Investigating the role of PINK1 in Parkinson’s disease

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Abstract

Parkinson’s disease is a common neurodegenerative movement disorder caused by preferential progressive loss of dopaminergic neurons. Mitochondrial dysfunction has been long implicated in Parkinson’s disease but it is unclear as to how mitochondrial dysfunction is instigated to cause dopaminergic neuronal cell death in Parkinson’s disease. The recent identification of an autosomal recessive familial Parkinson’s disease causative gene that encodes a unique mitochondrial protein kinase, PINK1, provides a link between mitochondrial dysfunction and Parkinson’s disease.

Missense and truncating mutations identified in PINK1 familial Parkinson’s disease indicates that the impairment or loss of PINK1 function is a cause of dopaminergic neurodegeneration in Parkinson’s disease. Indeed, overexpression of PINK1 is shown to suppress neuronal cell death induced by mitochondrial dysfunction in vitro. In addition, recent studies suggest that PINK1 promotes cell survival by mediating mitophagy of defective mitochondria with compromised mitochondrial potential. As PINK1 is a mitochondrial protein kinase, PINK1 is expected to phosphorylate key mitochondrial proteins to promote cell survival during mitochondrial stress to prevent Parkinson’s disease. Thus, my PhD project aimed to investigate the protein substrates and downstream mediators of PINK1 to facilitate the elucidation of the physiological function of PINK1 to understand how the loss of PINK1 function could instigate dopaminergic neurodegeneration in Parkinson’s disease.

In this PhD thesis, bioinformatic analysis of the amino acid sequence of PINK1 revealed that PINK1 has a mitochondrial targeting sequence, a transmembrane domain, a protein serine/threonine kinase domain and a unique C-terminal tail. The bioinformatic analysis on the effects of the missense mutations associated with PINK1 familial Parkinson’s disease predicted that these mutations were likely to occlude the mitochondrial localisation of PINK1, perturb the kinase activity, substrate recognition or structural integrity of PINK1 to cause the pathogenesis of PINK1 familial Parkinson’s disease.
To investigate the protein substrates of PINK1, *Sf9* insect cell and *Pichia* yeast recombinant protein overexpression systems were used in an attempt to generate soluble pure preparation of catalytically active recombinant PINK1 proteins. Using *Sf9*-generated recombinant PINK1 protein, biochemical approaches, such as Kinase Substrate Tracking and Elucidation (KESTREL), were explored to investigate the protein substrates and downstream mediators of PINK1. In addition, the phosphorylation of the putative protein substrates, Parkin and TRAP1, by PINK1 were investigated using the recombinant PINK1 protein generated by the *Sf9* overexpression system. However, the phosphorylation of putative mitochondrial proteins, Parkin and TRAP1 by the recombinant PINK1 protein was not observed under the experimental conditions used in this PhD project.

Besides these biochemical approaches, a proteomic approach was also adopted to investigate the protein substrates and downstream mediators of PINK1 in BE2-M17 dopaminergic neuroblastoma cells. Preliminary proteomic analysis of the mitochondria isolated from BE2-M17 cells revealed that the protein levels of Leucine-Rich Pentatricopeptide-Repeat Motif Containing Protein and LON Protease Homolog are down-regulated during the overexpression of PINK1. These proteins are involved in regulating the expression of proteins encoded by the mitochondrial genome and may also participate in the mitophagy of compromised mitochondria mediated by PINK1. The overexpression of PINK1 in BE2-M17 cells also up-regulates the protein level of Dihydrolipoyl Dehydrogenase, which may promote the activity of mitochondrial complex I and contribute to the pro-survival function of PINK1 during the inhibition of mitochondrial complex I.

Several studies have presented conflicting evidence of sub-mitochondrial localisation of PINK1. Herein, PINK1 is shown to reside on the cytosolic side of the outer membrane of the mitochondria using an *in vitro* mitochondrial import assay and Proteinase K sensitivity assay. Furthermore, the transmembrane domain of PINK1 was detected by mass spectrometry in the proteolytically processed PINK1 protein isolated from the
mitochondria, indicating that processed mitochondrial PINK1 protein is anchored to the cytosolic side of the outer membrane of the mitochondria.

In this PhD project, the specificity of the pro-survival function of PINK1 was examined by treating BE2-M17 dopaminergic neuroblastoma with an array of mitochondrial toxins and oxidative stress agents. Preliminary analysis revealed that the overexpression of PINK1 only promotes the cell survival of BE2-M17 cells during cellular stress induced by the inhibition of mitochondrial complex I. However, the overexpression of PINK1 does not seem to influence the cellular level of reactive oxygen species in BE2-M17 cells during the inhibition of mitochondrial complex I.

In summary, my study provides experimental evidence indicating that PINK1 resides on the surface of the mitochondria and retains its transmembrane domain. Preliminary proteomic analysis of the effects of PINK1 overexpression in BE2-M17 dopaminergic neuroblastoma identified several mitochondrial proteins that are up-regulated or down-regulated upon the overexpression of PINK1, which may be involved in the mitophagy of defective mitochondria mediated by PINK1. The identification of these mitochondrial proteins provides a conceptual framework for future investigation into the role of PINK1 in the pathogenesis of PD.
Declaration

This is to certify that:

i. the thesis comprises only my original work towards PhD except where indicated in the *Preface*,

ii. due acknowledgement has been made in the text to all other material used,

iii. the thesis is less than 100,000 words in length, exclusive of tables, figures and references.

Chou Hung Sim

February, 2011
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Preface

Part of this thesis was written as a continuation of the research findings of my original work published in *Human Molecular Genetics*. Chapter 3 was a continuation of that data as published in *Human Molecular Genetics* mentioned below.

The following publications are associated with this thesis:


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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CTP</td>
<td>Cytidine triphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>TTP</td>
<td>Thymidine triphosphate</td>
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<td>dATP</td>
<td>Deoxyadenosine triphosphate</td>
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<tr>
<td>dCTP</td>
<td>Deoxycytidine triphosphate</td>
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<tr>
<td>dGTP</td>
<td>Deoxyguanosine triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>Deoxythymidine triphosphate</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide Triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>FAD⁺</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FADH₂</td>
<td>Flavin adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-Transferase</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nickel-Nitrilotriacetic acid</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Sf9</td>
<td><em>Spodoptera frugiperda</em> 9 (silky army worm)</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
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1 Chapter 1 - Introduction

1.1 Parkinson’s disease is a costly incurable disease

Parkinson’s disease (PD) is the second most common neurological disabling disorder in Australia. Its total economic costs\(^1\) to Australia and United Kingdom were AUD 6.8 billion and £3.3 billion respectively in 2005 \([1, 2]\). The prevalence of PD in Australia has increased by 42.5% since 1966 and is forecasted to increase by 3% per annum due to demographic ageing and in turn increase the social and economic costs due to the high cost of healthcare and loss of productivity \([1, 3]\).

This debilitating disease results from the progressive and predominant degeneration of dopaminergic neurons in the *substantia nigra pars compacta*\(^2\) (SNpc) of the midbrain \([4, 5]\). The loss of these neurons upsets the dopamine-mediated neural circuits in the basal ganglia and in turn disrupts the communication between the brain and muscles. This neuronal disruption renders the PD patients unable to initiate or stop a muscular movement properly and to display a spectrum of movement deficits called hypokinesia\(^3\). They also suffer non-motor deficits, such as autonomic dysfunction, cognitive degeneration, insomnia, anxiety, depression, impaired proprioception\(^4\), constipation, gastric dysmotility and urinary incontinence \([6]\).

These clinical PD symptoms only develop when the dopamine level in *corpus striatum*\(^5\) falls below 70% of the healthy level; by then PD patients have already lost 50-

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\(^1\) The net value of the burden of disease and the financial cost of Parkinson’s disease.

\(^2\) *Substantia nigra* (Latin for “black substance”) *pars compacta* is a portion of the midbrain thought to be involved in certain aspects of movement and attention.

\(^3\) Hypokinesia describes a spectrum of movement disorders: Akinesia (inability to initiate movement), Bradykinesia (slowness in executing movement), Freezing (inability to move muscles in any desired direction), Rigidity (resistance to passive movement throughout the whole range of motion) and Postural instability (unable to maintain upright posture).

\(^4\) Awareness of bodily position in three-dimensional space.

\(^5\) *Corpus striatum* is a striped mass of white and grey matter located in front of the thalamus in each cerebral hemisphere of the brain. It consists of caudate nucleus and lenticular nucleus.
70% of their dopaminergic neurons in the midbrain, which make early clinical diagnosis of PD difficult [4, 7]. To date, there is no drug or treatment that can replenish or halt this neurodegeneration. Current PD treatments, such as Levodopa, a dopamine precursor, and the invasive surgical intervention of deep brain stimulation, can only provide temporary relief for PD symptomatic motor deficit and some non-motor deficits [8-11]. Therefore, it is important to identify the underlying causes of PD to facilitate the development of PD treatments, a cure and preventative drugs and regimes.

1.2 Mitochondrial dysfunction is involved in Parkinson’s disease

Although PD has been observed and treated since medieval times, its etiology even today is still poorly understood [12]. It was only in the 1950s that the Nobel Prize winner, Arvid Carlsson and other scientists identified the aforementioned biochemical changes in the brains of PD patients and subsequently developed the first effective PD therapeutic drug, Levodopa [7, 13-16]. Even armed with the neurotransmitter deficit knowledge for PD at that time, they did not know, nor do we now know, what could have triggered the degeneration of SNpc dopaminergic neurons in PD patients.

In 1977, a 23 year old graduate, Barry Kidston attempted to synthesise an opioid analgesic drug, 1-methyl-4-phenyl-4-propionoxypiperidine \(^6\) (MPPP) for recreational usage based on drug synthesis work by Albert Ziering [17]. Instead of synthesising the drug at -30°C, Kidston performed the synthesis at room temperature and accidentally co-synthesised a neurotoxin, 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) [18]. He and several other drug abusers developed chronic PD symptoms several days after injecting the MPTP-contaminated MPPP [19]. This neurotoxin is metabolised to 1-methyl-4-phenylpyridium ion (MPP\(^7\)), a mitochondrial complex I (MC-I) inhibitor, by monoamine oxidase B (MAO-B) in the brain and is selectively taken up by dopaminergic neurons via their dopamine transporter (DAT) to confer selective death of dopaminergic neurons via their dopamine transporter (DAT) to confer selective death of dopaminergic neurons.

\(^6\)MPPP is an analog of meperidine and reversed ester of pethidine that has 70% potency of morphine.
neurons in both primates and rodents, particularly more detrimental in older animals [19-37]. These early observations highlight the first potential implication of mitochondrial dysfunction in the pathogenesis of idiopathic PD and prompted other research groups to investigate the possible involvement of impaired MC-I in idiopathic PD. Indeed, reduced MC-I activity of up to 30% in SNpc, skeletal muscles and platelets were observed in these patients [38-43]. However, the underlying cause of this observed systemic impairment of MC-I in idiopathic PD patients and how it instigates progressive yet preferential degeneration of SNpc dopaminergic neurons are still unknown.

Consistent with the reduced systemic MC-I activity in PD patients, a systemic MC-I inhibitor, rotenone also causes preferential degeneration of SNpc neurons in rodents [44-54]. These rotenone-treated rodents suffered up to 80% reduction of dopamine in their midbrain and displayed motor deficits similar to PD patients [49]. Like PD patients, these rodents were responsive to Levodopa treatment. Using slice preparation of rat brain, Bywood et al showed that both rotenone and MPP⁺ preferentially damage the dendrites of SNpc dopaminergic neurons [46]. This observation is consistent with the observed predominant degeneration of SNpc dopaminergic neurons in post-mortem study of PD patients [55]. As rotenone is a naturally occurring pesticide present in several tropical and subtropical plants, it is conceivable that prolonged exposure to environmental systemic mitochondrial toxins is a main contributing cause of idiopathic PD [50, 56-62]. In agreement with this, Fleming et al detected an organochlorine insecticide, Dieldrin in the brain samples from 30% of their PD patients but not in their normal controls [63]. Several other population epidemiological studies have also indicated that occupational exposure to metals and pesticides increase the risk for PD [57-62]. However, it is unclear how these environmental factors could trigger mitochondrial dysfunction to cause PD and why dopaminergic neurons are particularly vulnerable to MC-I impairment rather than other neuronal and cell types. Nonetheless, these findings suggest mitochondrial dysfunction is a major cause of the degeneration of SNpc dopaminergic neurons in PD.
1.3 Mitochondrion is unique to each cell type

Besides being the ubiquitous indispensible energy generator in eukaryotes, the mitochondrion is also involved in many vital cellular processes and tissue functions, such as cell cycle, differentiation, trafficking of synaptic vesicles, viral defence, apoptosis, calcium homeostasis, muscular contraction, and neurotransmission [64-87]. The morphology, dynamic behaviour, spatial arrangement, and cellular distribution of the mitochondria vary drastically between cell types and are important to their specific physiological functions [88-91]. In cardiac muscles, the mitochondria are fixed in a lattice of parallel rows surrounding the contractile myofilaments to fuel the ATP and Ca\(^{2+}\)-consuming muscular contraction [92-94]. Imaging of the mitochondrial inner membrane potential revealed that the electrical continuity of this mitochondrial arrangement provides the basis for calcium tunnelling (Ca\(^{2+}\) wave propagation) [95-97]. This electrical continuity also allows the subsarcolemmal cardiac mitochondrial populations proximal to capillaries to receive oxygen and mitochondrial energy fuels from the blood and to maintain the H\(^{+}\) gradients in the less nutrient accessible intermyofibrillar mitochondrial populations via this interconnecting mitochondrial network [98]. On the other hand, the mitochondrial arrangement and distribution in neurons are much more mobile, dynamic, and dependent on neurotrophic factors and calcium influx [77, 78]. Unlike cardiac mitochondria, neuronal mitochondria migrate to nerve growth factor stimulated regions to provide ATP, Ca\(^{2+}\) buffering, and signalling platforms during axonal stimulation [77]. Although the mitochondrion is a universal powerhouse, its cellular role and behaviour in each cell type is actually unique and crucial to the respective cell type’s physiological function. However, it is unclear if the susceptibility of SNpc dopaminergic neurons towards MC-I inhibition is due to the specific properties of its mitochondria.
1.4 Neuronal mitochondria are heterogeneous and synaptic mitochondria are vulnerable to mitochondrial complex I inhibition

The cellular distribution of neuronal mitochondria is not random but rather often targeted to specific regions that typically have high-energy demand and calcium fluctuation. In axons, mitochondria gather at the initial segment of axon and the nodes of Ranvier to satisfy the metabolic demands of Na⁺/K⁺ ATPases, which maintain critical ionic gradients [99-101]. They also concentrate at pre-synaptic terminals to supply ATP to support synaptic vesicle loading, mobilisation and recycling, and maintain calcium homeostasis by direct Ca²⁺ sequestration and providing ATP to plasma membrane Ca²⁺ ATPases [80, 102-108]. To access these areas, neuronal mitochondria rely on anterograde and retrograde transportation systems to move forth and back along the axons. Interestingly, most mitochondria with high membrane potential are anterogradely transported to the ATP demanding axonal regions [109]. On the other hand, those with low membrane potential are preferentially transported back to the cell body, which presumably is a mechanism for rejuvenating energetically compromised mitochondria and removing severely damaged mitochondria [109-111]. In agreement with this, MPP⁺ inhibits fast kinesin-1 mediated anterograde transport of mitochondria in isolated squid axoplasm, indicating that the impaired MC-I function in PD could limit synaptic mitochondrial localisation and in turn disrupt the synaptic activity of dopamine-mediated neural circuits in the basal ganglia [112].

Besides higher membrane potential, mitochondrial fission⁷ is also important for synaptic mitochondrial localisation. Normal synaptic mitochondria are smaller than their somatic counterparts, and the disruption of a fission protein, dynamin related protein 1 (Drp) results in neurons with elongated mitochondria accumulated in the cell body and largely missing synaptic mitochondria due to unbalanced mitochondrial fission and fusion⁸ [78, 80, 108, 113]. On the other hand, the disruption of a fusion protein,

⁷ The division of a mitochondrion into two or more smaller mitochondria.
⁸ The combination of two mitochondria into a single larger mitochondria.
mitofusin 2 (Mfn2) causes abnormal clustering of small fragmented mitochondria in the
cell body and proximal axons due to excessive fission [114]. Despite having higher
membrane potential, synaptic mitochondria are more susceptible to mitochondrial
dysfunction caused by MC-I inhibition. The inhibition of MC-I of non-synaptic
mitochondria can reach up to 70% before oxidative phosphorylation and ATP production
are severely affected, whereas the tolerance of synaptic mitochondria towards MC-I
inhibition is only 25% [115-117]. It is unclear whether the lower tolerance of synaptic
mitochondria was due to their smaller sizes. Given that there is a reported decrease of
30% in MC-I activity in PD patients, these observations therefore suggest that the
synaptic mitochondria of PD patients are incapable of supporting the metabolic demands
of synaptic activity in dopamine-mediated neural circuits. As prolonged synaptic
inactivity due to locomotor inactivity has been shown to reduce synaptic mitochondrial
localisation in both pigeons and rats, the synaptic inactivity resulting from dysfunctional
synaptic mitochondria in PD patients may contribute further to the disease progression
and motor deficits [118, 119].

Besides their vulnerability towards MC-I inhibition, synaptic mitochondria have been
reported to have lower Ca\(^{2+}\) buffering capacity and more susceptibility to Ca\(^{2+}\) overload
than their larger non-synaptic counterparts [113]. In addition, synaptic mitochondria
have lower cardiolipin content and the oxidation of cardiolipin\(^9\) is known to instigate the
opening of mitochondrial permeability transition pore and/or truncated Bid-mediated
mitochondrial membrane permeation to release apoptotic factors, such as cytochrome c
[120-131]. Collectively, synaptic mitochondria seem to be predisposed towards
mitochondrial dysfunction and are more likely to instigate apoptosis in PD. Indeed,
axonal degeneration of SNpc dopaminergic neurons is observed in PD patients and
MPTP-treated squirrel monkeys [26, 27, 131, 132]. However, it is unknown as to why
synaptic mitochondria of SNpc dopaminergic neurons are more susceptible to
mitochondrial dysfunction than other neuronal types.

\(^9\) Cardiolipin is a mitochondrial inner membrane phospholipid that is involved in assembly of electron
transport chain and sequestering intermembrane space cytochrome c.
1.5 **SNpc dopaminergic neurons are more susceptible to apoptosis**

SNpc dopaminergic neurons have an average of 41% less mitochondria mass than other neurons and their average mitochondria size is 36% smaller [133]. They also exhibit lower glucose utilisation and slower firing of action potential [134-137]. Thus, Liang *et al* postulated that the low metabolic demands of SNpc dopaminergic neurons predispose them to have lower mitochondria mass, and make them more susceptible to mitochondria-mediated apoptosis than other neuronal types when mitochondrial function is impaired by age-related or environmental-induced stress [133]. This hypothesis could explain why the systemic inhibition of MC-I by rotenone in rodents causes preferential loss of dopaminergic neurons and not other neuronal types or tissues [47, 50, 52]. Moreover, dopamine could impose additional oxidative pressure on dopaminergic neurons, as its metabolism via enzymatic and non-enzymatic reactions could generate reactive oxygen species (ROS) and predispose SNpc neurons to axonal degeneration and apoptosis in PD [138-144].

1.6 **Lewy bodies are found in most PD patients**

Besides mitochondrial dysfunction, eosinophilic aggregates of filamentous proteins termed Lewy bodies are commonly found in the cytosol of SNpc dopaminergic neurons of PD patients and these are thought to play a major role in PD pathogenesis [145]. However, the formation of these Lewy bodies and how they could cause idiopathic PD are still poorly understood. This is further complicated by the fact that Lewy bodies are not present in all idiopathic PD patients and are also found in some healthy elderly controls [146-148]. Several research groups hypothesise that Lewy bodies confer neurotoxicity in a fashion analogous to the toxic amyloid aggregation of Aβ peptides found in Alzheimer’s disease patients [149-152]. This hypothesis could explain the absence and presence of Lewy bodies in some idiopathic PD patients and healthy
individuals respectively, as the precursors of Lewy bodies seem to be the pathogenic species and are sequestered by forming Lewy bodies in healthy neurons. Consistent with this notion, SNpc neurons containing Lewy bodies in PD patients appeared to be less susceptible to apoptosis than those lacking Lewy bodies [153]. In addition, Lewy bodies appeared to be inert and did not seem to perturb the morphology and function of the SNpc neurons [154]. Nonetheless, the mechanism behind the formation of Lewy bodies, whether Lewy bodies themselves or their precursors confer the observed neurotoxicity to SNpc neurons in PD and whether mitochondria are also involved in these processes remain to be determined.

1.7 Genetic contributions towards understanding idiopathic PD

Although the occurrence of PD is mainly idiopathic, mutations in several genes have been linked to clinical PD syndromes that are indistinguishable from idiopathic PD [155-159]. These genes may participate in pathogenic cellular pathways contributing to idiopathic PD. Indeed, one of these genes, α-synuclein highlights the importance of studying these genes in understanding idiopathic PD. α-Synuclein is a major component of Lewy bodies in idiopathic PD; both mutations and gene duplication of α-synuclein in the autosomal dominant familial PD were shown to accelerate the formation of protofibrils and oligomers of α-synuclein and in turn aggravate the formation of Lewy bodies [150, 151, 157, 160-164]. Besides causing Lewy bodies, the protofibrils and oligomers of α-synuclein are also implicated in instigating mitochondrial dysfunction in PD. They have been hypothesised to depolarise mitochondria and release cytochrome c by forming pores within the mitochondria in a manner similar to pore-forming bacterial toxins [165-168]. Thus, the formation of Lewy bodies may involve mitochondrial dysfunction in idiopathic PD. Hence, it is important to determine the functions of these PD causative genes, such as α-synuclein, to understand how mitochondrial dysfunction may arise and cause idiopathic PD.
1.8 **PINK1 is a key gene for understanding mitochondrial dysfunction in PD**

The recent identification of an autosomal recessive familial PD causative gene, *PINK1* could provide important insights into the enigmatic relationship between mitochondria and idiopathic PD [169, 170]. Residing in position 36 of chromosome 1, this gene encodes a novel nuclear-encoded mitochondrial protein kinase of 581 amino acids called PTEN (phosphatase and tensin homolog) -induced kinase 1 (PINK1). PINK1 has a canonical N-terminal mitochondrial targeting sequence (MTS), a putative transmembrane domain, a protein serine/threonine kinase domain and a regulatory C-terminus [169, 171-174]. Most of the missense mutations identified in *PINK1* familial PD are mapped onto residues that are essential to the structural integrity and enzymatic activity of PINK1’s kinase domain [169, 171-173, 175-179]. Hence, these mutations would probably disrupt the kinase activity of PINK1 in these *PINK1* familial PD patients and consequently instigate PD pathogenesis. In agreement with this notion, we and other research groups have demonstrated that these mutations do impair the kinase activity of PINK1 in vitro [171-173]. Thus, the impairment of PINK1’s kinase activity is the likely cause of PD in these *PINK1* familial PD patients.

As a nuclear-encoded mitochondrial protein, PINK1 should be targeted to the mitochondrion via its helical amphiphilic MTS and imported into the mitochondrion in an unfolded confirmation by mitochondrial import machinery, such as the Translocase of Outer Membrane (TOM) complex. Specific mitochondrial peptidases will then proteolytically process this unfolded PINK1 to allow proper folding and localisation of physiological functional PINK1 in the mitochondrion. In agreement with this, many studies have reported that PINK1 resides predominantly within the mitochondria of cell lines, rodent and human brains in several processed forms, using immunocytochemical microscopy and subcellular fractionation [169, 171, 172, 175-183]. Several PD-associated missense mutations have also been identified in the MTS of PINK1 [169, 184-187]. These mutations are mapped onto residues that are highly conserved among the vertebrates and are potentially important for the mitochondrial localisation of PINK1.
The expression of PINK1 seems to be ubiquitous, as it is highly transcribed in heart, skeletal muscle and testis but at lower levels in brain, placenta, liver, kidney, pancreas, prostate, ovary and small intestine [188]. Despite the lower transcriptional level of PINK1 in brain, functional abnormalities in other PINK1-expressing tissues have not been reported in these PINK1 familial PD patients. Hence, the ubiquitous loss of PINK1’s kinase activity in these patients appears to affect the brain predominantly. In addition, these patients responded well to Levodopa treatment, which indicates that loss of PINK1’s kinase activity impaired their dopamine-mediated neural circuits of the basal ganglia [170, 189-192]. As both PINK1 protein and mRNA are broadly and uniformly expressed in both rodent and human brains, it is likely that SNpc dopaminergic neurons and their mitochondria are specifically affected by the loss of PINK1 in PINK1 familial PD [176, 193, 194].

1.9 Loss of PINK1 impairs dopamine-mediated neural circuits

Several research groups have generated PINK1-deficient mice to model the impact of PINK1’s absence on the dopamine-mediated circuits of PINK1 familial PD patients. Unexpectedly, both PINK1 knockdown and knockout mice did not suffer dopaminergic neuronal loss and PD-associated motor deficits [195-197]. These mice also display no perturbations in dopamine level in the striatum or in the expression of dopamine receptors [195, 196]. Thus, these observations implied that PINK1 is not essential to the development and maintenance of dopaminergic neurons, dopamine synthesis and possibly dopamine-mediated signalling. However, Kitada et al proved the latter otherwise. Using amperometry, they observed reduced evoked dopamine release in
striatal slices, decreased quantal size and release frequency of catecholamine in dissociated chromaffin cells of their PINK1 knockout mice [196]. As these PINK1 knockout mice have unperturbed numbers of dopaminergic neurons, and a normal level of striatal dopamine and expression levels of D1 and D2 dopamine receptors; these amperometric recordings therefore suggested that the loss of PINK1 in these mice impairs their presynaptic release of dopamine and might affect their dopamine-mediated neural circuits. In dopamine-mediated neural circuits, nigrostriatal projections induce long-term potentiation (LTP) and depression (LTD) at corticostriatal synapses by releasing dopamine. The inductions of LTP and LTD are dependent on the activation of D1 dopamine receptors and both D1 and D2 dopamine receptors respectively [198, 199]. In their electrophysiological experiments, Kitada et al recorded lower inductions of LTP and LTD by high-frequency stimulation at the corticostriatal synapses of PINK1 knockout mice [196]. This observation is consistent with the reduced dopamine release previously reported in these mice. It is unlikely that the lower inductions of LTP and LTD were due to unresponsive dopamine receptors, as Kitada et al showed that the impairments of LTP and LTD could be rescued with D1 and D2 dopamine receptor agonists (SFK38393 and quinpirole) and Levodopa [196]. This observed impaired release of dopamine in PINK1 knockout mice potentially parallels the dysfunctional dopamine signalling consequences resulting from dopaminergic denervation in idiopathic PD. In agreement with this, positron emission tomography (PET) and single photon emission computed tomography (SPECT) studies of PINK1 familial PD patients have observed suggestive pre-synaptic dopaminergic defects and loss of dopaminergic terminals in their striatum [170, 200, 201]. However, it is still contentious as to whether the impairment of dopamine release is the cause and prerequisite of dopaminergic denervation in PINK1 familial PD.

As Kitada et al made their observations from 9 month old mice and laboratory mice have an average lifespan of up to 3 years\(^\text{10}\), Gispert et al extended their study to 24 months old mice to investigate whether PD-like phenotype would surface in older PINK1

\(^{10}\) Average lifespan of a wild *Mus musculus* is 1 year due to predatory and stress environment.
knockout mice [197]. Indeed, they observed 30% and up to 55% lower spontaneous locomotor activity\textsuperscript{11} in their 16 months old and 24 months old PINK1 knockout mice respectively. Although this age-dependent PD-like reduction of spontaneous locomotor activity was indicative of reduced dopaminergic signalling in the striatum, Gispert \textit{et al} did not observe any morphological or biochemical changes in the dopaminergic neurons and other regions of the brain from these mice. Hence, this reduced locomotor activity seems to be attributed to the impaired dopamine release observed earlier by Kitada \textit{et al}.

As mentioned earlier, mitochondria concentrate at pre-synaptic terminals to support synaptic activity by providing ATP and maintaining calcium homeostasis. It is worth mentioning that Ca\textsuperscript{2+} influx is a key player in mobilising and releasing synaptic dopamine vesicles [202, 203]. As PINK1 is a mitochondrial protein kinase, PINK1 might influence the synaptic release of dopamine by modulating certain mitochondrial processes, such as ATP production and Ca\textsuperscript{2+} sequestration. It is worth mentioning that recent observations also link mitochondria-endoplasmic reticulum tethering to calcium homeostasis [204-211]. To examine if the loss of PINK1 could affect respiration, Gautier \textit{et al} measured the respiratory capacity of the striatal mitochondria of their PINK1 knockout mice [212]. In agreement with idiopathic PD, these mice suffered 20% reduction in MC-I activity at both state 3\textsuperscript{12} and state 4\textsuperscript{13} respiration [213]. They also showed that the loss of PINK1 did not significantly affect the activities of other mitochondrial complexes or the expression level of MC-I, II and III. Despite the reduced MC-I activity in these mice, the ATP level in their striatum was not compromised nor was their striatal mitochondrial morphology abnormal, even at the age of 24 months. Therefore, it is unlikely that the loss of PINK1 impairs synaptic dopamine release by creating an overall energy crisis in PINK1 knockout mice.

\textsuperscript{11} Gispert \textit{et al} described spontaneous locomotor activity as the parameters measured in open field test analysis of total distance, horizontal activity and movement time.

\textsuperscript{12} State 3 respiration describes the increase of the respiration to a high steady state rate and the increased reduction of pyridine nucleotides upon the addition of respiratory substrate, such as succinate and pyruvate.

\textsuperscript{13} State 4 respiration describes the decrease of the respiration to a slower steady state rate and the conversion of ADP to ATP is at its maximum.
To examine if the loss of PINK1 poses oxidative stress in the mitochondria, Gautier et al measured the enzymatic activity of aconitase derived from the striatum of their PINK1 knockout mice. Aconitase is an important mitochondrial enzyme in the Krebs cycle. Like MC-I, the enzymatic activity of aconitase is sensitive to oxidative stress due to its iron sulphide cluster [214]. Gautier et al observed 35% reduction of aconitase activity but unperturbed activity of another oxidative stress sensitive non-mitochondrial enzyme, glucokinase in the striatum of their PINK1 knockout mice. Thus, oxidative stress effects in these mice appeared to be detected largely in the mitochondria.

To test if the loss of PINK1 increases the production of mitochondrial ROS, Gautier et al measured the amount of hydrogen peroxide produced by mitochondria isolated from striatum of PINK1 knockout mice using Amplex red dye fluorescence assay. Unexpectedly, no enhancement of ROS production was observed in these mice, even under oxidative stress induced by mitochondrial stressors, paraquat, 6-hydroxydopamine, dopamine and rotenone. Neither, the levels of thiobarbituric acid reactive species (a marker for lipid peroxidation), protein carbonyls (a marker for protein oxidation) and 4-hydroxynonenal Michael adducts (marker for protein oxidation) were enhanced in striatum of these PINK1 knockout mice. Thus, the loss of PINK1 appears to pose subtle mitochondrial oxidative stress that is ample to impair the activities of MC-I and aconitase but unable to compromise the overall ATP level in the striatum of these mice.

Gispert et al suggested that subtle mitochondrial oxidative stress has a potentially detrimental consequence in the long term [197]. Gispert et al investigated if the mitochondrial protein import machinery was compromised in their PINK1 knockout mice. In their in vitro mitochondrial import assay, Gispert et al incubated liver mitochondria isolated from the mice with radiolabelled mitochondrial matrix precursor proteins, ornithine transcarbamylase and malate dehydrogenase, and inner membrane precursor protein, cytochrome c1. Up to 50% reduction of the import efficiencies of these mitochondrial precursor proteins were observed in mitochondria deriving from 18-22 months old PINK1 knockout mice. Even at 3 months old, the import efficiency of ornithine transcarbamylase was decreased by 20% in these mice. This age-dependent
impairment of mitochondrial protein import would eventually cause mitochondrial
dysfunction in PINK1 knockout mice due to decreasing ability to replace damaged
mitochondrial proteins. However, it seems that the lifespan of PINK1 knockout mice is
too short to accumulate sufficient mitochondrial damage to cause pathogenic
mitochondrial dysfunction attributing to PD pathogenesis.

Also of interest, Gispert et al did not observe reduced lifespan of their PINK1
knockout mice nor was this reported by other studies of PINK1-deficient mice [195-197,
212]. Furthermore, PINK1 is transcribed in embryonic testis during gestation and the
loss of PINK1 in PINK1-deficient mice did not affect embryonic development, as also
found for PINK1 familial PD patients [188]. These findings further support previous data
that the impact of PINK1 deficiency on mitochondria is subtle and that the function of
PINK1 is dispensable during development but poses a fitness cost in the longer term. As
the average age for PD onset of PINK1 familial PD patients is 31.6 years old and well
into adulthood, it is possible that many years of accumulated mitochondrial damage are
required to cause PD pathogenesis in these patients [189].

1.10 PINK1 suppresses oxidative stress

There is increasing evidence indicating that the accumulation of oxidative damage by
ROS is one of the main contributing causes of idiopathic PD [215-222]. Post-mortem
studies have detected evident signs of ROS damage in the midbrain of idiopathic PD
patients, which include increased lipid peroxidation, oxidative DNA damage and protein
carbonylation. These patients also suffered reduced activities of antioxidant proteins,
such as catalase, and decreased level of glutathione [223-228]. Thus, the antioxidant
capacity of the dopaminergic neurons of idiopathic PD patients appeared to be
overwhelmed by accumulating ROS instigated by unknown PD causative factors.
Whether PINK1 familial PD patients also suffer similar ROS damage and compromised
antioxidant capability remain to be determined.
In agreement with the subtle oxidative stress observed in PINK1-deficient mice, Hoepken et al reported that the primary fibroblasts of PINK1 familial PD patients bearing G309D mutation did not suffer significant oxidative DNA and protein damage but exhibited elevated level of lipid peroxidation of up to 2-fold [177]. Since ROS are highly reactive and short-lived molecules, the oxidative damage caused by these molecules is likely to be maximal in the immediate vicinity of its production site. As mentioned earlier, the mitochondrion is a potential pathogenic source of ROS in PD and oxidation of its cardiolipins was shown to disrupt MC-I activity [229]. Whether the observed lipid peroxidation in PINK1 familial PD patients was of mitochondrial origin remains to be determined. Nonetheless, it seems that the impaired kinase activity of PINK1 in these PINK1 familial PD patients is insufficient to impose oxidative damage on their proteins and genomic DNA. Since autopsy of these patients has not been reported, it is still uncertain as to whether their dopaminergic neurons suffer lipid peroxidation as found for their fibroblasts.

ROS are continuously generated during mitochondrial respiration and up to 2% of the total oxygen consumption is converted to ROS, presumably by MC-I and MC-III [230]. The failure to sequester these ROS can impair the enzymatic activities of iron-sulfide containing enzymes, such as aconitase and MC-I, and cause oxidation of cardiolipins due to their unsaturated fatty acids and proximity to the electron transport chain. As mentioned earlier, the oxidation of cardiolipins sensitize the cells towards apoptosis by promoting the opening of mitochondrial permeability transition pore complex to release apoptotic cytochrome c and disrupt mitochondrial potential [120-131, 229, 231-235]. Several cell culture studies have shown that the overexpression of PINK1 promotes cell survival by suppressing the release of cytochrome c and the loss of membrane potential induced by \( \text{H}_2\text{O}_2 \), staurosporine, MC-I and proteasome inhibitors [169, 175, 179, 180, 236]. Conversely, down-regulating the expression of PINK1 in these cells increases their susceptibility to MC-I inhibition and \( \text{H}_2\text{O}_2 \) [180, 237]. Thus, PINK1 appears to promote survival by preventing the accumulation of stress-induced ROS and in turn suppressing the release of cytochrome c in these studies. In agreement with this notion, the basal rate of mitochondrial ROS production in primary neurons of PINK1 knockout mice and NSCs
human neuroblastoma with down-regulated expression of PINK1 was approximately two fold higher than their respective wild-type controls [238, 239]. As rotenone treatment of these cells failed to enhance their mitochondrial ROS production, it suggested that these cells were already exposed to oxidative stress level attributed to impaired MC-I at basal conditions.

The antioxidant function of PINK1 was further supported by studies on Drosophila melanogaster. PINK1 is also expressed ubiquitously in the entire lifespan of Drosophila, implying that PINK1 is required during all developmental stages of the fly [240-242]. Like PINK1-deficient mice and PINK1 familial PD patients, PINK1-deficient flies were intact upon eclosion [240-242]. Hence, the function of PINK1 is also not essential to pre-adult developmental stages or could be compensated during the developmental stages preceding eclosion. However, these adult flies do suffer progressive degeneration of indirect flight muscles, dopaminergic neurons, photoreceptors and mitochondrial dysfunction [240-242]. In addition, they suffer impaired locomotor activity, reduced lifespan and infertility. Interestingly, overexpression of superoxide dismutase (SOD1, an antioxidant protein) in these flies could actually suppress the dopaminergic neuronal loss induced by PINK1 deficiency [242]. More intriguingly, a simple diet of antioxidant, vitamin E could also alleviate the ommatidial degeneration that was also observed in these PINK1-deficient flies [242]. Collectively, these observations suggested that the loss of PINK1 allows toxic accumulation of ROS in these flies as they aged. On a similar note, the fibroblasts of PINK1 familial PD patients were reported to have higher levels of activated antioxidant proteins, such as manganese superoxide dismutase and glutathione reductase, and oxidised glutathione [177, 243]. Thus, the antioxidant defence system of these patients appeared to be responding to an oxidative insult. Collectively, these observations suggested that the loss of PINK1 function allows toxic accumulation of ROS in these PINK1-deficient flies to cause severe tissue degeneration, particularly in energy-consuming tissues, such as indirect flight muscles [240-242, 244]. Moreover, the removal of accumulated ROS in these flies by either a simple diet of antioxidant or overexpression of an antioxidant protein can relieve some of the tissue degeneration
induced by PINK1 deficiency [242]. However, it is unclear as to how PINK1 exerts its antioxidant function.

A study by Pridgeon et al suggested that PINK1 might suppress the toxic accumulation of ROS by phosphorylating a mitochondrial chaperone, tumour necrosis factor receptor-associated protein 1 (TRAP1, also commonly known as heat shock protein 75) [180]. Using immuno-precipitation and subcellular fractionation, they showed that PINK1 interacted with TRAP1 in the mitochondrial intermembrane space of PC12 neuroblastoma. To investigate whether TRAP1 is a substrate of PINK1, Pridgeon et al performed in vitro kinase assay using recombinant PINK1 and TRAP1 immunoprecipitated from PC12 neuroblastoma. Indeed, TRAP1 could be phosphorylated by PINK1 but not significantly by PINK1 bearing kinase-inactivating or PD-associated mutation. Consistent with this observation, elevated phosphorylation of TRAP1 in PC12 neuroblastoma overexpressing PINK1 but not in cells overexpressing PINK1 bearing kinase-inactivating or PD-associated mutation was observed. In addition, this observed phosphorylation of TRAP1 by PINK1 could be enhanced by H2O2 but not with PINK1 bearing PD-associated mutations, G309D or L347P in PC12 neuroblastoma. Pridgeon et al postulated that TRAP1 plays a major role in mediating the pro-survival effect of PINK1 overexpression, as the overexpression of PINK1 in TRAP1-deficient PC12 neuroblastoma failed to suppress the release of cytochrome c triggered by H2O2 treatment. Although the mechanism by which TRAP1 and PINK1 mediate survival during oxidative stress is still unknown, it is worth mentioning that TRAP1 is one of the 15 proteins that were co-immunoprecipitated by PINK1 in PC12 neuroblastoma [180]. Therefore, PINK1 is likely to operate in a multimeric complex with TRAP1 and other proteins to prevent pathogenic accumulation of ROS.

1.11 Loss of PINK1 disrupts mitochondrial calcium homeostasis

As mentioned earlier, PINK1-deficient mice suffer reduced spontaneous locomotor activity attributed to impaired synaptic release of dopamine. Since Ca^2+ influx is a key
player in mobilising and releasing synaptic dopamine vesicles, the apparent antioxidant function of PINK1 may involve regulating calcium homeostasis [202, 203]. As excessive influx of Ca^{2+} into mitochondria is known to disrupt ATP synthesis by inhibiting the electron transport chain and altering mitochondrial potential, the reported reduced MC-I activity, mitochondrial potential and ATP level in cell cultures, flies, mice and patients induced by PINK1 deficiency or PD-associated mutations may result from dysfunctional mitochondrial Ca^{2+} homeostasis [169, 175, 179, 180, 212, 236, 240, 241, 244-248].

Indeed, recent studies by Marongiu et al and Gandhi et al suggested that PINK1 prevents Ca^{2+} overloading in the mitochondria and in turn curbs ROS production and/or accumulation [239, 248]. Marongiu et al showed that an inhibitor of mitochondrial Ca^{2+} uniporter, ruthenium red could rescue the perturbed neurite outgrowth, reduced mitochondrial potential and ATP level induced by PINK1 bearing PD-associated mutation, W437X, in a rat neuroblastoma, B103 [248]. Thus, it suggested that the PD-associated impairment of PINK1’s kinase activity disrupted the neurite outgrowth of these cells by causing excessive mitochondrial Ca^{2+} influx to instigate mitochondrial dysfunction. On the other hand, Gandhi et al observed mitochondrial influx of Ca^{2+} followed by mitochondrial depolarisation during KCl-induced opening of potential-sensitive plasma membrane calcium channels in PINK1 knockdown human neurons derived from fetal mescencephalic stem cells [239]. To investigate which Ca^{2+} channel was involved, they treated the wild-type control neurons with a specific inhibitor of mitochondrial Na^{+}/Ca^{2+} exchanger, CGP-37157. These neurons displayed mitochondrial influx of Ca^{2+} and mitochondrial depolarisation that were similar to that of KCl-treated PINK1 knockdown neurons. However, the mitochondria of wild-type neurons but not those from PINK1 knockout neurons were still able to export Ca^{2+} during Na^{+} stimulation and ruthenium red treatment. Thus, it indicated that PINK1 prevents excessive mitochondrial Ca^{2+} influx by enhancing mitochondrial export of Ca^{2+} via the Na^{+}/Ca^{2+} exchanger. It is worth mentioning that the mitochondrial Ca^{2+} uptake capacity^{14} of PINK1 knockout mouse neurons was approximately 16 folds lower than its wild-type.

^{14}The maximum Ca^{2+} concentration tolerated prior to the collapse of mitochondrial potential and the rapid disappearance of fluo-4 signal (Ca^{2+} fluorescence probe) from the mitochondria.
counterparts. Therefore, these PINK1 knockdown neurons may be more susceptible to mitochondrial Ca\textsuperscript{2+} overloading. Collectively, these observations suggested that PINK1 maintains the Ca\textsuperscript{2+} homeostasis in the mitochondria to protect its functional integrity in these cells by preventing Ca\textsuperscript{2+} overloading via Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger.

To investigate the influence of KCl-induced Ca\textsuperscript{2+} influx on ROS production, Gandhi et al measured the cytosolic ROS production in primary mouse cortical and midbrain neurons by assessing the oxidation rate of cytosolic hydroethidine. They reported that the oxidation rate of hydroethidine was increased by three fold in wild-type neurons during KCl treatment. Unexpectedly, the basal oxidation rate of hydroethidine of PINK1 knockout neurons was already on par with the KCl-treated wild-type neurons and remained unperturbed with KCl treatment. On further investigation, Gandhi et al showed that Nicotinamide Adenine Dinucleotide Phosphate Oxidase (NOX) inhibitors (DPI and AEBCF) or down-regulating the expression of NOX-2 could suppress the enhanced ROS production induced by KCl or PINK1 deficiency. Thus, these PINK1 knockout neurons were already exposed to ROS produced by NOX at basal conditions. Although Ca\textsuperscript{2+} influx is known to activate NOX in astrocytes, it is unclear how the mitochondria-residing PINK1 is involved in sequestering ROS generated by the plasma membrane-residing NOX during Ca\textsuperscript{2+} influx [249].

As mentioned earlier, PINK1-deficient mice suffered subtle mitochondrial oxidative stress that was ample to decrease the activities of iron-cluster containing enzymes and impair its mitochondrial import machinery. In addition, SNpc dopaminergic neurons are subjected to high fluxes of Ca\textsuperscript{2+} during neurotransmission and suffer higher rate of mitochondrial DNA mutation than other regions of the brain [202, 203, 250]. It is unclear whether the Ca\textsuperscript{2+} influx during dopamine vesicle mobilisation and release could cause pathogenic accumulation of ROS in the mitochondria to impair the dopamine release or damage mitochondrial DNA in PINK1-deficient mice.
1.12 The relationship between PINK1 and mitochondrial dynamics

Mitochondrial fission and fusion are important processes in mitochondrial biogenesis that determine the morphology, copy number, functionality and health of the mitochondria. This is achieved by the fusion of two or more mitochondria, which pool their internal contents, such as proteins, metabolites and even mitochondrial nucleoids, to compensate for individual deficiencies; the subsequent fission process often gives rise to a daughter with functional mitochondrial potential and another with lower mitochondrial potential [251-260]. The latter daughter is then removed by autophagy as a mechanism to dispose of damaged mitochondria [259, 261].

