NEW INSIGHTS INTO THE ROLE OF PTEN-INDUCED KINASE 1 (PINK1) IN PARKINSON DISEASE USING A DROSOPHILA MODEL

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NEW INSIGHTS INTO THE ROLE OF *PTEN-INDUCED KINASE 1 (PINK1)* IN PARKINSON DISEASE USING A DROSOPHILA MODEL

by

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Abstract

Parkinson disease (PD) is the most prevalent human neurodegenerative movement disorder. Hereditary and sporadic forms of PD share pathological features including oxidative stress, protein aggregation and mitochondrial dysfunction. *PINK1 (PTEN induced putative kinase 1)* encodes a serine-threonine kinase linked to autosomal recessive and sporadic PD. With no animal models available at the commencement of this thesis, it was our goal to study the gene within a Drosophila model of the disease. We identified a *Drosophila melanogaster* homologue of *PINK1*, and used transgenics to employ expression studies along with morphological, longevity and behavioural assays. We have found that *Pink1* is able to improve the effects of toxic proteins, including overcoming the effects of *α-synuclein* and *Gal4* overexpression. Our results suggest that increases in *α-synuclein* and *Pink1* together can have a synergistic effect, allowing for enhanced protection and increased functional longevity. Additionally, our studies have identified the possibility of a non-protective role for the Pink1/parkin pathway. We have found that an increase in *Pink1* or *parkin* is able to increase the damaging effects of the directed over-expression of *Foxo* in Drosophila, highlighting a possible role in the apoptotic pathway. Taken together, our research highlights the complex role of *Pink1*, where it likely functions in a Pink1/parkin pathway to operate in the ubiquitin-proteasomal system, regulate mitochondrial fission/fusion events, regulate membrane permeability during apoptosis, and other roles that may be independent of its kinase function. Further investigation into the Pink1 mechanism of action will be important for the future development of disease modifying therapies for Parkinson disease.

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Co-Authorship Statement

The following statement clarifies the roles played by multiple authors in the manuscript Chapters 2, 3, 4, 5 and Appendices 1, 2 and 3 of this thesis. In accordance with the requirements of the School of Graduate Studies, my role in the completion of these manuscript chapters is defined with respect to the following categories: *i) design and identification of the research proposal; ii) practical aspects of the research; iii) data analysis; iv) manuscript preparation.*

i) design and identification of the research proposal

BES initiated the research project through setting the research goal to identify a *PINK1* homologue in Drosophila and investigate its role in a Drosophila model of Parkinson disease. All experiments in Chapters 2, 3, 4, 5 and Appendices 1 and 2 were initiated, conceived and designed by AMT with critical review from BES. The Eyer program in Appendix 3 was conceived and designed by PNW with critical review from AMT.

ii) practical aspects of the research

Identification of the Drosophila *Pink1* homologue, bioinformatics, creation of the *UAS-Pink1* transgenic line, and all experiments were carried out and performed by AMT.

iii) data analysis

All data was collected and analysed by AMT.

iv) manuscript preparation

All manuscripts in Chapters 2, 3, 4, 5 and Appendices 1 and 2 were prepared by AMT with critical review from BES. The manuscript in Appendix 3 was equally prepared by PNW and AMT with critical review from BES.

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List of Abbreviations

AIF: apoptosis-inducing factor

ANT: adenine nucleotide translocator

Apaf-1: apoptotic protease-activating factor 1

ADP: adenosine diphosphate

ATP: adenosine triphosphate

Co Q: coenzyme Q

Cyt c: cytochrome c

CypD: cyclophilin D

DA: dopaminergic

Ddc: dopa decarboxylase

Endo G: endonuclease G

ETC: electron transport chain

GMR: Glass Multiple Reporter

IAP: inhibitor of apoptosis protein

IM: inner membrane

IMS: intermembrane space

INR: insulin receptor

MOMP: mitochondrial outer membrane permeability

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

OM: outer membrane

PD: Parkinson disease

PI3K: phosphatidylinositol 3-OH-kinase

PINK1: human PTEN induced putative kinase 1

Pink1: Drosophila PTEN induced putative kinase 1

PTP: permeability transition pore

RNAi: interfering RNA

ROS: reactive oxygen species

SOD: superoxide dismutase

TCA Cycle: tricarboxylic acid cycle

TH: tyrosine hydroxylase

UAS: upstream activation sequence

VDAC: voltage-dependent anion channel

XIAP: X-linked inhibitor of apoptosis protein

Chapter 1

Introduction and Overview

1. Parkinson Disease

Parkinson disease (PD) is the most prevalent human neurodegenerative movement disorder (Lees et al. 2009; Weintraub et al. 2008). As PD affects 1 to 2% of the population over the age of 65 years, age is considered the largest risk factor for the development and progression of Parkinson disease. PD is characterized by a progressive loss of dopaminergic (DA) neurons in the *substantia nigra pars compacta*, resulting in age-dependent increases in bradykinesia, muscular rigidity, gait abnormalities, resting tremor and non-motor symptoms including autonomic and cognitive disturbances. Currently there are no cures and few disease-modifying therapies for PD. Dopamine replacement via levodopa can temporarily improve motor symptoms, however, non-motor symptoms are largely unaffected and further motor complications can develop in patients (Poewe et al. 2010). Investigation into the underlying mechanisms of PD may provide the means to develop disease-modifying therapies.

Although sporadic forms of PD are believed to be more common, 5 to 15% of PD cases are of an inherited nature and share features with sporadic PD, including oxidative stress, protein aggregation and mitochondrial dysfunction (Dawson and Dawson 2003). Commonly favoured mechanisms in both forms of the disease include abnormal protein degradation, mitochondrial dysfunction, calcium imbalance, and inflammation (Gupta et al. 2008). It is generally believed that a combination of environmental factors and genetic susceptibility play a role in the development of the symptoms associated with PD. With the precise pathologic mechanisms of PD still unclear, the use of familial forms to

understand the genetic component may help us to understand sporadic forms of the disease.

The discovery of inherited forms of Parkinson disease has renewed excitement and focus in PD research, and has led to the emergence of an increasingly complex network of genes found to contribute to disease risk and progression. Currently, mutant forms of at least six genes are linked to familial forms of PD, including *α-synuclein*, *LRRK2, uchL1, DJ-1, parkin*, and *PINK1* (Gasser 2007). These genes highlight key cellular systems in the pathogenesis of PD, including proteasomal protein degradation, reduction of oxidative stress, and maintenance of mitochondrial function. Mutations in other genes, such as the mitochondrial protease OMI/HTRA2, are also involved in hereditary forms of the disease, where they can considerably increase the risk of developing PD (Meissner et al. 2011). While the identification of PD genes may potentially provide insight into the disease mechanisms, at present we do not fully understand their biological functions within the cell. Investigations into the various, and often multiple, functions of the associated proteins and identification of their molecular targets will be necessary for designing effective treatments in the future.

2. PINK1/parkin pathway

2.1 parkin

The *parkin* gene is associated with an autosomal recessive form of Parkinson disease, with an early onset typically between childhood and age 40 years (Lucking et al. 2000). Functioning as an E3 ubiquitin ligase, parkin interacts with ubiquitin-conjugating

enzymes to catalyze attachment of ubiquitin to protein targets, tagging these proteins for destruction by the proteosome (Bekris et al. 2010). Many ubiquitination substrates have been proposed for parkin. One such substrate is the α-synuclein protein, a major component of ubiquitin-rich inclusions known as Lewy bodies, often present in the remaining neurons of PD patients (Lees et al. 2009). The *α-synuclein* gene has been identified as central to the pathogenic mechanism of Parkinson disease, with links to oxidative stress and various aspects of mitochondrial dysfunction (Devi et al. 2008; Liu et al. 2009; Schapira and Gegg 2011). It has been suggested that α-synuclein aggregation through Lewy body formation is a neuronal detoxification response (Whitworth 2011). The presence of ubiquitin in these inclusions suggests that parkin not only tags α-synuclein for degradation, but that it may be involved in this protective, aggregation response.

Parkin is predominantly a cytosolic protein that can co-localize to synaptic vesicles, the Golgi complex, endoplasmic reticulum, and the mitochondrial outer membrane (Bekris et al. 2010). PD-linked mutations in *parkin* can result in the alteration of this localization, as well as modification of its solubility, aggregation and function. Drosophila *parkin* mutants exhibit motor deficits, associated with dramatic, widespread apoptotic degeneration of muscle tissue and male sterility (Whitworth 2011). This phenotype appears to be the result of a severe loss of mitochondrial integrity, suggesting a role for parkin in mitochondrial homeostasis (Greene et al. 2003). Mitochondrial defects are a common characteristic of sporadic PD and are associated with *parkin* mutations in humans, and in animal models of PD (Whitworth 2011). It is possible that parkin acts to

regulate mitochondrial integrity and that mitochondrial dysfunction is the main contributor to the loss of dopaminergic neurons in patients with *parkin* mutations.

2.2 PINK1

PINK1 (PTEN induced putative kinase 1) encodes a serine-threonine kinase that has been linked to autosomal recessive and some sporadic forms of Parkinson disease (Jendrach et al. 2009; Valente et al. 2004a; Valente et al. 2004b). Identified as a kinase, PINK1 contains a mitochondrial targeting sequence and has been shown to locate to the mitochondria (Silvestri et al. 2005; Valente et al. 2004a). Initial studies into the function of Pink1 in Drosophila have shown degeneration of indirect flight muscles and defective spermatid formation as a result of null mutations in *Pink1* (Clark et al. 2006; Park et al. 2006). The phenotypes are accompanied by mitochondrial defects, with one study showing mitochondrial swelling in the dopaminergic neurons of *Pink1* mutants (Park et al. 2006). These results, as well as others using an RNAi approach to target *Pink1* (Yang et al. 2006), suggest that the pathogenic mechanisms responsible involve mitochondrial dysfunction. Further support for this theory can be seen in cell culture studies reporting that *PINK1* mutants are unable to inhibit cytochrome c release under stress conditions (Wang et al. 2007), and that overexpression of *PINK1* results in a reduction of cytochrome *c* release from mitochondria under both basal and stress conditions (Petit et al. 2005). In particular, *PINK1* appears to be important in preventing oxidative damage, where mutations result in oxidative stress and a decrease in complex I activity (Hoepken et al. 2007). Complex I dysfunction is a common feature of sporadic PD and is thought to be the result of increased oxidative stress that causes an abnormally high rate of mutation

accumulation within the mitochondrial genome (Schapira 2008). PINK1 may confer protection of the mitochondria through several mechanisms including an interaction with molecular chaperones to regulate oxidative stress responses, activation of parkin to result in the tagging of toxic proteins, such as α-synuclein, for degradation, or through the interaction with proteins to preserve mitochondrial integrity (Deas et al. 2009). In addition to maintaining and stabilizing mitochondrial networks, it is becoming increasingly apparent that protection during cell stress is due to the involvement of PINK1 in mitochondrial fission/fusion events (Chu 2010; Jendrach et al. 2009). This involvement implicates PINK1 as a key regulator of the fission/fusion balance, using the process of mitophagy to specifically process and degrade dysfunctional mitochondrial fragments.

2.3 PINK1/parkin pathway

It has been suggested that PINK1 and parkin function in the same pathway and that this PINK1/parkin pathway is necessary for proper mitochondrial function and morphology. This is supported by the striking similarity between *Pink1* and *parkin* mutant phenotypes in Drosophila (Clark et al. 2006; Park et al. 2006; Yang et al. 2006), and mammalian systems (Dagda et al. 2009; Exner et al. 2007). Studies show that overexpression of either Drosophila or human *parkin* is able to rescue *Pink1* mutant phenotypes, but that *Pink1* overexpression does not influence *parkin* mutant phenotypes, suggesting that Pink1 operates upstream of parkin (Clark et al. 2006; Park et al. 2006; Yang et al. 2006). As a kinase, Pink1 may interact with parkin through a phosphorylation event, and although there is some evidence to support this in Drosophila (Kim et al. 2008), it has not been

supported in mammalian studies (Vives-Bauza et al. 2010). It is possible that PINK1 may provide protection through an interaction with parkin to result in the tagging of cystolic proteins for degradation, as neurodegenerative diseases are often characterized by the accumulation of toxic proteins. This role is supported by the ability of Pink1 to protect neurons from the dopaminergic neurotoxin MPTP (Haque et al. 2008), where the pathology of MPTP is attributed to the α-synuclein protein (Dauer et al. 2002; Klivenyi et al. 2006). The expression of *parkin* has been shown to rescue an α-synuclein-induced phenotype, presumably through the targeting of α-synuclein for degradation (Haywood and Staveley 2004, 2006). It is possible that the ability of Pink1 to protect against MPTP is due to an interaction with parkin that results in the targeting of toxic α-synuclein for degradation.

Increasing evidence points towards the importance of the PINK1/parkin pathway in maintaining mitochondrial function in the cell, segregating damaged or dysfunctional mitochondrial fragments and promoting their degradation by autophagy (Whitworth and Pallanck 2009). In this way, the PINK1/parkin pathway acts as a quality control mechanism to degrade damaged mitochondria so to prevent their dysfunction, increased ROS production and potential release of pro-apoptotic factors. Recruitment of parkin to the mitochondria, under various conditions, appears to be dependent on PINK1 (Whitworth and Pallanck 2009). Once at the mitochondria, parkin can initiate mitophagy through the ubiquitination of mitochondrial membrane proteins. Two proteins identified as targets for ubiquitination by parkin, VDAC1 (Geisler et al. 2010) and Drosophila mitofusin (Poole et al. 2008; Ziviani et al. 2010), have been recently reported.

Ubiquitination of VDAC1 likely results in the recruitment of autophagic machinery, while the ubiquitination of mitofusin may directly alter its function in mitochondrial fusion. Although there is growing evidence for a role of the PINK1/parkin pathway in mitophagy, there are indications that they are not obligatory components, but instead regulate mitochondrial fission/fusion only in a specific biological context (Whitworth and Pallanck 2009). Future studies into the conditions required for recruitment of parkin to the mitochondria will help us to understand how the PINK1/parkin pathway promotes neuronal survival.

3. Mitochondria and cell survival

Mitochondria perform a range of fundamental functions in eukaryotic cells, including supplying energy needed for the cell to do work, thereby maintaining cell function and survival. Additionally, mitochondria are involved in the biosynthesis of amino acids, vitamin cofactors, fatty acids, and iron-sulphur clusters, and are involved in aspects of cell signalling (Bowsher and Tobin 2001; Giacomello et al. 2007; Mackenzie and McIntosh 1999). More recently, mitochondria have been highlighted as a central mediator of signals involved in programmed cell death and their involvement in cell fate decisions has been demonstrated in numerous diseases.

The generation of ATP (adenosine triphosphate) through the mitochondrial respiratory chain is considered to be the primary role of the mitochondria (Figure 1). ATP is the most important molecule for capturing and transferring energy in cells, where the hydrolysis of its phosphoanhydride bonds results in a free energy change of -7 Kcal/mol

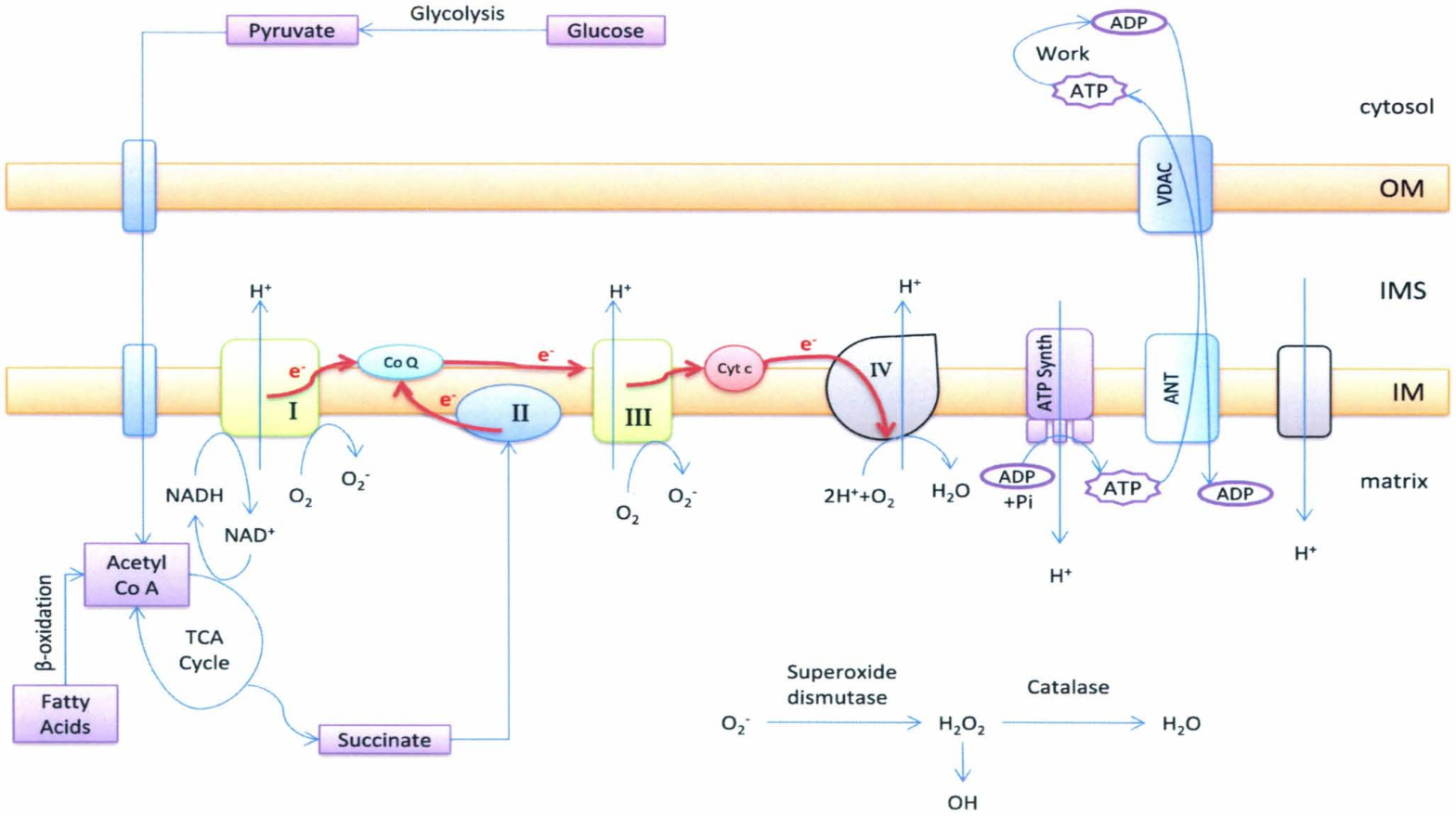

Figure 1. Mitochondrial Respiration and Oxidative Phosphorylation. ADP is phosphorylated to form ATP, driven by the transfer of electrons from NADH, through the ETC and to oxygen. NADH is supplied via the oxidation of glucose and fatty acids during glycolysis, β-oxidation and the TCA cycle. Energy released from the ETC is used to pump protons across the mitochondrial inner membrane, generating a proton motive force which is then used to power ATP synthesis via the return of protons down their gradient, passing through and providing energy to the ATP synthase.

(Gajewski et al. 1986). ATP enables work to be done in the cell and is involved in a variety of pathways necessary for cell function and survival. Some of these pathways include synthesis of macromolecules in the cell, moving substances across cell membranes and mechanical work such as chromatin assembly or contraction of muscle cells. ATP is used as an on-off switch for many proteins, thereby controlling enzymatic reactions and signalling within the cell (Brown 1992). In addition to the production of ATP, the membrane potential generated by the mitochondrial respiratory chain is essential for the import of mitochondrial proteins and movement of metabolites (Ricci et al. 2003). For these reasons, regulation of the respiratory chain is essential for the maintenance of membrane potential and the supply of ATP. It is also essential for the prevention of excessive electron loss that could otherwise result in the production of oxygen radicals in the mitochondria (Fariss et al. 2005). A decline in ATP production and increases in oxidative damage are important factors in mitochondrial dysfunction and initiation of apoptosis, such that defects in the respiratory machinery or its control have been implicated in the aetiology of numerous diseases, including Parkinson disease.

3.1 Oxidative Stress

Many neurodegenerative diseases have been linked, directly or indirectly, to oxidative stress (Sayre et al. 2008). This is largely due to the high energy-demanding nature of tissues in the nervous system. Deficiencies in the ability to combat or repair oxidative damage are particularly detrimental in these tissues due to the high level of oxygen metabolism and production of potentially damaging reactive oxygen species (ROS). ROS are an unavoidable consequence of mitochondrial respiration, with approximately 90% of

all ROS in the cell produced in the mitochondria (Balaban et al. 2005; Logan 2006). Mitochondrial ROS are generated at two major sites in the electron transport chain, complexes I and III (Figure 1), where there are large changes in the potential energy of the electrons (Chen et al. 2003; Fleury et al. 2002; Kushnareva et al. 2002). During the transfer of electrons along these complexes, single electrons can spontaneously leak into the mitochondrial matrix and react with molecular oxygen, forming a superoxide anion. This superoxide is normally converted to H_2O via superoxide dismutase and catalase (Balaban et al. 2005). However, when the rate of electron transport is limited due to the generation of a large proton gradient, an excess of free electrons occurs and the antioxidant enzymes, as well as non-enzymatic antioxidants, may become overwhelmed. This could lead to the generation of superoxide anions as well as other mitochondrial free radicals including hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH).

The highly reactive and short-lived nature of ROS results in localized impact, doing most damage to the proteins, lipids and nucleic acids in the mitochondria, where they are generated (Bras et al. 2005; Garrido et al. 2006; Wiseman and Halliwell 1996). This mitochondrial damage can have disastrous effects in the cell, where membrane disruption results in the release of pro-apoptotic compounds and any damage to the electron transport chain will deplete the cell of needed ATP as well as further amplify ROS generation. Accumulation of ROS can be alleviated by the activity of enzymatic and non-enzymatic antioxidants in the cell. In addition, there are mechanisms to help prevent ROS production, such as controlling over-reduction within the electron transport chain through the regulation of respiration and through "leaking" protons down their

concentration gradient via uncoupling proteins (Smith et al. 2004; Starkov 2006). It is important to realize that although ROS are typically thought of as having a toxic role in the cell, pro-oxidative and anti-oxidative systems are in a steady state, where an excess in either system has fatal consequences (Zorov et al. 2005). ROS should therefore be regarded more as part of the dynamic equilibrium that allows the mitochondria to initiate cellular life-and-death events.

Studies have shown that various metals, such as iron and copper, possess the ability to produce ROS in biological systems, and that disruption of metal ion homeostasis can lead to oxidative stress (Jomova and Valko 2011). The mechanism of metal-dependent ROS formation is highly influenced by the action of cellular antioxidants such as vitamin C, vitamin E, glutathione, and others. These antioxidants are capable of chelating metal ions, thereby reducing their ability to form ROS. Although metal-induced oxidative stress has been suspected previously in sporadic forms of PD, only recently have links been made with familial forms of PD. Studies in Drosophila have demonstrated a sensitivity of *parkin* mutants to iron and copper, with the administration of metal chelating agents significantly improving lifespan (Saini et al. 2010). In addition, dietary zinc markedly improved *parkin* mutant phenotypes (Saini and Schaffner 2010). Although the effect of zinc has been described as confusing, it is likely due to the involvement of zinc in antioxidant enzymes such as superoxide dismutase (SOD) and catalase. Other studies have provided evidence that substances such as coffee or tobacco can reduce the incidence of PD and potently suppress the age-dependent loss of dopaminergic neurons (Trinh et al. 2010). These findings have not been attributed to either caffeine or nicotine,

and the responsible components have yet to be identified. While various studies support the role of oxidative stress in the pathogenesis of PD, the lack of clear evidence that antioxidant therapies can provide benefit in this disease demonstrates the need for more therapeutic research in this area.

3.2 Increased mitochondrial membrane permeability

Mitochondrial membrane permeability resulting in the release of apoptotic factors into the cytosol is widely accepted as a "point of no return" during apoptosis. Even in apoptotic pathways that do not directly involve the mitochondria, such as the Fas pathway, pore formation in the mitochondrial membranes may be used to amplify the death signal. The precise mechanism of pore formation is still debated, as well as the importance of inner versus outer membrane permeability in the apoptotic cascade. One model suggests that outer membrane permeability is a consequence of the formation of a permeability transition pore (PTP) in the inner membrane, and therefore, the inner membrane permeability transition is the irreversible step during apoptosis (Garrido et al. 2006). Others argue that the mitochondrial outer membrane permeabilization (MOMP) is independent from the PTP, and is exclusively the product of cytoplasmic proteins belonging to the Bcl-2 family. Recent research has made connections between MOMP and mitochondrial fission, suggesting a causal relationship between the two. It is possible that PTP, MOMP and mitochondrial fission are all mechanisms involved in increased membrane permeability, operating independently or in cooperation to initiate mitochondrial fragmentation and/or apoptosis.

3.2.1 Permeability Transition Pore (PTP)

Increases in permeability observed across the mitochondrial inner membrane are widely attributed to the opening of protein channels identified as permeability transition pores (PTP)(Garrido et al. 2006; Green and Kroemer 2004; Mignotte and Vayssiere 1998). These pores span both the inner and outer membranes, allowing molecules smaller than 1.5 kDa to pass between the cytoplasm and the mitochondrial matrix in a non-selective manner (Bernardi et al. 1992). As ions and respiratory substrates equilibrate across the membranes, a reduction in membrane potential occurs, ATP synthesis is halted, and osmotic pressure results in the swelling of the mitochondrial matrix (Chipuk et al. 2006; Crompton 1999; Halestrap et al. 2002). This leads to an increased permeability of the outer membrane, liberation of contents from the inter-membrane space, and the eventual rupture of the outer mitochondrial membrane (Zoratti et al. 2005). To illustrate the importance of this phenomenon, it is thought that the opening of a single pore is sufficient to initiate all of these events.

