INVESTIGATING THE ROLE OF MITOCHONDRIA IN DISEASE AND AGING

By

Rehan Murtaza Baqri

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ABSTRACT

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Rehan Murtaza Baqri

Mitochondria are central regulators of multiple critical cellular processes. Mitochondrial dysfunction, therefore, severely compromises cellular integrity. Mutations in mitochondrial DNA (mtDNA), dysfunction of the electron transport chain, excessive oxidative stress, imbalance in mitochondrial turnover, disruption of the fusion/ fission machinery, impaired trafficking, and defective mitochondrial proteostasis are widely implicated in disease pathology and organismal aging. However, less is understood about the interdependence of these different aspects of mitochondrial biology. For example, it is unclear how mtDNA depletion impacts mitochondrial trafficking. Similarly, the effect of increased oxidative stress on mitochondrial proteostasis is unknown. In this dissertation, I have investigated the regulation of mitochondrial dynamics in the context of mtDNA disorders, and the regulation of the mitochondrial proteostasis machinery in the context of aging and healthy lifespan.

In Chapter 2, I will discuss the implications of blocking mtDNA replication on mitochondrial biogenesis, distribution and trafficking. We demonstrate that mtDNA depletion results in an upregulation of mitochondrial biogenesis and increased bidirectional axonal transport in *Drosophila*. Transport of other axonal cargoes is largely unaffected, indicating a specific regulation of mitochondrial axonal transport. We interpret these results in the context of

an SOS response that is activated to replenish the presynaptic terminal with functional mitochondria. These data uncover a novel contributory mechanism in distal neuropathy, often associated with mtDNA disorders.

In Chapter 3, I will present evidence that a mitochondrial chaperone TRAP1 is involved in regulating resistance to oxidative stress in *Drosophila*. Interestingly, there is only a marginal influence on lifespan, challenging the conventional belief that oxidative stress resistance is causally associated with longevity. Remarkably, dosage modulation of this mitochondrial chaperone has dramatic effects on the fitness and health of aging flies. This role of TRAP1 is mediated at least partially through the activation of the mitochondrial unfolded protein response. We discuss these results in the context of organismal aging and healthspan.

Taken together, this dissertation has uncovered novel mechanisms by which mitochondria deal with at least two different stressors, mtDNA depletion and oxidative stress. In Chapter 4, I will discuss my findings in the perspective of mitochondrial quality control and its larger implications in human diseases and health. I will also briefly explore unanswered questions that arise from my work and the directions of future research and avenues open.

To victims of terror and oppression across the world.
To all those children who never had the option of education.
To Sherlock Holmes and the Marlboro Man.

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KEY TO ABBREVIATIONS

AD	Alzheimer's disease
CMT	Charcot-Marie-Tooth
DVE	defective proventriculus
ER	endoplasmic reticulum
ETC	electron transport chain
HD	Huntington's disease
Hsp	heat shock protein
KSS	Kearn's Sayre syndrome
LHON	Leber Hereditary Optic Neuropathy
MELAS	Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-live events
MERF	Myoclonus Epilepsy Ragged-red Fibers
Miro	mitochondrial Rho-GTPase protein
MPT	mitochondrial permeability transition pore
mtDNA	mitochondrial DNA
mtGFP	GFP tagged mitochondria
OXPHOS	oxidative phosphorylation
PD	Parkinson's disease
PEO	Progressive external opthalmoplegia
PINK1	PTEN induced kinase 1
pol γ	mitochondrial DNA polymerase
RNAi	RNA interference

ROS	reactive oxygen species
sybGFP	GFP tagged synaptobrevin vesicles
TRAP1	Tumor necrosis factor Receptor Associated Protein 1
UAS	upstream activation sequences
UPR	unfolded protein response

Chapter 1
An Introduction to Mitochondria

Preface to Chapter 1

The following chapter is an introduction to topics relevant to work in this dissertation. Part 1 is an overview of mitochondrial structure, function and dynamics. Part 2 is a review of the biology of aging, the role of mitochondria in the aging process and stress response pathways implicated in the regulation of longevity. Part 3 is a discussion of *Drosophila* as a model system and key genetic tools utilized in this dissertation.

Part 1: Mitochondria

Sometime between one and two billion years ago a Eubacterium incorporated into an Archae-type host. Presumably, such encounters must have happened before and probably resulted in mutual damage. This time, the invader and the host discovered to their benefit that they could forge a symbiotic association. This mutually beneficial association evolved over time and present day mitochondria are believed to be the one-time-eubacteria-invaders.

The cell, structural unit of eukaryotic life, is a dynamic system with multiple critical processes operating in parallel. Mitochondria are central to several of these vital processes, chief among them being energy production, programmed cell death, cell cycle regulation, calcium signaling, and synaptic transmission in the case of nerve cells. Understandably, the critical nature of these cellular activities means that dysfunction or deregulation in any of them has severe pathological ramifications that result in diseases, aging, and/ or lethality. Thus, a comprehensive understanding of mitochondrial biology is paramount to a human health and longevity.

1.1. Mitochondrial Structure

1.1.1. Historical Review of Mitochondrial Structure

Altman reported the earliest scientific observation of mitochondria in 1890. He called them ‘bioblasts’ and observed that they have near universal presence in different cell types. Altman concluded that they were elementary organisms that reside in the cytoplasm and carry out vital functions. The name ‘mitochondrion’ itself was coined by Benda in 1898, deriving from the Greek *mitos* (thread) and *chondros* (granule) (L Ernster, 1981). In 1900, Michaelis discovered that the redox dye Janus Green B specifically stained mitochondria and this remained the primary means of visualizing mitochondria till 1952, when Palade published the first thin section electron micrographs wherein cristae and the double membranes were clearly observed for the first time (Palade, 1952). Palade concluded that the cristae were infoldings of the inner membrane. He was also the first to make reference to the mitochondrial matrix and the intermembrane space (Palade, 1956). It was also seen that mitochondria vary considerably in size, shape and number depending on cell type (Novikoff, 1961).

1.1.2. Current Knowledge of Mitochondrial Structure

Our present understanding of the mitochondrial ultrastructure is based primarily on high-resolution 3D images acquired by a high voltage electron microscope and then employing sophisticated reconstruction algorithms, a technique referred to as electron tomography. Recent evidence indicates that the cristae are not merely random folding of the inner membrane as initially thought. Rather, they are more like internal compartments formed by invagination of the membrane and there is some evidence to suggest that their specific morphology may regulate components of the apoptotic pathway, elimination of diffusion bottlenecks in ATP production,

and resistance to oxidative stress (Mannella, 2006). The lipid double membrane itself is rich with proteins that are involved in the translocation of other proteins, known as Translocases of the Inner Membrane (TIM) and Translocases of the Outer Membrane (TOM). The proteins involved in oxidative phosphorylation are enriched in the cristae membranes (Gilkerson et al., 2003). Two component proteins of the calcium uniporter, MICU1 and MCU, have been recently identified and found associated with the mitochondrial inner membrane (Perocchi et al., 2010; De Stefani et al., 2011).

1.2. Mitochondrial DNA

1.2.1. Historical Review of Mitochondrial DNA

A legacy of the endosymbiont origin of mitochondria is that they have their own circular, double stranded DNA. The earliest evidence of this came from dense metal staining fibers inside the mitochondrial matrix by Nass and Nass in 1962, revealing the presence of DNA within mitochondria (Nass and Nass, 2000). With advanced electron microscopy techniques and reliable biochemical isolation procedures, the closed circular nature of vertebrate mitochondrial DNA (mtDNA) and size of around 16 – 17 Kb was determined (Clayton et al., 1968; Clayton, 2000). Soon it was established that mtDNA could exist in thousands of copies in a cell and the entire genomic sequences of mouse and human mtDNA were reported (Anderson et al., 1981; Bibb et al., 1981). It was apparent from the studies that mtDNA has a compact organization and lacks introns. Human mtDNA has 16,569 base pairs that encodes 13 critical genes for oxidative phosphorylation, 22 tRNAs and 2 rRNAs (Anderson et al., 1981; Garesse and Kaguni, 2005).

In 1972, the mtDNA replication enzyme was identified as a RNA-dependent-DNA polymerase in HeLa cells (Fridlender et al., 1972), and its presence was subsequently localized

to the mitochondria (Bolden et al., 1977). Purification and biochemical analysis of the replicase from *Drosophila* embryos revealed that it to be a heterodimer of 125 and 35 kDa subunits (Wernette and Kaguni, 1986).

1.2.2. Current Knowledge of Mitochondrial DNA

The mtDNA organization and gene content discussed above is largely conserved among metazoans. Mammalian mtDNA has two strands, named as the heavy (H) and light (L) strands. These strands are replicated from independent origins of replication in a unidirectional and asymmetrical fashion that proceeds by displacement-loop mechanism (Bogenhagen and Clayton, 2003). However, recent evidence suggests that in some higher order animals, bidirectional replication of mtDNA is also possible (Bowmaker et al., 2003; Holt and Jacobs, 2003; Fish et al., 2004). Additional studies will resolve this controversy but for now it appears that two mechanisms are available for mtDNA replication in vertebrates, and a choice could be made depending on physiological circumstances (Garesse and Kaguni, 2005). In contrast, so far only one origin of mtDNA replication has been identified in *Drosophila* (Goddard and Wolstenholme, 1980).

Several proteins have been identified as critical for mtDNA replication and maintenance. The major proteins known to be involved in *Drosophila* are the catalytic and accessory subunits of mitochondrial DNA polymerase or pol γ, the mitochondrial single-stranded DNA binding protein mtSSB, and a mitochondrial DNA helicase homologous to the human Twinkle protein. In addition, several transcription factors such as A, B1 and B2, are also known to play important roles in primer synthesis and translation (Lefai et al., 2000; Maier et al., 2001; Iyengar et al., 2002; Matsushima et al., 2004; Tyynismaa et al., 2004; Matsushima et al., 2005). While there is

evidence that mtDNA plays an important role in regulation of mitochondrial fusion and fission, it's role in mitochondrial distribution remains unknown.

Recently, humanin, a 24 amino acid polypeptide that is transcribed from an open reading frame within the mitochondrial 16S ribosomal RNA has been identified in several animal models (Hashimoto et al., 2001). Endogenous humanin has been detected in wide range of rodent tissues such as testis, colon, brain, heart, etc (Muzumdar et al., 2009). Presently, the major role of humanin is believed to be promotion of cell survival. Humanin has been found to be involved in neuroprotection against Alzheimer's associated cell death, prion induced apoptosis and chemical-induced neuronal damage (Hashimoto et al., 2001; Mamiya and Ukai, 2001; Sponne et al., 2004). These observations underline the significance of humanin in the nervous system. Future studies will determine the *in vivo* regulation and additional physiological significance of humanin and other potential mtDNA encoded polypeptides.

In an evolutionary context, the integration of the mitochondrial endosymbiont has seen an evolving role of mtDNA in the cell. Genetic factors and selection pressure have meant that mtDNA is near exclusively maternally inherited. The mitochondrial genome still retains certain critical genes that are necessary for oxidative phosphorylation while the bulk of structural, functional and regulatory genes have been passed over to the nuclear genome (Wallace, 2007).

1.2.3. Mitochondrial DNA polymerase, pol γ

Pol γ is the sole mtDNA polymerase identified in animals and plays a critical role in repair, replication and recombination. It exists as a heterodimeric holoenzyme complex, comprising a large catalytic subunit pol γ–α, and a smaller accessory subunit pol γ-β. The pol γ holoenzyme is a catalytically efficient and processive DNA polymerase and partakes in

nucleotide selection and incorporation along with proofreading errors with its intrinsic 3' → 5' exonuclease activity (Wernette and Kaguni, 1986; Kaguni, 2004). It is now understood that pol-γ–α is responsible for its DNA polymerase and exonuclease activity whereas pol-γ–β enhances primer recognition and template-primer DNA binding (Lewis et al., 1996; Fan et al., 1999). Mutations in either the catalytic or accessory subunit of pol γ in *Drosophila* result in depletion of mtDNA and early lethality arising out of developmental defects (Iyengar et al., 1999; Iyengar et al., 2002).

1.3. Mitochondrial Function

1.3.1. Historical Review of Mitochondrial Function

After the initial period where mitochondrial studies were largely structural in nature, evolving biochemical techniques helped advance the understanding of mitochondrial function. Krebs had already discovered the citric acid cycle in 1937, and most of its components were subsequently identified (Krebs, 1937; Krebs and Johnson, 1937; Thauer, 1988). Several lines of evidence emerged in 1940s and 1950s that made it increasingly clear that mitochondria were central to cellular respiration. It was shown that the enzymes of the citric acid cycle, fatty acid oxidation and oxidative phosphorylation were present in mitochondrial fractions (Hogeboom et al., 1946; Kennedy and Lehninger, 1950). Multiple biochemical studies then revealed the kinetics and partial reactions of oxidative phosphorylation and the electron transfer system was reconstituted (Slater, 1953; Chance and Williams, 1955; Hatefi et al., 1962; Knowles et al., 1971; Thayer and Hinkle, 1975).

Another aspect of mitochondrial function was revealed when isolated mitochondria were found to accumulate and retain calcium (Rossi and Lehninger, 1964; Chance, 1965). Soon it was

established that mitochondria had a calcium uniporter and a Na^{+}/Ca^{2+} exchange pump, allowing calcium to cycle across the inner membrane through these transporters (Crompton and Heid, 1978; Crompton et al., 1978).

1.3.2. Energy Production

Generation of energy is the hallmark function of mitochondria. Energy is required for several critical cellular processes, including growth and differentiation. The mitochondrial ATP production machinery is composed of five enzyme complexes and the adenine nucleotide translocator (ANT) (Figure 1.1). Of the five enzyme complexes, mtDNA encodes a minority of polypeptides for four of them: Complexes I, III, IV and V. Nuclear DNA encodes the bulk of subunits in all these complexes and in addition, exclusively encodes Complex II and ANT (Shoffner and Wallace, 1990).

Complexes I, II, III and IV comprise the electron transfer chain. The Krebs cycle in the mitochondrial matrix generates NADH, which donates two electrons to NADH dehydrogenase and FMN on Complex I is reduced to $FMNH_2$, the electrons then pass through a series of iron-sulphur centers in Complex I and passed on to coenzyme Q. From here, electrons pass through another iron-sulphur cluster to a inner membrane anchored ubiquinone. Meanwhile, Complex II gets two electrons from succinate dehydrogenase of the TCA cycle and passes them on to FAD, then through another iron-culphur cluster and on to ubiquinone. Complex III receives electrons from the reduced ubiquinone. Thus, electron transport pathways from NADH and succinate converge in Complex III. After passing through multiple centers, electrons are then handed over to cytochrome c, a mobile electron carrier. Cytochrome c transports one electron at a time from Complex III to IV, where the electron reduces molecular oxygen to H_2O.

Importantly, the negative free energy of electron transfer through this chain is used towards pumping protons across the mitochondrial inner membrane at Complexes I, III and IV. This proton gradient (about -200 mV) is an important step in ATP generation. As the concentration of protons in the intermembrane space increases, the gradient pumps protons back inside the mitochondria through Complex V, where it is coupled to ATP synthesis by ATP synthase. Finally, the ANT exchanges this ATP for cytosolic ATP (Wallace, 1992; Smeitink et al., 2001; Larsson, 2010). Mitochondria contribute about 90% of cellular ATP, the rest is derived from glycolysis.

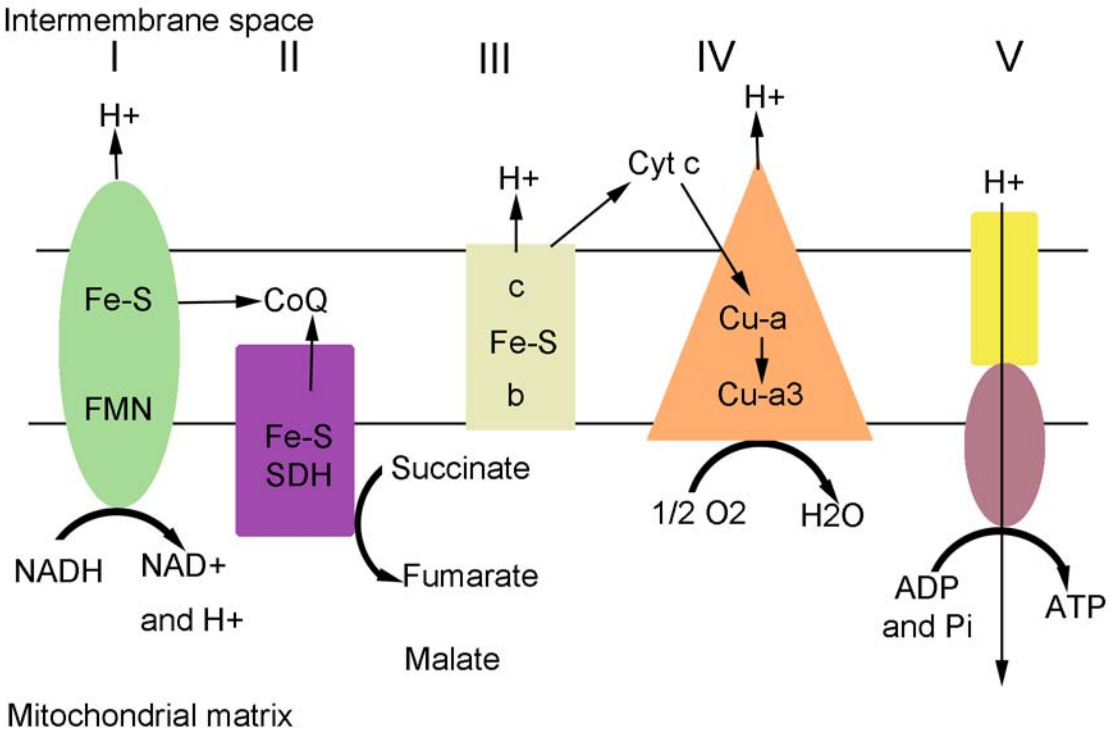

Figure 1.1 Energy production via oxidative phosphorylation in the mitochondrion. The mitochondrial ATP production machinery is composed of five enzyme complexes and the adenine nucleotide translocator (ANT). For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

1.3.3. Apoptosis

Apoptosis or programmed cell death is a distinctive and important means of eliminating cells, either during normal development, aging or as a defense mechanism (Elmore, 2007). Two main apoptotic pathways that have been identified so far: the extrinsic and intrinsic pathways that are triggered by specific signals and initiate an energy dependent cascade of events. Both pathways converge at caspase 3 and thereafter share the same execution pathway. These pathways were initially thought to function independently of one another but recent evidence indicates that there may be some crosstalk even before caspase 3 activation (Igney and Krammer, 2002).

The extrinsic pathway is activated by cell surface death receptors, while the intrinsic pathway is activated through the mitochondria. Factors such as oxidative stress, temperature stress, toxins, radiation, calcium overload, and impaired electron transport chain are known to activate the intrinsic pathway by facilitating the mitochondrial permeability transition that opens a non-specific pore in the inner mitochondrial membrane, permeable to all molecules of less than 1.5 kDa (Leung and Halestrap, 2008; Leung et al., 2008). This leads to the loss of mitochondrial membrane potential and release of two main groups of pro-apoptotic proteins from the intermembrane space into the cytosol.

The first group comprises cytochrome *c*, Smac/DIABLO, and the serine protease HtrA2/Omi (Du et al., 2000; Saelens et al., 2004). Cytochrome *c* activates Apaf-1 and procaspase-9, forming an apoptosome (Hill et al., 2003). This apoptosome leads to the activation of caspase-9 (Hill et al., 2004). Meanwhile, Smac/DAIBLO and HtrA2/Omi promote apoptosis by suppressing the inhibitors of apoptosis proteins (van Loo et al., 2002). The second group of proteins released from the mitochondria includes AIF, endonuclease G and CAD. AIF and

endonuclease G translocate to the nucleus and cause DNA fragmentation and cleave nuclear chromatin, respectively. At a later stage, they are joined in the nucleus by CAD, which precipitates the DNA fragmentation and chromatin damage (Enari et al., 1998; Joza et al., 2001; Li et al., 2001).

Apart from these major players in the apoptotic cascade, there are several other proteins that regulate intrinsic apoptosis. Most have been classified as members of the Bcl-2 family. The Bcl2 family is comprised of both pro-apoptotic (examples: Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, Blk) and anti-apoptotic proteins (examples: Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, BAG). These proteins regulate the mitochondrial membrane permeability transition and the subsequent release of cytochrome *c* (Elmore, 2007).

1.3.4. Calcium Signaling

Calcium regulates key cellular pathways directly and via secondary messenger systems. The concentration of calcium needs to be actively regulated in different tissues to ensure physiological functions, such as muscle contraction and neuronal firing. To this end, there are specialized systems such as mitochondria and the endoplasmic reticulum that can buffer excess cytosolic calcium and release it in a regulated manner.

Mitochondria have the capacity to accumulate impressive amounts of calcium, exceeding 1000 nmol/mg mitochondrial protein (Nicholls, 2005). Respiring mitochondria accumulate calcium through the calcium uniporter when extra-mitochondrial calcium becomes in excess of a certain *set point*, which is slightly variable for different tissue types. At the set point itself, the rate of influx is balanced by the rate of efflux through the Na^{+}/Ca^{2+} exchanger. The set point for isolated brain mitochondria is around 0.25 – 0.5 μM (Nicholls, 2009). Excessive calcium loading

by mitochondria can lead to the induction of the mitochondrial permeability transition (Leung and Halestrap, 2008; Leung et al., 2008). This mitochondrial permeability transition pore is recognized as a major step of cell death.

Calcium buffering by mitochondria is especially important for neurons as increased calcium load through NMDA receptor activation, tetanic stimulation and other physiological reasons, may potentially be toxic to the neuron. In stimulated neurons, mitochondria can reversibly sequester and release calcium to synchronize the quantal of vesicle release and minimizes depletion of releasable vesicles at the synapse (David and Barrett, 2003). Mitochondrial calcium buffering also facilitates the uptake of calcium through the voltage and receptor activated calcium channels as well as contributes to feedback inhibition pathways (Nicholls, 2009).

1.3.5. Cell Cycle Regulation

The cell cycle is an intricately synchronized process that is regulated by several checkpoints that respond to a wide variety of intracellular and extracellular signals (Johnson and Walker, 1999). It has been known for a while that mitochondrial energetics is essential for transition from G_1 to S phase. AMPK, the metabolic sensor of energy, has been implicated in arresting cells at the G_1-S transition in response to compromised ATP levels (Van den Bogert et al., 1988; Santamaria et al., 2006). Presumably, depleted ATP concentration may also impact the function of motors responsible for aligning and organizing the cytoskeleton during later stages of cell division (Walczak et al., 1996; Howell et al., 2001).

Recently, a more nuanced role of mitochondria has come to light in regulating the G_1-S transition. Mitochondria are seen to form a single, giant tubular network at this checkpoint. Interestingly, abolishing this unique mitochondrial morphology or sustaining it beyond the transition, prevented cells from progressing in the cell cycle (Mitra et al., 2009). Another indication of mitochondrial involvement in cell cycle progression came from the observation that a mutation in *Drosophila cytochrome c oxidase* led to a specific G_1 arrest (Mandal et al., 2005). There is some evidence that p53 is involved in both of these regulations (Finkel and Hwang, 2009). These lines of investigation are relatively recent and have opened new avenues to explore possible therapeutic interventions in cancer.

1.3.6. Synaptic Transmission

Synaptic transmission involves several ATP-dependent steps such as fueling the ion pumps, organizing cytoskeletal components, phosphorylation of multiple pathways, transport of vesicles, synaptic vesicle recycling, etc (Murthy and De Camilli, 2003; Vos et al., 2010). It is also heavily influenced by the cytosolic calcium concentration. These features place mitochondria as an important regulator of synaptic function and thereby, synaptic transmission.

At the *Drosophila* NMJ, defect in the ATP translocase leads to rapid inhibition of synaptic transmission in the temperature sensitive *sesB* mutants (Rikhy et al., 2003). Deficit in mitochondrial supply at the NMJ in *Miro* mutants also results in impaired neurotransmission (Guo et al., 2005). It was observed that mitochondrial ATP is required for mobilization of the reserve synaptic vesicle pool for release, and this lends some insight into the mechanism for the earlier observations (Verstreken et al., 2005). Interestingly, lower levels of ATP produced by

glycolysis seem sufficient for carrying out other steps of synaptic vesicle cycling (Calupca et al., 2001).

1.4. Mitochondrial Dynamics

1.4.1. Historical Review of Mitochondrial Dynamics

That mitochondria are dynamic organelles was noticed as early as 1914. Lewis and Lewis reported changes in position and shape of mitochondria in animal tissue cultures (Lewis and Lewis, 1914). In 1956, Frederic and Chevremont extended these observations to monitor mitochondrial displacement during mitosis and in response to varied experimental conditions. Over the next two decades, several groups independently confirmed the occurrence of mitochondrial division and fusion, and the shape modifications associated with these dynamics. These mitochondrial behaviors were thought to be controlled by their metabolic state (Novikoff, 1961; Bereiter-Hahn, 1978).

1.4.2. Current Knowledge of Mitochondrial Dynamics

With technological advances in optical microscopy in the 1990s, it became evident that a mitochondrion can undergo fission to give rise to two smaller distinct mitochondria and has the ability to fuse its double membrane with that of another mitochondrion and exchange intramitochondrial content. It was found that mitochondria go through constant morphological changes and the dynamic equilibrium between fusion and fission maintains the overall architecture of the mitochondrial population (Bereiter-Hahn and Voth, 1994; Nunnari et al., 1997; Rizzuto et al., 1998). Genetic manipulation of this equilibrium indicates that cells with high fusion to fission ratio have fewer mitochondria and those present, are tubular and elongated.

Conversely, cells with low fusion to fission ratio have more mitochondria that appear oval, smaller and fragmented (Detmer and Chan, 2007).

1.4.3. Molecular Machinery of Fusion

Genetic and molecular analyses in yeast, *Drosophila* and mammals have revealed some of the important regulators of mitochondrial fusion and fission. In 1997, fuzzy onions (FZO), a mitochondrial outer membrane GTPase was identified as a fusion factor in *Drosophila* (Hales and Fuller, 1997). The mammalian homologues of FZO, MFN1 and MFN2 were identified soon after and are grouped in the mitofusin family of GTPases. MFN1 and MFN2 are responsible for fusion of the outer mitochondrial membrane (Chen et al., 2003). OPA1 is another important regulator of the fusion process, the yeast orthologue of which is a dynamin related protein Mgm1 (Meeusen et al., 2006). OPA1 promotes fusion of the inner mitochondrial membrane through its functional interaction with MFN1. Knockdown of OPA1 levels by RNA interference results in mitochondrial fragmentation (Cipolat et al., 2004).

1.4.4. Molecular Machinery of Fission

The dynamin related GTPase Dnm1 in yeast, was the first protein identified to conclusively regulate mitochondrial fission (Bleazard et al., 1999), the mammalian homologue being DRP1 (Smirnova et al., 2001). Both Dnm1 and DRP1 are recruited from the cytosol to the mitochondrial surface, where they arrange into punctate spots to lead the fission process by regulated constriction (Shaw and Nunnari, 2002).

Elegant genetic, biochemical and cell biology approaches in yeast revealed three additional players in the fission machinery, Fis1, Mdv1 and Caf4 (Mozdy et al., 2000; Tieu and

Nunnari, 2000; Griffin et al., 2005). What emerged is a complex fission pathway wherein Fis1 binds indirectly to Dnm1, through either Mdv1 or Caf4, to recruit Dnm1 to the mitochondrial membrane (Griffin et al., 2005). Intriguingly, Fis1 is also known to regulate the fission of peroxisomes, the cellular compartments for hydrogen peroxide and lipid metabolism (Koch et al., 2005). This conservation of division machinery indicates that biogenesis and functions of mitochondria and peroxisomes may be more closely related than appreciated.

Recently, a tail-anchored outer membrane protein, Mff, has also been implicated in the fission process. Loss of Mff results in mitochondrial elongation. Interestingly, biochemical studies have established that Fis1 and Mff reside in complexes of different sizes, suggesting that they are part of two distinct fission pathways (Gandre-Babbe and van der Bliek, 2008). Future studies will shed light on how Mff regulates mitochondrial fission.

1.5. Mitochondrial Transport

1.5.1. Axonal transport

1.5.1.1. Historical Review of Axonal Transport

Translation of proteins takes place in the cytoplasm, at sites of ribosomal localization. These proteins need to be transported to sites of utilization such as the plasma membrane, different organelles and even translocation within the cytosol. The distinct morphology and physiology of neurons presents an especially unique challenge. Neuronal cell bodies send out axons that terminate in synapses and in certain cases these axons may be over a meter long, with a volume that is hundreds of times that of the cell body. Transport of proteins and organelles from the cell body to the synapse in such axons by passive diffusion alone could take over a decade. Thus, the need for an active and dedicated transport machinery.

In 1771, Van Leeuvenhook described the slow leaking of axoplasm from severed axons. This is the earliest recorded observation, which indicated that material inside the axon was free flowing. In 1906, F. H Scott reported that when the nerve root was severed, muscle contraction in the frog legs diminished earlier than when the root was still connected to the cell bodies in the dorsal root ganglion. He concluded that the cell body secretes a substance; distal *passage* of which is essential for stimulation (Dahlstrom, 2010).

In 1948, Weiss and Hiscoe published a landmark monograph where they reported their studies on almost 100,000 nerve constrictions in rats, rabbits, chicken and monkeys. On constricting the nerves, they reported that while the caliber of the portion distal to the constriction diminished, the region immediately proximal to the constriction enlarged and became swollen. They concluded that there is a column of axoplasm that is moving towards the distal end of the nerve and becomes *dammed up* at the constriction site. On releasing the constriction, the bolus of material moved down the axon at the rate of 1mm/ day, and eventually the enlarged region evens out, as does the caliber of the distal region (Weiss and Hiscoe, 1948). Subsequent use of radioactive tracers confirmed that proteins could be transported down the axon and the rates of transport were found similar to those reported by Weiss (1 – 5mm/ day) (Droz and Leblond, 1962). Grafstein, studying the fish optic system, made the seminal observation that proteins transported at faster rates were of particulate nature and predominantly destined for the synapse; those transported at slower rates were mostly soluble in nature (Grafstein, 1967; McEwen et al., 1971), providing some early insight into the composition of the slow and fast transported cargos.

Soon thereafter, it was established that slow axonal transport itself had two broadly distinct rates: slow component-a (0.1 – 1mm/ day) and slow component-b (2 – 10mm/day)

(Willard et al., 1974). What followed is a period of relative controversy in the field of slow axonal transport and several groups proposed different mechanisms for this phenomenon based on different observations in varied systems. Miller and Heidemann recently published an exhaustive review of the various observations and suggested mechanisms of slow axonal transport, and proposed a converging model that integrates “Stop and Go” transport (polymer delivery), microtubule based transport (soluble proteins), diffusion (soluble proteins) and low velocity transport (contributing to axonal elongation) (Miller and Heidemann, 2008).

Several early studies helped determine that there is also a faster component to axoplasmic transport. Lubinska and her group reported that Acetylcholine esterase accumulated in the sciatic nerve of rats at a faster rate than could be explained by slow transport, and suggested that it could be transported in both directions (Lubinska et al., 1963; Lubinska et al., 1964). Lasek and Miani used radioactive amino acids to label proteins and radioactive phosphatase to label phospholipids and found that material can move at the rate of up to 70mm/ day (Miani, 1963; Lasek, 1968). Sydney Och’s used isotope labeled precursors in cat sciatic nerve explants to estimate the rate of fast axonal transport to be about 410mm/ day (Ochs et al., 1969). Later, it was seen that proteins labeled in the middle of the axon were capable of moving in both directions (Fink and Gainer, 1980), firmly establishing that axonal transport was a bidirectional process.

Pioneering work by Dahlstrom revealed that microtubules were essential for the maintenance of fast axonal transport (Dahlstrom, 1968, 1971). He injected two mitotic inhibitors known to disrupt microtubule organization, colchicine and vinblastine, into the sciatic nerve and observed that regions above and below the injection site became swollen. Further, reducing temperature to below 4°C disrupts microtubule integrity and blocked fast axonal transport

(Cosens et al., 1976). Moreover, single microtubules were found to be capable of supporting bidirectional transport (Schnapp et al., 1985; Vale et al., 1985b). These studies confirmed the critical role of intact microtubules in fast axonal transport. It was also seen that when the axoplasm of the squid giant axon was extruded, fast transport of organelles continued unabated for up to 30 minutes, suggesting that transport was independent of action potentials (Brady et al., 1982).

Two breakthrough findings in the mid 1980s revealed the proteins involved in ATP-dependent microtubule-based anterograde and retrograde axonal transport, kinesin and dynein respectively (Brady, 1985; Vale et al., 1985a; Paschal et al., 1987). Also, Ochs and colleagues used cyanide and dinitrophenol, drugs that block the oxidative phosphorylation machinery, in explanted nerves and showed that axoplasmic transport came to a halt. This helped promote the idea that mitochondrial oxidative metabolism was critical and ATP was required for axonal transport (Ochs and Ranish, 1970; Ochs and Hollingsworth, 1971). However, it was subsequently discovered that cyanide also disrupts microtubule integrity (Friede and Ho, 1977), raising doubt over the interpretation of Ochs and colleagues. Thus, it remains to be answered whether oxidative phosphorylation is indeed critical for maintaining axonal transport.

1.5.1.2. Current Knowledge of Axonal Transport

Microtubules are dynamic components of the cytoskeleton, comprised of α and β tubulin dimers which impart them with polarity (Heidemann et al., 1981; Mitchison and Kirschner, 1988; Li et al., 2002). Specialized ATPase motors called motor proteins covert chemical energy of ATP hydrolysis to mechanical work and translocate along microtubule tracks. These molecular motors bind intracellular cargo and move them anterogradely from the neuronal cell

body towards the synapse. A similar mechanical arrangement moves material in the retrograde direction from the synapse towards the cell body.