Several studies using Drosophila and cell cultures have proposed that PINK1 maintains healthy mitochondrial population by regulating the dynamics between fission and fusion. In the absence of PINK1, the mitochondria of Drosophila were enlarged, swollen and aggregated [240, 241, 244, 262, 263]. Although the majority of these mitochondria suffered disorganised and fragmented cristae, mitochondria lacking inner membrane or outer membrane were also observed. These abnormal mitochondrial morphologies indicated that the loss of PINK1 might have caused excessive mitochondrial fusion in these PINK1-deficient flies. Indeed, the abnormal mitochondrial phenotype could be rescued by overexpressing fission protein, Drp1 or down-regulating the expression of fusion proteins, mitochondrial assembly regulatory factor (Marf) or optic atrophy 1 (Opa1), presumably by restoring the balance between mitochondrial fission and fusion [262-264]. Although overexpression of Drp1 or down-regulated expression of Opa1 promoted mitochondrial fission, flies overexpressing Drp1 or with down-regulated expression of Opa1 did not have significant changes in mitochondrial morphology [263]. Since PINK1 appeared to promote mitochondrial fission, flies overexpressing PINK1 should share similar mitochondrial phenotype as flies overexpressing Drp1 or with down-regulated expression of Opa1. However, the overexpression of PINK1 resulted in mitochondrial clustering and spherical non-aggregated mitochondria [263]. Thus, the gain of PINK1 function does not seem to have a simple direct relationship with mitochondrial dynamics of fission and fusion.
In disagreement with *Drosophila* studies, PINK1 was suggested to promote mitochondrial fusion in mammalian cells [182, 265-267]. Overexpression of PINK1 caused elongated and donut-shaped mitochondria in a human neuroblastoma SH-SY5Y, and neurons derived from rat fetal mesencephalic cells, N27(1RB2AN27) respectively [265, 268]. These abnormal mitochondrial morphologies were indicative of elevated mitochondrial fusion. In agreement with this notion, loss of PINK1 or expression of PINK1 bearing PD-associated mutations in mammalian cells and primary fibroblasts of PINK1 PD patients displayed a fragmented network of truncated mitochondria with reduced number of cristae and occasional missing cristae [182, 265-268]. Contrary to PINK1-deficient *Drosophila*, down-regulating Drp1 expression or overexpressing dominant negative mutant of Drp1 in these cells suppressed the abnormal mitochondrial morphology caused by PINK1 deficiency [266, 267]. Reciprocally, excessive mitochondrial fragmentation and truncation attributed by Drp1 overexpression in SH-SY5Y neuroblastoma could be complemented by overexpressing PINK1 [266]. On the other hand, promoting mitochondrial fusion by overexpressing a mitochondrial outer membrane fusion protein, mitofusin 2 (Mfn2) or the mitochondrial inner membrane fusion protein, Opa1 could also rescue the abnormal mitochondrial morphology in PINK1-deficient SH-SY5Y neuroblastoma [266]. Thus, these observations suggested that loss of PINK1 function causes excessive mitochondrial fission in mammalian cells.

Intriguingly, a study done by Sandebring *et al* suggested that this pro-fusion effect of PINK1 may play a role in promoting cell survival during MC-I inhibition [267]. Rotenone treatment has been shown to cause mitochondrial fragmentation and truncation in mammalian cells [269, 270]. Sandebring *et al* observed that this rotenone-induced abnormal mitochondrial phenotype could be rescued by overexpressing PINK1 in a human dopaminergic neuroblastoma, BE2-M17 [267]. Reciprocally, down-regulating the expression of PINK1 in these cells exaggerated the mitochondrial fragmentation and truncation attributed to rotenone treatment [267]. Thus, PINK1 may protect dopaminergic neurons against PD-associated insults, such as MC-I inhibition, by promoting mitochondrial fusion. In agreement with these findings, our group recently reported increased expression of PINK1 in the brain of transgenic mice overexpressing
human α-synuclein bearing PD-associated A53T mutation after chronic oral ingestion of rotenone [271].

As found for *Drosophila* studies, PINK1 does not have a simple direct relationship to mitochondrial dynamics of fission and fusion in mammalian cells. Contradictory to the aforementioned studies, Mai *et al* and Gegg *et al* reported that down-regulated expression of PINK1 in human endothelial cells and SH-SY5Y neuroblastoma did not cause abnormal mitochondrial morphological change in their experiments [272, 273]. Although overexpression of dominant negative mutant of Drp1 rescued the mitochondrial fragmentation and truncation instigated by PINK1 deficiency in previous studies, the enhanced ROS production attributed to PINK1 deficiency was not suppressed by the elevated mitochondrial fusion [268]. Nonetheless, it suggested that the ROS production was upstream of mitochondrial fragmentation and truncation in PINK1-deficient mammalian cells. It is worth mentioning again that PINK1 expression could influence mitochondrial ROS production, calcium influx, respiration and mitochondrial potential. Furthermore, mitochondria with low mitochondrial potential are less likely to undergo fusion but to be more likely primed for removal [259]. Hence, it is unclear whether the abnormal mitochondrial morphology was an indirect effect of PINK1 deficiency.

Accumulated age-associated oxidative damage to mitochondrial DNA of aged animals often results in large swollen mitochondria with reduced cristae and loss of inner membrane, which consequently reduces the efficiency of respiration and mitochondrial potential [274-278]. Coincidentally, these aged mitochondria resemble the mitochondria described in PINK1-deficient *Drosophila*. Furthermore, loss of mitochondrial DNA and reduced mitochondrial DNA synthesis has been reported in PINK1-deficient *Drosophila* and SH-SY5Y neuroblastoma [240, 273]. Whether this mitochondrial DNA abnormality can be attributed to ROS in a similar fashion as aged mitochondria is still unknown. Nonetheless, it probably would disrupt respiration and instigate ROS production. As the aforementioned pro-fusion and fission effects of PINK1 were mainly determined by mitochondrial morphology, further investigation needs to be carried out to substantiate PINK1’s direct involvement in mitochondrial fusion or fission, especially since the
morphology of the mitochondrion is greatly influenced by its energy state and oxidative stress.

1.13 Parkin is a downstream mediator of PINK1

As a mitochondrial protein kinase, PINK1 is expected to phosphorylate its protein substrates in the mitochondria to mediate the survival of dopaminergic neurons in response to PD-related apoptotic signals. Early genetic complementation studies in Drosophila indicated that a PD causative gene product, Parkin is a downstream mediator of PINK1. Both PINK1-deficient and Parkin-deficient flies share similar phenotypes, such as swollen mitochondria and flight muscle degeneration [240, 241, 244]. Overexpression of Parkin in PINK1-deficient flies could rescue the mutant phenotype but not vice-versa [240, 241, 244]. Furthermore, loss of Parkin in PINK1-deficient flies did not aggravate or give rise to additional phenotype [240, 241]. In agreement with these studies, Exner et al showed that the overexpression of Parkin in PINK1-knockdown HeLa cells also suppressed the mitochondrial fragmentation and truncation caused by PINK1 deficiency [182]. Thus, these observations indicated that PINK1 operated upstream of Parkin in a simple linear pathway, such that the overexpression of Parkin as the downstream mediator of PINK1/Parkin pathway could replace the loss of PINK1 function.

1.14 Parkin interacts with PINK1

The PD causative gene, Parkin encodes an E3 ubiquitin protein ligase of 465 amino acid residues that comprises an N-terminal ubiquitin-like (UBL) domain, a RING-finger motif (R1), in-between RING finger domain (IBR) and a C-terminal RING-finger motif (R2). Relatively similar to PINK1, Parkin is highly expressed in brain and at lower levels in heart, testis, skeletal muscles, kidney and liver [279-281]. Although Parkin resides
predominantly in the cytoplasm, spatial distributions of Parkin in nucleus and Golgi apparatus have also been reported [280, 282-284]. As an E3 ubiquitin protein ligase, Parkin labels its target protein substrates with ubiquitin for their proteasomal degradation by complexing with specific E2 enzymes [285-293]. Therefore, Parkin is likely to regulate the turnover rate of its substrates in these organelles. Since Parkin is also an autosomal recessive familial PD causative gene like PINK1, the accumulation of Parkin’s substrates due to the inability of Parkin to target its substrate for proteasomal degradation is a likely cause of Parkin familial PD. However, the mechanism by which Parkin could compensate the loss of PINK1’s function in PINK1/Parkin pathway is still poorly understood.

Early studies on the PINK1/Parkin pathway showed evidence of Parkin interacting with PINK1 directly in mammalian cell cultures and rat brain using immuno-precipitation [294-296]. Using truncated mutants of Parkin to immuno-precipitate PINK1, these studies mapped the region of Parkin that interacted with PINK1 in mammalian cell cultures. Initial study by Um et al showed that the linker residing between UBL and R1 of Parkin (101-206th residues of Parkin) was required for Parkin’s association with PINK1 in a human embryonic kidney cell line, HEK293 [294]. However, later similar studies by Shiba et al and Sha et al reported that the region containing R1 domain of Parkin (234-311th residues of Parkin) was essential for the immuno-precipitation of PINK1 in an African green monkey cell line, COS-1, and SH-SY5Y neuroblastoma respectively [295, 296]. Supporting their observations, Sha et al showed that a PD-associated T240R mutation of Parkin disrupted the interaction between Parkin and PINK1 but not the PD-associated T415N mutation [296]. As T415N mutation is known to abolish the E3 ligase activity of Parkin, the loss of PINK1’s association with Parkin bearing T240R mutation may be the cause of PD pathogenesis in Parkin familial PD [297, 298]. On the other hand, Um et al showed that PINK1 bearing triple kinase-inactivating mutation (K219A, D362A and D384A) was unable to associate with Parkin in HEK293 cells [294]. Thus, their study implied that the kinase activity of PINK1 is required for the association of PINK1 and Parkin. On the contrary, Sha et al reported that PD-associated mutations, G309D and L347P, and the triple kinase-inactivating mutation did not affect
the ability of PINK1 to interact with Parkin in SH-SY5Y neuroblastoma [296]. In addition, Sha et al reported that PINK1 lacking MTS was still able to associate with Parkin. Hence, they postulated that the interaction between PINK1 and Parkin occurs in the cytoplasm and is not dependent on PINK1’s kinase activity. Despite using similar techniques, it is unclear why the observation reported by Um et al is different from the studies done by Shiba et al and Sha et al. Nonetheless, this discrepancy may be attributed by different cell-type cultures used in these studies. As PD is a neurodegenerative disease, neuroblastoma is a more relevant model cell line for studying Parkin and PINK1 in the context of PD to avoid any non-neuronal specific observations.

1.15 PINK1 may phosphorylate Parkin and regulate its E3 ligase activity

Since PINK1 interacts with Parkin, Kim et al and Sha et al investigated whether PINK1 could phosphorylate Parkin [299, 300]. Initial study by Kim et al showed that immuno-precipitated PINK1 lacking the first 199 residues could phosphorylate a peptide of Parkin containing a conserved candidate phosphorylation site, T175, but not the peptide with T175A substitution [300]. It is worth mentioning that the truncation of the first 199 residues of PINK1 would result in the loss of a conserved ATP binding motif in the kinase domain of PINK1 (detailed description in Chapter 2). Similarly, Sha et al showed that PINK1 immuno-precipitated from SH-SY5Y neuroblastoma could phosphorylate immuno-precipitated Parkin in vitro using phospho-serine antibody [299]. This observed phosphorylation of Parkin was abrogated by PD-associated mutations, G309D and L347P, or the triple kinase-inactivating mutation of PINK1, substantiating that the observed phosphorylation of Parkin was due to PINK1’s kinase activity. In addition, they observed elevated phosphorylation of Parkin in SH-SY5Y neuroblastoma overexpressing PINK1 but not in cells overexpressing PINK1 bearing PD-associated mutation or triple kinase-inactivating mutation. Furthermore, rotenone treatment enhanced the phosphorylation of Parkin in the control cells and cells overexpressing PINK1. Thus, these observations suggested that the pro-survival effect of PINK1
overexpression could be attributed to the phosphorylation of Parkin in these cells during rotenone treatment.

As Parkin catalyses K48-linked poly-ubiquitination with E2 enzyme UbcH7 and K63-linked poly-ubiquitination with heterodimeric E2 enzyme UbcH13/Uev1a, Sha et al investigated whether the phosphorylation of Parkin by PINK1 could influence the mode of ubiquitination by Parkin [291-293]. They observed increased immuno-precipitation of UbcH13 with Parkin in SH-SY5Y neuroblastoma overexpressing PINK1 and UbcH13 but not in cells overexpressing PINK1 bearing triple kinase-inactivating mutation and UbcH13. Thus, it suggested that the phosphorylation of Parkin by PINK1 was required for UbcH13’s association with Parkin. On the other hand, the overexpression of PINK1 and UbcH7 did not result in enhanced interaction of UbcH7 with Parkin. In their in vitro ubiquitination assay, they also showed that the phosphorylated immuno-precipitated Parkin had increased K63-linked poly-ubiquitination E3 ligase activity towards a Parkin substrate, IκB kinase subunit γ (IKKγ) in the presence of UbcH13/Uev1a. To investigate whether PINK1 could regulate K63-ubiquitination E3 ligase activity of Parkin in SH-SY5Y neuroblastoma, an array of ubiquitin mutants containing lysine to arginine mutations was overexpressed to determine the specificity of ubiquitination mediated by Parkin in these cells. They observed increased poly-ubiquitination of IKKγ in cells overexpressing PINK1 and ubiquitin or ubiquitin mutant that only allows K63 linkage, but not in cells overexpressing other ubiquitin mutants. This observed elevated ubiquitination was abrogated by overexpressing PINK1 bearing PD mutation or triple kinase-inactivating mutation, or PINK1 deficiency. Thus, the kinase activity of PINK1 seemed to promote cell survival by increasing K63-ubiquitination E3 ligase activity of Parkin.

K63-linked poly-ubiquitination of IKKγ by Parkin was previously shown to regulate the IγKB kinase/nuclear factor (NF-γB) pathway, which modulates the transcription of several survival genes [292, 301-303]. Using NF-γB responsive luciferase reporter assay, Sha et al also observed increased NF-γB dependent transcription in SH-SY5Y neuroblastoma overexpressing PINK1 or Parkin but not in cells overexpressing PINK1
bearing PD-associated mutations or triple kinase-inactivating mutations. Similar to the phosphorylation of Parkin, rotenone treatment enhanced NF-\(\gamma\)B dependent transcription in these cells. Collectively, Sha et al and aforementioned studies indicated that the pro-survival effect of PINK1 is mediated by the phosphorylation of Parkin by PINK1 to activate NF-\(\gamma\)B dependent transcription via K63-linked poly-ubiquitination.

However, a recent study done by Vives-Bauza et al failed to observe phosphorylation of Parkin by PINK1 in SH-SY5Y neuroblastoma and mouse brain [304]. Using \(\gamma^{32}\)P-ATP autoradiography and phospho-serine and phospho-threonine antibodies, they did not observe phosphorylation of Parkin by PINK1 \textit{in vitro} and in SH-SY5Y neuroblastoma respectively. In addition, they did not observe phosphorylation-induced electrophoretic acidic shift of Parkin in wild-type mouse brain when compared to PINK1-deficient mice. Hence, these observations implied that PINK1 did not phosphorylate Parkin in their study. Nonetheless, they did observe the association of Parkin and PINK1 using fluorescence lifetime imaging microscopy by accessing the stabilities of the fluorescence tagged PINK1 and Parkin in HEK293T cells. Thus, this observation still substantiates the interaction of PINK1 and Parkin in PINK1/Parkin pathway in promoting cell survival. However, Parkin resides predominantly in the cytoplasm, whereas PINK1 is a mitochondrial protein kinase. It is unclear whether the association of PINK1 and Parkin occurs in the cytoplasm or in the mitochondria, especially when PINK1 is postulated to fold physiologically in the mitochondria based on the canonical behaviour of nuclear-encoded mitochondrial proteins containing MTS. Furthermore, the phosphorylation of Parkin by PINK1 is still controversial and how the phosphorylation of Parkin could affect the biology of mitochondria to promote survival during PD-related stress remains speculative.

\textbf{1.16 PINK1 recruits Parkin to mitochondria}

Although early studies did not observe significant mitochondrial localisation of Parkin, recent studies using cell cultures, rat brain and \textit{Drosophila} reported that Parkin
was specifically translocated to the mitochondria during mitochondrial depolarisation induced by ionophores, carbonyl cyanide \textit{m}-chlorophenylhydrazone (CCCP), carbonyl cyanide \textit{p}-trifluoromethoxyphenylhydrazone (FCCP) or valinomycin [304-311]. This mitochondrial translocation of Parkin seems to be specific and dependent on the mitochondrial potential, as the loss of mitochondrial $\Delta \text{pH}$ caused by H$^+$/K$^+$ anti-porter, nigericin or depletion of ATP by synthase inhibitor, oligomycin did not recruit Parkin to the mitochondria [311]. Since MC-I inhibition or mitofusin deficiency would result in cells with heterogeneous mitochondrial populations of functional and reduced potentials, the preferential translocation of Parkin to mitochondria with reduced potential would suggest the voltage dependency of its mitochondrial translocation [312, 313]. Indeed, Parkin was observed to translocate preferentially to the mitochondria with reduced potential in paraquat-treated or mitofusin-deficient cells [305]. In addition, Matsuda \textit{et al} observed that Parkin was selectively targeted to the mitochondria with weaker mitochondrial potential dependent staining by MitoTracker Red during mild exposure to CCCP [307]. Translocation studies by Vives-Bauza \textit{et al} and Narendra \textit{et al} indicated that the translocated Parkin resides on the surface of depolarised mitochondria, as the translocated Parkin was proteolysed during proteinase-K treatment of the mitochondria isolated from CCCP-treated HeLa cells [304, 308]. Furthermore, the addition of a cross-linker dithiobis[succinimidyl propionate] (DSP) was required to enhance the immuno-reactivity of Parkin in the isolated mitochondria of HeLa and SH-SY5Y cells [307]. Thus, these observations implied that the translocated Parkin was loosely attached to the surface of the depolarised mitochondria. This mitochondrial translocation of Parkin was abrogated by PD-associated mutations, K211N and T240R, indicating that these mutations reside in the amino acid region of Parkin responsible for its mitochondrial translocation during mitochondrial depolarisation [307, 309]. Therefore, the loss of the mitochondrial translocation of Parkin during mitochondrial depolarisation is likely to cause the PD pathogenesis in \textit{Parkin} familial PD patients bearing K211N or T240R mutation.

Recently, several studies have shown that PINK1 may mediate the mitochondrial translocation of Parkin induced by CCCP [307-310, 314]. The loss of PINK1 or the
overexpression of PINK1 bearing kinase-inactivating mutation in cell cultures was reported to abolish the translocation of Parkin to depolarised mitochondria during CCCP treatment or MC-I inhibition. Hence, the kinase activity of PINK1 is essential for the recruitment of Parkin to depolarised mitochondria. Relevant to PD pathogenesis in PINK1 familial PD, primary human dermal fibroblasts of PINK1 familial PD patients with Q456X truncating mutation were also unable to recruit Parkin to the mitochondria during CCCP treatment [314]. Despite the previous study by Sha et al which showed that PINK1 interacted with Parkin in the cytoplasm even in the absence of its MTS, cell cultures overexpressing PINK1 lacking MTS also failed to induce mitochondrial translocation of Parkin during CCCP treatment or MC-I inhibition [306-309, 314]. Thus, mitochondrial residing PINK1 is likely to serve as a signal relay to recruit Parkin during mitochondrial depolarisation. Indeed, the overexpression of PINK1 in cell cultures could recruit Parkin to the mitochondria even in the absence of mitochondrial depolarisation, substantiating that PINK1 operates upstream of Parkin in the PINK1/Parkin pathway [304, 308]. On the other hand, the overexpression of PINK1 bearing either PD-associated mutation W437X or L347P failed to instigate mitochondrial translocation of Parkin during basal condition or mitochondrial depolarisation [304]. Thus, the loss of Parkin recruitment to depolarised mitochondria is also a likely cause of PINK1 familial PD.

To show that PINK1 expression on the mitochondria’s surface is responsible for the mitochondrial recruitment of Parkin, Narendra et al generated a chimeric PINK1 protein with its MTS replaced with an outer mitochondrial membrane anchor (OMA) derived from OPA3 protein [308]. This OMA domain would target the chimeric PINK1 protein to the surface of the mitochondria in a manner similar to OPA3. In agreement with the hypothesis, the overexpression of this chimera resulted in the mitochondrial recruitment of Parkin regardless of the mitochondrial potential. To support this chimeric experiment, Narendra et al also generated another chimeric PINK1 protein with its MTS replaced with FRB domain of mammalian target of rapamycin (mTOR) and a chimeric protein, OMA-FKBP that comprises FK506 binding protein (FKBP) and OMA. In the presence of rapamycin derivative, AP21967, the FRB and FKBP would hetero-dimerise and
therefore the PINK1 chimera and OMA-FKBP would dimerise in the presence of each other. In the absence of AP21967, both PINK1 chimera and Parkin resided predominantly in the cytoplasm. Upon the addition of AP21967, the PINK1 chimera was recruited to the surface of mitochondria through OMA-FKBP and induced the mitochondrial translocation of Parkin regardless of mitochondrial depolarisation. Hence, these observations substantiated that the mitochondrial translocation of Parkin is dependent on the kinase activity of PINK1 on the surface of the mitochondria. However, it is unclear as to how PINK1 is triggered to induce the translocation of Parkin to depolarised mitochondria.

Several studies indicated that the initial observed voltage-dependency of Parkin’s mitochondrial translocation could be attributed to the mitochondrial turnover rate of PINK1 [304, 306-308]. Endogenous PINK1 was shown to accumulate in mitochondria with reduced potential during MC-I inhibition or CCCP treatment. The accumulation of PINK1 was unlikely to be caused by increased protein synthesis, as protein synthesis inhibitor, cycloheximide, had little effect on the accumulation of PINK1 in the depolarised mitochondria [308]. Matsuda et al postulated that PINK1 is consistently imported into the mitochondria and rapidly degraded by mitochondrial protein turnover system [307]. However, this consistent degradation of PINK1 ceased upon mitochondrial depolarisation. This hypothesis is plausible as the activities of several mitochondrial peptidases are dependent on ATP and therefore are regulated by the mitochondrial respiration [315-317]. In addition, mitochondrial import machinery is dependent on mitochondrial potential and respiration. Thus, mitochondrial depolarisation would inactivate the mitochondrial import of PINK1 and accumulate PINK1 on the surface of the depolarised mitochondria. In agreement with this notion, Matsuda et al recorded a prompt temporary decrease in full length PINK1 and increase in processed PINK1 abundance after removal of CCCP, which is potentially due to the re-activation of mitochondrial peptidases and import machinery [307]. Nonetheless, this hypothesis remains to be established.
1.17 PINK1 regulates mitophagy

Using MitoTracker Green\textsuperscript{15}, Mito-DsRed\textsuperscript{16}, mito-GFP\textsuperscript{17}, anti-MC-V antibody and anti-Tom20 antibody to visualise mitochondria, several studies reported that the prolonged exposure to CCCP resulted in the clearance of depolarised mitochondria in cell cultures \[308, 309\]. This CCCP-induced loss of mitochondria could be impeded by PINK1 deficiency, or mutation in either PINK1 or Parkin that abrogate the mitochondrial translocation of Parkin \[308\]. Thus, the PINK1-induced mitochondrial translocation of Parkin appeared to instigate the loss of these mitochondria. In agreement with this notion, co-overexpression of PINK1 and Parkin could trigger the loss of mitochondria in cell cultures even in the absence of CCCP treatment \[308\]. Whereas overexpression of Parkin itself was not able to induce mitochondrial loss, substantiating that PINK1-induced mitochondrial translocation of Parkin is necessary for the clearance of depolarised mitochondria during CCCP treatment. In agreement with this notion, the mitochondrial recruitment of Parkin by ectopic overexpression of the chimeric OMA-PINK1 protein lacking MTS was ample to instigate mitochondrial loss in the absence of mitochondrial depolarisation \[308\]. However, the mechanism by which PINK1 and Parkin clears depolarised mitochondria is still poorly understood.

As an E3 ligase, Parkin has been reported to ubiquitinate several proteins that may contribute to the clearance of depolarised mitochondria. Despite that ubiquitin usually resides in the cytoplasm, Matsuda \textit{et al} and Geisler \textit{et al} observed that ubiquitin co-localised with Parkin on depolarised mitochondria during CCCP treatment \[307, 309\]. However, Parkin bearing PD-associated E3 ligase inactivating mutations, T415N or G430D, abrogated this CCCP-induced mitochondrial translocation of ubiquitin \[307\]. Using an array of ubiquitin mutants, Geisler \textit{et al} showed that overexpression of ubiquitin mutant devoid of lysine inhibited the mitochondrial loss induced by CCCP treatment

\textsuperscript{15} A green fluorescent dye that labels mitochondrial lipid in a membrane potential independent manner and often used to measure total mitochondrial mass.

\textsuperscript{16} A mitochondrial targeting fusion protein that comprises of mitochondrial targeting sequence of subunit VIII of human cytochrome c oxidase and \textit{Discosoma sp}'s red fluorescent protein.

\textsuperscript{17} A mitochondrial targeting fusion protein that comprises of the first 34 amino acids of mitochondrial targeting sequence of PINK1 and green fluorescent protein.
On further investigation, they showed that the CCCP-induced mitochondrial loss required K27 or K63-linked ubiquitination, and K63R mutation of ubiquitin blocked both mitochondrial translocation of Parkin and the mitochondrial loss during CCCP treatment. Collectively, these observations suggested that Parkin labels depolarised mitochondria with ubiquitin for degradation, possibly by proteasomal pathway. As adaptor protein p62 (also known as SQSTM1 and sequestosome-1) is known to be involved in both proteasomal and autophagic pathways, Geisler et al investigated whether p62 was involved in the clearance of depolarised mitochondria during CCCP treatment. Indeed, down-regulated expression of p62 suppressed the clearance of mitochondria during CCCP treatment. However, it did not influence the translocation of Parkin to the mitochondria, indicating that p62 operates downstream of Parkin’s mitochondrial translocation and ubiquitination of the mitochondria. Nonetheless, it suggested that the ubiquitinated and depolarised mitochondria were removed via either proteasomal or autophagic pathway mediated by p62. On further examination, Geisler et al observed that a mitochondrial protein, voltage-dependent anion-selective channel protein 1 (VDAC1) was ubiquitinated by Parkin and not by its PD-associated mutants. Using the array of ubiquitin mutants again, they showed that VDAC1 was ubiquitinated predominantly by Parkin via K27-linked poly-ubiquitination and to lesser extent by K48- and K63-linkages. As the down-regulation of VDAC1 expression suppressed the loss of mitochondria induced by CCCP treatment, the ubiquitination of VDAC1 may serve as a signal for p62 to instigate the removal of depolarised mitochondria by either proteasomal or autophagic pathway.

Recent studies indicated that mitophagy is responsible for the removal of depolarised mitochondria, as Narendra et al showed that a chemical autophagy inhibitor, 3-methyladenine could suppress the loss of mitochondria during CCCP treatment [305]. Furthermore, several autophagic proteins are implicated in the PINK1/Parkin-mediated removal of depolarised mitochondria during CCCP treatment. A microtubule-associated autophagosome marker, light chain 3 (LC3) was found to co-localise with Parkin and PINK1 in depolarised mitochondria during CCCP treatment [304-306]. This mitochondrial recruitment of LC3 was abolished in cells overexpressing PINK1 lacking
MTS, or PINK1 bearing kinase-inactivating or PD-associated mutation [306]. As LC3 could be immuno-precipitated by PINK1, PINK1 may interact directly with LC3 to modulate the removal of depolarised mitochondria [306]. Nonetheless, these observations suggested that LC3 operates downstream of PINK1. However, it is unclear whether LC3 also operates downstream of Parkin in a similar manner as VDAC1. An early autophagy gene that encodes E1 ubiquitin activating enzyme, Atg7, was also implicated in the removal of depolarised mitochondria during CCCP treatment [306, 307]. Both CCCP treatment and co-overexpression of PINK1 and Parkin failed to induce mitochondrial loss in mouse embryonic fibroblasts (MEF) lacking Atg7 [306, 307]. Despite Atg7 being an E1 ubiquitin enzyme, it is unknown whether Atg7 cooperates with Parkin/UbcH13/Uev1a E2/E3 complex in the ubiquitination of depolarised mitochondria. Similarly, MEF lacking another autophagy gene, Atg5 that is involved in late stages of autophagosome formation, also did not suffer mitochondrial loss during CCCP treatment [305]. Thus, these observations substantiated that the PINK1/Parkin-mediated removal of depolarised mitochondria can be attributed to the autophagic pathway.

A recent study by Michiorri et al suggested a direct relationship between PINK1 and mitophagy [318]. An autophagy protein, Beclin1 was identified as a binding partner of PINK1 in yeast 2-hybrid experiments and could only be immuno-precipitated by full length PINK1 but not PINK1 lacking MTS or bearing C-terminal K496X truncating mutation in HeLa cells. Using confocal microscopy, Beclin1 and PINK1 were shown to co-localise in the mitochondria. However, the mitochondrial localisation of Beclin1 is independent of PINK1 expression. As Beclin1 is known to promote autophagosome formation and maturation, Michiorri studied the effects of PINK1 expression on starvation-induced autophagy [319, 320]. The number of control SH-SY5Y cells with LC3-positive vacuoles increased by three fold during starvation. However, the number of cells overexpressing PINK1 with LC3-positive vacuoles was already on a par with the starved control during basal conditions, suggesting that the starvation-induced autophagic pathway was already switched on in these cells. Nonetheless, starvation of these cells increased the number of cells containing LC3-positive vacuoles by another 40%. In disagreement with previous studies, Michiorri et al did not observe the co-localisation of
LC3 with PINK1 [305, 306]. Thus, it is unlikely that the observed increased LC3-positive vacuoles were due to increased mitochondrial recruitment of LC3 by overexpression of PINK1. Reciprocally, down-regulating the expression of PINK1 resulted in fewer cells with LC3-positive vacuoles. Although down-regulation of Beclin1 expression reduced starvation-induced autophagy in control cells and cells overexpressing PINK1, cells overexpressing PINK1 still displayed higher levels of LC3-positive vacuoles than control cells. Thus, these observations suggested that PINK1 modulates both autophagy and mitophagy triggered by starvation and mitochondrial depolarisation respectively.

1.18 Outstanding questions

Despite many studies investigating the cellular distribution of PINK1, the reports of the location of functional PINK1 have been inconsistent since its discovery. Initial studies on PINK1 showed that PINK1 resided in the mitochondria, namely on the outer membrane, inner membrane and the inter membrane space of the mitochondria [171, 176, 180]. These mitochondrial-residing forms of PINK1 are postulated to promote survival during cellular stress induced by MC-I inhibition, oxidative or proteasomal stresses [169, 175, 180, 237]. However, a significant portion of PINK1 has also been reported to reside in the cytoplasm by Haque et al [321]. In addition, they showed that PINK1 lacking MTS was still capable of promoting the survival of the mouse neuroblastoma, NIH 3T3 during MC-I inhibition, substantiating their claim that the functional form of PINK1 resides in the cytoplasm and not mitochondria. On the other hand, recent studies demonstrated that PINK1 resides on the surface of the mitochondria to induce mitophagy of dysfunctional mitochondria [174, 308]. Given these inconsistent reports, it is critical to determine the bona fide location of the functional PINK1 to facilitate understanding of the role of PINK1 in cell survival and mitochondrial biogenesis or mitophagy with respect to PD pathogenesis.
As a mitochondrial protein kinase, PINK1 is postulated to phosphorylate its protein substrates in the mitochondria to mediate the survival of dopaminergic neurons in response to PD-related stress. Thus far, I have shown that PINK1 is a protein serine/threonine kinase and other studies have reported that PINK1 phosphorylates TRAP1 and Parkin to promote cell survival following apoptotic insults [173, 180, 296, 300]. Despite Kim et al implicating T175 of Parkin as the phosphorylation site of PINK1, Vives-Bauza et al failed to observe phosphorylation of Parkin by PINK1 [300]. In addition, Narendra et al demonstrated that the introduction of a phospho-mimetic mutation, T175E into Parkin did not induce spontaneous mitochondrial translocation of Parkin to trigger mitophagy [308]. It is also still unknown how the function of TRAP1 may be modulated by the phosphorylation by PINK1. Hence, it is uncertain whether TRAP1 and Parkin are the bona fide protein substrates of PINK1. Although many studies have shown that PINK1 is involved in mitochondrial biogenesis, mitophagy and promoting cell survival, the exact mechanism by which PINK1 operates in these pathways to suppress PD pathogenesis is still unclear. Thus, identifying the protein substrates of PINK1 will facilitate the mapping of these pathways and provide novel insights into understanding of the role of PINK1 in PD pathogenesis.

This PhD study was undertaken to facilitate understanding of the role of PINK1 in PD pathogenesis and this thesis covers the following aspects:

i. Bioinformatic investigation of the structural features and the putative impact of PD mutations on the function of PINK1.

ii. Generation of catalytically active recombinant PINK1 protein for the investigation of putative protein substrates of PINK1 utilising insect and yeast recombinant protein overexpression systems.

iii. Investigation of putative protein substrates of PINK1 using kinase substrate tracking elucidation (KESTREL) and candidate protein substrate phosphorylation.

iv. Investigation of putative protein substrates and downstream mediators of PINK1 by 2-dimensional difference in gel electrophoresis (2D-DIGE)
comparison of the mitochondrial proteomes of BE2-M17 neuroblastoma with inducible overexpression of PINK1.

v. Investigation of the localisation of PINK1 in the mitochondria utilising \textit{in vitro} mitochondrial import assay and overexpression of PINK1 in an insect recombinant protein overexpression system.

vi. Investigation of the specificity of the cell survival effect of PINK1 following apoptotic insults using BE2-M17 neuroblastoma with inducible overexpression of PINK1.
Chapter 2 – Bioinformatic investigation of the structural features and the putative impact of Parkinson’s disease associated mutations on the function of PINK1

As mentioned in the previous chapter, PINK1 is a ubiquitous mitochondrial protein kinase of 581 amino acids (Figure 2.1). It has a canonical N-terminal mitochondrial targeting sequence (MTS), a putative transmembrane domain, a protein serine/threonine kinase domain and a regulatory C-terminus (Figure 2.1). Although many research groups have studied the topology and function of PINK1, their reports on the mitochondrial distribution and functional properties of PINK1, such as the ability to undergo auto-phosphorylation, have been conflicting. In this chapter, I analyse the amino acid sequence of PINK1 and aim to postulate the impact of the PD-associated mutations on the cellular distribution and functional properties of PINK1.

2.1 The mitochondrial targeting sequence of PINK1

2.1.1 Translocation of nuclear-encoded mitochondrial proteins

Although proteomic analysis reported that a human mitochondrion contains approximately 1000 different proteins, the mitochondrial genome only encodes 37 genes [322, 323]. The vast majority of the mitochondrial proteins are encoded by nuclear genes like PINK1. As the mitochondrion has two distinct lipid bi-layered membranes (outer and inner membranes) that define two aqueous compartments (inter-membrane space and matrix), the cell relies on several mechanisms to import the nuclear-encoded mitochondrial proteins through these physical barriers to their rightful places in the mitochondrion.

Most mitochondrial proteins residing in the outer membrane (OM) and the carrier proteins of the inner membrane (IM) of the mitochondrion do not have cleavable
mitochondrial targeting sequence (MTS) but possess poorly characterised intrinsic mitochondrial targeting sequences [324]. On the other hand, proteins residing in the inter-membrane space (IMS), IM and matrix usually have canonical cleavable N-terminal MTS. These MTS are usually 20-60 amino acids long and have the propensity to fold into an amphiphilic α-helix with a hydrophobic side and a positively charged side attributed by a series of lysines and arginines [325-327].

As folded proteins are too bulky to enter the protein transport channels of the mitochondrion, the cell has developed a unique mechanism to translocate nuclear-encoded mitochondrial proteins through the OM and IM of the mitochondrion. The nuclear-encoded mitochondrial proteins with MTS are translated by the ribosomes at the endoplasmic reticulum but they are maintained in an unfolded state immediately by the members of heat shock protein 70 (HSP70) and 90 (HSP90) protein families (Figure 2.2A) [328-334]. The positively charged side of the MTS of the unfolded mitochondrial precursor is directed to the acidic domains of the Translocase of OM (TOM) complex, which translocates the unfolded protein across the OM via this electrostatic interaction (Figure 2.2A) [335]. Subsequent translocations of the matrix and IM precursor proteins across IM are mediated by the Translocase of IM (TIM) complex and matrix HSP70 at the expense of ATP hydrolysis and mitochondrial potential (Figure 2.2B) [336-340].

After translocation, the MTS is no longer required by the precursor and the failure to remove the MTS has been shown to interfere with the subsequent sorting and/or protein folding and assembly [324, 341]. The MTS of matrix precursors are removed by a matrix peptidase called mitochondrial processing peptidase (MP-peptidase) (Figure 2.2B). Besides proteolytically processing the matrix precursors, MP-peptidase is also involved in the processing of IMS precursors because their MTS also contain a matrix-targeting sequence but followed by an IMS-sorting sequence (Figure 2.2C) [342-344]. Therefore, IMS precursors are also targeted to the TIM complex in a similar fashion as matrix precursors due to their matrix-targeting sequences. However, their IMS-sorting sequences are removed by IM peptidase (IM-peptidase) at the IMS side of the IM to release the processed protein into the IMS (Figure 2.2C) [345].
Although single proteolytic cleavage by MP-peptidase is ample for the complete removal of MTS of some mitochondrial precursors, the rest of the precursors require two sequential cleavages in the matrix by MP-peptidase followed by another matrix peptidase named mitochondrial intermediate peptidase (MI-peptidase) (Figure 2.2D). Thus, these precursors generally have longer MTS than matrix precursors that undergo single cleavage by MP-peptidase. Therefore, analysing the amino acid sequence of the N-terminus of PINK1 to determine the length of its MTS and predicting the cleavage sites of the mitochondrial peptidases would facilitate the identification of the \textit{bona fide} localisation of the functional PINK1 in the mitochondria.

2.1.2 Bioinformatic analysis of the mitochondrial targeting sequence of PINK1

PINK1 is evolutionarily conserved from the mammals to the simplest metazoan, \textit{Trichoplax aldhaerens}, which bears 27.5\% identity and 39.2\% similarity to human PINK1 (Figure 2.3). To date, a PINK1 orthologue has not been identified in unicellular eukaryotes, such as yeast. Hence, the function of PINK1 appears to be important and exclusive to the metazoans thus far. Therefore, multiple alignment analysis of PINK1 orthologues across the metazoans, from vertebrates to invertebrates would identify the evolutionarily conserved regions in the MTS of PINK1 that are potentially important to the mitochondrial translocation of PINK1.

The conserved alignments between the PINK1 homologues suggest that the MTS of human PINK1 is approximately 100 amino acids long and contains 18 basic residues (Table 2.1 and Figure 2.4). The helical wheel analysis of the first 98 residues of human PINK1 shows that these residues could form an amphipilic $\alpha$-helix that is characteristic of the canonical MTS, substantiating that the N-terminus of PINK1 is a MTS (Figure 2.5). The unusually long length of the MTS of PINK1 suggests that the MTS is cleaved more than once. Therefore, PINK1 may be an IMS, IM or matrix protein, depending on which
peptidase is responsible for the second cleavage after the initial processing by MP-peptidase (Figure 2.2C & 2.2D). In agreement with this postulation, experimental data have shown that PINK1 resides predominantly in the mitochondria and is processed to form several proteolytic products [171, 175, 178, 180, 181, 296, 314, 318, 346]. Thus, the identification of the mitochondrial peptidases responsible for processing PINK1 will facilitate the determination of the bona fide localisation of the functional PINK1 in the mitochondria.

2.1.3 Mapping of cleavage sites of mitochondrial peptidase in the mitochondrial targeting sequence of PINK1

Like many peptidases, the cleavage site motifs of MP-peptidase have a high level of amino acid sequence degeneracy because the structural conformation of the MTS also plays a major role in the substrate recognition of MP-peptidase (Table 2.2) [347, 348]. Fortunately, most of these motifs usually contain a conserved arginine that is recognised by MP-peptidase (Table 2.2). As the determinants of the cleavage sites in the MTS of human PINK1 should be evolutionarily conserved among PINK1 orthologues, it is possible to predict the cleavage site by aligning the known cleavage site motifs of MP-peptidase against the conserved alignments of PINK1 orthologues. To date, four cleavage site motifs of MP-peptidase have been identified and classified as depicted in Table 2.2 [347]. Among the four cleavage site motifs, only R-10 motif is linked to the sequential cleavages of matrix and IM precursors in the matrix by MP-peptidase and MI-peptidase (Table 2.2). Unlike other cleavage site motifs, R-10 motif is relatively conserved due to the cooperative proteolytic processing between MP-peptidase and MI-peptidase (Table 2.2). In R-10 cleavage, MP-peptidase recognises a conserved arginine and cleaves between the C-terminal neighbour of the conserved arginine and an invariant downstream hydrophobic residue, yielding a processing intermediate with the invariant hydrophobic residue at its N-terminus (Table 2.2) [347, 349]. This invariant hydrophobic residue is required to recruit MI-peptidase to the processing intermediate, which subsequently cleaves off eight amino acid residues from the N-terminus of the processing
intermediate to allow the maturation of the translocated precursor in the matrix [350-352]. Unfortunately, the cleavage site motif of IM-peptidase is still unknown and therefore the prediction of IM-peptidase processing of the MTS of PINK1 is not carried out in this study.

The conserved alignments of PINK1 orthologues with the cleavage motifs of MP-peptidase indicate that PINK1 has two potential R-10 cleavage sites in its MTS (Figure 2.4). These sites are mapped onto two conserved regions in the MTS of PINK1, which correspond to residues 8 to 18 and residues 67 to 77 of human PINK1. Both sites contain the conserved arginine and hydrophobic residue that are required for the sequential processing by MP-peptidase and MI-peptidase. Early cell culture studies on the mitochondrial localisation of PINK1 showed that the first 34 residues of human PINK1 fused to enhanced green fluorescent protein (EGFP) were sufficient to target the chimeric fusion protein into the mitochondria [178, 181]. As the disruption of the proteolytic processing of the MTS is known to cause rapid degradation of translocated mitochondrial precursors, this observation supports that the predicted R-10 cleavage site at residues 8 to 18 of human PINK1 is *bona fide* [353]. In a similar study, Silvestri *et al* showed that the first 93 residues and 156 residues of human PINK1 were also capable of importing enhanced cyan fluorescent protein (ECFP) into the mitochondria of HeLa cells [171]. Furthermore, cell fractionation analysis of the HeLa cells transfected with the chimeric ECFP fused with the first 156 residues of PINK1 indicated that the chimeric protein was exclusively processed in the mitochondria and was approximately 10kDa smaller than the unprocessed form. It is noteworthy that fusing a mature mitochondrial protein that is usually not processed by R-10 cleavage to the immediate C-terminus of the MTS of a mitochondria protein that is processed by R-10 cleavage has been shown to abrogate the R-10 processing of the MTS of such chimeric protein [350]. Thus, the observed processing of the chimeric ECFP suggests that the predicted R-10 cleavage site at residues 67 to 77 of PINK1 is also *bona fide*, as the R-10 cleavage site is not juxtaposed to the N-terminus of the ECFP. The approximate loss of 10kDa is equivalent to a theoretical loss of the first 98 amino acid residues of PINK1, whereas the loss of the first 77 residues is equivalent to a theoretical loss of 8kDa. As the resolution of the Western
blot performed by Silvestri et al was not sensitive enough to distinguish between 8kDa and 10kDa loss, it is uncertain as to whether there are additional processing sites downstream of this predicted R-10 cleavage site.

It is worth mentioning that a mitochondrial protein with two R-10 cleavage sites in its MTS or an R-10 cleavage site in a MTS that is longer than 50 amino acids has not been reported thus far. Nonetheless, a nuclear-encoded mitochondrial protein called Frataxin is reported to have a MTS of approximately 50 amino acids long and is processed by MP-peptidase exclusively in a two-step sequential manner via two R-3 cleavages [354]. Therefore, MP-peptidase may cleave the MTS of some mitochondrial precursors more than once, particularly on those precursors with long MTS. Although the major processed form of PINK1 is approximately 54kDa, other larger and smaller proteolytic forms of PINK1 (58kDa, 50kDa, 45kDa) have also been reported [171, 175, 178, 180, 181, 296, 314, 318, 346]. The conserved alignments of PINK1 orthologues indicate that the arginines 88 (R88) and 98 (R98) of human PINK1 are conserved and match the more degenerated R-2 and R-3 cleavage motifs of MP-peptidase (Figure 2.4). Thus, the smaller multiple proteolytic forms of PINK1 may arise from these predicted cleavage sites, whereas the larger proteolytic product may be attributed to the initial R-10 processing at residues 8 to 18 of human PINK1. Although the R88 and R98 of PINK1 are potential MP-peptidase cleavage sites, these conserved regions may not serve as cleavage determinants but may have important functional roles in the mitochondrial translocation and sorting of PINK1. In summary, PINK1 is likely to be a matrix or IM protein if PINK1 is indeed processed by MP-peptidase and MI-peptidase (Figure 2.2D).

2.1.4 The effects of PD-associated mutations

PD-associated G32R, P52L, L67F and A78V mutations reside in the regions of PINK1’s MTS that are at least conserved in mammalian PINK1 orthologues (Figure 2.4). G32R, L67F and A78V mutations replaced the original small amino acid residues with a larger residue that may affect the propensity of the MTS to retain its \( \alpha \)-helical structure or
the distribution of the positive charges on the helix. On the other hand, P52L mutation is likely to remove a potential kinked region that may be important to the structural integrity of the α-helical MTS. Thus, these mutations may affect the recruitment of PINK1’s MTS to the TOM complex or the subsequent translocation steps, which consequently reduce mitochondrial translocation of PINK1 or occlude PINK1 from the mitochondria.

