PTEN-INDUCED KINASE 1 (PINK1) AND ITS ROLE IN MITOCHONDRIAL FUNCTION AND DYNAMICS

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ABSTRACT

Parkinson disease (PD) is a neurodegenerative disorder with progressive loss of dopaminergic neurons in the substantia nigra. The majority of PD cases involve the sporadic form with unknown etiology, but mitochondrial dysfunction and oxidative stress are considered to play a role in disease pathogenesis. The discoveries of the genes parkin, PINK1 and DJ-1 that are linked to familial forms of parkinsonism have provided insight into the molecular mechanisms that cause disease. Recent findings implicate mitochondrial dysfunction associated with oxidative damage as a key molecular mechanism that compromises dopaminergic neurons in familial parkinsonism. Studies within this thesis characterize the mitochondrial function of PINK1 in human neuroblastoma cells. PINK1 prevents cell death mediated by mitochondrial toxins that inhibit complex one of the respiratory chain, which in turn contributes to oxidative stress. PINK1 protects against oxidative stress and secondary mitochondrial dysfunction; therefore, the role of PINK1 in mitochondrial function and morphology was further investigated.

Mitochondria are dynamic organelles that undergo the antagonizing events of fusion and fission. While fusion of mitochondrial membranes promotes cell survival, mitochondrial fission is associated with apoptosis. Defects in mitochondrial membrane
potential influence mitochondrial morphology in PINK1 deficient cells shifting phenotypes towards Dynamin-related protein 1 (Drp1)-mediated fission. PINK1 silencing results in mitochondrial fission by enhancing calcineurin activity, which promotes mitochondrial fragmentation via Drp1 dephosphorylation and increased Drp1 GTPase activity.

Exogenous expression of parkin in PINK1 deficient cells rescues mitochondrial fission, which confirms that PINK1 and parkin genetically interact to regulate mitochondrial morphology. Cells deficient in parkin or DJ-1 phenocopy PINK1 silencing and exhibit mitochondrial fission. Dephosphorylation of Drp1 is enhanced in DJ-1 deficient cells, suggesting that modulation of fission machinery may be dependent upon the generation of oxidative stress. PINK1, parkin and DJ-1 all act individually to limit the effects of agents that induce oxidative stress and trigger mitochondrial fission. Expression of PINK1 or parkin in DJ-1 deficient cells rescues mitochondrial phenotypes associated with DJ-1 silencing, which suggests DJ-1 lies upstream of the PINK1/parkin pathway to inhibit mitochondrial fission. These studies suggest that oxidative stress can influence mitochondrial shape, structure and function, which might contribute to parkinsonian phenotypes.
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CHAPTER ONE

Background and significance
INTRODUCTION

The studies in this thesis describe the mitochondrial function of gene products that are associated with autosomal recessive forms of parkinsonism. These rare, inherited mutations that cause parkinsonism may provide insight into the molecular mechanisms that contribute to both genetic and sporadic forms of Parkinson disease. Mitochondrial dysfunction has been implicated in sporadic Parkinson disease for more than 20 years; however, the identification of loss-of-function mutations in the PINK1 (PTEN-induced kinase 1) gene, which encodes a mitochondrial protein kinase, renewed interest in mitochondria as a pathological center for disease etiology. Data will be introduced in Chapter Three that describes the role of oxidative stress in PINK1 mRNA modulation and its relevance to parkinsonism using a neuron-like model. Chapter Four characterizes the functional effects of this PINK1 modulation and Chapter Five describes the implications of PINK1 in mitochondrial morphogenesis. Additionally, loss-of-function mutations in other recessive parkinsonism genes, parkin and DJ-1 also contribute to mitochondrial dysfunction. Chapter Six discusses the role of recessive parkinsonism genes in mitochondrial function, emphasizing how mitochondrial dynamics are altered by loss-of-function mutations.

To better understand the definition, pathology and etiology associated with Parkinson disease, these topics are discussed in section 1.1. A review of monogenic forms of autosomal recessive parkinsonism is provided in section 1.2. Proposed molecular pathways to parkinsonism are explored in sections 1.3 and 1.4. Possible regulatory molecular pathways that may provide clues for therapeutic design and
rationale for future studies in mitochondrial dysfunction found in Parkinson disease are shown in section 1.5. Lastly, the statement of purpose is defined in section 1.6.
1.1 INTRODUCTION TO PARKINSON DISEASE

1.1.1 Parkinson disease and parkinsonism defined

In 1817 a British physician, James Parkinson, noted clinical features of elderly male patients suffering from an unknown malady—involuntary tremor with lessened muscle power and a propensity for the trunk to lean forward with intact senses and intelligence. This case study, “An Essay on the Shaking Palsy,” described the clinical presentation he called *paralysis agitans* (Parkinson, 1817). William Gowers’ *Manual of Diseases of the Nervous System* also studied *paralysis agitans*, making observations on the amplitude and frequency of the tremor and problems in walking and balance. Gowers’ study emphasized rigidity, the expressionless face (flat affect), and monotonous speech in the disorder (Gowers, 1901). It was not until sixty years following Parkinson’s initial observation that a French neurologist, Jean-Martin Charcot, coined the term Parkinson disease (PD) to describe the same neurological disorder.

Presently, no diagnostic tests exist for PD and as such, physicians make diagnoses based upon a clinical examination. The current definition of the motor syndrome includes the presence of bradykinesia, gradual progressive muscle rigidity, resting tremors and postural instability. Initial clinical manifestations include mild periodic tremor and asymmetric rigidity. However, the symptoms become more severe, symmetrical and debilitating with disease progression. Individuals may also present non-motor symptoms such as fatigue, depression, anxiety, sleep disturbances, constipation, bladder problems and other autonomic disturbances (Fahn, 2003). Mild depression and mood changes have been suggested to be prodromic of PD (Stiasny-Kolster et al., 2005).

An atypical form of encephalitis caused a worldwide epidemic from 1918-1926
that resulted in millions of deaths. *Encephalitis lethargica* presented with flu-like symptoms—sleepiness, paralysis of eye movements, rigidity, slowness of movement, and behavioral changes. Most patients died in the acute phase, but those who survived were chronically disabled with symptoms resembling PD. This clinical phenotype resulting from *encephalitis lethargica* demonstrated that other syndromes could mimic the clinical definitions of PD. The discovery of PD-like syndromes paved the way for the distinction between PD and parkinsonism. Parkinsonism is a generic term that neurologists use to describe the clinical features of PD. Not all four features described in PD must be present for a parkinsonism diagnosis, but at least two of the cardinal symptoms should exist with at least one of them being bradykinesia or resting tremor (Fahn, 2000). Parkinson disease is primary parkinsonism, currently defined by the exclusion of known etiologies, and makes up the majority of all cases. But other classifications of parkinsonism exist (Fahn, 2000): secondary parkinsonism is composed of drug-, chemical-, prion-, or viral-induced parkinsonism; parkinsonism-plus syndromes include disorders resembling parkinsonism plus other neurological problems like multiple system atrophy (MSA) and progressive supranuclear palsy (PSP); lastly, when only one feature of parkinsonism is present in a hereditary degenerative disorder, such as Huntington’s disease or Wilson’s disease, they are classified as heredodegenerative disorders.

### 1.1.2 Pathology of PD

Neither Parkinson nor Charcot identified the precise brain area affected in PD. Further scrutiny of this area was not possible until *post mortem* examinations of the brain became available. The clinicopathology underlying Parkinson disease was first proposed
by a French neurologist, Edouard Brissaud, in 1895. He observed phenotypic similarities to PD in patients with lesions in the *substantia nigra* in the midbrain, suggesting damage to this area may cause the motor dysfunction seen in PD (Brissaud, 1895). However, it was Tretiakoff who confirmed this by examining nine encephalitic PD patients *post mortem* and found loss of pigmented cells of the *substantia nigra* and the presence of Lewy bodies (LBs) in the remaining cells (Tretiakoff, 1919). Frederick Lewy had previously described these spherical cytoplasmic inclusions in non-nigral areas (Forester, 1912). Forty years later Carlsson discovered the neurotransmitter dopamine (DA) was present in the mammalian brain with highest levels in the striatum (Carlsson, 1959, Carlsson et al., 1957, Carlsson et al., 1958, Sano et al., 1959). Dopamine levels are severely decreased in the striatum of patients with PD, as demonstrated by Ehringer and Hornykiewicz in 1960 (Ehringer and Hornykiewicz, 1960). The current pathological diagnosis of PD requires both the loss of dopaminergic cells in the *substantia nigra* and the presence LBs in surviving neurons in the presence of an intact striatum (Hughes et al., 2002, Hughes et al., 1992).

PD, parkinsonism and the parkinsonism-plus syndromes share degeneration of *substantia nigra pars compacta* (*SNpc*) dopaminergic neurons, resulting in a deficiency of striatal dopamine concentration (Fahn, 2000). Motor symptoms manifest after 50-60% of the dopamine-containing neurons in the *substantia nigra* are lost and when 70-80% of striatal dopamine is depleted (Seibyl et al., 1995). Positron emission tomography (PET) imaging and single photon emission computed tomography (SPECT) scanning can detect the progressive loss of [*F*-6-fluorodopa (*F*-dopa) and DA transporter ligand binding in the striatum (Benamer et al., 2000, Eidelberg et al., 1995, Morrish et al., 1996,
Vingerhoets et al., 1994). As the disease progresses, neuronal cell loss and the accumulation of non- and nigral LBs correlates with an increased risk of dementia (Braak et al., 2003a, Braak et al., 2003b, Jellinger, 2000). Roughly 20-40% of patients become demented during the course of the disease. Many patients show mild cognitive decline including impaired visual-spatial perception and attention, slowness in execution of motor tasks and impaired concentration. It is estimated that 40% of PD patients exhibit cognitive impairment, but this value may be underestimated (Jellinger, 2000, Jellinger, 2006).

The loss of pigmented dopaminergic neurons in the substantia nigra pars compacta is one of two pathological hallmarks of PD and the proximal cause of this movement disorder (Braak et al., 2003a, Braak et al., 2003b). The SNpc is located in the midbrain and is identifiable by dark pigmentation. This pigmentation is due to the production of neuromelanin, which is formed as a by-product of the synthesis of monoamine transmitters. During normal aging, significant numbers of SNpc neurons are found to degenerate (Mann et al., 1977). The most common current treatment for PD is to increase the level of dopamine in the SNpc by the administration of exogenous dopamine precursor L-3,4-dihydroxyphenylalanine (L-dopa). A limitation of L-dopa therapy is its waning effects, since dopamine is readily recycled. Initially L-dopa lessens PD symptoms, but over time its efficacy decreases and patients often develop dyskinesias or excess movements.

The second major pathological hallmark of Parkinson disease is the presence of Lewy bodies and Lewy Neurites (LN). These insoluble cytoplasmic inclusion bodies are found in dopaminergic neurons of the SNpc (Lewy, 1914), but can also be seen in other
types of neurons. For instance, LBs have been found in noradrenergic and cholinergic neurons in several brain regions but are rarely observed in motor neurons (Braak et al., 2003a). Lewy bodies have a dense central core surrounded by a halo of 7-10nm radiating fibrils, composed primarily of alpha-synuclein (Spillantini et al., 1997). Immunostaining experiments have identified the presence of various other proteins and lipids in LBs (Gai et al., 2000, Spillantini et al., 1997). Parkinson disease is therefore a complex disorder with two defining parts, parkinsonism and Lewy pathology.

1.1.3 Epidemiology of PD

The prevalence of PD globally is approximately 3-5 million people (Benmoyal-Segal and Soreq, 2006, de Rijk et al., 2000, de Rijk et al., 1997, Elbaz et al., 1999, von Campenhausen et al., 2005). More than 50,000 new cases are diagnosed each year in the United States (Lang and Lozano, 1998). PD incidence rises sharply after the fifth decade of life (Van Den Eeden et al., 2003), but it is primarily a disease of the elderly. PD affects approximately 1% of the population by the age of 65 increasing to 4-5% of the population by the age of 85 years (de Rijk et al., 2000, Lang and Lozano, 1998, Moghal et al., 1994). However, early-onset parkinsonism (onset at age 40 or younger) is estimated to account for 5-10% of all PD patients (Quinn et al., 1987). Gender differences have been reported with men having nearly two-fold greater rates than women after age adjustment (Rajput et al., 1984). Age- and gender-adjusted rates per 100,000 are highest among Hispanics (16.6), followed by non-Hispanic Whites (13.6), Asians (11.6), and African-Americans (10.2) illustrating the incidence of PD varies by race/ethnicity in the United States (Van Den Eeden et al., 2003). The average mortality
rate of Parkinson disease is approximately 1.5 times the general population (de Lau et al., 2005). The mean age at death is 73 years with average disease duration lasting 13 years (Marras et al., 2005). The most common causes of death in PD patients are pneumonia through lack of activity, cardiovascular disease or severe injury through falling (Hughes et al., 1993, Jellinger, 2005).

A major question surrounding Parkinson disease is its causation. The majority of PD cases are idiopathic, meaning they have no readily identifiable cause, whilst the minority of cases may be a circumstance of environment and/or genetics. This portion of the review regarding the epidemiology of PD will describe the known occupational and lifestyle risks associated with parkinsonism.

The identification of environmental toxins associated with early-onset parkinsonism has strengthened the notion of environment in Parkinson disease etiology. Chronic parkinsonism in young individuals was secondary to an accidental self-administration of the opioid analgesic meperidine, 1-methyl 4-phenyl 4-propionoxypiperidine (MPPP) contaminated with 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), a by-product of MPPP synthesis (Langston et al., 1983). Nigral cell loss and motor deficits were later recapitulated in mammalian animal models exposed to MPTP and were similar to PD associated with aging (Decamp and Schneider, 2004, Emborg et al., 2003, Langston et al., 1984, Przedborski and Vila, 2003). In general, however, rodent models exhibit mild forms of motor dysfunction, whereas non-human primate models more accurately mimic human disease. The MPTP finding renewed interest in the role of exogenous toxicants in the onset of parkinsonism, particularly compounds that may be similar to MPTP structurally or toxicologically,
including a number of pesticides and herbicides. A meta-analysis of 19 studies investigating the relationship between the onset of PD and pesticide exposure suggested a two-fold increased risk of PD with increased duration of exposure to pesticides (Priyadarshi et al., 2000). Despite this correlation, a dose-response relationship was not identified and no specific pesticide was attributed to disease onset. Some studies show an association between PD and overlapping risk factors such as farming, rural living and drinking well water (Ho et al., 1989, Koller et al., 1990). It has been proposed that the connection between PD and pesticides may be related to the leaching of chemicals from the soil into the ground water (Hubble et al., 1993, Metzler, 1982). However, the heterogeneous exposures of rural living make disease etiology difficult to ascertain. No pesticide administered to animal models has demonstrated the characteristic set of clinical and pathological criteria that is seen in human PD (Li et al., 2005a). In addition to farming, long-term occupational exposure to a combination of metals like manganese, copper, mercury, lead, iron, zinc, aluminium and amalgam have been reported as risk factors for PD (Gorell et al., 1997, Gorell et al., 1999, Zayed et al., 1990). However, exposure to these metals does not cause loss of dopaminergic neurons or the formation of Lewy bodies (Lai et al., 2002). Additional lifestyle factors may include caffeine and cigarette consumption. Strong epidemiological evidence suggests a lower risk of PD in smokers and coffee drinkers (Grandinetti et al., 1994, Hernan et al., 2002, Ross et al., 2000), although a limitation of these epidemiological studies is that they rely on the self-reporting and estimation of exposure. General weaknesses of epidemiology studies examining PD risk factors include limited case numbers, which decrease the statistical power of the study, and the unique experimental design of each study prohibits
reproduction of data. However, it may be entirely possible that the lifestyle and environmental exposures listed above do not contribute to the onset of Parkinson disease but may mimic parkinsonism. The etiology of PD remains complex and may include environmental risk factors as well as the strong effect of aging.

In addition to lifestyle and occupational exposures, medical circumstances may be factors in the etiology of parkinsonism. Secondary parkinsonism can be induced in patients taking neuroleptic drugs for the treatment of mental health disorders, such as schizophrenia and manic-depressive psychosis (Lang, 1989). Although this class of drugs effectively controls the psychiatric symptoms, the patients had parkinsonian features as a side effect that fortunately could be reversed by withdrawal of medication (Pranzatelli et al., 1994). It has also been suggested that the occurrence of PD may also be linked to head trauma and peripheral injury. These case-control studies are quite controversial because the association with PD varies depending upon the clinical definitions implied during patient accrueum; however, head trauma has been positively linked to PD (Seidler et al., 1996, Taylor et al., 1999). As previously mentioned, the observation that encephalitis lethargica preceded parkinsonism during the Spanish flu of 1910, suggests a putative post-infectious inflammatory cause of parkinsonism. Other infectious diseases like AIDS (Mintz et al., 1996), HSV-encephalitis (Solbrig and Nashef, 1993), influenza A (Isgreen et al., 1976), and Creutzfeldt-Jakob disease (Iida et al., 2001) are temporally associated with parkinsonism, mimicking its motor dysfunction through chronic inflammation. However, the parkinsonism in these conditions tends to resolve after infection and does not generally progress, an important contrast with sporadic PD.
Since the majority of Parkinson disease cases are sporadic, one might think the chief factors in its etiology are not genetic. However, over the last decade disease-associated genes have been identified accounting for ~10% of early-onset and 3% of late-onset PD cases. Twin studies to ascertain the genetic predisposition of Parkinson disease show similar concordance in monozygotic and dizygotic twin pairs suggesting that genetic factors play a minor role in the disease before 50 years of age (Duvoisin et al., 1981, Marsden, 1987, Marttila et al., 1988, Vieregge et al., 1992, Ward et al., 1983). However, these cross-sectional studies probably underestimated the role of genetics in disease development because they lack clinical follow-up and therefore, exclude the possibility of discordant disease onset (De Michele et al., 1995, Piccini et al., 1999, Tanner et al., 1999, Wirdefeldt et al., 2004). PET imaging studies measuring $^{18}$F-dopa uptake suggests decreased dopaminergic function may exist in the otherwise asymptomatic twin (Burn et al., 1992, Holthoff et al., 1994). In fact, the concordance rates in monozygotic twins were three times higher than in dizygotic twins using PET imaging as an outcome measure (58% versus 18%, respectively) (Piccini et al., 1997). Despite these analyses, twin studies have been unable to determine the contribution of genetics to the majority of PD cases. Familial transmission of PD has been illustrated by the discovery of genes and loci identified in multiple nuclear families and isolated populations that cause Parkinson disease.

The discovery of monogenic heritable forms of Parkinson disease has challenged the non-genetic etiological hypotheses of PD. Approximately 5 to 10% of patients carry a mutation in one of the known genes that cause autosomal dominant or recessive forms of PD. Numerous families have been described that exhibit Mendelian patterns of
inheritance and thirteen genetic linkages have been reported for PD (*PARK1-13*) [Table 1.1.3]. *PARK1-9* loci follow a typical Mendelian inheritance pattern, whereas *PARK10-12* represent susceptibility loci with presently undefined modes of transmission. Mutations identified in *α-synuclein* (*SCNA*), including its triplication were the first genetic association with PD (Kruger et al., 1998, Polymeropoulos et al., 1997, Singleton et al., 2003, Zarranz et al., 2004). Soon after the discovery of *SCNA* mutations in PD, this protein was found to be a major component of LBs (Spillantini et al., 1997). These observations suggested that overexpression of wild type *α-synuclein*, in the absence of other mutations, was sufficient to cause disease. The disease in these individuals is an autosomal early-onset dominant disease with extensive Lewy body pathology and glial cytoplasmic inclusions (Gwinn-Hardy et al., 2000). The role of *SCNA* in PD pathogenesis is beyond the scope of this review, but *SCNA* overexpression and its localization in Lewy bodies is widely considered an etiological factor in familial and sporadic cases of PD.
Table 1.1.3. Genetic loci implicated in parkinsonism

<table>
<thead>
<tr>
<th>Locus</th>
<th>Protein (gene)</th>
<th>Inheritance</th>
<th>Function</th>
<th>Age of onset</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARK1=</td>
<td>α-synuclein</td>
<td>Autosomal</td>
<td>Presynaptic signaling and membrane trafficking</td>
<td>30-60 years</td>
<td>PD/DLBD, dementia</td>
</tr>
<tr>
<td>PARK4 (4q)</td>
<td>(SNCA)</td>
<td>dominant</td>
<td></td>
<td></td>
<td>Lewy bodies</td>
</tr>
<tr>
<td>PARK2</td>
<td>Parkin</td>
<td>Autosomal</td>
<td>E3 ligase</td>
<td>~30 years</td>
<td>Parkinsonism, dystonia</td>
</tr>
<tr>
<td>(6q)</td>
<td></td>
<td>recessive</td>
<td></td>
<td></td>
<td>Slow course</td>
</tr>
<tr>
<td>PARK3</td>
<td>Unknown</td>
<td>Autosomal</td>
<td>Unknown</td>
<td>50-60 years</td>
<td>PD, dementia</td>
</tr>
<tr>
<td>(2p)</td>
<td></td>
<td>dominant</td>
<td></td>
<td></td>
<td>Lewy bodies, tangles and plaques</td>
</tr>
<tr>
<td>PARK6</td>
<td>PINK1</td>
<td>Autosomal</td>
<td>Mitochondrial kinase</td>
<td>30-50 years</td>
<td>Parkinsonism</td>
</tr>
<tr>
<td>(1p)</td>
<td></td>
<td>recessive</td>
<td></td>
<td></td>
<td>Slow course</td>
</tr>
<tr>
<td>PARK7</td>
<td>DJ-1</td>
<td>Autosomal</td>
<td>Involved in oxidative stress response</td>
<td>20-40 years</td>
<td>Parkinsonism, no dementia</td>
</tr>
<tr>
<td>(1p)</td>
<td></td>
<td>recessive</td>
<td></td>
<td></td>
<td>Pathology unknown</td>
</tr>
<tr>
<td>PARK8</td>
<td>LRRK2</td>
<td>Autosomal</td>
<td>Protein kinase</td>
<td>40-60 years</td>
<td>Parkinsonism, no dementia</td>
</tr>
<tr>
<td>(12p-q)</td>
<td></td>
<td>Dominant</td>
<td></td>
<td></td>
<td>Variable pathology, with and without Lewy</td>
</tr>
<tr>
<td>PARK9</td>
<td>ATP13A2</td>
<td>Autosomal</td>
<td>Lysosomal P-type ATPase</td>
<td>11-16 years</td>
<td>Parkinsonism with spasticity, supranuclear</td>
</tr>
<tr>
<td>(1p)</td>
<td></td>
<td>recessive</td>
<td></td>
<td></td>
<td>upgaze paresis, and dementia</td>
</tr>
<tr>
<td>PARK10</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Late</td>
<td>Typical PD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pathology unknown</td>
</tr>
<tr>
<td>PARK11</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Late</td>
<td>Typical PD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pathology unknown</td>
</tr>
<tr>
<td>PARK12</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Late</td>
<td>Typical PD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pathology unknown</td>
</tr>
</tbody>
</table>
1.2 AUTOSOMAL RECESSIVE PARKINSONISM

Mutations in *parkin (PARK2), PINK1 (PARK6)* and *DJ-1 (PARK7)* are all clearly associated with autosomal recessive parkinsonism. Mutations in these genes are associated with loss-of-function of their gene products and can lead to dopaminergic neuronal deterioration and parkinsonian phenotypes. The mapping and cloning of genes that cause inherited forms of PD has provided insight into molecular pathways that contribute to disease pathogenesis. Table 1.2.1 illustrates the mutations linked to recessive parkinsonism. These gene products are thought to be neuroprotective and may play an essential role in nigral neuron survival.

<table>
<thead>
<tr>
<th></th>
<th>PARK2</th>
<th>PARK6</th>
<th>PARK7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Locus</td>
<td>6q25-27</td>
<td>1p35-36</td>
<td>1p36</td>
</tr>
<tr>
<td>Gene</td>
<td>Parkin</td>
<td>PINK1</td>
<td>DJ-1</td>
</tr>
<tr>
<td># Pathogenic Mutations</td>
<td>&gt;100</td>
<td>&gt;20</td>
<td>&gt;9</td>
</tr>
<tr>
<td>Type of Mutations</td>
<td>Exonic deletion and duplication, insertion, nonsense, missense</td>
<td>Insertion, deletion, nonsense, missense</td>
<td>Exonic deletion, missense</td>
</tr>
<tr>
<td>Lewy body</td>
<td>(+) 4 cases</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
1.2.1 Parkin

The first gene associated with autosomal recessive parkinsonism was *parkin* (Kitada et al., 1998). Clinical manifestations of *parkin*-associated recessive parkinsonism were initially characterized as early-onset parkinsonism (before 40 years of age) that had a benign and slow course of disease and was L-dopa responsive (Lucking et al., 2000, Yamamura et al., 1973). Over the last ten years, many atypical clinical phenotypes, such as foot dystonia, hyper-reflexia, symmetrical onset and L-dopa induced dyskinesia, have been found to be associated with *parkin* mutations (Klein et al., 2000). However, affected individuals can present symptoms at a later age of onset that are clinically indistinguishable from idiopathic PD cases (Bonifati et al., 2001). Parkin patients usually survive 10-20 years following the onset of symptoms (Yamamura et al., 2000). Neuropathology of parkin patients exhibits severe neuronal loss and gliosis in the substantia nigra and locus coeruleus (Gouider-Khouja et al., 2003), consistent with a hallmark of PD. Despite the loss and dysfunction of these dopaminergic and noradrenergic neurons, reports indicate a lack of Lewy pathology (Hayashi et al., 2000, Khan et al., 2003, Takahashi et al., 1994) with the exception of four cases. These four parkin patients have exhibited α-synuclein positive LBs, two carried compound heterozygous *parkin* mutations (Farrer et al., 2001, Pramstaller et al., 2005) and the other two had homozygous deletions of exon three (Farrer et al., 2001, Sasaki et al., 2004).

*PARK2* mutations are the most common known cause of recessive parkinsonism, accounting for approximately 50% of familial cases where the age of onset is below 40 years (Abbas et al., 1999, Bertoli-Avella et al., 2005). The frequency of *parkin* mutations is inversely related to the age of onset—as the age of disease onset increases, the
frequency of *PARK2* mutations decreases (Periquet et al., 2003). The first mutation discovered in *PARK2* was a homozygous deletion of exons 3 through 7 in Japanese families with recessive parkinsonism (Kitada et al., 1998). Over 100 *parkin* mutations have now been described in patients with recessive parkinsonism including point mutations, multiplications, insertions and small exonic deletions. There are reports of cases that carry a single heterozygous *parkin* mutation that phenotypically resemble idiopathic PD with later age of onset and more asymmetric disease presentation. The consequences of carrying a heterozygous mutation is difficult to interpret and may be the result of haplo-insufficiency, a dominant negative effect, or the consequence of parkin susceptibility promoting disease when combined with another detrimental genetic or environmental event. Alternatively, the rare *parkin* variants may not be pathogenic and the association of single variants with sporadic PD may be a chance event. A case study reported the frequency of pathogenic *parkin* homozygous or compound heterozygous mutations to be similar, so whether heterozygosity is a risk factor for PD remains to be elucidated (Lincoln et al., 2003). However, the loss of one *parkin* allele may reduce parkin expression and enzymatic activity and correlate with the loss of striatal dopaminergic neurons by the decreased uptake of $^{18}$F-dopa via PET imaging (Khan et al., 2002a).

The *parkin* gene has 12 exons and encodes a 465 amino acid protein with a molecular mass of 52 kilodaltons. Parkin is comprised of an N-terminal ubiquitin-like (Ubl) domain (amino acids 1-76), two RING (Really Interesting New Gene) domains (amino acids 238-293 and 418-449) and an IBR (in-between ring) domain (amino acids 314-377). The presence of these domains indicates that parkin is a component of the
ubiquitin proteasome system (UPS). Parkin has homology to other E3 ubiquitin-protein ligases, which are responsible for targeting proteins for degradation (Shimura et al., 2000). The UPS acts dually to maintain the quality of proteins in the cell by promoting degradation of unfolded proteins and to temporally regulate the life span of short-lived proteins playing critical roles in signal transduction, metabolism and cell cycle regulation. Parkin works in tandem with Uba1 (ubiquitin-activating E1 enzyme) and various ubiquitin-conjugating E2 enzymes (Rankin et al., 2001, Shimura et al., 2000, Tanaka et al., 2001a). A high-energy thioester bond is formed between the ubiquitin-activating enzyme and ubiquitin. Next, the activated ubiquitin is transferred to the ubiquitin-conjugating enzyme. Lastly, glycine residues at the C-terminus of ubiquitin are covalently attached to the NH₂ group of a lysine residue on the protein substrate. The substrate is polyubiquitinated by the repetition of the above cycle and becomes recognizable by the proteasomal machinery and is subsequently degraded into small peptides and amino acids.

The formation of protein complexes may regulate normal localization and function of parkin. Sakata et al. showed that the Ubl domain of parkin interacts with the Rpn10 subunit of the 26S proteasome directing polyubiquitinated substrates for degradation (Sakata et al., 2003). One point mutation in parkin, R42P, causes a conformational change in the Ubl domain, impairing parkin binding to the proteasome (Sakata et al., 2003). It has been suggested that parkin interacts with the SCF complex (Skp1-Cullin-F-box protein) regulating its E3 ligase activity (Staropoli et al., 2003). Further E3 ligase enhancement results from parkin complexing with Hsp70 (Heat-shock protein 70) and CHIP (carboxyl terminus of the Hsc70-interacting protein) (Imai et al.,
2001). Some mutations in parkin have been shown to disrupt substrate binding (Sriram et al., 2005) and its E3 ligase activity (Sriram et al., 2005, Zhang et al., 2000), supporting the hypothesis that recessive mutations are a result of loss-of-function.

Mutations within PARK2, which inhibit its E3 ligase activity, prevent the degradation of its substrates and their accumulation may contribute to dopaminergic cell death. Proposed substrates of parkin include: cell division control-related protein 1 (CDCrel-1) (Zhang et al., 2000), parkin associated endothelin receptor-like receptor (Pael-R) (Imai et al., 2001), an O-glycosylated form of α-synuclein (αsp22) (Shimura et al., 2000), synphilin-1 (Chung et al., 2001), synaptotagmin XI (Huynh et al., 2003), cyclin E (Staropoli et al., 2003), α/β tubulin (Ren et al., 2003), poly-glutamine proteins (Tsai et al., 2003) and p38 t-RNA synthase (Corti et al., 2003). However, a limitation of this parkin substrate list is their in vitro discovery and lack of subsequent in vivo validation. Substrates escaping ubiquitination by parkin may accumulate in parkin-associated recessive parkinsonism brains leading to neuronal degeneration. For instance, increased levels of Pael-R (Takahashi et al., 2003), CDCrel-1 (Zhang et al., 2000), and αSp22 (Shimura et al., 2000) have been found in PD. Overexpression of Pael-R and its accumulation stresses the ER and induces cell death (Yang et al., 2003). The synaptic vesicle-associated protein, CDCrel-1 (Dong et al., 2003), can inhibit cellular dopamine release and mediate dopaminergic neuronal death. Toxicity attributable to Pael-R (Yang et al., 2003) and CDCrel-1 (Dong et al., 2003) can be overcome by simultaneous overexpression of wild type but not mutant parkin. Despite this phenotypic rescue, there is no accumulation of Pael-R or other parkin substrates in parkin null mice, indicating these substrates may not be true in vivo targets (Goldberg et al., 2003, Itier et al., 2003).
Typical recessive parkinsonism cases lack Lewy body pathology, but some studies have suggested \(\alpha\)-synuclein positive LBs may be a feature of parkin-associated disease (Farrer et al., 2001, Pramstaller et al., 2005, Sasaki et al., 2004). Synphilin-1, a putative parkin substrate has been shown to co-aggregate with \(\alpha\)-synuclein. If synphilin-1, \(\alpha\)-synuclein, and parkin are co-expressed, inclusion bodies form that immunostain positive for ubiquitin (Chung et al., 2001). The parkin substrate, p38 t-RNA synthase and its interactor far upstream binding element protein 1 (FBP1), accumulate in parkin deficient mouse brain and are found to be increased in brains of early-onset parkinsonism, sporadic PD and diffuse Lewy body disease (DLBD) patients (Ko et al., 2006). Furthermore, viral-mediated overexpression of p38 t-RNA synthase causes dopaminergic neuronal cell death (Ko et al., 2006). The possibility that these putative neurotoxic parkin substrates are removed by parkin-mediated polyubiquitination, resulting in their degradation, is an appealing proposition. However, no single currently identified substrate appears to explain both the specificity of dopaminergic cell death and the absence of Lewy body formation. One study has suggested that parkin is found in LBs of idiopathic PD brains, suggesting parkin need not be mutated to play a role in pathogenesis (Schlossmacher et al., 2002). The role of parkin in this pathway of degradation must be investigated more thoroughly to delineate the primary and secondary consequences of mutation.