The PTP forms at contact points between the inner and outer mitochondrial membranes, and is thought to be composed of proteins existing within these membranes as well as in the matrix (Figure 2). The exact composition of the PTP remains unknown, however it is typically thought to include the voltage-dependent anion channel (VDAC) from the outer membrane, the adenine nucleotide translocator (ANT) from the inner membrane, and cyclophilin D (CypD) from the matrix (Crompton 1999; Crompton et al. 2002; Green and Kroemer 2004). It should be noted that recent studies show that the ANT and CypD are not essential for PTP formation and that Complex I may be a major component of this

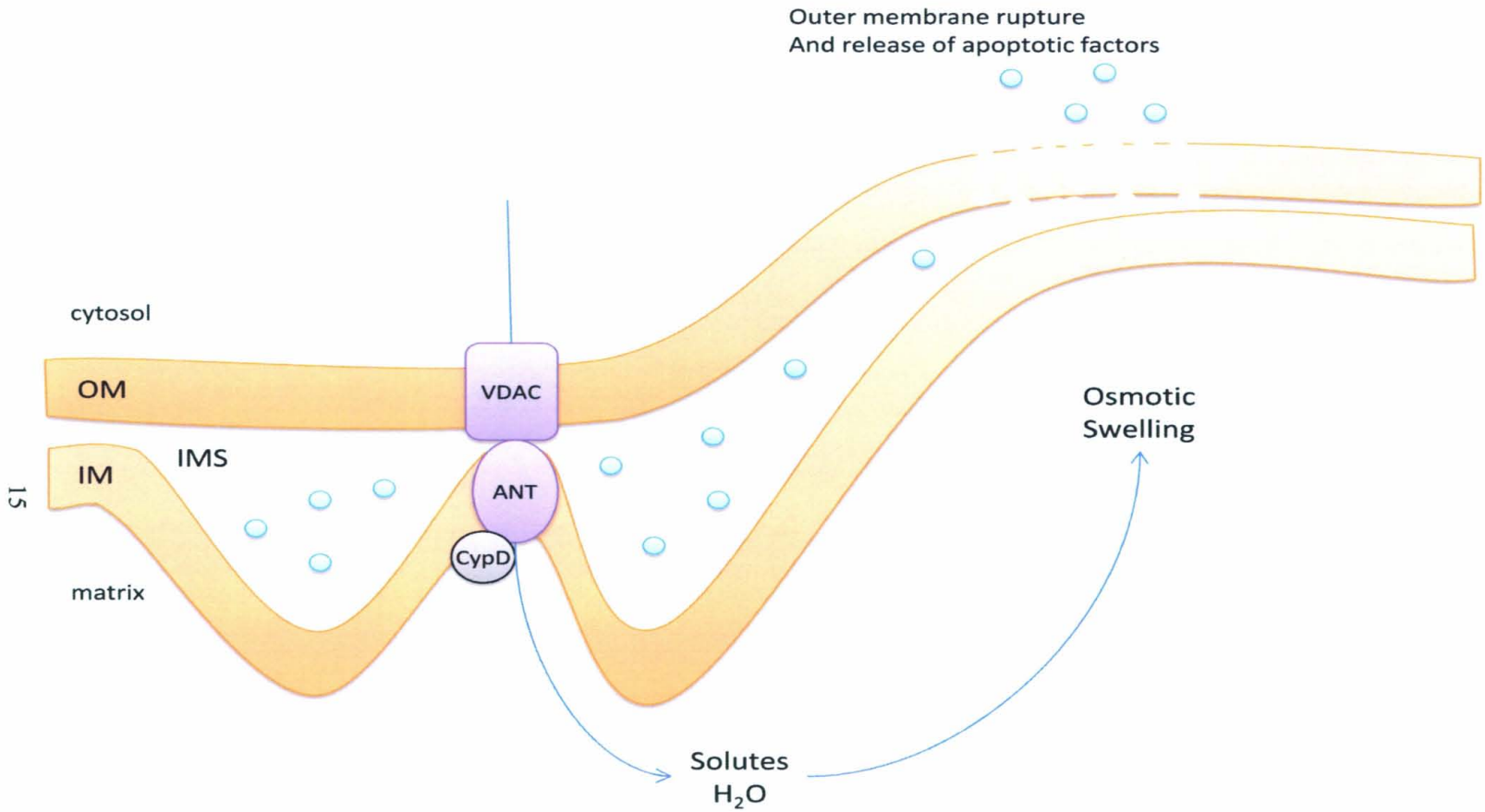

Figure 2. Permeability Transition Pore (PTP). Protein pore formation spanning inner and outer mitochondrial membranes, composed of mitochondrial proteins including VDAC, ANT and CypD. Molecules smaller than 1.5 kDa are able to pass between the cytoplasm and the mitochondrial matrix in a nonselective manner resulting in an increase in osmotic pressure, swelling of the mitochondrial matrix, increased outer membrane permeability and eventual liberation of apoptotic factors from the intermembrane space.

type of pore (Basso et al. 2005; Fontaine and Bernardi 1999; Kokoszka et al. 2004). It is possible that many mitochondrial proteins are able to contribute to PTP formation and that its composition is dependent on the initial stimulus.

The PTP has been shown to respond to various factors originating from both inside and outside the mitochondria. Three established activators of the PTP include increases in cytosolic Ca^{2+}, reactive oxygen species, and depolarization of the inner membrane (Giacomello et al. 2007; Kowaltowski et al. 2001; Matsuyama et al. 2000; Matsuyama and Reed 2000). The primary activating factor appears to be Ca^{2+}, and it is thought that other inducers and inhibitors of the PTP operate indirectly through alteration of Ca^{2+} levels (Fontaine and Bernardi 1999). The association of Ca^{2+} with cell death via the PTP appears to be in conflict with other studies that demonstrate the benefits of Ca^{2+} within the mitochondria. It has been well established that even a very large Ca^{2+} release in the cytosol, with subsequent accumulation in the mitochondria, does not trigger cell death but is able to increase mitochondrial respiration and ATP levels in the cell. It has been proposed that for Ca^{2+} to have damaging effects in the mitochondria, a "double hit" must occur where the apoptotic stimuli responsible for triggering Ca^{2+} release must have a dual target in the mitochondria, allowing for Ca^{2+} to act as an apoptotic signal (Pinton et al. 2001; Szalai et al. 1999). The ability of Ca^{2+} to both regulate respiration and aspects of apoptosis highlight the dual role of mitochondria with respect to cell survival and cell death.

3.2.2 Mitochondrial Outer Membrane Permeabilization (MOMP)

The activation of apoptosis in the mitochondrial pathway requires mitochondrial outer membrane permeabilization (MOMP), which may or may not be discrete from formation of the PTP. For MOMP to occur without PTP involvement, a coordinated effort between various Bcl-2 proteins must be employed (Garrido et al. 2006). During this process, multi-domain members of the Bcl-2 family, such as Bax or Bak, create pores in the outer membrane, resulting in the release of soluble proteins from the inter-membrane space into the cytosol (Figure 3). These inter-membrane proteins, such as cytochrome c, can then activate downstream apoptotic signals resulting in cell death.

The Bcl-2 protein family contains both pro-apoptotic and anti-apoptotic proteins. The pro-apoptotic proteins can be further broken down into those with multiple Bcl-domains, including Bax, Bak and Bok, and those with only one domain, including Bid, Bad, Bim and Puma, among others. For pro-apoptotic Bcl-2 proteins to initiate MOMP, the multi-domain proteins, usually Bax or Bak, must first be activated by single domain proteins, possibly by direct association (Kuwana et al. 2005; Letai et al. 2002). Once activated, the Bax/Bak proteins undergo oligomerization and insertion into the outer membrane, creating large multimeric pores and MOMP. Anti-apoptotic Bcl-2 proteins, including Bcl-2, Bcl-xL and Mcl-1, are able to prevent pore formation by binding to the single domain proteins, preventing their association with and activation of their multi-domain counterparts (Garrido et al. 2006). Not all members of the single domain Bcl-2 proteins are able to directly associate and activate Bax or Bak, but instead function to bind with the anti-apoptotic Bcl-2 proteins (Chen et al. 2005). By preferentially binding, these

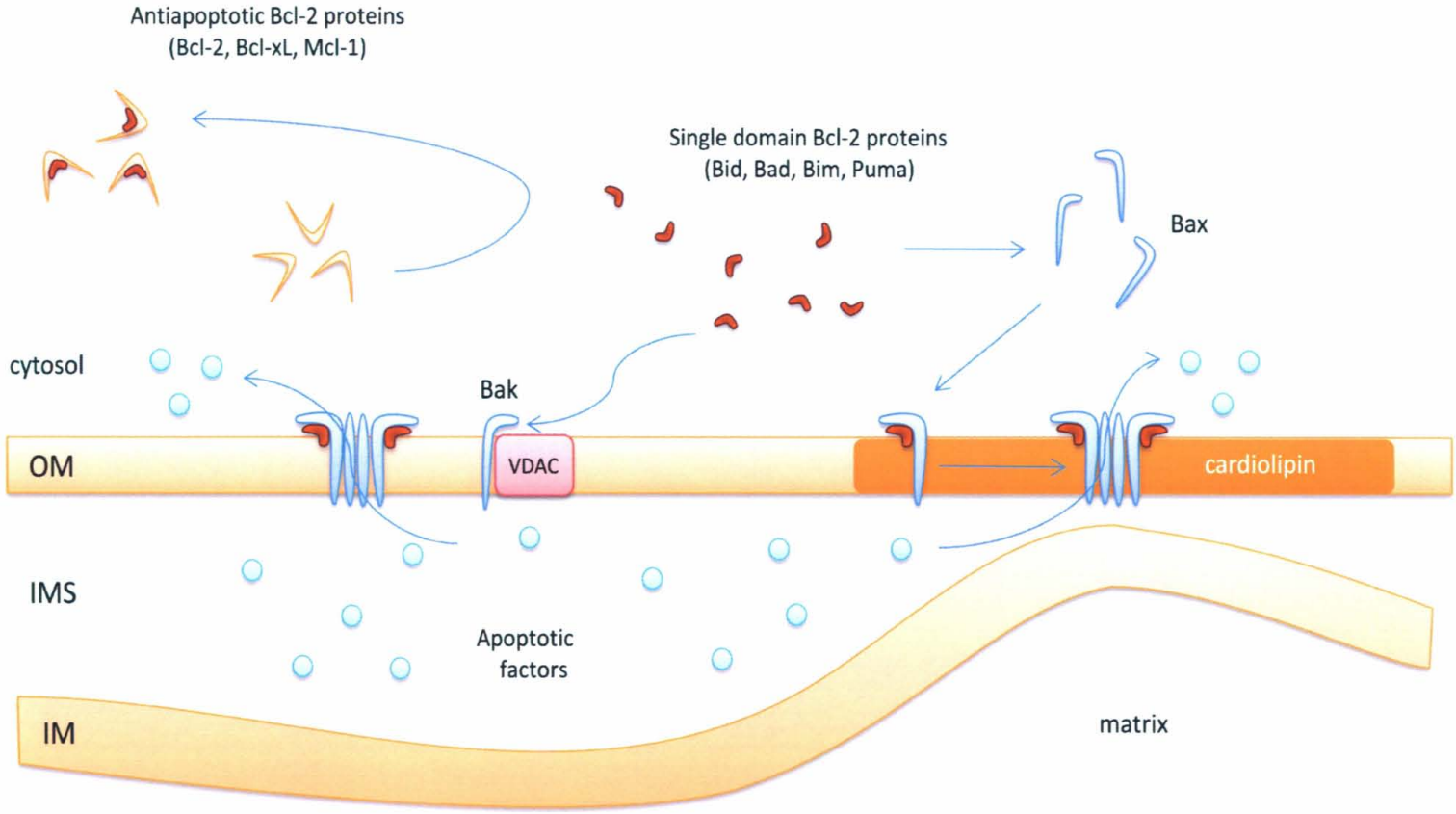

Figure 3. Mitochondrial Outer Membrane Permeabilization (MOMP). Single domain Bcl-2 proteins (Bid, Bad, Bim, Puma) activate multidomain, pro-apoptotic Bcl-2 proteins (Bax, Bak) resulting in their oligomerization and insertion into the outer membrane, creating large multimeric pores and MOMP. Anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-xL, Mcl-1) inhibit pore formation by binding to single domain Bcl-2 proteins, preventing their association with and activation of Bax/Bak. Activation of Bax takes place in the cytosol with membrane insertion occurring at localized concentrations of cardiolipin. Inactive Bak is present in the outer membrane, stabilized by VDAC until activation. During MOMP apoptotic factors are released from the intermembrane space into the cytosol.

single domain proteins are able to control anti-apoptotic activity and free up the other single domain proteins that may act directly on Bax/Bak and initiate pore formation.

Bax and Bak are essential for MOMP and cells lacking these proteins are resistant to cytochrome c release and apoptosis (Wei et al. 2001). Inactive Bax exists mainly in the cytosol, where, upon activation, a conformational change occurs that allows for its insertion into the outer membrane (Garrido et al. 2006). A strong association has been found between Bax membrane insertion and localized concentrations of cardiolipin (Lutter et al. 2000; Lutter et al. 2001). In this instance, it is thought that proteins in the outer membrane concentrate cardiolipin when stimulated by apoptotic signals originating from either inside or outside the mitochondria. Localization of cardiolipin at contact points then facilitate Bax insertion into the mitochondrial outer membrane leading to MOMP. Conversely, Bak resides within the outer membrane, but in an inactive state thought to be stabilized though interactions with outer membrane proteins such as VDAC (Cheng et al. 2003; Green 2005). It is not thought that these membrane proteins initiate activation of Bak, but that the single domain Bcl-2 proteins are able to disrupt the association between membrane proteins and Bak during times of cellular stress (Cheng et al. 2003). Taken together, it appears that the regulation of the single domain Bcl-2 proteins is the critical step in formation of the MOMP. These proteins are regulated by a variety of mechanisms in the cytosol, including cleavage by caspases in response to death receptor stimulation (Bid), inactivation by binding to the cytoskeleton (Bim), dephosphorylation (Bad) and upregulation by apoptotic stimulation (Puma) (Chipuk et al. 2006). Therefore, it is likely that multiple signal cascades are involved in MOMP, with

transcriptional and post-transcriptional regulation of the single domain Bcl-2 proteins at the heart of its activation.

3.2.3 Mitochondrial fission and MOMP

MOMP may rely on the activation of the molecular machinery involved in mitochondrial fission (Frank et al. 2001). Mitochondrial fission and fusion occur in normal, non-apoptotic cells and are essential for cellular function. In order to prevent apoptosis during normal cellular events, such as cell division, it is important that fission proceeds in a highly controlled manner such that leakage of soluble proteins from the inter-membrane space, as well as the matrix, is avoided. Morphological changes associated with fission have also been observed in MOMP during apoptosis. It has been shown that pro-apoptotic signals are able to influence the connectivity of the mitochondrial network, resulting in fragmentation and punctate mitochondrial morphology (Frank et al. 2001). Not only is mitochondrial fragmentation able to contribute to apoptosis, it is involved in its inhibition, depending on the nature of the initial signal. Many proteins involved in mitochondrial fission and fusion have been shown to have the ability to regulate MOMP, where the activation of those associated with fission (Drp-1, Endophilin and Fis) are connected with MOMP, and those associated with fusion (Fzo1 and Opa1) are connected with the inhibition of MOMP (Perfettini et al. 2005). As mentioned, studies indicate that protection via the PINK1/parkin pathway is largely due to its regulation of fission/fusion events (Chu 2010; Jendrach et al. 2009). Recruitment of parkin to the mitochondria results in the ubiquitination of mitofusin, a Drosophila mitochondrial fusion protein, resulting in decreased fusion and promotion of mitophagy (Gegg et al. 2010; Ziviani et al.

2010). As seen with other fission/fusion proteins, it is possible that PINK1/parkin may also be involved in MOMP as a means of triggering apoptosis under certain conditions.

3.3 Mitochondrial Apoptotic factors

The release of various pro-apoptotic proteins from the mitochondria into the cytosol is a well characterized event during apoptosis (Figure 4). Proteins released from the inter-membrane space may include caspase-independent factors such as nucleases and proteases, as well as caspase activators. The caspase dependent pathway involves the release of cytochrome c, as well as the release of proteins such as Omi/HtrA2 and Smac/DIABLO which help to prevent inhibition of the caspase cascade. The caspase independent pathway, including the AIF and Endo G proteins, typically involves direct action on cellular components including the initiation of DNA fragmentation. Although the mechanisms by which these mitochondrial factors are released are still debated, it is obvious that their release is vital in the decision between cell survival and cell death.

The first protein shown to be released from the mitochondria during apoptosis was cytochrome c; consequently it is one of the most investigated apoptotic proteins. Some studies indicate that once cytochrome c release is initiated, it continues until all cytochrome c has been released from all mitochondria within the cell (Goldstein et al. 2000). Other studies suggest that a subset of mitochondria retain their cytochrome c, possibly to provide the ATP required for completion of the apoptotic program (D'Herde et al. 2000). Normally a component of mitochondrial respiration, cytochrome c plays a dual role in the cell where, upon release from the inter-membrane space, it serves to initiate the caspase cascade. Once in the cytosol, cytochrome c forms an ATP dependent complex

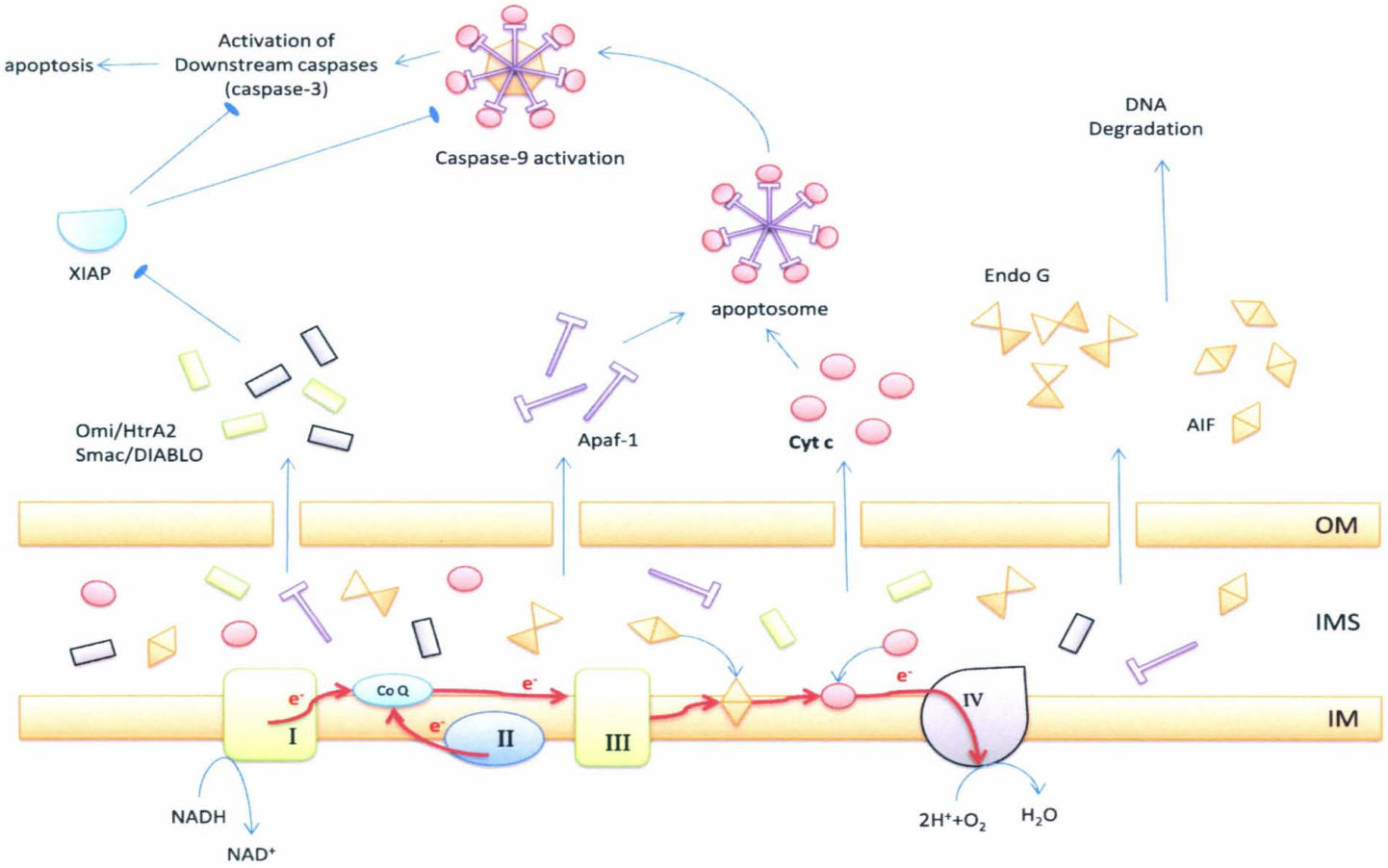

Figure 4. Release of Mitochondrial Apoptotic Factors. Release of Cyt c into the cytosol leads to formation of the apoptosome via association with Apaf-1. The apoptosome recruits and activates caspase-9, which can then activate other downstream effector caspases (caspase-3 and -7) and result in apoptosis. XIAP is able to inhibit caspase-3 and -9. Omi/HtrA2 and Smac/DIABLO bind XIAP preventing inhibition of the caspases. AIF and Endo G are released into the cytosol and translocate to the nucleus resulting in large scale DNA fragmentation. In non-apoptotic cells AIF may interact with Complex III the ETC, facilitating the transfer of an electron to Cyt c.

with apoptotic protease-activating factor 1 (Apaf-1), resulting in the formation of the apoptosome (Bras et al. 2005). This complex is able to recruit and activate seven dimers of caspase-9, which can then activate other downstream caspases. These effector caspases, including caspase-3 and -7, are able to activate DNases, inhibit DNA repair enzymes, and cleave cytoskeletal proteins, mediating the biochemical and morphological changes observed during apoptosis. The initiation of this cascade reaction is regulated by caspase inhibitors, including inhibitor of apoptosis proteins (IAPs) such as XIAP (Bratton et al. 2001). XIAP has been shown to bind the activated forms of caspase-3 and -9, thereby inhibiting the caspase cascade. Additional proteins released from the inter-membrane space during apoptosis, Omi/HtrA2 and Smac/DIABLO, are able to overcome the effects of XIAP through its binding and subsequent inactivation (Gao et al. 2007; Martins et al. 2002). It is important to note that recent findings have identified the release of cytochrome c as essential for cellular processes including formation of platelets, macrophage differentiation, Drosophila sperm cell differentiation and B-cell proliferation (Garrido et al. 2006). It is thought that these are able to avoid the apoptotic cascade through very specific cleavage of substrates by the caspases. The specific mechanisms behind these processes are still unknown, however it does suggest that release of cytochrome c is not only a regulator of cell death but of vital cellular processes.

A major contributor to caspase-independent apoptosis is apoptosis-inducing factor (AIF). When released from the inter-membrane space during apoptosis, AIF translocates through the cytosol to the nucleus via a nuclear localization signal (Susin et al. 2000; Susin et al. 1999). Once in the nucleus, AIF is able to bind DNA, resulting in large-scale DNA

fragmentation and peripheral chromatin condensation. As well, the mitochondrial endonuclease protein, Endo G, is able to work with both AIF and the caspase-activated DNase CAD/DFF40 (Bras et al. 2005). How these factors achieve chromatin condensation and nuclear degradation is not fully understood, and may involve nuclease activity, recruitment of other nucleases, or interaction with the DNA in such a way as to increase its susceptibility to nucleases. Although AIF has been shown to be caspase-independent, caspase-8 is able to induce a secondary release of AIF, and AIF is able to increase the release of cytochrome c (Cande et al. 2002; Cho and Choi 2002). This evidence of interaction between AIF and the caspase cascade indicates that these proteins do not always act in discrete pathways during apoptosis. The function of AIF in non-apoptotic cells remains elusive. Based on its similarity to prokaryotic oxidoreductases, it is thought that AIF may interact with Complex III of the electron transport chain, facilitating the transfer of an electron to cytochrome c (Cande et al. 2002). This proposed dual function of AIF coincides with that of cytochrome c, and it is probable that AIF may be involved in other cellular processes. It is very likely that more mitochondrial proteins will be identified in the future to have roles in both cell survival and cell death.

3.4 Mitochondrial theory of ageing

The mitochondria theory of ageing proposes that oxidation is the major contributor to cellular senescence and diseases associated with ageing. It is thought that ROS produced during mitochondrial respiration lead to senescence by damaging mtDNA, causing further declines in oxidative phosphorylation as well as other mitochondrial damage (Balaban et al. 2005; Dufour and Larsson 2004). During ROS production, a positive feedback loop

occurs where free-radical damage to mtDNA leads to respiratory dysfunction, which leads to increased leaking of free electrons into the matrix, and therefore more ROS and more mtDNA damage. Once cells accumulate enough damaged mitochondria, cell function is compromised and apoptosis may be initiated. Impairment of respiration is particularly detrimental in tissues that are critically dependent on large supplies of ATP, including the central nervous system, heart muscle, skeletal muscle and pancreatic islets (Chinnery and Turnbull 1997). These tissues have been shown to have accumulations of oxidative lesions in mtDNA, and can exhibit damage to mitochondrial proteins and lipids (Shigenaga et al. 1994). Given the sensitivity of these tissues, regulation of ROS production becomes of particular importance in maintaining cellular integrity.

The large amount of lifespan variation observed between species is of great interest when studying ageing in humans. Previous studies indicate that species with long lifespan tend to have low rates of ROS production, low levels of oxidative damage, and membranes that are resistant to oxidative damage (Pamplona et al. 2002). The low rates of ROS production are usually in response to low metabolic activity in these organisms, however there are exceptions. Birds, for example, have a very high metabolic activity associated with longevity, and insects show low metabolic activity with short lifespan (Dufour and Larsson 2004). Many of these exceptions can be explained by the efficiency of the ROS-eliminating systems in these organisms, where ROS production level still seems to be the underlying factor. In support of this theory, it has been found that increases in enzymatic antioxidants such as superoxide dismutase, or non-enzymatic antioxidants such as vitamin E, are able to increase lifespan in yeast, paramecium, nematodes, and mice (Dilova et al.

2007; Dufour and Larsson 2004). Ultimately, is appears that longevity is closely intertwined with energy production via the mitochondria, and the extent of ageing is dependent upon the efficiency of this process.

The cellular mechanisms that control energy conservation during times of reduced food availability may affect respiration such that ROS production is lowered and lifespan is prolonged (Shigenaga et al. 1994). The role of the mitochondria during calorie restriction is an active area of research that has yielded conflicting results. It has been shown that calorie restriction is able to promote mitochondrial respiration in mice and rats, and is able to do so with simultaneous reduction in ROS (Dilova et al. 2007; Lopez-Lluch et al. 2006). Up-regulation of mitochondrial respiration during calorie restriction has been demonstrated in other species, supporting the idea that increased respiration is an important feature in lifespan extension (Dilova et al. 2007). Conversely, several studies show that a decrease in mitochondrial function, in particular a decrease in respiration, extends lifespan (Dillin et al. 2002; Hansen et al. 2005; Lee et al. 2003). Calorie restriction is able to decrease respiration, and has been linked to decreases in superoxide production at complex I, as well as reduction of mtDNA damage (Dilova et al. 2007). It is important to note that although reduced mitochondrial respiration has been associated with increased lifespan, it has resulted in developmental delays as well as growth and behavioural alterations, possibly due to the catabolism of the organism's tissues (Dillin et al. 2002; Hansen et al. 2005; Lee et al. 2003). Apart from increasing / decreasing respiration, it has been suggested that calorie restriction acts to increase overall mitochondrial fitness via protection and maintenance of enzymes that normally decline

with age. Regardless of the action, for increases in longevity to occur in larger organisms, particularly those who have passed developmental stages, calorie restriction must be severe, making it difficult to maintain proper nutrient levels and a non-catabolic state. For this reason, calorie restriction, at present, is not a very practical application for lifespan extension in humans.

4. Goals of Study

In our ageing population, understanding degenerative diseases such as Parkinson disease is essential. A need for disease-modifying therapies for PD highlight the need for investigation into the underlying mechanisms of this disease. Although sporadic forms of PD are more common, inherited forms share features with the sporadic and can help us to understand disease pathogenesis and progression.

The fruit fly, *Drosophila melanogaster,* has proven to be an invaluable model system in the study of human disease, including PD (Whitworth 2011). As a relatively simple model organism, Drosophila are easy to genetically manipulate, yet they are complex enough to allow for behavioural assays. The conservation of genetic, molecular, and cell biology between Drosophila and humans has allowed Drosophila studies to address many biological puzzles relevant to human health.

