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# Investigating the mechanisms by which PINK1 protects against Parkinson's disease

Thesis presented by

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for the degree of

**Doctor of Philosophy**

**University College Cork**

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## Declaration

I declare that this thesis is my own, original work and has not been submitted, in whole or in part, for any other degree. The work was carried out under the supervision of Prof. Cora O'Neill and Prof. Aideen Sullivan between October 2015 and September 2019, in the School of Biochemistry and Cell Biology, University College Cork, Ireland.

_____

Signed: Rachel Furlong

October 2019

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## Abbreviations

Antisense oligonucleotides (ASO)

Autosomal dominant (AD)

ATP citrate lyase (ACL)

Autosomal recessive (AR)

Autosomal recessive juvenile Parkinsonism (ARJP)

calmodulin (CALMI)

Calpain small subunit (CAPSN1)

Calpain-2 catalytic subunit (CAPN2)

carbonyl cyanide m-chlorophenyl hydrazine (CCCP)

Catecholaminergic A-differentiated (CAD)

Deep brain stimulation (DBS)

Dopamine (DA)

Early onset PD (EOPD)

Endoplasmic reticulum (ER)

Epidermal growth factor receptor (EGFR)

fetal ventral midbrain (fVM)

Forkhead box-O class (FOXO)

Genome-wide association study (GWAS)

Glial cell derived neurotrophic factor (GDNF)

Glucagon-like peptide-1 (GLP-1)

Glycogen synthase kinase 3β (GSK3β)

Hexokinase II (HKII)

HtrA Serine Peptidase 2 (HtrA2)

induced pluripotent stem cells (iPSCs)

Insulin like Growth Factor-1 (IGF-1)

Insulin receptor substrate (IRS)

interferon (IFN)

interferon-activatable protein (IFI204)

Internal segment of the globus pallidus (GPi)

Levodopa (L-dopa)

Lewy bodies (LB)

Mammalian target of rapamycin complex 2 (mTORC2)

Mouse embryonic fibroblasts (MEFs)

1-methyl-4-phenylpyridine (MPP)

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

Nuclear factor-kappa B (NF-κB)

Outer mitochondrial membrane (OMM)

Parkinson's disease (PD)

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN)

Phosphatidylinositol (PI)

Phosphatidylinositol 4,5-bisphosphate ($PIP_2$)

Phosphatidylinositol 3,4,5-triphosphate ($PIP_3$)

Phosphoinositide-dependent kinase-1 (PDK-1)

Phosphoinositide 3-kinase (PI3-kinase)

Platelet-derived growth factor (PDGF)

Pleckstrin homology (PH)

Protein phosphatase 2A (PP2A)

protein S100-A (S100A6)

PTEN-induced putative kinase (PINK1)

Reactive oxygen species (ROS)

Receptor tyrosine kinase (RTK)

small ubiquitin-related modifier 2 (SUMO2)

Substantia nigra pars compacts (SNpc)

Substantia nigra pars reticulata (SNr)

Subthalamic nucleus (STN)

TNF receptor-associated protein 1 (TRAP1)

Toll-interacting protein (TOLLIP)

Tyrosine hydroxylase (TH)

Unified Parkinson's Disease Rating Scale (UPDRS)

Valosin-containing protein (VCP)

Ventral midbrain (VM)

## Abstract

PINK1 (PTEN-induced putative kinase-1) functions as a ubiquitous serine-threonine kinase, with pro-survival, neuroprotective and anti-stress signalling functions. Autosomal recessive mutations in PINK1, resulting in a loss of PINK1 function, cause early onset Parkinson's disease (PD). This thesis aimed to investigate the mechanisms by which PINK1 protects against PD, with a focus on PI3-kinase/Akt signalling. Akt signalling is central to cell survival, metabolism, protein and lipid homeostasis, and is impaired in PD. Akt activation is reduced in the PD brain, and by many PD-causing genes, including PINK1. However, it is not yet known how PINK1 regulates PI3-kinase/Akt signalling.

Chapter Two investigated the mechanisms by which PINK1 regulates Akt signalling in PINK1 modified mouse embryonic fibroblasts (MEFs). Our results reveal for the first time that PINK1 constitutively activates Akt in a PINK1-kinase dependent manner in the absence of growth factors and enhances Akt activation in normal growth medium. In PINK1 modified MEFs, agonist-induced Akt signalling failed in the absence of PINK1, due to significantly impaired PINK1 kinase-dependent increases in $PI(3,4,5)P_3$ at both the plasma membrane and Golgi. In the absence of PINK1, $PI(3,4,5)P_3$ levels did not increase in the Golgi, and there was significant Golgi fragmentation, a recognised characteristic of PD neuropathology. PINK1 kinase activity protected the Golgi from fragmentation in an Akt-dependent fashion. This demonstrates a new role for PINK1 as an upstream activator of Akt via PINK1 kinase-dependent regulation of its primary activator $PI(3,4,5)P_3$, providing novel mechanistic information on how loss of PINK1 impairs Akt signalling in PD.

Despite its discovery nearly two decades ago, there is a lack of cohesive information on PINK1 binding partners. Determining how PINK1 interacts with proteins involved in certain signalling pathways or cellular processes is crucial for understanding how loss of PINK1 function leads to PD. Chapter 3 aimed to assemble a comprehensive list of known PINK1-interacting proteins and to identify new candidates that are bound to PINK1, using bioinformatics analysis of existing data sets coupled with mass spectrometry approaches. This study identified PINK1-interacting proteins that are

heavily involved in receptor tyrosine kinase (RTK) signalling pathways, such as PI3-kinase/Akt, VEGF, PDGF, ERRB2/4 and EGF, indicating that PINK1 plays a key functional role through these pathways. Further analysis highlighted two PINK1-interacting proteins (PTEN and IRS4) that are involved in PI3-kinase/Akt signalling and which may mediate PINK1's regulation of this pathway through lipid binding with $PIP_3$. Using mass spectrometry approaches, several new binding proteins for PINK1 were identified, including RIPK3, calmodulin, EGFR, and interferon regulatory genes, all of which have been implicated in PD and as components of the Akt signalling system.

Accumulation of α-synuclein is central to the development of PD, and mutations in *SNCA*, which encodes α-synuclein, cause PD. There is a need to identify the mechanisms by which α-synuclein overexpression induces neurodegeneration in PD and to determine whether α-synuclein and PINK1 share common mechanisms. This information will be critical for future identification of therapeutic targets. In Chapter 4, we identified significant Golgi fragmentation, mitochondrial fission and reductions in neurite length in cultured ventral midbrain neurons, in response to overexpression of wildtype or mutant α-synuclein, and to deletion of PINK1. Increased Golgi fragmentation and mitochondrial fission induced by the PD risk genes were significantly correlated with reductions in neurite length. Levels of $PI(3,4,5)P_3$ were significantly decreased by overexpression of wildtype or mutant α-synuclein. α-synuclein overexpression in combination with knockdown of PINK1 induced a "dual-hit", to further reduce neurite length and increase Golgi fragmentation. This provides novel data showing that α-synuclein overexpression and PINK1 deletion converge to induce significant increases in Golgi fragmentation and mitochondrial fission, with concomitant decreases in neurite length and defects in PI3-kinase/Akt signalling.

This thesis therefore demonstrates that studies on the function of PD-causing genes such as PINK1 and α-synuclein enable the discovery of molecular pathways that may be involved in the pathogenesis of PD and could be targets for future therapies.

# Chapter 1: Introduction

## 1.1 Parkinson's disease

Parkinson's disease (PD) was first described in 1817 by James Parkinson (Parkinson, 2002) as a shaking palsy, the "Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace". He also noted the slowly progressive nature of the disease as "so slight and nearly imperceptible are the first inroads of this malady, and so extremely slow its progress, that it rarely happens, that the patient can form any recollection of the precise period of its commencement". His observations made two hundred years ago still hold true today. PD is now known to be the second most common age-related neurodegenerative disorder after Alzheimer's disease. It affects an estimated 10 million people worldwide (10,000 in Ireland) (Aron and Klein, 2011). The incidence rate of PD is 8-18 per 100,000 person-years and the prevalence is estimated at 0.3% of the whole population, 1% of individuals over 60 and 4% of those over 85 (de Lau and Breteler, 2006, Deng et al., 2013, Lee and Gilbert, 2016, Nussbaum and Ellis, 2003, Tanner and Goldman, 1996). The median age of onset is 60 years old, with mean disease duration of 15 years, although many people live with PD for a number of decades (Katzenschlager et al., 2008, Lees et al., 2009).

### 1.1.1 Clinical symptoms

The most typical clinical features of PD are the so-called cardinal motor dysfunctions such as bradykinesia, rigidity, gait instability and resting tremor (Jankovic, 2008, Moustafa et al., 2016). There are also secondary motor symptoms, including dysarthria, dysphagia, micrographia and hypomimia (Jankovic, 2008, Moustafa et al., 2016). Additionally, several non-motor symptoms occur in PD, including depression, dementia, fatigue, sleep disturbances, hyposmia and constipation (Schapira et al., 2017). The latter three symptoms have been reported to precede the cardinal motor dysfunctions of the disease (for review (Felice et al., 2016, Pfeiffer, 2018)). A definite diagnosis of PD cannot be confirmed until post-mortem, and at present diagnosis is based on clinical assessment, which uses the Unified Parkinson's Disease Rating Scale (UPDRS). The UPDRS was originally developed in the 1980s with the aim of providing an easy to

administer scale that could be used for all patients regardless of age, disease severity, or treatment (Goetz et al., 2008). The scale is composed of four core parts- I: behavioural issues, II: self-reported ability to perform daily living tasks, III: motor evaluation in clinic and IV: self-reported treatment complications (Goetz et al., 2008). This scale is also the most commonly used scale in clinical trials to determine the effectiveness of new treatments (Athauda et al., 2017, Mitchell et al., 2000, Whone et al., 2019a, Whone et al., 2019b).

### 1.1.2 Neuropathology

#### 1.1.2.1 Nigrostriatal degeneration and basal ganglia circuitry

The neuropathology of PD is characterised by progressive degeneration of dopamine (DA) neurons in the substantia nigra pars compacts (SNpc) and loss of DA neurotransmission in the striatum which leads the above-mentioned motor dysfunctions. By the time symptoms are detected ~60% of neurons have been lost in the striatum and ~40% in the SNpc (for review (Burke and O'Malley, 2013, Giguere et al., 2018, Toulouse and Sullivan, 2008). The motor circuitry of the basal ganglia has been implicated in PD pathophysiology. In the normal brain, motor function is carried out by a complex neural network composed of the basal ganglia, the striatum, the thalamus and the cortex (Fig. 1.1 a), (Alexander et al., 1986). SN dopaminergic neurons activate receptors in the striatum (caudate and globus pallidus), by releasing the neurotransmitter dopamine. Globus pallidus neurons project inhibitory neurons to the thalamus. The thalamus in turn projects excitatory neurons to the prefrontal and motor cortices. Signalling through this pathway causes increased inhibition of the globus pallidus, reducing inhibitory input to the thalamus, and resulting in glutamatergic excitation of the cortex and movement (Fig1.1a) (Calabresi et al., 2014). In PD, due to loss of nigrostriatal dopamine there is increased activity in the striatum. This increased activity leads to increased inhibition of the thalamus and therefore increased inhibition of the glutamatergic excitation of the motor cortex and a subsequent reduction in movement i.e. bradykinesia (Fig. 1.1b) (for review (Lewis and Barker, 2009, Zhai et al., 2018).

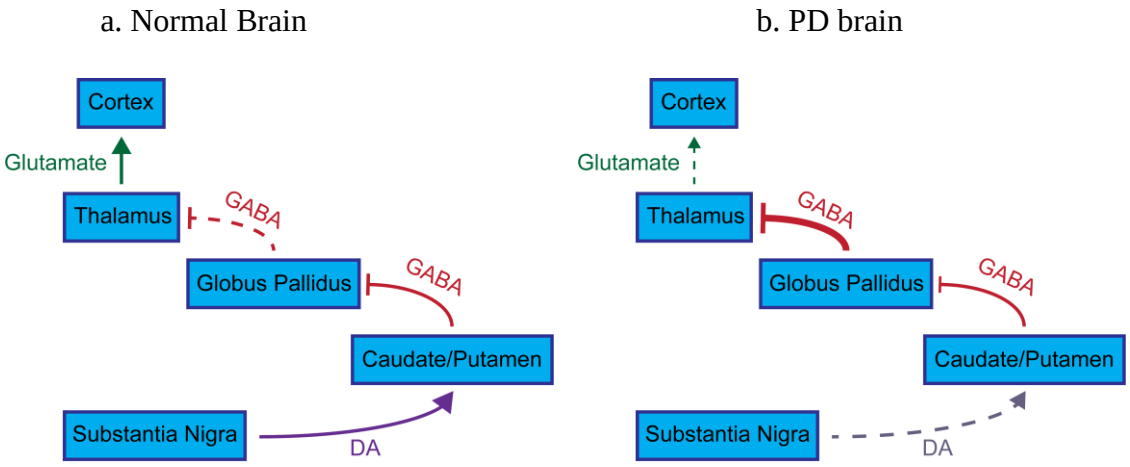



**Figure 1.1: Basal ganglia circuitry**

The changes in basal ganglia circuitry in the PD brain compared to the normal brain. The differing thickness of arrows represent the relative degree of activation in each projection. DA= Dopamine

#### 1.1.2.2 Lewy bodies

Lewy bodies (LB) are the major pathologic hallmark of PD. In 1912, these distinctive protein inclusions were first described by Freidrich Lewy in PD patients in neurons of the nucleus basalis of Meynert and the dorsal vagal nucleus (Lewy, 1912), and later they were reported in neurons of the SN and named after Lewy (Trétiakoff, 1919). They are composed of masses of 7-25 μm filaments containing a heavily phosphorylated α-synuclein core and a halo, in which ubiquitin is detected (Olanow and Brundin, 2013, Spillantini et al., 1998). One of the most common pathological staging strategies of PD, Braak staging, is based on the presence of LB pathology at autopsy in PD patients. This model suggests that the disease process starts in the lower brainstem, specifically in the dorsal motor nucleus of the vagus, as well as in anterior olfactory structures (Stage 1). The disease ascends rostrally through susceptible regions of the medulla, pontine tegmentum (Stage 2), midbrain and basal forebrain (Stage 3/4) and eventually into the cerebral cortex (Stage 5/6) (Braak et al., 2003a). The Braak hypothesis indicates that there is a progressive and stereotypical spread of α-synuclein from one brain area to the next, which correlates with the progression of motor and non-motor disease symptoms. Based on this hypothesis, it has been suggested that PD may be a prion-like disorder and that α–synuclein LB pathology may originate in the periphery in the gut enteric nervous system and nasal olfactory system (Braak et al., 2003a). This topic is further expanded in section 1.7, ("α-synuclein").

## 1.2 Treatment of Parkinson's disease

### 1.2.1 Neurotransmitter modulation

Currently there is no cure for PD, treatment is focused on the pharmacological management of symptoms, which attempts to maintain DA neurotransmission in the nigrostriatal system (Fig. 1.2). The most common therapy involves oral administration of the amino acid DA precursor levo-dopa (L-dopa), or DA agonists which activate DA receptors, such as pramipexole, rotigotine or ropinirole (for reviews see (Cotzias et al., 1969, Fahn, 2015, Yahr et al., 1969).

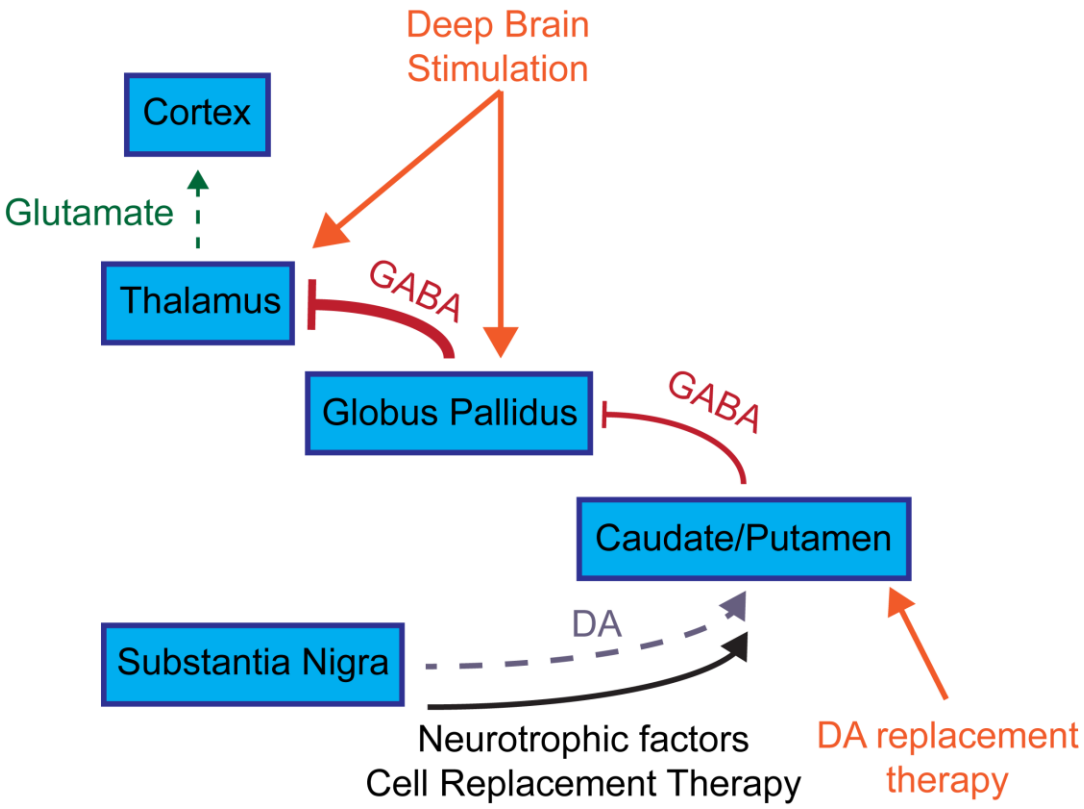



**Figure 1.2 Treatment of Parkinson's disease**

Current PD treatment is focused on the management of symptoms. Dopamine replacement therapy pharmacologically increases dopamine concentration. Deep brain stimulation targets the globus pallidus and subthalamic nucleus to normalise their inhibitory input on the cortex. The emerging therapies neurotrophic factor treatment and cell replacement therapy aim to protect remaining neurons, promote the regrowth of lost neurons and replace lost neurons with new dopamine neurons. DA=dopamine

Due to its oral administration, L-dopa can be rapidly and extensively converted to DA in the periphery particularly in the gut, which can cause a number of side effects and lead to problems in ensuring that effective concentrations cross the blood brain barrier to reach the brain. To prevent this, L-dopa is administered with a human dopa-decarboxylase inhibitor, such as carbidopa or benserazide (Montioli et al., 2016). These inhibitors block the synthesis of DA from L-dopa in the peripheral cells but cannot cross the blood brain barrier and so do not interfere with the generation of DA in the brain (Montioli et al., 2016). However, recent studies have shown that L-dopa is metabolized by gut microbes and that these microbial enzymes are not inhibited by the human dopa decarboxylase inhibitors, which may impact the efficacy of L-dopa in PD patients (for review (O'Neill, 2019), (Maini Rekdal et al., 2019, van Kessel et al., 2019)). Other drugs that are used in combination with L-dopa include catechol-O methyl transferase (COMT) inhibitors, such as entacapone or tolcapone, which prevent the breakdown of L-dopa and 3-O-methyl-dopa and monoamine oxidase (MAO-β) inhibitors which block the breakdown of DA to dihydroxyphenyl acetic acid, increasing the concentration of DA in the brain (Marsili et al., 2017, Siderowf and Kurlan, 1999).

As PD progresses and neurons continue to degenerate, patients' responses to DA replacement therapy becomes harder to manage because the therapeutic window narrows i.e. lower doses of DA start to result in a sub-therapeutic response and higher doses result in dyskinesias ((Calabresi et al., 2010, Contin and Martinelli, 2010), for review (Gupta et al., 2017, Salat and Tolosa, 2013). Prolonged DA replacement therapy can also cause serious behavioural problems, including impulse control disorders such as gambling or compulsive buying (Antonini et al., 2017, Vitale et al., 2019), and neuropsychiatric symptoms such as depression and anxiety (Fan et al., 2016, Schiavolin et al., 2017). A number of ongoing studies are seeking alternatives to oral delivery of these drugs, in order to reduce side effects; these include continuous infusion of L-dopa via percutaneous endoscopic gastrostomy tube, inhalation of L-dopa, and drugs with non-oral delivery options, such as apomorphine (DA agonist) and amantadine (N-methyl-D-aspartate (NMDA) receptor antagonist) (for review (Gupta et al., 2019).

### 1.2.2 Surgical interventions

In 1987, Dr. Alim-Louis Benabid implanted a wire containing four metal contacts at the tip and connected them to an external battery source into the untreated thalamus of an elderly man who had been previously treated with a contralateral ablation for a tremor. He reported akinesia alleviation in response to electrical stimulation, which was similar to that reported with DA drug therapies, but without dyskinesia (Benabid et al., 1994). Furthermore, he reported that chronic electrical stimulation of the subcortical brain was as effective as thalamotomy for tremor suppression (Benabid et al., 1994). This was the first deep brain stimulation (DBS) trial performed and inspired research on its use for a wide variety of movement disorders (Aum and Tierney, 2018, Oluigbo et al., 2012).

Today DBS for PD involves the implantation of an electrode into the subthalamic nucleus (STN) or the internal segment of the globus pallidus (GPi), followed by stimulation with a neurostimulator, located in the chest (Fig. 1.2). Insertions into both the STN and GPi provide improvements in the major motor symptoms of PD, including dyskinesia, and improvements in quality of life (Oyama et al., 2012, Peng et al., 2018). However, some differences have been reported between STN and GPi insertion. For example, STN stimulation results in increased level of depression and worsening cognition, but a lower requirement for DA agents (Follett et al., 2010). GPi stimulation leads to better control of axial motor symptoms, including speech and swallowing function (Follett et al., 2010, Odekerken et al., 2013, St George et al., 2010, Troche et al., 2014). Therefore, electrode implantation needs to be determined on a patient-to-patient basis. As gait and balance disturbances are not generally responsive to DBS at the STN or GPi, a number of other brain areas are currently under investigation; these include the pedunculopontine nucleus, the centromedian thalamus, and substantia nigra pars reticulata (SNr). Promising results have been reported, including alleviation of gait freezing following combined SNr/STN-DBS (Weiss et al., 2011, Weiss et al., 2013), and improvements in gait and posture following SNr DBS alone (Chastan et al., 2009).

At present, DBS is mainly considered for advanced PD patients who are no longer responsive to DA replacement therapies and are experiencing significant dyskinesia. To date, six large-scale, randomised, controlled trials have been performed using DBS in

PD (Deuschl et al., 2006, Okun et al., 2009, Okun et al., 2012, Schuepbach et al., 2013, Weaver et al., 2009, Williams et al., 2010). Each of these trials reported similar efficacy with improved quality of life and a reduction in the self-reported "off" time and in the severity of dyskinesias during the "on" state. A number of the trials also determined DBS in combination with brain stimulation plus the best medical therapy to be better than best medical therapy alone (Deuschl et al., 2006, Weaver et al., 2009, Williams et al., 2010).

The major limitation with DBS is that it is only considered for advanced, older PD patients. In the above trials, the mean age was 58.6 years old with mean disease duration of 12 years (Deuschl et al., 2006, Okun et al., 2009, Okun et al., 2012, Weaver et al., 2009, Williams et al., 2010). This means that only 2% of PD patients have the opportunity of receiving DBS. The EARLYSTIM trial provided compelling evidence for its use in a younger population (Schuepbach et al., 2013) and so should be considered in the future.

### 1.2.3 Emerging therapies

Although neurotransmitter modulation and DBS can increase the quality of life for the individual, they cannot stop the progression of the disease and their long-term use leads to numerous side-effects. Due to the aging population, the incidence of PD is on the rise with cases expected to double by 2030 (Dorsey et al., 2007, Dorsey ER, 2019). With no disease-modifying therapy available, this will have major impact on people living with PD, the population as a whole and lead to a significant increase in the financial burden on society. This highlights the need for new therapies and in particular for research to better understand the aetiology of PD. The two most prominent disease-modifying therapeutic strategies that are being investigated are treatment with neurotrophic factors, which aims to both protect the remaining neurons of the nigrostriatal DA pathway and regenerate those that have already been lost, and cell replacement therapy, which involves transplantation of new DA neurons to replace lost neurons (Fig. 1.2).

The most well-known neurotrophic factor that has been tested in clinical trials for PD is glial cell derived neurotrophic factor (GDNF). Preclinical data from both *in vitro* and *in*

*vivo* studies had showed promising results, with GDNF found to promote both the survival and growth of fetal ventral midbrain (fVM) neurons (Clarkson et al., 1997, Eggert et al., 1999, Granholm et al., 1997, Kholodilov et al., 2004, Lin et al., 1993, Sullivan et al., 1998, Tomac et al., 1995), for review (Hegarty et al., 2014)). However, placebo-controlled clinical trials showed no significant improvements in motor function in PD patients (Lang et al., 2006, Nutt et al., 2003, Whone et al., 2019a). Although the most recent trial failed to show significant improvement at the predetermined endpoint of 40 weeks (Whone et al., 2019a), researchers reported a 30% improvement in the 40-week open-label extension study that followed (Whone et al., 2019b). More research needs to be conducted in order to determine the mechanism of action of GDNF, which may explain why this factor was not effective in the clinic. Furthermore, there is a need to investigate other neurotrophic factors which are also showing promise, such as growth differentiation factor 5 (Costello et al., 2012, Hurley et al., 2004, O'Keeffe et al., 2004, O'Sullivan et al., 2010, Sullivan et al., 1997, Zhao et al., 2017b), and cerebral dopamine neurotrophic factor (Airavaara et al., 2012, Back et al., 2013, Lindholm et al., 2007, Voutilainen et al., 2011).

Research on cell replacement therapy for PD has been ongoing for over 30 years. Transplantation with fetal ventral midbrain (fVM) grafts yielded promising but variable results in PD patients (Freed et al., 2001, Lindvall et al., 1990, Lindvall et al., 1989, Lindvall et al., 1994, Mendez et al., 2002, Mendez et al., 2000, Olanow et al., 2003, Piccini et al., 1999, Piccini et al., 2000). There are a number of ethical and logistical concerns surrounding fVM transplants- their fetal origin, the number of foetuses required to treat each patient (three to four of the same developmental age, per graft), and post-mortem evidence of LB pathology in the transplant (Kordower et al., 2008, Li et al., 2008), for review (Stoker et al., 2017). However, a new trial involving fVM transplants, entitled 'TRANSEURO', with optimised patient selection and transplant preparation is now underway and there is a hope that it will yield better results (clinicaltrials.gov, 2019). This has also led the field to look for alternatives to fetal donors for transplantation strategies; *in vitro* and *in vivo* studies are now showing promising results, for human embryonic stem cell-derived induced pluripotent stem cells (iPSCs) (for reviews see (Alper, 2009, Kanemura et al., 2014)).

Other emerging therapies in clinical trials include glucagon-like peptide-1 (GLP-1) receptor agonists exenatide and longer-lasting agonists Lixisenatide and Liraglutide ((Athauda et al., 2017, Aviles-Olmos et al., 2013), ClinicalTrials.gov NCT03439943 and NCT02953665). GLP-1 is a gut peptide that regulates metabolism and also has potent neuroprotective and anti-inflammatory function (Aksoy et al., 2017, Long-Smith et al., 2013, Yuan et al., 2017), for review (Athauda and Foltynie, 2016)). Long-lasting GLP-1 agonists are used for the treatment of type 2 diabetes, and can restore insulin signalling with a mechanism that is believed to function via Akt signalling (for review (Athauda and Foltynie, 2016)). Thus far clinical trials with the GLP-1 agonist exenatide for PD have reached Phase II clinical trials and have shown effects on practically defined off-medication motor scores in PD, which were sustained beyond the period of exposure (Athauda et al., 2017). Lixisenatide and Liraglutide Phase II trials are still undergoing recruitment (ClinicalTrials.gov NCT03439943 and NCT02953665).

There are also a number of studies targeting the generation of α-synuclein, the major component on Lewy bodies as previously described in section 1.1.2.2 (for review (Savitt and Jankovic, 2019)). One of the most promising of these at present is antisense oligonucleotides (ASO) or short hairpin RNA which bind to their target mRNA sequence preventing it's translation to α-synuclein (Takahashi et al., 2015, Zharikov et al., 2015). These constructs have been further modified by the addition of amido-bridged nucleic acid which stabilises the construct and results in a more efficient knockdown (Yahara et al., 2012, Yamamoto et al., 2015). Most recently, these ASOs have been shown to be effective in *SNCA* transgenic mice by both reducing *SNCA* mRNA and α-synuclein protein levels and ameliorating the defects in motor behaviour observed (Uehara et al., 2019).

### 1.2.4 Lifestyle factors

As with all diseases there are a number of lifestyle factors that have been reported to impact upon disease symptomology. Participation in exercise has been strongly linked to improved quality of life in PD patients. Traditional forms of exercise such as endurance training (walking and cycling) (Flach et al., 2017, Mehrholz et al., 2015) and aquatic

exercise (Carroll et al., 2017) have been shown to improve motor disability as measured by UPDRS scores. Interestingly, these types of exercise have also been linked to improvements in non-motor symptoms such as sleeping, autonomic dysfunction, mood and cognition (Amara and Memon, 2018, David et al., 2015, Nascimento et al., 2014, Reynolds et al., 2016, Schmitz-Hubsch et al., 2006). Other promising avenues include partaking in dance classes such as tango and Irish set dancing, which also result in improved UPDRS scores (Dos Santos Delabary et al., 2018, Shanahan et al., 2017, Volpe et al., 2013). Group singing has also been shown to improve quality of life and to improve voice and respiratory impairment in PD patients (Abell et al., 2017, Stegemoller et al., 2017).

A number of studies have implicated good nutrition and a balanced diet in PD development and disease prognosis. Malnourished people with PD were found to have a poorer quality of life compared to well-nourished patients, and improved nutritional status resulted in an increase in their quality of life (Sheard et al., 2014). Overeating, obesity and a sedentary lifestyle and type 2 diabetes have been identified as potential risk factors for PD (Mattson, 2012, Procaccini et al., 2016). As with many diseases, certain dietary patterns, for example, the Mediterranean diet which is rich in fish oils and vegetables, have been shown to decrease the risk of PD (for review (Seidl et al., 2014)). In addition, with the current treatment of L-dopa, patients have to monitor and control dietary components, particularly to minimise protein intake to maximise L-dopa absorption in the gut (Virmani et al., 2016, Wang et al., 2017). Therefore, consideration of exercise and diet should be included as part of the treatment for PD patients.

## 1.3 Aetiology of Parkinson's disease

### 1.3.1 Known Risk factors

The main risk factor for PD is age (reviewed in (Reeve et al., 2014)). Reeve and co-workers suggested that known defects in a number of cell processes can occur with age and that these push the neurons of the SNpc towards vulnerability to toxic stimuli. These include mitochondrial dysfunction (for review (Bose and Beal, 2016, Park et al., 2018)), oxidative stress (reviewed by (Guo et al., 2018, Wei et al., 2018)), and α-synuclein

accumulation (reviewed by (Lehtonen et al., 2019)), key interlinked mechanisms that have been proposed to underlie PD neurodegeneration. There is also a higher risk of developing PD in the male sex. Haaxma et al. recorded the age of onset as 2.1 years later in women (53.4 years) than in men (51.3 years). More women presented with a tremor (67%) than men (48%). This is particularly important as the age of onset for patients presenting with a tremor was 3.6 years later and was associated with milder motor deterioration (38% by UPDRS-II) (Haaxma et al., 2007). There are also sex differences in the prevalence of non-motor symptoms in PD, that is, anxiety, depression, and constipation have been reported to be more prevalent in women, whereas men report more daytime sleepiness, drooling, and sexual symptoms (Martinez-Martin et al., 2012).

Both preclinical and clinical studies indicate that oestrogen plays a neuroprotective role against PD (Gillies et al., 2014, Rocca et al., 2008). Rocca and colleagues showed that women with who underwent oophorectomy before menopause were at increased risk of developing PD, and that it occurred twice as frequently in women with bilateral than in those that had unilateral oophorectomy (Rocca et al., 2008). A follow-up study is underway in the Mayo Clinic, with preliminary cohort data available now (Rocca et al., 2017) and neurodegenerative specific results pending. In addition, the POETRY study showed that oestrogen replacement therapy in post-menopausal women with advanced PD was associated with improvements in motor symptoms (Parkinson Study Group POETRY Investigators (2011). Exposure to certain viral infections such as influenza, measles and mumps have been shown to increase the risk of PD (Harris et al., 2012, Vlajinac et al., 2013). A higher incidence of PD is observed among patients with inflammatory bowel disease (IBD) than among individuals without IBD, and early exposure to anti-inflammatory anti-TNF therapy was associated with reduced PD incidence (Peter et al., 2018). This supports a role of systemic inflammation in the pathogenesis of PD. Certain lifestyles such as farm working have also been reported to increase risk for PD (Gunnarsson and Bodin, 2017, Van Maele-Fabry et al., 2012).

### 1.3.2 Genetic Risk Factors

In the majority of cases there is no explanation for why a person develops PD; this is known as idiopathic or sporadic PD. However, 15% of PD patients have a family history and in ~5-10% of patients, PD presents as a monogenic form of the disease with Mendelian inheritance which is either autosomal dominant or recessive. So far, 23 loci have been identified and linked to PD (Table 1) (Deng et al., 2018, Kalinderi et al., 2016, Tambasco et al., 2016). These disease-causing genes lead to familial PD; they include the autosomal dominant (AD) inherited genes, *SNCA* and *LRRK2*, and autosomal recessive (AR) inherited genes, *PRKN, DJ-1*, and *PINK1* ((Matsumine et al., 1997, Paisan-Ruiz et al., 2004, Polymeropoulos et al., 1997, Valente et al., 2004, van Duijn et al., 2001, Zimprich et al., 2004), reviewed by (Klein and Westenberger, 2012, Mullin and Schapira, 2015)). These genes and their functions are highlighted in the next sections, however as *PINK1* and *SNCA* are the major focus of this thesis their protein functions will be expanded upon most. It is worth noting that while genome-wide association study (GWAS) studies identify variants located close to *SNCA* and *LRRK2* as risk factors for idiopathic PD (Billingsley et al., 2018, Chang et al., 2017, Nalls et al., 2014, Simon-Sanchez et al., 2009), the greatest genetic risk factors identified for idiopathic PD are mutations in the glucocerebrosidase gene *(GBA)* (reviewed by (Balestrino and Schapira, 2018, O'Regan et al., 2017)).

The study of these risk genes and their mechanisms remains important today, as Polymeropoulos and co-workers remarked in their 1997 paper "even if the mutation…is directly related to only a small fraction of the total number of PD patients, it provides a clue that should lead to the understanding of the underlying pathways resulting in the symptoms of PD" (Polymeropoulos et al., 1997).

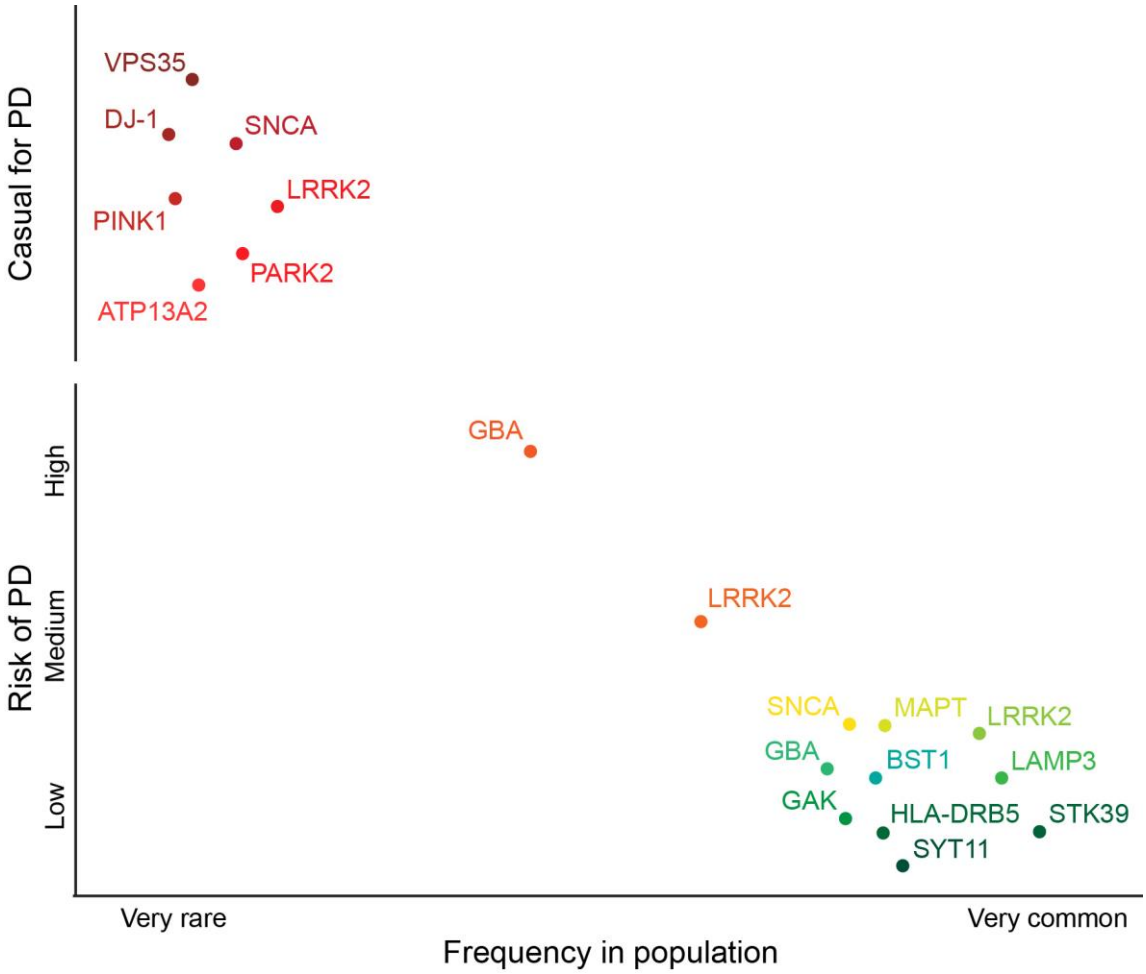



**Figure 1.3 Genetic architecture of PD**

Casual genes are shown in red, strong risk factors are shown in orange and weak risk factors are shown in green/yellow. Some genes, such as *LRRK2* and *SNCA*, are found in several or all of the categories (modified from Van Der Brug et al., 2015).

## Overview of major PD risk genes and their protein products

### 1.3.2.1 SNCA

The A53T mutation in the *SNCA* gene was the first PD causing gene mutation identified (Polymeropoulos et al., 1997), the same year as α-synuclein was discovered to be the major component of LBs (Spillantini et al., 1997). This mutation has been reported in a number of Italian and Greek families are well as a small number of individuals in Korea, Sweden, Poland, Spain, United Kingdom, France, Japan and China (Deng and Yuan, 2014, Polymeropoulos et al., 1996, Polymeropoulos et al., 1997, Xiong et al., 2016). Subsequently, five other point mutations were identified; A30P (Kruger et al., 2001, Kruger et al., 1998), E46K (Zarranz et al., 2004), H50Q (Appel-Cresswell et al., 2013, Proukakis et al., 2013), G51D (Kiely et al., 2013, Kiely et al., 2015, Lesage et al., 2013, Tokutake et al., 2014) and A53E (Pasanen et al., 2014). *SNCA* triplication was also identified in unrelated families with PD from America, France, Japan, Italy, and South-Africa (for review (Deng and Yuan, 2014)), and 47 *SNCA* duplication heterozygous cases from 32 families in Asia, Europe and America have been reported (Konno et al., 2016). Interestingly, a clear *SNCA* dosage-related PD disease phenotype has been observed. Triplication of *SNCA* or indeed four copies of *SNCA* (single allele triplication with a normal allele or duplication of both alleles) leads to an earlier age at disease onset, increased disease progression, and a limited lifespan (Ferese et al., 2015, Lesage and Brice, 2009, Singleton et al., 2003). In contrast, patients with *SNCA* duplication have milder clinical symptoms (Chartier-Harlin et al., 2004, Farrer et al., 2004, Konno et al., 2016, Lesage and Brice, 2009). Autosomal dominant mutations in *SNCA* account for an estimated 1-3% of all cases of familial PD (Deng et al., 2018). The role of α-synuclein, the protein encoded by the *SNCA* gene, is highlighted below.

#### 1.3.2.1.1 α-synuclein

##### 1.3.2.1.1.1 Structure and function of α-synuclein

As previously mentioned, the *SNCA* gene was the first gene linked to PD (Polymeropoulos et al., 1997), and α-synuclein is the protein product of this gene. α-synuclein is a 140-amino acid protein, with three domains- an N-terminal domain, a

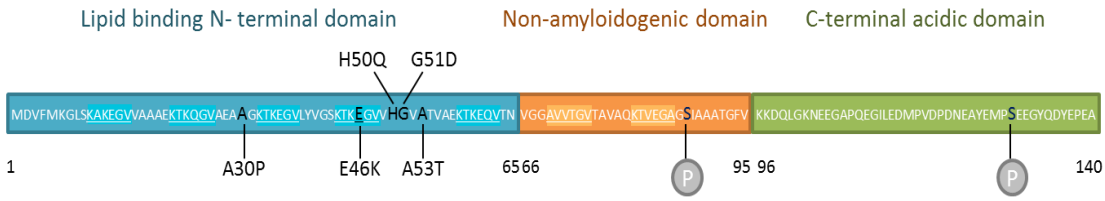



**Figure 1.4: α-synuclein structure**

α-synuclein contains an N-terminal domain, a non-amyloid-component of plaques (NAC) domain, and a C-terminal domain. PD-related mutations are marked at their position in the amino acid sequence (and described above), as are the phosphorylation sites at S87 and S129. KTKEGV imperfect repeats are highlighted which span through the amphipathic N terminal domain and the NAC domain. Amino acid residues at which each component starts and ends are indicated underneath.

non-amyloid-component of plaques (NAC) domain, and a C-terminal domain (Fig. 1.3). The N-terminal region contains all known clinical mutations (Dehay et al., 2015). Seven KTKEGV imperfect repeats spanning the N-terminal and NAC domains are responsible for the formation of two α-helices that interact with lipids (Bussell and Eliezer, 2003, Bussell et al., 2005, Cole et al., 2002, Eliezer et al., 2001, Nuscher et al., 2004, Perrin et al., 2000). The NAC domain has been shown to be responsible for the aggregation of α-synuclein by inhibiting its degradation and promoting its fibrillisation (Giasson et al., 2001). The native structure of α-synuclein is still being debated. Multiple studies have indicated that α-synuclein exists in a monomeric conformation (Binolfi et al., 2012, Chandra et al., 2003, Fauvet et al., 2012, Mor et al., 2016), however some studies have shown that native endogenous α-synuclein isolated from human red blood cells is present in an α-helically-folded tetramer of 58kDa (Bartels et al., 2011, Wang et al., 2011b). Further studies using mouse brain found α-synuclein present as an unstructured monomer that could form a coil in solution, and as an α-helical structure upon membrane binding (Burre et al., 2010, Burre et al., 2013). These differences could potentially be explained by different protocols e.g. denaturing reagents; further research is needed to determine the normal physiological structure of α-synuclein.

α-synuclein is expressed predominantly in the nervous system, where it is largely concentrated in presynaptic terminals, located in synaptic vesicles (Burre et al., 2018, Jakes et al., 1994). It is therefore unsurprising that the main documented functions of α-synuclein are associated with the nervous system, particularly at the synapse. α-synuclein has been shown to play a role in the compartmentalisation, storage and recycling of neurotransmitters (Allen Reish and Standaert, 2015). α-synuclein inhibits DA synthesis by reducing the phosphorylation state of tyrosine hydroxylase (TH), the rate-limiting enzyme of catecholamine biosynthesis, and stabilizing dephosphorylated inactive TH, thereby inhibiting TH expression and activity (Masliah et al., 2000, Peng et al., 2005, Perez et al., 2002, Wu et al., 2011, Yu et al., 2004). In support of this, a negative correlation has been reported between aging-related increases in α-synuclein expression in the SN and the expression of TH, in both monkeys and humans (Chu and Kordower, 2007). α-synuclein also increased the number of DA transporter molecules,

thus decreasing the amount of DA in the synaptic cleft (Al-Wandi et al., 2010, Lee et al., 2002). α-synuclein acts as a molecular chaperone through its interaction with the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) complex protein synaptobrevin-2 (Burre et al., 2015, Burre et al., 2010). It can rescue the neurodegenerative phenotype (nerve-terminal degeneration, motor impairment and cell death) induced by the knockout of the cysteine string protein α, another chaperone essential for synaptic health, by restoring SNARE complex levels in synaptic terminals and mice lacking both molecular chaperones have nerve terminal dysfunction and cell death (Chandra et al., 2005).