The R68 and R98 of PINK1 are evolutionarily conserved and therefore potentially serve as the positively charged residues that are critical to the recruitment of PINK1’s MTS to the TOM complex (Figure 2.4). PD-associated R68P and R98W mutations are likely to perturb the mitochondrial translocation of PINK1 by reducing the net number of positively charged residues in the MTS of PINK1. Nonetheless, these mutations may also grossly affect the α-helical structure of the MTS of PINK1 or its spatial distribution of positively-charged residues to disrupt the mitochondrial translocation of PINK1. If R98 was the cleavage determinant recognised by the MP-peptidase, R98W mutation would abrogate the processing of PINK1 and perturb the maturation of PINK1 in the mitochondria.

2.2 The transmembrane domain of PINK1

There are two distinct classes of transmembrane proteins. The predominant class belongs to the proteins with one or more α-helical transmembrane domains, with each transmembrane helix usually consisting of 17 to 25 amino acid residues with largely hydrophobic side chains [355, 356]. These transmembrane helices come in a variety of lengths and arrangements. They can be short, long, bent or discontinued in the centre of the membrane, inserted across the membrane at oblique angles, laid flat on the membrane or spanning only a portion of the membrane and then retreating to form a loop called a re-entrant loop [357]. The transmembrane domain in the other class is composed of 16-stranded β-barrel, which has only been identified in the bacterial outer porins thus far [358, 359].
2.2.1 Bioinformatic analysis of the putative transmembrane domain of PINK1

In this study, two bioinformatic α-helical transmembrane prediction programs, TopPred\(^\text{18}\) and HMMTOP\(^\text{19}\) were used to predict the transmembrane domain of PINK1 [360-363]. These programs utilise a prediction algorithm, which is based on the observations that α-helical transmembrane domains have high net hydrophobicity and the non-translocated loops are usually enriched in positively charged residues compared to the translocated loops [360]. Bioinformatic analysis of the amino acid sequence of human PINK1 by TopPred predicts that PINK1 has a 20 amino acid long α-helical transmembrane domain, which corresponds to residues 91 to 111 of human PINK1 (Figure 2.6). Conserved alignments of PINK1 orthologues indicate that this region is evolutionarily conserved and largely hydrophobic, supporting the prediction made by TopPred. In addition, this region bears similar distribution of hydrophobic residues to the putative transmembrane helices from the subunit mbhM of membrane bound hydrogenase in *Pelobacter propionicus* and the subunit H of NADH-quinone oxidoreductase in *Xanthomonas oryzae*. Furthermore, this conserved hydrophobic region is flanked by hydrophilic residues that are potentially exposed to aqueous environment. However, TopPred failed to detect transmembrane domain in fish PINK1 orthologues despite their homologies to mammalian PINK1 orthologues. Nonetheless, a transmembrane helix is predicted in zebrafish PINK1 by HMMTOP but not in salmon and puffer fish PINK1 orthologues. Although both prediction programs fail to detect a transmembrane helix in salmon and pufferfish PINK1 orthologues, the corresponding amino acid region in these orthologues are conserved with respect to the predicted transmembrane helix of the zebrafish PINK1 orthologue (Figure 2.6). Thus, salmon and pufferfish PINK1 orthologues are also likely to have the transmembrane helix predicted in the zebra fish PINK1 orthologue.

\(^{18}\) http://mobyle.pasteur.fr/cgi-bin/portal.py?form=toppred
\(^{19}\) http://www.enzim.hu/hmmtop/html/submit.html
2.2.2 Putative transmembrane domain of PINK1 is the ‘stop-transfer’ signal of the mitochondrial targeting sequence of PINK1

The putative transmembrane domain of PINK1 may also serve as a hydrophobic “stop-transfer” signal of the MTS of PINK1 (Figure 2.2C). “Stop-transfer” signal resides after a matrix-targeting signal and prevents further translocation of the mitochondrial precursor into matrix upon contact with the TIM complex (Figure 2.2C) [342, 364]. Subsequently, this “stop-transfer” signal may serve as an IM anchor for IM protein or is removed by IM-peptidase and/or MI-peptidase to release the mitochondrial protein into the IMS. If the putative transmembrane domain and R-10-processing of PINK1 were bona fide, PINK1 is likely to be an IM protein facing the IMS. Indeed, recent studies show that the putative transmembrane domain of PINK1 anchors PINK1 to the IM facing IMS and is essential to the proteolytic maturation of PINK1 in the mitochondria [365, 366]. Furthermore, Deas et al demonstrated that an IM protease called Presenilin-Associated Rhomboid-like protein (PARL) mediated the proteolytic maturation of PINK1 by cleaving between the conserved alanine 103 (A103) and the conserved phenylalanine 104 (F104) of PINK1 in its putative transmembrane domains (Figure 2.6) [365]. As Rhomboids are known to cleave within transmembrane domain by recognising helix-breaking residues, such as proline, Deas et al and Jin et al predicted that the mutations of conserved proline 95 (P95) of PINK1 and its surrounding residues would disrupt the cleavage of PINK1 by PARL (Figure 2.6) [365-368]. In agreement with their predictions, they observed that the mutations of these residues reduced the processing of PINK1 drastically in cell cultures and in turn increased the level of unprocessed PINK1 in the mitochondria [365, 366]. As Deas et al showed that the mutation of the conserved P95 of PINK1 to alanine promoted ROS production and reduced mitochondrial potential in SH-SY5Y neuroblastoma, it indicates that the PARL-mediated cleavage of PINK1 is important to the maturation of pro-survival form of PINK1 protein in the mitochondria [365].
Although the putative transmembrane domain of PINK1 is cleaved by PARL, Jin et al showed that the remaining transmembrane domain was sufficient to anchor PINK1 to the IM using *in vitro* mitochondrial import and Proteinase K sensitivity assays [366]. As the uncleaved PINK1 was also found on the IM of the mitochondria of PARL-knockout mouse embryonic fibroblasts, it indicates that the cleavage of PINK1 by PARL is required to allow the proper folding of PINK1 after its translocation to the IM [366]. It is worth mentioning again that incomplete removal of MTS is known to affect the folding and stability of translocated mitochondrial precursors, and thus PARL is likely to remove the MTS of PINK1 completely after PINK1’s translocation to the IM [324, 341, 353].

Although a PD-causative gene encoding a mitochondrial IMS protease, High temperature requirement protein A2 (HtrA2) also known as Omi stress-regulated endoprotease has been implicated in the pro-survival function of PINK1, studies by Deas et al and Jin et al reported that HtrA2 deficiency did not influence the proteolytic maturation of PINK1 in cell cultures [365, 366, 369, 370]. In agreement with their observations, a recent genetic interaction study by Tain et al suggested that HtrA2 operated downstream of PINK1 in *Drosophila* flies [371]. However, another independent genetic interaction study failed to detect significant genetic interaction between PINK1 and HtrA2 in *Drosophila* flies [372]. Thus, the functional link between PINK1 and HtrA2 remains debatable and requires further studies. Nonetheless, these studies suggest that HtrA2 is not involved in the proteolytic maturation of PINK1.

### 2.2.3 The effects of PD-associated mutations

PD-associated R98W and I111S mutations reside in the hydrophobic transmembrane helix of PINK1 (Figure 2.6). Arginine 98 (R98) and Isoleucine 111 (I111) of human PINK1 are conserved among vertebrates and are potentially important in maintaining the structural integrity of the α-helical transmembrane domain. Besides reducing the overall hydrophobicity of the transmembrane helix, I111S mutation may also destabilise the transmembrane helix that could prevent proper localisation of PINK1 in the mitochondria.
or induce mis-folding of PINK1. In addition, both R98W and I111S mutations may perturb the cleavage of the putative transmembrane domain of PINK1 by PARL. In agreement with this notion, Jin et al reported that the mutation of R98 to phenylalanine disrupted the cleavage of PINK1 by PARL and increased the levels of unprocessed PINK1 in HeLa cells [366].

2.3 The protein kinase domain of PINK1

2.3.1 PINK1 is a unique mitochondrial protein kinase

To date, there are only two other bona fide nuclear-encoded mitochondrial protein kinases with N-terminal MTS like PINK1 that have been reported. They are branched-chain α-ketoacid dehydrogenase kinase and pyruvate dehydrogenase kinase [373-378]. Although these mitochondrial protein kinases display protein-serine-specific kinase activity, their protein kinase domains are highly homologous to the prokaryotic histidine protein kinases. In contrast, the protein kinase domain of PINK1 resembles that of a eukaryotic protein serine/threonine kinase, such as Protein Kinase A, placing PINK1 as the only known nuclear-encoded mitochondrial protein that has a eukaryotic protein serine/threonine kinase domain and a MTS thus far.

2.3.2 The function of the protein kinase domain of PINK1

Eukaryotic protein kinases are involved in regulating a wide range of neuronal functions, ranging from cell survival to memory and learning [379-382]. The regulatory effects of protein kinases on these cellular functions are mediated by the transfer of γ-phosphate from ATP to the hydroxyl group of a specific alcoholic amino acid in the protein substrate catalysed by the protein kinase [383-385]. The resulting phosphorylated residue may alter the enzymatic activity, stability of the protein substrate, or even provide
a docking site for other proteins [386-401]. Thus, protein kinases provide a mechanism for the cell to modulate a large array of proteins to mediate appropriate cellular response to its immediate extracellular and/or intracellular environment. Hence, it is conceivable that PINK1 phosphorylates specific proteins to mediate its reported roles in cell survival, mitochondrial biogenesis and mitophagy. Genetic analysis of PINK1 familial PD patients revealed that most of the PD-associated mutations reside within the protein kinase domain of PINK1 (Figure 2.1) [169, 184-187, 190, 236, 402-424]. As PINK1 familial PD is inherited recessively, these mutations are postulated to abrogate the kinase activity of PINK1 and in turn disrupt the pathways that are modulated by PINK1.

2.3.3 The effects of PD-associated mutations on the protein kinase domain of PINK1

To understand the pathogenic effects of the PD-associated mutations on the kinase activity of PINK1, it is important to consider the general conserved structure of a eukaryotic protein kinase domain and its structural basis of catalysis. The protein kinase domain is characteristically folded into a two-lobe structure (Figure 2.7A). The catalysis of the phosphorylation of protein substrate occurs within the cleft between the lobes of the protein kinase domain (Figure 2.7B) [383-385]. Hanks and Hunter have aligned amino acid sequences deriving from sixty distinct eukaryotic protein kinases [383]. From the alignment of these protein kinases, they have defined 12 regions in the protein kinase domain that are never interrupted by large amino acid insertions and contain characteristic patterns of conserved amino acid residues. Subsequent sequence analysis of all known protein kinases revealed that these 12 regions are invariant or nearly invariant in most protein kinases. These conserved regions are termed subdomains (subdomain I to VIa and VIb to XI). Crystal structures of many protein kinases have revealed that these subdomains are critically involved in the catalysis of phosphorylation and/or maintaining the structural integrity of the protein kinase domain (Figure 2.7A) [383-385]. The phosphorylation of a protein substrate by a protein kinase is achieved by the optimal alignment of catalytically critical amino acid residues in these subdomains.
that line the catalytic cleft of the protein kinase domain (Figure 2.7C) [383-385]. The structural features and functions of these catalytically critical residues and the subdomains of the protein kinase domain, and how PD-associated mutations might affect the function of the protein kinase domain of PINK1 are discussed below.

2.3.3.1 Subdomain VIb has a conserved Asp in the catalytic loop which functions as the catalytic base

The phosphorylation of a protein substrate is catalysed by a conserved catalytic loop motif of His-Arg-Asp-Leu-Lys-X-X-Asn (HRDLKXXN) in subdomain VIb (Figure 2.7) [383-385]. The aspartate in this catalytic loop acts as the catalytic base, which accepts a proton from the hydroxyl group of the targeted alcoholic residue in the phosphorylation site of the protein substrate during phosphorylation (Figure 2.7C) [383-385]. Upon transferring the proton to the catalytic aspartate, the hydroxyl group of the targeted alcoholic amino acid launches a nucleophilic attack on the γ-phosphate of ATP and give rise to ADP and phosphorylated protein substrate as the final products of the enzymatic reaction (Figure 2.7C) [383-385]. This conserved HRDLKXXN motif corresponds to the residues 360 to 367 (HRDLKSDN) of PINK1 (Figure 2.8). As the lysine in the HRDLKXXN motif is usually substituted by an arginine or alanine in protein tyrosine kinases to accommodate the bulkier side chain of tyrosine from its substrate, the corresponding motif (HRDLKSDN) of PINK1 indicates that PINK1 is a protein serine/threonine kinase [383]. Indeed, I previously reported that the recombinant protein kinase domain of PINK1 only phosphorylated the serine and threonine residues and not tyrosine of an in vitro substrate, Histone H1 [173]. To allow phosphorylation to occur, appropriate positioning of the catalytically important aspartate 362 (D362) of PINK1, the γ-phosphate of ATP and the target hydroxyl group of the substrate are essential. Thus, PD-associated D362H mutation would abrogate the kinase activity of PINK1. On the other hand, PD-associated N367S and L369P mutations are likely to inactivate the kinase activity of PINK1 by disrupting the structural conformation of the catalytic loop and in turn occluding D362 of PINK1 from its catalytic position (Figure 2.8).
2.3.3.2 The ATP-binding subdomains

The proper positioning of ATP in the catalytic cleft of the protein kinase domain is predominately governed by the conserved residues in subdomains I, II and VII (Figures 2.7B and 2.7C) [383-385]. Subdomain I has a conserved β-hairpin motif of Gly-X-Gly-X-X-Gly-X-Val (GXGXXGXV) that anchors the adenosine moiety, α- and β- phosphates of ATP to the catalytic cleft (Figures 2.7B and 2.7C) [383-385]. This GXGXXGXV motif corresponds to the residues 163 to 170 of PINK1 (Figure 2.8). The anchoring of ATP in the catalytic cleft is also facilitated by an invariant lysine in subdomain II that forms hydrogen bonds to both α- and β- phosphates of ATP. Besides anchoring ATP, this lysine residue also restricts the movement of the phosphate backbone of ATP and therefore primes the ATP for the catalysis of the phosphorylation of the protein substrate (Figures 2.7B and 2.7C) [383-385]. This invariant lysine corresponds to the lysine 219 (K219) of PINK1 (Figure 2.8). The proper orientation of the phosphate backbone of the anchored ATP for the catalysis of phosphorylation is also mediated by a conserved Mg$^{2+}$-positioning motif in subdomain VII (Figures 2.7B and 2.7C) [383-385]. This Mg$^{2+}$-positioning motif has a highly conserved sequence of Asp-Phe-Gly (DFG), in which the aspartate chelates the Mg$^{2+}$ ion that bridges the β- and γ- phosphates of ATP (Figures 2.7B and 2.7C) [383-385]. This critical DFG motif corresponds to residues 384 to 386 of PINK1 (Figure 2.8). All these structural motifs act in concert to position and prime the γ-phosphate of ATP for the catalysis of phosphorylation of the protein substrate. Hence, PD-associated A168P mutation in the β-hairpin in subdomain I, PD-associated A217D mutation in subdomain II and PD-associated A383T, A385L, G386A and C388R mutations in subdomain VII of PINK1 are likely to perturb the recruitment and/or the positioning of ATP and in turn disrupt the catalysis of the phosphorylation of protein substrate (Figure 2.8). Indeed, I have previously shown that PD-associated mutation, G386A, in the DFG motif of the subdomain VII of PINK1 drastically reduced the kinase activity of recombinant PINK1 kinase domain [173].
2.3.3.3 The protein substrate binding subdomains

The catalysis of the phosphorylation of protein substrate by a protein kinase also requires proper positioning of the hydroxyl group of the target alcoholic amino acid in the protein substrate in close proximity to the γ-phosphate of ATP and the catalytic aspartate of the protein kinase (Figures 2.7B and 2.7C). The recruitment and orientation of a protein substrate to the protein kinase domain is mainly mediated by specific amino acid regions in subdomains VIII and IX that are unique to each protein kinase [383-385]. Subdomain VIII has a protein substrate binding loop, which interacts with the residues near the target phosphorylation site of the protein substrate (Figures 2.7B and 2.7C) [383-385]. This protein substrate binding loop usually consists of about 7 to 10 residues juxtaposed upstream of a conserved Ala-Pro-Glu (APE) motif (Figure 2.7B and 2.7C) [383]. This APE motif corresponds to residues 415 to 417 of PINK1 (Figure 2.8). Therefore, PD-associated mutations in these subdomains would affect the recruitment of protein substrate to the protein kinase domain of PINK1 or the proper positioning of the hydroxyl group of the protein substrate for phosphorylation. In agreement with this notion, I have previously shown that recombinant PINK1 protein kinase domain bearing PD-associated G409V mutation displayed lower kinase activity towards *in vitro* protein substrates, casein and Histone H1 [173].

2.3.4 Activation phosphorylation site resides in subdomain VIII

Besides playing a major role in the recognition of protein substrates, the phosphorylation of specific residues in subdomain VIII of some protein kinases are known to activate their kinase activity [425-427]. For example, the maximal kinase activity of protein kinase A (PKA-Cα) is achieved by the phosphorylation of its T197 in its subdomain VIII [425]. The oxygen atoms of the phosphate of this phosphorylated threonine form hydrogen bonds with the charged side chains of the neighbouring residues.
These electrostatic interactions between the phosphorylated threonine and its neighbours stabilise the catalytic conformation of the loop of subdomain VIII and in turn allows proper orientation of the protein substrate for phosphorylation. Thus, the loop in the subdomain VIII of protein kinase domain is also known as the activation loop. The start of the activation loop begins shortly after the conserved motif DFG in subdomain VII and ends before the conserved motif APE in subdomain VIII [383]. The length of the activation loop in most protein kinases typically ranges from 25 to 33 amino acids long [383]. The activation loop of PINK1 is 28 amino acids long and contains several alcoholic residues. Initial studies on PINK1 showed that the bacterial expressed recombinant protein kinase domain of PINK1 could undergo auto-phosphorylation, suggesting PINK1 has a phosphorylate-able activating residue in subdomain VIII. Multiple alignments of PINK1 homologues show there is a conserved alcoholic residue prior to the APE motif in the subdomain VIII. This residue corresponds to the serine 402 (S402) of PINK1 (Figure 2.8). However, it is uncertain as to whether S402 is indeed an auto-phosphorylation site and can activate PINK1’s kinase activity.

2.3.5 PINK1 has 3 unique insertion regions in its protein kinase domain

Conserved alignment analysis of PINK1 with respect to PKA-Cα reveals three insertion regions interspersed between the subdomains I and II, III and IV, and V and VIa of PINK1 (Figures 2.8 and 2.9). Interestingly, these insertion regions cluster together on one side of the protein kinase domain of PINK1 away from its catalytic cleft and on the same plane (Figure 2.9).

The first insertion region (IR-1) of 40 amino acid resides in between the subdomains I and II of PINK1 corresponding to the β-strands 2 (subdomain I) and 3 (subdomain II) of PKA-Cα (Figures 2.8 and 2.9) [428, 429]. The consensus prediction of the secondary structure of IR-1 by several secondary structure prediction programs indicate that IR-1 is composed of a disordered loop (Figure 2.8). In PKA-Cα, this region consists of 4 amino acids and forms a tight U-turn loop between its subdomains I and II, which enables the
subdomains I and II to anchor and orientate the ATP optimally for the phosphate-transfer reaction in the catalytic cleft (Figures 2.7 and 2.8) [428, 429]. It is worth mentioning that the typical length of this corresponding loop in most protein kinases is approximately 4 amino acids long [383]. Thus far, only a protein kinase called Meiosis Induction Protein Kinase (known as SME1 or IME2) derived from *Saccharomyces cerevisiae* is postulated to have a long intervening region between its subdomains I and II like that of PINK1. Although the structure of SME1 has not been determined, conserved alignment and secondary structure prediction analyses indicate that the intervening region of SME1 is approximately 30 amino acids long and adopts disordered loop conformation. Unfortunately, the function of this intervening loop of SME1 is unknown. Unlike PINK1, SME1 does not have other atypical long insertions in its protein kinase domain. Thus, it is unclear as to why PINK1 has a uniquely long loop in between subdomains I and II and other long insertion regions in its protein kinase domain.

The second insert region (IR-2) of 24 amino acids intercepts between the subdomains III and IV of PINK1 corresponding to the α-helix C (subdomain III) and β-strand 4 (subdomain IV) of PKA-Cα (Figures 2.8 and 2.9) [428, 429]. In contrast to IR-1, the secondary structure prediction programs consensually postulate that IR-2 adopts α-helical conformation (Figure 2.8). As the consensus prediction indicates that there is no interruption between the catalytically important α-helix in subdomain III and IR-2, it is uncertain as to whether IR-2 is actually part of the helical subdomain III of PINK1 (Figure 2.8). For most protein kinases, the typical length of the connecting loop between the α-helix in the subdomain III and the disordered loop in subdomain IV is approximately 2 amino acids long [383]. As the consensus secondary structure prediction of PINK1 indicates that IR-2 has a disordered loop of approximately two amino acids prior to the loop in the subdomains IV of PINK1, it supports that IR-2 is indeed part of the catalytic α-helix in subdomains III (Figure 2.8). In PKA-Cα, this α-helix (α-helix C) contains a catalytically important invariant glutamate that positions ATP optimally for the phosphate-transfer reaction by salt-bridging to a conserved ATP-binding lysine in subdomain II (Figure 2.7) [428, 429]. If IR-2 is indeed the extension of this catalytically important α-helix in the subdomain III of PINK1, IR-2 is likely to
protrude out of the protein kinase domain and sits directly beneath IR-1 (Figure 2.9). This may explain the exceptionally long length of IR-1, as it may interact with IR2 to maintain the structural arrangement and orientation of the catalytically important subdomains I, II and III of PINK1. It is worth mentioning that a protein kinase with an insertion region of more than 14 amino acids between its subdomains III and IV has not been reported thus far.

The third insertion region (IR-3) of 22 amino acids sits in between the subdomains V and VIa of PINK1 corresponding to the α-helix D (subdomain V) and the α-helix E (subdomain VIa) of PKA-Cα (Figure 2.8 and 2.9) [428, 429]. The consensus secondary structure prediction indicates that IR-3 is composed of a β-strand flanked by two disordered loops (Figure 2.8). In PKA-Cα, subdomain V is the linking region between the small and large lobes of its protein kinase domain (Figure 2.7) [428, 429]. The subdomain V of PKA-Cα is composed of a hydrophobic β-strand (β-strand 5) in the small lobe, a disordered loop and followed by an α-helix (α-helix D) in the large lobe (Figure 2.7) [428, 429]. Besides anchoring ATP to the catalytic cleft, the subdomain V of PKA-Cα is also involved in the recognition of the protein substrates of PKA-Cα by interacting with the N-terminus of the phosphorylation site of its protein substrate (Figure 2.7) [383, 428, 429]. On the other hand, the subdomain VIa of PKA-Cα folds into a long α-helix through its large lobe, presumably to provide structural support (Figures 2.7 and 2.9) [383, 428, 429]. The typical length of the intervening region between subdomains V and VIa for most protein kinases is approximately 2 to 5 amino acids long [383]. On a similar note with PINK1, a protein kinase called Proto-oncogene Tyrosine Protein Kinase Receptor Ret Precursor (RET) has a long insertion region of 24 amino acids in between its subdomains V and VIa. Structural studies reveal that this intervening region is likely to be a highly flexible and mobile disordered loop protruding away from the catalytic cleft, as they are unable to map this intervening region [430, 431]. Thus, it suggests that the IR-3 of PINK1 may adopt similar flexible and mobile loop conformation as the unmapped insertion region of RET.
As mentioned earlier, these insertion regions cluster together on one side of the protein kinase domain of PINK1. The spatial arrangement of these insertion regions on the surface of the protein kinase domain of PINK1 and their direct connections to catalytically important subdomains suggest that these insertion regions could serve as a docking site for regulatory proteins as a mechanism for regulating PINK1’s kinase activity. This mode of regulatory mechanism has been adopted by several protein kinases. For example, heme-regulated inhibitory kinase (HRI) has an insertion region of 153 amino acids between subdomains IV and V. This insertion region recruits heme to activate the kinase activity of HRI, presumably via stabilising the catalytically active conformation of its protein kinase domain [432]. Besides regulating the kinase activity, insertion regions are also known to be involved in the recruitment and recognition of protein substrates. For example, the loss of the insertion region of 24 amino acids between the subdomains II and III in Calcium-calmodulin Dependent Kinase Kinase (CaMKK) renders CaMKK incapable of phosphorylating and activating its protein substrate, Calcium-calmodulin Dependent Kinase 1 (CaM-KI) [433]. However, the loss of the insertion region does not affect CaMKK’s capability to phosphorylate and activate its other protein substrate, Protein kinase B (PKB), or its ability to undergo auto-phosphorylation [433]. Thus, it is important to determine whether the insertion regions of PINK1 could regulate its kinase activity or influence its substrate selectivity to understand the role of PINK1 in PD pathogenesis.

2.4 C-terminal tail

The C-terminal tail of a protein kinase often contains functional motifs that regulate its kinase activity, recruit protein substrates or regulatory proteins [434-436]. Initial biochemical characterisation of the kinase activity of PINK1 by Silvestri et al observed that the inclusion of the C-terminal tail of PINK1 decreased the kinase activity of their bacterial-expressed recombinant protein kinase domain of PINK1. On the other hand, my colleagues and I found that the inclusion of the C-terminal tail of PINK1 increased the kinase activity of our recombinant protein kinase domain of PINK1 isolated from the
baculovirus-Sf9 insect cell overexpression system. In addition, we reported that the C-terminal tail might play a role in substrate recognition, as the inclusion of the C-terminal tail to the protein kinase domain of PINK1 enhanced the phosphorylation of specific sites on an in vitro substrate, Histone H1 but not in another in vitro substrate, casein [173]. Although our observations are different from Silvestri et al, our differences may be attributed to the differential determination of the boundaries of the protein kinase domain or the choice of protein expression system in our studies that could potentially yield differently folded proteins. Nonetheless, the exact mechanism as to how the C-terminal tail could inhibit or promote the kinase activity, or influence the substrate recognition of PINK1 is still unknown.

To investigate the function of the C-terminal tail of PINK1, I have used Basic local alignment search tool (BLAST) to search for protein domains that bear similarity to the C-terminal tail of PINK1. However, the BLAST search failed to find a protein region of a non-PINK1 protein that bears similarity to the C-terminal tail of PINK1. Thus, I am unable to postulate the function of C-terminal tail of PINK1 from other existing studied proteins. Nonetheless, it suggests that the function and/or structure of the C-terminal tail of PINK1 are unique to the function of PINK1. As the secondary structure of the C-terminal tail of PINK1 is still unknown, I used several secondary structure prediction programs to predict the secondary structure of the C-terminal tail of PINK1. The consensus prediction of the programs indicates that the C-terminal tail of PINK1 is composed of three α-helices and three disordered loops (Figure 2.10). These predicted α-helices are evolutionarily conserved, supporting the consensus prediction of the secondary structure of the C-terminal tail of PINK1. It also suggests that these predicted α-helices are important to the function of PINK1. PD-associated A537T and N542S mutations reside in these predicted α-helices and may impair the function of the C-terminal tail of PINK1 by perturbing the structural integrity of these α-helices (Figure 2.10).
2.5 *PD-associated mutations indicate that intra-mitochondrial kinase activity of PINK1 prevents the pathogenesis of PINK1 familial PD*

Although the majority of the PD-associated mutations reside in the kinase domain of PINK1, PD-associated mutations are also found in other domains of PINK1 that could potentially occlude PINK1 from the mitochondria and/or from its physiological residence in the mitochondria, cause mis-folding or perturb the function of PINK1 in the mitochondria. Table 2.3 summarises the potential impact of PD-associated mutations on PINK1 protein in *PINK1* familial PD.
Chapter 3 – Investigation of putative protein substrates and downstream mediators of PINK1

As mentioned in Chapter 2, most PD-associated missense mutations reside in the conserved subdomains of PINK1’s kinase domain, which indicates that the impairment of the kinase activity of PINK1 is the cause of PINK1 familial PD. In agreement with this notion, I previously showed that PD-associated G386A and G409V mutations reduced the kinase activity of recombinant PINK1 protein corresponding to the kinase domain of PINK1 [173]. As a mitochondrial protein kinase, PINK1 is expected to phosphorylate mitochondrial proteins to prevent the pathogenesis of PINK1 familial PD in response to PD-associated stresses. However, the cellular function of PINK1 and the signalling pathway that PINK1 regulates to suppress the pathogenesis of PINK1 familial PD are still poorly understood. Thus, the identification of the physiological protein substrates of PINK1 will facilitate the elucidation of the cellular function of PINK1 and its role in the pathogenesis of PINK1 familial PD.

Theoretically, the protein substrates of PINK1 could be detected by incubating a mitochondrial extract with [γ-32P]-ATP and catalytically active recombinant PINK1 protein. However, endogenous mitochondrial protein kinases, such as pyruvate dehydrogenase kinase, and several other non-mitochondrial protein kinases, such as Protein Kinase A and Src kinase, would also phosphorylate their respective protein substrates in the mitochondrial extract upon incubation with [γ-32P]-ATP [437-439]. Therefore, the background phosphorylation by these endogenous protein kinases would greatly interfere with the detection of the mitochondrial proteins that were phosphorylated by the added recombinant PINK1 protein.

To overcome the background phosphorylation by endogenous protein kinases, Cohen and Knebel have developed an experimental approach called Kinase Substrate Tracking and Elucidation (KESTREL) and have successfully identified novel protein substrates for several protein kinases, such as Protein Kinase B and Aurora-A kinase [440-444]. This approach utilises several strategies to reduce the background
phosphorylation by the endogenous protein kinases (Figure 3.1). Firstly, conventional protein purification chromatography, such as ion-exchange chromatography, is performed on the protein extract of interest to separate the endogenous protein kinases partially from their substrates. Secondly, a low concentration of ATP is used in the kinase assay to limit the phosphorylation by the endogenous kinases. Thirdly, large amount of soluble recombinant protein kinase of interest is added to the chromatography fraction to overwhelm the background phosphorylation by the endogenous kinases and favour the phosphorylation of the substrates of the recombinant protein kinase in the presence of low ATP concentration. Fourthly, short reaction time is used in the phosphorylation assay to limit the background phosphorylation by the endogenous kinases. Lastly, the same assay is carried out in the absence of the recombinant protein kinase of interest to determine the proteins that are preferentially phosphorylated in the presence of the recombinant kinase. The resulting reactions are resolved by SDS-PAGE. Proteins that are preferentially phosphorylated in the presence of the recombinant protein kinase are then detected in the autoradiograms of the SDS-PAGE gel. The chromatography fraction containing the putative substrate of the recombinant protein kinase is then resolved by 2-dimensional gel electrophoresis to facilitate the identification of the putative protein substrates by mass spectrometry.

Previously, I have successfully generated catalytically active recombinant PINK1 proteins using *Spodoptera frugiperda* 9 (Sf9) insect cell recombinant protein overexpression system (Sf9 overexpression system) [173]. These recombinant PINK1 proteins displayed protein serine/threonine kinase activity towards *in vitro* protein substrate, Histone H1, demonstrating that PINK1 is a protein serine/threonine kinase. More importantly, I showed the C-terminal tail of PINK1 enhanced the catalytic activity of the PINK1 kinase domain towards Histone H1 by almost six fold; the C-terminal also influenced the phosphorylation site selectivity of the PINK1 kinase domain towards Histone H1. Thus, these observations suggest that the C-terminal tail of PINK1 is required to activate the catalytic activity of PINK1 kinase domain and is involved in the recruitment and recognition of its protein substrates. Thus, it is important to include the C-terminal tail of PINK1 in designing the recombinant PINK1 protein used for
KESTREL experiments to search for the putative protein substrates of PINK1. Unfortunately, the recombinant PINK1 proteins used in my previous study were purified by immuno-precipitation by anti-Flag antibody and could not be eluted off by Flag peptides. Hence, these agarose-immobilised recombinant PINK1 proteins are unsuitable for KESTREL experiments. In agreement with this notion, pilot KESTREL experiments using these immuno-precipitated recombinant PINK1 proteins yielded inconsistent and inconclusive data. This may be due to the difficulty in dispensing equal amount of immuno-precipitated recombinant PINK1 proteins into each chromatography fraction in a fast throughput fashion. In addition, the expression levels of these recombinant proteins in Sf9 overexpression system were poor and were insufficient to overwhelm the background phosphorylation by the endogenous protein kinases. Thus, I embarked on the following experiments to generate recombinant proteins suitable for KESTREL experiment and also used other experimental approaches to investigate the putative protein substrates and downstream mediators of PINK1:

- Generation of soluble catalytically active recombinant PINK1 protein for the investigation of putative protein substrates of PINK1 utilising Sf9 insect cell and Pichia yeast recombinant protein overexpression systems.

- Investigation of putative protein substrates of PINK1 using KESTREL and the phosphorylation of candidate protein substrates.

- Investigation of putative protein substrates and downstream mediators of PINK1 by in vitro phosphorylation of mitochondria isolated from BE2-M17 neuroblastoma with inducible overexpression of PINK1.

- Investigation of putative protein substrates and downstream mediators of PINK1 by 2-dimensional difference in gel electrophoresis (2D-DIGE) comparison of the mitochondrial proteomes of BE2-M17 neuroblastoma with inducible overexpression of PINK1.
3.1 Experimental procedures

3.1.1 Generation of pBacPAK9-recombinant PINK1 transfer vectors

Truncated PINK1 DNA constructs encoding the kinase domain and C-terminal tail of PINK1, PINK1(148-581)\(^{20}\) were amplified from the human PINK1 cDNA clone (OriGene Technology). Flag, Strep and 6x-His tags were introduced into their respective DNA constructs via polymerase chain reaction (PCR) using primers as listed in Table 3.1. The resulting PCR products were digested with their respective restriction enzymes and inserted into pBacPAK9 plasmid using molecular cloning protocols described by Sambrook et al [445].

To generate recombinant PINK1 protein with an N-terminal or C-terminal GST tag, the DNA sequence encoding GST’s amino acid sequence was amplified from a bacterial expression plasmid, pGEX-6p-3 (GE Healthcare) using primers as listed in Table 3.2. The resulting PCR products were digested with BamHI or NotI and inserted into pBacPAK9 plasmid to generate pBackPAK9 plasmids that introduce N-terminal and C-terminal GST tags to recombinant PINK1 protein respectively. Truncated PINK1 DNA constructs encoding PINK1(148-581) were amplified from the PINK1 cDNA clone using the primers listed in Table 3.1. The resulting PCR products were digested with their respective restriction enzymes and inserted into the pBacPAK9 plasmids containing N-terminal and C-terminal GST using molecular cloning protocols described by Sambrook et al [445].

The *Spodoptera frugiperda* 9 (*Sf*/9)-codon optimised human PINK1 DNA construct was purchased from GENEART with BamHI and NotI sites incorporated to its 5’-end and 3’ end respectively. In addition, DNA sequence encoding a Myc (EQKLISEEDL)\(^{21}\) and 6x-His tandem tag was introduced to the C-terminus of the encoded PINK1 protein. As the synthesised gene was delivered in pMA plasmid, the *Sf*/9-codon optimised PINK1

\(^{20}\) The numbers in PINK1 constructs denote PINK1 residue numbers of the N- and C-terminal boundaries.

\(^{21}\) Amino acid sequence of Myc tag.
DNA construct was excised and shuttled into pBacPAK9 plasmid via BamHI and NolI sites using molecular cloning protocols described by Sambrook et al [445]. Truncated PINK1 DNA constructs encoding PINK1(148-581) and the kinase domain of PINK1, PINK1(148-515) were amplified from the shuttled Sf9-codon optimised PINK1 DNA construct in pBacPAK9 plasmid using the primers listed in Table 3.1. The resulting PCR products were digested with their respective restriction enzymes and inserted into pBacPAK9 plasmid using molecular cloning protocols described by Sambrook et al [445]. The correct insertions and sequences of the PINK1 DNA constructs in the resulting pBacPAK9 plasmids were verified by restriction digestion and DNA sequencing.

3.1.2 Generation of PINK1 kinase inactive mutant using site-directed mutagenesis

The catalytic base, Asp-362 of PINK1 was mutated to alanine in human PINK1 cDNA construct and Sf9-codon optimised full-length PINK1 DNA construct by using QuikChange® II site-directed mutagenesis kit (Stratagene®) according to manufacturer’s instructions. The mutagenic sense and anti-sense primers used in the mutagenesis of human PINK1 cDNA were 5’ GCATCGCGCACAGAGCCCTGAAATCCGACAAC 3’ and 5’ GCATCGCGCACAGAGCCCTGAAATCCGACAAC 3’ respectively. The mutagenic sense and anti-sense primers used in the mutagenesis of Sf9-codon optimised PINK1 DNA construct were 5’ GTATCGCTACCCGTGCCTGAAGTCCGACAAC 3’ and 5’ TTGTCCCAGTTCCGACCGACGAGCTGTGACGATA 3’ respectively. The sequences of the mutated PINK1 DNA constructs were verified by DNA sequencing.

3.1.3 Generation of recombinant PINK1 baculovirus expression vector

To generate recombinant PINK1 baculovirus expression vector, a well of $1 \times 10^6$ Sf9 cells in 1.5 ml of serum-free Graces’ medium was transfected with pBacPAK9 plasmid.
containing the PINK1 DNA construct of interest and Bsu36I-linearised BacPAK6 viral DNA (Clontech) using Bacfectin® (Clontech) according to manufacturer’s instructions (Figure 3.2). The infected cell lysates were analysed by Western blotting to confirm the production of recombinant PINK1 protein.

3.1.4 Protein extraction of Sf9 cells overexpressing recombinant PINK1 protein

2 L of Sf9 cells (1 x 10⁶ cells/ml) were infected with the recombinant PINK1 baculovirus expression vector at a multiplicity of infection²² ≥2. At 50 hours after infection, the cells were harvested for protein purification by centrifugation at 1000 x g for 20 minutes at 4°C. The cell pellet was washed with serum-free Grace’s medium (Invitrogen®) and centrifuged at 1000 x g for 20 minutes at 4°C again. All subsequent protein purification procedures were performed at 4°C. The cell pellet was homogenised in 25 ml of Ni-NTA lysis buffer (50 mM Tris pH 7.5, 300 mM NaCl, 20 mM imidazole, 0.1 mg/ml soybean trypsin inhibitor, 0.1 mg/ml phenylmethylsulfonylflouride, 0.2 mg/ml benzamidine hydrochloride, 10 mM β-mercaptoethanol, 0.1 % NP-40) or GST lysis buffer (50 mM Tris pH 7.5, 300 mM NaCl, 1 mM EDTA, 0.1 mg/ml soybean trypsin inhibitor, 0.1 mg/ml phenylmethylsulfonylflouride, 0.2 mg/ml benzamidine hydrochloride, 10 mM β-mercaptoethanol, 0.1 % NP-40) using Avestin EmulsiFlex-C3 cell homogeniser. The homogenate was centrifuged at 100,000 x g for 30 minutes to sediment cell debris. The supernatant was collected and subjected to protein purification.

²² Multiplicity of infection refers to the virus to cell ratio. E.g., a multiplicity of infection of 1 means one virus per cell.
3.1.5 Generation of recombinant PINK1 protein in *Pichia pastoris* recombinant protein overexpression system

The PINK1 DNA construct encoding PINK1(148-581) with a N-terminal Flag tag and a C-terminal Strep and 6x-His tandem tag was excised from the pBacPAK9 plasmid generated in Section 3.1.1 and inserted into pPIC3.5K plasmid (Invitrogen) via *BamHI* and *NotI* sites using molecular cloning protocols described by Sambrook *et al* [445]. The pPIC3.5K plasmid was a generous gift from Prof. Roberto Cappai (The University of Melbourne). Restriction digestion and DNA sequencing verified the correct insertion and sequence of the PINK1 DNA construct in the resulting plasmid. The pPIC3.5K plasmid containing the PINK1 DNA construct was linearised by restriction enzyme, *PmeI* and transformed into *Pichia* histidinol dehydrogenase mutant (*His4−*) strain GS115 (Invitrogen) using Electroporator 2510 (Eppendorf) according to manufacturers’ instructions. *Pichia* strain GS115 was also a generous gift from Prof. Roberto Cappai (The University of Melbourne). The electroporated cells were plated onto Minimal Dextrose Medium (MD) agar plate (1.34 % yeast nitrogen base, 2% dextrose, 4 x 10⁻⁵ % biotin, 2 % agar) agar plate and incubated for 5 days at 30°C to screen for transformed *His4⁺* colonies. Each transformed colony was picked and grown in 6ml of Minimal Methanol (MM) media (1.34 % yeast nitrogen base and 4 x 10⁻⁵ % biotin) at 30°C. A final concentration of 0.5 % methanol was added to the culture when the OD₆₀₀ of the culture reached 1.0 to induce the overexpression of the recombinant PINK1 protein. As methanol is volatile, final concentration of 0.5 % methanol was added daily to replenish the evaporated methanol. The cultures were harvested after 72 hours to assess the expression level of the recombinant PINK1 protein by Western blotting.

3.1.6 Protein extraction of *Pichia* overexpressing recombinant PINK1 protein

The transformed *Pichia* bearing the recombinant PINK1 overexpression cassette was grown in either 400ml of MM media or Buffered Minimal Methanol (BMM) media
(1.34 % yeast nitrogen base and 4 x 10^{-5} % biotin, 100mM potassium phosphate, at pH 6.0) to OD_{600} of 1.0. 2 ml of methanol was added every 24 hours to induce and maintain the overexpression of recombinant PINK1 protein. The cells were harvested after 3 days for protein purification by centrifugation at 1000 x g for 20 minutes at 4°C. The cell pellet was washed with BMM media and centrifuged at 1000 x g for 20 minutes at 4°C again. All subsequent procedures were performed at 4 °C. The cell pellet was homogenised in 25 ml of Ni-NTA lysis buffer using Avestin EmulsiFlex-C3 cell homogeniser. The homogenate was centrifuged at 100,000 x g for 30 minutes to sediment cell debris. The supernatant was collected and subjected to protein purification.

3.1.7 Generation of recombinant Parkin and TRAP1 proteins in Escherichia coli recombinant protein overexpression system

The pMAL-p2T plasmid containing human Parkin fused with an N-terminal maltose binding protein DNA construct was a generous gift from Assoc. Prof. Kah-Leong Lim (National Neuroscience Institute, Singapore). The pET-26b(+) plasmid containing TRAP1 devoid of its first 59 residues (corresponding to its mitochondrial targeting sequence) and fused with a C-terminal 6x Histidines tag DNA construct was generously provided by Prof. Zusen Fan (National Laboratory of Biomacromolecules and Center for Infection and Immunity, Institute of Biophysics and Chinese Academy of Sciences, China). These plasmids were transformed into E. coli BL21 strain using molecular cloning protocol described by Sambrook et al [445].

The transformed cultures were grown in 1 L Luria Broth (LB) media containing 15 \mu g/ml kanamycin and 0.1mg/ml ampicillin with respect to pMAL-p2T-Parkin plasmid and pET-26(+) -TRAP1 plasmid. Final concentration of 0.4 mM isopropyl-1-thio-\beta-galactopyranoside (IPTG) was added when the OD_{600} of the culture reached 0.6 to induce the overexpression of the recombinant protein. The culture was incubated at 16°C for 18 hours prior to centrifugation at 3000 x g for 20 minutes at 4°C. All subsequent procedures were performed at 4°C. The cell pellet containing recombinant TRAP1
protein was homogenised in 25 ml of Ni-NTA lysis buffer and the cell pellet containing recombinant Parkin protein was homogenised in 25 ml of GST lysis buffer using Avestin EmulsiFlex-C3 cell homogeniser. The homogenate was centrifuged at 100,000 x g for 30 minutes to sediment cell debris. The supernatant was collected and subjected to protein purification.

3.1.8 Protein purification using Ni-NTA affinity chromatography

Protein solution was applied to a nickel-nitrilotriacetic acid (Ni-NTA) column (QIAGEN) pre-equilibrated with the Ni-NTA wash buffer (20 mM HEPES pH 7.0, 300 mM NaCl, 20 mM imidazole, 0.1 mg/ml phenylmethylsulfonylfluoride, 0.2 mg/ml benzamidine hydrochloride, 10 mM β-mercaptoethanol and 0.1% NP-40). The non-specifically bound proteins were removed by washing with the same buffer supplemented with 600 mM NaCl. Proteins specifically bound to the column were eluted with 30 ml of Ni-NTA elution buffer (20 mM HEPES pH 7.0, 100 mM NaCl, 250 mM imidazole, 0.1 mg/ml phenylmethylsulfonylfluoride, 0.2 mg/ml benzamidine hydrochloride, 10 mM β-mercaptoethanol and 0.1% NP-40). 1.5 ml fractions were collected. Aliquots from the eluted fractions were analysed by SDS-PAGE and Western blotting.

3.1.9 Protein purification using Strep-Tactin affinity chromatography

The elution fractions of the Ni-NTA affinity-purified recombinant PINK1 protein were pooled and applied to Strep-Tactin column (IBA BioTAGnology) pre-equilibrated with Strep-Tactin wash buffer (20 mM HEPES pH 8.0, 150 mM NaCl, 10 mM β-mercaptoethanol, 0.1 mg/ml phenylmethylsulfonylfluoride, 0.2 mg/ml benzamidine hydrochloride, 1 mM EDTA and 0.1% NP-40). The non-specifically bound proteins were removed by washing with the same buffer. Proteins specifically bound to the column were eluted with 10 ml of Strep-Tactin wash buffer supplemented with 3 mM
desthiobiotin (IBA BioTAGnology). 0.5 ml fractions were collected. Aliquots from the eluted fractions were analysed by SDS-PAGE and Western blotting.

