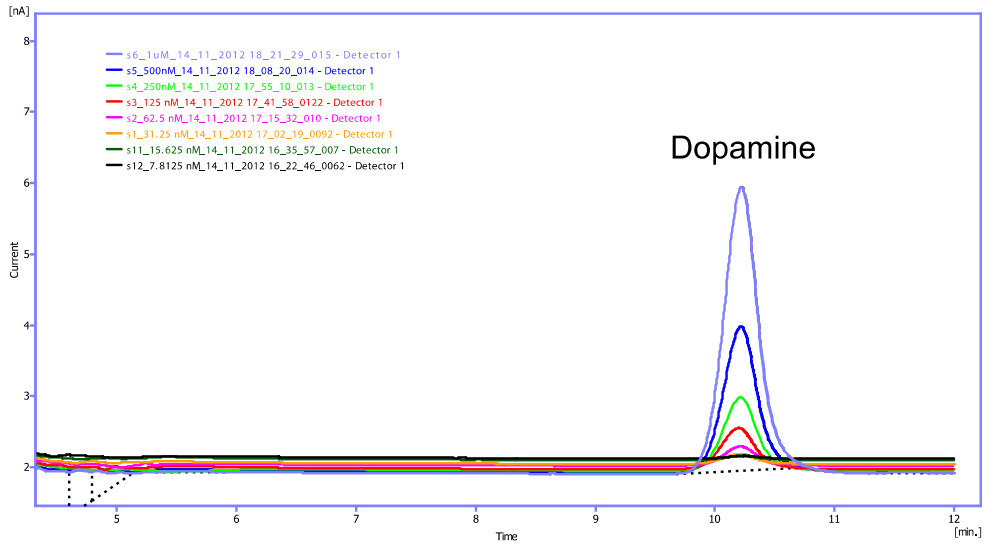



**Figure appendix 2.1: HPLC chromatograms of the dopamine reference standards.** The retention time was approximately 10.3 min.