In line with its functions in synaptic health and interactions with the DA system, a number of α-synuclein models have shown defects in these processes. For example, α/β/γ-synuclein triple knockout mice have a decreased life span and age-dependent synaptic dysfunction compared to wild-type mice (Burre et al., 2010). α-synuclein knockout mice also showed cognitive impairments in tests for working and spatial memory (Kokhan et al., 2012). α-synuclein-overexpressing rats (induced by injection of adeno associated virus (AAV)-α-synuclein or pre-formed fibrils of α-synuclein) have shown α-synuclein aggregation, DA degeneration and motor impairments, although the timelines and degree of symptom severity have varied (for review see (Ko and Bezard, 2017, Koprich et al., 2017, Visanji et al., 2016, Volpicelli-Daley et al., 2016)).

#### 1.3.2.1.1.2 Aggregation, degradation and spread

Instead of the α-helically folded monomer or tetramer described above, α-synuclein adopts a β-sheet amyloid conformation under pathological conditions. This β-sheet conformation is associated with the aggregation of α-synuclein, formation of fibrils and deposition of α-synuclein into LB (Conway et al., 1998, Ding et al., 2002, Greenbaum et al., 2005, Lashuel et al., 2002, Narhi et al., 1999, Yonetani et al., 2009). This β-sheet conformation is thought to be toxic to neurons and to play a role in neurodegeneration, but the exact nature of the toxic species (oligomer, protofibril, fibril, LB) remains unclear (Fig. 1.4). A number of factors have been shown to increase the fibrillisation of

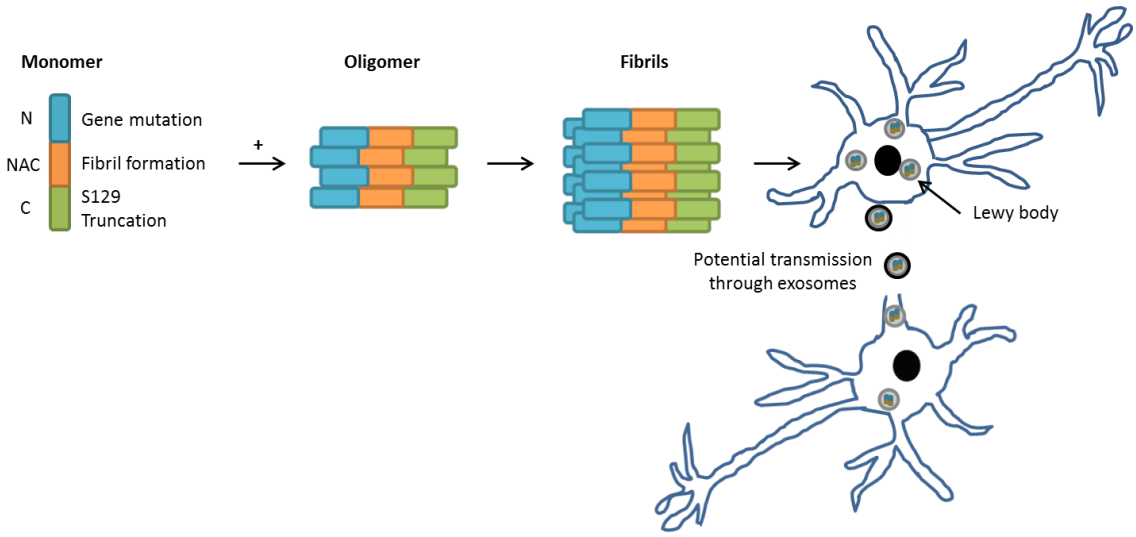



**Figure 1.5: α-synuclein aggregation and propagation pathway**

α-synuclein exists as a 140-amino acid protein with three domains- an N-terminal domain, non-amyloidogenic domain (NAC), and C-terminal domain. The two terminals play a role in aggregation, N-terminal gene mutations, Serine129 phosphorylation and C-terminal truncation increase aggregation. α-synuclein is toxic when aggregated into pathological oligomers, fibrils and LB. It is hypothesised that α-synuclein propagates via exosomes, as illustrated above.

α-synuclein, including PD-related mutations (Conway et al., 1998, Greenbaum et al., 2005, Lashuel et al., 2002, Narhi et al., 1999, Yonetani et al., 2009), phosphorylation at Serine 129 (Anderson et al., 2006, Chen and Feany, 2005, Kim et al., 2006, Samuel et al., 2016) and C-terminal truncation (Hoyer et al., 2004, Li et al., 2005).

It has been proposed that α-synuclein is degraded both by the ubiquitin proteasome pathway and by the autophagy-lysosomal system (Webb et al., 2003). Monoubiquitinated α-synuclein has been reported to be degraded by the proteasome, while polyubiquitinated α-synuclein and more complex formations such as aggregates are broken down by lysosomal degradation (Abeywardana et al., 2013, Ebrahimi-Fakhari et al., 2011, Shabek et al., 2012, Tofaris et al., 2011). In fact, a number of studies have shown that α-synuclein oligomers and protofibrils inhibit the ubiquitin proteasome pathway (Chen et al., 2005, Lindersson et al., 2004, Zhang et al., 2008). It is thought that defects in the degradation of α-synuclein may contribute to its accumulation, leading to increased neurotoxicity in PD (Ebrahimi-Fakhari et al., 2012).

Studies have shown that α-synuclein aggregates have the potential to self-propagate and spread. Pathological analysis of DA neurons in the SNpc of PD patients who had received fetal DA grafts showed the presence of LB and Lewy neurites after several years (Chu and Kordower, 2010, Li et al., 2008). This observation in combination with the studies of Braak showing the presence of α-synuclein through different brain areas as disease progresses led to the "prion like hypothesis" that α-synuclein could propagate from one cell to another (Braak et al., 2003a). Braak and colleagues initially proposed the following: stage 1 - 2, LB pathology is found in the dorsal motor nucleus of the vagus (DMN) and the olfactory system. Stages 3 - 4 sees LB pathology spreading to SNpc, basal forebrain magnocellular nucleus, subthalamic nucleus, and amygdala, and in the end stages 5 and 6 the pathological process approaches the cortex (Braak et al., 2003a). Braak and colleagues later hypothesised a role for the gut as the site or origin of α-synuclein pathology in PD (Braak et al., 2006, Braak et al., 2003b, Hawkes et al., 2007). They theorised that α-synuclein pathology emanates in the enteric nervous system and spreads along the vagus nerve to the DMN of the lower brainstem, followed by progression to the SN, hippocampus and cerebral cortex, as mentioned above (Braak

et al., 2006, Braak et al., 2004, Hawkes et al., 2007). In agreement with this pattern of disease progression hyposmia, constipation and sleep disturbances have been reported to precede motor symptoms, and cognitive symptoms such as depression and dementia tend to occur many years after the onset of motor symptoms (Felice et al., 2016, Pfeiffer, 2018).

Several subsequent *in vitro* and *in vivo* studies showed that aggregated α-synuclein could spread from cell to cell and region to region, resulting in progressive PD pathogenesis (Desplats et al., 2009, Hansen et al., 2011, Luk et al., 2012a, Luk et al., 2012b, Ulusoy et al., 2015). The exact mechanism of α-synuclein transfer remains unclear, however, a number of studies have hypothesised that extracellular vesicles may mediate spreading of harmful α-synuclein species. Emmanouilidou and colleagues suggested that α-synuclein is released by exosomes in a calcium-dependent manner (Emmanouilidou et al., 2010). Furthermore, Grey et al. showed that the addition of EV fractions from N2a neuroblastoma cells to recombinant α-synuclein monomers accelerated the formation of toxic α-synuclein oligomers equivalent to a low concentration of preformed α-synuclein fibrils (Grey et al., 2015). Moreover, α-synuclein with altered physiological functions or aggregation properties e.g. A53T mutation, can increase α-synuclein association with extracellular vesicles and increase its internalisation into recipient cells (Gustafsson et al., 2018). Another potential mechanism of neuron to neuron transfer is the spread of α-synuclein through tunnelling nanotubes (Ariazi et al., 2017, Gousset et al., 2009).

#### 1.3.2.2 LRRK2

Mutations in *LRRK2* with autosomal dominant inheritance were first reported by two independent studies in 2004 (Paisan-Ruiz et al., 2004, Zimprich et al., 2004). Greater than 80 variants have since been identified, accounting for 10% of patients with familial PD and a significant amount of those with idiopathic PD (Berg et al., 2005, Martin et al., 2014). Thus far, at least seven *LRRK2* mutations have been proven by linkage studies to be pathogenic (Corti et al., 2011, Rubio et al., 2012). On average, the G2019S mutation occurs in 1-3% of sporadic PD cases and 4-8% of familial cases, making it the most

common mutation of *LRRK2* (Kang and Marto, 2017). The discovery of the G2019S mutation was crucial for the study of PD, as it was the first mutation found to occur with high frequency and to be implicated in both familial and sporadic PD, indicating that there may be common pathogenic mechanisms linking these two forms of PD. Interestingly, the prevalence of the *LRRK2* mutations varies across populations (for review (Correia Guedes et al., 2010). Thus, the G2019S mutation responsible for 42% of familial and 35.7% of sporadic PD in Arab samples from North Africa (Lesage et al., 2006, Lesage et al., 2005) and 30% of familial PD in Ashkenazi Jewish populations (Ozelius et al., 2006). In contrast, only 6% of familial PD cases in Europe (Di Fonzo et al., 2005, Kachergus et al., 2005, Nichols et al., 2005) and 3% of sporadic PD in Europe and North America (Gilks et al., 2005) are linked to the G2019S mutation, and it is extremely rare in Asian populations (Kumari and Tan, 2009). The other six *LRRK2* mutations (R1441C, R1441G, R1441H, Y1699C, pG2019S, I2020T, N1437H) are far rarer worldwide, although there is a high frequency in PD cases with the R1441G mutation in the Basque region, accounting for 15% of PD patients from this region (Mata et al., 2005).

*LRRK2*-related PD can be clinically indistinguishable from sporadic PD. However, the severity and rate of progression of motor symptoms, occurrence of falls and dyskinesias, as well as non-motor symptoms including cognition and olfaction, are more benign in *LRRK2*-associated PD than in idiopathic PD (Healy et al., 2008). In *LRRK2* –related PD, the neuropathology is varied, and features LB pathology, tauopathy with neurofibrillary tangles and a number of pathological features of other neurodegenerative diseases including Dementia with Lewy Bodies and multiple system atrophy (Healy et al., 2008, Zimprich et al., 2004).

*LRRK2* is a complex kinase and PD-causing mutations are considered gain-of-function mutations, resulting in increases in kinase and GTPase activity (Henry et al., 2015), for review see (Martin et al., 2014). Investigation of LRKK2 function as of other PD risk genes is providing important information on mechanisms that may cause PD. *LRRK2* is thought to play a major role in a number of cellular signalling pathways and processes, including vesicular trafficking, cytoskeletal function, protein synthesis, the lysosomal

system, mitochondrial function and the immune system (for review (Civiero et al., 2018, Harvey and Outeiro, 2019, Wallings et al., 2015)). Therefore, it is not surprising that LRRK2 is expressed at low levels in neurons and has highest expression in immune cells (Gardet et al., 2010, Thevenet et al., 2011). Mutations in LRKK2 are also a risk factor for Crohn's disease LRKK2 also regulates the PI3-kinase/Akt pathway a major metabolic, survival and immune signalling pathway as discussed in later sections (see review (Wang et al., 2012)).

#### 1.3.2.3 PRKN

The gene responsible for autosomal recessive juvenile parkinsonism (ARJP) was located at chromosome 6q25.2-27 in 1997 (Matsumine et al., 1997), the same year as the *SNCA* gene was discovered. One year later, exon deletions in the *PRKN* gene were identified as the genetic cause of ARJP in several families in Japan. These deletions included four different homozygous intragenic deletional mutations, involving exons 3, 4 and 5 in 10 families (17 affected individuals), and a one-base deletion involving exon 5 in two families (2 affected individuals) (Hattori et al., 1998, Kitada et al., 1998). 268 variants of *PRKN* have been reported to date (for review (Deng et al., 2018)). These mutations include missense, nonsense, indels, exonic deletions, duplications, and triplications. *PRKN* is the most common cause of autosomal recessive early onset PD (EOPD), accounting for 50% of EOPD patients under 25 years, 3-7% of EOPD with age of onset 30-45 years and 19% of sporadic EOPD cases (Deng et al., 2006, Lucking et al., 2000, Periquet et al., 2003).

As mentioned earlier, mutations in *PRKN* cause ARJP which is a severe form of PD, often with an age of onset before 20 years of age. Apart from their age, patients with ARJP present with a typical PD syndrome indistinguishable from idiopathic PD with a slow progression. Pathologically there is a loss of both nigral and locus coeruleus neurons, but surprisingly in the majority of cases no LBs are present (although exceptions have been reported) (Farrer et al., 2001, Kitada et al., 1998, Matsumine et al., 1997).

PD causing mutations in *PRKN* cause loss of function of the encoded protein. *PRKN* encodes the parkin protein an E3 ubiquitin ligase with a crucial function in ubiquitination and subsequent protein degradation (for review (Seirafi et al., 2015)). Ubiquitination is a post-translational modification that marks a protein for degradation through the addition of ubiquitin (Hershko and Ciechanover, 1998, Seirafi et al., 2015). The role of the E3 ubiquitin ligases is to provide regulation of ubiquitination activity and specificity in the recognition of substrates (Deng et al., 2018, Dye and Schulman, 2007). Mutations in *PRKN* have been shown to cause E3 ligase dysfunction, which leads to impaired protein degradation and increased accumulation of toxic proteins (Kalinderi et al., 2016, Shimura et al., 2000). Parkin also interacts with PINK1 to regulate mitophagy, which is the most studied function of parkin. Parkin$^{-/-}$ *Drosophila* first drew attention to parkin's role in mitochondrial quality control as these flies demonstrated a severe mitochondrial phenotype (swollen, malformed, with disintegration of cisternae) (Greene et al., 2003). A similar phenotype was observed with *PINK1* deletion, which could be rescued by overexpression of parkin, indicating parkin acts downstream of PINK1 regulating mitochondrial quality control (Clark et al., 2006, Park et al., 2006). This will be further expanded upon in section 1.4.2.1. Studies of homozygous parkin knockout mice (*Prkn*$^{-/-}$) have found decreased abundance of numerous proteins that are involved in mitochondrial function and oxidative stress (Periquet et al., 2005). These have been supported by functional assays, which showed reductions in respiratory capacity of striatal mitochondria isolated from *Prkn*$^{-/-}$ mice (Palacino et al., 2004). *Prkn*$^{-/-}$ mice also exhibit nigrostriatal deficits including altered levels of extracellular DA in the striatum and decreased synaptic excitability of striatal neurons but no loss of DA neurons (Goldberg et al., 2003, Itier et al., 2003, Oyama et al., 2010, Palacino et al., 2004).

#### 1.3.2.4 DJ-1

The *PARK7* locus which encodes the DJ-1 protein was first reported in 2001 to be located at chromosome 1p36. The affected family were living in an isolated genetic population from the Netherlands, with multiple consanguinity loops identified and linked to EOPD (van Duijn et al., 2001). In 2003, two independent studies of unrelated

EOPD patients reported three homozygous mutations, a 14-kb deletion, p.L166P and p.M26I and a heterozygous mutation p.D149A (Abou-Sleiman et al., 2006, Bonifati et al., 2003). To date, at least 27 variants of PARK7 have been identified (for review (Deng et al., 2018)).

The DJ-1 protein is ubiquitously expressed in numerous cell types and predominantly localises to the cytosol, but it is also found in the nucleus and is associated with mitochondria under certain conditions (Wilhelmus et al., 2012). In studies of human brains, the majority of DJ-1 was found in astrocytes with a smaller amount in neurons (Bandopadhyay et al., 2004, van Horssen et al., 2010). Similarly, in the PD brain, DJ-1 is mainly present in astrocytes (both in reactive astrocytes and resting astrocytic processes) and to a lesser extent in neurons (Bandopadhyay et al., 2004). DJ-1 was found to be absent from α-synuclein-positive LBs and was very significantly reduced in the SN of patients with sporadic PD (Nural et al., 2009, Taipa et al., 2016), for review (Cookson and Bandmann, 2010).

Multiple studies have shown that DJ-1 can protect DA neurons against various insults, including rotenone (Liu et al., 2008), mutant α-synuclein (Liu et al., 2008, Zhou and Freed, 2005), hydrogen peroxide (Zhou and Freed, 2005), and 6-hydroxy-dopamine (6-OHDA) (Lev et al., 2009, Zhou and Freed, 2005). However, the exact function of the DJ-1 protein remains to be determined. Studies have indicated that it may act as an antioxidant and/or a molecular chaperone (Bonifati et al., 2003), although the mechanism remains elusive and reports have been conflicting, indicating that more research is needed to determine how DJ-1 could protect against PD (for review see (Wilhelmus et al., 2012)).

#### 1.3.2.5 PINK1

The locus known as *PARK6*, which is now known to be the *PINK1* gene, was originally reported in one Sicilian and one Italian family with autosomal recessive, early onset (32-48 years) parkinsonism (Valente et al., 2001). Valente and colleagues later identified homozygous mutations in *PINK1* at G309D, a missense mutation in a Spanish family, and at W437X, a truncating nonsense mutation in two Italian families (Valente et al.,

2004). To date, 111 point mutations, frameshift and truncating mutations of PINK1 have been found in families with familial PD (Bonifati et al., 2005, Ibanez et al., 2006, Valente et al., 2004), for review, (Arena and Valente, 2017). In addition, heterozygous *PINK1* mutations are the cause of 1-3% of all sporadic cases of PD with an early onset (Abou-Sleiman et al., 2006, Kumazawa et al., 2008). Similar to *LRRK2*, the penetrance of *PINK1* mutations was found to differ depending on the population, comprising 2-4% of cases of early onset PD in Caucasian populations and 4-9% in Asian populations (Schulte and Gasser, 2011).

The clinical presentation of *PINK1*-related PD includes early onset, prolonged response to levodopa (L-dopa), L-dopa-induced dyskinesia, and slow progression (Bentivoglio et al., 2001, Valente et al., 2001). A number of atypical symptoms have also been reported including dystonia, pyramidal signs and psychiatric disorders such as depression and anxiety (Arena and Valente, 2017, Bonifati et al., 2005). Neuropathological analysis indicates a typical LB pathology (Samaranch et al., 2010).

PINK1 (PTEN-induced putative kinase) encodes a serine threonine kinase and mutations are considered loss of function mutations, with the majority of mutations located in PINK1's kinase domain (Fig. 1.5) (Ibanez et al., 2006). The potential impact of this loss of function on the numerous cellular processes PINK1 regulates and its implications for PD will be discussed in the following section 1.4 "PINK1 and Parkinson's disease" and section 1.7 "Relationship between PINK1 and the PI3-kinase/Akt pathway".

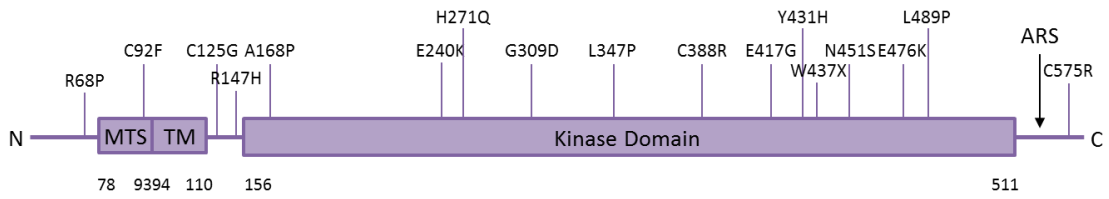



**Figure 1.6 Mutations in PINK1 that lead to PD.**

The PINK1 protein is predicted to consist of an N-terminal, non-canonical mitochondrial targeting sequence (MTS), a transmembrane domain (TM), a highly conserved serine/threonine kinase domain and a C-terminal autoregulatory sequence (ARS), discussed in 1.2. The majority of mutations reside in the kinase domain. Amino acid residues at which each component starts and ends are indicated underneath.

## 1.4 PINK1 and Parkinson's disease

Both *SNCA* and *LRKK2* gene mutations are inherited in autosomal dominant fashion. This can make understanding the mechanisms by which they contribute to PD less straightforward than understanding PD risk genes that are inherited in an autosomal recessive manner. For example, as mentioned above mutations in *LRRK2* are known to result in a gain-of-function for the LRRK2 protein, resulting in increases in kinase and GTPase activity (Henry et al., 2015), and potentially additional unknown functions. In contrast, when considering PINK1, PD-causing mutations result in loss of function of the encoded protein, making it easier to determine what pathways/processes could be defective in PD. This enables discovery of tissue/ cell and molecular pathways that lead to PD in systems in which PINK1 is modified, by deletion, overexpression and modulation of its major functional domains. The next sections overview PINK1 function and mechanisms by which loss of this function is believed to cause PD.

### 1.4.1 PINK1 Localisation and Structure

PINK1 was originally discovered in cancer cells as a gene induced by exogenous expression of PTEN and was thus named PTEN-induced putative kinase 1 (Unoki and Nakamura, 2001). This linked PINK1 to the major PI3-kinase/Akt cell survival and stress protective pathways from the first instance and demonstrated PINK1 was a serine threonine kinase. Following this, autosomal recessive mutations in PINK1 were discovered in a number of individuals with familial PD, drawing much attention to how loss of function of PINK1 could cause PD (Valente et al., 2004). The PINK1 gene contains eight exons spanning ~1.8 kilobases which encode a 581-amino acid protein (Valente et al., 2004). PINK1 is expressed ubiquitously and localises to the mitochondria and cytosol. The PINK1 protein is predicted to consist of an N-terminal, non-canonical mitochondrial targeting sequence (MTS), which is essential for mitochondrial localisation, and a C-terminal autoregulatory sequence (Fig. 1.5) (Cardona et al., 2011). The transmembrane domain and highly-conserved serine/threonine kinase domain are responsible for membrane tethering and cytosolic facing topology. Proteolysis downstream of the transmembrane domain and interaction

between the kinase domain and Hsp90 are reported to be required for retrograde transport of the PINK1 protein from the mitochondria to the cytosol, as removal of either of these domains results in an absence of cytosolic PINK1 and an increase in mitochondrial PINK1 (Lin and Kang, 2010, Weihofen et al., 2008).

The highest levels of PINK1 protein have been reported in the central nervous system, particularly in the SN, hippocampus and cerebellum, expressed mainly in neurons, with a lower level of expression in glial cells (Blackinton et al., 2007). Through immunohistochemical and fractionation studies on human and rat brains, PINK1 was shown to be localised to mitochondrial membranes (Gandhi et al., 2006) as well as non-mitochondrial cytosolic fractions. Outside of the nervous system, PINK1 was found to be most abundant in the heart, testis, and skeletal muscle (Unoki and Nakamura, 2001). Immunohistochemical analysis of PD brains revealed that 5-10% of all brainstem, α-synuclein-containing intraneuronal inclusions with morphological features of LBs contain PINK1 immunoreactivity (Gandhi et al., 2006).

### 1.4.2 Functions of PINK1

#### 1.4.2.1 PINK1 and mitochondrial function

Multiple studies have shown that PINK1 is a key regulator of mitochondrial health, including mitophagy, fission, fusion, bioenergetics and mitochondrial antigen presentation (Summarised Fig. 1.6) (Harper et al., 2018, Matheoud et al., 2019, Matheoud et al., 2016, O'Flanagan et al., 2016, Pilsl and Winklhofer, 2012, Youle and Narendra, 2011, Youle and van der Bliek, 2012). Regulation of the mitochondria by PINK1 has been shown to be crucial for maintaining the balance between cell survival and cell death (reviewed by (Bueler, 2010, Kitagishi et al., 2017, Leites and Morais, 2018)).

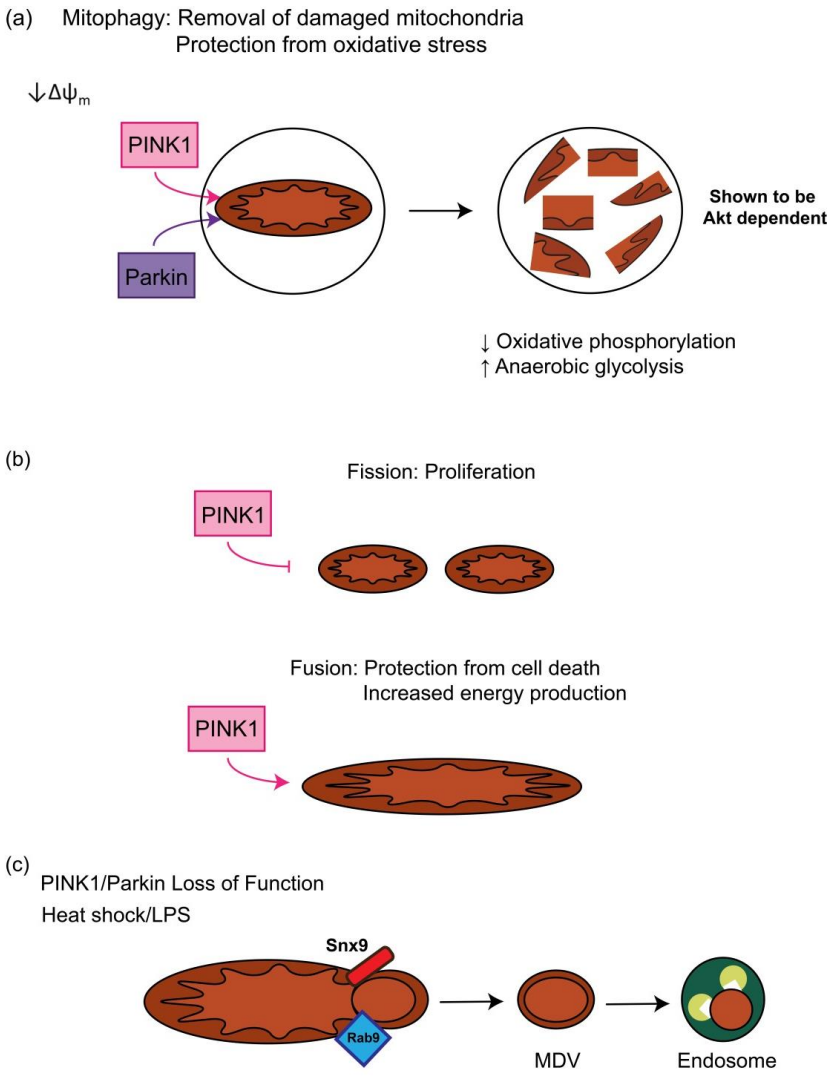



**Figure 1.7 PINK1 is a key regulator of mitochondrial health**

a. PINK1 is a major mediator of mitophagy in combination with parkin, clearing damaged mitochondria from the cell to maintain mitochondrial homeostasis b. PINK1 promotes mitochondrial fusion and prevents mitochondrial fission as shown in mouse models and human cell lines, protecting cells from apoptosis and stress .c. Inflammatory conditions trigger mitochondrial antigen presentation in immune cells, this leads to the formation of mitochondrial derived vesicles (MDVs) which require Rab9 and Sorting nexin 9 (Snx9). MDVs then fuse with the endosomal system promoting degradation of damaged mitochondria.

The most prominently described role for PINK1 is its regulation of mitophagy, the major quality control mechanism regulating mitochondrial numbers and clearance from the cell (Pallanck, 2010). PINK1 regulation of mitophagy has mostly been studied using *in vitro* systems where PINK1 and parkin are overexpressed and it is not clear if this translates to contexts in vivo (Goldberg et al., 2003, Kitada et al., 2007, Matheoud et al., 2019, Perez and Palmiter, 2005). Within this model system, PINK1 has been described to act as a molecular sensor which recruits the E3 ubiquitin ligase parkin to initiate mitophagy (Matsuda et al., 2010, Narendra et al., 2010). Under normal circumstances, PINK1 is reported to undergo two cleavage events, one by mitochondrial processing peptidase (Greene et al., 2012) and the second by presenilin associated rhomboid-like protease (Deas et al., 2011b) to change from a 63 kDa to a 52 kDa fragment which can be released from the mitochondria and degraded by the proteasome (Takatori et al., 2008). Upon mitochondrial membrane depolarisation, PINK1 has been shown to become stabilised on the outer mitochondrial membrane and to undergo autophosphorylation at Ser-228 and Ser-402 (Okatsu et al., 2012). This results in the recruitment of parkin to the mitochondria and it's phosphorylation by PINK1 (Sha et al., 2010). More recently, studies have shown that PINK1 directly phosphorylates ubiquitin at Serine 65 (Kane et al., 2014, Kazlauskaite et al., 2014, Koyano et al., 2014). It is now hypothesised that PINK1 phosphorylation of ubiquitin is a parkin activator, required for its translocation and that both phosphorylations are necessary for full activation of parkin E3 activity (Kane et al., 2014, Kazlauskaite et al., 2014, Koyano et al., 2014). This results in the ubiquitination of a number of outer mitochondrial membrane proteins that will in turn signal the organelle to be degraded by mitophagy (Chan et al., 2011, Okatsu et al., 2015, Ordureau et al., 2015, Ordureau et al., 2014, Shiba-Fukushima et al., 2014). PINK1-induced pSer65-Ub in mitochondria is also required for the recruitment of mitophagy adaptors to initiate mitophagy (Heo et al., 2015, Lazarou et al., 2015). Without PINK1 or parkin, mitophagy is impaired, which results in a build-up of damaged mitochondria that can cause the release of cytochrome c, reactive oxygen species (ROS) and $Ca^{2+}$, all of which are known to induce neuronal cell death (Chu, 2010). This could underlie part of the pathogenic mechanisms associated with inherited forms of PD which have mutations in either the parkin or PINK1 genes.

A novel, parkin-independent role has recently emerged for PINK1 in regulating autophagy. PINK1 was found to promote both basal and starvation-induced autophagy through its direct interaction with the key pro-autophagic protein Beclin1 (Michiorri et al., 2010). Furthermore, it was found that PINK1 selectively accumulates to specific sites of contact between the endoplasmic reticulum (ER) and mitochondria called mitochondria-associated membranes in response to mitochondrial depolarisation. There, PINK1 recruits Beclin1 to promote ER-mitochondria tethering and autophagosome formation (Gelmetti et al., 2017).

Deletion of PINK1 in *Drosophila* caused an extensive mitochondrial pathology that was identical to that induced by the ubiquitin ligase parkin (Clark et al., 2006, Park et al., 2006). Moreover, PINK1 could protect against these parkin-mediated pathologies, placing PINK1 upstream of parkin in the regulation of mitochondrial health (Clark et al., 2006, Park et al., 2006, Yang et al., 2006). Surprisingly, deletion of PINK1 in mouse models does not generally cause PD-like pathology or symptoms (Gautier et al., 2008, Gispert et al., 2009, Perez and Palmiter, 2005). This indicates that some primary stress may be necessary to induce PINK1 survival and neuroprotection in PD. This is in line with findings that depletion of PINK1 by RNAi in primary cortical neurons resulted in increased neuronal toxicity induced by the neurotoxin 1-methyl-4-phenylpyridine (MPP), a mitochondria complex 1 inhibitor that causes PD and is used to model PD *in vitro* and *in vivo.* Although wild-type PINK1 was shown to protect neurons against MPP prodrug- 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)- induced toxicity, neither the G309D mutation linked to inherited PD or a kinase-dead mutant K219M could not protect neurons in this way in both *in vitro* and *in vivo* contexts (Haque et al., 2008). Haque et al. further highlighted the role of cytoplasmic PINK1 in cell survival; a mutant that contained a deletion of the MTS was targeted to the cytoplasm but still provided protection against MPP-/MPTP-induced toxicity *in vitro* (Haque et al., 2008). In contrast to the mouse model, PINK1 knockout rats present progressive nigral neurodegeneration and significant motor deficits (Dave et al., 2014).

PINK1 has been implicated in mitochondrial metabolism, particularly in the regulation of complex I activity. Complex I is involved in the electron transport chain which

maintains mitochondrial membrane potential (for review (Lenaz et al., 2006)). In support of this, decreased complex I activity was observed in PINK1-null models, and a higher sensitivity of PINK1-deficient cells to complex I inhibitors (Abramov et al., 2011, Gautier et al., 2008, Haque et al., 2012, Morais et al., 2009). Morais and colleagues indicated one possible mechanism in which PINK1 could regulate complex I is through its direct phosphorylation of NADH dehydrogenase ubiquinone 1 alpha subcomplex 10 (NdufA10), a subunit required for ubiquinone reductase activity by complex I (Morais et al., 2014). PINK1 may also regulate complex I function by controlling the mitochondrial targeting and translation of certain respiratory chain complex mRNAs including the complex I subunits C-I 30 and ND75 (Gehrke et al., 2015).

DA neurons prepared from the SN of PINK1 knockout mice showed mitochondrial impairments including a loss of mitochondrial membrane potential and increased ROS compared to wild-type mice (Wang et al., 2011a). These impairments were rescued by overexpression of wild-type PINK1, but not by overexpression the PINK1 mutants G309D and E417G, in cultured PINK1$^{-/-}$ nigral DA neurons (Wang et al., 2011a). This indicates that PINK1 plays a role in maintaining mitochondrial membrane potential. Increased protection against mitochondrial stress could be mediated by PINK1 phosphorylation of HtrA Serine Peptidase 2 (HtrA2). HtrA2 is thought to activate a mitochondrial stress sensing pathway which would result in the removal of damaged mitochondrial proteins. In agreement, mice lacking HtrA2 displayed increased ROS levels and accumulation of misfolded proteins in brain mitochondria, and HtrA2 mutations lead to neuronal death in humans as well (Moisoi et al., 2009, Plun-Favreau et al., 2007). HtrA2 represents a susceptibility factor for PD, and reduced levels of phospho-HtrA2 were observed in the brains of PINK1-mutated PD patients (Plun-Favreau et al., 2007).

#### 1.4.2.2 PINK1 and cell survival and stress resistance

PINK1 regulates several non-mitochondrial systems central to survival, neuroprotection and stress-resistance (Dagda et al., 2009, Dagda et al., 2014, Dickey and Strack, 2011,

Fedorowicz et al., 2014, Ries et al., 2006, Steer et al., 2015, Xiong et al., 2009), for review (Arena and Valente, 2017, O'Flanagan and O'Neill, 2014). As part of a study aimed at identifying key genes that regulate cell survival and apoptosis, MacKeigan and colleagues conducted an extensive RNAi screen of the human kinase and phosphatase components of the genome and identified PINK1 as a major pro-survival kinase (MacKeigan et al., 2005). Furthermore, knockdown of PINK1 by siRNA sensitized BT474 breast carcinoma cells towards paclitaxel-induced cell death (MacKeigan et al., 2005). Cultured human DA neurons with the PINK1 protein knocked down by RNAi displayed characteristics of marked oxidative stress, widespread mitochondrial dysfunction and abnormal mitochondrial morphology (Wood-Kaczmar et al., 2008). This suggests that the cell may be attempting to remove mitochondria which have been damaged by oxidative stress, or have those which have been altered structurally, and to synthesise new mitochondria via mitochondrial biogenesis (Wood-Kaczmar et al., 2008).

Research from our team has shown that deletion of PINK1 diminishes survival, proliferation, and clonogenicity in mouse embryonic fibroblasts (MEFs) from PINK1 knockout animals and in BE(2)-M17 neuroblastoma cells stably transfected with short hairpin RNA against PINK1 (O'Flanagan et al., 2016, O'Flanagan et al., 2015). Moreover, these results revealed for the first time that PINK1 regulates the cell cycle through its control of mitochondria fission to fusion transitions at the key checkpoints G2/M and G0/G1, which are vital for cell death and survival, growth and stress resistance in cancer (O'Flanagan et al., 2016, O'Flanagan et al., 2015).

Cytochrome c release is part of the programmed cell death process which can be triggered by an increase in ROS. It is released from the mitochondria into the cytosol of the cell where, through its interaction with the apoptotic protease activating factor 1, it triggers the activation of a cascade of caspases which result in cell death (Cai et al., 1998). PINK1 has been shown to protect against oxidative-stress-induced cell death by suppressing cytochrome c release through its phosphorylation of the mitochondrial chaperone TNF receptor-associated protein 1 (TRAP1) in pheochromocytoma cells of the rat adrenal medulla (PC12) (Pridgeon et al., 2007). That study also demonstrated that

this ability to resist cell death through the phosphorylation of TRAP1 was impaired by the PD-linked PINK1 mutations at G309D, L347P and W437X, which indicates that dysregulation of oxidative-stress-induced programmed cell death, may be involved in the pathological processes involved in PD (Pridgeon et al., 2007).

PINK1 was also shown to play an anti-apoptotic role in SH-SY5Y cells (neuroblastoma cells used to model dopaminergic neurons), in a study that reported a PINK1-mediated reduction in cytochrome c release from mitochondria, which prevented subsequent caspase-3 activation (Petit et al., 2005). Overexpression of wild-type PINK1 resulted in marked reductions in both basal and staurosporine-induced caspase 3 activity in addition to reducing the levels of cleaved caspase-9, caspase-3, and caspase-7 (Petit et al., 2005). Similar to the findings in the study by Pridgeon et al., PD-related mutations and a kinase-inactive mutation in PINK1 resulted in a loss of the protective effect of PINK1 (Petit et al., 2005). More recently, studies using the proteosomal inhibitor MG132 showed that skin fibroblasts from patients with homozygous G309D-PINK1 mutations had enhanced caspase activation and vulnerability to apoptosis (Klinkenberg et al., 2010).

Bcl-xL is a member of the Bcl-2 family of anti-apoptotic proteins (for reviews, see (Gabellini et al., 2017, Tsujimoto, 1998). It has been shown to interact with Beclin 1, which recruits a number of autophagic proteins to form a pre-autophagosomal structure, preventing its assembly into this autophagy-promoting structure (Liang et al., 1998). Cleavage of Bcl-xL results in a toxic C-terminal fragment which induces apoptosis (Clem et al., 1998). In HEK293 cells, PINK1 was found to phosphorylate Bcl-xL at Ser-62 upon mitochondrial depolarization (Arena et al., 2013). The PINK1–Bcl-xL interaction did not disturb Beclin-1 release from Bcl-xL. Instead, PINK1 protected the cell from apoptosis by impeding the pro-apoptotic cleavage of Bcl-xL (Arena et al., 2013).

Under trophic deprivation SH-SY5Y cells and cultured cortical neurons with PINK1 knockdown showed reduced levels of key autophagic proteins including LC3-II and LAMP-2 (Parganlija et al., 2014). This downregulation of autophagic factors caused

increased apoptosis, which could be rescued by overexpression of PINK1 (Parganlija et al., 2014) further highlighting PINK1's role in protection from cell death.

#### 1.4.2.3 PINK1 and the immune system

Neuroinflammation in PD was first described when human leukocyte antigen D-related-positive reactive microglia were found in the SN of post-mortem PD brain tissue (McGeer et al., 1988). Furthermore, an increase in pro-inflammatory cytokines (CXCR4, CXCL12, TNFα, interleukin (IL)-1β and IL-6) has been reported in the SN of PD patients (Shimoji et al., 2009) as well as in the CSF (Nagatsu et al., 2000) and periphery (Qin et al., 2016). Activated microglia and alterations in astrocytes and infiltrating T-cells are found also in post-mortem PD brain tissue (Brochard et al., 2009, Duke et al., 2007). DA neurons are particularly vulnerable to neuronal cell death due to impaired redox homeostasis, and the production of these free radicals is exacerbated due to neuroinflammation, in addition to dopamine degradation, mitochondrial dysfunction, aging, glutathione depletion, and high levels of iron or Ca2+ (Dias et al., 2013, Meiser et al., 2013).

A number of studies have implicated PINK1 in immune system function. Gene expression profiling in PINK1-null mouse striatum revealed that the largest category of altered genes was that modulating innate immunity (Akundi et al., 2011). Loss of PINK1 in astrocytes resulted in increased concentrations of inflammatory cytokines including TNFα and IL-1β, increased nitric oxide production and increased inflammation-induced NF-κB signalling, leading to enhanced death in neurons co-cultured with these astrocytes (Sun et al., 2018). $PINK1^{-/-}$ mouse cortical brain slices that were obtained from early postnatal period (P7) using an organotypic slice culture method which mimics brain injury (method (Huuskonen et al., 2005)) showed elevated TNFα, IL-6 and IL-1β levels, in conjunction with increased NFκB signalling compared to wild type mice (Kim et al., 2013a). In support of this, PINK1 was found to interact with key components of Toll-like receptor signalling TRAF6, TAK1 and IRAK1, in the IL-1β-mediated signalling pathway, consequently up-regulating NF-κB signalling in mouse embryonic fibroblasts (Lee and Chung, 2012, Lee et al., 2012). PINK1 deletion

in association with diverse stressors accelerates the impact of impaired mitophagy and may be necessary to induce PD phenotypes *in vivo* that can couple with inflammatory and immune pathways (Sliter et al., 2018). Thus, exhaustive exercise and mitochondrial DNA mutations coupled with PINK1 and parkin deletion induce PD pathophysiology *in vivo* via activation of the cGAS–STING pathway to cause inflammation that can induce cell death (Sliter et al., 2018)

PINK1 and parkin play a key role in adaptive immunity by repressing presentation of mitochondrial antigens (Matheoud et al., 2016). Matheoud and colleagues further showed that intestinal infection with gram-negative bacteria in PINK1$^{-/-}$ mice results in mitochondrial antigen presentation and autoimmune mechanisms that elicit the establishment of cytotoxic mitochondria-specific CD8+ T cells in the periphery and in the brain (Matheoud et al., 2019). These mice show a decrease in density of dopaminergic axonal varicosities in the striatum and are affected by motor impairment (pole test), both of which are reversed by L-Dopa treatment (Matheoud et al., 2019). This indicates that PINK1 is a repressor of the immune system and an initial stressor or immune challenge, particularly in the gut, may act as a triggering event in Parkinson's disease, highlighting the importance of the gut-brain axis in PD.

#### 1.4.2.4 PINK1 and membrane trafficking

Rab GTPases play a major role in endocytic and vesicle trafficking and are critical for neuronal function (Ng and Tang, 2008). Lai and colleagues have shown that PINK1 regulates the phosphorylation of serine 111 (Ser$^{111}$) in a family of Rab GTPases, namely Rab8A, 8B and 13. This is abolished in PINK1 knockout as well as PINK1 mutant patient derived cells, indicating that this site is dependent on PINK1 (Lai et al., 2015). Previously, Rab8A, 8B and 13 have been localised to early endosomes, recycling endosomes, and vesicles (for review (Wandinger-Ness and Zerial, 2014). Therefore, PINK1's interaction with Rabs may be involved in trafficking within the cell for example mitochondrial trafficking. Rab GTPases may represent a molecular nexus between the PINK1 signalling pathway and other PD-linked genes. This is particularly relevant here because the second gene of interest in this thesis α-synuclein has also been

shown to interact with Rabs including Rab1 and Rab8a (Lee et al., 2011, Yin et al., 2014).

In summary, it is clear from studies of PD patients with PINK1 mutations, as well as the many *in vitro* and *in vivo* studies, that PINK1 is necessary for mitochondrial function, cellular survival and stress resistance in cells. The PI3-kinase/Akt signalling system is a key overarching signalling pathway that regulates many of the aforementioned systems that PINK1 controls. A number of studies show PINK1 can regulate and be regulated by PI3-kinase/Akt signalling. Furthermore, the PI3-kinase/Akt system is regulated by many PD risk genes and is dysfunctional in the PD brain. This positions the PI3-kinase/Akt system as a critical node of PD pathogenesis. However, it is not clear which elements of the pathway become impaired in PD or as a result of PD-causing mutations including PINK1. The following sections overview this area.