1.2.2 PINK1

Another autosomal recessive gene whose mutations are associated with early-onset parkinsonism is PINK1 (PARK6), the phosphatase and tensin homolog (PTEN)-induced kinase 1. PINK1 was originally discovered as a gene transcriptionally
transactivated by PTEN (Unoki and Nakamura, 2001). PINK1 patients have parkinsonism with asymmetrical onset and L-dopa responsiveness, but the course of disease is slower and the age of onset is earlier (between the third and fifth decades) than idiopathic PD. Some PINK1 mutations exhibit a phenotype similar to parkin-associated recessive parkinsonism with hyperkinesia and foot dystonia at onset (Ibanez et al., 2006, Valente et al., 2004b). Post mortem studies of PINK1 patients are not available due to the low prevalence of mutations, although PINK1 has been shown to localize with a small proportion (5-10%) of LBs in sporadic PD (Gandhi et al., 2006), DLBD and in cytoplasmic glial inclusions of MSA patients (Murakami et al., 2007). ¹⁸F-dopa PET imaging has confirmed a loss of presynaptic dopaminergic neurons in these patients (Khan et al., 2002b).

The first PINK1 mutations, missense G309D and truncating W437X, were identified in patients of Spanish and Italian origin with recessive parkinsonism (Valente et al., 2004a). Over 20 pathological mutations have now been identified in PINK1 including point mutations, truncations and whole gene heterozygous deletions (Klein et al., 2006, Marongiu et al., 2007, Zadikoff et al., 2006). The frequency of PINK1 mutations is 1-9% of recessive parkinsonism cases (Healy et al., 2004, Li et al., 2005c, Rohe et al., 2004, Tan et al., 2006, Tan et al., 2005, Valente et al., 2004b). Up to 5% of sporadic early-onset PD cases have heterozygous PINK1 mutations (Bonifati et al., 2005, Valente et al., 2004b, Abou-Sleiman et al., 2006). ¹⁸F-dopa PET imaging and SPECT studies of heterozygous mutant asymptomatic persons illustrated decreased dopaminergic function (Kessler et al., 2005, Khan et al., 2002b). Haploinsufficiency of PINK1, as was suggested with parkin, could be a risk factor in parkinsonism. Age of onset is the major
phenotypic difference between homozygous and heterozygous PINK1 mutations, whereas onset takes place a decade later in the latter (Hatano et al., 2004, Healy et al., 2004, Rogaeva et al., 2004).

PINK1 consists of eight exons, encoding a 581-amino acid protein with a predicted molecular mass of 62.8 kilodaltons. PINK1 mRNA is expressed ubiquitously, but the highest expression levels are found in the heart, skeletal muscle, and testes while intermediate expression is observed in the brain (expression is primarily neuronal not glial in the hippocampus, SNpc and cerebellar Purkinje cells (Blackinton et al., 2007)), liver, kidney and pancreas (Unoki and Nakamura, 2001, Taymans et al., 2006). The PINK1 protein has a large kinase domain with homology to serine/threonine kinases (Valente et al., 2004a) and exhibits autophosphorylation activity in vitro (Nakajima et al., 2003, Beilina et al., 2005, Silvestri et al., 2005). An N-terminal mitochondrial-targeting signal (MTS) is sufficient for mitochondrial import of PINK1 (Muqit et al., 2006, Silvestri et al., 2005) but PINK1 also localizes to the cytosol (Beilina et al., 2005, Haque et al., 2008, Lin and Kang, 2008, Takatori et al., 2008, Weihofen et al., 2008, Zhou et al., 2008). PINK1 has been reported to localize to the outer mitochondrial membrane (OMM) (Gandhi et al., 2006), the mitochondrial intermembrane space (IMS) (Plunj-Favreau et al., 2007, Pridgeon et al., 2007, Silvestri et al., 2005) and the inner mitochondrial membrane (IMM) (Gandhi et al., 2006, Lin and Kang, 2008, Muqit et al., 2006, Pridgeon et al., 2007, Silvestri et al., 2005). The controversial subcellular and submitochondrial localization of PINK1 raises a question as to the authenticity of reported substrates. Two putative mitochondrial substrates, tumor necrosis factor type 1 receptor associated protein 1 (TRAP1) (Pridgeon et al., 2007) and the serine protease
Omi/HtrA2 (high temperature requirement protein A2) (Plun-Favreau et al., 2007), have been identified. TRAP1 has been shown to localize primarily in the mitochondrial matrix, but it has also been found in the IMS (Pridgeon et al., 2007) and at extramitochondrial sites (Cechetto et al., 2000). HtrA2 is released from the intermembrane space of the mitochondria during apoptosis to the cytosol where it interacts with the inhibitor of apoptosis protein (IAP) (Strauss et al., 2005). Recently, it was shown that PINK1 topology relies on the presence of a transmembrane domain located after the MTS to insert the N-terminal tail into the outer mitochondrial membrane with the kinase domain facing the cytoplasm (Zhou et al., 2008). This suggested orientation would imply that physiological PINK1 substrates would be localized in the outer mitochondrial membrane or cytosol.

Recessive mutations are predicted to inactivate PINK1 and lead to a loss of protein kinase function. Mutations within the PINK1 protein have been identified both inside and outside the boundaries of the kinase domain. Pathogenic PINK1 mutations in the kinase domain exhibit decreased \textit{in vitro} kinase activity. The G309D and L347P PINK1 mutations have been associated with decreased kinase activity (Beilina et al., 2005) and may markedly reduce or alter phosphorylation of its substrates. The L347P mutation showed a loss of protein stability (Beilina et al., 2005, Moriwaki et al., 2008) and decreased TRAP1 phosphorylation in cultured cells (Pridgeon et al., 2007). However, it is difficult to say whether mutations outside the kinase domain affect kinase activity. C-terminal regions of many protein kinases contain functional motifs that control catalytic activity of the kinase domain and binding of regulatory proteins and substrates (Jeffrey et al., 1995, Niefind et al., 1998, Nolen et al., 2001). Deletion of the
C-terminal region resulted in reduced kinase activity of recombinant PINK1 when expressed in baculovirus-infected insect cells (Sim et al., 2006). However, this C-terminal deletion showed enhanced kinase activity when recombinant PINK1 was expressed in *E.coli* (Silvestri et al., 2005). *PINK1* C-terminal truncations identified produce clinical phenotypes similar to missense *PINK1* mutations (Rohe et al., 2004).

A neuroprotective effect of PINK1 has been demonstrated in cell culture models under various forms of cellular stress. PINK1 has been shown to protect against cell death induced by proteasome inhibition (Muqit et al., 2006, Valente et al., 2004a, Wang et al., 2007) and oxidative damage with rotenone (Deng et al., 2005) and MPP⁺ (Deng et al., 2005, Haque et al., 2008, Tang et al., 2006). The ability of PINK1 to protect against oxidative stress has been well characterized, as loss of function mutations (Hoepken et al., 2008, Hoepken et al., 2007, Pridgeon et al., 2007, Valente et al., 2004a) or PINK1 silencing (Clark et al., 2006, Gautier et al., 2008, Wang et al., 2006, Wood-Kaczmar et al., 2008) enhances susceptibility to cell death mediated by oxidative damage. PINK1 has also been shown to inhibit or block apoptosis induced by staurosporine, a general kinase inhibitor (Gelmetti et al., 2008, Petit et al., 2005, Wood-Kaczmar et al., 2008). In the presence of PINK1, the release of cytochrome c from the mitochondria is decreased (Petit et al., 2005, Piccoli et al., 2008b, Pridgeon et al., 2007, Wang et al., 2007) and downstream caspase activation and nuclear fragmentation is prevented (Petit et al., 2005) following the induction of apoptosis. Neuroprotection is abrogated when mutations (E240K, L489P) are present in *PINK1* (Valente et al., 2004a) further characterizing the loss of PINK1 function with these rare, hereditary mutations.
1.2.3 *DJ-1*

Another autosomal recessive parkinsonism-associated gene is *DJ-1 (PARK7)*. Clinically, *DJ-1* patients resemble *PINK1*-associated recessive parkinsonism with asymmetric, early-onset (20-40 years of age) with slow disease progression and respond well to dopamine replacement therapy. Similar to *parkin* (Khan et al., 2003) and *PINK1* (Criscuolo et al., 2006, Ephraty et al., 2007, Steinlechner et al., 2007) patients, few *DJ-1* patients also display the co-morbid association of psychiatric disorders and focal dystonia (Bonifati et al., 2003, van Duijn et al., 2001). To date, no neuropathological data exist from *DJ-1* patients to determine the presence of Lewy bodies or Lewy neurites, but *DJ-1* has been shown to co-localize with glial cytoplasmic inclusions in MSA and tau inclusions in tauopathies like Pick’s disease, corticobasal degeneration, progressive supranuclear palsy and Alzheimer’s disease (Bandopadhyay et al., 2004, Neumann et al., 2004, Rizzu et al., 2004). Patients carrying homozygous *DJ-1* mutations have decreased $^{18}$F-dopa uptake shown via PET imaging, similar to *parkin* and *PINK1*. Unlike *parkin* and *PINK1* patients asymptomatic heterozygous *DJ-1* cases, have normal $^{18}$F-dopa metabolism (Dekker et al., 2004). Pathogenic *DJ-1* mutations in early-onset PD are extremely rare with a frequency estimate of 1-2% (Abou-Sleiman et al., 2003). Two homozygous mutations in *DJ-1* were first identified in 2003 in two consanguineous families with recessive parkinsonism from the Netherlands and Italy (Bonifati et al., 2003). The first mutation was a large genomic deletion of the first five exons whilst the second was a missense, L166P, mutation. Since 2003 at least seven more pathogenic mutations, including homozygous and heterozygous missense mutations and exonic deletions, have been connected with *DJ-1*-associated parkinsonism (Annesi et al., 2005,
Clark et al., 2004, Hague et al., 2003, Hedrich et al., 2004, Hering et al., 2004, Tang et al., 2006). The R98Q mutation is found in 1% of the general population but does not associate with recessive parkinsonism (Hague et al., 2003, Hedrich et al., 2004).

*DJ-1* contains eight exons and encodes a 189-amino acid dimeric, single domain protein (Bonifati et al., 2003) belonging to the ThiJ/Pfpl superfamily (Bandyopadhyay and Cookson, 2004, Lucas and Marin, 2007, Tao and Tong, 2003). DJ-1 is ubiquitously expressed in peripheral tissues and the brain (Bader et al., 2005, Galter et al., 2007, Shang et al., 2004), immunostaining positively in neurons and glia (Bandopadhyay et al., 2004) localizing to the cytosol, mitochondria and nucleus (Bonifati et al., 2003, Hod et al., 1999, Miller et al., 2003).

Recessive mutations in *DJ-1* can affect protein stability. It has been demonstrated that the L166P mutation destabilizes DJ-1 (Gorner et al., 2004, Gorner et al., 2007, Herrera et al., 2007, Hulleman et al., 2007, Malgieri and Eliezer, 2008, Miller et al., 2003, Moore et al., 2003) and promotes protein degradation via the UPS (Miller et al., 2003). DJ-1 instability also occurs with the M26I mutation in mammalian cells (Blackinton et al., 2005, Hulleman et al., 2007, Takahashi-Niki et al., 2004). The pathogenic DJ-1 mutations M26I, E64D and D149A show lower binding to mRNA compared to wild-type DJ-1 protein (van der Brug et al., 2008). The consequence of abrogated DJ-1 function may increase neuronal susceptibility to toxic insult and apoptosis (Canet-Aviles et al., 2004, Gorner et al., 2007, Meulener et al., 2006, Taira et al., 2004).

The ability of DJ-1 to respond to oxidative stress has been well described (Canet-Aviles et al., 2004, Mitumoto et al., 2001). Overexpression of DJ-1 is neuroprotective.
against oxidative toxic insults (Canet-Aviles et al., 2004, Meulener et al., 2005, Ved et al., 2005). DJ-1 silencing in vitro and in vivo results in reactive oxygen species (ROS) accumulation (Andres-Mateos et al., 2007, Taira et al., 2004, Takahashi-Niki et al., 2004). Additionally, the role of DJ-1 in mediating cellular oxidation was supported by increased glutathione synthesis following oxidative stress with exogenous DJ-1 (Liu et al., 2008b, Zhou and Freed, 2005). Under paraquat-induced oxidative stress in cultured cells, DJ-1 undergoes an acidic shift in isoelectric point (Canet-Aviles et al., 2004, Mitumoto et al., 2001). This change in the accumulation of acidic isoforms has been confirmed post mortem in PD brains (Bandopadhyay et al., 2004, Choi et al., 2006). Crystallization of the DJ-1 protein revealed that under oxidizing conditions, a cysteine residue at position 106 forms a cysteine-sulfin acid (Wilson et al., 2003, Witt et al., 2008) that is stabilized by a neighboring glutamic acid (E18) (Wilson et al., 2005). This relationship is evolutionarily conserved in all PfPI family members (Wilson et al., 2005). DJ-1 translocates from the cytosol to the outer mitochondrial membrane (Canet-Aviles et al., 2004) under conditions of oxidative stress. However, the protection C106 provides against oxidative stress is abrogated with mutation and C106 mutants fail to relocalize to the mitochondria (Canet-Aviles et al., 2004). It has also been shown that a pool of DJ-1 localizes to the mitochondrial matrix and the intermembrane mitochondrial space but no alterations in oxidation were observed (Zhang et al., 2005). Localization of DJ-1 is also altered when mutations, E46K and D149A, are present (Blackinton et al., 2005, Xu et al., 2005). A mitochondrial localization sequence has not been identified in the DJ-1 protein, so it remains unknown how DJ-1 is imported into the mitochondria. In vitro (Canet-Aviles et al., 2004) and in vivo (Meulener et al., 2006) evidence suggests the C106
residue, the most sensitive to modification (Kinumi et al., 2004), is compulsory to protect against cell loss under oxidative conditions.

DJ-1 was initially described as a protein interactor of c-myc capable of transforming mouse NIH3T3 cells with activated ras, suggesting DJ-1 may be a mitogen-dependent oncogene product (Nagakubo et al., 1997). DJ-1 was also shown to interact with androgen receptor interacting protein 3 (PIASxα) and potentially could transactivate androgen-response genes (Takahashi et al., 2001). Additional protein interactors of DJ-1 including DJBP (Niki et al., 2003), PSF and p54nrb (Xu et al., 2005), may further implicate DJ-1 as a transcriptional regulator. DJ-1 has also been proposed to interact with nucleic acid binding proteins that include the RNA helicase Abstrakt (Sekito et al., 2005) and the mouse homolog of DJ-1 was shown to regulate RNA-protein interactions (Hod et al., 1999). DJ-1 has been shown to suppress apoptosis by modulating the PTEN/Akt pathway (Davidson et al., 2008, Yang et al., 2005b) and influences the expression of Bax (a pro-apoptotic protein) (Bretaud et al., 2007) by repressing transcription of p53 (Fan et al., 2008).

The possibility that DJ-1 is involved in all of these functional roles seems unlikely, and it is more probable that a single intrinsic biochemical function underlies its multiple effects. Recently, van der Brug et al. re-examined the regulatory role of DJ-1 in RNA binding to explain its extensive functions. DJ-1 RNA binding targets found in vitro and in vivo were classified into three groups, namely genes involved in mitochondrial function, oxidative stress response and cell survival (van der Brug et al., 2008). Exposure to oxidative stress resulted in decrease of bound mRNA to DJ-1 and increased protein
levels of transcripts associated with DJ-1. The regulation of these transcripts by DJ-1 may explain the pleiotrophic effects of this small, single domain protein.

1.3 MOLECULAR PATHWAYS TO PARKINSONISM

Oxidative damage (Dexter et al., 1989, Shimura-Miura et al., 1999, Sofic et al., 1992, Yoritaka et al., 1996, Youdim et al., 1989) and biochemical aberrations within the mitochondria (Hattori et al., 1991, Mizuno et al., 1994, Mizuno et al., 1989, Schapira et al., 1989) and the proteasome (McNaught et al., 2003, Spillantini et al., 1997) have been proposed to be associated with parkinsonism pathogenesis. The identification of recessive parkinsonism genes and their protein localization may support the idea that these molecular pathways are important in the causation of parkinsonism. One possibility is that parkinsonism associated genes converge to form a single pathogenic pathway leading to the ubiquitous loss of dopaminergic neurons. The role of oxidative damage in dopaminergic cell loss is especially important as it may be related to the combinatorial consequences of mitochondrial and ubiquitin-proteasome (UPS) dysfunction.

1.3.1 Ubiquitin proteasome dysfunction

The proteasome, a barrel shaped multi-protein complex, is an ATP-dependent, proteolytic system located in the cytosol and the nucleus. Ubiquitinated proteins are degraded by the 26S proteasome that contains two main types of subcomplexes, a barrel-like core particle (20S) protease unit and two regulatory particles capping both ends of the barrel (19S). Polyubiquitinated proteins interact with the 19S portion of the
proteasome and are unravelled in an ATP-dependent process allowing unfolded proteins to translocate to the core particle for degradation. Sporadic PD patients have decreased proteasome activity (Blandini et al., 2006, McNaught et al., 2002, McNaught et al., 2004) and the inability of the proteasome to degrade proteins in a timely fashion could lead to the accumulation of cytotoxic proteins. Evidence implicating a direct role of the UPS in parkinsonism is the association of genetic mutations in parkin (Kitada et al., 1998) and subsequent demonstration that parkin functions as a ubiquitin ligase associated with proteasomal degradation (Imai et al., 2000, Shimura et al., 2000, Zhang et al., 2000). Moreover, pathogenic mutations in parkin compromise its E3 ligase activity, which directly links UPS dysfunction to dopaminergic neuron survival (Imai et al., 2000, Shimura et al., 2000, Zhang et al., 2000). Post-translational modification of E3 ligases can alter its activity in response to cellular stress or subcellular localization (Fang et al., 2000). For instance, nitric oxide-mediated S-nitrosylation of parkin can modify its E3 ligase activity. One study suggests S-nitrosylation decreases (Chung et al., 2004) the ability of parkin to target proteins for degradation while another suggests its ability and neuroprotection is enhanced (Yao et al., 2004) by this modification. Yao et al. proposed initial E3 ligase activity was upregulated with S-nitrosylation and enhanced neuroprotection—implying that parkin would be autoubiquitinated by increased E3 ligase activity following its modification (Yao et al., 2004). The activity of parkin would subsequently be inhibited by its autoubiquitination, further impairing its ability to degrade substrate proteins. Dopamine also covalently modifies parkin to functionally inactivate its E3 ligase activity increasing its insolubility in vitro and in vivo (LaVoie et
al., 2005). Parkin appears to preserve proteasome function to prevent the accumulation of toxic substrates that may contribute to the loss of dopaminergic neurons.

1.3.2 Mitochondrial dysfunction

Mitochondria are subcellular, membrane-enclosed, organelles that are essential for cell survival in eukaryotes. These dynamic organelles are fundamental for energy production, providing substrates for intracellular metabolic pathways. Mitochondria also influence cellular signalling and metabolic pathways. Mitochondria possess their own genome, encoding 13 genes whose protein products are subunits of the respiratory chain or the oxidative phosphorylation system (OXPHOS). Electron transport and ATP synthesis by oxidative phosphorylation act continuously within a mitochondrion. The electron transport chain, organized into five separate enzyme complexes I-V, resides in the inner mitochondrial membrane and is the common pathway by which electrons, derived from energy rich molecules, flow to oxygen. Complexes I to IV each contain part of the electron transport chain, whereas complex V catalyzes the synthesis of ATP. Complex I functions as a redox center, accepting or donating electrons, to mobile electron carriers including coenzyme Q and cytochrome c, resulting in the oxidation of NADH.

Defects in mitochondrial metabolism are implicated in many common diseases of aging. For instance, a link between parkinsonism and mitochondria was proposed when complex I activity was found to be diminished in the substantia nigra (Schapira et al., 1989) and peripheral tissues of parkinsonism patients (Krige et al., 1992, Parker et al., 1989, Wallace et al., 1992). Inhibition of electron transport produces damaging reactive
oxygen species and diminishes the capacity to generate ATP, which has profound effects on the cell. For instance, reactive oxygen species are capable of damaging nuclear and mitochondrial DNA (mtDNA) through oxidation and modify cysteine and methionine residues in proteins, like DJ-1 (Ooe et al., 2005). Furthermore, vital cellular processes like transcription, translation and protein turnover are dependent upon the production of ATP. Continuous oxidative insult induces cell death, which increases the opening of outer membrane permeability transition pores, leading to a loss of mitochondrial membrane potential and the subsequent release of pro-apoptotic factors, such as cytochrome c (Tsujimoto and Shimizu, 2007). Mitochondrial complex I inhibitors, like rotenone and MPTP, lead to neuronal cell death and a phenotype resembling parkinsonism in animal models (Betarbet et al., 2000, Langston et al., 1983, Langston et al., 1984, Liou et al., 1996, Ricaurte et al., 1986). Furthermore, animals chronically infused with rotenone exhibit selective dopaminergic neurodegeneration of the substantia nigra (Betarbet et al., 2000, Sherer et al., 2003) and fibrillar cytoplasmic inclusions (Betarbet et al., 2000). However, the selective loss of dopaminergic neurons following complex I inhibition in animal models remains puzzling. Decreased complex I activity in parkinsonism patients is not restricted to dopaminergic neurons, as it is also diminished in peripheral tissues (Blake et al., 1997) and it is unclear why neurons in the substantia nigra are more susceptible to cell death in cases of parkinsonism.

Mitochondrial connections have now been identified for three autosomal recessive genes associated with parkinsonism. These familial forms of parkinsonism along with mutations in mitochondrial genes may explain selective SNpc vulnerability to oxidative stress. Discoveries of mtDNA abnormalities in parkinsonism patients (Rana et
al., 2000, Swerdlow et al., 1998, Swerdlow et al., 1996) have demonstrated mitochondrial dysfunction is associated with disease pathogenesis (Bender et al., 2006). Mutations in the nuclear encoded mitochondrial gene DNA Polymerase γ (POLG) have been identified in parkinsonism and parkinsonism-associated chronic progressive external ophthalmoplegia (CPEO) families (Luoma et al., 2004). Furthermore, increased somatic mtDNA deletions have been reported to correlate with neurodegeneration that results from aging or parkinsonism and has functional effects on respiratory chain activity (Bender et al., 2006, Kraytsberg et al., 2006). Consistent with the aging etiology of parkinsonism, mitochondrial mutations in the SNpc are rarely exhibited in young subjects but by geriatric age nearly all SNpc neurons have mtDNA deletions (Bender et al., 2006, Kraytsberg et al., 2006). However, consistent mtDNA mutations have not been identified in either familial or sporadic forms of parkinsonism (Tan et al., 2000). Moreover, genetic association studies investigating the link between mitochondrial deletions and the risk of sporadic parkinsonism are conflicting. Variations in mitochondrial content contributing to parkinsonism have been evaluated by mtDNA haplotypes. mtDNA haplogroups U, K, J and T from a Finnish population share a common maternal ancestor that is defined by the presence of 73A and 14766A polymorphisms (Finnila et al., 2001, Macaulay et al., 1999). One group suggested reduced risk of parkinsonism in individuals with the haplotype cluster, UKJT (Pyle et al., 2005), whereas van der Walt et al. showed lower risk was limited to haplotypes J and K (van der Walt et al., 2003). A third group (with a smaller sample size) suggested individuals with haplotypes J and T had an increased risk for parkinsonism (Ross et al., 2003).
One theory to explain the susceptibility of mtDNA and the preferential loss of dopaminergic neuron in parkinsonism is the toxicity caused by dopamine synthesis and metabolism. End products of dopamine oxidation reactions produce free radicals and dopamine adducts, both of which can damage DNA and covalently modify proteins. Enzymatic oxidation of free dopamine in the cytoplasm via monoamine oxidase B can generate reactive oxygen species before forming neuromelanin (Sulzer et al., 2000). Conversely, non-enzymatic and spontaneous oxidation of dopamine can create reactive superoxides and DOPA quinones (Graham, 1978). However, the formation of DA quinones by auto-oxidation resulting in ROS (Asanuma et al., 2004, Asanuma et al., 2003, Pardo et al., 1995) is not limited to dopaminergic neurons (Braak et al., 2003a, Braak et al., 2003b) and these quinones formed are themselves cytotoxic (Emdadul Haque et al., 2003). Dopaminergic neurons perhaps undergo a higher basal oxidative burden making them more sensitive than other neuronal cell types. However, while dopamine neurons in the SNpc are lost in parkinsonism, neurons in the mesolimbic dopamine pathway are unaffected (Hwang et al., 2003). Moreover, non-catecholaminergic neurons can be affected in parkinsonism (Rinne, 1991) and especially in DLBD (Tiraboschi et al., 2000). Lewy pathology in PD is not restricted to the substantia nigra (Braak et al., 2003a, Braak et al., 2003b). Therefore, although dopamine metabolism may be a precipitating factor in parkinsonism, it is unlikely to explain all of the known pathological events in this disorder.

Recently mtDNA mutations in subunits of mitochondrial complex I were found in a parkinsonism patient with a homozygous nonsense mutation, W437X, in PINK1 (Piccoli et al., 2008a). Examination of fibroblasts from this patient showed constitutive
overproduction of reactive oxygen species and altered complex I enzymatic activity (Piccoli et al., 2008a). Proteins with iron sulfide clusters that are involved in oxidative phosphorylation and the electron transport chain are sensitive to oxidative stress (Gardner and Fridovich, 1991) and prolonged oxidative damage can result in mitochondrial dysfunction. Decreased ATPase activity in the W437X PINK1 patient fibroblasts when cultured with galactose causes cells to utilize oxidative phosphorylation instead of relying on glycolytic ATP, which suggests a basal oxidative phosphorylation deficit in these cells (Piccoli et al., 2008b). Complex I respiratory defects were also found in primary fibroblasts isolated from patients homozygous for G309D PINK1 (Piccoli et al., 2008b). Coupled with sensitivity to oxidative stress, PINK1 silencing also results in mitochondrial respiratory dysfunction. PINK1 knockout mice exhibit impaired striatal mitochondrial respiration and decreased aconitase activity (Gautier et al., 2008). In addition, these mice display respiratory defects and mitochondrial dysfunction that result following exposure to short-term (H$_2$O$_2$) or long-term (aging) oxidative stress (Gautier et al., 2008). Together these data demonstrate that PINK1 plays a role in protecting against oxidative stress and/or mitochondrial dysfunction.

1.3.3 Genetic interaction of PINK1 and parkin

Regardless of the impact of mitochondrial mutations in the onset of PD, there is substantial evidence that mutations in the recessive parkinsonism genes parkin, PINK1 and DJ-1, all contribute to dysfunctional mitochondria. The functional consequences of mutant parkin and PINK1 will be more thoroughly described as they are linked in a common pathway. Despite the lack of gross mitochondrial morphological defects found
in *PINK1* knockout mice (Kitada et al., 2007), three groups concurrently reported abnormal mitochondrial phenotypes in PINK1 loss of function *Drosophila* mutants (Clark et al., 2006, Park et al., 2006, Yang et al., 2006). The groups all reported a genetic interaction between *parkin* and *PINK1* that modulates mitochondrial morphology in *Drosophila*. The phenotype associated with *PINK1* knockout in *Drosophila* was found to be similar to that of *parkin* knockout. Independently, these *PINK1*- and *parkin*-null flies have altered mitochondrial morphology consisting of fragmentation and disintegration with loss of cristae, flight defects attributable to the degeneration of flight-wing muscle, sensitivity to oxidative stress (Greene et al., 2003, Pesah et al., 2004) and male sterility due to ineffective spermatid maturation (Clark et al., 2006, Park et al., 2006). The phenotypes are suggested to be the direct or indirect consequence of loss of PINK1 on mitochondrial dysfunction. When *Drosophila* mutants overexpressing human *parkin* were crossed with *PINK1* knockout *Drosophila*, these transgenic flies could rescue the *PINK1*-null phenotypes of male sterility and mitochondrial abnormalities (Clark et al., 2006, Park et al., 2006). However, *PINK1* transgenic flies could not ameliorate the *parkin*-null phenotype, suggesting that *PINK1* lies upstream of *parkin* in a common genetic pathway. Also the phenotype of double *parkin/PINK1* null flies resembled that of single mutants instead of an additive phenotype, suggesting that *PINK1* and *parkin* do not act in parallel (Clark et al., 2006, Park et al., 2006). This data was confirmed in mammalian cells where fragmented and truncated mitochondrial phenotypes associated with the loss of PINK1 could be rescued by overexpression of parkin (Exner et al., 2007). Additionally, altered cristae as seen in PINK1 fly mutants were observed and glucose deprivation promoted abnormal mitochondrial phenotypes, further supporting
mitochondrial morphological and metabolic defects associated with PINK1 silencing (Exner et al., 2007). Despite the questionable absence of nigral neuronal degeneration, the identification of a common genetic pathway for PINK1 and parkin suggests a causal role of mitochondrial dysfunction in disease etiology.

A current controversy surrounds the localization of PINK1 and parkin, which can occupy different subcellular sites. Mammalian parkin has been shown to localize to the cytosol with a fraction at the outer mitochondrial membrane (Darios et al., 2003). Kuroda et al. suggests that parkin preferentially localizes to the mitochondrial matrix, and its biogenesis is enhanced through an association with mitochondria transcription factor A in dividing cells (Kuroda et al., 2006). However, the majority of parkin is cytoplasmic in most cells, including neurons (Cookson et al., 2003, Hase et al., 2002). As previously described, PINK1 is found in both the mitochondria (Beilina et al., 2005, Gandhi et al., 2006, Plun-Favreau et al., 2007, Lin and Kang, 2008, Muqit et al., 2006, Pridgeon et al., 2007, Silvestri et al., 2005) and the cytoplasm (Beilina et al., 2005, Haque et al., 2008, Lin and Kang, 2008, Takatori et al., 2008, Weihoffen et al., 2008, Zhou et al., 2008). Whether PINK1 and parkin proteins physically interact is unclear. It is plausible that they associate in the cytoplasm before or after PINK1 is transported to the mitochondria or perhaps they interact after mitochondrial import and processing of PINK1. Alternatively, PINK1 and parkin might modify one another indirectly. For example, they may share substrates, such as the mitochondrial proteins released into the cytoplasm under stress conditions or other proteins mediating cell death and/or survival. Parkin may only ubiquitylate substrates that have been phosphorylated by PINK1. It is also possible
that parkin can only function as an ubiquitin ligase after phosphorylation by PINK1 whose kinase activity is upregulated in response to oxidative stress.

1.3.4 Cell death and parkinsonism

In addition to metabolism, a secondary function of mitochondria is in the control of cell death. The role of mitochondria in cell death is closely linked in both their physiology and pathology. Irrespective of end-stage cell death (necrotic, apoptotic, autophagic or other types), the integrity of mitochondrial membranes is a decisive factor in determining the fate of a cell. Mitochondrial membrane permeabilization is often considered the “point of no return” in events leading to apoptosis. During apoptosis, the permeability of the outer mitochondrial membrane increases allowing soluble, intermembrane space proteins to be released into the cytoplasm. The induction of outer mitochondrial membrane permeabilization can be detected by the shift in subcellular localization of intermembrane space proteins, such as cytochrome c, apoptosis-inducing factor (AIF) and other proteins (but not limited to) procaspases, Smac/DIABLO, Omi/HtrA2 and EndoG to the cytosol (Adrain et al., 2001, Patterson et al., 2000, Susin et al., 1999). Overexpression of PINK1 and parkin in cultured cells has shown that these proteins can prevent pro-apoptotic events such as the release of cytochrome c and caspase activation, and that this anti-apoptotic effect is abrogated with pathogenic mutations in either gene (Darios et al., 2003, Gelmetti et al., 2008, Petit et al., 2005, Pridgeon et al., 2007).