Research for this thesis began shortly following initial reports of an association between mutations in *PINK1* and sporadic early-onset parkinsonism (Valente et al. 2004a; Valente et al. 2004b). With no animal models at the time, it was my goal to identify a Drosophila *PINK1* homologue and study the gene within a Drosophila model of the disease. In

particular, I wished to study the effects of *Pink1* in an *α-synuclein*-induced Drosophila model of PD, which had been previously used in our laboratory to study the biological role of *parkin*. At this time no links between *Pink1* and other PD genes had been identified. Therefore, my aim was to investigate the role of Pink1 in the Drosophila model in comparison to *parkin*. Moreover, I sought to use expression studies to investigate the possible protective role of *Pink1* and interactions it may have with other genes or pathways.

To explore possible roles of Pink1 in the Drosophila model, I identified, characterized and cloned a Drosophila homologue of human *PINK1 (Pink1)* and generated transgenic Drosophila that can conditionally express *Pink1* under the control of the Gal4-responsive UAS enhancer element. Expression studies were carried out using various neuronal and ubiquitous *Gal4* transgenes to express genes in a tissue specific manner. Biological parameters were analyzed to observe the effects of gene expression including morphological, longevity and behavioural effects. Initial results suggested that Pink1 was able to impart protection against protein toxicity, possibly through an interaction with parkin. To further evaluate the protective role of these proteins, I performed studies using *Gal4* and *Foxo* overexpression to induce cell stress. Results from these studies demonstrate complex roles for *Pink1* and *parkin*, and suggest that they may not act exclusively in cell protection. It is my hope that these findings shed light on the function of Pink1 in the cell, and help us better understand some of the mechanisms that underlie the pathogenesis of Parkinson disease.

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Chapter 2

Pink1 suppresses *α-synuclein* induced phenotypes in a Drosophila model of Parkinson disease

A version of this chapter has been published in Genome (Todd and Staveley 2008)

Abstract

Parkinson disease (PD) is the most prevalent human neurodegenerative movement disorder and is characterized by a selective and progressive loss of the dopaminergic neurons. Mutations in the genes parkin and PTEN-induced putative kinase 1 (PINK1) result in autosomal recessive forms of PD. It has been suggested that parkin and Pink1 function in the same pathway in Drosophila, with Pink1 acting upstream of parkin. Previous work in our laboratory has shown that parkin can rescue an α-synuclein-induced PD-like phenotype in Drosophila. To investigate the ability of Pink1 to protect against α-synuclein-induced toxicity, we performed longevity, mobility, and histological studies to determine whether Drosophila Pink1 can rescue the α-synuclein phenotypes. We found that overexpression of Pink1 results in the rescue of the α-synuclein-induced phenotype of premature loss of climbing ability, suppression of degeneration of the ommatidial array, and suppression of α-synuclein-induced developmental defects in the Drosophila eye. These results mark the first demonstration of Pink1 counteracting PD phenotypes in a protein toxicity animal model, and they show that Pink1 is able to impart protection against potentially harmful proteins such as α-synuclein that would otherwise result in cellular stress.

Introduction

Parkinson disease (PD) is the most prevalent human neurodegenerative movement disorder and affects 1-2% of the population over the age of 65 years (Weintraub et al. 2008). Characterized by a progressive loss of dopaminergic (DA) neurons in the *substantia nigra pars compacta*, PD results in age-dependent increases in bradykinesia, muscular rigidity, gait abnormalities, and resting tremor. Pathological characteristics of PD often include the presence of ubiquitin-positive and α-synuclein-enriched inclusions, known as Lewy bodies, in the remaining neurons. Currently, mutant forms of six genes have been linked to familial forms of PD, including α-synuclein, LRRK2, uchL1, DJ-1, parkin, and PINK1 (Gasser 2007). Although sporadic forms of PD are believed to be more common, 5-15% of PD cases are of an inherited nature, and many of these familial forms share features with sporadic PD, including oxidative stress, protein aggregation and mitochondrial dysfunction (Dawson and Dawson 2003). For this reason, understanding the mechanisms behind familial forms of PD may also help us to understand sporadic forms of PD.

Mutations in the gene PINK1 (PTEN induced putative kinase 1) result in a form of autosomal recessive PD and mutations in PINK1 have been detected in sporadic cases of the disease (Valente et al. 2004a; Valente et al. 2004b). Identified as a kinase, PINK1 has been shown to locate to the mitochondria and is hypothesized to be involved in mitochondrial protection. Mutations in PINK1, and in the Drosophila homologue Pink1, show substantial mitochondrial defects in sensitive tissues (Clark et al. 2006; Exner et al. 2007; Park et al. 2006; Yang et al. 2006). Studies support a role for PINK1 in the inhibition of the release of cytochrome c from the mitochondria (Petit et al. 2005; Wang et al. 2007) and indicate that PINK1 is important in

preventing oxidative damage, since mutations result in oxidative stress and a decrease in complex I activity (Hoepken et al. 2007). Complex I dysfunction is a common feature of sporadic PD, resulting in increased oxidative stress within the mitochondria and eventual mitochondrial dysfunction (Schapira 2008). Additionally, there is evidence supporting the involvement of PINK1 in the maintenance of the mitochondrial membrane potential and therefore mitochondrial function (Wood-Kaczmar et al. 2008). It is becoming clear that PINK1 has a role in mitochondrial protection, but the underlying mechanism and the relationship of PINK1 with other genes involved in PD are unknown.

Overexpression of the gene encoding α-synuclein has been shown to be an inherited cause of PD, and a transgenic α-synuclein model in Drosophila has been successfully used to mimic the degenerative processes seen in PD (Feany and Bender 2000; Whitworth et al. 2006). Moreover, α-synuclein pathology appears to be mediated by mitochondrial dysfunction (Hsu et al. 2000; Martin et al. 2006). Previous work in our laboratory has shown the ability of parkin overexpression to rescue an α-synuclein induced PD-like phenotype in Drosophila, presumably through targeting the α-synuclein protein for degradation (Haywood and Staveley 2004). Additionally, Pink1 has recently been shown to protect neurons from the dopaminergic neurotoxin MPTP (Haque et al. 2008), where the pathology of MPTP is attributed to the α-synuclein protein (Dauer et al. 2002; Klivenyi et al. 2006). It has been suggested that parkin and Pink1 function in the same pathway and that this Pink1/parkin pathway is necessary for proper mitochondrial function and morphology (Clark et al. 2006; Park et al. 2006; Poole et al. 2008). To further investigate the possibility of Pink1 acting in this pathway, we have performed longevity, mobility and histological studies to determine

the effects of overexpression of Drosophila Pink1 and its ability to rescue the α-synuclein phenotypes.

Materials and Methods

Bioinformatic sequence analysis

The *Drosophila melanogaster* homologue of PINK1 was identified with the tblastn algorithm (Altschul et al. 1990; Camacho et al. 2009) of the National Center for Biotechnology Information (NCBI) using the theoretical translation of human PINK1, NM_032409.2. The *Mus musculus* (BC067066.1), *Danio rerio* (BC165767.1), *Caenorhabditis elegans* (NM_062616.3), and *Anopheles gambiae* (XM_313587.4) homologues of Pink1 were identified with the tblastn algorithm using the theoretical translation of the *Drosophila melanogaster* homologue GH20931 cDNA. A multi-alignment of the homologues was constructed using the ClustalW2 package of the European Bioinformatics Institute (Larkin et al. 2007). Mitochondrial targeting motifs were identified using MitoProt of the Institute of Human Genetics (Claros and Vincens 1996). The kinase domains and active sites were identified using InterProScan of the European Bioinformatics Institute (Quevillon et al. 2005).

Molecular biology

A clone of the *Drosophila melanogaster* Pink1 cDNA (GH20931) was obtained from Research Genetics (Rubin et al. 2000) and was sub-cloned into the pUAST vector to generate the *UAS-Pink1* transgene. Transgenic lines were created using heat shock π as a source of transposase and standard injection techniques (Binari et al. 1997) into w^{1118} *Drosophila melanogaster* embryos.

Fly stocks and culture

Dr. M. Feany (Harvard Medical School) generously provided *UAS-α-synuclein* flies (Feany and Bender 2000) and Dr. J. Hirsh (University of Virginia) the *Ddc-Gal4* flies (Li et al. 2000). The *GMR-Gal4* flies (Freeman 1996) were obtained from the Bloomington Drosophila Stock Center at Indiana University. The *UAS-α-synuclein; UAS-Pink1* line was generated using standard techniques. To drive expression of the transgenes, *Ddc-Gal4* (for expression in the dopaminergic neurons) or *GMR-Gal4* (for expression in the eye) homozygous females were crossed to w^{1118} males (control) or *UAS-α-synuclein* with or without *UAS-Pink1*.

Ageing analysis

Two hundred adult males of each genotype were collected under gaseous carbon dioxide and aged upon standard cornmeal/yeast/molasses/agar media, at 25°C, in upright standard plastic shell vials. Flies were maintained in non-crowded conditions with 1 to 20 individuals per vial. Flies were scored for viability every two days and transferred to fresh media. Resulting survival curves were compared via logrank test.

Locomotion assay

Forty adult males of each genotype were assayed for climbing ability (Todd and Staveley 2004). Flies were maintained on standard cornmeal/yeast/molasses/agar media at 25°C and were assayed every seven days. Climbing ability was determined via non-linear curve fit within a 95% confidence interval (CI).

Scanning electron microscopy of the Drosophila eye

Flies of each genotype were aged on standard cornmeal/yeast/molasses/agar media at 29°C. These were then frozen at -70°C and examined under a dissecting microscope.

Flies were mounted, desiccated overnight and coated in gold before photography at 150 times magnification with a Hitachi S-570 SEM. Area of the eye was measured based on the presence of ommatidia. Ommatidia with an area greater than 150% of normal ommatidia were classified as fused or enlarged. For each condition at least six representative flies were analyzed.

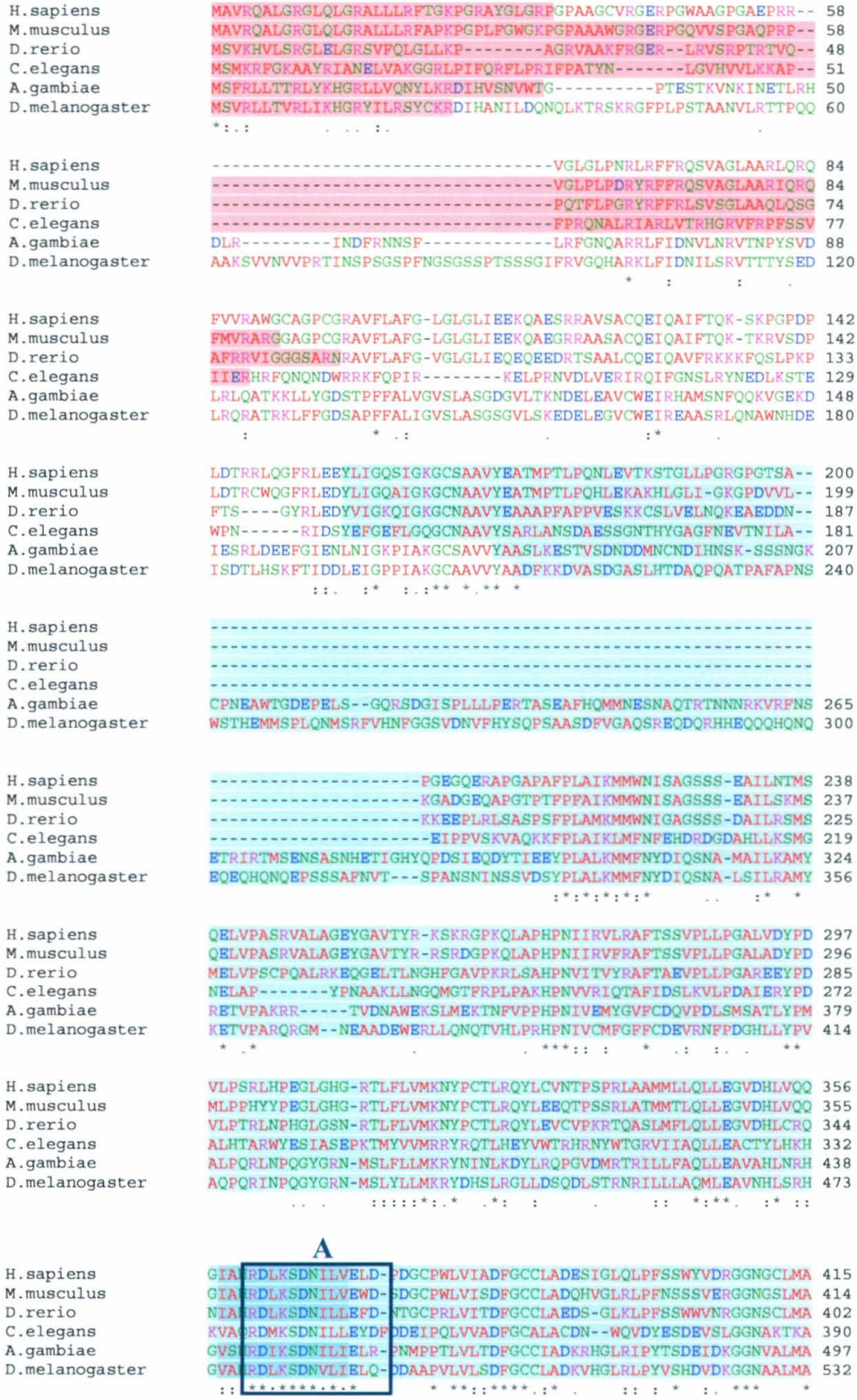
Histological examination of Drosophila adult retinas

Adult flies were aged on standard cornmeal/yeast/molasses/agar media at 25°C. Heads of the flies were removed, fixed in Karnovsky's fixative and embedded in epon. Tangential retinal sections were prepared for 8 flies of each genotype, at a thickness of 0.5 μm. Sections were stained with toluidine blue, examined by light microscopy and photographed at magnification of 800 times. The extent of ommatidial disruption is represented by the percentage of ommatidia exhibiting vacuolization and architectural distortion.

Results

The Drosophila melanogaster Pink1 retains functional domains found in mammalian, fish, and invertebrate homologues

Drosophila Pink1 encodes a theoretical protein of 721 amino acids (Figure 1). Alignment of Drosophila Pink1 with vertebrate and invertebrate homologues using ClustalW2 reveal that both the mitochondrial targeting motif and the serine/threonine protein kinase domain are well conserved in their relative sequence / position in the protein. Although there is variation in length of the mitochondrial targeting motif, all homologues score 91% and above by MitoProt for predicted location to the mitochondria.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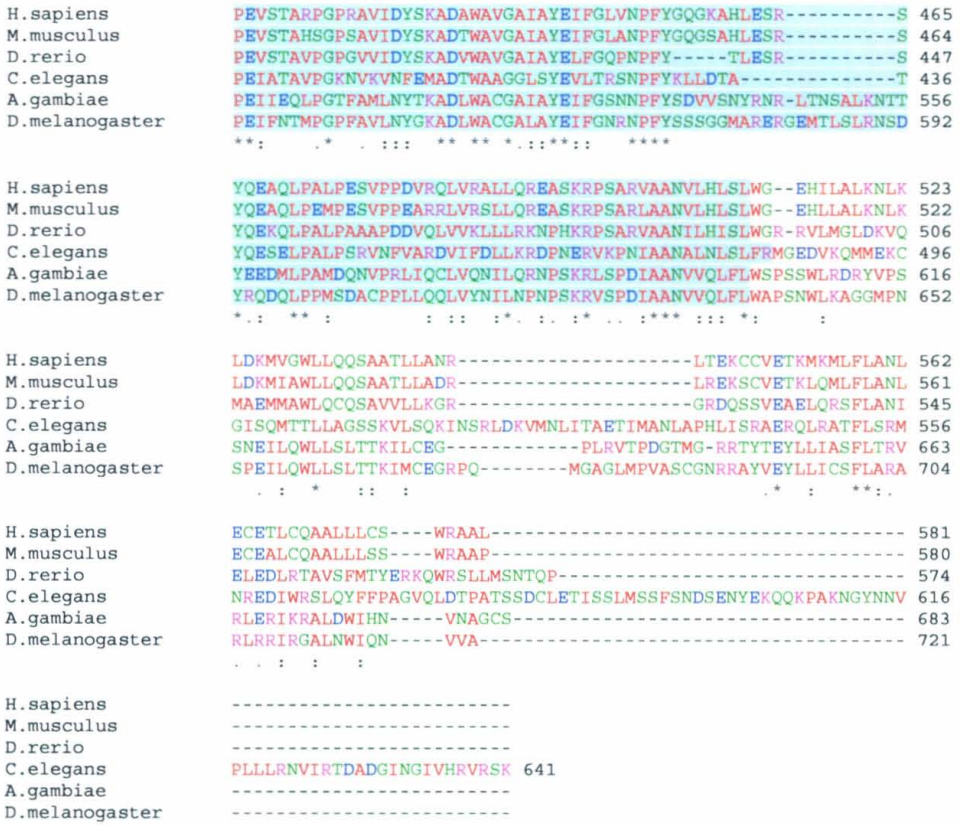

Figure 1. Pink1 sequence alignments of *Homo sapiens*, *Mus musculus*, *Danio rerio*, *Caenorhabditis elegans*, *Anopheles gambiae*, and *Drosophila melanogaster*. Red shading indicates the mitochondrial targeting motif. The serine/threonine protein kinase, catalytic domain is indicated by the blue shading, with the active site outlined in box A. Similarity is indicated as identical (*), conserved substitutions (:), and semi-conserved substitutions (.). Amino acid colours indicate acidic (blue), basic (magenta), basic-hytroxyl / amine (green), and small-hydrophobic including aromatic (red).

Pink1 rescues the α-synuclein induced phenotype of premature decreased climbing ability

Drosophila melanogaster exhibit a strong negative geotactic response and, when stimulated, will immediately climb towards the top of a vial (Le Bourg and Lints 1992). This response decreases with age to result in shorter, more sporadic climbs as well as an increased number of failed climbing attempts. Transgenic flies that express α-synuclein, in response to either a pan-neural (elav-Gal4) or a dopaminergic neuronal (Ddc-Gal4) transgene, prematurely develop this locomotor dysfunction (Figure 2A)(Feany and Bender 2000). This results in a functional impairment that provides an experimentally versatile Drosophila model of Parkinson disease. Our results show that within this α-synuclein-induced model, directed overexpression of Pink1 to the dopaminergic neurons results in the complete suppression of the premature loss in climbing ability (Figure 2A). These results suggest that with the expression of Pink1, the dopaminergic neurons are able to overcome the damaging effects of α-synuclein. Directed overexpression of Pink1 alone does not have a significant effect on climbing ability (Figure 2A). Neuronal expression of α-synuclein and / or overexpression of Pink1 do not alter lifespan (Figure 2B), indicating that changes observed are due to differences in mobility and are not due to premature senescence.

Pink1 suppresses α-synuclein-induced developmental defects in the eye

GMR-Gal4 heterozygotes raised at 25°C have a rough eye phenotype that is enhanced when the flies are raised at 29°C (Figure 3A) (Kramer and Staveley 2003). At 29°C, the severity of this rough eye phenotype increases with the expression of α-synuclein (Figure 3C, E), to suggest that the presence of α-synuclein further interferes with normal ommatidial development. By overexpressing Pink1 with α-synuclein in the

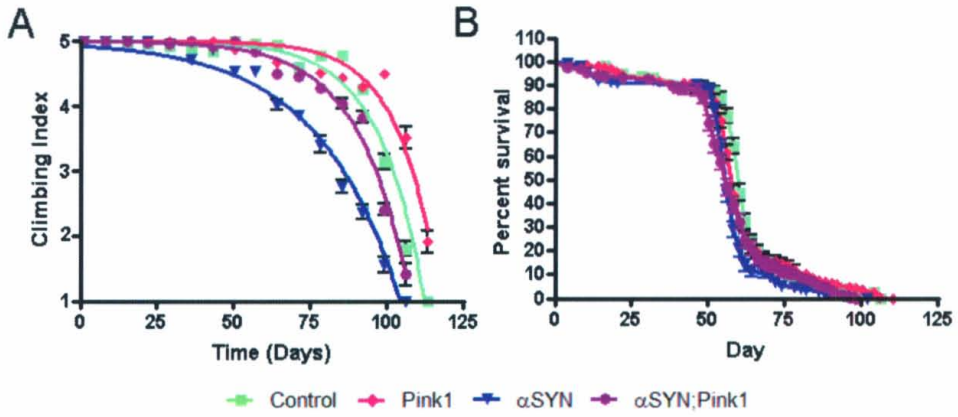

Figure 2. Pink1 rescues the α-*synuclein* induced phenotype of premature decreased climbing ability. Overexpression of *Pink1* in the dopaminergic neurons via *Ddc-Gal4* results in increased mobility and rescue of the *α-synuclein* induced phenotype (A). Longevity of flies expressing *Pink1* and / or *α-synuclein* do not differ from control flies (B). Genotypes are (Control) w^{1118}; +/+; *Ddc-Gal4/+*, (Pink1) w^{1118}; +/+; *UAS-Pink1/Ddc-Gal4*, (αSYN) w^{1118}; *UAS*-α-*synuclein/+*; *Ddc-Gal4/+*, and (αSYN;Pink1) w^{1118}; *UAS*-α-*synuclein/+*; *UAS-Pink1/Ddc-Gal4*. Longevity is shown as percent survival ($p<0.05$ as determined by log rank). Climbing ability was determined via non-linear curve fit (CI = 95%). Error bars indicate standard error.

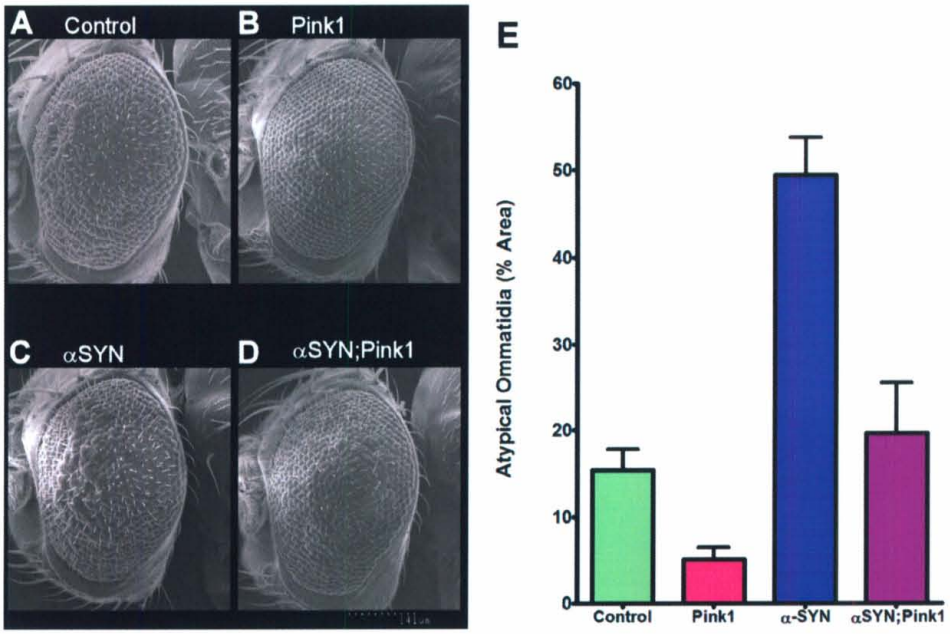

Figure 3. Pink1 suppresses α-synuclein induced developmental defects in the eye. Rough eye phenotype present at 29°C with *GMR-Gal4* (A). Ultra-structure disarray is increased with α-*synuclein* expression and is suppressed with overexpression of *Pink1* as compared to both α-*synuclein* and the control (A-E). The genotypes are (A) w^{1118}; *GMR-Gal4/+*, (B) w^{1118}; *GMR-Gal4/+*; *UAS-Pink1/+*, (C) w^{1118}; *GMR-Gal4/UAS-α-synuclein*, and (D) w^{1118}; *GMR-Gal4/UAS-α-synuclein*; *UAS-Pink1/+*. (A-D) scanning electron micrographs (SEM). (E) Percent of eye area containing fused or enlarged (>150%) ommatidia. Error bars indicate standard error.

developing eye, a partial suppression of the rough eye phenotype is observed, such that the level of ultra-structure disruption is reduced to control levels (Figure 3D, E). Additionally, overexpression of Pink1 partially suppresses the rough eye phenotype of the control (Figure 3B, E). These results suggest that overexpression of Pink1 is not only able to counteract the effects of α-synuclein in the developing eye, but also, to some extent, the effects of Gal4.

Pink1 suppresses α-synuclein-induced degeneration of the ommatidial array

To observe neuronal degeneration over time, the α-synuclein model was used to observe degeneration of the Drosophila ommatidial array. As previously described, cross-sections of the retinas of Drosophila expressing α-synuclein in the developing eye are intact at three days old, with premature deterioration of the retina at thirty days old (Figure 4C, G, I) (Feany and Bender 2000). As compared to the control (Figure 4A, E, H), overexpression of Pink1 in the developing eye (Figure 4B, F, I) results in no significance difference in retinal degeneration. When Pink1 is overexpressed within the α-synuclein model, intact retinas are seen at three-days-old and this morphology is maintained at thirty-days-old (Figure 4D, H, I). These results demonstrate the ability of Pink1 overexpression to suppress the α-synuclein-dependent degeneration of the ommatidial array, further supporting the participation of Pink1 in overcoming the detrimental effects of the α-synuclein protein.

Discussion

The α-synuclein protein is a central pathogenic mechanism leading to oxidative stress (Dauer et al. 2002), and α-synuclein accumulation has been associated with mitochondrial Complex I dysfunction (Betarbet et al. 2006; Fornai et al. 2005). Even

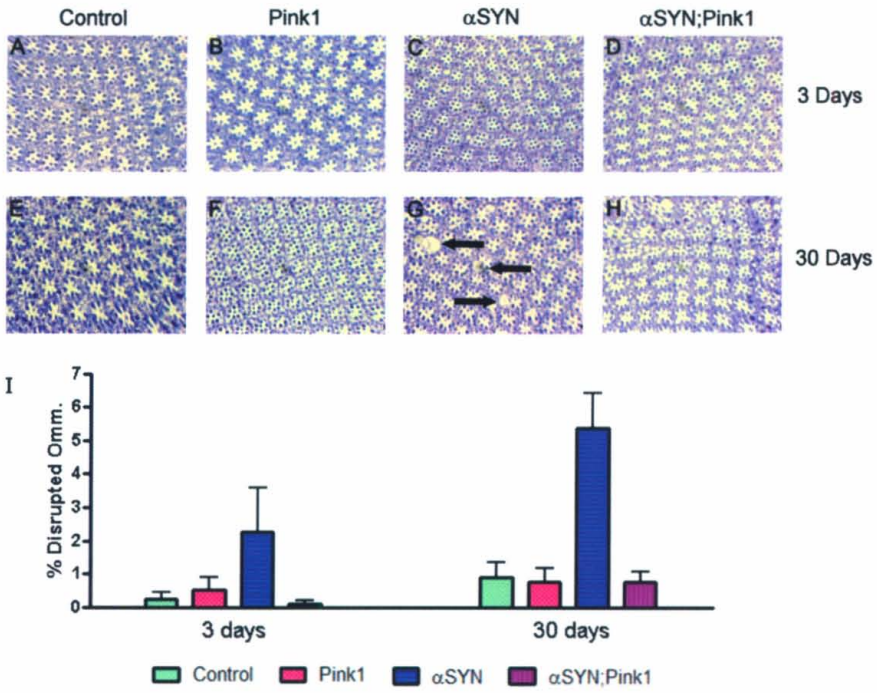

Figure 4. Expression of Pink1 suppresses α-synuclein-induced retinal degeneration. Flies expressing α-*synuclein* have intact retinas at 3 days old with deterioration of the retina at 30 days old (C, G, I). Flies overexpressing *Pink1* with α-*synuclein* show intact retinas at 3 and 30 days old (D, H, I). The genotypes are (Control) w^{1118}; *GMR-Gal4/+*, (Pink1) w^{1118}; *GMR-Gal4/+*; *UAS-Pink1/+*, (αSYN) w^{1118}; *GMR-Gal4/UAS-α-synuclein*, and (αSYN;Pink1) w^{1118}; *GMR-Gal4/UAS-α-synuclein*; *UAS-Pink1/+*. (A-D) 3 day old flies, (E-H) 30 day old flies. (I) Percent of disrupted ommatidia present in the ommatidial array. Error bars indicate standard error. Arrows indicate degeneration of the ommatidial architecture.

a slight elevation in α-synuclein levels can lead to neuronal toxicity (Ibanez et al. 2004), and it has been suggested that α-synuclein is able to disrupt a range of normal cellular functions through the formation of cellular protein aggregates (Mukaetova-Ladinska and McKeith 2006). In addition, oligomerized forms of the protein may also lead to toxicity by forming pores within cellular membranes (Volles et al. 2001). Although the exact pathogenesis of α-synuclein remains unclear, it has been successfully used to mimic the degenerative processes seen in Parkinson disease.