### 1.5 The PI3-kinase/Akt signalling pathway

The phosphoinositide 3-kinase (PI3-kinase)/Akt signalling pathway is a major signalling hub for cell survival, metabolism and proteostasis (Manning and Toker, 2017), key systems that fail in PD. It is activated primarily by insulin and insulin like Growth Factor-1 (IGF-1) and in this context often referred to as the IIS (insulin and IGF-1 signalling) pathway (for reviews see (Brunet et al., 2001, Liang and Slingerland, 2003, O'Neill, 2013). Both the insulin and IGF-1 receptors (IGF-1R and IR, respectively) are tyrosine kinase receptors; upon activation by insulin or IGF-1, these receptors trigger tyrosine phosphorylation of the insulin receptor substrate (IRS), predominantly IRS-1 and IRS-2. The activated form of IRS in turn activates PI3-kinase, which phosphorylates the membrane lipid phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to generate phosphatidylinositol 3,4,5-triphosphate ($PIP_3$) (Fig. 1.7) (Kenyon, 2010, Taniguchi et al., 2006). It is worth noting that the PI3-kinase Class I protein is responsible for this generation of $PIP_3$ and is composed of a regulatory subunit (p85α, p55α, p50α, p85β, p50γ, p101, p84 or p87) and a catalytic subunit (p110α, p110β, p110γ, p110δ) (for review (Jean and Kiger, 2014). As $PIP_3$ levels increase, Akt is recruited to the membrane, the interaction of Akt with $PIP_3$ results in a conformational change in Akt

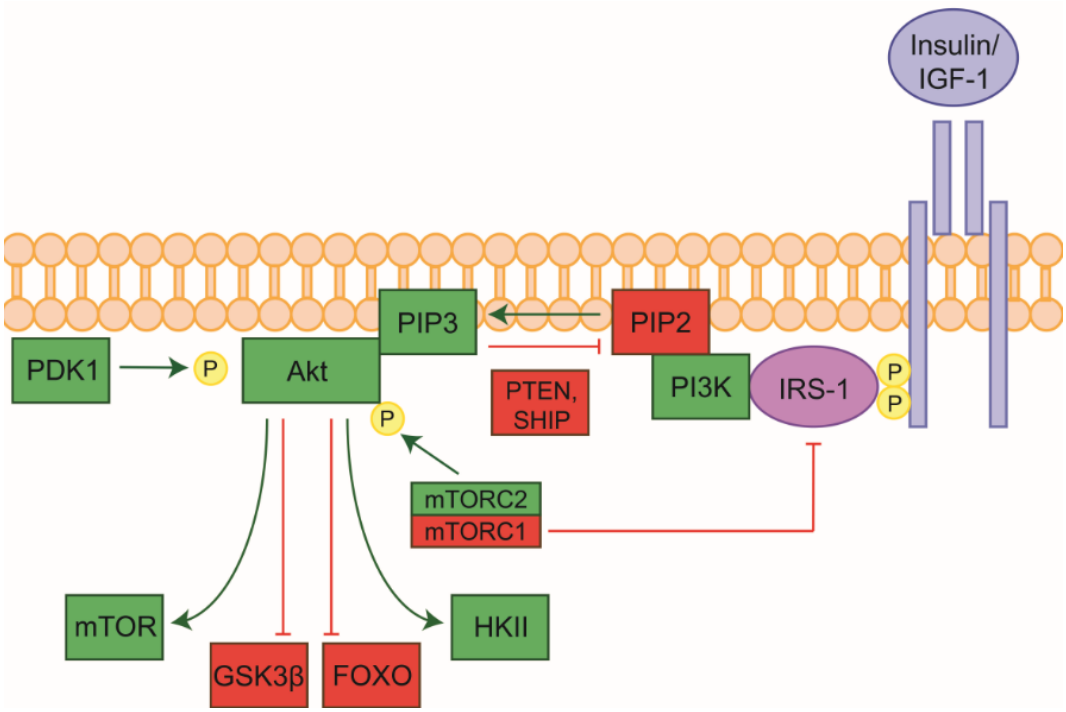



**Figure 1.8 The major signalling molecules in the PI3-kinase/Akt pathway.**

Insulin/IGF activate their respective receptors which in turn phosphorylate the IRS. This activates PI3-k which phosphorylates $PIP_2$ to $PIP_3$, increasing levels of $PIP_3$ recruit Akt to the membrane. Phosphorylation by PDK-1 at Thr308, and by mTORC2 at Ser473 are required for full activation of Akt. Akt targets a vast number of downstream signalling molecules such as mTOR, GSK3β, and FOXO. PTEN dephosphorylates $PIP_3$ to $PIP_2$, PP2A and PHLPPs dephosphorylate Akt at Thr308 and Ser473, respectively. Molecules activated by PI3-k/Akt signalling are shown in green, and molecules inactivated are shown in red.

exposing its activation loop which phosphoinositide-dependent kinase-1 (PDK-1) can now access (Calleja et al., 2007). For full activation of Akt, it must be phosphorylated by PDK-1 at $Thr^{308}$ (increases activity 100-fold) and by mTORC2 at $Ser^{473}$ (allows for maximal Akt activation) (Fig. 1.7) (Alessi et al., 1996, Franke, 2008, Risso et al., 2015). Importantly, recent studies have shown that $PIP_3$ can equilibrate between endomembranes via membrane trafficking routes and can also be generated within endomembranes *in situ* (Braccini et al., 2015, Ebner et al., 2017, Jethwa et al., 2015, Salamon and Backer, 2013, Sato et al., 2003). The observation that Akt localises to endomembranes, in addition to the plasma membrane, has led to the modification of Akt signalling models to incorporate compartment-specific patterns of activation (Ebner et al., 2017, Manning and Toker, 2017).

Akt targets and phosphorylates a vast number of downstream signalling molecules, estimated at > 200 proteins to modify their function, which include: mTOR, glycogen synthase kinase 3β (GSK3β) and Forkhead box-O class (FOXO) transcription factors (Fig. 1.7). Akt phosphorylates and activates mTOR, a Ser/Thr kinase with 2 distinct protein complexes, mTORC1 and mTORC2 (Loewith et al., 2002, Shimobayashi and Hall, 2014). mTORC1 activation increases protein translation and synthesis via phosphorylation of its main effector proteins, inhibition of eukaryotic translation initiation factor 4E (eIF-4E)-binding protein 1 and activation of 70-kDa ribosomal S6 protein kinase (Sabatini, 2006) in addition to enhancing RNA translation through the S6 ribosomal protein (Jewell et al., 2013, Ma and Blenis, 2009, Volarevic and Thomas, 2001). As previously mentioned, mTORC2 is required for Akt activation, and it has also been linked to cytoskeletal regulation and cell survival, and mTORC1 inhibits autophagy (Jacinto et al., 2004, Rabanal-Ruiz et al., 2017). GSK3β is inactivated by phosphorylation of $Ser^9$ upon Akt activation, this results in enhanced lipid synthesis and induction of glycogen synthesis (Cross et al., 1995, Golpich et al., 2015). A number of additional functions of GSK3β have since been described, for example, it is now known to play a key role in cellular processes including cell differentiation, proliferation and growth (Force and Woodgett, 2009, Shin et al., 2011). In addition, inhibition of GSK-3β was found to suppress the transcriptional activity of nuclear factor-kappa B (NF-κB) and to enhance the transcriptional activity of cAMP response element binding protein in the

liver, which resulted in reduced inflammation and hepatic cell apoptosis (Zhang et al., 2014). This indicates that inhibition of GSK3β by Akt could protect cells from apoptosis by regulating inflammatory pathways (Golpich et al., 2015). FOXO is also inactivated by Akt; FOXO transcription factors are phosphorylated by Akt at three conserved residues (e.g. FOXO1 at $Thr^{24}$, $Ser^{256}$, and $Ser^{319}$), resulting in 14-3-3 binding (Manning and Cantley, 2007). This has been found to correlate with its nuclear export which is hypothesised to be the result of decreased affinity of FOXO for its target DNA sequence and a potential conformational change which exposes nuclear export sequences (Brunet et al., 2002, Cahill et al., 2001). FOXO transcription factors are thereafter retained in the cytoplasm due to masking of the nuclear localisation signal which ultimately results in inhibition of the transcriptional activity of FOXO (Greer and Brunet, 2008, Kloet and Burgering, 2011). In the context of the PI3-kinase/Akt signalling axis, FOXO acts as the main opposing force to Akt, when cells are deprived of nutrients and the PI3-kinase/Akt pathway is not stimulated by insulin or IGF-1, FOXO is necessary for the transcription of stress response genes and repair systems (O'Neill, 2013).

In addition to FOXO signals, there are a number of different means by which the PI3-kinase/Akt signalling pathway is negatively regulated. PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a member of the protein-tyrosine phosphatase gene superfamily, which acts as an antagonist of PI3-kinase as it dephosphorylates $PIP_3$ to $PIP_2$ (Hers et al., 2011). Downstream of PI3-kinase in the PI3-kinase/Akt pathway Akt is inactivated by dephosphorylation at $Thr^{308}$ by protein phosphatase 2A (PP2A), and at $Ser^{473}$ by PH domain leucine-rich repeat protein phosphatases (Bayascas and Alessi, 2005, Gao et al., 2005). Finally, sustained activation of mTOR leads to mTORC1-mediated serine phosphorylation of IRS-1, which results in its inactivation and the dissociation of PI3-kinase/Akt from the IR/ IGF-1Rs which has been attributed to causing insulin resistance (Moloney et al., 2010, Tanti and Jager, 2009).

## 1.6 The PI3-kinase/Akt system in PD

### 1.6.1 The PI3-kinase/Akt system in the PD brain

Understanding the connection between PD and the PI3-kinase/Akt pathway is of central importance for PD research, as this pathway is a master regulator of cell survival, metabolism and proteostasis (for review see (Manning and Toker, 2017), key systems that fail in PD. Studies have shown Akt activation is impaired in post-mortem tissue from PD patients, with markedly reduced levels of both total Akt and phospho-Akt (activated Akt) in the DA neurons of the SNpc (Malagelada et al., 2008, Timmons et al., 2009). Kim and colleagues demonstrated regrowth of axons from dopaminergic neurons to their target, the striatum, after an adeno-associated virus (AAV)-mediated transduction of surviving endogenous dopaminergic neurons of the SN with constitutively active forms of the Akt (Kim et al., 2011). Abnormal levels of downstream signalling events have also been reported. Levels of GSK-3β phosphorylated at $Tyr^{216}$ (which hyperphosphorylates tau to produce toxic pathological forms of phosphorylated tau) were higher in the striatum of PD patients (Wills et al., 2010). Elevated levels of mTOR have also been found in the striatum of PD brains (Wills et al., 2010). In agreement, a further study reported alterations in mTOR, EIF2, EIF4 and p70S6K signaling in the SN of PD patients (Dijkstra et al., 2015). These changes were reported from Braak stage 0 to 6, indicating their involvement throughout the progression of PD pathology (Dijkstra et al., 2015). This indicates that immune response and protein translation signalling are impaired via defects in Akt signalling during disease progression.

Studies have linked regulation of the PI3-kinase/Akt signalling pathway to DA levels in the mouse brain. In one study, DA was found to induce PI3-kinase-independent activation of Akt in striatal neurons (Brami-Cherrier et al., 2002). Hyperdopaminergia in DA transporter knockout rodents resulted in reduced Akt activity and a concomitant activation of both GSK3α and GSK3β due to a reduction in their phosphorylation by Akt (Beaulieu et al., 2004). These defects could be rescued by inhibiting dopamine synthesis using the irreversible TH inhibitor α-methyl-para-tyrosine (Beaulieu et al., 2004). Similarly, elevated DA levels have been reported to inactivate Akt and activate

GSK3β in the rat frontal cortex (Sutton and Rushlow, 2012) and the developing zebrafish brain (Souza et al., 2011). This could be attenuated by decreasing DA levels with the DA receptor antagonist raclopride (Sutton and Rushlow, 2012). This indicates that a DA is involved in the regulation of Akt activation and that its levels need to be tightly regulated to avoid impairments in PI3-kinase/Akt signalling in PD.

### 1.6.2 PD risk genes and the PI3-kinase/Akt system

Several PD risk genes have been shown to regulate the PI3-kinase/Akt signalling system, these include *PINK1* (Akundi et al., 2012, Boonying et al., 2019, Contreras-Zarate et al., 2015, Ellis et al., 2013, Hauser et al., 2017, Mei et al., 2009, Murata et al., 2011, O'Flanagan et al., 2015, Sanchez-Mora et al., 2012, Soutar et al., 2018), *PRKN* (Fallon et al., 2006, Gupta et al., 2017), *DJ-1* (Jaramillo-Gomez et al., 2015, Kim et al., 2005, Kim et al., 2011, Zhang et al., 2016), *LRKK2* (Chuang et al., 2014, Ohta et al., 2011) and *SNCA* (Chung et al., 2011, Seo et al., 2002). The interaction between PINK1 and the PI3-kinase/Akt pathway will be expanded upon in a later section (1.7).

EGFR signalling via the PI3-kinase/Akt pathway is reduced in the parkin knockout mouse brain (Fallon et al., 2006). It is hypothesised that EGF stimulates parkin binding to both Eps15 and the EGF-R and that this interaction impairs the ability of Eps15 ubiquitin-interacting motifs to bind to the ubiquitinated EGFR, thereby delaying EGFR internalisation and degradation and prolonging PI3-kinase/Akt signalling (Fallon et al., 2006). Therefore, a loss of parkin function, as occurs in PD, will prevent this prolonged signal resulting in reduced PI3-kinase/Akt signalling. Parkin has also been implicated in the regulation of PTEN inhibition by S-nitrosylation (Gupta et al., 2017). Therefore, loss of parkin function will relieve the inhibition on PTEN resulting in activated PTEN and decreased PI3-kinase/Akt signalling (Gupta et al., 2017). LRRK2 has been found to directly phosphorylate and activate Akt1 at $Ser^{473}$ and LRRK2 disease-associated mutations R1441C, G2019S and I2020T exhibited reduced interaction with, and phosphorylation of, Akt1 in SH-SY5Y cells (Ohta et al., 2011). Furthermore, in *Drosophila*, LRRK2-activated Akt further phosphorylates and inhibits FOXO1 and this signalling cascade was found to be critical for LRRK2-mediated protection from

apoptosis (Chuang et al., 2014). DJ-1 has been identified as a negative regulator of PTEN ensuring the maintenance of its phosphorylation and inactivation at $Ser^{380}$ (Jaramillo-Gomez et al., 2015, Kim et al., 2005, Sitaram et al., 2009) and a positive regulator of Akt by phosphorylating and activating Akt at $Ser^{473}$ in catecholaminergic A-differentiated (CAD) cells and renal cell carcinoma (Jaramillo-Gomez et al., 2015, Sitaram et al., 2009) and $Thr^{308}$ in SH-SY5Y cells (Zhang et al., 2016). One study with α-synuclein showed that at nanomolar concentrations, α-synuclein protected neurons against serum deprivation, oxidative stress, and excitotoxicity through the PI3/Akt signaling pathway (Seo et al., 2002). However, overexpression of α-synuclein as can occur in PD, resulted in suppressed IGF-1-induced Akt activation in SK-N-SH neuroblastoma cells (Chung et al., 2011), and cytotoxicity in in SH-SY5Y cells (Seo et al., 2002).

### 1.6.3Targeting the PI3-kinase/Akt system in PD preclinical models

Activation or normalisation of the PI3-kinase pathway represents a therapeutic avenue which should be further researched to benefit individuals with PD (as highlighted by (Burke, 2007)). Maintaining Akt phosphorylation to actively promote PI3-kinase/Akt signalling could both prevent disease progression and promote axon growth and sprouting. New axons could replace connections that have been lost as a result of PD and could potentially reverse symptoms (Greene et al., 2011). However, caution must be exercised as elevated Akt activity promotes cell survival, proliferation and metastasis (Franke, 2008, Steelman et al., 2008). Manipulation of Akt activity must be limited to neurons which have been affected by PD, in order to prevent the development of malignancies (Greene et al., 2011). Studies have shown that constitutive activation of Akt protects against DA neuron loss in the 6-OHDA neurotoxin lesioned preclinical model of PD (Kim et al., 2011, Ries et al., 2006). Kim et al. further reported regrowth of axons from those DA neurons that had survived the neurotoxic lesion, to their striatal target region (Kim et al., 2011). As previously mentioned, GLP-1 agonists can restore insulin signalling with a mechanism that is believed to function via Akt signalling (for review (Athauda and Foltynie, 2016). Pre-clinical trials yielded positive results (for

review see (Batista et al., 2019), which lead to clinical trials that are now at phase II and showing positive results for treatment of motor symptoms (Athauda et al., 2017).

## 1.7 Relationship between the PINK1 protein and PI3-kinase/Akt signalling.

### 1.7.1 PINK1 and the central signalling molecule Akt

Several studies have shown that the pro-survival and stress resistance function of PINK1 occurs via Akt activation (Summarised Fig 1.8). Akt activation by PINK1 confers protection against several cell stressors, including rotenone (Murata et al., 2011), calyculin A, FK506, staurosporine (Akundi et al., 2012), and ceramide (Contreras-Zarate et al., 2015, Sanchez-Mora et al., 2012), and Akt signalling regulates PINK1-dependent mitophagy (Deas et al., 2011a, Hauser et al., 2017, Matsuda et al., 2010, McCoy et al., 2014, Soutar et al., 2018). It is clear that studying PINK1 with respect to Akt function will provide a window of opportunity to understand how the PI3-kinase/Akt system becomes deregulated in PD, thereby providing better opportunities to identify specific targets in the PI3-kinase/Akt pathway that can normalise the pathway in PD therapeutics.

While it is clear that PINK1 can activate Akt and that PINK1 deletion reduces Akt activation (Akundi et al., 2012, Boonying et al., 2019, Contreras-Zarate et al., 2015, Ellis et al., 2013, Hauser et al., 2017, Maj et al., 2010, Mei et al., 2009, Murata et al., 2011, O'Flanagan and O'Neill, 2014, Sanchez-Mora et al., 2012, Soutar et al., 2018), the mechanism by which this occurs and its dependence on PINK1 kinase activity remain unclear. In SH-SY5Y cells, overexpression of PINK1 was found to activate mTORC2, more specifically Rictor which is a component of mTORC2, resulting in increased phosphorylation of Akt at $Ser^{473}$ (Murata et al., 2011). This phosphorylation of Akt was found to be essential for the activation of Akt and for the protection of SH-SY5Y cells from numerous cytotoxic agents (Murata et al., 2011). In this study, PINK1-induced Akt activation was reported to occur independently of PI3-kinase, via PINK1-mediated

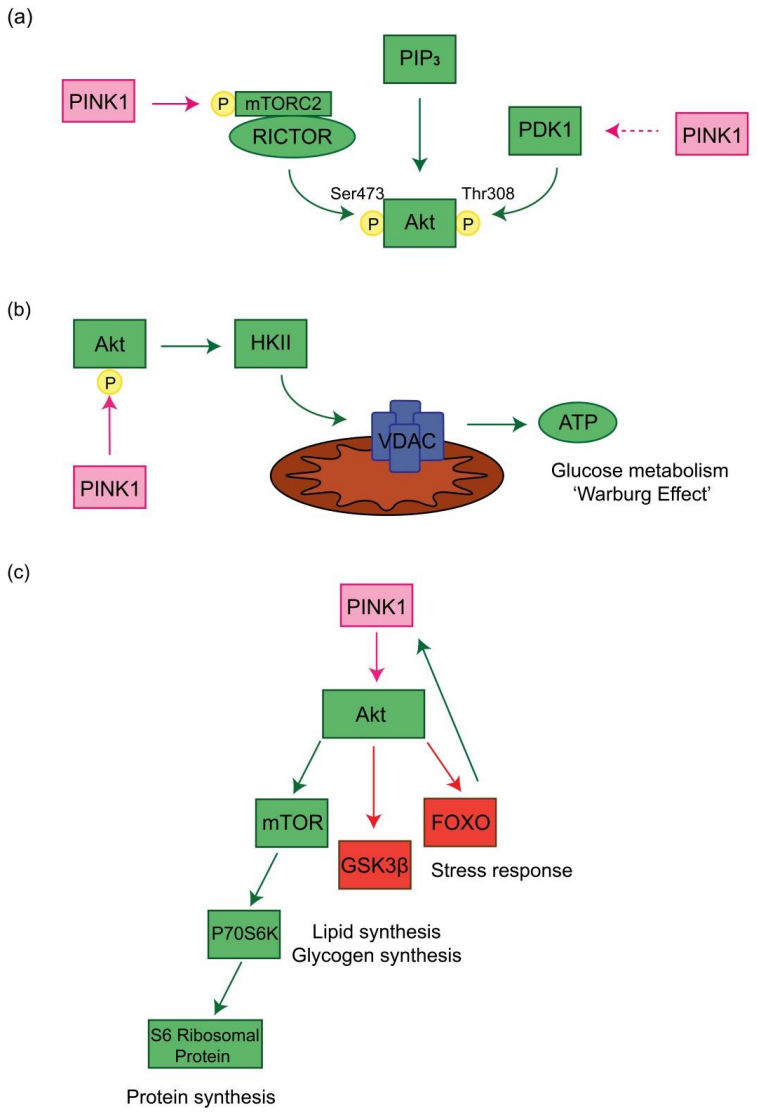



**Figure 1.9 PINK1 interactions with the PI3-kinase/Akt pathway**

a. Overexpression of PINK1 activates mTORC2 increasing phosphorylation of Akt at $Ser^{473}$. b. PINK1 has been shown to increase HKII levels, and its localisation to the mitochondrial membrane. c. $PINK1^{-/-}$ results in reduced phosphorylation of ribosomal protein S6 and GSK3β. FOXO3a has been shown to induce human PINK1 transcription and PINK1 contains a number of FOXO binding elements on its promoter region.

activation of mTORC2 via Rictor (Murata et al., 2011). In contrast, subsequent studies revealed that MEFs from PINK1$^{-/-}$ mice had reduced IGF-1- and insulin-induced activation of Akt, with reduced phosphorylation at both Akt activation residues, Thr$^{308}$ and Ser$^{473}$ (Akundi et al., 2012). These studies indicate that PINK1 promotes Akt activation via a mechanism upstream of Akt phosphorylation (Akundi et al., 2012, Contreras-Zarate et al., 2015, Ellis et al., 2013, Maj et al., 2010, Manning and Toker, 2017, Mei et al., 2009, Murata et al., 2011, Sanchez-Mora et al., 2012). Thus, conflicting mechanisms have been reported and there is no clear information on whether Akt activation requires PINK1 kinase activity. Several additional interactions between PINK1 and the PI3-kinase/Akt pathway have been reported. PINK1 has been shown to increase signalling responses via the PI3-kinase/Akt signalling pathway in response to stimulation with both IGF-1 and insulin ((Akundi et al., 2012, Contreras-Zarate et al., 2015). Conversely, silencing or mutating PINK1 has also been described to impair Akt signalling in a number of models such as human cell lines (SH-SY5Y and prostate cancer cell lines PC3 and DU145) (Murata et al., 2011), MEFs (Akundi et al., 2012), mouse primary cortical neurons (Akundi et al., 2012), CAD cells (Contreras-Zarate et al., 2015), zebrafish (Anichtchik et al., 2008) and PD patient fibroblasts (Maj et al., 2010). PINK1 deletion has also been described recently to reduce phosphorylation of IGF-1R and IGF-1R trafficking in CAD cells (Contreras-Zarate et al., 2015).

### 1.7.2 PINK1 and HKII

Downstream signalling molecules in the PI3-kinase/Akt signalling axis have also been described to be regulated by PINK1. PINK1 has been shown to increase Hexokinase II (HKII) levels (the initialising enzyme of glycolysis), and their localisation to the mitochondrial membrane through phosphorylation of HKII by Akt (Contreras-Zarate et al., 2015, McCoy et al., 2014). Upon phosphorylation by Akt, HKII associates with the voltage-dependent anion channel in the mitochondria, which provides HKII with numerous ATPs. As a result, HKII is ready to phosphorylate glucose molecules as they become available. This creates a very efficient and increased rate of glycolysis, often referred to as the Warburg effect, which allows cells to survive adverse conditions

(Stiles, 2009). HKII is also essential (in combination with PINK1) for recruitment of parkin to depolarised mitochondria (McCoy et al., 2014).

### 1.7.3 PINK1 and FOXO3a

The major transcription factor FOXO3a is activated when the PI3-kinase/Akt system is inactive and has been shown to upregulate PINK1 mRNA expression (Mei et al., 2009). In line with this, a number of FOXO-binding elements have been found in the region of the PINK1 promoter (Mei et al., 2009). FOXO3a has been shown to induce human PINK1 transcription in cancer cells when growth factors and cytokines were withdrawn and when PI3-kinase/Akt signalling was blocked by inhibition of PI3-kinase with LY-294002 (Mei et al., 2009). Cells are able to continually adapt to varying conditions in their environment owing to this switch between FOXO and Akt signalling (Kloet and Burgering, 2011). Through FOXO activation and the subsequent transcription of gene networks involved in cell survival, stress response and repair systems, the PI3-kinase/Akt pathway is constantly primed for activation if and when the source of cellular stress should subside. PINK1 has been proposed to protect cells from short term cellular stress, increasing cell survival due to activation by FOXO3a (Mei et al., 2009). Overexpression of NF-κB has also been shown to upregulate PINK1 expression in HEK293 cells and SH-SY5Y cells (Duan et al., 2014).

### 1.7.4 PINK1 and biomolecule synthesis

In $PINK1^{-/-}$ MEFs, IGF-1-induced phosphorylation of ribosomal protein S6 is lower than in wild-type MEFs (Akundi et al., 2012). IGF-1 induces protein synthesis through activation of a signalling cascade involving Akt, mTOR and p70S6kinase, which culminates in phosphorylation of the S6 ribosomal protein (reviewed by (Ma and Blenis, 2009)). This indicates that silencing of PINK1 interferes with IGF-1-induced protein synthesis (Akundi et al., 2012). As previously mentioned, GSK3β is inactivated by phosphorylation on $Ser^{9}$ by Akt; this action is required for the induction of glycogen synthesis and enhanced lipid synthesis. IGF-1 stimulation has been reported to increase levels of p-$GSK3\beta^{Ser9}$ in wildtype, but not $PINK1^{-/-}$ MEFs (Akundi et al., 2012,

Contreras-Zarate et al., 2015) implying that GSK3β signalling is impaired by ablation of PINK1.

### 1.7.5 PINK1 and PTEN

PINK1 was first described and named due to its increased expression in response to PTEN (the major negative regulator of the PI3-kinase/Akt pathway) (Unoki and Nakamura, 2001). Subsequently, Siddall and co-workers further described the relationship between PINK1 and PTEN. They reported that deletion of PTEN resulted in a decrease in PINK1 expression in H9c2 myoblast cells and in $PTEN^{+/-}$ mouse hearts compared to that in $PTEN^{+/+}$ mouse hearts (Siddall et al., 2008). Further studies implicated PINK1 in the protection of cardiomyocytes from ischemia-reperfusion injury in the HL-1 cardiac muscle cell line (Siddall et al., 2013). Loss of PINK1 increased vulnerability of these cells to ischemia-reperfusion injury and overexpression of PINK1 reduced cell death as a result of this simulated ischemia-reperfusion injury (Siddall et al., 2013). However, it is worth acknowledging that the original study that discovered and named PINK1 found that exogenous expression of PTEN induced expression of the PINK1 gene (Unoki and Nakamura, 2001); this indicates that PTEN and PINK1 may have different interrelationships, depending on the cellular context. Another link between PINK1 and PTEN was described recently, as a longer form of PTEN, PTEN-L, was found to negatively regulate PINK1-induced phosphorylation of pSer65-Ub to inhibit PINK1-parkin-mediated mitophagy (Wang et al., 2018b).

This literature review highlights the need for new disease modifying therapies to protect against neuronal cell death and promote the regrowth of neurons in PD. It is clear that PINK1 is a major neuroprotective, anti-stress protein which enhances cell survival, protein health, and mitochondrial function, and that it interacts with the PI3-kinase/Akt pathway to elicit many of these effects. The discovery of potential mechanisms involving PINK1 regulation of the activation of PI3-kinase/Akt signalling would identify new treatment targets for PD that could protect against neuronal cell death and promote the regrowth of neurons and therefore is the focus of this thesis.

## 1.8 Aims and thesis structure

This thesis aimed to examine the function of the PD-causing gene PINK1, with a focus on PI3-kinase/Akt signalling to enable the discovery of new therapeutic targets, molecular pathways and cell systems that may be involved in the pathogenesis of PD. The aims of this thesis were examined in research work described in three results chapters which are written in manuscript form and listed below. The materials and methods employed are described in the relevant results chapter.

### Chapter 2 The Parkinson's gene PINK1 activates Akt via PINK1 kinase-dependent regulation of the phospholipid PI(3,4,5)$P_3$

In this chapter we employed PINK1-modifed cell systems as a tool to discover the primary mechanism by which PINK1 regulates Akt signalling.

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### Chapter 3 An investigation of binding proteins for the Parkinson's risk gene PINK1 identifies new candidates that regulate survival signalling, inflammatory responses and Akt signalling

This chapter aimed to assemble a comprehensive list of known PINK1-interacting proteins and to identify new candidates bound to PINK1 under normal growth conditions. Bioinformatic and mass spectrometry analysis were employed to allow for the discovery of novel PINK1-interacting proteins and identify PINK1 regulated signalling pathways.

### Chapter 4 α-synuclein overexpression and loss of PINK1 induce mitochondrial fission and Golgi fragmentation and reduce neurite length in midbrain dopaminergic neurons.

Chapter 4 aimed to expand the knowledge on mitochondrial dynamics, Golgi fragmentation and PI3-kinase/Akt signalling in PD and how this was regulated by both PINK1 and α-synuclein through analysis of cultured ventral midbrain dopaminergic neurons.

Submitted to Parkinsonism and Related Disorders September 2019

# Chapter 2

# Chapter 2 The Parkinson's gene PINK1 activates Akt via PINK1 kinase-dependent regulation of the phospholipid PI(3,4,5)$P_3$

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## 2.1 Summary statement:

Loss of PINK1 kinase function as occurs in Parkinson's disease, causes impaired activation of Akt, as a result of defects in phospholipid PI(3,4,5)$P_3$ regulation.

## 2.2 Abstract:

Akt signalling is central to cell survival, metabolism, protein and lipid homeostasis, and is impaired in Parkinson's disease (PD). Akt activation is reduced in the PD brain, and by many PD-causing genes, including PINK1 (PTEN-induced putative kinase-1). This study investigated the mechanisms by which PINK1 regulates Akt signalling. Our results reveal for the first time that PINK1 constitutively activates Akt in a PINK1-kinase dependent manner in the absence of growth factors and enhances Akt activation in normal growth medium. In PINK1 modified MEFs, agonist-induced Akt signalling failed in the absence of PINK1, due to significantly impaired PINK1 kinase-dependent increases in PI(3,4,5)$P_3$ at both plasma membrane and Golgi. In the absence of PINK1, PI(3,4,5)$P_3$ levels did not increase in the Golgi, and there was significant Golgi fragmentation, a recognised characteristic of PD neuropathology. PINK1 kinase activity protected the Golgi from fragmentation in an Akt-dependent fashion. This study demonstrates a new role for PINK1 as a primary upstream activator of Akt via PINK1 kinase-dependent regulation of its primary activator PI(3,4,5)$P_3$, providing novel mechanistic information on how loss of PINK1 impairs Akt signalling in PD.

## 2.3 Keywords:

Akt / Parkinson's disease / PINK1 / PI(3,4,5)$P_3$ ($PIP_3$)

## 2.4 Introduction:

PINK1 (phosphatase and tensin homologue (PTEN)-induced kinase 1) was identified as a gene upregulated by overexpression of the tumor suppressor PTEN, the major negative regulator of phosphoinositide-3-kinase (PI3-kinase)/Akt signalling (Unoki and Nakamura, 2001). Subsequently, loss of function mutations in PINK1 were reported to cause autosomal recessive early onset Parkinson's disease (PD) (Valente et al., 2004), leading to a major research effort to understand PINK1 function. PINK1, a ubiquitous serine-threonine kinase, has pro-survival, neuroprotective and anti-stress signalling functions (Arena et al., 2013, Haque et al., 2008, Li et al., 2017, MacKeigan et al., 2005, Wood-Kaczmar et al., 2008, Yang et al., 2018). Multiple studies, from our and other labs, have shown that PINK1 is a key regulator of mitochondrial health, including mitophagy, fission, fusion, bioenergetics and mitochondrial antigen presentation (Harper et al., 2018, Matheoud et al., 2016, O'Flanagan et al., 2015, Pilsl and Winklhofer, 2012, Youle and Narendra, 2011, Youle and van der Bliek, 2012). Although these are the most widely documented functions of PINK1, PINK1 also regulates several other non-mitochondrial systems central to survival, neuroprotection and stress-resistance (Dagda et al., 2009, Dagda et al., 2014, Dickey and Strack, 2011, Fedorowicz et al., 2014, Ries et al., 2006, Steer et al., 2015, Xiong et al., 2016), for review (Arena and Valente, 2017, O'Flanagan and O'Neill, 2014). These include PI3-kinase/Akt signalling (Akundi et al., 2012, Contreras-Zarate et al., 2015, Ellis et al., 2013, Maj et al., 2010, Sanchez-Mora et al., 2012), the cell cycle (O'Flanagan et al., 2015), protein kinase A (Dagda et al., 2014, Dickey and Strack, 2011), NF-κB (Lee and Chung, 2012, Lee et al., 2012, Sha et al., 2010) ubiquitination, proteasomal degradation (Xiong et al., 2009) and macroautophagy (Dagda et al., 2009, Fedorowicz et al., 2014).

Understanding the interactions between PINK1 and the PI3-kinase/Akt pathway is of central importance for PD research, as this pathway is a master regulator of cell survival, metabolism and proteostasis, key systems that fail in PD (Manning and Toker, 2017). In concordance, Akt activation is impaired in postmortem tissue from the substantia nigra of PD patients (Malagelada et al., 2008, Timmons et al., 2009), and constitutive activation of Akt protects against dopaminergic neuron loss in a preclinical model of PD

(Ries et al., 2006). Notably, several PD genes, including PINK1, converge to crosstalk with the PI3-kinase/Akt signalling system (Chuang et al., 2014, Fallon et al., 2006, Gupta et al., 2017, Jaramillo-Gomez et al., 2015, Kim et al., 2005, Ohta et al., 2011, Zhang et al., 2016). Akt activation by PINK1 confers protection against several cell stressors including rotenone (Murata et al., 2011), calyculin A, FK506, staurosporine (Akundi et al., 2012), and ceramide (Contreras-Zarate et al., 2015, Sanchez-Mora et al., 2012). Akt-induced inhibition of FOXO3a can reduce PINK1 mRNA levels (Mei et al., 2009), and Akt signalling regulates PINK1-dependent mitophagy (Deas et al., 2011a, Hauser et al., 2017, Matsuda et al., 2010, McCoy et al., 2014, Soutar et al., 2018).

Akt activation mechanisms and Akt signalling are the subject of intensive investigation in cell biology in health and disease, (for review (Manning and Toker, 2017). Accumulation of the membrane lipid phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$ or $PIP_3$), via agonist-induced PI3-kinase phosphorylation of $PI(4,5)P_2$, is an essential prerequisite for Akt activation. $PIP_3$ acts by recruiting inactive cytosolic Akt to membranes by interacting with its pleckstrin homology (PH) domain (Bellacosa et al., 1998). This interaction induces a conformational change in Akt, essential for Akt activation, allowing phosphorylation at two regulatory residues, $Ser^{473}$ by mammalian target of rapamycin complex 2 (mTORC2) (Alessi et al., 1996, Sarbassov et al., 2005, Stokoe et al., 1997), and $Thr^{308}$, by phosphoinositide dependent kinase 1 (PDK1) (Calleja et al., 2007, Stokoe et al., 1997). Termination of PI3-kinase-$PIP_3$-Akt signalling is predominantly due to dephosphorylation of $PIP_3$ to $PI(4,5)P_2$ by the phosphatase PTEN (Maehama and Dixon, 1998, Stokoe et al., 1997). It is increasingly recognised that $PIP_3$ can equilibrate between endomembranes via membrane trafficking routes, and can also be generated within endomembranes in situ (Braccini et al., 2015, Ebner et al., 2017, Jethwa et al., 2015, Salamon and Backer, 2013, Sato et al., 2003). The observation that Akt localises to endomembranes, in addition to the plasma membrane, has led to the modification of Akt signalling models to incorporate compartment-specific patterns of activation (Ebner et al., 2017, Manning and Toker, 2017).

While it is clear that PINK1 can activate Akt (Akundi et al., 2012, Boonying et al., 2019, Contreras-Zarate et al., 2015, Ellis et al., 2013, Hauser et al., 2017, Maj et al.,

2010, Mei et al., 2009, Murata et al., 2011, O'Flanagan and O'Neill, 2014, Sanchez-Mora et al., 2012, Soutar et al., 2018), and that PINK1 deletion reduces Akt activation, the mechanism by which this occurs and its dependence on PINK1 kinase activity remain unclear. Initial studies revealed that PINK1 markedly enhanced the phosphorylation of Akt at $Ser^{473}$ but not $Thr^{308}$, inducing Akt activation that was essential for protection from a variety of cytotoxic agents (Murata et al., 2011). PINK1-induced Akt activation was reported to occur independently of PI3-kinase, via PINK1-mediated activation of mTORC2 via Rictor (Murata et al., 2011). In contrast, subsequent studies revealed that mouse embryonic fibroblasts (MEFs) from $PINK1^{-/-}$ mice had reduced IGF-1- and insulin-induced activation of Akt, with reduced phosphorylation at both Akt activation residues, $Thr^{308}$ and $Ser^{473}$, and protection from apoptosis and metabolic dysfunction (Akundi et al., 2012). Together, these studies show that PINK1 promotes Akt activation via a mechanism upstream of Akt phosphorylation (Akundi et al., 2012, Contreras-Zarate et al., 2015, Ellis et al., 2013, Maj et al., 2010, Manning and Toker, 2017, Mei et al., 2009, Murata et al., 2011, Sanchez-Mora et al., 2012). However, various mechanisms have been proposed and there is no clear information on whether Akt activation requires PINK1 kinase activity.

In this study, we employed PINK1-modifed cell systems (Morais et al., 2009, O'Flanagan et al., 2015) to investigate the mechanism by which PINK1 activates Akt. We identify PINK1 as a major upstream activator of Akt, and provide a new mechanism involving PINK1 kinase-dependent regulation of the plasma membrane and endomembrane localisation of $PIP_3$, the essential lipid activator of Akt. This study significantly advances knowledge on the mechanisms by which loss of function of PINK1 impairs Akt activation in PD.

## 2.5 Materials and Methods

### 2.5.1 Generation of PINK1$^{-/-}$ mice and derived MEF cell lines

The cell lines used in these experiments are Mouse Embryonic Fibroblast (MEF) cells, as previously employed and described (Glasl et al., 2012, Morais et al., 2009, O'Flanagan et al., 2015). PINK1$^{-/-}$ knockout and PINK1$^{+/-}$ heterozygous knockout mice were generated by Wolfgang Wurst and Daniela Vogt-Weisenhorn (Helmholtz Center, Munich, Germany) and immortalised MEFs were generated as previously described (Morais et al., 2009). In brief, PINK1$^{+/-}$ mice were interbred to generate mutant mice and wild-type littermate controls. Embryonic day 13 mice were dissected, heads and red organs removed and used for genotyping. The rest of the bodies were chopped up in cell culture dishes containing Dulbecco's modified Eagle's medium supplied with 50% fetal bovine serum and 1% penicillin/streptomycin. Cultures were expanded and serum decreased to 10% fetal bovine serum after the attainment of consistent growth. Afterward cultures were immortalised by transfection with simian virus 40 (SV40) large T-antigen. PINK1$^{-/-}$ MEFs were stably transfected with a plasmid containing human PINK1 (hPINK1 construct Origene (Rockville, MD, USA)), and the triple kinase dead hPINK1$_{K219A/D362A/D384A}$ mutants using site-directed mutagenesis (Stratagene, Santa Clara, CA, USA). The triple kinase dead mutation for PINK1 is not known to affect the stability/conformation of the PINK1 (Pridgeon et al., 2007). Cells were routinely monitored for mycoplasma and bacterial/yeast contaminations.

### 2.5.2 RT-PCR

PINK1 deletion/expression was confirmed in MEF cell lines employed in this study using RNA extraction and RT-PCR analysis (Supp. 2.4A). RNA was extracted using the PureLink RNA Mini Kit (Thermo Scientific) according to the instructions of the manufacturer. cDNA synthesis was performed with the QuantiTect reverse transcription kit (Qiagen) using 1µg of RNA. The primers and conditions used for RT-PCR are shown in supplementary information, this was performed using the GoTaq G2 Green Master Mix kit (Promega).

### 2.5.3 Cell Culture and stimulation/inhibition of the PI3-kinase/Akt pathway

MEFs were cultured in Dulbecco's Modified Eagle Medium: High glucose (DMEM-Hi), supplemented with 10% fetal bovine serum (FBS) and 2 mM L-Glutamine. Cells were cultured at $37^0$C in a humidified incubator supplemented with 5% CO2. In IGF-1 stimulation experiments, cells were plated overnight with complete media, followed by incubation with serum-free DMEM-Hi for 24 h. After 24 h, 10 ng/ml IGF-1 was added at the specified time-points (2, 10 or 60 min). In experiments for inhibition of PI3-kinase, cells were plated overnight with complete media, incubated with serum-free DMEM-Hi for 24 h, followed by incubation with 10 μM LY294002 for 30 min and subsequent stimulation with 10 ng/ml IGF-1 for 10 min. In experiments for MK2206 inhibition, cells were plated overnight with complete media, serum deprived for 24 h and then incubated with 1μM for MK2206 for 1 or 4 h. For Lysotracker and $PIP_3$ colocalisation analysis (Supp. 2.3), 75 nM of Lysotracker was added to the cells in culture for 1 h before fixation with 3% PFA for immunocytochemistry. BE(2)-M17 cells, transduced with PINK1 shRNA (PINK1A8, PINK1C2) or with control shRNA (Control 1, Control 2), were kindly provided by Mark Cookson and Alexandra Beilina NIA, Bethesda, MD, USA and were transduced and cultured as previously described (Sandebring et al., 2009).

### 2.5.4 Plasmids

The GFP-Akt-PH construct was kindly provided by Martin Lowe (University of Manchester, Manchester, UK). pEGFP-Akt was a gift from Julian Downward (Addgene plasmid # 39531; http://n2t.net/addgene:39531 ; RRID:Addgene_39531) (Watton and Downward, 1999). MEFs were seeded in 24-well plates to be 70% confluent the following day. Cells were then transfected with GFP-Akt-PH or GFP-Akt using Lipofectamine™ as recommended by the manufacturer's instructions.

### 2.5.5 Western Immunoblotting

The following primary antibodies, dilutions and sources were used: Akt (1:1,000) (Cell Signalling #9272), Akt 1 (1:1,000) (Cell Signalling #2938), Akt 2 (1:1,000) (Cell

Signalling #3063), Akt 3 (1:1,000) (Cell Signalling #8018), P-Akt pSer437 (1:500) (Cell Signalling #4060), P-Akt pThr308 (1:500) (Cell Signalling #9275), P-PDK1Ser241 (1:500) (Cell Signalling #3061), P-PI3-kinase p85Tyr458/p55Tyr199 (1:500) (Cell Signalling #4228), PI3-kinase p85α (1:1,000) (Cell Signalling #4257), PI3-kinase p110α (1:500) (BD Transduction Laboratories 611399), PTEN (1:500) (Cell Signalling #9552), P-PTENSer380 (1:500) (Cell Signalling #9551), RICTOR (1:500) (Cell Signalling #2114), β-actin (1:16,000) (Sigma Aldrich A5441). Primary antibodies were detected using horseradish peroxidase (HRP)-conjugated isotype-specific anti-rabbit IgG or anti-mouse IgG (1:1000, DAKO, Cambridgeshire, UK) secondary antibodies.

Western immunoblotting was carried out as described previously (Griffin et al., 2005, Moloney et al., 2010, O'Flanagan et al., 2015). Briefly, proteins were extracted, separated by SDS-PAGE (20μg/lane) and transferred electrophoretically to nitrocellulose membranes using a wet transfer apparatus and transfer buffer consisting of 48 mM Tris base, 39 mM glycine and 20% ethanol. After blocking for 1 h in 5% milk or 30 min in 5% Bovine Serum Albumin (phospho antibodies), cells were incubated in primary antibody solutions overnight at 4ºC. The blot underwent three 10-min washes in 1X TBX-T prior to secondary antibody incubation. Cells were incubated in secondary antibody solution for 1 h at RT followed by three 5-min washes in 1X TBS-T. Immunoreactive proteins were detected with enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK). All quantifications are illustrated by histograms which represent the densitometry of each protein normalised to β-Actin for an n = 3 as determined by ImageJ software.

### 2.5.6 Immunofluorescence and confocal microscopy

The following primary antibodies and dilutions were used: Phosphatidylinositol (3,4,5)-trisphosphate (1:150) (Tebu-Bio 117Z-P345B), Giantin (1:300) (kindly provided by Martin Lowe, University of Manchester), GM130 (1:4,000) (BD biosciences 610822), GRP-78-BIP (1:800) (Abcam Ab21685), His (1:400) (Abcam #9108) Rab 11a (1:100) (Zymed 715300), Rab14 (1:500) (Sigma R0656) and TGN46 (1:500) (Serotek UK AHP500). Primary antibodies were detected using Cy3 anti-rabbit IgG (1:400) (Jackson

laboratories 711-165-152) or Alexa Fluor 488 conjugated anti-mouse IgG (1:400) (Invitrogen A11001) secondary antibodies.