### 3.1.10 Protein purification using glutathione affinity chromatography

Protein solution was applied to a glutathione-sepharose™ column (QIAGEN) pre-equilibrated with the glutathione-sepharose wash buffer (20 mM HEPES pH 7.0, 300 mM NaCl, 10 mM β-mercaptoethanol, 0.1 mg/ml phenylmethylsulfonylflouride, 0.2 mg/ml benzamidine hydrochloride, 1 mM EDTA and 0.1 % NP-40). The non-specifically bound proteins were removed by washing with the same buffer supplemented with 600 mM NaCl. Proteins specifically bound to the column were eluted with 30 ml of glutathione-sepharose wash buffer supplemented with 30 mM reduced glutathione. 1.5 ml fractions were collected. Aliquots from the eluted fractions were analysed by SDS-PAGE and Western blotting.

### 3.1.11 Protein purification using amylose affinity chromatography

Protein solution was applied to an amylose resin column (New England BioLabs) pre-equilibrated with the glutathione-sepharose wash buffer. The non-specifically bound proteins were removed by washing with the same buffer supplemented with 600 mM NaCl. Proteins specifically bound to the column were eluted with 30 ml of glutathione-sepharose wash buffer supplemented with 10 mM maltose. 1ml fractions were collected. Aliquots from the eluted fractions were analysed by SDS-PAGE and Western blotting.
3.1.12 Protein purification using Mono Q anion-exchange column chromatography

The protein solution was dialysed at 4°C for 12 hours in the Mono Q column buffer (25 mM HEPES pH 7, 0.1 mg/ml phenylmethylsulfonylfluoride, 0.2 mg/ml benzamidine hydrochloride, 0.1 % NP-40, 1 mM EDTA and 10 mM β-mercaptoethanol) prior to loading onto the MonoQ column. Proteins bound to the column were eluted with 40 ml of linear gradient of 0 to 1 M NaCl in the MonoQ column buffer at a rate of 0.25ml/min. 0.5 ml fractions were collected. Aliquots from these fractions were analysed by SDS-PAGE and Western blotting.

3.1.13 Protein kinase activity assay

A 20 µl aliquot of the elution fraction of recombinant pink1 was incubated in kinase assay buffer (20 mM Tris pH 7.0, 50 µM Na3VO4, 10 mM MgSO4 and 1 mM MnSO4) and 25 µM [γ-P32] ATP (specific radioactivity of 1000-3500 cpm/pmole) with 2 µg of Histone H1 (Calbiochem®), Myelin Basic Protein (Sigma-Aldrich), or 20 µl of purified recombinant Parkin protein or recombinant TRAP1 protein for 1 hour at 30°C. The reaction was stopped by the addition of 5x SDS-PAGE sample loading buffer (0.3 M Tris-HCl pH 6.8, 25 % glycerol, 10 % SDS, 8 % β-mercaptoethanol and 0.0125 bromophenol blue) and heated at 95°C for 5 minutes. The resulting reaction was resolved by SDS-PAGE and exposed to Phosphorimager (Molecular Dynamics).

3.1.14 Isolation of mitochondria from mouse tissues

Mouse brain, liver or testes derived from 10 mice were minced and homogenised in mitochondrial isolation buffer (20 mM HEPES pH 7.5, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA and 0.1 mg/ml phenylmethylsulfonylfluoride) at 4°C. All
subsequent procedures were performed at 4°C. The homogenate was centrifuged at 600 x g for 15 minutes to remove nuclei fraction. The supernatant was centrifuged again at 600 x g for 15 minutes to ensure that all nuclei were removed. To sediment the mitochondria, the resulting supernatant was centrifuged at 6000 x g for 10 minutes. The mitochondria pellet was washed with mitochondrial isolation buffer and centrifuged at 6000 x g for 10 minutes to collect the mitochondrial pellet.

### 3.1.15 Hydroxyapatite chromatography of mouse brain mitochondrial extract

The mouse brain mitochondria pellet was homogenised in 5 ml of Lysis buffer (50 mM Tris pH 7.0, 0.1 mg/ml soybean trypsin inhibitor, 0.2 mg/ml benzamidine hydrochloride, 1% n-octyl β-D-glucopyranoside and 0.1 mg/ml phenylmethylsulfonylfluoride). The homogenate was centrifuged at 20,000 x g for 1 hour to sediment cell debris. The supernatant was collected and subjected to hydroxyapatite (HAP) chromatography. Proteins bound to the column were eluted with 10 ml of linear gradient of 0 to 0.3 M potassium phosphate in MonoQ column buffer (Section 3.1.12) at a rate of 0.25 ml/min and 0.5 ml fractions were collected.

### 3.1.16 Kinase Substrate Tracking and Elucidation (KESTREL) procedure

10μl Aliquots of each eluted fraction from HAP chromatography of mouse brain mitochondria were incubated in kinase assay buffer, 1 mM [γ-P32] ATP (specific radioactivity of 1000-3500 cpm/pmole) and in the absence or presence of 20 μl of the recombinant PINK1 protein preparation purified by Ni-NTA and glutathione affinity chromatographies at room temperature for 10 minutes in a final volume of 35 μl. The reactions were stopped by the addition of 5x SDS-PAGE sample loading buffer and heated at 95°C for 5 minutes. The resulting reaction was resolved by SDS-PAGE and exposed to Phosphorimager.
3.1.17 *In vitro* phosphorylation of the isolated mitochondria by recombinant PINK1 protein

A 20 μl aliquot of purified *Sf9*-codon optimised recombinant PINK1(148-515) protein was added to 30 μg isolated mitochondria resuspended in 20 μl of *in vitro* phosphorylation buffer (20 mM Tris pH 7.5, 150 mM MgCl₂, 10 mM KCl, 100 μM Na₃VO₄, 440 mM mannitol, 140 mM sucrose, 0.1 mg/ml phenylmethylsulfonylfluoride and 50 μM [γ-³²P] ATP with specific radioactivity of 1000-3500 cpm/pmole). The mixture was incubated at 30°C for 30 minutes in the presence or absence of 0.1 % Triton X-100. The reaction was stopped by the addition of 5x SDS-PAGE sample loading buffer and heated at 95°C for 5 minutes. The resulting reaction was resolved by SDS-PAGE and exposed to Phosphorimager.

For alkaline phosphatase treated mitochondria, 30 μg of isolated mitochondria was incubated in 20 μl of dephosphorylation buffer (20 mM Tris pH 7.5, 150 mM MgCl₂, 10 mM KCl, 220 mM mannitol, 70 mM sucrose, 0.1 mg/ml phenylmethylsulfonylfluoride) with 2 active units of alkaline phosphatase (New England BioLabs © Inc) at 30°C for 30 minutes in the presence or absence of 0.1 % Triton X-100. The dephosphorylation was stopped by the addition of Na₃VO₄ to a final concentration of 100 μM. The Triton X-100-untreated dephosphorylated mitochondria were collected by centrifuging at 10,000 x g for 5 minutes. A 20 μl aliquot of purified recombinant PINK1(148-515) protein and 20 μl of *in vitro* phosphorylation buffer were added to the mitochondria pellet. The mitochondrial pellet was resuspended and incubated at 30°C for 30 minutes. For Triton X-100 treated dephosphorylated mitochondria, 20 μl aliquot of the elution fraction of recombinant PINK1 containing 50 μM [γ-³²P] ATP was added and incubated at 30°C for 30 minutes. The reactions were stopped by the addition of 5x SDS-PAGE sample loading buffer and heated at 95°C for 5 minutes. The resulting reactions were resolved by SDS-PAGE and exposed to Phosphorimager.
3.1.18 Expression of recombinant PINK1 protein in lentiviral-mediated mammalian recombinant protein inducible overexpression system

Lenti-X™ Tet-On® Advanced Inducible Expression System developed by Clontech was used to generate lentiviral stably transduced mammalian cell lines with inducible overexpression of recombinant PINK1 protein. The PINK1 DNA construct encoding full-length PINK1 with a C-terminal Strep- and 6x Histidines tandem tag was excised from the pBacPAK9 plasmid generated in Section 3.1.1 and inserted into pLVX-Tight-Puro plasmid (Clontech) via BamHI and NotI sites using molecular cloning protocols described by Sambrook et al [445]. The recombinant PINK1 overexpression lentiviral vector and inducer lentiviral vector were generated according to manufacturer’s instructions.

HEK293T embryonic kidney cell line and HT-1080 fibrosarcoma were cultured in 90 % Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS). BE2-M17 dopaminergic neuroblastoma was cultured in OptiMEM I media (Invitrogen) supplemented with 10 % FBS, 1mM sodium pyruvate and 1x MEM Non-Essential Amino acids (Invitrogen) containing 0.1mM of glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline and L-serine. BE2-M17 cell line was generously provided by Prof. Matthew Farrer (Mayo Clinic, Jacksonville USA). The cell lines were transduced with the recombinant PINK1 overexpression lentiviral vector and inducer lentiviral vector according to the manufacturer’s instructions. After 24 hours, the transduced cells were selected by using 400 to 800 μg/ml G418 (Clontech) and 1μg/ml puromycin (Clontech). 1μg/ml doxycycline was added to the media to induce the overexpression of recombinant PINK1 protein in the cell lines stably transduced with the recombinant PINK1 overexpression lentiviral vector and inducer lentiviral vector. The doxycycline-treated transduced cells were analysed by Western blotting to confirm the production of recombinant PINK1 protein.
3.1.19 Generation of BE2-M17 cell line stably transduced with shRNAmir targeting PINK1

The pGIPZ lentiviral plasmid containing DNA sequence encoding a green fluorescent protein (GFP) and a shRNAmir that targets human and mouse PINK1 (V2LMM_34228) and a non-silencing pGIPZ lentiviral shRNAmir control (RHS4346) plasmid were purchased from Open Biosystems. The PINK1-targeting shRNAmir sequence in V2LMM_34228 is 5’ TGCTGTTGACAGTGAGCGCGCCATCAAGATGATGTGAA TTAGTGAAGCCACAGATGTAATTCCACATCTTTGATGGCATGCCTACTGCCC TCGGA 3’. Red, blue and green fonts denote sense and anti-sense regions of PINK1 DNA sequence and hairpin loop sequence respectively. To generate shPINK1mir lentiviral vectors, HEK293FT cells were transfected with pGIPZ, pMD2.G (Addgene) and psPAX2 (Addgene) plasmids using Lipofectamine 2000 (Invitrogen). pMD2.G (Addgene) and psPAX2 (Addgene) plasmids were generous gifts from Assoc. Prof. Andrew Hill (The University of Melbourne). After 16 hours post-transfection, the media was replaced with fresh DMEM supplemented with 10 % FBS. After 72 hours, the media was centrifuged at 3000 x g for 15min to remove cells and cell debris. 200 μl of the viral supernatant was used to transduce 0.5 x 10^5 BE2-M17 cells in 2 ml of OptiMEM I media supplemented with 10 % FBS, 1mM sodium pyruvate and 1x MEM Non-Essential Amino acids in the presence of 2 μg/ml polybrene. After 16 hours post-transduction, the media was replaced with fresh media containing 1 μg/ml puromycin to select GFP-expressing transduced cells.

3.1.20 Comparative quantitative real-time polymerase chain reaction analysis of BE2-M17 cell lines

Each BE2-M17 cell line was grown to confluence in 6-well Cell Culture Plate (BD Science) in the absence or presence of 1 μg/ml doxycycline. The total RNA of each cell line was extracted using TRIozol® regent (Invitrogen) according to manufacturer’s instructions. First-strand cDNA synthesis was performed using 5 μg of the extracted total
RNA, 50 μM oligo(dT)$_{20}$ (Invitrogen), dNTP mix containing 10 mM dATP, dGTP, dCTP and dTTP (Invitrogen), and SuperScript™ III Reverse Transcriptase (Invitrogen) accordingly to manufacturer’s instructions.

To assess the level of PINK1 mRNA, quantitative real-time polymerase chain reaction (qRT-PCR) was performed on the synthesised cDNA corresponding to 100ng of total RNA using PINK1 sense primer (5’ AGCGAGAGGCCAGCAAGAGA 3’) and PINK1 anti-sense primer (5’ CAGCCAACCATCTTGTCTAACTT 3’), Platinum® SYBR® green qPCR SuperMIX UDG (Invitrogen) and StepOnePlus™ Real-Time PCR System (Applied Biosystems) according to the manufacturers’ instructions. To quantify the relative amount of the housekeeping β-actin mRNA in each sample, qRT-PCR was performed on the synthesised cDNA corresponding to 100 ng of total RNA using β-actin sense primer (5’ AGGATGCAGAAGGAGATCACTG 3’) and β-actin anti-sense primer (5’ GATCCACACGGAGTACTTGCG 3’), Platinum® SYBR® green qPCR SuperMIX UDG and StepOnePlus™ Real-Time PCR System according to the manufacturers’ instructions. The experiment was repeated 3 times. To calculate the relative PINK1 mRNA level per 1000 β-actin mRNA in each cell line, the following formula was used:

$$\frac{\text{Relative PINK1 mRNA level}}{\text{per 1000 β-actin mRNA}} = \frac{2^{\text{Cycle threshold of PINK1}}}{2^{\text{Cycle threshold of β-Actin}}}$$

3.1.21 Purification of recombinant PINK1 protein from stably transduced BE2-M17 cells overexpressing PINK1 using urea-denaturing Ni-NTA affinity chromatography

Stably transduced BE2-M17 cell line overexpressing PINK1 was grown to confluence in a 100 mm culture dish in absence or presence of 1 μg/ml doxycycline prior to cell harvesting. The media was removed and 1ml of guanidinium lysis buffer (6 M guanidine hydrochloride, 1 % Triton X-100, 20 mM sodium phosphate pH 7.8 and 500 mM NaCl) was added to lyse the cells. The lysate was centrifuged at 20,000 x g for 15 minutes to remove the cell debris. 20 μl of Ni-NTA agarose was added to the supernatant and
incubated for an hour. The Ni-NTA agarose was spun down by centrifuging at 800 x g for 5 minutes. The Ni-NTA agarose was washed with 1ml of denaturing binding buffer (8 M urea, 20 mM sodium phosphate pH 7.8 and 500 mM NaCl). The Ni-NTA agarose was spun down by centrifuging at 800 x g for 5 minutes. The Ni-NTA agarose was washed with 3 times with 1 ml of denaturing wash buffer (8 M urea, 20 mM sodium phosphate pH 6.0 and 500 mM NaCl). The Ni-NTA agarose was spun down by centrifuging at 800 x g for 5 minutes. Proteins bound to Ni-NTA agarose were analysed by Western blotting.

3.1.22 In vitro $^{32}$P-labelled putative protein substrates and downstream mediators of PINK1 in the mitochondria isolated from BE2-M17 cell lines

Each BE2-M17 cell line was grown to confluence in a 100 mm dish and washed with Phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na$_2$HPO$_4$, 1.76 mM KH$_2$PO$_4$, pH 7.4). To induce the overexpression of recombinant PINK1 protein or the PINK1 kinase-inactivating mutant, 1 µg/ml doxycycline was added 4 hours prior to the cell harvesting. In 5 ml of PBS, the cells were scrapped off from the dish using a rubber policeman. The cells were spun down by centrifuging at 1000 x g for 10 minutes. 1 ml of hypotonic cell homogenisation medium (CHM) (150 mM MgCl$_2$, 10 mM KCl and 10 mM Tris-Cl pH 6.7) was used to resuspend the cell pellet and incubated on ice for 5 minutes. All subsequent procedures were performed at 4°C. The mixture was passed through a 26 G syringe twice and 1 ml of 2x osmotic buffer (40 mM HEPES pH 6.7, 440 mM mannitol, 140 mM sucrose, 0.2 mg/ml phenylmethylsulfonylfluoride, 150 mM MgCl$_2$ and 10 mM KCl) was added to neutralise the hypotonic environment. The homogenate was centrifuged at 600 x g for 15 minutes to remove nuclei fraction. The supernatant was centrifuged at 1000 x g for 10 minutes to collect heavy mitochondria fraction. The resulting supernatant was then centrifuged at 3000 x g for 10 minutes to harvest the lighter mitochondria fraction. The supernatant was then centrifuged at 6000 x g for 10 minutes to collect the lighter mitochondria fraction. The resulting supernatant
was centrifuged at 10,000 x g for 10 minutes to harvest the lighter mitochondria fraction. The supernatant was then centrifuged at 15,000 x g for 10 minutes to collect the lighter mitochondria fraction. The resulting supernatant was centrifuged at 20,100 x g for 10 minutes to collect the lightest mitochondria fraction. The mitochondrial pellets were resuspended in 30 µl of \textit{in vitro} assay buffer (10 mM Tris-Cl pH 6.7, 150 mM MgCl$_2$, 10 mM KCl, 220 mM mannitol, 70 mM sucrose, 0.1 mg/ml phenylmethylsulfonylflouride, 25 µM [γ-P$^{32}$] ATP with specific radioactivity of 1000-3500 cpm/pmole and 50 µM Na$_3$VO$_4$) and incubated at 30°C for 30 minutes. The reactions were stopped by the addition of 5x SDS-PAGE sample loading buffer and heated at 95°C for 5 minutes. The resulting reactions were resolved by SDS-PAGE and exposed to Phosphorimager.

### 3.1.23 Two-dimensional gel electrophoresis of mitochondrial proteomes isolated from stably transduced BE2-M17 cells overexpressing PINK1

The stably transduced BE2-M17 cells overexpressing PINK1 were grown to confluence in five 150 mm dishes and washed with PBS. To induce the overexpression of PINK1, 1 µg/ml doxycycline was added for 4 hours to another set of five 150 mm dishes of the stably transduced BE2-M17 culture prior to cell harvesting. 10 ml of PBS was added to each dish to scrap the cells off from the dish using a rubber policeman and pooled with respect to the experimental set. The cells were collected by centrifuging at 1000 x g for 5 minutes. 5 ml of hypotonic CHM was used to resuspend the cell pellet before incubation on ice for 5 minutes. All subsequent procedures were performed at 4°C. The mixture was passed through a 26 G syringe twice and 5 ml of 2x osmotic buffer was added to neutralise the hypotonic environment. The homogenate was centrifuged at 600 x g for 15 minutes to remove nuclei fraction. The resulting supernatant was centrifuged at 6000 x g for 10 minutes to collect the mitochondrial pellet. The mitochondrial pellet was washed with 10ml of mitochondrial isolation buffer and centrifuged at 6000 x g for 10minutes. The mitochondrial pellet was resuspended in 500 µl of 2-D protein extraction buffer (40 mM Tris pH 7.0, 2 % SDS and 60 mM dithiothreitol). The homogenate was heated at 95°C for 5 minutes and centrifuged at
20,100 x g for 10 minutes to remove insoluble debris. The proteins in the supernatant were precipitated by using 2-D Clean-Up Kit (GE Healthcare) according to manufacturer’s instructions. The protein precipitate was solubilised in 200 μl of Sample Preparation solution (7 M urea, 2 M thiourea, 4 % CHAPS and 20 mM dithiothreitol) and incubated at room temperature for 5 minutes. The protein solution was centrifuged at 20100 x g for 10 minutes to remove insoluble precipitate. The protein concentration in the supernatant was quantified by 2-D Quant Kit (GE Healthcare) according to manufacturer’s instructions.

For 7cm Immobiline DryStrip gel pH 3-11 Non-Linear (GE Healthcare), 6 μg protein from the supernatant was added to a final volume of 125 μl with DeStreak Rehydration solution (GE Healthcare) containing 0.5 % pH 3-11NL IPG buffer (GE Healthcare). The sample solution was loaded onto 7 cm Immobiline DryStrip gel pH 3-11 Non-Linear by rehydrating the isoelectric focusing (IEF) gel strip in the sample solution overnight. For 24 cm Immobiline DryStrip gel pH 3-11 Non-Linear (GE Healthcare), 60 μg protein from the supernatant was added to a final volume of 450 μl with DeStreak Rehydration solution (GE Healthcare) containing 0.5 % pH 3-11NL IPG buffer. The sample solution was loaded onto 24 cm Immobiline DryStrip gel pH 3-11 Non-Linear by rehydrating the isoelectric focusing (IEF) gel strip in the sample solution overnight.

The sample-containing IEF gel strips were resolved by IEF using Ettan IPGphor II Isoelectric Focusing system (GE Healthcare) on the following day according to manufacturer’s instructions. The 7 cm and 24 cm IEF gel strips were incubated in 5 ml and 15 ml of SDS equilibration buffer (6 M urea, 75 mM Tris-HCl pH 8.8, 29.3 % glycerol, 2 % SDS and 0.002 % bromophenol blue) containing 10 mg/ml of dithiothreitol for 15 minutes respectively before discarding the buffers. 5 ml and 15 ml of SDS equilibration buffer containing 25 mg/ml iodoacetamide were added to the 7 cm and 24 cm IEF gel strips respectively and incubated for 15 minutes before discarding the buffers. The proteins in 7 cm IEF gel strip were resolved by size in the second dimension using Novex® 4-20 % Tris-Glycine ZOOM® Gels 1.0 mm IPG well (Invitrogen) according to the manufacturer’s instructions. The proteins in 24 cm gel strip were resolved by size in
the second dimension using DALT Precast Gel Cassette (GE Healthcare) and Ettan DALTsix system (GE Healthcare) according to the manufacturer’s instructions. The gels were then silver-stained to visualise the protein spots.

3.1.24 Protein identification by mass spectrometry

The protein spot of interest was excised and washed with 200 µl of MS Washing solution (50 mM NH₄HCO₃ and 50 % acetonitrile) twice for 15 minutes. The washing solution was removed and 200 µl of 100 % acetonitrile was added to the protein spot to dehydrate the gel piece. The solution was discarded and the gel piece was air-dried. The gel piece was then rehydrated in 50 µl of 50 mM NH₄HCO₃ containing 0.8 µg sequencing grade trypsin (Promega) and incubated overnight at 37°C. The tryptic peptides-containing solution was collected on the following day. 50 µl of a solution containing 50 % acetonitrile and 1 % formic acid was added to the gel piece and sonicated for 30 minutes to extract the remaining peptides residing in the gel piece. Both peptide solutions were pooled and evaporated to a volume of approximately 15 µl. The peptide solution was then subjected to nanoCHIP 3D Ion Trap mass spectrometry.

3.1.25 Two-dimensional differential gel electrophoresis of mitochondrial proteomes isolated from stably transduced BE2-M17 cells overexpressing PINK1

Mitochondria were isolated from the stable transduced BE2-M17 cells overexpressing PINK1 grown in the absence or presence of doxycycline as described in Section 3.1.23. However, the mitochondria were solubilised in 100 µl of two-dimensional differential gel electrophoresis (2D-DIGE) lysis buffer (30 mM Tris pH 8.5, 7 M urea, 2 M Thiourea and 4 % CHAPS). The homogenate was centrifuged at 20100 x g for 10 minutes to remove insoluble debris. The protein concentration of the supernatant was quantified by 2-D Quant Kit according to the manufacturer’s instructions. 50 µg of mitochondrial proteins
derived from the cells with induced overexpression of PINK1 was labelled with 400 pmol of Cy5 dye (GE Healthcare) according to the manufacturer’s instructions. 400 pmol of Cy3 dye (GE Healthcare) was used to label mitochondrial proteins isolated from the cell line grown in the absence of doxycycline according to the manufacturer’s instructions. The Cy3- and Cy5-labelled samples were pooled with 370 µl of DeStreak Rehydration solution containing 0.5 % pH 3-11NL IPG buffer. The solution was then loaded onto a 24 cm Immobiline DryStrip gel pH 3-11 Non-Linear and incubated overnight to allow the rehydration of the isoelectric focusing (IEF) gel strip. The sample was resolved by IEF in the first dimension and by size in the second dimension on the following day using Ettan IPGphor II Isoelectric Focusing system, DALT Precast Gel Cassette and Ettan DALTsix system as described in Section 3.1.23.

The fluorescence images of the Cy3- and Cy5-labelled proteins in the 2-D DIGE gel were captured by Typhoon Variable Mode Imager (GE Healthcare) according to manufacturer’s instructions. DeCyder 2D software (GE Healthcare) was then used to analyse the protein spots on the Cy3 and Cy5 fluorescent gel images acquired from the Typhoon Variable Mode Imager. The physical gel was then silver-stained to visualise the protein spots. Silver-stained protein spots of interests were excised and identified by mass spectrometry as described in Section 3.1.24.
3.2 Results

3.2.1 Expression of recombinant PINK1 proteins in Sf9 insect cell recombinant protein overexpression system

3.2.1.1 Recombinant PINK1 protein with Strep and 6x-His tags

Although I have previously generated recombinant PINK1 proteins in Sf9 overexpression system for biochemical characterisation, these recombinant proteins were unsuitable for KESTREL experiments. The expression levels of these recombinant PINK1 proteins were insufficient to provide an ample pure preparation of recombinant PINK1 protein for KESTREL experiments. In addition, these recombinant PINK1 proteins were prone to proteolysis and were degraded during the second protein purification chromatography. Thus, it was also technically difficult to obtain a pure preparation of recombinant PINK1 protein for KESTREL experiments using multiple protein purification chromatographies.

As the C-terminal tail plays an important role in activating the kinase activity of PINK1 and substrate recognition, it is important to generate a recombinant PINK1 protein containing the kinase domain and C-terminal tail of PINK1 for KESTREL experiments. To facilitate the protein purification of the recombinant PINK1 protein, a tandem Strep and 6x-His tag was introduced to the C-terminus of the recombinant PINK1 protein to expedite the protein purification process and improve the purity of the preparation of the recombinant protein by using tandem Ni-NTA and Strep-Tactin affinity protein purification chromatography. This recombinant PINK1 protein was generated in Sf9 overexpression system and purified using Ni-NTA affinity chromatography and followed by Strep-Tactin affinity chromatography. As shown in Figure 3.3, the initial purification of the recombinant PINK1 protein by Ni-NTA affinity chromatography was insufficient to yield a pure preparation of recombinant PINK1 protein for KESTREL experiments. The elution fractions of Ni-NTA affinity
chromatography were then pooled and purified using Strep-Tactin affinity chromatography. Unexpectedly, the recombinant PINK1 protein did not bind to Strep-Tactin column (Figure 3.4). Similar to my previous study, subsequent protein purification attempt using a high-resolution anion-exchange chromatography, Mono Q system, resulted into the proteolysis of the recombinant PINK1 protein. As the Ni-NTA affinity chromatography alone was insufficient to yield a pure preparation of the recombinant PINK1 protein, this recombinant PINK1 protein was unsuitable for KESTREL experiments (Figure 3.3).

3.2.1.2 Recombinant PINK1 protein with GST tag

Since Ni-NTA affinity purification was insufficient to yield a pure preparation of 6x-His tagged recombinant PINK1 protein, a GST-tag was introduced to the C-terminus of the recombinant PINK1 protein to allow a single-step protein purification by glutathione affinity protein purification chromatography. However, the expression level of the GST fusion protein in the Sf9 overexpression system was poor (Figure 3.5). Nonetheless, the glutathione affinity chromatography yielded a more purified preparation of the recombinant PINK1 protein than that was achieved with Ni-NTA affinity chromatography alone (Figure 3.5). Unfortunately, attempts to concentrate the recombinant PINK1 protein preparation by centrifugal filtration of the elution fractions of the glutathione affinity chromatography resulted in proteolysis of the recombinant PINK1 protein. Thus, this recombinant PINK1 protein was also not suitable for KESTREL experiments.

3.2.2 Expression of recombinant PINK1 protein in Pichia yeast recombinant protein overexpression system

To overcome the poor expression of the recombinant PINK1 proteins faced in Sf9 overexpression system, an alternative eukaryotic recombinant protein overexpression
system using *Pichia* yeast (*Pichia pastoris* overexpression system) was adopted to improve the expression level of the recombinant PINK1 protein. A DNA construct encoding the kinase domain and the C-terminal tail of PINK1 was inserted into the *Pichia* overexpression plasmid (pPIC3.5K) containing a *His*\(^+\) gene, and transformed into *His*\(^-\) *Pichia* mutant. As this plasmid does not have a yeast origin of replication, the resulting *His*\(^+\) transformants were due to recombination between the plasmid and the *Pichia* genome (Figure 3.6). It is worth mentioning that the expression level of the recombinant protein in *Pichia* overexpression system is dependent on the number of plasmids inserted into the *Pichia* genome. Thus, every transformant would have different expression level of the recombinant PINK1 protein due to the different amount of plasmids inserted into the *Pichia* genome (Figure 3.6). However, large amount of inserted plasmids may not necessarily give high protein expression for some proteins. Hence, the expression level of the recombinant PINK1 protein was assessed in each of the transformants using Western blot. As shown in Figure 3.7, several transformants displayed high expression level of recombinant PINK1 proteins. Among these transformants, transformant 14 has the highest expression level of the recombinant PINK1 protein. Thus, transformant 14 was selected to generate recombinant PINK1 protein for KESTREL experiments.

As *Pichia* can be grown in Minimal Methanol media (MM media) or Buffered Minimal Methanol media (BMM media), transformant 14 was cultured in both media to investigate which media gave the best protein yield of the recombinant PINK1 protein. It is worth mentioning that the pH of the MM media may reach pH 4 during fermentation. The latter media has a buffered pH of 6.0, which may prevent the mis-folding of proteins that are sensitive to low pH. As GST-tag seems to yield poor expression of recombinant PINK1 protein in *Sf9* overexpression system, the tandem Strep and 6x-His tag was introduced to this recombinant PINK1 protein to expedite the protein purification process by using Ni-NTA and Strep-tactin affinity chromatography. As shown in Figure 3.8 and Figure 3.9, the initial protein purification by Ni-NTA affinity chromatography indicates that the culture grew on BMM media has better protein yield of the recombinant PINK1 protein than the culture grew on MM media. Similar to the recombinant PINK1 protein generated in *Sf9* overexpression system, the *Pichia*-generated recombinant PINK1 protein
also did not bind to the Strep-tactin column. Subsequent attempt to purify the Ni-NTA purified recombinant PINK1 protein using Mono Q system also resulted into the proteolysis of the recombinant PINK1 protein. Nonetheless, the protein yield and the purity of the Ni-NTA affinity preparation of the Pichia-generated recombinant PINK1 protein were better than its Sf9-generated counterparts.

As Pichia is a lower eukaryotic unicellular organism, it may lack the machinery to fold or post-translationally modify the recombinant PINK1 protein of higher eukaryotic origin. Furthermore, it is unclear whether PINK1 undergoes other post-translation modification besides mitochondrial proteolytic processing. Thus, it is uncertain as to whether the recombinant PINK1 protein generated in the Pichia overexpression system was folded properly and catalytically active. To assess the catalytic activity of the recombinant PINK1 protein, a kinase assay was performed on the recombinant PINK1 protein using Histone H1 as an in vitro protein substrate in the presence of [γ-P\textsuperscript{32}-ATP. Unexpectedly, the recombinant PINK1 could not phosphorylate Histone H1, suggesting that the recombinant PINK1 protein was incorrectly folded or not catalytically active (Figure 3.10). Hence, the Pichia-generated recombinant PINK1 protein could not be used for KESTREL experiments.

### 3.2.3 Expression of recombinant PINK1 protein with GST and 6x-His tags in Sf9 insect cell recombinant protein overexpression system

As the recombinant PINK1 protein generated in the Pichia overexpression system was not catalytically active, the Sf9 overexpression system was again pursued to generate catalytically active recombinant PINK1 protein. As the recombinant PINK1 protein generated in Sf9 overexpression system was prone to proteolysis, 6x-His and GST tags were introduced into the recombinant PINK1 protein to expedite the purification of the recombinant PINK1 protein and improve the purity of the preparation. As shown in Figure 3.5, the glutathione affinity chromatography also co-purifies Sf9’s GST during the purification of the recombinant GST-PINK1 fusion protein. Thus, the Sf9-generated
recombinant PINK1 protein in this experiment was initially subjected to Ni-NTA affinity chromatography to remove GST and this was followed by glutathione affinity chromatography to remove the rest of the contaminating proteins. As shown in Figures 3.11 and 3.12, a relatively pure preparation of soluble recombinant PINK1 protein was obtained by using this rapid protein purification approach. Furthermore, the recombinant PINK1 protein could phosphorylate \textit{in vitro} protein substrates, Histone H1 and Myelin Basic Protein, demonstrating that the purified recombinant PINK1 protein is catalytically active (Figure 3.13). However, the attempt to concentrate the recombinant PINK1 protein for KESTREL experiments using centrifugal concentration caused the proteolysis of the recombinant PINK1 protein. Despite the low concentration in the glutathione affinity-purified preparation, the purified catalytically active recombinant PINK1 protein may be sufficient for KESTREL experiments.

### 3.2.3.1 KESTREL using mouse brain mitochondrial extract

To investigate the putative substrates of PINK1 that are involved in PD, mitochondria were isolated from mouse brain and solubilised by a strong non-ionic detergent, n-octyl \(\beta\)-D-glucopyranoside (ODG), to include mitochondrial membrane proteins for the KESTREL experiment. As hydroxyapatite (HAP) column binds to both acidic and basic proteins due to its surface mosaic of positively charged calcium ions and negatively charged phosphate ions, the mitochondrial extract was subjected to HAP chromatography to fractionate the entire spectrum of the extracted mitochondrial proteins (Figure 3.14) \[446\]. A pilot KESTREL experiment was performed with the HAP chromatography fractions and the purified recombinant PINK1 protein. However, no preferential phosphorylation of the mitochondrial protein by the recombinant PINK1 protein was observed (Figure 3.15). As it is not possible to concentrate the purified recombinant PINK1 protein without proteolysis, it is unclear as to whether the lack of preferential phosphorylation of the mitochondrial proteins by the recombinant PINK1 protein was due to the insufficient amount of recombinant PINK1 protein added to overwhelm the
background phosphorylation or the abundance of the protein substrate of PINK1 was too low to be detected by the KESTREL approach.

### 3.2.4 Expression of recombinant PINK1 proteins with Sf9-optimised codons

The PINK1 DNA constructs generated for the protein expression of the recombinant PINK1 proteins in Sf9 overexpression system thus far were derived from human PINK1 cDNA. It is plausible that the Sf9 cells could not translate the PINK1 DNA construct efficiently due to the usage of human codons that are not optimised for the translation in Sf9 cells. Furthermore, it is unclear as to whether the mRNA transcript of the PINK1 DNA construct contains secondary RNA structure that may interfere with the protein translation in Sf9 cells. In an attempt to improve the expression of the recombinant PINK1 protein in Sf9 overexpression system, the codons of the human PINK1 DNA construct were replaced with the codons that are optimised for the translation in Sf9 cells (Figure 3.16). In addition, potential secondary RNA structures were avoided when designing this new PINK1 DNA construct. Using this new Sf9 codon-optimised PINK1 DNA construct, a DNA construct encoding the kinase domain and the C-terminal tail of PINK1 was generated and expressed in the Sf9 overexpression system (Figure 3.17). However, the expression level of this recombinant PINK1 protein was as poor as the previous recombinant PINK1 proteins. To investigate whether the removal of the C-terminal tail of PINK1 could improve the expression level of recombinant PINK1 protein, a Sf9-optimised PINK1 DNA construct encoding the kinase domain of PINK1 was generated and expressed in Sf9 overexpression system (Figure 3.17). To yield a purer preparation of the recombinant PINK1 protein from the Ni-NTA affinity chromatography, a 12x-His tag was fused to the C-terminus of the recombinant PINK1 protein to increase the stringency of the wash during the protein purification (Figure 3.17). As shown in Figure 3.17, the expression level of this recombinant PINK1 protein was better than the recombinant PINK1 proteins generated previously in Sf9 overexpression system. In addition, the inclusion of the 12x-His tag improved the purity of the preparation of the recombinant PINK1 protein from the Ni-NTA affinity chromatography.
3.2.4.1 Investigating the phosphorylation of candidate protein substrates, Parkin and TRAP1 by recombinant PINK1 protein

Although several studies have indicated that Parkin and TRAP1 are the protein substrates of PINK1, the residues that are phosphorylated by PINK1 in these proteins are still unknown [180, 299, 300]. In addition, it is unclear at to whether PINK1 regulates the E3 ligase activity and the ATPase activity of Parkin and TRAP1 via phosphorylation respectively. To investigate the phosphorylation of these proteins by PINK1, the recombinant Parkin and TRAP1 proteins were generated in an *Escherichia coli* bacterial recombinant protein overexpression system and purified by amylose affinity and Ni-NTA affinity chromatographies respectively (Figure 3.18). These recombinant proteins were used as candidate protein substrates in the kinase assay with the recombinant PINK1 protein in the presence of \([\gamma-P^{32}]\)-ATP. As shown in Figure 3.19, the recombinant PINK1 protein did not phosphorylate Parkin and TRAP1. Consistent with my previous study, the wild-type recombinant PINK1 protein could phosphorylate the *in vitro* protein substrate, Histone H1 but the recombinant PINK1 protein bearing the kinase inactivating D362A mutation was inactive towards Histone H1 (Figure 3.19). Thus, these observations indicated that the recombinant PINK1 protein used in the kinase assay was catalytically active but could not phosphorylate the recombinant Parkin and TRAP1 proteins. It is worth considering the reported results of phosphorylation of Parkin and TRAP1 by PINK1 were observed in mammalian cells [180, 299, 300]. Although recombinant Parkin and TRAP1 proteins expressed in bacterial overexpression system were previously shown to be catalytically active in several studies, the bacterial expressed recombinant Parkin and TRAP1 proteins in this study may lack the eukaryotic post-translation modifications that are required for the phosphorylation by PINK1 [293, 447-450]. Hence, future studies should investigate whether the recombinant Parkin and TRAP1 proteins generated in *Sf9* overexpression system can be phosphorylated by the recombinant PINK1 protein.
3.2.4.2 Investigating the phosphorylation of putative mitochondrial protein substrates of PINK1

As recent studies reported that PINK1 resided on the surface of depolarised mitochondria to initiate mitophagy, PINK1 is likely to phosphorylate its protein substrates on the outer membrane (OM) of the mitochondria to instigate the mitophagy of the depolarised mitochondria. To investigate the putative OM protein substrates of PINK1, intact mitochondria were isolated from mouse brain, liver and testes. It is worth mentioning that PINK1 is highly expressed in the testes [188]. These isolated intact mitochondria were subjected to kinase assays with the recombinant PINK1 protein kinase domain in the presence of [γ-P³²]-ATP to label the potential OM protein substrates of PINK1 (Figure 3.20). To enhance the detection of the potential OM protein substrates of PINK1, the isolated mitochondria were treated with alkaline phosphatase prior to the kinase assay to dephosphorylate OM proteins in a parallel experiment (Figure 3.20). High concentration of sodium orthovanadate was added to inhibit the alkaline phosphatase during the kinase assay with the recombinant PINK1 protein to prevent the dephosphorylation of the OM proteins. However, the preferential phosphorylation of mitochondrial proteins in the presence of the recombinant PINK1 protein was not observed, as shown in Figure 3.21. To investigate the putative protein substrates of PINK1 in other mitochondrial compartments, the isolated mitochondria were lysed using non-ionic detergent, Triton X-100 and subjected to kinase assays with the recombinant PINK1 protein (Figure 3.20). Similarly, a parallel experiment with alkaline phosphatase was carried out to enhance the detection of the potential substrates of PINK1 (Figure 3.20). However, preferential phosphorylation of mitochondrial proteins in the presence of the recombinant PINK1 protein was again not observed under these conditions (Figure 3.21). As the recombinant PINK1 protein is catalytically active, it is unclear as to whether the background phosphorylation by the endogenous protein kinases masked the phosphorylation by the recombinant PINK1 protein.
3.2.5 Expression of full-length recombinant PINK1 protein in BE2-M17 dopaminergic neuroblastoma

As the search for the putative mitochondrial protein substrates of PINK1 using Sf9-generated recombinant PINK1 proteins has been unsuccessful thus far, stably transduced human cell lines, HEK293T embryonic kidney cell line, HT-1080 fibrosarcoma and BE2-M17 dopaminergic neuroblastoma overexpressing PINK1 were generated to investigate the putative substrates and downstream mediators of PINK1. These cell lines were transduced with lentivirus containing a doxycycline-inducible PINK1 overexpression cassette, which overexpress PINK1 in the presence of doxycycline (Figure 3.22). Despite an overnight induction of PINK1 overexpression, the overexpression of recombinant PINK1 protein was not observed in these cell lines under these conditions (Figure 3.23). As BE2-M17 neuroblastoma expresses a full complement of dopamine synthesis enzymes and has measurable amounts of dopamine, BE2-M17 neuroblastoma is a relevant cell line for investigating the role of PINK1 in the pathogenesis of PINK1 familial PD [451-454]. Thus, the mRNA levels of PINK1 in BE2-M17 cell lines were examined using quantitative real-time PCR (qRT-PCR). The qRT-PCR analysis of these cell lines overexpressing wild-type PINK1 and the PINK1 mutant bearing kinase inactivating D362A mutation indicated that the level of PINK1 mRNA increased up to 20 fold during the overnight induction by doxycycline (Figure 3.24). Thus, the absence of recombinant PINK1 protein during the induction by doxycycline was not due to the lack of exogenous PINK1 mRNA. Consistent with these observations, Sandebring et al reported a 4 fold increase of PINK1 mRNA in their BE2-M17 cell lines overexpressing PINK1 and the PINK1 mutants bearing PD-associated G309D mutation and triple kinase inactivating mutations [267]. As the overexpression of PINK1 promoted cell survival during the inhibition of mitochondrial complex I (MC-I), they also tested whether the induced overexpression of PINK1 could promote the survival of the BE2-M17 cell lines during MC-I inhibition induced by rotenone. In agreement with that notion, their BE2-M17 cells overexpressing PINK1 were less susceptible to MC-I inhibition but not the cells overexpressing the PINK1 mutant bearing PD-associated G309D mutation or triple kinase inactivating mutations. As the BE2-M17 cell lines overexpressing PINK1 in this study had up to 20 fold increase in PINK1 mRNA level during induction by doxycycline,
these cell lines are likely to have improved cell survival during MC-I inhibition like those generated by Sindebring et al.

As the Sf9-generated recombinant PINK1 proteins are prone to proteolysis, it is possible that the recombinant PINK1 protein generated in BE2-M17 neuroblastoma was degraded shortly after the induction by doxycycline. To determine the optimal induction time, a time-course study on the induced expression of PINK1 in BE2-M17 cells overexpressing wild-type PINK was carried out. To enrich and prevent the rapid degradation of recombinant PINK1 protein during the protein extraction for the analysis by Western blot, Ni-NTA agarose beads were used to purify and concentrate recombinant PINK1 protein via its C-terminal 6x His-tag under urea-denaturing conditions. The time-course analysis indicated that the protein expression of recombinant PINK1 protein peaked at 4 hours of PINK1 overexpression induced by doxycycline (Figure 3.25). Unexpectedly, the protein level of recombinant PINK1 declined at 8 hours of induction. Consistent with the aforementioned qRT-PCR experiment, qRT-PCR analysis of BE2-M17 cells indicated that the PINK1 mRNA level was increased by 10 fold at 4 hours of induced overexpression of wild-type PINK1 (Figure 26). Thus, these observations suggested that the protein level of PINK1 was tightly regulated in BE2-M17 cells and the excessive accumulation of PINK1 protein was prevented by rapid turnover of PINK1 protein after 4 hours of induced PINK1 overexpression. Thus, all subsequent experiments performed with BE2-M17 cell lines overexpressing PINK1 and the PINK1 mutant bearing kinase inactivating D362A mutation were carried out at 4 hours of induction by doxycycline to investigate the protein substrates, downstream mediators and function of PINK1.

3.2.5.1 Investigating the putative substrates and downstream mediators of PINK1

To investigate the putative substrates and downstream mediators of PINK1, the mitochondria were isolated from the BE2-M17 cell lines and treated with [γ-P³²]-ATP. As ATP is diffusible through the mitochondrial membranes, PINK1 and the endogenous
protein kinases would phosphorylate their respective substrates using \([\gamma-P^{32}]\)-ATP during the treatment. Thus, mitochondrial proteins that are preferentially phosphorylated in BE2-M17 cells overexpressing PINK1 but not in the cells overexpressing the PINK1 kinase inactive mutant are potential substrates or downstream mediators of PINK1. As the size of the mitochondria range from 0.4\(\mu\)m to 2.5\(\mu\)m, using differential centrifugation could separate the mitochondria according to their sizes [455]. Thus, the mitochondrial fraction spun down at lower centrifugal force would have lesser contamination of smaller sized organelles, such as lysosomes and microsomes [455]. However, the autoradiograms of the SDS-PAGE of the \([\gamma-P^{32}]\)-ATP-treated mitochondria did not detect preferential phosphorylation of the mitochondrial proteins in the cells overexpressing PINK1 (Figure 3.27). It is plausible that the background phosphorylation by the endogenous protein kinases masked the proteins that might be preferentially phosphorylated during the overexpression of PINK1 or that the abundance of these proteins was too low to be detected by this experimental approach.