This study marks the first demonstration of Pink1 counteracting the phenotypic effects of α-synuclein toxicity in an animal model. The ability of Pink1 to suppress the effects of α-synuclein are similar to those observed with parkin (Haywood and Staveley 2004), supporting the theory that Pink1 and parkin act in a common pathway (Clark et al. 2006; Park et al. 2006; Poole et al. 2008). It is possible that Pink1 may activate parkin directly or indirectly, thereby activating the ubiquitin-proteasomal system, to result in the tagging of α-synuclein for degradation. Pink1 may operate to protect the mitochondria through an interaction with molecular chaperones, since PINK1 has been shown to phosphorylate the mitochondrial chaperone TRAP1 (Hsp75), thus protecting mitochondria against oxidative stress and preventing cytochrome c release (Pridgeon et al. 2007). In support of chaperone involvement, α-synuclein toxicity in Drosophila is able to be partially rescued by co-expression of molecular chaperones, namely Hsp70 (Auluck et al. 2002). Although the identification of PINK1 targets is in its infancy, the ability of Pink1 to suppress phenotypes in a toxic protein model may suggest a broad role for Pink1 in mitochondrial protection and the existence of targets that serve to protect against toxic proteins such as α-synuclein.

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Chapter 3

Expression of *Pink1* with *α-synuclein* in the dopaminergic neurons of Drosophila leads to increases of both lifespan and healthspan

A version of this chapter has been submitted to Genetics and Molecular Research (Todd and Stavely 2011)

Abstract

Overexpression of the gene coding *α-synuclein* has been shown to be an inherited cause of Parkinson disease. Our laboratory has previously used the genes *parkin* and *Pink1* to rescue *α-synuclein*-induced phenotypes within a Drosophila model. To further investigate the effect of *Pink1* in this model, we have performed longevity and behavioural studies using various driver lines to express the *α-synuclein* and *Pink1* genes. Our findings show that overexpression of *Pink1* and overexpression of *Pink1* with *α-synuclein* result in an increased lifespan when driven with the *TH-Gal4* transgene. This increase in longevity is accompanied with increased healthspan, as measured by mobility, suggesting that this is an example of improved functional ageing. Our results indicate that, in the dopaminergic cells targeted by *TH-Gal4,* increased expression of *α-synuclein* and *Pink1* together have a synergistic effect, allowing for enhanced protection and increased survival of the organism.

Introduction

Parkinson disease (PD) affects 1-2% of the population over the age of 65 years, where age is the largest risk factor for the development and progression of the disease (Lees et al. 2009). PD is characterized by a progressive loss of dopaminergic neurons in the *substantia nigra pars compacta,* often including the presence of ubiquitin-positive and α-synuclein-enriched inclusions, known as Lewy bodies, in the remaining neurons. Several genes have been linked to familial forms of PD, including the genes encoding α-synuclein and PTEN induced putative kinase 1 (PINK1).

PINK1 has been shown to locate to the mitochondria and is hypothesized to be involved in mitochondrial protection (Deas et al. 2009). Mutations in *PINK1*, and in the Drosophila homologue *Pink1*, show substantial mitochondrial defects in sensitive tissues, with the inability to inhibit cytochrome c release under stress conditions (Clark et al. 2006; Exner et al. 2007; Park et al. 2006; Wang et al. 2007; Yang et al. 2006). As a protective protein, PINK1 may confer protection of the mitochondria through several mechanisms (Deas et al. 2009), including an interaction with molecular chaperones to regulate oxidative stress responses, activation of the parkin E3 ubiquitin ligase to result in the tagging of toxic proteins, such as α-synuclein, for degradation, or through the initiation of fission events to remove dysfunctional mitochondria via mitophagy.

Overexpression of the gene encoding α-synuclein has been shown to be an inherited cause of PD, and a transgenic *α-synuclein* model in Drosophila has been successfully used to mimic the degenerative processes seen in PD (Feany and Bender 2000; Whitworth et al. 2006). Previous work in our laboratory has shown the ability of

parkin overexpression and *Pink1* overexpression to rescue an *α-synuclein*-induced PD-like phenotype in *Drosophila melanogaster*, presumably through the targeting of the α-synuclein protein for degradation (Haywood and Staveley 2004; Todd and Staveley 2008). To further investigate the effect of *Pink1* in this model, we have performed longevity and behavioural studies using several neuronal and ubiquitous drivers to express the *α-synuclein* and *Pink1* transgenes.

Materials and Methods

Fly stocks and culture

Dr. M. Feany (Harvard Medical School) generously provided *UAS-α-synuclein* flies (Feany and Bender 2000) and Dr. J. Hirsh (University of Virginia) provided the *Dopa decarboxylase-Gal4 (Ddc-Gal4)* flies (Li et al. 2000) and the *Tyrosine Hydroxylase-Gal4 (TH-Gal4)* flies (Friggi-Grelin et al. 2003). The *UAS-Pink1* transgenic line was created previously using the GH20931 *Drosophila melanogaster Pink1* clone (Todd and Staveley 2008). The *UAS-α-synuclein*; *UAS-Pink1* line was generated using standard techniques. The w^{1118} flies were obtained from Dr. Howard Lipshitz at the Hospital for Sick Children in Toronto. The $GawB^{C739}$-*Gal4*, $GawB^{V55}$-*Gal4*, *Elav-Gal4*, *arm-Gal4* and *UAS-GFP* were obtained from the Bloomington Drosophila Stock Center at Indiana University. All flies were cultured on standard cornmeal/yeast/molasses/agar media at 25°C.

Ageing analysis

Two hundred adult males of each genotype were collected under gaseous carbon dioxide and aged upon standard cornmeal/yeast/molasses/agar media, at 25°C, in upright standard plastic shell vials. Flies were maintained in non-crowded conditions

with 1 to 20 individuals per vial. Flies were scored for viability every two days and transferred to fresh media. Resulting survival curves were compared via logrank test.

Locomotion assay

Forty adult males of each genotype were assayed for climbing ability (Todd and Staveley 2004). Flies were maintained on standard cornmeal/yeast/molasses/agar media at 25°C and were assayed every seven days. Climbing ability was determined via non-linear curve fit within a 95% confidence interval (CI).

Results

Overexpression of *Pink1* increases lifespan when driven with *TH-Gal4* (Figure 1A). In addition, overexpression of *Pink1* with *α-synuclein,* using the *TH-Gal4* driver, results in a dramatic increase in lifespan. The results suggest that increases in expression of *α-synuclein* and *Pink1* together can have a synergistic effect, allowing for enhanced protection and increased survival. When assessing the extension of lifespan observed in Figure 1A, it is necessary to determine if this is accompanied by improved healthspan, or functional ageing, in the surviving individuals. *Drosophila melanogaster* exhibit a strong negative geotactic climbing response, allowing for the conduct of mobility assays within the *α-synuclein*-induced model (Haywood and Staveley 2004; Todd and Staveley 2008). When we assessed the climbing ability of flies expressing *Pink1,* using the *TH-Gal4* driver (Figure 1B), there was a rescue of the characteristic *α-synuclein*-induced phenotype of premature loss of climbing ability. In addition, flies expressing *Pink1* with *α-synuclein* using the *TH-Gal4* driver showed significant increase of climbing ability in the surviving flies as compared to other genotypes of the same age. This suggests that the increase in longevity is an

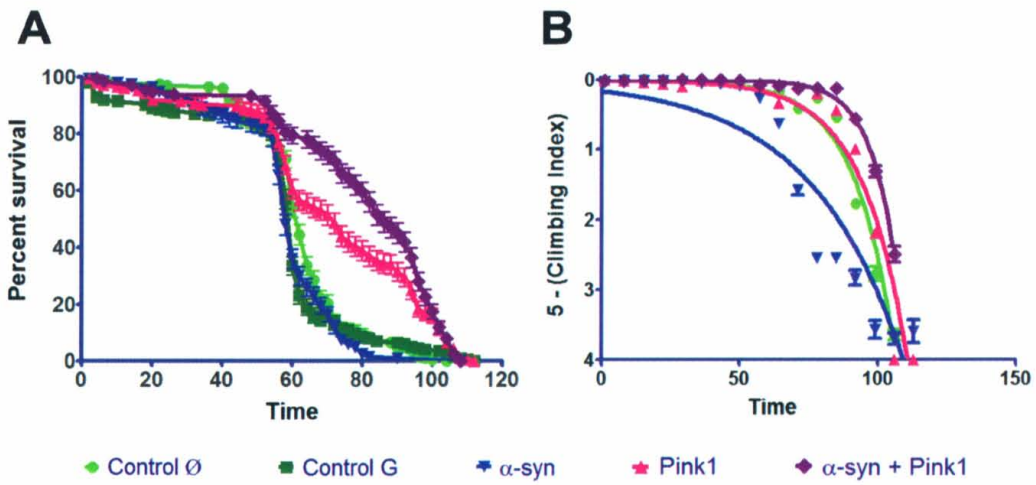

Figure 1. Effect of *Pink1* and *α-synuclein* expression on longevity (A) and mobility (B) when driven with *TH-Gal4*. Overexpression of *Pink1* using the *TH-Gal4* driver shows an increase in lifespan (mean = 72 days) as compared to controls (mean = 61±1 days), $p<0.0001$ (A). Overexpression of *Pink1* with *α-synuclein* using the *TH-Gal4* driver shows a dramatic increase in lifespan (mean = 86 days) as compared to controls (mean = 61±1 days), $p<0.0001$. Overexpression of *Pink1* results in a rescue of the *α-synuclein*-induced premature loss of climbing ability when driven with *TH-Gal4* (B). Overexpression of *Pink1* with *α-synuclein,* using *TH-Gal4,* shows significantly increased climbing ability above that of controls (Non-linear curve fit comparison, CI=95%). Survival curves were compared using the Log-rank Test. Genotypes expressed include w^{1118} (Control Ø), *UAS-GFP/+* (Control G), *UAS-α-synuclein/+* (α-syn), *UAS-Pink1/+* (Pink1), *UAS-α-synuclein/+; UAS-Pink1/+* (α-syn + Pink1). Error bars indicate standard error of the mean.

example of healthy ageing, and that the lifespan differences observed between *Pink1* with *α-synuclein*, versus *Pink1* expression alone, is a synergistic effect.

To investigate the effect of *α-synuclein* and *Pink1* expression in other cell populations, longevity studies were performed using a variety of established constitutive drivers (Figure 2). The significant increases in lifespan shown with *TH-Gal4* (Figure 1A) were not observed when using other drivers, including neuronal (*GawB^{C739}-Gal4, GawB^{V55}-Gal4, Elav-Gal4*) and ubiquitous (*Arm-Gal4*) drivers (Figure 2A, B, C and D respectively). This suggests that the increases in longevity are dependent on expression within the dopaminergic neurons targeted by *TH-Gal4.*

Discussion

Our findings show that expression of *Pink1* with *α-synuclein* has a synergistic effect when driven with the *TH-Gal4* transgene, leading to increased longevity in Drosophila. Healthspan, as measured by climbing ability over time, is also enhanced, suggesting an improvement of functional ageing in these flies, opposed to longevity alone. This protective effect is of interest, as α-synuclein is involved in a central pathogenic mechanism for Parkinson disease and has been linked to various aspects of mitochondrial dysfunction (Schapira and Gegg 2011). Previous studies indicate that accumulation of α-synuclein in the mitochondria of mammalian dopaminergic neurons leads to reduced mitochondrial complex I activity and increased production of reactive oxygen species (ROS) (Devi et al. 2008; Liu et al. 2009). It is important to note that ROS also act as signalling molecules, and can be involved in a number of pro-survival pathways, including regulation of autophagy (Scherz-Shouval and Elazar 2007; Weber and Reichert 2010). In this respect, α-synuclein can be involved in the turnover of

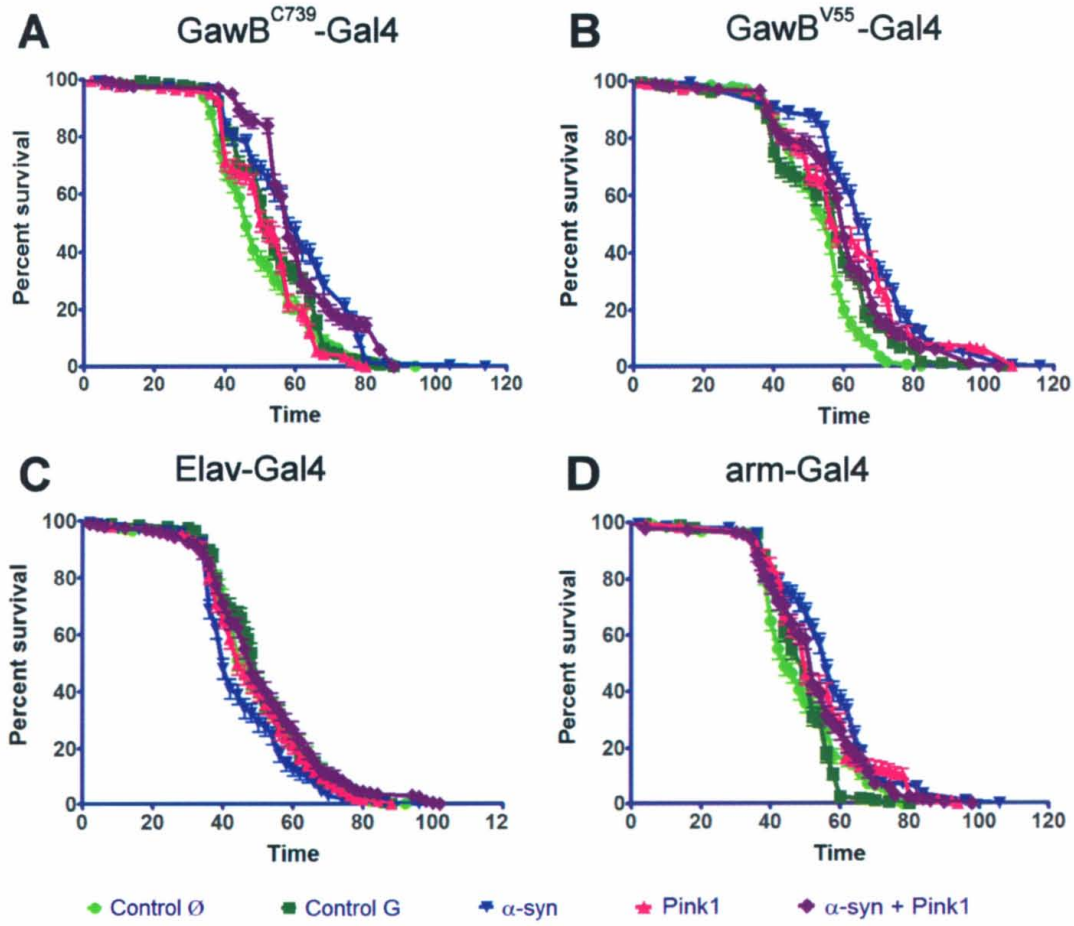

Figure 2. Effect of *Pink1* and *α-synuclein* expression on longevity when driven with *GawB*^{C739}*-Gal4* (A), *GawB*^{V55}*-Gal4* (B), *Elav-Gal4* (C) and *arm-Gal4* (D). Survival curves were compared using the Log-rank Test. Genotypes expressed include w^{1118} (Control Ø), *UAS-GFP/+* (Control G), *UAS-α-synuclein/+* (α-syn), *UAS-Pink1/+* (Pink1), *UAS-α-synuclein/+; UAS-Pink1/+* (α-syn + Pink1). Error bars indicate standard error of the mean.

mitochondria by autophagy, or mitophagy, acting in unison with Pink1 in a pro-survival role via the removal of defective mitochondria.

The increases in lifespan shown with *TH-Gal4* were not observed when using other drivers, including neuronal (*Elav-Gal4, GawB^{C739}-Gal4, GawB^{V55}-Gal4*) and ubiquitous (*Arm-Gal4*) drivers. Within the cell, tyrosine hydroxylase enzyme catalyzes the conversion of l-tyrosine to l-dopa, which is the initial and rate-limiting step in the biosynthesis of catecholamines such as dopamine. These dopaminergic neurons are particularly sensitive, perhaps exacerbated by the metabolic stress created by sustained Ca^{2+} entry during signalling (Surmeier et al. 2010). *Pink1* may have a more pronounced protective effect in this particular cell type, as Pink1 acts in a general protective role but can also directly regulate calcium flux through the mitochondria (Deas et al. 2009). Interestingly, previous work in our laboratory expressing *α-synuclein* and *Pink1* using *Ddc-Gal4* (Todd and Staveley 2008) has not shown the synergistic effect seen in this study, using *TH-Gal4*. Dopa decarboxylase catalyzes the last step of dopamine synthesis, l-dopa to dopamine, and the last step of serotonin synthesis, l-tryptophan to serotonin. The discrepancy between results seen when using the *TH-Gal4* driver and the *Ddc-Gal4* driver may be due to differences in the amount of Pink1 produced, or possibly due to differing coverage of the dopaminergic neurons. There is growing evidence that although all dopaminergic neuron clusters in the fly brain seem to be targeted by the *TH-Gal4* driver, they are not covered equally, and that *Ddc-Gal4* likely does not target all dopaminergic neuronal clusters (Yarali and Gerber 2010). This incomplete overlap of dopaminergic neurons targeted by the *TH-Gal4* and *Ddc-Gal4* transgenes may indicate that there is a particular dopaminergic cell cluster responsible for the increased lifespan observed in

this study. It will be important for future studies to examine the differences between the *TH-Gal4* and *Ddc-Gal4* drivers with respect to ageing and functional longevity.

Our results suggest that increases in *α-synuclein* and *Pink1* together can have a synergistic effect, allowing for enhanced protection and increased functional longevity in Drosophila. This may be a result of the upregulation of pro-survival mechanisms via Pink1, in response to an increase in ROS signalling due to *α-synuclein* overexpression. The restriction of these results to *TH-Gal4*-expressing cells likely reflects the existence of a dopaminergic cell cluster that is particularly sensitive to changes in *Pink1* and *α-synuclein* expression. Moreover, the results indicate the need for future examination of this particular cell population, where findings may shift therapeutic efforts towards a particular dopaminergic cluster.

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Chapter 3 Supporting Information

Table S1. Lifetime survival data presented at 20 day age-intervals (I). Table indicates number entering the age-interval (Nx) and number dead within the age interval (Dx). Genotypes expressed include w^{1118} (Control Ø), *UAS-GFP/+* (Control G), *UAS-α-synuclein/+* (α-syn), *UAS-Pink1/+* (Pink1), *UAS-α-synuclein/+; UAS-Pink1/+* (α-syn + Pink1).

A. TH-Gal4

Control Ø			Control G			α-syn			Pink1			α-syn + Pink1		
I	Nx	dx	I	Nx	dx	I	Nx	dx	I	Nx	dx	I	Nx	dx
0	200	0	0	200	0	0	200	0	0	200	0	0	200	0
20	179	21	20	196	4	20	193	7	20	184	16	20	190	10
40	174	26	40	192	8	40	173	27	40	181	19	40	188	12
60	67	133	60	108	92	60	72	128	60	121	79	60	159	41
80	17	183	80	19	181	80	5	195	80	78	122	80	119	81
100	8	192	100	1	199	100	2	198	100	32	168	100	35	165
120	0	200	120	0	200	120	0	200	120	0	200	120	0	200

B. GawB^{C739}-Gal4

Control Ø			Control G			α-syn			Pink1			α-syn + Pink1		
I	Nx	dx	I	Nx	dx	I	Nx	dx	I	Nx	dx	I	Nx	dx
0	200	0	0	200	0	0	200	0	0	200	0	0	200	0
20	198	2	20	196	4	20	196	4	20	195	5	20	195	5
40	163	37	40	137	63	40	167	33	40	143	57	40	194	6
60	65	135	60	43	157	60	94	106	60	45	155	60	81	119
80	4	196	80	4	196	80	3	197	80	0	200	80	29	171
100	0	200	100	0	200	100	2	198				100	0	200
						120	0	200						

C. GawB^{V55}-Gal4

Control Ø			Control G			α-syn			Pink1			α-syn + Pink1		
I	Nx	dx	I	Nx	dx	I	Nx	dx	I	Nx	dx	I	Nx	dx
0	200	0	0	200	0	0	200	0	0	200	0	0	200	0
20	194	6	20	198	2	20	199	1	20	194	6	20	195	5
40	150	50	40	175	25	40	182	18	40	172	28	40	168	32
60	73	127	60	40	160	60	128	72	60	91	109	60	91	109
80	12	188	80	1	199	80	25	175	80	24	176	80	16	184
100	1	199	100	0	200	100	9	191	100	12	188	100	2	198
120	0	200				120	0	200	120	0	200	120	0	200

D. Elav-Gal4

Control Ø			Control G			α-syn			Pink1			α-syn + Pink1		
I	Nx	dx	I	Nx	dx	I	Nx	dx	I	Nx	dx	I	Nx	dx
0	200	0	0	200	0	0	200	0	0	200	0	0	200	0
20	197	3	20	193	7	20	194	6	20	194	6	20	193	7
40	141	59	40	146	54	40	96	104	40	131	69	40	142	58
60	42	158	60	47	153	60	25	175	60	42	158	60	53	147
80	3	197	80	4	196	80	3	197	80	3	197	80	9	191
100	0	200	100	0	200	100	0	200	100	0	200	100	1	199
												120	0	200

E. arm-Gal4

Control Ø			Control G			α-syn			Pink1			α-syn + Pink1		
I	Nx	dx	I	Nx	dx	I	Nx	dx	I	Nx	dx	I	Nx	dx
0	200	0	0	200	0	0	200	0	0	200	0	0	200	0
20	196	4	20	194	6	20	197	3	20	197	3	20	195	5
40	164	36	40	130	70	40	160	40	40	170	30	40	155	45
60	5	195	60	34	166	60	74	126	60	58	142	60	52	148
80	0	200	80	0	200	80	14	186	80	7	193	80	5	195
						100	2	198	100	0	200	100	0	200
						120	0	200						

Chapter 4

Pink1 and *parkin* demonstrate multifaceted roles when co-expressed with *Foxo*

A version of this chapter has been submitted to European Journal of Neuroscience (Todd and Staveley 2011)

Abstract

Pink1 has been linked to both autosomal recessive and sporadic forms of Parkinson disease. The Pink1 protein is thought to be involved in mitochondrial protection by interacting with parkin to prevent oxidative damage, through maintenance of mitochondrial integrity and regulation of mitophagy. To further investigate the roles of Pink1/parkin during cell stress, we have performed co-expression studies to determine the effects *Pink1* and *parkin* on the *Foxo*-induced phenotype of developmental defects in the Drosophila eye. Although direct and indirect links have been made between Pink1/parkin and insulin receptor (INR) pathway components PTEN, Akt and Foxo, the effects of Pink1/parkin in the INR pathway have been largely overlooked. Overexpression of *Foxo* in the Drosophila eye leads to a characteristic phenotype of reductions in cell size and cell number. We looked at the ability of *Pink1* and *parkin,* as protective proteins, to rescue this phenotype. We examined directed expression of *Pink1, parkin, Pink1* or *parkin* mutants, and *Pink1* or *parkin* interfering RNAs (RNAi) with the overexpression of *Foxo* in the developing eye of Drosophila. Unexpectedly, our findings show that reduction of *Pink1* suppresses the effects of *Foxo* overexpression, where co-overexpression with *Pink1* or *parkin* increases the severity of the phenotype. This suggests that *Pink1* and *parkin* are able to increase the effects of *Foxo*. Contrary to the view that Pink1 and parkin act exclusively as protective proteins in the cell, it is likely that the Pink1/parkin pathway is involved in aspects of cell fate decisions other than degrading toxic proteins and maintaining mitochondrial integrity.

Introduction

Pink1 (PTEN induced putative kinase 1) encodes a serine-threonine kinase which has been linked to autosomal recessive and some sporadic forms of Parkinson disease (Jendrach et al. 2009; Valente et al. 2004a; Valente et al. 2004b). Targeted to the mitochondria, Pink1 is thought to be involved in mitochondrial protection by preventing oxidative damage and maintaining mitochondrial integrity, where loss of function of Pink1, in humans and in *Drosophila melanogaster*, show substantial mitochondrial defects in sensitive tissues (Clark et al. 2006; Exner et al. 2007; Hoepken et al. 2007; Park et al. 2006; Yang et al. 2006). In addition to maintaining and stabilizing mitochondrial networks, it is becoming increasingly apparent that protection during cell stress is due to the involvement of Pink1 in mitochondrial fission/fusion events (Chu 2010; Jendrach et al. 2009). This involvement implicates Pink1 as a key regulator of the fission/fusion balance, using the process of mitophagy to specifically process and degrade dysfunctional mitochondrial fragments.

The E3 ubiquitin ligase, parkin, acts downstream of Pink1 and is thought to operate in a Pink1/parkin pathway necessary for proper mitochondrial integrity and function (Clark et al. 2006; Park et al. 2006; Poole et al. 2008; Yang et al. 2008). In this role, recruitment of parkin to the mitochondria by Pink1 results in the ubiquitination of various mitochondrial proteins, promoting mitophagy (Gegg et al. 2010; Geisler et al. 2010; Vives-Bauza et al. 2010; Ziviani et al. 2010). In contrast, studies have found that loss of parkin or Pink1 function can result in increased fission, promoting mitophagy (Dagda et al. 2009; Lutz et al. 2009). Although the fission/fusion decision is not fully understood, it does suggest

that the protective properties of parkin are not only due to tagging of cytosolic proteins for degradation, but by direct interaction with Pink1 to maintain mitochondrial homeostasis.