Culture medium was removed from each of the wells. Cells were washed with 1X PBS in-between each of the following steps: fixation in 0.5 ml 3% paraformaldehyde (PFA) for 15 min at room temperature, quenching in 1 ml of 50 mM NH4Cl/PBS for 15 min, permeabilisation in 1 ml buffer (0.05% Saponin/PBS, 0.2% BSA/PBS) for 5 min. Coverslips were transferred to a humid box and primary antibodies were added at appropriate dilutions in 5% BSA/PBS. Primary antibodies were incubated for 2 h at room temperature. Then cells were washed twice in 1X PBS. Cells were incubated in secondary antibody solutions in 5% BSA/PBS, as well as DAPI/PBS (1:10,000) for 1 h at room temperature. Cells were washed twice with 1X PBS for 5 min. Coverslips were mounted onto microscope slides using Mowiol mounting media. Cells were dried overnight and fluorescence images were acquired using Zeiss LSM 510 Meta confocal microscope fitted with a 63x/1.4 plan apochromat lens (Jena, Germany).

For GFP-Akt-PH analysis, each experiment was performed in duplicate. For the two coverslips per condition > 200 cells were counted. The threshold of intensity used was fluorescence in the cytosol as the baseline and any fluorescence intensity level above that at the plasma membrane (PM) was judged as an accumulation at the PM. To quantify the localisation of Akt at the PM the total number of transfected cells and the number of cells with Akt at the plasma membrane were counted (determined as described above). From these values the percentage of cells with Akt at the PM was then calculated for n = 3 and the representative histogram generated in GraphPad. To determine fluorescence intensity at the plasma membrane a line plot was drawn through the cell using the plot profile analysis in Carl Zeiss Zen 2.1 image analysis software, as described previously (Lindsay and McCaffrey, 2009). The X-axis represents distance along the line and the Y-axis is the pixel intensity. Pearson's colocalisation coefficient was calculated using Zeiss ZEN 2009 software as described previously (Lindsay et al., 2013).

### 2.5.7 Mass spectrometry

Mass spectrometry was used to measure inositol lipid levels essentially as previously described (Clark et al., 2011), using a QTRAP 4000 (AB Sciex) mass spectrometer and employing the lipid extraction and derivitisation method described for cultured cells, with the modification that 10 ng C17:0/C16:0 PtdIns(3,4,5)$P_3$ internal standard (ISD) and 10 ng C17:0/C16:0 PtdIns ISD were added to primary extracts, and that final samples were dried in a Speedvac concentrator rather than under liquid N2. Measurements were conducted in duplicate for three separate experiments, on $3x10^5$ cells per sample. PIP, $PIP_2$ and $PIP_3$ response ratios were calculated by dividing PIP, $PIP_2$ and $PIP_3$ response areas by the corresponding response areas of $PIP_2$ (for PIP and $PIP_2$) and $PIP_3$ ($PIP_3$ only) ISD in each sample. PIP, $PIP_2$, and $PIP_3$ responses were normalised to PI response ratio to account for any cell input variability.

### 2.5.8 Crystal Violet staining

Crystal violet staining was used for assaying cell survival in response to treatment with MK2206. Cells were fixed with 4% PFA for 20 min, followed by staining with 0.05% crystal violet in 20% ethanol for 30 min. Cells were washed with $dH_20$ and let to dry overnight and images were acquired using the Odyssey imaging system.

### 2.5.9 Statistical analysis

All data were analysed using GraphPad Prism. Data are expressed as means ± the standard error of the mean (SEM). Statistical analysis was carried out using one-way ANOVA, followed by a post-hoc Tukey test if the ANOVA indicated significance. Differences were considered significant at $p < 0.05$.

## 2.6 Results:

### 2.6.1 PINK1 activates Akt in a PINK1 kinase-dependent manner

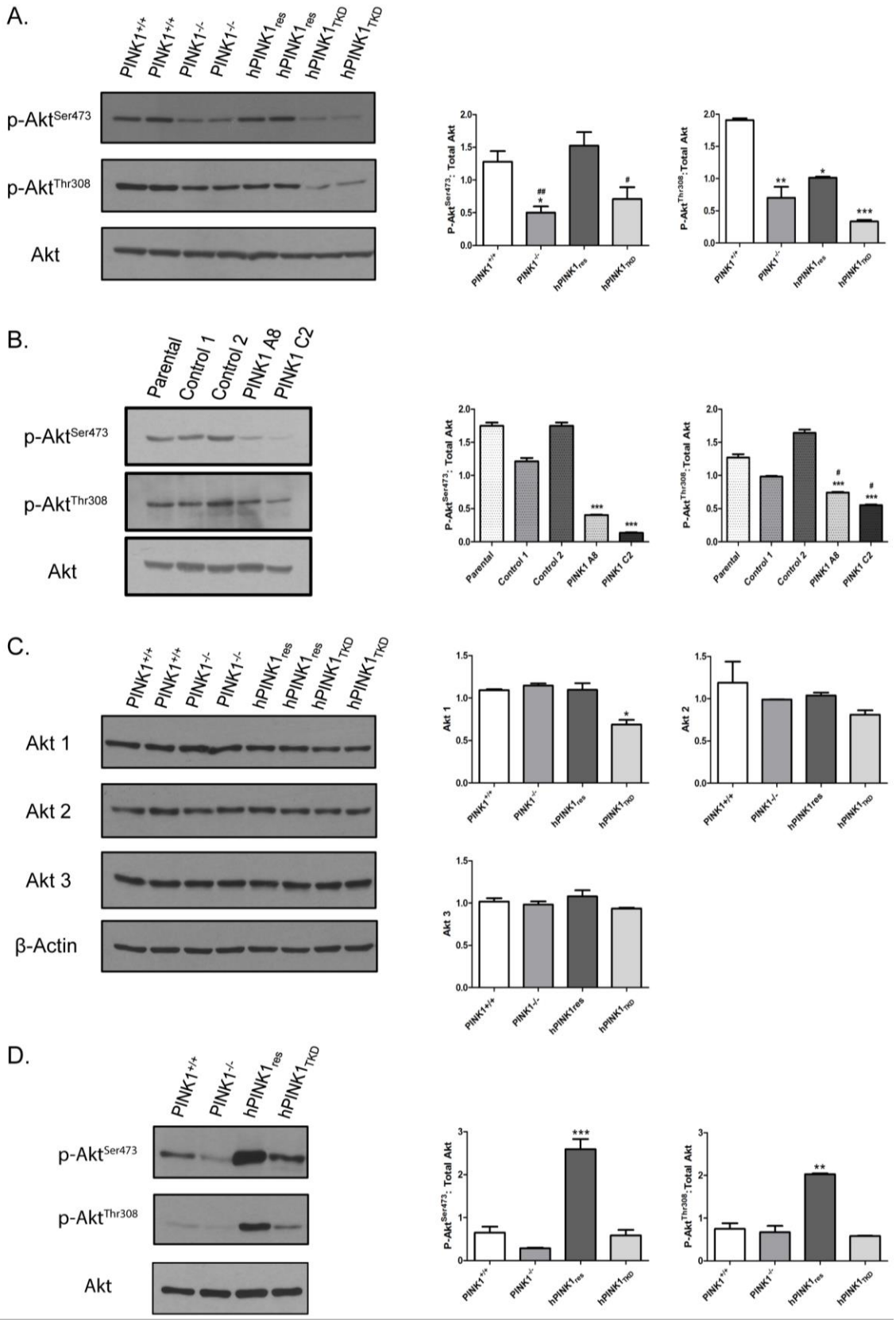
Initial results show that terminally-differentiated mouse embryonic fibroblasts (MEFs) derived from $PINK1^{-/-}$ mice (Morais et al., 2009, O'Flanagan et al., 2015) displayed significantly reduced Akt phosphorylation at both $Ser^{473}$ and $Thr^{308}$ activating residues, when cultured in normal growth medium (Fig. 2.1A). Total Akt levels were equivalent in $PINK1^{-/-}$ and $PINK1^{+/+}$ cells, indicating that deletion of PINK1 significantly reduced activation of Akt (phospho-Akt/Akt ratio). These findings were confirmed in BE(2)-M17 neuroblastoma cells transduced with shRNA to PINK1 (Fig. 2.1B). Stable expression of human PINK1 ($hPINK1_{res}$) in $PINK1^{-/-}$ MEFs fully restored phosphorylation at the $Ser^{473}$ residue ($p < 0.05$), and partially restored phosphorylation at $Thr^{308}$ ($p = 0.09$) (Fig. 2.1A). In contrast, a triple kinase dead PINK1 mutant ($hPINK1_{TKD}$) did not restore phosphorylation at either residue (Fig. 2.1A). Deletion of PINK1 did not alter Akt 1, 2, 3 isoform levels, however expression of $hPINK1_{TKD}$ resulted in a significant decrease in the level of Akt1, which indicates PINK1 kinase activity can specifically regulate the levels of Akt1 (Fig. 2.1C). We further demonstrate that PINK1 overexpression in $PINK1^{-/-}$ MEFs resulted in constitutive activation of Akt at both activating residues, in the absence of serum, whereas Akt activation was markedly impaired in wild type, $PINK1^{-/-}$ and $hPINK1_{TKD}$ mutants (Fig. 2.1D). Total Akt levels were similar in all cell lines (Fig. 2.1D). Together, these data show that PINK1 activates Akt by inducing phosphorylation at both the mTORC2-dependent $Ser^{473}$ and PDK1-dependent $Thr^{308}$ activation sites, without affecting Akt isoform levels. Additionally, these results indicate that PINK1 can constitutively activate Akt, even in the absence of serum, and that this requires PINK1 kinase activity.

### 2.6.2 Plasma membrane localisation of Akt is enhanced by PINK1 kinase activity

GFP-Akt-PH transfection allows visualisation of Akt at the plasma membrane (Calleja et al., 2007, Ebner et al., 2017, Meyer et al., 2012), which is known to be critical for Akt activation (Bellacosa et al., 1998). We next examined whether increased Akt activation

**Figure 2.1 PINK1 is a major regulator of Akt activation.**

A Representative immunoblot analysis showing phosphorylation of Akt at $Ser^{473}$ and $Thr^{308}$ in $PINK1^{+/+}$, $PINK1^{-/-}$, $hPINK1_{res}$ and $hPINK1_{TKD}$ MEFs, which were grown for 24h in DMEM-Hi supplemented with 10% FBS. B Representative immunoblot analysis showing phosphorylation of Akt at $Ser^{473}$ and $Thr^{308}$ in BE M17s, which were grown for 24h in DMEM-Hi supplemented with 10% FBS. C Representative immunoblot analysis showing levels of Akt 1, 2, and 3 in $PINK1^{+/+}$, $PINK1^{-/-}$, $hPINK1_{res}$ and $hPINK1_{TKD}$ MEFs, which were grown for 24h in DMEM-Hi supplemented with 10% FBS. D Representative immunoblot analysis showing phosphorylation of Akt at $Ser^{473}$ and $Thr^{308}$ in $PINK1^{+/+}$, $PINK1^{-/-}$, $hPINK1_{res}$ and $hPINK1_{TKD}$ MEFs, which were grown in the absence of serum. Data information: In A-C, data are presented in corresponding graphs as mean ± SEM (n = 3 biological replicates for each). *=p<0.05, **=p<0.01, and ***=p<0.0001 with respect to $PINK1^{+/+}$ MEFs. #=p<0.05 ##=p<0.01 with respect to $hPINK1_{res}$ MEFs (One-way ANOVA). In D, data are presented in corresponding graphs as mean ± SEM (n = 3 biological replicates). **=p<0.01, and ***=p<0.0001 with respect to $PINK1^{+/+}$, $hPINK1_{res}$ and $hPINK1_{TKD}$ MEFs.


A.
PINK1+/+
PINK1+/+
PINK1-/-
PINK1-/-
hPINK1res
hPINK1res
hPINK1TKD
hPINK1TKD
p-AktSer473
p-AktThr308
Akt
P-AktSer473: Total Akt
P-AktThr308: Total Akt
B.
Parental
Control 1
Control 2
PINK1 A8
PINK1 C2
C.
Akt 1
Akt 2
Akt 3
β-Actin
D.

induced by PINK1 was mechanistically associated with increased recruitment of Akt to the plasma membrane. Our results show that, under normal growth conditions, GFP-Akt-PH was localised to the plasma membrane at a number of focal points, where clear areas with increased GFP-Akt-PH were evident in PINK1$^{+/+}$ and hPINK1$_{res}$ cells, but not in PINK1$^{-/-}$ and hPINK1$_{TKD}$ MEFs (Fig. 2.2A). This was visualised by analysis of the fluorescence intensity along a line that bisected the cell, where sharp peaks of fluorescence intensity were observed where the line crosses the plasma membrane in PINK1$^{+/+}$ and hPINK1$_{res}$ cells but not in PINK1$^{-/-}$ and hPINK1$_{TKD}$ MEFs (Supp. 2.1A). The percentage of cells with GFP-Akt-PH at the plasma membrane was quantified, revealing a significant reduction in PINK1$^{-/-}$ and hPINK1$_{TKD}$ MEFs ($p < 0.0001$) in comparison to PINK1$^{+/+}$ and hPINK1$_{res}$ MEFs (Fig. 2.2B).

This impairment in GFP-Akt-PH localisation to the plasma membrane was also evident in PINK1$^{-/-}$ and hPINK1$_{TKD}$ cells in response to the agonist IGF-1, a major activator of Akt (Fig. 2.3). In unstimulated cells, GFP-Akt-PH was distributed diffusely throughout the cytoplasm in all four MEF cell lines (Fig. 2.3A). After 2-min stimulation with IGF-1, a rapid and significant increase in the percentage of cells with GFP-Akt-PH at the plasma membrane was evident in both PINK1$^{+/+}$ and hPINK1$_{res}$ MEFs. This was significantly lower ($p < 0.0001$), remaining at baseline levels, in both PINK1$^{-/-}$ or hPINK1$_{TKD}$ MEFs (Fig. 2.3A, B). The percentage of cells displaying plasma membrane localisation of GFP-Akt-PH in the PINK1$^{-/-}$ and hPINK1$_{TKD}$ cells increased following 10-min IGF-1 stimulation but remained significantly lower than that observed in PINK1$^{+/+}$ and hPINK1$_{res}$ MEFs cell lines ($p = 0.0002$) (Fig. 2.3B). Plasma membrane localisation of full length GFP-Akt is also enhanced by PINK1 kinase activity in the same manner, verifying that this is not due to truncation of Akt (Supp. 2.2). Thus, it can be concluded that the localisation of Akt at the plasma membrane, which is known to be critical for Akt activation, is regulated by PINK1 kinase activity, both under normal growth conditions and in response to short-term stimulation with IGF-1.

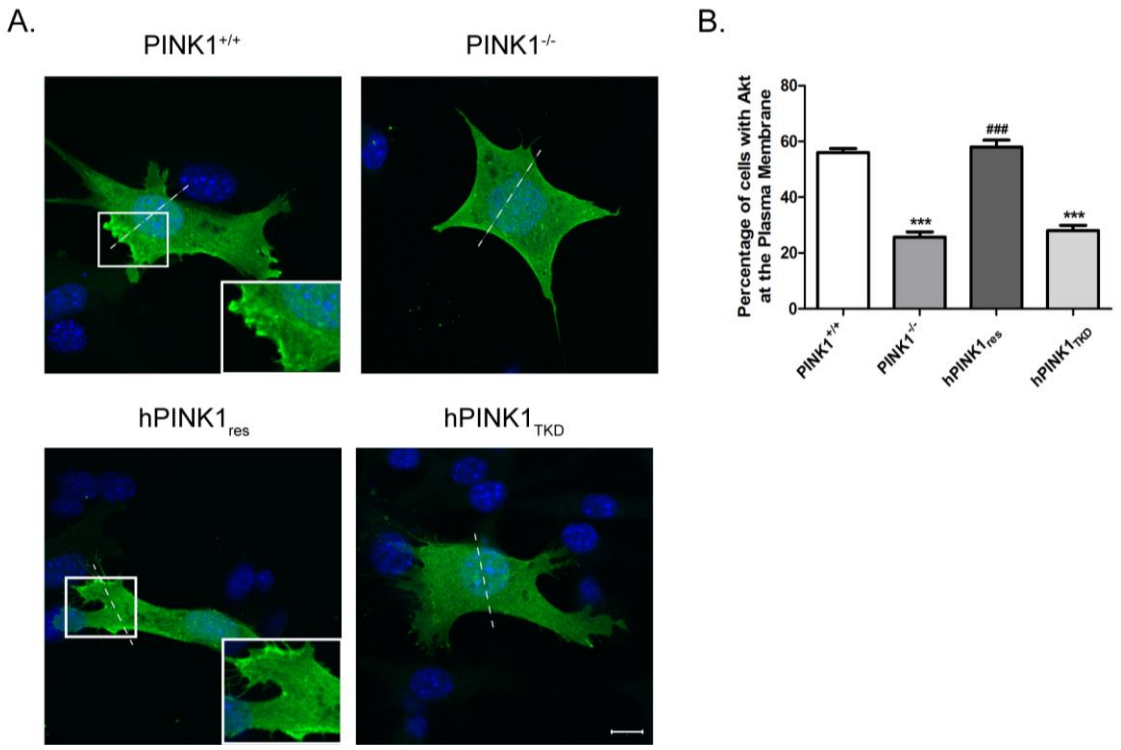
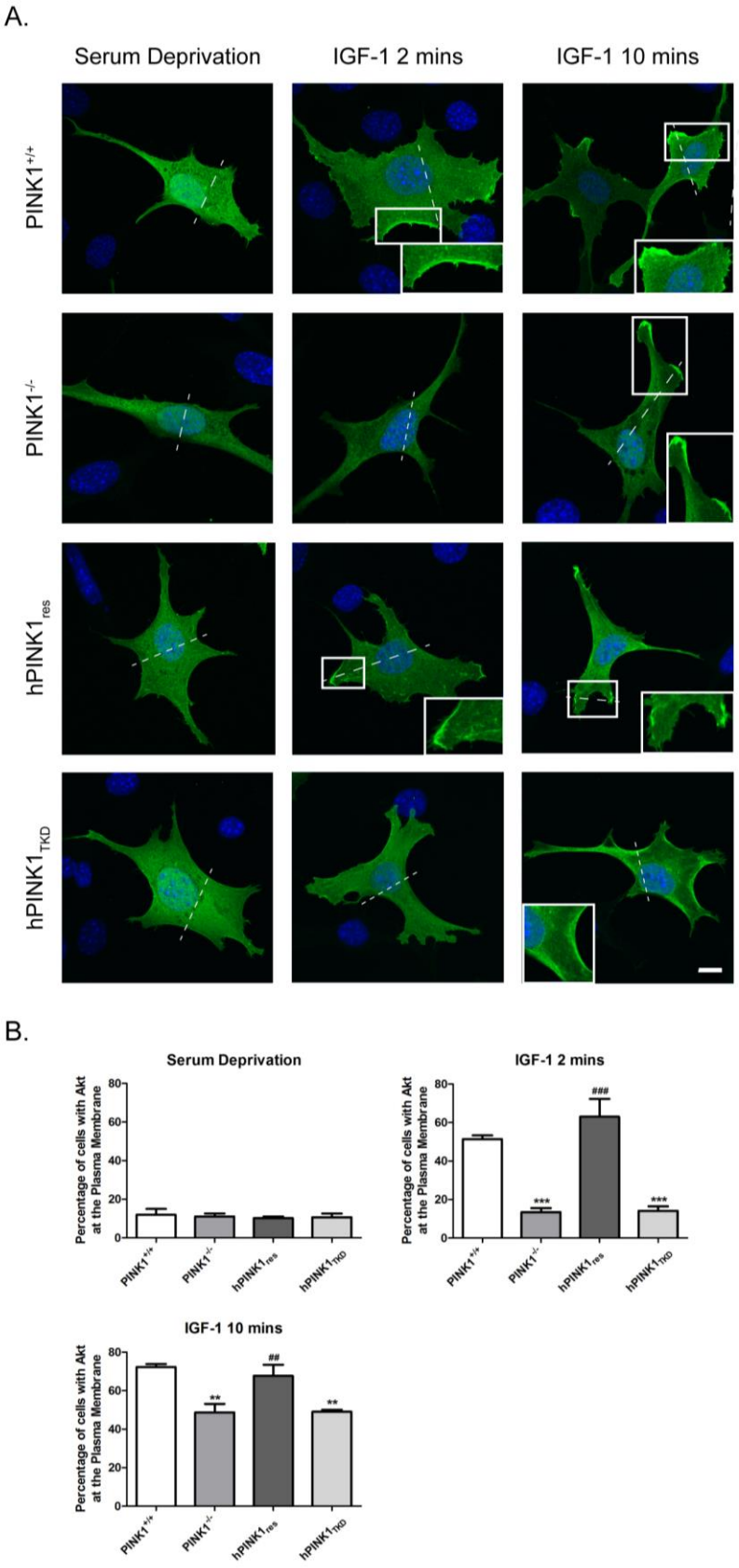



**Figure 2.2 PINK1 regulates the localisation of Akt to the plasma membrane.**

A Representative confocal images showing GFP-Akt-PH localisation in PINK1$^{+/+}$, PINK1$^{-/-}$, hPINK1$_{res}$ and hPINK1$_{TKD}$ MEFs, which were grown in DMEM-Hi supplemented with 10% FBS, transfected with GFP-Akt-PH for 24h and stained with DAPI (blue). Scale bars 10 μm. B Histogram depicting the percentage of transfected cells with Akt accumulations at the plasma membrane. Data information: In B data are presented as mean ± SEM (n = 3 biological replicates for each). ***=$p<0.0001$ with respect to PINK1$^{+/+}$ MEFs. ###=$p<0.0001$ with respect to PINK1$^{-/-}$ MEFs and hPINK1$_{TKD}$ MEFs (One-way ANOVA).

**Figure 2.3 PINK1 decreases the time taken for Akt to get to the plasma membrane in response to IGF-1 stimulation.**

A Representative confocal images showing GFP-Akt-PH localisation in $PINK1^{+/+}$, $PINK1^{-/-}$, $hPINK1_{res}$ and $hPINK1_{TKD}$ MEFs, following transfection with GFP-Akt-PH for 24 h and stained with DAPI (blue). Serum-starved cells were stimulated with 10 ng/ml IGF-1 for the indicated times (n = 3 biological replicates). Scale bars 10 μm. B Histograms depicting the percentage of transfected cells with Akt accumulations at the plasma membrane. Data information: In B data are presented as mean ± SEM (n = 3 biological replicates). **=p<0.01, and ***=p<0.0001 with respect to $PINK1^{+/+}$ MEFs. ##=p<0.01 and ###=p<0.0001 with respect to $PINK1^{-/-}$ and $hPINK1_{TKD}$ MEFs (One-way ANOVA).


A.
Serum Deprivation
IGF-1 2 mins
IGF-1 10 mins
PINK1+/+
PINK1-/-
hPINK1res
hPINK1TKD
B.
Serum Deprivation
IGF-1 2 mins
IGF-1 10 mins
Percentage of cells with Akt at the Plasma Membrane
PINK1+/+
PINK1-/-
hPINK1res
hPINK1TKD
###
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##

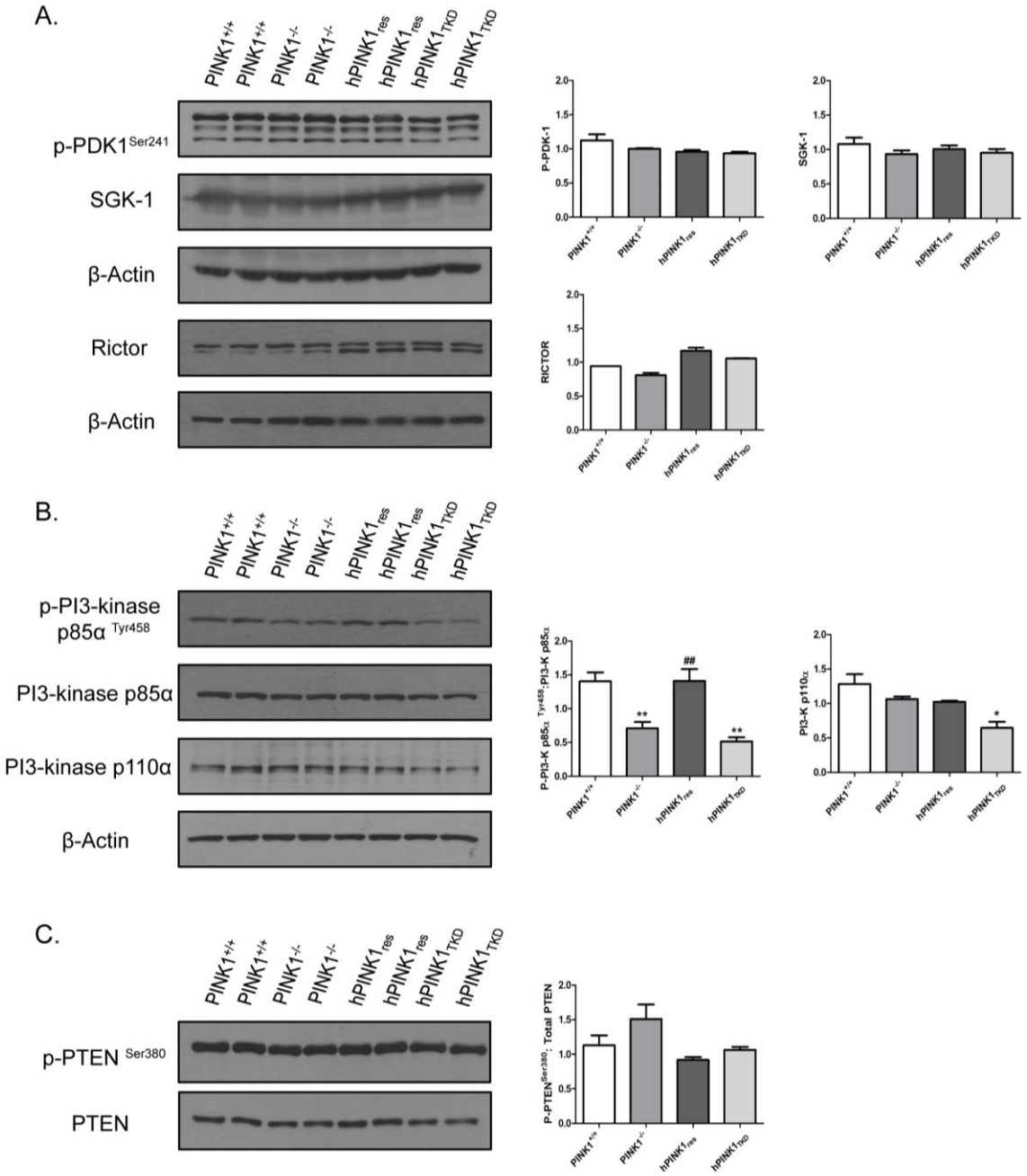
### 2.6.3 PI3-kinase p85α subunit phosphorylation is reduced in the absence of PINK1 kinase activity

Next we sought to identify mechanisms by which PINK1 activates Akt. Firstly we determined whether PINK1 regulated Akt activity by enhancing either PDK1- and /or mTORC2- induced phosphorylation of $Akt^{Thr308}$ and $Akt^{Ser473}$, respectively. PDK1 activation was assessed indirectly by measuring PDK-$1^{Ser241}$ phosphorylation (necessary for PDK-1 activation (Casamayor et al., 1999, Komander et al., 2005, Wick et al., 2003), SGK-1 (protein downstream of PDK-1 and regulated by its activation (Castel et al., 2016, Hall et al., 2012)) and mTORC2 via Rictor levels (Sarbassov et al., 2004). Comparative western immunoblot analysis revealed there was no significant alteration in $PDK1^{Ser241}$, SGK-1 or Rictor levels when comparing wild-type, $PINK1^{-/-}$, $hPINK1_{res}$ or $hPINK1_{TKD}$ cells (Fig. 2.4A).

Phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$ or $PIP_3$) accumulation in membranes is essential for the recruitment of Akt to membrane compartments for subsequent mTORC2- and PDK1-induced activation of Akt via phosphorylation of Akt at $Ser^{473}$ and $Thr^{308}$, respectively (Alessi et al., 1996, Manning and Toker, 2017). As PINK1 enhances Akt phosphorylation at both $Ser^{473}$ and $Thr^{308}$ sites, we hypothesised that PINK1 may regulate PI3-kinase and PTEN, the primary enzymes that control the synthesis and degradation of $PIP_3$, respectively (Hers et al., 2011). The levels of the PI3-kinase catalytic subunit, pl10α, were not altered by PINK1 expression (Fig. 2.4B). However, activation of the regulatory subunit PI3-kinase p85 (as measured by the phospho-p85 (pTyr458) (Aweida et al., 2018, Ibrahim et al., 2019, Pedrosa et al., 2019, Zheng et al., 2019) /p85α ratio) was significantly decreased by PINK1 deletion. This p85 activation was restored in $hPINK1_{res}$, but not in $hPINK1_{TKD}$ (Fig. 2.4B). In contrast, neither total PTEN levels, nor its phosphorylation at $Ser^{380}$ (inactivating residue), were significantly altered by PINK1 knockout or overexpression (Fig. 2.4C). Together these data indicate that activation of Akt by PINK1 may be mechanistically linked to PI3-kinase phosphorylation, specifically the p85 regulatory subunit.

**Figure 2.4 Phosphorylation and activation of PI3-kinase is PINK1 kinase-dependent.**

A-C Representative immunoblot analysis showing relative expression of proteins which regulate Akt activation in $PINK1^{+/+}$, $PINK1^{-/-}$, $hPINK1_{res}$ and $hPINK1_{TKD}$ MEFs, which were grown in DMEM-Hi supplemented with 10% FBS. Data information: In A-C, data are presented in corresponding graphs as mean ± SEM (n = 3 biological replicates for each). *=$p < 0.05$ and **=$p < 0.01$ with respect to $PINK1^{+/+}$ MEFs and ##=$p < 0.01$ with respect to $PINK1^{-/-}$ and $hPINK1_{TKD}$ MEFs (One-way ANOVA).


A.
PINK1+/+
PINK1+/+
PINK1-/-
PINK1-/-
hPINK1res
hPINK1res
hPINK1TKD
hPINK1TKD
p-PDK1 Ser241
SGK-1
β-Actin
Rictor
β-Actin
P-PDK-1
SGK-1
RICTOR
B.
p-PI3-kinase p85α Tyr458
PI3-kinase p85α
PI3-kinase p110α
β-Actin
P-PI3-K p85α Tyr458:PI3-K p85α
PI3-K p110α
C.
p-PTEN Ser380
PTEN
P-PTENSer380: Total PTEN

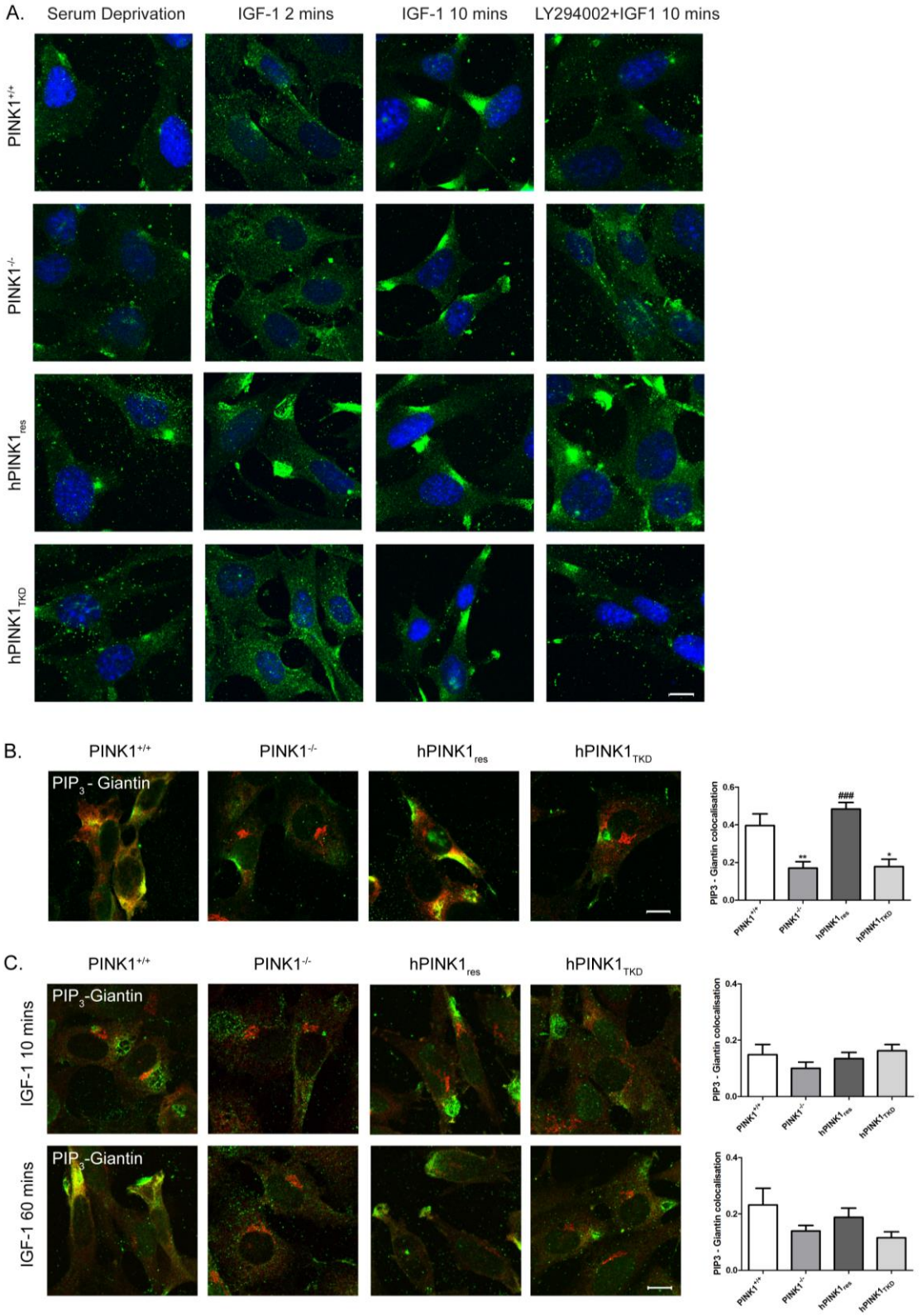
### 2.6.4 PINK1 kinase activity regulates the dynamic subcellular localisation of $PIP_3$ at the plasma membrane and Golgi in response to IGF-1.

Initial rapid plasma membrane and subsequent longer-term endomembrane localisation of $PIP_3$, particularly in the Golgi and ER, in response to agonists / growth factors are critical determinants of compartment-specific Akt activation (Ebner et al., 2017, Jethwa et al., 2015, Manning and Toker, 2017, Sato et al., 2003). We employed a $PIP_3$-specific monoclonal antibody (Braccini et al., 2015, Moult et al., 2010, Papakonstanti et al., 2007, Wang et al., 2010) to determine whether PINK1 modulated the subcellular localisation of $PIP_3$ under normal growth conditions and in response to short- (2, 10 min) and longer-term (60 min) IGF-1 stimulation. Following 24 h serum deprivation, $PIP_3$ was diffusely distributed throughout all cells (Fig. 2.5A). Non-uniform accumulations of $PIP_3$ at the plasma membrane in response to IGF-1 were evident in $hPINK1_{res}$ after 2 min, but not in $PINK1^{+/+}$, $PINK1^{-/-}$, or $PINK1_{TKD}$ MEFs (Fig. 2.5A). After 10 min of IGF-1 stimulation, $PIP_3$ was observed at specific plasma membrane domains in all four cell lines (Fig. 2.5A). Importantly, PI3-kinase inhibition, by pretreatment with LY294002 (10 μM), prevented production of $PIP_3$ at the plasma membrane in all three cell types. However, hPINK1res-expressing cells had distinctly increased $PIP_3$ levels near the plasma membrane, in the presence of LY294002 10 μM (Fig. 2.5A) and also using increased concentrations of LY294002 50 μM (data not shown).

We observed that $PIP_3$ localises predominantly in the perinuclear region during normal growth conditions (Fig. 2.5B) compared to its localisation in distinctive loci at the plasma membrane following 10-min stimulation with IGF-1 (Fig. 2.5A). To determine the subcellular localisation of $PIP_3$ in this perinuclear region, we performed double immunofluorescence co-localisation analysis of $PIP_3$ with a number of selective endomembrane markers (Rab14: early endosomes, Rab11a: recycling endosomes, GRP-78-BIP: endoplasmic reticulum, Giantin: medial-Golgi, TGN46: trans-Golgi and Lysotracker: lysosomes) (Supp. 2.3). We further sought to determine whether PINK1 deletion modified the localisation of $PIP_3$ within any specific endomembrane compartment. The results revealed a selective localisation of $PIP_3$ to the medial-Golgi,

**Figure 2.5 $PIP_3$ localisation is PINK1 kinase-dependent.**

A Representative confocal images showing $PIP_3$ localisation in $PINK1^{+/+}$, $PINK1^{-/-}$, $hPINK1_{res}$ and $hPINK1_{TKD}$ MEFs. Serum-starved cells were stimulated with 10 ng/ml IGF-1 for the indicated times, or pre-incubated with 10 μM LY294002 and subsequently stimulated with 10 ng/ml IGF-1, and immunostained for $PIP_3$ (green) and DAPI (blue) (n = 3 biological replicates). Scale bar 10 μm. B Representative confocal images showing $PIP_3$ localisation in $PINK1^{+/+}$, $PINK1^{-/-}$, $hPINK1_{res}$ and $hPINK1_{TKD}$ MEFs, which were grown in DMEM-Hi supplemented with 10% FBS, and immunostained for $PIP_3$ (green) and Giantin (red), the accompanying histogram shows the colocalisation percentage of $PIP_3$ and Giantin in each cell type (n = 3 biological replicates). Scale bar 10 μm. C Representative confocal images showing $PIP_3$ localisation in $PINK1^{+/+}$, $PINK1^{-/-}$, $hPINK1_{res}$ and $hPINK1_{TKD}$ MEFs. Serum-starved cells were stimulated with 10 ng/ml IGF-1 for the indicated times, and immunostained for $PIP_3$ (green) and Giantin (red), the accompanying histogram shows the colocalisation percentage of $PIP_3$ and Giantin (n = 3 biological replicates). Scale bar 10 μm. Data information: In B and C, data are presented as mean ± SEM. *=$p < 0.05$, **=$p < 0.01$ with respect to $PINK1^{+/+}$ MEFs and $hPINK1_{res}$ MEFs, ###=$p < 0.0001$ with respect to $PINK1^{-/-}$ MEFs (One-way ANOVA).


A.
Serum Deprivation
IGF-1 2 mins
IGF-1 10 mins
LY294002+IGF1 10 mins
PINK1+/+
PINK1-/-
hPINK1res
hPINK1TKD
B.
PINK1+/+
PINK1-/-
hPINK1res
hPINK1TKD
PIP3 - Giantin
PIP3 - Giantin colocalisation
0.6
0.4
0.2
0.0
**
###
*
C.
IGF-1 10 mins
IGF-1 60 mins
PIP3-Giantin

as the medial-Golgi protein, Giantin, displayed the greatest colocalisation with $PIP_3$ (42.9% in $PINK1^{+/+}$ and 48.3% $hPINK1_{res}$ cells) (Fig. 2.5B). Notably, there was a significant reduction in the colocalisation of $PIP_3$ and Giantin in $PINK1^{-/-}$ and $hPINK1_{TKD}$ MEFs (16.9% and 17.8%, respectively, $p < 0.0001$) (Fig. 2.5B). Furthermore, the colocalisaton of $PIP_3$ within the medial-Golgi was selective, since $PIP_3$ colocalisation with the trans-Golgi marker, TGN46, and all other endomembrane markers was significantly lower than that measured for Giantin (15-20%) and moreover, was not altered by PINK1 deletion or PINK1 kinase activity (Supp. 2.3). The only exception to this was a significant reduction of $PIP_3$ colocalisation with Rab14 in $PINK1^{-/-}$ cells ($p < 0.0005$) compared to wild-type MEFs, although the colocalisation of $PIP_3$ was rather low in these cells (27%). Moreover, the reduced colocalisation of $PIP_3$ with Rab 14 was only partially rescued by overexpression of PINK1 and was not PINK1 kinase-dependent (Supp. 2.3).

Interestingly a time lag for $PIP_3$ localisation to the Golgi after PDGF, EGF and insulin stimulation has been described (Ebner et al., 2017). We sought to determine if this was also apparent in response to IGF-1 and furthermore whether this was regulated by PINK1. Our results show that following short-term IGF-1 stimulation (10 min) the colocalisation of $PIP_3$ with Giantin was low in all cell lines ($PINK1^{+/+}$ 17.6%, $PINK1^{-/-}$ 10%, $hPINK1_{res}$ 15.7%, $hPINK1_{TKD}$ 17.4%) (Fig. 2.5C). However, longer-term stimulation with IGF-1 (60 min) resulted in a moderate increase in the colocalisation of $PIP_3$ with Giantin in wildtype and $hPINK1_{res}$ cells (26% and 20% respectively), but not in $PINK1^{-/-}$ and $hPINK1_{TKD}$ MEFs (13.9% and 11.5% respectively) (Fig. 2.5C). Together, these results indicate that $PIP_3$ localises to the medial-Golgi under normal growth conditions, and that PINK1 kinase activity plays a key role in regulating the localisation of $PIP_3$ to the medial-Golgi.

### 2.6.5 PINK1 colocalises with $PIP_3$ and protects against Golgi fragmentation with Akt dependency

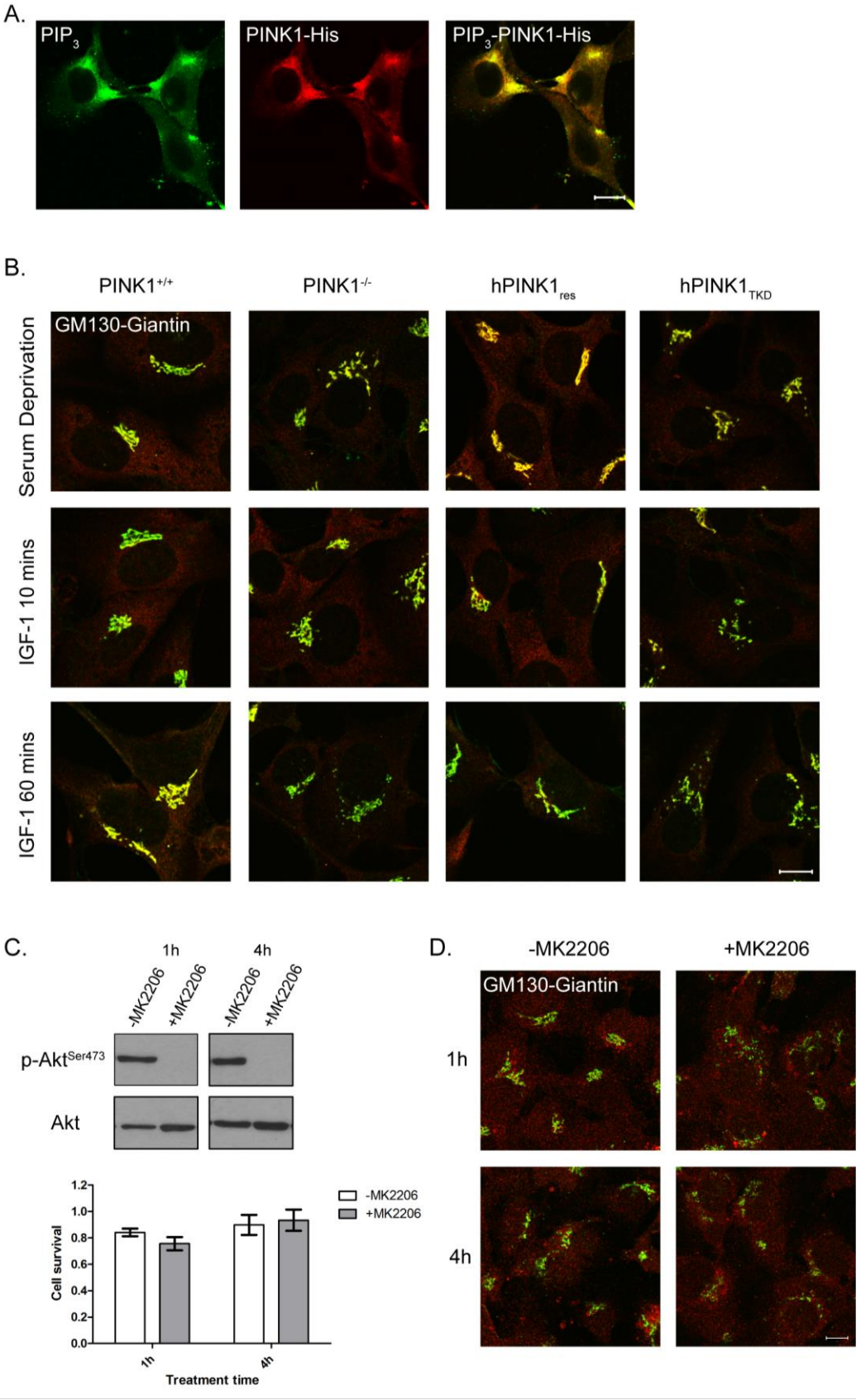
PINK1 has been reported to have a dynamic subcellular localisation including within the cytosol (Dagda et al., 2009, Dagda et al., 2014, Dickey and Strack, 2011, Fedorowicz et

al., 2014, Ries et al., 2006, Steer et al., 2015, Xiong et al., 2009), with increased localisation of PINK1 at the mitochondria when mitochondria are depolarised (Kane et al., 2014, Kazlauskaite et al., 2014, Kondapalli et al., 2012, Koyano et al., 2014, Matsuda et al., 2010, Shiba-Fukushima et al., 2012, Vincow et al., 2013, Ziviani et al., 2010). Due to our discovery that PINK1 could modulate $PIP_3$ localisation both at the plasma membrane and the medial-Golgi in a PINK1-kinase dependent fashion, we investigated the possible colocalisation of overexpressed His-tagged $hPINK1_{res}$ and $PIP_3$. Our results reveal a clear colocalisation of His-tagged $hPINK1_{res}$ and $PIP_3$ (Fig. 2.6A).