An alternate approach was used to investigate the protein substrates and downstream mediators of PINK1 by comparing the mitochondrial proteomes of the doxycycline-treated and untreated BE2-M17 cell line overexpressing wild-type PINK1. Initial analysis of the mitochondrial proteomes using 7cm two-dimensional gel (2D-gel) electrophoresis detected an up-regulated protein spot in the cells overexpressing PINK1 (Figure 3.28). Analysis of this protein spot by mass spectrometry could only suggest that this protein spot was sideroflexin-1 due to the small protein amount available in the protein spot. Nonetheless, this experiment showed that the overexpression of PINK1 affected the expression levels of the mitochondrial proteins in BE2-M17 cells. To increase the protein sample loading and the sensitivity of the 2D-gel electrophoresis, 24cm 2D-gel electrophoreses were performed on the mitochondria isolated from the doxycycline-treated and untreated BE2-M17 cell line overexpressing PINK1. Expectedly, the larger protein sample loaded in 24cm 2D-gel electrophoresis increased the number of protein spots detected by silver staining (Figure 3.29). However, it was technically challenging to identify the up- and down-regulated protein spots confidently between two 2D-gels. To overcome this problem, a preliminary 24cm two-dimensional differential gel
electrophoresis (2D-DIGE) of the mitochondria isolated from the doxycycline-treated and untreated BE2-M17 cell line overexpressing PINK1 was carried out. Using Decyder program developed by GE healthcare, 31 down-regulated protein spots and 12 up-regulated protein spots were detected (Figure 3.30 and Table 3.3). Despite the increased protein sample loaded in the 2D-DIGE, only a few of these protein spots could be identified by mass spectrometry confidently. Out of the 12 up-regulated protein spots, only one protein spot was identified confidently by mass spectrometry. This protein was Dihydrolipoyl Dehydrogenase (DLD) (Figure 3.31). On the other hand, 4 down-regulated protein spots were identified. Two of the identified protein spots were Leucine-Rich Pentatricopeptide-Repeat Motif Containing Protein (LRPPRC) (Figure 3.32 and 3.33). It is worth mentioning that LRPPRC has 7 acetylated lysine residues and the acetylation of each lysine residues would cause an acidic shift during isoelectric focusing [456]. Thus, LRPPRC may exist in 8 different post-translational modified forms in the cell. In agreement with this notion, 6 unidentified down-regulated protein spots were juxtaposed to the two identified protein spots. However, the overexpression of PINK1 did not seem to influence the acetylation profile of LRPPRC but down-regulated the overall protein expression of LRPPRC. The other down-regulated protein spots were LON Protease Homolog (LONP1) and Tumor Necrosis Factor Type 1 Receptor-Associated Protein (TRAP1) (Figures 3.34 and 3.35). To identify the rest of the up- and down-regulated protein spots confidently, future study should analyse the mitochondrial lysates of the BE2-M17 cell line overexpressing PINK1 using IEF strips of narrower pH range in the 2D-DIGE to increase the loading of the protein sample and the detection of the protein spots of low abundance.
3.3 Discussion

The attempts to search for putative protein substrates and downstream mediators of PINK1 using biochemical approaches were limited by the poor expression of recombinant PINK1 protein in the Sf9 insect cell recombinant protein overexpression system. Although several affinity tags were used to expedite the purification of recombinant PINK1 proteins, a single affinity purification step was insufficient to yield a pure preparation of recombinant PINK1 protein. Furthermore, the incorporation of a GST tag to the C-terminus of the recombinant PINK1 protein decreased the expression level of the recombinant PINK1 protein in the Sf9 overexpression system (Figure 3.5). It is plausible that the introduced affinity tag may interact non-specifically with the PINK1 polypeptide during protein folding and in turn promote protein mis-folding and degradation of the recombinant PINK1 protein. Nonetheless, the incorporation of N-terminal GST tag and C-terminal 6x-His tag to the recombinant PINK1 protein did expedite the purification process and yield a relatively pure and catalytically active recombinant PINK1 protein. Unfortunately, the resulting recombinant PINK1 protein could not be concentrated due to its susceptibility to degradation and/or protein aggregation. Thus, the unconcentrated protein solution of this recombinant PINK1 protein has limited use in the biochemical approaches adopted in this study to search for putative protein substrates and downstream mediators of PINK1. Although the Pichia yeast recombinant protein overexpression system provided a reasonable expression level of recombinant PINK1 protein, the Pichia-generated recombinant PINK1 protein was catalytically inactive. As mentioned in Section 2.1.2, a PINK1 orthologue has not been identified in unicellular organisms and may exist only in metazoans. Thus, Pichia may lack the cellular machinery to fold and/or post-translationally modify the recombinant PINK1 protein physiologically to generate catalytically active PINK1 protein, which may only be possible in metazoans.

Although the search for the putative protein substrates and downstream mediators of PINK1 using biochemical approaches was unsuccessful, the proteomic approach using 2D-DIGE comparison of the mitochondrial proteomes of the BE2-M17 cell line
overexpressing PINK1 identified four candidate mitochondrial proteins that are potentially involved in the pro-survival function of PINK1. These proteins are DLD, LRPPRC, LONP1 and TRAP1.

The protein expression of DLD was up-regulated during the overexpression of PINK1 in BE2-M17 cells. DLD is a component of pyruvate dehydrogenase complex, α-ketoglutarate dehydrogenase complex, branched-chain α-keto acid dehydrogenase complex and the mitochondrial glycine cleavage system in the matrix of the mitochondria. It catalyses the oxidation of dihydrolipoate by transferring two electrons to the co-factor, FAD⁺; the resulting FADH₂ in DLD then reduces NAD⁺ to NADH to provide the metabolic fuel to the MC-I. Interestingly, reduced DLD expression has been reported to increase the vulnerability of the heterozygous DLD-knockout mice towards the MC-I inhibition induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [457]. In addition, the degeneration of their substantia nigra pars compacta dopaminergic (SNpc) neurons was more severe than the control mice during the treatment with MPTP [457]. As DLD operates upstream of MC-I in the metabolic pathway, the reduced expression of DLD in the heterozygous DLD-knockout mice is likely to deprive the MC-I of its substrate, NADH, and increase the susceptibility of the SNpc neurons towards MC-I inhibition caused by MPTP. Thus, the reduced activity of DLD may be involved in the reduced MC-I activity observed in idiopathic patients. In agreement with this notion, a post-mortem study of idiopathic PD patients showed that the activity of α-ketoglutarate dehydrogenase complex was reduced by 50% in their cerebellum [458]. In addition, the expression of the α-ketoglutarate dehydrogenase complex in the SNpc neurons of the idiopathic PD patients decreased with the progression of PD [459]. As the overexpression of PINK1 in BE2-M17 cells increased the expression of DLD, PINK1 may exert its pro-survival function by promoting the production of NADH to sustain the activity of MC-I during MC-I inhibition. Besides generating NADH, Chen et al reported that DLD also catalyses the reduction of nitrated DNA and protein adducts using dihydrolipoic acid, NADH, or ubiquinol as cofactor [460]. Thus, it is also plausible that PINK1 exerts its pro-survival function by up-regulating the expression of DLD to reduce the oxidative damage caused by the MC-I inhibition. It is noteworthy that the genetic
mutations in DLD cause severe brain damage after birth and motor deficits, such as hypotonia and ataxia, in Maple Syrup Urine disease and Pyruvate dehydrogenase deficiency disease [461].

In contrast to DLD, the protein expression of LRPPRC was down-regulated during the overexpression of PINK1 in BE2-M17 cells. LRPPRC is a matrix protein that regulates the protein translation of Cytochrome c Oxidase subunits 1 and 3 of the mitochondrial complex IV (MC-IV) and other unknown mitochondrial proteins encoded in the mitochondrial genome [462-464]. The occlusion of LRPPRC from the mitochondria due to a genetic missense mutation impairs the assembly of the MC-IV and cause a mitochondrial neurodegenerative disorder called French-Canadian Type Leigh’s syndrome [463, 465, 466]. Besides causing French-Canadian Type Leigh’s syndrome, LRPPRC deficiency is also implicated in idiopathic PD. Recent studies reported that the expression of LRPPRC was reduced in the blood and SNpc neurons of idiopathic PD patients [467, 468]. Although LRPPRC deficiency impairs mitochondrial respiration, recent studies suggest that LRPPRC is involved in the mitophagy mediated by PINK and Parkin [464, 469, 470]. Xie et al reported that the depletion of LRPPRC in HeLa cells activates LC3-mediated autophagy, indicating that the down-regulated protein expression of LRPPRC may prime BE2-M17 cells overexpressing PINK1 for autophagy [469]. In agreement with this notion, recent proteomic studies on HEK293 cells overexpressing Parkin or PINK1 identified LRPPRC as one of the interacting partners of Parkin and PINK1 [470, 471]. Collectively, these observations suggest that PINK1 recruits Parkin to depolarised mitochondria to reduce the protein level of LRPPRC to execute mitophagy. However, it is also plausible that the observed down-regulated protein expression of LRPPRC in the BE2-M17 cells overexpressing PINK1 was a compensatory mechanism to cope with the increased expression of DLD to maintain healthy mitochondrial respiration rate by reducing the protein level of MC-IV in the BE2-M17 cells overexpressing PINK1.

Similar to LRPPRC, the protein expression of LONP1 was also down-regulated in the BE2-M17 cells overexpressing PINK1. LONP1 is an ATP-dependent serine protease that
digests denatured and oxidised proteins in the matrix, especially on proteins containing iron sulphide clusters, such as aconitase and MC-I [472-476]. Besides removing damaged proteins, LONP1 also regulates the replication of the mitochondrial DNA and the mitochondrial expression of the mitochondrial proteins by degrading the mitochondrial regulatory proteins residing on their respective mitochondrial promoters [477, 478]. The loss of LONP1 due to a genetic nonsense mutation causes mild mental retardation in a family [479]. Although ATP stimulates the activity of LONP1, Stanyer et al reported that ATP-stimulated LONP1 was 5 fold more sensitive to peroxynitrite treatment than the unstimulated LONP1 [480]. As DLD may promote the activity of MC-I in BE2-M17 cells overexpressing PINK1, LONP1 may be damaged by the increased reactive oxygen species generated from increased mitochondrial respiration [230]. Furthermore, DLD may also facilitate the removal of nitrated LONP1 using dihydrolipoic acid, NADH, or ubiquinol [460]. As LONP1 also regulates the protein expression of the mitochondrial genome, it is conceivable that PINK1 may indirectly regulate the expression of mitochondrial proteins via LONP1 to initiate mitophagy.

The protein expression of TRAP1 was down-regulated during the overexpression of PINK1 in BE2-M17 cells. TRAP1 is a mitochondrial inter-membrane space chaperone that bears homology to the heat shock protein 90 family [180, 447, 481-483]. Although the function of TRAP is still poorly understood, TRAP1 has been reported to be an antagonist of the ROS generation in Granzyme M-mediated apoptosis [448]. In addition, Pridgeon et al reported that PINK1 phosphorylated TRAP1 to promote the survival of PC-12 neuroblastoma during the oxidative stress treatment with H2O2 [180]. As TRAP1 is a reported substrate of PINK1, it is unclear why the expression of TRAP1 was decreased in BE2-M17 cells overexpressing PINK1. It is worth mentioning that TRAP1 has 4 acetylated lysine residues, 1 phosphorylated tyrosine residue and 1 phosphorylated serine residue [456, 484-486]. As TRAP1 may exist in 7 post-translation modified forms that may be resolved by IEF, it is uncertain as to whether the overexpression of PINK1 in BE2-M17 cells favoured a particular post-translation modification of TRAP1, such as the

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23 A reactive product of the reaction of nitric oxide with superoxide, and is one of the reactive nitrogen species.
phosphorylation of its serine residue. Hence, future study should use IEF strips of narrower range in the 2D-DIGE analysis of the mitochondria of BE2-M17 cell line overexpressing PINK1 to investigate whether the overexpression of PINK1 could influence the post-translation modification of TRAP1 and the detection of other proteins of abundance. Future study should also confirm the perturbed expressions of protein spots identified in the 2D-DIGE analysis by labelling the mitochondria isolated from cells with and without induced overexpression of PINK1 with alternate order of dye labelling. In addition, Western Blotting could be used to verify the expression levels and modified forms of these proteins if antibodies were available.
Chapter 4 – Investigation of the localisation of PINK1 in the mitochondria

Although many studies have investigated the cellular distribution of PINK1, the reports of the cellular distribution and sub-mitochondrial localisation of functional PINK1 have been inconsistent since its discovery as a causative gene of PINK1 familial PD. Initial extensive study by Silvestri et al used several experimental approaches to determine the cellular distribution of PINK1 [171]. Using immuno-fluorescence microscopy, they showed that that PINK1 co-localised with mitochondrial proteins, AFG3-like protein 2 (AFG3L2) and Mitochondrial Import Receptor subunit TOM20 (TOM20) but not with the endoplasmic reticulum protein, calnexin, or the lysosomal protein, Lysosome-associated Membrane Glycoprotein 1 (LAMP1) in HeLa cells overexpressing PINK1. In agreement with their observation, many other studies have also reported that PINK1 co-localised with the mitochondria in mammalian cell cultures using immuno-fluorescence microscopy [169, 175, 180, 318]. However, the resolution of immuno-fluorescence microscopy is insufficient to determine the exact location of PINK1 in the mitochondria.

To overcome the limiting resolution of immuno-fluorescence microscopy, Silverstri et al and Muqit et al used immuno-gold electron microscopy to analyse the distribution of PINK1 in the mitochondria of HeLa and COS-7 cells overexpressing PINK1 [171, 178]. Although immuno-gold microscopy indicated that PINK1 resided on the cristae of these mitochondria, this technique was unable to establish whether PINK1 was facing the inter-membrane space (IMS) or the matrix. Nonetheless, it implied that PINK1 resided on the inner membrane (IM). To assess whether PINK1 is residing on the IMS-side of the IM, Silvestri et al treated the mitochondria isolated from HeLa cells overexpressing PINK1 with trypsin [171]. The trypsin treatment of these mitochondria failed to digest PINK1, substantiating that PINK1 was an intra-mitochondrial protein. Furthermore, the selective rupture of the OM of these mitochondria by detergent exposed the intra-mitochondrial PINK1 to digestion during trypsin treatment. In addition, the proteolytic profile of PINK1 in the mitochondria with ruptured OM was similar to that of an IMS-facing inner
membrane protein, Mitochondrial Import Inner Membrane Translocase Subunit TIM23 (TIM23) but not the matrix-facing IM protein, α-subunit of F\textsubscript{1}F\textsubscript{0} ATP synthase. Collectively, these observations indicated that PINK1 resided on the IMS-side of the IM.

Besides being an IMS-facing IM protein, later studies by Gandhi et al and Pridgeon et al reported that PINK1 was also an OM protein and IMS protein respectively [176, 180]. Using sub-mitochondrial fractionation, Gandhi et al showed that PINK1 was evenly distributed between the OM and IM fractions of the mitochondria isolated from rat brain [176]. Despite using a similar technique, Pridgeon et al observed that PINK1 resided in the IMS and IM fractions isolated from the mitochondria of PC12 cells overexpressing PINK1 [180]. Although these studies consistently reported that PINK1 is an IM protein, it is uncertain as to whether other observed sub-mitochondrial localisations of PINK1 were due to experimental artefacts arising from the experimental techniques, cell culture and/or overexpression system used in the studies.

In contradiction to previous studies, Zhou et al reported that PINK1 was not an IM protein [174]. Treating mitochondria isolated from SH-SY5Y neuroblastoma overexpressing PINK1 with increasing concentration of proteinase K, Zhou et al observed that PINK1 shared a similar proteolytic profile as a cytosol-facing OM protein, Apoptosis regulator Bcl-2 (BCL2) but not TIM23 (IM protein), Endonuclease G (IMS protein) or Heat Shock Protein 60 (matrix). Thus, this observation indicated that PINK1 was a cytosol-facing OM protein. Although there is no further direct experimental evidence indicating that PINK1 is a cytosol-facing OM protein, a recent study by Narendra et al indicated that functional PINK1 resided on the surface on the mitochondria [308]. As described in Chapter 1, they showed that the ectopic overexpression of chimeric PINK1 on the surface of the mitochondria could recruit Parkin to the mitochondria regardless of the mitochondrial potential to induce mitophagy in HeLa cells [308]. These observations implied that PINK1 cooperated with cytosolic Parkin on the surface of the mitochondria to mediate mitophagic function of PINK1. This substantiated that PINK1 resided on the cytosolic side of the OM as described by Zhou et al. Although these studies supported the findings that functional PINK1 resided
on the surface of the mitochondria, it is unclear as to why PINK1 bears a canonical MTS that resembles that of an intra-mitochondrial protein.

Several studies have shown that the MTS of PINK1 could target green (GFP) and cyan (CFP) fluorescence proteins into the mitochondria of cell cultures [171, 178, 181, 487]. Lin et al also showed that the Proteinase K treatment of the mitochondria isolated from HeLa cells overexpressing the first 150 residues of PINK1 fused to the GFP failed to digest the chimeric protein, indicating that the chimeric GFP was imported into the mitochondria by the first 150 residues of PINK1 [487]. In addition, Silvestri et al reported that the resulting intra-mitochondrial chimeric fluorescence protein was approximately 8kDa smaller than the unprocessed chimeric protein, corresponding to the loss of the MTS [171]. Thus, these studies demonstrated that the MTS of PINK1 functioned similarly as a canonical MTS as described in Chapter 2. Silvestri et al also performed an in vitro mitochondrial import assay to assess the capability of in vitro translated S^{35}-labelled unfolded PINK1 polypeptide to translocate into respiring mitochondria isolated from HeLa cells [171]. They observed that the S^{35}-labelled PINK1 polypeptide was imported into the mitochondria and insensitive to the Proteinase K treatment of these mitochondria. Furthermore, the imported PINK1 was processed to an approximately 55kDa form, corresponding to the loss of its MTS. Thus, it implied that PINK1 has a functional canonical MTS, which is subject to canonical mitochondrial processing after translocating into the mitochondria. Although the study by Zhou et al implied that PINK1 was a cytosol-facing IM protein, it is noteworthy that no other nuclear-encoded mitochondrial protein with a long MTS of up to 100 amino acids has been reported to reside on the cytosolic side of the OM of the mitochondria thus far. However, recent experimental evidence as described in Chapter 1 has indicated that PINK1 recruits the cytosolic Parkin to the surface of the mitochondria to mediate mitophagy, substantiating that PINK1 is a cytosol-facing OM protein. Thus, it is important to determine the bona fide distribution of PINK1 in the mitochondria to facilitate understanding of the role of PINK1 in mitophagy and PD pathogenesis.
To investigate the *bona fide* distribution of PINK1 in mitochondria, I used the following approaches to investigate the localisation of PINK1 in mitochondria:

- Determining the sub-mitochondrial location of *in vitro*-translated S\(^{35}\)-labelled nascent PINK1 in an *in vitro* mitochondrial import assay using respiring mitochondria isolated from yeast, mouse liver and brain.
- Determining the distribution of PINK1 in mitochondria isolated from *Sf9* insect cells overexpressing human PINK1.
- Investigating the N-terminal boundary of processed PINK1 derived from the mitochondria isolated from *Sf9* insect cells overexpressing human PINK1.
4.1 Experimental procedures

4.1.1 *In vitro* generation of $^{35}$S-labelled PINK1 polypeptide

*Bam* HI and *Eco* RI restriction sites were introduced to the 5’ and 3’ ends of the PINK1 cDNA construct during PCR using sense primer (5’ TATATATAGGATCCGCC ACCATGGCGGTGCACAGGCGCTGGGC 3’) and anti-sense primer (5’ TATATATAGAATTCTCACTTGTGTCGTCGTCGTCCTTTGTTAGTCCATGCCACAG GGCTGCCCTCCATGAGCAGAGG 3’). The resulting PCR product was digested with *Bam* HI and *Eco* RI restriction enzymes and inserted into pSP64 plasmid using molecular cloning protocols described by Sambrook *et al* [445]. pSP64 plasmid was a generous gift from Dr. Kip Gabriel (Monash University). The correct insertion and sequence of the PINK1 DNA construct in the resulting pSP64 plasmid was verified by restriction digestion and DNA sequencing.

The pSP64 plasmid containing PINK1 DNA construct was linearised using *Sca* I restriction enzyme and purified using phenol/chloroform extraction and ethanol precipitation as described by Sambrook *et al* [445]. To generate *in vitro* mRNA transcript of PINK1, 5 active units of SP6 polymerase (Promega) was added to a microfuge tube containing 5 μg of linearised pSP64 plasmid containing PINK1 DNA construct, 20 active units of SUPERase-In™ (Ambion Inc), 5 μM 7-methylguanosine cap GTP, 5 μM of ATP, CTP and TTP, 500 μM dithiothreitol, 40 mM Tris pH 7.9, 6 mM MgCl$_2$, 2 mM spermidine and 10 mM NaCl in a final volume of 50 μl (Figure 4.1). The reaction mixture was incubated at 40°C for 15 minutes. 5 μl of 5 mM GTP was then added to the reaction mixture and incubated at 40°C for 90 minutes to allow the extension of the PINK1 transcript.

To generate $^{35}$S-labelled PINK1 polypeptides, 5 μl of the *in vitro* transcription reaction was added to a microfuge tube containing 50 μl of rabbit reticulocyte lysate, 1 μM isoleucine, 1 μM leucine, 1 μM lysine, 1 μM phenylalanine, 1 μM threonine, 1 μM
tryptophan, 1 μM valine, 1 μM alanine, 1 μM asparagine, 1 μM aspartic acid, 1 μM cysteine, 1 μM glutamic acid, 1 μM glutamine, 1 μM glycine, 1 μM proline, 1 μM serine, 1 μM tyrosine, 1 μM arginine and 1 μM histidine, 1 μM S35-labelled methionine, 20 active units of SUPERase-In™ and incubated at 30°C for 40 minutes (Figure 4.1). The 2 μl of the resulting reaction was resolved by SDS-PAGE and exposed to Phosphorimager

4.1.2 In vitro mitochondrial import assay

Mitochondria used in in vitro mitochondrial import assay were isolated from mouse brain and liver as described in Section 3.1.14. Yeast mitochondria were isolated from Saccharomyces cerevisiae and were generously provided by Dr. Kip Gabriel (Monash University). The in vitro mitochondrial import assay was performed by adding 5 μl of the reticulocyte lysate containing the synthesised S35-labelled PINK1 polypeptide to 50 μg mitochondria in 100 μl of import assay buffer (10 mM sodium succinate, 1 mM dithiothreitol, 200 mM HEPES pH 7.5, 250 mM sucrose, 5 mM MgCl2, 80 mM KCl, 2 mM ATP and 2 mM NADH) in the absence or the presence of 10 μM antimycin A, 20 μM oligomycin and 2 μM valinomycin. The assay was carried out at 30°C for 30 minutes and stopped by the addition of 10 μM antimycin A, 20 μM oligomycin and 2 μM valinomycin. To determine the sub-mitochondrial location of the imported S35-labelled PINK1 protein, the parallel stopped import reactions were subjected to several treatments. To degrade OM proteins, 2.5 μg Proteinase K was added to the stopped import reaction (Figure 4.1). To degrade OM and IMS proteins, 600 μl of 10 mM HEPES and 2.5 μg Proteinase K were added to another stopped reaction and vortexed to shear the OM of the mitochondria (Figure 4.1). To degrade mitochondrial proteins, 0.05 % Triton X-100 and 2.5 μg Proteinase K were added to the stopped import reaction to solubilise the mitochondrial membranes (Figure 4.1). The Proteinase K treatments were performed on ice for 10 minutes. 2 mM phenylmethylsulfonylflouride was added to each reaction to stop further digestion by Proteinase K. The mitochondria were collected by centrifuging at 10000 x g for 10 minutes at 4°C. The mitochondrial pellets were washed twice with mitochondrial isolation buffer (Section 3.1.14). The mitochondrial pellets were
resuspended in SDS-PAGE sample loading buffer and resolved by SDS-PAGE (Figure 4.1). The S\textsuperscript{35}-labelled PINK1 proteins were detected by autoradiogram using Phosphorimager.

4.1.3 Generation of recombinant PINK1-GFP fusion protein in \textit{Saccharomyces cerevisiae}

\textit{Bam} HI and \textit{Eco} RI restriction sites were introduced to the 5’ and 3’ ends of the PINK1 cDNA construct during PCR using sense primer (5’ TATATATAGGATCCGCCACCATGGCGGTGCGACAGGCGCTGGGC 3’) and anti-sense primer (5’ TATATATAAGAATTCCTTCTGTCGTCTCGTCGTCGTCCTTTGATGCATGC CCGACAGGGCTGCCCTCCATGAGCAGAGG 3’). Due to the anti-sense primer, the resulting PINK1 PCR product does not have a stop codon. In addition, the anti-sense primer also introduced a serine-glycine linker to fuse the C-terminal tail of PINK1 to a GFP encoded in pGFP-C-Fus plasmid. The pGFP-C-Fus plasmid was a generous gift from Dr. Kip Gabriel (Monash University), which contains an essential yeast gene \textit{URA3}. The resulting PCR product was digested with \textit{Bam} HI and \textit{Eco} RI restriction enzymes and inserted into pGFP-C-Fus plasmid using molecular cloning protocols described by Sambrook \textit{et al} [445]. The correct insertion and sequence of the PINK1 DNA construct in the resulting pGFP-C-Fus plasmid was verified by restriction digestion and DNA sequencing.

\textit{Saccharomyces cerevisiae} \textit{URA3} strain SY4741 was generously provided by Dr. Kip Gabriel (Monash University). The yeast was cultured in 5ml of yeast peptone dextrose (YPD) media (1 % yeast extract, 2 % peptone and 2 % glucose) overnight at 30°C. The yeast was harvested by centrifuging at 3000 x g for 1min. The yeast pellet was washed twice with sterile distilled water and was then resuspended in 1ml of sterile 100 mM lithium acetate. The yeast was spun down by centrifuging at 3000 x g for 1 minute and resuspended in 500 \textmu l of sterile 100 mM lithium acetate. The suspension was incubated for 5 minutes at room temperature. 50 \textmu l of the suspension was added to 350 \textmu l of
transforming solution (70 % PEG 3600, 100 mM lithium acetate, 286 µg/ml salmon sperm carrier) containing 1.5 µg pGFP-C-FUS plasmid containing PINK1 DNA construct. The mixture was vortexed and incubated at 30°C for 30 minutes. The mixture was then incubated at 42°C for 30 minutes to induce heat shock. The yeast was harvested by centrifuging at 3000 x g for 1 minute and plated onto *URA3* selective synthetic defined (SD) agar plate (0.67 % yeast nitrogen base, 2 % glucose and 2 % agar, pH 5.5) supplemented with 20 mg/L of each of the following: adenine sulphate, L-tryptophan, L-histidine, L-arginine, L-methionine, L-tyrosine, L-leucine, L-isoleucine, L-lysine, L-phenylalanine, L-aspartic acid, L-glutamic acid, L-valine, L-threonine and L-serine. The agar plate was incubated at 30°C for two days. Several transformed colonies were picked and cultured in 10 ml of the *URA3* selective SD media at 30°C overnight. The cells were harvested on the following day and the expression of recombinant PINK1 protein was analysed by Western blotting.

### 4.1.4 Proteinase K sensitivity assay

200 ml of *Sf9* cells (1 x 10⁶ cells/ml) were infected with the recombinant PINK1 baculovirus expression vector carrying DNA construct encoding *Sf9*-codon optimised full-length PINK1 with a C-terminal Myc and 12x-His tandem tag at a multiplicity of infection ≥2. At 50 hours after infection, the cells were harvested by centrifuging at 1000 x g for 5 minutes at 4°C. The cell pellet was washed with 50 ml of PBS and centrifuged at 1000 x g for 5 minutes at 4°C again. All subsequent protein purification procedures were performed at 4°C.

5ml of CHM (Section 3.1.22) was used to resuspend the cell pellet before incubation for 5 minutes. The mixture was passed through a 26 G syringe twice and 5 ml of 2x osmotic buffer (section 3.1.22) was added to neutralise the hypotonic environment. The homogenate was centrifuged at 600 x g for 15 minutes to remove nuclei fraction. The resulting supernatant was centrifuged at 6000 x g for 10 minutes to isolate the mitochondria. The mitochondrial pellet was washed with 10 ml of mitochondrial
isolation buffer (Section 3.1.14) and centrifuged at 6000 x g for 10 minutes to collect the mitochondrial pellet.

To determine the sub-mitochondrial location of the recombinant PINK1 protein, the isolated mitochondria were subjected to several treatments similar to the in vitro mitochondrial import assay (Figure 4.1). To degrade OM proteins, 2.5 μg Proteinase K was added to 50 μg mitochondria resuspended in 100 μl of osmotic buffer (20 mM HEPES pH 6.7, 220 mM mannitol, 70 mM sucrose, 150 mM MgCl₂ and 10 mM KCl). To degrade OM and IMS proteins, 600 μl of 10 mM HEPES and 2.5 μg Proteinase K were added to 50 μg mitochondria resuspended in 100 μl of osmotic buffer. To degrade mitochondrial proteins, 0.05 % Triton X-100 and 2.5 μg Proteinase K were added to 50 μg mitochondria resuspended in 100 μl of osmotic buffer. The Proteinase K treatments were performed on ice for 10 minutes. 2 mM phenylmethylsulfonylflouride was added to stop further digestion by Proteinase K. The mitochondria were collected by centrifuging at 10,000 x g for 10 minutes at 4°C. The mitochondrial pellets were washed twice with mitochondrial isolation buffer and subjected to Western blotting.

4.1.5 Mass spectrometry of Sf9-generated mitochondrial recombinant PINK1 protein

Mitochondria were isolated from a 2 L culture of Sf9 cells overexpressing full-length PINK1 as described in the above section. The mitochondrial pellet was solubilised in 10 ml of guanidinium lysis buffer (Section 3.1.21). The lysate was centrifuged at 3000 x g for 15 minutes to remove insoluble debris. The supernatant was applied to a Ni-NTA column pre-equilibrated with guanidinium lysis buffer. The non-specifically bound proteins were removed by washing with denaturing binding buffer and denaturing wash buffer (section 3.1.21). Proteins specifically bound to the column were eluted with 20 ml of denaturing elution buffer (8 M urea, 500 mM NaCl, and 20 mM NaH₂PO₄ pH4.0). The elution was concentrated to approximate final volume of 300 μl using Amicon Ultra-
15 Centrifugal Filter (Millipore) by centrifugation. The concentrate was then resolved by SDS-PAGE.

Protein bands that were smaller than 62 kDa were excised and washed with MS Washing solution (Section 3.1.24) twice for 15 minutes. The washing solution was removed and 200 µl of 100% acetonitrile was added to dehydrate the gel piece. The solution was discarded and the gel piece was air-dried. 200 µl of 50 mM NH₄HCO₃ containing 10 mM dithiothreitol was added to the gel piece and incubated for 30 minutes. The solution was discarded and 200 µl of 50 mM NH₄HCO₃ containing 20 mM iodoacetamide was added to alkylate the proteins. The reaction mixture was incubated at room temperature in the dark for 1 hour. The solution was discarded and the gel piece was washed twice with 200 µl of 100 mM NH₄HCO₃ containing 50% acetonitrile for 15 minutes. 200 µl of 100% acetonitrile was added to the gel piece to dehydrate the gel. The acetonitrile was removed and the gel piece was air-dried. The gel piece was then rehydrated in 50 µl of 50 mM NH₄HCO₃ containing 0.8 µg sequencing grade trypsin (Promega) and incubated overnight at 37°C. The tryptic peptides-containing solution was collected on the following day. 50 µl of 50% acetonitrile in 1% formic acid was added to the gel piece and sonicated for 30 minutes to extract the remaining peptides residing in the gel piece. Both peptide solutions were pooled and evaporated to approximately 15 µl. The peptide solution was then subjected to nanoCHIP 3D Ion Trap mass spectrometry.

4.1.6 Phosphorylation of Parkin and TRAP1 using mitochondria isolated from Sf9 cells overexpressing recombinant PINK1 protein

Mitochondria were isolated from 200 ml cultures of Sf9 cells overexpressing full-length PINK1 and the PINK1 mutant bearing kinase inactivating D362A mutation as described in the aforementioned section. 50 µg of mitochondria was resuspended in 20 µl of in vitro assay buffer (Section 3.1.22). 20 µl of purified recombinant Parkin protein or recombinant TRAP1 protein was added to the suspension and incubated at 30°C for 30
minutes. The reactions were stopped by the addition of 10 μl of 5x SDS-PAGE sample loading buffer and heated at 95°C for 5 minutes. The resulting reactions were resolved by SDS-PAGE and exposed to Phosphorimager.
4.2 Results

4.2.1 In vitro mitochondrial import assay

In vitro mitochondrial import assay is one of the common approaches used to determine the sub-mitochondrial location of a mitochondrial protein [488-491]. In addition, it measures the mitochondrial import efficiency of the mitochondrial protein. Briefly, the sub-mitochondrial localisation and import efficiency of a protein are assessed by incubating an unfolded in vitro translated S$^{35}$-labelled mitochondrial precursor polypeptide with respiring mitochondria. By using mitochondria derived from respective available yeast (Saccharomyces cerevisiae) mutants, in vitro mitochondrial import assay also allows the identification of the mitochondrial peptidases and proteins involved in importing and processing the S$^{35}$-labelled mitochondrial precursor polypeptide. It is noteworthy that most of the loss-of-function mutations in the proteins involved in mitochondrial import or processing in these yeast mutants are often lethal in metazoans and even in cell cultures. Thus, the usage of yeast mitochondria in the assay provides valuable tools for identifying the mitochondrial peptidases and proteins that are involved in importing and processing PINK1.

Initial in vitro translation of S$^{35}$-labelled PINK1 was thought to be unsuccessful, as a resolved S$^{35}$-labelled protein band of 62kDa corresponding to the intact PINK1 protein was not detected in the autoradiogram of the SDS-PAGE gel (Figure 4.2). However, S$^{35}$-labelled protein aggregates were observed in the wells of the stacking portion of the SDS-PAGE gel (Figure 4.2). As early studies on PINK1 indicate that PINK1 is a component of aggresome in PD and is inherently insoluble, the observed S$^{35}$-labelled protein aggregates might be aggregated PINK1 caused by rapid cooling during the loading to SDS-PAGE gel after the boiling treatment to break the di-sulfide bonds in the loading sample [171, 172, 176, 178]. To overcome the suspected protein aggregation due to rapid and drastic cooling, the loading sample of the rabbit reticulocyte lysate containing the translated S$^{35}$-labelled PINK1 was heated to 70°C and cooled down slowly prior to the
loading to SDS-PAGE. The occlusion of the boiling treatment indeed overcame the apparent protein aggregation of $^{35}$S-labelled PINK1, as the SDS-PAGE could resolve the translated product as a 62kDa protein band (Figure 4.2). Thus, it indicated that the observed protein aggregate was indeed $^{35}$S-labelled PINK1 and the boiling treatment of the loading sample was the cause of the protein aggregation of $^{35}$S-labelled PINK1. Hence, the $^{35}$S-labelled PINK1 is unlikely to be an insoluble protein aggregate after the in vitro translation reaction and therefore it is suitable for in vitro mitochondrial import assay.

Although Silvestri et al previously showed that PINK1 was an intra-mitochondrial protein using in vitro mitochondrial assay, they did not determine the location of the imported PINK1 in the mitochondria. In addition, the mitochondria used in their assay were derived from HeLa cells, which may contribute to the different sub-mitochondrial localisation of PINK1 observed in SH-SY5Y neuroblastoma by Zhou et al as mentioned earlier. Thus, it is uncertain whether the discrepancy of the reported sub-mitochondrial localisations of PINK1 in these studies is due to possible artefacts from cell cultures. In my study, in vitro mitochondrial import assay was initially carried out using mitochondria isolated from Saccharomyces cerevisiae because of the available array of yeast mutants that could be used for identifying the mitochondrial peptidases and the proteins involved in importing and processing PINK1. However, the intra-mitochondrial localisation of $^{35}$S-labelled PINK1 was not observed in the in vitro mitochondrial import assay using yeast mitochondria (Figure 4.3). Despite the final washing step in the assay, $^{35}$S-labelled PINK1 was still detected in the in vitro mitochondrial import reaction that was not treated with Proteinase K (Figure 4.3). Thus, it implied that the observed $^{35}$S-labelled PINK1 was attached to the surface of the yeast mitochondria. This observation is in agreement with the study by Zhou et al that reported that PINK1 was a cytosol-facing OM protein [174]. However, it is plausible that $^{35}$S-labelled PINK1 was unable to translocate into the yeast mitochondria due to missing unknown mammalian import factors. Relevant to this, a helicobacter toxin subunit p55 can only be imported into mammalian mitochondria but not yeast mitochondria [492].
To avoid possible limitations and artefacts posed by the mitochondria deriving from yeast and mammalian cell cultures, I investigated whether S\textsuperscript{35}-labelled PINK1 could be imported into the mitochondria isolated from mouse liver. It is worth mentioning that liver mitochondria are a common source of mammalian mitochondria used for in vitro mitochondrial import assay [488-491]. Similar to the in vitro mitochondrial import assay using yeast mitochondria, S\textsuperscript{35}-labelled PINK1 was found to attach to the surface of the liver mitochondria and was not imported into the mitochondria (Figure 4.4). As the liver has a slightly lower expression level of PINK1 than the brain, it is plausible that the liver mitochondria are not as well equipped with import factors that are required for the mitochondrial import of PINK1 as the brain mitochondria [188]. To examine the validity of this notion, the in vitro mitochondrial import assay was repeated with mitochondria isolated from mouse brain. Similar to the findings made with liver mitochondria, S\textsuperscript{35}-labelled PINK1 was also found attached to the surface of the brain mitochondria (Figure 4.5). Therefore, it suggests that the observed association of PINK1 with the cytosolic side of the OM in these in vitro mitochondrial import assays was not tissue-specific or even organism-specific. In agreement with the study by Zhou et al, these observations support the previous report that PINK1 resides on the surface of the mitochondria [174].

### 4.2.2 Live mitochondrial localisation of PINK1 in yeast.

As the observation made by in vitro mitochondrial import assay using yeast mitochondria was identical to that of mouse mitochondria, I attempted to express recombinant PINK1 fused to a GFP at its C-terminal in yeast to study the real-time mitochondrial translocation behaviour of PINK1 in live yeast. As mentioned earlier, the availability of the yeast mutants allows investigation of the unusual mitochondrial import behaviour of PINK1 in a manner which otherwise is not possible in mammalian cell cultures or animals. The PINK1-GFP chimeric DNA construct was constructed in a plasmid called pGFP-C-Fus, which contains an essential yeast gene coding for orotidine 5-phosphate decarboxylase (\textit{URA3}). Thus, this plasmid was transformed into \textit{URA3}\textsuperscript{-} yeast mutant to express the chimeric PINK1 protein. Despite the \textit{URA3}\textsuperscript{-} phenotype of the
yeast mutant being complemented after the transformation, the expression of PINK1 was not detected in these transformants (Figure 4.6). Thus, the investigation of the mitochondrial translocation of PINK1 in live yeast was not pursued further in my study. Furthermore, it is uncertain as to whether PINK1 expressed in yeast could behave physiologically and similarly to its *bona fide* mammalian counterparts.

### 4.2.3 Sub-mitochondrial localisation of PINK1 in *Sf9* cells

As the yeast overexpression system was not favourable for expression of PINK1 in my study, I then endeavoured to express recombinant PINK1 in mammalian cell cultures to study the sub-mitochondrial localisation of PINK1. However, the expression level of PINK1 in mammalian cell cultures was not ample for biochemical analysis of the sub-mitochondrial localisation of PINK1, as described in Chapter 3. Fortunately, the expression level of recombinant PINK1 in *Sf9* cells was ample for Western blot analysis after using *Sf9*-codon optimised human PINK1 construct, as described in chapter 3. Thus, the sub-mitochondrial localisation study of PINK1 was carried out using *Sf9* cells overexpressing PINK1.

Using differential centrifugation, the mitochondria were isolated from *Sf9* cells overexpressing PINK1 and subjected to treatments similar to those in *in vitro* mitochondrial assay to determine the sub-mitochondrial location of PINK1 in these mitochondria. Proteinase K treatment of the intact mitochondria and mitoplasts of the *Sf9* cells overexpressing PINK1 resulted in the digestion of the recombinant PINK1, indicating that the recombinant PINK1 was not residing in the mitochondria (Figure 4.7). In the absence of Proteinase K treatment, PINK1 was detected in intact form of approximately 62kDa and processed form of 55kDa form (Figure 4.7). Thus, these observations were similar to the results of *in vitro* mitochondrial import assay, which indicated that PINK1 resided on the surface of the mitochondria (Figures 4.3 to 4.5). In agreement with this notion, the proteolytic profile of PINK1 was similar to that of the OM transmembrane protein, Voltage-dependent anion-selective Channel Protein 1.
(VDAC1) in the Proteinase K-treated mitochondria and mitoplasts (Figures 4.7). Although α-tubulin is not a mitochondrial protein, α-tubulin resides on the surface of the mitochondria via its association with VDAC1, dynein and kinesin motors [112, 493-500]. The mitoplast treatment of the Sf9 mitochondria resulted in the drastic loss of the more loosely-attached α-tubulin but not the OM transmembrane protein, VDAC1 (Figures 4.7). The retention of VDAC1 after the mitoplast treatment was likely due to the remaining ruptured OM attached to the mitoplast. In agreement with this notion, the Proteinase K treatment of the mitoplasts resulted in the digestion of VDAC1. Similar to VDAC1, the immuno-reactivity of PINK1 was decreased partially during mitoplast treatment (Figure 4.7). Thus, these observations suggested that the recombinant PINK1 protein was tightly associated to the OM and the association was likely to be mediated by its putative transmembrane domain, similar to VDAC1 (Figure 4.7).

As most of the cytochrome c proteins were not lost during the mitoplast treatment, it indicated that the rest of the cytochrome c proteins were still associated to the IM (Figure 4.7). Thus, the isolated Sf9 mitochondria were likely to be healthy and the observed OM localisation of PINK1 was unlikely to be caused by impaired mitochondrial import machinery resulting from compromised/apoptotic mitochondria. Furthermore, the observed OM localisation of PINK1 is consistent to the results of in vitro mitochondrial import assay and the findings reported by Zhou et al. (Figures 4.3 to 4.5) [174].

4.2.4 Determining the N-terminal boundary of processed PINK1

Although PINK1 does not seem to translocate into mitochondria, the processing of PINK1 was still observed in Sf9 mitochondria (Figure 4.7). Identification of the N-terminal boundary of processed PINK1 will facilitate the design of the recombinant PINK1 protein corresponding to the processed PINK1 form for biochemical characterisation in future. As PINK1 is not translocated into the mitochondria, it is unlikely that PINK1 is processed by the canonical mitochondrial peptidases as described in Chapter 2. Thus, identifying the N-terminal boundary of processed PINK1 will also
facilitate the identification of the protease responsible for processing PINK1 on the surface of the mitochondria.

Edman degradation was attempted to sequence the N-terminus of the processed form of PINK1 of approximately 55kDa purified from the mitochondria of Sf9 cells overexpressing PINK1. Unfortunately, the amount of processed PINK1 purified from the mitochondria isolated from 2L of Sf9 culture was insufficient for Edman degradation to be carried out. Thus, the processed PINK1 was trypsinised and analysed by mass spectrometry (Figure 4.8). Mass spectrometry detected six peptides and the most N-terminal peptide contains the conserved arginine 98 and the putative transmembrane domain of PINK1 (Figure 4.8). Although it is uncertain as to whether this is the actual N-terminal boundary of the processed PINK1, this observation indicates that the processed PINK1 in this experimental system still retains its transmembrane domain. It is worth mentioning that the theoretical molecular weight of the first 97 residues of PINK1 is 10kDa, which the loss of these residues would give rise to a 52kDa truncated PINK1 protein similar to the processed PINK1 protein in Sf9 cells. Collectively, the observations made in this study supports the finding that PINK1 resides on the surface of the mitochondria with its transmembrane domain inserted into the OM, as described by Zhou et al [174].

4.2.5 Investigating the phosphorylation of Parkin and TRAP1 by PINK1

As presented in Chapter 3, the phosphorylation of TRAP1 and Parkin by recombinant PINK1 protein was not observed. It is uncertain as to whether the lack of the phosphorylation of TRAP1 and Parkin was due to missing mitochondrial factors and/or residues prior to the kinase domain of PINK1. As the kinase domain of PINK1 is likely to reside on the surface of the mitochondria, mitochondria isolated from Sf9 cells overexpressing PINK1 were used to investigate whether recombinant Parkin and TRAP1 proteins could be phosphorylated by mitochondria-associated PINK1 from Sf9 cells.
However, the phosphorylation of Parkin and TRAP1 by the mitochondria containing wild-type PINK1 was not observed (Figure 4.9).

4.3 Discussion

In this study, experimental observations made in in vitro mitochondrial import assay and the mitochondria isolated from Sf9 cells overexpressing PINK1 both indicated that PINK1 resided on the surface of the mitochondria. In agreement with this notion, Narendra et al showed that the ectopic overexpression of the kinase domain and C-terminal tail of PINK1 on the surface of the mitochondria via the OM anchor domain of Optical Atrophy 3 protein (OPA3) could instigate the mitochondrial translocation of Parkin and in turn initiate mitophagy even in the absence of mitochondrial depolarisation, as described in Chapter 1 [308]. Thus, it indicated that the putative transmembrane domain of PINK1 might anchor the kinase domain and the C-terminal tail of PINK1 to the OM in a similar fashion as the PINK1 chimeras in their study. Supporting this notion, the putative transmembrane domain was retained in the processed form of recombinant PINK1 in the mitochondria of Sf9 cells overexpressing PINK1.