Under conditions of oxidative stress or starvation, Foxo transcription factors are activated, targeting genes that promote cell cycle arrest, stress resistance, or apoptosis (Greer and Brunet 2005). To further investigate the roles of Pink1/parkin during cell stress, we have performed expression studies to determine the effects of *Pink1* and *parkin* on the *Foxo*-induced phenotype of developmental defects in the Drosophila eye. The involvement of Pink1/parkin in the insulin receptor (INR) pathway has been largely overlooked, but clues to possible interactions include: interaction of PTEN with Pink1 (Unoki and Nakamura 2001) and DJ-1 (Kim et al. 2005; Kim et al. 2009), an indirect interaction with Akt through parkin (Fallon et al. 2006), an interaction with Akt through DJ-1 (Aleyasin et al. 2010; Yang et al. 2005), and transactivation of *Pink1* by Foxo (Mei et al. 2009; Sengupta et al. 2011). We hypothesized that through an interaction with the INR pathway, or through mitochondrial protective effects, *Pink1* and *parkin* would be capable of alleviating the detrimental effects of *Foxo* overexpression. In contrast, our findings show that reduction of *Pink1* is able to suppress the effects of *Foxo* overexpression, where co-overexpression of *Foxo* with *Pink1* or *parkin* results in an increased severity of the *Foxo*-induced phenotype. These findings suggest a complex role for the Pink1/parkin pathway.

Materials and Methods

Fly stocks and culture

The *UAS-Pink1* transgenic line was created from the GH20931 *Drosophila melanogaster* Pink1 clone (Todd and Staveley 2008). The *UAS-murine Foxo1* (*UAS-Foxo*) and *UAS-murine Foxo1*^{AA} (*UAS-Foxo*^{AA}) transgenes are described in Kramer *et al.* (2003) and the *GMR-Gal4 UAS-Foxo* and *GMR-Gal4;UAS-Foxo*^{AA} lines were established through standard means. *UAS-parkin* was created previously in our laboratory (Haywood and Staveley 2004). The *Pink1*^{B9} mutant line was provided by Dr. J. Chung (Park et al. 2006). The *UAS-Pink1*^{RNAi} and *UAS-parkin*^{RNAi} lines were provided by Dr. B. Lu (Yang et al. 2006; Yang et al. 2003). The *UAS-GFP* control was obtained from the Bloomington stock centre. The *parkin*^{45} mutant line was provided by Dr. L. Pallanck (Greene et al. 2003). All crosses were performed using standard techniques. All flies were cultured on standard cornmeal/yeast/molasses/agar media at 25°C.

Scanning electron microscopy of the Drosophila eye

Flies were aged three days past eclosion on standard cornmeal/yeast/molasses/agar media at 25°C. Flies were then frozen at -80°C and examined under dissecting microscope. Flies were mounted, desiccated overnight and coated in gold before photography at 170 times magnification with a Hitachi S-570 SEM. Area of the eye was measured as per the ocular area, regardless of the presence of ommatidia. This was determined by outlining the ocular margin and/or ridge bristles indicating the postocular area. Eye areas and ommatidial counts were compared using GraphPad Prism 5, using unpaired *t*-test.

Results

parkin increases the severity of the Foxo-induced phenotype

Overexpression of *Foxo* in the developing Drosophila eye results in a characteristic phenotype with reductions in cell number and area (Kramer et al. 2003). When co-overexpressed with *parkin*, there is a significant increase in the severity of the *Foxo*-induced phenotype (Figure 1), including a significant reduction in number of ommatidia and overall area of the eye ($p<0.0001$, $df=31$). This suggests that the addition of *parkin* further reduces the number of viable cells available during eye development. Co-overexpression with *Pink1* shows no significant increase in the *Foxo*-induced reduction of ommatidia ($p=0.1150$, $df=29$) and area ($p=0.2335$, $df=29$) (Figure 1).

Reduction in Pink1 decreases the severity of the Foxo-induced phenotype

Overexpression of *Foxo* in a *Pink1* mutant background ($Pink1^{B9}$) results in a significant increase in ommatidia number ($p=0.0008$, $df=21$) and eye area ($p=0.0015$, $df=21$) (Figure 2). In addition, co-overexpression of *Foxo* with $Pink1^{RNAi}$ shows an even greater effect, with significant increases in ommatidia number and area ($p<0.0001$, $df=30$) (Figure 2). These results suggest that the absence or depletion of Pink1 during eye development is able to alleviate the detrimental effects of *Foxo*. Overexpression of *Foxo* in a *parkin* mutant background ($parkin^{45}$) or co-overexpression with $parkin^{RNAi}$ resulted in apparent synthetic lethality with no surviving progeny. This implies that the broad protective functions of parkin are necessary to maintain a viable organism during this development.

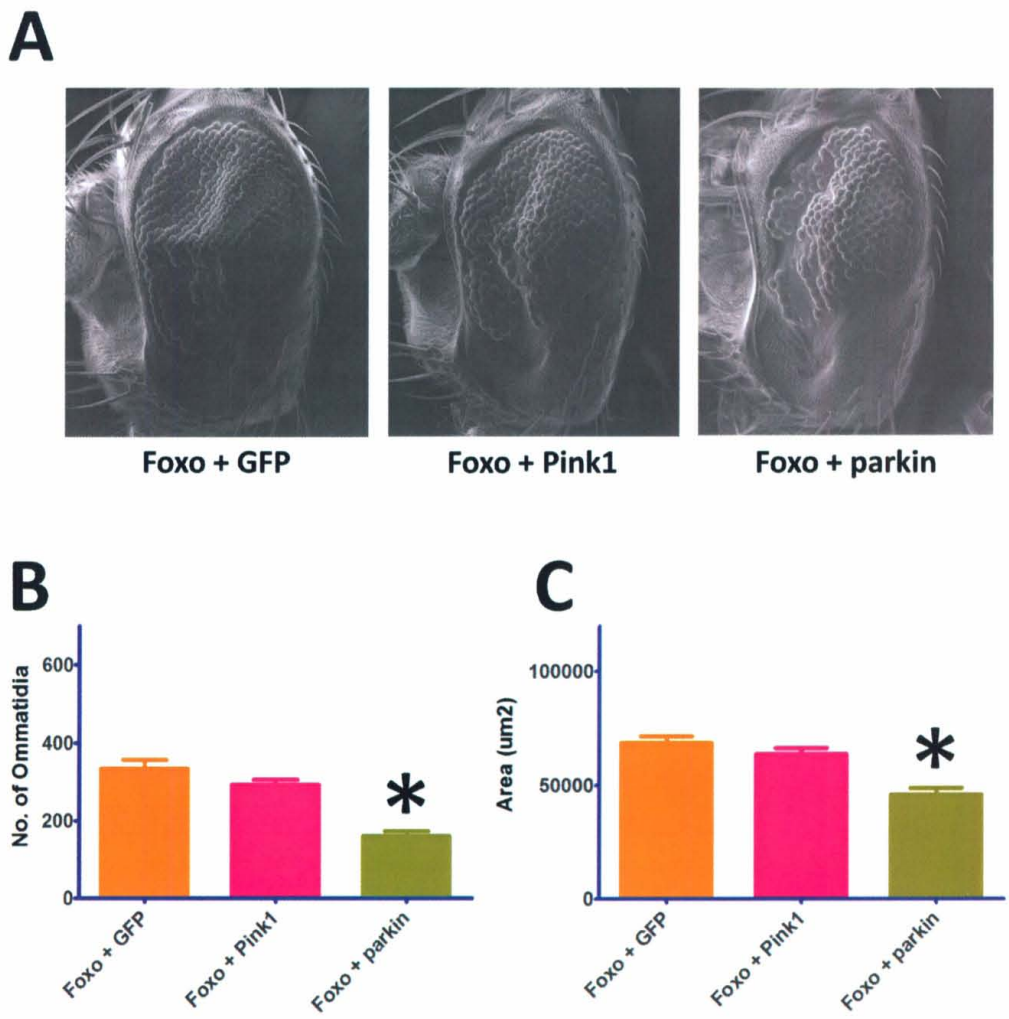

Figure 1. *parkin* increases the severity of the *Foxo*-induced phenotype. Co-overexpression of *Foxo* with *parkin* shows a significant reduction in number of ommatidia and overall area of the eye. Co-overexpression of *Foxo* with *Pink1* shows no significant increase in the Foxo-induced reduction of ommatidia and area. Genotypes shown include *GMR-Gal4 UAS-Foxo/UAS-GFP* (Foxo + GFP), *GMR-Gal4 UAS-Foxo/+;UAS-Pink1/+* (Foxo + Pink1), *GMR-Gal4 UAS-Foxo/+;UAS-parkin/+* (Foxo + parkin). Error bars indicate standard error of the mean.

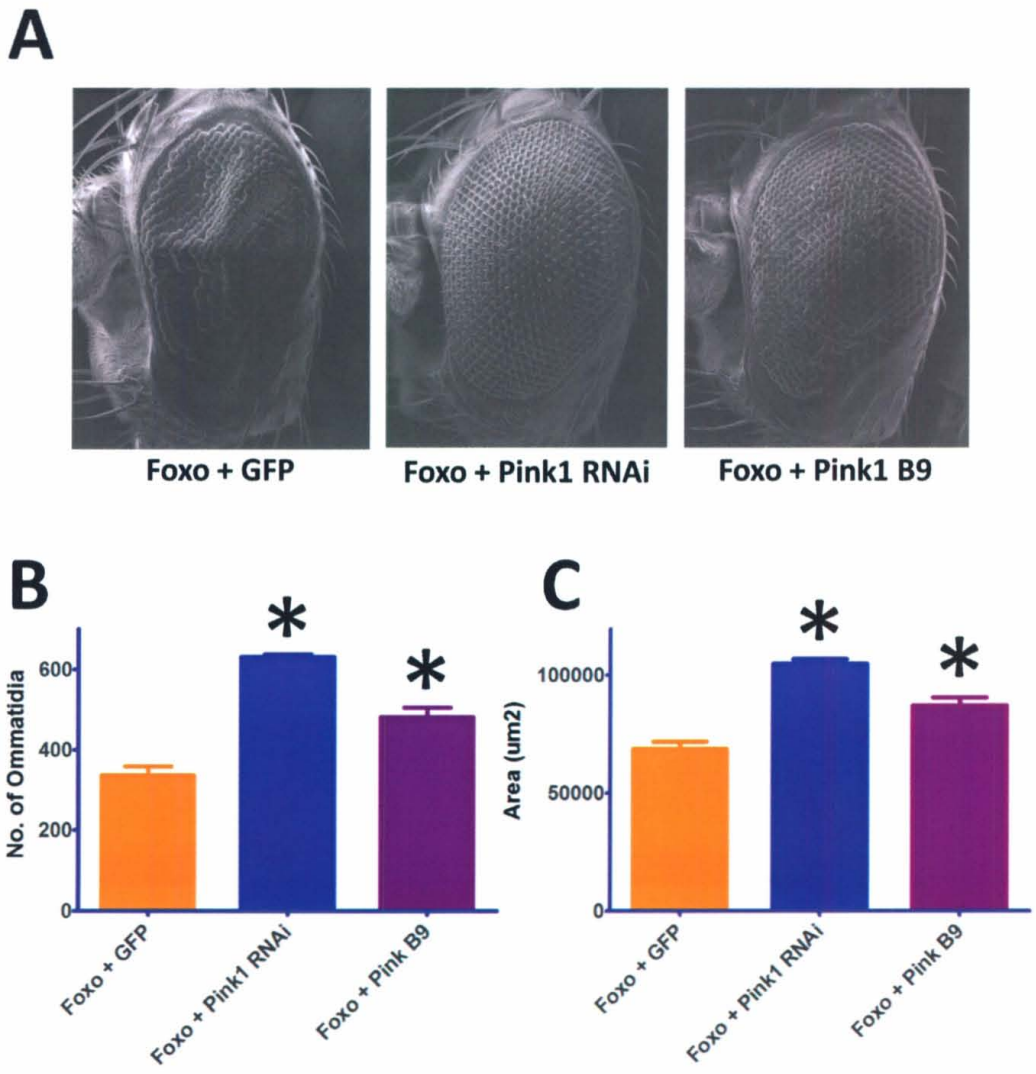

Figure 2. Reduction in *Pink1* decreases the severity of the *Foxo*-induced phenotype. Overexpression of *Foxo* in the *Pink1* mutant background (Pink1 B9) results in a significant increase in ommatidia number and eye area. Co-overexpression of *Foxo* with $Pink1^{RNAi}$ (Pink1 RNAi) shows significant increases in ommatidia number and area. Overexpression of Foxo in a *parkin* mutant background (*GMR-Gal4 UAS-Foxo/+;*$parkin^{45}/parkin^{45}$) or co-overexpression with $parkin^{RNAi}$ (*GMR-Gal4 UAS-Foxo/+;*$parkin^{RNAi}$/+) resulted in apparent synthetic lethality. Genotypes shown include *GMR-Gal4 UAS-Foxo/UAS-GFP* (Foxo + GFP), *GMR-Gal4 UAS-Foxo/+;UAS-*$Pink1^{RNAi}$/+ (Foxo + Pink1 RNAi), $Pink1^{B9}$/*y;GMR-Gal4 UAS-Foxo/+* (Foxo + Pink1 B9). Error bars indicate standard error of the mean.

Effects of Pink1 and parkin on the Foxo-induced phenotype are independent of Akt signalling

The constitutively active version of *Foxo* ($Foxo^{AA}$) contains an alanine substitution at the T1 (T24A) and S1 (S253A) Akt phosphorylation sites (Biggs et al. 1999). Using $Foxo^{AA}$, the severity of the *Foxo*-induced phenotype was seen to increase with *Pink1* or *parkin* co-overexpression (Figure 3). Co-overexpression of *Pink1* with $Foxo^{AA}$ results in significant decreases in number of ommatidia and eye area ($p<0.0001$, $df=30$). Co-overexpression of *parkin* with $Foxo^{AA}$ also results in significant decreases in number of ommatidia ($p=0.0090$, $df=29$) and eye area ($p=0.0190$, $df=29$). The apparent rescue of the *Foxo*-induced phenotype, seen when co-overexpressing *Foxo* with $Pink1^{RNAi}$(Figure 2), is maintained when using the constitutively active version, $Foxo^{AA}$ (Figure 4). Co-overexpression of $Foxo^{AA}$ with $Pink1^{RNAi}$ results in a dramatic increase in ommatidia number and eye area ($p<0.0001$, $df=30$). These results indicate that the *Pink1/parkin* interaction with *Foxo* is independent of Akt signalling. In contrast, there is no significant difference in ommatidia number ($p=0.2131$, $df=29$) or eye area ($p=0.8027$, $df=29$) when $Foxo^{AA}$ is overexpressed in the $Pink1^{B9}$ mutant background (Figure 4). As seen with *Foxo* overexpression, co-overexpression of $Foxo^{AA}$ with $parkin^{RNAi}$ resulted in apparent synthetic lethality with no surviving progeny.

Discussion

Under cell stress conditions, Foxo transcription factors are activated, targeting genes that promote cell cycle arrest, stress resistance, or apoptosis (Greer and Brunet 2005). The

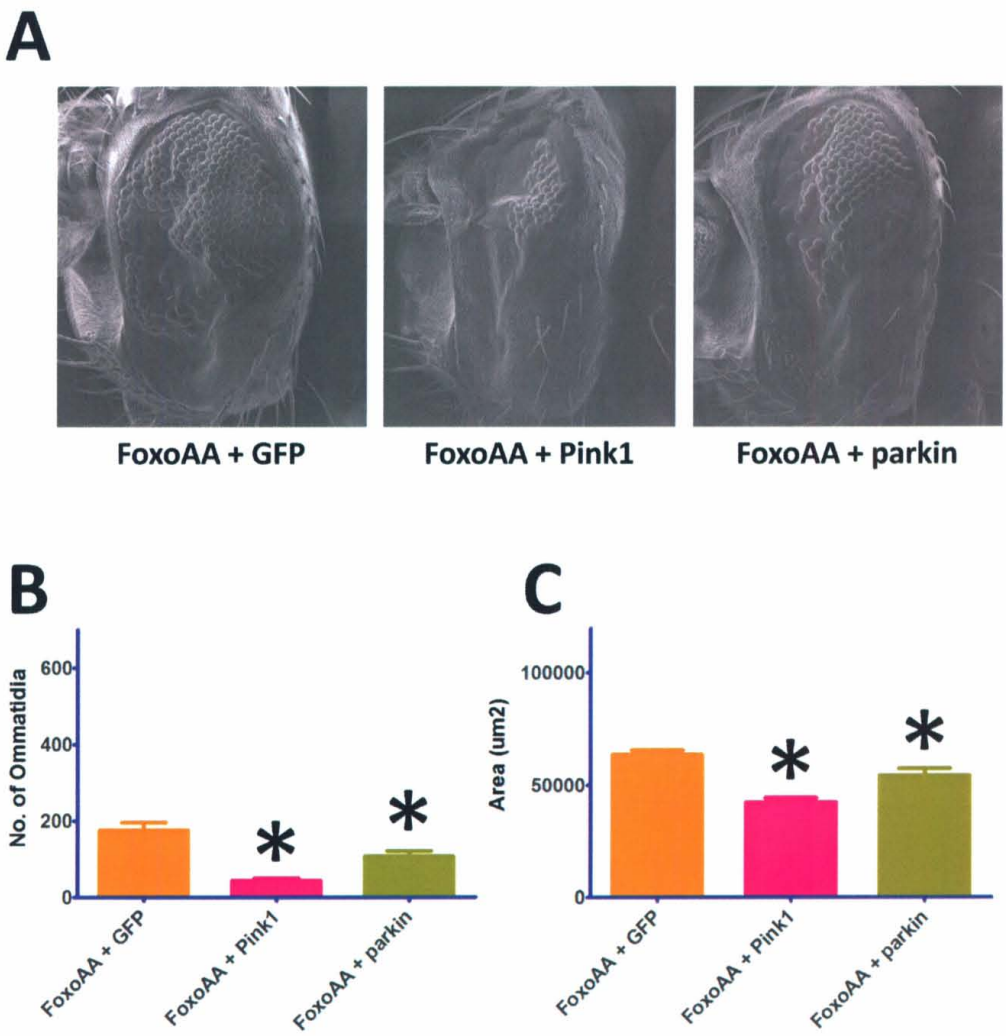

Figure 3. Effects of *Pink1* and *parkin* on the *Foxo*-induced phenotype, independent of Akt signalling. Co-overexpression of $Foxo^{AA}$ (FoxoAA) with *Pink1* results in significant decreases in number of ommatidia and eye area. Co-overexpression of $Foxo^{AA}$ with *parkin* results in significant decreases in number of ommatidia and eye area. Genotypes shown include *GMR-Gal4/UAS-GFP;UAS-*$Foxo^{AA}$/+ (FoxoAA + GFP), *GMR-Gal4/+; UAS-*$Foxo^{AA}$*/UAS-Pink1* (FoxoAA + Pink1), *GMR-Gal4/+; UAS-*$Foxo^{AA}$*/UAS-parkin* (FoxoAA + parkin). Error bars indicate standard error of the mean.

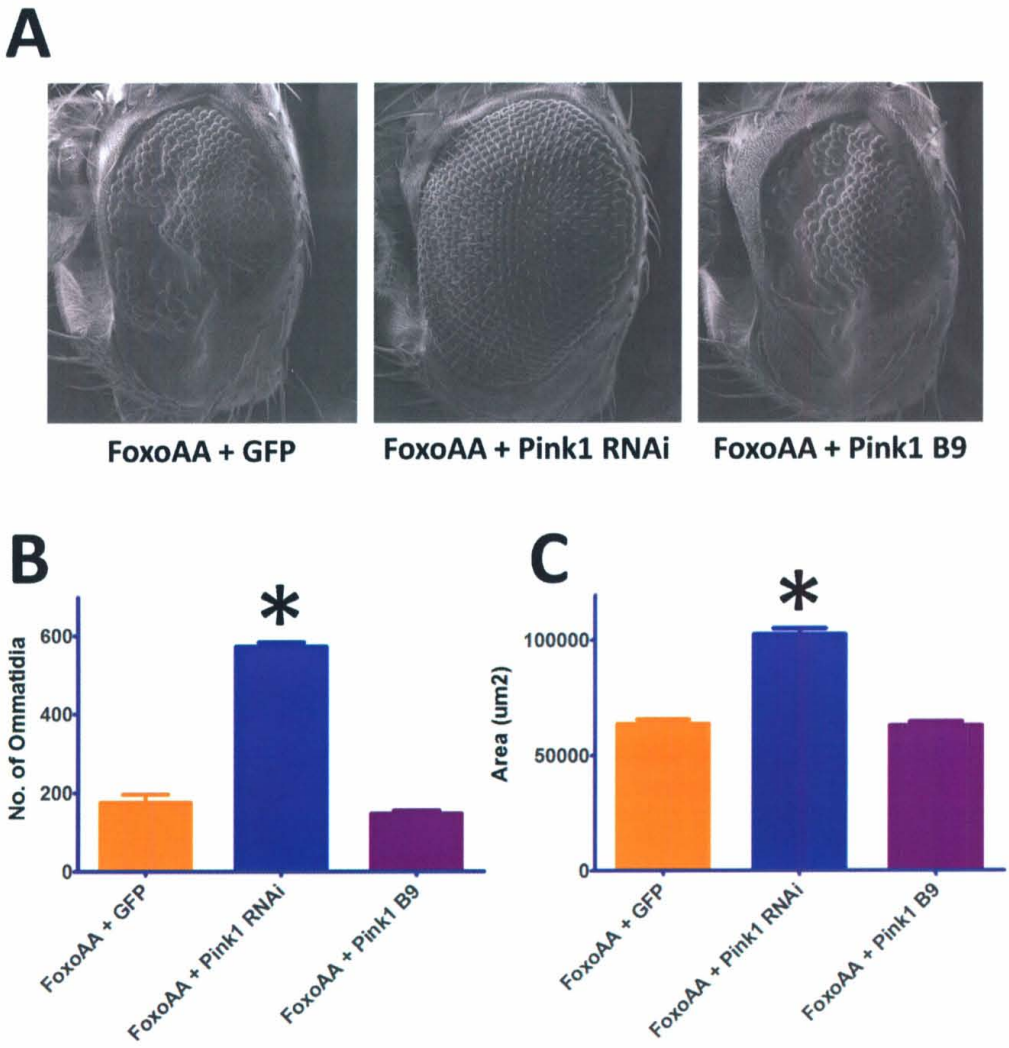

Figure 4. Effects of reductions in *Pink1* on the *Foxo*-induced phenotype, independent of Akt signalling. Co-overexpression of $Foxo^{AA}$ (FoxoAA) with $Pink1^{RNAi}$ (Pink1 RNAi) results in a dramatic increase in ommatidia number and eye area. Overexpression of $Foxo^{AA}$ in the *Pink1* mutant background (Pink1 B9) is not significantly different from the control. Co-overexpression of $Foxo^{AA}$ with with $parkin^{RNAi}$ (*GMR-Gal4/+; UAS-*$Foxo^{AA}$*/*$parkin^{RNAi}$*/+*) resulted in apparent synthetic lethality. Genotypes shown include *GMR-Gal4/UAS-GFP;UAS-*$Foxo^{AA}$*/+* (FoxoAA + GFP), *GMR-Gal4/+;UAS-*$Foxo^{AA}$*/UAS-*$Pink1^{RNAi}$ (FoxoAA + Pink1 RNAi), $Pink1^{B9}$*/y;GMR-Gal4/+;UAS-*$Foxo^{AA}$*/+* (FoxoAA + Pink1 B9). Error bars indicate standard error of the mean.

transactivation of *Pink1* by Foxo (Mei et al. 2009; Sengupta et al. 2011) suggests that there may be recruitment of the Pink1/parkin pathway to help maintain mitochondrial homeostasis during these types of cell stress. As opposed to the general tagging of toxic proteins for degradation, studies indicate that the Pink1/parkin pathway confers cell protection by regulating fission/fusion events, triggering mitophagy to degrade dysfunctional mitochondrial fragments when they cannot be repaired (Chu 2010; Jendrach et al. 2009). Studies have shown that recruitment of parkin by Pink1 to depolarized mitochondria results in the ubiquitination of mitochondrial proteins VDAC1 (Geisler et al. 2010) and mitofusin (Gegg et al. 2010; Ziviani et al. 2010), leading to recruitment of autophagic proteins or decreasing mitochondrial fusion, thereby promoting mitophagy. It is hypothesized that when this process is impaired, by mutations in either Parkin or Pink1, an accumulation of defective mitochondria results, leading to the neurodegeneration seen in Parkinson disease (Vives-Bauza et al. 2010). In contrast, our results indicate that Pink1/parkin may be involved in aspects of cell fate other than protection. Our findings show that co-overexpression of *Pink1* or *parkin* results in an increased severity of the *Foxo*-induced phenotype, and that a reduction in *Pink1* is able to improve the phenotype. This suggests that there may be a more complex role for the Pink1/parkin pathway under differing cell stress conditions.

Many transcriptional targets of Foxo have been identified, including molecules involved in metabolism, oxidative stress resistance, cell cycle arrest and apoptosis (van der Horst and Burgering 2007). Previous phenotypic studies using the Drosophila eye have identified the pro-apoptotic *Hid* gene as one responsible target, where overexpression of

Hid causes dramatic eye degeneration (Kanao et al. 2010; Wilson et al. 2002). Hid functions by inactivating IAPs (inhibitor of apoptosis proteins), which in turn results in caspase activation and apoptosis. This is similar to the role of HtrA2/Omi and Smac/DIABLO in humans. Other components of mitochondrial dependent apoptosis are also functionally conserved between Drosophila and mammals (Igaki and Miura 2004). These include homologues and functional orthologues to BCL-2 proteins, IAPs, Apaf, AIF, EndoG, VDAC, caspases, and cytochrome c, among others. Interactions between these homologues and Hid, or other Foxo targets, may explain the results observed in our study. One possible link could be the involvement of VDAC during mitofission events, where recruitment of parkin to the mitochondrial membrane results in the ubiquitination of VDAC1 (Geisler et al. 2010). VDAC is a major component of the permeability transition pore (PTP), and is involved with mitochondrial outer membrane permeabilization (MOMP) through interactions with pro-apoptotic Bcl-2 proteins Bax and Bak (Green and Kroemer 2004). With both the permeability transition pore and mitochondrial outer membrane permeabilization implicated as initiators of apoptosis, mitofission events triggered by ubiquitination of VDAC must be controlled so to prevent release of apoptotic factors from the mitochondria. Compounding factors such as the effects of Hid in the *Foxo*-induced phenotype, may result in overwhelming instability during increases in *Pink1* or *parkin* expression, making this degree of control impossible. In this instance, the Pink1/parkin pathway may actively participate in the initiation of apoptosis, whereas during other types of cell stress, they maintain their traditional protective roles.

Expression of the constitutively active version of *Foxo* ($Foxo^{AA}$) with co-overexpression of *Pink1, parkin* or $Pink1^{RNAi}$ seems to indicate that the *Pink1/parkin* effect on the *Foxo*-induced phenotype is independent of Akt signalling. In contrast, the change in significance when expressing $Foxo^{AA}$ in the $Pink1^{B9}$ mutant background suggests that there is Akt involvement. This may indicate that there is a role for *Pink1* in the cell that is independent of its kinase function, and that this additional role is somehow involved in the Akt signalling pathway. In this respect, the apparent rescuing effect of the *Foxo*-induced phenotype during decreases in *Pink1* expression would be partially due to the decrease in kinase activity, and partially due to the presence of the Pink1 protein. Future studies looking into an additional role for Pink1, apart from its kinase function, may yield new interactions and targets in the Pink1/parkin pathway.