Mitochondrial membrane potential ($\Delta \psi_m$) results from the charge imbalance across the inner mitochondrial membrane as the respiratory chain builds up the proton
gradient that is required for oxidative phosphorylation. One mechanism resulting in the decrease of $\Delta \psi_m$ results from the permeabilization of the inner mitochondrial membrane by oxidative stress or mitochondrial Ca\textsuperscript{2+} overload. Inner mitochondrial membrane permeabilization results in increased mitochondrial matrix volume, reduced matrix electron density and the disorganization of the cristae—the internal compartments formed by the inner membrane of a mitochondrion (Nieminen et al., 1997, Petronilli et al., 1999). Mitochondrial membrane potential is higher with wild-type PINK1 overexpression and is decreased by the presence of the G309D mutation after treatment with the proteasome inhibitor MG-132 (Valente et al., 2004a), heterozygous PINK1 mutations (Abou-Sleiman et al., 2006) or PINK1 silencing (Exner et al., 2007). Parkin can also affect $\Delta \psi_m$ (Mortiboys, 2008) and decrease mitochondrial swelling once parkin has translocated to the outer mitochondrial membrane (Darios et al., 2003). Inactivation of DJ-1 has also been shown to impair mitochondrial function and increase sensitivity to oxidative stress (Chen et al., 2005b, Kim et al., 2005b), whereas overexpression of DJ-1 protects cells from mitochondrial damage (Canet-Aviles et al., 2004, Li et al., 2005b). Furthermore, OMI/HtrA2 a quality control serine protease within the intermembrane space of mitochondria and a pro-apoptotic factor binding to inhibitor of apoptosis proteins when released into the cytosol via Bax and Bak, pro-apoptotic proteins (Strauss et al., 2005). Expression of OMI/HtrA2 G399S and A141S mutations in culture promotes mitochondrial swelling, decreased membrane potential and increased proteasome-inhibitor induced cell death (Strauss et al., 2005). OMI/HtrA2 was formerly considered a PD associated gene (Strauss et al., 2005) but recently these mutations were also identified in neurologically normal controls (Simon-Sanchez and Singleton, 2008). HtrA2 has been
proposed to be an indirect substrate for PINK1 (Plun-Favreau et al., 2007). PINK1 enhances phosphorylation and activation of the mitochondrial protease HtrA2 by the stress activated MAP (mitogen activated protein) kinase p38γ (Plun-Favreau et al., 2007). Oxidative stress induces phosphorylation and activation of p38γ by upstream kinases such as MEKK3. When PINK1 binds to HtrA2, PINK1 enhances phosphorylation of Ser142 of HtrA2 by p38γ (Plun-Favreau et al., 2007). The phosphorylated HtrA2 then protects neuronal cells from undergoing apoptosis induced by neurotoxins and oxidative stress.

Cytochrome c has been proposed to be released via mitochondrial core components (Narita et al., 1998), including the adenine nucleotide translocation (ANT) or VDAC (voltage-dependent anion channel), which is responsible for metabolite flux across the mitochondrial outer membrane during apoptosis (Colombini, 1983). The mitochondrial response to cellular stress and modulation of apoptosis by PINK1, parkin and OMI/HtrA2 suggest they might share a common pathway. If parkin lies downstream of PINK1, one might speculate that OMI/HtrA2 is downstream and initiates apoptosis when oxidative stress is no longer tolerable to the cell. Additionally, it has been shown that PINK1 binds and phosphorylates TRAP1 (Pridgeon et al., 2007). Upon phosphorylation, TRAP1 is activated and suppresses ROS generation and protects cells from apoptosis induced by Granzyme M (Hua et al., 2007). As reactive oxygen species induce opening of mitochondrial permeability transition pores, PINK1 and TRAP1 may indirectly block this event and prevent the release of cytochrome c and other proapoptotic proteins.
Bcl-2 family proteins regulate the release of cytochrome c and other pro-apoptotic factors through the outer mitochondrial membrane (Adams and Cory, 2007, Chipuk and Green, 2008). The Bcl-2 family has a cell death dichotomy—Bcl-2, Bcl-xl, and Mcl-1 family members inhibit apoptosis whereas Bax and Bak activate apoptosis. Cytochrome c release and apoptosis is initiated when BH3-only domain proteins, Puma and Bim, bind and inhibit pro-survival Bcl-2 family proteins. This protein inhibition allows pro-apoptotic Bax and Bak to permeabilize the outer mitochondrial membrane inducing apoptotic events (Youle and Strasser, 2008). However upon the induction of cytochrome c release, the mitochondria fragment into smaller, spherical units—a process called mitochondrial fission. Inhibition of mitochondrial fission prohibits the release of cytochrome c and delays cell death, thus providing a link between mitochondrial morphogenesis and the induction of apoptosis. The next section will highlight the processes that regulate mitochondrial morphology and how they participate in outer mitochondrial membrane permeabilization and apoptosis.

1.4 MITOCHONDRIAL MORPHOGENESIS MACHINERY

Mitochondria are dynamic organelles that continually undergo the opposing processes of fusion and fission to maintain a distinct morphology. The balance between fission and antagonizing fusion events regulates mitochondrial morphology, which includes controlling the shape, number and length of mitochondria. The shape of the mitochondria corresponds to the metabolic status (Rossignol et al., 2004) and the health of the cell (Youle and Karbowski, 2005). The process of fusion is necessary for maintaining mitochondrial function, including mitochondrial inner membrane potential, respiration, and genomic content (Chen et al., 2003). Fission events mediate apoptosis by
regulating the release of pro-apoptotic factors from the intermembrane space to the cytosol. Proteins involved in mitochondrial dynamics originally identified in yeast are highly conserved in mammals. The fission mediators include Drp1 (Dnm1 in yeast) and Fis1, while the mediators of fusion are Mitofusins 1 and 2 (Mfn) (Fzo1 in yeast) and Opa1 (Mgm1 in yeast) (Westermann, 2008). However, additional proteins have been found in mammals that do not appear in yeast, such as Bax (Karbowski et al., 2006) and Endophilin B1 (Karbowski et al., 2004), also influence mitochondrial dynamics. Notably, yeast also has mitochondrial dynamics players that have not been identified in mammals (Coonrod et al., 2007). Figure 1.4.1 illustrates the localization of mammalian proteins involved in mitochondrial dynamics.

1.4.1 Mitochondrial fission in mammalian cells

In mammalian cells, the large GTPase, dynamin-related protein 1 (Drp1) mediates division of the mitochondrial network (Hoppins et al., 2007, Okamoto and Shaw, 2005). Drp1 belongs to the conserved dynamin large GTPase superfamily that controls membrane tubulation and fission (Praefcke and McMahon, 2004). Dynamin, a Drp1 homolog, is assembled in the cytosol and forms spirals around endosomes to mediate fission from the plasma membrane. Helices of dynamin mediate lipid tubule scission by constricting and twisting the anchored tubules upon GTP cleavage (Danino et al., 2004, Roux et al., 2006). Presumably acting like dynamin, Drp1 oligomerizes into ring-like structures at division sites on the outer mitochondrial membrane to initiate fission in a GTP-dependent manner (Ingerman et al., 2005). However unlike dynamin, Drp1 contains a carboxy-terminal GTPase effector domain (GED), which facilitates intra- and
intermolecular interactions that regulate GTPase activity (Chang and Blackstone, 2007, Zhu et al., 2004). Dominant negative mutations that block Drp1 GTPase activity result in an elongated mitochondrial phenotype through the inhibition of GTP-mediated fission (Otsuga et al., 1998, Smirnova et al., 2001).

Soluble Drp1 is mostly cytosolic, but it is recruited upon apoptotic stimulation to the mitochondria by an unknown process and forms punctate foci at sites of mitochondrial fission (Smirnova et al., 2001, Wasiak et al., 2007). Although, it has been shown that actin filaments and microtubules are integral in the recruitment of Drp1 to mitochondria (De Vos et al., 2005, Varadi et al., 2004), Drp1 recruitment to the outer mitochondrial membrane has been shown to occur via a transient interaction with Fis1 (Yoon et al., 2003, Yu et al., 2005). Fis1, a tetratricopeptide domain protein contains a C-terminal transmembrane domain that localizes to the outer mitochondrial membrane and its N-terminus, which protrudes into the cytosol, is necessary for mitochondrial fission (Yoon et al., 2003) and the interaction with Dnm1 (Dohm et al., 2004, Suzuki et al., 2003). It has been suggested that Fis1 acts as a limiting factor in mitochondrial fission, supporting the notion that Fis1 acts as a receptor for Drp1 on the outer mitochondrial membrane (Yoon et al., 2003). In yeast, Fis1 recruits Dnm1 to the mitochondria via adaptor proteins, Mdv1 (Mitochondrial division 1) or Caf4 (Caffeine-resistant 4) (Griffin et al., 2005, Mozdy et al., 2000, Tieu and Nunnari, 2000) but mammalian orthologs of these adaptor proteins have not yet been identified. However, Drp1 can assemble on mitochondria in the absence of Fis1 (Lee et al., 2004, Stojanovski et al., 2004), leaving the mechanism of Drp1 localization to mitochondria open to further investigation.
Membrane-associated ring- (C3HC4) V (MarchV) is a mitochondrial E3 ligase that plays a role in the regulation of mitochondrial morphology (Karbowski et al., 2007, Nakamura et al., 2006, Yonashiro et al., 2006). This protein contains an N-terminal RING finger domain and four transmembrane domains that tether MarchV to the OMM. There is some controversy concerning the function of MarchV. One group reports that inhibition of MarchV activity by expression of a dominant negative mutant results in mitochondrial fragmentation (Nakamura et al., 2006, Yonashiro et al., 2006); however, another group reported that MarchV inhibition increases mitochondrial length and connectivity (Karbowski et al., 2007). RING mutants of MarchV localize in foci with Drp1 on the mitochondrial surface whereas, wild-type MarchV localizes evenly around the mitochondria. Therefore, this E3 ligase seems to play a role in mitochondrial fission, presumably promoting the disassembly or turnover of Drp1.

Another component of mammalian mitochondrial fission is Endophilin B1 (also called EndoB1, Bif-1 and SH3GLB) (Cuddeback et al., 2001, Farsad et al., 2001, Pierrat et al., 2001); a cytosolic protein that transiently interacts with Bax on the mitochondria and heterodimerization is enhanced with the induction of apoptosis (Cuddeback et al., 2001, Pierrat et al., 2001, Takahashi et al., 2005). Like Endophilin A1, EndoB1 binds dynamin and mediates division of the endosome, splits bilayers of lipids into tubules (Farsad et al., 2001) and regulates the curvature of the membrane (Peter et al., 2004). EndoB1 knockdown by RNA interference in cells results in the dissociation of the outer and inner mitochondrial membranes and possibly inhibits OMM fission, which causes the mitochondria to appear elongated (Karbowski et al., 2004). Double EndoB1 and Drp1 knockdown results in a mitochondrial phenotype that is identical to the single Drp1
knockdown, which suggests that EndoB1 acts downstream of Drp1, translocating to and from the OMM to regulate mitochondrial fission (Karbowski et al., 2004).

1.4.2 Mitochondrial fusion in mammalian cells

Fusion proteins that promote elongation and reticular mitochondrial phenotypes antagonize mitochondrial fission. Three large GTPases from the dynamin family, Mitofusin 1 (Mfn1), Mitofusin 2 (Mfn2) and Optic atrophy protein 1 (Opa1), are conserved from yeast to mammals and mediate mitochondrial fusion. It has been demonstrated that Mfn1 and Mfn2 regulate fusion of the outer mitochondrial membrane while Opa1 is responsible for fusion of the inner mitochondrial membrane (Meeusen et al., 2006, Meeusen et al., 2004). Although both Opa1 and Mfn1/2 are necessary for mitochondrial fusion no direct interactions between the two proteins have observed in mammalian cells. Although, Mfn1 has been reported to be required for Opa1-dependent inner mitochondrial membrane fusion (Cipolat et al., 2004).

Mitofusins 1 and 2 localize to the outer mitochondrial membrane through two transmembrane domains (Santel and Fuller, 2001) orienting their N-terminal GTPase domains and C-terminal coiled-coil regions towards the cytosol (Rojo et al., 2002) and facilitate the docking of adjacent mitochondria through a tethering mechanism (Koshiba et al., 2004). Trans-mitochondrial tethering occurs when the C-terminal coiled-coil regions of Mfn1 and Mfn2 interact (Chen et al., 2003, Ishihara et al., 2004, Koshiba et al., 2004, Meeusen et al., 2004). The GTPase activity of the mitofusins is necessary for fusion activity in addition to the maintenance of mitochondrial membrane potential (Hales and Fuller, 1997, Hermann et al., 1998, Santel and Fuller, 2001). Some
experiments suggest that Mfn1 and Mfn2 proteins are functionally redundant (Chen et al., 2003); however, Mfn1 and Mfn2 seem to have specialized functions despite their similarities in protein structure (Cipolat et al., 2006, Eura et al., 2003). It has been shown that increased GTPase activity of Mfn1 mediates more efficient GTP-dependent mitochondrial tethering than Mfn2 (Ishihara et al., 2004). Using co-immunoprecipitation, anti-apoptotic Bcl-2 and Bcl-xL have been shown to specifically interact with Mfn2 to enhance mitochondrial fusion (Delivani et al., 2006). Mitofusin interactors also include the pro-apoptotic Bcl-2 family members Bax and Bak, which have been shown to interact with both Mfn1 and Mfn2 (Brooks et al., 2007). It has been proposed that mitofusins and Bcl-2 family proteins interact to regulate mitochondrial fusion in healthy cells (Karbowski et al., 2006).

Opa1 is located in the inner mitochondrial membrane, facing the intermembrane space (Olichon et al., 2002) and is required for fusion but not mitochondrial docking (Chen et al., 2005a, Cipolat et al., 2004, Olichon et al., 2003). A single gene encodes mammalian Opa1 with 8 transcript variants resulting from alternative splicing (Delettre et al., 2001). Opa1 splice variants are differentially proteolyzed and yield various long and short isoforms that influence membrane association (Satoh et al., 2003). Longer isoforms, which retain the N-terminal transmembrane segment and the MTS, are more tightly connected to the inner mitochondrial membrane when compared to the shorter isoforms lacking residues required for membrane insertion (Ishihara et al., 2004). Numerous proteases have been identified in Opa1 processing that include paraplegin (Ishihara et al., 2004), PARL (presenilin-associated rhomboid-like) (Cipolat et al., 2006, McQuibban et al., 2003) and Yme1 (yeast nuclear gene iAAA protease) (Griparic et al.,
2007, Song et al., 2007). The identification of multiple splice variants of Opal in mammalian cells suggests that many versions of Opal may be necessary to differentially regulate mitochondrial morphology with respect to damaging cellular triggers. Cleavage of Opal is enhanced under conditions of decreased inner mitochondrial membrane potential suggesting its activity is coordinated with the energetic state of the mitochondria, supporting the notion that high membrane potential is required for fusion (Duvezin-Caubet et al., 2006, Grįparic et al., 2004, Ishihara et al., 2004, Meeusen et al., 2006).

1.4.3 Post-translational modifications in mitochondrial morphogenesis

Post-translational modification is the chemical modification of a protein that can include the addition of other proteins such as SUMOylation and ubiquitylation, or the addition of functional groups like phosphorylation. In addition to MarchV-dependent ubiquitination of Drp1, phosphorylation of Drp1 has also been shown to regulate mitochondrial fission. Drp1 activity is enhanced by cyclin-dependent kinase 1/cyclin B phosphorylation of Ser618 (human Drp1; Ser585 in rat Drp1) that results in mitochondrial fragmentation during mitosis (Taguchi et al., 2007). However, cAMP-dependent protein kinase A (PKA) inactivates Drp1 GTPase activity by phosphorylation of Ser637 (human Drp1; Ser656 in rat Drp1) which results in mitochondrial fusion by blocking fission activity (Chang and Blackstone, 2007, Cribbs and Strack, 2007). Additionally, expression of a phosphomimetic mutant Drp1 S656D in cells results in an elongated mitochondrial phenotype (Cribbs and Strack, 2007). Elimination of the conserved PKA phosphorylation site with a S656A mutation in Drp1 leads to
mitochondrial fragmentation in cells (Cribbs and Strack, 2007). Therefore, Drp1 activity can be differentially regulated by phosphorylation, which alters mitochondrial morphology.

SUMOylation also regulates Drp1 activity and mitochondrial morphology. Drp1 has been shown to interact with SUMO1 (Harder et al., 2004) and overexpression of SUMO1 enhances Drp1 SUMOylation, resulting in mitochondrial fission (Harder et al., 2004). The cytosolic SUMO protease, SENP5, desumoylates Drp1 and can reverse mitochondrial fission caused by SUMO1 (Zunino et al., 2007). SUMOylation of Drp1 enhances its activity, positively regulating mitochondrial fission. During apoptosis, more Drp1 protein associates with the outer mitochondrial membrane and Drp1 SUMOylation increases (Wasiak et al., 2007), resulting in increased mitochondrial fission.

The mitofusins, which regulate fusion events in yeast (Fzo1) and mammals, may be regulated by ubiquitination. During the mating of yeast, mitochondria fragment and the levels of Fzo1 decrease. Chemical inhibition (Neutzner and Youle, 2005) or genetic inactivation (Escobar-Henriques et al., 2006) of the proteasome attenuates the loss of Fzo1. Fzo1 is removed from the outer mitochondrial membrane and degraded following lysine 48-dependent ubiquitination to regulate its function in a process similar to ERAD (ER-associated degradation) removal and degradation of membrane spanning proteins. Mammalian mitofusin protein levels increase after exposure to proteasome inhibitors in culture (Karbowski et al., 2007), suggesting the ubiquitin proteasome pathway regulates these fusion proteins.

Future studies should elucidate the importance of parkinsonism genes in mitochondrial dynamics like fusion and fission events and in phosphorylation-dependent
signalling cascades modulating cell growth and survival. In the absence of fusion, mitochondria become fragmented because there is no balance to fission events. The possible interaction of fusion proteins mitofusins and Opa1 and the fission protein Drp1 with PINK1 and parkin should be investigated further. These dynamic proteins are essential for mitochondrial morphology, cell growth, membrane potential and respiration (Ephraty et al., 2007). Interestingly, cell models of mutant parkin and PINK1 exhibit defects in all of these categories and either PINK1 or parkin could possibly regulate the activity of these dynamic proteins under basal or stress conditions.
Figure 1.4.1  Mitochondrial fission and fusion machinery

A schematic of the localization of the proteins involved in mitochondrial dynamics is illustrated. Mitochondrial fission divides a mitochondrion into two daughter units by the coordinated actions of Drp1, Endophilin B1, Fis1 and MarchV. Tethering of two mitochondria results in mitochondrial fusion of the membranes mediated by Mfn and Opa1. Bcl-2 family proteins, Bax and Bak are involved primarily in the induction of apoptosis, which influences mitochondrial dynamics.
1.5 MODELS FOR PATHWAY REGULATION

While the precise function of the recessive PD-associated genes remains to be defined, some downstream survival signalling pathways have been linked with their putative function. Common survival signalling components within the Bcl-2 family, c-Jun terminal kinase (JNK), and PTEN pathways have been associated with these loss-of-function genes. *Parkin* null fly phenotypes can be alleviated by inactivation of the pro-apoptotic JNK pathway (Greene et al., 2003). Moreover, JNK activation is thought to transcriptionally activate pro-apoptotic Bcl-2 family members, like Bax. DJ-1 has been shown to decrease Bax expression through transcriptional regulation of p53 thus inhibiting cell death (Bretau et al., 2007, Fan et al., 2008). Overexpression of Buffy, the Bcl-2 homologue in Drosophila, protects PINK1 mutant *Drosophila* (Clark et al., 2006, Park et al., 2006). Buffy acts as an anti-apoptotic protein that is capable of maintaining mitochondrial membrane integrity (Quinn et al., 2003). Parkin is also modified by its interactor, BAG5 (Bcl-2-associated anthanogene 5), which inhibits its E3 ligase activity and promoting aggregation of parkin (Kalia et al., 2004). Additionally, parkin ubiquitylates and degrades components of the pro-apoptotic signalling cascade (Kalia et al., 2004) and/or intracellular apoptotic in vitro stimuli like PAEL-R (Yang et al., 2003). Lastly, PTEN signalling has been associated with the recessive parkinsonism genes. PTEN is the antagonist to PI3K (phosphoinositide-3-kinase), which downregulates the pro-survival kinase Akt. RNAi-mediated knock down of *DJ-1* or *parkin* in *Drosophila* suppresses Akt activation (Yang et al., 2006). In contrast, enhanced PI3K signalling in these knockdown animals reduced cell death, whereas decreased PI3K expression improved cell survival. Furthermore, DJ-1 has been shown to negatively regulate the
tumor suppressor activity of PTEN (Davidson et al., 2008, Yang et al., 2005b) and influence mRNA transcripts in the PTEN/Akt pathway (van der Brug et al., 2008). PINK1 is also closely linked to PTEN since it was identified as a gene transcriptionally transactivated by the overexpression of PTEN (Unoki and Nakamura, 2001). Cytosolic parkin and PINK1 may interact directly or indirectly with DJ-1 in cytoplasmic signalling cascades associated with PTEN.

Interestingly, the two pathways to parkinsonism pathogenesis—mitochondrial dysfunction and ubiquitin proteasome impairment—may not be mutually exclusive. A close interrelationship exists between oxidative stress, mitochondrial dysfunction and the ubiquitin proteasome system. Figure 1.5.1 illustrates the converging pathways associated with parkinsonism. For instance, oxidative damage to the mitochondrial membrane may increase the production of ROS—with no counterbalance of ROS removal, ROS levels accumulate and ATP production may decrease lowering the efficiency of the ATP-dependent proteasome. Rotenone, a complex I inhibitor, treatment in culture has directly demonstrated this process, decreasing ATP production ~20% (as a result of ROS accumulation) and drastically decreasing proteasome activity (Shamoto-Nagai et al., 2003). Pathological characteristics of systemic and chronic inhibition of complex I recapitulate PD phenotypes, which include nigral neuronal degeneration and Lewy body-like inclusions (McNaught et al., 2004); significant ventral midbrain reduction of 20S protease activity (Betarbet et al., 2000, Sherer et al., 2003); and an increase in ubiquitin-conjugated proteins, suggesting the 26S proteasome is impaired. Conversely, mutations within or overexpression of α-synuclein has been shown to impair the 26S proteasome (Chen et al., 2006, Ghee et al., 2000, Kuroda et al., 2006, Snyder et al., 2003). 26S
inhibition results in the accumulation of putative cytotoxic proteins (McNaught et al., 2006, Tanaka et al., 2001b), which subsequently may lead to mitochondrial damage and the enhancement of pro-apoptotic factors leading to cell death. Proteasome inactivation may create a feedforward mechanism promoting ROS generation as a consequence of failing to degrade damaged proteins.

There is some evidence linking UPS dysfunction to secondary mitochondrial damage. The proteasome inhibitor MG-132 mediates the release of cytochrome c from the intermembrane space to the cytosol through mitochondrial membrane depolarization, allowing other pro-apoptotic molecules to be activated and cell death proceeds (Dewson et al., 2003, Emanuele et al., 2002, Qiu et al., 2000, Wagenknecht et al., 2000). Commencement of either proteasomal stress or mitochondrial dysfunction may result in mild defects but through feedforward or feedback mechanisms dopaminergic neurons in the SNpc might be selectively vulnerable to cell loss. Putative molecular links between mitochondrial dysfunction and UPS impairment are the electron transport chain, mitochondrial integrity and oxidative stress. Further research should examine the interplay of the recessive genes associated with parkinsonism with these potential pathways.
**Figure 1.5.1  Converging pathways in parkinsonism**

Parkinsonism is a complex disorder with many proposed etiologies, yet these associations may intersect in common pathways that lead to depletion of dopaminergic neurons. This figure summarizes the contribution of oxidative stress, mitochondrial dysfunction and impairment of the proteasome in dopaminergic cell death associated with parkinsonism. *Parkin* mutations and oxidative stress can inhibit parkin-mediated ubiquitination of specific substrates leading to their accumulation. These substrates may promote cell death through proteasomal or mitochondrial dysfunction. PINK1 and DJ-1 promote cell survival, either directly or indirectly by protecting mitochondria from various forms of oxidative stress.
1.6 Statement of purpose

The studies described in this thesis address the function and potential signaling pathways that regulate mitochondrial morphology and function in recessive parkinsonism associated genes, *parkin*, *DJ-1* and *PINK1*. When this project commenced, the roles of these recessive parkinsonism genes in mitochondrial function were unknown. Additionally, a fourth recessive gene ATP13a2 was not yet associated with hereditary early-onset parkinsonism (Ramirez et al., 2006). Previous studies provided information on the mitochondrial localization of PINK1 (Beilina et al., 2005, Valente et al., 2004a) and the association of DJ-1 (Blackinton et al., 2005, Bonifati et al., 2003, Canet-Aviles et al., 2004, Li et al., 2005b, Miller et al., 2003, Zhang et al., 2005) or parkin (Darios et al., 2003) with the outer mitochondrial membrane under stressful stimuli. Characterization of mitochondrial dysfunction in recessive parkinsonism was also limited. Decreased mitochondrial membrane potential was associated with a PINK1 pathogenic mutation G309D (Valente et al., 2004a), parkin-deficient models displayed defective mitochondria (Greene et al., 2003, Pesah et al., 2004) and reduced mitochondrial function (Palacino et al., 2004) and DJ-1 was shown to prevent mitochondrial dysfunction enhanced by oxidation (Ooe et al., 2005). Additionally, all three recessive parkinsonism-associated genes were shown to protect against apoptosis (Greene et al., 2003, Petit et al., 2005, Valente et al., 2004a, Xu et al., 2005).

The major focus of this work is on PINK1, a mitochondrial kinase that promotes protection against mitochondrial dysfunction and oxidative stress. However, it is unknown how overexpression of PINK1 in cellular models can afford protection against oxidative stressors that inhibit complex I, such as rotenone. It was hypothesized that this
protection might be associated with changes in the abundance of the PINK1 mRNA transcript, a proxy measure of increased protein expression, following exposure to oxidative stress. Therefore, the first aim was to examine PINK1 mRNA transcriptional changes observed in neuroblastoma cells following oxidative stress.

All three recessive parkinsonism-associated genes have been shown to be associated with mitochondrial function and protection against oxidative stress. The second aim of the study was to examine the mitochondrial morphological changes following PINK1 modulation that include measuring rates of fission and fusion directly in living cells. The mechanism(s) involved in PINK1-dependent maintenance of morphology and cell viability are not defined. As PINK1 is a kinase, it is likely that there are key substrate(s) yet to be identified that affect mitochondrial morphology. Cells deficient in PINK1 exhibit a phenotype of fragmented mitochondria that resemble cells lacking fusion proteins involved in mitochondrial dynamics (Chen et al., 2003). Therefore, the third aim of the current study was to address the underlying mechanism by which PINK1 influences mitochondrial morphology. Additionally, mitochondrial morphology was characterized using live cell imaging in DJ-1 and parkin deficient cells to determine if all three recessive parkinsonism genes are linked in a single pathway to regulate mammalian mitochondrial phenotypes.
CHAPTER TWO

Materials and methods
2.1 Materials and methods for Chapter Three

2.1.1 Cell culture

*Lentivirus generation*

A cDNA entry clone encoding full-length human PINK1 (residues 1-581) was purchased from GeneCopeia (Germantown, MD). In addition to the pathogenic G309D PINK1 mutation, single K219A, D362A, D384A and triple K219A/D362A/D384A mutations to abolish kinase activity were introduced into the PINK1 entry using Quickchange Multi site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. PINK1<sub>WT</sub>, PINK1<sub>KD</sub>, and PINK1<sub>G309D</sub> variants were transferred by Gateway recombination technology (Invitrogen) from PINK1 Gateway entry clones to the pLenti6-DEST/V5 vector (Invitrogen). For expression in mammalian cells, the full length PINK1 cDNA was transferred into the destination vectors pcDNA-DEST47 or pcDNA-DEST53 using Gateway recombination technology (Invitrogen) according to manufacturer’s instructions. The pLenti6-DEST/V5 vector is a lentiviral expression vector containing components for the viral packaging of the gene of interest and a C-terminal V5 tag for detection. Viral vector stocks were produced by transient transfection of HEK-293FT cells, plated on 10cm dishes, with 12µg of Virapower packaging mix (Invitrogen) containing gag/pol plasmid (pLP1), a plasmid encoding the envelope G glycoprotein (pLP/VSVG), a plasmid containing regulatory Rev element (pLP2) and 3µg of pLenti6-DEST/PINK1/V5 variant or pLenti6/GW/LacZ, which encodes the control protein β-galactosidase. Transfections were performed using a 1:3 ratio of DNA to Lipofectamine-2000 (Invitrogen). Supernatants were harvested 48 hours after transfection by centrifugation at 3000 rpm for 15 minutes. Titers of the viral preparations, expressed as a
number of transducing units per milliliter (TU/ml), were determined by transducing parental M17 cells with serial viral dilutions for 48 hours. For the control LacZ viral preparation, transduced cells were incubated in X-gal solution and blue colonies were counted. The titers of the PINK1 viral preparations were calculated by immunocytochemistry with V5 tag antibodies. Titers were approximately 2 x10⁵ TU/ml for unconcentrated virus.

*Generation of M17 PINK1 stable cell lines*

M17 cells (5 x 10⁵ total) growing in Opti-MEM media (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS) were transduced with approximately 10⁵ TU of PINK1-WT, KD, G309D or LacZ virus per plate. The following day, media-containing virus was replaced with complete culture medium and cells were incubated at 37°C overnight. The next day, cells were treated with 5μg/ml blasticidin (Invitrogen) to select for stably transduced M17 cells. This blasticidin concentration was determined in preliminary experiments to be sufficient to kill untransduced M17 cells within 10 days of treatment. During the selection process, media-containing blasticidin was replaced every 3-4 days. Twelve days after selection, distinct antibiotic resistant colonies were screened in PINK1-WT, KD, G309D, and LacZ plates. The mock transduced well did not have any live cells. Approximately ten individual colonies for each sample were picked, expanded and characterized for transgene protein expression by western blotting and immunocytochemistry.
To knockdown endogenous PINK1, two separate shRNA constructs (target sequences 5’-GCTGGAGGAGTATCTGATAGG, starting at nucleotide 550 of human PINK1 and 5’-GGGAGCCATCGCCCTATGAAAT, starting at nucleotide 1411) and a control shRNA (5’- CCTAGACGCGATAGTATGGAC) were made. These sequences were cloned into pLenti6, packaged at a multiplicity of infection (MOI) of 5 and were used to transduce M17 cells as above. Knockdown was confirmed using quantitative real-time polymerase chain reaction methods described below.

2.1.2 MTT toxicity assay

M17 neuroblastoma cells stably transduced with PINK1WT, PINK1KD, PINK1G309D or LacZ were seeded in 96 well plates at 2 x 10⁴ cells/well. On the following day, cells were exposed to different concentrations of rotenone and 1-methyl-4-phenylpyridinium (MPP⁺). Eight wells were used per concentration. Forty-eight hours after treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Aldrich) reagent was added to a final concentration of 0.5 μg/μl and cells were incubated at 37°C for 1 hour. During this time, dark blue crystals form within the healthy cells. Subsequently, a solubilization solution containing 20% SDS (sodium dodecyl sulfate) and 50% dimethylformamide (pH 4.7) was added and cells were resuspended with shaking at 37°C for 2 hours. Absorbance of the converted dye was measured using a multi-well scanning spectrophotometer at a wavelength of 590nm.
2.1.3 Quantitative real-time polymerase chain reaction (qRT-PCR)

Control and PINK1 shRNA cells were seeded at a density of $3 \times 10^5$ cells/well in a 6-well plate 24 hours prior to rotenone treatment. Total RNA was isolated from cells using TRIzol (Invitrogen). One microgram quantities of total RNA were reverse transcribed separately with SuperScript III reverse transcriptase (Invitrogen) by using oligo (dT) primers. cDNA templates were diluted 10-fold before use in qRT-PCR.

Table 2.1.3 Primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Primer Orientation</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human PINK1</td>
<td>Forward</td>
<td>5'-AGACGCTTGAGGGCTTTC-3'</td>
</tr>
<tr>
<td>Human PINK1</td>
<td>Reverse</td>
<td>5'-GGCAATGAGGATGGTTGAGG-3'</td>
</tr>
<tr>
<td>Human β-actin</td>
<td>Forward</td>
<td>5'-AGAAGGATTCATAGGGTGACG-3'</td>
</tr>
<tr>
<td>Human β-actin</td>
<td>Reverse</td>
<td>5'-CATGTCGATCAGGTTGAC-3'</td>
</tr>
<tr>
<td>Mouse PTEN</td>
<td>Forward</td>
<td>5'-ATAGCCCTAAACCAGAAACG-3</td>
</tr>
<tr>
<td>Mouse PTEN</td>
<td>Reverse</td>
<td>5'-TGAAAACCTCGATGCTGA-3'</td>
</tr>
<tr>
<td>Mouse PINK1</td>
<td>Forward</td>
<td>5'-CCAGACAAGGATTGGTAAAGC-3'</td>
</tr>
<tr>
<td>Mouse PINK1</td>
<td>Reverse</td>
<td>5'-GCAAGATGTTGATGGCC-3'</td>
</tr>
<tr>
<td>Mouse β-actin</td>
<td>Forward</td>
<td>5'-ACACTGTGCCTCTACGAG-3'</td>
</tr>
<tr>
<td>Mouse β-actin</td>
<td>Reverse</td>
<td>5'-TCAGCTGTTGTTGAGAAGC-3'</td>
</tr>
</tbody>
</table>

Real-time quantitative PCR was performed and analyzed by using the Applied Biosystems 7900 system (Baptista et al., 2003).
2.1.4 Tissue homogenization

Two-month old C57BL/6 male mice were injected subcutaneously for three consecutive days with 2mg/kg rotenone dissolved in DMSO. Animals were sacrificed up to four weeks following treatment [treatment and dissection performed by Dr. Charbel Moussa, Georgetown University]. Sham treated mice were used as controls. Whole brain hemispheres were flash frozen after dissection. Using a mortar and pestle tissue grinder, tissues were homogenized. Homogenates were then stabilized in TRIZol (Invitrogen) and total RNA was extracted for qRT-PCR [as described in 2.1.3].