Although these observations indicate that PINK1 resides on the surface of the mitochondria to mediate mitophagy, it is unclear as to why PINK1 bears a MTS similar to that of a canonical intra-mitochondrial protein. In particular, previous studies have demonstrated that the MTS of PINK1 could direct cytosolic fluorescence proteins derived from Aequorea victoria into the mitochondria [171, 178, 181, 487]. In addition, early studies on PINK1 showed that PINK1 was an IM protein using multiple experimental approaches [171, 176, 180]. It is worth mentioning that some of these approaches were similar to the approaches used in my study, such as the in vitro mitochondrial import assay coupled with protease-treatment of isolated mitochondria. In contrast, my findings and other studies indicated that PINK1 was an OM protein [174, 487]. Thus, it is unclear as to why there is a discrepancy in the reports of the sub-mitochondrial location of PINK1 between different studies.
Matsuda et al postulated a hypothetical model that could explain the discrepancy of the sub-mitochondrial location of PINK1 observed in different studies (Figure 4.10) [307]. As the endogenous PINK1 was shown to accumulate in the mitochondria with compromised potential, Matsuda et al proposed that PINK1 is consistently imported into the mitochondria in a canonical manner similar to other intra-mitochondrial proteins and rapidly degraded by an unknown mitochondrial PINK1 protein turnover mechanism (Figure 4.10) [307]. It is worth mentioning again that the observed accumulation of PINK1 during mitochondrial depolarisation was not due to increased protein synthesis of PINK1, as the protein synthesis inhibitor, cycloheximide, had little effect on the accumulation of PINK1 in the depolarised mitochondria [308]. In agreement with this proposal, the activities of several mitochondrial proteases are dependent on mitochondrial respiration, supporting the view that normal rapid degradation of PINK1 could be due to the activities of energy-sensitive mitochondrial proteases in healthy mitochondria [315-317].

Several studies have shown that the MTS is essential for the mitochondrial translocation of PINK1; the removal of the MTS would abolish the mitochondrial translocation of PINK1 and in turn abrogate the recruitment of Parkin to the mitochondria and mitophagy [306-309, 314]. Later studies indicated that PINK1 might be indeed imported into the mitochondria, as demonstrated by early studies of PINK1 [174, 487]. These studies observed that the removal of the putative transmembrane domain of PINK1 resulted in the accumulation of PINK1 in the matrix, indicating that the putative transmembrane domain functions as a ‘stop-transfer’ signal. As mentioned in Chapter 2, the ‘stop-transfer’ signal prevents the import of IM and IMS proteins into the matrix. Indeed, early studies found that PINK1 resided in the IMS and IM but not matrix [171, 176, 180]. Furthermore, recent studies showed that the transmembrane domain of the IM-residing PINK1 was cleaved by the IM protease, PARL and this PARL-mediated cleavage was essential to the PINK1-mediated suppression of ROS production induced by rotenone [365, 366]. In addition, Deas et al demonstrated that PARL cleaves between A103 and F104 in the transmembrane domain of the IM-residing PINK1 [365]. Although
the mass spectrometry analysis of the processed PINK1 protein in this study shows that the processed OM-residing protein retains at least its conserved R98 and the putative transmembrane domain, the observed differential processing of PINK1 in the study by Deas et al and this study could be attributed to the different sub-mitochondrial localisation of the analysed PINK1 proteins. It is possible that the observed differential processing of PINK1 proteins may be due to the use of the insect cell overexpression system in this study. It is worth mentioning that an IMS protein, NADH cytochrome B5 reductase (MCR1) has a bipartite MTS, which targets the mitochondrial protein to the OM in mitochondria with compromised potential [501-503]. After the mitochondrial translocation, the IMS-residing MCR1 is processed by IM-peptidase but the OM-residing MCR1 remains unprocessed [501-503]. Thus, it is conceivable that the MTS of PINK1 may have similar bipartite properties as MCR1, which contributes to the observed different localisation and processing of PINK1 in S/9 cells.

As PINK1 is shown to accumulate on the surface of depolarised mitochondria to initiate Parkin-mediated mitophagy, I postulate that the putative transmembrane domain of PINK1 functions as a sensor to remove defective mitochondria with compromised potential via mitophagy (Figure 4.10) [308]. The putative transmembrane domain of PINK1 may function as a ‘difficult-to-import’ region of PINK1 that could only be imported into healthy mitochondria; whereby compromised mitochondria lack the potential gradient to drive the canonical mitochondrial import machinery to import PINK1 into the mitochondria (Figure 4.10). Thus, this hypothesis could explain the discrepancy on the reports of the sub-mitochondrial location of PINK1 by different studies, especially when cell cultures rely predominantly on glycolysis and often bear altered mitochondrial biology, known as the Warburg phenotype [504-508]. In agreement with this hypothesis, endogenous PINK1 was found to reside in both OM and IM of the mitochondria isolated from healthy rat brain but not in cell culture studies [171, 174, 176, 180, 487]. Hence, it provides a possible explanation on why Silvestri et al observed IM localisation of PINK1 in HeLa cells and my study observed OM localisation of PINK1 in S/9 cells [171]. However, it is unclear as to why the OM localisation of PINK1 was observed in my in vitro mitochondrial import assay while intra-mitochondrial
localisation of PINK1 was observed in the similar assay performed by Silvestri et al, despite the use of mitochondria isolated from mouse organs in this study while Silvestri et al used mitochondria isolated from HeLa cells [171]. Although the conditions used in our assays were similar, the discrepancy between our observations may be due to possible differential energetic state of the mitochondria used in our assays if the mitochondrial import of PINK1 was sensitive to mitochondrial potential. In particular, it is conceivable that the energy health of the isolated mitochondria is poorer than its non-isolated counterparts. Nevertheless, whether PINK1 could only be imported into healthy mitochondria and rapidly degraded by mitochondrial proteases in healthy mitochondria remains to be established and should be investigated in future studies to understand the role of PINK1 in PD pathogenesis.
Chapter 5 – Investigation of the specificity of the cell survival effect of PINK1 overexpression following apoptotic insults

The discovery of 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) as the causative agent of the chronic Parkinson’s disease (PD) symptoms of 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP) abusers in the 1980s indicated that the impairment of mitochondrial complex I (MC-I) might be a cause of idiopathic PD [19-37]. Consistent with this notion, systemic reduced MC-I activity was observed in idiopathic PD patients [38-43]. However, the cause of the systemic impairment of MC-I in idiopathic patients is still unknown.

Later studies on the role of impaired MC-I in PD reported that a systemic MC-I inhibitor, rotenone could cause preferential degeneration of Substantia nigra pars compacta (SNpc) dopaminergic neurons in rodents, in support of systemic inhibition of MC-I being involved in the pathogenesis of idiopathic PD [44-54]. It is worth mentioning that rotenone is a naturally occurring pesticide present in several tropical and subtropical plants and is used as poison bait for hunting fish by the Native Americans. Thus, several research groups postulated that prolonged exposure to environmental mitochondrial toxins, like rotenone, could be a main contributing cause of idiopathic PD [50, 56-62]. In agreement with this notion, Fleming et al detected an organochlorine insecticide, Dieldrin in the brain samples from 30% of their PD patients but not in their normal controls [63]. Several other population epidemiological studies have also indicated that occupational exposure to metals and pesticides increase the risk for PD [57-62]. However, it is unclear how these environmental factors could trigger mitochondrial dysfunction to cause PD and why dopaminergic neurons are particularly more vulnerable to these factors than other neurons and tissues.

Recent studies indicate that the PD symptoms of PINK1 familial PD are indistinguishable from that of idiopathic PD; these PINK1 familial PD patients suffer canonical PD-associated motor deficits, reduced MC-I activity, Lewy body pathology and
are responsive to Levodopa treatment [170, 189, 509]. As PINK1 is a mitochondrial protein kinase, understanding the role of PINK1 in the pathogenesis of PINK1 familial PD may help to identify the environmental factors that trigger the mitochondrial dysfunction in idiopathic PD. Several studies have observed that the expression of PINK1 promotes cell survival in cell cultures during stress induced by MC-I inhibitors, proteasomal inhibitors, oxidative stress and staurosporine [169, 175, 179, 180, 236]. However, the mechanism by which PINK1 promotes survival is still poorly understood.

To investigate the role of PINK1 in PD pathogenesis, I undertook the study of the specificity of the pro-survival function of PINK1 by treating BE2-M17 dopaminergic neuroblastoma with the following toxins in this study: Rotenone (Mitochondrial complex I inhibitor), antimycin A (Mitochondrial complex III inhibitor), sodium Azide (Mitochondrial complex IV inhibitor), oligomycin (Mitochondrial complex V inhibitor), valinomycin (Mitochondrial potential uncoupler), hydrogen peroxide (Oxidative stress agent), 6-Hydroxydopamine (PD-associated oxidative stress agent) and glutamate (Excitotoxic agent).
5.1 Experimental procedures

5.1.1 MTT viability assay

Each BE2-M17 cell line was seeded in a well of Clear 96-well Microtest™ Plate (BD Falcon™) at a cell density of $4 \times 10^5$ cells/well in 100 μl of culture media for each toxin treatment. To induce the overexpression of recombinant PINK1 protein, 1μg/ml doxycycline was added to the media during the seeding. At 4 hours post-seeding, the cells were checked for cell attachment under the microscope prior to toxin treatments. Desired concentrations of rotenone (Sigma-Aldrich), antimycin A (Sigma-Aldrich), oligomycin (Sigma-Aldrich), sodium azide (Sigma-Aldrich), valinomycin (Sigma-Aldrich), hydrogen peroxide (Sigma-Aldrich), 6-hydroxydopamine (Sigma-Aldrich) and glutamate (Sigma-Aldrich) were freshly prepared and added to each well in the dark. All subsequent procedures were performed in the dark. The culture plate was incubated at 37°C with 5 % CO₂ for 24 hours. 20 μl of 5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) in OptiMEM I media was added to each well and a ‘blank’ well containing 100 μl of OptiMEM I media. The plate was incubated at 37°C with 5 % CO₂ for 1 hour. The media was discarded and the plate was air-dried. 100 μl of DMSO was added to each well to solubilise the purple formazan produced by the mitochondrial reductase in viable cells. The culture plate was then incubated at 37°C for 10min to facilitate the solubilisation of the formazan. The absorbance of the formazan was measured at wavelength 570 nm by using FLUOstar Optima microtiter plate reader (BMG Technologies). The experiment was repeated 11 times.
5.1.2 Assessment of ROS level in stably transduced BE2-M17 cells overexpressing PINK1 using DHR123 and NAO fluorescent probes

Stably transduced BE2-M17 cell line was seeded in a 24-well culture plate at \(1 \times 10^4\) cells per well. To induce the overexpression of recombinant PINK1 protein, 1µg/ml doxycycline was added to the media during the seeding. At 4 hours post-seeding, the cells were checked for cell attachment under the microscope prior to the treatment with rotenone. A final concentration of 5 nM rotenone was added to each well in the dark to induce the inhibition of MC-I. All subsequent procedures were performed in the dark. The plate was incubated at 37°C with 5 % CO\(_2\) for 24 hours. A final concentration of 20 µM dihydrorhodamine 123 (DHR123) (maxima excitation: 507 nm and maxima emission: 529 nm) (Marker Gene Technology Inc) or 2 µg/ml of 10-N-Nonyl acridine orange (NAO) (maxima excitation: 495 nm and maxima emission: 519 nm) (Enzo Life Sciences) was added to each well and incubated at 37°C with 5 % CO\(_2\) for 30 minutes. The cells were then washed with fresh media and visualised by using Leica AF6000 fluorescence microscope with the GFP filter cube (excitation filter: 470 nm +/- 50 nm, dichromatic mirror: 500 nm and suppression filter: 525 nm +/- 25 nm).
5.2 Results

5.2.1 Investigating the pro-survival function of PINK1 during mitochondrial stress

To investigate the role of PINK1 in the pathogenesis of PINK1 familial PD, an array of mitochondrial toxins was used to treat BE2-M17 dopaminergic neuroblastoma cell lines generated in Chapter 3 to investigate whether PINK1 mediates its pro-survival effect against a specific mitochondrial stress. These mitochondrial toxins were rotenone, antimycin A, sodium azide, oligomycin and valinomycin, which specifically inhibit MC-I, MC-III, MC-IV, MC-V and depolarise mitochondrial potential respectively.

5.2.1.1 PINK1 promotes survival during mitochondrial complex I inhibition

The treatment of BE2-M17 cells with this array of mitochondrial toxins indicated that PINK1 might play a role in promoting survival during the impairment of MC-I (Figures 5.1 to 5.5). The pro-survival function of PINK1 was most prominent at the lowest tested concentration of rotenone. However, the pro-survival effect of PINK1 expression diminished with increasing concentration of rotenone.

At 5nM rotenone treatment, the cells overexpressing PINK1 were less susceptible to MC-I inhibition than those overexpressing the PINK1 mutant bearing kinase inactivating D362A mutation (PINK1 inactive mutant) (Figure 5.1). This observation is consistent with several studies that the kinase activity of PINK1 is required to promote cell survival during MC-I inhibition [237, 267, 510, 511]. On a similar note, the cells with down-regulated expression of PINK1 mediated by PINK1-targeting lentiviral shRNAAmir (PINK1 shRNA) were more susceptible to the 5nM rotenone treatment than the cells overexpressing PINK1 and the control cells (Figure 5.1). In agreement with this observation, several previous studies have shown that PINK1 deficiency increases the
susceptibility of cell cultures towards rotenone treatment [237, 510, 511]. However, the

cells overexpressing PINK1 inactive mutant were slightly less susceptible to MC-I

inhibition than the cells overexpressing PINK1 shRNA (Figure 5.1). This may be due to

the pro-survival effects of PINK1 overexpression that were not dependent on PINK1’s

kinase activity, such as protein interactions with PINK1’s pro-survival mediators, or non-

specific pro-survival effects from the overexpression system used in this study. Thus,

future study should include cells overexpressing an inert protein, such as GFP, as a

control to detect possible non-specific pro-survival effects from the overexpression

system used. Unexpectedly, the viability of the control cells was not significantly
different from the cells overexpressing PINK1 (Figure 5.1). As described in Chapter 3,

the recombinant PINK1 protein was degraded rapidly after 4 hours of induced

overexpression of PINK1 in the inducible cell line overexpressing PINK1. Thus, it is

uncertain as to whether these cells and the control cells shared similar protein level of

PINK1 during the 16 hour long rotenone treatment.

When treated with 60nM rotenone, the cells overexpressing PINK1 were less

susceptible to MC-I inhibition than the control cells (Figure 5.1). Hence, the endogenous

protein level of PINK1 in the control cells was probably insufficient to promote cell

survival as well as the cells overexpressing PINK1. In disagreement with the

observations made with 5nM rotenone treatment, the viability of the cells overexpressing

PINK1 inactive mutant was not statistically different from the control cells and the cells

overexpressing PINK1 (Figure 5.1). It is unclear whether this observation could be

attributed to the pro-survival effects of PINK1 overexpression that were not dependent on

PINK1’s kinase activity. Nonetheless, the cells overexpressing PINK1 shRNA were

more susceptible to MC-I inhibition than the cells overexpressing PINK1 (Figure 5.1).

Although the mean viability of the cells overexpressing PINK1 shRNA was lower than

the rest of the cell lines, the viability difference between these cells was not statistically

significant (Figure 5.1). Collectively, these observations suggest that the pro-survival

effect of PINK1 overexpression is weaker during the treatment with 60nM rotenone than

the treatment with 5nM rotenone, resulting in smaller viability differences between the

cell lines that are statistically less resolved (Figure 5.1). Nonetheless, the subtle
decreased viability of the cells overexpressing PINK1 shRNA may be due to insufficient knock-down expression of PINK1 by the lentiviral PINK-targeting shRNA mir as shown in Figure 3.24.

The pro-survival effect of PINK1 was the least pronounced at the highest tested concentration of rotenone. Although the cells overexpressing PINK1 were less susceptible to MC-I inhibition induced by 250nM rotenone than the cells overexpressing PINK1 shRNA, the viability of the cells overexpressing PINK1 was not significantly different from the rest of the treated cell lines (Figure 5.1). Thus, it suggests that the high concentration of 250nM rotenone has reached the threshold of the pro-survival effect of PINK1 overexpression. As the cells overexpressing PINK1 shRNA were consistently more susceptible to MC-I inhibition than the cells overexpressing PINK1 in each rotenone treatment, it suggests that the protein level of PINK1 in these cells is consistently lower than the cells overexpressing PINK1 (Figure 5.1). Therefore, this data supports that the proposal that the expression of PINK1 promotes cell survival during MC-I inhibition. It is unlikely that these observations were due to lentiviral transfection, as the viabilities of the cells with un-induced overexpression of PINK1 and the cells transduced with lentiviral non-silencing shRNA mir were similar to the control cells.

5.2.1.2 PINK1 does not promote cell survival during the inhibition of mitochondrial complex III, IV or V, or mitochondrial depolarisation

Unexpectedly, the overexpression of PINK1 did not promote the survival of BE2-M17 cells during mitochondrial stress induced by antimycin A, sodium azide, oligomycin or valinomycin (Figures 5.2 to 5.5). The overexpression of PINK1 inactive mutant and PINK1 shRNA also did not increase the mortality during mitochondrial stresses induced by these mitochondrial toxins (Figures 5.2 to 5.5). Hence, PINK1 does not seem to promote cell survival during mitochondrial stress induced by the impairments of MC-III, MC-IV or MC-V, or mitochondrial depolarisation under the conditions used in this study.
5.2.2 Investigating the pro-survival effect of PINK1 during oxidative stress

Studies by Kim et al and Pridgeon et al reported that the expression of PINK1 in dopaminergic SN4741 neuroblastoma and PC-12 neuroblastoma promoted cell survival during oxidative stress induced by hydrogen peroxide [180, 510]. However, the viability assay in the current work indicated that PINK1 did not promote cell survival during oxidative stress induced by hydrogen peroxide or the PD-associated 6-hydroxydopamine, even at low concentrations (Figures 5.6 and 5.7). Hence, PINK1 did not seem to promote cell survival during oxidative stress in BE2-M17 cells. It is plausible that the viability assay used in this study may not be sensitive and consistent enough to resolve the subtle viability differences between the different BE2-M17 cell lines used in this study. Nonetheless, the failure to observe the pro-survival effect of PINK1 expression during oxidative stress treatment in this study may be attributed to the different cell line and culture conditions used as compared to the aforementioned studies.

5.2.3 Investigating the pro-survival effect of PINK1 during excitotoxicity

To investigate whether PINK1 could promote cell survival during glutamate-induced excitotoxicity, the BE2-M17 cell lines were treated with 12mM and 25mM glutamate. Similar to the observations made with oxidative stress treatment, the expression of PINK1 did not influence the survival of these cell lines significantly during the exposure to glutamate (Figure 5.8). Thus, PINK1 may not promote cell survival during glutamate-induced excitotoxicity.
5.2.4 Investigating the pro-survival function of PINK1 during mitochondrial complex I inhibition

Besides compromising mitochondrial respiration, the impairment of MC-I is also known to cause toxic generation of reactive oxygen species (ROS) [120-131, 512-516]. As mentioned in Chapter 1, post-mortem studies have observed ROS damage in the midbrain of idiopathic PD patients, which includes lipid peroxidation, oxidative DNA damage and protein carbonylation [215-222]. To investigate whether PINK1 promotes cell survival by suppressing or sequestering the ROS generated by MC-I inhibition, a ROS-sensitive fluorescent probe, dihydrorhodamine 123 (DHR123) was used to assess the cellular ROS level in the cell lines overexpressing PINK1 and the PINK1 inactive mutant during the treatment with 5nM rotenone. DHR123 is a membrane-permeable dye, which reacts with peroxynitrite, hydrogen peroxide and hypochlorous acid to form membrane-impermeable fluorescent rhodamine 123 (R123) [517-524]. Thus, DHR123 is an ideal ROS probe to investigate whether PINK1 prevents oxidative damage induced by the inhibition of MC-I.

Although the overexpression of PINK1 promoted the survival of BE2-M17 cells, there was no gross difference in the fluorescent intensity of R123 between the cells with induced overexpression of PINK1 and endogenous expression of PINK1 during the treatment with 5nM rotenone (Figure 5.9). Thus, PINK1 may not promote cell survival during MC-I inhibition by suppressing ROS production or sequestering ROS. Nonetheless, it is possible that this assay was not sensitive enough to resolve the possible subtle difference in ROS level between cells overexpressing PINK1 and cells expressing endogenous level of PINK1.

Although the midbrain tissue from idiopathic patients had oxidative DNA damage, protein carbonylation and lipid peroxidation, PINK1 familial PD patients bearing G309D mutations displayed only elevated lipid peroxidation. As PINK1 is a mitochondrial protein kinase, it is plausible that the loss of PINK1’s kinase activity allowed the lipid peroxidation of the inner membrane (IM) of the mitochondria in these patients. It is
worth mentioning again that the inhibition of MC-I is known to generate toxic level of ROS due to the electron leakage from the respiratory complexes at the inner membrane [512]. As ROS are highly reactive and short-lived molecules, the oxidative damage caused by these molecules is likely to be maximal in the immediate vicinity of its production site. Thus, the ROS generated by the inhibited MC-I might be sequestered immediately by the oxidation-susceptible IM lipid, cardiolipin. Thus, these ROS may not be detected by DHR123 in the earlier experiment. To eliminate this possible problem, a cardiolipin-specific fluorescent probe called 10-N-Nonyl acridine orange (NAO) was used to assess whether the overexpression of PINK1 could prevent the lipid peroxidation of IM to promote cell survival during the treatment with 5nM rotenone. NAO has high affinity towards cardiolipin but low affinity towards oxidised cardiolipin [525-528]. Therefore, the decrease in NAO fluorescence intensity would suggest lipid peroxidation of the IM. However, there was no gross difference in the fluorescence intensity of NAO between cells overexpressing PINK1 and cells expressing endogenous level of PINK1 during the treatment with 5nM rotenone, indicating that PINK1 may not promote cell survival during MC-I inhibition by suppressing the oxidation of the IM of mitochondria (Figure 5.10). Nevertheless, it is also possible that this assay was not sufficiently sensitive to resolve possible subtle difference in the oxidation state of the IM between these cells. It is noteworthy that the inhibition of MC-III and V are known to generate toxic level of ROS [513, 529-536]. As this study did not observe pro-survival effect of PINK1 overexpression during the inhibition of MC-III and MC-V, these observations suggest that PINK1 does not promote cell survival by preventing oxidative damage.
5.3 Discussion

The treatments of BE2-M17 cell lines with the array of mitochondrial toxins, oxidative stress agents and excessive glutamate indicate that PINK1 only promotes cell survival during MC-I inhibition. As there is no difference detected in the cellular ROS level and the oxidation status of the IM between the cells overexpressing PINK1 and cells expressing endogenous level of PINK1, it is unlikely that PINK1 promotes cell survival during MC-I inhibition by suppressing ROS-mediated damage. Furthermore, PINK1 did not promote cell survival during the ROS-generating MC-III and MC-V inhibition in this study. As the expression of PINK1 did not influence the viability of these cell lines during the treatments with low concentrations of non-MC-I toxins, it also implies that PINK1 is at least not directly involved in suppressing apoptosis per se. It is worth mentioning that the levels of PINK1 protein in these cell lines were not assessed before and during the toxin treatments due to the difficulties in detecting low level of PINK1 expression using Western blot with the current available antibodies. Thus, it is unclear as to whether the observed lack of pro-survival effect of PINK1 overexpression in the cells treated with non-MC-I toxins was due to the rapid degradation of PINK1 protein in BE2-M17 cells described earlier in Chapter 3. Furthermore, the viability experiments designed in this study could only detect the pro-survival effect of PINK1 overexpression, if the induced overexpression of PINK1 protein was sustained during the toxin treatment or the pro-survival effect of PINK1 persisted after the degradation of PINK1 protein.

Recent studies by Morais et al and Hoepken et al reported that PINK1-deficient Drosophila flies and mice and PINK1 familial PD patients suffered reduced MC-I activity, indicating that PINK1 may regulate the activity of MC-I. Furthermore, the protein composition of the respiratory chain and the activities of other mitochondrial complexes were normal in these PINK1-deficient animals and PINK1 familial PD patients. Thus, PINK1 may promote the survival of the BE2-M17 cells overexpressing PINK1 and control cells during the treatment of 5nM rotenone by increasing MC-I activity. In agreement with this notion, the overexpression of PINK1 up-regulates the expression of dihydrolipoyl dehydrogenase in BE2-M17 cells during basal conditions as
reported in Chapter 3. It is worth mentioning again that dihydrolipoyl dehydrogenase catalyses the flavin-mediated oxidation of dihydrolipoate to generate NADH. Furthermore, patients suffering genetic mutations in this gene displayed motor deficits, such as hypotonia and ataxia [461]. As DLD also catalyses the reduction of nitrated DNA and protein adducts using dihydrolipoic acid, NADH, or ubiquinol as cofactor, the up-regulated expression of DLD may also reduce the oxidative damage caused by MC-I inhibition [460]. However, the up-regulated expression of DLD in BE2-M17 cells overexpressing PINK1 did not seem to promote cell survival during the inhibition of MC-III and V, which are known to generate toxic level of ROS [513, 529-536]. Thus, the overexpression of PINK1 may promote cell survival during MC-I inhibition by supplying more NADH to the inhibited MC-I to maintain mitochondrial respiration rather than reducing oxidative damage in BE2-M17 cells. This may explain why there was no difference in the measured cellular ROS level between the cells overexpressing PINK1 and the cells expressing endogenous level of PINK1 and why the pro-survival effect of PINK1 diminishes with increasing concentrations of rotenone. Future proteomic and microarray studies should be carried out to investigate whether PINK1 could regulate the activity of MC-I to promote cell survival during MC-I inhibition by regulating the expression of metabolic proteins to facilitate the identification of the PD-causative environmental factors.
Chapter 6 – Summary

6.1 The transmembrane domain of PINK1 may govern the mitochondrial residence of PINK1 in healthy and compromised mitochondria

As described in Chapter 1, recent studies show that PINK1 accumulates on the surface of depolarised mitochondria to recruit Parkin to mediate the clearance of these compromised mitochondria by mitophagy [304, 306-310, 314]. In addition, studies by Zhou et al and Narendra et al indicate that PINK1 resides on the surface of the mitochondria with its transmembrane domain inserted into the outer membrane (OM) to mediate its pro-mitophagy function [174, 308]. Consistent with these studies, the experimental observations made in the in vitro mitochondrial import assay and the mitochondria isolated from Sf9 cells overexpressing PINK1 in this study show that PINK1 indeed resides on the surface of the mitochondria. Furthermore, the mass spectrometry analysis of the proteolytically processed recombinant PINK1 protein isolated from the mitochondria of Sf9 cells overexpressing PINK1 indicates that the transmembrane domain of PINK1 is retained in the processed recombinant protein. As the removal of the transmembrane domain of PINK1 is shown to accumulate PINK1 in the mitochondrial matrix of cultured cells, I postulate that the transmembrane domain of PINK1 functions as a sensor to remove defective mitochondria with compromised mitochondrial potential via mitophagy (Figure 4.10) [174, 487]. The transmembrane domain may function as a switch that governs the abundance of PINK1 on the mitochondria, which usually permits the mitochondrial import and the subsequent degradation of PINK1 protein in healthy mitochondria (Figure 4.10). However, the compromised mitochondria lack the mitochondrial potential gradient to drive the canonical mitochondrial import machinery to import PINK1 protein into the mitochondria for degradation (Figure 4.10). Consequently, PINK1 protein was able to accumulate on the surface of the mitochondria to recruit Parkin for the mitophagy of the defective mitochondria (Figure 4.10). In agreement with this postulation, recent studies
show that PINK1 is indeed imported into healthy mitochondria and the transmembrane domain of the translocated IM-residing PINK1 is cleaved by the IM protease, PARL [365, 366]. Furthermore, Narendra et al reported that the protein synthesis inhibitor, cycloheximide, had little influence on the accumulation of PINK1 on the surface of the depolarised mitochondria, indicating that the accumulation of PINK1 on the depolarised mitochondria was caused by decreased turnover of PINK1 protein [308]. It is worth mentioning again that the bipartite MTS of MCR1 is known to target the nascent MCR1 polypeptide to the IMS of healthy mitochondria and the OM of mitochondria with compromised potential [501-503]. Thus, it is conceivable that the MTS of PINK1 has similar potential-sensitive bipartite properties as MCR1.

6.2 PINK1 may regulate the expression of mitochondria-encoded proteins to initiate the mitophagy of compromised mitochondria

The involvement of PINK1 in mitophagy is further substantiated by the preliminary 2-dimensional difference in gel electrophoresis (2D-DIGE) analysis of BE2-M17 dopaminergic neuroblastoma cells overexpressing PINK1 reported in this thesis. The 2D-DIGE analysis revealed that the protein expression of an autophagy-associated mitochondrial translation regulatory protein, Leucine-Rich Pentatricopeptide-Repeat Motif Containing Protein (LRPPRC) was down-regulated during the overexpression of PINK1 in BE2-M17 cells. As LRPPRC is a Parkin and PINK1-binding protein and its depletion in HeLa cells activates LC3-mediated autophagy, it is plausible that PINK1 recruits Parkin to depolarised mitochondria to facilitate proteasomal degradation of LRPPRC and/or to prevent the mitochondrial import of LRPPRC into the matrix (Figure 6.1) [469-471]. Although the mechanism by which LRPPRC mediates its autophagic function is unknown, the down-regulated expression of LRPPRC by PINK1 overexpression is likely to influence the mitochondrial expression of the mitochondria-encoded proteins involved in the initiation of mitophagy. Besides LRPPRC, the protein level of a mitochondrial transcription regulatory protein, LON Protease Homolog (LONP1) was also down-regulated during the overexpression of PINK1 in BE2-M17
As LONP1 is known to regulate the mitochondrial transcription of the mitochondria-encoded proteins by digesting the transcription regulatory proteins, the down-regulated expression of LONP1 may act with LRPPRC to alter the expression of the mitochondria-encoded proteins to prepare the mitochondrion for mitophagy [477, 478]. Thus, future studies should investigate the mitochondria-encoded proteins regulated by LRPPRC and LONP1 to understand how PINK1 interplays with Parkin, LRPPRC and LONP1 to facilitate the clearance of potential-compromised mitochondria by mitophagy.

6.3 PINK1 may promote cell survival by promoting the activity of mitochondrial complex I

Besides mitophagy, the investigation of the specificity of the cell survival effect of PINK1 overexpression using an array of mitochondrial toxins and oxidative stress agents shows that PINK1 only promotes the survival of BE2-M17 cells during the inhibition of mitochondrial complex I (MC-I) under the conditions used in this study. The 2D-DIGE analysis of BE2-M17 cells revealed that the protein expression of Dihydrolipoyl Dehydrogenase (DLD) was up-regulated during the overexpression of PINK1. Thus, PINK1 may promote the survival of BE2-M17 cells during the inhibition of MC-I by increasing the production of NADH to compensate for the impaired activity of MC-I to maintain healthy mitochondrial respiration. Although DLD is also known to repair DNA and protein adducts damaged by reactive nitrogen species, the overexpression of PINK1 did not promote the survival of BE2-M17 cells during the oxidative stress induced by the mitochondrial complex III and mitochondrial complex V inhibitors or the oxidative stress agents, 6-hydroxydopamine and hydrogen peroxide [460]. In addition, the expression of LONP1 is down-regulated in BE2-M17 cells overexpressing PINK1. It is worth mentioning that LONP1 is known to degrade oxidised and mis-folded mitochondrial proteins in the matrix [472-476]. Thus, the down-regulated expression of LONP1 in the cells overexpressing PINK1 indicates that LONP1 is not involved in the degradation of the matrix proteins damaged by the inhibition of MC-I. As the protease involved in the
turnover of DLD is unknown, it is plausible that LONP1 regulates the protein level of DLD and its depletion could result in the accumulation of DLD in the matrix. Thus, future comparative analysis of the mitochondrial proteome of BE2-M17 cells overexpressing PINK1 using 2D-DIGE should use narrower pH range to facilitate the identification of the substrates and downstream mediators of PINK1 to investigate the involvement of MC-I in the clearance of potential compromised mitochondria by mitophagy.

6.4 Summary

In summary, my study provides experimental evidence indicating that PINK1 resides on the surface of the mitochondria and retains its transmembrane domain. Although the investigation of putative protein substrates of PINK1 using active recombinant PINK1 enzyme has proven difficult, the search for putative protein substrates of PINK1 using Sf9-codon optimised recombinant PINK1 protein kinase domain is still ongoing in our laboratories. I am hopeful that the improved expression level of the recombinant PINK1 enzyme by the usage of Sf9-optimised codons will allow us to overcome the technical challenges of their purification for future investigation of protein substrates of PINK1 and biochemical characterisation. Preliminary proteomic analysis of the effects of PINK1 overexpression in BE2-M17 dopaminergic neuroblastoma identified several mitochondrial proteins that are up-regulated and down-regulated upon the overexpression of PINK1. The identification of these mitochondrial proteins provides a conceptual framework for future investigation into the understanding of the role of PINK1 in the pathogenesis of PD.
7 References


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Figure 2.1. Schematic diagram of the protein domains of PINK1 and the distribution of PD-associated missense mutations in PINK1

PINK1 consists of an undetermined mitochondrial targeting sequence (MTS) of up to 98 amino acids, a putative α-helical transmembrane domain (TM) of 12 amino acids, a protein serine serine/threonine kinase of 357 amino acids and a C-terminal tail of 69 amino acids with possible roles in regulating the kinase activity or/and the recruitment of protein substrate. There are at least 68 missense mutations identified in homozygous recessive familial PINK1 PD pedigrees. The majority of the mutations reside in the protein kinase domain of PINK1, followed by the mitochondrial targeting sequence of PINK1.
7 missense mutations
MTS
	number

2 missense mutations
TM

52 missense mutations
Kinase

4 missense mutations
Tail

1 99 111 156 512 581
Figure 2.2. Models depicting the mitochondrial translocation of mitochondrial protein precursors and their proteolytic processing by mitochondrial peptidases

A) **Entry into the mitochondrion.** As the mitochondrial precursor protein is translated by the ribosome at the endoplasmic reticulum/cytosol, chaperone members of heat shock protein 70 and 90 families bind to the newly translated polypeptide to prevent protein folding. This unfolded mitochondrial precursor is translocated to the Translocase of Outer Membrane (TOM) complex via a poorly understood mechanism, which resides on the outer membrane (OM) of the mitochondrion. Positive charges on the mitochondrial targeting sequence (MTS) of the mitochondrial precursor interact with negatively charged acidic residues on the TOM complex to initiate translocation of the unfolded mitochondrial precursor into the mitochondrion. In some cases, mitochondria reside juxtaposed to the translating ribosome and the mitochondrial precursor is simultaneously imported into the mitochondria while it is translated.

B) **Translocation of matrix and inner membrane precursors.** After translocating through the OM, the N-terminus of the MTS of matrix and inner membrane (IM) precursors is targeted to the Translocase of Inner Membrane (TIM) complex. The mitochondrial potential provides the driving force to pull the mitochondrial precursor through the TIM complex, together with matrix heat shock protein 70 (HSP70) at the expense of ATP hydrolysis. After translocation, mitochondrial processing peptidase (MP-peptidase) cleaves off the matrix-targeting sequence of the MTS of matrix and IM precursors. Matrix proteins with only one cleavage site for MP-peptidase in their MTS will fold into their native conformation upon cleavage. Other translocated precursors undergo further processing depending on the information residing in their remaining MTS.

C) **Translocation of inter-membrane space and inner membrane precursors.** After the translocation of the matrix-targeting portion of the MTS of the inter-membrane space (IMS) and IM precursors, MP-peptidase cleaves off the translocated portion of the MTS. The IMS-sorting signal (IMS-S) in the MTS of these precursors prevents further translocation into the matrix. Inner membrane peptidase (IM-peptidase) cleaves off the IMS-S of IMS proteins to release these proteins into IMS to allow native folding of these proteins. In the case of some IM precursors, this IMS-S is not cleaved off and serves as an IM anchor.

D) **Translocation of matrix and inner membrane precursors.** After translocating into the matrix, MP-peptidase cleaves off the matrix-targeting portion of the MTS of some matrix and IM precursors. The processing of these precursors by MP-peptidase expose a hydrophobic residue in their remaining MTS that is recognised by Mitochondrial Intermediate peptidase (MI-peptidase), which cleaves off the remaining MTS to allow the maturation of these mitochondrial proteins in the matrix. After processing by MI-peptidase, IM precursors are translocated back into the IM via an unknown mechanism.
Figure 2.3. Phylogram Tree of PINK1 orthologues.

Figure 2.4. Alignments of the mitochondrial targeting sequences of PINK1 orthologues, mapping of putative cleavage sites of mitochondrial processing peptidase and the distribution of PD-associated mutations.

Single letter amino acid code is used and gaps are indicated by dashes. The consensus line is given according to the following code: uppercase letters, invariant residues; lowercase letters, nearly invariant residues; o, conserved non-polar residues; *, conserved polar residues; +, conserved small residues with near neutral polarity. The nature of the residues is depicted with the following colour code: green, polar residue; red, hydrophobic residue; magenta, basic residue; blue, acidic residue. Cleavage site in R-10 motif (xRx↓(F/L/I)(S/T/x)(S/T/G)xxx↓) is depicted as a space. Residue number of each PINK1 orthologue is depicted at the end of the sequence. The positions of the missense mutations associated with PINK1 familial Parkinson’s disease are indicated by red arrows and fonts.
Table 2.1. Comparison table of the mitochondrial targeting sequence of PINK1 orthologues.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Possible longest MTS Length (Residues)</th>
<th>Number of Lysine and Arginine Residues</th>
<th>Intact Length</th>
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<td>581</td>
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<td><em>Fugu rubripes</em></td>
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<td>19</td>
<td>564</td>
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<tr>
<td><em>Trichoplax adhaerens</em></td>
<td>103</td>
<td>18</td>
<td>558</td>
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<td><em>Nasonia vitripennis</em></td>
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<td><em>Apis mellifera</em></td>
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<td>578</td>
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<tr>
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<td><em>Tribolium castaneum</em></td>
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<td><em>Culex quinquefasciatus</em></td>
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<td><em>Anopheles gambiae</em></td>
<td>103</td>
<td>18</td>
<td>683</td>
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<tr>
<td><em>Aedes aegypti</em></td>
<td>106</td>
<td>19</td>
<td>667</td>
</tr>
</tbody>
</table>
Figure 2.5. Bioinformatic analysis of the spatial distribution of the basic residues on the mitochondrial targeting sequence of Human PINK1.

A) Amino acid sequence of the first 98 residues of Human PINK1. Single letter amino acid code is used. The following colours depict the nature of the residues: green, polar residue; red, hydrophobic residue; magenta, basic residue; blue, acidic residue. Residue number of PINK1 is depicted at the end of the sequence.

B) Helical wheel analysis of the first 98 residues of Human PINK1 using Helical Wheel Projections Program written by Don Armstrong and Raphael Zidovetzki, University of California, Riverside. Single letter amino acid code is used and residue number of PINK1 is depicted beside its corresponding amino acid. The natures of the residues are depicted in geometrical shapes. Hydrophilic residues are depicted as circles, hydrophobic residues are depicted as diamonds, negatively charged residues are depicted as triangles, and positively charged residues are depicted as pentagons. Hydrophobicity is color-coded: the most hydrophobic residue is green, and the amount of green is decreasing proportionally to the hydrophobicity, with zero hydrophobicity coded as yellow. Hydrophilic residues are coded red with pure red being the most hydrophilic residue, and the amount of red decreasing proportionally to the hydrophilicity. The potentially charged residues are light blue and marked with +.

C) Schematic illustration of the spatial distribution of the positive charges on the mitochondrial targeting sequence of Human PINK1
Table 2.2. List of cleavage motifs of Mitochondrial Processing Peptidase.

Amino Acid Sequence of Cleavage Site of Mitochondrial Processing Peptidase

<table>
<thead>
<tr>
<th>Name of Cleavage Motif</th>
<th>Amino Acid Sequence of Cleavage Site of Mitochondrial Processing Peptidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-2</td>
<td>$xRx \downarrow x (S/x)$</td>
</tr>
<tr>
<td>R-3</td>
<td>$xRx (Y/x) \downarrow (S/A/x) \times$</td>
</tr>
<tr>
<td>R-none</td>
<td>$xx \downarrow x (S/x)$</td>
</tr>
<tr>
<td>R-10</td>
<td>$xRx \downarrow (F/L/I) (S/x) (S/T/x) (S/T/G) x\times \downarrow$</td>
</tr>
</tbody>
</table>
Figure 2.6. Alignments of the putative transmembrane domains of PINK1 orthologues and the distribution of PD-associated missense mutations.

Yellow highlighted regions are putative transmembrane domains predicted by transmembrane prediction program, TopPred written by Heijne et al, Institut Pasteur "Logiciels et Banques de Données" Team and the Ressource Parisienne en Bioinformatique Structurale (http://mobyle.pasteur.fr/cgi-bin/portal.py?form=toppred). Underlined region is the putative transmembrane domain predicted by transmembrane prediction program, HMMTOP written by G.E. Tusnády, Institute of Enzymology at Hungarian Academy of Sciences (http://www.enzim.hu/hmmtop/). Single letter amino acid code is used and gaps are indicated by dashes. The consensus line is given according to the following code: uppercase letters, invariant residues; lowercase letters, nearly invariant residues; o, conserved non-polar residues; *, conserved polar residues; +, conserved small residues with near neutral polarity. The nature of the residues is depicted with the following colour code: green, polar residue; red, hydrophobic residue; magenta, basic residue; blue, acidic residue. Residue number of each protein is depicted at the end of the sequence. The positions of the missense mutations associated with PINK1 familial Parkinson’s disease are indicated by red arrows and fonts. Green arrows and fonts indicate the positions of missense mutations with unclear association with PINK1 familial Parkinson’s disease.
Consensus

Homo sapiens  GLAARLQ----------RQFVVRAWGCAGPCGRAVFLAFGLGLIEEKQAESRRAVSACQEIQAIF 132
Pongo abelii   GLAARLQ----------RQFVARWGCAGPCGRAVFLAFGLGLIEEKQAESRRAVSACQEIQAIF 132
Macaca mulatta GLAARLQ----------RQFLVRAWGCAGPCGRAVFLAFGLGLIEEKQAESRRAVSACQEIQAIF 132
Boa taurus    GLAARLQ----------RQFVARWGGAGPCGRAVFLAFGLGLIEEKQAESRRAVSACQEIQAIF 132
Mus musculus  GLAARIQ----------RQFMVRARGGAGPCGRAVFLAFGLGLIEEKQAEGRRAASACQEIQAIF 132
Moinidelphis domestica GLAARLQ----------RQLVLRARGAGAPCGRAVFLAFGLGQEQ1LDDSRASAPCQFIQAF 122
Danio rerio   GLAQQNSAGFRRGGLPSARPRAAVFLAGGQEQQEEDRTSAALCQEIQAVF 122
Fugu rubripes GLAAQLQSGGFRRFGGSGSPRNRAGGSGSPRNRAVFLAFGLGVGLIEQQLDDDRRSAATCQEIQAVF 122
Trichoplax adhaerens ----ISNNHLHLKASATLVTLTPGFLIHLLLLQTVAGFDN-BQQRQQLVVSQSL1 141
Nasonia vitripennis --------TLAADLRRRAASRLLFGGDSAPFFALVGISLASGTGILTKEDELEGVCEIREAV 140
Apis mellifera ------SLSADLRRRAASRLVFGGDSAPFFALVGISLASGTGILTKEDELEGVCEIREAV 142
Acrythosiphon pisum ------SLSALRRRAARLVPDDPSAPFFALVGISLASGTGILTKEDELEGVCEIREAV 142
Tribolium castanum -------SLSALRRRAARLVPDDPSAPFFALVGISLASGTGILTKEDELEGVCEIREAV 142
Culex quinquefasciatus --------PYSADLRRRAARLVMYGDSTPFFALVGISLASGDGMLTNDNELEAVCWEIREAV 137
Anopheles gambiae --------PYSADLRRRAARLVMYGDSTPFFALVGISLASGDGMLTNDNELEAVCWEIREAV 137
Aedes aegypti --------PYSADLRRRAARLVMYGDSTPFFALVGISLASGDGMLTNDNELEAVCWEIREAV 137
Pediculus humanus corporis TILNSDLKKAAARFRLYSLQKAFARFLYGCGCAPITALGYCGLSGGLTDEDEGICWEIREAV 135
Membrane bound hydrogenase YVASNQKVQFLFGGGLGGLGFGFLLYLLK 262
NADH-quinone oxidoreductase AAQWSTIALGFALVMIAASGSQ 191
Figure 2.7. Conserved structural features of a protein kinase domain.

A) Crystal structure of the kinase domain of Protein Kinase A (PDB ID: 1ATP) with its substrate analog bound to its active site. The kinase domain of Protein Kinase A is folded into a small lobe and a large lobe. The N-terminal small lobe comprises of conserved subdomains I to IV and the C-terminal large lobe comprises of conserved subdomain V to XI. The linker between the two lobes is represented by subdomain V. These conserved subdomains are represented by the highlighted regions.

B) Critical subdomains involved in protein phosphorylation. Subdomain I (dark green) anchor ATP by spatial hydrophobic and electrostatic interactions to reduce the entropy of phosphorylation reaction. The optimal orientation of $\gamma$-phosphate of ATP for the phosphorylation reaction is facilitated by an invariant lysine from subdomain II (orange) and Mg$^{2+}$-chelating aspartate from subdomain VII (blue). The binding and orientation of protein substrate are mediated by residues that are characteristic to each protein kinase in subdomain VIII (pale green) and subdomain IX (yellow). The catalysis of protein phosphorylation is mediated by catalytically critical aspartate in subdomain VIb (magenta).