In conclusion, our results suggest that *Pink1* and *parkin* are able to increase the effects of *Foxo* in Drosophila, highlighting a role for both Pink1 and parkin in regulating cell death. In addition, the constitutively active version of *Foxo* allows us to exclude a general requirement for Akt when increasing the expression of *Pink1/parkin,* however, suggests that there may be an additional role for Pink1 apart from its kinase function, which warrants further investigation. The possibility remains that other interactions between proteins linked with the Pink1/parkin and INR pathways, such as the interaction of PTEN with DJ-1 (Kim et al. 2005; Kim et al. 2009) may be involved in some way. Further studies into the role of Pink1/parkin in mitochondrial fission/fusion events, and additional mitochondrial targets for Pink1/parkin, may uncover underlying mechanisms that mediate the shift towards apoptosis. Moreover, it is likely that the Pink1/parkin pathway is

involved in various aspects of cell fate decisions, contrary to the view that Pink1 and parkin act exclusively as protective proteins.

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Chapter 4 Supporting Information

Supplementary Results

parkin increases the severity of the Foxo-induced phenotype at 29°C

Co-overexpression of *Foxo* with *parkin* results in an increase in the severity of the *Foxo*-induced phenotype (Figure S1), showing a significant reduction in number of ommatidia ($p=0.0005$, $df=32$) and overall area of the eye ($p<0.0001$, $df=32$). Co-overexpression with *Pink1* does not significantly affect the number of ommatidia ($p=0.0642$, $df=32$) or area ($p=0.5958$, $df=32$) (Figure S1). These results are in agreement with those observed at 25°C.

Reduction in Pink1 decreases the severity of the Foxo-induced phenotype at 29°C

Overexpression of *Foxo* in a *Pink1* mutant background ($Pink1^{B9}$) results in a significant increase in ommatidia number ($p=0.0335$, $df=27$) and eye area ($p=0.0424$, $df=27$) (Figure S1). In addition, co-overexpression of *Foxo* with $Pink1^{RNAi}$ shows an even greater effect, with significant increases in ommatidia number and area ($p<0.0001$, $df=31$) (Figure S1). Overexpression of *Foxo* in a *parkin* mutant background ($parkin^{45}$) or co-overexpression with $parkin^{RNAi}$ resulted in apparent synthetic lethality. These results are in agreement with those observed at 25°C.

Effects of Pink1 and parkin on the Foxo-induced phenotype are independent of Akt signalling

Co-overexpression of *Pink1* with $Foxo^{AA}$ results in significant decreases in number of ommatidia and eye area ($p<0.0001$, $df=18$) (Figure S2). Co-overexpression of *parkin* with $Foxo^{AA}$ also results in significant decreases in number of ommatidia ($p=0.0002$, $df=25$) and eye area ($p<0.0001$, $df=25$). Co-overexpression of $Foxo^{AA}$ with $Pink1^{RNAi}$ results in an increase in ommatidia number ($p<0.0001$, $df=26$) and eye area ($p=0.0037$, $df=26$). No significant difference was observed in ommatidia number ($p=0.0770$, $df=24$) or eye area ($p=0.1684$, $df=25$) when $Foxo^{AA}$ is overexpressed in the $Pink1^{B9}$ mutant background (Figure S2). Co-overexpression of $Foxo^{AA}$ with $parkin^{RNAi}$ resulted in apparent synthetic lethality. These results are in agreement with those observed at 25°C.

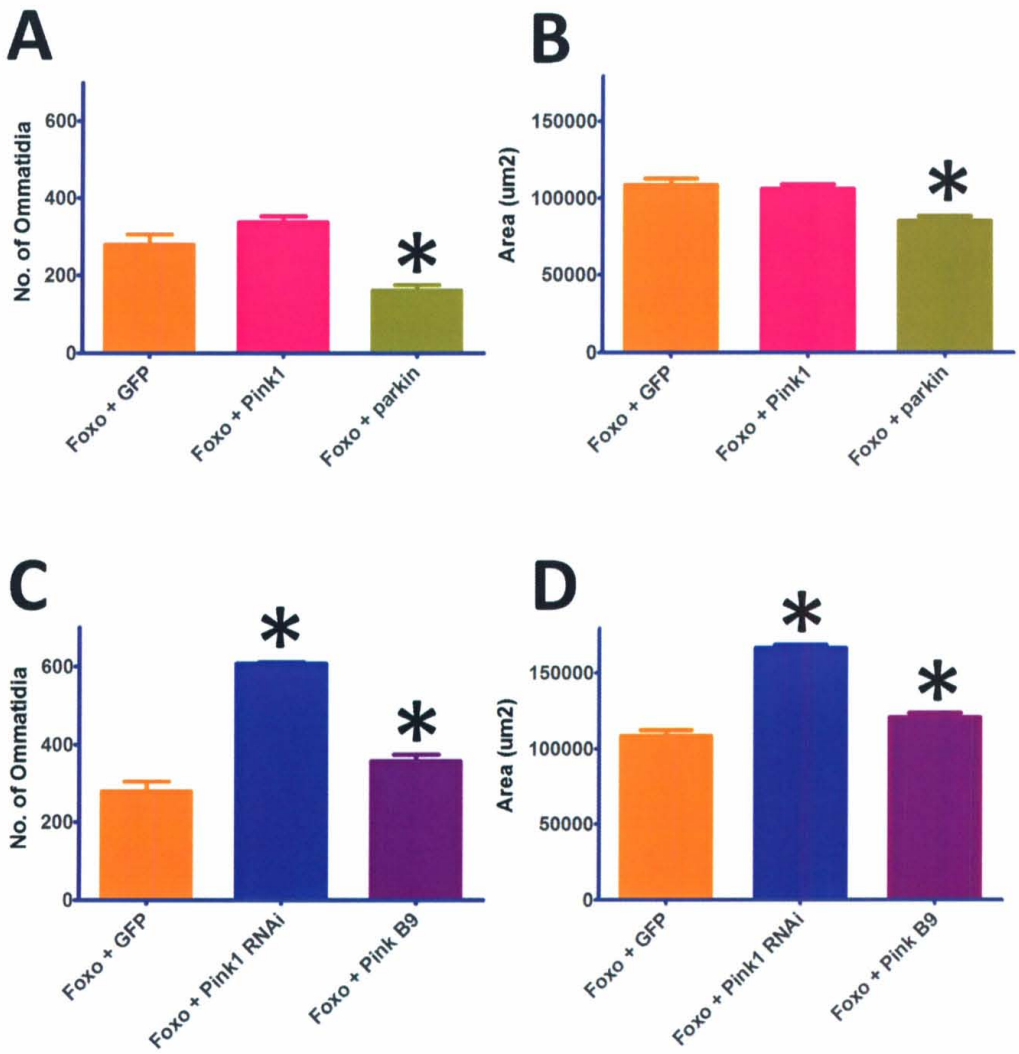

Figure S1. The severity of the *Foxo*-induced phenotype is increased by *parkin* and decreased with reductions in *Pink1*. Co-overexpression of *Foxo* with *parkin* at 29°C shows a significant reduction in number of ommatidia (A) and overall area of the eye (B). Co-overexpression of *Foxo* with *Pink1* shows no significant change in the Foxo-induced reduction of ommatidia and area (A,B). Overexpression of *Foxo* in the *Pink1* mutant background (Pink1 B9) results in a significant increase in ommatidia number and eye area (C,D). Co-overexpression of *Foxo* with $Pink1^{RNAi}$ (Pink1 RNAi) shows significant increases in ommatidia number and area (C,D). Overexpression of Foxo in a *parkin* mutant background (*GMR-Gal4 UAS-Foxo/+;*$parkin^{45}$*/*$parkin^{45}$) or co-overexpression with $parkin^{RNAi}$ (*GMR-Gal4 UAS-Foxo/+;*$parkin^{RNAi}$*/+*) resulted in apparent synthetic lethality. Genotypes shown include *GMR-Gal4 UAS-Foxo/UAS-GFP* (Foxo + GFP), *GMR-Gal4 UAS-Foxo/+;UAS-Pink1/+* (Foxo + Pink1), *GMR-Gal4 UAS-Foxo/+;UAS-parkin/+* (Foxo + parkin), *GMR-Gal4 UAS-Foxo/+;UAS-*$Pink1^{RNAi}$*/+* (Foxo + Pink1 RNAi), $Pink1^{B9}$*/y;GMR-Gal4 UAS-Foxo/+* (Foxo + Pink1 B9). Error bars indicate standard error of the mean.

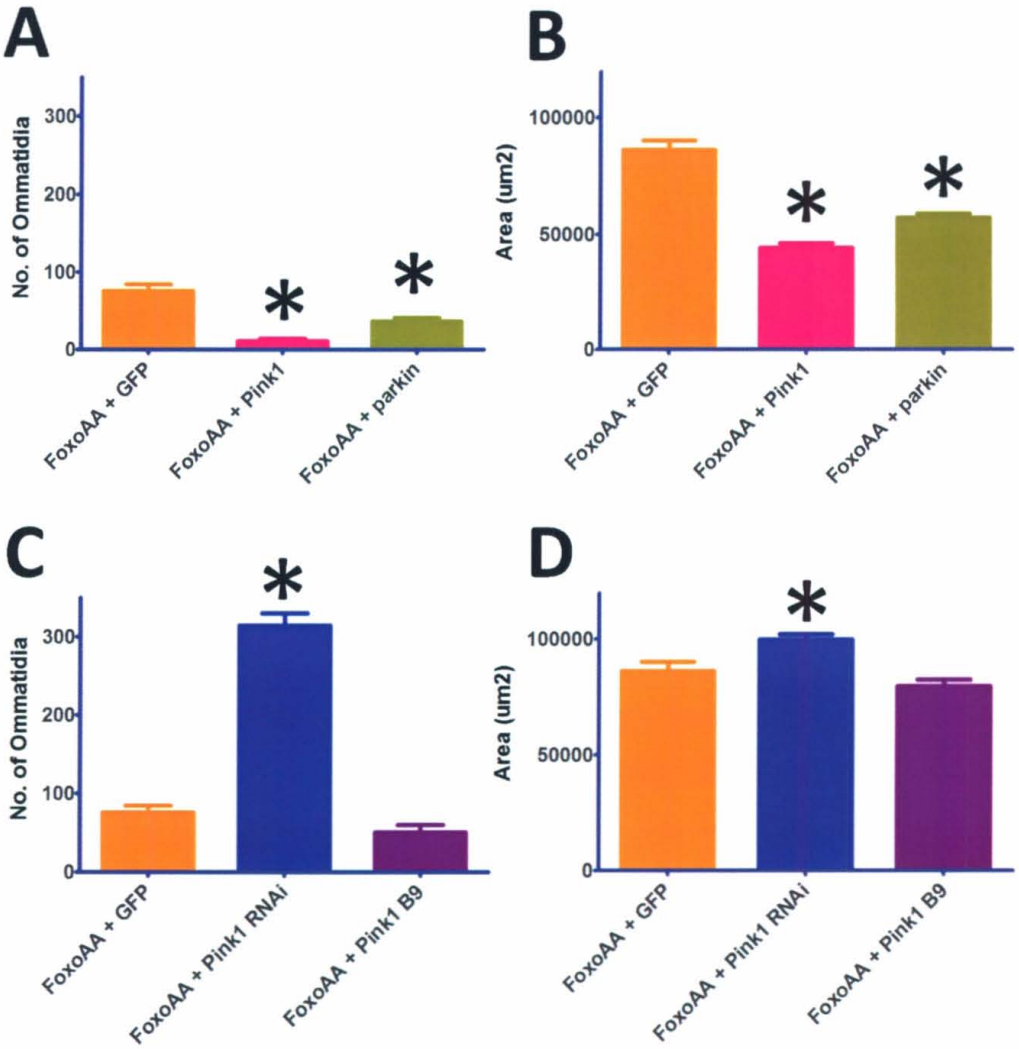

Figure S2. Effects of *Pink1* and *parkin* on the *Foxo*-induced phenotype, independent of Akt signalling. Co-overexpression of $Foxo^{AA}$ (FoxoAA) with *Pink1* results in significant decreases in number of ommatidia and eye area (A,B). Co-overexpression of $Foxo^{AA}$ with *parkin* results in significant decreases in number of ommatidia and eye area (A,B). Co-overexpression of $Foxo^{AA}$ (FoxoAA) with $Pink1^{RNAi}$ (Pink1 RNAi) results in a dramatic increase in ommatidia number and eye area (C,D). Overexpression of $Foxo^{AA}$ in the *Pink1* mutant background (Pink1 B9) shows no significant change in the $Foxo^{AA}$ phenotype (C,D). Co-overexpression of $Foxo^{AA}$ with with $parkin^{RNAi}$ (*GMR-Gal4/+; UAS-*$Foxo^{AA}$*/parkin*^{RNAi}*/+*) resulted in apparent synthetic lethality. Genotypes shown include *GMR-Gal4/UAS-GFP;UAS-*$Foxo^{AA}$*/+* (FoxoAA + GFP), *GMR-Gal4/+; UAS-*$Foxo^{AA}$*/UAS-Pink1* (FoxoAA + Pink1), *GMR-Gal4/+; UAS-*$Foxo^{AA}$*/UAS-parkin* (FoxoAA + parkin), *GMR-Gal4/+;UAS-*$Foxo^{AA}$*/UAS-*$Pink1^{RNAi}$ (FoxoAA + Pink1 RNAi), $Pink1^{B9}$*/y;GMR-Gal4/+;UAS-*$Foxo^{AA}$*/+* (FoxoAA + Pink1 B9). Error bars indicate standard error of the mean.

Chapter 5

Pink1 rescues *Gal4*-induced developmental defects in the Drosophila eye

Abstract

Parkinson disease pathology often includes the presence in the remaining neurons of ubiquitin-positive, α-synuclein-enriched inclusions known as Lewy bodies. *Pink1 (PTEN induced putative kinase 1)*, identified as PARK6, encodes a serine-threonine kinase involved in mitochondrial protection, that works with parkin to ubiquitinate various proteins, promoting mitophagy and possibly tagging cystolic proteins for degradation. Previous work in our laboratory has examined the adverse effects of expressing toxic proteins, including *Gal4,* and has shown the ability of *parkin* to rescue a *Gal4*-induced phenotype. To further investigate the role of Pink1 in protection against the toxic effects of proteins, we have performed expression studies to determine the effects of increases and decreases of Pink1 on the *Gal4*-induced phenotype of developmental defects in the Drosophila eye. Our results show that *Pink1* is able to rescue the *Gal4*-induced phenotype, suggesting that Pink1 can protect against the effects of the Gal4 protein. However, we have found that reductions in *Pink1* or *parkin* do not have harmful effects and are not able to induce a phenotype when combined with low level *Gal4* expression. This suggests that the effects of low level Gal4 are independent of *Pink1* or *parkin,* or that there is an alternative mechanism to alleviate the low level effects of Gal4. Our findings highlight the protective role of Pink1 against toxic proteins, and suggests that further investigation into the Pink1 mechanism of action during differing types of cell stress is warranted.

Introduction

Parkinson disease (PD) is the most prevalent neurodegenerative movement disorder (Weintraub et al. 2008). Characterized by a progressive loss of dopaminergic neurons, PD pathology often includes the presence of Lewy bodies, ubiquitin-positive and α-synuclein-enriched inclusions, in the remaining neurons. Although sporadic forms of PD are believed to be more common, many familial forms share features with sporadic PD, including protein aggregation and mitochondrial dysfunction (Dawson and Dawson 2003). *Pink1 (PTEN induced putative kinase 1)* encodes a serine-threonine kinase which has been linked to autosomal recessive and some sporadic forms of Parkinson disease (Jendrach et al. 2009; Valente et al. 2004a; Valente et al. 2004b). Targeted to the mitochondria, Pink1 is involved in mitochondrial protection, as loss of function of Pink1 results in substantial mitochondrial defects in sensitive tissues (Clark et al. 2006; Exner et al. 2007; Hoepken et al. 2007; Park et al. 2006; Yang et al. 2006). It is increasingly apparent that both Pink1 and parkin, acting in the same pathway, are necessary for proper mitochondrial integrity and function (Poole et al. 2008; Yang et al. 2008). The parkin E3 ubiquitin ligase acts downstream of Pink1. In mitochondrial protection, the recruitment of parkin to the mitochondria by Pink1 results in the ubiquitination of various mitochondrial proteins, promoting mitophagy (Gegg et al. 2010; Geisler et al. 2010; Vives-Bauza et al. 2010; Ziviani et al. 2010). In addition, Pink1 may have a protective role apart from the mitochondria, where an interaction with parkin could result in the tagging of cystolic proteins for degradation. This may be an important, but largely

overlooked role, as neurodegenerative diseases are often characterized by the accumulation of toxic proteins.

Our laboratory has examined the adverse effects of expressing proteins that can produce toxicity, including α-synuclein and the Gal4 transcription factor (Haywood and Staveley 2004, 2006; Haywood 2006; Kramer and Staveley 2003). We have shown the ability of *parkin* and *Pink1* to rescue an *α-synuclein*-induced phenotype (Haywood and Staveley 2004, 2006; Todd and Staveley 2008), and the ability of *parkin* overexpression to rescue a *Gal4*-induced phenotype (Haywood 2006). The suppression of the effects of Gal4 is presumably through its targeting for proteosomal degradation by parkin. To further investigate the role of Pink1 in protection against toxic proteins, we have performed expression studies to determine the effects of *Pink1* on the *Gal4*-induced phenotype of developmental defects in the Drosophila eye. Our results show that *Pink1* is able to rescue the *Gal4*-induced phenotype, suggesting that Pink1 can protect against the effects of the Gal4 protein.

Materials and Methods

Fly stocks and culture

The *UAS-Pink1* transgenic line was created from the GH20931 *Drosophila melanogaster* Pink1 clone (Todd and Staveley 2008). The $Pink1^{B9}$ mutant line (Park et al. 2006) was provided by Dr. J. Chung. The $UAS\text{-}Pink1^{RNAi}$ and $UAS\text{-}parkin^{RNAi}$ lines (Yang et al. 2006; Yang et al. 2003) were provided by Dr. B. Lu. *UAS-parkin* was created previously in our laboratory (Haywood and Staveley 2004). The $parkin^{45}$ mutant line (Greene et al.

2003) was provided by Dr. L. Pallanck. The *GMR-Gal4* flies (Freeman 1996) were obtained from the Bloomington Drosophila Stock Center at Indiana University. All crosses were performed using standard techniques. All flies were cultured on standard cornmeal/yeast/molasses/agar media.

Scanning electron microscopy of the Drosophila eye

Flies were aged three days past eclosion on standard cornmeal/yeast/molasses/agar media at either 25°C or 29°C. Flies were then frozen at -80°C and examined under dissecting microscope. Flies were mounted, desiccated overnight and coated in gold before photography at 170 times magnification with a Hitachi S-570 SEM. Area of disruption was determined by the presence of fused or enlarged (>150%) ommatidia.

Results

Pink1 is able to rescue the Gal4-induced rough eye phenotype

High levels of *Gal4* expression in the developing Drosophila eye result in a characteristic rough eye phenotype (Kramer and Staveley 2003). At 25°C, *GMR-Gal4* homozygotes have a rough eye phenotype characterized by an 81% ommatidial disruption of the eye area (Figure 1). Co-overexpression with one copy of the *Pink1* transgene results in a significant reduction of the *Gal4*-induced phenotype, reducing the disruption to 5% (95% CI). Co-overexpression with two copies of the *Pink1* transgene results in a further, significant reduction of the *Gal4*-induced phenotype, near control levels (0.5% disruption,

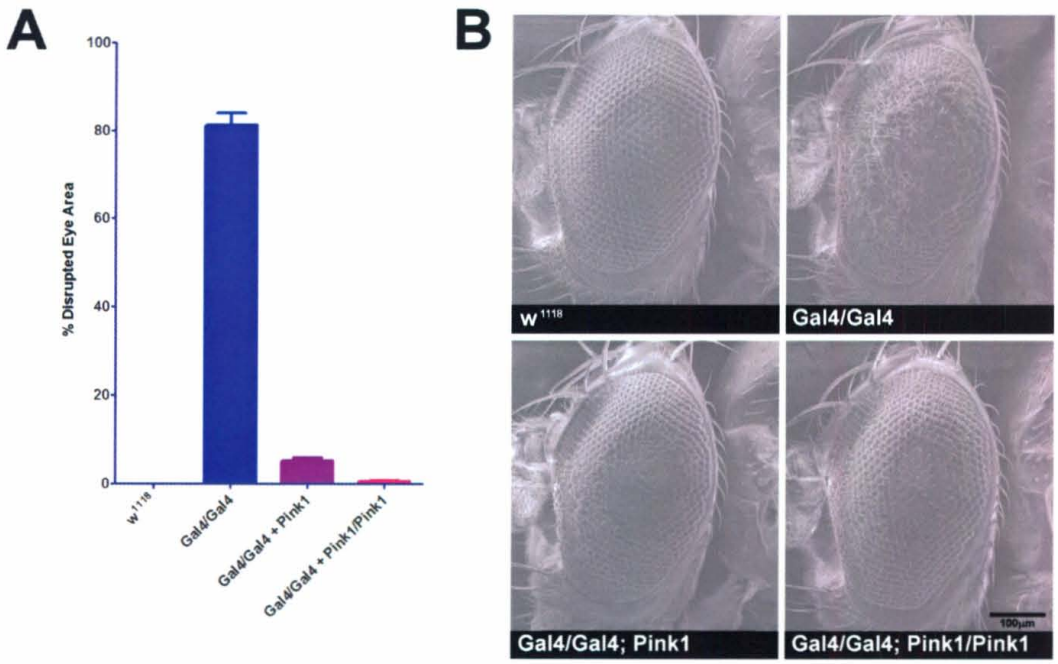

Figure 1. *Pink1* decreases the severity of the *Gal4*-induced phenotype. *GMR-Gal4* homozygotes show a characteristic rough eye phenotype. Co-overexpression with one or two copies of the *Pink1* transgene results in a significant reduction of the *Gal4*-induced phenotype. Genotypes shown include w^{1118} (w^{1118}), *GMR-Gal4/GMR-Gal4* (Gal4/Gal4), *GMR-Gal4/GMR-Gal4; UAS-Pink1/+* (Gal4/Gal4; Pink1), *GMR-Gal4/GMR-Gal4; UAS-Pink1/UAS-Pink1* (Gal4/Gal4; Pink1/Pink1). Flies were raised at 25°C. Error bars indicate standard error of the mean.

95% CI). These results suggest that an increase in *Pink1*, in a dose dependent manner, during eye development is able to alleviate the detrimental effects of *Gal4* expression.

GMR-Gal4 heterozygotes show a mild rough eye phenotype at 29°C

Previous work in our laboratory indicates a mild *Gal4*-induced phenotype in *GMR-Gal4* heterozygotes at 29°C, with intermediate levels of apoptosis (Kramer and Staveley 2003). Our results with *GMR-Gal4* heterozygotes at 29°C show this mild phenotype, with an unevenness of the ommatidial surface, and no visible fusing of ommatidia or enlargement greater than 150% (Figure 2). Due to the subtle nature of the phenotype, it is difficult to determine if co-overexpression of either one or two copies of the *Pink1* transgene has an effect. Therefore, possible protective effects of *Pink1* overexpression on the *GMR-Gal4* heterozygotes cannot be detected using this phenotype.

Reductions in Pink1 or parkin are not able to induce a rough eye phenotype in GMR-Gal4 heterozygotes

It was of interest to determine if reductions in *Pink1* or *parkin* have an effect on the subtle phenotype observed in the *GMR-Gal4* heterozygotes at 29°C (Kramer and Staveley 2003)(Figure 2). Our results show no appreciable change in eye morphology during co-overexpression of $parkin^{RNAi}$, $Pink1^{RNAi}$ or when *GMR-Gal4* heterozygotes are expressed in a *Pink1* mutant background ($Pink1^{B9}$)(Figure 2). These results suggest that reductions in *Pink1* or *parkin* are not sufficient to induce the *Gal4*-induced phenotype. Expression in a *parkin* mutant background resulted in apparent synthetic lethality (*GMR-Gal4/+;*

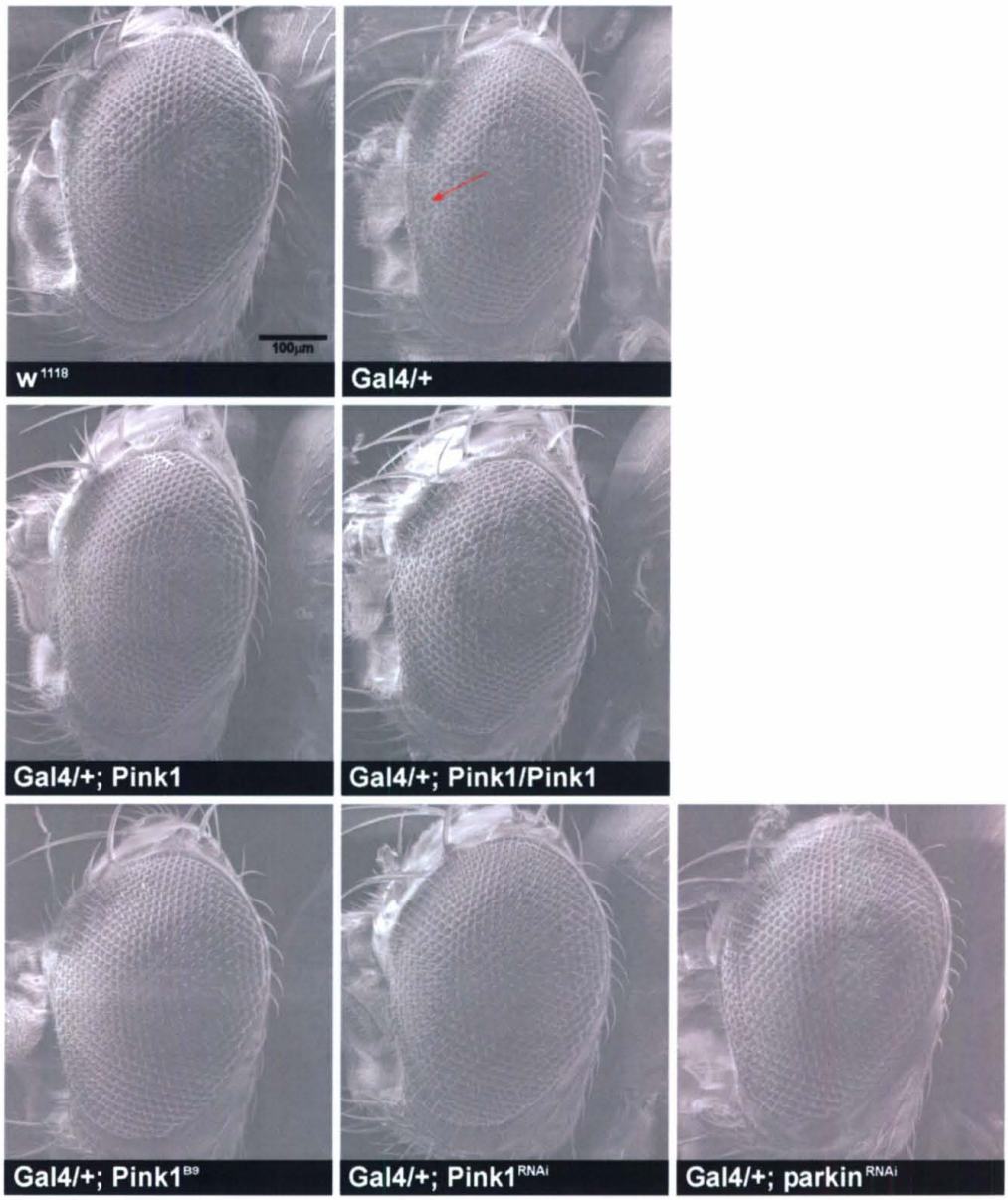

Figure 2. Reductions in *Pink1* or *parkin* do not induce a rough eye phenotype in *GMR-Gal4* heterozygotes. *GMR-Gal4* heterozygotes show a mild phenotype of unevenness in the ommatidial surface, with no visible fusing of ommatidia or enlargement over 150% (red arrow). No changes were observed with co-overexpression of *Pink1*, $parkin^{RNAi}$, $Pink1^{RNAi}$ or when expressed in the $Pink1^{B9}$ mutant background. Expression in a *parkin* mutant background resulted in apparent synthetic lethality (*GMR-Gal4/+;* $park^{45}/park^{45}$). Genotypes shown include w^{1118} (w^{1118}), *GMR-Gal4/+* (Gal4/+), *GMR-Gal4/+; UAS-Pink1/+* (Gal4/+; Pink1), *GMR-Gal4/+; UAS-Pink1/UAS-Pink1* (Gal4/+; Pink1/Pink1), *GMR-Gal4/+;* $UAS\text{-}parkin^{RNAi}$/+ (Gal4/+;$parkin^{RNAi}$), $Pink1^{B9}$/y; *GMR-Gal4/+* (Gal4/+; $Pink1^{B9}$), *GMR-Gal4/+;* $Pink1^{RNAi}$/+ (Gal4/+; $Pink^{RNAi}$). Flies were raised at 29°C.

park^{45}/*park*^{45}). This implies that the broad protective functions of parkin are necessary to maintain a viable organism during this development.