2.1.5 mRNA stability assay

To estimate mRNA half-life, control shRNA and PINK1 shRNA cells were either treated with DMSO or rotenone for 24 hours. The cells then were treated with Actinomycin D (2μg/ml) (Sigma) for the times shown (up to 8h). PINK1 and GAPDH mRNA levels were measured by qRT-PCR, normalized to 18S rRNA levels, and plotted on a logarithmic scale to calculate the time required for each mRNA to reach one-half of its initial abundance. Primers for human PINK1 are listed above, but primers for GAPDH and 18S rRNA were obtained from Applied Bysystems.

2.2 Materials and methods for Chapter Four

2.2.1 Cell culture

Stably transduced M17 neuroblastoma cells were used as previously described (2.1.1).

2.2.2 Transformations
Per transformation reaction, 1μg of DNA was pipetted into 15μl of TOP10 *Escheria Coli* competent cells (Invitrogen), mixed by gentle tapping and incubated for thirty minutes on ice. Cells were then heat-shocked at 42°C for thirty seconds, followed by incubation on ice for two additional minutes. Two hundred and fifty microliters of room temperature SOC medium (Invitrogen) was added and the cells were shaken at 37°C for one hour at 225 rpm. One hundred microliters of the transformed cell suspension was spread onto 50μg/ml LB/kanamycin (Invitrogen) or 50μg/ml LB/ampicillin (Invitrogen) plates and incubated overnight at 37°C.

### 2.2.3 DNA purification for plasmids

Subsequent to transformation and growth of bacterial colonies in antibiotic selection plates, 5ml of LB/kanamycin or LB/ampicillin (50μg/ml) was inoculated with an individual colony from the plate with a pipet tip. The culture was incubated overnight at 37°C at 225 rpm shaking and samples were centrifuged to pellet the cells. The cell pellet was then purified using Mini-prep DNA purification kits (Qiagen) according to the manufacturer’s instructions. For large-scale plasmid DNA purification, 200μl of the 5ml culture was added to either 200ml LB/kanamycin or LB/ampicillin (50μg/ml) to incubate overnight in 37°C at 225 rpm. The cell pellet was then obtained by centrifugation and purified using Maxi-prep DNA purification kit (Qiagen) according to the manufacturer’s instructions.
Table 2.2.3 Plasmids used in Chapter Four

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>(Fusion) protein for detection</th>
<th>Antibiotic resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mito-YFP</td>
<td>YFP</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>Mito-paGFP</td>
<td>GFP</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>pCMV/DsRed-Express</td>
<td>RFP</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>pAd/Empty Vector</td>
<td>GFP</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>pAd/PINK1(_{ΔNt})</td>
<td>FLAG/GFP</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>pAd/PINK1(_{WT}) (full length)</td>
<td>FLAG/GFP</td>
<td>Ampicillin</td>
</tr>
</tbody>
</table>

### 2.2.4 Immunoblot analysis of stable cells lines overexpressing PINK1

M17 neuroblastoma cells stably transduced with PINK1\(_{WT}\), PINK1\(_{KD}\), PINK1\(_{G309D}\) or LacZ were seeded in 6-well plates at 3 x 10^5 cells/well 24 hours prior to harvesting. Cells were harvested in 1x phosphate buffered saline (PBS) by scraping and centrifuged at 4°C for three minutes at 1200 rpm. Cell pellets were lysed by resuspension in 1x Cell Lysis Buffer (Cell Signaling) containing a protease inhibitor cocktail (Roche). Intact cells and insoluble proteins were precipitated by centrifugation (4°C, 20 minutes at 10,000 rpm). Protein concentrations were determined by performing a BCA assay (Pierce) on the supernatants of the cleared cell lysates. Twenty micrograms of lysates were resolved on a 4-20% Tris-HCl SDS-PAGE gel (Criterion) and transferred to Immobilon polyvinylidene fluoride (PVDF) membranes (Millipore) for immunoblot analysis. Membranes were washed briefly in TBST [1x TBS (Biorad) containing 0.1% Tween 20 (Biorad)] prior to blocking for one hour in TBST supplemented with 5% non-fat dry milk (Carnation). Immunoblotting was performed using rabbit polyclonal anti-V5 antibody
(Invitrogen) diluted 1:5000 in blocking buffer and followed by donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase (Jackson Immunoresearch Labs). Immuneactive protein bands were detected with an enhanced chemiluminescence (ECL) plus reagent (Amersham Pharmacia). The membrane was stripped for one hour at 37°C using Pierce Restore Western Blot Stripping Buffer and reprobed with mouse monoclonal anti-β-actin antibody (Sigma, 1:5000) to show equal loading.

2.2.5 Analysis of mitochondrial membrane potential (Δψₘ) by TMRE

Cells (2 x 10⁵) were seeded into LabTekII borosilicate chambers (Nunc) in OptiMEM media (Invitrogen) supplemented with 10% FBS 24 hours prior to live cell imaging. Cells were washed once with 1x PBS and stained with 100nM TMRE (tetramethylrhodamine ethyl ester) in media for 30 minutes at 37°C. Cells were then washed with 1x PBS, media was replaced and kept at 37°C until images were captured on an inverted Zeiss LSM510 microscope with a 100x Plan-Apochromat 1.4/Oil DIC objective lens (Carl Zeiss) using LSM 510 software (Zeiss MicroImaging). Images were analyzed using MetaMorph software (Molecular Devices). Rotenone (500nM exposure for 1 hour) was used as a positive control for mitochondrial membrane depolarization. TMRE was calculated as a percentage of mean fluorescence intensity when normalized to the mean fluorescence intensity of the control cell line when set to 100%.

2.2.6 Quantitative real-time PCR

The same protocol listed in 2.1.3 was used.
2.2.7 Mitotracker staining imaged with confocal microscopy

M17 cells (2 x 10^5) were grown on 22-mm coverslips coated with poly-L-lysine. Mitochondria were stained with 200nM Mitotracker CMXRos (Molecular Probes, Inc.) for 30 minutes at 37°C, washed once with 1x PBS and fixed with 4% (w/v) paraformaldehyde. Cells were permeabilized with 1x PBS plus 0.1% (v/v) Triton X-100 for 10 minutes at room temperature. Excess PFA was quenched with 0.1M Glycine in 1x PBS at room temperature for 20 minutes. Coverslips were mounted with ProLong Gold Antifade reagent (Molecular Probes, Inc.) prior to imaging on a Zeiss 510 microscope with a 100x Plan-Apochromat 1.4/Oil DIC objective lens (Carl Zeiss). Counts for mitochondrial morphologies were performed by counting Mitotracker stained cells in each of three independent experiments. The observer was blind to transfection/treatment status.

2.2.8 Mitotracker staining combined with PINK1-FLAG immunofluorescence

COS7 cells (2 x 10^5) were grown for 24 hours on 22-mm coverslips coated with poly-L-lysine prior to transient transfection (24 hours) of 2.5μg of pAd/Empty vector, pAd/PINK1_{AN1}-FLAG and pAd/PINK1_{AN1}-FLAG plasmids using Lipofectamine 2000 (Invitrogen). Mitochondria were stained with 200nM Mitotracker CMXRos (Molecular Probes, Inc.) for 30 minutes at 37°C, washed once with 1x PBS and fixed with 4% (w/v) paraformaldehyde in 1x PBS. Cells were permeabilized with 1x PBS plus 0.1% (v/v) Triton X-100 for 10 minutes at room temperature. Excess PFA was quenched with 0.1M Glycine in 1x PBS at room temperature for 20 minutes. Coverslips were blocked for one
hour at room temperature with 10% FBS in 1x PBS containing 0.1% Triton X-100. Immunostaining with mouse monoclonal anti-FLAG M2 antibody (Sigma) diluted 1:200 in blocking buffer was performed overnight at 4°C in a humidified chamber tray. Coverslips were washed with 1x PBS and the secondary goat anti-mouse IgG Alexa Fluor 647 antibody was diluted 1:200 in blocking buffer and added to the coverslips for one hour. Cells were washed again with 1x PBS and mounted with ProLong Gold Antifade reagent (Molecular Probes, Inc.) prior to imaging on a Zeiss 510 microscope with a 100x Plan-Apochromat 1.4/Oil DIC objective lens (Carl Zeiss). Overlay images were generated using LSM 510 software (Zeiss MicroImaging).

2.2.9 Fluorescence recovery after photobleaching (FRAP) using live cell imaging with confocal microscopy

FRAP was performed as previously described (Karbowsk1 et al., 2006, Szabadkai et al., 2004). Cells were transiently transfected with 0.5μg of mitochondrial matrix-localized YFP (mitoYFP) using Lipofectamine-2000 (Invitrogen) and seeded into LabTekII borosilicate chambers (Nunc) in phenol red- and serum-free Opti-MEM (Invitrogen). Circular regions of interest (ROI), 2.5μm in diameter, were imaged on an inverted Zeiss 510 microscope with a 100x Plan-Apochromat 1.4/Oil DIC objective lens (Carl Zeiss) before and after photobleach with 4 iterations of 514nm laser set to 100% power. Scans were taken in 0.25 second intervals, for a total of 40 images and the fluorescence intensity in imaged ROIs was digitized with LSM 510 software (Zeiss MicroImaging). Curves were corrected for both non-specific photobleaching (NSPB) that occurred during imaging and background, and normalized to the first image in the series. Each FRAP
curve represents the average of ≥30 measurements representative of results obtained in 2-
3 separate experiments. Mobile fractions were calculated as follows (t-time, i-initial
time): Mobile Fraction = [(FRAPt-Background)/FRAPi][(NSPBt-Background)/NSPBi].

2.2.10 Photoactivation using live cell imaging with confocal microscopy

Assays for mitochondrial fusion rates were performed using modifications of a technique
described previously (Karbowskii et al., 2006). Cells were transfected for 24 hours with
0.5μg photoactivatable mitochondrially targeted GFP (mito-paGFP) and 1.0μg
pCMV/DsRed-Express using Lipofectamine 2000 (Invitrogen). Cells were imaged using
a 63x plan-apochromat 1.4/Oil DIC objective lens (Zeiss). DsRed positive cells were
selected for mitochondrial photoactivation. After collecting baseline fluorescence
images, two circular, 15μm, regions were photoactivated in each cell using a 413nm laser
set to 50% power output and 100% excitation. Fluorescence images were then taken of
the same cells (n=9-10 per line) at 15 minute intervals over one hour using a 488nm laser
with 5% excitation and 70% laser power, with a GFP filter for emission. Fusion of
mitochondria was estimated from the loss of photoactivated mito-paGFP fluorescence,
averaged across both regions of interest and normalized to the initial measurement, which
was set at 100% (MetaMorph software, Molecular Devices).

2.2.11 Assay for fusion capacity

Stably transduced M17 cells (3 x 10^5) were transiently transfected with 0.5μg of mito-
YFP using Lipofectamine-2000 (Invitrogen) and seeded into LabTekII borosilicate
chambers (Nunc) in phenol red- and serum-free Opti-MEM (Invitrogen). Baseline FRAP
measurements [as described above] were taken prior to treatment with 10µM CCCP (m-chloro carbonyl cyanide phenyl hydrazone; Sigma) for 1.5 hours to induce mitochondrial membrane depolarization. FRAP analysis was performed immediately following the CCCP treatment and after a washout with 1x PBS and a 6 hour recovery period in Opti-MEM media.

2.3 Materials and methods for Chapter Five

2.3.1 Cell Culture

Stably transduced M17 neuroblastoma cells were used as described in 2.1.1.

2.3.2 Transformations

Transformations were performed as described in 2.2.2.

2.3.3 DNA purification for plasmids

DNA was purified as described in 2.2.3.
Table 2.3.3 Plasmids used in Chapter Five

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2.3.4 FRAP and immunoblot analysis of transiently transfected fusion proteins

For FRAP: Control and PINK1 shRNA cells (2 x 10^5 cells/well) were co-transfected in suspension with 0.5μg mito-YFP and 2.0μg pcDNA3.1/Opa-myc or pCMV/Mfn2-FLAG using Lipofectamine 2000 (Invitrogen) for 24 hours prior to imaging in LabTekII borosilicate chambers (Nunc) using a 1:3 ratio of DNA to transfection reagent. FRAP was performed as previously described [2.2.9].

For immunoblot: Control and PINK1 shRNA cells (3 x 10^5 cells/well) were reverse transfected with 2.0μg pcDNA3.1/Opa-myc or pCMV/Mfn2-FLAG for 24 hours prior to harvest. Cells were harvested in 1x PBS by scraping and centrifuged at 4°C for three minutes at 1200 rpm. Cell pellets were lysed by resuspension in 1x Cell Lysis Buffer
(Cell Signaling) containing a protease inhibitor cocktail (Roche). Intact cells and insoluble proteins were precipitated by centrifugation (4°C, 20 minutes at 10,000 rpm). Protein concentrations were determined by performing a BCA assay (Pierce) on the supernatants. Twenty micrograms of protein was resolved on a 10% Tris-HCl SDS-PAGE gel (Criterion) and transferred to PVDF for immunoblot analysis. Membranes were washed briefly in TBST [1x TBS (Biorad) containing 0.1% Tween 20 (Biorad)] prior to blocking for one hour in TBST supplemented with 5% non-fat dry milk (Carnation). Immunoblotting was performed using the following antibodies: mouse monoclonal anti-c-myc antibody (Roche) diluted 1:1000 in blocking buffer; mouse monoclonal anti-FLAG M2 (Sigma) diluted 1:1000 in blocking buffer; or mouse monoclonal anti-β-actin (Sigma) diluted 1:5000 in blocking buffer followed by an incubation with donkey anti-mouse secondary antibody conjugated to horseradish peroxidase (Jackson Immunoresearch Labs). Immunoreactive protein bands were detected with an ECL plus reagent (Amersham Pharmacia).

2.3.5 Drp1 protein knockdown using RNA interference for FRAP and immunoblot

Control and PINK1 shRNA cells (4 x 10⁶) were seeded into 160mm dishes transfected with 4.0μg pREP4/Drp1 or GFP siRNA using Lipofectamine-2000 (Invitrogen) according to the manufacturer’s instructions. Drp1 RNAi construct was a generous gift from Dr. Richard Youle. Opti-MEM medium (Invitrogen) supplemented with 10% (v/v) FBS and 5μg/ml blasticidin (Invitrogen) was changed after 24 hours to include 300 μg/ml hygromycin (Invitrogen) antibiotic selection for 4 days.
Prior to seeding for FRAP, cells were washed twice with 1x PBS to remove any remaining hygromycin or phenol-red containing medium. Cells were transfected in suspension with 0.5μg mito-YFP using Lipofectamine 2000 (Invitrogen) 24 hours prior to FRAP analysis [previously described 2.2.9].

Protein knockdown was confirmed by immunoblot. Remaining cells not used for FRAP analysis, were lysed by resuspension in 1x Cell Lysis Buffer (Cell Signaling) containing a protease inhibitor cocktail (Roche). Protein concentrations were determined by performing a BCA assay (Pierce). Ten micrograms of lysates were resolved on a 10% Tris-HCl SDS-PAGE gel (Criterion) and transferred to PVDF for immunoblot analysis. Membranes were washed briefly in TBST [1x TBS (Biorad) containing 0.1% Tween 20 (Biorad)] prior to blocking for one hour in TBST supplemented with 5% non-fat dry milk (Carnation). Immunoblotting was performed using mouse monoclonal anti-Drp1 antibody (BD Translabs) diluted 1:1000 in blocking buffer and mouse monoclonal anti-β-actin (Sigma) diluted 1:5000 in blocking buffer followed by an incubation with donkey anti-mouse secondary antibody conjugated to horseradish peroxidase (Jackson Immunoresearch Labs). Immunoreactive protein bands were detected with an ECL plus reagent (Amersham Pharmacia).

2.3.6 Mitotracker staining with Drp1-YFP fluorescence

Control and PINK1 shRNA cells (2 x 10^5) were grown for 24 hours on 22-mm coverslips coated with poly-L-lysine prior to transient transfection (24 hours) with 2.5μg of pDEST53/Drp1-YFP using Lipofectamine 2000 (Invitrogen). Mitochondria were stained
with 200nM Mitotracker CMXRos (Molecular Probes, Inc.) for 30 minutes at 37°C, washed once with 1x PBS and fixed with 4% (w/v) paraformaldehyde in 1x PBS. Cells were permeabilized with 1x PBS plus 0.1% (v/v) Triton X-100 for 10 minutes at room temperature. Excess PFA was quenched with 0.1M Glycine in 1x PBS at room temperature for 20 minutes. Cells were washed again with 1x PBS and mounted with ProLong Gold Antifade reagent (Molecular Probes, Inc.) prior to imaging on a Zeiss 510 microscope with a 100x Plan-Apochromat 1.4/Oil DIC objective lens (Carl Zeiss). Overlay images were generated using LSM 510 software (Zeiss MicroImaging).

2.3.7 Subcellular fractionation and immunoblot analysis of Drp1-YFP overexpression

Control and PINK1 shRNA cells (1 x 10⁶) were seeded into 10cm dishes prior to a 24-hour transfection with 4.0μg of pDEST53/Drp1-YFP using Lipofectamine 2000 (Invitrogen). Total cell lysates were harvested and mitochondrial and cytosolic separations were obtained using the mitochondrial isolation kit for cultured mammalian cells (Pierce), according to the manufacturer’s instructions. Protein concentrations were determined by performing a BCA assay (Pierce). Ten micrograms of lysates were resolved on a 10% Tris-HCl SDS-PAGE gel (Criterion) and transferred to PVDF for immunoblot analysis. Membranes were washed briefly in TBST [1x TBS (Biorad) containing 0.1% Tween 20 (Biorad)] prior to blocking for one hour in TBST supplemented with 5% non-fat dry milk (Carnation). Immunoblotting was performed using mouse monoclonal anti-Drp1 antibody (BD Translabs) diluted 1:1000 in blocking buffer followed by donkey anti-mouse secondary antibody conjugated to horseradish
peroxidase (Jackson Immunoresearch Labs). Immunoreactive protein bands were
detected with an ECL plus reagent (Amersham Pharmacia). The membrane was stripped
for one hour at 37°C using Pierce Restore Western Blot Stripping Buffer and reprobed
with mouse monoclonal anti-Hsp60 antibody (Stressgen, 1:1000) to show purity of
fractionation.

2.3.8 Mitotracker staining combined with HA-Drp1 immunofluorescence

Following FRAP analysis, chambers containing control and PINK1 shRNA cells
transiently co-transfected with mito-YFP and pGW/HA-Drp1 were stained with 200nM
Mitotracker CMXRos (Molecular Probes, Inc.) for 30 minutes at 37°C, washed once with
1x PBS and fixed with 4% (w/v) paraformaldehyde in 1x PBS. Cells were permeabilized
with 1x PBS plus 0.1% (v/v) Triton X-100 for 10 minutes at room temperature. Excess
PFA was quenched with 0.1M Glycine in 1x PBS at room temperature for 20 minutes.
Coverslips removed from the chamber were blocked with 10% FBS in 1x PBS containing
0.1% Triton X-100 for one hour at room temperature. Immunostaining with mouse
monoclonal anti-HA antibody (Roche) diluted 1:200 in blocking buffer was performed
overnight at 4°C in a humidified chamber tray. Coverslips were washed with 1x PBS and
the secondary goat anti-mouse IgG Alexa Fluor 647 antibody was diluted 1:200 in
blocking buffer and added to the coverslips for one hour. Cells were washed again with
1x PBS and mounted with ProLong Gold Antifade reagent (Molecular Probes, Inc.) prior
to imaging on a Zeiss 510 microscope with a 100x Plan-Achromat 1.4/Oil DIC
objective lens (Carl Zeiss). Overlay images were generated using LSM 510 software
(Zeiss MicroImaging).
2.3.9 FRAP and immunoblot analysis of transiently transfected fission proteins

For FRAP: Control and PINK1 shRNA cells (2 x 10^5 cells/well) were co-transfected in suspension with 0.5μg mito-YFP and 2.0μg pcMV/Fis1-FLAG or pGW1/Drp1-HA using Lipofectamine 2000 (Invitrogen) for 24 hours prior to imaging in LabTekII borosilicate chambers (Nunc). FRAP was performed as previously described [2.2.9].

For immunoblot: Control and PINK1 shRNA cells (3 x 10^5 cells/well) were transfected in suspension with 2.0μg pcMV/Fis1-FLAG or pGW1/Drp1-HA for 24 hours prior to harvest. Cells were harvested in 1x PBS by scraping and centrifuged at 4°C for three minutes at 1200 rpm. Cell pellets were lysed by resuspension in 1x Cell Lysis Buffer (Cell Signaling) containing a protease inhibitor cocktail (Roche). Intact cells and insoluble proteins were precipitated by centrifugation (4°C, 20 minutes at 10,000 rpm). Protein concentrations were determined by performing a BCA assay (Pierce) on the supernatants. Ten micrograms of lysates were resolved on a 4-20% Tris-HCl SDS-PAGE gel (Criterion) and transferred to PVDF for immunoblot analysis. Membranes were washed briefly in TBST [1x TBS (Biorad) containing 0.1% Tween 20 (Biorad)] prior to blocking for one hour in TBST supplemented with 5% non-fat dry milk (Carnation). Immunoblotting was performed using the following antibodies: mouse monoclonal anti-HA antibody (Roche) diluted 1:1000 in blocking buffer; mouse monoclonal anti-FLAG M2 (Sigma) diluted 1:1000 in blocking buffer; or mouse monoclonal anti-β-actin (Sigma) diluted 1:5000 in blocking buffer followed by an incubation with donkey anti-mouse secondary antibody conjugated to horseradish peroxidase (Jackson Immunoresearch.
Immunoreactive protein bands were detected with an ECL plus reagent (Amersham Pharmacia).

2.3.10 Phosphoprotein enrichment and immunoblot analysis of mitochondrial dynamics proteins

Control and PINK1 shRNA cells (4 x 10^6) were seeded into 160mm dishes 24 hours prior to harvesting. Cells were harvested by scraping with 1x TBS and subjected to centrifugation to collect cell pellet for Phosphoprotein enrichment (Qiagen)(Greggio et al., 2007). After lysis (Qiagen Phosphoprotein reagents) and a centrifugation step to clear the lysate, the protein content of the cells was adjusted to 0.1 mg/ml after performing a BCA assay (Pierce). The protocol to purify phosphorylated proteins was then followed according to the manufacturer’s instructions. Ten micrograms of phosphorylated eluates and total lysates were resolved on a 4-20% Tris-HCl SDS-PAGE gel (Criterion) and transferred to PVDF for immunoblot analysis. Membranes were washed briefly in TBST [1x TBS (Biorad) containing 0.1% Tween 20 (Biorad)] prior to blocking for one hour in TBST supplemented with 5% non-fat dry milk (Carnation). The following antibodies were used at given dilutions: mouse monoclonal anti-Drp1 (BD Translabs), 1:1000; mouse monoclonal anti-Opa1 (BD Translabs), 1:500; rabbit polyclonal anti-Omi/HtrA2 (Cell Signaling), 1:1000; rabbit polyclonal anti-Fis1 (Biovision) 1:1000; monoclonal anti-DJ (Stressgen), 1:1000. Mfn1 and Mfn2 rabbit polyclonal antibodies were a generous gift from Dr. Richard Youle. Phospho-S637 Drp1 rabbit polyclonal antibody was kindly provided by Dr. Craig Blackstone. Donkey anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Jackson Immunoresearch Labs) were
then used. Immunoreactive protein bands were detected with an ECL plus reagent (Amersham Pharmacia). The signals were scanned using a StormScan860 densitometer and quantified using Image-Quant software (GE Healthcare) where protein expression of phosphorylated Drp1 was compared to total Drp1 signal.

2.3.11 Subcellular fractionation and immunoblot analysis of endogenous Drp1

Control and PINK1 shRNA cells (1 x 10⁶) were seeded into 10cm dishes prior harvest. Total cell lysates were harvested and mitochondrial and cytosolic separations were obtained using the mitochondrial isolation kit for cultured mammalian cells (Pierce), according to the manufacturer’s instructions. Protein concentrations were determined by performing a BCA assay (Pierce). Ten micrograms of lysates were resolved on a 10% Tris-HCl SDS-PAGE gel (Criterion) and transferred to PVDF for immunoblot analysis. Membranes were washed briefly in TBST [1x TBS (Biorad) containing 0.1% Tween 20 (Biorad)] prior to blocking for one hour in TBST supplemented with 5% non-fat dry milk (Carnation). Immunoblotting was performed using mouse monoclonal anti-Drp1 antibody (BD Translabs) diluted 1:1000 in blocking buffer followed by an incubation with donkey anti-mouse secondary antibody conjugated to horseradish peroxidase (Jackson Immunoresearch Labs). Immunoreactive protein bands were detected with an ECL reagent (Amersham Pharmacia). The membrane was stripped for one hour at 37°C using Pierce Restore Western Blot Stripping Buffer and reprobed with mouse monoclonal anti-Hsp60 antibody (Stressgen, 1:1000) to show purity of fractionation.

2.3.12 BMH crosslinking to examine Drp1 oligomerization
Control and PINK1 shRNA cells (1 x 10^6) were seeded into 10 cm dishes. Prior to harvest, cells were incubated with bis(maleimido)hexane (BMH, Pierce) crosslinker (20 μM, 30 min) followed by two rinses with DTT (20 mM) to quench the crosslinking reaction. Cells were harvested in 1x PBS by scraping and centrifuged at 4°C for three minutes at 1200 rpm. Cell pellets were lysed by resuspension in 1x Cell Lysis Buffer (Cell Signaling) containing a protease inhibitor cocktail (Roche). Protein concentrations were determined by performing a BCA assay (Pierce) on the supernatants of the cleared cell lysates. Ten micrograms of lysates were resolved on a 4-20% Tris-HCl SDS-PAGE gel (Criterion) and transferred to PVDF for immunoblot analysis. Membranes were washed briefly in TBST [1x TBS (Biorad) containing 0.1% Tween 20 (Biorad)] prior to blocking for one hour in TBST supplemented with 5% non-fat dry milk (Carnation). Protein multimers were detected by immunoblotting with mouse monoclonal anti-Drp1 antibody (BD Translabs) diluted 1:1000 in blocking buffer followed by an incubation with donkey anti-mouse secondary antibody conjugated to horseradish peroxidase (Jackson Immunoresearch Labs). Immunoreactive protein bands were detected with an ECL reagent (Amersham Pharmacia).

2.3.13 Drp1 GTPase assays

Control and PINK1 shRNA cells (4 x 10^6) were seeded into 160 mm dishes. Cells were harvested in 1x PBS by scraping and centrifuged at 4°C for three minutes at 1200 rpm. Cell pellets were lysed by resuspension in 1x Cell Lysis Buffer (Cell Signaling) containing a protease inhibitor cocktail (Roche) for 30 minutes by inversion at 4°C. Intact cells and insoluble proteins were precipitated by centrifugation (4°C, 20 minutes at
10,000 rpm). Supernatants were immunoprecipitated with 4μg anti-Drp1 antibody (BD Translabs) overnight with rocking at 4°C. Lysates were then incubated with protein G sepharose beads (Amersham) for 2 hours at 4°C. Beads were washed 5 times with 1x PBS supplemented with 300mM NaCl and 1% Triton X-100, once in assay buffer (20mM HEPES pH 7.2, 2mM MgCl2, 1mM DTT, 0.005% BSA), re-suspended in 40μl of the same buffer and α³²P-GTP (5 μCi; GE healthcare) was added to each reaction. Samples were incubated at room temperature with vigorous shaking and 1μl aliquots removed at time points from 0-120 minutes and spotted onto TLC plates (Sigma). Samples were then subjected to rising thin layer chromatography under 1M formic acid with 1.2M LiCl for two hours. Plates were dried for 5 minutes and radioactive bands were detected by autoradiography using a phosphorscreen. [³²P]-GDP and [³²P]-GTP spots were identified using a StormScan860 PhosphorImager with ImageQuant software (GE Healthcare). GTPase activity was expressed as loss of GTP at each time point.

2.3.14 Immunoblotting for calcineurin

Control and PINK1 shRNA cells (1 x 10⁶) were seeded into 10cm dishes. Cells were harvested in 1x TBS by scraping and centrifuged at 4°C for three minutes at 1200 rpm. Post-nuclear supernatants were then purified by gel filtration (Biomol kit). Proteins were resolved on a 10% Tris-HCl SDS-PAGE gel, transferred to PVDF, and immunoblot analysis was performed. Mouse monoclonal anti-calcineurin α-subunit (BD Translabs) and mouse monoclonal anti-β-actin (Sigma) antibodies were used. The blot was developed with a peroxidase-labeled secondary donkey anti-mouse antibody (Jackson Immunochemicals) using an ECL plus reagent (Amersham Pharmacia) with a
StormScan860 densitometer. The relative intensity of immunoreactive bands was measured using ImageQuant software (GE Healthcare) and normalized to β-actin.

2.3.15 Calcineurin enzyme assay
Control and PINK1 shRNA cells (1 x 10^6) were seeded into 10cm dishes. Cells were harvested in 1x TBS by scraping and centrifuged at 4°C for three minutes at 1200 rpm. Post-nuclear cell extracts were desalted by gel filtration to remove excess phosphate and nucleotides. Samples were used in the cellular calcineurin (PP2B) phosphatase assay according the manufacturer’s instructions (Biomol). The detection of free-phosphate released is based on malachite green assay using human recombinant calcineurin as a positive control. Following background subtraction, absorbance data collected at 620nm (OD620nm) data was converted into the amount of phosphate released using standard curve line-fit data, where the amount of phosphate released=(OD620nm-Yint)/slope. To determine the contribution of PP2B, activity from samples treated with EGTA were subtracted from the total phosphatase activity for each sample.

2.3.16 FRAP, phosphoprotein-enrichment and immunoblot analysis of FK506 treated cells
For FRAP: Control and PINK1 shRNA cells (2 x 10^5 cells/well) were reverse transfected with 0.5µg mito-YFP using Lipofectamine 2000 (Invitrogen) for 24 hours. Prior to imaging in LabTekII borosilicate chambers (Nunc), cells were treated with DMSO (vehicle) or 1µM FK506 (Sigma) for 1 hour or 3 hours. FRAP was performed as previously described [2.2.9].

-80-
For phosphoprotein-enrichment and immunoblot: Control and PINK1 shRNA cells (4 x 10^6) seeded in 160mm dishes were treated for 1 hour with 1μM FK506 (Sigma) or DMSO prior to harvesting. Cells were harvested by scraping with 1x TBS and subjected to centrifugation to collect cell pellet for Phosphoprotein enrichment (Qiagen)(Greggio et al., 2007). After lysis (Qiagen Phosphoprotein reagents) and a centrifugation step to clear the lysate, the protein content of the cells was adjusted to 0.1 mg/ml after performing a BCA assay (Pierce). The protocol to purify phosphorylated proteins was then followed according to the manufacturer’s instructions. Protein concentrations were determined by performing a BCA assay (Pierce). Ten micrograms of phosphorylated eluates and total lysates were resolved on a 10% Tris-HCl SDS-PAGE gel (Criterion) and transferred to PVDF for immunoblot analysis. Membranes were washed briefly in TBST [1x TBS (Biorad) containing 0.1% Tween 20 (Biorad)] prior to blocking for one hour in TBST supplemented with 5% non-fat dry milk (Carnation). Immunoblotting was performed with mouse monoclonal anti-Drp1 (BD Translabs, 1:1000), rabbit polyclonal anti-pS637-Drp1 (gift from Craig Blackstone, 1:500), and mouse monoclonal anti-DJ-1 (Stressgen, 1:4000). Donkey anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Jackson Immunoresearch Labs) were then applied at a dilution of 1:5000. Immunoreactive protein bands were detected with an ECL reagent (Amersham Pharmacia). The signals were scanned using a StormScan860 densitometer and quantified using Image-Quant software (GE Healthcare) where protein expression of phosphorylated Drp1 was compared to total Drp1 signal.
2.4 Materials and methods for Chapter Six

2.4.1 Cell culture

Primary fibroblast cells derived from five patients with homozygous or compound heterozygous mutations in the parkin gene were cultured continuously in Minimum Essential Medium with 10% FBS, 100IU/ml penicillin, 100μg/ml streptomycin, 1mM sodium pyruvate, 2mM L-glutamine, 0.1mM amino acids, 50μg/ml uridine and 1X MEM vitamins.

M17 neuroblastoma cells were used as previously described (2.1.1). Clonal M17 cell lines stably expressing different DJ-1 shRNAs were constructed with the Invitrogen BLOCK-iT Lentiviral system, according to the manufacturer's instructions. DJ-1 target sequences (GGAAGTAAAGTTACAACACA or GGTCATTACACCTACTCTGAG) and one nonsense control sequence (GCCTAGACGCGATAGTATGGA) were used. DJ-1 protein knockdown was confirmed by western blot using monoclonal anti-DJ-1 antibody, 1:3000 (Stressgen).

2.4.2 Transformations

The same protocol was used as described in 2.3.2.