C) Schematic representation of critical subdomains involved in protein phosphorylation. Subdomains I (dark green), II (orange) and VII (blue) clamp down ATP in the catalytic cleft, while subdomains VIII (pale green) and IX (yellow) recruit and position the protein substrate in the catalytic cleft. This contributes to the loss of entropy of phosphorylation reaction. The catalytic aspartate in subdomain VIb (magenta) acts as a catalytic base by accepting the proton from the hydroxyl group from the targeted serine, threonine or tyrosine in the substrate (pale purple). This increases the nucleophilicity of the hydroxyl group and facilitates the nucleophilic attack on $\gamma$-phosphate of ATP to result in the phosphorylation of substrate.
Figure 2.8. Alignments of the protein kinase domains of PINK1 orthologues and the catalytic subunit of Protein Kinase A and the distribution of PD-associated mutations.

Single letter amino acid code is used and gaps are indicated by dashes. The consensus line is given according to the following code: uppercase letters, invariant residues; lowercase letters, nearly invariant residues; o, conserved non-polar residues; *, conserved polar residues; +, conserved small residues with near neutral polarity; Ø, conserved aromatic residues. The nature of the residues is depicted with the following colour code: green, polar residue; red, hydrophobic residue; magenta, basic residue; blue, acidic residue. Residue number of each PINK1 orthologue is depicted at the end of the sequence. The missense mutations associated with PINK1 familial Parkinson’s disease are indicated by red fonts. Green fonts indicate missense mutations with unclear association with PINK1 familial Parkinson’s disease. The secondary structure is depicted with the following illustration: green arrow represents β-strand and red cylinder represents α-helix. The predicted secondary structure of Human PINK1 is the consensus prediction made by the following secondary structure prediction programs:

APSSP (http://imtech.res.in/raghava/apssp/),
CFSSP (http://www.biogem.org/tool/chou-fasman/),
GOR (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html),
HNN (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_nn.html),
Jpred3 (http://www.compbio.dundee.ac.uk/www-jpred/),
JUFO (http://www.meilerlab.org/web/view.php),
NetSurfP (http://www.cbs.dtu.dk/services/NetSurfP/),
NNPREDICT (http://www.empharm.ucsf.edu/~nomi/nnpredict.html),
PORTER (http://distill.ucd.ie/porter/),
PROF (http://www.aber.ac.uk/~phi/www/prof/),
PSIpred (http://bioinf.cs.ucl.ac.uk/psipred/),
SOPMA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html),
Scratch Protein Predictor (http://scratch.proteomics.ics.uci.edu/index.html).
Conserved Subdomain
Protein Kinase A
Secondary Structure
PIN1 Predicted Structure

Homo sapiens
---LQGFRLEYELIQQGICGCQAYEATMPTPLQNL-EVYKSTG-LLGQGGT3AQEGQQEAFQAPAFFJIALPMM9W1SNQAQLSSEALINCQKVLVLSATVAILAEGAVTYV-258
Pongo abelii
---WQGFRLEYELIQQGICGCQAYEATMPTPLQNL-EVYKSTG-LLGQGGT3AQEGQQEAFQAPAFFJIALPMM9W1SNQAQLSSEALINCQKVLVLSATVAILAEGAVTYV-258
Bos taurus
---WQGFRLEYELIQQGICGCQAYEATMPTPLQNL-EVYKSTG-LLGQGGT3AQEGQQEAFQAPAFFJIALPMM9W1SNQAQLSSEALINCQKVLVLSATVAILAEGAVTYV-258
Mus musculus
---WQGFRLEYELIQQGICGCQAYEATMPTPLQNL-EVYKSTG-LLGQGGT3AQEGQQEAFQAPAFFJIALPMM9W1SNQAQLSSEALINCQKVLVLSATVAILAEGAVTYV-258

Secondary Structure
PINK1 Predicted Structure

Homo sapiens
----LQGFRLEYELIQQGICGCQAYEATMPTPLQNL-EVYKSTG-LLGQGGT3AQEGQQEAFQAPAFFJIALPMM9W1SNQAQLSSEALINCQKVLVLSATVAILAEGAVTYV-258
Pongo abelii
----WQGFRLEYELIQQGICGCQAYEATMPTPLQNL-EVYKSTG-LLGQGGT3AQEGQQEAFQAPAFFJIALPMM9W1SNQAQLSSEALINCQKVLVLSATVAILAEGAVTYV-258
Bos taurus
----WQGFRLEYELIQQGICGCQAYEATMPTPLQNL-EVYKSTG-LLGQGGT3AQEGQQEAFQAPAFFJIALPMM9W1SNQAQLSSEALINCQKVLVLSATVAILAEGAVTYV-258
Mus musculus
----WQGFRLEYELIQQGICGCQAYEATMPTPLQNL-EVYKSTG-LLGQGGT3AQEGQQEAFQAPAFFJIALPMM9W1SNQAQLSSEALINCQKVLVLSATVAILAEGAVTYV-258

Acrythosiphon pisum
---LNSSLGLKQFELGSVIAKGNNAVVYEARKIEK-----------------DRDPSDVIKLQGS----SNTMYPLAVKMMFNYDAESNAMSILRAMSRETVPSRSVQHDFSRSDQQW240
Trichoplax adhaerens
-ISCSCDTTNSYKVGPRLGSRSCASAVYATKCSCG-------------------------------------KEYAMKMMFNYGN-SKDDFRRKFLQAYCQLSLFPRTPVTRFQEME---232
Conserved Subdomain

Protein Kinase A

Secondary Structure

PINK1 Predicted Structure

Homo sapiens

Pongo abelii

Macaca mulatta

Bos taurus

Mus musculus

Molukusalus domestica

Salmo salar

Danio rerio

Fugu rubripes

Nasonia vitripennis

Apis mellifera

Pediculus humanus corporis

Tribolium castaneum

Acrythosiphon pisum

Trichoplax adhaerens

PD Missense Mutations

L268V
H271Q
R276Q
R279H
A280T
P296L
L305P
G309D
T313M
V317I
M318L
P322L
A339T
A340T
M341I
M342V
L347P

Insertion Region 2

Insertion Region 3

Hydrophobic patch likely involved in structural integrity

Anchors adenine and ribose rings of ATP, and possible substrate interaction

Hydrophobic helix important for structural integrity
**Conserved Subdomain**

Protein Kinase A

Secondary Structure

PINK1 Predicted Structure

**Homo sapiens**

**Pongo abelii**

**Macaca mulatta**

**Bos taurus**

**Mus musculus**

**Nemobius domesticus**

**Salmo salar**

**Panio rerio**

**Fugu rubripes**

**Nasonia vitripennis**

**Apis mellifera**

**Pediculus humanus corporis**

**Tribolium castaneum**

**Acrythosiphon pisum**

**Trichoplax adhaerens**

**Activating Phosphorylation site**

Conserved D serves as catalytic base during phosphotransfer. Also involves in substrate recognition.

Orientates γ-phosphate of ATP.


Conserved D stabilises hDok.xN catalytic loop in subdomain VIb. Also involved in substrate recognition.

**PD missense mutations**

D362H  N367S  L369P

A383T  F385L  G386A  C388R

G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q

G409V  G411S  P416R  E417G

S419P  G395V  P399L  R407Q
Conserved Subdomain

Protein Kinase A
Secondary Structure

PINK1 Predicted Structure

Homo sapiens
Pongo abelii
Macaca mulatta
Bos taurus
Mus musculus
Moinodelphis domestica
Salmo salar
Danio rerio
Pangu rubripes
Masonia vitripennis
Apis mellifera
Pediculus humanus corporis
Tribolium castaneum
Acrythosiphon pisum
Trichoplax adhaerens

Involved in structural integrity
Conserved R salt-bridges with the conserved E in subdomain VIII and involved in structural integrity

PD missense mutations

R464H
E476H
L489P
P498L

Conserved R salt-bridges with the conserved E in subdomain VIII and involved in structural integrity

X

XI

Conserved Subdomain

Protein Kinase A

Secondary Structure

PINK1 Predicted Structure

Homo sapiens
Pongo abelii
Macaca mulatta
Bos taurus
Mus musculus
Moinodelphis domestica
Salmo salar
Danio rerio
Pangu rubripes
Masonia vitripennis
Apis mellifera
Pediculus humanus corporis
Tribolium castaneum
Acrythosiphon pisum
Trichoplax adhaerens

Involved in structural integrity
Conserved R salt-bridges with the conserved E in subdomain VIII and involved in structural integrity

PD missense mutations

R464H
E476H
L489P
P498L
Figure 2.9. Illustration of the spatial distribution of the insertion regions in the protein kinase domain of PINK1

Crystal structure of the kinase domain of Protein Kinase A (PDB ID: 1ATP) with its substrate analog bound to its active site is used as a model to depict the spatial distribution of the insertion regions in the protein kinase domain of PINK1. Subdomains and insertion regions are highlighted as follow: subdomain I (green), subdomain II (yellow), subdomain III (red), subdomain IV (aqua), subdomain V (brown), subdomain VIa (blue) and insertion regions (black).

A.i) Side view of Protein kinase A with the corresponding regions to the insertion regions of PINK1.

A.ii) Schematic representation of the distribution of the insertion regions of PINK1 in Figure A.i. Black line represents insertion region.

B.i) Top view of Protein kinase A with the corresponding regions to the insertion regions of PINK1.

B.ii) Schematic representation of the distribution of the insertion regions of PINK1 in Figure B.i. Black line represents insertion region.

C.i) Top back view of Protein kinase A with the corresponding regions to the insertion regions of PINK1.

C.ii) Schematic representation of the distribution of the insertion regions of PINK1 in Figure C.i. Black line represents insertion region.

D.i) Bottom view of Protein kinase A with the corresponding regions to the insertion regions of PINK1.

D.ii) Schematic representation of the distribution of the insertion regions of PINK1 in Figure D.i. Black line represents insertion region.
B.i)

B.ii)
C.i) 

1st insertion loop

2nd insertion loop

3rd insertion loop

C.ii)
D.i)  
1\textsuperscript{st} insertion loop

D.ii)  
2\textsuperscript{nd} insertion loop

D.ii)  
3\textsuperscript{rd} insertion loop
Figure 2.10. Alignments of the C-terminal tails of PINK1 orthologues, prediction of the secondary structure of the C-terminal tail of PINK1 and the distribution of PD-associated missense mutations.

Single letter amino acid code is used and gaps are indicated by dashes. The consensus line is given according to the following code: uppercase letters, invariant residues; lowercase letters, nearly invariant residues; \( \omega \), conserved non-polar residues; \( * \), conserved polar residues; \( + \), conserved small residues with near neutral polarity; \( \varnothing \), conserved aromatic residues. The nature of the residues is depicted with the following colour code: green, polar residue; red, hydrophobic residue; magenta, basic residue; blue, acidic residue. Residue number of each PINK1 orthologue is depicted at the end of the sequence. The positions of the missense mutations associated with PINK1 familial Parkinson’s disease are indicated by red arrows and fonts. Green arrows and fonts indicate the positions of missense mutations with unclear association with PINK1 familial Parkinson’s disease. The secondary structure is depicted with the following illustration: red cylinder represents \( \alpha \)-helix. The predicted secondary structure of Human PINK1 is derived from the consensus prediction made by the following secondary structure prediction programs:

- APSSP (http://imtech.res.in/raghava/apssp/),
- CFSSP (http://www.biogem.org/tool/chou-fasman/),
- GOR (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html),
- HNN (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_nn.html),
- Jpred3 (http://www.compbio.dundee.ac.uk/www-jpred/),
- JUFO (http://www.meilerlab.org/web/view.php),
- NetSurfP (http://www.cbs.dtu.dk/services/NetSurfP/),
- NNPPREDICT (http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html),
- PORTER (http://distill.ucd.ie/porter/),
- PROF (http://www.aber.ac.uk/~phi/www/prof/),
- PSIPred (http://bioinf.cs.ucl.ac.uk/psipred/),
- SOPMA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html),
**Consensus Predicted Human PINK1 Secondary Structure**

**Consensus**

- **Homo sapiens**
- **Pongo abelii**
- **Macaca mulatta**
- **Bos taurus**
- **Mus musculus**
- **Moinodophis domestica**
- **Salmo salar**
- **Danio rerio**
- **Fugu rubripes**
- **Aedes aegypti**
- **Culex quinquefasciatus**
- **Anopheles gambiae**
- **Tribolium castaneum**
- **Acrythosiphon pisum**
- **Pediculus humanus corporis**
- **Trichoplax adhaerens**

**Subdomain XI**

- **DS15N**
- **A574T**
- **N542S**

**Consensus Predicted Human PINK1 Secondary Structure**

- **Homo sapiens**
- **Pongo abelii**
- **Macaca mulatta**
- **Bos taurus**
- **Mus musculus**
- **Moinodophis domestica**
- **Salmo salar**
- **Danio rerio**
- **Fugu rubripes**
- **Aedes aegypti**
- **Culex quinquefasciatus**
- **Anopheles gambiae**
- **Tribolium castaneum**
- **Acrythosiphon pisum**
- **Pediculus humanus corporis**
- **Trichoplax adhaerens**

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**C575R**

**213**
Table 2.3. List of missense mutations identified in PINK1 familial Parkinson’s disease and their potential effects

Homozygous missense mutations associated with PINK1 familial Parkinson’s disease are indicated by red fonts. Green fonts indicate the missense mutations with unclear association with PINK1 familial Parkinson’s disease.
<table>
<thead>
<tr>
<th>Missense Mutation</th>
<th>Affected Domain</th>
<th>Potential Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>G32R</td>
<td>MTS</td>
<td>Destabilise amphiphilic helical structure of MTS</td>
<td>[184]</td>
</tr>
<tr>
<td>P52L</td>
<td>MTS</td>
<td>Destabilise amphiphilic helical structure of MTS</td>
<td>[185]</td>
</tr>
<tr>
<td>L67F</td>
<td>MTS</td>
<td>Destabilise amphiphilic helical structure of MTS</td>
<td>[186]</td>
</tr>
<tr>
<td>R68P</td>
<td>MTS</td>
<td>Destabilise amphiphilic helical structure and reduce net positive charge of MTS</td>
<td>[537]</td>
</tr>
<tr>
<td>A78V</td>
<td>MTS</td>
<td>Destabilise amphiphilic helical structure of MTS</td>
<td>[187]</td>
</tr>
<tr>
<td>C92F</td>
<td>MTS</td>
<td>Destabilise amphiphilic helical structure of MTS</td>
<td>[537]</td>
</tr>
<tr>
<td>R98W</td>
<td>MTS/ Transmembrane domain</td>
<td>Destabilise amphiphilic helical structure and net positive charge of MTS and PARL-mediated cleavage</td>
<td>[186]</td>
</tr>
<tr>
<td>I111S</td>
<td>Transmembrane domain</td>
<td>Reduce overall hydrophobicity of transmembrane helix</td>
<td>[186]</td>
</tr>
<tr>
<td>Q115L</td>
<td>Linker between transmembrane and kinase domains</td>
<td>Unclear</td>
<td>[412]</td>
</tr>
<tr>
<td>C125G</td>
<td>Linker between transmembrane and kinase domains</td>
<td>Unclear</td>
<td>[191]</td>
</tr>
<tr>
<td>Q126P</td>
<td>Linker between transmembrane and kinase domains</td>
<td>Unclear</td>
<td>[410]</td>
</tr>
<tr>
<td>R147H</td>
<td>Linker between transmembrane and kinase domains</td>
<td>Unclear</td>
<td>[402]</td>
</tr>
<tr>
<td>A168P</td>
<td>Subdomain I of kinase domain</td>
<td>Destabilise interactions with adenine ring, α- and β-phosphates of ATP</td>
<td>[537]</td>
</tr>
<tr>
<td>G193R</td>
<td>Insertion region 1 of kinase domain</td>
<td>Unclear</td>
<td>[422]</td>
</tr>
<tr>
<td>P196L</td>
<td>Insertion region 1 of kinase domain</td>
<td>Unclear</td>
<td>[412]</td>
</tr>
<tr>
<td>P209A</td>
<td>Insertion region 1 of kinase domain</td>
<td>Unclear</td>
<td>[415]</td>
</tr>
<tr>
<td>A217D</td>
<td>Subdomain II of kinase domain</td>
<td>Disrupt ATP orientation and/or destabilise the salt-bridge between subdomains II and III</td>
<td>[416]</td>
</tr>
<tr>
<td>E231G</td>
<td>Subdomain III of kinase domain</td>
<td>Destabilise the salt-bridge between subdomains II and III</td>
<td>[419]</td>
</tr>
<tr>
<td>M237V</td>
<td>Subdomain III of kinase domain</td>
<td>Destabilise the salt-bridge between subdomains II and III</td>
<td>[419, 538]</td>
</tr>
<tr>
<td>E240K</td>
<td>Subdomain III of kinase domain</td>
<td>Abrogate the salt-bridge between subdomains II and III</td>
<td>[419]</td>
</tr>
<tr>
<td>A244G</td>
<td>Subdomain III of kinase domain</td>
<td>Destabilise the salt-bridge between subdomains II and III</td>
<td>[413]</td>
</tr>
<tr>
<td>T257I</td>
<td>Insertion region 2 of kinase domain</td>
<td>Unclear</td>
<td>[186]</td>
</tr>
<tr>
<td>L268V</td>
<td>Insertion region 2 of kinase domain</td>
<td>Unclear</td>
<td>[420]</td>
</tr>
<tr>
<td>H271Q</td>
<td>Subdomain IV of kinase domain</td>
<td>Disrupt structural integrity of kinase domain</td>
<td>[190]</td>
</tr>
<tr>
<td>R276Q</td>
<td>Subdomain IV of kinase domain</td>
<td>Disrupt structural integrity of kinase domain</td>
<td>[186]</td>
</tr>
<tr>
<td>R279H</td>
<td>Subdomain IV of kinase domain</td>
<td>Disrupt structural integrity of kinase domain</td>
<td>[539]</td>
</tr>
<tr>
<td>A280T</td>
<td>Subdomain IV of kinase domain</td>
<td>Disrupt structural integrity of kinase domain</td>
<td>[540]</td>
</tr>
<tr>
<td>P296L</td>
<td>Subdomain V of kinase domain</td>
<td>Destabilise interactions with adenine and ribose rings of ATP and/or substrate interaction</td>
<td>[537]</td>
</tr>
<tr>
<td>L305P</td>
<td>Insertion region 3 of kinase domain</td>
<td>Unclear</td>
<td>[402]</td>
</tr>
<tr>
<td>G309D</td>
<td>Insertion region 3 of kinase domain</td>
<td>Unclear</td>
<td>[169]</td>
</tr>
<tr>
<td>T313M</td>
<td>Insertion region 3 of kinase domain</td>
<td>Unclear</td>
<td>[423]</td>
</tr>
<tr>
<td>V317I</td>
<td>Insertion region 3 of kinase domain</td>
<td>Unclear</td>
<td>[411]</td>
</tr>
<tr>
<td>M318L</td>
<td>Insertion region 3 of kinase domain</td>
<td>Unclear</td>
<td>[419]</td>
</tr>
<tr>
<td>P322L</td>
<td>Insertion region 3 of kinase domain</td>
<td>Unclear</td>
<td>[186]</td>
</tr>
<tr>
<td>A339T</td>
<td>Subdomain V1α of kinase domain</td>
<td>Destabilise Helix E of kinase domain</td>
<td>[419]</td>
</tr>
<tr>
<td>A340T</td>
<td>Subdomain V1α of kinase domain</td>
<td>Destabilise Helix E of kinase domain</td>
<td>[421]</td>
</tr>
<tr>
<td>M341I</td>
<td>Subdomain V1α of kinase domain</td>
<td>Destabilise Helix E of kinase domain</td>
<td>[415]</td>
</tr>
<tr>
<td>M342V</td>
<td>Subdomain V1α of kinase domain</td>
<td>Destabilise Helix E of kinase domain</td>
<td>[187]</td>
</tr>
<tr>
<td>L347P</td>
<td>Subdomain V1α of kinase domain</td>
<td>Destabilise Helix E of kinase domain</td>
<td>[190]</td>
</tr>
<tr>
<td>Missense Mutation</td>
<td>Affected Domain</td>
<td>Potential Effects</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------------</td>
<td>------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>D362H</td>
<td>Subdomain VIb of kinase domain</td>
<td>Abrogate kinase activity</td>
<td>[419]</td>
</tr>
<tr>
<td>N367S</td>
<td>Subdomain VIb of kinase domain</td>
<td>Destabilise catalytic loop</td>
<td>[184]</td>
</tr>
<tr>
<td>L369P</td>
<td>Subdomain VIb of kinase domain</td>
<td>Destabilise catalytic loop</td>
<td>[191]</td>
</tr>
<tr>
<td>A383T</td>
<td>Subdomain VII of kinase domain</td>
<td>Destabilise interaction with γ-phosphate of ATP</td>
<td>[411]</td>
</tr>
<tr>
<td>F385L</td>
<td>Subdomain VII of kinase domain</td>
<td>Destabilise interaction with γ-phosphate of ATP</td>
<td>[184]</td>
</tr>
<tr>
<td>G386A</td>
<td>Subdomain VII of kinase domain</td>
<td>Destabilise interaction with γ-phosphate of ATP</td>
<td>[191]</td>
</tr>
<tr>
<td>C388R</td>
<td>Subdomain VII of kinase domain</td>
<td>Destabilise interaction with γ-phosphate of ATP</td>
<td>[405]</td>
</tr>
<tr>
<td>C395V</td>
<td>Activation loop of subdomain VIII of kinase domain</td>
<td>Affect substrate recognition</td>
<td>[186]</td>
</tr>
<tr>
<td>P399L</td>
<td>Activation loop of subdomain VIII of kinase domain</td>
<td>Affect substrate recognition</td>
<td>[236]</td>
</tr>
<tr>
<td>R407Q</td>
<td>Activation loop of subdomain VIII of kinase domain</td>
<td>Affect substrate recognition</td>
<td>[236]</td>
</tr>
<tr>
<td>G409V</td>
<td>Subdomain VIII of kinase domain</td>
<td>Affect substrate recognition and/or destabilise the salt-bridge between subdomains VIII and XI</td>
<td>[191]</td>
</tr>
<tr>
<td>G411S</td>
<td>Subdomain VIII of kinase domain</td>
<td>Affect substrate recognition and/or destabilise the salt-bridge between subdomains VIII and XI</td>
<td>[417]</td>
</tr>
<tr>
<td>P416R</td>
<td>Subdomain VIII of kinase domain</td>
<td>Affect substrate recognition and/or destabilise the salt-bridge between subdomains VIII and XI</td>
<td>[418]</td>
</tr>
<tr>
<td>E417G</td>
<td>Subdomain VIII of kinase domain</td>
<td>Abrogate the salt-bridge between subdomains VIII and XI and/or affect substrate recognition</td>
<td>[190]</td>
</tr>
<tr>
<td>S419P</td>
<td>Subdomain VIII of kinase domain</td>
<td>Affect substrate recognition and/or destabilise the salt-bridge between subdomains VIII and XI</td>
<td>[418]</td>
</tr>
<tr>
<td>P425S</td>
<td>Linker between subdomains VIII and IX of kinase domain</td>
<td>Destabilise interactions between subdomains VIII and IX</td>
<td>[419]</td>
</tr>
<tr>
<td>Y431H</td>
<td>Subdomain IX of kinase domain</td>
<td>Destabilise the catalytic loop in subdomain VIb and/or affects substrate recognition</td>
<td>[411]</td>
</tr>
<tr>
<td>W437R</td>
<td>Subdomain IX of kinase domain</td>
<td>Destabilise the catalytic loop in subdomain VIb and/or affects substrate recognition</td>
<td>[187]</td>
</tr>
<tr>
<td>G440E</td>
<td>Subdomain IX of kinase domain</td>
<td>Destabilise the catalytic loop in subdomain VIb and/or affects substrate recognition</td>
<td>[414]</td>
</tr>
<tr>
<td>I442T</td>
<td>Subdomain IX of kinase domain</td>
<td>Destabilise the catalytic loop in subdomain VIb and/or affects substrate recognition</td>
<td>[537]</td>
</tr>
<tr>
<td>N451S</td>
<td>Subdomain IX of kinase domain</td>
<td>Destabilise the catalytic loop in subdomain VIb and/or affects substrate recognition</td>
<td>[411]</td>
</tr>
<tr>
<td>R464H</td>
<td>Subdomain X of kinase domain</td>
<td>Destabilise structural integrity of kinase domain</td>
<td>[537]</td>
</tr>
<tr>
<td>E476K</td>
<td>Subdomain XI of kinase domain</td>
<td>Destabilise the salt-bridge between subdomains VIII and XI</td>
<td>[537]</td>
</tr>
<tr>
<td>L489P</td>
<td>Subdomain XI of kinase domain</td>
<td>Destabilise the salt-bridge between subdomains VIII and XI</td>
<td>[419]</td>
</tr>
<tr>
<td>P498L</td>
<td>Subdomain XI of kinase domain</td>
<td>Destabilise the salt-bridge between subdomains VIII and XI</td>
<td>[424]</td>
</tr>
<tr>
<td>D525N</td>
<td>C-terminal tail</td>
<td>Unclear</td>
<td>[537]</td>
</tr>
<tr>
<td>A537T</td>
<td>C-terminal tail</td>
<td>Unclear</td>
<td>[186]</td>
</tr>
<tr>
<td>N542S</td>
<td>C-terminal tail</td>
<td>Unclear</td>
<td>[187]</td>
</tr>
<tr>
<td>C575R</td>
<td>C-terminal tail</td>
<td>Unclear</td>
<td>[411]</td>
</tr>
</tbody>
</table>
Figure 3.1. Overview for the KESTREL approach to search for putative mitochondrial protein substrates of PINK1

Mitochondria isolated from mouse brains are extracted using detergent and subjected to protein purification chromatography. Each chromatography fraction is then treated with [γ-32P]-ATP in the presence and absence of catalytically active recombinant PINK1 protein. The resulting phosphorylated proteins are resolved and detected by the autoradiogram of the SDS-PAGE gel. The chromatography fraction with mitochondrial proteins that are preferentially phosphorylated in the presence of the recombinant PINK1 protein is treated with ATP again in the presence and absence of the recombinant PINK1 protein. The resulting protein mixture is then resolved by 2-dimensional gel electrophoresis to facilitate the identification of the mitochondrial that are preferentially phosphorylated by the recombinant PINK1 protein. It is worth mentioning that the phosphorylation of alcoholic residue will cause an acidic shift of the pI of the protein.
Protein purification chromatography

Autoradiogram of SDS-PAGE gel

Fraction X  Y  Z
-/+ PINK1

Preferentially phosphorylated by PINK1

Kinase assay

+ [γ-32P]-ATP
-/+ PINK1

Fraction Z

Kinase assay

+ [γ-32P]-ATP
-/+ PINK1

2D gel electrophoresis

Acidic mobility shift due to phosphorylation

Identification by mass spectrometry

Kinase assay

+ [γ-32P]-ATP
-/+ PINK1

Autoradiogram of SDS-PAGE gel

pH 3  pH 11

pH 3  pH 11

- PINK1  + PINK1

Identification by mass spectrometry
Table 3.1. The list of primers used to generate PINK1 DNA construct in pBacPAK9 plasmid.

Red fonts denote DNA sequence encoding PINK1 protein sequence. Blue fonts denote DNA sequence encoding recombinant tag. Underlined denotes restriction site.
<table>
<thead>
<tr>
<th>PINKI DNA Construct</th>
<th>Sense Primer</th>
<th>Anti-Sense Primer</th>
<th>5' Restriction Site</th>
<th>3' Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flag-PINKI(148-581)-6xHis</td>
<td>5’AGGGAATTCGATGGACTACAAAAAGAGACGAGACGACGACGACGAGGTTGCAAGGGCTTTGCGGCTG</td>
<td>5’ATGTCTTCTGCAATGATGATGGTGATGGTGATGGTGCTGGTTCTTTGCAACTGAGGTCGAGGTCGAGG</td>
<td>Eco RI</td>
<td>Xho 1</td>
</tr>
<tr>
<td>Flag-PINKI(148-581)-GST</td>
<td>5’AGGGAATTCGATGGACTACAAAAAGAGACGAGACGACGACGAGGTTGCAAGGGCTTTGCGGCTG</td>
<td>5’GGGCCCCTCGAGCAGGGCTGCCCTCCCATGAGCAGAGG</td>
<td>Eco RI</td>
<td>Xho 1</td>
</tr>
<tr>
<td>GST-PINKI(148-581)-6xHis</td>
<td>5’AGGGAATTCGATGGACTACAAAAAGAGACGAGACGACGACGAGGTTGCAAGGGCTTTGCGGCTG</td>
<td>5’ATGTCTTCTGCAATGATGATGGTGATGGTGATGGTGCTGGTTCTTTGCAACTGAGGTCGAGG</td>
<td>Xho 1</td>
<td>Not 1</td>
</tr>
<tr>
<td>S/S-codon optimised Flag-PINKI(148-581)-myc-12xHis</td>
<td>5’AGGGAATTCGATGGACTACAAAAAGAGACGAGACGACGACGAGGTTGCAAGGGCTTTGCGGCTG</td>
<td>5’ATGTCTTCTGCAATGATGATGGTGATGGTGATGGTGCTGGTTCTTTGCAACTGAGGTCGAGG</td>
<td>Bam HI</td>
<td>Not 1</td>
</tr>
<tr>
<td>S/S-codon optimised Flag-PINKI(148-515)-12xHis</td>
<td>5’AGGGAATTCGATGGACTACAAAAAGAGACGAGACGACGACGAGGTTGCAAGGGCTTTGCGGCTG</td>
<td>5’ATGTCTTCTGCAATGATGATGGTGATGGTGATGGTGCTGGTTCTTTGCAACTGAGGTCGAGG</td>
<td>Bam HI</td>
<td>Not 1</td>
</tr>
</tbody>
</table>
Table 3.2. The list of primers used to generate pBacPAK9 plasmid containing N-terminal GST-tag or C-terminal GST-tag

Red fonts denote DNA sequence encoding GST. Green fonts denote stop codon. Underlined denotes restriction site.
<table>
<thead>
<tr>
<th></th>
<th>Sense Primer</th>
<th>Anti-Sense Primer</th>
<th>5’ Restriction Site</th>
<th>3’ Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminal GST-tag in pBacPAK9</td>
<td>5’CCTGGGATCCATGTCCTATACCTGTTATTGCTGAAAATTAG 3’</td>
<td>5’TGGGATCCAGGGCCCCTGG 3’</td>
<td><strong>Bam HI</strong></td>
<td><strong>Bam HI</strong></td>
</tr>
<tr>
<td>C-terminal GST-tag in pBacPAK9</td>
<td>5’CCTGATAGCCGCGCCGATGTCCCCTATACTAGTTATTGGAATTAG 3’</td>
<td>5’TCCATTGCGGGCCTGCCCATTGGAAATAG 3’</td>
<td><strong>Not I</strong></td>
<td><strong>Xho I</strong></td>
</tr>
</tbody>
</table>
The pBacPAK9 transfer vector contains a polyhedrin promoter upstream of PINK1 coding sequence. An essential gene (ORF1629) essential for baculoviral replication is located downstream of PINK1 coding sequence in pBacPAK9. On the other hand, Bsu361 digested BacPAK6 DNA is a cryptic linearised baculoviral DNA, which has partially deleted ORF1629 gene and could not propagate in Sf9 cells. These DNA molecules are introduced into Sf9 cells via transfection. In the Sf9 cell, the viral replicating function of BacPAK6 is restored by transferring PINK1 coding sequence and the ORF1629 gene to the linearised BacPAK6 via a double homologous recombination event. Besides generating infectious baculovirus, the polyhedrin promoter upstream of PINK1 coding sequence is activated at the late stage of the infection (>24 hr post-infection) and drives the strong transcription of PINK1 coding sequence to give rise to strong protein expression of recombinant PINK1 protein at 24-60 hr post-infection.
Transfect into *S*9 *cells*

Viable baculoviral DNA

Viral propagation

Overexpression of PINK1

Baculovirus

*Bsul361*-linearised pBacPak6 (Baculoviral genome with partial deleted ORF1629)
Figure 3.3. Ni-NTA affinity chromatography elution profile of recombinant PINK1 protein containing the kinase domain and C-terminal tail of PINK1.

A) Schematic diagram of PINK1(148-581) DNA construct used to generate the recombinant PINK1 protein containing the kinase domain and C-terminal tail of PINK1 in Sf9 overexpression system.

B) Coomassie blue stained 10% SDS-PAGE gel and Western blot of Ni-NTA affinity chromatography eluted protein fractions using antibody targeting Flag tag (purchased from Sigma-Aldrich). Protein solution was applied to Ni-NTA column pre-equilibrated with the Ni-NTA wash buffer. The non-specifically bound proteins were removed by washing with the same buffer supplemented with NaCl. Proteins specifically bound to the column were eluted with Ni-NTA elution buffer.
A) Flag — Kinase — Tail — STREP 6His

B) Coomassie-stained SDS-PAGE gel

IB: Anti-Flag

Theoretical size of Flag-148-581-Strep-6His

Unknown post-translation modification of Flag-148-581-Strep-6His

Flag-148-581-Strep-6His

Degraded Flag-148-581-Strep-6His

Lyse Flow through Wash 1 (1M NaCl) Wash 2 (1M NaCl) Elution 1 Elution 2 Elution 3 Elution 4 Elution 5 Elution 6 Elution 7 Elution 8 Elution 9 Elution 10

175kDa - 82kDa - 62kDa - 47.5kDa - 32.5kDa - 25kDa - 16kDa -
Figure 3.4. Strep-Tactin affinity chromatography elution profile of the Ni-NTA affinity-purified recombinant PINK1 protein.

Western blot of the protein purification profile of the Ni-NTA affinity-purified recombinant PINK1 protein after Strep-Tactin affinity chromatography using antibody targeting Flag tag. The elution fractions of the Ni-NTA affinity-purified recombinant PINK1 protein were pooled and applied to Strep-Tactin column pre-equilibrated with Strep-Tactin wash buffer. The non-specifically bound proteins were removed by washing with the same buffer. Proteins specifically bound to the column were eluted with Strep-Tactin wash buffer supplemented with 3 mM desthiobiotin.
Flow-through
Wash 1 (0M NaCl)
Wash 2 (0.15M NaCl)
Wash 3 (0.3M NaCl)
Wash 4 (0M NaCl)
Elution 1
Elution 2
Elution 3
Elution 4
Elution 5
Elution 6
Elution 7
Elution 8
Elution 9

IB: Anti-Flag

- Aggregated Flag-148-581-Strep-6his
- Flag-148-581-Strep-6his

175kDa -
82kDa -
62kDa -
47.5kDa -
32.5kDa -
25kDa -
16kDa -
Figure 3.5. Glutathione affinity chromatography elution profile of recombinant PINK1 protein containing the kinase domain and C-terminal tail of PINK1.

A) Schematic diagram of the PINK1(148-581) DNA construct used to generate the recombinant PINK1 protein containing the kinase domain and C-terminal tail of PINK1 in Sf9 overexpression system.

B) Western blot of Sf9 cells overexpressing the recombinant PINK1 using antibody targeting Flag tag.

C) Coomassie blue stained 10% SDS-PAGE gel and Western blot of glutathione affinity chromatography eluted protein fractions using antibody targeting Flag tag. Protein solution was applied to a glutathione-sepharose™ column equilibrated with the glutathione-sepharose wash buffer. The non-specifically bound proteins were removed by washing with the same buffer supplemented with NaCl. Proteins specifically bound to the column were eluted with glutathione-sepharose wash buffer supplemented with 30 mM reduced glutathione.
A) 

B) 

Sf9 cells overexpressing Flag-148-581-GST

Un-infected Sf9 cells

Flow-through

Wash 2 (0.5M NaCl)

Wash 4 (0M NaCl)

Elution 1

Elution 2

Elution 3

Elution 4

Elution 5

Elution 6

Elution 7

Elution 8

Elution 9

Elution 10

Coomassie-stained SDS-PAGE gel

Theoretical size of Flag-148-581-GST

Sf9 cell's GST

IB: Anti-Flag

Theoretical size of Flag-148-581-GST

**IB: Anti-Flag**

**Theoretical size of Flag-148-581-GST**

**IB: Anti-Flag**
The protein expression level of recombinant PINK1 in *Pichia* yeast recombinant protein overexpression system is dependent on the number of copies of the overexpression cassette inserted into *Pichia*’s genome.

The plasmid, pPIC3.5K contains an overexpression cassette that drives the overexpression of the recombinant PINK1 protein using AOX1 promoter. This plasmid also contains an essential gene, *HIS4*, which is required for *HIS4* *Pichia* mutant to grow on minimal media after transformation. The multiple insertion of the overexpression cassette into the genome of the *Pichia* mutant is mediated by multiple consecutive events of single recombination.
1st crossover

2nd crossover

Pichia's genome

Pichia's genome

pPIC3.5K
Figure 3.7. Screening for *Pichia* transformants with high protein expression of the recombinant PINK1 protein.

A) Schematic diagram of PINK1(148-581) DNA construct used to generate the recombinant PINK1 protein containing the kinase domain and C-terminal tail of PINK1 in *Pichia* overexpression system.

B) Western blot of *Pichia* transformants overexpressing recombinant PINK1 protein using antibody targeting Flag tag.
Selected for protein expression

A) Flag-148-581-Strep-6His

B) Degraded Flag-148-581-Strep-6His

IB: Anti-Flag
Figure 3.8. Ni-NTA affinity chromatography elution profile of recombinant PINK1 protein generated by *Pichia* transformant 14 grown on Minimal Methanol media.

Coomassie blue stained 10% SDS-PAGE gel and Western blot of Ni-NTA affinity chromatography eluted protein fractions using antibodies targeting Flag tag, residues 258-274 of PINK1 (purchased from Imgenex) and residues 511-526 of PINK1 (generated using PINK1 peptide corresponding to residues 511 to 526). Protein solution was applied to Ni-NTA column pre-equilibrated with the Ni-NTA wash buffer. The non-specifically bound proteins were removed by washing with the same buffer supplemented with NaCl. Proteins specifically bound to the column were eluted with Ni-NTA elution buffer.
Flow-through
Wash 1 (0M NaCl)
Wash 2 (1M NaCl)
Wash 3 (1M NaCl)
Wash 4 (0M NaCl)
Elution 1
Elution 2
Elution 3
Elution 4
Elution 5
Elution 6
Elution 7
Elution 8
Elution 9

Coomassie-stained SDS-PAGE gel

IB: Anti-Flag

IB: Anti-PINK1 (258-274)

IB: Anti-PINK1 (511-526)
Figure 3.9. Ni-NTA affinity chromatography elution profile of recombinant PINK1 protein generated by *Pichia* transformant 14 grown on “Buffered” Minimal Methanol media.

Coomassie blue stained 10% SDS-PAGE gel and Western blot of Ni-NTA affinity chromatography eluted protein fractions using antibodies targeting Flag tag, residues 258-274 of PINK1 and residues 511-526 of PINK1 (affinity-purified using PINK1 peptide corresponding to residues 511 to 526). Protein solution was applied to Ni-NTA column pre-equilibrated with the Ni-NTA wash buffer. The non-specifically bound proteins were removed by washing with the same buffer supplemented with NaCl. Proteins specifically bound to the column were eluted with Ni-NTA elution buffer.
Lysate
Flow-through
Wash 2 (1M NaCl)
Wash 3 (1M NaCl)
Wash 4 (0M NaCl)
Elution 1
Elution 2
Elution 3
Elution 4
Elution 5
Elution 6
Elution 7
Elution 8
Elution 9

Contaminants

Possible degraded Flag-148-581-Strep-6his from the N-terminus

IB: Anti-Flag
Flag-148-581-Strep-6his
Flag-148-581-Strep-6his
Flag-148-581-Strep-6his

IB: Anti-PINK1 (258-274)
Flag-148-581-Strep-6his
Flag-148-581-Strep-6his
Flag-148-581-Strep-6his

IB: Affinity-purified anti-PINK1 (511-526)
Flag-148-581-Strep-6his
Flag-148-581-Strep-6his
Flag-148-581-Strep-6his

Coomassie-stained SDS-PAGE gel
Figure 3.10. The *Pichia*-generated recombinant PINK1 protein could not phosphorylate *in vitro* substrate, Histone H1.

Autoradiogram and Coomassie blue stained 10% SDS-PAGE gel of the kinase assay with Ni-NTA affinity purified recombinant PINK1 protein from Buffered Minimal Methanol-cultured *Pichia* transformant 14 and Histone H1.
Autoradiogram of SDS-PAGE gel

Flag-148-581-Strep-6His + Histone H1

Coomassie-stained SDS-PAGE gel

Flag-148-581-Strep-6His

Histone H1
Figure 3.11. Ni-NTA affinity chromatography elution profile of recombinant GST-PINK1 fusion protein containing the kinase domain and C-terminal tail of PINK1.

A) Schematic diagram of PINK1(148-581) DNA construct used to generate the recombinant GST-PINK1 fusion protein containing the kinase domain and C-terminal tail of PINK1 in *Sf9* overexpression system.

B) Coomassie blue stained 10% SDS-PAGE gel and Western blot of Ni-NTA affinity chromatography eluted protein fractions using antibodies targeting residues 258-274 of PINK1 and GST tag. Protein solution was applied to Ni-NTA column pre-equilibrated with the Ni-NTA wash buffer. The non-specifically bound proteins were removed by washing with the same buffer supplemented with NaCl. Proteins specifically bound to the column were eluted with Ni-NTA elution buffer.
A) GST 148 Kinase Tail STREP 6His

B) Coomassie-stained SDS-PAGE gel

IB: Anti-GST
IB: Anti-PINK1 (258-274)

Unknown post-translation modification of Flag-148-581-Strep-6His
GST-148-581-Strep-6His

Unknown post-translation modification of Flag-148-581-Strep-6His
GST-148-581-Strep-6His
Figure 3.12. Glutathione affinity chromatography elution profile of Ni-NTA affinity-purified recombinant GST-PINK1 fusion protein.

Coomassie blue stained 10% SDS-PAGE gel and Western blot of glutathione affinity chromatography eluted protein fractions using antibody targeting residues 258-274 of PINK1. Elution fractions of the Ni-NTA affinity chromatography were pooled and solution was applied to a glutathione-sepharose™ column equilibrated with the glutathione-sepharose wash buffer. The non-specifically bound proteins were removed by washing with the same buffer supplemented with NaCl. Proteins specifically bound to the column were eluted with glutathione-sepharose wash buffer supplemented with 30 mM reduced glutathione.
Flow-through
Wash 1 (0M NaCl)
Wash 2 (1M NaCl)
Wash 4 (0M NaCl)
Elution 1
Elution 2
Elution 3
Elution 4
Elution 5
Elution 6
Elution 7
Elution 8
Elution 9
Elution 10

175kDa - 82kDa - 62kDa - 47.5kDa - 32.5kDa - 25kDa - 16kDa -

IB: Anti-PINK1 (258-274)

Coomassie-stained SDS-PAGE gel

GST-148-581-Strep-6His

GST-148-581-Strep-6His
Figure 3.13. Recombinant GST-PINK1 fusion protein is catalytically active.

A) Autoradiogram of the kinase assay with the recombinant GST-PINK1 fusion protein with Histone H1.

B) Autoradiogram of the kinase assay with the recombinant GST-PINK1 fusion protein with Myelin Basic Protein (MBP).
A) Histone H1 + GST-148-581-Strep-6His

Phosphorylated contaminants in GST-148-581-Strep-6His

Histone H1

Autoradiogram

- 175kDa
- 82kDa
- 62kDa
- 47.5kDa
- 32.5kDa
- 25kDa
- 16kDa

B) GST-148-581-Strep-6His + MBP

Phosphorylated contaminants in GST-148-581-Strep-6His

MBP

Autoradiogram

- 62kDa
- 47.5kDa
- 32.5kDa
- 25kDa
- 16kDa
Figure 3.14. Hydroxyapatite chromatography elution profile of n-octyl \( \beta \)-D-glucopyranoside-solubilised mitochondria isolated from mouse brains.

Coomassie blue stained 10% SDS-PAGE gel of hydroxyapatite chromatography eluted protein fractions. Protein solution of mouse brain mitochondria was applied to hydroxyapatite column. Proteins bound to the column were eluted with linear gradient of 0 to 0.3 M potassium phosphate in MonoQ column buffer.
Coomassie-stained SDS-PAGE gels
Figure 3.15. Preferential phosphorylation of mitochondrial proteins by recombinant GST-PINK1 fusion protein is not detected in KESTREL.

Autoradiograms of kinase assays with hydroxyapatite-purified mitochondrial fractions in the presence and absence of the recombinant GST-PINK1 fusion protein.
Figure 3.16. The DNA sequence difference between PINK1 DNA sequence and Sf9 codon-optimised PINK1 DNA sequence.

Conserved alignment of PINK1 DNA sequence and Sf9 codon-optimised PINK1 DNA sequence using ClusterW program. “*” denotes identical base. Yellow highlight denotes Start codon. Red highlight denotes Stop codon. Aqua highlight denotes Myc tag coding sequence. Green highlight denotes 12x-His tag coding sequence.
Figure 3.17. Ni-NTA affinity chromatography elution profile of Sf9 codon-optimised recombinant PINK1 fusion protein containing the kinase domain of PINK1.