Golgi fragmentation is a known characteristic of PD neuropathology (Fujita et al., 2006, Gosavi et al., 2002, Rendon et al., 2013). Because of our findings that PINK1 could colocalise with $PIP_3$ and regulate levels of $PIP_3$ in the medial-Golgi, we were interested to determine if PINK1 regulated Golgi morphology. We employed both Giantin and GM130 to examine Golgi morphology and we found that $PINK1^{-/-}$ MEFs demonstrated significant levels of Golgi fragmentation during serum deprivation (Fig. 2.6B). Notably, Golgi fragmentation was rescued by hPINK1 overexpression and was PINK1 kinase-dependent (Fig. 2.6B). We further showed that this aberrant Golgi morphology was not altered by short- (10 min) or long-term (60 min) stimulation with IGF-1 in the absence of PINK1 kinase activity. We next hypothesised that increased PINK1 kinase-induced localisation of $PIP_3$ in the medial-Golgi protected against Golgi fragmentation via Akt activation. To investigate this, we treated $hPINK1_{res}$ MEFs with the selective Akt inhibitor MK2206 (Yap et al., 2011). Low concentrations (1 μM) of MK2206 prevented Akt activation with no significant effect on cell survival in $hPINK1_{res}$ MEFs (Fig. 2.6C). Furthermore, PINK1-induced protection from Golgi fragmentation, evident in $hPINK1_{res}$ MEFs, was completely blocked by Akt inhibition (Fig. 2.6D). Together, these results indicate that PINK1 kinase activity promotes Akt activity, that it dynamically regulates its major activating lipid $PIP_3$, particularly at the medial-Golgi, and that PINK1 kinase-dependent Akt activation protects against Golgi fragmentation.

**Figure 2.6 PINK1 colocalises with $PIP_3$ and protects against Golgi fragmentation in an Akt dependent manner.**

A Representative confocal images showing $PIP_3$ colocalising with PINK1 in $hPINK1_{res}$ MEFs, which were grown in DMEM-Hi supplemented with 10% FBS, and stained with antibodies to $PIP_3$ (green) and His (red) (n = 3 biological replicates). Scale bar 10 μm. B Representative confocal images showing GM130 and Giantin localisation in $PINK1^{+/+}$, $PINK1^{-/-}$, $hPINK1_{res}$ and $hPINK1_{TKD}$ MEFs. Serum-starved cells were stimulated with 10 ng/ml IGF-1 for the indicated times, and immunostained for GM130 (green) and Giantin (red) (n = 3 biological replicates). Scale bar 10 μm. C Representative immunoblot analysis showing phosphorylation of Akt at $Ser^{473}$ and $Thr^{308}$ in $hPINK1_{res}$ MEFs incubated with 1μM MK2206 for the indicated times. Histogram depicting cell survival as measured by crystal violet staining in $hPINK1_{res}$ MEFs incubated with 1μM MK2206 for the indicated times. D Representative confocal images showing GM130 and Giantin localisation in $hPINK1_{res}$ MEFs. Serum-starved cells were incubated with 1μM MK2206 for the indicated times, and immunostained for GM130 (green) and Giantin (red) (n = 3 biological replicates). Scale bar 10 μm.


A.
PIP3
PINK1-His
PIP3-PINK1-His
B.
PINK1+/+
PINK1-/-
hPINK1res
hPINK1TKD
GM130-Giantin
Serum Deprivation
IGF-1 10 mins
IGF-1 60 mins
C.
1h
4h
-MK2206
+MK2206
-MK2206
+MK2206
p-AktSer473
Akt
Cell survival
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1h
4h
Treatment time
-MK2206
+MK2206
D.
-MK2206
+MK2206
GM130-Giantin
1h
4h

### 2.6.6 PINK1 can modulate the cellular levels of $PIP_3$ and $PIP_2$

Finally, we were interested to determine whether PINK1 could directly regulate total cellular phosphatidylinositol (PI) levels. Using advanced mass-spectrometry approaches (Kielkowska et al., 2014) we measured total levels of phosphatidylinositol (PI), phosphatidylinositol phosphate (PIP), $PIP_2$ and $PIP_3$ in wild type and PINK1-modified MEFs under normal growth conditions, following 24 h serum deprivation and upon 10-min IGF-1 stimulation. $PIP_3$ levels were significantly reduced in $PINK1^{-/-}$ MEFs in normal growth medium (but not in serum deprivation conditions or in response to IGF-1 stimulation); this was partially rescued by $hPINK1_{res}$ but was not kinase-dependent (Supp. 2.4B). $PIP_2$ levels were significantly lower in $PINK1^{-/-}$ cells in response to IGF-1 stimulation (but not in normal growth medium or under serum deprivation conditions) and this was partially rescued by $hPINK1_{res}$ but was not kinase-dependent (Supp. 2.4C). There were no significant differences in levels of PI and PIP between $PINK1^{+/+}$, $PINK1^{-/-}$, $hPINK1_{res}$ and $hPINK1_{TKD}$ cells (Supp. 2.4D, E). Thus, PINK1 kinase activity can selectively modulate the total cellular levels of $PIP_2$ and $PIP_3$, under certain conditions. However, this does not appear to directly underpin the regulation of normal and IGF-1-induced subcellular localisation of $PIP_3$ by PINK1 kinase activity.

## 2.7 Discussion

The serine threonine kinase PINK1 has been extensively investigated since its discovery as an autosomal recessive PD-causing gene (Valente et al., 2004). PINK1 activates and interacts with PI3-kinase/Akt signalling to induce cell survival, mitochondrial integrity and stress resistance (Akundi et al., 2012, Boonying et al., 2019, Contreras-Zarate et al., 2015, Ellis et al., 2013, Hauser et al., 2017, Maj et al., 2010, Mei et al., 2009, Murata et al., 2011, O'Flanagan et al., 2015, O'Flanagan and O'Neill, 2014, Sanchez-Mora et al., 2012, Soutar et al., 2018). Notably, Akt activity is significantly reduced in both *in vitro* PD models and human dopaminergic PD substantia nigral neurons (Malagelada et al., 2008, Timmons et al., 2009). However, the mechanisms by which Akt activation becomes impaired due to loss of PINK1 function in PD are unclear. In this study, we show that PINK1 is a primary upstream activator of Akt during normal growth, and constitutively activates Akt in the absence of growth factors. We reveal for the first time that promotion of Akt activation by PINK1 is PINK1 kinase-dependent, and that PINK1 kinase activity enhances Akt recruitment to the plasma membrane during normal growth and in response to IGF-1. We show that PINK1 regulates the phosphorylation of PI3-kinase p85, colocalises with its product $PIP_3$, the lipid essential for Akt membrane recruitment, vital for Akt activation (Manning and Toker, 2017). Furthermore, we show that PINK1 dynamically increases $PIP_3$ levels at the plasma membrane and Golgi in response to short- and longer-term IGF-1 stimulation, respectively, and during normal growth. We demonstrate that PINK deletion induces Golgi fragmentation, a known characteristic of PD neuropathology. Importantly, we show that PINK1 overexpression restores normal Golgi morphology and that this depends on activation of PINK1 kinase and of Akt. Together these data provide novel insights into mechanisms by which loss of PINK1 function causes Akt signalling defects in PD.

Full Akt activation requires mTORC2-induced phosphorylation of Akt at $Ser^{473}$ in the C-terminal hydrophobic motif (Alessi et al., 1996, Sarbassov et al., 2005, Stokoe et al., 1997) and PDK-1 phosphorylation of Akt at $Thr^{308}$ in the activation loop (Calleja et al., 2007, Stokoe et al., 1997), for review (Manning and Toker, 2017). MEFs derived from $PINK1^{-/-}$ mice display impaired IGF-1-dependent Akt phosphorylation at both

$Ser^{473}$/$Thr^{308}$ activating residues (Akundi et al., 2012). The present study is the first to reveal that phosphorylation at both Akt-activating residues in $PINK1^{-/-}$ MEFs can be rescued by PINK1 overexpression and is PINK1 kinase-dependent. Our results further indicate that the impact of PINK1 appears to be more prominent for phosphorylation at $Ser^{473}$ than $Thr^{308}$. Notably, the activation of Akt is extremely diminished in the absence of $Ser^{473}$ phosphorylation (Alessi et al., 1996, Yang et al., 2002). One recent study concluded that $PIP_3$ activation of Akt is predominantly due to recruitment of Akt to $PIP_3$ in membranes to facilitate PDK1 and mTORC2 catalysed Akt phosphorylation (Chu et al., 2018). However, in contrast, another recent study found that $PIP_3$ binding allosterically activates Akt and that dissociation from $PIP_3$ is rate limiting for Akt dephosphorylation (Ebner et al., 2017). It is thus possible that our results indicate that PINK1 inhibits $PIP_3$ dissociation that favours Akt phosphorylation at $Ser^{473}$ over $Thr^{308}$ by more effectively blocking $Ser^{473}$ dephosphorylation. The more pronounced effect of PINK1 on $Ser^{473}$ phosphorylation may be further due to regulation of a number of properties reported for $Ser^{473}$ phosphorylation (Chu et al., 2018, Lucic et al., 2018, Manning and Toker, 2017) or possible regulation by mTORC2 (Murata et al., 2011).

Furthermore, our results demonstrate that PINK1 can constitutively activate Akt, increasing phosphorylation at both activating residues, even in the absence of growth factors, and that this is dependent on PINK1 kinase activity. This places PINK1 kinase as a primary upstream activator of Akt, in agreement with studies which showed that PINK1 is necessary for maximal responses of Akt to growth factors (Akundi et al., 2012, Contreras-Zarate et al., 2015, Ellis et al., 2013, Maj et al., 2010, Manning and Toker, 2017, Mei et al., 2009, Murata et al., 2011, Sanchez-Mora et al., 2012), preventing apoptosis, promoting cell survival and protecting against several cell stressors (Akundi et al., 2012, Contreras-Zarate et al., 2015, Murata et al., 2011, Sanchez-Mora et al., 2012). Importantly, it has been shown that PINK1 mRNA expression is induced by FOXO3a, a major stress-resistant transcription factor, which is up-regulated in the absence of Akt activation when growth factors and nutrients are absent (Mei et al., 2009). Our results showing that PINK1 can activate Akt constitutively when growth factors and nutrients are absent may indicate a role for this pathway in the short-term protection necessary to sustain cells until growth factors are

available. The PI3-kinase/Akt pathway is intimately linked with mitochondrial respiration, prevention of mitochondrial apoptosis, and metabolic rewiring via enhanced glycolysis, including in cancer cells (for review (O'Flanagan and O'Neill, 2014)). PINK1 has been most studied as a key regulator of mitochondrial health, particularly in the regulation of mitophagy via the PINK1/parkin mitophagy pathway (Harper et al., 2018, Kane et al., 2014, Kawajiri et al., 2010, Kazlauskaite et al., 2014, Kondapalli et al., 2012, Matsuda et al., 2010, Pilsl and Winklhofer, 2012, Soutar et al., 2018, Vincow et al., 2013, Youle and Narendra, 2011, Youle and van der Bliek, 2012). Previous studies showed that inhibition of Akt with MK2206 blocks the PINK1/parkin pathway (Hauser et al., 2017, McCoy et al., 2014). More recent studies show that Akt signalling regulates PINK1-dependent mitophagy (Soutar et al., 2018). In these contexts, Akt activation was placed upstream of PINK1 and it was recognised that the reciprocal regulation of PINK1 by Akt and Akt by PINKI is important in mitophagy regulation (Soutar et al., 2018).

Our major aim was to determine the mechanism by which PINK1 promotes Akt activation. We hypothesised that PINK1 may induce both PDK-1 phosphorylation of Akt at $Thr^{308}$ and mTORC2-induced phosphorylation of Akt at $Ser^{473}$ (Alessi et al., 1996, Sarbassov et al., 2005, Stokoe et al., 1997). Interestingly, previous studies showed that PINK1 can induce mTORC2 activation of $Ser^{473}$ in the absence of $Thr^{308}$ phosphorylation. This was indicated to be via Rictor, and to occur independently of PI3-kinase (Murata et al., 2011). In contrast, we found that PINK1-induced increased phosphorylation of Akt at both $Ser^{473}$ and $Thr^{308}$ residues was not associated with altered levels of active PDK1 or of Rictor. The discrepancies between these studies regarding the mechanisms by which PINK1 can activate Akt may be due to cell- or agonist-specific effects.

Dynamic, rapid, and transient agonist-induced accumulation of Akt at the plasma membrane is essential for growth factor- and agonist-induced Akt activation (for review (Manning and Toker, 2017)), and can be monitored by GFP-Akt-PH transfection (Calleja et al., 2007, Ebner et al., 2017, Meyer et al., 2012). Our results provide novel data showing that PINK1 significantly accelerates this recruitment of GFP-Akt-PH and GFP-Akt to the plasma membrane with PINK1 kinase-dependency, both in normal

growth media and in response to shorter-term IGF-1 stimulation. The recruitment of Akt to the plasma membrane occurs via its PH domain and relies predominantly on increased, localised and rapid production of $PIP_3$ (Calleja et al., 2007, Manning and Toker, 2017). These initial rapid plasma membrane and subsequent longer-term endomembrane localisations of $PIP_3$, particularly in the Golgi and ER, in response to agonists / growth factors are critical determinants of compartment-specific Akt activation (Ebner et al., 2017, Jethwa et al., 2015, Manning and Toker, 2017, Sato et al., 2003). These results drew us to explore the possibility that PINK1 may regulate the production of $PIP_3$ within the plasma membrane and endomembranes. We show that PINK1 kinase activity accelerates the production of $PIP_3$ towards the plasma membrane in response to short-term stimulation by IGF-1 (2 and 10 min). Interestingly, several cells expressing $hPINK1_{res}$ had distinct areas of $PIP_3$ immunoreactivity near the plasma membrane, even in the presence of the PI3-kinase inhibitor. There may be both PI3-kinase dependent and independent mechanisms responsible for the PINK1-induced $PIP_3$ increases. The changes in PI3-kinase phosphorylation indicate that activation of Akt by PINK1 may be mechanistically linked to the p85 regulatory subunit. However, p85α/β has many functions that do not involve PI3-kinase activation, for example, p85α can regulate PTEN (Chagpar et al., 2010, Cheung et al., 2015, Rabinovsky et al., 2009) and the PI3-kinase p110α subunit catalytic activity can occur independent of p85α/β (Thorpe et al., 2017, Tsolakos et al., 2018).

We further show that His-tagged PINK1 displays a very strong co-localisation with $PIP_3$, throughout the cell during normal growth, further supporting PINK1's function as a regulator of $PIP_3$ within both plasma- and endo-membranes. A previous study using spatio-temporal examination of $PIP_3$ production in living cells, following ligand stimulation, demonstrated that $PIP_3$ levels increase to a larger extent in endomembranes (ER and Golgi) than at the plasma membrane (Sato et al., 2003). In agreement, we show that $PIP_3$ is predominantly localised in the perinuclear region during normal growth. We performed a detailed analysis to determine the endomembrane localisation of $PIP_3$ and demonstrate a strong selective co-localisation of $PIP_3$ with Giantin in the medial-Golgi compartment. Importantly, we further show that PINK1 deletion significantly and selectively reduces the localisation of $PIP_3$ to the medial-Golgi, during normal growth,

and moderately in response to longer-term IGF-1 stimulation (60 min), and show that this is PINK1 kinase-dependent. In agreement with our study, a time-lag for $PIP_3$ localisation to the Golgi has been shown following PDGF, EGF and insulin stimulation of Akt in HeLa and MCF-7 cells (Ebner et al., 2017). The Golgi plays a pivotal role in the compartmentalisation of cell signalling initiating at the plasma membrane (Peng et al., 2014). Our results thus indicate a new role for PINK1 kinase activity in the regulation of $PIP_3$ responses in the plasma membrane, Golgi and endomembranes, which are known to be important for both compartmentalised and temporal regulation of Akt substrate phosphorylation (Ebner et al., 2017, Jethwa et al., 2015, Manning and Toker, 2017, Sato et al., 2003). Furthermore, $PIP_3$ generation in situ, primarily in the Golgi and ER, has been shown to depend on endocytosis of activating receptor tyrosine kinases. Interestingly, in this respect it has been reported that PINK1 deficiency disturbs the intracellular localisation of the IGF-1 receptor (Contreras-Zarate et al., 2015).

Although we did not detect Akt in the Golgi network we do think there is potential it is also present here. The main tools employed were GFP-Akt and GFP-Akt-PH constructs, and we do not see any Golgi localisation with either of these. However, these constructs may favour the plasma membrane and the concentration of Akt may be higher at the plasma membrane than in endomembrane compartments in these PINK1 modified MEFs. Thus, the availability of more sensitive tools such as improved Akt antibodies may uncover Akt in the Golgi, and the endosomal-ER-Golgi network.

In healthy cells, the Golgi apparatus is a ribbon-like structure made up of flattened, parallel cisternae which are interconnected in the perinuclear region (Nakagomi et al., 2008) as we observe in wild type MEFs. Numerous studies have shown that during cell stress or apoptotic cell death, Golgi stacks disassemble into tubulovesicular clusters, a process known as fragmentation of the Golgi apparatus (Alvarez-Miranda et al., 2015, Chiu et al., 2002, Fan et al., 2008, Lane et al., 2002, Machamer, 2003). Golgi fragmentation has been reported in nigral neurons in postmortem samples of PD brain (Fujita et al., 2006) and in in vitro PD models (Gosavi et al., 2002, Rendon et al., 2013). Furthermore, bioinformatics modelling has linked stress-induced Golgi fragmentation to a number of neurodegenerative processes (Alvarez-Miranda et al., 2015). We reveal for

the first time that PINK1 deletion causes significant Golgi fragmentation, and importantly, we show that this is prevented by PINK1 overexpression and dependent on PINK1 kinase activity. This is a significant new function for PINK1 and furthermore is linked mechanistically to impaired Akt signalling caused by PINK1 deletion. Thus, we show that Akt inhibition in cells overexpressing PINK1 prevents the ability of PINK1 to protect against Golgi fragmentation. Interestingly, inhibition of $PI(4,5)P_2$ synthesis in GH3 cells has been reported to lead to Golgi fragmentation, linking phosphoinositides with the structural integrity of the Golgi apparatus (Siddhanta et al., 2003).

In this study, we demonstrate that impaired activation of Akt, via loss of PINK1 kinase function, as occurs in PD, centers on defects in $PIP_3$ regulation upstream of Akt activation. Our mass spectrometry analysis showed that total levels of $PIP_3$ and $PIP_2$, but not PIP and PI, lipids were modulated by PINK1 deletion in some contexts, but not regulated in a PINK1 kinase-dependent manner. This infers that PINK1 kinase regulates selective membrane pools of $PIP_3$ rather than total $PIP_3$ levels. It is important to note that several risk genes for PD regulate the PI3-kinase/Akt signalling axis, including *PRKN* (Fallon et al., 2006, Gupta et al., 2017), *DJ-1* (Jaramillo-Gomez et al., 2015, Kim et al., 2005, Zhang et al., 2016), *LRKK2* (Chuang et al., 2014, Ohta et al., 2011) and *SNCA* (Cheung et al., 2015, Seo et al., 2002). Importantly, defects in Akt signalling occur in nigral neurons in the PD brain (Malagelada et al., 2008, Timmons et al., 2009), drawing attention to the relevance of targeting this vital survival and metabolic system for neuroprotection in PD. Phosphoinositide-protein interactions are at the hub of both Akt and other major signalling axes and play integral roles in vesicular trafficking. These are key systems known to be regulated by PD risk genes (Cao et al., 2017, Krebs et al., 2013, Vanhauwaert et al., 2017) and to become impaired in PD (Kutateladze, 2010, Wenk and De Camilli, 2004). Our study provides new mechanistic insights for a modulation of the core $PIP_3$-Akt regulatory machinery by PINK1 kinase activity, presenting a novel mechanism that may underlie impaired activation of the vital Akt signalling machinery in PD. This draws attention to the integration of phosphoinositide and protein networks in the understanding and therapeutic targeting of key systems, including Akt, that become defective in the neurodegenerative processes underlying PD.

## 2.8 Acknowledgments

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## 2.9 Author contributions

Rachel Furlong: study conception, investigation, performed experiments, data and evidence collection, formal analysis, and manuscript preparation.

Andrew Lindsay: manuscript preparation, commentary, critical review.

Karen E. Andersson: mass spectrometry for phosphoinositide analysis, commentary, critical review

Phillip T. Hawkins: mass spectrometry for phosphoinositide analysis, commentary, critical review

Aideen Sullivan: study conception, supervision, manuscript preparation, commentary, critical review.

Cora O Neill: study conception, supervision, manuscript preparation, commentary, critical review.

## 2.10 Conflict of Interest

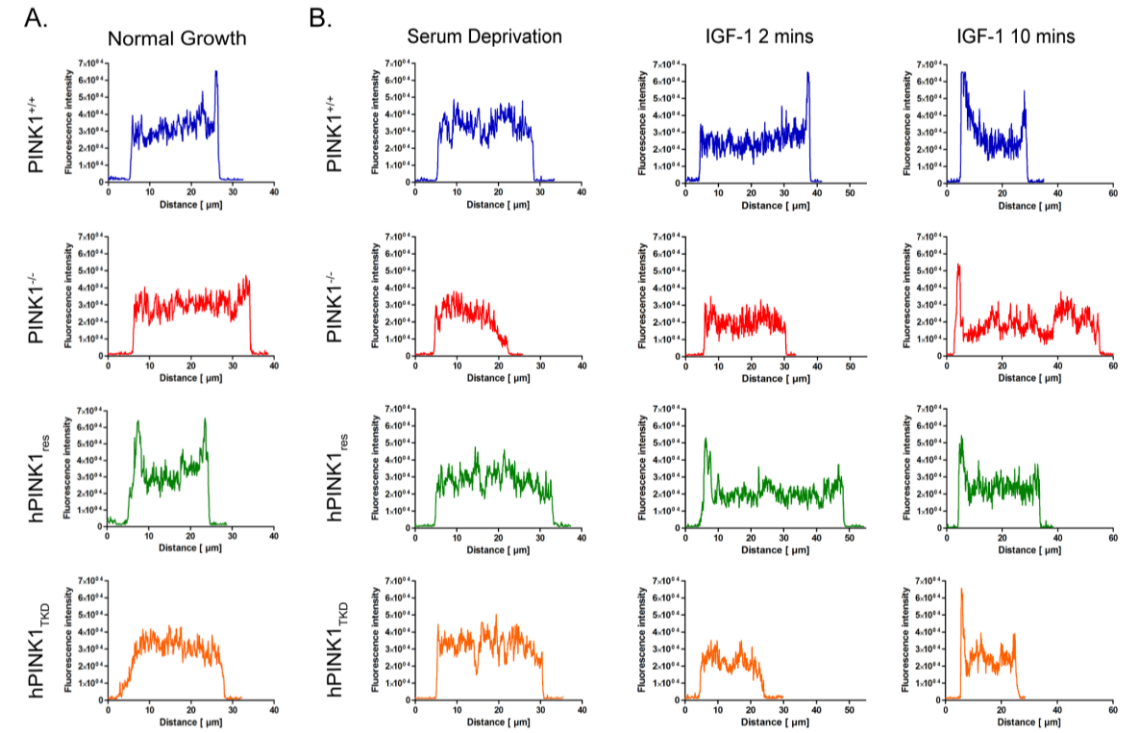
The authors declare no conflict of interest.

## 2.11 Supplementary Materials and Methods:

### 2.11.1 RT-PCR

| Primer Set | Sequences | Conditions |
|---|---|---|
| mPINK1 | F: 5’ GCTGATCGAGGAGAAGCAG 3’<br>R: 5’ GATAATCCTCCAGACGGAAGC 3’ | 95°C 15 min, [94°C 30 secs, 60°C 30 secs, 72°C 30 secs] 35 cycles. |
| hPINK1 | F: 5’ AGACGCTTGCAGGGCTTTC 3’<br>R: 5’ GGCAATGTAGGCATGGTGG 3’ | 95°C 15 min, [94°C 30 secs, 50°C 30 secs, 72°C 30 secs] 35 cycles. |

## 2.12 Supplementary Results:

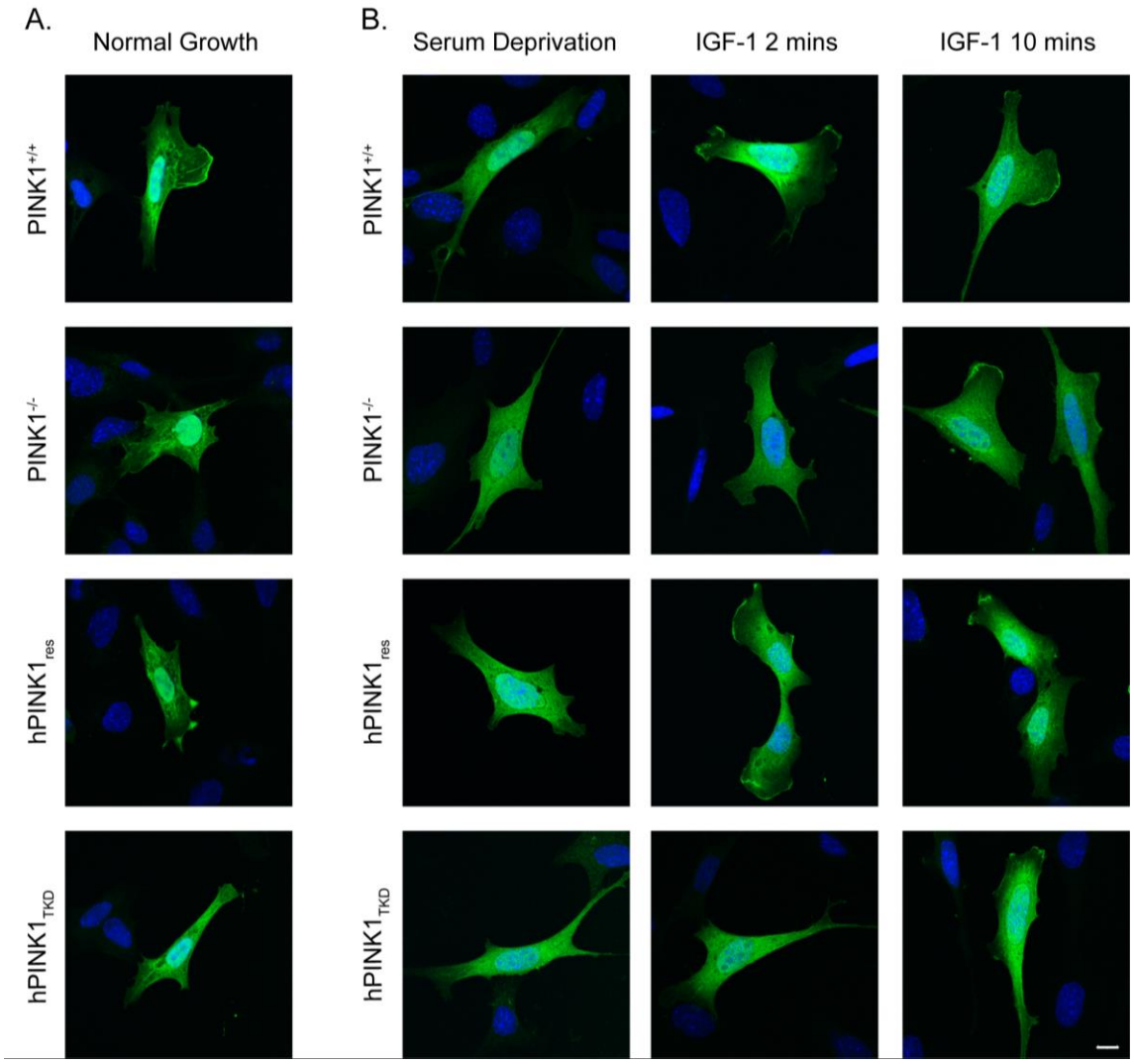



**Supplemental Figure 2.1 Increases in fluorescence intensity at the plasma membrane show that PINK1 decreases the time taken for Akt localisation to the plasma membrane in response to IGF-1 stimulation.**

Representative line plots indicating the fluorescence intensity of GFP-Akt-PH along the white lines shown in Figure 2 (A) and 3 (A). Normal growth MEFs were grown in DMEM-Hi supplemented with 10% FBS. Serum-starved cells were stimulated with 10 ng/ml IGF-1 for the indicated times and subsequently stimulated with 10 ng/ml IGF-1.

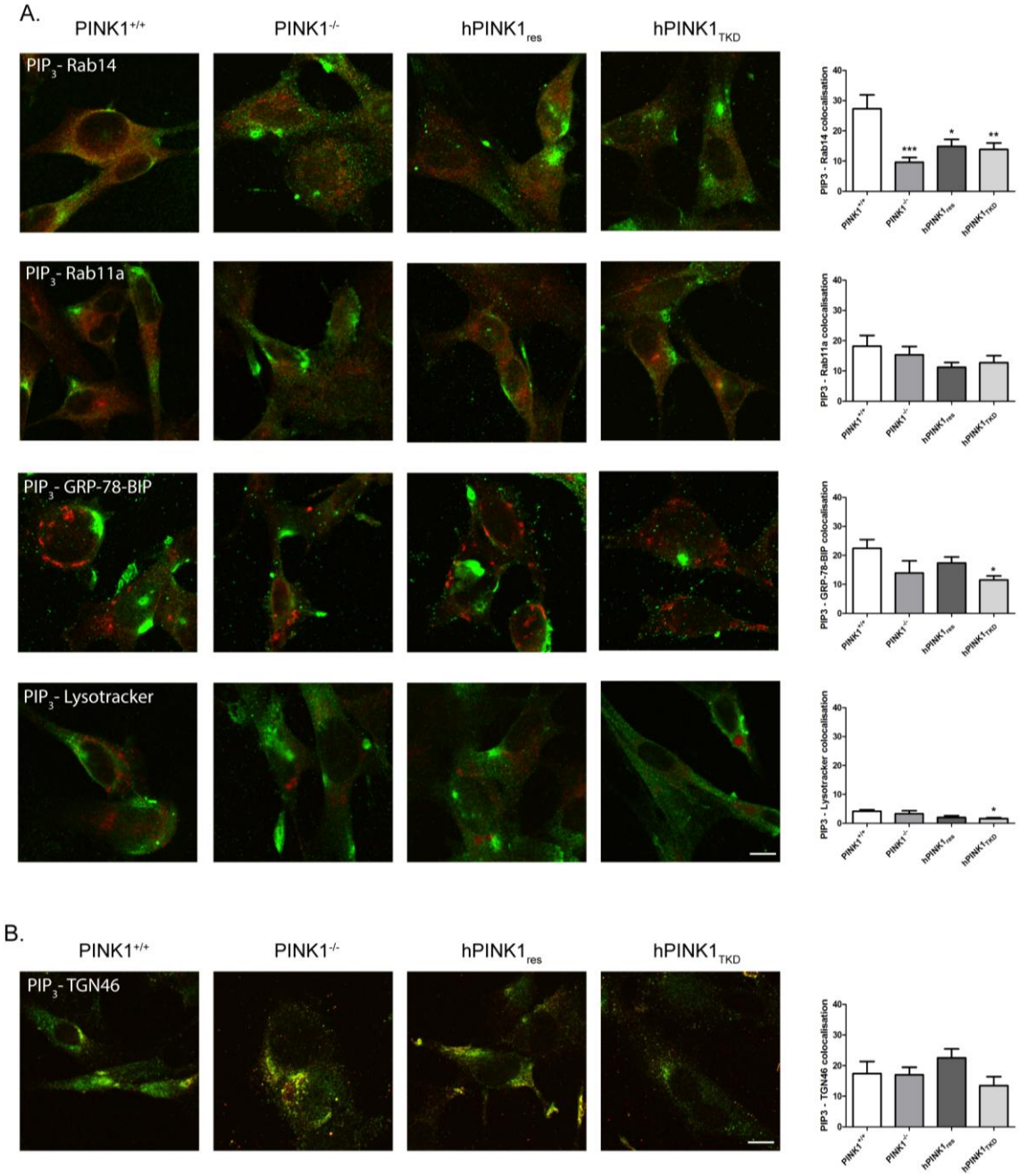
**Supplemental Figure 2.2 PINK1 regulates the localisation of Akt to the plasma membrane under normal growth conditions and decreases the time taken for Akt to get to the plasma membrane in response to IGF-1 stimulation.**

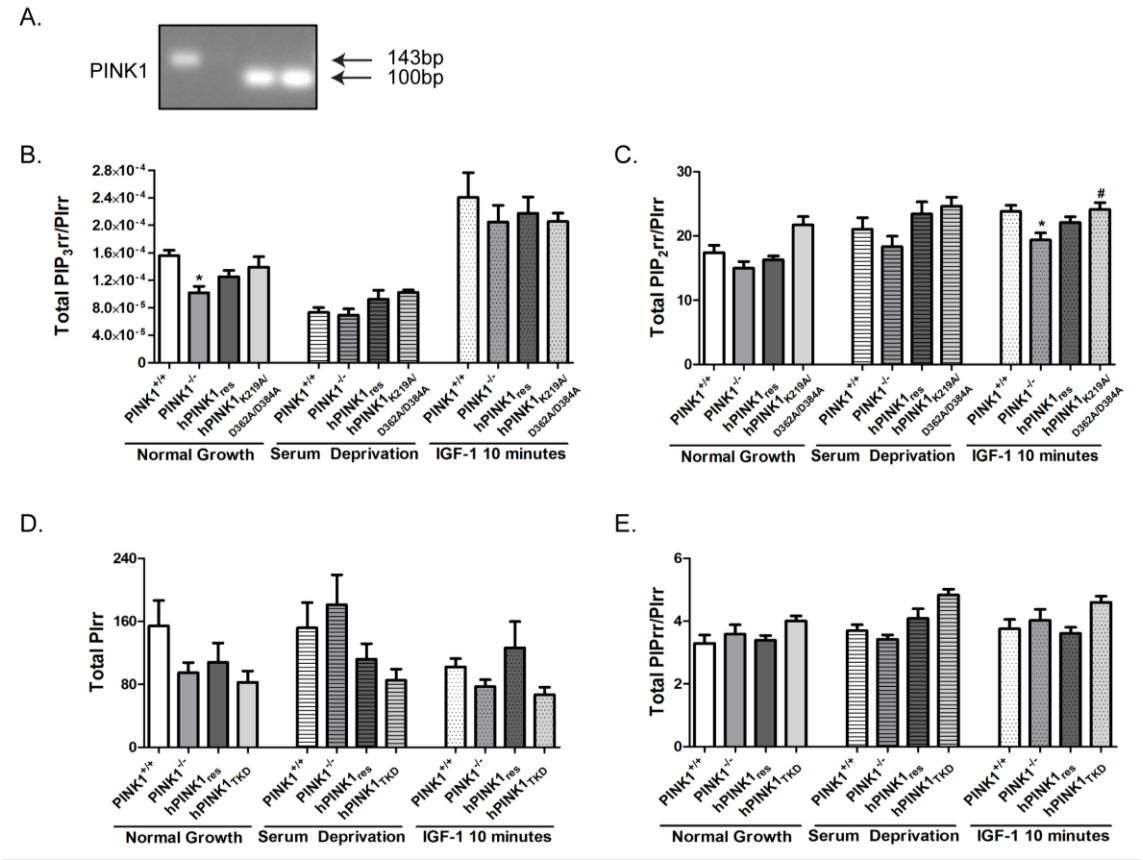
A Representative confocal images showing GFP-Akt localisation in $PINK1^{+/+}$, $PINK1^{-/-}$, $hPINK1_{res}$ and $hPINK1_{TKD}$ MEFs, which were grown in DMEM-Hi supplemented with 10% FBS, transfected with GFP-Akt for 24h and stained with DAPI (blue). B Representative confocal images showing GFP-Akt localisation in $PINK1^{+/+}$, $PINK1^{-/-}$, $hPINK1_{res}$ and $hPINK1_{TKD}$ MEFs, following transfection with GFP-Akt for 24 h and stained with DAPI (blue). Serum-starved cells were stimulated with 10 ng/ml IGF-1 for the indicated times (n = 3). Scale bar 10 μm.


A.
Normal Growth
B.
Serum Deprivation
IGF-1 2 mins
IGF-1 10 mins
PINK1+/+
PINK1-/-
hPINK1res
hPINK1TKD

**Supplemental Figure 2.3 Colocalisation analysis using endomembrane markers Rab14, Rab11a, GRP-78-BIP and Lysotracker reveals selective localisation of $PIP_3$ to the medial-Golgi.**

Representative confocal images showing $PIP_3$ colocalisation with endomembrane markers in $PINK1^{+/+}$, $PINK1^{-/-}$, $hPINK1_{res}$ and $hPINK1_{TKD}$ MEFs, which were grown in DMEM-Hi supplemented with 10% FBS. A, B Cells were immunostained for $PIP_3$ (green) and Rab 14, Rab 11a, GRP-78-BIP, Lysotracker and TGN46 (red). Scale bar 10 μm. Data information: In A, B, data are presented in corresponding graphs as mean ± SEM (n=3 for each). *=$p < 0.05$, **=$p < 0.01$, and ***=$p < 0.0001$ with respect to $PINK1^{+/+}$ MEFs (One-way ANOVA).


A.
PINK1+/+
PINK1-/-
hPINK1res
hPINK1TKD
PIP3 - Rab14
PIP3 - Rab11a
PIP3 - GRP-78-BIP
PIP3 - Lysotracker
PIP3 - Rab14 colocalisation
PIP3 - Rab11a colocalisation
PIP3 - GRP-78-BIP colocalisation
PIP3 - Lysotracker colocalisation
B.
PINK1+/+
PINK1-/-
hPINK1res
hPINK1TKD
PIP3 - TGN46
PIP3 - TGN46 colocalisation




**Supplemental Figure 2.4: PINK1 modification in MEFs, PINK1 modulates $PIP_3$ and $PIP_2$ levels.**

A. Agarose Gel showing PINK1 deletion and re-expression as confirmed by RNA extraction and RT-PCR analysis. Total $PIP_3$ (B), $PIP_2$ (C), PI (D), and PIP (E) levels as measured by mass spectrometry in $PINK1^{+/+}$, $PINK1^{-/-}$, $hPINK1_{res}$ and $hPINK1_{TKD}$ MEFs grown in DMEM-Hi supplemented with 10% FBS, serum deprived for 24h or stimulated with 10 ng/ml IGF-1 for 10 min (n = 3). Data information: In B-E, data are presented in corresponding graphs as mean ± SEM. *=$p < 0.05$ with respect to $PINK1^{+/+}$ MEFs, #=$p < 0.05$ with respect to $PINK1^{-/-}$ MEFs (Two-way ANOVA).

# Chapter 3

# Chapter 3: An investigation of binding proteins for the Parkinson's risk gene PINK1 identifies new candidates that regulate survival signalling, inflammatory responses and Akt signalling.

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**To be submitted**

## 3.1 Abstract

PINK1 functions as a ubiquitous serine-threonine kinase, with pro-survival, neuroprotective and anti-stress signalling functions. Autosomal recessive mutations in PINK1, resulting in a loss of PINK1 function, cause early onset Parkinson's disease (PD). Despite its discovery nearly two decades ago, there is a lack of cohesive information on PINK1 binding partners. Determining how PINK1 interacts with proteins involved in certain signalling pathways or cellular processes is crucial for understanding how loss of PINK1 function leads to PD. This study aimed to assemble a comprehensive list of known PINK1-interacting proteins and to identify new candidates that are bound to PINK1 using bioinformatics analysis of existing data sets coupled with mass spectrometry approaches. Our study identified PINK1-interacting proteins that are heavily involved in receptor tyrosine kinase (RTK) signalling pathways, such as PI3-kinase/Akt, VEGF, PDGF, ERRB2/4 and EGF, indicating that PINK1 plays a key functional role through these pathways. Further analysis highlighted two PINK1-interacting proteins (PTEN and IRS4) that are involved in PI3-kinase/Akt signalling and which may mediate PINK1's regulation of this pathway through lipid binding with $PIP_3$. Using mass spectrometry approaches, this study identifies several new binding proteins for PINK1, including, RIPK3, calmodulin, EGFR, and interferon regulatory genes which have been implicated in PD and as components of the Akt signalling system. Analysing proteins that bind to PD-causing genes such as PINK1 and investigation of their function in health and disease is vital to understanding how loss of PINK1 function leads to PD pathogenesis.



## 3.2 Keywords:

PINK1, Receptor tyrosine kinase, $PIP_3$, Akt

## 3.3 Introduction

The PINK1 protein was originally discovered in cancer cells as a gene induced by exogenous expression of PTEN, and thus named PTEN-induced putative kinase 1 (Unoki and Nakamura, 2001). Subsequently, autosomal recessive loss of function mutations in *PINK1* (*PARK6*) were identified in familial PD (Valente et al., 2004). This has drawn significant attention to determining how loss of PINK1 function could cause PD. PINK1 is a ubiquitous serine-threonine kinase and regulates both mitochondrial and non-mitochondrial systems central to pro-survival, neuroprotection, and stress resistance (for review (Arena and Valente, 2017, O'Flanagan and O'Neill, 2014)). Although PINK1 was discovered nearly two decades ago, there is limited knowledge on PINK1's binding partners and substrates. Thus, according to the iRefWeb database, there are only twelve known direct interactors of PINK1 (Wodak lab/iRefWeb, n. d., Table 1). Cohesive information on PINK1's interactions with proteins involved in certain signalling pathways or cellular processes is vital to further the understanding on how loss of PINK1 function leads to PD pathogenesis.

The most well-documented substrate and interacting protein of PINK1 is parkin, an E3 ubiquitin ligase, encoded by the *PRKN (PARK2)* gene, mutations in which also cause autosomal recessive forms of PD (Kitada et al., 1998). The PINK1-parkin interaction is best described in the regulation of mitophagy during severe mitochondrial stress (for reviews (Harper et al., 2018, Klinkenberg et al., 2010, Pickles et al., 2018, Pickrell and Youle, 2015, Ryan et al., 2015)). In this system, damaged mitochondria with decreased mitochondrial membrane potential (ΔψM) induce stabilisation of PINK1 in the outer mitochondrial membrane (OMM) with PINK1 autophosphorylation (Jin et al., 2010, Narendra et al., 2010, Yamano and Youle, 2013), and PINK1-induced translocation of parkin to the OMM from the cytosol (Narendra et al., 2008). In the OMM PINK1 phosphorylates ubiquitin at $Ser^{65}$ ($pSer^{65}$-Ub) (Kane et al., 2014, Kazlauskaite et al., 2014, Koyano et al., 2014) and parkin at $Ser^{65}$ in its ubiquitin like domain, enabling maximal parkin phosphorylation by PINK1 (Iguchi et al., 2013, Kondapalli et al., 2012, Shiba-Fukushima et al., 2012). This activates parkin leading to enhanced ubiquitination of proteins in the OMM targeting them for degradation (Okatsu et al., 2015, Ordureau et

al., 2015, Ordureau et al., 2014, Shiba-Fukushima et al., 2014). PINK1-induced pSer$^{65}$-Ub in mitochondria is also required for the recruitment of mitophagy adaptors to initiate mitophagy (Heo et al., 2015, Lazarou et al., 2015). The PINK1-parkin mitophagy pathway has further identified a number of direct and indirect substrates for PINK1 including Miro (Birsa et al., 2014, Tsai et al., 2014, Weihofen et al., 2009), Mfn (Chen and Dorn, 2013, McLelland et al., 2018) and Drp1 (Pryde et al., 2016).