2.4.3 DNA purification for plasmids

The same protocol was used as described in 2.3.3.
Table 2.4.3 Plasmids used in Chapter Six

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2.4.4 FRAP analysis of control and parkin patient fibroblasts

A series of fibroblasts from control and parkin patient fibroblasts were obtained from Dr. Oliver Bandmann (University of Sheffield, UK). Control and parkin patient fibroblast cells (2 x 10^5 cells/well) were transfected in suspension with 0.5μg mito-YFP using Lipofectamine 2000 (Invitrogen) for 24 hours. Prior to imaging in LabTekII borosilicate chambers (Nunc), cells were treated with DMSO (vehicle) or 100nM rotenone (Sigma) for 72 hours. FRAP was performed as previously described [2.2.9].

2.4.5 FRAP and immunoblot analysis of neuroblastoma cells after parkin and PINK1 overexpression

For FRAP: M17, control or PINK1 shRNA cells (2 x 10^5 cells/well) were co-transfected in suspension with 0.5μg mito-YFP and 2.0μg pcDNA3.1/parkin-myc or pDEST40/PINK1-V5 using Lipofectamine 2000 (Invitrogen) and treated with DMSO or
100nM rotenone for 24 hours prior to imaging in LabTekII borosilicate chambers (Nunc). FRAP was performed as previously described [2.2.9].

For immunoblot: M17, control or PINK1 shRNA cells (3 x 10^5 cells/well) were reverse transfected with 2.0μg pcDNA3.1/parkin-myc or pDEST40/PINK1-V5 using Lipofectamine 2000 (Invitrogen) in a 6-well plate 24 hours prior to harvest. Cells were harvested in 1x PBS by scraping and centrifuged at 4°C for three minutes at 1200 rpm. Cell pellets were lysed by resuspension in 1x Cell Lysis Buffer (Cell Signaling) containing a protease inhibitor cocktail (Roche). Intact cells and insoluble proteins were precipitated by centrifugation (4°C, 20 minutes at 10,000 rpm). Protein concentrations were determined by performing a BCA assay (Pierce) on the supernatants. Ten micrograms of lysates were resolved on a 10% Tris-HCl SDS-PAGE gel (Criterion) and transferred to PVDF for immunoblot analysis. Membranes were washed briefly in TBST [1x TBS (Biorad) containing 0.1% Tween 20 (Biorad)] prior to blocking for one hour in TBST supplemented with 5% non-fat dry milk (Carnation). Immunoblotting was performed using mouse monoclonal anti-c-myc antibody (Roche, 1:1000) or rabbit polyclonal anti-V5 antibody (Invitrogen, 1:5000). Proteins were detected using donkey anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Jackson Immunoresearch Labs) with an ECL plus reagent (Amersham Pharmacia). The membrane was stripped for one hour at 37°C using Pierce Restore Western Blot Stripping Buffer and reprobed with mouse monoclonal anti-β-actin antibody (Sigma, 1:5000) to show equal loading.
2.4.6 FRAP and immunoblot analysis of DJ-1 deficient cells

For FRAP: Control and DJ-1 shRNA cells (2 x 10^5 cells/well) were transfected in suspension with 0.5µg mito-YFP using Lipofectamine 2000 (Invitrogen). Cells were treated with DMSO, 100nM rotenone (Sigma), 200µM paraquat (Sigma), 100µM MPP⁺ (1-methyl-4-phenylpyridinium, Sigma), 100nM staurosporine (Sigma), 10µM 2-oxo-L-thiazolidine-4-carboxylic acid (OTCA, Sigma) or 100µM glutathione ethyl ester (GSHEE, Sigma) for 24 hours prior to imaging in LabTekII borosilicate chambers (Nunc). FRAP was performed as previously described [2.2.9].

For immunoblot of DJ-1 knockdown: Control and DJ-1 shRNA cells (3 x 10^5) were seeded in a 6-well plate 24 hours prior to harvest. Cells were harvested in 1x PBS by scraping and centrifuged at 4°C for three minutes at 1200 rpm. Cell pellets were lysed by resuspension in 1x Cell Lysis Buffer (Cell Signaling) containing a protease inhibitor cocktail (Roche). Intact cells and insoluble proteins were precipitated by centrifugation (4°C, 20 minutes at 10,000 rpm). Protein concentrations were determined by performing a BCA assay (Pierce) on the supernatants. Ten micrograms of lysates were resolved on a 4-20% Tris-HCl SDS-PAGE gel (Criterion) and transferred to PVDF for immunoblot analysis. Membranes were washed briefly in TBST [1x TBS (Biorad) containing 0.1% Tween 20 (Biorad)] prior to blocking for one hour in TBST supplemented with 5% non-fat dry milk (Carnation). Immunoblotting was performed using mouse monoclonal anti-DJ-1 antibody (Stressgen, 1:1000). Proteins were detected using donkey anti-mouse secondary antibody conjugated to horseradish peroxidase (Jackson Immunoresearch Labs) with an ECL plus reagent (Amersham Pharmacia). The membrane was stripped for
one hour at 37°C using Pierce Restore Western Blot Stripping Buffer and reprobed with mouse monoclonal anti-β-actin antibody (Sigma, 1:5000) to show equal loading.

### 2.4.7 FRAP and immunoblot analysis of DJ-1 deficient cells following PTEN/Akt modulation

For FRAP: Control and DJ-1 shRNA cells (2 x 10^5 cells/well) were transfected in suspension with 0.5μg mito-YFP using Lipofectamine 2000 (Invitrogen). Cells were treated with DMSO, 10μM 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT, Calbiochem) or 18μM LY294002 (Calbiochem) for 24 hours prior to imaging in LabTekII borosilicate chambers (Nunc). FRAP was performed as previously described [2.2.9].

For immunoblot: Control and DJ-1 shRNA cells (3 x 10^5 cells/well) were treated with DMSO, 10μM DMAT or 18μM LY294002 for 24 hours prior to harvest. Cells were harvested in 1x PBS by scraping and centrifuged at 4°C for three minutes at 1200 rpm. Cell pellets were lysed by resuspension in 1x Cell Lysis Buffer (Cell Signaling) containing a protease inhibitor cocktail (Roche). Intact cells and insoluble proteins were precipitated by centrifugation (4°C, 20 minutes at 10,000 rpm). Protein concentrations were determined by performing a BCA assay (Pierce) on the supernatants. Ten micrograms of lysates were resolved on a 4-20% Tris-HCl SDS-PAGE gel (Criterion) and transferred to PVDF for immunoblot analysis. Membranes were washed briefly in TBST [1x TBS (Biorad) containing 0.1% Tween 20 (Biorad)] prior to blocking for one hour in TBST supplemented with 5% non-fat dry milk (Carnation). Immunoblotting was
performed using the following antibodies: rabbit polyclonal anti-PTEN (Cell Signaling, 1:1000); mouse monoclonal anti-phospho-PTEN (S380/T382/T383) (Cell Signaling, 1:1000); rabbit polyclonal anti-Akt (Cell Signaling, 1:1000); and mouse monoclonal anti-phospho-Akt (T308) (Cell Signaling, 1:1000). Proteins were detected using donkey anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch Labs) with an ECL plus reagent (Amersham Pharmacia). The membranes were stripped for one hour at 37°C using Pierce Restore Western Blot Stripping Buffer and reprobed with mouse monoclonal anti-β-actin antibody (Sigma, 1:5000) to show equal loading.

2.4.8 FRAP, phosphoprotein-enrichment and immunoblot analysis following the inhibition of fission or the promotion of fusion in DJ-1 deficient cells

For FRAP: Control and DJ-1 shRNA cells (2 x 10⁵ cells/well) were co-transfected in suspension with 0.5µg mito-YFP and 2.0µg pcDNA3.1/Opa1-myc, pCMV/Mfn1-FLAG or pGW1/Drp1 K38A-myc using Lipofectamine 2000 (Invitrogen) for 24 hours prior to imaging in LabTekII borosilicate chambers (Nunc). FRAP was performed as previously described [2.2.9].

For immunoblot of transfection: Control and DJ-1 shRNA cells (3 x 10⁵) seeded in a 6-well plate were transfected in suspension with 2.0µg pcDNA3.1/Opa1-myc, pCMV/Mfn1-FLAG or pGW1/Drp1 K38A-myc using Lipofectamine 2000 (Invitrogen) for 24 hours prior to harvest. Cells were harvested in 1x PBS by scraping and centrifuged at 4°C for three minutes at 1200 rpm. Cell pellets were lysed by resuspension in 1x Cell
Lysis Buffer (Cell Signaling) containing a protease inhibitor cocktail (Roche). Intact cells and insoluble proteins were precipitated by centrifugation (4°C, 20 minutes at 10,000 rpm). Protein concentrations were determined by performing a BCA assay (Pierce) on the supernatants. Ten micrograms of lysates were resolved on a 4-20% Tris-HCl SDS-PAGE gel (Criterion) and transferred to PVDF for immunoblot analysis. Membranes were washed briefly in TBST [1x TBS (Biorad) containing 0.1% Tween 20 (Biorad)] prior to blocking for one hour in TBST supplemented with 5% non-fat dry milk (Carnation). Immunoblotting was performed using the following antibodies: mouse monoclonal anti-c-myc (Roche, 1:1000); mouse monoclonal anti-FLAG M2 (Sigma, 1:1000); mouse monoclonal anti-DJ-1 (Stressgen, 1:1000). Proteins were detected using donkey anti-mouse secondary antibody conjugated to horseradish peroxidase (Jackson Immunoresearch Labs) with ECL plus reagent (Amersham Pharmacia). The membranes were stripped for one hour at 37°C using Pierce Restore Western Blot Stripping Buffer and reprobed with mouse monoclonal anti-β-actin antibody (Sigma, 1:5000) to show equal loading.

For phospho-protein enrichment and immunoblot: Control and DJ-1 shRNA cells (4 x 10⁶) were seeded into 160mm dishes 24 hours prior to harvesting. Cells were harvested by scraping with 1x TBS. Phosphoprotein enrichment (Qiagen) was performed and validated as described previously (Greggio et al., 2007). Protein concentrations were determined by performing a BCA assay (Pierce). Ten micrograms of phosphorylated eluates and total lysates were resolved on a 10% Tris-HCl SDS-PAGE gel (Criterion) and transferred to PVDF for immunoblot analysis. Membranes were washed briefly in TBST
[1x TBS (Biorad) containing 0.1% Tween 20 (Biorad)] prior to blocking for one hour in TBST supplemented with 5% non-fat dry milk (Carnation). Immunoblotting was performed with mouse monoclonal anti-Drp1 (BD Translabs, 1:1000), mouse monoclonal anti-DJ-1 (Stressgen, 1:1000) and rabbit polyclonal anti-phospho-ERK1/2 (Cell Signaling, 1:1000). Proteins were detected using donkey anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Jackson Immunoresearch Labs) with an ECL plus reagent (Amersham Pharmacia). The signals were scanned using a StormScan860 densitometer and quantified using Image-Quant software (GE Healthcare) where protein expression of phosphorylated Drp1 was compared to total Drp1 signal.

2.4.9 FRAP and immunoblot analysis in DJ-1 deficient cells after parkin or PINK1 overexpression

For FRAP: Control and DJ-1 shRNA cells (2 x 10^5 cells/well) were co-transfected in suspension with 0.5μg mito-YFP and 2.0 μg pcDNA3.1/parkin-myc or pDEST40/PINK1-V5 using Lipofectamine 2000 (Invitrogen) for 24 hours prior to imaging in LabTekII borosilicate chambers (Nunc). FRAP was performed as previously described [2.2.9].

For immunoblot of transfection: Control and DJ-1 shRNA cells (3 x 10^5) seeded in a 6-well plate were transfected in suspension with 2.0μg pcDNA3.1/parkin-myc or pDEST40/PINK1-V5 using Lipoectamine 2000 (Invitrogen) for 24 hours prior to harvest. Cells were harvested in 1x phosphate buffered saline (PBS) by scraping and
centrifuged at 4°C for three minutes at 1200 rpm. Cell pellets were lysed by resuspension in 1x Cell Lysis Buffer (Cell Signaling) containing a protease inhibitor cocktail (Roche). Intact cells and insoluble proteins were precipitated by centrifugation (4°C, 20 minutes at 10,000 rpm). Protein concentrations were determined by performing a BCA assay (Pierce) on the supernatants. Ten micrograms of lysates were resolved on a 4-20% Tris-HCl SDS-PAGE gel (Criterion) and transferred to PVDF for immunoblot analysis. Membranes were washed briefly in TBST [1x TBS (Biorad) containing 0.1% Tween 20 (Biorad)] prior to blocking for one hour in TBST supplemented with 5% non-fat dry milk (Carnation). Immunoblotting was performed using the following antibodies: mouse monoclonal anti-c-myc (Roche, 1:1000); rabbit polyclonal anti-V5 (Invitrogen, 1:1000); mouse monoclonal anti-DJ-1 (Stressgen, 1:1000). Proteins were detected using donkey anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Jackson Immunoresearch Labs) with an ECL plus reagent (Amersham Pharmacia). The membranes were stripped for one hour at 37°C using Pierce Restore Western Blot Stripping Buffer and reprobed with mouse monoclonal anti-β-actin antibody (Sigma, 1:5000) to show equal loading.
CHAPTER THREE

Stress-induced modulation of PINK1 and its relevance to parkinsonism
3.1 Summary

Mitochondria isolated from PD patients exhibit decreased complex I activity and increased levels of reactive oxygen species (Schapira et al., 1989, Swerdlow et al., 1996). Human exposure to the complex I inhibitor MPTP leads to selective dopaminergic cell death and development of a parkinsonism phenotype (Langston et al., 1983). These mitochondrial toxins are used to generate animal models of PD that exhibit selective dopaminergic neurodegeneration of the \textit{SNpc} (Langston et al., 1984) and in some cases accumulation of cytoplasmic inclusions (Betarbet et al., 2000). This chapter illustrates that PINK1 is neuroprotective against mitochondrial toxins that cause oxidative stress in cultured mammalian cells. PINK1 mRNA expression is upregulated after induction of oxidative stress induced by rotenone \textit{in vitro} and \textit{in vivo} possibly affording protection against mitochondrial dysfunction. Additionally, no alterations in PINK1 mRNA stability are seen following rotenone treatment suggesting transcriptional changes are explained by increased PINK1 mRNA expression. These data suggest that PINK1 promotes cell survival, which could potentially impact mitochondrial function and morphology.
3.2 Results

3.2.1 Cell viability of stable neuroblastoma cells overexpressing PINK1 after exposure to mitochondrial toxins

Previous studies have shown that overexpression of PINK1 in cultured mammalian cells provides neuroprotection against oxidative stress (Deng et al., 2005, Haque et al., 2008, Hoepken et al., 2008, Hoepken et al., 2007, Pridgeon et al., 2007, Tang et al., 2006, Valente et al., 2004a, Wood-Kaczmar et al., 2008). MTT assays were performed to examine the function of the stable neuroblastoma cell lines overexpressing wild-type PINK1, a recessive mutant G309D (Kessler et al., 2005) and an artificial variant previously shown to lack kinase activity, here on referred to as kinase dead (Beilina et al., 2005) when exposed to mitochondrial toxins for 48 hours. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. Cell viability was calculated by the absorbance measured at a wavelength of 590nm after solubilization of formazan. After correcting for the blank, results were expressed as a percentage of the mean when normalized to each untreated cell line. Overexpression of wild-type PINK1 was shown to protect against complex I inhibitors, rotenone (Fig. 3.2.1.A) and MPP⁺ (Fig. 3.2.1.B), that induce oxidative stress through reactive oxygen species generation. Stable cell lines expressing LacZ or the recessive mutant or kinase dead forms of PINK1 failed to protect against these mitochondrial toxins.
Figure 3.2.1  Cell viability of stable neuroblastoma cells overexpressing PINK1 after exposure to mitochondrial toxins

(A-B) MTT assays show PINK1 overexpressing cells (pink lines, upwards triangles) have greater viability after exposure to rotenone for 48 hours compared to cells expressing LacZ (blue squares), G309D PINK1 (green circles) or kinase dead [KD] PINK1 (black squares). The difference between cell lines was significant by two-way ANOVA ($P<0.0001$) as was the effect of rotenone ($P<0.0001$) and there was a significant ($P<0.0001$) interaction between the two parameters. A similar protective effect was seen for MPP$^+$ (B; $P<0.0001$ for MPP$^+$ concentration and $P<0.0001$ for cell line). Each point represents the mean percentage of $n=8$ measurements, normalized to untreated cells in the same cell line and blank corrected. Error bars indicate the percent standard error of the mean (SEM).
Figure 3.2.1  Cell viability of stable neuroblastoma cells overexpressing PINK1

after exposure to mitochondrial toxins

A

B

MTT (% control)

[Rotenone] (nM)

[MPP+] (µM)

LacZ
PINK1 WT
PINK1 G309D
PINK1 KD
3.2.2 *In vitro* PINK1 mRNA expression after exposure to rotenone

To examine the role of PINK1 in oxidative stress and survival, stable neuroblastoma cell lines that express either a short hairpin RNA (shRNA) directed against PINK1 or a scrambled control sequence were generated. These cells were exposed to rotenone and relative PINK1 mRNA expression was measured using comparative quantitative real-time PCR (qRT-PCR) with β-actin mRNA normalization. Relative PINK1 mRNA expression was measured by qRT-PCR following exposure to various concentrations of rotenone for 24 hours (Fig. 3.2.2.A). Relative PINK1 mRNA expression was increased 3.8-fold when control shRNA cells were treated with 50nM rotenone for 24 hours when compared to vehicle-treated control shRNA cells (Fig. 3.2.2.B; two-way ANOVA ($P<0.0001$) cell line, ($P=0.0195$) treatment, ($P=0.0096$) interaction). Figure 3.2.2.B shows the time dependence of PINK1 mRNA induction. PINK1 mRNA expression increases 4.3-fold when control shRNA cells were treated with 50nM rotenone for 48h when compared to the vehicle-treated control shRNA cells (Fig. 3.2.2.B; two-way ANOVA ($P<0.0001$) time, ($P<0.0001$) treatment, ($P<0.0001$) interaction). Optimized time and concentration of rotenone exposure shows relative PINK1 mRNA expression is increased 1.8-fold in control shRNA cells treated with 50nM rotenone for 48 hours (Fig. 3.2.1.C; two-way ANOVA ($P<0.0001$) cell line, ($P=0.0080$) treatment, ($P=0.0012$) interaction). No induction of PINK1 was seen following rotenone treatment in PINK1 shRNA cells. These data show that rotenone induces PINK1 mRNA expression *in vitro.*
Figure 3.2.2  *In vitro* PINK1 mRNA expression after exposure to rotenone

(A) Total RNA was extracted, reverse-transcribed into cDNA and analyzed by qRT-PCR to examine PINK1 mRNA expression following a 24-hour treatment with various concentrations of rotenone in shRNA-mediated knockdown of PINK1 or control scrambled sequence in M17 cells. Relative mRNA expression was normalized to β-actin and data points are shown as the mean gene expression (three independent experiments performed in quadruplicate). Error bars indicate the SEM. Results were analyzed using two-way ANOVA with Bonferroni post-tests: ($P<0.0001$) cell line, ($P=0.0195$) treatment, ($P=0.0096$) interaction between treatment and cell line. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, not significant.

(B) A time course was performed in the control shRNA cells to determine maximal PINK1 mRNA induction. Total RNA was extracted, reverse-transcribed into cDNA and analyzed by qRT-PCR after exposure to 50nM rotenone for up to 48 hours. Data points are shown as the mean gene expression normalized to β-actin of n=3 samples per cell line and/or treatment with quadruplicate cDNA samples. Error bars indicate the SEM. Using two-way ANOVA (($P<0.0001$) time, ($P<0.0001$) treatment, ($P<0.0001$) interaction), results were analyzed with Bonferroni post-tests *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, not significant.

(C) Using the optimal time and concentration of rotenone, control and PINK1 shRNA cells were treated 50nM rotenone treatment for 48 hours. Total RNA was extracted, reverse-transcribed into cDNA and analyzed by qRT-PCR. Bars show the mean gene expression normalized to β-actin of n=6-9 samples per cell line and treatment with quadruplicate cDNA samples. Error bars indicate the SEM. Using two-way ANOVA


\((P<0.0001)\) cell line, \((P=0.0080)\) treatment, \((P=0.0012)\) interaction), results were analyzed with Bonferroni post-tests *\(, P<0.05\); **\(, P<0.01\); ***\(, P<0.001\); ns, not significant.
Figure 3.2.2  *In vitro* PINK1 mRNA expression after exposure to rotenone

A

$\text{PINK1/\beta$-actin mRNA}$

$\text{Rotenone [nM]}$

- Control shRNA
- PINK1 shRNA

B

$\text{PINK1/\beta$-actin mRNA}$

$\text{Time (h)}$

- DMSO
- 50nM Rotenone

C

$\text{PINK1/\beta$-actin mRNA}$

- Control shRNA
- PINK1 shRNA

- DMSO
- 50nM Rotenone
3.2.3  *In vivo* PTEN and PINK1 mRNA expression after exposure to rotenone

To confirm that induction of PINK1 mRNA expression also occurs *in vivo*, a chronic administration of rotenone was given subcutaneously to C57BL/6 male mice and cDNA templates were created from the extracted RNA from whole hemisphere tissue. Relative PINK1 mRNA expression from rotenone treated brain tissue was measured using comparative quantitative real-time PCR (qRT-PCR) with β-actin mRNA normalization. Quantitative real time-PCR data showed PINK1 mRNA expression was increased 2.0-fold when two weeks lapsed following the last rotenone injection when compared to the sham-treated mice (Fig. 3.2.3.A; \(P=0.0162\)). Since *PINK1* was originally discovered as a gene induced in a *PTEN* responsive manner, the relative mRNA expression of PTEN was also measured. Relative PTEN mRNA expression is also increased 2.0-fold at this time point when compared to the sham-treatment mouse (Fig. 3.2.3.B; \(P=0.0084\) by one-way ANOVA). Together these data demonstrate that PTEN and PINK1 mRNA expression is induced *in vitro* and *in vivo* by rotenone exposure.
Figure 3.2.3 *In vivo* PTEN and PINK1 mRNA expression after exposure to rotenone

Two-month-old male C57BL/6 mice were subcutaneously injected with 2mg/kg rotenone for three consecutive days and sacrificed up to three weeks following treatment. Results were compared to a sham-treated mouse sacrificed at the final time point. PTEN (A) and PINK1 (B) mRNA expression was examined using qRT-PCR after total RNA was extracted and reverse transcribed from mouse whole hemisphere brain tissues (N=3) and analyzed by qRT-PCR with quadruplicate cDNA samples. Bars show the mean gene expression when normalized to β-actin. Error bars indicate the SEM. Using one-way ANOVA (F$_{4,10}$=6.32, $P<0.0084$), results were analyzed with Dunnett’s multi-comparison post-tests when compared to the sham-treated sample (*, $P<0.05$; **, $P<0.01$, ***, $P<0.001$).
Figure 3.2.3  *In vivo* PTEN and PINK1 mRNA expression after exposure to rotenone
3.2.4 PINK1 mRNA stability in neuroblastoma cells after exposure to rotenone

Figures 3.2.2 and 3.2.3 illustrated that PINK1 mRNA expression was upregulated in response to oxidative stress induced by rotenone. It has been shown that the regulation of mRNA stability is a critical component in pathways whereby tissues respond to toxins, stress or infection (Ross, 1995), therefore the stability of PINK1 mRNA was examined. Using the transcriptional inhibitor Actinomycin D (2μg/ml), control shRNA neuroblastoma cells pretreated with 50nM rotenone for 48 hours were cultured with the inhibitor and harvested at different times thereafter to measure PINK1 mRNA turnover using qRT-PCR. After normalizing PINK1 to 18S rRNA expression levels, the percentage of remaining mRNA in control shRNA cells with and without rotenone exposure was calculated after normalization to the zero hour time point of Actinomycin D treatment. Figure 3.2.4.A shows the half-life of PINK1 mRNA is approximately 6 hours and its stability remained unchanged following a 48-hour exposure to 50nM rotenone. No significant differences were found in mRNA levels of PINK1 (Fig. 3.2.4.A; (P=0.9431) treatment, (P<0.0001) time, (P=0.9948) interaction) or GAPDH (Fig. 3.2.4.B; (P=0.8136) treatment, (P=0.9843) time, (P=0.9918) interaction) following treatment using two-way ANOVA analysis with Bonferroni post-tests. Previous studies in various cell types have estimated the half-life of a long-lived message such as GAPDH around 20-24 hours (Laubach et al., 1997, Yague et al., 2003). Since the stability of PINK1 mRNA was not decreased following rotenone treatment, changes in relative PINK1 mRNA expression levels when measured by qRT-PCR indicate changes are inferred to be due to increased activity of the PINK1 promoter.
Figure 3.2.4  PINK1 mRNA stability in neuroblastoma cells after exposure to rotenone

To estimate PINK1 mRNA stability, control shRNA neuroblastoma cells were treated with vehicle (DMSO) (downward triangles) or 50nM rotenone (squares) for 48 hours and then were treated with Actinomycin D (2 µg/ml) for the times shown. Total RNA was extracted and reverse transcribed to measure PINK1 (A) and GAPDH (B) mRNA levels by qRT-PCR. After 18S rRNA normalization, results were plotted on a logarithmic scale to calculate the time required for each mRNA to reach one-half of its initial abundance (50%, dashed line). Quantitative real time-PCR results represent the mean values ± SEM from three independent experiments performed in quadruplicate. Using two-way ANOVA, results were analyzed with Bonferroni post-tests *, P<0.05; **, P<0.01; ***, P<0.001; ns, not significant.
Figure 3.2.4  PINK1 mRNA stability in neuroblastoma cells after exposure to rotenone
3.3 Discussion

Patients with parkinsonism exhibit complex I deficiencies in the *substantia nigra* (Schapira et al., 1989). The chronic use of the complex I inhibitor rotenone in animal models has been shown to recapitulate parkinsonism phenotypes (Betarbet et al., 2000, Langston et al., 1983, Langston et al., 1984, Liou et al., 1996, Ricautre et al., 1986). Rotenone, which induces continuous oxidative insults through the production of reactive oxygen species, has significant implications for cell survival. Data presented in this chapter illustrates that PINK1 can protect against mitochondrial dysfunction in neuronal-like cultured cells. It was hypothesized that this neuroprotection was afforded by changes in the levels of the PINK1 mRNA transcript, a proxy measure of changes in protein expression. The mitochondrial toxin rotenone was used in cellular and animal models to induce changes in relative PINK1 gene expression. Prior to mutations being associated with parkinsonism, PINK1 was shown to be upregulated by the tumor suppressor gene, *PTEN* (phosphatase and tensin homologue). Using qRT-PCR, rotenone induces PTEN and PINK1 mRNA expression in a mouse model of chronic exposure. However, no mRNA induction is seen in neuroblastoma cell lines stably expressing a short hairpin RNA against PINK1. PINK1 mRNA expression is increased during the inhibition of complex I activity, which implies that mitochondrial function could be altered by decreased ATP anabolism.

As fibroblasts isolated from PINK1 patients exhibit decreased complex I activity (Hoepken et al., 2007, Piccoli et al., 2008b) and PINK1 protects against mitochondrial dysfunction and oxidative stress, it is tempting to speculate that upregulation of PINK1 mRNA expression promotes the activation of cell survival signaling pathways. The
functional relationship between PINK1 transcriptional regulation and cell survival remains to be elucidated; however, data presented in this chapter suggests indirectly that PINK1 promoter activity may be increased following exposure to oxidative stress.

It is possible that PINK1 activity and function may be influenced by changes in upstream PTEN signaling pathways, since PTEN mRNA levels are also increased following rotenone exposure. PTEN acts to regulate the cell cycle and signals cells to stop dividing and undergo apoptosis. PTEN is a phosphatase that dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) to the product phosphatidylinositol (4,5)-diphosphate (PIP₂) (Maehama and Dixon, 1998). Dephosphorylation of PIP₃ results in the inhibition of Akt through PI3K signaling. Akt is involved in cellular processes that inhibit apoptosis, thereby promoting cell survival (Crowder and Freeman, 1998, Datta et al., 1997, Philpott et al., 1997). Akt can also prevent apoptosis that is induced by oxidative stress (Kang et al., 2003). In a mouse model of parkinsonism using MPP⁺, reactive oxygen species were decreased and apoptosis was prevented in cells overexpressing an active form of Akt (Salinas et al., 2001). Akt signaling has been shown to influence mitochondrial function through the regulation of Bad (Bcl-2 associated death protein). Bad is thought to initiate apoptosis following heterodimer formation with Bcl-xL, displacing pro-apoptotic Bax from Bcl-xL (Yamaguchi and Wang, 2001). Unassociated Bax then translocates to the mitochondria to promote cytochrome c release.Akt phosphorylates Bad to inhibit cytochrome c release and the activation of apoptosis, a similarity shared with some models of PINK1 overexpression in culture (Petit et al., 2005, Wang et al., 2007). Alternatively, anti-apoptotic activity of Akt may also be mediated directly at the level of the mitochondrial membrane and alter
mitochondrial integrity through pathways that are independent of Bel-xL (Plas et al., 2001). Modulation of the inner mitochondrial membrane potential via Akt has been shown to prevent apoptosis (Dijkers et al., 2002). Transcription factors upregulated by alterations in the PI3K/Akt survival pathway could potentially increase PINK1 expression that results from oxidative stress. Future studies should address the genomic elements within the PINK1 promoter that influence gene expression and promote cell survival.

The mechanisms responsible for regulating the PINK1 gene locus, besides rare mutations, give insight into how alterations in PINK1 may influence mitochondrial function. Examination of transcriptional regulation from the PINK1 locus could provide information concerning the neuroprotective role of PINK1. A novel splice variant of PINK1 (svPINK1) that is homologous to the regulatory C-terminus of the PINK1 kinase domain has been predicted (Scheele et al., 2007b). Moreover, a cis-transcribed non-coding natural anti-sense PINK1 (naPINK1) RNA transcript has been shown to overlap in sequence with the svPINK1 and partially overlaps with the 3’ end of the PINK1 transcript (Browser, Zhang et al., 2007). Scheele et al. demonstrated the naPINK1 transcript regulates the PINK1 locus by stabilizing the svPINK1 transcript during mitochondrial biogenesis in human neuroblastoma cells and skeletal muscle tissue (Scheele et al., 2007b). During physical inactivity the PINK1 locus has an opposing pattern of regulation—the naPINK1 transcript is upregulated while the PINK1 transcript is downregulated, suggesting the expression of the PINK1 locus could be influenced by metabolic needs (Scheele et al., 2007a).
In eukaryotes, nuclear mRNA precursor (pre-mRNA) splicing occurs via the U2-dependent spliceosome that is comprised of five uridine-rich small nuclear ribonucleoproteins (snRNPs), U1, U2, U4, U5, U6 snRNPs and other non-snRNP proteins (Reed, 2000). Spliceosome formation is initiated by the recognition of the U1 snRNP to the last three nucleotides at the end of the exon (the 5’ splice site) (Reed, 2000). The pathogenic PINK1 missense mutation E417G has now been identified as an exonic splicing mutation at the 5’ splice site (Sahashi et al., 2007). This splicing mutation found in PINK1 compromised U1 snRNP binding to the 5’ splice site, which affected pre-mRNA splicing and resulted in skipping of the mutation-harboring exon (Sahashi et al., 2007). It is important to discriminate aberrant splicing from other types of mutations to better understand disease mechanism.