1.5.1.3. Kinesin Mediated Axonal Transport

Members of the kinesin superfamily mediate axonal transport in the anterograde direction. To date, 14 members have been identified with 40% amino acid homology within the kinesin motor domain (Miki et al., 2005). Conventional kinesin was the first member to be identified in a biochemical fractionation of squid and mammalian nervous tissue to identify proteins that generate microtubule based motility (Vale et al., 1985a). In 2004, a standardized kinesin nomenclature was adopted to unify proposed nomenclature from different experimental models (Lawrence et al., 2004). Kinesin 1 (previously known as KIF5), kinesin 2 (heterotrimeric kinesin) and kinesin 3 (unc104/KIF1) are the major motor proteins implicated in plus-end transport of varied intracellular cargos such as organelles, synaptic vesicle precursors, mRNA, and cytoskeletal elements (Yonekawa et al., 1998; Brendza et al., 2000; Prahlad et al., 2000; Takeda et al., 2000; Baqri et al., 2006; Pilling et al., 2006).

Kinesins are highly processive motors and can translocate at average speeds of 0.6 – 0.8 μm/ sec *in vitro* (Gennerich and Vale, 2009). This ability of a single motor to maintain interaction with the microtubule to continue translocation for a while before stalling, is due to a synchronized hand over hand step mechanism wherein the two motor domains alternate their catalytic cycles of ATP hydrolysis such that at least one motor domain is in physical interaction with the microtubule. The step size of each cycle is 8 nm (Valentine and Gilbert, 2007).

1.5.1.4. Dynein Mediated Axonal Transport

Cytoplasmic dynein is the motor responsible for mediating retrograde axonal transport (axonemal dynein is involved in bending of cilia and flagella). It is a large protein complex composed of two identical heavy chains and several associated chains (Vale, 2003). Like kinesin, dynein uses a similar hand over hand step mechanism; however its stepping behavior is more irregular (Reck-Peterson et al., 2006) and responds differentially to the extent of the load. In response to heavier load, dynein takes smaller but more powerful steps by regulating ATP binding at secondary sites in the motor head (Mallik et al., 2004). Further, recent *in vitro* evidence has indicated that dynein can translocate bidirectionally under induced force conditions (Ross et al., 2006; Gennerich et al., 2007). In general, less is understood about dynein's processivity and directionality than kinesin. Deciphering the atomic structure of dynein is likely to be the key to understanding the mechanism of dynein-mediated transport.

A multisubunit protein called dynactin associates with cytoplasmic dynein and this interaction is critical for retrograde transport. Disruption of the dynactin complex by targeting the dynamitin subunit nearly abolished dynein mediated transport (Burkhardt et al., 1997). It has recently emerged that dynactin enhances the processivity of dynein rather than influence the attachment to cargo (King and Schroer, 2000; Haghnia et al., 2007).

1.5.2. Mitochondrial Axonal Transport

As noted earlier, since 1914 mitochondria were known to change positions in the cell (Lewis and Lewis, 1914). Video enhanced microscopy revealed that mitochondria can be transported in both directions (Cooper and Smith, 1974). In 1986, Forman observed that the same mitochondrion could reverse directionality at times during transport (Dahlstrom, 2010). It is now established that mitochondrial axonal transport is vital. Given that several critical cellular

processes including axonal transport are dependent on ATP and that mitochondria are the major source of cellular ATP, proper distribution and positioning of mitochondria attains great importance. In accordance, mitochondrial enrichment has been observed at the synapse, active growth cones, nodes of Ranvier, myelination boundaries and sites of axonal protein synthesis (Boldogh and Pon, 2007).

Long distance transport of mitochondria displays a saltatory profile with frequent reversals in direction, at instantaneous velocities of 0.3 – 1.5 μm/ min. They are able to dock and remain stationary for prolonged periods of time, and later undock and resume transport either in the original direction or the reverse (Hollenbeck and Saxton, 2005). Careful statistical analysis of mitochondrial docking events suggest that they are uniformly distributed throughout the axon with moving mitochondria docking within gaps between other stationary mitochondria (Miller and Sheetz, 2004). Moreover, mitochondria are capable of undergoing fusion and fission at any point during their journey. This complex transport behavior of mitochondria indicates more sophisticated machinery at work as compared to vesicles, other organelles and cytoskeletal elements.

1.5.2.1. Motor Machinery for Mitochondrial Axonal Transport

Anterograde axonal transport of mitochondria is driven by kinesin 1, while dynein mediates their retrograde transport (Pilling et al., 2006). Inhibition of kinesin 1 leads to abnormal perinuclear clustering of mitochondria in non-neuronal cells (Tanaka et al., 1998). Importantly, mutations in the kinesin heavy chain in *Drosophila* lead to a near complete cessation of axonal transport in the segmental nerves (Pilling et al., 2006). There is some evidence to suggest that kinesin 3 may also have a role to play in long distance mitochondrial transport: purified kinesin 3

was able to translocate mitochondria *in vitro* (Nangaku et al., 1994). However, there is no evidence of kinesin 3 being involved in mitochondrial transport *in vivo* (Barkus et al., 2008). Thus, further conclusive studies are warranted.

In contrast, dynein is the only known motor to be involved in retrograde axonal transport of mitochondria. A genetic screen for proteins involved in transport identified the *roadblock* mutant in *Drosophila* that has a mutation in the dynein light chain and exhibits severe disruption of retrograde transport (Bowman et al., 1999). Similarly, mutation in the dynein heavy chain disrupts retrograde axonal transport of mitochondria in *Drosophila* segmental nerves (Pilling et al., 2006). More recently, it was shown that the dynein light chain LC8 regulates mitochondrial transport in mice (Chen et al., 2009), suggesting that the role of cytoplasmic dynein in mediating retrograde mitochondrial transport is conserved in higher animals. Interestingly, inhibition of kinesin 1 in *Drosophila* motor neurons also results in disruption of dynein mediated retrograde transport of mitochondria, indicating a functional link and inter-dependence between kinesin and dynein motor activity (Pilling et al., 2006).

1.5.2.2. Regulation of Mitochondrial Transport

The complex mitochondrial transport behaviors discussed above point to a refined regulation machinery. Indeed, we are now starting to discover some of these regulatory mechanisms. Staining of mitochondria with the potential sensitive dye JC1 reveals that there is a high correlation between the direction of transport and mitochondrial membrane potential. Mitochondria with a high membrane potential predominantly move in the anterograde direction while mitochondria with low membrane potential tend to move retrogradely (Miller and Sheetz, 2004). Recently, another group observed a similar bias in mitochondrial directionality with JC1,

however they did not see a correlation between membrane potential and transport direction with a different dye TMRM (Verburg and Hollenbeck, 2008).

There are several adaptor proteins that help mediate specific aspects of kinesin mediated mitochondrial transport. Syntabulin, a syntaxin binding protein that links syntaxin containing vesicles to the kinesin heavy chain, also associates and co-translocates with mitochondria *in vivo*. Knockdown of syntabulin results in impaired mitochondrial distribution in distal neuronal processes (Cai et al., 2005). Recently, a role for syntaphilin, a neuron-specific protein, has been suggested in mitochondrial docking. Mutant syntaphilin dramatically increases the number of motile mitochondria and alters the ratio of motile versus stationary mitochondria in mice neurons, suggesting its role in mitochondrial docking (Kang et al., 2008).

The best-studied mitochondrial adaptor proteins Milton and Miro were identified in independent genetic screens in *Drosophila* to identify novel regulators of axonal transport and synaptic function. Milton is a mitochondria-associated protein required for kinesin-mediated transport. Loss of Milton results in complete cessation of anterograde mitochondrial transport and disruption of synaptic function (Stowers et al., 2002). Milton exclusively binds to the heavy chain of kinesin 1. In fact, deletion of the sole light chain of kinesin 1 in *Drosophila* does not hinder mitochondrial transport (Glater et al., 2006). The mammalian orthologues of Milton are TRAK1 and TRAK2, which are also important for mitochondrial transport but have evolved to be involved in transport of some vesicular cargo as well (Brickley et al., 2005). These studies strongly indicate that Milton acts as an adaptor that links the heavy chain of kinesin 1 to the mitochondrial surface to facilitate anterograde axonal transport.

Mitochondrial Rho-GTPase protein or Miro is another critical regulator of anterograde mitochondrial transport. Loss of Miro results in severe reduction in the number of motile

mitochondria in the axon (Guo et al., 2005). It is an integral mitochondrial outer-membrane protein and contains two calcium binding EF hand domains (Fransson et al., 2006). The EF hands on Miro are thought to be the calcium sensors that inhibit mitochondrial motility and allow mitochondria to dock at regions of high calcium in order to buffer excess intracellular calcium. Two mechanisms have been proposed for how Miro does this. The first model proposes that calcium binding to the EF hands inhibits direct interaction between Miro and kinesin resulting in the uncoupling of mitochondria from the motor machinery (Macaskill et al., 2009). The second model proposes that Miro binds to Milton, and Milton binds to kinesin. High calcium permits Miro to bind directly to the motor domain of kinesin, thereby uncoupling the complex from the transport machinery. Thus, kinesin switches from an active state where it is bound to Miro via Milton, to an inactive state where it binds Miro directly in the presence of high calcium (Wang and Schwarz, 2009). Additional studies are needed to resolve the mechanism of transport regulation by Miro.

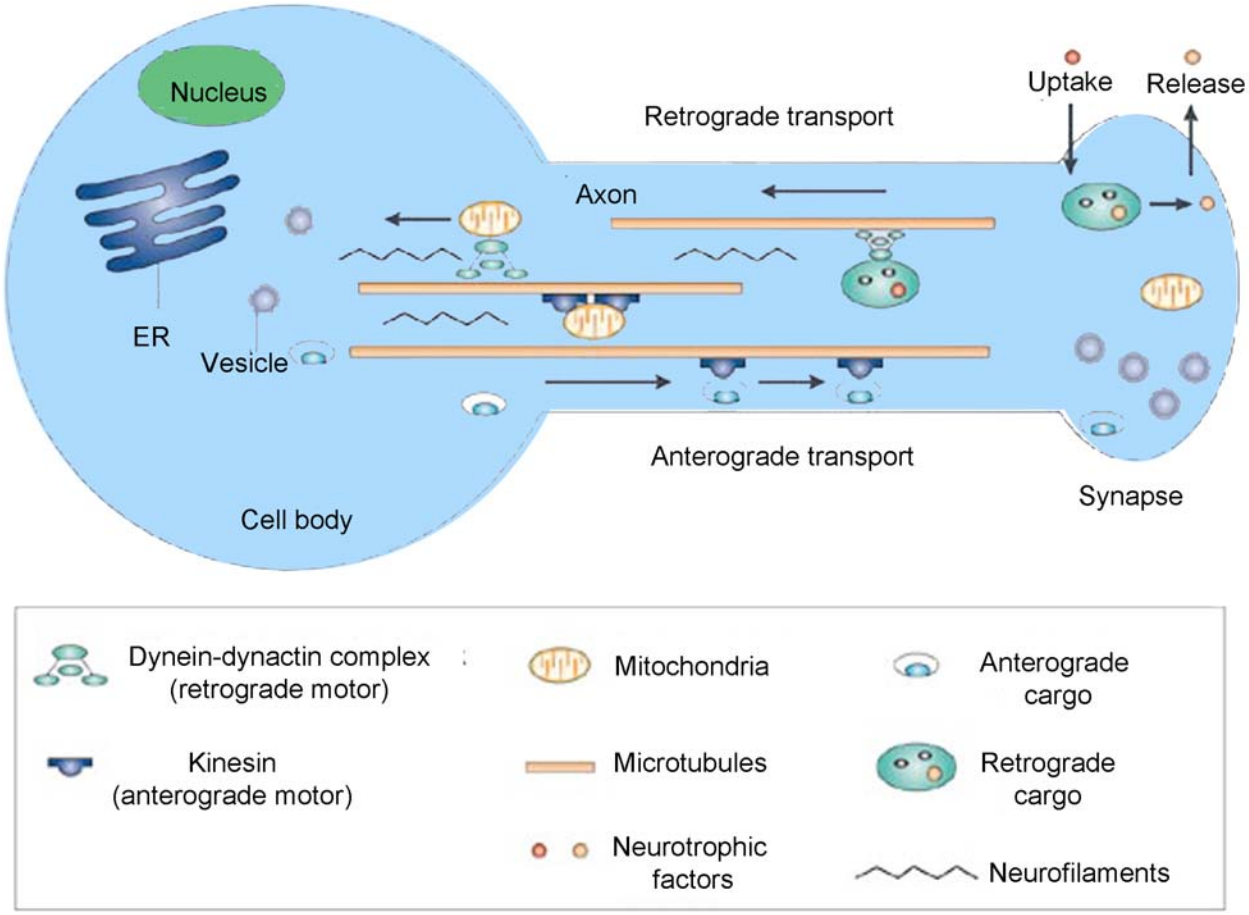

Figure 1.2 Axonal transport of mitochondria. Kinesin 1 and the dynein-dynactin complex mediate mitochondrial transport over microtubule tracks. Other axonal cargoes are transported in a similar energy dependent manner. Modified from (Pasinelli and Brown, 2006)

1.6. Mitophagy/ Mitochondrial Autophagy

1.6.1. Historical review of Autophagy

Cells have an intrinsic quality control mechanism that can deal with dysfunctional organelles and aggregated proteins. In the late 1950s, Clark and Novikoff independently noted the presence of mitochondria within membrane bound compartments (Clark, 1957; Novikoff, 1959). These compartments, or dense bodies as they were called, were found to contain lysosomal enzymes (Novikoff and Essner, 1962). In 1963, at a conference on lysosomes, de Duve proposed the term 'autophagy', deriving from the Greek *auto* (self) and *phagy* (eat) (Yang and Klionsky, 2010). Subsequent studies demonstrated that glucagon induces autophagy and that insulin inhibits it, indicating a role of nutrient deprivation in this process (Deter et al., 1967; Pfeifer, 1978). These results are the basis for our current understanding of autophagy as a catabolic process that works as backup storage system for nutrients. These landmark discoveries also established that mitochondria undergo autophagy.

1.6.2. Current Knowledge of Mitophagy/ Mitochondrial Autophagy

Autophagy involves engulfment of the target by a double membrane structure known as the autophagosome (Nakatogawa et al., 2009). Thereby, defective cytosolic components such as protein aggregates are sequestered. This autophagosome then fuses with the lysosome and cargo is degraded, providing the option of reusing the nutrients under conditions of starvation (Klionsky and Emr, 2000). Second targets of autophagy are dysfunction organelles that could be potentially toxic and their removal may be undertaken independent of nutrient availability. Over 30 different autophagy-related (Atg) proteins are known to regulate autophagy (Yang and Klionsky, 2010).

Loss of membrane potential due to the mitochondrial permeability transition (MPT) can lead to selective autophagy of mitochondria (Lemasters et al., 1998). This selective degradation of mitochondria is termed 'mitophagy' (Kim et al., 2007). CsA and NIM811, blockers of the MPT, suppress mitochondrial depolarization and subsequent proliferation of auophagosomes (Elmore et al., 2001; Rodriguez-Enriquez et al., 2006). Mitophagy is known to be a relatively fast process, occurring within 5 mins and is mediated by the yeast protein Atg8 (mammalian homologue is LC3). Atg8/ LC3 is an ubiquitin-like protein that integrates with the isolation membrane of the autophagosome and helps recruit the target mitochondrion (Nakatogawa et al., 2007; Noda et al., 2010).

Mitochondrial fission occurs prior to mitophagy, presumably to divide mitochondrial into manageable sizes for the autophagsome to encapsulate them. Loss of the fission protein Mdm38 decreases mitophagy (Nowikovsky et al., 2007). Further, selective fusion of damaged mitochondria was also observed important for mitophagy to proceed (Twig et al., 2008). These results indicate the mitochondrial dynamics have an intricate relationship with mitophagy. Interestingly, it is been observed that just a portion of a mitochondrion can also be sequestered for mitophagy (Kim et al., 2007), suggesting a more complicated interplay between the fission and mitophagy pathways than is currently understood. Mitophagy has also been observed when energetic requirements are altered. For example, uth1 – an outer mitochondrial membrane protein in yeast, mediates the elimination of excess mitochondria when energetic requirements are low (Camougrand et al., 2004).

Interestingly, recent evidence has implicated the Parkinson's disease proteins, parkin and PINK1, in mitophagy of mitochondria that have undergone permeability transition. Mitochondrial permeability transition causes a reversal of ATP synthase operation, resulting in

depolarization of mitochondrial membrane potential (Forte and Bernardi, 2005). Parkin is normally a cytosolic E3 ubiquitin ligase and is recruited selectively to mitochondria that are damaged (Narendra et al., 2008). This translocation of parkin is mediated by the activity of PINK1 (Narendra et al., 2010; Vives-Bauza et al., 2010). Accordingly, overexpression of parkin in cybrid osteosarcoma cells promotes selective mitophagy of dysfunctional mitochondria with mutations in cytochrome oxidase subunit I, thus enriching the population of wild-type mitochondria and rescuing cellular function (Suen et al., 2010). Further, induced expression of PINK1 leads to an upregulation of parkin translocation to the mitochondria and promotes mitophagy (Narendra et al., 2010).

1.7. Mitochondrial Life Cycle

As evident from the discussion so far, mitochondria are dynamic organelles that have important functional and regulatory roles to play: their turnover is regulated as per the cellular metabolic requirements, they undergo fusion and fission, they are transported to sites of utilization, and they are cleared by selective autophagy when damaged. These distinct aspects of mitochondrial biology are tightly regulated and finely coordinated to preserve cellular integrity.

Work done by several groups, including the Miller laboratory, has guided us in proposing a model for the mitochondrial life cycle (Figure 1.3). While this model attempts to integrate our current knowledge of mitochondrial biology in neurons, it may very well explain the life cycle of mitochondria in all cell types with a few minor adaptations.

A) The first step in the mitochondrial life cycle is the birth of a new mitochondrion. Mitochondrial biogenesis takes place by replication of mitochondrial DNA followed by

fission of existing mitochondria to divide some protein content and mitochondrial DNA into the two new mitochondria. Proteins like Drp1 and Fis1 are important mediators of this process (Baloh, 2008). Majority of the mitochondrial proteins are encoded by the nuclear genome and are imported into the mitochondria through the translocases of the outer and inner membrane. The import machinery is assisted by molecular chaperones such as mtHsp70 and Hsp60, which also help in folding the proteins in the mitochondrial matrix (Wiedemann et al., 2004). Mitochondrial biogenesis predominantly takes place in the neuronal cell body (Davis and Clayton, 1996; Magnusson et al., 2003). Although, there is some evidence that like protein translation, a portion of biogenesis can also occur out in the axon (Amiri and Hollenbeck, 2008). Members of the PGC-1 family of regulated coactivators, especially PGC-1α, play an important role in regulating mitochondrial biogenesis through modulation by the energy sensors such as AMPK and SIRT1 (Scarpulla, 2011)

B) These newly synthesized mitochondria are 'healthy' as they have high membrane potential and functional oxidative phosphorylation machinery (Martinez-Diez et al., 2006; Huttemann et al., 2008). They are transported out into the axon by kinesin 1 along microtubules to regions of high energy demand (Miller and Sheetz, 2004). Milton and Miro participate in this trafficking by serving as adaptors to link kinesin 1 with the mitochondrion (Chen and Chan, 2009; MacAskill and Kittler, 2010)

C) These healthy mitochondria distribute uniformly in the axon and dock at regions of unmet energy demand (Miller and Sheetz, 2004). High concentration of local calcium is indicative of lower mitochondrial density and is a putative signal for mitochondria to halt. Miro binds calcium through its EF hand domains and uncouples mitochondria from

kinesin either by allowing Miro to circumvent Milton and bind the kinesin motor domain, or by directly uncoupling Miro from kinesin (Macaskill et al., 2009; Wang and Schwarz, 2009). Proteins like syntaphilin facilitate the docking of these stationary mitochondria (Kang et al., 2008).

D) Docked mitochondria perform their cellular functions of energy generation and calcium buffering (Nicholls and Budd, 2000; Kiryu-Seo et al., 2010).

E) Healthy mitochondria may encounter damaged mitochondria in the axon and undergo fusion with them to help 'repair and restore' functionality by exchange of proteins and mitochondrial DNA. Healthy mitochondria may also fuse with other healthy mitochondria to ensure enhanced functionality to deal with demanding conditions (Chen and Chan, 2005). Proteins such as MFN1, MFN2 and OPA1 mediate these events (Chen and Chan, 2009).

F) After a certain point, under the influence of stressors such as increased reactive oxygen species, metabolic toxins or calcium overload, these mitochondria undergo the mitochondrial permeability transition and lose their membrane potential, thereby losing their functionality as well (Leung and Halestrap, 2008).

G) PINK1 begins to accumulate in these mitochondria with low potential, and leads to the selective recruitment of parkin (Vives-Bauza et al., 2010). Parkin serves as a flag for the autophagosome machinery and these damaged mitochondria are encapsulated by a membrane and targeted for mitophagy (Youle and Narendra, 2011).

H) Lysosomal degradation may take place in the axon (Wang et al., 2006). Alternatively, the autophagosomes may be cleared from the axon and sent back to the neuronal cell body

for completion of degradation (Miller and Sheetz, 2004). The dynein-dynactin complex mediates this retrograde transport.

I) Mitochondria that have not undergone excessive damage and may be salvaged are also sent back *towards* the cell body where the probability of finding healthy mitochondria is higher. Damaged mitochondria ‘repair’ themselves by undergoing fusion with the healthy ones (Miller and Sheetz, 2004; Frederick and Shaw, 2007).

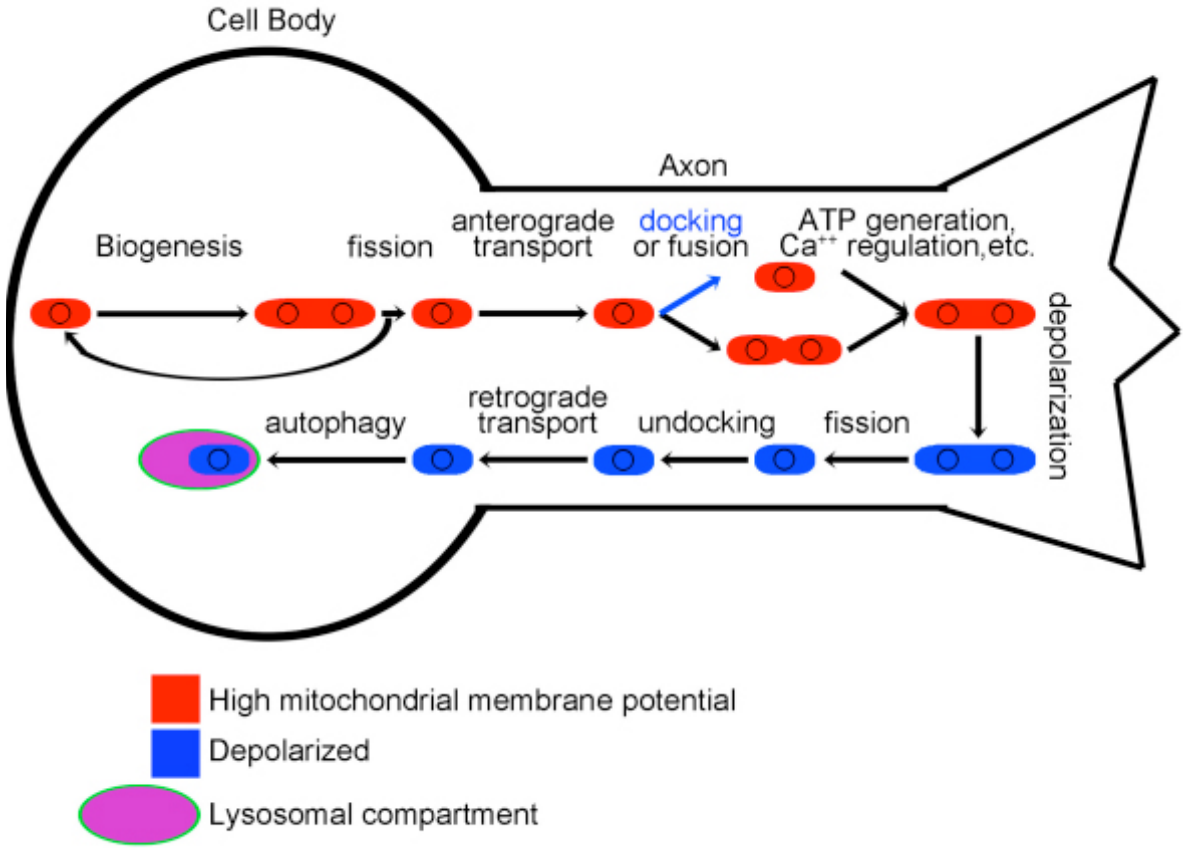

Figure 1.3 The life cycle of mitochondria in neurons. Mitochondrial DNA replication and biogenesis largely occurs in the cell body. Healthy mitochondria with high membrane potential are predominantly transported in the anterograde direction, and they uniformly distribute themselves at regions of unmet energy demands. Following damage, low membrane potential mitochondria are transported retrogradely towards the cell body to ensure clearance. Damaged mitochondria may also undergo fusion with healthy mitochondria for repair purposes.

1.8. Mitochondria in Disease

The central role of mitochondria in multiple cellular processes means that mitochondrial dysfunction is heavily implicated in diseases. These diseases may involve mitochondrial DNA mutations, mitochondrial dysfunction, and/ or disruption of mitochondrial dynamics. Although, most mitochondrial disorders considerably overlap in terms of cause and effect, the clinical symptoms presented are often wide-ranging and distinct. In fact, the nature of diseases involving mitochondrial dysfunction varies from metabolic disorders to neurodegeneration. Interestingly, most mitochondrial diseases have severe neural pathology, reinforcing the importance of mitochondrial function in neurons. For the sake of simplicity, I have classified different diseases based on which aspect of mitochondrial biology is the focal point.

1.8.1. Disorders of Mitochondrial DNA

Mutations, deletions and depletion of mtDNA result in severe disease pathology. Surprisingly however, most mutations result in very distinct syndromes with little overlap in clinical symptoms. This may in part be explained by the fact that different regions present accumulation of mutations in some of the different syndromes. For example, mutations of Kearns Sayre Syndrome (KSS) are plentiful in the choroid plexus (Tanji et al., 2000), Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-live events (MELAS) mutations abound in the walls of cerebral arterioles (Betts et al., 2006), and the olivary nucleus of the cerebellum is rich with the Myoclonus Epilepsy Ragged-red Fibers (MERF) mutation (Tanji et al., 2001). Why these mutations are enriched in specific regions remains elusive, but their occurrence likely influences the clinical manifestations.

1.8.1.1. Diseases arising out of mutations

Mutations in any one of the 13 essential components of the oxidative phosphorylation machinery result in respiratory chain defects (Wallace, 1992). Multiple mutations in mtDNA are known to cause Leigh syndrome, a debilitating neurodegenerative disorder of infancy that results from dysfunctional oxidative phosphorylation (Kirby et al., 2003; Sarzi et al., 2007). Several identified mutations in mtDNA encoded complex I genes cause Leber Hereditary Optic Neuropathy (LHON). Interestingly, while most LHON mutations are homoplasmic (in all cells), pathology is observed only in the retinal ganglion cells, resulting in blindness in young adults (Carelli et al., 2007). LHON is maternally inherited and men are more frequently afflicted, indicating an X-linked modifier effect (Hudson et al., 2005).

Interestingly, several of the diseases caused by mtDNA mutations are also caused by mutations in nuclear DNA (nDNA) encoded genes of the oxidative phosphorylation machinery. For example, mutations in nDNA encoded complex I or II genes and genes involved in assembly of the complexes such as SURF1 and NDUFA12L, also manifest in symptoms of Leigh syndrome (DiMauro and Schon, 2008). These results suggest a complex interplay between nuclear DNA and mtDNA-encoded genes to modulate unique pathophysiologies (Carelli et al., 2007).

1.8.1.2. Diseases arising out of deletions

Certain clinical disorders are caused by multiple deletions in mitochondrial genes; for example, Progressive External Opthalmoplegia (PEO). Patients suffering from PEO present themselves with ptosis, ataxia and sensorimotor neuropathy. While specific mutations are also associated with PEO, multiple deletions in some genes essential for the maintenance and

replication of mtDNA are the major clinical cause. Examples of these genes include *PEO1* – encoding Twinkle, and *POLG* – encoding the catalytic subunit of pol γ (Spinazzola and Zeviani, 2009).

Disorders linked to mutations in *POLG* can have either autosomal recessive or autosomal dominant inheritance. Autosomal recessive inheritance is observed in Alpers syndrome, a drastic mtDNA depletion disorder that manifests in hepatocerebral disease and mortality in early childhood (Naviaux and Nguyen, 2004). Interestingly, polymorphisms and deletions in *POLG* have recently been implicated in Parkinsonism (Davidzon et al., 2006). Mitochondrial Neurogastrointestinal Encephalopathy (MNGIE) is caused by mutations in *thymidine phosphorylase*, which leads to impairment of mtDNA stability and results in multiple deletions and point mutations in mtDNA (Hirano et al., 2005).

1.8.1.3. Diseases arising out of depletion

As described earlier, mutations in *POLG* also result in depletion of mtDNA, and Alpers syndrome is an example of such a severe occurrence. Mutations in *DGUOK* that encodes the enzyme deoxyguanosine kinase, *TK2* that encodes the mitochondrial thymidine kinase, and *SUCLA2* that encodes a subunit of succinyl-CoA synthetase, all lead to dramatic depletion of mtDNA and result in encephalopathy (Elpeleg et al., 2005; DiMauro and Schon, 2008). While mutations in the 13 polypeptide genes of mtDNA are obviously deleterious, there are several instances of disease pathology because of mutations in tRNAs encoded by mtDNA. The T14709C mutation in $tRNA^{Glu}$ results in diabetes, and mutations in $tRNA^{Lys}$ manifest in multiple lipomas (DiMauro and Schon, 2008).

1.8.2. Disorders of Mitochondrial Function

Mitochondria are central regulators of several cellular processes. The critical nature of mitochondrial functions also means the result of any perturbation or dysfunction is severe. The most obvious examples are diseases that afflict the respiratory chain and energy generation capacity of mitochondria. Majority of these diseases have been covered in the section on mtDNA disorders. Among other prominent diseases of the respiratory chain is Friedreich ataxia, caused by abnormal frataxin. Loss of fraaxin results in impaired mitochondrial iron storage and defects in complexes I, II and III (Chantrel-Groussard et al., 2001). Also, mutation in *SPG7,* the gene encoding paraplegin, may result in accumulation of defective respiratory chain subunits that physically impairs the import of nuclear encoded proteins (Claypool et al., 2006). This condition is referred to as Hereditary spastic paraplegia. Recently, altered mitochondrial function has emerged as an important regulator of diabetes-related pathology (Friederich et al., 2009).

Altered mitochondrial function has been widely observed in cancer cells. Disrupted respiratory chain activity, decreased oxidation of NADH-linked substrates, and excessive mtDNA mutations are characteristic features of cancer (Carew and Huang, 2002). Moreover, the central role of mitochondria in mediating apoptosis places them at the center of cancer treatment strategies. More recently, the aneuploidy stress response and the unfolded stress response have focused attention on the role of mitochondria in regulating cell division of cancer cells and their subsequent survival (Williams and Amon, 2009; Siegelin et al., 2011).

Hyperphosporylated tau tangles are a hallmark feature of Alzheimer's disease (AD) pathology. Rotenone, which blocks the transfer of electrons from complex I to ubuiquinone causes acute accumulation of hyperphosphorylated tau in neurons (Hoglinger et al., 2005). So does complex I inhibitor annonacin, indicating mitochondrial dysfunction has an important role

to play in AD pathology (Escobar-Khondiker et al., 2007). Further, reduced α-ketoglutarate dehydrogenase complex activity in the brain is strongly associated with decreased energy metabolism in AD (Gibson et al., 1988).

Mitochondrial dysfunction has also been associated with psychiatric disorders. Several MELAS and PEO patients present themselves with clinical depression and other psychiatric problems (DiMauro and Schon, 2008). An important role for mitochondria has also been proposed in impaired calcium homeostasis and repressed energy metabolism in Bipolar disorder (Kato et al., 2003; Konradi et al., 2004). A role for *DISC1*, disruption of which is implicated in schizophrenia, is suggested in regulating mitochondrial dynamics (Shao et al., 2008).

While huntingtin, the protein impaired in Huntington's disease (HD), is not a mitochondrial protein, recent evidence has strongly associated with mitochondrial dysfunction with the pathology of HD. Biochemical analysis reveals that activities of complexes II and III are significantly reduced in postmortem HD brains. Further, polyglutamine accumulations disrupt the calcium buffering ability of mitochondria leading to induction of the permeability transition (Choo et al., 2004; Seong et al., 2005). Interestingly, mutant huntingtin has now been seen to inhibit PGC1-α activity and thus, impair mitochondrial biogenesis (Greenamyre, 2007). Similarly, impaired SOD1 activity is implicated in Amyotrophic lateral sclerosis. SOD1 is responsible for dealing with the stress of reactive oxygen species, a byproduct of oxidative phosphorylation. SOD1 mutant mice show decreased respiratory chain activity and calcium homeostasis (Liu et al., 2004). The role of mitochondria in these diseases will be further discussed in the next section.

1.8.3. Disorders of Mitochondrial Dynamics

Mitochondria are dynamic organelles that undergo fusion, fission, and long-range transport. Impairment of the dynamic behavior of mitochondria has severe disease ramifications, especially in the morphologically unique and energetically demanding neurons.

1.8.3.1. Diseases arising out of impaired fusion

Mutations in the fusion protein OPA1 are the most common cause of heritable optic neuropathy, Autosomal Dominant Optic Atrophy, which involves the degeneration of retinal ganglion cells (Delettre et al., 2000; Fuhrmann et al., 2010). The exact nature of OPA1 mediated pathology is still unclear, but presumably occurs because of the critical role of mitochondrial fusion in maintaining functionality. Notably, some patients with *OPA1* mutations also have higher levels of mtDNA deletions (Amati-Bonneau et al., 2008), emphasizing the role of fusion in maintaining mtDNA integrity.