PINK1 deletion in association with diverse stressors accelerates the impact of impaired mitophagy and may be necessary to induce PD phenotypes *in vivo* that can couple with inflammatory and immune pathways (Sliter et al., 2018). Thus, exhaustive exercise and mitochondrial DNA mutations coupled with PINK1 and parkin deletion induce PD pathophysiology *in vivo* via activation of the cGAS–STING pathway to cause inflammation that can induce cell death (Sliter et al., 2018). Functional links between mitochondria and the immune and inflammatory system are further strengthened by studies linking PINK1-parkin function to effective NF-kB signalling (Lee and Chung, 2012, Sha et al., 2010) and the regulation of mitochondrial antigen presentation (Matheoud et al., 2016, Matheoud et al., 2019). PINK1 also interacts with key inflammatory and immune regulators including TRAF6 (tumor necrosis factor receptor–associated factor 6), TAK1 ((TGF-β)-activated kinase) and SARM1 (sterile α and Toll/interleukin 1 receptor (TIR) motif containing 1) (Lee et al., 2012, Murata et al., 2013). PINK1 positively modulates TRAF6, an E3-ubiquitin ligase and TAK1 in the IL-1β-mediated signaling pathway, up-regulating their downstream inflammatory events (Lee et al., 2012). PINK1 and TRAF6 also form an interacting complex via PINK1's interaction with SARM1 (Murata et al., 2013). This enables TRAF-6 ubiquitination and stabilization of PINK1 promoting PINK1 interaction with TOM20 in the OMM thereby recruiting parkin to damaged mitochondria (Murata et al., 2013).

PINK1 plays multiple roles in regulating mitochondrial quality control systems and non-mitophagy related mitochondrial functions independent of parkin (for review (Leites and Morais, 2018)). These include: regulation of mitochondrial bioenergetics via PINK1 phosphorylation of the Complex I subunit NdufA10 (Morais et al., 2014), and regulation of mitochondrial fission/fusion transitions (O'Flanagan et al., 2015, Youle and van der

Bliek, 2012). Our previous work has shown that PINK1 regulates the cell cycle via regulation of mitochondrial fission- fusion transitions that couple to mitosis (O'Flanagan et al., 2015). In addition, PINK1 phosphorylates and regulates mitochondrial associated proteins that promote cell survival and protect against cell stress including TRAP1 (TNF receptor-associated protein 1) (Pridgeon et al., 2007), the anti-apoptotic BCL protein, BCL-xL (Arena et al., 2013) and HtrA2 (high temperature regulated A2) (Plun-Favreau et al., 2007).

PINK1 regulates several non-mitochondrial systems central to cell survival, neuroprotection and stress resistance. These include PI3-kinase/Akt signalling (Akundi et al., 2012, Ellis et al., 2013, Furlong et al., 2019, Maj et al., 2010, Murata et al., 2011, Sanchez-Mora et al., 2012), protein kinase A ((Dagda et al., 2014, Dickey and Strack, 2011), including via VCP (valosin-containing protein) (Wang et al., 2018a)), ubiquitination, proteasomal degradation (Kovalchuke et al., 2019, Tang and Zhang, 2019, Xiong et al., 2009), Rab-GTPase trafficking proteins (Lai et al., 2015), and macroautophagy (Dagda et al., 2009, Fedorowicz et al., 2014). Ubiquitin function is central to protein health and turnover and to all major signalling pathways that promote cell health. Importantly, PINK1 is the first kinase identified to phosphorylate ubiquitin for review (Tang and Zhang, 2019), (Kane et al., 2014, Kazlauskaite et al., 2014, Koyano et al., 2014). Ubiquitin phosphorylation is critical for remodelling the ubiquitin system and the regulation of cell fate (Tang and Zhang, 2019). $pSer^{65}$-Ub, the PINK1 substrate site, is not limited to the mitochondria (Kovalchuke et al., 2019, Tang and Zhang, 2019). The mechanistic links between PINK1 and the ubiquitin system are supported by studies showing PINK1 can be modified by the ubiquitin-like protein NEDD8 (neural-precursor-cell expression down regulated-8) (Choo et al., 2012) to protect against neurodegenerative PD-like phenotypes induced by PINK1 knockdown (Choo et al., 2012).

PINK1 regulation of PI3-kinase/Akt is very well described and of key importance as it is a master regulator of cell survival, metabolism, and proteostasis including the ubiquitin proteasomal system and autophagy (for review (Manning and Toker, 2017)). Many PD risk genes as well as PINK1 converge to regulate PI3-kinase/Akt signalling (Chuang et

al., 2014, Fallon et al., 2006, Gupta et al., 2017, Jaramillo-Gomez et al., 2015, Kim et al., 2005, Ohta et al., 2011, Zhang et al., 2016) and Akt activation is impaired in the PD brain (Malagelada et al., 2008, Timmons et al., 2009). Notably, PINK1-induced Akt activation protects against several cell stressors (Akundi et al., 2012, Contreras-Zarate et al., 2015, Murata et al., 2011, Sanchez-Mora et al., 2012), and Akt signalling regulates PINK1 levels (Mei et al., 2009). Furthermore, Akt regulates PINK1-dependent mitophagy (Soutar et al., 2018). PTEN is the major negative regulator of Akt and functions to dephosphorylate phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$) or $PIP_3$ to phosphatidylinositol (4,5)-bisphosphate or $PI(4,5)P_2$, thus reducing levels of $PIP_3$, the vital primary activator of Akt. The mechanistic link between Akt, mitophagy, and ubiquitin function are highlighted by recent findings revealing that PTEN-L, a longer form of PTEN, negatively regulates PINK1-induced phosphorylation of $pSer^{65}$-Ub to inhibit PINK1-parkin-mediated mitophagy (Wang et al., 2018b). PTEN-L dephosphorylation of $pSer^{65}$-Ub also occurs in non-mitochondrial compartments, linking Akt/PTEN signalling to PINK1's role as a key ubiquitin kinase. We have recently shown that PINK1 activates Akt via PINK1 kinase-dependent regulation of $PIP_3$ (Furlong et al., 2019). Studies generally only consider protein interactors for PD risk genes including PINK1. However, it is not known whether PINK1 may interact with $PIP_3$ or other major phosphoinositides that regulate signalling and trafficking (for review see (Balla, 2013)).

From the above it is clear that PINK1 has a pivotal role in major cell signalling systems governing the balance between cell survival and death in response to stress and whose loss of function causes PD. Within this context there is very limited information available that cohesively combines information on PINK1 binding proteins, and no knowledge exists concerning the possible binding of PINK1 to phosphoinositide lipids. Furthermore, many studies examine PINK1 binding proteins in the context of cell stress, thus the PINK1 interactome under normal growth conditions is not well defined. Therefore, in this study we aimed to assemble a comprehensive list of known PINK1-interacting proteins and to identify new candidates that are bound to PINK1 under normal conditions using bioinformatic analysis of existing data sets coupled with mass spectrometry approaches. The results of this study identify key known and novel

binding proteins for PINK1 and highlight PINK1 regulation of central survival signalling pathways. This characterisation of the networks of proteins known to interact with PINK1, allows for the identification of novel pathogenic proteins or pathways, which may be targets for therapeutic development in PD.

## 3.4 Materials and Methods

### 3.4.1 Generation of PINK1$^{-/-}$ mice and derived MEF cell lines

The cell lines used in these experiments are Mouse Embryonic Fibroblast (MEF) cells as employed and previously described (Furlong et al., 2019, Glasl et al., 2012, Morais et al., 2009, O'Flanagan et al., 2015). PINK1$^{-/-}$ knockout and PINK1$^{+/-}$ heterozygous knockout mice were generated by Wolfgang Wurst and Daniela Vogt-Weisenhorn (Helmholtz Center, Munich, Germany) and immortalized MEFs were generated as previously described (Morais et al., 2009). In brief, PINK1$^{+/-}$ mice were interbred to generate mutant mice and wild-type littermate controls. Embryonic day 13 mice were dissected, heads and red organs removed and used for genotyping. The rest of the bodies were chopped up in cell culture dishes containing Dulbecco's modified Eagle's medium with 50% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cultures were expanded and the serum level was decreased to 10% FBS after the attainment of consistent growth. Afterward cultures were immortalized by transfection with simian virus 40 (SV40) large T-antigen. PINK1$^{-/-}$ MEFs were stably transfected with a plasmid containing human PINK1 (hPINK1 construct Origene (Rockville, MD, USA)) using site-directed mutagenesis (Stratagene, Santa Clara, CA, USA). PINK1 deletion/expression was confirmed using RNA extraction and RT-PCR analysis.

### 3.4.2 Cell Culture

MEFs were cultured in Dulbecco's modified eagle medium: high glucose (DMEM-Hi), supplemented with 10% FBS and 2 mM L-glutamine. Cells were cultured at 37$^0$C in a humidified incubator supplemented with 5% $CO_2$.

### 3.4.3 Protein purification

Purification of the GST protein was performed using a pGEX-3X plasmid and GST-PINK1 using a pGST-PINK1-FL plasmid. The GST–PINK1 and GST constructs were generated from pcDNA3.1 by PCR using a sense oligonucleotide carrying the restriction site BamHI at position 1 and 77 of the corresponding plasmid and an antisense

oligonucleotide, EcoRI, in frame with the GST protein (kindly supplied by E.M. Valente, see (Michiorri et al., 2010)). The GST-tagged constructs were expressed in the *Escherichia coli* strain BL21 and GST-tagged proteins were produced under the induction of 0.1mM isopropyl thiogalactopyranoside (IPTG; Sigma I6758) overnight at 16°C. Proteins were purified by affinity chromatography using glutathione agarose (Sigma G4510) as per manufacturer's instructions. Briefly, cultures were pelleted by centrifugation, resuspended, sonicated and lysed in 1% v/v TritonX-100/PBS for 30 min rotating. The clarified supernatant was incubated with glutathione agarose rotating for 1 h at 4°C. GST and GST-PINK1 were eluted with 10 mM reduced glutathione. Protein preparations were analysed by Coomassie blue staining of SDS-PAGE and western immunoblotting with an anti-GST antibody (1:2,000) (Sigma G7781).

### 3.4.4 Phospholipid binding assay

The interaction of PINK1 with phospholipids was tested by protein-lipid blot overlay assays. Commercially available PIP-strips were used (P-6001; Echelon Biosciences, Salt Lake City, UT). PIP strips contain a hydrophobic membrane spotted with 100 pmol of eight phosphoinositides (phosphatidylinositol, phosphatidylinositol (3)-phosphate, phosphatidylinositol (4)-phosphate, phosphatidylinositol (5)-phosphate, phosphatidylinositol (3,4)-bisphosphate, phosphatidylinositol (3,5)-bisphosphate, PI(4,5)$P_2$, $PIP_3$) and seven other biological important lipids (lysophosphatidic acid, lysophosphocholine, phosphatidylethanolamine, phosphatidylcholine, sphingosine 1-phosphate, phosphatidic acid, phosphatidylserine). Membranes were blocked in a solution of 3% BSA/PBS-T for 1 h, followed by incubation with GST or GST-PINK1 purified proteins (0.5µg/ml) for 1 h, both at room temperature (RT). The membrane underwent three 5 min washes in 1X PBS-T after incubation with the protein of interest and also between each of the following steps: primary antibody incubation for 1 h at RT, anti-GST antibody (1:2,000; Sigma G7781), secondary antibody incubation for 1 h at RT, anti-rabbit-HRP (1:1,000; Cell Signalling 7074). Immunoreactive proteins were detected using an Enhanced Chemiluminescence kit (Amersham Biosciences, Little Chalfont, UK).

### 3.4.5 Pull down of PINK1 from whole cell lysates

Protein lysates from $hPINK1_{res}$ MEFs which overexpress His-hPINK1 and $PINK1^{-/-}$ MEF controls (2.5 mg per sample) were diluted in a final volume of 800 µl. Lysates were incubated with cobalt agarose overnight rotating at 4ºC. Agarose beads were recovered by centrifugation at 3,000 RPM for 3 min at 4ºC and subsequently washed three times with ice-cold lysis buffer containing inhibitors. Proteins were eluted by increasing concentrations of imidazole (10 mM-200mM). Proteins were analysed by Coomassie staining of SDS-PAGE and by western immunoblotting. Protein was eluted with 40 mM imidazole and n = 3 analysed by mass spectrometry.

### 3.4.6 Mass Spectrometry

Mass spectrometry analysis was performed by the Proteomics Core Facility in the European Molecular Biology Laboratory (EMBL), Heidelberg, Germany. Samples were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and subsequently by a tandem mass tag (TMT) workflow as previously described (Pereira et al., 2019).

### 3.4.7 Coomassie blue staining

Proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue overnight at 4°C. Gels were destained using destaining buffer (25% Ethanol/7% Acetic Acid/$dH_2O$), until individual bands were visible.

### 3.4.8 Western Immunoblotting

Primary antibodies used were: GST (1:2,000; Sigma G7781) and His (1:1,000; Abcam #9108). These were detected using horseradish peroxidase (HRP)-conjugated isotype-specific anti-rabbit IgG or anti-mouse IgG secondary antibodies (1:1000; DAKO, Cambridgeshire, UK).

Western immunoblotting was carried out as described previously (Moloney et al., 2010, O'Flanagan et al., 2015). Briefly, proteins were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes using a wet transfer apparatus and transfer buffer consisting of 48 mM Tris base, 39 mM glycine and 20% ethanol. After blocking for 30 min in 5% BSA, cells were incubated in primary antibody solutions overnight at 4ºC. The immunoblot underwent three 10-min washes in 1X TBX-T prior to secondary antibody incubation. Cells were incubated in secondary antibody solution for 1 h at RT followed by three 5-min washes in 1X TBS-T. Immunoreactive proteins were detected using Enhanced Chemiluminescence (Amersham Biosciences, Little Chalfont, UK).

### 3.4.9 Bioinformatics analysis

iRefWeb was used to compile a list of known PINK1 interacting proteins from the current literature (Table 1). The iRefWeb database was chosen to analyse the known PINK1 interactome as it contains protein interaction data consolidated from 10 databases: BIND, BioGRID, CORUM, DIP, IntAct, HPRD, MINT, MPact, MPPI and OPHID. It also provides a score (MI score) for the reliability of an interaction using criteria such as the number of supporting publications, the scale of the corresponding studies (high- or low-throughput) and the detection methods used in the original experiments. This list of proteins from the literature and the list obtained by Mass Spectrometry analysis following His pulldown were analysed using two free online databases: the Database for Annotation, Visualisation and Integrated Discovery (DAVID; https://david.ncifcrf.gov/) and Reactome. DAVID was used to assess enriched gene ontology terms. The three categories graphed from the gene ontology database included subcellular localisation of molecules and reactions (Cell Component (CC)), molecular functions (MF), and the larger biological processes (BP). The fold enrichment of all categories with a Benjamini value of $p < 0.005$ (Literature) and $p < 0.05$ (Mass Spectrometry) were graphed. Reactome was used as a database of biological pathways. The categories graphed from Reactome were overall cell events, and cell signalling.

### 3.4.10 Statistical analysis

All data were analysed using GraphPad Prism. For DAVID bioinformatics analysis, fold enrichment of all categories with a Benjamini value of $p < 0.005$ were considered to be significant and were graphed. Reactome data is presented as the entities ratio, entities found (number of interacting molecules that are common between the submitted dataset and the pathway of interest) normalised to entities total (entities found/total entities).

## 3.5 Results

### 3.5.1 PINK1-interacting proteins are involved in mitophagy, mitochondria-related functions, receptor tyrosine kinase signalling, protein binding and inflammation

We first sought to identify and interrogate the protein interactome of PINK1 employing the iRefWeb database. A list of 46 PINK1-interacting proteins was compiled from previously-published data. These proteins were categorised as either a direct interaction (Table 3.1), an associative interaction (Table 3.2) or a potential complex (Table 3.3) with PINK1. In order to analyse the role of PINK1 and its identified interactome within the cell, we employed Reactome and DAVID software. By calculating the entities ratio (entities found/entities total) using Reactome, we showed that the PINK1 interactome is highly present in cell events such as mitophagy, DNA replication and repair, programmed cell death, cell cycle, and cell responses to external stimuli (Fig. 3.1A). Using the same calculations with the Reactome database, we found that the key signalling pathways in which PINK1 interacting proteins are enriched are certain RTK pathways, including insulin like growth factor (IGF)-1R, $PIP_3$-activating Akt, vascular endothelial growth factor (VEGF) and erb-b2 receptor tyrosine kinase 2/4 (ERBB2/4) signalling (Fig. 3.1B).

The PINK1 interactome was further analysed using DAVID. Gene ontology analysis revealed that the PINK1-interacting proteins were most significantly enriched in the cytosol, mitochondria, mitochondrial outer membrane translocase complex, integral component of the mitochondrial outer membrane and the extracellular exosome (Fig. 3.1C). The major molecular functions enriched by PINK1-interacting proteins on DAVID were ubiquitin protein ligase binding, protein binding, protein kinase binding and unfolded protein binding (Fig. 3.1D). The major biological processes identified were mitochondrial-associated processes including protein targeting to mitochondria, protein import into the mitochondrial outer membrane, and as per Reactome mitophagy and inflammation associated processes such as T cell receptor signalling, activation of NF-kB signalling and kinase activity, and MyD88-dependent toll like receptor signalling (Fig. 3.1E).

**Table 3.1: Direct Interactions with PINK1**

List of proteins reported to directly interact with PINK1 as determined from the Reactome database. The MI score reports the measure of confidence in molecular interactions annotated from the literature (MI scale: 1 = binary interaction, literature showing direct interaction, 0.5 = mainly non-direct, low throughput studies, 0.25 = non-direct, high throughput studies) and the publications and methods used to analyse these interactions are also reported.

| Protein | MI Score | Publications | Methods |
|---|---|---|---|
| PARK2/Parkin | 1 | Kondapalli et al., 2012 | t7 phage display |
| | | Um et al., 2009 | enzymatic study |
| | | Xiong et al., 2009 | enzymatic study |
| | | Sha, Chin and Li 2009 | t7 phage display |
| | | Vives-Bauza et al., 2010 | Identification by antibody, bimolecular fluorescence complementation enzymatic study |
| | | Shiba et al., 2009 | enzymatic study |
| | | Lazarou et al., 2013 | t7 phage display |
| PARK7, Protein/nucleic acid deglycase DJ-1 | 0.80 | Xiong et al., 2009 | enzymatic study |
| TRAP1 | 0.78 | Pridgeon et al., 2007 | bimolecular fluorescence complementation, biosensor, cleavage reaction |
| MARK2, MAP/ microtubule affinity regulating kinase 2 | 0.72 | Matenia et al., 2012 | t7 phage display fluorescence technology |
| TRAF6, tumour necrosis factor receptor-associated factor 6 | 0.59 | Murata et al., 2013 | t7 phage display (Also mentions SARM1) |
| PGAM5 | 0.55 | Imai et al., 2010 | t7 phage display |
| UBC12, NEDD8-conjugating enzyme | 0.55 | Choo et al., 2012 | t7 phage display |
| SYUA | 0.55 | Um et al., 2009 | enzymatic study |
| M3K7, Mitogen-activated protein kinase kinase kinase 7 (TAK1) | 0.55 | Lee et al., 2012 | t7 phage display |
| FBX7, F-box only protein 7 | 0.55 | Burchell et a., 2013 | enzymatic study |
| SNCAIP Synphilin-1 | 0.43 | Bandopadhyay et al., 2005 | fluorescence technology (colocalisation) |
| SENP8, Sentrin-specific protease 8 | 0.43 | Choo et al., 2012 | t7 phage display |

**Table 3.2: Associative Interactions with PINK1**

List of proteins reported to indirectly interact with PINK1 as determined from the Reactome database, deemed associative interactions. The MI score reports the measure of confidence in molecular interactions annotated from the literature (MI scale: 1 = binary interaction, literature showing direct interaction, 0.5 = mainly non-direct, low throughput studies, 0.25 = non-direct, high throughput studies) and the publications and methods used to analyse these interactions are also reported.

| Protein | MI Score | Publications | Methods |
|---|---|---|---|
| Park2, Parkin | 1 | Um et al., 2009 (physical association) | static light scattering |
| | | Sha et al., 2010 (physical association) | static light scattering |
| | | Shiba et al., 2009 (physical association) | static light scattering |
| | | Zheng and Hunter, 2013 (physical association) | static light scattering |
| | | Xiong et al., 2009 (physical association) | static light scattering |
| | | Geisler et al., 2010 (physical association) | static light scattering |
| Tom20, Mitochondrial import receptor subunit TOM20 | 0.82 | Kawajiri et al., 2010 (colocalisation) | molecular weight estimation by coomassie staining, phage display |
| | | Lazarou et al., 2012 (physical association) | static light scattering |
| | | Murata et al., 2013 (physical association) | static light scattering |
| UBC, Ubiquitin C | 0.82 | Emanuele et al., 2011 (high throughput) (physical association) | static light scattering |
| | | Tang et al., 2006 (physical association) | static light scattering |
| | | Xiong et al., 2009 (physical association) | static light scattering |
| | | Lee et al., 2011 (physical association) | static light scattering |
| | | Murata et al., 2013 (physical association) | static light scattering |
| | | Um et al., 2009 (physical association) | static light scattering |
| HS90A – heat shock protein 90-alpha 1 | 0.81 | Imai et al., 2010 (physical association) | static light scattering |
| | | Moriwaki et al., 2008 (physical association) | static light scattering |
| | | Weihofen et al., 2008 (physical association) | static light scattering |

| | | | |
|---|---|---|---|
| | | Taipale et al., 2012 (physical association) | static light scattering |
| PARK7, Protein/nucleic acid deglycase DJ-1 | 0.8 | Xiong et al., 2009 (physical association) | static light scattering |
| | | Tang et al., 2006 (physical association) | static light scattering |
| MARK2, microtubule affinity regulating kinase 2 | 0.72 | Matenia et al., 2012 (physical association) | static light scattering |
| HS90B- heat shock protein 90-beta | 0.68 | Taipale et al., 2012 (physical association) | Phage display, peptide mass fingerprinting |
| | | Weihofen et al., 2008 (physical association) | static light scattering |
| MIRO1, Mitochondrial Rho GTPase 1 | 0.68 | Wang et al., 2011 (physical association) | bimolecular fluorescence complementation, electrophoretic mobility-based method, cleavage reaction |
| | | Liu et al., 2012 (physical association) | static light scattering |
| MLP3B, Microtubule-associated proteins 1A/1B light chain 3B | 0.64 | Kawajiri et al., 2010 (colocalisation)<br><br>(physical association) | molecular weight estimation by coomassie staining, phage display cleavage reaction, bimolecular fluorescence complementation |
| CDC37, Hsp90 co-chaperone CDC37 | 0.63 | Moriwaki et al., 2008 (physical association) | static light scattering |
| TRAF6 tumour necrosis factor receptor-associated factor 6 | 0.59 | Lee et al., 2012 (physical association) | static light scattering |
| PGAM5, PGAM family member 5 / mitochondrial Serine/threonine-protein phosphatase | 0.55 | Imai et al., 2010 (physical association) | static light scattering |
| TBA1A, Tubulin alpha-1A chain | 0.55 | Imai et al., 2010 (physical association) | static light scattering |

| | | | |
|---|---|---|---|
| UBC12, NEDD8-conjugating enzyme | 0.55 | Choo et al., 2012 (physical association) | static light scattering |
| SYUA | 0.55 | Um et al., 2009 (physical association) | static light scattering |
| M3K7, Mitogen-activated protein kinase kinase kinase 7 (TAK1) | 0.55 | Lee et al., 2012 (physical association) | static light scattering |
| FBX7, F-box only protein 7 | 0.55 | Burchell et a., 2013 (physical association) | static light scattering |
| IRS4, Insulin Receptor Substrate 4 | 0.48 | Imai et al., 2010 (physical association) | static light scattering |
| KIF11, Kinesin Family Member 11 | 0.48 | Imai et al., 2010 (physical association) | static light scattering |
| | | Weihofen et al., 2008 (physical association) | static light scattering |
| HSP74, heat shock 70kDa protein 4 | 0.48 | Imai et al., 2010 (physical association) | static light scattering |
| | | Moriwaki et al., 2008 (physical association) | static light scattering |
| TBB5, Tubulin beta chain | 0.28 | Imai et al., 2010 (physical association) | static light scattering |
| PRKDC, Protein Kinase, DNA-Activated, Catalytic Polypeptide | 0.28 | Imai et al., 2010 (physical association) | static light scattering |
| PRS10, 26S protease regulatory subunit S10B | 0.28 | Imai et al., 2010 (physical association) | static light scattering |
| RL40, Ubiquitin-60S ribosomal protein L40 | 0.28 | Vives-Bauza et al., 2010 | bimolecular fluorescence complementation |
| PSMD2, 26S proteasome non-ATPase regulatory subunit 2 | 0.28 | Imai et al., 2010 (physical association) | static light scattering |
| PTEN | 0.28 | Crokett et al., 2005 (physical association) | static light scattering |
| PSMD1, Proteasome 26S Subunit, Non-ATPase 1 | 0.28 | Imai et al., 2010 (physical association) | static light scattering |
| MAP1B, Microtubule | 0.28 | Imai et al., 2010 | static light scattering |

| | | | |
|---|---|---|---|
| Associated Protein 1B | | (physical association) | |
| RICTR, Rictor | 0.28 | Murata et al., 2011 (physical association) | static light scattering |
| IRAK1, Interleukin-1 receptor-associated kinase 1 | 0.28 | Lee and Chung 2012 (physical association) | static light scattering |
| TOLIP, toll interacting protein | 0.28 | Lee and Chung 2012 (physical association) | static light scattering |
| TOM40, main component of the preprotein translocase of the outer membrane of mitochondria | 0.28 | Lazarou et al., 2012 (physical association) | static light scattering |
| PARL, mitochondrial protease presenilin-associated rhomboid-like | 0.28 | Shi et al., 2011 (physical association) | static light scattering |
| NEDD8 | 0.28 | Choo et al., 2012 (physical association) | static light scattering |
| MLP3A, Microtubule-associated proteins 1A/1B light chain 3A | 0.28 | Kawajiri et al., 2010 (physical association) | static light scattering |
| MAPKAP1, Mitogen-Activated Protein Kinase Associated Protein 1) | 0.28 | Murata et al,. 2011 (physical association) | static light scattering |
| TOM22, Mitochondrial import receptor subunit TOM22 | 0.28 | Lazarou et al., 2012 (physical association) | static light scattering |
| HTRA2, Serine protease HTRA2 | 0.28 | Plun-Favreau et al., 2007 (physical association) | static light scattering |
| Akt1 | 0.28 | Murata et al,. 2011 (physical association) | static light scattering |
| CSN8, COP9 signalosome complex subunit 8 / CSN complex subunit 8. | 0.28 | Choo et al., 2012 (physical association) | static light scattering |
| TOM70, Mitochondrial import receptor subunit TOM70 | 0.28 | Lazarou et al., 2012 (physical association) | static light scattering |
| VIME, Vimentin, type III intermediate filament protein. | 0.28 | Murata et al., 2013 (physical association) | static light scattering |

| Protein | MI Score | Publications | Methods |
|---|---|---|---|
| Parkin, Park7, PINK1 | 0.94 | Xiong et al., 2009 (association) | polyclonal antibody western blot, phosphotransferase assay |
| Tom20, Park2, PINK1 | 0.82 | Kawajiri et al., 2010 (colocalisation) | molecular weight estimation by coomassie staining, phage display |
| Miro, Milton, PINK1 | 0.28 | Weihofen et al., 2009 (association) | bimolecular fluorescence complementation |

**Table 3.3: Potential protein complexes with PINK1**

List of proteins reported to form potential complexes with PINK1 as determined from the Reactome database. The MI score reports the measure of confidence in molecular interactions annotated from the literature (MI scale: 1 = binary interaction, literature showing direct interaction, 0.5 = mainly non-direct, low throughput studies, 0.25 = non-direct, high throughput studies) and the publications and methods used to analyse these interactions are also reported.

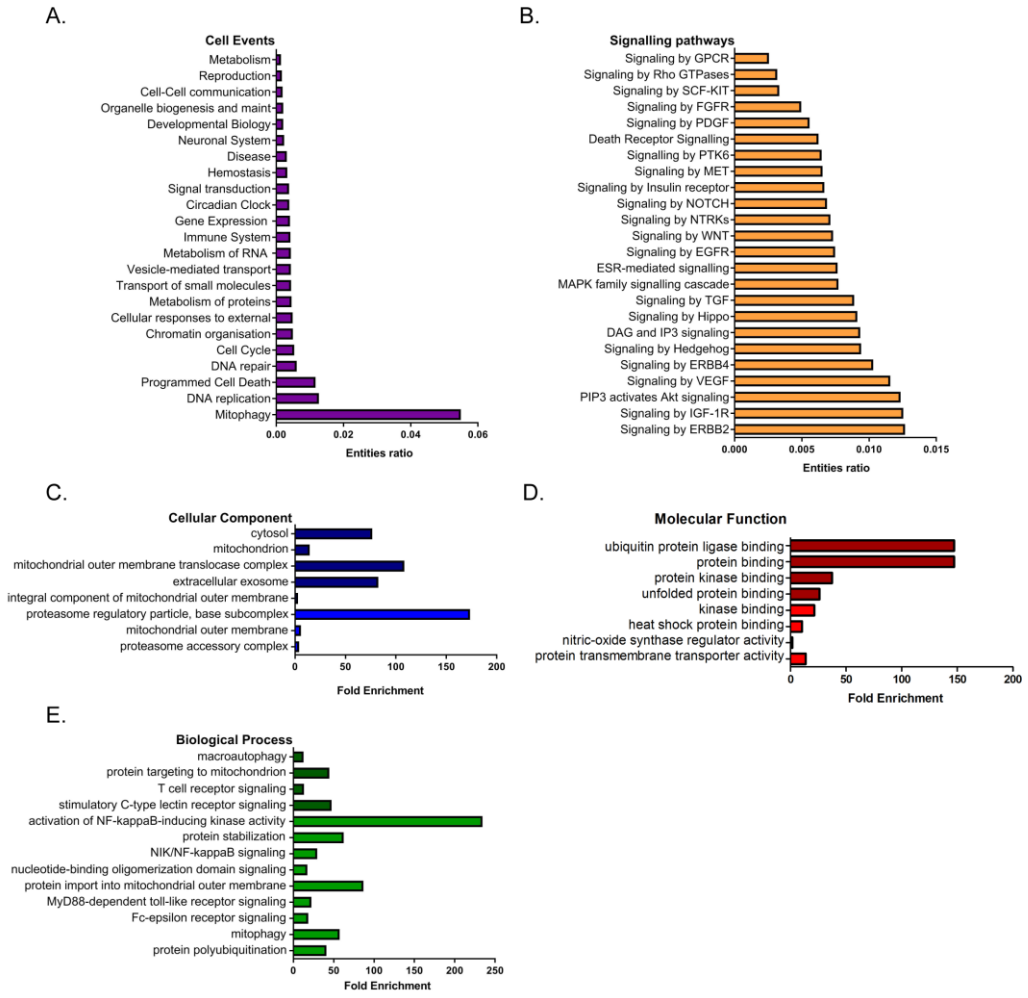



**Figure 3.1. PINK1-interacting proteins are involved in mitophagy, mitochondria-related functions, receptor tyrosine kinase signalling, protein binding and inflammation.**

Entities ratio of PINK1-interacting proteins identified by iRefWeb involved in (A) cell events and (B) signalling pathways, as determined by Reactome. Fold enrichment of PINK1-interacting proteins identified by iRefWeb involved in (C) cellular component, (D) molecular function and (E) biological process, as determined by gene ontology using in DAVID. For DAVID analysis, all graphed values were below p = 0.005 as determined by Benjamini test. (Darker colours indicated where significance was below p = 0.001). Significant hits for DAVID analysis ranked from top to bottom of each graph C, D, E.

### 3.5.2 PINK1 interacts with four known phosphoinositide lipid-binding proteins

Our previous work has shown that PINK1 kinase activity can modulate the core $PIP_3$-Akt regulatory machinery by increasing $PIP_3$ levels in the plasma membrane and in endomembranes, particularly the Golgi (Chapter 2, (Furlong et al., 2019)). However, it is not yet known how PINK1 regulates $PIP_3$ production. We therefore next cross-referenced the list obtained from Reactome with a database of proteins with known phosphoinositide (PI) lipid-binding domains (Somogyi et al., unpublished) in order to identify potential proteins that can bind both to PINK1 and to the PI family of lipids. Four proteins were identified: phosphatase and tensin homolog (PTEN), insulin receptor substrate 4 (IRS-4), toll-interacting protein (TOLLIP) and RAC-alpha serine/threonine protein kinase 1 (AKT1) (Table 3.4). This indicates potential cross-talk between PINK1 and specific PI binding proteins.

### 3.5.3 PINK1 cannot directly bind to $PIP_3$ or any other member of the phosphatidylinositol family.

Our recent study (Chapter 2, Furlong et al., 2019) suggests that PINK1 could interact with PIs. We have shown that PINK1 kinase activity increases levels of $PIP_3$ at the plasma membrane and Golgi concomitant with Akt activation (Chapter 2, (Furlong et al., 2019)). We hypothesised that PINK1 could bind directly to $PIP_3$ or its substrate $PIP_2$ to effect $PIP_3$ production. In order to investigate this, we purified GST and GST-PINK1 proteins. Coomassie staining showed efficient purification of GST (Fig. 3.2A) and GST-PINK1 (Fig. 3.2B). This was confirmed by western immunoblotting (Fig. 3.2A, B bottom panel). To determine whether PINK1 could bind to $PIP_3$, $PIP_2$ or any other member of the PI family, a protein-lipid overlay assay was performed on a PIP strip, which contained PI and all seven PIPs, as well as other primary cellular lipids (Fig. 3.2C). These results did not identify any positive interaction between PINK1 and PIs indicating PINK1 cannot directly bind to any of these PIs (Fig. 3.2D). This suggests that the effect of PINK1 on $PIP_3$ production results from its interaction with another protein(s) that regulate $PIP_3$ production, such as those identified above.

| Gene Name | Lipid Binding Domain | Protein name |
|---|---|---|
| PTEN | C2 | Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN (Phosphatase and tensin homolog) (Mutated in multiple advanced cancers ) |
| IRS4 | PH | Insulin receptor substrate 4 (IRS-4) (160 kDa phosphotyrosine protein) |
| TOLLIP | C2 | Toll-interacting protein |
| AKT1 | PH | RAC-alpha serine/threonine-protein kinase) (Protein kinase B) (PKB) |

**Table 3.4: Proteins identified with lipid binding domains**

Proteins identified by iRefWeb software as PINK1 interacting proteins cross-referenced with a database of proteins with known phospholipid binding domains (Somogyi et al., unpublished).

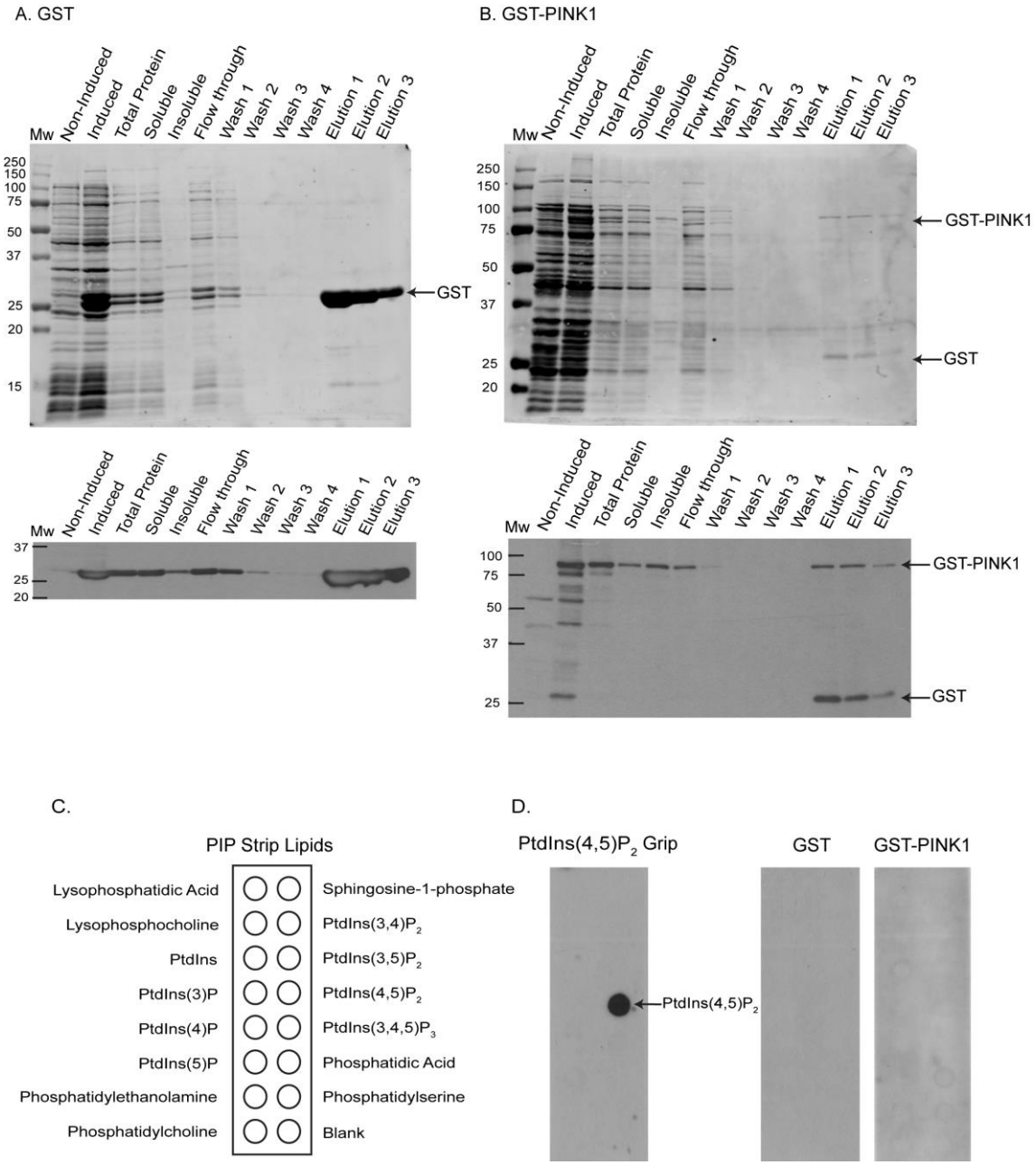



**Figure 3.2. PINK1 cannot directly bind to $PIP_3$ or any other member of the phosphatidylinositol family.**

(A, B) Coomassie blue staining and western immunoblot analysis showing efficient purification of GST and GST-PINK1 proteins. (C) Schematic of PIP strip. (D) Purified GST-tagged proteins incubated with a PIP strip and immunoreactive protein ($PI(4,5)P_2$ control) detected with enhanced chemiluminescence. n = 3 replicates for each

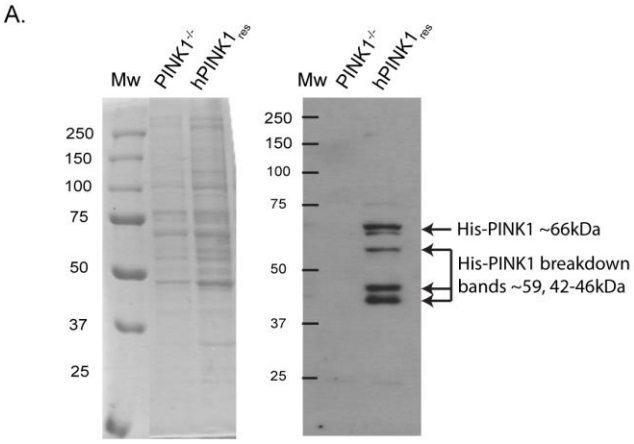
### 3.5.4 His-PINK1 pull down shows that PINK1 interacts with key signalling molecules that regulate cell survival, ubiquitination, calcium and inflammation

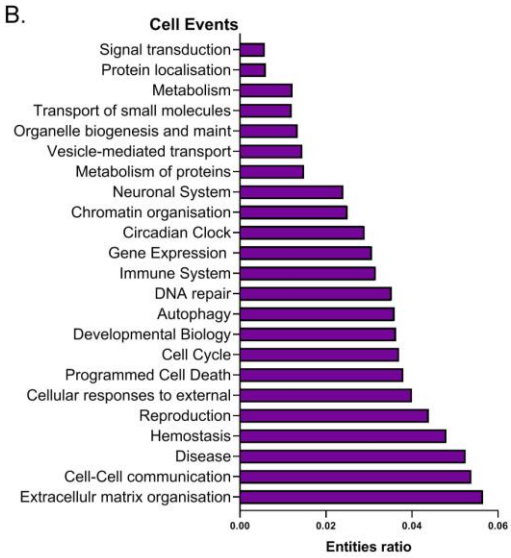
Our bioinformatic data analysis of the iRefWeb database allowed us to cohesively identify candidate binding proteins of PINK1 and position them in their most prominent cellular processes and signalling pathways. However, the literature to date (on which these software are based) has frequently selectively focused on the mitochondria, and more specifically PINK1/parkin mediated mitophagy during extreme mitochondrial stress induced by mitochondrial uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP) (Jin et al., 2010, Narendra et al., 2010, Yamano and Youle, 2013). We therefore next aimed to isolate the PINK1 protein under normal cellular conditions and identify proteins or protein complexes with which it interacts. A PINK1 pull down assay was performed on PINK1$^{-/-}$ MEFs with stable expression of a His-tagged human PINK1 (hPINK1$_{res}$). These cells were previously employed and shown to restore several key mitochondrial and non-mitochondrial functions of PINK1, including mitochondrial quality control, cell cycle regulation and Akt activation when compared to PINK1$^{-/-}$ MEFs (Furlong et al., 2019, Morais et al., 2009, O'Flanagan et al., 2015). His-PINK1 was efficiently pulled down and eluted from cobalt resin by imidazole as shown by western immunoblot analysis (Fig. 3.3A). The His-PINK1 pull down and its full complement of interacting proteins obtained (Fig. 3.3A) were processed by LC-MS/MS. A total of 37 proteins were identified by LC-MS/MS in the hPINK$_{res}$ MEF samples distinct from the PINK1$^{-/-}$ control MEFs (Table 3.5).

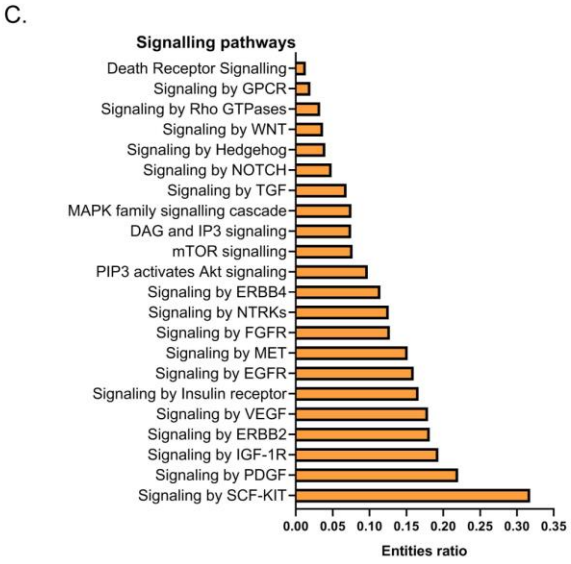
The LC-MS/MS identified fourteen of these interacting proteins as hits, due to the presence of at least three peptide sequences from the interacting protein and the remaining twenty-three interacting proteins as candidates due to the presence of a one peptide sequence from the interacting protein (Table 3.5). In the total list of interacting proteins there were three protein matches between the iRefWeb database PINK1 interacting protein list and the LC-MS/MS results PARK7 (DJ-1), heat shock protein 70kDa 4 (HSP74) and vimentin (VIME). Additionally, a protein already identified as PINK1 interacting proteins but not in iRefWeb database was present in the candidate list, namely transitional endoplasmic reticulum ATPase or valosin-containing protein

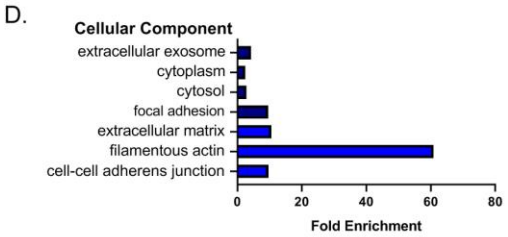
**Figure 3.3: Proteins identified by mass spectrometry analysis of His-PINK1 are enriched in similar cell events, signalling pathways and cellular components as are the known PINK1-interacting proteins.**

(A) Coomassie blue staining and western immunoblot analysis showing His-PINK1 pull down in PINK1$^{-/-}$ and hPINK1$_{res}$ MEFs, as indicated. His-tag localised on the C-terminal of the PINK1 protein. Entities ratio of PINK1-interacting proteins identified by LC-MS/MS involved in (B) cell events and (C) signalling pathways, as determined by Reactome. (D) Fold enrichment of PINK1-interacting proteins identified by LC-MS/MS involved in a cellular component, as determined by gene ontology analysis using DAVID. For DAVID analysis, all graphed values were below p=0.05 as determined by Benjamini test. Significant hits for DAVID analysis ranked from top to bottom of graph, C.