Data presented in this chapter illustrates a complex I deficiency can influence PINK1 transcription levels that are associated with cell survival. Patients with nuclear-encoded complex I mutations demonstrate a relationship between complex I deficiency and mitochondrial dysfunction (Koopman et al., 2007). Changes in mitochondrial metabolism, structure and oxidative stress are paralleled in drug-induced models of parkinsonism and human complex I deficiencies. As seen with rotenone exposure, mutation-induced decreases in the cellular amount and activity of complex I have been shown to enhance reactive oxygen species (Koopman et al., 2007). Increased oxidative stress exposure changes mitochondrial shape and number—promoting mitochondrial fission (Jendrach et al., 2008, Knott et al., 2008). Depolarization of the mitochondrial membrane by oxidative stress results in fragmentation of the mitochondrial network (Pletjushkina et al., 2006). Regulation of the mitochondrial network, therefore, is
important for maintaining the health and function of mitochondria. The next chapter will characterize PINK1 mitochondrial function and morphology following oxidative stress.
CHAPTER FOUR

Characterization of mitochondrial morphology and function

following modulation of PINK1
4.1 Summary

Chapter Four examined the role of PINK1 in mitochondrial function. Two cell models were used to study PINK1 function within the mitochondria. The first model utilized stable PINK1 mammalian cell lines that express the wild type form, a recessive mutant G309D, or an artificial variant lacking kinase activity. A second model was employed to mimic the activity of PINK1 loss-of-function mutations by knocking down endogenous PINK1 gene expression in mammalian cells. Decreased $\Delta \psi_m$ was seen in PINK1 deficient cells, in agreement with results in the literature (Abou-Sleiman et al., 2006, Exner et al., 2007, Valente et al., 2004a, Wood-Kaczmar et al., 2008). Depolarization of the mitochondrial membrane can influence mitochondrial morphology (Chen et al., 2003), so the shape of the mitochondrial network was examined using immunocytochemistry, fluorescence recovery after photobleaching (FRAP), and photoactivation experiments. All techniques showed that cells deficient in PINK1 exhibit abnormal mitochondrial phenotypes consistent with mitochondrial fission. Furthermore, mitochondrial connectivity was maintained by the presence of PINK1 and its kinase activity. Exogenous or endogenous PINK1 could protect against rotenone toxicity promoting connectivity of the mitochondrial network seen by FRAP. These observations indicated that PINK1 either promotes mitochondrial fusion or inhibits mitochondrial fission. Photoactivation assays were used to assess differences in mitochondrial fusion in the absence of PINK1. PINK1 deficient cells have deficits in mitochondrial fusion that were associated with decreased mitochondrial membrane potential. Additionally, mitochondrial fusion was dependent upon PINK1 kinase activity. Using a mitochondrial uncoupler, cells deficient in PINK1 kinase activity showed a decreased capacity for
mitochondrial fusion. Together these data demonstrated that PINK1 indirectly influences mitochondrial morphology and function.
4.2 Results

4.2.1 Mitochondrial function in stable cell lines expressing PINK1

To address the function of PINK1 in living cells, two different cell models were used. The first model utilized stable cells from dopaminergic neuroblastoma M17 cells that were generated using lentiviruses that overexpress wild type PINK1, a recessive mutant, G309D and an artificial variant previously shown to lack kinase activity (Beilina et al., 2005) (Fig. 4.2.1.A-B). Because PINK1 mutations are recessive, cells stably transduced with either of two shRNA sequences directed against PINK1 or with a scrambled shRNA used as control (Fig. 4.2.1.C-D) were examined as a model of PINK1 silencing. PINK1 overexpression lines had equivalent expression of protein, assessed via western blotting (Fig. 4.2.1.A). No available antibodies screened to date are able to detect endogenous PINK1 without having additional protein bands (data not shown), so qRT-PCR was used to confirm decreased PINK1 mRNA expression in the shRNA lines. Expression was decreased by >80% with the first PINK1 shRNA sequence (A) and by a similar amount with a second shRNA sequence (C) when compared to the control shRNA cells (Fig. 4.2.1.C).

PINK1 can protect against rotenone toxicity (Deng et al., 2005, Petit et al., 2005) and is important in maintaining mitochondrial membrane potential ($\Delta \psi_m$) (Exner et al., 2007, Wood-Kaczmar et al., 2008) in mammalian cells. These previous observations were used to confirm that the PINK1 constructs behaved as expected. Cells expressing the wild type form of PINK1 but not kinase dead or the pathogenic mutation maintained $\Delta \psi_m$ estimated using TMRE (tetramethylrhodamine ethyl ester) fluorescent staining in living cells (Fig. 4.2.1.B; $P<0.0001$ for cell lines by one-way ANOVA), which was found
to be similar to that of previous studies (Abou-Sleiman et al., 2003, Valente et al., 2004a). In contrast, cells expressing PINK1 shRNA showed decreased $\Delta \psi_m$ (Fig. 4.2.1.D, 28% with sequence A and 25% with sequence C; $P<0.0001$ for cell lines using one-way ANOVA), again consistent with recent reports (Exner et al., 2007). It was confirmed that rotenone depolarizes mitochondrial membrane potential and decreased TMRE fluorescence 64% when compared to the untreated control cells (Fig. 4.2.1.B/D). Collectively, these results suggested that PINK1, via its kinase activity, influenced mitochondrial function that included maintaining mitochondrial membrane potential.
Figure 4.2.1 Mitochondrial function in stable cell lines expressing PINK1

(A) Whole cell lysates from stable neuroblastoma cell lines generated using lentiviral transduction were resolved on a 4-20% SDS-PAGE gel and transferred to PVDF membrane. A control lentivirus expressing LacZ is shown in lane 1, lanes 2-4 are wild type (WT), G309D and kinase dead (KD) PINK1. PINK1 is V5-tagged at the C-terminus, thus the precursor (arrow) and mature (closed arrowhead) forms of PINK1 are visible after immunoblotting with anti-V5 antibody. In the lower panel, the blot was reprobed with β-actin (open arrowhead) to show equal loading. Molecular weight markers on the right are in kilodaltons.

(B) Mitochondrial membrane potential ($\Delta \psi_m$) was measured using TMRE staining in live cells stably overexpressing LacZ, PINK1 WT, PINK1 G309D or PINK1 KD. Results are expressed as the mean percentage of TMRE fluorescence with background correction when compared to the mean fluorescence intensity in the LacZ control cell line. Error bars show the SEM (n=8 independent experiments of 10 observations per line). Rotenone (500nM, 1 hour) was used to confirm mitochondrial membrane depolarization and subsequent loss of TMRE fluorescent staining. The differences between the cell lines were significant by one-way ANOVA ($F_{4,323}=770.5$, $P<0.0001$, $n=8$ independent experiments of 10 observations per line) using Dunnett’s multi-comparison post-tests with LacZ comparison (*, $P<0.05$; ***, $P<0.0001$; ns, $P>0.05$).

(C) Total RNA was extracted and reverse transcribed from control and PINK1 shRNA cell lines to be examine PINK1 mRNA expression by qRT-PCR. Bars show the mean relative PINK1 expression when normalized to β-actin and error bars indicate the SEM between experiments. PINK1 shRNA sequences (A) and (C) decreased endogenous
mRNA expression. The differences between the cell lines were significant by one-way ANOVA ($F_{2,15}=162.3$, $P<0.0001$, $n=6$ independent experiments each with three biological replicates) using Dunnett’s multi-comparison post-tests when compared to the control shRNA cell line; ***, $P<0.0001$.

(D) Mitochondrial membrane potential ($\Delta \psi_m$) was measured using TMRE staining in live cells stably expressing an shRNA directed against PINK1 or a control scrambled sequence. Results are expressed as the mean percentage of TMRE fluorescence with background correction when compared to the mean fluorescence intensity in the control shRNA cell line; error bars show the SEM ($n=8$ independent experiments of 10 observations per line). Rotenone (500nM, 1 hour) was used to confirm mitochondrial membrane depolarization and subsequent loss of TMRE fluorescent staining. An additional shRNA sequence against PINK1 was used to show clonal effects do not contribute to the loss of mitochondrial membrane potential when PINK1 protein is knocked down. The differences between control and PINK1 shRNA were significant by one-way ANOVA ($F_{3,316}=970.3$, $P<0.0001$, $n=8$ independent experiments of 10 observations per cell line) using Dunnett’s multi-comparison post-tests with control shRNA comparison (***, $P<0.0001$).
Figure 4.2.1 Mitochondrial function in stable cell lines expressing PINK1
4.2.2 Effects of PINK1 knockdown on mitochondrial morphology

PINK1 silencing resulted in deficits in mitochondrial function; therefore, mitochondrial morphology was also examined in these stable PINK1 deficient cell lines. Following Mitotracker CMXRos labeling, control and PINK1 shRNA cells were fixed and imaged by confocal microscopy in a blinded fashion. Using the same approach as that outlined by Exner et al (Exner et al., 2007) the mitochondrial morphologies of PINK1 deficient cells were divided into intact, truncated and fragmented classifications (Fig. 4.2.2.A). Cells were also treated for 24 hours with 100nM rotenone, which has previously been shown to cause mitochondrial fission (Barsoum et al., 2006), to examine the influence of oxidative stress on mitochondrial morphology. It was determined that when PINK1 was knocked down the number of cells containing truncated and fragmented mitochondria increased (12% and 2% when compared to control shRNA cells, respectively), and that rotenone had an additive effect (Fig. 4.2.2.B). These results showed there was mild mitochondrial morphology defects in PINK1 deficient cells in agreement with previous studies (Exner et al., 2007, Ichishita et al., 2008) that suggest PINK1 silencing alters mitochondrial phenotypes.
Figure 4.2.2 Effects of PINK1 knockdown on mitochondrial morphology

(A) Control shRNA neuroblastoma cells were stained with 200nM Mitotracker CMXRos for 30 minutes at 37° C and fixed with 4% paraformaldehyde for confocal imaging. Cells were assigned to one of three morphological categories (from top to bottom), intact, truncated or fragmented. The scale bar in the lower panel is 2μm, applies to all fluorescence micrographs.

(B) Using confocal imaging to assess mitochondrial morphology, blinded counts of 45-130 cells were performed in control or PINK1 shRNA lines with or without exposure to 100nM rotenone for 24 hours. Cells with truncated (orange) or fragmented (red) mitochondria are expressed as a percentage of the total counted. Error bars indicate the SEM between experiments (n=3). The difference between mitochondrial phenotypes was significant two-way ANOVA (P<0.0001), as was the effect of cell lines (P=0.0161) but the interaction was not significant (P=0.5635).
Figure 4.2.2 Effects of PINK1 knockdown on mitochondrial morphology
4.2.3 PINK1 expression and its effects on mitochondrial connectivity

Having established that deficiencies in mammalian PINK1 result in defects in mitochondrial function and morphology, fluorescence recovery after photobleaching (FRAP) was used to provide a quantitative estimate of functional mitochondrial connectivity in living cells. Cells were transiently transfected with mito-YFP (mitochondrial matrix localized yellow fluorescent protein) and treated with DMSO or 100nM rotenone for 24 hours prior to FRAP analysis. Using live cell imaging, a region of interest within the YFP-expressing mitochondria was photobleached and the fluorescence intensity of that region was measured over a 12 second interval. This curve of fluorescence recovery over time was normalized to the first image taken in the series and corrected for both non-specific photobleaching (NSPB) and background fluorescence.

Cells expressing wild type, but not kinase dead, PINK1 maintained connected mitochondrial morphology in the presence of rotenone as assessed via live cell confocal microscopy (Fig. 4.2.3.A). Using FRAP, a loss of fluorescence upon photobleaching that partially recovered over time was observed in control conditions (Fig. 4.2.3.B). Rotenone treatment in control M17 cell lines lowered the recovery of fluorescence, consistent with induction of mitochondrial fission. The same effect was seen in cell lines expressing recessive G309D or kinase dead PINK1. However, FRAP curves revealed that cells expressing wild type PINK1 was similar with or without rotenone treatment, showing that PINK1 functionally protects against rotenone-induced mitochondrial fission (Fig. 4.2.3.B). To analyze mitochondrial connectivity, the mobile fraction of mito-YFP was calculated from the raw FRAP data using the following formula: Mobile Fraction =
[(FRAP_{	ext{Background}})/\text{FRAP}]/[(\text{NSPB}_{\text{Background}})/\text{NSPB}]. This calculation provides a ratio of fluorescent recovery to the fluorescent bleach with background subtraction. It was shown that PINK1 protected against mitochondrial damage in a kinase-dependent fashion (Fig. 4.2.3.C). The difference between rotenone treated and untreated cells was significant for control M17 \((P<0.01)\), G309D and kinase dead PINK1 stable lines \((P<0.05)\) but not for wild type PINK1.

To confirm that this effect was also seen with endogenous PINK1, cells stably expressing PINK1 shRNA and a scrambled control shRNA were examined (Fig. 4.2.3.D-F). PINK1 knockdown cells had a partially fragmented morphology and a lower basal FRAP mobile fractions (mean\(\pm\)SEM: 0.31\(\pm\)0.02, sequence A; mean\(\pm\)SEM: 0.30\(\pm\)0.03, sequence C) compared to controls (mean\(\pm\)SEM: 0.58\(\pm\)0.03), and poorer recovery after rotenone exposure. Under the rotenone conditions, mitochondria were swollen as well as fragmented but only in the absence of PINK1 (Fig. 4.2.3.D). The effect was confirmed with an independent shRNA sequence and a different shRNA control cell line, thus excluding a non-specific shRNA effect (Fig. 4.2.3.D). Together these data show that exogenous or endogenous PINK1 protects against rotenone-induced fission in a kinase-dependent manner.
Figure 4.2.3  PINK1 expression and its effects on mitochondrial connectivity

(A) Living cells were imaged by confocal microscopy after transfection with mito-YFP and exposure to DMSO or 100nM rotenone for 24 hours. Cells expressing PINK1 (upper panels) had longer mitochondria compared to kinase dead (lower panels). Rotenone (right panels) induced mitochondrial fragmentation in control lines but not in lines expressing PINK1. Scale bar in the lower right panel is 2 μm, applies to all fluorescence micrographs.

(B) FRAP was performed in M17 parental cells or stable cell lines expressing wild type, G309D or kinase dead PINK1 after transfection with mito-YFP and exposure to DMSO or 100nM rotenone for 24 hours. FRAP curves showing that all cells exhibited more recovery under control conditions (open symbols) than after 24h exposure to 100nM rotenone (closed symbols) with the exception of lines stably expressing wild type PINK1 (red upwards triangles) where rotenone did not have an effect. In this experiment, control cells (blue circles) are parental M17 cells. Cells expressing G309D (green diamonds) or kinase dead (black downward triangles) PINK1 responded to rotenone. Curves were normalized to the first image in the series and corrected for both non-specific photobleaching (NSPB) that occurred during imaging and background. Each point is the average of >30 separate measurements and is representative of duplicate experiments for each line/treatment.

(C) Mobile fraction of mito-YFP was measured from the indicated cell lines shown in (B) [colors as in B, filled boxes are rotenone treated]. Mobile fractions were calculated as follows: Mobile Fraction = [(FRAP_t-Background)/FRAP_t][NSPB_t-Background]/NSPB_t. The box indicates the upper and lower quartiles, central line indicates the median and
range bars indicate the 10th to 90th percentile range. Cells lying outside of this range are shown as single points and the ‘+’ indicates the mean. Differences between treatments were significant overall by one-way ANOVA (F_{7,227}=6.632, P<0.0001, n=30 from duplicate experiments) and Student-Newman Kuells’ post-hoc test was used to evaluate rotenone effects in each line (*, P<0.05; **, P<0.01; ns, P>0.05).

(D) Living cells were imaged by confocal microscopy after transfection with mito-YFP and exposure to DMSO or 100nM rotenone for 24 hours. Induction of mitochondrial fission was seen in cell lines expressing a control shRNA construct (upper panels) after rotenone treatment. Cells expressing a shRNA sequence (A) against PINK1 (lower panels) showed basal evidence of fission, with some fragmented mitochondria visible, and exaggerated responses to rotenone and mitochondrial swelling. Scale bar in the lower right panel is 2 µm, applies to all fluorescence micrographs.

(E) FRAP was performed in control and PINK1 shRNA cell lines after transfection with mito-YFP and exposure to DMSO or 100nM rotenone for 24 hours. FRAP curves showed that all cells have more fluorescence recovery with DMSO treatment (open symbols) than after a 24-hour exposure to 100nM rotenone (closed symbols). In this experiment, control shRNA cells (blue circles) responded to rotenone, but cells expressing shRNAs [sequence A, pink1 squares; sequence C, black squares] directed against PINK1 had the lowest FRAP curves. Curves were normalized to the first image in the series and corrected for both non-specific photobleaching (NSPB) that occurred during imaging and background. Each point is the average of >30 separate measurements and is representative of duplicate experiments for each line/treatment.
(F) Mobile fraction of mito-YFP was measured from the indicated cell lines shown in (E) [colors as in E, filled boxes are rotenone treated]. Mobile fractions were calculated as follows: Mobile Fraction = [(FRAP_{t}-Background)/FRAP_{t}]/[(NSPB_{t}-Background)/NSPB_{t}]. Similar results were obtained using a cell line stably expressing a second PINK1 shRNA sequence (C) indicating these effects are not due to any potential non-specific shRNA effects. The box indicates the upper and lower quartiles, central line indicates the median and range bars indicate the 10^{th} to 90^{th} percentile range. Cells lying outside of this range are shown as single points and the ‘+’ indicates the mean. Differences between treatments were significant overall by one-way ANOVA (F_{5,162}=22.44, P<0.0001, n=30 cells, representative of at least two experiments per cell line) and Student-Newman Kuells’ post-hoc test was used to evaluate rotenone effects in each line (*, P<0.05; ***, P<0.001).
Figure 4.2.3  PINK1 expression and its effects on mitochondrial connectivity
4.2.4 Effect of cytoplasmic PINK1 on mitochondrial connectivity

PINK1 contains a putative mitochondrial localization sequence (Valente et al., 2004a). To test whether the mitochondrial targeting sequence was essential for the protective effects of PINK1, a PINK1 mutant (PINK1_{ΔN1}) lacking the first 111 amino acids from the N-terminus was used. Plasmids containing either GFP alone or with full length PINK1-FLAG or PINK1_{ΔN1}-FLAG were transiently transfected for 24 hours into COS7 (African green monkey kidney) cells. GFP-positive cells were evaluated for exogenous PINK1 localization by FLAG immunostaining. Overexpression of full length PINK1 showed both cytoplasmic and mitochondrial localization in M17 cells (Fig. 4.2.4.A). However, targeting of PINK1 to mitochondria was impaired by the N-terminal deletion; there was little if any costaining of the PINK1_{ΔN1}-FLAG and mitotracker signals (Fig. 4.2.4.A). The PINK1 mutant localization appeared diffuse and cytoplasmic indicating that the deletion of the first 111 amino acids of PINK1 was sufficient to prevent mitochondrial targeting.

It has been demonstrated that cytoplasmic PINK1 can protect against MPP⁺ toxicity (Haque et al., 2008). Therefore, it was hypothesized that the PINK1_{ΔN1} mutant could also prevent rotenone-induced mitochondrial fission like the full-length version of PINK1. M17 cells were transiently transfected as above and FRAP was performed following a 24 hour incubation with 100 nM rotenone. No significant differences were observed in cells expressing either full length or mutant PINK1 before or after rotenone treatment. However, rotenone treated cells expressing the empty vector had lower mobile fraction (1.5-fold decrease) when compared to the untreated (Fig. 4.2.4.B; P<0.001).
Transient expression of exogenous full length PINK1 as well cytoplasmic PINK1 could protect against rotenone-induced mitochondrial fission (Fig. 4.2.4.B).
Figure 4.2.4  Effect of cytoplasmic PINK1 on mitochondrial connectivity

(A) Immunostaining of COS7 cells transiently transfected for 24 hours with a plasmid expressing full length (FL) PINK1 or an N-terminal deletion (ΔNt) of PINK1, which lacks the mitochondrial targeting sequence, or the empty vector containing a GFP tag. Cells were incubated with 200nM Mitotracker CMXRos for 30 minutes at 37° C, fixed and an anti-FLAG antibody was used to visualize PINK1. The scale bar in the lower right panel is 5μm and applies to all fluorescence micrographs.

(B) FRAP curves of parental M17 cells transiently co-transfected with mito-YFP and empty vector (black diamonds), full length PINK1 (pink boxes) or N-terminal deletion of PINK1 (blue circles). Open symbols are untreated cells, whereas closed symbols are cells treated with 100nM rotenone for 24 hours. Each point is the average of >30 separate measurements and is representative of duplicate experiments for each line/treatment.

(C) FRAP mobile fraction values of parental M17 cells transiently co-transfected with mito-YFP and vector (black boxes), full length PINK1 (pink boxes) or N-terminal deletion of PINK1 (blue boxes). Open boxes are untreated cells and closed boxes are cells treated with 100 nM rotenone for 24 hours. The box indicates the upper and lower quartiles, central line indicates the median and range bars indicate the 10th to 90th percentile range. Cells lying outside of this range are shown as single points and the ‘+’ indicates the mean. Differences between treatments were significant overall by one-way ANOVA (F5,165=3.816, P<0.0001; n=30 cells from duplicate experiments) and Student-Newman Kuells’ post-hoc test was used to evaluate rotenone effects in each line (**, P<0.01; ns, P>0.05).
Figure 4.2.4 Effect of cytoplasmic PINK1 on mitochondrial connectivity
4.2.5 Effect of PINK1 deficiency on mitochondrial fusion

While the previous data suggested that PINK1 deficient cells showed lower mitochondrial connectedness after rotenone exposure, but did not distinguish between the possibilities that there was increased fission or that there was decreased fusion. To address this, the rate of mitochondrial fusion was measured using photoactivatable GFP, where fusion results in a loss of GFP fluorescence over time, in control and PINK1 deficient cells. Control and PINK1 shRNA cells were transiently co-transfected for 24 hours with red fluorescent protein derived from a variant of Discosoma (dsRED) and a mitochondrial targeted photoactivatable GFP (mito-pAGFP), a variant of Aequorea victoria GFP that fluoresces upon photoactivation at 405nm. Cells positively expressing cytoplasmic dsRED were photoactivated in two regions of interest and fluorescence was measured every 15 minutes for a period of one hour. Fusion of mitochondria was estimated from the loss of mito-pAGFP fluorescence, averaged across both photoactivated regions of interest and normalized to the initial measurement (t=0), which was set to 100%. PINK1 deficient cells retained fluorescence in the photoactivated area to a greater extent than control cell lines, indicating a defect in fusion (Fig. 4.2.5.A). The difference between cell lines was significant by two-way ANOVA ($P<0.001$), as was the effect of time ($P<0.001$) but the interaction was not significant ($P=0.6996$). The loss of fluorescence was quantified and although a control shRNA did not affect fusion compared to the parental cells, an shRNA directed to PINK1 lowered the rate of fusion, indicated by a higher rate of retention of fluorescence signal (Fig. 4.2.5.B). In summary, fusion assays indicated that PINK1 silencing resulted in mitochondrial fusion deficits.
Figure 4.2.5  Effect of PINK1 deficiency on mitochondrial fusion

(A) Control shRNA (upper panels) or PINK1 shRNA (lower panels) cells were co-transfected with mito-pAGFP and dsRED to assess mitochondrial fusion. Two regions of interest within dsRED positive transfected cells were photoactivated and images of single cells were taken at 15-minute intervals for an hour post-photoactivation. When cells were fusion-competent, decreases in fluorescence were seen over time in the region of photoactivation. Note that the fluorescence intensity was equal across the cell in the control line in the upper panel by 30 minutes, whereas the PINK1 deficient cell retained areas of higher intensity, indicating decreased fusion. Sequential images of the single cells (0, 15, 30, 45 and 60 minutes as indicated above the images) after photoactivation are shown in glow scale with color intensities ranging from red (high fluorescence intensity) to blue (low fluorescence intensity). Each scale bar is 5 μm and applies to all fluorescence micrographs in that series.

(B) Quantification of experiments as in (A) showed the loss of fluorescence over time for parental M17 cells (black triangles), control shRNA (blue circles) or PINK1 shRNA (pink squares) cell lines following photoactivation. Each point is shown as the mean with error bars indicating the SEM (n=9-10 observations per time point). The difference between cell lines was significant by two-way ANOVA ($P<0.001$), as was the effect of time ($P<0.001$).
Figure 4.2.5  Effect of PINK1 deficiency on mitochondrial fusion
4.2.6 Effect of PINK1 kinase activity on mitochondrial fusion

It has been previously shown that loss of mitochondrial membrane potential caused by treatment with the protonophore CCCP (m-chlorocarboxylicyanide phenylhydrazone) induces rapid mitochondrial fission. Within minutes, mitochondria of cells treated with CCCP become fragmented, dispersed, and swollen (Griparic et al., 2007). This mitochondrial decoupler has been used to assess the dependence of membrane potential in mitochondrial fusion by imaging mitochondrial morphology before and after CCCP treatment using confocal microscopy (Griparic et al., 2007). Reticular mitochondria that returned to their basal phenotype following CCCP treatment showed mitochondria could repolarize and fuse. This method was used to examine the capacity for mitochondrial fusion in the stable PINK1 overexpressing cell lines, since an intact mitochondrial membrane potential is required for fusion, and to further support photoactivation assay data. FRAP assays showed that cells expressing kinase active PINK1 (M17 and PINK1\textsubscript{WT}) exhibited higher mobile fraction values basally (mean\textpm SEM: 0.54\textpm 0.02 and 0.61\textpm 0.04, respectively), indicating greater mitochondrial connectivity than cells stably overexpressing a kinase dead variant of PINK1 (PINK1\textsubscript{KD}) (mean\textpm SEM: 0.43\textpm 0.05) (Fig. 4.2.6.C). However, following a 1.5-hour induction with 10\textmu M CCCP, all cell types demonstrated a fragmented mitochondrial phenotype (Fig. 4.2.6.A). This CCCP-induced fission observed in FRAP assays was overcome after washout with 1x phosphate buffered saline (PBS) and a 6-hour recovery period, except in cells lacking PINK1 kinase activity (Fig. 4.2.6.C). Unlike mitochondria of M17 or PINK1\textsubscript{WT} cells, the mitochondria of PINK1\textsubscript{KD} cells do not recover from mitochondrial decoupling to basal levels (recovery, mean\textpm SEM: 0.28\textpm 0.03) of the mobile fraction (Fig.
4.2.6.C). Differences between treatments were significant overall by one-way ANOVA ($P<0.0001$). Perhaps the kinase dead version of PINK1 acts as a dominant negative in these assays that estimate the capacity for fusion (Fig. 4.2.6.C). Together, these data suggest that PINK1-dependent mitochondrial fusion is dependent on kinase activity.
Figure 4.2.6  Effect of PINK1 kinase activity on mitochondrial fusion

(A) Living cells were imaged by confocal microscopy after a 24-hour transfection with mito-YFP. Cells expressing PINK1 (middle panels) had longer mitochondria compared to control lines (kinase dead, lower panels; parental M17, upper panels). CCCP (10μM) applied for 1.5 hours induced mitochondrial fragmentation by mitochondrial membrane depolarization is shown in the second series of micrographs. Cells were imaged following a washout with 1x PBS and recovery period of 6 hours (third series of micrographs). Scale bar in the upper left panel is 2 μm, applies to all fluorescence micrographs.

(B) M17, PINK1WT, and PINK1KD cells were transiently transfected with mito-YFP for 24 hours prior to FRAP. FRAP curves revealed that cells expressing kinase active PINK1 (M17 and PINK1WT) showed more recovery under basal conditions relative to PINK1KD cells. Following exposure to CCCP (10μM, 1.5 hours), all cell types had lower recovery of fluorescence indicating a loss of connectivity. CCCP-induced fission was overcome following a recovery period and protonophore wash out. In M17 (black circles) and PINK1WT (red circles) cells mitochondrial fluorescence recovered near basal levels, but PINK1KD (black circles) cells had decreased fluorescence when compared to its basal levels. Curves were normalized to the first image in the series and corrected for both non-specific photobleaching (NSPB) that occurred during imaging and background. Each point is the average of >30 separate measurements and is representative of duplicate experiments for each line/treatment.

(C) Mobile fraction of mito-YFP was measured in M17 (blue), PINK1WT (red) and PINK1KD (black). Mobile fractions were calculated as follows: Mobile Fraction =
[(FRAP₁-Background)/FRAP₁][(NSPB₁-Background)/NSPB₁]. The box indicates the upper and lower quartiles, central line indicates the median and range bars indicate the 10th to 90th percentile range. Cells lying outside of this range are shown as single points and the ‘+’ represents the mean. These box plots summarize the mobile fraction values of 24-30 cells under basal, CCCP and recovery conditions described in (B) when analyzed by FRAP. Differences between treatments were significant overall by one-way ANOVA (F₈, ₂₄₈=19.57, P<0.0001, 30 observations from duplicate experiments) and Student-Newman Kuells’ post-hoc test was used to evaluate treatment effects in each line (*P<0.05; ** P<0.01; ***P<0.001).
Figure 4.2.6  Effect of PINK1 kinase activity on mitochondrial fusion

A

Untreated  10μM CCCP, 1.5h  Recovery, 6h

M17

PINK1_{WT}

PINK1_{KD}

B

Normalized Fluorescence

Time (s)

C

Mobile Fraction

Untreated

10μM CCCP

Recovery
4.2.7 Fusion capacity of cells deficient in PINK1

Stable cell lines overexpressing PINK1 demonstrated that kinase activity was required for mitochondrial fusion using FRAP. Moreover, PINK1KD cells exhibited decreased mitochondrial membrane potential (Fig. 4.2.1.B) and cannot recover mitochondrial phenotypes seen prior to CCCP-induced fission. However, it remained unknown if a basal loss in mitochondrial membrane potential, as seen in PINK1 deficient cells (Fig. 4.2.1.E), would be sufficient to inhibit mitochondrial fusion following further mitochondrial decoupling by CCCP. To test this idea, mitochondrial fusion capacity of the shRNA series was estimated using FRAP following the induction of fission by 10μM CCCP for 1.5 hours. The mitochondrial phenotype in PINK1 deficient cells was basally fragmented, but following membrane depolarization the truncated morphology was exaggerated leaving them swollen (Fig. 4.2.7.A). Unlike PINK1KD cells, cells deficient in PINK1 (both shRNA sequences, A and C) have a similar mitochondrial mobile fraction after recovery from CCCP-induced fission (mean±SEM: A, 0.28±0.02; C, 0.31±0.03) when compared to basal values of the PINK1 shRNA cells (mean±SEM: A, 0.32±0.03 C, 0.37±0.04) (Fig. 4.2.7.C) and differences between treatments were significant overall (P<0.0001 by one-way ANOVA). These data demonstrate that loss of mitochondrial membrane potential in PINK1 deficient cells does not necessarily account for the loss of mitochondrial fusion, as trends shown before membrane decoupling were maintained in the recovery stages. It seems PINK1 deficient cells still retain the capacity for fusion despite a decrease in mitochondrial membrane potential. However, this does not extricate the detrimental affects of mitochondrial membrane depolarization in the regulation of mitochondrial morphology.
Figure 4.2.7  Fusion capacity of cells deficient in PINK1

(A) Living cells were imaged by confocal microscopy after transfection with mito-YFP for 24 hours. Cells expressing PINK1 (control shRNA, top panels) had longer mitochondria compared to PINK1 deficient cell lines (two clones shown, sequence A-middle panels; sequence C-lower panels). 10μM CCCP applied for 1.5 hours induced fragmentation by mitochondrial membrane depolarization is shown in the second series of micrographs. Following a washout with 1x PBS and recovery period of 6 hours (third series of micrographs), cells were imaged. Scale bar in the upper left panel is 2 μm, applies to all fluorescence micrographs.

(B) Control and PINK1 shRNA cells were transiently transfected with mito-YFP for 24 hours prior to FRAP. FRAP curves showing that cells expressing PINK1 (control shRNA) showed more recovery under basal conditions and after 1.5h exposure to 10μM CCCP all cell types had lower recovery of fluorescence indicating a loss of connectivity. CCCP-induced fission was overcome following a recovery period and protonophore wash out with 1x PBS. Control shRNA cells (blue circles) mitochondrial fluorescence recovered near basal levels, but PINK1 shRNA (magenta and red circles) had decreased fluorescence when compared to its basal levels. Curves were normalized to the first image in the series and corrected for both non-specific photobleaching (NSPB) that occurred during imaging and background. Each point is the average of >30 separate measurements and is representative of at least duplicate experiments for each line/treatment.

(C) Mobile fraction of mito-YFP was measured in the indicated cell lines (colors as in B). Mobile fractions were calculated as follows:  Mobile Fraction = [(FRAP-
Background)/FRAP_i[(NSPB_i-Background)/NSPB_i]. The box indicates the upper and lower quartiles, central line indicates the median and range bars indicate the 10th to 90th percentile range. Cells lying outside of this range are shown as single points and the ‘+’ represents the mean. These box plots summarize the mobile fraction values of 24-30 cells under basal, CCCP and recovery conditions described in (B) when analyzed by FRAP. Differences between treatments were significant overall by one-way ANOVA (F_{8,250}=15.19, P<0.0001) and Student-Newman Kuells’ post-hoc test was used to evaluate treatment effects in each line (***, P<0.001).
Figure 4.2.7  Fusion capacity of cells deficient in PINK1
4.3 Discussion

Mitochondrial shape can be influenced by variations in metabolic stability, which includes changes in energy substrates, inhibition of oxidative phosphorylation (OXPHOS), pathological mutations or cellular physiology (Benard and Rossignol, 2008). It has been reported that PINK1 mutations result in decreased complex I activity that results in ATP depletion in culture (Hoepken et al., 2007) —defects that resemble rotenone exposure. In addition, it has been shown that PINK1 deficient cells have mildly depolarized mitochondrial membrane potential. Decreased membrane potential in neurons increases apoptotic sensitivity, alters mitochondrial morphology and decreases mitochondrial motility (Safiulina et al., 2006). PINK1 has been shown to protect against mitochondrial toxins and FRAP assays measuring fission show that overexpression of PINK1 can prevent loss of mitochondrial connectivity associated with increased oxidative stress, loss of membrane potential and [presumably] ATP depletion caused by rotenone exposure. Exogenous or endogenous PINK1 can protect against rotenone-induced fission. More specifically, cells expressing PINK1 kinase activity and an N-terminal deletion of the MTS of the PINK1 protein domain are sufficient to protect against rotenone-induced fission events. These results show that the normal function of PINK1 in mammalian cells is to limit mitochondrial damage and, secondarily, induction of mitochondrial fission.