Another important disease caused by defect in mitochondrial fusion is Charcot-Marie-Tooth type 2A (CMT2A). CMT2A afflicted patients exhibit distal motor and sensory impairment as a consequence of degenerating peripheral nerves. Nearly 40 mutations in human *MFN2* have been associated with CMT2A (Zuchner et al., 2006). Ultrastructural analysis of mitochondria in diseased nerves reveals abnormal inner and outer membrane morphology and swelling, indicative of mitochondrial dysfunction (Verhoeven et al., 2006). It is interesting that impaired OPA1 and MFN2 are both responsible for defective fusion; yet cause two very distinct forms of neuropathy. The most parsimonious explanation would be to point out that OPA1 is an inner membrane fusion protein while MFN2 is an outer membrane fusion protein. A comprehensive answer to this conundrum remains elusive at this time.

1.8.3.2. Diseases arising out of impaired fission

A recessive form of CMT disease is CMT4A, and is caused by defect in ganlioside-induced differentiation association protein 1(GDAP1). GDAP1 is an integral outer membrane protein that influences mitochondrial fission and therefore, regulates mitochondria function (Niemann et al., 2005; Niemann et al., 2009). Although the role of Drp1 has not been directly established in any heritable disease, one case of neonatal lethality has been ascribed to it (Waterham et al., 2007). Unless the severity of defective mitochondrial fission proteins is such that even embryonic survival is not possible, more disease cases attributed to it should emerge with improving diagnostic assays.

However, there is considerable evidence for the involvement of impaired fission in several disorders, mostly neuronal in nature. PINK1 and parkin, proteins implicated in PD, actively regulate mitochondrial fission and fragmentation, characteristic features in animal models of PD (Exner et al., 2007; Yang et al., 2008). Aβ peptides have been seen to induce mitochondrial fragmentation, implicating deregulation of fission in AD pathology (Barsoum et al., 2006). Similarly, high glucose also leads to fragmented mitochondria. This evidence becomes especially relevant when taken together with the fact that diabetic rats exhibit increased Drp1 expression (Leinninger et al., 2006).

1.8.3.3. Diseases arising out of motility

Mitochondria need to be distributed appropriately to all parts of the cell. The long axonal processes of neurons are especially susceptible to impaired mitochondrial transport. However, other essential cargos such as dense core vesicles, ER etc, are also transported by similar machinery as mitochondria, so care should be taken not to classify global transport impairments

into mitochondrial disorders. Inevitably, in all animal models, complete blockage of axonal transport results in lethality.

Mutant MFN2 significantly impairs mitochondrial transport in axons, suggesting abnormal mitochondrial trafficking is an important contributor to CMT2 disease associated neuropathy (Baloh et al., 2007). Further, N-terminal of mutant huntingtin associates with mitochondria and disrupts mitochondrial transport (Orr et al., 2008). This results in impaired mitochondrial distribution and contributes to HD pathology. Also, in a mouse presenilin 1 mutant model of AD, axonal transport is impaired (Pigino et al., 2003). There is some evidence that presenilin 1 may localize to the mitochondrial inner membrane, thus directly implicating impaired mitochondrial mobility in AD (Ankarcrona and Hultenby, 2002). More recently, impaired mitochondrial transport has been observed in a *Drosophila* model of Friedrich Ataxia (Shidara and Hollenbeck, 2010). Finally, disruption of mitochondrial axonal transport has been reported in mutant SOD1 animals, suggesting an important role of mitochondrial distribution in the neuropathology of ALS (De Vos et al., 2007).

It is important to consider that mitochondria are the major source of ATP, and cytoskeleton based transport is mediated by ATPase motors. Hence, any significant disruption of mitochondrial function, including but not limited to mtDNA abnormalities and misregulation of the fusion-fission machinery, could result in widespread disruption of transport and directly or indirectly contribute to disease pathology. Thus, mitochondrial dynamics should be treated as a multifactorial yet interdependent behavior and the contribution of mitochondria in diseases should be assessed accordingly.

Part 2: Aging

1.9. Biology of Aging

Aging is a complex process that involves a progressive decline in physiological, cognitive and reproductive function over time, ultimately resulting in mortality (Narasimhan et al., 2009). Aging is deeply connected to the onset of several diseases such as type 2 diabetes, arthritis, cancer and neurodegenerative disorders. Until recently, aging was thought of as an inevitable functional decline that manifests towards the end of an organism's reproductive phase. However, it is now appreciated that the lifespan of an organism is determined by a combination of genetic and environmental factors. Indeed, over the last three decades, research from model organisms such as yeast, worms and flies has identified hundreds of genes that regulate longevity (Narasimhan et al., 2009). These genes primarily fall into pathways that regulate energy metabolism, nutrient sensing and stress resistance. Remarkably, polymorphisms in a number of these genes have now also been correlated with human longevity (Bonafe et al., 2003; Suh et al., 2008). These genes encode proteins that regulate metabolic, protein homeostasis and stress response pathways (Kenyon, 2005). Additionally, extrinsic factors such as a reduction in food intake, termed as dietary restriction, can significantly delay age-associated decline across phylogeny. Thus, our perception of the aging process today is that of one with extraordinary plasticity (Kenyon, 2005; Narasimhan et al., 2009). Importantly, with improvements in healthcare and sanitation, humans are now living longer than ever before. As a consequence several countries around the world are faced with a growing population that is above the age of 60. Therefore, a better understanding of the molecular mechanisms that modulate lifespan have not only biological but also immense economic implications.

1.9.1. Mitochondria in Aging

Mitochondria, as central regulators of multiple cellular processes, have been tightly linked to the aging process (Sohal and Weindruch, 1996; Singh, 2004; Lesnefsky and Hoppel, 2006). There are at least three fundamental levels at which mitochondrial function is thought to affect organismal aging: (a) Role of reactive oxygen species, (b) Mitochondrial DNA mutations, and (c) Role of apoptosis. These links are not mutually exclusive and there is considerable crosstalk amongst them (Figure 1.4).

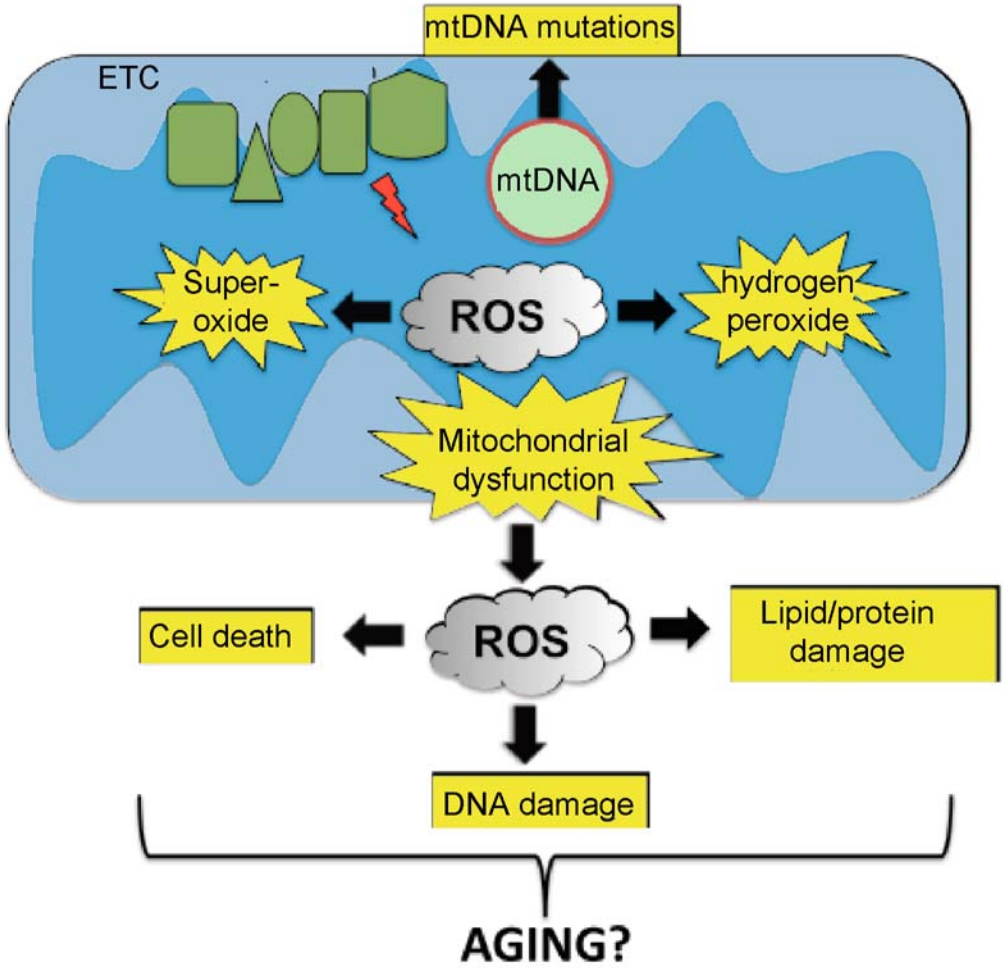

Figure 1.4. Mitochondria are central regulators of aging. Function and dysfunction of the electron transport chain, accumulation of mtDNA mutations, and excessive production of reactive oxygen species are known to regulate longevity.

1.9.2. Mitochondrial ROS and Aging

Several theories have been proposed to explain how and why aging occurs. Among these, the Free Radical Theory of Aging proposed by Harman in 1956 most strongly implicates the role of mitochondria. This theory posits that mitochondria generate high levels of reactive oxygen species (ROS), such as superoxide radicals and hydrogen peroxide as a by-product of oxidative phosphorylation, which damage cellular macromolecules and gradually impair cellular function. According to this theory, accumulation of such damage drives the physiological decline associated with age (Harman, 1956). Indeed, ROS have been shown to target lipid components of organelle and cell membranes, lead to oxidation of amino acids and even induce double stranded breaks in DNA (Bennett, 2001).

Fortunately, the cell has several mechanisms to counter oxidative stress, including the expression of antioxidant enzymes such as superoxide dismutases, catalases as well as glutathione superoxides, which act to offset the harmful effects of the superoxide radicals (Vendelbo and Nair, 2011). Genetic screens from model organisms have identified several mitochondrial and stress-response genes that modulate longevity (Lee et al., 2003). In worms, RNAi or mutation of genes such as *clk-1,* the gene encoding demethoxyubiquinone hydroxylase, or the complex III iron-sulfur protein *isp-1* cause defects in mitochondrial function and a concomitant increase in the resistance to oxidative stress (Hekimi and Guarente, 2003). Independent studies have identified several other mitochondrial genes, including components of the electron transport chain (ETC) that when knocked down by RNAi not only decreased ATP production, but surprisingly also increased lifespan (Dillin et al., 2002; Lee et al., 2003). In *Drosophila,* an RNAi study exploring the effects of knocking down 53 ETC-associated genes identified several genes that extend lifespan (Copeland et al., 2009). Importantly, transcription

factors such as FOXO and Nrf2, that act as master regulators of anti-oxidative and other stress responses in the cell also regulate longevity (Narasimhan et al., 2009). Further, long-lived mutants from both mitochondrial, as well as other pathways such as insulin/IGF-1 signaling show elevated levels of antioxidant enzymes and resistance to oxidative stress (Honda and Honda, 1999).

Accepted at face value, Harman's theory would suggest that enhanced management of mitochondrial ROS should also increase lifespan. Indeed, mice that overexpressed catalase in their mitochondria (mCAT) were shown to have an increased lifespan, delayed age-associated pathologies, reduced mtDNA deletions with age and reduced levels of ROS (Schriner et al., 2005). In the same study, the authors crossed these mCAT mice to mice overexpressing superoxide dismutase 1 and 2 (SOD1 and SOD2) respectively. The resulting double transgenic progeny had an even further extension in lifespan, supporting the idea that antioxidant mechanisms play a role in regulating longevity (Schriner et al., 2005).

However, the data with mutant mice is rather contradictory. While single or double mutant combinations in the SOD1, SOD2, glutathione peroxidase(s), thioredoxin or methionine sulfoxide reductase genes significantly affect oxidative stress, only SOD1 mutants demonstrate a decrease in lifespan (Perez et al., 2009). In *C. elegans*, while the supplementation of superoxide dismutase mimetics can significantly reduce oxidative stress and extend lifespan, mutations in the five superoxide dismutase genes, *sod-1, sod-2, sod-3, sod-4* and *sod-5* show no decrease in lifespan (Van Raamsdonk and Hekimi, 2009; Yen et al., 2009). Finally, in *Drosophila*, overexpression of SOD and catalase together results in an overall increase in lifespan that is not observed with expressing either transgene alone, while in another study overexpression of the

Mn/Cu SOD gene alone was enough to confer lifespan extension (Orr and Sohal, 1994; Sun et al., 2002).

The observed increase in lifespan when ETC function is impaired is especially intriguing. It is known that metabolic rate itself does not correlate directly with lifespan (Yen et al., 2004). Further, it has been shown that mitochondria produce more ROS when operating at low metabolic rates (Aguilaniu et al., 2005). Also, mutations in the gene Mclk1 in mice reduce ubiquinone synthesis, which in turn reduces the flow of electrons during oxidative phosphorylation (Kayser et al., 2004). While animals have an increased lifespan, reduced ATP synthesis and show resistance to oxidative stress; paradoxically, mitochondrial ROS damage is increased (Lapointe and Hekimi, 2008).

More recent studies have indicated that ROS in the cell are not always harmful and instead they may act as important second messengers in different signaling pathways. For example, hydrogen peroxide is now appreciated to be important for regulating cell survival through activation of anti-apoptotic signaling pathways (Groeger et al., 2009). Thus, the correlation between ROS, mitochondria, and longevity has been surprisingly difficult to decipher and remains to be further explored.

1.9.3. Mitochondrial DNA Mutations and Aging

Mitochondria are the only other source of cellular DNA other than the nucleus. Mitochondrial function is sustained by the function of genes encoded by both mtDNA as well as nuclear DNA. Yet, mtDNA is significantly more susceptible to ROS-induced damage, in large measure because of the absence of histones and physical proximity to mitochondrial ROS (Mandavilli et al., 2002). Over time, such damage can lead to defects in mtDNA

replication/repair and subsequently, the accumulation of mtDNA mutations. As a consequence mitochondrial function is impaired, and the cellular energy demands are unmet (Oliveira et al., 2010).

What implications do mtDNA mutations have for the aging process? Studies using mice in whom pol γ–α has a replacement with a proofreading-deficient catalytic core, called the 'mtDNA mutator' mice, have shown that they demonstrate several age-related ailments and also have a reduced lifespan (Trifunovic et al., 2004; Trifunovic, 2006). Curiously, while these mice have reduced electron transport chain function and reduced ATP levels, overall ROS levels are not significantly increased (Khrapko et al., 2006). Further, the load of mtDNA mutations found in these mice is at least an order of magnitude higher than what is commonly found in an aging human population (Kraytsberg et al., 2009). Hence, the physiological relevance of the mutator mice remains controversial.

More recently, a study was undertaken to identify reasons for the observed premature aging in HIV patients that have been successfully treated with anti-retroviral drugs. The researchers found that these patients had abnormally high and progressively accumulating somatic mtDNA mutations. Interestingly, these mutations accumulated not because of increased mutagenesis, but increased turnover of mtDNA which lead to clonal expansion of preexisting mutations (Payne et al., 2011). Finally, as pointed out earlier, fusion and fission are important regulators of mtDNA content and integrity. Thus, certain disorders related to defects in mitochondrial dynamics also exhibit some premature aging phenotype (Kowald and Kirkwood, 2011).

1.9.4. Apoptosis and Aging

Mitochondria are central regulators of apoptosis and deregulation of apoptosis is a hallmark feature of several disorders such as neurodegenerative diseases, metabolic diseases and cancer. The onset of these diseases is often associated with age, thus emphasizing the role of programmed cell death in the aging process.

Apart from the pathological association of apoptosis with aging, several direct lines of evidence are also available. Increased ROS damage in SOD1 RNAi results in the induction of p53, which in turn can lead to apoptosis (Macip et al., 2003). Interestingly, profound induction of apoptosis is also observed in the mtDNA mutator mice and could significantly contribute to the premature aging phenotype (Kujoth et al., 2005). Further, decreased median lifespan is observed in conjunction with increased apoptosis in *Drosophila* larvae overexpressing pol $\gamma-\alpha$ (Martinez-Azorin et al., 2007).

1.10. Lifespan to Healthspan – redefining the focus of aging research

The earliest known work of literature, *Epic of Gilgamesh*, describes a man's quest for immortality and youth. In the *Homeric Hymn to Aphrodite*, Eos asked Zeus to grant Tithonus with immortality, but forgot to ask for eternal health. Since time immemorial, man has been obsessed with immortality and eternal health.

Our understanding of known modulators of aging has largely been defined by using lifespan as readout. Immortality is still distant, but extension of lifespan has been achieved in some model systems. But the question is, whether living long is good enough? Lifespan extension does not necessarily translate into healthy extra years. Importantly, several of the lifespan extension animal models are not healthy through their life. In worms, mitochondrial

mutants such as *isp-1* and *nuo-2* live long, but are slow growing, have a reduced brood size and demonstrate a number of other abnormalities (Rea et al., 2007). In the context of human aging, age-related disability, frailty and onset of disease are major social and economic burden. Thus, it is important to ensure that any possible therapeutic interventions that are proposed do not only extend the lifespan of an animal, but also result in a concomitant increase in healthspan.

Healthspan itself could be defined as the normal span of physiological, reproductive, motor and cognitive abilities of an organism (Figure 1.6). In most animals, healthspan commences at conception, starting with an early period of development progressing towards the reproductive phase. Here, peak vitality in reproductive and other abovementioned abilities is achieved. This is followed by a period of progressive decline in vitality, concluding in morbidity (Larrick and Mendelsohn, 2010). Ideally, any therapeutic intervention of note should be able to maximize the period of vitality where an organism is able to operate at maximum capacity.

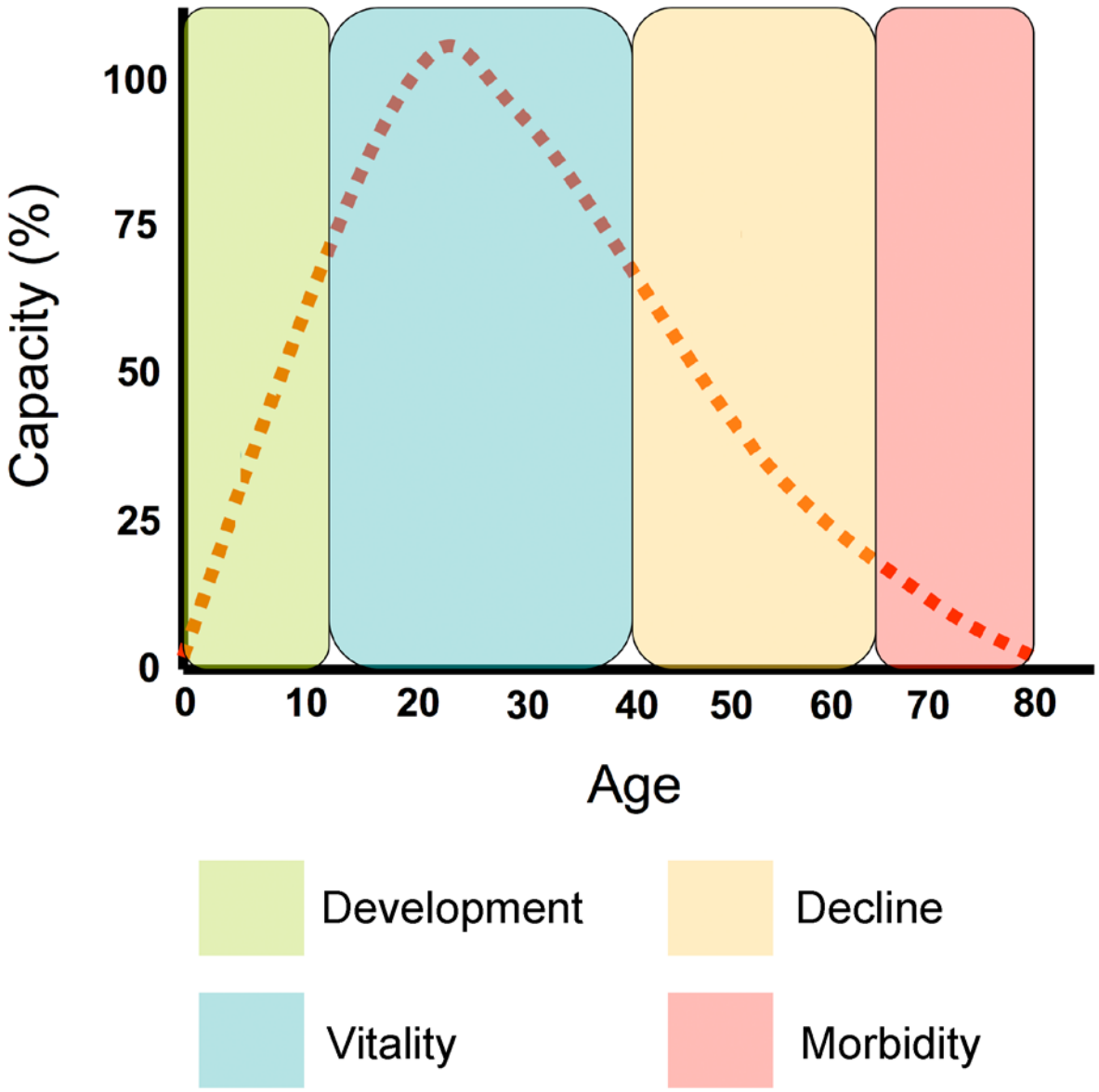

Figure 1.5 Healthspan or healthy lifespan of humans. In almost all animals, there is an initial period of development, followed by a peak in vitality of physiological, cognitive, motor and reproductive capacity. This is followed by a period of steady decline, which ends in morbidity. Ideally, therapeutic and genetic interventions should aim at shifting the curve towards the right, instead of simply aspiring to extend maximal lifespan, which may end up extending morbidity.

1.11. Proteostasis in Aging

The cellular proteome is tightly regulated to ensure that proteins are synthesized, folded, and compartmentalized correctly. In the event of misregulation of these steps or damage to the protein, there are cellular mechanisms to repair or degrade them (Balch et al., 2008). Alterations of protein homeostasis, or proteostasis, are the underlying feature of several age-associated diseases. Accordingly, ROS-linked protein damage and/ or aggregation is considered an important contributor to the aging process (Morimoto, 2008). Two established modulators of proteostasis are molecular chaperones and stress response pathways.

1.11.1. The Role of Chaperones

Molecular chaperones or heat shock proteins (HSP) are rapidly induced in response to elevated stress stimuli such as temperature. In addition, certain other chaperones that are localized in either in the cytosol or in specific cellular compartments are also recruited to deal with the stress (Bukau et al., 2006). Genes encoding molecular chaperones can be placed in three categories: constitutively expressed and regulated in development and growth, constitutive and inducible, and strictly inducible under stress. On the basis of molecular weight, chaperones are broadly classified into five families: HSP100, HSP90, HSP70, HSP60 and small HSPs (Liberek et al., 2008). These may be cytosolic, such as Hsp60, Hsp70 and Hsp90, and be responsible for cellular homeostasis. Or they may be compartment specific, such as ER chaperones, and essential for the normal functioning of the organelle. In either case, if a protein cannot be folded properly, it is eventually targeted for degradation (Liberek et al., 2008).

Changes in chaperone content and induction capacity are observed in several age-associated diseases as well as aging. Decreased transcriptional upregulation of Hsp70 is seen in

response to varied stress stimuli in aged animals (Hall et al., 2000). In addition, long-lived animal models in the insulin/IGF-1 signaling or the dietary restriction pathways were found to have increased Hsp16 expression (Wadhwa et al., 2005). Further, cells from human centenarians also exhibit remarkably preserved chaperone upregulation in response to stress (Marini et al., 2004). Accordingly, increased chaperone induction results in extension of lifespan and protection against stress in several species (Lithgow et al., 1995; Khazaeli et al., 1997; Tatar et al., 1997; Shama et al., 1998). Overexpression of small HSPs has usually been seen to be beneficial in lifespan extension. For example, overexpression of Hsp16 resulted in a marginal lifespan increase of 11% in worms (Walker and Lithgow, 2003), while Hsp27 resulted in a 30% extension in *Drosophila* (Wang et al., 2004). Overexpression of mitochondrial Hsp22 extends lifespan by 30% in *Drosophila* and confers resistance to oxidative stress (Morrow et al., 2004a), while loss of mitochondrial Hsp22 decreases lifespan (Morrow et al., 2004b).

Conversely, overexpression of larger HSPs such as cytosolic Hsp70 and mitochondrial Hsp60 did not increase lifespan in *Drosophila* and yeast (Tatar et al., 1997; Wadhwa et al., 2005), while overexpression of mitochondrial Hsp70 only resulted in a minor increase of 10% in worms (Morrow et al., 2010). Importantly, overexpression of Hsp70 in *Drosophila* cells is deleterious for growth (Feder et al., 1992). These observations suggest that overexpression of larger HSP may not be as beneficial in extending lifespan as smaller HSP probably because their concentration is carefully titrated in the cell and/ or because of their size, overexpression may itself be stressful.

1.11.1.1. The Mitochondrial Chaperone TRAP1

Tumor necrosis factor Receptor Associated Protein 1 (TRAP1) is a member of the HSP90 family and is the mitochondrial analogue of Hsp75 (Felts et al., 2000). Tumor necrosis factor–α (TNF- α) binds two distinct receptors, type I and type II TNF receptors (TNFR1 and TNFR 2). The TNF-α /TNFR1 signaling pathway is involved in several transduction cascades such as JNK, NFkB, and caspases (Baud and Karin, 2001). TRAP1 was originally identified as a binding partner of the intracellular domain of TNFR1 (Song et al., 1995). Interestingly, while TRAP1 retains ATPase activity like Hsp90; it lacks chaperone activity *in vitro* and does not bind the typical HSP90 co-chaperones p23 or HOP (Felts et al., 2000). Also, TRAP1 binds retinoblastoma protein via a unique LxCxE motif during mitosis (Chen et al., 1996), and with tumor suppressors EXT1 and EXT2 (Simmons et al., 1999).

Like several other chaperones, TRAP1 expression is upregulated in cells under stress. Specifically, TRAP1 is found expressed at higher levels in tumor cells where it is supposed to play a pro-survival role by inhibiting apoptosis (Kang et al., 2007; Costantino et al., 2009). Indeed, TRAP1 protects against apoptosis induced by β-Hydroxyisovalerylshikonin (β -HIVS) (Masuda et al., 2004) and granzyme M (Hua et al., 2007). *In vitro* evidence also suggests that TRAP1 regulates ROS levels and protects cells against oxidative damage (Gesualdi et al., 2007; Hua et al., 2007; Im et al., 2007). Further, overexpression of TRAP1 in rat brain was found to be neuroprotective against ischemic injury (Volobоueva et al., 2008; Xu et al., 2009). In *Dictyostelium*, Dd-TRAP1 is localized in the cortex of cells and translocates to the mitochondria to induce a prestarvation response that promotes spore differentiation during development (Morita et al., 2004; Morita et al., 2005). Altogether, these observations indicate that TRAP1 has

distinct physiological roles than other HSP90 members and is not only required to directly deal with cellular stress, but also appears to be involved in regulating response pathways.

Intriguingly, TRAP1 is the only identified substrate of the PD gene, PINK1. Phosphorylation of TRAP1 by PINK1 prevents the release of cytochrome *c* from mitochondria and inhibits the apoptotic cascade (Pridgeon et al., 2007). This may well be a novel mechanism by which cell death is mediated in Parkinson's disease. However, this result has not been verified or tested *in vivo* by other groups yet. Moreover, it is unclear why dopaminergic neurons would be more vulnerable to TRAP1 mediated apoptosis. In addition, TRAP1 is also believed to interact with cyclophilin D, a key mediator of the mitochondrial permeability transition (Kang et al., 2007). Thus, TRAP1 has emerged as a central mediator of several critical survival pathways. Further studies are warranted to understand the molecular basis for TRAP1 mediated stress resistance, the physiological relevance of TRAP1 *in vivo* and its role in mitochondrial quality control.

1.11.2. The Role of Stress Response Pathways

The best-studied form of stress response is the activation of heat shock factors (HSF) in response to thermal stress. These factors induce expression of HSP by either promoting transcription or post-transcriptionally regulating stress-induced mRNA stability (Theodorakis and Morimoto, 1987), or by translational control (Theodorakis et al., 1988), or by stress-induced changes in activity and localization of the chaperones (Welch and Suhan, 1986). Multiple HSFs are expressed in mammals, for example HSF1, HSF2, and HSF4; while only HSF1 is expressed in *Drosophila*. (Anckar and Sistonen, 2007). Here, I will focus on two stress response pathways involving signaling between the endoplasmic reticulum and nucleus in one, and between

mitochondria and nucleus in the other. The overall goal of both these pathways is to upregulate the expression of chaperones.

1.11.2.1. The Endoplasmic Reticulum Unfolded Protein Response

The concept of proteostasis hinges heavily on the ability of the cell to deal with misfolded proteins and clearance of damaged proteins. If untended, this would have broad scale consequences on multiple cellular processes. Beside the cytosol, the lumen of the endoplasmic reticulum (ER) is an active site for protein folding by chaperones. Under certain circumstances, factors such as increased protein synthesis or increased misfolded proteins may exceed the capacity of the chaperone machinery to deal with them, resulting in ER stress. Other factors that trigger ER stress are changes in calcium stores, oxidative stress and alteration of the redox balance in the ER lumen (Ron and Walter, 2007). This stress induces a cascade of signaling events towards an effort to correct the imbalance, called the ER Unfolded Protein Response (UPR).

The ER-UPR acts at three distinct levels. First, the import of unfolded polypeptides into the ER lumen is decreased. Second, the capacity of ER is increased to handle the extra load. Third, if the load cannot be handled, apoptosis is initiated. Multiple studies have identified the basic players and mechanism of ER-UPR in eukaryotes. Inositol-requiring protein 1 (IRE1) oligomerizes in the plane of the stressed ER and mediates the sequence specific cleavage of transcription factor X-box binding protein 1 (XBP1) which allows its translocation to the nucleus. Spliced *XBP1* mRNA encodes a transcriptional activator XBP1s, while the unspliced mRNA encodes an inhibitor of the UPR, XBP1u. In mammals, an additional pathway has also been reported wherein phosphorylated IRE1 recruits Tumor necrosis factor Receptor Associated

Factor 2 (TRAF2), which activates the JNK pathway. Both known pathways upregulate the transcription of chaperones and promote ER associated degradation (Urano et al., 2000; Papa et al., 2003; Yoshida et al., 2006; Ron and Walter, 2007; Uemura et al., 2009).

1.11.2.2. The Mitochondrial Unfolded Protein Response

Like the cytosol and the ER, mitochondria have their own stress response machinery. This is crucial for mitochondrial homeostasis as a vast majority of mitochondrial proteins are encoded by the nucleus and are imported into the mitochondria as unfolded polypeptides, where mitochondrial chaperones fold them in the matrix. Understandably, an imbalance in unfolded protein load and available chaperones has severe consequences. Hence, the presence of a dedicated mitochondrial stress response machinery, called the mitochondrial UPR (mt-UPR). The first evidence of the presence of signaling between the mitochondria and nucleus to regulate stress response came from the observation that deletion of mtDNA resulted in increased Hsp60 and Hsp70 levels (Martinus et al., 1996). Further, induced expression of a misfolded protein in the mitochondrial matrix also results in increased Hsp60, Hsp70 as well as the protease ClpP (Zhao et al., 2002). Despite the knowledge of its presence, relatively little is known about the mt-UPR mechanism. Recent studies in *C. elegans* have identified some of the key proteins that modulate mt-UPR signaling (Figure 1.6).

Excess of unfolded proteins in the mitochondrial matrix leads to the induction of a signaling cascade, which eventually activates the ATP-dependent protease ClpXP. ClpXP degrades the protein and the peptides are released into the cytosol with the help of the peptide exporter HAF-1. In yeast, Mdl1 has been identified as required for the peptide efflux. These peptides act as a signal and cause the worm transcription factor ZC376.7 to translocate to the

nucleus. In addition, expression of ubuiqutin like protein 5 (UBL-5) is enhanced. UBL-5 associates with DVE-1 and this complex also translocates to the nucleus and aids ZC376.7 to promote the transcription of genes such as mtHsp70 and Hsp60, which are then imported back into the mitochondria to deal with the stress of unfolded proteins (Young et al., 2001; Arnold et al., 2006; Haynes et al., 2007; Haynes and Ron, 2010; Haynes et al., 2010; Wiseman et al., 2010). Other regulators of the mt-UPR remain to be identified. In fact, how the known regulators interact with each other and the chaperone machinery is not well understood.

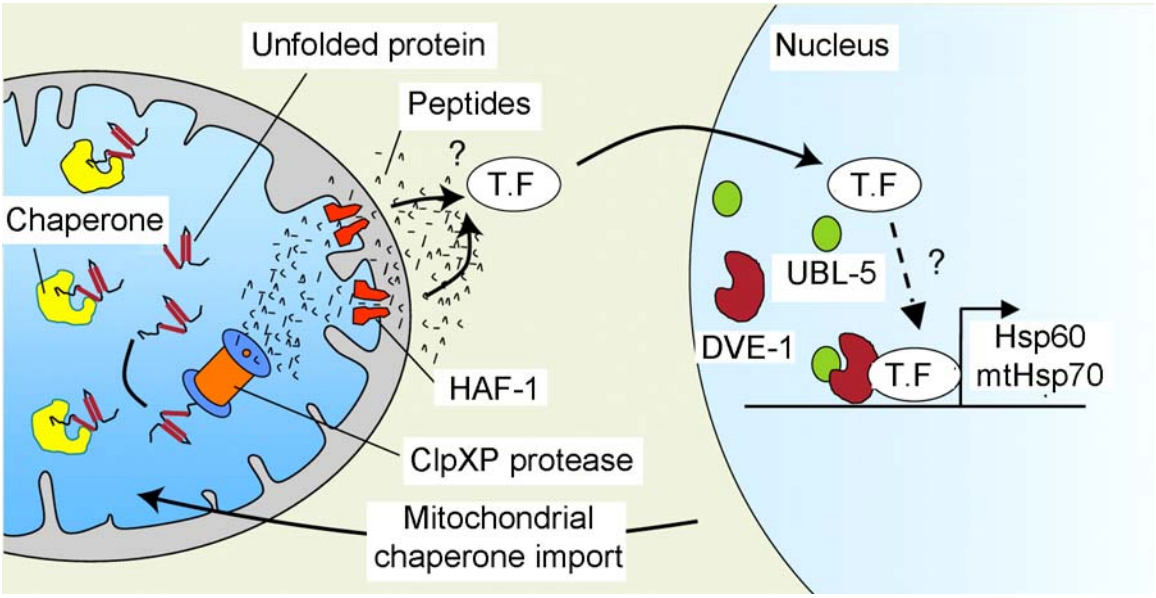

Figure 1.6 The mitochondrial Unfolded Protein Response in *C. elegans*. Excess unfolded proteins in the mitochondrial matrix initiate a signaling cascade between mitochondria and the nucleus that results in an upregulation of chaperone expression. Adapted from (Haynes and Ron, 2010).