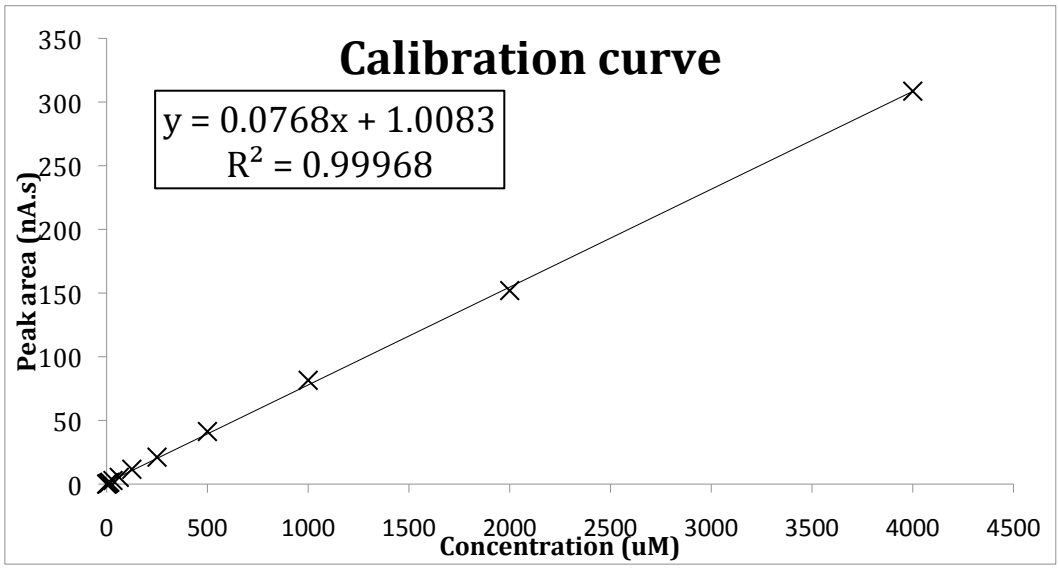



**Figure appendix 2.2: Calibration curve of dopamine standards obtained from HPLC data.**

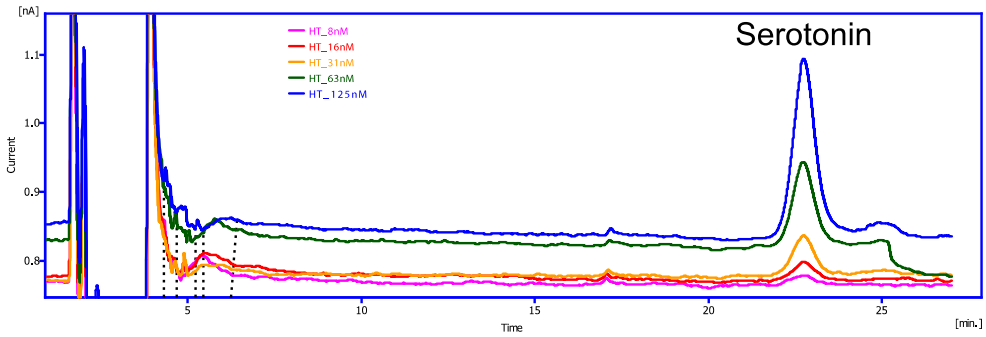



**Figure appendix 2.3: HPLC chromatograms of serotonin reference standards.** The retention time was approximately 23 min.

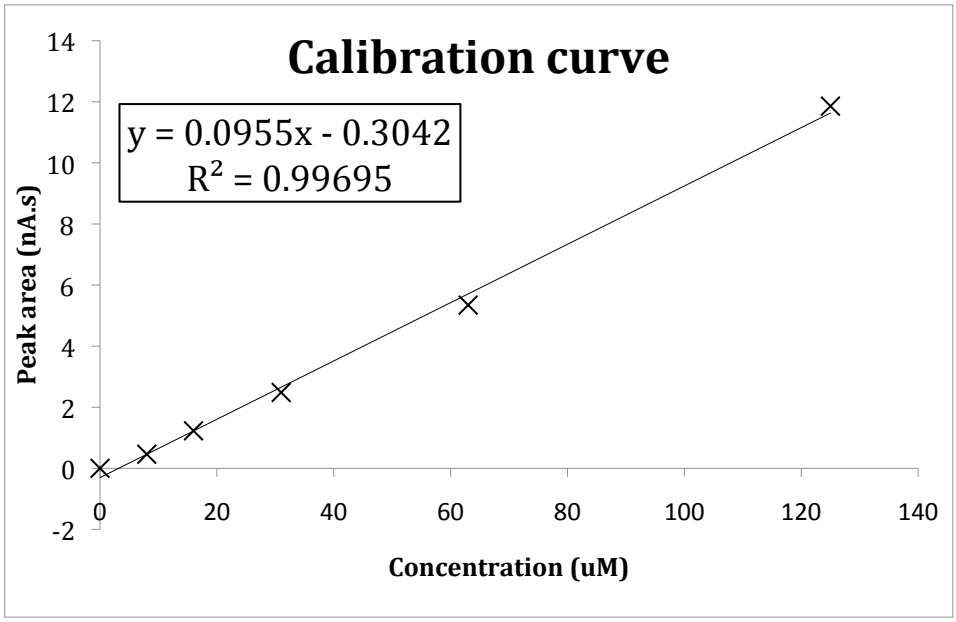



**Figure appendix 2.4: Calibration curve of dopamine standards obtained from HPLC data.**

# Publications

**Costa, A. C.**, Loh, S.H.Y., and Martins, L.M.; *Drosophila Trap1* protects against mitochondrial dysfuntion in a PINK1/Parkin model of Parkinson's disease. *Cell Death and Disease*. In press.

**Costa, A. C.**, Martins, L. M. and Loh, S. H. Y. (2012). Emerging Concepts Linking Mitochondrial Stress Signalling and Parkinson's Disease. In Martins, L. M. and Loh, S. H. Y. (Eds.), *Neurodegeneration* (pp. 77-92). Rijeka: Intech.

De Castro, I.P. *, **Costa, A. C.** *, Tufi, R., Moisoi, N., Lam, D., Dinsdale, D., Loh, S.H.Y., and Martins, L.M.; *Drosophila ref(2)*P is required for the *parkin*-mediated suppression of mitochondrial dysfunction in *Pink1* mutants. *Human Molecular Genetics*. (Under review).
* Joint first author

De Castro, I.P., **Costa, A. C.**, Lam, D., Tufi, R., Fedele, V., Moisoi, N., Dinsdale, D., Deas, E., Loh, S.H.Y., and Martins, L.M. (2012); Genetic analysis of mitochondrial protein misfolding in *Drosophila melanogaster*. *Cell Death and Differentiation* **19**: 1308-1316.

**Ana Carina Costa**. "*Drosophila Trap1 interacts genetically with Pink1"*. "MiPSummer 2012", 7 to 13 July 2012, Cambridge, United Kingdom. (oral communication).

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