CCCP experiments shown in this chapter illustrate that fission was a driven by the loss of mitochondrial membrane potential. Mitochondrial depolarization by CCCP caused elongated and tubular forms of mitochondria to swell and fragment forming spherical structures. However, PINK1 deficiencies fail to repolarize membrane potential
following decoupling and mitochondria in cells with decreased PINK1 kinase activity remain fragmented. Data from this chapter confirmed that alterations in mitochondrial morphology in PINK1 deficient cells are a consequence of loss of mitochondrial membrane potential ($\Delta \psi_m$) (Exner et al., 2007, Wood-Kaczmar et al., 2008). Since an intact membrane potential is required for mitochondrial fusion (Legros et al., 2002), it is proposed that this loss of $\Delta \psi_m$ is a primary trigger for increased fission in the PINK1 deficient cell model.

In addition to maintaining effects on viability, fission and fusion sustain a healthy mitochondrial population and influence biogenesis, distribution and subcellular localization of the organelle. For instance, a high fusion-to-fission ratio results in fewer mitochondria with a reticular morphology whereas cells with a low fusion-to-fission ratio have numerous mitochondria with a phenotype that is more spherical or fragmented (Detmer and Chan, 2007). The shape of the organelles affects ability of cells to distribute mitochondria to specific subcellular locations. This is particularly important in energetically demanding neurons where mitochondria are recruited along cytoskeletal tracks to active pre- and post-synaptic sites (Chang et al., 2006, Li et al., 2004). Mitochondria with high $\Delta \psi_m$ move preferentially in the anterograde direction towards distal regions with high-energy demands, whereas low $\Delta \psi_m$ move in the retrograde direction towards the soma perhaps for cell death or repair (Miller and Sheetz, 2004). PINK1 kinase deficient cells demonstrate a fragmented phenotype, which might promote mitochondrial trafficking in a healthy cell, but these mitochondria maintain low $\Delta \psi_m$ and have deficits in mitochondrial fusion compared to control cells. Therefore, the loss of $\Delta \psi_m$ may be a critical trigger for the balance of fission/fusion in PINK1 deficient cells.
Chapter Five will address the role of PINK1 in mitochondrial dynamics, the process that regulates mitochondrial fusion and fission.
CHAPTER FIVE

Role of PINK1 in mitochondrial dynamics
5.1 Summary

While Chapter Four addressed the influence of PINK1 on mitochondrial function and morphology, it also raised the possibility that PINK1 may be involved in mitochondrial morphogenesis. Mitochondria are dynamic organelles that undergo changes via fusion and fission to regulate a number of cellular energetic processes. Chapter Five addresses whether the mechanism contributing to altered mitochondrial dynamics associated with the loss of PINK1 involves the fission/fusion machinery. PINK1 deficient cells exhibit a fragmented mitochondrial phenotype that resembles cells deficient in fusion proteins (Chen et al., 2005a). Overexpression of the fusion proteins, Opa1 (optic atrophy 1) and Mfn2 (mitofusin 2), rescue FRAP deficits caused by the loss of PINK1, suggesting PINK1 normally functions to promote fusion or inhibit fission. Dynamin-related protein 1 (Drp1) RNAi in PINK1 deficient cells also results in the elongation of the formerly fragmented mitochondrial network. Moreover, PINK1 deficient cells show increased fragmentation after expression of the fission GTPase Drp1 but not after expression of Fis1 (fission 1 [mitochondrial outer membrane] homolog \textit{S. cerevisiae}), a proposed receptor for Drp1 at the mitochondrial surface. Since PINK1 was shown to protect against rotenone-induced fission (Fig. 4.2.3), stable cell lines overexpressing PINK1 are used to demonstrate if PINK1 could protect against overexpression of fission proteins. PINK1 overexpression could protect against Drp1-mediated but not Fis1-mediated fission. Together these data demonstrate that PINK1 has an effect on Drp1- but not Fis1-dependent fission events.

Increased fission in PINK1 deficient cells is the result of Drp1 dephosphorylation. It has been shown that phosphorylation of Drp1 negatively regulates fission by inhibiting
GTPase activity. Increased Drp1 GTPase activity was observed with dephosphorylation of Drp1 in PINK1 deficient cells. Moreover, decreased Drp1 phosphorylation enhanced fission in PINK1 deficient cells as a consequence of calcineurin activation. The calcineurin inhibitor FK506 blocks both Drp1 dephosphorylation and loss of mitochondrial integrity in PINK1 deficient cells. These studies suggest loss of PINK1 results in decreased $\Delta \psi_m$ and calcineurin activation which increase fission by activation of Drp1.
5.2 Results

5.2.1 Effects of the fusion proteins Opa1 and Mfn2 on mitochondrial connectivity in PINK1 deficient cells

Collectively, the results presented in Chapter Four suggested that PINK1 protected against rotenone-induced changes in mitochondrial morphology that were consistent with fission. To better understand the role of PINK1 in mitochondrial dynamics, the function of morphogenesis proteins was examined in a PINK1 deficient background. To address the mechanism involved, a series of proteins involved in mitochondrial fusion or fission were expressed and the basal mitochondrial deficits seen in PINK1 shRNA cell lines were used as a quantifiable phenotype. First, either of two GTPases involved in mitochondrial fusion, Opa1 and Mfn2 were investigated to see if basal fragmentation associated with the loss of PINK1 could be rescued by their transient overexpression. Control and PINK1 shRNA cells were transiently co-transfected with mito-YFP and Opa1-myc or Mfn2-FLAG for 24 hours prior to FRAP. Morphologically, both Opa1 and Mfn2 proteins caused mitochondrial elongation that mitigated the fragmented phenotype seen in PINK1 deficient cells (Fig. 5.2.1.A). Immunoblot analysis (performed in tandem with FRAP experiments) of control and PINK1 shRNA cells transiently transfected with Opa1-myc or Mfn2-FLAG show equal protein expression in both cell lines (Fig. 5.2.1.D). Either fusion protein could rescue the loss of mitochondrial connectivity in PINK1 knockdown cells (Fig. 5.2.1.B-C).
Figure 5.2.1  Effects of the fusion proteins Opa1 and Mfn2 on mitochondrial connectivity in PINK1 deficient cells

(A) Control (upper panels) or PINK1 shRNA (lower panels) cells were imaged with confocal microscopy after a 24 hour transfection with mito-YFP alone (UT, left panels) or transfected with mito-YFP and the fusion proteins Opa1-myc (middle panels) or Mfn2-FLAG (right panels). Expression of Opa1 or Mfn2 in PINK1 deficient cells increased mitochondrial length. Scale bar is 2 µm, applies to all panels.

(B) Control and PINK1 shRNA cells were transiently co-transfected with mito-YFP and Opa1-myc for 24 hours prior to FRAP. FRAP curves were used to show that Opa1 improved connectivity of PINK1 shRNA cells (pink1 squares; open symbols are untransfected, closed symbols are with Opa1) but had only no effect on the control shRNA cells (blue circles). Curves were normalized to the first image in the series and corrected for both non-specific photobleaching (NSPB) that occurred during imaging and background. Each point is the average of >30 separate measurements and is representative of at least duplicate experiments for each line/treatment.

(C) FRAP mobile fraction of mito-YFP was measured from control and PINK1 shRNA cells transiently co-transfected with mito-YFP and Opa1-myc or Mfn2-FLAG. Mobile fractions were calculated as follows: Mobile Fraction = [(FRAPi-Background)/FRAPi][((NSPBi-Background)/NSPBi]. The box indicates the upper and lower quartiles, central line indicates the median and range bars indicate the 10th to 90th percentile range. Cells lying outside of this range are shown as single points and the ‘+’ indicates the mean. Differences between treatments were significant overall by one-way ANOVA (F5,165=3.18, P=0.0092, n=30 from duplicate experiments) and Student-
Newman Kuells’ *post-hoc* test was used to evaluate treatment effects in each line \*, 
P<0.05.

(D) Whole cell lysates from control and PINK1 shRNA cells transiently transfected for 
24 hours with Opa1-myc or Mfn2-FLAG were resolved on a 10% SDS-PAGE gel and 
transferred to PVDF. Immunoblotting for anti-myc or anti-FLAG showed equivalent 
protein expression in both cell lines. The immunoblot was also probed for β-actin to 
show equal loading. Molecular weight markers listed on the right are in kilodaltons.
Figure 5.2.1  Effects of the fusion proteins Opa1 and Mfn2 on mitochondrial connectivity in PINK1 deficient cells

A

UT     Opa1-myc     Mfn2-FLAG
Control shRNA

Control shRNA

PINK1 shRNA

PINK1 shRNA + Opa1

B

Normalized Fluorescence

Time (s)

C

Mobile Fraction

D

Control shRNA

PINK1 shRNA

Opa1-myc

Mfn2-FLAG

IB: FLAG

IB: myc

IB: β-actin
5.2.2 Effect of Drp1 RNAi on mitochondrial connectivity in PINK1 deficient cells

The rescue by fusion proteins suggested that PINK1 deficient cells have increased mitochondrial fission. Based on the above results, it was predicted that loss of Drp1 would antagonize the effects of PINK1 deficiency. Control and PINK1 shRNA cell lines were transfected with an siRNA directed against Drp1 or a non-target gene (GFP) for 4 days prior to FRAP analysis. Confocal microscopy of transient mito-YFP expression showed the mitochondrial network was elongated in PINK1 shRNA lines after Drp1 RNAi (Fig. 5.2.2.A). In these experiments, the Drp1 knockdown was to ~25% of normal levels and has a similar efficacy in the control and PINK1 shRNA lines (Fig. 5.2.2.B). Partial knockdown of the fission protein Drp1 prevented mitochondrial defects associated with loss of PINK1 expression. Drp1 knockdown rescued the loss of fluorescence recovery over time in PINK1 shRNA cell lines (Fig. 5.2.2.C). Calculating the mobile fraction of mito-YFP, the difference between control shRNA and PINK1 shRNA lines was significant ($P<0.05$, one-way ANOVA) after expression of a control siRNA directed against a non-target gene (GFP). However, the mobile fraction in PINK1 shRNA cells was significantly different after exposure to the siRNA against Drp1 ($P<0.01$ by one-way ANOVA; Fig. 5.2.2.D). These results suggested that the effects of loss of PINK1 could be rescued by knockdown of Drp1.
Figure 5.2.2 Effect of Drp1 RNAi on mitochondrial connectivity in PINK1 deficient cells

(A) Confocal imaging of control and PINK1 shRNA cells transfected with mito-YFP for 24 hours after GFP and Drp1 transient knockdown. The mild morphological defects in cells stably expressing shRNA against PINK1 (lower panels) compared to cells expressing a control shRNA (upper panels) were rescued by knockdown of Drp1 (right panels, compared to untreated cells on the left). Scale bar in the lower right panel is 2 μm, applies to all fluorescence micrographs.

(B) Control and PINK1 shRNA cells were transiently transfected with Drp1 or GFP siRNA. Whole cell lysates were separated on a 10% SDS-PAGE gel and transferred to PVDF for immunoblot analysis. Transfectants were analyzed with anti-Drp1 antibody. Exposure of control shRNA (lane 1,2) or PINK1 shRNA (lane 3,4) stable cell lines to Drp1 siRNA (lanes 2,4) for four days results in approximately 75% knockdown of Drp1 protein (arrow) compared to a non-specific GFP siRNA (lanes 1 and 3). The immunoblot was also probed with an antibody to β-actin (open arrowhead) to show equal loading. Molecular weight markers are in kilodaltons.

(C) Control and PINK1 shRNA cells were transiently transfected with mito-YFP for 24 hours after Drp1 and GFP knockdown prior to FRAP. FRAP curves over time show that although under basal conditions there was a difference between the control shRNA line (blue circles) and the PINK1 shRNA line (purple squares), these differences were corrected after the cells were exposed to a siRNA against Drp1 (closed symbols). Curves were normalized to the first image in the series and corrected for both non-specific photobleaching (NSPB) that occurred during imaging and background. Each point is the
average of >30 separate measurements and is representative of at least duplicate experiments for each line/treatment.

(D) Mobile fraction of mito-YFP was measured in control shRNA (blue) or PINK1 shRNA (purple) cell lines either after expression of a control siRNA (against GFP, open boxes) or after expression of an siRNA against Drp1 (filled boxes). Mobile fractions were calculated as follows: Mobile Fraction = [(FRAP_t-Background)/FRAP_t][(NSPB_t-Background)/NSPB_t]. The box indicates the upper and lower quartiles, central line indicates the median and range bars indicate the 10th to 90th percentile range. Cells lying outside of this range are shown as single points and the ‘+’ indicates the mean. Boxplots summarize data from n=24-30 cells and are representative of duplicate experiments. Differences between treatments were significant overall by one-way ANOVA (F_{3,115}=5.53, P=0.0014) and Student-Newman Kuells’ post-hoc test was used to evaluate the differences between cell lines. *P<0.05; **P<0.01.
Figure 5.2.2 Effect of Drp1 RNAi on mitochondrial connectivity in PINK1 deficient cells
5.2.3 Effect of the fission GTPase Drp1 on mitochondrial morphology in PINK1 deficient cells

The rescue of mitochondrial phenotypes seen in PINK1 deficient cells by increasing fusion or inhibiting fission suggested that PINK1 limited fission. To further address this, YFP-Drp1 was transiently expressed in the control and PINK1 shRNA cell lines and mitochondria were imaged with confocal microscopy when YFP was reliably detected and non-transfected (YFP negative) cells were used in the same cultures as controls. In both control and PINK1 shRNA lines, YFP-Drp1 was recruited to foci on the surface of mitochondria as described previously (Karbowski et al., 2002). Twenty-four hours after transfection, PINK1 deficient lines showed more disrupted mitochondrial networks compared to control lines or untransfected cells (Fig. 5.2.3.A-D). Control and PINK1 shRNA cell transfectants were separated by subcellular fractionation and immunoblotted with anti-Drp1 antibody. The amount of YFP-Drp1 recruited to mitochondrial fractions was similar in control and PINK1 deficient lines (Fig. 5.2.3.E).

To provide a quantitative measure of this effect, FRAP experiments were performed in control and PINK1 shRNA cells co-transfected with mito-YFP and a HA-tagged Drp1 construct (to avoid interference of the YFP tag on FRAP). As with YFP-Drp1, HA-Drp1 induced fission, especially in PINK1 shRNA lines. There was a small amount of loss of FRAP over time in the control shRNA lines but a more pronounced enhancement of the basal FRAP deficit in PINK1 shRNA lines by overexpression of Drp1 (Fig. 5.2.3.G). As in the rotenone experiments, the mobile fraction of mito-YFP was calculated in these experiments and the PINK1 shRNA cells were sensitive to Drp1 overexpression; the mobile fraction of PINK1 shRNA cells had a 1.8-fold decrease after
ectopic Drp1 expression when compared to untransfected PINK1 shRNA cells (Fig. 5.2.3.H). Expression of the HA-tagged Drp1 was confirmed using confocal microscopy after FRAP (Fig. 5.2.3.F), >90% of mito-YFP positive cells were HA-Drp1 positive and again no differences were seen in Drp1 localization between cell lines. These data suggest that Drp1 overexpression sensitizes PINK1 deficient cells to mitochondrial fission.
Figure 5.2.3  Effect of the fission GTPase Drp1 on mitochondrial morphology in PINK1 deficient cells

(A-D) Control shRNA (A,B) or PINK1 shRNA (C,D) were transfected with YFP-Drp1 (green in upper panels) and stained with 200nM Mitotracker CMXRos for 30 minutes at 37° C (red in upper panels; lower panels show Mitotracker only). In these transient transfections, non-transfected (YFP-) with transfected (YFP+) cells were imaged in the same microscope field to allow strict comparison, hence there is some staining for YFP-Drp1 in the YFP- panels. At 24 hours after transfection, PINK1 shRNA lines (D) showed a greater degree of mitochondrial damage than control shRNA lines. Scale bar in the lower panel of (D) is 10 μm and applies to all photomicrographs.

(E) Control shRNA (lanes 1,2,5 and 6) and PINK1 shRNA (lanes 3,4,7 and 8) cells were transfected with YFP (lanes 1,3,5,7) or YFP-Drp1 (lanes 2,4,6,8). Total cell lysates (lanes 1-4) or mitochondrially-enriched fractions (lanes 5-8) were separated on a 4-20% SDS-PAGE gel, transferred to PVDF and immunoblotted with anti-Drp1 antibody (upper panel; filled arrowhead shows YFP-Drp1, arrow indicates endogenous Drp1) or anti-Hsp60 antibody as a loading control (lower panel, open arrowhead). Markers on the right are in kilodaltons.

(F) Immunocytochemistry was performed following FRAP examination of transient HA-Drp1 and mito-YFP transfection in control and PINK1 shRNA cells. Cells were stained with 200nM Mitotracker CMXRos for 30 minutes at 37° C prior to fixation and anti-HA immunostaining was then performed. Scale bar in the lower panel is 10 μm and applies to all fluorescence micrographs.
(G) To provide a quantitative evaluation of the effect of Drp1 overexpression, control cells (blue circles) and PINK1 shRNA cells (pink squares) were analyzed via FRAP. Open symbols are only mito-YFP transfected cells; closed symbols are co-transfected with Drp1 for 24 hours. Curves were normalized to the first image in the series and corrected for both non-specific photobleaching (NSPB) that occurred during imaging and background. Each point is the average of >30 separate measurements and is representative of at least duplicate experiments for each line/treatment.

(H) Mobile fraction of mito-YFP was measured in control shRNA (blue) or PINK1 shRNA (purple) cell lines after transfection of mito-YFP alone (open boxes) or Drp1 co-transfection (filled boxes). Mobile fractions were calculated as follows: Mobile Fraction = [(FRAP_r-Background)/FRAP_r][(NSPB_r-Background)/NSPB_r]. The box indicates the upper and lower quartiles, central line indicates the median and range bars indicate the 10th to 90th percentile range. Cells lying outside of this range are shown as single points and the ‘+’ indicates the mean. Boxplots summarize data from n=24-30 cells and are representative of duplicate experiments. Differences between treatments were significant overall by one-way ANOVA (F_{5,162}=12.98, \textit{P}<0.0001) and Student-Newman Kuells’ \textit{post-hoc} test was used to compare each line with and without Drp1 expression. *\textit{P}<0.05; ** \textit{P}<0.01; ***\textit{P}<0.001.
Figure 5.2.3  Effect of the fission GTPase Drp1 on mitochondrial morphology in PINK1 deficient cells
5.2.4 Effect of Fis1 overexpression on mitochondrial morphology in PINK1 deficient cells

PINK1 deficient cells exhibited mitochondrial fragmentation that was enhanced by overexpression of the fission mediator Drp1. It was predicted that increased expression of PINK1 would protect against Drp1-induced fission like rotenone exposure. M17 and stable PINK1\textsubscript{WT} cells were transiently co-transfected with mito-YFP and HA-Drp1 24 hours prior to FRAP. Elongated and tubular mitochondrial phenotypes were seen in parental M17 and stable PINK1\textsubscript{WT} cells and this morphology was maintained in cell lines stably overexpressing PINK1 after ectopic expression of Drp1 (Fig. 5.2.4.A). FRAP experiments confirmed that PINK1 protected against Drp1 overexpression, as no significant differences in mobile fraction were identified in stable PINK1\textsubscript{WT} cells with and without Drp1 transfection (Fig. 5.2.4.B). Fis1, which is the proposed mitochondrial outer membrane receptor for Drp1 and causes fission (Stojanovski et al., 2004), was also expressed in stable cell lines overexpressing PINK1\textsubscript{WT}. Surprisingly, PINK1 did not protect against Fis1-induced fission as mitochondrial fragmentation was observed in both M17 and stable PINK1\textsubscript{WT} cells following transient transfection (Fig. 5.2.4.A). PINK1\textsubscript{WT} cells mobile fractions were decreased with Fis1 overexpression (mean±SEM: 0.40±0.03 with Fis1; 0.61±0.04 without Fis1) (Fig. 5.2.4.B). Immunoblotting for HA-Drp1 and Fis1-FLAG in M17 and stable PINK1\textsubscript{WT} cells indicated that the two proteins were expressed equally and therefore did not contribute to Drp1 specific effects (Fig. 5.2.4.C). To explore this phenomenon further, Fis1 was transiently overexpressed in control and PINK1 deficient cells. In both morphological (Fig. 5.2.4.C) and quantitative (Fig. 5.2.4.E) measurements by FRAP, PINK1 deficient cells did not demonstrate enhanced
fission when Fis1 was overexpressed. Fluorescence recovery of mito-YFP after photobleaching was similar between PINK1 shRNA cells with and without Fis1 overexpression and Fis1 transfected control shRNA cells (Fig. 5.2.4.D). These results suggested that PINK1 had an effect on Drp1- but not Fis1-dependent fission.
5.2.4 Effect of Fis1 overexpression on mitochondrial morphology in PINK1 deficient cells

(A) Parental M17 (upper panels) and stable PINK1<sub>WT</sub> (lower panels) cells were transfected with mito-YFP alone or co-transfected with mito-YFP and Fis1-FLAG or HA-Drp1 plasmids for 24 hours and mitochondrial morphology was imaged using confocal microscopy. Scale bar is 2 µm and applies to all panels.

(B) Parental M17 or stable PINK1<sub>WT</sub> overexpressing cells were transfected alone with mito-YFP or cotransfected with mito-YFP and HA-Drp1 (upward triangles) or Fis1-FLAG (downward triangles) transiently for 24 hours. FRAP was performed and mobile fractions were derived as follows: Mobile Fraction = [((FRAP<sub>T-Background</sub>)/FRAP<sub>I</sub>)/((NSPB<sub>T-Background</sub>)/NSPB<sub>I</sub>)]. The box indicates the upper and lower quartiles, central line indicates the median and range bars indicate the 10<sup>th</sup> to 90<sup>th</sup> percentile range. Cells lying outside of this range are shown as single points and the ‘+’ indicates the mean. Boxplots summarize data from n=24-30 cells. Both Drp1 and Fis1 increased fission in parental M17 cells (P<0.01 and P<0.001 respectively). PINK1 blocks the effects of Drp1 but not Fis1 (P<0.001 for Fis1). Differences between treatments were significant overall by one-way ANOVA (F<sub>5,163</sub>=15.60, P<0.0001, representative of duplicate experiments) and Student-Newman Kuells’ post-hoc test was used to compare each treatment. *P<0.05; ** P<0.01; ***P<0.001.

(C) Parental M17 or stable PINK1<sub>WT</sub> overexpressing cells were untransfected or transfected with HA-Drp1 or Fis1-FLAG transiently for 24 hours. M17 and PINK1<sub>WT</sub> transfectants were separated on a 4-20% SDS PAGE gel, transferred to PVDF and analyzed by immunoblot. Immunoblotting with anti-HA antibody and anti-FLAG
antibody showed overexpression of HA-Drp1 and Fis1-FLAG was equivalent. β-actin is shown as a loading control. Markers on the right are in kilodaltons.

(D) Control (upper panels) and PINK1 (lower panels) shRNA cell lines were transfected with mito-YFP and Fis1-FLAG (right panels) or mito-YFP alone (UT, left panels) for 24 hours and mitochondrial morphology was imaged using confocal microscopy. Scale bar is 2 µm and applies to all panels.

(E) Control cells (blue circles) and PINK1 shRNA cells (pink squares) were analyzed via FRAP. Open symbols are only mito-YFP transfected cells; closed symbols are co-transfected with Fis1-FLAG for 24 hours. FRAP curves showed that Fis1 decreases recovery in control (circles) but not PINK1 (squares) shRNA cell lines. Curves were normalized to the first image in the series and corrected for both non-specific photobleaching (NSPB) that occurred during imaging and background. Each point is the average of >30 separate measurements and is representative of at least duplicate experiments for each line/treatment.

(F) Mobile fractions of mito-YFP was measured in control shRNA (blue) or PINK1 shRNA (purple) cell lines with (open boxes) and without (filled boxes) Fis1 overexpression. Mobile fractions were calculated as follows: Mobile Fraction = [(FRAP_{t-Background})/FRAP_{t}][(NSPB_{t-Background})/NSPB_{t}]. The box indicates the upper and lower quartiles, central line indicates the median and range bars indicate the 10^{th} to 90^{th} percentile range. Cells lying outside of this range are shown as single points and the ‘+’ indicates the mean. Boxplots summarize data from n=24-30 cells. The loss of mobile fraction between control and PINK1 shRNA was significant by one-way ANOVA (F_{4,140}=5.13, P=0.0007, representative of duplicate experiments) as was the
effect of Fis1 in control lines ($P<0.001$). However, Fis1 had no effect in PINK1 shRNA lines ($P>0.05$). Student-Newman Kuells’ post-hoc test was used to evaluate the differences between control shRNA and PINK1 shRNA after each treatment. *$P<0.05$; ** $P<0.01$; ***$P<0.001$. 
5.2.4 Effect of Fis1 overexpression on mitochondrial morphology in PINK1 deficient cells
5.2.5 Phosphorylation and GTPase activity of Drp1 in PINK1 deficient cells

The increased fission in the PINK1 deficient cell lines could be a result of Drp1 activity, which is influenced by post-translational modification, recruitment to mitochondria and oligomerization state. Previous studies have shown that Drp1 activity is downregulated by serine phosphorylation (Chang and Blackstone, 2007), which inhibits mitochondrial fission. It was hypothesized that Drp1 phosphorylation might be altered in PINK1 deficient cells. To determine Drp1 phosphorylation status, whole cell lysates from control and PINK1 shRNA cells were purified on PhosphoProtein Purification columns (Qiagen). Proteins that carry a phosphate group on any amino acid are bound with a high specificity, while proteins without phosphate groups do not bind the column and are found in the flow-through fraction. After phosphoprotein enrichment, eluates and total lysates from both cell lines were separated by gel electrophoresis and transferred to PVDF. Phospho-enrichment was confirmed by immunoblotting with anti-Drp1 phospho-S637. DJ-1, which is not basally phosphorylated (Rahman-Roblick et al., 2008), was used as a negative control. The following proteins were examined by immunoblotting for endogenous Drp1, Opa1, Mfn1/2, HtrA2 and Fis1 (Fig. 5.2.5.A). Of all the proteins examined, only Drp1 showed a loss of phosphorylation in the absence of PINK1 (Fig. 5.2.5.A). Quantification across multiple experiments showed an ~30% loss of endogenous Drp1 phosphorylation in PINK1 deficient cells when compared to control shRNA cells (Fig. 5.2.5.B). An antibody specific for phosphorylated Drp1 showed an ~34% decrease in phosphorylated Drp1 at serine residue 637 in PINK1 shRNA cells when compared to controls, a conserved site that regulates mitochondrial fission (Fig. 5.2.5.B) (Chang and Blackstone, 2007, Cribbs and Strack, 2007).
To confirm increased fission associated with the loss of PINK1 was dependent upon Drp1 phosphorylation, the effects of PINK1 loss on the mitochondrial association and oligomerization of endogenous Drp1 were also tested (Fig. 5.2.5.C and 5.2.5.D). A potential explanation for enhanced fission in PINK1 shRNA cells might be increased recruitment of Drp1 to the mitochondria. Subcellular fractionation of control versus PINK1 shRNA cells were analyzed by western blotting and showed total and mitochondrial protein expression levels of endogenous Drp1 were not influenced by the loss of PINK1 (Fig. 5.2.5.C). One might also speculate that the loss of PINK1 might prevent Drp1 oligomer formation, which influences GTPase activity. Oligomers of Drp1 were produced by crosslinking the native monomeric protein with bismaleimidohexane (BMH) in control and PINK1 shRNA cells. BMH-crosslinking experiments did not detect any alterations in Drp1 oligomerization in PINK1 deficient cells (Fig. 5.2.5). Together these results suggest that the increased fission found in PINK1 deficient cells is linked to Drp1 phosphorylation.

Previous studies have shown that Drp1 phosphorylation inhibits its GTPase activity, thus preventing fission (Chang and Blackstone, 2007). Endogenous Drp1 was immunoprecipitated from control and PINK1 shRNA cells to examine the GTPase activity of Drp1. Using in vitro assays, GTPase activity was increased (19% less GTP remained in PINK1 deficient cells compared to controls after 120 minutes in the reaction buffer) in cells expressing PINK1 shRNA, suggesting that the absence of PINK1-dependent phosphorylation increased Drp1 GTPase activity (Fig. 5.2.5.E; P<0.01 for cell lines).
Figure 5.2.5 Phosphorylation and GTPase activity of Drp1 in PINK1 deficient cells

(A) Control and PINK1 shRNA cell lines were purified on a PhosphoProtein Purification column (Qiagen). Total lysates and eluates from control shRNA lines (lanes 1,2) or either of two PINK1 shRNA lines (lanes 3-6) were separated on a 4-20% SDS-PAGE gel, transferred to PVDF and immunoblotted for proteins indicated on the left of the blots. Phospho-enrichment (phospho, lanes 1,3,5) was compared to total lysates (total, lanes 2,4,6). Drp1 was found in both phospho-enriched and total fractions, but less was seen in the PINK1 deficient cells (upper blot). Immunoblotting with anti-Drp1 phospho-S637 confirmed the phosphopurification was efficient (second blot) while DJ-1 was probed as a negative control for phosphorylated fractions (bottom blot). The GTPases Opa1, Mfn1 and 2, the protease Omi/HtrA2, and Fis1 were examined as indicated. Blots are representative of triplicate independent experiments and purifications. Molecular weight markers on the right of all blots are in kilodaltons.

(B) Quantification of Drp1 as a ratio of the phospho-enriched and total fractions shows that there is an ~30% decrease in phospho-Drp1 that was consistent between both shRNA sequences (n=3) when protein levels were measured by densitometry. Although there was a trend, this did not reach statistical significance by one-way ANOVA (P=0.08). Quantification of pS637-Drp1 as a ratio of the phospho-enriched and total fractions shows that there was an ~34% decrease in phospho-Drp1 that is consistent between both shRNA sequences (n=3) when protein levels were measured by densitometry. Using one-way ANOVA (F_{2,6}=17.63, P=0.0031), results were analyzed with Student-Newman Kuells’ post-hoc test (**P<0.01). For both graphs, the bars indicate the mean phosphorylated to total ratio of endogenous Drp1 protein. Error bars indicate SEM.
(C) Subcellular fractions from control and PINK1 shRNA cells were purified using a mitochondria isolation kit (Pierce). Fractions were separated on a 10% SDS-PAGE gel, transferred to PVDF and subjected to Drp1 immunoblotting. Recruitment of endogenous Drp1 (upper panel) to mitochondrial fractions (lanes 3-4; lanes 1-2 show total lysates and 5-6 show cytosolic fractions) was similar in control (lanes 1,3,5) and PINK1 (lanes 2,4,6) shRNA cell lines. The purity of the mitochondrial fraction was determined by using the anti-Hsp60 antibody. Blot is representative of duplicate experiments. Molecular weight markers on the right of the blots are in kilodaltons.

(D) Prior to harvest, control and PINK1 shRNA cells were crosslinked with BMH (20μM, 30 min) to form endogenous Drp1 oligomers. The crosslinking reaction was quenched with two rinses of DTT (dithiothreitol) (20mM) and cell lysates were separated on a 4-20% SDS-PAGE gel, transferred to PVDF and Drp1 multimers were detected by immunoblotting with anti-Drp1 antibody. Oligomerization status of Drp1 was not affected by PINK1 shRNA (lanes 2,4) compared to control lines (lanes 1,3). Lysates were crosslinked with BMH (lanes 1,2) or not treated to show equivalent loading of Drp1 (lanes 3,4). The arrowhead shows Drp1 oligomers, whereas, the arrow shows monomeric Drp1. Markers on the right of the blot are in kilodaltons.