Part 3: *Drosophila* as a Model System

Drosophila is widely regarded as a premier genetic model for studying diseases and aging. Flies have a short lifecycle of about 10-11 days, are easy and inexpensive to maintain, and have high fecundity. While these are attractive features for any model system, what makes *Drosophila* outstanding is the array of sophisticated tools available for advanced molecular genetic manipulations. The sequenced *Drosophila* genome contains approximately 13, 767 genes, located on four pairs of chromosomes: an X/Y pair, and three autosomal pairs (Adams et al., 2000). Relatively simple techniques allow stable germ-line transformation, transposable element mutagenesis, RNAi-mediated gene silencing, and spatial and temporal control over gene expression. Importantly, majority of the mammalian genes and pathways are conserved in flies.

Drosophila has been used to model several human diseases, including neurodegenerative disorders and mitochondrial disorders. They have a relatively sophisticated nervous system with about 300, 000 neurons. In the embryo and larva, there is a single ventral ganglion, akin to the vertebrate spinal cord, from which segmental nerves project to the peripheral muscles on the body wall. These nerves are approximately 5 microns in diameter and each contains a combined total of approximately 85–90 motor neuron and sensory neuron axons (Leiserson et al., 2000). The translucent body wall of larvae has allowed researchers to conduct elegant cell biological assays in living and intact animals (Miller et al., 2005). Furthermore, flies are capable of processing complex motor behaviors and higher order learning making them amenable to advanced behavioral assays (Sokolowski, 2001).

1.12.1. Gal4/ UAS System

The powerful Gal4/ UAS is a bipartite system that allows tissue specific (targeted) expression of a gene of interest. It consists of the 881 amino acid yeast transcription factor Gal4, which directly and specifically binds the Upstream Activating Sequences (UAS). The UAS elements are analogous to eukaryotic enhancer elements and their Gal4 binding is essential for transcriptional activation of downstream genes. For targeted gene expression, lines containing the UAS element controlling the gene of interest are mated with lines expressing Gal4 in a tissue-specific pattern under the control of a genomic promoter, called the driver. The resulting progeny, that contain both driver and UAS element, express the gene of interest in a spatial pattern that reflects the transcriptional profile of the genomic promoter that drives Gal4 (Brand and Perrimon, 1993). This system provides an excellent means to overexpress/ knockdown an endogenous gene or expresses a reporter gene in specific tissues (Duffy, 2002). Further, using promoters that are active during specific developmental phases can also exert some degree of temporal control.

1.12.2. Gene-Switch Gal4/ UAS System

An advanced manipulation of the Gal4/ UAS system is achieved by using its inducible forms. One such refined technique is the Gene-Switch Gal4/ UAS system that uses a hormone responsive chimera that includes the DNA binding domain of Gal4 fused to the p65 activation domain and a mutant human progesterone receptor ligand-binding domain. Under normal conditions the Gene-Switch is OFF, the p65 activation domain binds the progesterone receptor and inhibits Gal4 binding to UAS. Only in the presence of the ligand, mifepristone or RU486, the Gene-Switch will turn ON. RU486 binds to the progesterone receptor ligand-binding domain

and this allows a conformational change that frees Gal4 to bind to the UAS element and promote transcriptional activation (Osterwalder et al., 2001; Roman et al., 2001). This tool allows tight temporal regulation along on addition of RU486 along with the traditional spatial control offered by the Gal4/ UAS system.

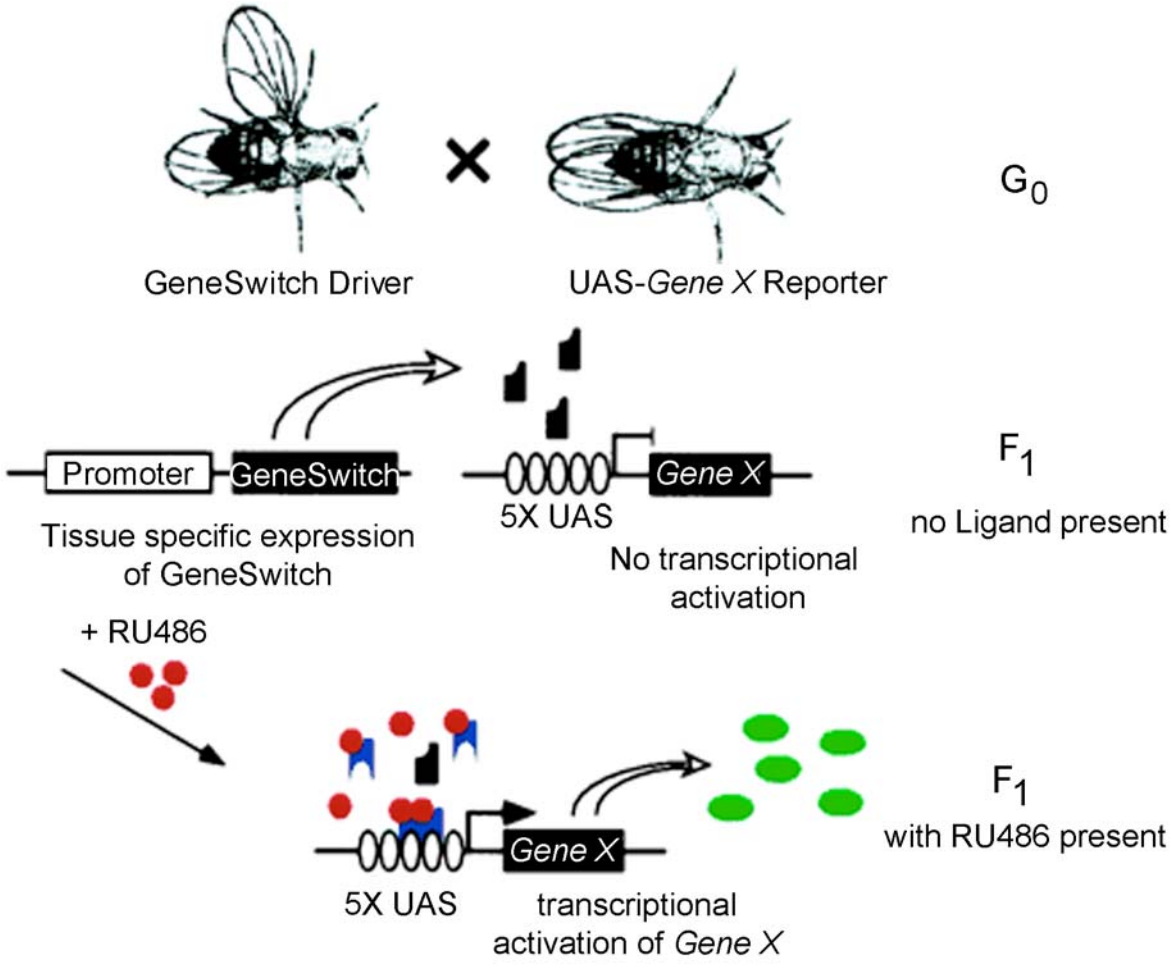

Figure 1.7 GeneSwitch Gal4 – A modified Gal4/ UAS system. This genetic manipulation allows temporal control over gene expression in addition to spatial control. Chimeric Gal4 ensures that transcription is initiated only in the presence of the ligand, RU486 (Osterwalder et al., 2001).

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Chapter 2
Disruption of Mitochondrial DNA Replication in *Drosophila* Increases Mitochondrial Fast Axonal Transport *In Vivo*

Preface to Chapter 2

This chapter describes the regulation of mitochondrial biogenesis and axonal transport in response to depletion of mitochondrial DNA in *Drosophila*. The results of this study are discussed in the context of the mitochondrial life cycle and a novel compensatory mechanism to deal with mitochondrial dysfunction.

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Abstract

Mutations in mitochondrial DNA polymerase (pol γ) cause several progressive human diseases including Parkinson's disease, Alper's syndrome, and progressive external ophthalmoplegia. At the cellular level, disruption of pol γ leads to depletion of mtDNA, disrupts the mitochondrial respiratory chain, and increases susceptibility to oxidative stress. Although recent studies have intensified focus on the role of mtDNA in neuronal diseases, the changes that take place in mitochondrial biogenesis and mitochondrial axonal transport when mtDNA replication is disrupted are unknown. Using high-speed confocal microscopy, electron microscopy and biochemical approaches, we report that mutations in pol γ deplete mtDNA levels and lead to an increase in mitochondrial density in *Drosophila* proximal nerves and muscles, without a noticeable increase in mitochondrial fragmentation. Furthermore, there is a rise in flux of bidirectional mitochondrial axonal transport, albeit with slower kinesin-based anterograde transport. In contrast, flux of synaptic vesicle precursors was modestly decreased in pol γ-α mutants. Our data indicate that disruption of mtDNA replication does not hinder mitochondrial biogenesis, increases mitochondrial axonal transport, and raises the question of whether high levels of circulating mtDNA-deficient mitochondria are beneficial or deleterious in mtDNA diseases.

2.1 Introduction

Mitochondrial DNA (mtDNA) depletion, deletion and/ or point mutations are implicated in many diseases. Mutations in DNA polymerase gamma (pol γ), the mtDNA replicase (Kaguni, 2004), cause several progressive diseases including Parkinson's disease (Luoma et al., 2004; Luoma et al., 2007), Alpers' syndrome (Naviaux and Nguyen, 2004), and progressive external ophthalmoplegia (Longley et al., 2005). Pol γ is the sole DNA polymerase responsible for mtDNA replication in animals. It is a highly accurate and processive protein complex comprising a 125 kD catalytic subunit (pol γ–α) and a 35 kD accessory subunit (pol γ–β) in *Drosophila*, encoded by the genes *tamas* and *pol γ–β*, respectively. Both subunits have been cloned and characterized extensively in *Drosophila* (Lewis et al., 1996; Wang et al., 1997) and in numerous other systems including humans (Kaguni, 2004). At a cellular level, alteration of pol γ expression leads to depletion of mtDNA, disrupts the mitochondrial respiratory chain, and increases susceptibility to oxidative stress (Iyengar et al., 2002; Lewis et al., 2003; Mansouri et al., 2003; Smeitink et al., 2006; Martinez-Azorin et al., 2007).

Mitochondria are distributed in neurons through a dynamic combination of transport and stopping (docking) events (Miller and Sheetz, 2004). Fast axonal transport of mitochondria is carried out by molecular motors, conventional kinesin and cytoplasmic dynein, that utilize ATP to perform their function (Brady, 1985; Vale et al., 1985; Paschal et al., 1987; Pilling et al., 2006). Healthy mitochondria have a high mitochondrial membrane potential that provides the electrochemical energy to drive ATP synthesis through oxidative phosphorylation (Senior, 1988). Accordingly, the role of mitochondrial ATP production in regulating fast axonal transport has been the subject of several studies. Early *in vitro* studies designed to disrupt oxidative phosphorylation have suggested that it is essential for the maintenance of axonal transport (Ochs

and Hollingsworth, 1971). However, other studies have presented conflicting results. For example, uncoupling agents CCCP and FCCP block all cytoplasmic transport while another uncoupler DNP has no effect (Hollenbeck et al., 1985); complex III inhibitor antimycin increases retrograde transport with little effect on anterograde transport (Miller and Sheetz, 2004), as does the complex I inhibitor annonacin (Escobar-Khondiker et al., 2007). As the disruption of axonal trafficking is implicated in neuronal degeneration and is observed in diseases like Alzheimer's disease, Huntington's disease, spinobulbar muscular atrophy, Charcot-Marie-Tooth disease, *etc.* (Ferreirinha et al., 2004; Chang and Reynolds, 2006; Baloh et al., 2007), the influence of genetic impairment of oxidative phosphorylation on axonal transport is especially relevant. We hypothesized that mutations in the accessory and catalytic subunits of pol γ would disrupt fast axonal transport. To investigate the influence of depleted mtDNA content on axonal trafficking and mitochondrial biogenesis, we studied transport dynamics in pol γ mutants of *Drosophila*.

2.2 Results

2.2.1 mtDNA is depleted in mutants of pol γ

We disrupted mtDNA replication in *Drosophila* using mutations in the two subunits of pol γ, *pol γ–*$^{\beta 1/\beta 2}$ (accessory subunit mutant alleles) and *tam*^{3}/ *tam*^{9} (catalytic subunit mutant alleles) (Iyengar et al., 1999; Iyengar et al., 2002). To verify that mtDNA content is decreased in these backgrounds, we stained muscles of crawling third instar *Drosophila* larvae with the fluorescent dye PicoGreen (Figure 2.1). PicoGreen reliably labels mtDNA and has been used to detect mtDNA depletion in living human cells and rat liver mitochondria (Ashley et al., 2005). Because muscles are large, flat, and contain multiple nuclei that serve as internal controls for dsDNA, they are excellent for quantitative assessment of mtDNA depletion.

We measured mtDNA depletion in muscles 6/ 7 in abdominal segments A3 - A6. Muscle nuclei were stained brightly and displayed similar pixel intensity in wildtype, *pol γ–*$^{\beta 1/\beta 2}$ and *tam*^{3}/ *tam*^{9} mutants, suggesting that PicoGreen had been incorporated equally and sufficiently into dsDNA. Numerous mtDNA nucleoids were visible in the wildtype muscles, but were nearly absent at identical exposure levels in *pol γ–*$^{\beta 1/\beta 2}$ and *tam*^{3}/ *tam*^{9} mutants (Figure 2.1). The normal mitochondrial distribution and patterning in wildtype muscles is seen by co-immunostaining with an antibody against mitochondrial complex V. This patterning was severely disrupted in *pol γ–*$^{\beta 1/\beta 2}$ and *tam*^{3}/ *tam*^{9} mutants. In particular, some *tam*^{3}/ *tam*^{9} mutants had visibly higher mitochondrial density and mitochondria appeared to be tightly packed instead of arranged in normal banded patterns. Quantitative analysis of PicoGreen staining in muscles reveals a significant decrease in density of mtDNA nucleoids in *pol γ–*$^{\beta 1/\beta 2}$ and *tam*^{3}/ *tam*^{9} mutants as

compared to controls (Figure 2.2 C). Further, the fluorescent intensity of PicoGreen stain is also reduced significantly (Figure 2.2 C), suggesting lower mtDNA content in the existing nucleoids. This is the first study to visualize directly mtDNA depletion in muscles using tissue staining.

In order to validate these results using a molecular analysis, we evaluated mtDNA content and integrity in the pol γ mutants by quantitative Southern blotting. Total DNA from wildtype and mutant pol γ larvae late in the third instar was isolated and digested with *Xho*I, which cleaves *Drosophila* mtDNA once. A mtDNA probe encoding the ATPase 6 gene was used to determine mtDNA copy number and integrity, and a multiple-copy genomic probe of the nuclear histone gene cluster was used as a DNA loading control. Multiple analyses of mtDNA content demonstrated severe mtDNA depletion in pol γ mutants; mtDNA was nearly undetectable in *pol γ-α* mutant larvae (Figure 2.2 A). Based on prior work that estimates that 2 – 10 molecules of mtDNA are found in each mitochondrion (Legros et al., 2004; Ashley et al., 2005), we make the conservative estimate that more than 90% of the mitochondria in *pol γ–*$^{\beta 1/\beta 2}$ and *tam*^{3}/ *tam*^{9} mutant backgrounds lack mtDNA.

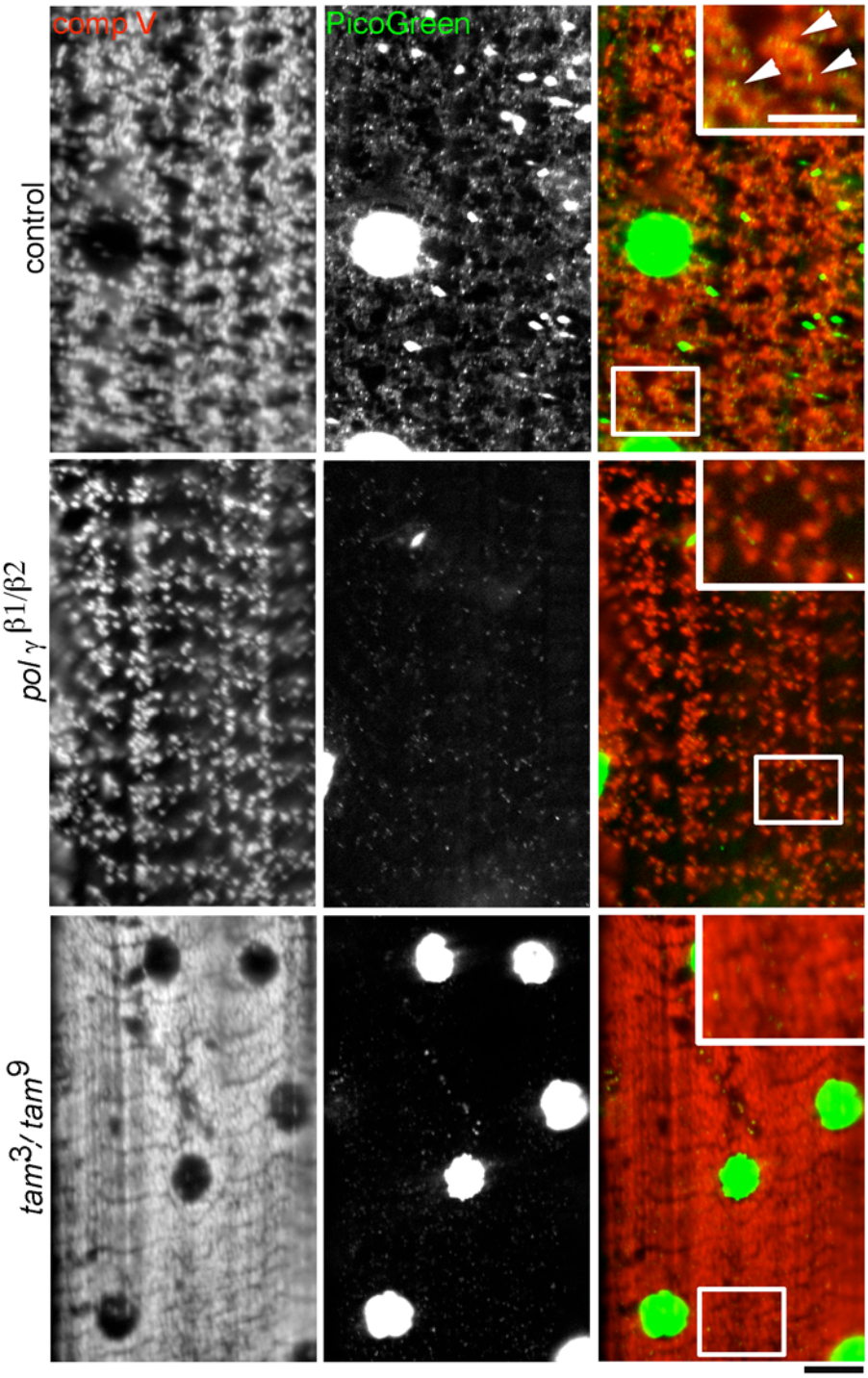

Figure 2.1. Mutations in the catalytic and accessory subunits of DNA polymerase γ impair mtDNA replication and decrease mtDNA content

An antibody against mitochondrial complex V (red) and the dye PicoGreen (green) are used to label mitochondria and mtDNA respectively, in muscles of wildtype control, *pol* γ*–*$^{\beta 1/\beta 2}$ and *tam*^{3}/ *tam*^{9} crawling 3rd instar *Drosophila* larvae. Regular mitochondrial distribution is disrupted *pol* γ*–*$^{\beta 1/\beta 2}$ and *tam*^{3}/ *tam*^{9}mutants and number of mtDNA nucleoids are significantly reduced. PicoGreen also labels the dsDNA of muscle nuclei that serves as internal control for the staining. Muscle nuclei appear smaller in the pol γ mutants. Because of the relatively high concentration of dsDNA in muscle nuclei, they appear saturated at offset levels required to visualize the smaller mtDNA nucleoids. Insets in the RGB merge show digitally magnified regions from the boxes and arrowheads indicate presence of mtDNA in control muscles and are absent in muscles of pol γ mutants. *tam*^{3}/ *tam*^{9}larvae have visibly higher mitochondrial density. Scale bars equal 10 mm.

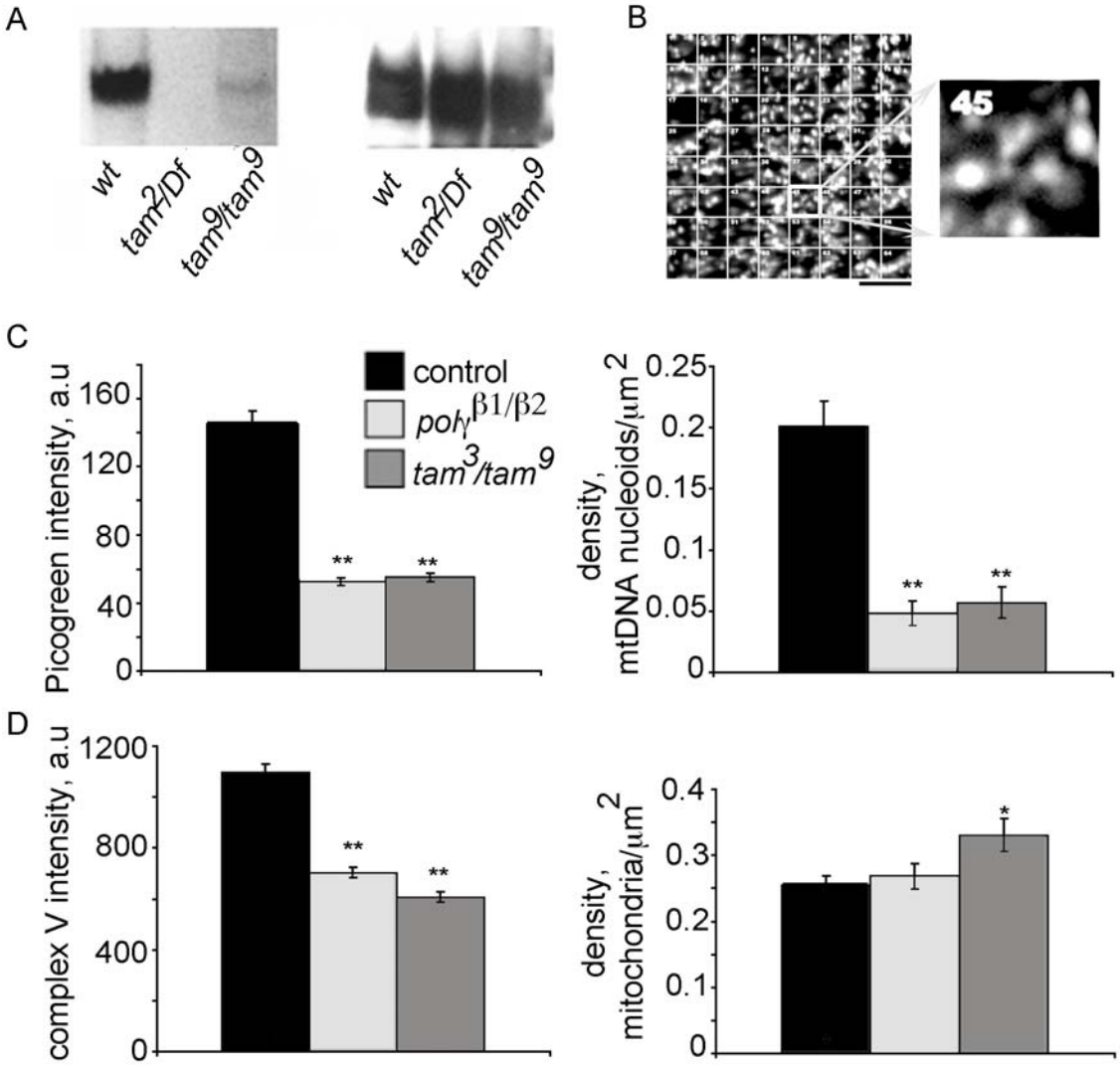

Figure 2.2 Mitochondrial density is higher in *tam*^{3}/ *tam*^{9}mutants whereas density of mtDNA nucleoids is reduced

(A) Southern blot analysis of total DNA extract, hybridized with ^{32}P-labeled DNA probes from the ATPase 6 gene of mtDNA shows a decrease in pol γ mutants compared to wildtype controls. A histone gene cluster (*his* genes) is used as a nuclear DNA control. (B) Density of mitochondria

and mtDNA nucleoids is measured by dividing a 512 X 512 pixel frame of the muscle into a grid of 64 regions of interest (ROIs). A random list of numbers (1-64) was generated and the number of mitochondria / mtDNA nucleoids was counted in those ROIs for each frame. (C) Intensity of PicoGreen stained mtDNA (in arbitrary units, a.u); as well as density of mtDNA nucleoids was reduced significantly in pol γ mutant muscles. Numbers at the base of columns represent number of mtDNA nucleoids sampled and number of animals used, respectively. (D) Intensity of complex V stained mitochondria is reduced significantly in both pol γ mutants (in arbitrary units, a.u); while average mitochondrial density is increased moderately in tam^{3}/ tam^{9} mutant muscles. Numbers at the base of columns represent number of mitochondria sampled and number of animals used, respectively. Error bars represent 95% confidence intervals. * indicate $p<0.05$ and ** indicate $p<0.001$ from Student's t-test.

2.2.2 Lysosomal dsDNA clusters are absent in *pol γ–$^{β1/β2}$* and *tam^{3}/ tam^{9}* mutants

Interestingly, control muscles regularly displayed numerous dense extra-nuclear clusters of dsDNA that did not colocalize with mitochondria, and these clusters were absent or rarely seen in *pol γ–$^{β1/β2}$* and *tam^{3}/ tam^{9}* mutants (Figure 2.3). We investigated further the nature of these clusters by co-immunostaining with several probes and antibodies and found them to colocalize completely with anti-spin antibody (Figure 2.3, arrowheads). *Spin (Spinster)* encodes a multipass transmembrane protein that is localized to a late endosomal/ lysosomal compartment (Sweeney and Davis, 2002). Expectedly, not all anti-spin compartments have dsDNA but all dsDNA clusters colocalize with anti-spin, indicating that the extra-nuclear dsDNA clusters were always present inside lysosomes, whereas all lysosomes do not contain dsDNA clusters.

2.2.3 Mitochondrial density is higher in muscles of *tam^{3}/ tam^{9}* mutants

Mitochondrial density increases in the liver when pol γ is disrupted in humans (Naviaux et al., 1999). Mitochondrial mass is also increased when mitochondrial transcription factor B2 is downregulated in *Drosophila* (Adan et al., 2008). To determine whether mutations in pol γ altered mitochondrial density in *Drosophila*, we studied the spatial distribution of mitochondria in muscles 6/ 7 in abdominal segments A3 - A6 of *tam*^{3}/ *tam*^{9} and *pol γ–$^{β1/β2}$* larvae. A single confocal plane from immunolabeled images was cropped into 512 X 512 pixel frames and divided into a 64-square grid. A random list of squares was generated for sampling; the mitochondria in each square were counted and divided by the area to yield density in units of mitochondria/ mm^{2} (Figure 2.2 B). Average mitochondrial density registered a slight increase in *pol γ–$^{β1/β2}$* mutants and was significantly higher in *tam*^{3}/ *tam*^{9} mutants (Figure 2.2 D). Further,

quantitative analysis of the average fluorescent intensity of anti-complex V staining is significantly reduced *pol* γ–$^{\beta 1/\beta 2}$ and *tam*^{3}/ *tam*^{9} mutants (Figure 2.2 D). Mitochondrial complex V, also called ATP synthase, is responsible for ATP synthesis. Reduction in anti-complex V staining suggests that in *pol* γ–$^{\beta 1/\beta 2}$ and *tam*^{3}/ *tam*^{9} mutants the overall health and ATP generation capacity of mitochondria is compromised. Together, our data demonstrate that the average mitochondrial density in muscles is not decreased in both mutants of mtDNA replication, and in fact may increase in *tam*^{3}/ *tam*^{9} mutants.

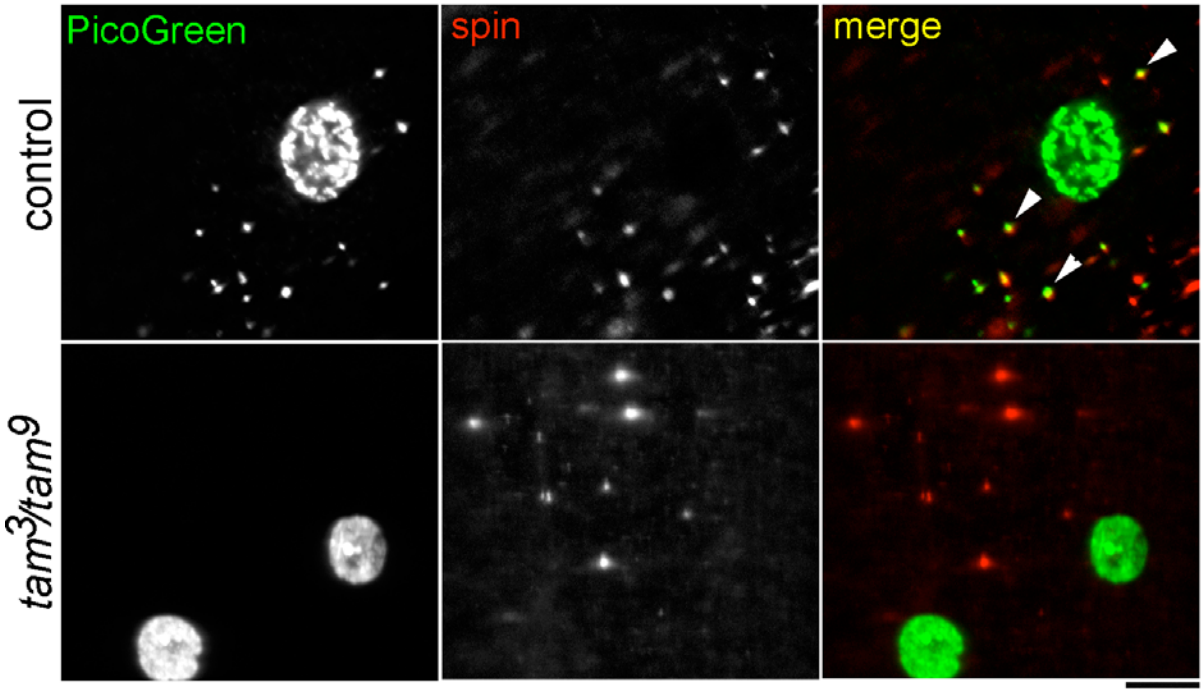

Figure 2.3. Lysosomal clusters of double-stranded DNA are absent in muscles of pol g mutant larvae

Immunolabeling of late endosomal/ lysosomal compartments with anti-spin antibody (red) reveals the presence of extra-nuclear dsDNA clusters (green) in these compartments in control muscle cells (arrowheads), presumably from mitochondria undergoing mitophagy. These dsDNA clusters are absent in the lysosomes of *tam*^{3}/ *tam*^{9} mutant muscles. Scale bar equals 10 μm.

2.2.4 Mitochondrial density is higher in the proximal nerves of *tam*^{3}/ *tam*^{9} mutants

To evaluate the effect of mtDNA depletion on mitochondrial distribution in neurons, we measured mitochondrial density in the segmental nerves of flies with the genotypes *pol* $\gamma-^{\beta1/\beta2}$; *UAS-mtGFP, D42Gal4/+* and *tam*^{3}/ *tam*^{9}; *UAS-mtGFP, D42-Gal4/+*. *UAS-mtGFP* encodes the S65T spectral variant of GFP fused at the N-terminus with the 31 amino acid mitochondrial import sequence from human cytochrome *c* oxidase subunit VIII. The *D42*Gal4 driver predominantly expresses in motor neurons along with a few body wall sensory neurons, and the salivary glands in the larva (Sanyal, 2009). As the cell bodies of motor neurons are located in the ventral nerve cord, regions of the segmental nerve that are close to the ventral nerve cord correspond to the proximal axonal regions of the motor neurons. 3-D reconstructed images from the proximal, medial and distal nerve region of *UAS-mtGFP; D42*Gal4 expressing animals were used for the analysis (Figure 2.4 A and B). Quantification of average mitochondrial density reveals a significant increase in the proximal segmental nerves of *tam*^{3}/ *tam*^{9} mutants while proximal nerves of *pol* $\gamma-^{\beta1/\beta2}$ mutants have mitochondrial densities similar to control. The medial and distal nerves in the same animals did not show any difference from control; in fact, *pol* $\gamma-^{\beta1/\beta2}$ mutants registered a slight reduction in mitochondrial density in the medial and distal regions (Figure 2.4 C).