A) Schematic diagram of the Sf9 codon-optimised DNA constructs used to generate the recombinant PINK1 proteins in Sf9 overexpression system.

B) Coomassie blue stained 10% SDS-PAGE gel and Western blot of Ni-NTA affinity chromatography eluted protein fractions of recombinant PINK1 protein containing the kinase domain of PINK1 using antibody targeting Flag tag. Protein solution was applied to Ni-NTA column pre-equilibrated with the Ni-NTA wash buffer. The non-specifically bound proteins were removed by washing with the same buffer supplemented with NaCl. Proteins specifically bound to the column were eluted with Ni-NTA elution buffer.
A) 

Flag-148-515-12His

B) 

Coomassie-stained SDS-PAGE gels

IB: Anti-Flag
Figure 3.18. Protein purification profiles of recombinant Parkin and TRAP1 proteins generated from *Escherichia coli* bacterial recombinant protein overexpression system.

A) Amylose affinity chromatography elution profile of recombinant Maltose-binding-protein-Parkin fusion protein. Coomassie blue stained 10% SDS-PAGE gel and Western blot of amylose affinity chromatography eluted protein fractions using antibody targeting R2 domain of human and mouse Parkin (purchased from Signet). Protein solution was applied to an amylose resin column pre-equilibrated with the glutathione-sepharose wash buffer. The non-specifically bound proteins were removed by washing with the same buffer supplemented with NaCl. Proteins specifically bound to the column were eluted with glutathione-sepharose wash buffer supplemented with 10 mM maltose.

B) Ni-NTA affinity chromatography elution profile of recombinant TRAP1 protein. Coomassie blue stained 10% SDS-PAGE gel and Western blot of Ni-NTA affinity chromatography eluted protein fractions using antibody targeting recombinant TRAP1 protein (purchased from Abcam). Protein solution was applied to Ni-NTA column pre-equilibrated with the Ni-NTA wash buffer. The non-specifically bound proteins were removed by washing with the same buffer supplemented with NaCl. Proteins specifically bound to the column were eluted with Ni-NTA elution buffer.
A) Lysate Flow-through
Wash 1 (0M NaCl)
Wash 2 (0.5M NaCl)
Elution 1
Elution 2
Elution 3
Elution 4
Elution 5
Elution 6
Elution 7
Elution 8
Elution 9
Elution 10

Coomassie-stained SDS-PAGE gel

175kDa - 82kDa - 62kDa - 47.5kDa - 32.5kDa - 25kDa - 16kDa

IB: Anti-Parkin

Maltose-binding protein-Parkin
Degraded maltose-binding protein-Parkin

B) Lysate
Flow-through
Wash 1 (0M NaCl)
Wash 3 (0.5M NaCl)
Elution 1
Elution 2
Elution 3
Elution 4
Elution 5
Elution 6
Elution 7
Elution 8

Coomassie-stained SDS-PAGE gel

175kDa - 82kDa - 62kDa - 47.5kDa - 32.5kDa - 25kDa - 16kDa

IB: Anti-TRAP1

Contaminants

Maltose-binding protein-Parkin
Degraded maltose-binding protein-Parkin

TRAP1-6His

TRAP1-6His

Contaminants
Figure 3.19. *Sf9* codon-optimised recombinant PINK1 protein did not phosphorylate recombinant Parkin and TRAP1 proteins.

 Autoradiogram of 10% SDS-PAGE gel of the kinase assays with the recombinant PINK1 protein with Parkin, TRAP1 and Histone H1. Parallel kinase assays were performed with *Sf9*-codon optimised recombinant PINK1 protein bearing kinase inactivating D362A mutation. “WT” denotes wild-type *Sf9* codon-optimised recombinant PINK1 protein. “D362A” denotes *Sf9* codon-optimised recombinant PINK1 protein bearing kinase inactivating D362A mutation.
TRAP1 + D362A
TRAP1 + WT
Histone H1 + D362A
Histone H1 + WT

Parkin + D362A
Parkin + WT

Maltose binding protein-Parkin
TRAP1-6His

Histone H1

Autoradiograms
To search for putative mitochondrial outer membrane (OM) protein substrates of PINK1, kinase assays using *Sf9* codon-optimised recombinant PINK1 protein were performed with the mitochondria isolated from mouse brain, liver and testes and resolved using SDS-PAGE. Proteins that were preferentially phosphorylated in the presence of the recombinant PINK1 proteins are putative OM protein substrates of PINK1. To search for putative intra-mitochondrial protein substrates of PINK1, parallel kinase assays were performed in the presence of detergent, Triton X-100, to rupture the OM and inner membrane of the mitochondria. The resulting kinase reactions were also resolved using SDS-PAGE to detect the mitochondrial proteins that are preferentially phosphorylated in the presence of the recombinant PINK1 protein.
Figure 3.21. *In vitro* phosphorylation of the mitochondria isolated from mouse brain, testes and liver using *Sf9* codon-optimised recombinant PINK1 protein.

A) Autoradiogram of the kinase assays with mitochondria isolated from mouse brain and *Sf9* codon-optimised recombinant PINK1 protein.

B) Autoradiogram of the kinase assays with mitochondria isolated from mouse testes and *Sf9* codon-optimised recombinant PINK1 protein.

C) Autoradiogram of the kinase assays with mitochondria isolated from mouse liver and *Sf9* codon-optimised recombinant PINK1 protein.

“WT” denotes wild-type *Sf9* codon-optimised recombinant PINK1 protein. “D362A” denotes *Sf9* codon-optimised recombinant PINK1 protein bearing kinase inactivating D362A mutation. “Mito” denotes intact mitochondria. “Mito lysate” denotes Triton X-100-solubilised mitochondria. “Dephosphorylated” denotes that intact mitochondria or Triton X-100-solubilised mitochondria were treated with alkaline phosphatase prior to the kinase assay; sodium orthovanadate was added before the kinase assay to inhibit alkaline phosphatase.
A) Mi to lysate
Mi to lysate + WT
Mi to lysate + D362A
Dephosphorylated mitolysate
Dephosphorylated mitolysate + WT
Dephosphorylated mitolysate + D362A
Mito + WT
Mito + D362A
Dephosphorylated mito
Dephosphorylated mito + WT
Dephosphorylated mito + D362A

B) Brain
Liver
Testes

C) Brain
Liver
Testes

Autoradiograms
Figure 3.22. Expression of recombinant PINK1 protein in a lentiviral-mediated mammalian recombinant protein inducible overexpression system.

A) In the absence of doxycycline, the tetracycline-controlled transactivator, RtTA-Advanced developed by ClonTech is not recruited to the modified tetracycline response element (TRE) and unable to drive the transcription of the overexpression cassette containing PINK1 gene.

B) In the presence of doxycycline, RtTA-Advanced is recruited to the modified TRE to recruit transcription factors to a modified minimal CMV promoter via its three minimal transcription "F”-type activation domains derived from herpes simplex virus VP16 (min VP16) to activate the transcription of the overexpression cassette containing PINK1 gene.
Figure 3.23. Overexpression of recombinant PINK1 protein is not detected in Human cell lines stably transduced with lentiviral PINK1 inducible overexpression cassette after 16 hour exposure to doxycycline.

A) Schematic diagram of the PINK1 DNA construct used to overexpress recombinant PINK1 proteins in mammalian cells.

B) Western blot of stably transduced HEK293T embryonic kidney cell line, HT-1080 fibrosarcoma and BE2-M17 dopaminergic neuroblastoma with lentiviral overexpression cassette containing wild-type PINK1 (WT) or the PINK1 mutant bearing kinase inactivating mutation, D362A (D362A) using antibodies targeting residues 258-274 of PINK1 and α-tubulin (purchased from Abcam). “Dox” denotes 16 hour exposure to doxycycline.
A)  

MTS → TM → Kinase → Tail → STREP

B)  

IB: Anti-PINK1 (258-274)  

IB: Anti-α-tubulin
Figure 3.24. PINK1 mRNA increased in BE2-M17 cell lines stably transduced with lentiviral PINK1 inducible overexpression cassette after 16 hour exposure to doxycycline.

Quantitative real-time PCR analysis of PINK1 mRNA level with respect to β-actin mRNA in BE2-M17 cell lines. “WT” denotes BE2-M17 cell line stably transduced with lentiviral inducible overexpression cassette containing PINK1. “D362A” denotes BE2-M17 cell line stably transduced with lentiviral overexpression cassette containing PINK1 mutant bearing kinase inactivating D362A mutation. “ShPINK1mir” denotes BE2-M17 cell line stable transduced with lentiviral shRNAmir targeting PINK1 mRNA. “pGIPz NSC” denotes BE2-M17 cell line stably transduced with lentiviral non-silencing shRNA. “Dox” denotes 16 hour exposure to doxycycline. Data represent geometric mean ± 95% confidence interval of three independent experiments. Bonferroni multiple comparison test was used to compare all pairs of columns at P<0.001.
A)

B)

M17 Cell lines
Figure 3.25. Time course of doxycycline-induced protein expression of PINK1 in BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette.

Western blot of Ni-NTA affinity-purified recombinant PINK1 protein from denatured cell lysates of BE2-M17 cell line stably transduced lentiviral PINK1 inducible overexpression cassette using antibody targeting residues 511-526 of PINK1. A PINK1 antibody-reactive protein of expected mobility was detected followed by Ni-NTA enrichment after 4 hours of induction by doxycycline. “Whole lysate” denotes the guanidine hydrochloride- and Triton X-100-treated cell lysate prior to centrifugation to remove insoluble debris. “Insoluble Pellet” denotes insoluble fraction of the cell lysate after the protein extraction using guanidine hydrochloride and Triton X-100. “Unbound” denotes the supernatant resulting from the soluble protein fraction of the cell lysate treated with Ni-NTA beads. “Ni-NTA beads” denotes the Ni-NTA beads treated with the soluble protein fraction of the cell lysate.
IB: Affinity-purified anti-PINK1 (511-526)

Coomassie-stained SDS-PAGE gel after transfer

Unbound Ni-NTA beads

Un-induced 30min 4h 8h 24h 48h

Non-specific bands

Unknown post-translational modification of PINK1

Degraded PINK1 from its N-terminus

IB: Affinity-purified anti-PINK1 (511-526)

Coomassie-stained SDS-PAGE gel after transfer

Un-induced 30min 4h 8h 24h 48h

Non-specific bands

Unknown post-translational modification of PINK1

Degraded PINK1 from its N-terminus
Figure 3.26. PINK1 mRNA increased in BE2-M17 cell lines stably transduced with lentiviral PINK1 inducible overexpression cassette after 4 hour exposure to doxycycline.

Quantitative real-time PCR analysis of PINK1 mRNA level with respect to β-actin mRNA in BE2-M17 cell lines. “WT” denotes BE2-M17 cell line stably transduced with lentiviral inducible overexpression cassette containing PINK1. “D362A” denotes BE2-M17 cell line stably transduced with lentiviral overexpression cassette containing PINK1 mutant bearing kinase inactivating D362A mutation. “ShPINK1mir” denotes BE2-M17 cell line stably transduced with lentiviral shRNAmir targeting PINK1 mRNA. “pGIPz NSC” denotes BE2-M17 cell line stably transduced with lentiviral non-silencing shRNA. “Dox” denotes 4 hour exposure to doxycycline. Data represent geometric mean ± 95% confidence interval of three independent experiments. Bonferroni multiple comparison test was used to compare all pairs of columns at P<0.001.
Figure 3.27. *In vitro* P\(^{32}\)-labelling of putative protein substrates and downstream mediators of PINK1 in the mitochondria isolated from BE2-M17 cell line stably transduced with inducible PINK1 overexpression cassette.

Mitochondria were extracted from BE2-M17 cells overexpressing PINK1 after 4 hours of exposure to doxycycline. The isolated mitochondria were treated with [\(\gamma-P^{32}\)]-ATP and resolved using 10% SDS-PAGE. “WT” denotes BE2-M17 cell line stably transduced with lentiviral inducible overexpression cassette containing PINK1. “D362A” denotes BE2-M17 cell line stably transduced with lentiviral overexpression cassette containing PINK1 mutant bearing kinase inactivating D362A mutation. “ShPINK1mir” denotes BE2-M17 cell line stably transduced with lentiviral shRNAmiR targeting PINK1 mRNA. “pGIPz NSC” denotes BE2-M17 cell line stably transduced with lentiviral non-silencing shRNA. “Dox” denotes 4 hour exposure to doxycycline.
Hypotonic and mechanical lysis

 Autoradiograms
Figure 3.28. Two-dimensional gel electrophoresis of mitochondrial proteome of BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette.

A) Silver-stained 7cm two-dimensional gel electrophoresis of mitochondrial proteomes of BE2-M17 cell line stably transduced with lentiviral PINK1 inducible overexpression cassette. “WT” denotes BE2-M17 cell line stably transduced with lentiviral inducible overexpression cassette containing PINK1. “Dox” denotes 4 hour exposure to doxycycline.

B) Close-up view of an up-regulated protein spot in the doxycycline-treated BE2-M17 cells.

C) Result for identity of induced spot following tryptic digestion, nanoCHIP 3D Ion Trap mass spectrometry and Mascot MS/MS Ion search. The theoretical weight and pI of sideroflexin-1 matched the migration of the induced spot on the gel, adding to identity confidence.
A) WT WT + Dox

Silver-stained 7cm 2D gels

B)

C) Sideroflexin-1, 36kDa, pI 9.22

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Figure 3.29. Two-dimensional gel electrophoresis of mitochondrial proteome from BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette.

Silver-stained 24cm two-dimensional gel electrophoresis of mitochondrial proteomes of BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette. “WT” denotes BE2-M17 cell line stably transduced with lentiviral inducible overexpression cassette containing PINK1. “Dox” denotes 4 hour exposure to doxycycline.
Silver-stained 24cm 2D gels
Figure 3.30. Two-dimensional differential gel electrophoresis of mitochondrial proteome of BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette.

A) 24cm two-dimensional differential gel electrophoresis (2D-DIGE) of mitochondrial proteomes of BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette. Green outlined protein spots indicate the protein spots of similar levels in the cells with basal expression and induced overexpression of PINK1.

B) 24cm two-dimensional differential gel electrophoresis (2D-DIGE) of mitochondrial proteomes of BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette. Blue outlined protein spots indicate the protein spots of increased expression in the cells with induced overexpression of PINK1.

C) 24cm two-dimensional differential gel electrophoresis (2D-DIGE) of mitochondrial proteomes of BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette. Red outlined protein spots indicate the protein spots of decreased expression in the cells with induced overexpression of PINK1.

“WT” denotes BE2-M17 cell line stably transduced with lentiviral inducible overexpression cassette containing PINK1. “Dox” denotes 4 hour exposure to doxycycline. Protein spots that are identified by mass spectrometry are indicated by arrow and its protein spot number.
A) Similar expression

B) Increased expression

C) Decreased expression

Cy3 stain

Cy5 stain
Table 3.3. Summary table of protein spots detected in the two-dimensional differential gel electrophoresis of mitochondrial proteome of BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette.

“WT” denotes BE2-M17 cell line stably transduced with lentiviral inducible overexpression cassette containing PINK1. “Dox” denotes 4 hour exposure to doxycycline.
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<th>Protein expression levels of WT + dox with respect to WT</th>
<th>No. of protein spots</th>
<th>Percentage of the mitochondrial proteome</th>
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<tr>
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<tr>
<td>Increased expression</td>
<td>12</td>
<td>0.606%</td>
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Figure 3.31. Protein spot 1147 is up-regulated during the overexpression of PINK1 in BE2-M17 cells.

A) Close-up view of protein spot 1147 in the 2D-DIGE of the mitochondrial proteome of BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette.

B) DeCyder program comparative analysis of the protein level of protein spot 1147 in the cells with basal expression and induced overexpression of PINK1.

C) Result for identity of up-regulated protein spot 1147 following tryptic digestion, nanoCHIP 3D Ion Trap mass spectrometry and Mascot MS/MS Ion search. The theoretical weight and pI of Dihydrolipoyl dehydrogenase matched the migration of the induced spot on the gel, adding to identity confidence.

“WT” denotes BE2-M17 cell line stably transduced with lentiviral inducible overexpression cassette containing PINK1. “Dox” denotes 4 hour exposure to doxycycline.
A) WT

B)

C) Dihydrolipoyl dehydrogenase, 54kDa, pI 7.95 (Spot 1147, +1.59 fold)

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Figure 3.32. Protein spot 531 is down-regulated during the overexpression of PINK1 in BE2-M17 cells.

A) Close-up view of protein spot 531 in the 2D-DIGE of the mitochondrial proteome of BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette.

B) DeCyder program comparative analysis of the protein level of protein spot 531 in the cells with basal expression and induced overexpression of PINK1.

C) Result for identity of down-regulated protein spot 531 following tryptic digestion, nanoCHIP 3D Ion Trap mass spectrometry and Mascot MS/MS Ion search. The theoretical weight and pI of Leucine-rich PPR motif-containing protein matched the migration of the induced spot on the gel, adding to identity confidence.

“WT” denotes BE2-M17 cell line stably transduced with lentiviral inducible overexpression cassette containing PINK1. “Dox” denotes 4 hour exposure to doxycycline.
**Leucine-rich PPR motif-containing protein.** 158kDa, pI 5.81 (Spot 531, -2.45 fold)

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Figure 3.33. Protein spot 543 is down-regulated during the overexpression of PINK1 in BE2-M17 cells.

A) Close-up view of protein spot 543 in the 2D-DIGE of the mitochondrial proteome of BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette.
B) DeCyder program comparative analysis of the protein level of protein spot 543 in the cells with basal expression and induced overexpression of PINK1.
C) Result for identity of down-regulated protein spot 543 following tryptic digestion, nanoCHIP 3D Ion Trap mass spectrometry and Mascot MS/MS Ion search. The theoretical weight and pI of Leucine-rich PPR motif-containing protein matched the migration of the induced spot on the gel, adding to identity confidence.

“WT” denotes BE2-M17 cell line stably transduced with lentiviral inducible overexpression cassette containing PINK1. “Dox” denotes 4 hour exposure to doxycycline.
| Leucine-rich PPR motif-containing protein, 158kDa, pI 5.81 (Spot 543, -2.62 fold) |
|---|---|---|---|---|---|---|---|---|
| Observed | Mr(expt) | Mr(calc) | Score | Expect | Rank | Unique | Peptide |
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| 692.4900 | 1382.9654 | 1382.7293 | 55 | 0.00084 | 1 | U | K.VIEQLEPAVEK.I |
| 738.4500 | 1474.8854 | 1473.8377 | 18 | 7.7 | 2 | U | K.MVFINNIALAQIK.N |

A) WT WT + Dox

B) [Images of Western blots showing WT and WT + Dox conditions]

C) [Images of protein visualizations]
Figure 3.34. Protein spot 700 is down-regulated during the overexpression of PINK1 in BE2-M17 cells.

A) Close-up view of protein spot 700 in the 2D-DIGE of the mitochondrial proteome of BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette.

B) DeCyder program comparative analysis of the protein level of protein spot 700 in the cells with basal expression and induced overexpression of PINK1.

C) Result for identity of down-regulated protein spot 700 following tryptic digestion, nanoCHIP 3D Ion Trap mass spectrometry and Mascot MS/MS Ion search. The theoretical weight and pI of LON protease homolog matched the migration of the induced spot on the gel, adding to identity confidence.

“WT” denotes BE2-M17 cell line stably transduced with lentiviral inducible overexpression cassette containing PINK1. “Dox” denotes 4 hour exposure to doxycycline.
**A**)

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**B**)

| ![Image](image3.png) | ![Image](image4.png) |

**C**)

Lon protease homolog, 106kDa, pl 6.01 (Spot 700, -2.97 fold)

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Figure 3.35. Protein spot 1130 is down-regulated during the overexpression of PINK1 in BE2-M17 cells.

A) Close-up view of protein spot 1130 in the 2D-DIGE of the mitochondrial proteome of BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette.

B) DeCyder program comparative analysis of the protein level of protein spot 1130 in the cells with basal expression and induced overexpression of PINK1.

C) Result for identity of down-regulated protein spot 1130 following tryptic digestion, nanoCHIP 3D Ion Trap mass spectrometry and Mascot MS/MS Ion search. The theoretical weight and pI of TRAP1 matched the migration of the induced spot on the gel, adding to identity confidence.

“WT” denotes BE2-M17 cell line stably transduced with lentiviral inducible overexpression cassette containing PINK1. “Dox” denotes 4 hour exposure to doxycycline.
### A)

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### B)

![Images of protein spots](image1)

### C)

**TRAP1, 80kDa, pI 8.3 (Spot 1130, -2.38 fold)**

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Figure 4.1. The workflow of *in vitro* mitochondrial import assay for PINK1

*In vitro* transcription was carried out by incubating linearised pSP64 plasmid containing PINK1-Flag DNA construct, ribonucleotide tri-phosphates and SP6 polymerase. The SP6 promoter residing in the plasmid recruits the SP6 polymerase to initiate transcription of the PINK1 DNA construct. The resulting reaction was then incubated with [S\textsuperscript{35}]-Methionine, other amino acids and rabbit reticulocyte lysate to translate the PINK1 mRNA generated in the previous reaction. To examine whether the nascent PINK1 protein could be imported into the mitochondria, the translation reaction was incubated with isolated respiring mitochondria for 30 mins to allow the mitochondrial import of PINK1 to occur. After 30 mins, some mitochondrial import reactions were treated with Proteinase K. A reaction was treated with Proteinase K but in a hypotonic solution to selectively rupture the outer membrane of the mitochondria to expose the proteins in the inter-membrane space for Proteinase K digestion. Another parallel reaction was treated with Proteinase K in the presence of Triton X-100 to allow the rupture of both outer and inner membranes of the mitochondria to expose matrix proteins for Proteinase K digestion. The last reaction was not treated with Proteinase K. After the Proteinase K treatment, all the reactions were centrifuged to collect the mitochondria and remove S\textsuperscript{35}-labelled PINK1 polypeptides that were not associated with the mitochondria. The resulting mitochondrial pellets were washed and collected. The resulting mitochondrial pellets were then resolved using SDS-PAGE and autoradiograms of the SDS-PAGE gels were taken.
**In vitro Transcription**

- Linearised pSP64 plasmid
- + rNTP
- + SP6 Polymerase

**In vitro Translation**

- PINK1 mRNA
- + S^35-Met
- + Amino Acids
- + Rabbit Reticulocyte Lysate

**In vitro Mitochondrial Protein Import**

- S^35-labelled PINK1 Polypeptide
- + Respiring Mitochondria

**Proteinase K treatment**

- Intact Mitochondria
- Mitoplasting via hypotonic treatment
- Solubilised Mitochondria via detergent treatment

**Proteolysis**

- Proteolysis of OM proteins
- Proteolysis of OM, IMS and IMS-facing IM proteins
- Proteolysis of all mitochondrial proteins

**Centrifuge and wash with import assay buffer**

**SDS-PAGE**
Figure 4.2. *In vitro* translated $S^{35}$-labelled nascent PINK1 polypeptide aggregates upon heat treatment at 100°C

Linearised pSP64 plasmid containing PINK1-Flag construct was transcribed using SP6 polymerase. The resulting *in vitro* mRNA was translated with $[S^{35}]$-methionine and other amino acids using rabbit reticulocyte lysate. The translation reaction was boiled or heated to 70°C and then resolved by 8% SDS-PAGE. Autoradiogram of the SDS-PAGE gel was obtained using Phosphorimager.
**In vitro Transcription**

Linormed p2216 plasmid

+ ATP
+ SP6 Polymerase

**In vitro Translation**

+ S35-Met
+ Amino Acids
+ Rabbit Reticolocyte Lysate

**In vitro Mitochondrial Protein Import**

+ Boiling Mitochondria

**Proteinase K treatment**

**Autoradiogram**

- 175kDa
- 82kDa
- 62kDa
- 47.5kDa
- 32.5kDa
- 25kDa

**Stacking Gel**

**Separating Gel**

Boiled S35-labelled PINKI polypeptide
70°C-heated S35-labelled PINKI polypeptide

Aggregated S35-PINK1

S35-PINK1

Centrifuge and wash with import assay buffer

SDS-PAGE
Figure 4.3. *In vitro* mitochondrial import assay of PINK1 using yeast mitochondria

Autoradiogram of 10% SDS-PAGE gel containing the *in vitro* mitochondrial import reaction using yeast mitochondria. ‘Mito’ denotes isolated mitochondria. ‘PK’ denotes Proteinase K treatment. ‘-Δψ mito’ denotes mitochondria depolarised by antimycin A, valinomycin and oligomycin. ‘TX-100’ denotes Triton X-100.
Autoradiogram

- Proteolysis of OM proteins
- Proteolysis of OM, IMS and IMS-facing IM proteins
- Proteolysis of OM proteins
- Proteolysis of OM proteins

35S-labeled PINK1

Proteolysis of all mitochondrial proteins

Intact yeast mitochondria

Mito + PK

Mitoplast treatment with PK

TX-100 treatment with PK

Mito + S35-labeled PINK1

Mito + S35-labeled PINK1 + PK

Mito + S35-labeled PINK1 + PK

Mito + S35-labeled PINK1

Proteolysis of OM proteins

-175kDa

-155kDa

-135kDa

-115kDa

-95kDa

-75kDa

-55kDa

-35kDa

-15kDa

-5kDa
Figure 4.4. *In vitro* mitochondrial import assay of PINK1 using mouse liver mitochondria

Autoradiogram of 10% SDS-PAGE gel containing the *in vitro* mitochondrial import reaction using liver mitochondria. ‘Mito’ denotes isolated mitochondria. ‘PK’ denotes Proteinase K treatment. ‘Δψ mito’ denotes mitochondria depolarised by antimycin A, valinomycin and oligomycin. ‘TX-100’ denotes Triton X-100.
Mitoplast treatment with PK
TX-100 treatment with PK
Mito + PK
Mito + S\(^{35}\)-labelled PINK1

 Autoradiogram

Proteolysis of OM proteins
Intact liver mitochondria
Proteolysis of OM proteins
Proteolysis of OM proteins
Proteolysis of OM, IMS and IMS-facing IM proteins

 Autoradiogram

Proteolysis of OM proteins

 Autoradiogram

Proteolysis of OM proteins

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Proteolysis of OM proteins

 Autoradiogram
Figure 4.5. *In vitro* mitochondrial import assay of PINK1 using mouse brain mitochondria

 Autoradiogram of 10% SDS-PAGE gel containing the *in vitro* mitochondrial import reaction using brain mitochondria. ‘Mito’ denotes isolated mitochondria. ‘PK’ denotes Proteinase K treatment. ‘-ΔΨ mito’ denotes mitochondria depolarised by antimycin A, valinomycin and oligomycin. ‘TX-100’ denotes Triton X-100.
Mitoplast treatment with PK
TX-100 treatment with PK
Mitoplast + S<sup>35</sup>-labelled PINK1 + PK
Mito + S<sup>35</sup>-labelled PINK1 + PK
Mito + PK
S<sup>35</sup>-labelled PINK1

Proteolysis of OM proteins
Proteolysis of OM, IMS and IMS-facing IM proteins
Proteolysis of all mitochondrial proteins

Intact brain mitochondria

Autoradiogram
Figure 4.6. PINK1-Flag-GFP was not expressed in a yeast overexpression system.

A) PINK1-Flag-GFP DNA construct in pGFP-C-Fus plasmid
B) Western blot of cell lysates of yeast transformed with pGFP-C-Fus plasmid containing PINK1-Flag-GFP and pGFP-C-Fus plasmid.
A) 

MTS | TM | Kinase | Tail | Flag | GFP

B) 

Theoretical position of PINK1-Flag-GFP

IB: Anti-GFP
Figure 4.7. PINK1 resides on the surface of the mitochondria of Sf9 cells

A) Sf9-codon-optimised PINK1 DNA construct in recombinant baculovirus.
B) Western blot of isolated mitochondria from Sf9 cells overexpressing Sf9-codon-optimised PINK1 using antibodies targeting residues 511-526 of PINK1, α-tubulin, VDAC1 (purchased from Sigma-Aldrich) and cytochrome c (purchased from Abcam). ‘Mito’ denotes isolated mitochondria. ‘PK’ denotes Proteinase K treatment. ‘TX-100’ denotes Triton X-100.
A)  

MTS  TM  Kinase  Tail  Myc  12His  

B)  

IB: Affinity-purified anti-PINK1 (511-526)  
IB: Anti-αTubulin  
IB: VDAC1  
IB: Anti-Cytochrome c  

Proteolysis of OM proteins  
Leakage of IMS proteins  
Proteolysis of OM, IMS and IMS-facing IM proteins  
Intact S9 mitochondria
Figure 4.8. Processed PINK1 contains putative transmembrane domain

A) Workflow for the investigation of the N-terminal boundary of processed PINK1 isolated from the mitochondria of $S_f9$ cells overexpressing $S_f9$-codon optimised PINK1-Myc-12x-His.

B) Coomassie-stained 10% SDS-PAGE gel containing concentrated elution of denaturing Ni-NTA affinity chromatography. The processed PINK1 of approximately 55kDa was analysed by mass spectrometry (NanoCHIP 3D Ion Trap). PINK1 peptide containing putative transmembrane domain of PINK1 is indicated by red amino acid sequence.
2L culture of SF9 cells overexpressing PINK1-Myc-12His

Isolation of mitochondria via differential centrifugation

Guanidine hydrochloride and detergent extraction

Urea-denaturing Ni-NTA affinity chromatography

Concentration of elution fractions

10% SDS-PAGE

Mass Spectrometry

A) M/S Peptide Coverage

R. AVFALGGLGLIEEK. Q    (R98 to Q115)
R. LEYLIQSIGK. G    (R152 to G165)
R. AFGAFPLA. K    (R207 to M220)
R. AFPSVPLLPGALVDYPDVLPSR. L    (R279 to L303)
R. SQAQALPESVPQDR. Q    (R464 to Q484)
K. MVGWLQQSAATLARN. L    (K526 to L544)

B) Concentrated Ni-NTA affinity Purified urea-denatured PINK1-Myc-12His

Comassie-stained SDS-PAGE gel

PINK1 region

Guanidine hydrochloride and detergent extraction

Urea-denaturing Ni-NTA affinity chromatography

Concentration of elution fractions

10% SDS-PAGE

Mass Spectrometry

B) Concentrated Ni-NTA affinity Purified urea-denatured PINK1-Myc-12His
Figure 4.9. PINK1 on the surface of Sf9 mitochondria is unable to phosphorylate recombinant Parkin and TRAP1

A) Autoradiogram of the phosphorylation reactions with the mitochondria isolated from Sf9 cells overexpressing PINK1 or PINK1 mutant bearing kinase-inactivating D362A mutation and Parkin or TRAP1 resolved by 10% SDS-PAGE

B) Western blots of Proteinase K treated mitochondria isolated from Sf9 cells overexpressing wild-type PINK1 or PINK1 mutant bearing kinase-inactivating D362A mutation using antibody targeting residues 258-274 of PINK1. The Western blots indicated that both PINK1 proteins resided on the surface of the isolated Sf9 mitochondria.

Figure 4.10. PINK1 is degraded rapidly in healthy mitochondria but mediates mitophagy in potential-compromised mitochondria.

PINK1 is constantly imported into the mitochondria and probably to the inner membrane of the mitochondria due to its canonical mitochondrial targeting sequence and putative transmembrane domain. In potential-compromised mitochondria, the mitochondrial import machinery lacks the potential gradient to import the putative transmembrane domain of PINK1, resulting in the folding of PINK1 on the surface of the mitochondria. The folded PINK1 then recruits Parkin to the mitochondria via its kinase activity to initiate mitophagy to clear the unhealthy mitochondria to prevent pathogenic generation of ROS and apoptosis.
Mitophagy

Rapid degradation of PINK1

Compromised potential

Healthy mitochondrion

Accumulation of PINK1

Recruitment of Parkin

No Mitophagy

Tail

MTS

Kinase

Parkin

Mitophagy

Healthy mitochondrion

No Mitophagy

Tail

MTS

Kinase
Figure 5.1. PINK1 promotes cell survival during the inhibition of mitochondrial complex I.

A) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 5nM rotenone.
B) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 60nM rotenone.
C) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 250nM rotenone.

“WT + Dox” denotes BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette with 4 hours of induced overexpression of PINK1 upon rotenone treatment.

“D362A + Dox” denotes BE2-M17 cell line stably transduced with lentiviral inducible PINK1 kinase inactive mutant overexpression cassette with 4 hours of induced overexpression of PINK1 upon rotenone treatment.

“ShPINK1mir” denotes BE2-M17 cell line stably transduced with lentiviral PINK1-targeting ShRNAmir.

“Control M17” denotes control BE2-M17 cell line.

“WT” denotes BE2-M17 cell line stably transduced with inducible lentiviral PINK1 overexpression cassette.

“D362A” denotes BE2-M17 cell line stably transduced with inducible lentiviral PINK1 kinase inactive mutant overexpression cassette.

“pGIPz NSC” denotes BE2-M17 cell line stably transduced with lentiviral ShRNAmir with scrambled sequence (Non-silencing control).

Data represent geometric mean ± 95% confidence interval of 11 independent experiments. Bonferroni multiple comparison test was used to compare all pairs of columns at P<0.001. “N.S” denotes “Not Significant”.
A) 5nM Rotenone

B) 60nM Rotenone

C) 250nM Rotenone
Figure 5.2. PINK1 does not promote cell survival during the inhibition of mitochondrial complex III.

A) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 250nM antimycin A
B) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 60nM antimycin A
C) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 5nM antimycin A

“WT + Dox” denotes BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette with 4 hours of induced overexpression of PINK1 upon rotenone treatment.
“D362A + Dox” denotes BE2-M17 cell line stably transduced with lentiviral inducible PINK1 kinase inactive mutant overexpression cassette with 4 hours of induced overexpression of PINK1 upon rotenone treatment.
“ShPINK1mir” denotes BE2-M17 cell line stably transduced with lentiviral PINK1-targeting ShRNAmir.
“Control M17” denotes control BE2-M17 cell line.
“WT” denotes BE2-M17 cell line stably transduced with inducible lentiviral PINK1 overexpression cassette.
“D362A” denotes BE2-M17 cell line stably transduced with inducible lentiviral PINK1 kinase inactive mutant overexpression cassette.
“pGIPz NSC” denotes BE2-M17 cell line stably transduced with lentiviral ShRNAmir with scrambled sequence (Non-silencing control).

Data represent geometric mean ± 95% confidence interval of 11 independent experiments. Bonferroni multiple comparison test was used to compare all pairs of columns at P<0.001. “N.S” denotes “Not Significant”.
A) 250nM Antimycin A

B) 60nM Antimycin A

C) 5nM Antimycin A

Abs of Formazan in Treated/Untreated cells

M17 Cell Lines

N.S

B)

Abs of Formazan in Treated/Untreated cells

M17 Cell Lines

N.S

C)

Abs of Formazan in Treated/Untreated cells

M17 Cell Lines

N.S
Figure 5.3. PINK1 does not promote cell survival during the inhibition of mitochondrial complex IV.

A) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 90mM sodium azide.
B) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 5mM sodium azide.
C) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 0.5mM sodium azide.

“WT + Dox” denotes BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette with 4 hours of induced overexpression of PINK1 upon rotenone treatment.

“D362A + Dox” denotes BE2-M17 cell line stably transduced with lentiviral inducible PINK1 kinase inactive mutant overexpression cassette with 4 hours of induced overexpression of PINK1 upon rotenone treatment.

“ShPINK1mir” denotes BE2-M17 cell line stably transduced with lentiviral PINK1-targeting ShRNAmir.

“Control M17” denotes control BE2-M17 cell line.

“WT” denotes BE2-M17 cell line stably transduced with inducible lentiviral PINK1 overexpression cassette.

“D362A” denotes BE2-M17 cell line stably transduced with inducible lentiviral PINK1 kinase inactive mutant overexpression cassette.

“pGIPz NSC” denotes BE2-M17 cell line stably transduced with lentiviral ShRNAmir with scrambled sequence (Non-silencing control).

Data represent geometric mean ± 95% confidence interval of 11 independent experiments. Bonferroni multiple comparison test was used to compare all pairs of columns at P<0.001.

“N.S” denotes “Not Significant”.

320
Figure 5.4. PINK1 does not promote cell survival during the inhibition of mitochondrial complex V.

A) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 2μM oligomycin.
B) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 1μM oligomycin.
C) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 250nM oligomycin.

“WT + Dox” denotes BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette with 4 hours of induced overexpression of PINK1 upon rotenone treatment.

“D362A + Dox” denotes BE2-M17 cell line stably transduced with lentiviral inducible PINK1 kinase inactive mutant overexpression cassette with 4 hours of induced overexpression of PINK1 upon rotenone treatment.

“ShPINK1mir” denotes BE2-M17 cell line stably transduced with lentiviral PINK1-targeting ShRNAmir.

“Control M17” denotes control BE2-M17 cell line.

“WT” denotes BE2-M17 cell line stably transduced with inducible lentiviral PINK1 overexpression cassette.

“D362A” denotes BE2-M17 cell line stably transduced with inducible lentiviral PINK1 kinase inactive mutant overexpression cassette.

“pGIPz NSC” denotes BE2-M17 cell line stably transduced with lentiviral ShRNAmir with scrambled sequence (Non-silencing control).

Data represent geometric mean ± 95% confidence interval of 11 independent experiments. Bonferroni multiple comparison test was used to compare all pairs of columns at P<0.001. “N.S” denotes “Not Significant”.

322
Figure 5.5. PINK1 does not promote cell survival during mitochondrial depolarisation.

A) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 250nM valinomycin.
B) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 60nM valinomycin.
C) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 5nM valinomycin.

“WT + Dox” denotes BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette with 4 hours of induced overexpression of PINK1 upon rotenone treatment.

“D362A + Dox” denotes BE2-M17 cell line stably transduced with lentiviral inducible PINK1 kinase inactive mutant overexpression cassette with 4 hours of induced overexpression of PINK1 upon rotenone treatment.

“ShPINK1mir” denotes BE2-M17 cell line stably transduced with lentiviral PINK1-targeting ShRNAmir.

“Control M17” denotes control BE2-M17 cell line.

“WT” denotes BE2-M17 cell line stably transduced with inducible lentiviral PINK1 overexpression cassette.

“D362A” denotes BE2-M17 cell line stably transduced with inducible lentiviral PINK1 kinase inactive mutant overexpression cassette.

“pGIPz NSC” denotes BE2-M17 cell line stably transduced with lentiviral ShRNAmir with scrambled sequence (Non-silencing control).

Data represent geometric mean ± 95% confidence interval of 11 independent experiments. Bonferroni multiple comparison test was used to compare all pairs of columns at P<0.001. “N.S” denotes “Not Significant”. 
Figure 5.6. PINK1 does not promote cell survival during oxidative stress induced by hydrogen peroxide.

A) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 6mM hydrogen peroxide.
B) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 1mM hydrogen peroxide.
C) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 0.4mM hydrogen peroxide.

“WT + Dox” denotes BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette with 4 hours of induced overexpression of PINK1 upon rotenone treatment.

“D362A + Dox” denotes BE2-M17 cell line stably transduced with lentiviral inducible PINK1 kinase inactive mutant overexpression cassette with 4 hours of induced overexpression of PINK1 upon rotenone treatment.

“ShPINK1mir” denotes BE2-M17 cell line stably transduced with lentiviral PINK1-targeting ShRNAmir.

“Control M17” denotes control BE2-M17 cell line.

“WT” denotes BE2-M17 cell line stably transduced with inducible lentiviral PINK1 overexpression cassette.

“D362A” denotes BE2-M17 cell line stably transduced with inducible lentiviral PINK1 kinase inactive mutant overexpression cassette.

“pGIPz NSC” denotes BE2-M17 cell line stably transduced with lentiviral ShRNAmir with scrambled sequence (Non-silencing control).

Data represent geometric mean ± 95% confidence interval of 11 independent experiments. Bonferroni multiple comparison test was used to compare all pairs of columns at P<0.001. “N.S” denotes “Not Significant”. 

326
A) 6mM H₂O₂

B) 1mM H₂O₂

C) 0.4mM H₂O₂
Figure 5.7. PINK1 does not promote cell survival during oxidative stress induced by 6-hydroxydopamine.

A) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 1.4mM 6-hydroxydopamine.

B) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 45 μM 6-hydroxydopamine.

C) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 20 μM 6-hydroxydopamine.

“WT + Dox” denotes BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette with 4 hours of induced overexpression of PINK1 upon rotenone treatment.

“D362A + Dox” denotes BE2-M17 cell line stably transduced with lentiviral inducible PINK1 kinase inactive mutant overexpression cassette with 4 hours of induced overexpression of PINK1 upon rotenone treatment.

“ShPINK1mir” denotes BE2-M17 cell line stably transduced with lentiviral PINK1-targeting ShRNAmir.

“Control M17” denotes control BE2-M17 cell line.

“WT” denotes BE2-M17 cell line stably transduced with inducible lentiviral PINK1 overexpression cassette.

“D362A” denotes BE2-M17 cell line stably transduced with inducible lentiviral PINK1 kinase inactive mutant overexpression cassette.

“pGIPz NSC” denotes BE2-M17 cell line stably transduced with lentiviral ShRNAmir with scrambled sequence (Non-silencing control).

Data represent geometric mean ± 95% confidence interval of 11 independent experiments. Bonferroni multiple comparison test was used to compare all pairs of columns at P<0.001. “N.S” denotes “Not Significant”.
A) 1.4mM 6-Hydroxydopamine

B) 45μM 6-Hydroxydopamine

C) 20μM 6-Hydroxydopamine
Figure 5.8. PINK1 does not promote cell survival during excitotoxicity.

A) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 25mM L-glutamate.

B) Relative viability of BE2-M17 cell lines during the 16 hour treatment with 12mM L-glutamate.

“WT + Dox” denotes BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette with 4 hours of induced overexpression of PINK1 upon rotenone treatment.

“D362A + Dox” denotes BE2-M17 cell line stably transduced with lentiviral inducible PINK1 kinase inactive mutant overexpression cassette with 4 hours of induced overexpression of PINK1 upon rotenone treatment.

“ShPINK1mir” denotes BE2-M17 cell line stably transduced with lentiviral PINK1-targeting ShRNAmir.

“Control M17” denotes control BE2-M17 cell line.

“WT” denotes BE2-M17 cell line stably transduced with inducible lentiviral PINK1 overexpression cassette.

“D362A” denotes BE2-M17 cell line stably transduced with inducible lentiviral PINK1 kinase inactive mutant overexpression cassette.

“pGIPz NSC” denotes BE2-M17 cell line stably transduced with lentiviral ShRNAmir with scrambled sequence (Non-silencing control).

Data represent geometric mean ± 95% confidence interval of 11 independent experiments. Bonferroni multiple comparison test was used to compare all pairs of columns at P<0.001. “N.S” denotes “Not Significant”.
A) 25mM L-Glutamate

![Graph showing Abs of Formazan in Treated/Untreated cells for different cell lines under 25mM L-Glutamate treatment.]

B) 12mM L-Glutamate

![Graph showing Abs of Formazan in Treated/Untreated cells for different cell lines under 12mM L-Glutamate treatment.]

M17 Cell Lines

N.S

Abs of Formazan in Treated/Untreated cells
Figure 5.9. PINK1 does not influence cellular ROS level.

Fluorescence microscopy of DHR123-treated BE2-M17 cell lines after 16 hour treatment with 5nM rotenone.
“WT + Dox” denotes BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette with 4 hours of induced overexpression of PINK1 upon rotenone treatment.
“WT” denotes BE2-M17 cell line stably transduced with inducible lentiviral PINK1 overexpression cassette.
WT + dox  WT  WT + dox  WT

Rhodamine123 Fluorescence

Phase Contrast

5nM Rotenone  0nM Rotenone
Figure 5.10. PINK1 does not influence cellular ROS level.

Fluorescence microscopy of NAO-treated BE2-M17 cell lines after 16 hour treatment with 5nM rotenone.
“WT + Dox” denotes BE2-M17 cell line stably transduced with lentiviral inducible PINK1 overexpression cassette with 4 hours of induced overexpression of PINK1 upon rotenone treatment.
“WT” denotes BE2-M17 cell line stably transduced with inducible lentiviral PINK1 overexpression cassette.
WT

WT + dox

NAO Fluorescence

Phase Contrast

0nM Rotenone

5nM Rotenone
Figure 6.1. PINK1 is degraded rapidly in healthy mitochondria but mediates mitophagy in potential-compromised mitochondria by regulating the mitochondrial expression of proteins.

PINK1 is constantly imported into the mitochondria and probably to the inner membrane of the mitochondria due to its canonical mitochondrial targeting sequence and putative transmembrane domain. In potential-compromised mitochondria, the mitochondrial import machinery lacks the potential gradient to import the putative transmembrane domain of PINK1, resulting in the folding of PINK1 on the surface of the mitochondria. The folded PINK1 then recruits Parkin to the mitochondria. The mitochondrial translocated Parkin and PINK1 interact with a mitochondrial translation regulatory protein, LRPPRC, to prevent its matrix localisation, possibly by proteasomal degradation. In addition, the protein expression of a mitochondrial transcription regulatory protein, LONP1 is down-regulated by the accumulation of PINK1 on the surface of the potential-compromised mitochondria. The mitochondrial depletion of LRPPRC and LONP1 result in the expression of mitochondria-encoded proteins involved in mitophagy of the potential-compromised mitochondrion.
Healthy mitochondrion

Rapid degradation of PINK1

No Mitophagy

Compromised potential

Accumulation of PINK1

Recruitment of Parkin

Parkin intercepts LRPPRC

Occlusion of LRPPRC and LONP1

Mitophagy
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