A.
Mw
PINK1-/-
hPINK1res
250
150
100
75
50
37
25
His-PINK1 ~66kDa
His-PINK1 breakdown
bands ~59, 42-46kDa


B.
Cell Events
Signal transduction
Protein localisation
Metabolism
Transport of small molecules
Organelle biogenesis and maint
Vesicle-mediated transport
Metabolism of proteins
Neuronal System
Chromatin organisation
Circadian Clock
Gene Expression
Immune System
DNA repair
Autophagy
Developmental Biology
Cell Cycle
Programmed Cell Death
Cellular responses to external
Reproduction
Hemostasis
Disease
Cell-Cell communication
Extracellulr matrix organisation
0.00
0.02
0.04
0.06
Entities ratio


C.
Signalling pathways
Death Receptor Signalling
Signaling by GPCR
Signaling by Rho GTPases
Signaling by WNT
Signaling by Hedgehog
Signaling by NOTCH
Signaling by TGF
MAPK family signalling cascade
DAG and IP3 signaling
mTOR signalling
PIP3 activates Akt signaling
Signaling by ERBB4
Signaling by NTRKs
Signaling by FGFR
Signaling by MET
Signaling by EGFR
Signaling by Insulin receptor
Signaling by VEGF
Signaling by ERBB2
Signaling by IGF-1R
Signaling by PDGF
Signaling by SCF-KIT
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
Entities ratio


D.
Cellular Component
extracellular exosome
cytoplasm
cytosol
focal adhesion
extracellular matrix
filamentous actin
cell-cell adherens junction
0
20
40
60
80
Fold Enrichment

| Protein Identified | Name |
|---|---|
| **Hits** | |
| VIME | Vimentin |
| RPL23A | Ribosomal protein L23a |
| RIPK3 | Receptor-interacting serine/threonine-protein kinase 3 |
| RPL22L1 | 60S ribosomal protein L22-like 1 |
| DAP | Death-associated protein |
| GATM | Glycine amidinotransferase, mitochondrial |
| FABP5 | Fatty acid-binding protein, epidermal |
| CAPNS1 | Calpain small subunit 1 |
| NUDC | Nuclear migration protein nudC |
| TMSB4X | Isoform Short of Thymosin beta-4 |
| MCAM | Cell surface glycoprotein MUC18 |
| LGALS9 | Galectin |
| CALM1 | Calmodulin |
| HN1L | HN1-like protein |
| **Candidates** | |
| PARK7 | Protein deglycase DJ-1 |
| EGFR | Epidermal growth factor receptor |
| EPS8L2 | Epidermal growth factor receptor kinase substrate 8-like protein 2 |
| HSPA4L | Heat shock 70 kDa protein 4L |
| SUMO2 | Small ubiquitin-related modifier 2 |
| VCP | Transitional endoplasmic reticulum ATPase |
| TMSB10 | Thymosin beta-10 |
| CRIP2 | Crip2 protein |
| PLEC | Plectin |
| ACLY | ATP-citrate synthase |
| PDAP1 | 28 kDa heat- and acid-stable phosphoprotein |
| ISG15 | G1p2 protein |
| AHNAK2 | Protein Ahnak2 |

| | |
|---|---|
| PTMA | Prothymosin alpha |
| ANP32B | Acidic leucine-rich nuclear phosphoprotein 32 family member B |
| IFI204 | Interferon-activable protein 204 |
| CAPN2 | Calpain-2 catalytic subunit |
| S100A6 | Protein S100-A6 |
| FLNA | Filamin, alpha |
| AKR1B1/AKR1B3 | Aldose reductase |
| BRD2 | Bromodomain-containing protein 2 |
| TGM2 | Protein-glutamine gamma-glutamyltransferase 2 |
| C5 | Complement C5 |

**Table 3.5: Proteins identified from mass spectrometry analysis of His-PINK1 pull down**

Proteins identified to be enriched by LC-MS/MS analysis of His-PINK1 pulldown with $hPINK_{res}$ MEFs compared to $PINK1^{-/-}$ control. Hits = three or more peptide match identified by LC-MS/MS, Candidates = peptide match by LC/MS/MS.

(VCP) (Wang et al., 2018a). We further identified several novel proteins not previously identified as PINK1-interacting proteins (Table 3.5). These included receptor proteins e.g. epidermal growth factor receptor (EGFR) and its substrate epidermal growth factor receptor kinase substrate 8-like protein 2 (EPS8L2), ubiquitin related protein (small ubiquitin-related modifier 2 (SUMO2)), calcium regulation binding proteins and enzymes (calpain small subunit (CAPSN1), calpain-2 catalytic subunit (CAPN2) and calmodulin (CALMI)), ribosomal protein 60S ribosomal protein L22-like 1 (RPL22L1), inflammatory/immune related proteins (interferon-activatable protein (IFI204), ISG15, protein S100-A (S100A6) and C5 complement (C5)) (Table 3.5), and other proteins that regulate cell survival and response to stress (See Table 3.5).

### 3.5.5 Proteins identified by mass spectrometry analysis of His-PINK1 are enriched in similar cell events, signalling pathways and cellular components as the known PINK1-interacting proteins.

To further analyse the role of PINK1 and the interacting proteins that we identified by LC-MS/MS we employed DAVID and Reactome software. We identified the PINK1 interactome to be highly present in cell events such as cell-cell communication, cell responses to external stimuli, programmed cell death and the cell cycle as seen with the known PINK1 interacting proteins (Fig. 3.3B). We also show enrichment for a wider range of cellular processes with less favour for mitochondrial associated functions, with new prominent events such as extracellular matrix organisation, developmental biology, autophagy and disease (Fig. 3.3B). The key signalling pathways in which PINK1-interacting proteins are implicated are certain RTKs including IGF-1, platelet-derived growth factor (PDGF), ERBB2, VEGF, insulin, epidermal growth factor (EGF) receptor signalling and SCF-KIT signalling (Fig. 3.3C) as determined by Reactome. Gene ontology analysis using DAVID revealed enrichment in the cytosol, cytoplasm and extracellular exosome cell components (Fig. 3.3D). It is worth noting that a number of inflammation-related biological processes were enriched, such as I-kB kinase/NF-kB signalling, although they did not reach statistical significance.

### 3.5.6 Novel PINK1 binding proteins are linked to Akt signalling and PD

As our, and other work clearly indicates that PINK1 activates the Akt signalling axis (see introduction), we hypothesise that the regulation of Akt by PINK1 may be central to many cellular functions of PINK1 including regulation of cell survival, stress resistance, immune and mitochondrial function. We thus next analysed the newly identified PINK1 interacting proteins with respect to the Akt signalling axis and also PD. Our results reveal that several of the new PINK1 interacting proteins activate Akt, stabilise Akt or are actual substrates of Akt where they function to promote cell survival, stress resistance, immune and mitochondrial function. Of the proteins identified the following from the hit list have been reported to activate Akt: FAB5 (Lv et al., 2019), CAPSN1 (Bertoli et al., 2009), MCAM (Colomb et al., 2017), LGALS9 (Colomb et al., 2017), CALM1 (Agamasu et al., 2017, Zhang et al., 2018), RPL22L1 (Oh et al., 2010). From the candidate list the following proteins have been reported to activate Akt: PARK7 (Lin et al., 2018, Zhang et al., 2016), EGFR (Oda et al., 2005), TMSB10 (Zhang et al., 2017b), ANP32B (Yang et al., 2016), CAPN2 (Abeyrathna et al., 2016, Ho et al., 2012, Miao et al., 2017), S100A6 (Li et al., 2018), BRD2 (Zhang et al., 2017a), Complement C5 (Bosmann et al., 2011, Fattahi et al., 2017, Hu et al., 2018). Furthermore, FLNA inhibits Akt signalling (Campos et al., 2016), aldose reductase interacts with the kinase domain of Akt-1 (Zhao et al., 2017a) and ISG15 indirectly activates Akt (Kaur et al., 2008, Tian et al., 2015). RIPK3 has been reported to inactivate (Li et al., 2010) and activate Akt (Imamura et al., 2018) and to induce Akt-dependent phosphorylation of ATP citrate lyase (ACL) (Imamura et al., 2018). Finally three of the PINK1 interacting proteins identified are substrates of Akt, these are Vimentin (Zhu et al., 2011), ACL (Berwick et al., 2002, Covarrubias et al., 2016, Lee et al., 2014) and VCP (Vandermoere et al., 2006).

Furthermore, several of these newly identified PINK1 interacting proteins have been previously implicated in PD pathogenesis. These include: RIPK3 (Iannielli et al., 2018, Lee et al., 2019). CALM1 (Martinez et al., 2003, Yang et al., 2018, Zhang et al., 2017c), EGFR (Choi et al., 2013, Fallon et al., 2006, O'Keeffe et al., 2009), ISG15 (Im et al., 2016, Li et al., 2019), CAPN2 (Knaryan et al., 2014, Samantaray et al., 2013), FABP5 (Sharon et al., 2001), VCP (Kim et al., 2013b, Wang et al., 2018a), Vimentin (Muqit et

al., 2004, Tanaka et al., 2004) and SUMO (Eckermann, 2013, Krumova et al., 2011, Rott et al., 2017, Rousseaux et al., 2018). Together these data indicate that the PINK1 interactome plays a role in cell survival which may be mediated by the Akt signalling pathway and these proteins can be pursued further to confirm these links.

### 3.6 Discussion

In this study we aimed to assemble a comprehensive list of known PINK1-interacting proteins and to identify new candidates that are bound to PINK1 under normal conditions interrogating both existing data-sets coupled with experimental mass spectrometry approaches. The results of the study identify key known and several novel binding proteins for PINK1 and highlight PINK1's regulation of central systems that regulate cell survival, immune and mitochondrial systems, including Akt signalling. This characterisation of the networks of proteins known to interact with PINK1, allows for the identification of novel pathogenic proteins or pathways, which may be targets for therapeutic development for PD.

Analysis of PINK1-interacting proteins using iRefWeb, allows us to examine the currently-known PINK1 interactome. We determined that PINK1-interacting proteins are enriched in cellular processes particularly mitophagy, as well as protein targeting to mitochondria and protein import into the mitochondrial outer membrane and cellular components including the mitochondria and cytosol. This is unsurprising as the most well-documented roles of PINK1 are in the mitochondria, during extreme mitochondrial stress induced by CCCP (Jin et al., 2010, Narendra et al., 2010, Yamano and Youle, 2013), so while this may be true it may also reflect a bias in the current literature. In contrast, our LC-MS/MS analysis showed enrichment for a wider range of cellular processes with less favour for mitochondrial associated functions. These included extracellular matrix organisation, cell to cell communication, developmental biology, autophagy and disease. This indicates that PINK1 plays an important role in a number of cellular processes beyond the mitochondria.

Several PD risk genes, including PINK1, converge to crosstalk with the PI3-kinase/Akt signalling system (Chuang et al., 2014, Fallon et al., 2006, Furlong et al., 2019, Gupta et al., 2017, Jaramillo-Gomez et al., 2015, Kim et al., 2005, Ohta et al., 2011, Zhang et al., 2016). Our initial bioinformatics analysis of the PINK1 interactome identified from current literature highlighted the role of PINK1 in additional RTK signalling pathways, including VEGF and ERBB2/4 signalling. In agreement with this, our mass spectrometry analysis showed enrichment in the RTK signalling pathways VEGF and

ERBB2, however, we also identified enrichment in the PDGF, insulin and EGF signalling pathways. We also identified involvement of the SCF-KIT signalling pathway in the PINK1 interactome. This pathway has been shown to be involved in the regulation of mitochondrial function and energy expenditure (Huang et al., 2014), and like the RTKs, in cell survival, migration, and proliferation (Lennartsson and Ronnstrand, 2012). This indicates that PINK1 and other PD risk genes could regulate a number of cell growth/survival pathways in addition to the PI3-kinase/Akt pathway, which could have implications for the pathogenesis of PD and therefore need to be studied further.

However it is important to note that Akt is activated by numerous pathways, has a broad range of downstream targets and is widely studied in a broad range of diseases. Therefore this may be a reflection of how extensive Akt research is and reflect a bias in the current literature for cell signalling pathways.

This study aimed to identify proteins having the ability to interact with PINK1 in such a way as to regulate the PI3-kinase/Akt pathway at the level of $PIP_3$. This is due to our recent finding that PINK1 kinase activity activates Akt by regulating $PIP_3$ in both the plasma membrane and Golgi (Chapter 2, (Furlong et al., 2019)). We first determined that PINK1 could not directly bind to $PIP_2$, $PIP_3$ or any other phosphoinositide proteins. This implied that there must be additional protein(s) involved in PINK1's regulation of $PIP_3$. Cross-referencing the list of known PINK1-interacting proteins with a database of proteins containing known lipid-binding domains (Somogyi et al., unpublished) showed that two of the four (PTEN and IRS4) were proteins that are known to be involved in PI3-kinase/Akt signalling upstream of $PIP_3$. This highlights their potential as candidates for mediators of PINK1's regulation of this pathway. This is of further interest due to findings the PTEN-L, is a major de-ubiquitinase enzyme that negatively regulates PINK1 induced-phosphorylation of ubiquitin at $Ser^{65}$ (Wang et al., 2018b). The balance between PINK1, PTEN and Akt may be of functional importance in regulating Akt signalling. The identification of TOLLIP in both the known PI lipid-binding proteins and known PINK1 interacting protein lists indicates that normal interaction between PINK1 and Tollip also relies on PI integrity. Tollip like PTEN contains a C2 PI

interacting domain. Tollip is a key mediator of IL-1β inflammatory signalling, and PINK1 binding to Tollip increases upon IL-1β signalling (Lee and Chung, 2012). This places PINK1, PIs and Tollip as positive regulators of inflammatory tone. Interestingly, Tollip can also negatively regulate Akt in immune signalling (Diao et al., 2016) and is an early regulator of the inflammatory response in the substantia nigra (Humbert-Claude et al., 2016).

Mass spectrometry analysis of proteins that bound to His-PINK1 expressed in PINK1$^{-/-}$ MEFs compared to PINK1$^{-/-}$ cells, uncovered a number of PINK1-interacting proteins that may be implicated in PINK1 function in healthy cells, as well as in dysfunction in PD. Only some of these have been previously described to be interacting proteins including heat shock protein 70kDa 4 (HSP74), Vimentin and PARK7. PARK7 (DJ-1) has been previously reported to bind to both PINK1 (as found in our study) and parkin, forming a complex. This complex promotes ubiquitination and degradation of un-/misfolded parkin substrates, including parkin itself and synphilin-1 in both neuroblastoma cells and human brain lysates (Xiong et al., 2009). PD-causing mutations in PINK1 and parkin impaired this degradation function. This indicates that impaired functioning of this complex, leading to impaired degradation function, may contribute to PD pathogenesis (Xiong et al., 2009).

Another hit on our mass spectrometry screen for PINK1-interacting proteins was vimentin, a type III intermediate filament protein. Vimentin has previously been shown to bind to PINK1 by liquid chromatography–mass spectrometry (Murata et al., 2013), although the function of this interaction it not yet known. Interestingly, multiple studies have shown that vimentin plays a key role in the nervous system, including stimulating axonal elongation (Dubey et al., 2004, Yabe et al., 2003), neuronal proliferation in an *in vivo* model of Huntington's disease (Mazurova et al., 2006), and alcohol-induced neurodegeneration (Kelso et al., 2011). Vimentin has previously been linked to PD, an increased level of cleaved vimentin has been reported in the substantia nigra of PD patients (Tong et al., 2015), and vimentin has also been detected with both α-synuclein and parkin in aggresomes (Konno et al., 2012, Lee et al., 2002, Muqit et al., 2004,

Tanaka et al., 2004). Thus the interaction between PINK1 and vimentin is of interest to many aspects of nervous system functioning and should be further examined.

VCP was another PINK1-interacting protein discovered by our mass spectrometry analysis. This supports recent findings which have shown VCP to be essential for mitochondrial quality control by PINK1/parkin and this function to be impaired by VCP mutations (Kim et al., 2013b). Pathogenic VCP mutations induced mitochondrial uncoupling and reduced ATP levels in patient fibroblasts (Bartolome et al., 2013). Furthermore, Wang and colleagues showed that the interaction of PINK1 with VCP and its co-factor p47 promotes dendritic arborisation in cortical primary neurons (Wang et al., 2018a). VCP has been reported to be involved in Golgi fragmentation and reassembly during mitosis ((Acharya et al., 1995, Rabouille et al., 1995), for review (Wang et al., 2004). These are two major functions we have shown to be impaired by loss of PINK1 and to regulated by PINK1 kinase activity (Furlong et al., 2019, O'Flanagan et al., 2015). Thus, VCP may be an important candidate for PINK1-induced organelle (Golgi and mitochondria) and cell cycle control. Interestingly, VCP is a downstream target of Akt which phosphorylates VCP on Ser-351, Ser-745 and Ser-747 and this phosphorylation is required for growth factor-induced anti-apoptotic signalling mediated by the Akt/NF-κB pathway (Vandermoere et al., 2006). VCP has also been linked to several neurodegenerative disorders, including Huntington's disease (Guo et al., 2016), amyotrophic lateral sclerosis (Hall et al., 2017) and spinocerebellar ataxia Type 3 (Ristic et al., 2018). Therefore, the interaction between VCP and PINK1 may have implications for PD pathogenesis linking PINK1, Akt, Golgi fragmentation, mitophagy, inflammation and neuronal integrity, making it a good candidate for further research in this area.

RIPK3 plays a functional role in necroptosis, a form of programmed cell death and neuroinflammation implicated in PD (Yuan et al., 2019), in mitochondrial dysfunction in dopaminergic neurons (Iannielli et al., 2018) and has recently been reported to be regulated by another PD risk gene, parkin (Lee et al., 2019). RIPK3 activates (Imamura et al., 2018, Liu et al., 2014) and inactivates (Li et al., 2010) Akt and induces Akt dependent phosphorylation of ACL (Imamura et al., 2018). Notably we also identify

ACL as a potential PINK1 interacting protein. ACL is a substrate of Akt (Berwick et al., 2002, Covarrubias et al., 2016, Lee et al., 2014) and is a major metabolic enzyme that couples citrate/mitochondrial bioenergetics with Acetyl-CoA production, lipogenesis and histone acetylation. The potential interaction of PINK1 with RIPK3 and ACL with respect to Akt signalling, necroptosis and mitochondrial function is deserved of future attention.

Our mass-spectrometry analysis also identified several calcium regulatory proteins as PINK1 interacting proteins. These include CALM1, CAPSN1 and CAPN2 and S100A6. PINK1 has already been shown to suppress α-synuclein-induced neuronal injury via CALM1 (Yang et al., 2018). CALM1 can in turn regulate PINK1-parkin induced mitophagy pathways (Zhang et al., 2017c). Interestingly CALM1 has been reported to regulate PI3-kinase and thus Akt via binding to its regulatory subunit p85α/β (Yang et al., 2018). In our analysis, we hypothesise that PINK1 regulates $PIP_3$/Akt signalling at the level of PI3-kinase p85 (Furlong et al., 2019) which interestingly also regulates PTEN (Chagpar et al., 2010, Cheung et al., 2015, Rabinovsky et al., 2009).

We also identified several immune related proteins as PINK1 interacting proteins: including ISG15, IFI204 and C5. ISG15, is a ubiquitin-like protein which is one of the major type 1 interferon (IFN) effectors. Akt activation is essential for induction of ISG15 induced IFN antiviral response (Kaur et al., 2008). ISG15 is conjugated to target proteins via the activation of E1-E3 enzyme and balanced by removal by UPS18 regulated degradation of EGFR (Xu et al., 2015), another PINK1 interactor we identified here. Importantly ISG15 has been shown to be a "hub protein" in a 6-OHDA rat model of PD transcriptome sequence analysis (Li et al., 2019), links to mitophagy (Desai et al., 2018) and positively regulates the E3 ligase activity of parkin (Im et al., 2016). IFI204 also functions in the IFN response (Chen et al., 2019), and autophagy activation via cGAS-STING response (Storek et al., 2015), a pathway implicated in balancing PINK1-parkin survival/death pathways (Sliter et al., 2018). Together this draws attention to functional links between PINK1, Akt, EGFR and interferon responses in PD pathogenesis.

This study has examined all previously-reported PINK1 interacting proteins and identified several new PINK1 interacting proteins that are involved in diverse processes that regulate cell survival and stress resistance, many of which center on Akt signalling. These interactions need to be further validated by methods such as co-immunoprecipitation with endogenous and overexpressed proteins, bimolecular fluorescence complementation or T7 phage display. Determining how PINK1 interacts with these novel proteins and signalling systems and how they function in health and disease is vital to understanding the mechanisms involved in PD pathogenesis caused by loss of PINK1 function.

### 3.7 Acknowledgements

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### 3.8 Author contributions

Rachel Furlong: study conception, investigation, performed experiments, data and evidence collection, formal analysis, and manuscript preparation.

Aideen Sullivan: study conception, supervision, manuscript preparation, commentary, critical review.

Cora O' Neill: study conception, supervision, manuscript preparation, commentary, critical review.

### 3.9 Conflict of Interest

The authors declare no conflict of interest.

# Chapter 4

# Chapter 4: α-synuclein overexpression and loss of PINK1 induce mitochondrial fission and Golgi fragmentation and reduce neurite length in midbrain dopaminergic neurons.

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**This chapter lists thirty references due to this reference limit in the Parkinsonism and Related Disorders journal to which it has been submitted 26/09/19**

## 4.1 Abstract:

### Introduction

Accumulation of α-synuclein is central to the development of Parkinson's disease (PD), and mutations in *SNCA*, which encodes α-synuclein, cause PD. Loss of function mutations in PTEN-induced putative kinase-1 (*PINK1*) also lead to PD. There is a need to identify the mechanisms by which α-synuclein overexpression and PINK1 loss induce neurodegeneration in PD, to determine whether they share common mechanisms and to identify therapeutic targets.

### Methods

We employed embryonic day (E) 14 rat ventral midbrain (VM) cultures to investigate the effects of overexpression of wildtype or mutant (A53T) α-synuclein and of siRNA deletion of PINK1 on neurite length, organelle health and PI3-kinase/Akt signalling, defects in which have been implicated in PD neuropathology.

### Results

We identified significant Golgi fragmentation, mitochondrial fission and reduced neurite length in response to overexpression of wildtype or mutant α-synuclein, and to deletion of PINK1. Increased Golgi fragmentation and mitochondrial fission induced by the PD risk genes were significantly correlated with reductions in neurite length. Levels of phosphatidylinositol 3,4,5 triphosphate ($PIP_3$), the primary activator of Akt, were significantly decreased by overexpression of wildtype or mutant α-synuclein. α-synuclein overexpression in combination with knockdown of PINK1 induced a "dual-hit", to further reduce neurite length and increase Golgi fragmentation.

### Discussion

This study provides novel data showing that α-synuclein overexpression and PINK1 deletion converge to induce significant increases in Golgi fragmentation and mitochondrial fission, with concomitant decreases in neurite length and defects in PI3-kinase/Akt signalling. This highlights the need for further studies on these converging mechanisms in the pathogenesis and treatment of PD.

## 4.2 Keywords:

α-synuclein, PINK1, midbrain dopaminergic neurons, neurite growth, Golgi fragmentation, mitochondrial fission

## 4.3 Introduction:

The neuropathology of PD is characterised by the progressive degeneration of midbrain dopamine (mDA) neurons and loss of DA neurotransmission in the striatum (for review see (O'Keeffe and Sullivan, 2018)). The major pathological hallmark of PD is the appearance of Lewy bodies and Lewy neurites, which are primarily composed of α-synuclein. Six PD-causing point mutations in the gene encoding α-synuclein, *SNCA*, have been identified, as well as duplication and triplication of the *SNCA* gene, all of which have been associated with autosomal dominant PD (for review see (Lesage and Brice, 2009). Linkage and genome-wide association studies have also identified genetic risk loci and variants in *SNCA* for idiopathic forms of PD. Interestingly, a clear *SNCA* dosage-related disease phenotype has been observed. Triplication of *SNCA* leads to an earlier age at disease onset, increased disease progression and a limited lifespan, in contrast to patients with *SNCA* duplication who have milder clinical symptoms (for review see (Lesage and Brice, 2009).

Overexpression of α-synuclein has been linked to axonal degeneration in dopamine neurons. Specifically, reduced neurite growth, connectivity and spine formation, and increased neuronal toxicity, have been reported in both cultured rat mDA neurons and human induced pluripotent stem cell (iPSC)-derived neurons with *SNCA* triplication or α-synuclein overexpression (Lin et al., 2016, Oliveira et al., 2015, Skibinski et al., 2017). Moreover, iPSC-derived mDA neurons carrying the A53T mutation have been reported to develop neurite degeneration (Kouroupi et al., 2017). There is much evidence to support a role for axonal degeneration as an early pathological process in PD (for review, see (O'Keeffe and Sullivan, 2018). The fact that α-synuclein overexpression can lead to decreased neurite length and axonal degeneration highlights

the need to identify the mechanisms involved in this process, in order to aid the development of therapeutic targets for early intervention in PD.

A number of organelle abnormalities have been reported in degenerating neurons in the PD brain, in cells from PD patients and in preclinical models of PD. Increased mitochondrial fission, which is strongly associated with apoptosis, was observed in skin fibroblasts of PD patients (Exner et al., 2007). There is solid evidence for mitochondria-mediated oxidative stress and defects in mitochondrial morphology and function in mDA neurons derived from PD patients' iPSCs (Byers et al., 2011, Tolo et al., 2018). Mitochondrial fission/fusion balance has been shown to be critical for cell cycle progression, cell division and growth (for review see (Serasinghe and Chipuk, 2017)). We have shown that deletion of *PINK1* leads to increased mitochondrial fission and a failure of progression though the cell cycle (O'Flanagan et al., 2015). However, there is limited information regarding the effect of α-synuclein overexpression on mitochondrial dynamics, such as fission/fusion balance. Overexpression of α-synuclein in primary cultures of cortical neurons has been shown to increase mitochondrial thiol oxidation and activate caspases, resulting in diminished neuronal excitability, decreased frequency of action potentials and neuronal loss (Tolo et al., 2018). Furthermore, iPSC-derived neurons with *SNCA* triplication showed elevated levels of markers of oxidative stress, including monoamine oxidase-A (MAO-A) and heme oxygenase 2 (HMOX2), as well as sensitivity to peroxide-induced oxidative stress (Byers et al., 2011). It is therefore important to examine whether α-synuclein overexpression also induces mitochondrial deficits in neuronal models of PD, whether this may be influenced by loss of PINK1, and how this relates to neurite growth.

Golgi fragmentation, another essential failure in organelle fission and fusion dynamics, is another known characteristic of PD neuropathology (Gosavi et al., 2002, Rendon et al., 2013). A tight correlation has been reported between the presence of prefibrillar α-synuclein aggregates and Golgi fragmentation in non-neuronal COS-7 cells that had been transduced with adeno-associated virus (AAV)-α-synuclein (Gosavi et al., 2002). Additionally, neuritic degeneration and Golgi fragmentation were reported in primary cultures of dorsal root ganglia (DRG) neurons overexpressing α-synuclein (Lee et al.,

2006). In contrast, another study reported that the Golgi remained compact in response to A53T-α-synuclein overexpression in normal rat kidney (NRK) and PC12 cells (Thayanidhi et al., 2010). Our recent studies have discovered that deletion of PINK1 causes Golgi fragmentation (Chapter 2, (Furlong et al., 2019)), indicating that this may be a common denominator in PD neuropathology. It is important to investigate whether α-synuclein overexpression also induces Golgi deficits in dopaminergic neuronal models, whether this may be influenced by loss of PINK1, and how this relates both to mitochondrial defects and to neurite growth.

Akt signalling has been implicated in α-synuclein-induced neurotoxicity, and reduced Akt activation occurs in neurons in the PD brain (Timmons et al., 2009). Akt, the central kinase in this pathway, is a master regulator of cell survival, metabolism, proteostasis, organelle health and trafficking, which are key systems that fail in PD. Accumulation of the membrane lipid $PIP_3$, via agonist-induced PI3-kinase phosphorylation of $PI(4,5)P_2$, is the most critical prerequisite for Akt activation, recruiting inactive cytosolic Akt to membrane domains (for review see (Manning and Toker, 2017)). It has been reported that overexpression of wild-type α-synuclein or mutant (A53T or A30P) α-synuclein suppresses IGF-1-induced Akt activation in SK-N-SH cells (Chung et al., 2011). In another study, α-synuclein overexpression suppressed neuronal differentiation by blocking GSK3β inactivation, which is induced by Akt, and by increasing PP2A activity, a negative regulator of Akt activation (Kim et al., 2018). Importantly, many PD risk genes impair Akt signalling (reviewed by (Wang et al., 2012)). These include PINK1, whose loss of function significantly impairs Akt activation (Akundi et al., 2012, Furlong et al., 2019). Defects in Akt activation induced by loss of PINK1 function are mechanistically linked to impaired mitophagy (Soutar et al., 2018). Our recent work revealed that loss of PINK1 kinase activity reduces Akt activation due to an inability to increase $PIP_3$ at the plasma membrane and in the Golgi (Chapter 2, (Furlong et al., 2019)). Furthermore, overexpression of human PINK1 in cells where PINK1 had been deleted was found to restore normal Golgi morphology in an Akt-dependent manner (Chapter 2, (Furlong et al., 2019)). In combination, these studies link impairment of Akt activation to both mitochondrial and Golgi pathology in PD. However, it is unclear whether there is a link between changes in Akt activation and neurite growth, and

alterations in mitochondrial and Golgi pathology, which are induced by overexpression of wild-type or mutant α-synuclein. Furthermore, it is not known whether such α-synuclein-induced effects would be further regulated by deletion of PINK1, as occurs in PD.

In the present study, we aimed to expand the knowledge on mitochondrial dynamics, Golgi fragmentation and PI3-kinase/Akt signalling in PD by examining the effects of overexpression of wild-type or mutant (A53T) α-synuclein, in cultures of E14 rat VM, the brain area affected by PD. We employed neurite length as a read-out of α-synuclein pathology at the cellular level (Hegarty et al., 2016). We hypothesised that PINK1 knockdown in cultured VM neurons would cause defects in $PIP_3$ synthesis and Golgi and mitochondria integrity, such as we have previously found in PINK1-modified mouse embryonic fibroblasts (MEFs) (Furlong et al., 2019, O'Flanagan et al., 2015) and further aimed to determine how this would be regulated by overexpression of α-synuclein in a "dual hit" model system.

Our results reveal for the first time that overexpression of either wildtype or mutant α-synuclein and deletion of PINK1 converge to induce significant increases in Golgi fragmentation and mitochondrial fission, with concomitant decreases in neurite length. $PIP_3$ levels were significantly decreased by overexpression of either wildtype or mutant α-synuclein. We also report that Golgi and mitochondrial pathology were negatively correlated with neurite length. Furthermore, a "dual-hit" combining overexpression of α-synuclein with deletion of PINK1 induced further reductions in neurite length and further increases in Golgi fragmentation. Together, these results advance the current knowledge on the mechanisms by which overexpression/accumulation of α-synuclein and loss of function of PINK1 impairs neuronal function in PD.

## 4.4 Materials and Methods:

### 4.4.1 Cell Culture

SH-SY5Y cells and E14 rat VM were cultured as previously described (Hegarty et al., 2016), under license with full ethical approval. For the preparation of VM cultures, E14 embryos were obtained by laparotomy from date-mated female Sprague–Dawley rats following decapitation under terminal anesthesia induced by inhalation of isoflurane (Isoflo®). Dissected VM tissue was incubated in a 0.1 % trypsin-Hank's balanced salt solution for 5 min, at 37 °C with 5 % $CO_2$. Fetal bovine serum (FBS) was then added to the tissue. The tissue was resuspended in 1 ml of differentiation media (Dulbecco's modified Eagle's medium/F12, 33 mM D-glucose, 1 % l-glutamine, 1 % FCS, supplemented with 2 % B27) using a P1000 Gilson pipette, then carefully triturated using a 25-gauge needle and syringe. Cell density was estimated using a haemocytometer. Cells were plated on poly-d-lysine (Sigma)-coated 24-well tissue culture plates at a density of $1.5 \times 10^4$ cells per well in 500 µl of differentiation media at 37 °C with 5 % $CO_2$.

### 4.4.2 Plasmids

SH-SY5Y cells were seeded at a density of $5 \times 10^4$ cells per well and allowed to reach ≥ 70% confluency prior to transfection. E14 VM cultures were seeded at a density of $1.5 \times 10^5$ cells per well. The TransIT-X2® Dynamic Delivery System (Mirus Bio) was used according to the manufacturer's instructions. The TransIT-X2:DNA complex was prepared using serum-free medium, TransIT-X2 reagent and 0.5 µg/µl stock of a control EGFP plasmid (Control-GFP) or EGFP-α-synuclein-Wild-Type (αSynWT-GFP) or EGFP-α-synuclein-A53T (αSynA53T-GFP) plasmids. These were a kind gift from David Rubinsztein (Addgene plasmid #40823; 25)). Cells were transfected for 48 h before further experimentation.

### 4.4.3 siRNA

Cells were transfected with 50 nM PINK1 siRNA (Sigma) or negative control siRNA

(siNeg) (Sigma) using the TransIT-X2® Dynamic Delivery System (Mirus Bio). The PINK1 target sequences were: 5′CGCAAATGTGCTTCATCTA′3 and 5' CCTATGAAATCTTCGGGCT 3' (siRNA68 and siRNA69 respectively). Cells were transfected for 48 h before further experimentation.

### 4.4.4 RT-PCR

PINK1 knockdown was confirmed using RNA extraction and RT-PCR analysis as described previously (Furlong et al., 2019) RNA was extracted using the PureLink RNA Mini Kit (Thermo Scientific) according to manufacturer's instructions. cDNA synthesis was performed with the QuantiTect reverse transcription kit (Qiagen) using 1µg of RNA. RT-PCR was performed using the GoTaq G2 Green Master Mix kit (Promega). Primer sequences: Forward: 5' GCTGATCGAGGAGAAGCAG 3' Reverse: 5' GATAA TCCTCCAGA CGGAAGC 3'

### 4.4.5 Immunofluorescence and confocal microscopy

The following primary antibodies and dilutions were used: phosphatidylinositol (3,4,5)-trisphosphate (1:200; Tebu-Bio 117Z-P345B), GM130 (1:4,000; BD biosciences 610822), Tom20 (1:400; Santa Cruz sc-11415). Primary antibodies were detected using Cy3 anti-rabbit IgG (1:400; Jackson laboratories 711-165-152) or Cy3 anti-mouse IgG (1:200; Jackson laboratories 715-165-150) secondary antibodies.

Culture medium was removed from each of the wells. Cells were washed with 1X PBS between each of the following steps: fixation in 0.5 ml 4% paraformaldehyde (PFA) for 15 min at room temperature, quenching in 1 ml of 50 mM $NH_4Cl$/PBS for 15 min, permeabilisation in 1 ml buffer (0.05% Saponin/PBS, 0.2% BSA/PBS) for 5 min. Coverslips were transferred to a humid box and primary antibodies were added at appropriate dilutions in 5% BSA/PBS. Primary antibodies were incubated for 2 h at room temperature. Cells were washed twice in 1X PBS, then incubated in secondary antibody solutions in 5% BSA/PBS, as well as DAPI/PBS (1:10,000) for 1 h at room temperature. Cells were washed twice with 1X PBS for 5 min. Coverslips were mounted

onto microscope slides using Mowiol mounting media. Cells were dried overnight and fluorescence images were acquired using Zeiss LSM 510 Meta confocal microscope fitted with a 63x/1.4 plan apochromat lens (Jena, Germany).

### 4.4.6 Assessment of neurite length, organelle health, and PINK1 immunofluorescence intensity

For neurite length analysis, Control-GFP/αSynWT-GFP/αSynA53T-GFP-positive neurons were analysed. Neurite length was measured using ImageJ software; the lengths of five randomly-selected neurites were measured in three randomly-selected fields per n number. For Golgi and mitochondria fragmentation, Control-GFP/αSynWT-GFP/αSynA53T-GFP-positive neurons were analysed. GM130-immunostaining was used to determine the percentage and number (Golgi only) of fragments, which were quantified from each neuron in three randomly-selected fields per n number. The percentages of mitochondria with decreases in both mitochondrial interconnectivity and elongation (indicating major fission and fragmentation) in the region of the cell soma and proximal early axon were quantified by determining the percentage of TOM20-immunostained cells with multiple unconnected fragments under the size of 3 μm in this region. This was quantified from each neuron in three randomly-selected fields per n number. $PIP_3$ fluorescence intensity in transfected primary cultures of VM neurons was analysed using Zeiss software by measuring the fluorescence intensity of $PIP_3$ staining that overlapped with GFP. The intensity of PINK1 immunofluorescence staining in individual GFP-positive neurons was measured using ImageJ software to confirm siRNA-mediated knockdown of PINK1. For all experiments, each n number was run in duplicate and 3 biological repeats were performed.

### 4.4.7 Statistical analysis

All data were analysed using GraphPad Prism. Data are expressed as means ± the standard error of the mean (SEM). Statistical analysis was carried out using one-way ANOVA (α-synuclein overexpression experiments) or two-way ANOVA (α-synuclein overexpression and PINK1 knockdown experiments) followed by a post-hoc Tukey test

if the ANOVA indicated significance. Differences were considered significant at $p < 0.05$. Correlations were examined using Pearson correlation analysis.

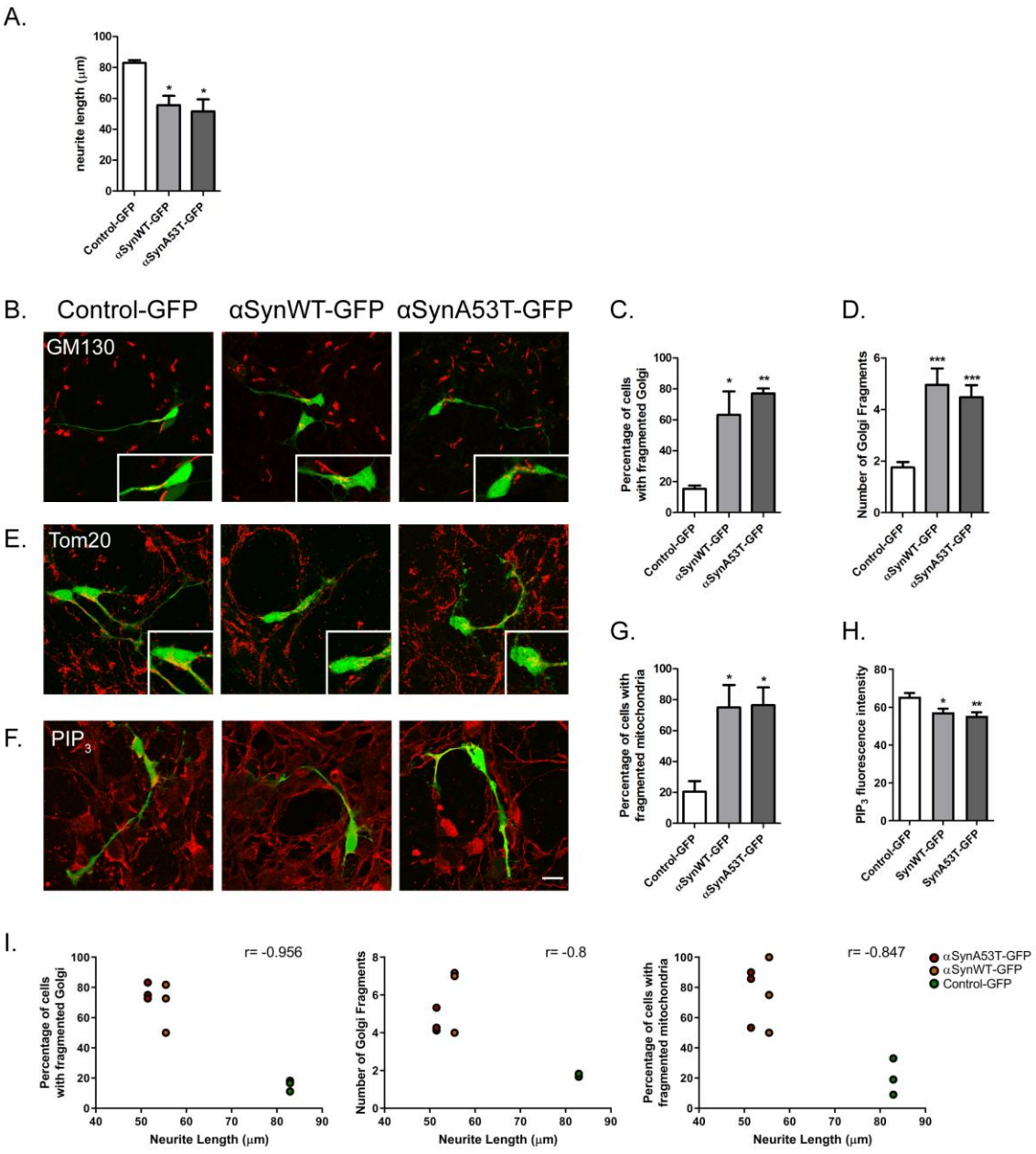
**4.5 Results:**

Initial experiments were performed on SH-SY5Y neuroblastoma cells transfected with αSynWT-GFP or αSynA53T-GFP constructs. Higher levels of Golgi and mitochondrial fragmentation, and decreases in $PIP_3$ levels, were found in α-synuclein-transfected neurons compared to controls (Supp. 4.1). We then performed all subsequent experiments on E14 rat VM cultures transfected with αSynWT-GFP or αSynA53T-GFP. There was a significant reduction in neurite length in both αSynWT-GFP- and αSynA53T-GFP-transfected neurons compared to control-GFP-transfected neurons ($p < 0.05$; Fig. 4.1A, B, E, F). Overexpression of αSynWT-GFP or αSynA53T-GFP also resulted in detrimental effects on organelle health. In transfected cells, there were significantly higher levels of Golgi fragmentation ($p < 0.05$) and increases in the numbers of Golgi fragments ($p < 0.001$), compared to controls (Fig. 4.1B-D). The percentages of mitochondria with decreases in both mitochondrial interconnectivity and elongation (indicating major fission and fragmentation) in the region of the cell soma and proximal early axon were also increased in response to αSynWT-GFP or αSynA53T-GFP overexpression ($p < 0.05$; Fig. 4.1E, G). The fluorescence intensity of $PIP_3$ was significantly lower in both wildtype- and A53T α-synuclein-transfected neurons, compared to controls ($p < 0.05$ and $p < 0.01$ respectively; Fig. 4.1F, H), indicating that there may be impaired PI3-kinase/Akt signalling. There were no significant differences in neurite length, in Golgi or mitochondrial fragmentation, or in $PIP_3$ levels between αSynWT-GFP- and αSynA53T-GFP-transfected neurons, indicating that overexpression of wildtype α-synuclein was sufficient to cause these defects and that the A53T α-synuclein mutation did not result in a more severe phenotype. Neurite length was strongly negatively correlated with Golgi fragmentation, with number of Golgi fragments and with mitochondrial fragmentation ($r = -0.956$, -0.8, and -0.847, respectively; Fig. 4.1I).

We next considered whether the phenotypes induced by α-synuclein overexpression in E14 rat VM cultures could be mimicked by PINK1 knockdown. PINK1 knockdown using either of two PINK1 siRNA constructs, siRNA68 or siRNA69, was confirmed by

**Figure 4.1 Overexpression of wild-type or A53T α-synuclein in cultured E14 rat VM causes decreased neurite length, increased mitochondrial fission, increased Golgi fragmentation and decreased $PIP_3$ fluorescence intensity.**

(A) Total neurite length of transfected cells in primary cultures of E14 rat VM following 48 h transfection with wild-type or A53T α-synuclein. Representative confocal images of (B) GM130-, (E) Tom20- and (F) $PIP_3$-immunostained primary cultures of E14 rat VM, following 48 h transfection with control or α-synuclein, as indicated. Scale bar = 10 μm. Corresponding quantification of (C) Golgi and (G) mitochondria fragmentation, (D) number of Golgi fragments and (H) $PIP_3$ fluorescence intensity. (I) Pearson correlation analysis of neurite length with Golgi and mitochondria fragmentation and number of Golgi fragments. All data are presented as mean ± SEM (n = 3 for each). *= $p < 0.05$, **= $p < 0.01$, and ***= $p < 0.0001$ with respect to Control-GFP (One-way ANOVA).