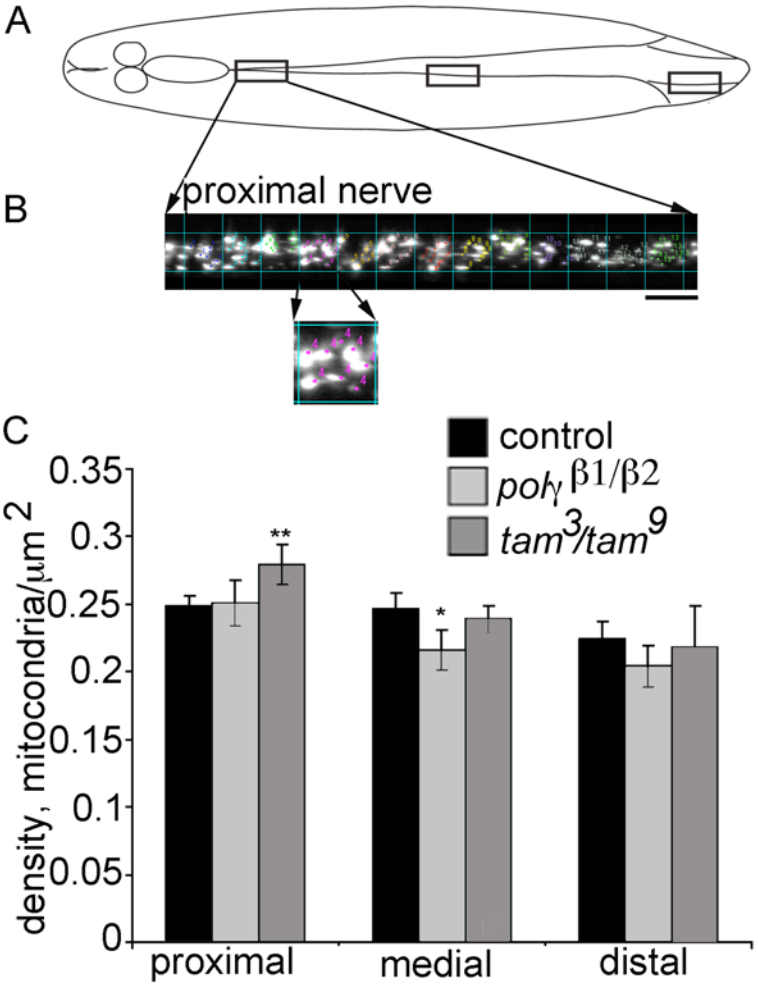

Figure 2.4. Mitochondrial density is increased in the proximal nerves of *tam*^{3}/ *tam*^{9} mutant larvae

(A) Schematic illustration of the neuronal organization of the 3rd instar *Drosophila* larva. The region in box P represents the area imaged for proximal, M for medial and D for distal nerve analysis. (B) The nerve was divided into 100 μm^{2} bins along its length and individual mitochondria counted in each bin and averaged over the area to yield mitochondrial density/ μ2.

Scale bar equals 10 μm. (C) Mitochondrial density is significantly increased in proximal region of nerves of tam^{3}/ tam^{9} mutant larvae compared to wildtype controls. Density along the medial nerve and distal nerve remains unchanged in tam^{3}/ tam^{9} mutants. Error bars represent 95% confidence intervals. * indicates $p<0.05$ and ** indicates $p<0.001$ from Student's t-test.

2.2.5 Mitochondrial ultrastructure is preserved in *pol* $\gamma-^{\beta 1/\beta 2}$ and *tam*^{3}/ *tam*^{9} mutants

Mitochondrial fragmentation has been characterized by an increase in numbers of small round mitochondria and by mitochondria with abnormal cristae (Knott et al., 2008). To determine if mitochondria were fragmented or otherwise abnormal, muscle fibers, the segmental and intersegmental nerves, the small nerve branches within the body wall muscles, and neuromuscular junctions were examined using transmission electron microscopy. Third instar larvae prior to the wall climbing stage were selected for study to avoid any age-related degeneration that might occur in association with metamorphosis.

Mitochondria in *Drosophila* muscle are typically arranged in large masses along with glycogen granules between the sarcolemma and the columns of myofibrils. These groups of mitochondria are distributed irregularly around the circumference and along the length of the fiber, so that they may or may not be present in any given plane of section. There may be a thick layer on one side of the fiber and not on the other (Figure 2.5 A, arrow). Mitochondria and glycogen also surround each muscle fiber nucleus and each neuromuscular junction. Smaller (cross-sectional diameter) mitochondria are found along the sarcoplasmic reticulum among the columns of myofilaments at the level of z-bands. No qualitative differences were detected in this general pattern of distribution between Canton-S and *D42*Gal4 controls or among *pol* $\gamma-^{\beta 1/\beta 2}$ and *tam*^{3}/ *tam*^{9} mutants. The appearance of individual mitochondria also did not differ between mutants and controls. We found no examples either of mitochondria with crystalline formations or with striking abnormalities in the cristae (Figure 2.5), features that are typically associated with mitochondrial fragmentation (Knott et al., 2008). Due to the small sample size, we did not conduct a statistical analysis of muscle fiber mitochondrial density.

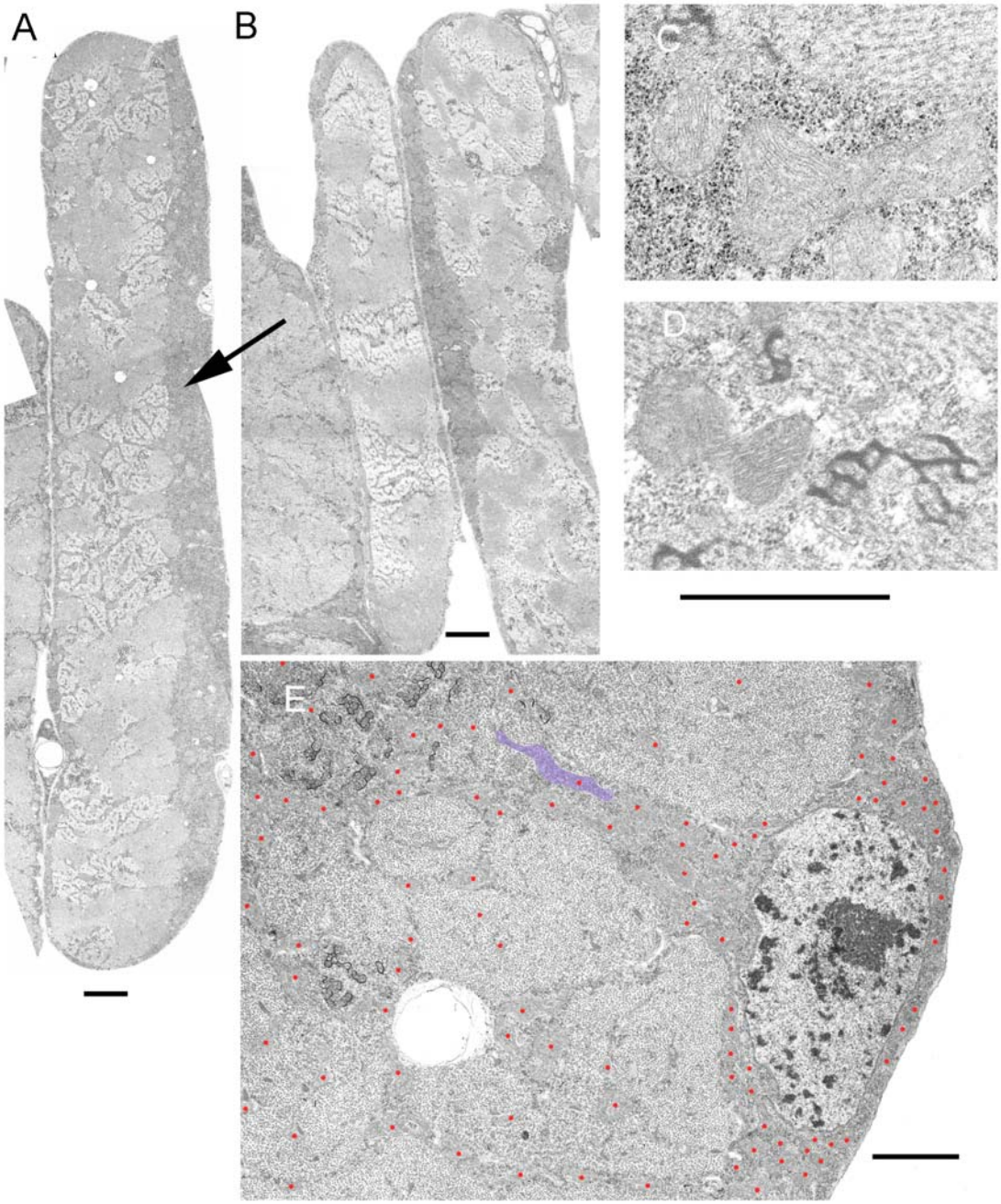

Figure 2.5. Mitochondrial morphology and distribution in larval muscles

(A) (muscle fiber 7) and **(B)** (muscle fibers 15 and 28) are montages of low power electron micrographs to illustrate whole muscle cells. (A and C) are from Canton S, and (B, D, and E)

from *tam*^{3}/ *tam*^{9}. In (A) and (B) the dark bands (arrow) along the edges of fibers consist of mitochondria and glycogen granules. The thickness of this layer can be seen to vary along the same fiber and between adjacent fibers particularly on (B). Mitochondria and glycogen are also found in streaks radiating inward from the edges of the fibers, largely in the vicinity of the Z bands (C, D, and E); their distribution gives rise to the banded patterns seen with immunolabeling (Figure 2.1). **(E)** Illustrates the increase in clusters of mitochondria around nuclei; the individual mitochondria have been indicated with a red dot at high magnification so their distribution can be visualized. The shape of a single mitochondrion was overlaid with blue to indicate relative size. No qualitative changes were detected between the structures of control **(C)** and *tam*^{3}/ *tam*^{9} mutant mitochondria **(D).** Scale bars equal 2 μm for (A and B), 1 μm for (C, D, and E).

In nerves, the mitochondria in the axons and glia are smaller in diameter than those in muscle fibers. Typically only one profile of a mitochondrion is present in any given cross-section of a particular axon although exceptions are seen in which 5 or more are present in both control and mutant animals. Several to a dozen microtubules (depending on axon diameter) are also present, and occasionally clusters of glycogen granules are seen within axons or in glia. No qualitative differences in the structure of axonal or glial mitochondria were detected between the control and the mutant animals and cristae appeared normal (Figure 2.6). In neuromuscular junctions a mitochondrion may or may not appear in any given cross section; no difference in morphology in these mitochondria was seen between mutant and control animals. Together, these morphological observations suggest that mitochondria remaining in nerves and muscles at this stage are structurally normal. The similar range of sizes observed in the sampled populations together with the fluorescence observations suggest that mitochondrial fragmentation is not occurring in pol γ mutants, but modest increases in fission cannot be ruled out.

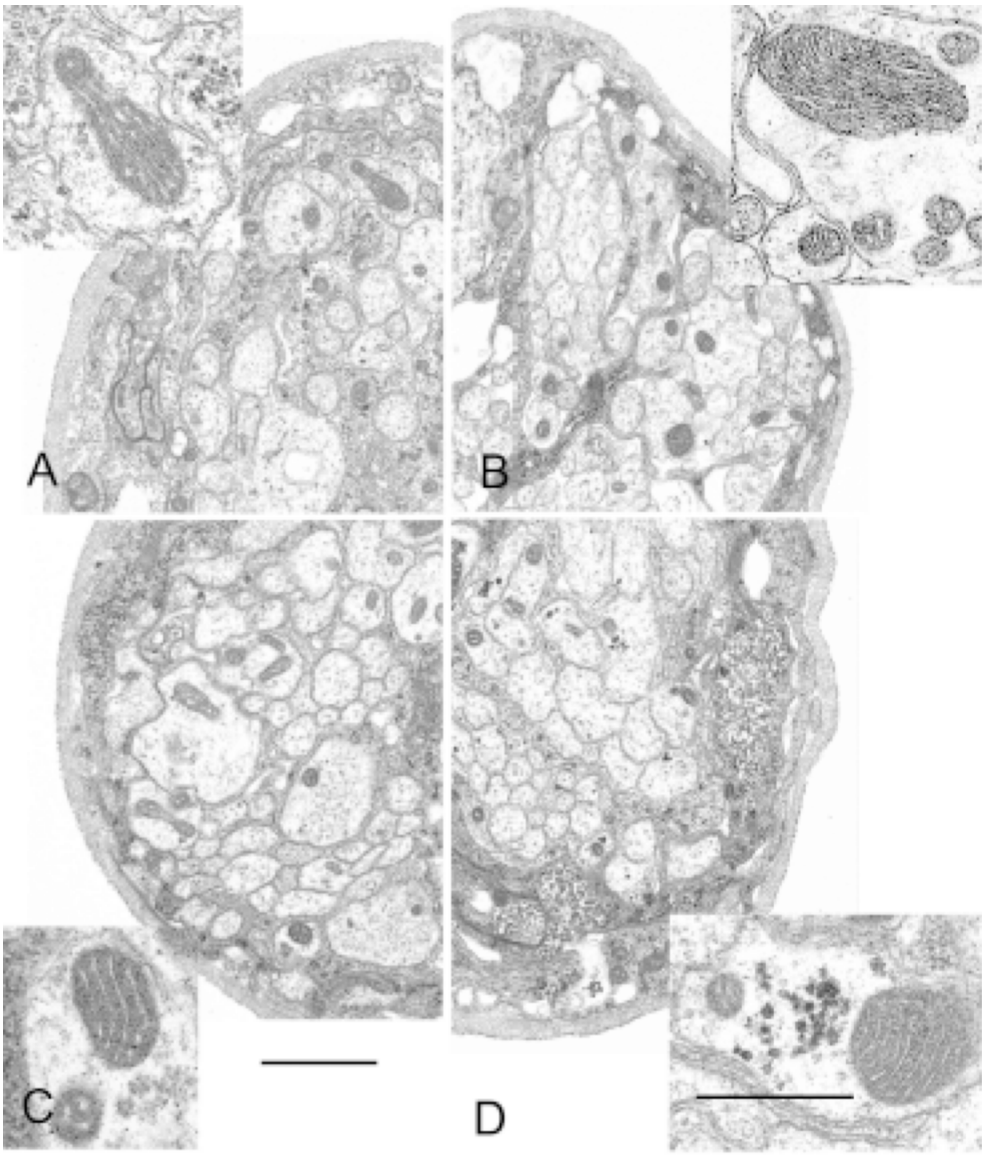

Figure 2.6. Mitochondrial ultrastructure in segmental nerves

Each quadrant shows a portion of a segmental nerve from each of the four types of animals that were examined. No differences in the nerve structure were noticed between control and mutant

larvae. **(A)** Background control (*UAS-mtGFP; D42*Gal4 in wildtype background); **(B**) Canton S; **(C)** tam^{3}/ tam^{9}; and **(D)** *pol* γ–$^{\beta 1/\beta 2}$. Cross-sections of mitochondria (insets) are found in about 1 out of every 5 axonal profiles in controls. The relative number of mitochondrial profiles in axons appeared to be slightly increased in *pol* γ–$^{\beta 1/\beta 2}$ and tam^{3}/ tam^{9} mutants, to around 1 out of 3 in a sample of 3 nerves each. Oblique or longitudinal sections show that axonal mitochondria can be quite long and threadlike, and range in diameter from 100 to 800 nm. Both large and small diameter mitochondria may be found side by side in the same axon (B, C, D). Glycogen granules are seen in some of the large diameter axons (D). Scale bars equal 1 μm for nerve quadrants and 0.5 μm for insets.

2.2.6 Mitochondrial flux increases when mtDNA replication is impaired

To determine if neuronal mitochondrial transport was altered *in vivo*, we used the same methodology we developed for observing the transport of synaptic vesicle precursors in *Drosophila* (Miller et al., 2005). In brief, intact third instar larvae were anesthetized and mounted between a slide and coverslip and time lapse images of GFP-labeled mitochondria were acquired (Figure 2.7 A and B; see Materials and methods). The mitochondrial label (*UAS-mtGFP*) was expressed in motor neurons using the *D42*Gal4 driver. In control and mutant backgrounds, the number of GFP-labeled mitochondria that crossed a specific point across the nerve were counted, and the total was then divided by the 'time of observation' to yield 'flux' in terms of mitochondria/ min. To measure these dynamics we generated kymographs, which are graphical records of transport with distance on the X-axis and time on the Y-axis (Figure 2.7 C). Mitochondrial flux registered a striking increase in both directions in *pol* γ*–*$^{\beta 1/\beta 2}$ and *tam*^{3}*/ tam*^{9} mutants as compared to control animals (Figure 2.8 A, supplemental videos 1 and 2 on PLoS One). Consistent with the varying severity of *pol* γ*–*$^{\beta 1/\beta 2}$ and *tam*^{3}*/ tam*^{9} mutant phenotypes, heterozygous *pol* γ*–*$^{\beta 1}$*/ Cyo* does not show any significant difference from control, whereas a single copy of the mutation in the heterozygous *tam*^{3}*/ Cyo* animals is sufficient for a significant increase in bidirectional flux. The average flux values with standard deviations are summarized in Table 2.1. These data provide the first direct evidence that bidirectional mitochondrial transport increases when mitochondrial DNA replication is blocked.

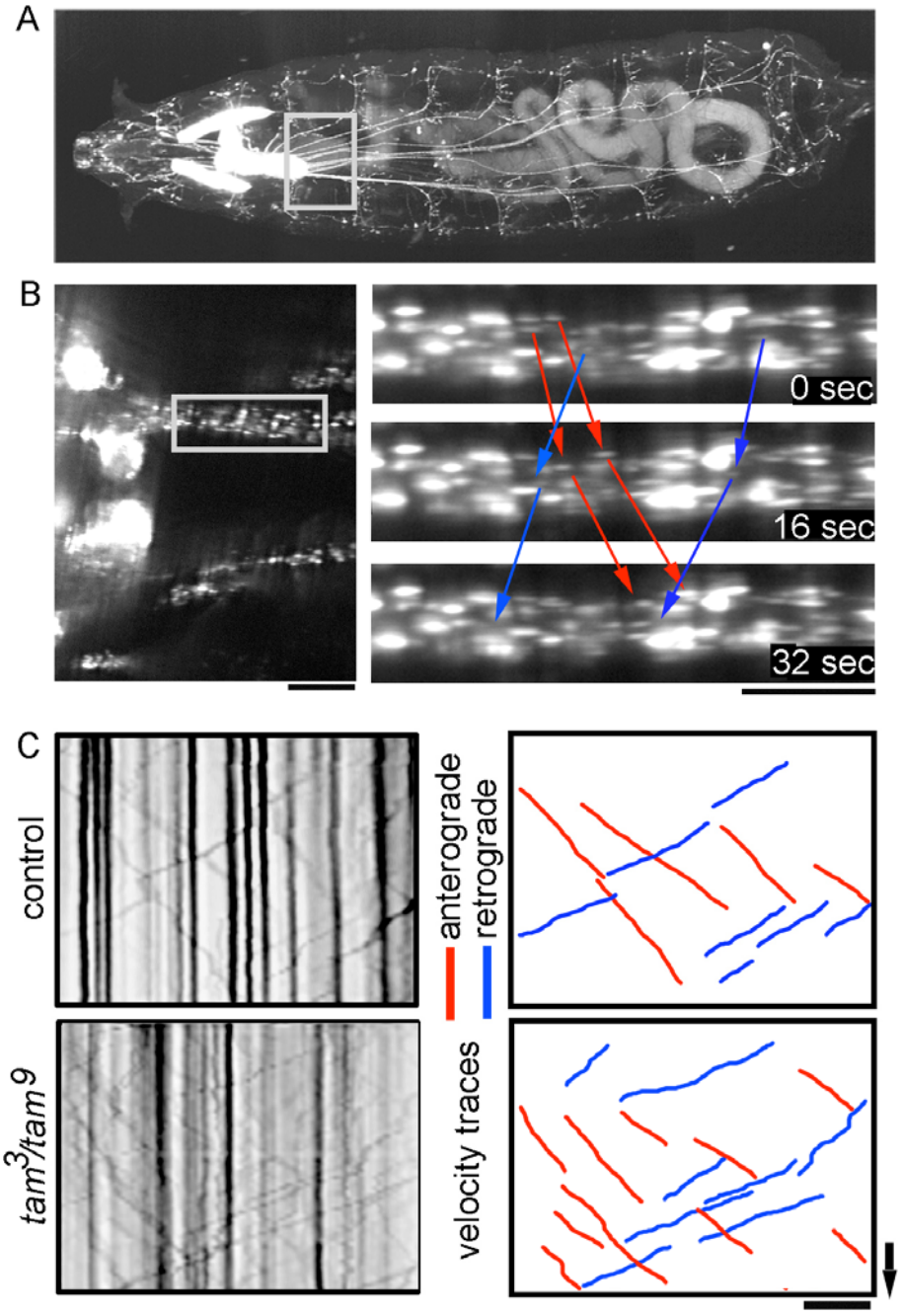

Figure 2.7. Measurement of flux and velocity of axonal transport from *Drosophila* segmental nerves

(A) 3-D reconstruction of a 3rd instar *Drosophila* larva expressing *UAS-mtGFP* in segmental nerves under the *D42*GAL4 driver. Mitochondria can be visualized in the CNS, salivary glands and the entire length of segmental nerves. There is some auto-fluorescence in the gut. A single optical plane from the proximal region of the two medial nerves was used for time lapse imaging as described in the methods section. (**B)** Kymographs were generated as described in the methods section. Dark vertical lines denote docked mitochondria; hand traced red lines depict mitochondria moving in the anterograde direction while blue lines depict retrograde movement. The slope of these lines yields velocity of transport. Arrow represents 20 s and the scale bar equals 10 μm.

Axonal Transport of Mitochondria					
FLUX (mitochondria/ min)	control	*pol γ–$^{\beta}$/ Cyo*	*tam^{3}/ Cyo*	*pol γ–$^{\beta1/\beta2}$*	*tam^{3}/ tam^{9}*
Anterograde	10.15 ± 2.48	10.17 ± 2.33	13.66 ± 2.56	16.31 ± 5.47	18.20 ± 4.87
Retrograde	9.52 ± 2.73	10.56 ± 2.10	13.00 ± 2.40	16.18 ± 5.79	17.38 ± 4.49
VELOCITY (μm/ s)	control	*pol γ–$^{\beta}$/ Cyo*	*tam^{3}/ Cyo*	*pol γ–$^{\beta1/\beta2}$*	*tam^{3}/ tam^{9}*
Anterograde	0.08 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
Retrograde	0.07 ± 0.01	0.07 + 0.01	0.07 + 0.01	0.07 + 0.01	0.07 + 0.01
Axonal Transport of Synaptic Vesicle Precursors					
FLUX (syn. vesicles / min)	control			*pol γ–$^{\beta1/\beta2}$*	*tam^{3}/ tam^{9}*
Anterograde	39.07 ± 6.36			37.81 ± 6.25	34.59 ± 4.53
Retrograde	35.46 ± 5.45			32.87 ± 6.10	30.48 ± 6.32
VELOCITY (μm/ s)	control			*pol γ–$^{\beta1/\beta2}$*	*tam^{3}/ tam^{9}*
Anterograde	0.08 ± 0.02			0.08 ± 0.02	0.08 ± 0.01
Retrograde	0.06 ± 0.01			0.06 + 0.01	0.06 + 0.01

Table 2.1. Flux and velocity values for bidirectional transport of mitochondria and synaptic vesicle precursors in Drosophila larvae. Errors indicate standard deviation.

Next, we measured the propensity of mitochondria to remain stationary or remain motile in the same nerve regions used to measure flux. We found that there was no significant change in the number of docked mitochondria in control and tam^{3}/ tam^{9} mutants (Figure 2.8 B). The ratio of docked versus moving mitochondria increases in tam^{3}/ tam^{9} mutants because of higher number of motile mitochondria.

2.2.7 Velocity of anterograde mitochondrial transport is reduced whereas retrograde velocity is unchanged

To determine if depletion of mtDNA altered the more subtle aspects of transport, we measured the velocity of mitochondrial transport in *pol* γ*–*$^{\beta 1/\beta 2}$ and *tam*^{3}/ *tam*^{9} mutants, as well as heterozygous flies with single copy of the mutations. In all conditions, we found that kinesin-based anterograde velocity was reduced significantly whereas dynein-based retrograde velocity was maintained at the same rate as control animals (Figure 2.8 C). The average velocity values with standard deviations are summarized in Table 2.1. Although this impairment in axonal transport is minimal, it could contribute to peripheral neuropathy over a sustained time period as observed with some POLG1 mutations (Davidzon et al., 2006). These results raise the question if decreased anterograde velocity is due to a global disruption of axonal transport such as an alteration in ATP or ADP levels [19,37], the formation of 'clogs' or blocks along the axon (Goldstein, 2003), or disruption of the integrity of microtubules (Friede and Ho, 1977).

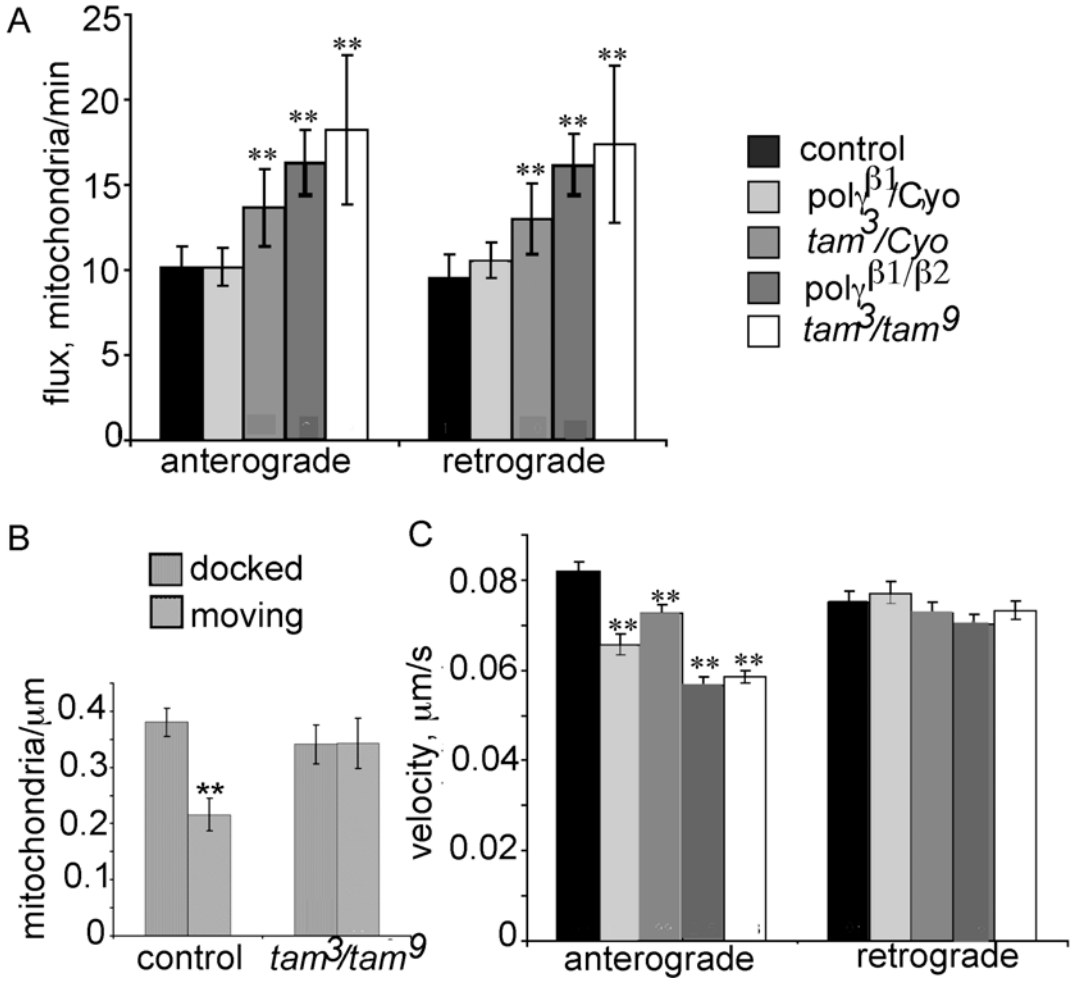

Figure 2.8. Mutations in pol γ increase mitochondrial flux in both directions but decrease only anterograde velocity

(A) Bidirectional mitochondrial flux is increased significantly in segmental nerves of heterozygous *tam*^{3} and heteroallelic combination of *pol γ–*$^{\beta1/\beta2}$ and *tam*^{3}/ *tam*^{9}; numbers at the base of columns indicate number of nerves assayed. **(B)** There is no significant change in the

density of docked mitochondria in *tam*^{3}/ *tam*^{9} mutants; numbers at the base of columns indicate number of nerves assayed. **(C)** Average velocity of anterograde run is decreased significantly in heterozygous and heteroallelic pol γ mutants while average velocity of retrograde runs is affected; numbers at the base of columns indicate number of mitochondria traced. All error bars represent 95% confidence intervals. * indicates $p<0.05$ and ** indicates $p<0.001$ from Student's t-test.

2.2.8 Synaptic vesicle precursor transport is largely unaffected in *pol* $\gamma-^{\beta1/\beta2}$ and *tam*^{3}/ *tam*^{9} mutants

To determine if the observed elevation of bidirectional flux and decrease in the velocity of kinesin mediated transport was global, we observed transport of synaptic vesicle precursors in *pol* $\gamma-^{\beta1/\beta2}$ and *tam*^{3}/ *tam*^{9} mutants (supporting videos 3 and 4). SNARE protein synaptobrevin (Vesicle Associated Membrane Protein) tagged to GFP was expressed in motor neurons using the *D42*Gal4 driver, and its axonal transport was assayed in the segmental nerves using time lapse confocal imaging in crawling third instar *Drosophila* larvae (Figure 2.9 A). Measurement of flux and velocity revealed that axonal transport of synaptic vesicle precursors was not altered severely by disruption of mtDNA replication. Although, *tam*^{3}/ *tam*^{9} mutants showed a slight decrease in flux, this transport behavior is in contrast to the elevation in bidirectional mitochondrial transport and could potentially be an indicator of depleted ATP levels. There was no significant difference in the velocity of anterograde and retrograde synaptic vesicle precursor transport in *pol* $\gamma-^{\beta1/\beta2}$, *tam*^{3}/ *tam*^{9} and control larvae (Figure 2.9 B and C). The average flux and velocity of synaptic vesicle precursors with standard deviations are summarized in Table 1. We conclude that disruption of mitochondrial DNA replication does not cause global impairment of axonal transport.

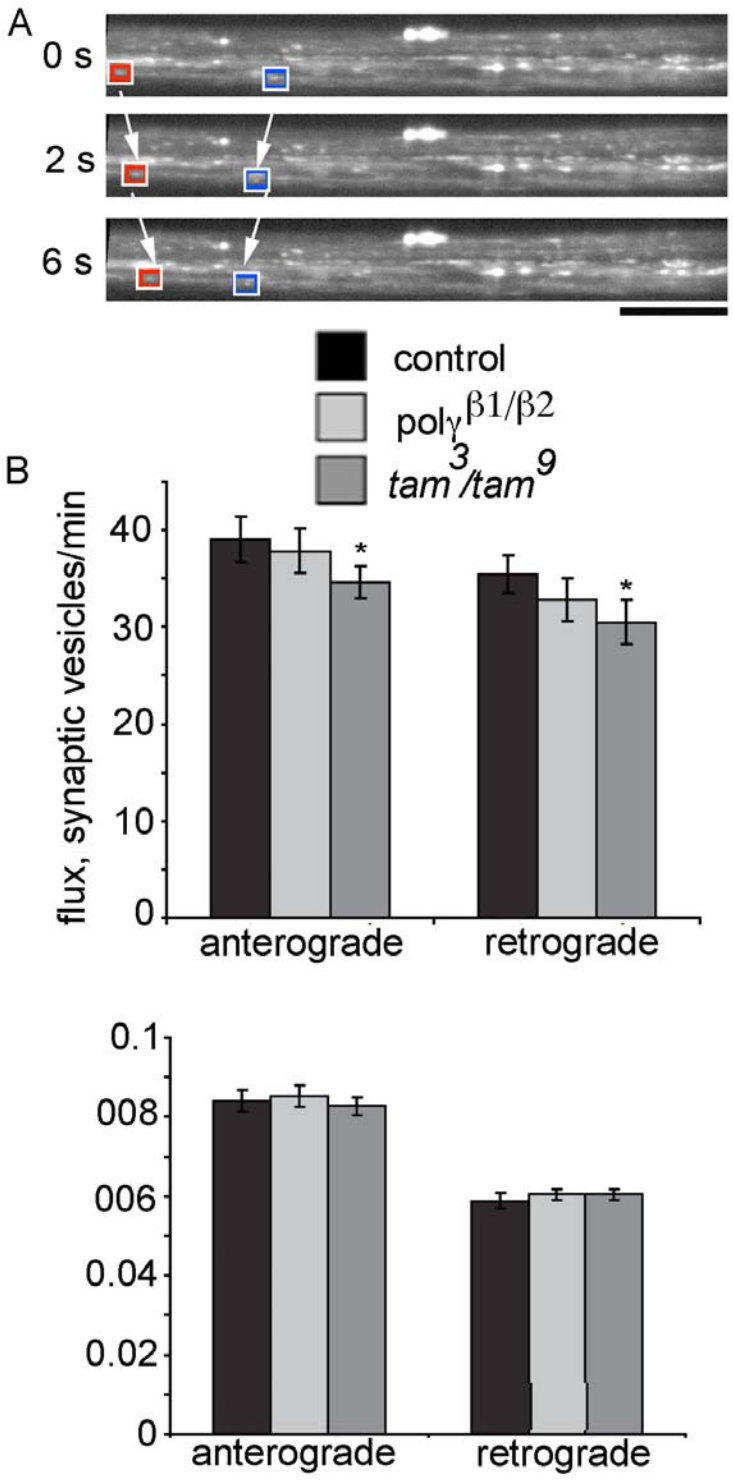

Figure 2.9. Flux of synaptic vesicle transport is moderately reduced in *tam*^{3}/ *tam*^{9} mutants whereas velocity remains unchanged

(A) *UAS-n-sybGFP* is expressed in segmental nerves of *Drosophila* larvae using the *D42*GAL4 driver. Time lapse imaging of proximal region of medial nerves allows visualization of synaptic vesicle transport. Red box indicates anterograde while blue box indicates retrograde moving vesicle. **(B)** There is a moderate decrease in bidirectional synaptic vesicle flux in tam^{3}/ tam^{9} mutants while *pol* $\gamma^{\beta 1/\beta 2}$ mutants show no significant difference in flux compared to wildtype controls. Numbers at the base of columns represent number of nerves assayed. **(C)** There is no significant change in average velocity of synaptic vesicle transport in either direction in the pol γ mutants. Numbers at the base of columns indicate number of synaptic vesicles traced. Error bars represent 95% confidence intervals. * indicates $p<0.05$ from Student's t-test.