Discussion

Increase in *Pink1* expression during eye development is able to alleviate the detrimental effects of *Gal4* expression in a dose dependent manner. The ability of *Pink1* to counteract the effects of *GMR-Gal4* is similar to previous results in our laboratory with *parkin* (Haywood 2006), supporting the theory that Pink1 is acting via parkin. Pink1 may interact with parkin to activate the ubiquitin-proteasomal system, resulting in the tagging of Gal4 for degradation. Alternatively, Pink1 may operate to protect the mitochondria from the effects of Gal4, recruiting parkin to the membrane to remove damage via mitophagy. Studies have shown that recruitment of parkin by Pink1 to depolarized mitochondria results in the ubiquitination of mitochondrial proteins VDAC1 (Geisler et al. 2010) and mitofusin (Gegg et al. 2010; Ziviani et al. 2010), leading to recruitment of autophagic proteins or decreases in mitochondrial fusion. It is hypothesized that when this process is impaired, by mutations in either *parkin* or *Pink1*, an accumulation of defective mitochondria results, leading to the neurodegeneration seen in Parkinson disease (Vives-Bauza et al. 2010). Pink1 may impart mitochondrial protection by interacting with other molecular chaperones at the mitochondrial membrane. For example, the phosphorylation of TRAP1 (Hsp75) by Pink1 has been shown to protect against oxidative stress and prevent cytochrome c release (Pridgeon et al. 2007). Identification of PINK1 targets is still in early stages. It is likely that PINK1 will be

identified to interact with various proteins, and serve to protect against multiple stressors such as toxic proteins, oxidative stress and mitochondrial dysfunction.

The *GMR-Gal4* heterozygotes exhibit a mild phenotype at 29°C, with intermediate levels of apoptosis in the eye imaginal discs (Kramer and Staveley 2003). It was thought that this mild *Gal4*-induced effect may be increased with reductions in *Pink1* or *parkin*, inducing a measurable rough eye phenotype. Our results show that these reductions are not sufficient to induce a rough eye phenotype, implying that either the levels of Gal4 are not enough to cause developmental defects, or that low levels of *Pink1* or *parkin* may be sufficient to protect against low levels of *Gal4*. The lack of an effect observed using the $Pink1^{B9}$ mutant may suggest that a functional kinase is not necessary for Pink1 to participate in protection against the effects of Gal4. As interactions with the *Foxo*-induced phenotype have suggested that *Pink1* may have a role independent of its kinase function, investigations into other aspects of the Pink1 protein may shed light on unexplored roles or interactions.

In conclusion, our results show that *Pink1* is able to decrease the effects of *Gal4* in Drosophila, highlighting a protective role for Pink1 against the effects of toxic proteins. Our results suggest that low levels of *Pink1* or *parkin* may be sufficient to protect against low levels of *Gal4,* and that a functional kinase may not be necessary for Pink1 to participate in this protection. It is likely that Pink1 has a diverse role in cell protection, including interaction with parkin in the ubiquitin-proteasomal system, regulation of mitochondrial fission/fusion events, and other roles that may be independent of its kinase

function. Further investigation into the Pink1 mechanism of action will be important in revealing different protective roles that are dependent upon the type of cell stress.

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Chapter 6

Discussion and Conclusions

Pink1 is able to alleviate the effects of toxic proteins

Aggregation and accumulation of toxic proteins is a unifying feature of various neurodegenerative disorders (Kopito and Ron 2000). The aggregation of α-synuclein is a central pathogenic mechanism for Parkinson disease and has been linked to various aspects of mitochondrial dysfunction (Lee and Trojanowski 2006; Schapira and Gegg 2011). The ability of *Pink1* to counteract the effects of *α-synuclein* are similar to those observed with *parkin* (Haywood and Staveley 2004), supporting the theory that Pink1 and parkin act in a common pathway where Pink1 can act through parkin to confer a protective role against toxic proteins. This is further supported by our results showing the ability of *Pink1* to alleviate the detrimental effects of *Gal4* expression in a dose dependent manner. The exact mechanism of how Pink1 protection against toxic proteins occurs is still unclear. Pink1 may activate parkin directly or indirectly, thereby activating the ubiquitin-proteasomal system, resulting in the tagging of α-synuclein for degradation. Alternatively, Pink1 may confer mitochondrial protection through the recruitment of parkin to the mitochondrial membrane, leading to the eventual segregation and mitophagy of damaged mitochondria (Chu 2010; Whitworth and Pallanck 2009). Pink1 could act independently of parkin, through an interaction with molecular chaperones such as TRAP1 (Pridgeon et al. 2007), protecting the mitochondria from oxidative stress caused by the presence of toxic proteins. Oxidative stress is another unifying characteristic of neurodegenerative diseases, and is implicated in the pathogenesis of α-synuclein (Devi et al. 2008; Liu et al. 2009). Oxidative stress in the α-synuclein model may also play a role in initiating mitophagy, where the ROS produced can act as signalling molecules in the

regulation of autophagy (Scherz-Shouval and Elazar 2007; Weber and Reichert 2010). Our results suggest that increases in *α-synuclein* and *Pink1* together can have a synergistic effect in a specific population of dopaminergic cells, allowing for enhanced protection and increased functional longevity in Drosophila. This may be a result of the upregulation of pro-survival mechanisms via Pink1, in response to an increase in ROS signalling due to *α-synuclein* overexpression. Regardless of the mechanism, *Pink1* is able to counteract the effects of toxic proteins resulting in cell protection and, in some cases, increased survival of the organism. This role of Pink1 has been largely overlooked in the research community and warrants further investigation.

Pink1 is able to increase lifespan and healthspan

Late age onset is the most striking feature of human neurodegenerative disorders, with age considered to be the largest risk factor for the development and progression of Parkinson disease (Lees et al. 2009; Weintraub et al. 2008). Studies with various neurodegenerative diseases have revealed mechanistic links between ageing and the onset of toxic protein aggregation (Dillin and Cohen 2011). Additionally, increased lifespan in some models has been attributed to either the reduction of protein aggregates, or the protective aggregation of toxic cytoplasmic proteins. Our results suggest that combined increases in *α-synuclein* and *Pink1* can have a synergistic effect of increased longevity, but only in a specific population of dopaminergic cells targeted with the *TH*-Gal4 driver. It is likely that the protective effects of *Pink1* on the *α-synuclein* phenotype are due to downstream tagging of α-synuclein for destruction, and/or maintenance of overall mitochondrial function in the cell through the mitophagy of damaged mitochondria.

Interestingly, the increases in lifespan were not observed when using other neuronal and ubiquitous drivers, and are not observed when using *Ddc-Gal4* to drive expression in the dopaminergic neurons. There is growing evidence that the *TH-Gal4* and *Ddc-Gal4* drivers have differing coverage of the dopaminergic neurons (Yarali and Gerber 2010). Our results therefore suggest that a specific dopaminergic cell cluster, targeted by *TH*-Gal4 but not by *Ddc*-Gal4, is responsible for the observed increases in longevity. Identification of this neuronal population could shift future therapeutic efforts towards a particular dopaminergic cluster.

Pink1 and parkin have complex roles in cell fate decisions

The Pink1/parkin pathway is commonly viewed exclusively as a protective pathway. In contrast, our results show that an increase in *Pink1* or *parkin* is able to increase the damaging effects of the directed over-expression of *Foxo* in Drosophila, highlighting a role for the Pink1/parkin pathway in regulating cell death. This role may be dependent on the type of cell stress, where Foxo transcription factors are often involved in nutritional stress, targeting genes promoting cell cycle arrest, stress resistance, or apoptosis (Greer and Brunet 2005). The transactivation of *Pink1* by Foxo (Mei et al. 2009; Sengupta et al. 2011) was assumed to promote stress resistance, via maintenance of mitochondrial homeostasis. However, our results suggest that recruitment of the Pink1/parkin pathway may not always be a protective mechanism. It is possible that, under certain conditions, Pink1/parkin serve to initiate formation of the permeability transition pore (PTP) and/or mitochondrial outer membrane permeabilization (MOMP). In this instance, Pink1/parkin

may actively participate in the initiation of apoptosis, whereas during other types of cell stress, they maintain their traditional protective roles.

Our expression studies using *Foxo* suggest that there may be an additional role for Pink1 apart from its kinase function. Using an RNAi approach to target Pink1 appears to improve the *Foxo* phenotype, independent of Akt involvement. Using the $Pink1^{B9}$ mutant, containing a nonfunctional kinase, improves the *Foxo* phenotype to a lesser degree, and appears to be dependent on Akt involvement. This discrepancy suggests that there may be a second mechanism at work. If Pink1 is able to confer protection without a functioning kinase, but it is dependent on phosphorylation of Foxo by Akt, then this protective role may still be fulfilled when using the $Pink1^{B9}$ mutant. This is an interesting prospect and future studies looking into non-kinase functions for Pink1 may yield new interactions and targets in the Pink1/parkin pathway.

Final thoughts and future directions

It was the purpose of this thesis to use expression studies in a Drosophila model to investigate the protective role of *Pink1* and interactions it may have with other genes or pathways. It has become clear that Pink1 plays a role in mitochondrial protection. Due to the importance of mitochondria in maintaining cell sustenance, it is understandable that mitochondrial dysfunction has detrimental effects in the cell. What is not so widely known is the intricate signalling that occurs within, and exterior to, the mitochondria, that can result in either its increased functionality, or in the initiation of apoptosis. The

mitochondria act as major players in cell fate decisions, and the role of Pink1 with mitochondria may be more complex than previously thought.

With the identification of PINK1 targets is in its infancy, it of interest to determine if there are other PD related genes that *Pink1* is involved with. Five Pink1/parkin-interacting genes have recently been identified in Drosophila (Fernandes and Rao 2011). Further investigations into these and other gene interactions could be accomplished with Drosophila expression studies, as many PD genes have homologues in Drosophila. Alternatively, this model can also allow for relatively easy transgenic expression of genes that have yet to have a Drosophila homologue identified. It is likely that PINK1 is able to interact with various proteins that may or may not be linked to PD, proteins that serve to protect against toxic proteins, membrane permeability, or oxidative stress. It is also possible that proteins involved in apoptosis may interact with Pink1, where studies looking at the involvement of Pink1 with Bcl-2 proteins would be of particular interest.

It is likely that Pink1 has a diverse role in cell fate decisions, including functioning in a Pink1/parkin pathway to operate in the ubiquitin-proteasomal system, regulate mitochondrial fission/fusion events, regulate membrane permeability during apoptosis, and other roles that may be independent of its kinase function. Further investigation into the Pink1 mechanism of action will be important in revealing different protective roles that may be dependent upon the type of cell stress.

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Appendix 1

Novel Assay and Analysis for Measuring Climbing Ability in Drosophila

A version of this appendix has been published in Drosophila Information Services (Todd and Staveley 2004)

Introduction

The locomotor activity of Drosophila has been commonly used to investigate mobility in ageing Drosophila and especially in Drosophila models of neurodegenerative diseases ((Feany and Bender 2000; Haywood and Staveley 2004; Le Bourg and Lints 1992). Climbing ability is a commonly used assay to measure locomotor activity. A frequently applied method uses empty, standard size vials in which flies are evaluated as having the ability to climb an eight centimetre distance or not. Our novel method employs a long, thin tube in which flies are graded for various levels of climbing. Our approach to analyze climbing is based upon an index that 1) is sensitive to differences in climbing ability and 2) uses non-linear fit curves that allow for easy comparison of data. The following describes the assay design and analysis, along with a demonstration of how the assay operates using data that was produced while investigating locomotor effects in a *Drosophila melanogaster* model of Parkinson disease.

Materials and Methods

Drosophila Strains and Culture

The *UAS-TH* and *UAS-Ddc* (True et al. 1999) lines were generously provided by Dr. S.B. Carroll (University of Wisconsin). The double *Ddc-GAL4* driver line was generated by standard genetic means from $Ddc\text{-}GAL4^{36}$ and $Ddc\text{-}GAL4^{43D}$ (Li et al. 2000), provided by Dr. J. Hirsh (University of Virginia). The w^{1118} line was provided by Dr. H. Lipshitz (University of Toronto). Individuals were generated for the assay by crossing w^{1118} females to males of the *UAS-TH*, *UAS-Ddc* and the double *Ddc-GAL4* lines. All flies

were raised upon standard cornmeal/yeast/molasses/agar medium at 25°C in standard plastic shell vials.

Assay Design

The climbing apparatus (Figure 1) consists of a 30 cm long glass tube, with a diameter of 1.5 cm. The tube is held in place by a plastic funnel that acts as both a base for the tube and as a means for transferring flies into the apparatus. Starting from the base, the glass tube is divided into a series of five sections, each 2 cm in height (scored 1-5), with an additional buffer zone in the upper portion of the apparatus. Sponges are placed in each end of the tube to prevent escape yet allow air exchange.

Due to the natural negative geotaxis displayed by Drosophila, the flies climb up the sides of the apparatus after being tapped down to the bottom. Flies are allowed ten seconds to climb after being tapped down and are given a score based upon the sections reached. The flies are scored ten times (trials) per climbing session, from which a climbing index is calculated as follows:

$$\textbf{Climbing index} = \Sigma(nm) / N$$

Where **n** = number of flies at a given level, **m** = the score for that level (1-5) and **N** = total number of flies climbed for that trial.

For climbing analysis, fifty male progeny from each genotype under investigation are collected within 24 hours of eclosion and separated into groups of ten individuals.

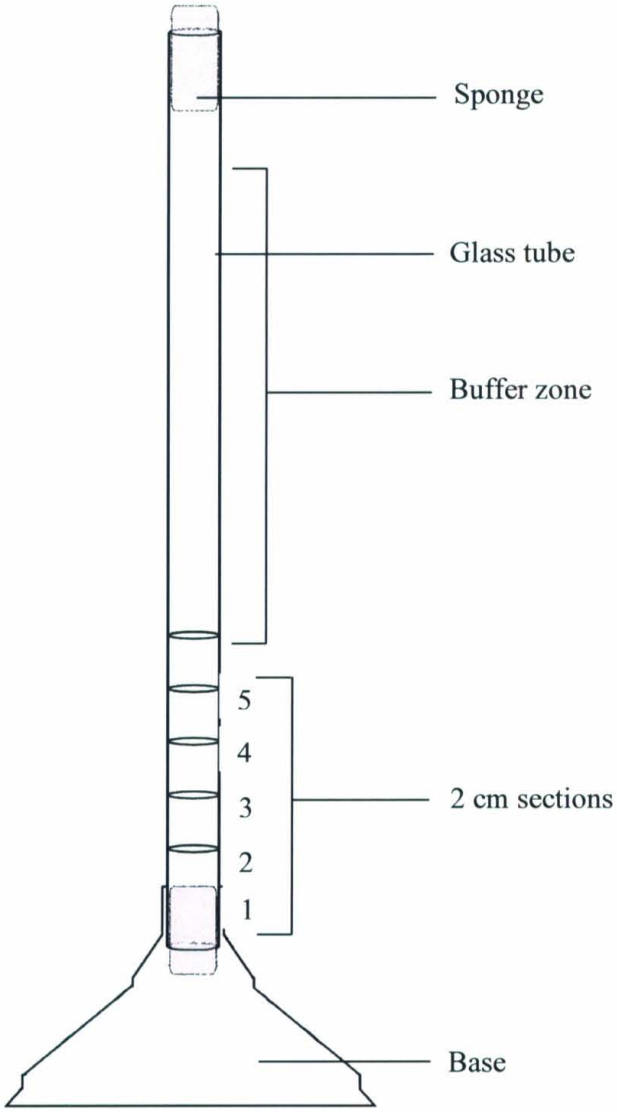

Figure 1: Schematic representation of the climbing apparatus. The apparatus consists of a 30 cm long glass tube, with a diameter of 1.5 cm. The tube is held in place by a plastic base that also acts as a funnel for transferring flies into the apparatus. The glass tube is divided into five, 2 cm sections (1-5) with a buffer zone at the top of the apparatus. The ends of the tube are plugged with sponges to prevent escape of flies.

Starting at day one after eclosion, each group is tested for climbing ability and is continually tested every five days throughout their lifespan.

Statistical Analysis

Data from the climbing assay was analyzed using the GraphPad Prism 4.02 program (GraphPad Software Inc.). Climbing data were compared using curve fit comparison in which the climbing index was subtracted from five and a non-linear regression curve fit was performed. The resulting slopes of the fitted curves for each set of data were compared with 95% confidence intervals.

The model generated for the climbing data using a non-linear regression curve fit is as follows:

$$\textbf{5- Climbing index} = \mathbf{C}\, e^{\mathbf{Kt}}$$

or $$\textbf{Climbing index} = \mathbf{5} - (\mathbf{C}\, e^{\mathbf{Kt}})$$

Where **C** = constant, **K**= the slope of the fitted curve and **t** = time in days.

Results (Sample Analysis)

As a part of ongoing experiments looking at dopamine synthesis in relation to a model of Parkinson disease, *tyrosine hydroxylase (TH)* and *dopa-decarboxylase (Ddc)* (True et al. 1999) were expressed using the UAS/GAL4 system for ectopic gene expression (Brand and Perrimon 1993) and assayed for climbing ability. The climbing results from the double *Ddc-GAL4* flies and the *UAS-TH* and *UAS-Ddc* flies are presented (Figure 2). A comparison of the *UAS-TH* flies with the *UAS-Ddc* flies (Figure 2a) shows a small but

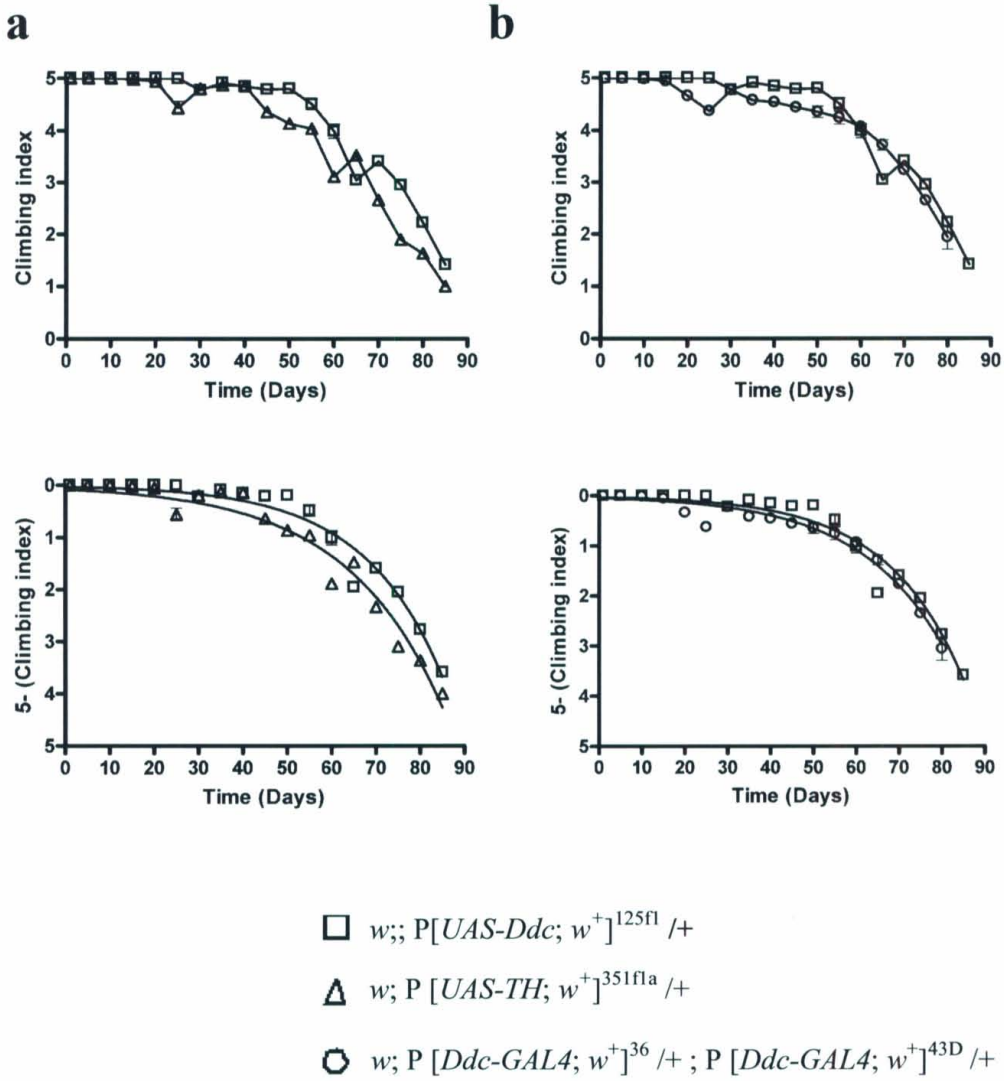

Figure 2: Comparison of the climbing ability showing curves where a significant difference is present (a) and where there is not a significant difference (b) The top graph in each is the non-fitted data where the bottom graph is the fitted curve. Error bars in the non-fitted graphs are standard error of the mean; most error bars are within the area of the data point symbol.

significant difference in climbing ability. The slopes for the fitted curves (Table 1) are 0.056 and 0.046 respectively, and do not overlap within a 95% confidence interval (95% CI). In contrast, comparison of the *UAS-TH* flies with the double *Ddc-GAL4* flies (Figure 2b, Table 1) does not show a significant difference in climbing ability within a 95% CI with slopes of 0.056 and 0.050 respectively.

Comparison to the Non-graded Method

A common method for analysis of climbing ability in Drosophila is a similar apparatus with a single line at the 8 cm mark (Feany and Bender 2000; Haywood and Staveley 2004) in which flies are scored as being able or unable to reach the 8 cm distance. This type of assay is usually performed in empty 9.5 x 2.7 cm vials. The 1.5 cm tube proposed here presents less opportunity for individuals to fly in the apparatus. In addition, the non-graded method results in less sensitivity when detecting differences in climbing ability. Figure 3 shows a comparison of the graded method proposed here (Figure 3a) with the non-graded method (Figure 3b) using the non-linear curve fit analysis for each. From these graphs, it is apparent that the non-graded method is unable to detect climbing ability when nearing the end of lifespan. In the non-graded method flies appear as unable to climb when 70-80 days old, when they are actually able to climb between two and six centimeters at this age, as shown in the graded method.

The non-graded method is also unable to discern the difference in climbing ability between the *UAS-TH* flies and the double *Ddc-GAL4* flies (Tables 2 and 3). In the graded method (Table 2), the slope of the curve for the *UAS-TH* flies has a 95% CI of 0.076-

Table 1: Comparison of the non-linear fitted curves for climbing ability showing slopes (K), standard error (SE), confidence intervals (CI), and generated models for each of the fitted curves.

Genotype	Slope (K)	Standard error (SE)	95% Confidence intervals (CI)	Generated model (Climbing index = $5\text{-}Ce^{Kt}$)
w; P [*UAS-TH*; w^+]^{351fla} /+	0.056	0.0018	0.052 to 0.059	Climbing index = $5\text{-}(0.032\ e^{0.056t})$
w;; P[*UAS-Ddc*; w^+]^{125fl} /+	0.046	0.0012	0.043 to 0.048	Climbing index = $5\text{-}(0.087\ e^{0.046t})$
w; P [*Ddc-GAL4*; w^+]^{36} /+ ; P [*Ddc-GAL4*; w^+]^{43D} /+	0.050	0.0020	0.046 to 0.054	Climbing index = $5\text{-}(0.053\ e^{0.050t})$

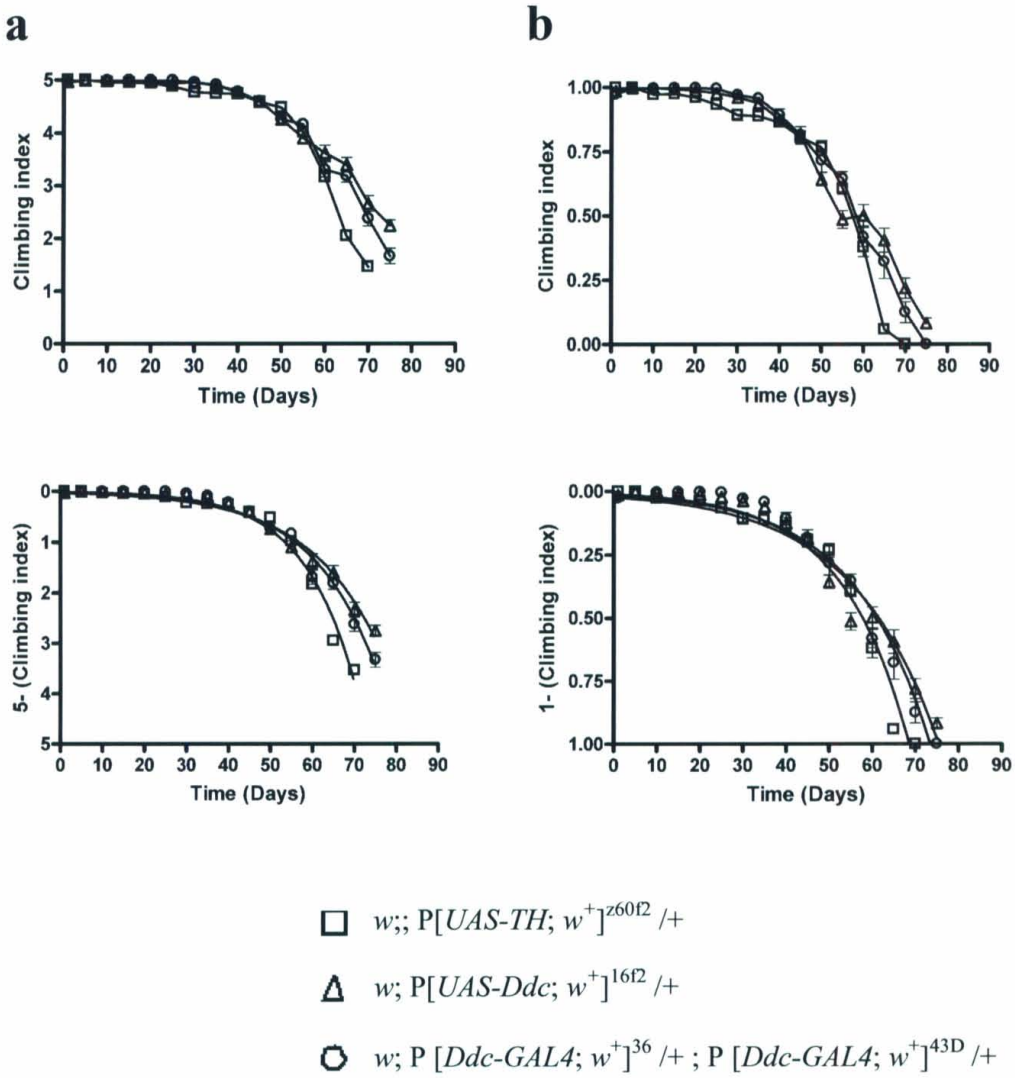

Figure 3: Comparison of graded (a) and non-graded (b) climbing methods using non-linear curve fit analysis. The top graph in each is the non-fitted data where the bottom graph is the fitted curve. Error bars in the non-fitted graphs are standard error of the mean; most error bars are within the area of the data point symbol.