A.
neurite length (μm)
Control-GFP
αSynWT-GFP
αSynA53T-GFP
B.
Control-GFP
αSynWT-GFP
αSynA53T-GFP
GM130
C.
Percentage of cells with fragmented Golgi
D.
Number of Golgi Fragments
E.
Tom20
F.
PIP3
G.
Percentage of cells with fragmented mitochondria
H.
PIP3 fluorescence intensity
SynWT-GFP
SynA53T-GFP
I.
r= -0.956
r= -0.8
r= -0.847
Neurite Length (μm)
αSynA53T-GFP
αSynWT-GFP
Control-GFP

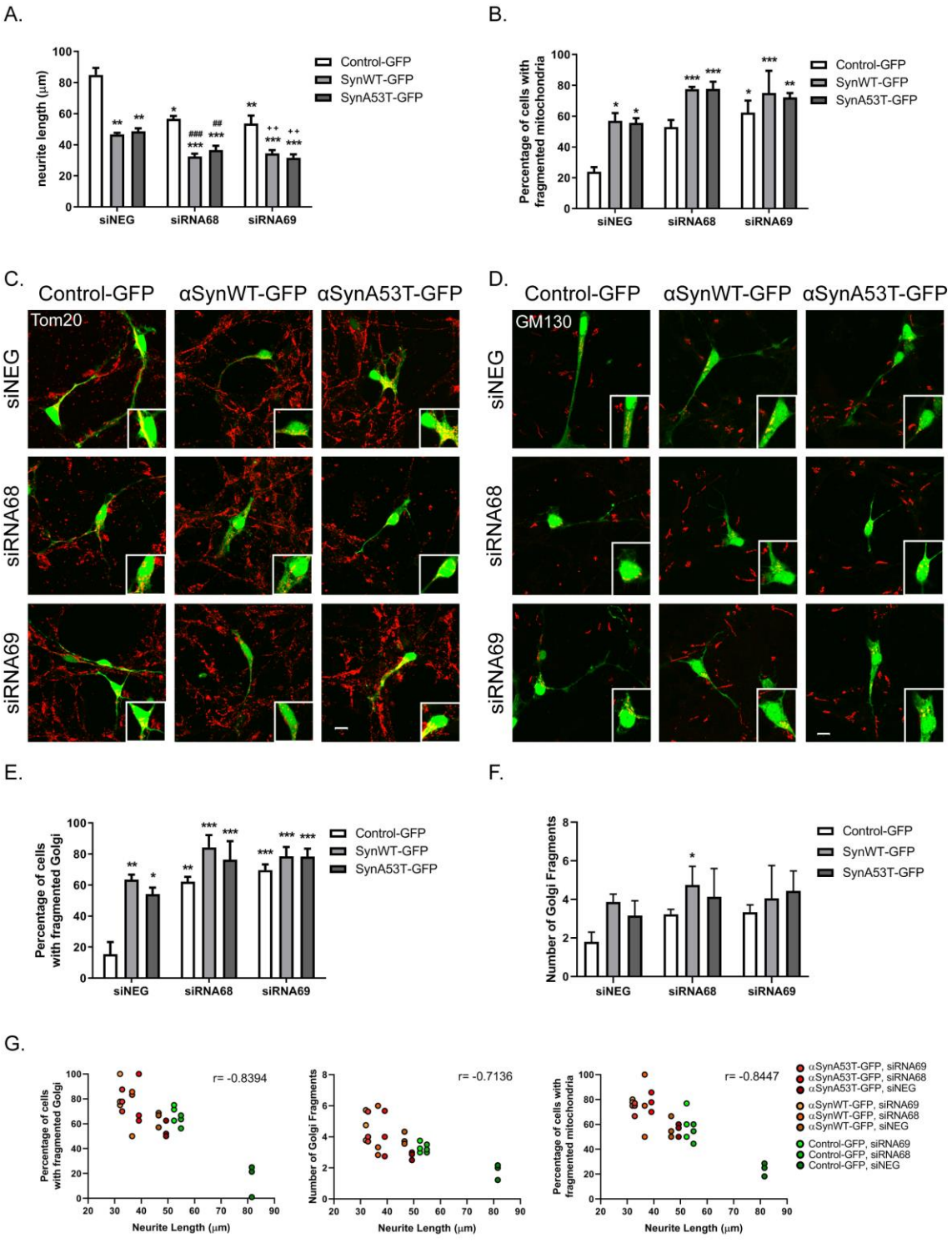
RT-PCR for PINK-1 on the entire culture samples (30% reduction, low due to low transfection efficiency; Supplementary Fig. 4.1D) and by analysis of PINK-1 immunofluorescence intensity in individual GFP-positive neurons that had been transfected (63% and 65% reductions in siRNA68- and siRNA6-transfected neurons, respectively; Supplementary Fig. 4.1E). PINK1 knockdown significantly decreased neurite length ($p < 0.05$, $p < 0.01$; Fig. 4.2A, C, D), increased mitochondrial fragmentation ($p < 0.05$; Fig. 4.2B, C) and increased Golgi fragmentation ($p < 0.01$, $p < 0.001$; Fig. 4.2D, E). PINK1 knockdown also caused a 15% decrease in the fluorescence intensity of $PIP_3$ in VM neurons (Fig. 4.3A, B), similar to that observed with α-synuclein overexpression, although this did not reach statistical significance.

Next, we investigated the effects of a possible "dual hit" induced by both α-synuclein overexpression in combination with PINK1 knockdown. We found that neurite length was significantly decreased in neurons overexpressing αSynWT-GFP or αSynA53T-GFP with either siRNA68- or siRNA69-induced knockdown of PINK1, compared to Control-GFP- and siNeg-transfected neurons ($p < 0.001$), and to neurons overexpressing α-synuclein or siRNA68/69 alone (post-hoc test $p < 0.01$, interaction effect $p = 0.02$; Fig. 4.2A). The combination of both α-synuclein overexpression and PINK1 knockdown resulted in a slightly higher percentage of cells displaying mitochondrial fission over that induced by either α-synuclein overexpression or PINK1 knockdown alone, although this did not reach statistical significance (Fig. 4.2B, C). However, there was a trend towards a significant increase in the percentage of cells with Golgi fragmentation in neurons overexpressing αSynWT-GFP or αSynA53T-GFP with PINK1 knockdown through siRNA68 or siRNA69, compared to either α-synuclein overexpression or PINK1 knockdown alone (interaction effect $p = 0.06$; Fig. 4.2E). There was also a trend towards a further increase in the number of Golgi fragments in neurons overexpressing αSynWT-GFP or αSynA53T-GFP with PINK1 knockdown through siRNA68 or siRNA69, although this did not reach statistical significance (Fig. 4.2F). Neurite length was strongly negatively correlated with Golgi fragmentation, with number of Golgi fragments and with mitochondrial fragmentation ($r = -0.8394$, $-0.7136$ and $-0.8447$ respectively; Fig. 4.2G). The 15% decrease in $PIP_3$ levels in VM neurons induced by PINK1 knockdown was further decreased by the combination of α-synuclein

overexpression and PINK1 knockdown (3-8%), although this was not statistically significant (Fig. 4.3A, B). Together, these results indicate that α-synuclein overexpression and PINK1 knockdown, both alone and in combination, result in substantial detrimental effects in E14 VM neurons.

**Figure 4.2 Overexpression of wild-type or A53T α-synuclein and PINK1 knockdown in combination causes decreased neurite length, fragmentation of mitochondria and Golgi, and increased numbers of Golgi fragments.**

(A) Total neurite length of transfected cells in primary cultures of E14 rat VM following 48 h transfection with α-synuclein or siRNAs, as indicated. Representative confocal images of (C) Tom20- and (D) GM130-immunostained primary cultures of E14 rat VM, following 48 h transfection with α-synuclein or siRNAs, as indicated. Scale bar = 10 μm. Corresponding quantification of (B) mitochondria and (E) Golgi fragmentation, and (F) number of Golgi fragments. (G) Pearson correlation analysis of neurite length with Golgi and mitochondria fragmentation and number of Golgi fragments. All data is presented as mean ± SEM (n = 3 for each). *= $p < 0.05$, **=$p < 0.01$, and ***= $p < 0.0001$ with respect to Control-GFP and siNeg, ##= $p < 0.01$ and ###= $p < 0.001$ with respect to Control-GFP and siRNA68, ++= $p < 0.01$ with respect to Control-GFP and siRNA69 (Two-way ANOVA).


A.
neurite length (μm)
Control-GFP
SynWT-GFP
SynA53T-GFP
siNEG
siRNA68
siRNA69
B.
Percentage of cells with fragmented mitochondria
C.
Control-GFP
αSynWT-GFP
αSynA53T-GFP
Tom20
D.
GM130
E.
Percentage of cells with fragmented Golgi
F.
Number of Golgi Fragments
G.
r= -0.8394
r= -0.7136
r= -0.8447
Neurite Length (μm)
αSynA53T-GFP, siRNA69
αSynA53T-GFP, siRNA68
αSynA53T-GFP, siNEG
αSynWT-GFP, siRNA69
αSynWT-GFP, siRNA68
αSynWT-GFP, siNEG
Control-GFP, siRNA69
Control-GFP, siRNA68
Control-GFP, siNEG

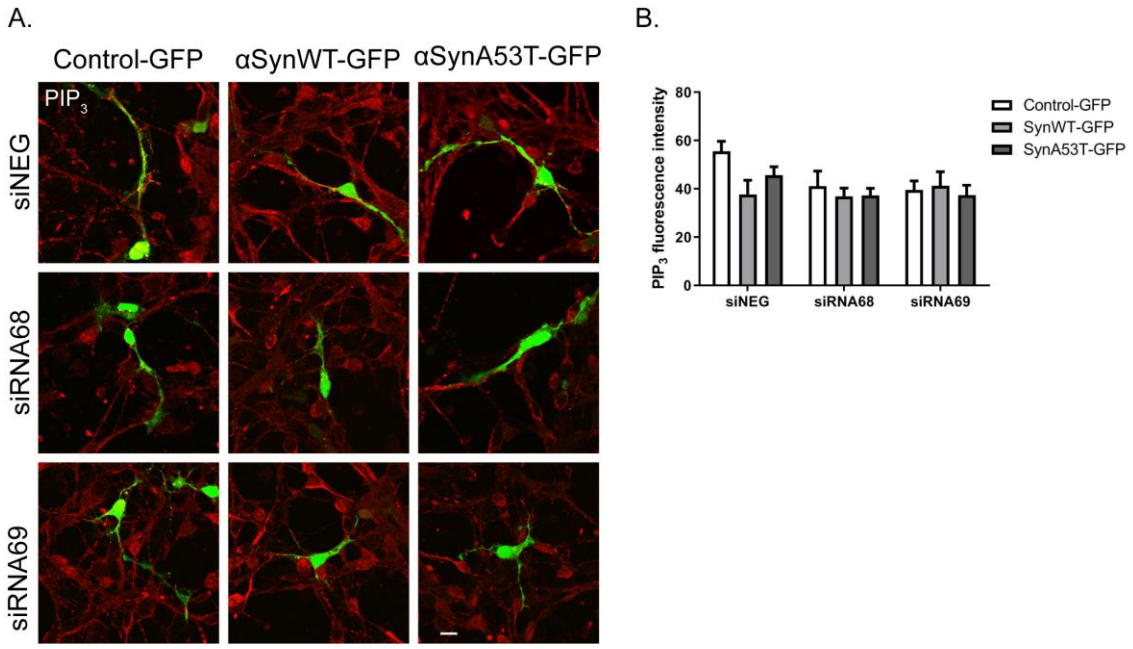



**Figure 4.3 Overexpression of wild-type or A53T α-synuclein and PINK1 knockdown in combination causes slight decreases in $PIP_3$ levels.**

(A) Representative confocal images of $PIP_3$-immunostained primary cultures of E14 rat VM following 48 h transfection with α-synuclein or siRNAs, as indicated. Scale bar = 10 µm. (B) Corresponding quantification of $PIP_3$ fluorescence intensity in transfected neurons. All data is presented as mean ± SEM (n = 3 for each). Statistical analysis was performed using Two-way ANOVA.

## 4.6 Discussion

Our findings show that overexpression of α-synuclein and loss of PINK1, as occurs in PD, cause a number of pathological features in cultured VM neurons including decreased neurite length, increased fragmentation of mitochondria and Golgi, and decreased $PIP_3$ synthesis. Furthermore, we show that increased mitochondrial fission and Golgi fragmentation were strongly correlated with decreased neurite length induced by both of these PD risk genes. In our "dual hit" PD model, where overexpression of α-synuclein was combined with deletion of PINK1, results in augmented detrimental effects, that is, further significant decreases in neurite length in addition to some increases in Golgi fragmentation. This indicates that α-synuclein and PINK1 may elicit their pathological effects through related pathways which impact both vital organelle health and neuritic function.

This study has shown for the first time that α-synuclein overexpression in cultured E14 rat VM neurons causes both Golgi fragmentation and mitochondrial fission, and that Golgi fragmentation is further exacerbated when the PD risk gene, PINK1, is also modified. Impaired mitochondrial quality control, with defects in mitophagy, fission, fusion and bioenergetics, is a key feature of PD pathogenesis. Nonetheless, an impact of overexpression of α-synuclein on mitochondrial fission in cultured dopaminergic neurons has not previously been reported. Moreover, our results reveal that the effects of α-synuclein or PINK1 on mitochondrial morphology is not amplified when both genes are simultaneously modified, indicating that these two genes act via common pathways to affect mitochondrial fission. Moreover, we show that these effects are accompanied by significant Golgi fragmentation, which is much less examined in PD pathogenesis than mitochondrial changes. Together, this indicates that impaired regulation of fission/fusion transitions in major organelles deserves further attention as a neuropathological mechanism in PD. Notably, it has been hypothesised that Golgi dispersal may be a means to propagate an α-synuclein-induced Golgi-based stress signal (Wang and Hay, 2015). Exactly how Golgi fragmentation is initiated in response to α-synuclein and PINK1 knock-down remains unclear. One study on PC12 cells indicated that Golgi disruption precedes microtubule disruption and inhibition of ER/Golgi

transport, implying that Golgi disruption is not a result of a trafficking block (Rendon et al., 2013). Golgi fragmentation was postulated to act as an early independent branch of the pathology regulated by Rab1, 2, 8 and SNARE-dependent mechanisms (Rendon et al., 2013). Importantly, studies have shown that PINK1 can modulate Rab8 (Lai et al., 2015) and that α-synuclein interacts with Rab1 and Rab8a (Lee et al., 2011, Yin et al., 2014). It would be interesting to examine whether blocking Golgi fragmentation and dispersal may protect against α-synuclein-induced neurotoxicity in VM cultures, and to explore any involvement of Rab1, 2, 8 or SNARE.

Our previous work has shown that impaired Akt signalling in PINK1-modified MEFs is caused by defects in $PIP_3$ synthesis at endomembranes (Furlong et al., 2019). Here, we report for the first time that $PIP_3$ levels are significantly decreased in neurons with α-synuclein overexpression. This is in agreement with the suppression of IGF-1-induced Akt activation reported in SK-N-SH cells following overexpression of wild-type α-synuclein or mutant (A53T or A30P) α-synuclein (Chung et al., 2011). Impaired signalling has been reported at additional levels of the PI3-kinase/Akt pathway in response to α-synuclein, including impaired GSK3β inactivation, increased PP2A activation (Kim et al., 2018), and decreased insulin sensitivity leading to insulin resistance (Chang et al., 2018). This indicates that the PI3-kinase/Akt pathway may be critical for both PINK1- and α-synuclein-induced neurodegeneration and highlights the need for more research in this area. In this respect, we have shown recently that overexpression of PINK1 can protect against Golgi fragmentation induced by loss of PINK1 in an Akt- and PINK1-kinase dependent manner (Furlong et al., 2019).

It was not possible to stain these neurons for a marker of dopaminergic neurons, for example with TH, as we do not have reliable antibodies for Golgi and mitochondrial staining that can work in combination with a TH antibody, nor did we have access to a confocal microscope which could record all three channels simultaneously. Therefore, it is possible that there may be additional neuronal populations transfected here such as glutamatergic and GABAergic neurons, which are known to be present in the VM region (Nair-Roberts et al., 2008), and this should be analysed further in future studies.

It is notable that there were no significant differences between overexpression of wildtype or mutant A53T α-synuclein, indicating that overexpression of wildtype α-synuclein can cause these pathological defects to the same degree as a mutated form. This is interesting as it suggests that the findings we report here could be of importance for both inherited and idiopathic forms of PD, as α-synuclein build up in Lewy bodies is reported in both. Our study provides new insights into VM neuron pathology in response to α-synuclein overexpression and PINK1 deletion and highlights the need for further integration of knowledge on cell survival signalling and organelle defects in neurodegeneration.

### 4.7 Acknowledgments

This work was funded by the Irish Research Council Government of Ireland Postgraduate Scholarship Programme, GOIPG/2015/3467. The Molecular Cell Biology group, UCC, provided access to a Zeiss 510 Confocal microscope, funded by an SFI Programme Grant to Mary W. McCaffrey.

### 4.8 Author contributions

Rachel Furlong: study conception, investigation, performed experiments, data and evidence collection, formal analysis, and manuscript preparation.

Gerard O'Keeffe: manuscript preparation, commentary, critical review.

Cora O'Neill: study conception, supervision, manuscript preparation, commentary, critical review.

Aideen Sullivan: study conception, supervision, manuscript preparation, commentary, critical review.

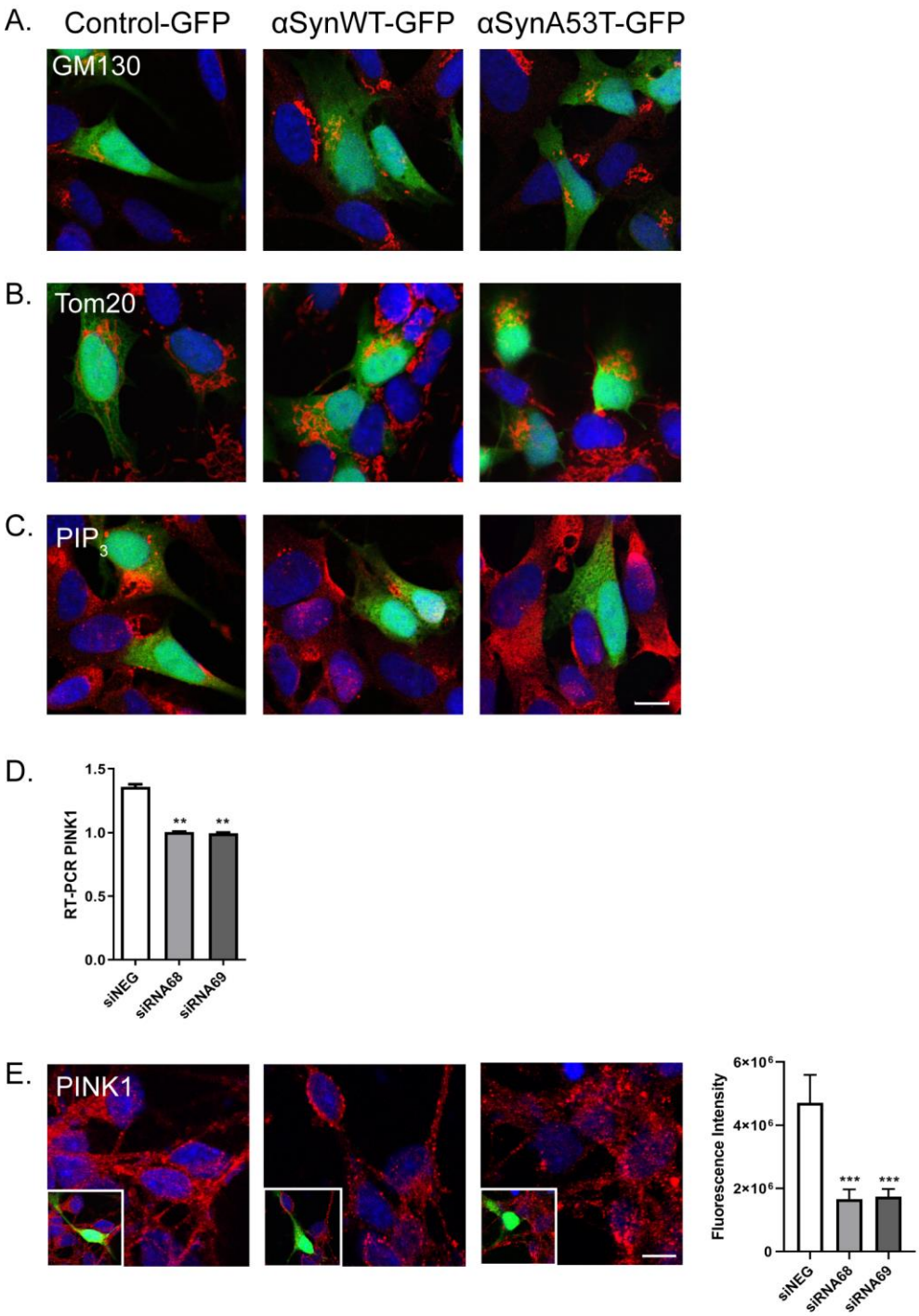
### 4.9 Conflict of Interest

The authors declare no conflict of interest.

## 4.10 Supplementary Results

**Supplemental Figure 4.1 Overexpression of wild-type or A53T α-synuclein in SH-SY5Y cells causes fragmentation of mitochondria and Golgi, and decreased $PIP_3$ fluorescence intensity.**

Representative confocal images of (A) GM130-, (B) Tom20- and (C) $PIP_3$-immunostained SH-SY5Y cells following 48 h transfection with α-synuclein, as indicated. Scale bar = 10 µm. (D) siRNA-mediated knockdown of PINK1 as confirmed by RNA extraction and RT-PCR analysis of E14 rat VM cultures. (E) siRNA-mediated knockdown of PINK1 as confirmed by quantification of the intensity of PINK1 immunofluorescence in GFP-positive neurons. Scale bar 10 µm.


A.
Control-GFP
αSynWT-GFP
αSynA53T-GFP
GM130
B.
Tom20
C.
PIP3
D.
RT-PCR PINK1
1.5
1.0
0.5
0.0
**
**
siNEG
siRNA68
siRNA69
E.
PINK1
Fluorescence Intensity
6×10⁶
4×10⁶
2×10⁶
0
***
***
siNEG
siRNA68
siRNA69

# Chapter 5: Discussion

## 5.1 General Discussion

The neuropathology of PD is characterised by progressive degeneration of DA neurons which leads to motor dysfunctions (Jankovic, 2008, Moustafa et al., 2016). Current therapies treat these motor symptoms but do not stop the progression of the disease. Therefore, a treatment that can slow down or prevent neuronal death remains elusive. In the majority of cases there is no explanation for why a person develops PD, ~5-10% of patients present with monogenic forms of the disease with Mendelian inheritance. These patients hold the key to discovering the mechanisms that cause PD pathology and identifying novel treatment targets. This is especially true when considering *PINK1*, where PD-causing mutations result in loss of function of the encoded protein. This enables the discovery of molecular pathways that lead to PD, in systems in which PINK1 is modified and is the focus of this thesis.

The most widely documented function of PINK1 is as key regulator of mitochondrial health, including mitophagy, fission, fusion, bioenergetics and mitochondrial antigen presentation (Harper et al., 2018, Matheoud et al., 2016, O'Flanagan et al., 2015, Pilsl and Winklhofer, 2012, Plun-Favreau et al., 2007, Youle and Narendra, 2011, Youle and van der Bliek, 2012). However, PINK1 has also been reported to play a role in PI3-kinase/Akt signalling (Akundi et al., 2012, Contreras-Zarate et al., 2015, Ellis et al., 2013, Maj et al., 2010, Sanchez-Mora et al., 2012). This pathway is a master regulator of cell survival, metabolism and proteostasis, key systems that fail in PD (for review (Manning and Toker, 2017)). While it is clear that PINK1 can activate Akt, and that PINK1 deletion reduces Akt activation (Akundi et al., 2012, Boonying et al., 2019, Contreras-Zarate et al., 2015, Ellis et al., 2013, Hauser et al., 2017, Maj et al., 2010, Mei et al., 2009, Murata et al., 2011, O'Flanagan and O'Neill, 2014, Sanchez-Mora et al., 2012, Soutar et al., 2018), the mechanism by which this occurs and its dependence on PINK1 kinase activity is unclear.

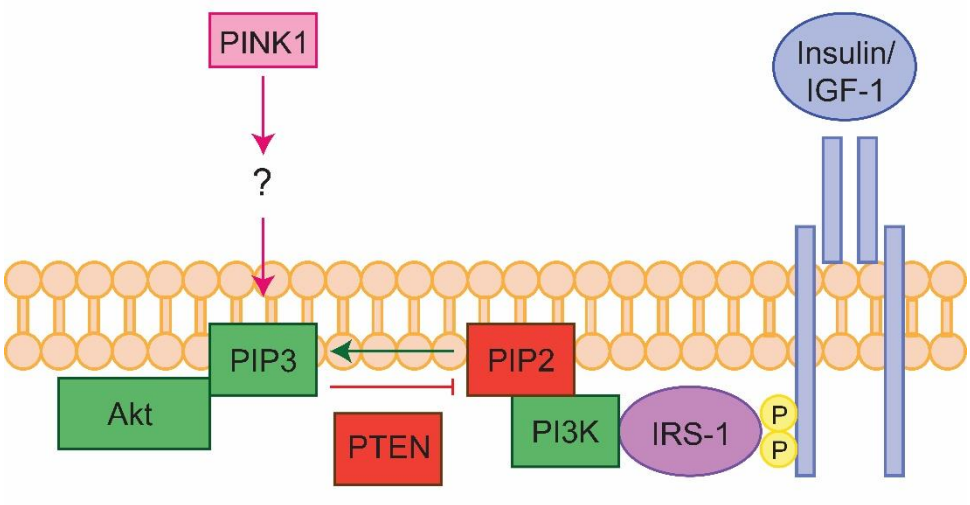
In Chapter 2 of this thesis we aimed to determine at what level of the PI3-kinase/Akt pathway PINK1 was regulating its activation. Chapter 3 aimed to identify potential mechanisms by which PINK1 could interact with $PIP_3$, Akt signalling and broader signalling networks. Here, bioinformatic software including iRefWeb, Reactome and

DAVID and wet lab interaction analysis involving lipid overlay assays and pull-down assays with mass spectrometry were employed. Finally, Chapter 4 aimed to expand the knowledge on mitochondrial dynamics, Golgi fragmentation and PI3-kinase/Akt signalling in PD by examining the effects of overexpression of α-synuclein in cultures of VM neurons. We further aimed to determine how this would be affected by both overexpression of α-synuclein and siRNA-mediated knockdown of PINK1 in a "dual hit" model system.

Results from Chapter 2 demonstrate a new role for PINK1 as a primary upstream activator of Akt via PINK1 kinase-dependent regulation of its primary activator $PIP_3$. This provides novel mechanistic information on how loss of PINK1 impairs Akt signalling in PD. However, it was not clear how PINK1 interacted with $PIP_3$ to affect its production. It was possible that PINK1 itself may be phosphorylating $PIP_2$ to produce $PIP_3$, that it may be interacting with known kinases or phosphatases that regulate the production of $PIP_3$ such as PI3-kinase or PTEN, or interacting with a novel protein that can also regulate the production of $PIP_3$. Analysis from Chapter 3 determined that PINK1 cannot bind directly to $PIP_3$, indicating that the effect of PINK1 on $PIP_3$ production results from its interaction with another protein(s) or from the formation of a protein complex (Fig. 5.1). $PIP_3$ production is dependent on PI3-kinase phosphorylation of $PI(4,5)P_2$ and in Chapter 2 we showed that PINK1 regulated the phosphorylation of the p85 regulatory subunit of PI3-kinase. The relationship between the p85 regulatory subunits, the p110α catalytic component of PI3-kinase and PI3-kinase activation is complex with numerous phosphorylation sites on PI3-kinase p85 proposed to activate and inactivate p110α (Marshall et al., 2019, Tsolakos et al., 2018). Therefore, further study of the impact of PINK1 on various activating tyrosine phospho-epitopes of p85α/β subunits and also inactivating Ser/Thr phospho-epitopes of p85α/β would be extremely valuable. The tools at present for specific PI3-kinase isoforms and their phosphorylation sites remain limited, future development of more specific antibodies that work for both western blotting and immunofluorescence would greatly assist the characterisation of PI3-kinase involvement in PINK1 regulation of $PIP_3$.

**Figure 5.1 PINK1 acts as a primary upstream activator of Akt via PINK1 kinase-dependent regulation of $PIP_3$**

PINK1 cannot bind directly to $PIP_3$. The effect of PINK1 on $PIP_3$ production results from its interaction with another protein(s) or from the formation of a protein complex, this is indicated by "?". Molecules activated by PI3-kinase signalling are shown in green and molecules inactivated are shown in red.

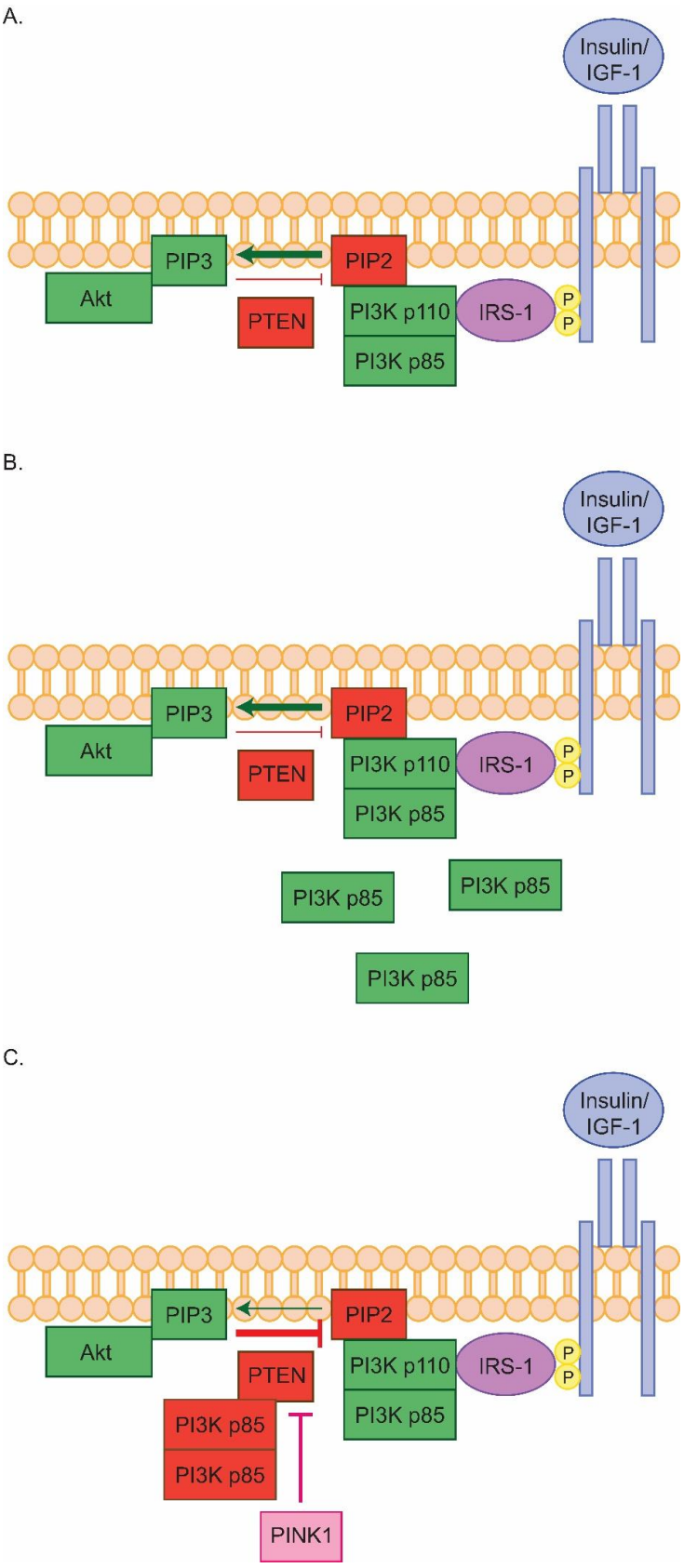

PINK1
?
Insulin/
IGF-1
PIP3
PIP2
Akt
PTEN
PI3K
IRS-1
P
P

Interestingly, recent studies have shown that the PI3-kinase p110α subunit catalytic activity can occur independent of p85α/β (Thorpe et al., 2017, Tsolakos et al., 2018). PI3-kinase p85α/β also performs a number of functions that do not involve PI3-kinase activation, including interactions with Rho GTPases, PTEN and Rab5 (for review (Marshall et al., 2019, Mellor et al., 2012)). This indicates that PI3-kinase p85α could regulate the PI3-kinase/Akt signalling pathway at multiple levels, with PI3-kinase activation (p110α-mediated), deactivation (PTEN-mediated) and receptor trafficking/signalling (Rab5-mediated) functions (Mellor et al., 2012). Termination of PI3-kinase-$PIP_3$-Akt signalling is predominantly due to dephosphorylation of $PIP_3$ to $PI(4,5)P_2$ by the phosphatase PTEN (Maehama and Dixon, 1998, Stokoe et al., 1997). The interaction between PINK1 and PTEN signalling has been highlighted since PINK1's discovery as it was named PTEN-induced putative kinase (Unoki and Nakamura, 2001). Recent studies show the PTEN-L, a longer form of PTEN de-ubiquitinates the $pSer^{65}$-Ub site phosphorylated by PINK1 (Wang et al., 2018b). Interestingly, PTENα has been shown to interact with canonical PTEN to increase PINK1 protein levels and promote energy production (Liang et al., 2014). Furthermore, PI3-kinase p85α can regulate PTEN (Chagpar et al., 2010, Cheung et al., 2015, Rabinovsky et al., 2009). It has been shown that when p85α levels are in excess of p110α, the p85α protein homodimerises (Cheung et al., 2015) and binds directly to PTEN in response to growth factor stimulation (Chagpar et al., 2010, Cheung et al., 2015, Rabinovsky et al., 2009). This interaction improves PTEN stability and stimulates the catalytic activity of the PTEN protein (Chagpar et al., 2010, Cheung et al., 2015, Rabinovsky et al., 2009). The PTEN-PI3-kinase p85α interaction is important in the context of our study as we have highlighted (in Chapter 3) that PTEN can bind to both PINK1 and to the phospholipid family of lipids. This indicates that there is a connection between PI3-kinase p85α, PTEN, $PIP_3$ and PINK1 or that the proteins could form a complex, which could play a role in PINK1 kinase-dependent regulation of $PIP_3$ and PI3-kinase/Akt signalling.

We hypothesise that PINK1 could act as a negative regulator of the stable PTEN-PI3-kinase p85α complex that dephosphorylates $PIP_3$ by binding to PTEN, phosphorylating

**Figure 5.2 Hypothesis; PINK1 binds to the PTEN-PI3-kinase p85α complex, inactivating PTEN leading to activation of the pathway and production of $PIP_3$.**

A. Insulin/IGF-1 activate their respective receptors which in turn phosphorylate the insulin receptor substrate (IRS). This activates PI3-kinase which phosphorylates $PIP_2$ to $PIP_3$ and increases levels of $PIP_3$, resulting in recruitment Akt to the membrane. B. PI3-kinase p85α levels are in excess of PI3-kinase p110α C. As a result, the p85α protein homodimerises (Cheung et al., 2015) and binds directly to PTEN in response to growth factor stimulation (Chagpar et al., 2010, Rabinovsky et al., 2009, Cheung et al., 2015). This interaction improves PTEN stability and stimulates the catalytic activity of the PTEN protein, turning off PI3-kinase/Akt signalling. We hypothesise that PINK1 then phosphorylates and inactivates PTEN to resulting in activation of PI3-kinase/Akt signalling.


A.
Insulin/
IGF-1
PIP3
PIP2
Akt
PTEN
PI3K p110
IRS-1
P
P
PI3K p85
B.
Insulin/
IGF-1
PIP3
PIP2
Akt
PTEN
PI3K p110
IRS-1
P
P
PI3K p85
PI3K p85
PI3K p85
PI3K p85
C.
Insulin/
IGF-1
PIP3
PIP2
Akt
PTEN
PI3K p110
IRS-1
P
P
PI3K p85
PI3K p85
PI3K p85
PINK1

it and thus leading to its inactivation as shown in Fig. 5.2. This is supported by the fact that the PINK1 regulation of Akt activation is dependent on PINK1 kinase function (Chapter 2). We did not find any changes in PTEN phosphorylation at $Ser^{380}$ (Fig. 2.4C) or total PTEN levels (Fig. 2.4C) in Chapter 2. However, another study has shown that the $Thr^{366}$ phosphorylation residue can be phosphorylated to inhibit PTEN function and alter its localisation (Moult et al., 2010). Therefore, PINK1 may be involved in regulating PTEN activation at another phosphorylation site. It would be important to examine the $Thr^{366}$ phosphorylation site and, more broadly to perform in vitro kinase assays, to determine whether PINK1 could regulate PTEN phosphorylation and inactivation.

PI3-kinase plays a role in several RTK pathways beyond the insulin/IGF-1 (IIS) pathway including the EGF, ERRB2/4, PDGF, VEGF, FGFR and c-MET signalling pathways (for review see (Lemmon and Schlessinger, 2010) that were found to be enriched in our Reactome analysis of PINK1 interacting proteins (Chapter 3). PI3-kinase is involved in Akt activation through the production of $PIP_3$, as previously described, and this leads to downstream signalling through a number of different proteins in each RTK pathway with a similar output, that is, promoting cell survival. Therefore, the regulation of PI3-kinase/Akt activation by PINK1, potentially through a complex with PI3-kinase p85α, PTEN and $PIP_3$, will also have implications for numerous RTK pathways and diverse survival signalling. In support of this, one study has shown impaired EGFR signalling, decreased EGFR expression and EGFR-induced Akt activation, in astrocytes cultured from PINK1-knockout mice (Choi et al., 2013).

It is important to note that the observation that PINK1 acts as a primary upstream activator of Akt via PINK1 kinase-dependent regulation of its primary activator $PIP_3$ has implications for signalling at both the plasma membrane and endomembranes. Recent evidence shows $PIP_3$ can equilibrate between endomembranes via membrane trafficking routes, that $PIP_3$ can be generated within endomembranes *in situ* and that Akt can localise to endomembranes, in addition to the plasma membrane (Braccini et al., 2015, Ebner et al., 2017, Jethwa et al., 2015, Salamon and Backer, 2013, Sato et al., 2003). Together these findings led to the modification of Akt signalling models to incorporate

compartment-specific patterns of activation (Ebner et al., 2017, Manning and Toker, 2017). In support of this new model, we show that PINK1 regulates the localisation of $PIP_3$ at the plasma membrane in response to short term IGF-1 stimulation, and to the Golgi under basal conditions and in response to long term IGF-1 stimulation (Chapter 2).

Another interesting finding of this thesis is the significant impairment of organelle fission to fusion transitions. We report significant Golgi fragmentation and mitochondrial fission in response to PINK1 knockout in MEFs and siRNA-mediated knockdown in cultured VM neurons (Chapter 2 and 4). In order to determine if these findings were common to other PD-causing mutations we examined the effect of overexpression of α-synuclein in cultured VM neurons. In line with the rest of our cell line studies, we found increased Golgi fragmentation, number of Golgi fragments and mitochondrial fission in cultured VM neurons in response to α-synuclein overexpression. Interestingly, VM neurons displayed impaired production of $PIP_3$ (as seen with PINK1$^{-/-}$ MEFs in Chapter 2), as well as a decrease in neurite length, in response to overexpression of α-synuclein or to loss of PINK1. The combined effect in our 'dual hit' model, where α-synuclein was overexpressed and PINK1 deleted, resulted in added detrimental effects, namely further significant decreases in neurite length in addition to a trend towards a further increase in Golgi fragmentation. Strikingly, there were strong negative correlations between the decreases in neurite length and the increases in both Golgi fragmentation and mitochondrial fission, in response to α-synuclein overexpression and PINK1 deletion. This indicates that α-synuclein and PINK1 may elicit their pathological effects through related pathways which impact upon both vital organelle health and neuritic function. Together, this shows that impaired regulation of fission/fusion transitions in major organelles, and not just mitochondria, is deserved of increased attention as a neuropathological mechanism in PD.

The limitation of the organelle analysis in this project is that it was all counted by eye and by one researcher. For future studies with more resources and personnel it would be optimal to develop automated software that could characterise fragmented Golgi and

mitochondrial fission, or if this was not possible, it would be advisable to have the morphology counted by a second researcher.

To further analyse the interaction between PINK1 and α-synuclein, it would be interesting to determine if overexpression of PINK1 in the α-synuclein-overexpressing VM neurons could rescue any of the phenotypes reported in Chapter 4, as enabling PINK1 or the vital cell systems it regulates could be a key therapy for PD. One recent study on primary cortical neurons reported that PINK1 interacted with α-synuclein through PINK1's kinase domain, blocking α-synuclein localisation to the mitochondria and alleviating α-synuclein-induced decreases in cell viability and cytotoxicity (Liu et al., 2017). Potentially this interaction could also rescue defects in neurite length and organelle health. As we have previously shown that PINK1 protects against Golgi fragmentation through the PI3-kinase/Akt signalling pathway in mouse embryonic fibroblasts (Chapter 2, (Furlong et al., 2019)), activation of this pathway with a constitutively-active Akt may also rescue the above-mentioned phenotypes. In addition, Wang and Hay hypothesised that Golgi dispersal may be a means to propagate an α-synuclein-caused Golgi-based stress signal (Wang and Hay, 2015). It would therefore be important to determine if blocking Golgi fragmentation and dispersal could remediate against α-synuclein-induced neuronal defects.

Proteomic approaches to elucidate PINK1's mechanism of action such as the pulldown: LC-MS/MS assay performed in Chapter 3 and subsequent bioinformatic analysis have the advantage of identifying unexpected novel binding partners for PINK1. This offers a valuable approach for defining new therapeutic targets in pathologies such as PD. Further studies of the novel PINK1-interacting proteins identified in this thesis work (Chapter 3) should yield insightful findings regarding PD pathogenesis and PINK1 function. The major challenge for these types of studies is that interactions between proteins are transient and some only occur in certain cellular contexts e.g. stress-induced mitophagy or at particular times of development or maturation. In using such techniques, we are thus trying to determine PINK1-interacting proteins that exist at a snapshot in time and therefore may be missing more candidates which are not identified in this screen. Our approach sought to identify PINK1 binding proteins under normal

cell growth conditions. Importantly, we chose to do this in cell systems and conditions in which we and others have discovered that PINK1 regulates the cell cycle, Akt signalling and mitochondrial quality control (Furlong et al., 2019, Morais et al., 2009, O'Flanagan et al., 2015). The novel PINK1 interacting proteins CALM1, RIPK3, ISG15 and ACL are of particular interest to us for future studies as is VCP, also identified in one previous study to be a PINK1 interacting protein (Wang et al., 2018a). These proteins all regulate or are regulated by Akt to elicit functional roles in programmed cell death, neuroinflammation, immune response and mitochondrial quality control (as highlighted in 3.6).

Bioinformatic analysis is only as good as the databases on which it is based. iRefWeb is a good tool to assemble the known interactome of a protein. However, when using this database we are relying on it being up to date and unfortunately upon surveying the literature further we did find one protein that had been shown to interact with PINK1 which was missing from the iRefWeb database, VCP. This paper (Wang et al., 2018a) was published 6 months before our analysis indicating there may be a delay in uploading current papers. Therefore iRefWeb must be used in combination with a further survey of the literature. Another limitation of this bioinformatic analysis is that the Reactome software does not have statistical testing, the output is based on a frequency of hits in a pathway or process. DAVID, in contrast, runs a Benjamini statistical test and only significant fold enrichment values are graphed making this database more stringent.

VCP is of interest to future studies as it is described as a target of Akt (Vandermoere et al., 2006) and is required for growth factor-induced anti-apoptotic signalling mediated by the Akt/NF-κB pathway (Vandermoere et al., 2006). Furthermore, VCP has been reported to be involved in Golgi fragmentation and reassembly during mitosis (Acharya et al., 1995, Rabouille et al., 1995), for review (Wang et al., 2004). We have previously shown that PINK1 kinase-dependent Akt activation protects against Golgi fragmentation (Chapter 2, (Furlong et al., 2019)) and that PINK1 regulates mitosis via mitochondrial quality control (O'Flanagan et al., 2015). Thus VCP could be the mediator of Akt activation and protection from Golgi fragmentation (as reported in Chapter 2 and 4). Moreover, PINK1 interaction with VCP and its co-factor p47 promotes dendritic

arborisation in cortical primary neurons (Wang et al., 2018a). Therefore, it would be interesting to determine if this interaction played a role in the reduced neurite length phenotype that we observed (Chapter 4) in response to overexpression of α-synuclein and loss of PINK1.

We have therefore identified a number of further research questions arising from this work that will be examined in our lab. In addition to these mechanistic studies in the PINK1 and α-synuclein modified MEFs and VM neuron PD models employed, it would be beneficial to determine if these defects occur in iPSCs from PD patients with *PINK1* or *SNCA* mutations and *PINK1* or *SNCA* knockout rodent models.

## 5.2 Conclusion

In summary, this thesis demonstrates that studies on the function of PD-causing genes such as PINK1 and α-synuclein using genetically modified systems, enables the discovery of key neuroprotective targets for PD. There are currently no disease modifying therapies for PD patients and treatment focuses on the motor symptoms but cannot prevent neuronal death. Thus, identifying the defective nodes of a cell survival signalling system involved in PD pathogenesis represents a significant advancement.

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