2.3 Discussion

Disruption of axonal transport and mitochondrial function are associated with many neurological diseases. While acute disruption of oxidative phosphorylation by mitochondrial poisons (CCCP in particular) can lead to a dramatic blockade of axonal transport (Ochs and Hollingsworth, 1971; Miller and Sheetz, 2004), there have been no studies that have examined the effect of mutations in genes that disrupt mitochondrial ATP synthesis on axonal transport *in vivo.* Here, we impaired genetically the mtDNA replication machinery to study axonal transport in *Drosophila*. Animal mtDNA encodes 13 essential polypeptides that constitute subunits of the oxidative phosphorylation complexes (Shoffner and Wallace, 1994). We began with the hypothesis that depletion of mtDNA would dramatically impair mitochondrial and synaptic vesicle precursor transport in axons. Surprisingly, we found that when mtDNA is depleted in pol γ mutants, bidirectional flux of mitochondrial transport is almost doubled, and there is a significant increase in mitochondrial density along the proximal axons. Furthermore, the flux of synaptic vesicle precursors is maintained at comparable levels suggesting that there is an organelle-specific regulation of transport in mtDNA-depleted mutants.

The observed transport profiles of mitochondria and synaptic vesicle precursors suggest that normal oxidative phosphorylation is not critical for the sustenance of axonal transport. When mitochondrial ATP generation is disrupted, there is a consistent decrease in cellular ATP concentration: ~30% reduction is observed in *Drosophila* (Adan et al., 2008), a ~10-40% reduction in human cell lines (Liu et al., 2006a), and a ~40-50% reduction in cerebellar granule cells (Atlante et al., 2005). Nonetheless, this is not as dramatic as would be expected based on the contribution that mitochondrial oxidative phosphorylation normally makes to ATP generation (*i.e.*, ~90% of total ATP produced).

It is important to note that glycolysis is upregulated under oxidative stress and/ or when oxidative phosphorylation is disrupted (Gott et al., 1990a; Liu et al., 2006b). For example, depletion of mitochondrial transcription factor B2 (*TFB2M*) of the mtDNA transcription machinery leads to a metabolic shift towards glycolysis in *Drosophila* that partially restores ATP levels (Adan et al., 2008). Similarly, transcription factor A (*Tfam*) knockout mice display increased gene expression of several glycolytic enzymes (Hansson et al., 2004b). Thus it is likely that glycolysis compensates for defective oxidative phosphorylation to sustain axonal transport in *pol* γ*–*$^{\beta 1/\beta 2}$ and *tam*^{3}/ *tam*^{9} mutants.

The observed increase in bidirectional mitochondrial flux was coupled with higher mitochondrial density in muscles and proximal nerves of *tam*^{3}/ *tam*^{9} mutants. Higher density could be a consequence of two phenomena: increased mitochondrial fragmentation or addition of new mitochondria. To test these possibilities, we looked at electron micrographs of muscles and nerves from pol g mutants. There was no sign of the morphological features (damaged or missing cristae) that typically accompany unusual mitochondrial fragmentation. Further, we did not see a significant difference in density of docked mitochondria in the nerves of *tam*^{3}/ *tam*^{9} mutants. Together these data suggest that the observed increase in density and transport was due to addition of new mitochondria, albeit without mtDNA. This increase in mitochondrial number is consistent with the increased biogenesis observed in mitochondrial myopathies (Gott et al., 1990b; Heddi et al., 1999; Hansson et al., 2004a). It would be logical for diseased cells to have a checkpoint to safeguard against proliferation of defective mitochondria. Yet our data, like those obtained previously in other systems, suggests that mitochondrial biogenesis increases when mitochondrial function is compromised.

A possible explanation for the increase in bidirectional mitochondrial transport is that it is a simple reflection of a greater mitochondrial density in the nerves of the mutant animals. In the control animals we found an anterograde flux of 10.15 +/-2.48 mito/min (average +/- s.d.) and a density of 0.25 +/- 0.01 mito/μm^2 (average +/- s.d.) in the proximal nerves. In the *tam*^{3}/ *tam*^{9} animals we found an anterograde flux of 18.20 +/- 4.87 mito/min (average +/- s.d.) and a density of 0.28 +/- 0.01 mito/μm^2 (average +/- s.d.). Whereas the increase in density was statistically significant in the *tam*^{3}/ *tam*^{9} animals, it was small as compared to the increase in flux: the flux increased by 79% and the density increased by 12%. Furthermore, examination of the density of transported and docked mitochondria shows that there is little difference between the levels of docked mitochondria, but a significant increase in the number of transported mitochondria (Fig. 2.8 B). Together these data argue strongly that bidirectional mitochondrial flux is increased when mtDNA replication is inhibited.

An additional factor that may influence mitochondrial flux is that mtDNA replication might require mitochondria to dock. If so, inhibition of mtDNA replication would naturally lead to elevated transport. This raises the question of "what percentage of mitochondria undergo mtDNA replication at a given time?" Based on EM analysis of mtDNA molecules in *Drosophila* (Goddard and Wolstenholme, 1978), ~1% of mtDNA molecules are replicating. Because mitochondria are typically thought to contain ~10 copies of mtDNA, approximately 10% of all mitochondria might have replicating mtDNA. Whereas some mtDNA replication occurs along the axon, it appears that the majority occurs in the neuronal cell body (Davis and Clayton, 1996; Magnusson et al., 2003; Amiri and Hollenbeck, 2008). Thus, one could assume that along the

axon somewhat less than 10% of all mitochondria have replicating mtDNA. Thus, whereas it is possible that a decrease in mtDNA replication could lead to an increase in mitochondrial axonal transport, it would be expected to represent only a modest increase.

We previously demonstrated using the dye JC1 that polarized mitochondria are transported in the anterograde direction and depolarized mitochondria are transported in the retrograde direction along the axon (Miller and Sheetz, 2004). Our interpretation of this data was that following biogenesis newly synthesized mitochondria are transported out into the axon and damaged mitochondria are transported back to the cell body for degradation. While a recent study confirmed these results with JC-1, the investigators failed to see a correlation between membrane potential and direction of transport with the dye TMRM (Verburg and Hollenbeck, 2008). If we consider the results of the TMRM study at face value and assume that mitochondria, regardless of membrane potential, move randomly along the axon, the increased bidirectional transport of mitochondria could be interpreted simply as evidence that suggests an overall increase in mitochondrial trafficking when mtDNA replication is inhibited. Alternatively, a speculative interpretation based on the JC-1 data and our current results would suggest that inhibition of mtDNA replication increases bidirectional mitochondrial transport because biogenesis and degradation are both increased. In either case, elevated transport suggests an SOS response is occurring in a futile attempt to supply the axon with functional mitochondria.

Despite the increase in mitochondrial flux, we observed that velocity of kinesin mediated mitochondrial transport was reduced in pol γ mutants. Whereas flux reflects the demand and regulation of the cargo, transport velocity signifies motor activity. The precise reason for slower kinesin mediated mitochondrial transport is unclear. Kinesin velocity is known to be proportional to ATP concentration (Fisher and Kolomeisky, 2001). It is possible that kinesin-1, the main

mitochondrial motor protein (Pilling et al., 2006), is more sensitive to fluctuations in ATP concentration than cytoplasmic dynein. This could also account for a previous report that damaged mitochondria in cultured neurons from SOD1 mutant mice have decreased velocity of anterograde but not retrograde axonal transport (De Vos et al., 2007). In contrast, kinesin-3 is the major motor for synaptic vesicle transport (Klopfenstein and Vale, 2004; Goldstein et al., 2008). Kinesin-1 and kinesin-3 have dissimilar biophysical properties, adaptors and structure (Hirokawa and Noda, 2008). Conceivably, kinesin-3 may be less vulnerable to perturbations in ATP concentration than kinesin-1, explaining the normal velocity of synaptic vesicle precursor transport in *pol* γ–$^{\beta 1/\beta 2}$ and *tam*^{3}/ *tam*^{9} mutants. Although this impairment in anterograde mitochondrial transport is not severe by itself, it could contribute to peripheral neuropathy over a sustained time period as observed with some *POLG1* mutations (Davidzon et al., 2006) and AZT treatment (Samuels, 2006).

Mitophagy is an integral part of normal turnover and life cycle of mitochondria (Kundu and Thompson, 2005; Samuels, 2006). In muscles of wildtype *Drosophila*, we noticed extra-nuclear dsDNA clusters localized to late endosomes/ lysosomes, presumably from mitochondria that have been submitted for mitophagy. These clusters were conspicuous by their absence in *pol* γ–$^{\beta 1/\beta 2}$ and *tam*^{3}/ *tam*^{9} mutant muscles. The lack of any other known source of cellular dsDNA besides the nucleus and mitochondria suggests that they may be mtDNA aggregates from mitochondria that are destined for mitophagy. The absence of such lysosomal dsDNA clusters in *pol* γ–$^{\beta 1/\beta 2}$ and *tam*^{3}/ *tam*^{9} mutants clearly corroborates the severe depletion of mtDNA in these mutants. However, decreased mitophagy in *pol* γ–$^{\beta 1/\beta 2}$ and *tam*^{3}/ *tam*^{9} mutants remains an alternate explanation.

Interestingly, in all our studies we find that *tam*^{3}/ *tam*^{9} mutants have a more severe phenotype than *pol γ–*$^{\beta 1/\beta 2}$. In fact, even the heterozygous *tam* mutants displayed greater severity than the *pol γ–β* hetrozygotes. *tam (tamas)* encodes the catalytic subunit pol γ–α of the pol γ complex and is responsible for its DNA polymerase and exonuclease activities whereas *pol γ–β* encodes the accessory subunit pol γ–β, and enhances primer recognition and template-primer DNA binding (Kaguni, 2004). A simple explanation for the difference in phenotypic severity could be that the mutations in the *tam* alleles are more disruptive than the specific mutations in the *pol γ–β* alleles. Alternatively, because the two subunits have clearly different functions, it is possible that deleterious mutations in the catalytic core have more serious consequences for the animal.

Defects of mtDNA are an important cause of neuropathy. Although much is known about the pathological ramifications of mtDNA defects in muscles, very little is understood about its cellular impact on neurons *in vivo* (Betts et al., 2004). Based on our results, we postulate a speculative mechanistic model for the progressive neuropathy observed in mtDNA diseases: **1)** mutation or depletion of mtDNA leads to an increase in mitochondrial biogenesis to compensate for mitochondrial dysfunction; **2)** bidirectional mitochondrial transport is elevated in an attempt to supply the axon with mitochondria and as a consequence, the nerve is populated with dysfunctional mitochondria; **3)** these mitochondria consume cytosolic ATP to maintain their membrane potential (McKenzie et al., 2007) and potentially generate ROS, which increase cellular stress and lead to disrupted neuronal function and eventually cell death (Beal, 2005); **4)** and in addition, subtle transport alterations such as reduction in kinesin-based mitochondrial transport and slight reduction in the flux of synaptic vesicles, contribute to distal neuropathy.

Together, these cellular modifications could account for the sustained progression of neuronal pathology in mtDNA diseases. This model raises the intriguing question of whether cellular stress would be decreased in mtDNA diseases if mitochondrial biogenesis is intentionally inhibited, or if mitochondria lacking mtDNA have an undiscovered neuroprotective role.

This is the first study to monitor directly mitochondrial trafficking *in vivo* when mtDNA replication is genetically disrupted. While future studies are warranted to document conclusively the relevance of this work to mtDNA disease, our current findings provide potentially important and counter-intuitive insights into the biology of mitochondria in neurons.

2.4 Materials and methods

Drosophila stocks and culture

Standard cornmeal fly medium was used and all stocks maintained at 25°C. *UAS-n-SybGFP* flies were obtained from Dr. Mani Ramaswami, University of Arizona, Tucson and *UAS-mtGFP* line from Dr. William Saxton, University of California, Santa Cruz. All other stocks were obtained from the Bloomington Stock Center, Indiana. Canton-S flies were used as the wildtype control strain unless otherwise mentioned. *UAS-mtGFP* was expressed in segmental nerves using the *D42*Gal4 driver (Pilling et al., 2006). The *D42* driver predominantly expresses in motor neurons along with a few body wall sensory neurons, and the salivary glands in the larva (Sanyal, 2009). Mutations in the 34D locus on the second chromosome of *Drosophila* cause a disruption of the 125-kDa catalytic subunit (pol γ–α) of mitochondrial pol γ (Iyengar et al., 1999), encoded by the gene *tamas*. For our analysis, we used two hypomorphic alleles of this gene, tam^{3}/ tam^{9} that have a glutamate to alanine conversion at nucleotide position 1783, and a 5-bp deletion beginning at nucleotide position 3371, respectively. The gene encoding the accessory subunit of pol γ in *Drosophila* (pol γ–β) is *pol γ–β,* located in the 34D subdivision of the left arm of the second chromosome, 3.8 kb distal from the gene encoding the catalytic subunit. The mutant allele $pol\ \gamma\text{–}\beta^{1}$ is an EMS-induced mutation resulting in a glutamate substitution of a highly conserved glycine residue in the N-terminal domain of the accessory subunit. The $pol\ \gamma\text{–}\beta^{2}$ allele is a spontaneous mutation caused by an in-frame 74-bp insertion in the N-terminal domain that creates a premature stop (Iyengar et al., 2002). Both pol γ mutants are homozygous lethal at the late larval third instar stage. To avoid phenotypic expression of any

unknown background mutations, we used heteroallelic mutant combinations of *tam*^{3}/ *tam*^{9} for pol γ–α studies and *pol γ–β*^{1}*/ pol γ–β*^{2} for pol γ–β.

Immunohistochemistry and PicoGreen staining

Larvae were dissected in Ca^{2+} free saline and fixed for 20 min in 4% paraformaldehyde. Next, they were rinsed in phosphate-buffered saline (PBS) with 0.1% Tween-20 and after 30 min of blocking in 10% normal goat serum, incubated overnight at 4°C in primary antibody solution, followed by washes in PBST with three changes, and further incubation in PicoGreen dye and fluorescently-conjugated secondary antibody solution in PBST for 2 h at room temperature. Then the samples were washed in PBS and mounted under a cover glass. We used mouse anti-mitochondrial complex V monoclonal antibody at 1:500 (MitoSciences, Eugene, OR, USA), guinea pig anti-spin at 1:250 (Graeme Davis, University of California, San Francisco) and PicoGreen dye (Invitrogen, USA) at 1:200. The following fluorescently labeled secondary antibodies were used: goat anti-mouse Alexa 568 (Invitrogen, USA) and goat anti-guinea pig Alexa 568 (Molecular Probes, USA).

Mitochondrial and mtDNA nucleoid density

For measuring density, 3D reconstruction images of segmental nerves and muscle fibres 6/ 7 in abdominal segments A3 – A6 in third instar *Drosophila* larvae were used. For muscles, a 55 mm X 55 mm single frame stained with anti-complex V antibody or PicoGreen was divided into a grid of 64 regions of interest (ROI). A random list of numbers (1-64) was generated on Microsoft Excel and the number of mitochondria / mtDNA nucleoids was counted in eight of the

corresponding ROI number for each frame and averaged over the area to yield density/ μm^2. (Figure 2.2 B). For segmental nerves, *UAS-mtGFP* driven by *D42*Gal4 was imaged in the proximal, medial and distal regions of the segmental nerves; the regions were divided into 100 μm^2 bins and total mitochondria counted and averaged over that area to yield mitochondria/ μm^2 (Figure 4 A and B).

Southern blotting

Total DNA was purified from third instar larvae by standard methods. DNA (3 μg) was cleaved with *Xho*I, which cuts mtDNA once, fractionated in a 0.8% agarose gel/ TBE and transferred to a nylon membrane (Amersham Pharmacia Biotech). Hybridization was carried out for 16 h at 65°C in 10 mM sodium phosphate pH7.4/ 0.5% SDS. Filters were washed three times for 10 min at room temperature with 4 × SSC containing 0.1% SDS, once for 30 min at 65°C with 0.1 × SSC containing 0.1% SDS. Blots were probed with radiolabeled DNAs for the mitochondrial gene ATPase 6 and the nuclear histone gene cluster.

Electron Microscopy

The nerves and muscles of four different strains of *Drosophila* were examined: *pol* γ*–*$\beta 1/\beta 2$ (n = 3), *tam*^{3}/ *tam*^{9} (n = 3), Canton-S (n=2), and animals with *UAS-mtGFP; D42*Gal4 in wildtype background (n=2). Third instar larvae, 90-100 hrs after egg laying, were dissected in cold, Ca^{2+} free saline (125mM NaCl, 4mM $MgCl_2$, 10mM $NaHCO_3$, 2mM NaH_2PO_4, 5mM trehalose, 40mM sucrose, 10mM Hepes, pH 7.2). After pinning in Sylgard dishes, they were rinsed in fresh, cold, Ca^{2+} free saline and then fixed for one hour with cold fixative (4%

paraformaldehyde w/v and 1% glutaraldehyde v/v in Millonig's phosphate buffer, ph 7.2). They were then unpinned and fixed for an additional hour in fresh fixative in scintillation vials. After 12 hours rinse with 5 changes, in 0.1M phosphate buffer, they were postfixed for two hours with 1% OsO_4 in 0.1M phosphate buffer. Following an overnight rinse in 0.1M phosphate buffer, samples were dehydrated through a graded series of ethanol solutions, then propylene oxide. They were infiltrated overnight in 50% Poly/Bed 812 Araldite resin in proplylene oxide, and then 8 hours in 100% Poly/Bed 812. After placing in flat embedding molds, the resin was hardened for two days at 60^{o}C. Animals were sectioned perpendicular to the long axis and thick sections photographed in order to map and identify the individual muscles. Thin sections were photographed with a JEOL 100CX electron microscope at 60kV. Negatives were digitized at 600, 1200, 2400 or 3200 pixels per inch with an Epson V750 Pro flat bed scanner equipped with a negative carrier.

Image acquisition and analysis of axonal transport

Crawling third instar *Drosophila* larvae were selected and anaesthetized in halocarbon oil 700 (Sigma) with 10-25% chloroform, titrated to levels just sufficient to inhibit significant muscular contraction. The larvae were then mounted between a slide and coverslip and were imaged for no more than 15 minutes at ~25°C (Figure 2.7 A). All images were acquired on a swept field confocal microscope with NIS software using a Nikon TE2000-E inverted microscope and a PlanApo 60X oil objective, NA 1.4. The aperture and exposure were set at 70-slit and 100 ms, respectively, and images were captured at 2 s intervals for total time of 7 min for a time-lapse series.

NIS files were opened in ImageJ, and frames were aligned using the StackReg plugin with rigid body settings. The two medial nerves at the base of the ventral nerve cord were selected for each analysis (Figure 2.7 B), cropped and rotated, using TJ Rotate with cubic-B-spline interpolation, so that the axons were always oriented horizontally with the cell body on one side and the synapse on the other. These images were re-sliced and z-projected using the sum-slices option to generate kymographs. The kymographs were opened in Adobe Photoshop, image color depth was converted from 16 bits/ pixel to 8 bits/ pixel and color inverted to facilitate better visibility of transport events.

For the flux, total numbers of transport events in each direction were counted at three different positions along each axon at different time points and an average was taken. This was considered one data point of transport in units of mitochondria/ min or synaptic vesicles/ min. For velocity of transport, lines were hand-traced over the path of mitochondria or synaptic vesicle precursors on the kymographs, on different layers in Adobe Photoshop and the slope calculated for each (Figure 2.7 C). These results were then exported to Microsoft Excel to calculate average velocity of transport in units of mm/ s.

For measurement of docked mitochondria, total numbers of mitochondria that remained stationary during the entire duration of observation were counted and divided by the length of nerve imaged to yield docked mitochondria/ μm. Data from 10-12 nerves was averaged in each case. These observations were made in the same regions in which flux was measured.

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Chapter 3
TRAP1, a Novel Modulator of the Mitochondrial UPR, Promotes Oxidative Stress Resistance and Extends Healthspan in *Drosophila*

Preface to Chapter 3

This chapter describes the role of the mitochondrial chaperone, TRAP1, in regulating oxidative stress resistance, lifespan and healthspan in *Drosophila*. The identification of TRAP1 as a novel regulator of the mitochondrial unfolded protein response provides mechanistic insight to its physiological role.

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Rehan M. Baqri, Arielle V. Pietron, Douglas H. Roossien, Rewatee H. Gokhale, Brittany A. Turner, Laurie S. Kaguni, Alexander W. Shingleton, & Kyle E. Miller

"TRAP1, a Novel Modulator of the Mitochondrial UPR, Promotes Oxidative Stress Resistance and Extends Healthspan in *Drosophila*" (Manuscript in preparation)

Abstract

The molecular mechanisms influencing healthspan are unclear (Tatar, 2009; Yu and Driscoll, 2011), but mitochondrial function, resistance to oxidative stress and proteostasis are recurring themes. Tumor necrosis factor Receptor Associated Protein 1 (TRAP1) is a mitochondrial analogue of Hsp75 and a member of the Hsp90 family (Felts et al., 2000). TRAP1 regulates the level of reactive oxygen species (ROS) *in vitro* (Gesualdi et al., 2007; Hua et al., 2007; Im et al., 2007), and is found expressed at higher levels in tumor cells where it is thought to play a pro-survival role (Kang et al., 2007; Costantino et al., 2009). While TRAP1-directed compartmentalized protein folding is a promising target for cancer therapy, its role at the organismal level is unknown (Altieri et al., 2011). Here we report in *Drosophila* that depletion and overexpression of TRAP1 decrease and increase oxidative stress resistance and healthspan respectively, but have little effect on lifespan. In addition, loss or overexpression of TRAP1 enhances translocation of the homeobox protein Dve from the cytosol to the nucleus, paired with increased expression of *Hsp60, mtHsp70* and a putative protease, *CG5045*. Together these results indicate that alterations in TRAP1 dosage induce the mitochondrial unfolded protein response (UPR^{mt}). These studies provide the first evidence for UPR^{mt} in *Drosophila* and suggest that TRAP1 is integral to maintaining mitochondrial proteostasis and regulating healthspan.

Introduction

Aging is a multifaceted process that is accompanied by the progressive inability to deal with cellular stress. Evidence from diverse model systems implicates hundreds of genes that regulate longevity, which primarily fall within pathways that regulate energy metabolism, nutrient sensing and stress resistance (Narasimhan et al., 2009). Mitochondria, as central regulators of multiple cellular processes, are known to modulate aging at several levels such as accumulation of mitochondrial DNA mutations, dysfunction of the electron transport chain and generation of reaction oxygen species (ROS). In particular, chronic exposure to ROS induced-oxidative stress or decreased anti-oxidant defenses damage cellular macromolecules and are correlated with the aging process (Finkel and Holbrook, 2000; Cho et al., 2011). Fortunately, the cell has several mechanisms to counter stress, including the activation of coordinated stress response pathways and chaperone networks, impairment of which are implicated in aging and age-related disorders (Morimoto, 2008).

Heat shock proteins (Hsp) help to cope with the stress of misfolded proteins and aggregates and play key roles in stress resistance in *Drosophila, C. elegans* and mammals (Calderwood et al., 2009). Long-lived and stress resistant animal models in the insulin/IGF-1 signaling or the dietary restriction pathways have increased Hsp16 expression (Wadhwa et al., 2005). Cells from human centenarians also exhibit remarkably preserved HSP70 synthesis in response to stress (Marini et al., 2004). Changes in cytosolic Hsp levels and their induction capacity are observed in several age-associated diseases as well as aging itself (Tatar et al., 1997; Tower, 2011). However, the role of mitochondrial Hsps in the aging process largely remains elusive.

Age related impairment of locomotor, reproductive and physiological functions is a near universal phenomenon in animals. A steering implication of aging research is that interventions that increase longevity also increase the healthspan of an organism. However, the molecular mechanisms that regulate healthspan are not understood. Tumor necrosis factor Receptor Associated Protein 1 (TRAP1) is a mitochondrial analogue of Hsp75 and a member of the Hsp90 family (Felts et al., 2000). It was originally identified as a binding partner of the intracellular domain of TNFR1 (Song et al., 1995). TRAP1 is found expressed at higher levels in tumor cells where it is thought to play a pro-survival role by inhibiting apoptosis (Kang et al., 2007; Costantino et al., 2009). *In vitro* evidence suggests that TRAP1 regulates ROS levels and protects cells against oxidative damage (Gesualdi et al., 2007; Hua et al., 2007; Im et al., 2007). Recent evidence has uncovered the role of mitochondrial proteostasis in healthspan regulation (Luce and Osiewacz, 2009). Because TRAP1 is a mitochondrial chaperone, we sought to determine the role of TRAP1 in oxidative stress resistance, lifespan and healthspan in *Drosophila* and uncover its mechanistic basis.

Results & Discussion

In vitro evidence indicates that loss of TRAP1 results in increased accumulation of cellular ROS (Hua et al., 2007). To determine whether TRAP1 regulates ROS levels *in vivo*, we assayed ROS levels in the brains of adult *Drosophila* with MitoSox, a cell permeable dye that fluoresces when oxidized by superoxides and is rapidly and selectively targeted to the mitochondria. There is a marked increase in MitoSox staining in the brains of *TRAP1* deletion mutant, *w; TRAP1Δ4/ TRAP1Δ4 ; +/+*, but not when TRAP1 was overexpressed using the constitutive and ubiquitous driver *Actin*Gal4 (Figure 3.1 A and B). Mitochondrial distribution was not significantly altered in the segmental nerves of these strains, indicating that increased MitoSox staining in the mutants was not due to changes in mitochondrial density (Figure 3.1 C).

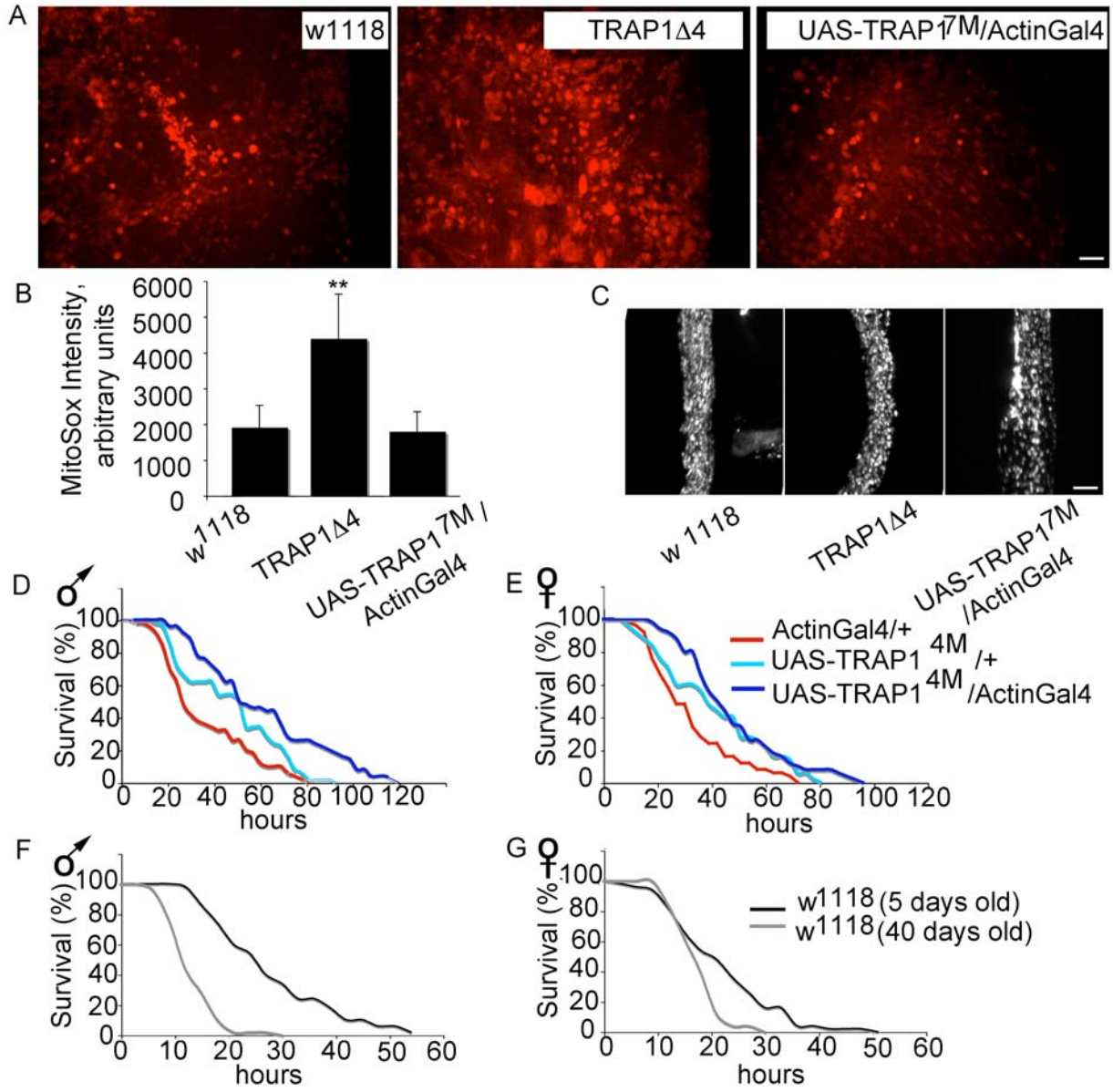

Figure 3.1. *TRAP1* regulates ROS levels and oxidative stress resistance *in vivo*

(A) Optic lobes of young adult *Drosophila* brains stained with Mitosox. **(B)** MitoSox staining is significantly increased in *w; TRAPΔ4/TRAPΔ4; +/+* mutants. While staining is marginally reduced in overexpression flies *w; +/+; UAS-TRAP1*^{7M}*/Actin*Gal4, it is not significantly

different from wildtype. Error bars denote standard deviation of means. N = 3-5. (**) indicate p < 0.001 from Student's t-test. **(C)** Mitochondrial distribution appears normal in mutant and overexpression animals as determined by immunostaining with antibody against ComplexV in segmental nerves of *Drosophila* larvae. In all cases, scale bars equal 10 μm. **(D and E)** Oxidative stress resistance is increased by ubiquitous overexpression of TRAP1 in males, in an independent transgenic strain. **(D)** Median survival on 20 mM paraquat of male 5-days old *w*; *+/+; UAS-TRAP1*^{4M}*/Actin*Gal4 (61.62 ± 3.92 hrs) is significantly more than median survival of *w; +/+; Actin*Gal4/+ males (36.18 ± 2.76 hrs; p<0.0001), and *w; +/+; UAS-TRAP1*^{4M}/+ (47.76 ± 3.1 hrs; p=0.016). **(E)** Median survival of female *w*; *+/+; UAS-TRAP1*^{4M}*/Actin*Gal4 (47.8 ± 2.7 hrs) is significantly more than *w; +/+; Actin*Gal4/+ females (32.4 ± 2.2 hrs; p<0.0001), but comparable to median survival of *w; +/+; UAS-TRAP1*^{4M}/+ females (41.3 ± 3.0 hrs; p=0.24). **(F and G)** Oxidative stress resistance declines with age in both sexes. Young and old *w*^{1118}*; +/+; +/+* were subjected to identical treatment with 20 mM paraquat. **(F)** Median survival of young males (28.4 ± 1.6 hrs) is significantly higher than old males (13.8 ± 0.6 hrs; p<0.001). **(G)** Median survival of young females (22.4 hrs $\pm$ 1.4) is significantly higher than old females (18.1 $\pm$ 0.6; p=0.018). Statistical significance was determined using the standard chi-squared based log-rank test.

Increased ROS levels are associated with oxidative damage and diminished stress resistance in several animal models (Sohal, 2002). To examine if TRAP1 regulates resistance to oxidative stress, we exposed 5-day old *TRAP1Δ4* mutant flies to paraquat and assayed for survival. Paraquat is a methyl viologen that undergoes redox cycling to generate superoxide (Bus and Gibson, 1984). Young *TRAP1Δ4* mutant males were significantly more susceptible to oxidative stress as compared to males of the w^{1118} wildtype background strain (Figure 3.2 A). In contrast, resistance to oxidative stress was significantly increased when TRAP1 was ubiquitously overexpressed in males of two independent transgenic strains, *w; +/+; UAS-TRAP1*^{7M}*/Actin*Gal4 and *w: +/+; UAS-TRAP1*^{4M}*/Actin*Gal4 (Figure 3.2 B and 3.1 D). Interestingly, this response was sex specific: female flies in both the mutant and overexpression strains did not show pronounced changes in oxidative stress resistance (Figure 3.2 C, D, and 3.1 E).

To verify the specificity of the deletion mutant and efficacy of the *UAS-TRAP1* construct, we rescued loss of function by over-expressing TRAP1 in the *TRAP1Δ4/ TRAP1Δ4* mutant background. Median survival on paraquat was significantly enhanced in *w; TRAP1Δ4/ TRAP1Δ4; UAS-TRAP1*^{7M}*/Actin*Gal4 flies as compared to *w; TRAP1Δ4/ TRAP1Δ4*; *+/+* mutants (Figure 3.2 E). These results suggest strongly that TRAP1 is an important regulator of ROS *in vivo*, and regulates resistance to oxidative stress in young adults.