Table 2: Comparison of the non-linear fitted curves for climbing ability using the graded method showing slopes (K), standard error (SE), confidence intervals (CI), and generated models for each of the fitted curves.

Genotype	Slope (K)	Standard error (SE)	95% Confidence intervals (CI)	Generated model (Climbing index = $5\text{-}Ce^{Kt}$)
w;; P[*UAS-TH*; w^+]^{z60f2} /+	0.080	0.0022	0.076 to 0.084	Climbing index = $5\text{-}(0.014\ e^{0.080t})$
w; P[*UAS-Ddc*; w^+]^{16f2} /+	0.059	0.0024	0.054 to 0.063	Climbing index = $5\text{-}(0.036\ e^{0.059t})$
w; P [*Ddc-GAL4*; w^+]^{36} /+ ; P [*Ddc-GAL4*; w^+]^{43D} /+	0.065	0.0024	0.061 to 0.070	Climbing index = $5\text{-}(0.026\ e^{0.065t})$

Table 3: Comparison of the non-linear fitted curves for climbing ability using the non-graded method showing slopes (K), standard error (SE), confidence intervals (CI), and generated models for each of the fitted curves.

Genotype	Slope (K)	Standard error (SE)	95% Confidence intervals (CI)	Generated model (Climbing index = $5\text{-}Ce^{Kt}$)
w;; P[*UAS-TH*; w^+]^{z60f2} /+	0.064	0.0019	0.060 to 0.068	Climbing index = $5\text{-}(0.012\ e^{0.064t})$
w; P[*UAS-Ddc*; w^+]^{16f2} /+	0.050	0.0020	0.046 to 0.054	Climbing index = $5\text{-}(0.023\ e^{0.050t})$
w; P [*Ddc-GAL4*; w^+]^{36} /+ ; P [*Ddc-GAL4*; w^+]^{43D} /+	0.056	0.0021	0.052 to 0.060	Climbing index = $5\text{-}(0.016\ e^{0.056t})$

0.084, and does not overlap with the 95% CI for the double *Ddc-GAL4* flies of 0.061-0.070. In contrast, analysis of the same data using the non-graded method (Table 3) results in overlapping confidence intervals for the slopes of the *UAS-TH* and the double *Ddc-GAL4* cohorts, with 95% CI of 0.060-0.068 and 0.052-0.060 respectively. Therefore, if using the non-graded method in this analysis, the difference in the climbing ability between these two genotypes would not have been detected.

Discussion

Evaluation of Assay

We propose that this novel, graded climbing assay is an accurate method for measuring climbing ability in Drosophila. The narrow glass tube used in the climbing apparatus is able to discourage Drosophila from flying upward within the tube, and therefore, the index is a measure of climbing ability only. The narrow tube also helps to prevent flies from falling long distances during the ten second climbing trial, making it easier to score the highest point reached by each fly.

As early as by day twenty, and throughout the remainder of the assay, flies were occasionally observed to move in horizontal circles in one graded section of the tube. This usually occurred in the lowest section of the tube, and although these flies were very mobile, their horizontal movement resulted in a low score and consequently a lower climbing index for the genotype. Horizontal movement, as well as downward climbing and complete lack of climbing, was often observed to increase as the trials progressed from one through ten during a given session. These climbing behaviours contributed to

the change in the slope of the fitted line with increasing trials as shown in Table 4. However, it should also be noted that with each trial, the 95% Confidence Interval decreases (Table 4) and indicates that with the addition of each trial the fitted slope of the line becomes more accurate.

Conclusions

The climbing assay is an accurate measure of climbing ability in Drosophila. The novel apparatus and longer testing period is more sensitive to differences as compared to the commonly used non-graded method. The number of trials was determined to be appropriate, however concerns remain about fatigue as a result of the continuous repetition of the trials (with no break in between each trial). This is a problem in both the proposed graded and non-graded methods and may be resolved by providing a controlled break period between trials. Alternatively, a combination of a decrease in the number of trials and an increase in the overall number of flies tested may reduce the possibility of fatigue. In summary, the combination of our novel apparatus and analysis provides an accurate assay for climbing ability in Drosophila.

Table 4: The effect of number of trials performed during the climbing assay on the resulting slope (K) of the non-linear fitted line and the confidence interval for climbing ability.

Trial #	Genotype		
	w;; P[*UAS-TH*; w^+]^{z60f2} /+	*w*; P[*UAS-Ddc*; w^+]^{16f2} /+	*w*; P [*Ddc-GAL4*; w^+]^{36} /+ ; P [*Ddc-GAL4*; w^+]^{43D} /+
1	K=0.10 ± 0.022	K=0.073 ± 0.015	K=0.073 ± 0.025
2	K=0.098 ± 0.012	K=0.076 ± 0.0087	K=0.079 ± 0.014
3	K=0.096 ± 0.0088	K=0.075 ± 0.0070	K=0.077 ± 0.011
4	K=0.093 ± 0.0068	K=0.074 ± 0.0072	K=0.075 ± 0.0091
5	K=0.091 ± 0.0061	K=0.069 ± 0.0072	K=0.073 ± 0.0074
6	K=0.090 ± 0.0055	K=0.066 ± 0.0062	K=0.073 ± 0.0065
7	K=0.087 ± 0.0051	K=0.064 ± 0.0057	K=0.071 ± 0.0060
8	K=0.083 ± 0.0049	K=0.063 ± 0.0055	K=0.069 ± 0.0053
9	K=0.082 ± 0.0045	K=0.061 ± 0.0050	K=0.067 ± 0.0050
10	K=0.080 ± 0.0042	K=0.059 ± 0.0046	K=0.065 ± 0.0046

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Appendix 2

Co-expression of *α-synuclein* does not affect the reduction of lifespan resulting from overexpression of *phosphatidylinositol 3-OH-kinase (PI3K)* in Drosophila dopaminergic neurons

A version of this appendix has been published in Drosophila Information Services (Todd and Staveley 2010)

Introduction

The phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR pathway is a ubiquitous and evolutionarily conserved signalling cascade that functions in cell growth, survival, proliferation, migration and metabolism (Endersby and Baker 2008). PI3K is a lipid kinase that phosphorylates the three position of the inositol ring of phosphatidylinositols and phosphoinositides (Engelman et al. 2006). A major downstream target of PI3K signalling is the serine-threonine kinase AKT, which, once activated, results in an anti-apoptotic or cell survival signal. Cell culture experiments have demonstrated the rescue of α-synuclein toxicity with activation of the PI3K pathway (Kao 2009), suggesting that activation of this pathway may have broad protective effects.

In *Drosophila melanogaster*, as in mammals, PI3K plays a role in the conserved insulin signalling pathway, regulating metabolism, growth and lifespan (Oldham and Hafen 2003). This pathway is negatively regulated by a PTEN orthologue. Previous experiments show that deletion of *PI3K* in Drosophila results in a reduction in cell size and cell number, whereas deletion of *PTEN* leads to an increase in cell size and organ size (Engelman et al. 2006). In contrast, we have previously shown an unexpected reduction in lifespan with overexpression of *PI3K* in the dopaminergic neurons of Drosophila (Saunders et al. 2003). Although unexpected, other studies have shown apoptosis with overexpression of *PI3K* (Klippel et al. 1998; Vanhaesebroeck et al. 2001). This suggests a sensitivity in specific cell types may exist, likely sensitivity to the deregulation of the cell cycle.

Given the conflicting role of PI3K in the survival of dopaminergic neurons, we looked at the reproducibility of, and the possible effect that an additional stressor, in the form of *α-synuclein* overexpression, may have on the reduced lifespan previously observed.

Materials and Methods

Fly stocks and culture

Dr. M. Feany (Harvard Medical School) generously provided *UAS-α-synuclein* flies (Feany and Bender 2000), Dr. J. Hirsh (University of Virginia) the *Ddc-Gal4* flies (Li et al. 2000), Dr. Sally Leevers (Ludwig College, London) the *UAS-PI3K-dp110* ($PI3K^{+}$) and *UAS-PI3K-dp110*^{D954A} ($PI3K^{DN}$) flies (Leevers et al. 1996), and the w^{1118} line was provided by Dr. H. Lipshitz (University of Toronto). All flies were raised upon standard cornmeal/yeast/molasses/agar medium at 25°C in standard plastic shell vials.

Ageing assay

Approximately 300 adult males of each genotype were collected under gaseous carbon dioxide and aged upon standard cornmeal/yeast/molasses/agar media, at 25°C, in upright standard plastic shell vials. Flies were maintained in non-crowded conditions with one to twenty individuals per vial. Flies were scored for viability every two days and transferred to fresh media according to established protocol (Staveley et al. 1990). Log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test were used to compare resulting survival curves.

Results and Discussion

Overexpression of *PI3K*$^{+}$ using the *Ddc-Gal4* tissue specific driver showed a dramatic reduction of lifespan, with a median survival of 46 days as compared to 80 days seen in the control (Log-rank and Wilcoson: $p<0.0001$) (Figure 1). In contrast, expression of the *PI3K*^{DN} showed a normal life span, median survival of 78 days, as compared to the control (Wilcoson: $p=0.4495$). As mentioned previously, these results are in contrast to the typical role of the PI3K signalling pathway, as an initiator of cell growth and proliferation. This is likely due to sensitivity in the dopaminergic cells with respect to deregulation of cell cycle controls. In post-mitotic cells, including those in Drosophila, PI3K signalling has been shown to control cell growth (cell size) as opposed to proliferation (Engelman et al. 2006). Cells may be triggered to undergo apoptosis due to failure to progress through cycle (Klippel et al. 1998; Vanhaesebroeck et al. 2001). It also appears that Drosophila lifespan is limited by metabolic activity and oxidative damage to the nervous system in particular, opposed to other tissues (Shmookler Reis et al. 2009). Therefore, targeting of *PI3K* expression to these sensitive cells may show an effect that is otherwise unnoticed during expression in other tissues.

The PI3K pathway involves many levels of regulation including various negative feedback mechanisms (Reviewed in Engelman et al. 2006). In addition, activation of downstream transcription factors can have various effects including the activation of protective proteins such as PTEN induced punitive kinase 1 (Pink1) (Mei et al. 2009). *Pink1* has been shown to rescue an *α-synuclein* induced phenotype in Drosophila (Todd and Staveley 2008), presumably through downstream tagging and degradation of α-

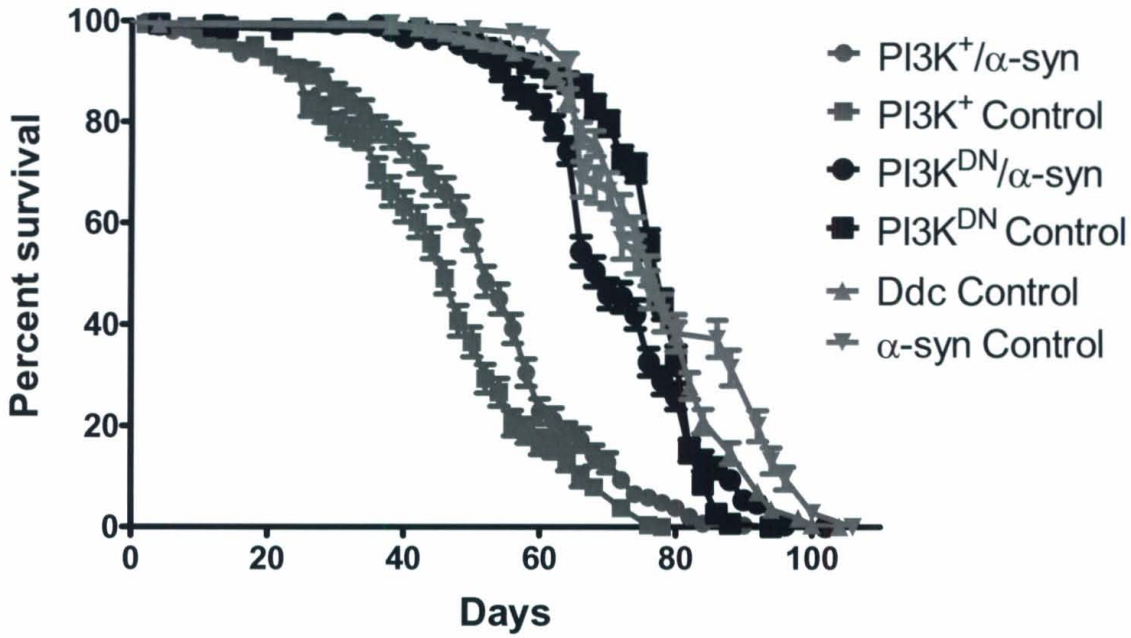

Figure 1. Survival curves of Drosophila overexpressing PI3K+ or PI3KDN with or without the co-overexpression of α-synuclein, in the dopaminergic neurons, aged on a standard medium. Genotypes are UAS-PI3K+/ UAS-α-synuclein; Ddc-Gal4/+ (PI3K+/ α-syn)(n=289), UAS-PI3K+/ +; Ddc-Gal4/+ (PI3K+ Control)(n=263), UAS-PI3KDN/ UAS-α-synuclein; Ddc-Gal4/+ (PI3KDN/ α-syn)(n=298), UAS-PI3KDN/+; Ddc-Gal4/+ (PI3KDN Control)(n=294), +/+; Ddc-Gal4/+ (Ddc Control)(n=287), and UAS-α-synuclein/+; Ddc-Gal4/+ (α-syn Control)(n=175). Error bars indicate standard error of the mean.

synuclein. It was not found that this additional cell stressor of α-synuclein overexpression contributes to the PI3K induced reduction in longevity. Overexpression of *α-synuclein* with $PI3K^{DN}$ using the *Ddc-Gal4* tissue specific driver does not have a substantial effect on lifespan, median survival 70 days, as compared to overexpression of $PI3K^{DN}$ alone, median survival 78 days (Log-rank: p=0.0677)(Figure 1). Overexpression of *α-synuclein* with $PI3K^{+}$ shows a slight increase in lifespan, median survival 52 days, compared to overexpression of $PI3K^{+}$ alone, median survival 46 days (Log-rank and Wilcoson: $p<0.0001$). This however is not rescued to the level of the *α-synuclein* or *Ddc-Gal4* controls (Log-rank and Wilcoson: $p<0.0001$). The engagement of protective proteins such as Pink1 during α-synuclein cytotoxicity may not have enough of an effect to increase the *PI3K* phenotype, alternatively, the detrimental effects of *PI3K* may be too severe on its own, or is initiated before *α-synuclein* induced cytotoxicity takes effect.

In conclusion, it appears that, in dopominergic neurons, overexpression of $PI3K^{+}$ results in a severe decrease in lifespan, likely due to the sensitivity of this particular cell type coupled with the inability of these cells to progress through cell cycle, thereby triggering apoptosis. A substantial difference was not observed with the addition of *α-synuclein* overexpression, and therefore, no conclusion can be made regarding possible links between the PI3K signalling pathway and α-synuclein cytotoxicity.

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Appendix 3

Eyer: Automated Counting of Ommatidia using Image Processing Techniques

A version of this appendix has been published in Drosophila Information Services (Woodman et al. 2011)

Introduction

The UAS/GAL4 ectopic expression system (Brand and Perrimon 1993) is a widely used tool for the overexpression of transgenes in Drosophila melanogaster. This bipartite expression system allows for the expression of transgenes in specific tissues, including the Drosophila eye using the glass multiple reporter (GMR)-GAL4 driver line (Freeman 1996). During this expression, it is of interest to measure differences in morphological characteristics such as ommatidia number, ommatidial area and overall area of the eye. This measurement is both time consuming and labour intensive given the number of Drosophila required for analysis and the limitations of currently available programs. Here, we describe a novel program designed for simple, morphological analysis such as counting and area measurements. Although initially designed for analysis of the Drosophila eye, this program could be used for analysis of various tissues, and could be used for other applications apart from expression studies.

Design of Program

Software

The Eyer software was developed as the user interface for a segmentation algorithm optimized for the Drosophila eye. It was written for the Windows platform using Visual Studio C# and WPF. The image processing routines used by Eyer are performed by the AForge.NET open-source imaging library and the Vincent-Soille immersion watershed algorithm. The program was developed using images at a resolution of 1.33 pixels per

micrometer. This equates to ommatidia that are approximately 20 pixels in diameter at the center of the eye in adult Drosophila.

User interface

The Eyer software is a single purpose application, which allows for a completely tailored interface to make the eye analysis task as quick and easy as possible. Instead of opening individual image files, a folder is selected for editing, and all images are shown as a list of thumbnails. The user can switch between active images by clicking on its thumbnail. An image can be easily panned and zoomed via mouse or keyboard. All measurements carried out on an image are automatically recorded in an associated text file and stored with the image in the original folder.

Algorithm

There are several challenges presented to standard segmentation algorithms by the images of Drosophila eyes. The convex nature of the eye results in ommatidia size and shape variations across the eye leading to partially obscured ommatidia around the edge. The lighting used when taking SEM images can vary over the surface of the eye and also from image to image. Light spots and shadows cause the intensity of an ommatidium to be unpredictable. The bristles that grow between Drosophila ommatidia reflect intense light, creating further challenge for a segmentation algorithm. These bristles, from an image processing perspective, appear to split many of the ommatidia while following along the edge of others. For these reasons, the goal set for the development of the segmentation algorithm was not to completely segment the eye, but to segment the largest area possible

with the highest accuracy. The portion of the eye not segmented by the software is then completed manually.

The solution to the segmentation problem described above required a set of processes that remove color variation and highlight the ommatidia edges uniformly. Once that had been accomplished, an immersion watershed algorithm was used to separate and mark each ommatidium. The complete algorithm can be described in five steps. (1) *Contrast Enhancement:* This is achieved with a combination of histogram equalization, and contrast stretching. (2) *Edge Detection:* Both horizontal and vertical edges are detected with a standard edge detection routine. The edge detection results are added to the contrast-improved image to further distinguish the lines around the ommatidia from the centers. (3) *Local Thresholding:* Removal of all color intensity to create a black and white image. The typical conversion method uses a threshold value, where intensities above that value are white, and below are black. Since the intensity of the ommatidia varies over the surface of the eye, an adaptive local threshold has to be used, where the value of the threshold changes for each pixel, dependent on the surrounding area. After this step most edges are visible, however many ommatidia still appear joined. (4) *Watershed:* The watershed algorithm is able to separate connected blobs. This step processes the image as if it is a topographic relief, identifying locations where water would pool if poured onto the image. (5) *Blob Analysis:* The resulting watershed lines are then used to segment the eye. The list of segmented objects is filtered by size and shape by a blob analysis package to determine which objects are valid ommatidia.

Sample Analysis and Discussion

Drosophila

Flies of various genotypes were aged 3 days past eclosion and frozen at –80 °C. Flies were mounted, desiccated overnight, and coated in gold before photography at 150 times magnification with a Hitachi S-570 scanning electron microscope as per standard methods.

Area

To measure area, or to segment and image for counting purposes, the region of interest (ROI) must be defined by the user. The ROI tool creates a point based polygon where points can be easily added and removed while defining the area. The polygon can be made with as many points as needed, resulting in a clearly defined area without the labour intensive process of using a freehand tool. The total area of the eye is calculated in real time and recorded in the associated text file as mentioned.

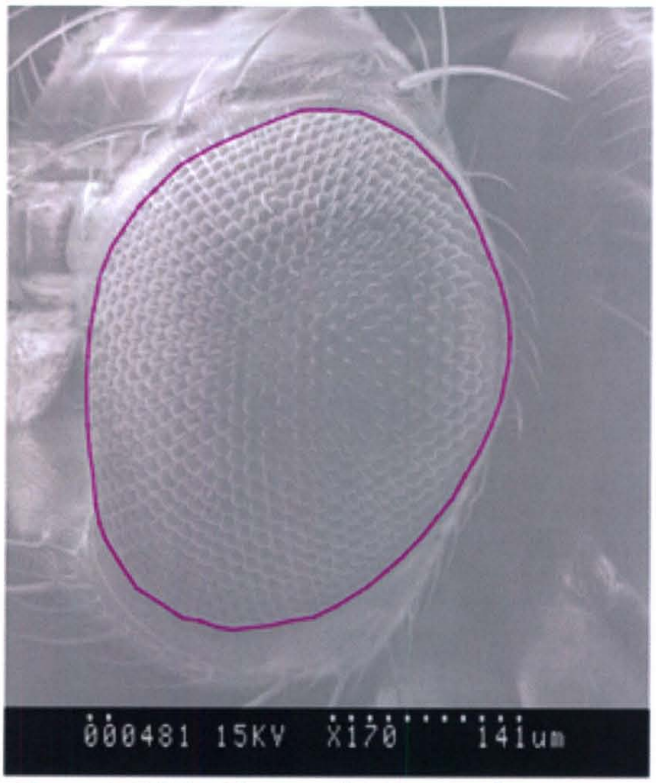
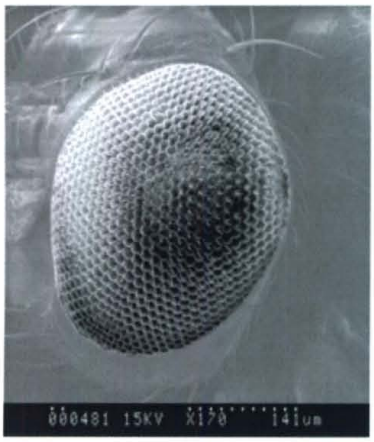


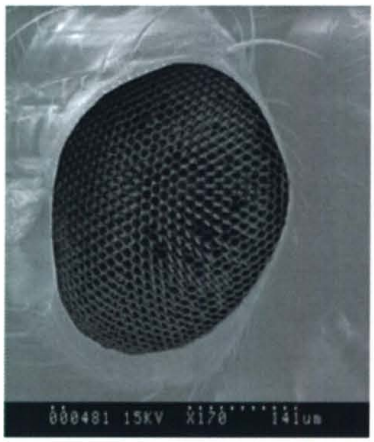
Figure 1: Original Image with User Defined ROI

Counting

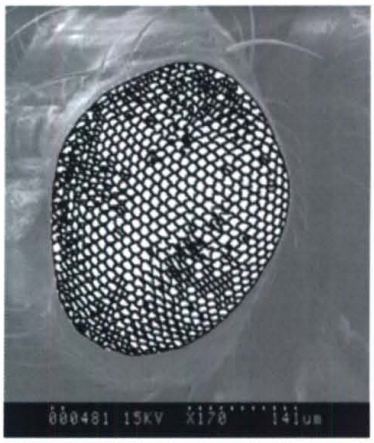
The resolution of the image affects the performance and speed of the segmentation required for counting. Higher resolutions will result in a higher segmentation percentage, with a reduction in speed.



A: Contrast Enhancement



B: Edge Detection



C: Watershed Segmentation

Figure 2: Image Processing Stages

The contrast enhancement performed by Eyer can be applied to the entire image separately to improve the visibility of detail on the subject. This was found to be useful when examining features such as the overall area of the eye.

After the segmentation process, identified ommatidia are marked with red dots. In this example, segmentation took 4.8 seconds to complete, and it completed with 561 of 688 ommatidia correctly marked. 4 dots were placed incorrectly. The 4 false positives and remaining unmarked ommatidia can be completed manually before the results are saved.

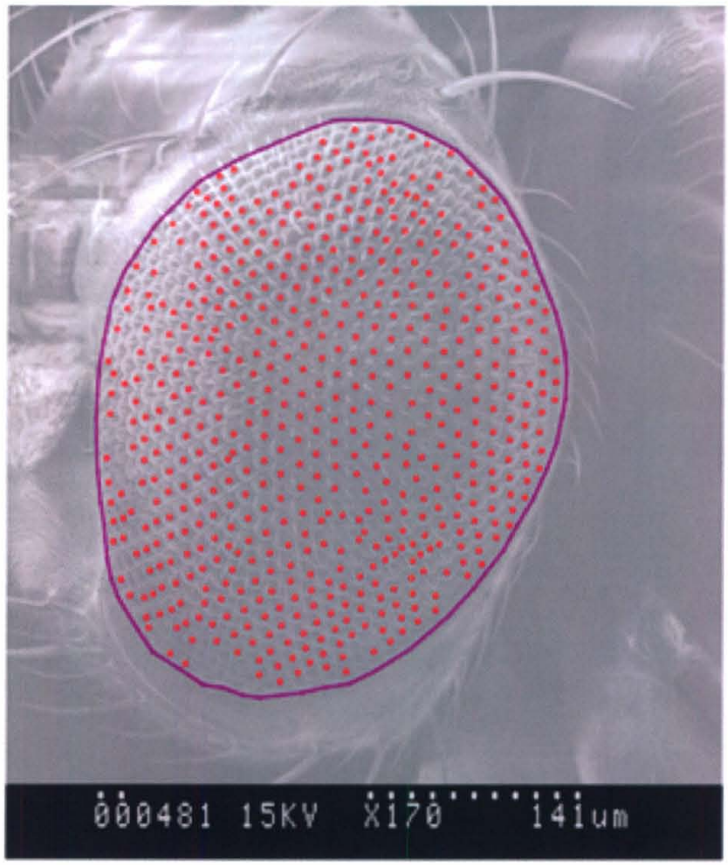


Figure 3: Identified Ommatidia Marked by Eyer

Conclusions

Morphological analysis in Drosophila is widely used during expression studies to investigate gene and protein function. In the Drosophila eye, measurement of characteristics such as ommatidia number, ommatidial area and area of the eye are often time consuming and labour intensive. Our novel program, Eyer, has been shown to be a valuable tool for simple morphological analysis of the Drosophila eye. This program could reduce the time needed for morphological analysis of various Drosophila tissues, as well as tissues in other organisms.

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