Consistent with previous reports (Parashar et al., 2008)**,** we also found that oxidative stress resistance declined as a function of age in wild-type flies (Supplementary Figure 3.1 F and G). To determine whether TRAP1 over-expression in older flies could confer protection against oxidative stress, we transiently over-expressed TRAP1 in 40-day old flies using the GeneSwitch-

*tub5*Gal4 driver and assessed oxidative stress resistance against paraquat. Median survival was significantly increased in *UAS-TRAP1*^{7M}/ *GS-tub5*Gal4 males maintained on RU-486 as compared to control flies of the same genotype, sex and age maintained on the vehicle (Figure 1 F). These results suggest that a high transient dosage of TRAP1 is sufficient to improve resistance to oxidative stress in older flies. Together, these data implicate TRAP1 as an important modulator of oxidative stress resistance in aging *Drosophila*.

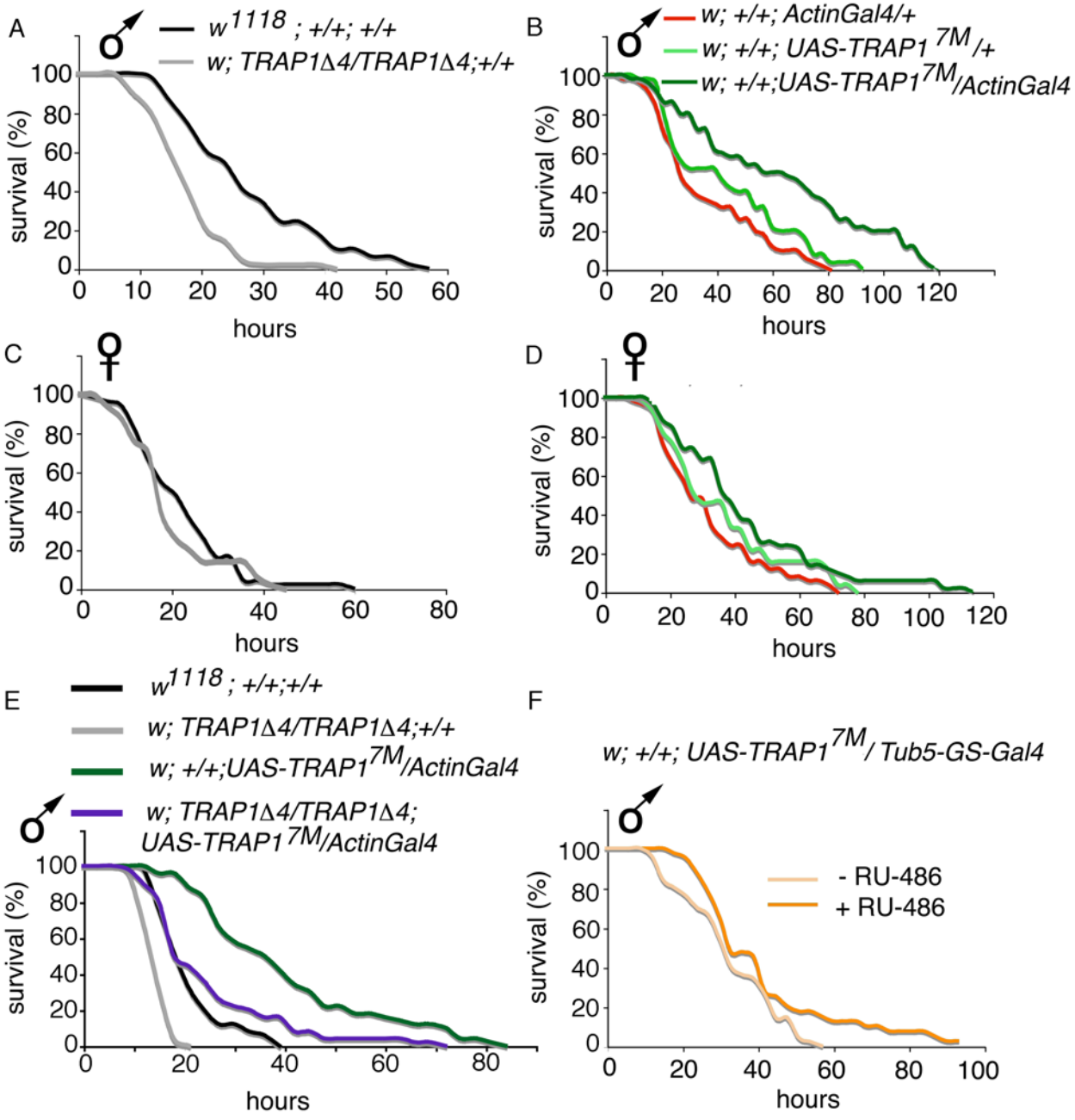

Figure 3.2. TRAP1 regulates oxidative stress resistance in young and old *Drosophila* males.

(A–E) Survival curves of young flies (5 days) of indicated genotypes exposed to 20 mM paraquat. **(A)** Median survival of male *w; TRAP1Δ4/ TRAP1Δ4; +/+* (18.5 hrs ± 0.9) is significantly less than that of w^{1118}*; +/+; +/+* (28.4 hrs ± 1.6; p<0.0001). **(B)** Median survival

of male *w; +/+; UAS-TRAP1*^{7M}*/Actin*Gal4 (63.2 hrs ± 4.8) is significantly more than that of *w; +/+; UAS-TRAP1*^{7M}*/ +* (43.9 hrs ± 3.2; p=0.0002), and that of *w; +/+; Actin*Gal4 (36.2 ± 2.8; p<0.0001)**. (C)** Median survival of female *w; TRAP1Δ4/ TRAP1Δ4; +/+* (20.3 hrs ± 1.4) is comparable to that of *w*^{1118}*; +/+; +/+* (22.4 hrs ± 1.4; p=0.4). **(D)** Median survival of female *w; +/+; UAS-TRAP1*^{7M}*/Actin*Gal4 (43.9 hrs ± 3.3) is significantly higher than that of *w; +/+; Actin*Gal4/+ (32.4 hrs ± 2.2; p=0.004) but comparable to that of *w; +/+; UAS-TRAP1*^{7M}*/ +* (36.9 hrs ± 2.7; p=0.1). **(E)** Median survival of young *w; TRAP1Δ4/ TRAP1Δ4; UAS-TRAP1*^{7M}*/Actin*Gal4 males (25.26 hrs ± 1.88) is significantly higher than *w; TRAP1Δ4/ TRAP1Δ4; +/+* (15 hrs ± 0.4; p<0.0001). **(F)** Median survival is increased in old (40 days) *w; +/+; UAS-TRAP1*^{7M}*/ GS-tub5*Gal4 males maintained on 5 mM RU-486 (41.47 hrs ± 2.99), relative to control flies of the same genotype, sex and age maintained on vehicle (32.88 ± 1.78; p = 0.03). In all cases, errors denote standard deviation. Statistical significance was determined using the standard chi-squared based log-rank test.

While recent reports indicate that ROS levels may not regulate longevity directly, overexpression of chaperones and enhanced resistance to oxidative stress are both associated with lifespan extension (Orr and Sohal, 1994; Walker and Lithgow, 2003; Sanz et al., 2010). It has been reported previously in multiple model systems that overexpressing small heat shock proteins (Hsps) such as Hsp16, Hsp 27 and Hsp22 leads to a sizeable extension in lifespan (Walker and Lithgow, 2003; Morrow et al., 2004; Wang et al., 2004). However, overexpression of larger Hsps such as Hsp70 and Hsp60 do not affect lifespan dramatically (Tatar et al., 1997; Wadhwa et al., 2005), and in the case of induced Hsp70 expression can even be deleterious for growth in *Drosophila* cells (Feder et al., 1992). To examine whether TRAP1-mediated oxidative stress resistance is associated with alteration in longevity, we conducted lifespan analyses. Survival of TRAP1 mutant males and females is not significantly different from controls (Figure 3.3 A and B). We did observe a statistically significant increase in median lifespan in one of the TRAP1 overexpressing strains, *UAS-TRAP1*^{7M}*/Actin*Gal4 (Figure 3.3 D). However, this effect was relatively minor (~ 4–14% increase in median lifespan), unlike the substantial increase in oxidative stress resistance in TRAP1 overexpressing flies (~ 44% increase in median survival in males). Moreover, it was not significant in the females and altogether absent in the independent overexpression strain, *UAS-TRAP1*^{4M}*/Actin*Gal4 (Figure 3.3C and Figure 3.4). Together, these observations suggest that knockout or overexpression of TRAP1 has little effect on lifespan.

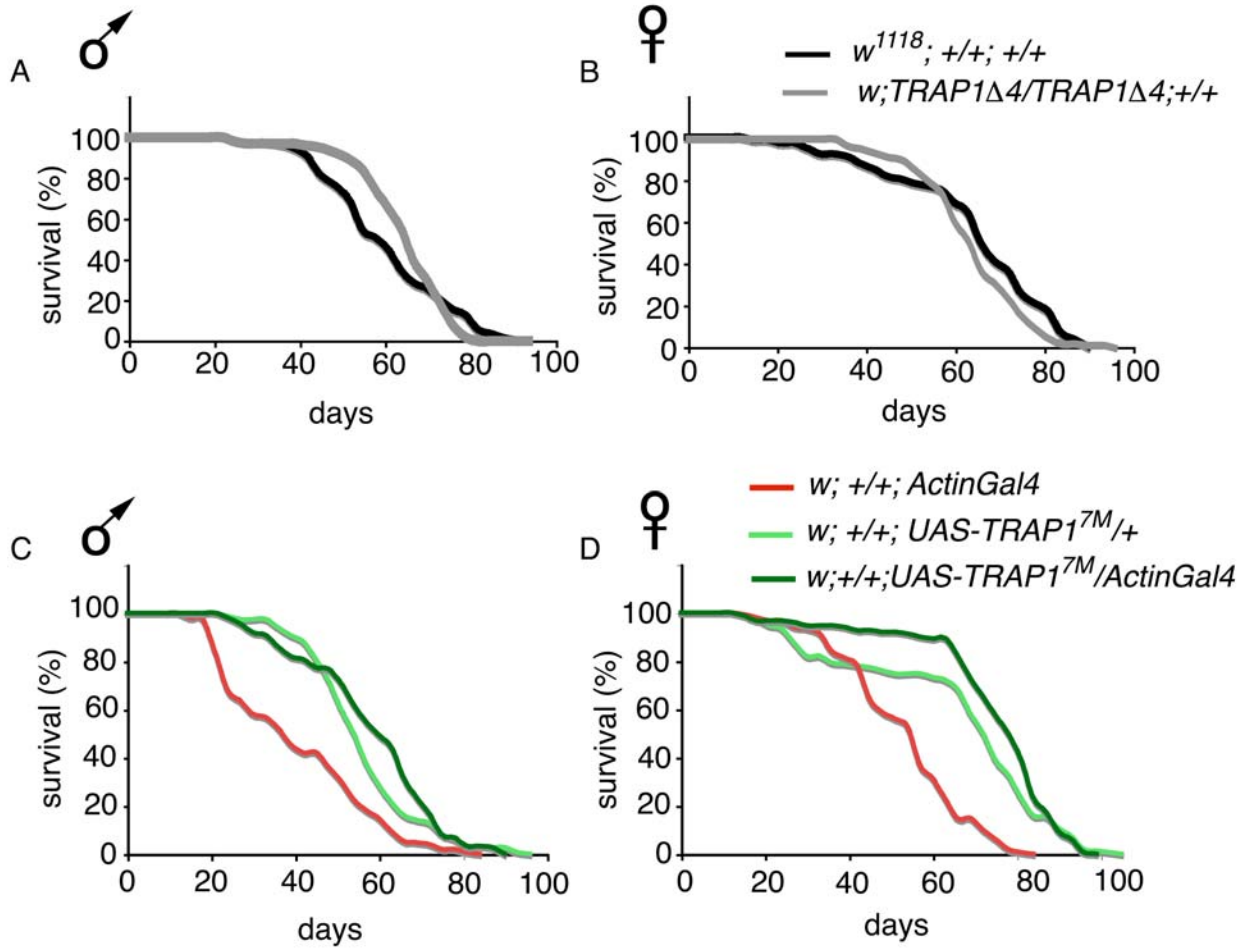

Figure 3.3. TRAP1 has a minor influence on lifespan.

Lifespan curves of indicated genotypes. **(A)** Median lifespan of male w^{1118}*; +/+; +/+* (60.4 ± 1.4 days) is comparable to that of *w; TRAP1Δ4/ TRAP1Δ4; +/+* (64.9 ± 1.1 days; p=0.48). **(B)** Median lifespan of female w^{1118}*; +/+; +/+* (64.6 ± 1.44 days) is comparable to *w; TRAP1Δ4/ TRAP1Δ4; +/+* (64.32 ± 0.98 days; p=0.08). **(C)** Median lifespan of male *w; +/+; UAS-TRAP1*^{7M}*/Actin*Gal4 (58.6 ± 1.3 days) is significantly more than *w; +/+; Actin*Gal4/+ (40.7 ± 1.4 days; p<0.001), and marginally more than that of *w; +/+; UAS-TRAP1*^{7M}*/ +* (56.5 ± 1.1

days; p=0.05). **(D)** Median lifespan of female *w; +/+; UAS-TRAP1*^{7M}/*Actin*Gal4 (75.5 ± 1.4 days) is significantly more than that of *w; +/+; Actin*Gal4/+ (53.5 ± 1.2 days; p<0.001), and marginally more than that of *w; +/+; UAS-TRAP1*^{7M}/ + (66.5 ± 1.9; p=0.044). In all cases, errors denote standard deviation. Statistical significance was determined using the standard chi-squared based log-rank test.

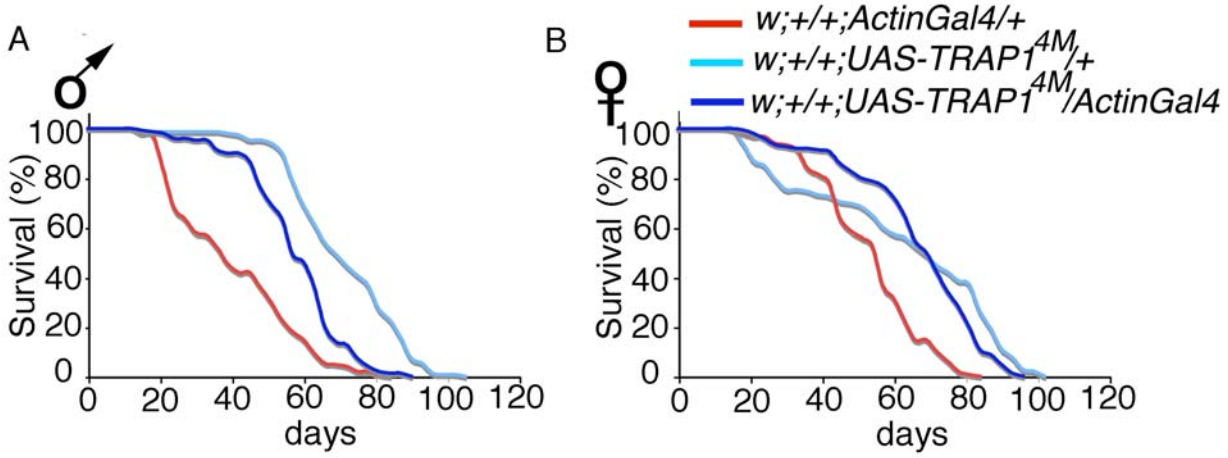

Figure 3.4. Ubiquitous overexpression of TRAP1 does not affect lifespan in an independent transgenic strain

(A) Median lifespan of male *w; +/+; UAS-TRAP1*^{4M}*/Actin*Gal4 (58.2 ± 1.1 days) is significantly more than median lifespan of *w; +/+; Actin*Gal4/+ (40.7 ± 1.4 days; p<0.001), but significantly less than median lifespan of *w; +/+; UAS-TRAP1*^{4M}/+ (71.3 ± 1.3 days; p<0.001). Thus, due to its lifespan being intermediate to the two control strains, we conclude that there is no significant alteration of lifespan in this overexpression strain. **(B)** Median lifespan of female *w; +/+; UAS-TRAP1*^{4M}*/Actin*Gal4 (67.2 ± 1.5 days) is significantly more than *w; +/+; Actin*Gal4/+ (53.5 ± 1.2 days; p<0.001), but comparable to median lifespan of *w; +/+; UAS-TRAP1*^{4M}/+ (62.8 ± 2.2 days; p=0.06). Statistical significance was determined using the standard chi-squared based log-rank test.

Recent evidence has uncovered the role of mitochondrial proteostasis in healthspan regulation (Luce and Osiewacz, 2009). Because TRAP1 is a mitochondrial chaperone, we sought to determine whether TRAP1 would regulate healthspan. In *Drosophila*, negative geotaxis (the ability of flies to climb vertically when startled) is an established paradigm to assess age-related locomotor impairment (Rhodenizer et al., 2008). We found in a longitudinal study that locomotor performance in TRAP1 mutant flies was decreased relative to control flies at older ages (Figure 3.5 A and B). In contrast, male and female TRAP1 overexpressing flies (*UAS-TRAP1*^{7M}/*Actin*Gal4 and *UAS-TRAP1*^{4M}/*Actin*Gal4) climb significantly better than the driver alone and transgene alone controls at 60 and 70 days of age (Figure 3.5 C and D, Figure 3.6 A and B). As a second indicator of healthspan, we determined the fertility of TRAP1 mutant and overexpression flies. TRAP1 mutant females show a severely compromised fecundity at all age-points (Figure 3.5 E). In contrast, overexpression of TRAP1 results in increased fecundity (Figure 3.5 F). In addition, TRAP1 mutant males tend to be less fertile than control males through all age-points (Figure 3.6 C), whereas males overexpressing TRAP1 produced significantly more progeny at older ages than control strains (Figure 3.6 D). Together, these results suggest strongly that over-expression of TRAP1 extends healthspan in both male and female *Drosophila*.

Increased antioxidant activity exclusively in the nervous system has been reported to extend lifespan and enhance stress resistance (Parkes et al., 1998; Mattson et al., 2002), presumably through a cell non-autonomous mechanism (Durieux et al., 2011). To test whether overexpressing TRAP1 in the nervous system alone would be beneficial, we used the pan-neuronal driver *elav*Gal4 for tissue specific overexpression. While overexpression of TRAP1

exclusively in the nervous system was insufficient to confer oxidative stress resistance (Figure 3.6 E and F), or extend lifespan (Figure 3.6 G and H), it improved locomotor performance of both sexes at older age-points in *w; +/+; UAS-TRAP1*^{4M}*/elav*Gal4 strain (Figure 3.5 G and H). Given previous work indirectly implicating TRAP1 in Parkinson's disease via its interaction with PINK1 (Pridgeon et al., 2007), these data raise the question of whether augmenting TRAP1 expression would be beneficial in human neurological disease.

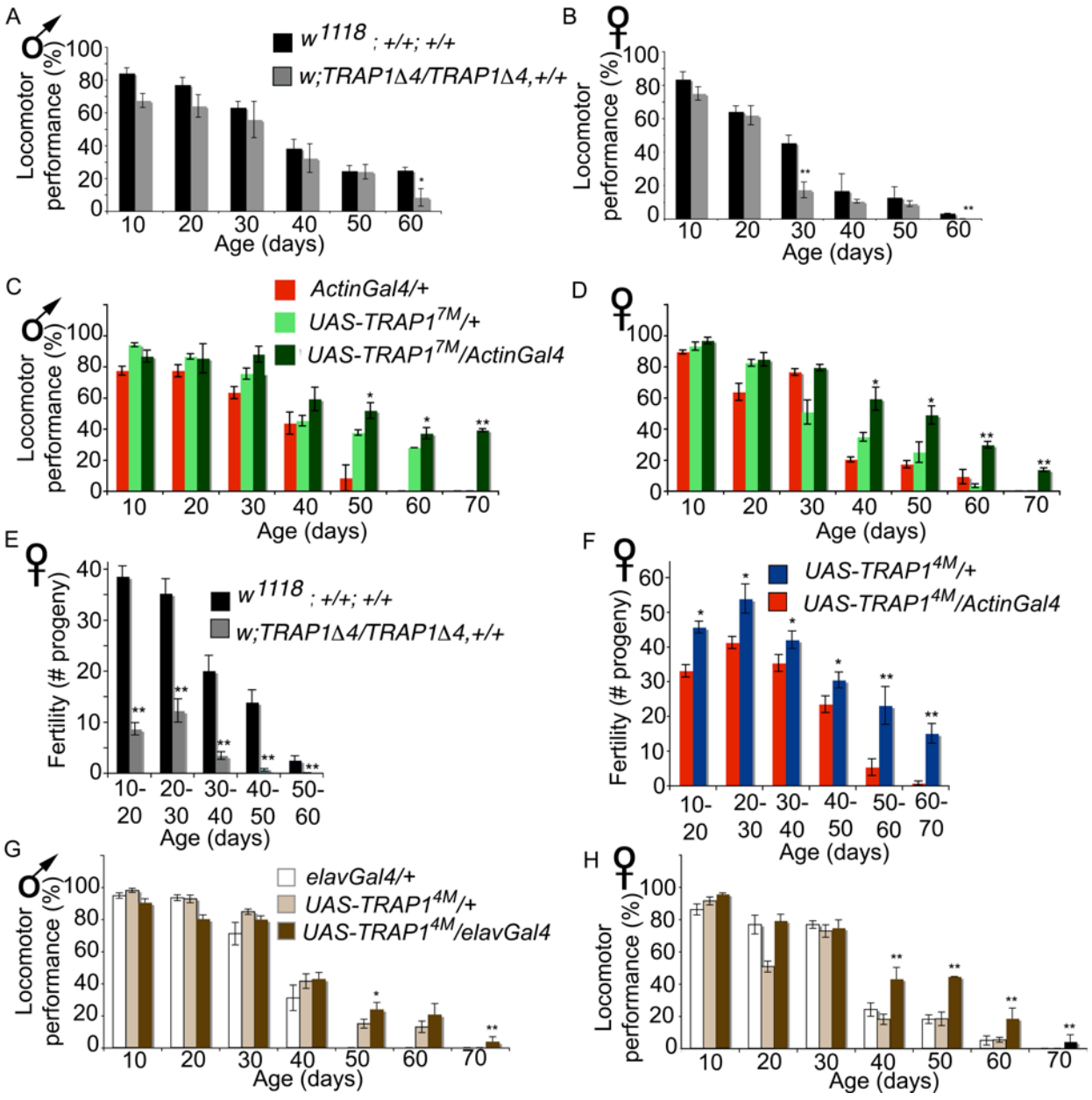

Figure 3.5. TRAP1 modulates healthspan.

(A and **B)** TRAP1 mutant flies tend to climb more poorly than control in the negative geotaxis assay and the difference becomes significant at older ages. There is a significant drop in

performance of mutant females at 30 days. By 60 days, fewer male and female *w; TRAP1Δ4/TRAP1Δ4; +/+* flies climbed successfully as compared to w^{1118}*; +/+; +/+*. **(C** and **D)** Locomotor performance after 40 days of age is maintained remarkably in *w; +/+; UAS-TRAP1*^{7M}*/Actin*Gal4 males and females on ubiquitous overexpression of TRAP1. **(E)** *w; TRAP1Δ4/TRAP1Δ4; +/+* females produce fewer progeny than control at all stages of their life. **(F)** *w; +/+; UAS-TRAP1*^{7M}*/Actin*Gal4 females produce more progeny than control at all stages of their life. In all cases error bars denote standard error of means; (*) indicates $p < 0.05$, (**) indicates $p < 0.001$. **(G and H)** Male and female flies overexpressing TRAP1 exclusively in the nervous system displayed significant maintenance of locomotor ability at older age-points in *w; +/+; UAS-TRAP1*^{4M}*/ elav*Gal4; (*) indicate $p<0.05$, (**) indicate $p<0.001$.

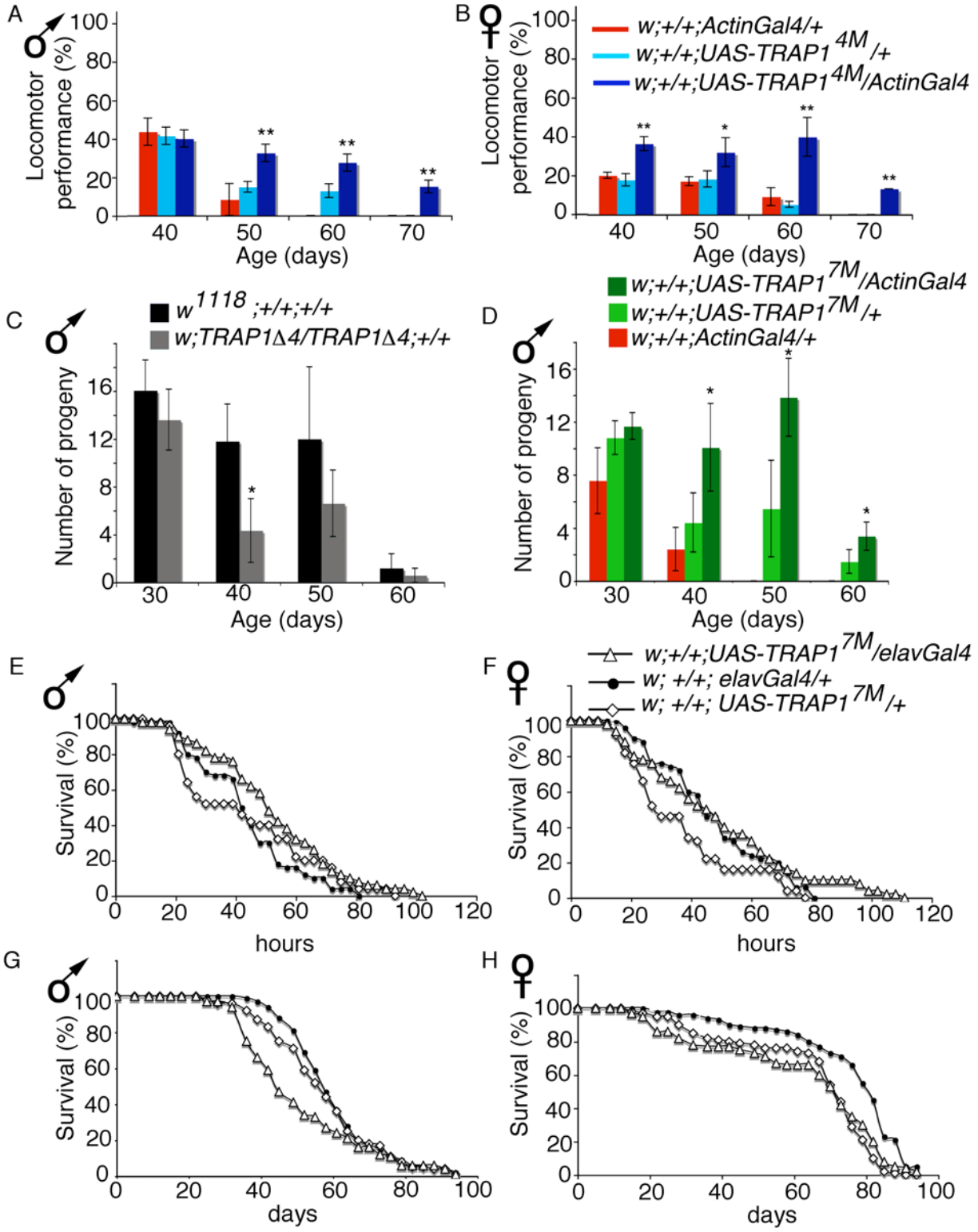

Figure 3.6. Ubiquitous overexpression of TRAP1 extends healthspan while neuronal-specific overexpression has no effect on oxidative stress resistance or lifespan.

(Figure 3.6 continued) (A and B) Locomotor performance in the negative geotaxis assay of males and females of *w; +/+; UAS-TRAP1*^{4M}*/Actin*Gal4 flies ubiquitously overexpressing TRAP1, is improved in older flies as compared to transgene alone and driver alone controls of the same age. **(C)** Loss of TRAP1 has a marginal effect on male fertility. *w; TRAP1Δ4/TRAP1Δ4; +/+* males exhibit a significant reduction in progeny at 40-days of age. **(D)** Overexpression of TRAP1 in *w; +/+; UAS-TRAP1*^{7M}*/Actin*Gal4 results in males producing significantly more offspring as compared to controls at older age. Error bars indicate standard error of means; (*) indicate $p<0.05$, (**) indicate $p<0.001$. **(E)** Median survival of male *w; +/+; UAS-TRAP1*^{7M}*/ elav*Gal4 (52.7 ± 3.0 hrs) is comparable to *w; +/+; UAS-TRAP1*^{7M}*/+* (43.9 ± 3.2 hrs; p=0.2). **(F)** Median survival of female *w; +/+; UAS-TRAP1*^{7M}*/ elav*Gal4 (49 ± 3.6 hrs) is higher than *w; +/+; UAS-TRAP1*^{7M} */+* (36.9 ± 2.7 hrs; p=0.01), but comparable to *w; +/+; elav*Gal4*/ +* (47.6 ± 2.6 hrs; p=0.5). **(G)** Median lifespan of male *w; +/+; UAS-TRAP1*^{7M} */elav*Gal4 (50.8 ± 1.3 days) is significantly more than *w; +/+; elav*Gal4/+ (56.9 ± 1.1; p=0.009), but significantly less than *w; +/+; UAS-TRAP1*^{7M} */+* (56.5 ± 1.1 days; p=0.02). **(H)** Median lifespan of female *w; +/+; UAS-TRAP1*^{7M} */elav*Gal4 (70.7 ± 2.1 days) is more than *w; +/+; UAS-TRAP1*^{7M} */+* (66.5 ± 1.9 days; p=0.0018), but comparable to *w; +/+; elav*Gal4/+ (76.7 ± 1.4 days; p=0.82). Statistical significance was determined using the standard chi-squared based log-rank test.

The mitochondrial unfolded protein response (UPR^{mt}) is a protective response pathway between mitochondria and the nucleus that is initiated in response to a mitochondrial stress signal (Haynes and Ron, 2010). Induction of the UPR^{mt} results in enhanced mitochondrial proteostasis in mammalian cell culture and *C. elegans* (Zhao et al., 2002; Yoneda et al., 2004) However, the presence of this stress response pathway in *Drosophila* has not been established previously. Because TRAP1 is part of the mitochondrial proteostasis machinery, we hypothesized that it is involved in the regulation of the UPR^{mt}. As part of the UPR^{mt} in worms, transcription factors such as DVE-1 and UBL-5 translocate from the cytoplasm to the nucleus to promote the transcription of chaperones (Haynes and Ron, 2010). In adult wildtype flies, Dve, the *Drosophila* homolog of DVE-1, is localized predominantly in the cytoplasm of neuronal cell bodies (Figure 3.7 A). In contrast, Dve in TRAP1 mutants displayed a diffuse staining pattern (Figure 3.7 B) indicating nuclear translocation (Figure 3.7 D), and induction of the UPR^{mt}. In worms, this nuclear translocation of transcription factors promotes the expression of protective chaperones and proteases for more efficient proteostasis (Haynes and Ron, 2010). To test if TRAP1 regulates the UPR^{mt}, we examined expression levels of genes known to be associated with the UPR^{mt}, including *Hsp60, mtHsp70* and a putative *Drosophila* protease *CG5045* that is 77% identical to the worm ClpP (Flybase). In males, loss of TRAP1 led to strong upregulation of *Hsp60, mtHsp70* and *CG5045* (Figure 3.7 E, G and I). As with resistance to oxidative stress, this response is sex-specific: in females only *Hsp60* was increased significantly (Figure 3.7 F, H and J). These results suggest that depletion of TRAP1 leads to induction of a robust UPR^{mt}, presumably in response to increased level of unfolded proteins. In the TRAP1 overexpression

strains we also found translocation of Dve into the nucleus in some cells, but it was not as consistent as in the TRAP1 mutant flies (Figure 3.7 C and D). Similarly expression of the UPR^{mt} genes were modestly elevated in the overexpression strain and displayed a similar sex dependent transcription profile as mutants (Figure 3.7 E–J). These results suggest that overexpression of TRAP1 also induces a moderate UPR^{mt} response in males.

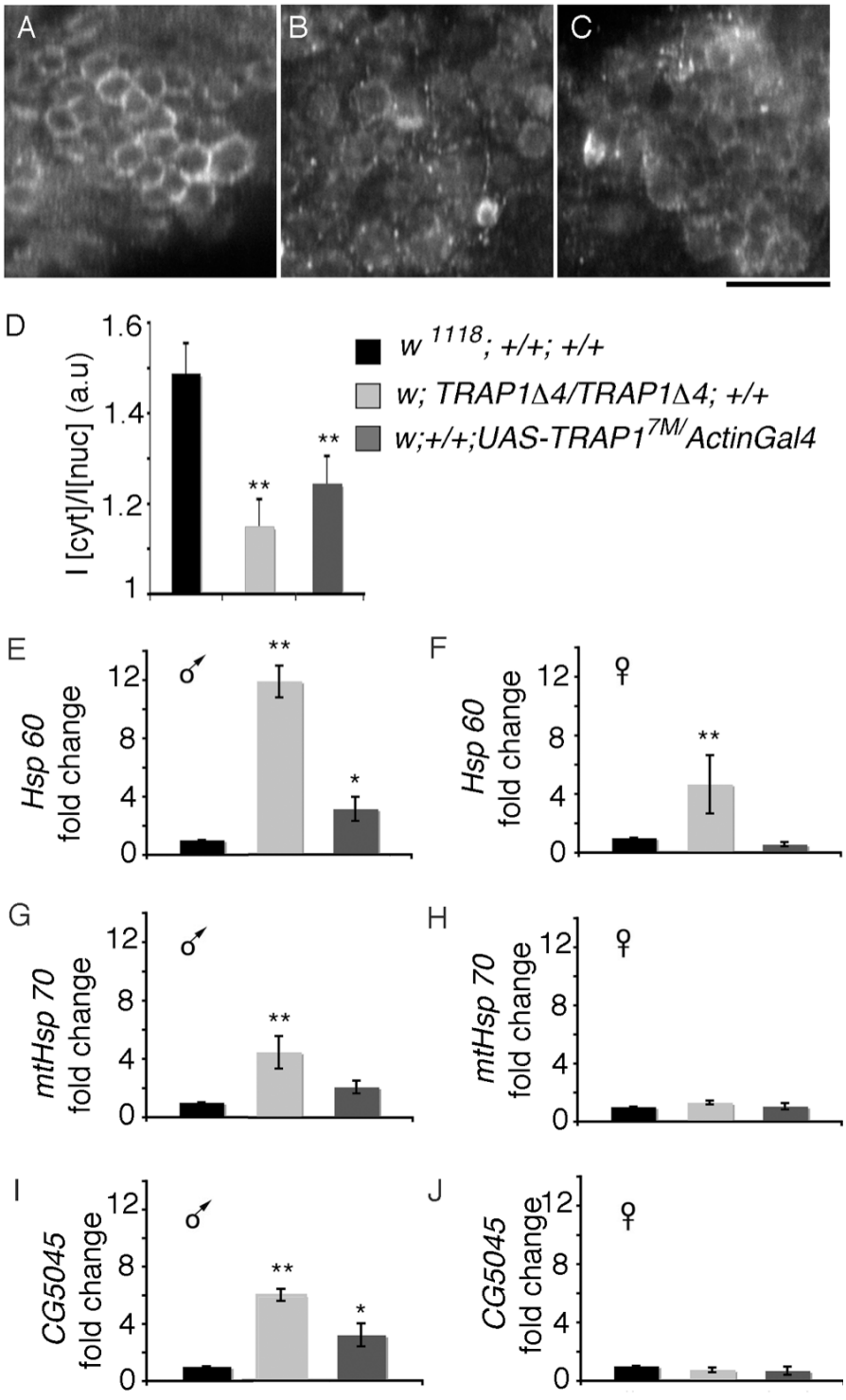

Figure 3.7. Loss and overexpression of TRAP1 induces nuclear translocation of Dve and promotes transcription of UPR^{mt} genes.

(Figure 3.7 continued) (**A**) Anti-Dve antibody reveals a tight cytosolic localization of Dve in neuronal cell bodies in brains of adult w^{1118}*; +/+; +/+* males. (**B**) Dve staining appears diffuse in neuronal cell bodies of male *w; TRAP1Δ4/TRAP1Δ4, +/+* flies. (**C**) Dve staining appears diffuse in neuronal cell bodies of male *w; +/+; UAS-TRAP1*^{7M}*/Actin*Gal4 flies. Scale bar equals 10 μm. (**D**) Ratio of staining intensity of Dve in cytoplasm and nucleus in indicated genotypes as noted by A, B and C respectively from above. (**E, G** and **I**) Male *w; TRAP1Δ4/TRAP1Δ4, +/+* and *w; +/+; UAS- TRAP1*^{7M} */Actin*Gal4 flies exhibit significantly increased mRNA expression of *Hsp60, mtHsp70* and *CG5045* as compared to w^{1118}*; +/+; +/+*. (**F, H** and **J**) Female *w; TRAP1Δ4/TRAP1Δ4; +/+* exhibit significantly increased mRNA expression of *Hsp60* as compared to w^{1118}*; +/+; +/+*, but not that of *mtHsp70* and *CG5045*. In all cases, data are presented as fold change from control. Error bars denote standard error of means; (*) indicates $p < 0.05$, (**) indicates $p < 0.001$.

Our finding that both loss and overexpression of TRAP1 activate the UPR^{mt} is perhaps perplexing. Because TRAP1 is a chaperone, induction of the UPR^{mt} in *TRAP1* mutants could be a direct result of excess unfolded proteins. While this response pathway may allow the *TRAP1* mutants to have a near normal lifespan, the absence of TRAP1 function has obvious negative consequences on health. The induction of the UPR^{mt} in TRAP1 overexpressing flies suggests a more complex mechanism. One possibility is that the mild stress of over-abundant TRAP1 in the mitochondrial matrix indirectly induces the UPR^{mt} and may be responsible for the improved fitness of these flies at older age, via a physiologically-beneficial stress response (Wu and Kaufman, 2006). Alternatively but not mutually exclusive, given the potential role of TRAP1 in multiple signaling pathways involving Rb, myc, TNF, and cyclophilin D (Song et al., 1995; Chen et al., 1996; Coller et al., 2000; Kang et al., 2007), overexpression of TRAP1 may lead to efficient protein folding, direct suppression of apoptosis, and the coordinated induction of other UPR genes through a TRAP1 mediated stress response. In the context of a minimal proteostasis boundary (Powers et al., 2009), enhanced mitochondrial protein quality control in TRAP1 overexpression flies would shift the proteostasis boundary towards health.

Though the possibility of crosstalk between UPR^{mt} signaling and other stress responses has been raised, the identity of the sensing mechanisms remain unknown. The link with the endoplasmic reticulum (ER)-UPR is especially interesting in consideration of its elaborate stress response machinery, as well as the extensive physical association of the ER with mitochondria (Altieri, 2011; Malhotra and Kaufman, 2011). Among other proteins, Xbp1 and Ire-1 are important regulators of the ER-UPR (Ron and Walter, 2007). Interestingly, in male TRAP1 mutant and overexpression flies the expression of *Xbp1* is upregulated whereas that of *Ire-1*

remains unchanged (Figure 3.8). These findings indicate partial coupling of the UPR^{mt} and ER-UPR pathways, potentially through TRAP1. Our data suggest that in response to stress, coordinated response pathways are activated in conjunction with transcriptional upregulation of a subset of genes, ultimately resulting in enhanced proteostasis.

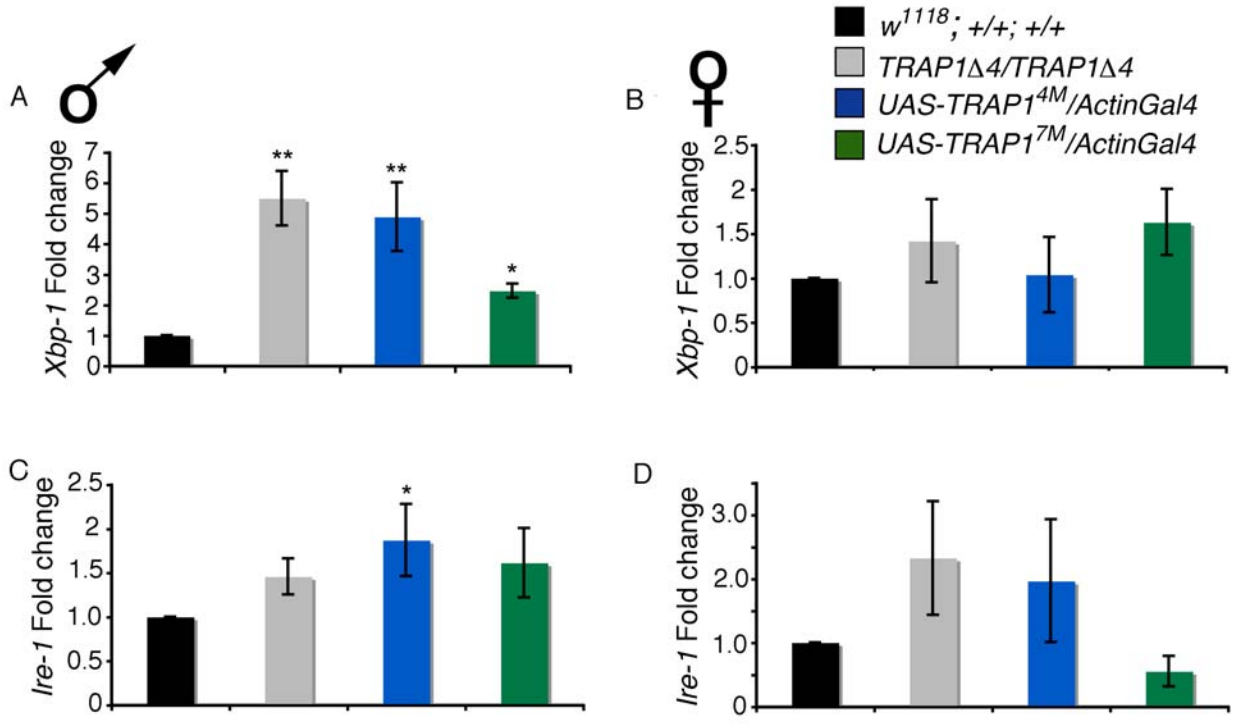

Figure 3.8. Loss and overexpression of TRAP1 induces transcription of the ER-UPR gene *Xbp-1* in males.

(**A** and **B**) Males of the mutant *w; TRAP1Δ4/TRAP1Δ4, +/+* strain, and of the overexpression strains *w; +/+; UAS-TRAP1*^{4M}*/Actin*Gal4 and *w; +/+; UAS-TRAP1*^{7M}*/Actin*Gal4, exhibit significantly increased mRNA expression *Xbp-1* as compared to w^{1118}*; +/+; +/+*, whereas females are unchanged. (**C** and **D**) mRNA expression of *Ire-1* is largely unchanged in males and females of the mutant and overexpression strains. While a small significant increase occurs in the *UAS-TRAP1*^{4M} */Actin*Gal4 strain, no effect is observed in the *UAS-TRAP1*^{7M} */Actin*Gal4 strain.

In all cases, data are presented as fold change from control. Error bars denote standard error of means; (*) indicates $p < 0.05$, (**) indicates $p < 0.001$.

The role of TRAP1 in activation of the UPR^{mt} has broad clinical relevance. TRAP1 is reported to be expressed at higher levels in cancer cells where it has been suggested to play a pro-survival role by inhibiting apoptosis (Kang et al., 2007; Costantino et al., 2009; Leav et al., 2010). Hsp90 molecules, including TRAP1, antagonize the cyclophilin D-dependent mitochondrial permeability transition, and this cytoprotective pathway is a potential target in cancer therapy (Green and Kroemer, 2004; Kang et al., 2007). Our results demonstrate that complete loss of function of TRAP1 has little to no effect on organismal lifespan (Figure 3.3) and thus, highlight the TRAP1-mediated stress response pathways as viable therapeutic targets for cancer.

Our data indicate that the effects of TRAP1 are sex specific. Sex specificity has been reported previously in stress survival studies involving mitochondrial proteins (Magwere et al., 2006; Mourikis et al., 2006). One possible explanation is that mitochondria in female *Drosophila* have dissimilar bioenergetics from males, with higher oxygen consumption, higher hydrogen peroxide production and lower levels of catalase, along with higher mtDNA copy number (Yin et al., 2004; Ballard et al., 2007). In our oxidative stress survival assays (Figure 3.2), TRAP1 mutant and overexpression animals displayed considerable sex-dependence. In contrast, effects on healthspan (locomotor activity and fecundity) were observed in both sexes (Figure 3.5). Oxidative stress resistance itself was correlated directly with the sex-specificity in expression of *mtHsp70* and *CG5045*, but not *Hsp60* (Figure 3.7). This suggests a potential difference in the extent of involvement of these genes in determining resistance to oxidative stress as compared to healthspan. These findings reveal a novel mechanism wherein sex-specific differences in activation of the UPR^{mt} may underlie the sex specificity in resistance to oxidative stress.

The biochemical and functional properties of TRAP1 have been studied *in vitro*, yet this is the first *in vivo* investigation of its physiological relevance and impact on an organism. Our work provides the first evidence for the presence of the UPR^{mt} in *Drosophila*, confirming the conservation of this stress response pathway across phylogeny, and it identifies *TRAP1* as a novel regulator of the UPR^{mt}. Furthermore, we provide direct evidence that *TRAP1* regulates oxidative stress resistance in males but not females, underscoring the importance of considering sex in the development of molecular therapeutics. Finally, we show that overexpression of TRAP1 ubiquitously or in the nervous system extends healthspan in both sexes.

Materials and Methods

Drosophila Stocks and Culture

All fly stocks were maintained on standard cornmeal medium, unless stated otherwise, at 25°C and 60% humidity under 12 hr light: dark cycles. *w; TRAP1Δ4/ Cyo; +/+* flies were obtained from Dr. Jessica Treisman, New York University. A full-length deletion of the *TRAP1* locus resulting from an imprecise p-element excision was confirmed by qPCR in *w; TRAP1Δ4/ TRAP1Δ4; +/+* flies. To generate transgenic overexpression strains, *Drosophila TRAP1* cDNA was inserted in pUAST plasmid and microinjected into w^{1118}*; +/+; +/+* embryos (BestGene Inc). Insertions were verified by inverse PCR and two independent strains were used, *w; +/+; UAS* $TRAP1^{4M}$*/+* and *w; +/+; UAS* $TRAP1^{7M}$*/+*. Overexpression in the *w; +/+; UAS* $TRAP1^{4M}$*/Actin*Gal4 and *w; +/+; UAS* $TRAP1^{7M}$*/Actin*Gal4 was confirmed by qPCR. All strains were backcrossed 5-6 generations in the w^{1118}*; +/+; +/+* background. w^{1118}*; +/+; +/+*, *Actin*Gal4 and *elav*Gal4 drivers were obtained from the Bloomington Stock Center, Indiana. Geneswitch-*tub5*Gal4 flies were a gift from Dr. Scott Pletcher (University of Michigan).

Lifespan Analysis and Oxidative Stress Resistance

w^{1118}*; +/+; +/+* strain was used as the background control for *TRAP1* mutants. For the TRAP1 overexpressing flies, the transgene alone and the driver alone strains were used as controls. In both lifespan and oxidative stress assays, significant alteration was deemed only when $p<0.05$ relative to all control strains. Statistical analysis was performed using JMP software and significance was determined using the standard chi-squared based log rank test.

Each lifespan analysis was conducted with a cohort of 100 male and 100 female flies, collected within 1 day of each other (0 $\pm$ 1), for each genotype. Data presented are pooled from two independent experiments. Flies were allowed to mate for at least 2 days before sexes were separated and subsequently maintained in groups of 10. Survival was scored every 3 days, when they were also transferred to fresh food.

Each oxidative stress assay was conducted with a cohort of 50 male and 50 female flies, collected and sorted as described above. For stress assays in young adults, 5-day old flies were starved by transferring to a bottle with filter paper saturated with ddH_2O for 3 hr. Thereafter, they were shifted to vials stacked with 5 equal size filter papers saturated with 20 mM paraquat in 5% sucrose solution. Vials were replenished every 12 hr to avoid starvation induced mortality and flies on 5% sucrose alone were used as controls. Survival was scored every 3 hr until all flies were dead. For transient TRAP1 overexpression in older adults, 50 *w; +/+; UAS-TRAP1*^{7M}*/ GS-tub5*Gal4 were collected, sorted and maintained on normal food for 35 days, before moving them for 5 days to food laced with 200µl of 125 mM RU486 (Sigma) dissolved in ethanol. An equal number of flies of the same age, sex and genotype were simultaneously maintained on food with 200µl of ethanol as controls. Older flies were not starved before the assay, and 5 mM paraquat in 5% sucrose was used to assess oxidative stress resistance. All survival assays were repeated at least 2 times with different cohorts and representative data are presented.

Locomotor ability

Locomotor ability was assessed using the negative geotaxis assay in a longitudinal study. A total of 50 male and 50 female flies of each genotype were assayed every 10 days, starting with 10-

day old flies. On the day of experiment, flies were transferred to empty 2.5 X 9.5 cm vials. After 120 sec of acclimatization, flies were gently tapped to the bottom three times and allowed to climb the walls. The number of flies that successfully crossed 7 cm in 10 sec was scored. Each experiment was conducted with 5 independent groups, and each group was tested three times after a 5 min interval between each test. Statistical significance between w^{1118} and *TRAP1* mutant flies was determined by Student's t-test. For analysis of overexpression strains and their controls, one-way ANOVA was performed with Dunnett's post hoc comparison using SPSS software.

Fertility

To assess female fertility, 5 virgin females of each genotype were allowed to mate with 5 w^{1118} males, aged 3 days. Flies were transferred to fresh food every 3 days and total number of progeny eclosed from each vial was counted once every 3 days for 9 days. Fresh 3-day old w^{1118} males were replaced at every time-point. Brood size was defined as the total progeny eclosed over 60 days. All genotypes were tested in parallel and three independent repeats were performed. To determine the fertility of males, 1 male of each genotype was allowed to mate with 3 virgin w^{1118} females aged 3 days. Parent flies were removed after 3 days and total number of progeny eclosed from each vial was counted. Fresh 3-day old virgin w^{1118} females were replaced at every time-point. The experiment was conducted with 5 males of each genotype in parallel and was repeated twice. Statistical significance was determined when $p<0.05$, using Student's t-test.

ROS measurement

ROS levels were measured using MitoSox (Invitrogen). Adult brains were dissected in cold HBSS, transferred to 5 μM MitoSox and incubated for 30 min at room temperature. Brains were washed 3 times with cold PBS and mounted in Vectashield (Invitrogen) between a slide and a coverslip placed on double-sided tape to prevent squashing. For quantification purposes, z-stacks were acquired through the entire thickness of the brain, and total fluorescent intensity was measured from 3D reconstruction images using ImageJ (NIH).

Immunohistochemistry

Drosophila larvae were dissected and stained as described previously (Baqri et al., 2009). Adult *Drosophila* brains were dissected in cold PBS, fixed overnight in 0.8% paraformaldehyde at 4°C, and washed thrice in PBS with 0.5% Triton and 0.5% BSA (PBST) for 15 min each. Samples were blocked in 10% BSA for 2 hrs, and incubated overnight in primary antibody at 4°C. Samples were washed four times with PBST for 30 min each, and incubated with secondary antibody for 12 hrs at 4°C. After three 30 min washes with PBST and a 15 min wash with PBS, brains were mounted in Vectashield (Invitrogen). We used mouse anti-mitochondrial complex V monoclonal antibody at 1:500 (MitoSciences) and rabbit anti-Dve antibody at 1:1000 (Nakagoshi et al., 1998). All comparative images were acquired on a Nikon TE swept field microscope at identical laser settings, exposure and aperture. Fluorescently labeled secondary antibodies used were goat anti-mouse Alexa 568 (Invitrogen) and goat anti-rabbit Alexa 488 (Invitrogen). To assess Dve localization, intensity profile was mapped across the diameter of each cell and the ratio of the maxima in the cortex and in the nucleus was determined. Measurements were made in comparable regions of 3 – 4 adult brains for each genotype.

Quantitative PCR

Total RNA was reverse transcribed using the Taqman kit by Applied Biosystems (Roche), according to manufacturer's recommendations. The quantitative PCR was done using the Power SYBr Master Mix (Applied Biosystems). Gene expression was normalized based on 28*S* RNA expression from three replicate experiments. The following primers were used –

CG5045 fwd: 5' – GAT CAT GCT GAA AAC CGC TG – 3'

CG5045 rev: 5' – CGT GAG AAT ATG TCG TAG GCC – 3'

Hsp60 fwd: 5' – CAC AGA AAA GTC AAG CGA ACT G – 3'

Hsp60 rev: 5' – GAA ACT GGC AAA CGG AAC ATC – 3'

Xbp-1 fwd: 5' – GCC TGG ACC ATC TAA CCT G – 3'

Xbp-1 rev: 5' – GTC TGT CAG CTC CTT GAT CTC – 3'

mtHsp70 fwd: 5' – AAG TGT CGC TCG AAC TGC – 3'

mtHsp70 rev: 5' – GAG GTC AGG AAA GCC ACT TC – 3'

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Chapter 4
Mitochondrial Quality Control in Human Disease and Aging – A Perspective

Conclusion

Research from the past several decades has focused on understanding the various aspects of mitochondrial biology. Undoubtedly, these organelles are critical regulators of cellular function. The wide diversity of cellular processes that are influenced by mitochondria and the several known mechanisms by which mitochondria can influence these processes goes to establish how deeply mitochondria are implicated in disease pathology and aging. The work presented in this dissertation has shed light on two novel mechanisms by which mitochondria are able to deal with different cellular stressors. Depletion of mtDNA and increased oxidative stress have obvious deleterious effects in an organism. In response to these stressors, mitochondria are able to regulate their dynamics and proteostasis machinery respectively, to mount varied rescue mechanisms.. Admittedly, there may be considerable cross talk and interdependence between these mechanisms and they may well operate in coordinated synchrony under any stress condition.

Future Directions of Research

In Chapter 2, we demonstrated that mitochondrial density is increased in the muscles and proximal nerves of *Drosophila* when mtDNA replication is impaired. Because there is no noticeable evidence of mitochondrial fragmentation, we conclude that this increase in density is largely due to addition of new mitochondria by upregulated biogenesis. Further, we determined that in mutants of mtDNA replication, axonal transport of mitochondria is nearly doubled in both

anterograde and retrograde directions. I discussed these results in the context of an SOS response wherein mitochondrial biogenesis is increased in a futile attempt to supply the axon with functional mitochondria, which exhausts the already depleted ATP resources and contributes to sustained progression of neuronal pathology in mtDNA diseases. Our model raises the question whether deliberate inhibition of mitochondrial biogenesis will be beneficial in mtDNA disorder-associated neuropathy. Future work must aim to knockdown *Spargel*, the *Drosophila* homologue of PGC-1 (Lee et al., 2011), in the pol γ mutants to test this hypothesis directly.

Further, we established that oxidative phosphorylation is not essential for the maintenance of mitochondrial axonal transport, as had been previously proposed (Ochs and Hollingsworth, 1971). Presumably, the small amount of maternally inherited mtDNA along with a metabolic shift towards glycolysis that has been observed in certain mitochondrial mutants (Hansson et al., 2004; Adan et al., 2008), is sufficient to sustain axonal transport till the third larval instar stage. It would be worthwhile to transiently inhibit glycolysis in the pol γ mutants using food laced with chemical inhibitors such 2-deoxy-D-glucose. This would help determine the contribution of glycolytically derived ATP in axonal transport when oxidative phosphorylation is inhibited.

We also observed clusters of mtDNA nucleoids in muscles of wildtype larvae but not in those of mtDNA replication mutants and these clusters colocalized with a late endosomal/ early lysosomal marker, spin. This could simply be a reflection of depleted mtDNA in the mutants, or they may indicate changes in mitochondrial turnover and mitophagy. It would be interesting to look at axonal transport of GFP labeled mitochondria and RFP labeled endosomes/ lysosomes simultaneously in the mtDNA replication mutants. Such an analysis would not only address the

regulation of mitophagy in these animals but importantly, would also shed light on a critical part of the mitochondrial life cycle in neurons: clearance of defective mitochondria from the axon. In neurodegenerative diseases such as Alzheimer's, autophagic vacuoles selectively accumulate in focal axonal swellings (Morfini et al., 2009). Inhibtion of lysosomal proteolysis disrupts axonal transport and causes neuropathy (Lee et al., 2011). These recent observations suggest that efficient degradation and clearance of mitochondria may be a critical, but presently understudied, phenomenon.

In Chapter 3, we observed sex specificity to oxidative stress response in TRAP1 mutant and overexpressing flies. Male TRAP1 mutants are significantly less resistant to oxidative stress while females do not show a robust decline in stress resistance. Similarly, male flies overexpressing TRAP1 were significantly more resistant to oxidative stress while females did not show an appreciable difference. Such sex specificity is not uncommon in stress survival studies involving mitochondrial proteins and has been previously observed in *Drosophila*. (Mourikis et al., 2006). Further, treatment with superoxide dismutase/ catalase mimetics show improved oxidative stress resistance with considerable sex specific effects (Magwere et al., 2006). While, the different stress response profiles in males and females could be a major contributor to this discrepancy, it may in part also be due to the dissimilar bioenergetic profiles in *Drosophila* males and females (Ballard et al., 2007). Future studies are warranted to explore the role of TRAP1 in mitochondrial bioenergetics. This is especially relevant in light of recent evidence that mutations in the electron transport chain result in activation of UPR^{mt} (Durieux et al., 2011).

Recently, it was reported that alteration of mitochondrial function in a single tissue may modulate longevity cues in distal tissues in *C. elegans* (Durieux et al., 2011). Accordingly, knockdown and overexpression of TRAP1 may be ensured in random cells using mosaic analysis in *Drosophila*, and its effect on UPR^{mt} activation may be assessed in neighboring cells by verifying DVE localization. This study will uncover the cell-autonomous versus cell-non-autonomous nature of UPR^{mt} induction.

The association of TRAP1 with PINK1 makes it an extremely interesting candidate to investigate in the pathogenesis of PD (Pridgeon et al., 2007). *Drosophila* have a well defined dopaminergic system (Drobysheva et al., 2008) and are an established model system for PD pathology (Clark et al., 2006; Park et al., 2006). Genetic epistasis studies would help dissect the position and role of TRAP1 in the PINK1/parkin pathway. If TRAP1 is indeed downstream of PINK1, then overexpression of TRAP1 in PINK1 mutants could rescue PD pathology by protecting against cellular stress. Further, this system will be ideal to investigate the potential role of UPR^{mt} in regulating PD pathology.

Mitochondrial Quality Control

Mitochondrial function is tightly regulated in the cell. Mitochondria are transported within the cell to sites of energy requirement. They constantly undergo cycles of fusion and fission, and this in turn affects multiple aspects of mitochondrial function and distribution (Liesa et al., 2009; Seo et al., 2010). Mutations in proteins that regulate mitochondrial function have been implicated in neurodegenerative diseases and more recently, metabolic disorders (Chen and Chan, 2009; Liesa

et al., 2009). Decline in mitochondrial functions is also associated with aging and age-related disorders (Sohal and Weindruch, 1996; Lesnefsky and Hoppel, 2006). To this effect, there are several mechanisms to repair, if not counter, defects in mitochondrial function to ensure that homeostasis is maintained for the various cellular processes. Mitochondria themselves actively mediate this quality control process on at least three levels: molecular, organellar, and cellular.

1) Molecular Quality Control – Mitochondria can 'repair' mild dysfunction

Mild deficits in mitochondrial function or an intrinsic defect in mitochondrial proteostasis can be repaired by the mitochondrion at a molecular level. Mitochondria attempt to repair mild deficits by induction of the mitochondrial unfolded protein response (UPR^{mt}). A signaling cascade is initiated between mitochondria and the nucleus that allows increased expression of chaperones such as mtHsp70, Hsp60 etc. These proteins endeavor to repair and/ or counter the damage of aggregated proteins, misfolded proteins and ROS-mediated damage to macromolecules (Haynes and Ron, 2010). The mitochondrial proteome is complex as it consists of nuclear DNA and mtDNA encoded proteins. Understandably, UPR^{mt} may have a wider role in regulating cellular integrity than currently appreciated and could be playing an important role not just in the regulation of longevity and health, but also in containing disease pathology. Interestingly, TRAP1, the novel regulator of UPR^{mt} that our work has uncovered, is found expressed at higher levels in cancer cells and is believed to contribute to their robustness (Leav et al., 2010).

2) Organellar Quality Control – Mitochondria 'restructure or perish' if the damage is severe

Another mechanism to repair defective mitochondria is through the fusion/fission machinery. Damaged mitochondria may undergo fusion with healthy mitochondria to exchange intra-mitochondrial content and/ or fission to divest and segregate damaged content (Chen and Chan, 2005). Proteins such as MFN1 and MFN2 are involved in fusion of damaged mitochondria to healthy, while OPA1, Fis1 and Drp1 are involved in segregating damaged mitochondria. Failure of the fusion/ fission machinery is implicated in several human diseases such as Autosomal Dominant Optic Atrophy (Delettre et al., 2000), CMT2A (Zuchner et al., 2006), CMT4A (Niemann et al., 2009), PD (Exner et al., 2007), AD (Barsoum et al., 2006) and Type 2 diabetes (Leinninger et al., 2006). While fusion/ fission have not been directly implicated in the aging process, their efficacy conceivably declines with age and could be an important contributing factor to some of the adult onset diseases listed above. The resultant pathology associated with defective fusion/ fission underlines the importance of these behaviors to repair defective mitochondria.

In case the damage is more severe and cannot be repaired, the damaged mitochondria are targeted for mitophagy and cleared (Lemasters et al., 1998). Mitophagy involves the engulfment of the damaged or fragmented mitochondria and lysosome-mediated degradation. PINK1 and parkin are known regulators of mitochondrial clearance and defects in quality control are implicated in PD pathogenesis (Narendra et al., 2010) and HD (Krainc, 2010). Several recent studies have also identified a link between reduced autophagy and the aging process (Lemasters, 2005). Indeed, enhanced autophagic clearance mediated by rapamycin treatment is seen to slow down symptoms of Hutchison-Gilford progeria or accelerated aging (Cao et al., 2011).

3) Cellular Quality Control – Mitochondria 'kill' the cell if damage is extensive

As the final line of quality control when mitochondrial damage is extensive and cannot be contained by molecular or organellar actions, the mitochondrial permeability transition pore is initiated and the apoptotic cascade is initiated to ensure that the cell perishes instead of risking a systemic failure. Of course, mitochondrial mediated programmed cell death is an important component of normal development and physiology (Olson and Kornbluth, 2001). However, it achieves pathological proportions in disease conditions. The deregulation of apoptosis is a hallmark feature of several disorders such as neurodegenerative diseases, metabolic diseases and cancer. The onset of these diseases is often associated with age, thus emphasizing the role of programmed cell death in the aging process.

Perspective on the mitochondrial quality control mechanisms relevant to this dissertation

The work presented in this dissertation addresses several key aspects of mitochondrial quality control in *Drosophila*. Using mutants of mtDNA replication, we have demonstrated a novel mechanism by which neurons may initiate a rescue response in an attempt to repair/ fix mitochondrial deficits. Biogenesis of mitochondria is upregulated and more mitochondria are transported into the axon to compensate for the dysfunction. Also, the increased retrograde mitochondrial transport indicates elevated clearance of damaged mitochondria. While these repair mechanisms are futile in our mutants, they suggest a novel interaction between mitochondria and nucleus in the face of mtDNA depletion to coordinate biogenesis and trafficking. Furthermore, as these mutations are lethal at an early developmental stage, it follows that the extensive mitochondrial dysfunction cannot be offset by the repair attempts. System wide cell death, accelerated because of energy deficits and collapse of key cellular processes,

ensures lethality.

In addition, using mutant and transgenic *Drosophila*, we modulated the dosage of the mitochondrial chaperone TRAP1. Conceivably, the mild stress induced to these genetic manipulations is not sufficient to cause systemic failure. Mitochondria attempt, and largely succeed, in restoring this impairment by upregulating the transcription of other molecular chaperones such as *Hsp60* and *mtHsp70*. These studies have identified TRAP1 as a novel regulator of UPR^{mt}. Our findings on the influence of TRAP1 on stress resistance, longevity and health, along with the association of TRAP1 with PINK1 and it's elevated presence in cancer cells, make it a key component of mitochondrial quality control that warrants further investigated.

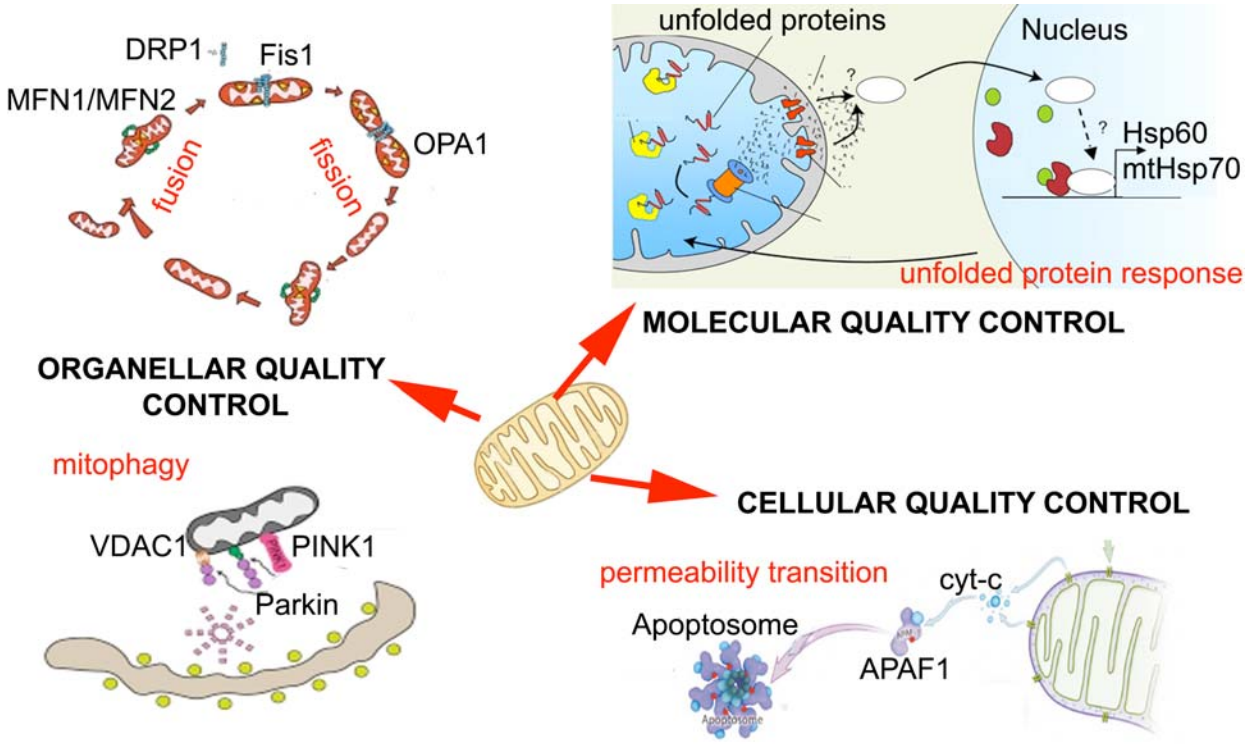

Figure 4.1 Mitochondrial Quality Control is regulated at least at three levels. (1) Molecular: Mild dysfuction triggers stress response pathways to amend the perturbation. (2) Under more severe damage, mitochondria undergo fusion/ fission in an attempt to repair. If damage is beyond repair, mitochondria are flagged for mitophagy and moved towards the lysosomal degradation machinery. (3) Severe mitochondrial dysfunction leads the permeability transition pore which initiates the apoptotic cascade. All levels of quality control are aimed at retaining an eventual state of cellular or systemic homeostasis. Modified from (Williams, 2006; Haynes and Ron, 2010; de Castro et al., 2011)

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