(E) Precleared lysates from control and PINK1 shRNA cells were immunoprecipitated with 4 μg anti-Drp1 antibody. GTPase activity of Drp1 immunopurified from control (circles) or PINK1 (squares) shRNA cell lines was followed over time and expressed as percentage of GTP converted to GDP. Data was corrected for background and normalized to mean values obtained from each cell line at time t=0 with amount of GTP remaining set to 100%. The difference between the lines was significant (P<0.01 by two-
way ANOVA). Each point is the mean of 3 replicates and is representative of two independent experiments; error bars show SEM.
Figure 5.2.5  Phosphorylation and GTPase activity of Drp1 in PINK1 deficient cells

A

IB: Drp1
IB: pS637-Drp1
IB: Opa1
IB: Min1
IB: Min2
IB: HtrA2
IB: Fis1
IB: DJ-1

B

IB: Drp1
IB: Total Drp1

C

IB: Drp1
IB: Hsp60

D

IB: Drp1

E

% CTTI staining

shRNA control
shRNA PINK1

Time (min)
5.2.6 Effect of calcineurin on Drp1 phosphorylation in PINK1 deficient cells

Calcineurin has been proposed as a candidate phosphatase for Drp1 (Cribbs and Strack, 2007). The protein level of the calcineurin catalytic α subunit was similar in PINK1 deficient cells compared to controls (Fig. 5.2.6.A-B; 92±5.2% of controls after correction for β-actin, n=3). Cellular calcineurin activity was measured using the Cellular Calcineurin Phosphatase Assay (Biomol) in control and PINK1 shRNA cells using post-nuclear extracts desalted by gel filtration to remove excess phosphate and nucleotides. Desalted extracts were assayed in calmodulin-containing buffers supplemented with and without 20mM EGTA (ethylene glycol tetraacetic acid). Calcineurin requires calcium for its activity, thus the EGTA buffered samples represent total phosphatase activity less calcineurin. Phosphatase activity was expressed as the release of free phosphate from a calcineurin substrate peptide. Following background subtraction, protein absorbance was measured at a wavelength of 620nm using spectrophotometry and data collected at this optical density were converted into the amount of phosphate released using standard curve line-fit data, where the amount of phosphate released=(OD620nm-Yint)/slope. The contribution of calcineurin was determined by subtracting total phosphatase activity (Pi released) from the activity of EGTA buffered samples (CaN=Total-EGTA buffered samples). The amount of cellular CaN activity was 2.5-fold higher in PINK1 deficient cells compared to controls (Fig. 5.2.6.C; F_{2,27}=79.28, P<0.0001 by one-way ANOVA).

These results suggested that mitochondria in PINK1 deficient cells might have increased cytoplasmic levels of Ca^{2+} after depolarization, which would be predicted to stimulate calcineurin activity and may account for the decrease in observed phosho-
Drp1. If this is correct, then one might predict that the calcineurin inhibitor FK506 would block dephosphorylation of Drp1 and consequently rescue the FRAP defects in these PINK1 deficient cells. Control or PINK1 shRNA cells were treated with 1μM FK506 for 1 hour and extracts were separated using the PhosphoProtein Purification column (Qiagen) [as described in Fig. 5.2.5] to examine phosphorylated versus total proteins when immunoblotting for Drp1 (Fig. 5.2.6.D, shows the PINK1 deficient cells). Quantification confirmed the ~30% decrease in the ratio of phosphorylated to total Drp1 in PINK1 deficient cells compared to controls as in Fig. 5.2.5.B ($P=0.007$ by one-way ANOVA, $n=6$) and showed that this measure of Drp1 phosphorylation was increased to a level similar to controls after FK506 treatment (Fig. 5.2.6.E).

Control or PINK1 shRNA cells transfected with mito-YFP for 24 hours and were treated with 1μM FK506 for 1-3 hours before mitochondria were imaged using confocal microscopy (Fig. 5.2.6.F). FRAP analysis was performed on control and PINK1 shRNA cells treated with and without 1μM FK506. Quantitatively, the lower mobile fractions seen in PINK1 deficient mitochondria were increased 1.4 to 1.6–fold by FK506 treatment (Fig. 5.2.6.G) such that by 3 hours the difference between control and PINK1 shRNA lines was not significant. The effect of FK506 in PINK1 shRNA lines was significant at 1 hour ($P<0.05$) and 3 hours ($P<0.001$ by one-way ANOVA, $n=30$ cells measured over two experiments). Therefore, inhibition of calcineurin rescues the PINK1 phenotype, likely through Drp1.
Figure 5.2.6  Effect of calcineurin on Drp1 phosphorylation in PINK1 deficient cells

(A) Control and PINK1 shRNA post-nuclear supernatants purified by gel filtration were separated on a 10% SDS-PAGE gel, transferred to PVDF and immunoblotted for the catalytic α subunit of calcineurin. Protein levels of the catalytic α subunit of calcineurin (arrow) were similar in control (lanes 1,2) and PINK1 shRNA (lanes 3,4) cell lines. The immunoblot was probed with β-actin (open arrowhead) to show equal loading. Molecular weight markers on the right are in kilodaltons.

(B) Quantification of immunoblots examining catalytic α subunit of calcineurin protein expression measured by densitometry when compared to β-actin levels in control and PINK1 shRNA cells. Bars indicate the mean and SEM is shown. There were no significant differences in the mean between cell lines when analyzed by Student t-test (ns, P>0.05; n=3).

(C) Calcineurin enzyme activity was measured in post-nuclear supernatants desalted by gel filtration from control and PINK1 shRNA cells using calmodulin-containing buffers supplemented with and without 20mM EGTA (ethylene glycol tetraacetic acid). Activity was expressed as the release of Pi from a calcineurin substrate peptide, corrected for activity seen in the presence of EDTA to chelate calcium. Following background subtraction, 620nm absorbances obtained by spectrophotometry were converted into the amount of phosphate released using standard curve line-fit data, where the amount of phosphate released=(OD620nm-Yint)/slope. The contribution of calcineurin was determined by subtracting total phosphatase activity (Pi released) from the activity of EGTA buffered samples (CaN=Total-EGTA buffered samples). Bars indicate the mean
and SEM is shown. Differences between the cell lines were significant by one-way ANOVA (F_{2,27}=79.28, P<0.0001, n=5). *, P<0.05; ***, P<0.001; ns, not significant by ANOVA with Student-Newman Kuells’ post-hoc test.

(D) Cell extracts from PINK1 shRNA lines, treated either with DMSO as a vehicle (lanes 1,2) or 1μM FK506 for 1 hour (lanes 3,4), were enriched for phosphoproteins (lanes 1,3) or compared with total lysates (lanes 2,4). Samples were separated on a 10% SDS-PAGE gel, transferred to PVDF and immunoblotted for Drp1 (arrow) showed enhanced accumulation in the phosphofraction after FK506 treatment.

(E) Quantification of endogeneous Drp1 protein expression in (E) (n=6) confirmed a lower relative amount of phospho-Drp1 in PINK1 shRNA cells compared to controls (*, P<0.05) but this difference was not significant after treatment with FK506. Bars indicate the mean and error bars represent SEM. Differences between treatments were significant overall by one-way ANOVA (F_{3,19}=5.47, P=0.007). *, P<0.05; ***, P<0.001; ns, not significant by one-way ANOVA with Student-Newman Kuells’ post-hoc test.

(F) Control and PINK1 shRNA cells were transiently transfected (24 hours) with mito-YFP prior to FRAP. Mitochondrial morphology in control shRNA (upper panels) and PINK1 shRNA cells (lower panels) was demonstrated by mito-YFP expression. Cells were treated with vehicle only (control, left panels) or 1 μM FK506 for 1 hour (middle panels) or 3 hours (right panels). Increasing the duration of FK506 treatment improves mitochondrial connectedness, especially in the PINK1 shRNA line. Scale bar is 2 μm and applies to all panels.

(G) Control and PINK1 shRNA cells were transfected with mito-YFP 24 hours prior to FRAP. Cells (blue boxes are control shRNA lines, magenta boxes are PINK1 shRNA
lines) were treated either with DMSO (open boxes) or 1µM FK506 for 1 hour (stippled) or 3 hours (spotted). FRAP curves (data not shown) were generated and mobile fractions were derived as follows: Mobile Fraction = [(FRAP<sub>i</sub>-Background)/FRAP<sub>i</sub>][((NSPB<sub>i</sub>-Background)/NSPB<sub>i</sub>). The box indicates the upper and lower quartiles, central line indicates the median and range bars indicate the 10<sup>th</sup> to 90<sup>th</sup> percentile range. Cells lying outside of this range are shown as single points and the ‘+’ indicates the mean. Boxplots summarize data from n=24-30 cells from duplicate experiments. Differences between treatments were significant overall by one-way ANOVA (F<sub>5, 165</sub>=10.51, P<0.0001) and Student-Newman Kuells’ post-hoc test was used to compare each treatment. *P<0.05; ** P<0.01; ***P<0.001.
Figure 5.2.6 Effect of calcineurin on Drp1 phosphorylation in PINK1 deficient cells

A

IB: Calcineurin α
IB: β-actin

1 2 3 4

-50 -37

B

Calcineurin α subunit/β-actin

Control shRNA PINK1 shRNA

C

Ca²⁺ Activity

Phosphate Released (pmol)

Control shRNA PINK1 shRNA Cal³⁺ (ves oh)

D

DMSO

+FK506

Phospho total

Phospho total

1 2 3 4

-75

-25

E

pDrp1/Total Drp1

DMSO +FK506 DMSO +FK506

Control shRNA PINK1 shRNA

F

+ 1 μM FK506

DMSO 1h 3h

Control shRNA

PINK1 shRNA

G

Mobile Fraction

Control shRNA PINK1 shRNA FK506 1 μM 1h

Control shRNA FK506 1 μM 3h

Control shRNA FK506 1 μM 3h

PINK1 shRNA FK506 1 μM 3h

-180-
5.3 Discussion

Under conditions of oxidative stress, the recessive parkinsonism associated gene PINK1 seems to maintain mitochondrial function under stressful conditions. Neuronal cell loss has been linked to mitochondrial dysfunction and PINK1 seems to be neuroprotective against degeneration of nigral cells. These results show that PINK1 in mammalian cells limits mitochondrial damage and, secondarily, induction of mitochondrial fission. Evidence provided in this chapter suggests that this is due to decreased phosphorylation of Drp1 through an indirect mechanism involving calcineurin.

Previous studies have suggested that shifting the balance of mitochondrial dynamics towards fusion and away from fission is a protective response (Detmer and Chan, 2007). A dominant negative (K38A) form of Drp1 prevents stress-induced fission and neuronal cell death (Barsoum et al., 2006). The data presented in this chapter suggest PINK1 inhibits mitochondrial fission, which has close links to apoptosis (reviewed in (Herzig and Martinou, 2008)). These results further support previous reports that suggest PINK1 is neuroprotective (Deng et al., 2005, Petit et al., 2005, Wood-Kaczmar et al., 2008, Haque et al., 2008).

PINK1 deficient cells mitochondria have lower \( \Delta \psi_m \) and have a decreased rate of mitochondrial fusion compared to control cells. Loss of \( \Delta \psi_m \) has been shown to enhance \( Ca^{2+} \) release from mitochondria to the cytosol where \( Ca^{2+} \)-calmodulin dependent kinases and phosphatases are activated (Nakanishi and Okazawa, 2006). Calcineurin, an abundant \( Ca^{2+} \)- and calmodulin-dependent protein phosphatase known to dephosphorylate Drp1 was examined (Cribbs and Strack, 2007). Data in this chapter show that as a consequence of enhanced calcineurin activity, the GTPase activity of Drp1 is higher in
PINK1 deficient cells, consistent with fission. Neither the mitochondrial recruitment nor the oligomerization status of Drp1 is affected by PINK1 knockdown. Phosphorylation does not change intermolecular interactions of Drp1 (which would otherwise block oligomer formation) but does affect intramolecular interactions and GTPase activity (Chang and Blackstone, 2007). These results suggest that loss of PINK1 results in decreased $\Delta \psi_m$, activates calcineurin locally around mitochondria and increases fission by activation of Drp1.

One might suggest that mitochondria in PINK1 deficient cells may have a decreased ability to regulate calcium. Reticular mitochondria actively sequester $\text{Ca}^{2+}$ but these stores are not homogenously distributed and the ratio of fusion-to-fission can alter local buffering of $\text{Ca}^{2+}$ and ATP production. Since mitochondrial membrane depolarization decreases ATP catabolism, mitochondria with decreased $\Delta \psi_m$ might have secondary alterations in $\text{Ca}^{2+}$ dynamics. It has been shown that increases in cytoplasmic $[\text{Ca}^{2+}]$ usually decrease or halt mitochondrial motility (Gallo et al., 2006). Moreover, Drp1-dependent fission is influenced by changes in calcium levels (Han et al., 2008). Therefore, altered $\text{Ca}^{2+}$ dynamics and rates of fission and fusion are likely to be linked.

The immediate kinase substrate for PINK1 that maintains mitochondrial function is not known. However, characterization of PINK1 topology (Zhou et al., 2008) suggests the kinase domain faces the cytoplasm, which would make PINK1-dependent phosphorylation of cytoplasmic mitochondrial morphogenesis proteins plausible. A similar lack of *in vitro* phosphorylation was reported for Omi/HtrA2, a mitochondrial protease whose phosphorylation state is influenced by PINK1 levels (Plun-Favreau et al., 2007). However, the balance of data presented here suggests that regulation of
phosphorylation by PINK1 could involve indirect mechanisms, such as calcineurin activation. Perhaps FK506 can block the loss of phosphorylation of Omi/HtrA2 or the other proposed PINK1 substrate, TRAP1 (Pridgeon et al., 2007). TRAP1 phosphorylation has not been assessed as only a minor amount is found in the phospho-fraction. A lower molecular fragment of HtrA2 seen in the phosphorylated fraction of our cells, suggests HtrA2 may be regulated by both phosphorylation and proteolysis.

These data differ from Drosophila models, where increased Drp1 dosage suppresses effects of PINK1 deficiency (Poole et al., 2008, Yang et al., 2008), although they are consistent with work in C elegans (Ichishita et al., 2008). There are several possible reasons for this apparent discrepancy. First, regulation of Drp1 by PINK1 in mammals and Drosophila may differ. Phosphorylation of the conserved serine residue at position 129 in the PD protein synuclein shows that preventing phosphorylation blocks toxicity in flies (Chen and Feany, 2005) but has the opposite effect in rats (Gorbatyuk et al., 2008). Second, FRAP data presented in this chapter relied on transient manipulation of fission or fusion proteins to avoid compensation by long-term expression used in fly models. Although fusion and fission are regarded as independent events, recent data suggests they are linked (Twig et al., 2008), so chronic promotion of fission could change the rate of fusion. Lastly, differences might be attributable to the tissue type and its developmental stage, where triggers for fusion or fission may vary.

These results support that PINK1 plays a critical role in the balance of fission and fusion in response to cellular stress, limiting the effects of agents that trigger mitochondrial fission. This will allow the investigation of pathways relevant to dopaminergic cell death such as the influence of other recessive parkinsonism genes, DJ-
1 and parkin. Chapter Six will address the role of recessive parkinsonism genes in mitochondrial dynamics.
CHAPTER SIX

Role of recessive parkinsonism genes in mitochondrial dynamics
6.1 Summary

The previous data chapters highlight the role of PINK1 in mitochondrial function and morphology. However, genetic links between PINK1 and parkin suggest they share a common pathway to regulate mitochondrial phenotypes. Chapter Six reveals the similarities in mitochondrial morphology when either of these recessive parkinsonism genes are silenced in mammalian cells. Fibroblasts isolated from parkin patients exhibit a mitochondrial phenotype that resembles mitochondrial fission. Additionally, oxidative stress-induced fission can be overcome in mammalian cells when parkin is overexpressed. These characteristics mimic those seen in PINK1 deficient cells and cells with increased PINK1 expression, respectively. Overexpression of parkin in a PINK1 deficient background can rescue mitochondrial fission associated with the loss of PINK1. Collectively, these results suggest that the genetic pathway reported in Drosophila genetic studies that regulates mitochondrial morphology (Clark et al., 2006, Park et al., 2006) is conserved in mammalian cells.

Furthermore, cells deficient in DJ-1 also exhibit similar defects in mitochondrial morphology. DJ-1 knockdown results in fragmentation of the mitochondrial network and a loss of connectivity similar to that seen with the loss of PINK1 or parkin. It was determined that DJ-1 shRNA induced fission is driven by oxidation and not by apoptosis. Increasing intracellular glutathione can rescue the DJ-1 deficient phenotypes that are the result of enhanced oxidative stress. Additionally, overexpression of mitochondrial fusion proteins Mfn1 and Opa1 or downregulation of the fission protein Drp1 can shift DJ-1 deficient mitochondrial phenotypes towards fusion. Evidence shown in this chapter suggests that fission characterized in DJ-1 deficient cells is a secondary response to
oxidative stress, which modulates Drp1 activity and function through post-translational modification. Lastly, it was determined that all three recessive parkinsonism genes play a role in maintaining mitochondrial morphology, an aspect of mitochondrial dynamics. Mitochondrial fission in DJ-1 deficient cells can be rescued by exogenous expression of PINK1 or parkin, suggesting DJ-1 lies upstream of PINK1/parkin in a pathway to regulate mitochondrial morphology.
6.2 Results

6.2.1 Analysis of mitochondrial morphology in parkin deficient fibroblasts

In *Drosophila*, mutations in PINK1 and parkin alter mitochondrial morphology (Clark et al., 2006, Park et al., 2006, Yang et al., 2006). Moreover, PINK1 lies upstream of parkin in a genetic pathway that has been shown to regulate mitochondrial phenotypes. Data presented in Chapters Four and Five illustrate that loss of mammalian PINK1 induced mitochondrial fission and PINK1 could protect against oxidative stress-induced fission and changes in Drp1 GTPase activity. In *Drosophila* knockout models of parkin and PINK1, mitochondrial phenotypes were identical, so it was hypothesized that parkin silencing would display a similar phenotype to that of PINK1 deficient cells. Mammalian parkin mutants were examined to see if they also exhibit a fragmented mitochondrial phenotype using FRAP assays. A series of fibroblasts from parkin mutation patients and controls were obtained from Dr. Oliver Bandmann, University of Sheffield, UK. These were provided in a blinded fashion and the code broken after FRAP experiments were completed.

Fibroblasts isolated from control and parkin mutation patients were transfected with mito-YFP for 24 hours and FRAP was performed to examine mitochondrial connectivity. Additionally, control and parkin deficient fibroblasts were treated with 100nM rotenone for 72 hours to assess the ability of parkin to protect against oxidative stress-induced fission. Parkin deficient cells showed basal fragmentation of the mitochondrial network and after rotenone exposure this phenotype was enhanced (Fig. 6.2.1.A). FRAP curves demonstrated that parkin deficient cells had lower recovery of mito-YFP fluorescence than controls under basal conditions and the recovery was
decreased further following rotenone treatment (Fig. 6.2.1.B). To provide an overall summary measure of the FRAP results, the mobile fraction of mito-YFP was calculated over time (Fig. 6.2.1.C). Using this as a metric, the effect of genotype and rotenone were both significant (two-way ANOVA, $P_{\text{genotype}}=0.0036$, $P_{\text{rotenone}}=0.006$). Quantification of FRAP mobile fractions showed that parkin deficient cells had less mitochondrial connectivity than controls under basal conditions (mean±SEM: controls, 0.58±0.03; patients, 0.44±0.05) (Fig. 6.2.1.C). Mobile fraction values of control fibroblasts were decreased following treatment with rotenone (mean±SEM: controls untreated, 0.58±0.03; controls treated, 0.46±0.04) and parkin deficient cells had decreased mobile fractions compared to controls following treatment (mean±SEM: controls treated, 0.46±0.04; patients treated, 0.34±0.02), indicating sensitivity to rotenone (Fig. 6.2.1.C). Overall, these results showed that parkin deficient fibroblasts have a basal defect in functional connectivity that was enhanced by rotenone.
Figure 6.2.1 Analysis of mitochondrial morphology in parkin deficient fibroblasts

(A) Mitochondrial morphology in mito-YFP transfected control (left panels) and parkin deficient (right panels) fibroblasts was altered by exposure to rotenone (lower panels), consistent with the induction of fission. Scale bar is 2 μm and applies to all panels.

(B) Fluorescence recovery after photobleaching (FRAP) curves in controls (n=4; open symbols) and parkin deficient cell lines (n=5; closed symbols) either without treatment (circles) or after exposure to 100nM rotenone for 72 hours (squares). Curves were normalized to the first image in the series and corrected for both non-specific photobleaching (NSPB) that occurred during imaging and background. Each point is the average of >30 separate measurements and is representative of at least duplicate experiments for each line/treatment. Error bars indicate the SEM between individual cell lines.

(C) Fibroblasts were transfected with mito-YFP and FRAP was performed. Mobile fractions were calculated as follows: Mobile Fraction = \frac{[(\text{FRAP}_i-\text{Background})/\text{FRAP}_i][(\text{NSPB}_i-\text{Background})/\text{NSPB}_i]}{[(\text{FRAP}_o-\text{Background})/\text{FRAP}_o][(\text{NSPB}_o-\text{Background})/\text{NSPB}_o]}. The mobile fraction of mito-YFP derived from FRAP is lower in parkin deficient lines (n=5) (filled bars) than in controls (n=4) (open bars); 100nM rotenone treatment for 72 hours enhanced these effects. Each cell line was analyzed with 30 separate measurements. Two-way ANOVA shows a significant effect of both genotype and treatment (Pgenotype=0.0036, Protenone=0.006); Bonferroni post-tests show that the effect of rotenone is significant in the patient lines (*, P<0.05).
Figure 6.2.1  Analysis of mitochondrial morphology in parkin deficient fibroblasts
6.2.2  Effect of parkin on mitochondrial fission in PINK1 deficient cells

Since parkin deficient fibroblasts exhibited a similar phenotype to cells lacking PINK1, it seemed likely that the proposed *Drosophila* genetic interaction between PINK1 and parkin might be conserved in mammalian cells. The next step was to determine if parkin overexpression could compensate for the loss of PINK1 and/or protect against rotenone-induced mitochondrial fission in neuroblastoma cells. M17 neuroblastoma cells were transiently co-transfected for 24 hours with either PINK1-V5 or parkin-myc and mito-YFP to assess mitochondrial connectivity basally and under oxidative stress induced by rotenone (24 hours, 100nM) (Fig. 6.2.2.A-B). Exogenous PINK1 or parkin could protect the mitochondria from rotenone-induced fission in neuroblastoma cells as no significant differences were observed in mobile fraction values between treatments (Fig. 6.2.2.B). To determine if ectopic expression of parkin could rescue mitochondrial fragmentation associated with the loss of PINK1, PINK1 shRNA cells were transiently co-transfected with wild-type myc-parkin and mito-YFP to examine mitochondrial morphology (Fig. 6.2.2.D) and functional connectedness using FRAP (Fig. 6.2.2.E). It was determined that transient parkin overexpression could rescue the mitochondrial fragmentation caused by PINK1 silencing.
Figure 6.2.2  Effect of parkin on mitochondrial fission in PINK1 deficient cells

(A) Living M17 cells were imaged after transfection with wild-type PINK1 or wild-type parkin and mito-YFP. Cells expressing PINK1-V5 (upper panels) or parkin-myc (lower panels) had elongated mitochondria and maintained the mitochondrial network after 24 hours of 100nM rotenone exposure (right panels). PINK1 and parkin overexpression could protect against rotenone-induced fragmentation (right panels). Scale bar in the upper right panel is 2 µm and applies to all fluorescence micrographs.

(B) Mobile fraction of mito-YFP was measured by FRAP in the indicated cell lines (blue squares are M17 parental cells, red squares are PINK1 transient transfections and black boxes indicate parkin transient transfected cells, filled boxes are rotenone treated). Mobile fractions were calculated as follows: Mobile Fraction = [(FRAP-Background)/FRAP]([(NSPB-Background)/NSPB]). Summaries are of approximately 60 cells (from duplicate experiments of n=30). The box indicates the upper and lower quartiles, the mean is indicated by a ‘+’, central line indicates the median and range bars indicate the 10th to 90th percentile range. Cells lying outside of this range are shown as single points. Differences between treatments were significant overall by one-way ANOVA (F5,336=18.54, P<0.0001) and Student-Newman Kuells’ post-hoc test was used to evaluate rotenone effects in each line. *P<0.05; ** P<0.01; ***P<0.001; ns, not significant (P>0.05).

(C) Whole cell lysates from M17 cells transiently transfected with myc-parkin or PINK1-V5 were separated on a 10% SDS-PAGE gel, transferred to PVDF and analyzed by immunoblot. Immunoblotting with anti-myc or anti-V5 antibody showed equivalent protein expression between the cell lines for FRAP. In the lower panel, the blot was
reprobed with β-actin (open arrowhead) to show equal loading. Molecular weight markers on the right are in kilodaltons.

(D) Control and PINK1 shRNA cells were transiently co-transfected with mito-YFP and myc-parkin for 24 hours prior to confocal imaging to examine mitochondrial morphology. Elongation of the mitochondrial network was maintained in cell lines expressing a control shRNA construct following transient expression of myc-parkin (upper right panel). Cells expressing a shRNA against PINK1 (lower panels) showed some basal evidence of fission, with some fragmented mitochondria visible but the mitochondrial phenotype was rescued by exogenous parkin expression (lower right panel). Scale bar in the upper right panel is 2 μm, applies to all fluorescence micrographs.

(E) Mobile fraction of mito-YFP was measured in control and PINK1 shRNA cell lines following FRAP (blue square, control shRNA cells; red square, PINK1 shRNA cells; black box, parkin transient transfected PINK1 shRNA cells). Mobile fractions were calculated as follows: Mobile Fraction = [(FRAP_1-Background)/FRAP_1][(NSPB_1-Background)/NSPB_1]. Mitochondrial connectivity of PINK1 shRNA cells was increased to levels of control shRNA cells following the transient overexpression of myc-parkin. Summaries are of approximately 60 cells (from duplicate experiments of n=30). The box indicates the upper and lower quartiles, the mean is indicated by a ‘+’, central line indicates the median and range bars indicate the 10th to 90th percentile range. Cells lying outside of this range are shown as single points. Differences between treatments were significant overall by one-way ANOVA (F_{2,161}=38.86, P<0.0001) and Student-Newman
Kuells’ *post-hoc* test was used to evaluate the effect of parkin in each line. *P<0.05; **P<0.01; ***P<0.001; ns, not significant (P>0.05).

(F) Whole cell lysates from control and PINK1 shRNA cells transiently transfected with myc-parkin were separated on a 10% SDS-PAGE gel, transferred to PVDF and analyzed by immunoblot. Immunoblotting with anti-myc antibody showed parkin protein was expressed in the PINK1 shRNA cell line for FRAP. In the lower panel, the blot was reprobed with β-actin (open arrowhead) to show equal loading. Molecular weight markers on the right are in kilodaltons.
Figure 6.2.2  Effect of parkin on mitochondrial fission in PINK1 deficient cells
6.2.3 Analysis of mitochondrial morphology in DJ-1 deficient cells

PINK1 and parkin deficiencies in mammalian cells exhibited mitochondrial fragmentation that was further enhanced with exposure to oxidative stress. Another recessive parkinsonism gene DJ-1 has been shown to protect against oxidative stress and translocate to the mitochondria under oxidant challenge. To characterize DJ-1 silencing in mammalian cells, two independent M17 neuroblastoma cell lines expressing short hairpin RNA (shRNA) against DJ-1 (van der Brug et al., 2008) were transfected with mitochondrial YFP and examined by FRAP. Qualitatively, both DJ-1 deficient cell lines had morphological differences with a slight shortening in mitochondrial length and some evidence of increased fragmentation when compared to control shRNA lines (Fig. 6.2.3.A). Fluorescence recovery after photobleaching was used to quantify the differences in mitochondria morphology between cell lines (Fig. 6.2.3.B). The two different DJ-1 shRNA cell lines (mean±SEM: clone 1, 0.44±0.02; clone 2, 0.43±0.02) showed a lower recovery of fluorescence than either control cell line (mean±SEM: clone 1, 0.59±0.02; clone 2, 0.55±0.02) (Fig. 6.2.3.C). These results demonstrated that DJ-1 deficient cells displayed mitochondrial fragmentation, a phenotype also observed in PINK1 and parkin silencing.
Figure 6.2.3  Analysis of mitochondrial morphology in DJ-1 deficient cells

(A) Stably transduced DJ-1 shRNA and control shRNA M17 neuroblastoma cells were transiently transfected with mito-YFP, then living cells were imaged after 24 hours. Mitochondria were highly connected in control shRNA cells (upper panels), but appeared more fragmented across two different clones for DJ-1 shRNA clones (lower panel). Scale bar in the lower right panel is 2 μm, applies to all fluorescence micrographs.

(B) Whole cell lysates from control and DJ-1 shRNA cells were separated on a 4-20% SDS-PAGE gel, transferred to PVDF and analyzed by immunoblot. Immunoblotting with anti-DJ-1 antibody (arrow) showed knockdown of endogenous DJ-1 was sufficient in both clones. In the lower panel, the blot was reprobed with β-actin (open arrowhead) to show equal loading. Molecular weight markers on the right are in kilodaltons.

(C) FRAP was used to examine mitochondrial connectivity in control and DJ-1 shRNA cells transiently transfected with mito-YFP for 24 hours. Curves show decreased fluorescence recovery in DJ-1 deficient cells (red and purple symbols) compared to control lines (black and grey symbols). Curves were normalized to the first image in the series and corrected for both non-specific photobleaching (NSPB) that occurred during imaging and background. Each point is the average of >60 separate measurements and is representative of at least duplicate experiments for each line/treatment.

(D) Mobile fraction values were calculated from control (red and purple boxes) and DJ-1 shRNA (black and grey boxes) cells after FRAP. Mobile fractions were calculated as follows: Mobile Fraction = [(FRAP_t-Background)/FRAP_t][((NSPB_t-Background)/NSPB_t)]. Mobile fraction of mito-YFP was lower in DJ-1 deficient mitochondria compared to controls. Summaries are of approximately 60 cells (from duplicate experiments of n=30).
The box indicates the upper and lower quartiles, the mean is indicated by a ‘+’, central line indicates the median and range bars indicate the 10th to 90th percentile range. Cells lying outside of this range are shown as single points. Differences between treatments were significant overall by one-way ANOVA (F3,229=9.11, P<0.0001, n=60 from duplicate experiments) and Student-Newman Kuells’ post-hoc test was used to evaluate the effect of DJ-1. *P<0.05; ** P<0.01; ***P<0.001; ns = not significant (P>0.05).
Figure 6.2.3  Analysis of mitochondrial morphology in DJ-1 deficient cells
6.2.4 Effects of oxidation on mitochondrial morphology in DJ-1 deficient cells

Given the reports of increased oxidative stress in DJ-1 deficient cells (Andres-Mateos et al., 2007, Taira et al., 2004, Takahashi-Niki et al., 2004), oxidative stress was examined as a possible contributor to the observed mitochondrial phenotypes. Control and DJ-1 shRNA cells were treated for 24 hours with sublethal concentrations of the complex I inhibitors, rotenone and MPP⁺; the superoxide generator, paraquat; and the apoptosis inducing kinase inhibitor, staurosporine prior to FRAP analysis. Oxidation enhanced mitochondrial fragmentation in the rotenone, MPP⁺ and paraquat treatments (Fig. 6.2.4.A-B). No additional fission was observed in DJ-1 knockdown cells with staurosporine treatment, suggesting the enhancement of mitochondrial fragmentation was specific to oxidative stressors and not a response to apoptosis. However, staurosporine treatment did not change mitochondrial morphology in the control shRNA lines, which suggests that perhaps the optimal time and concentration of treatment was not utilized in this experiment. Previous studies using staurosporine suggest that this apoptosis inducer fragments the mitochondria to either promote fission (Alirol et al., 2006) or inhibit fusion (Karbowski et al., 2004) during programmed cell death. Therefore, this data cannot exclude the possibility that DJ-1 may play a role to prevent mitochondrial morphological changes caused by the induction of apoptosis, but it can be concluded that DJ-1 shRNA induced fission was driven by oxidation and/or ATP depletion.
Figure 6.2.4  Effects of oxidation on mitochondrial morphology in DJ-1 deficient cells

(A) Mito-YFP transiently transfected DJ-1 and control shRNA cell lines were treated with 100nM rotenone, 200µM paraquat, 100µM MPP\(^+\) or 100nM staurosporine for 24 hours and then imaged using confocal microscopy. Increased mitochondrial fragmentation was observed with all oxidative toxins but not with staurosporine treatment. Images show representative cells for each treatment and cell type. Scale bar is 2 µm and applies to all panels.

(B) Control and DJ-1 shRNA cells were transiently transfected with mito-YFP and treated with 100nM rotenone, 200µM paraquat, 100µM MPP\(^+\) or 100nM staurosporine for 24 hours prior to FRAP. FRAP curves were generated (data not shown). Mobile fraction values were calculated in control and DJ-1 shRNA cells following the respective treatments. Mobile fractions were calculated as follows: Mobile Fraction = [(FRAP\(_f\)-Background)/FRAP]\/[(NSPB\(_f\)-Background)/NSPB]. Quantification of mobile fraction values for treatments confirmed mitochondrial fragmentation was enhanced in DJ-1 shRNA cell lines following oxidative stress. Summaries are of approximately 60 cells (from duplicate experiments of n=30). The box indicates the upper and lower quartiles, the mean is indicated by a ‘+’, central line indicates the median and range bars indicate the 10\(^{th}\) to 90\(^{th}\) percentile range. Cells lying outside of this range are shown as single points. Differences between treatments were significant overall by one-way ANOVA (F\(_{9,578}\)=10.79, P<0.0001, n=60 from duplicate experiments) and Student-Newman Kuells’ post-hoc test was used to evaluate drug effects in each line. *P<0.05; ** P<0.01; ***P<0.001; ns = not significant (P>0.05).
Figure 6.2.4  Effects of oxidation on mitochondrial morphology in DJ-1 deficient cells
6.2.5 Effects of anti-oxidants and PTEN/Akt modulation on DJ-1 shRNA induced fission

It has been shown recently that DJ-1 binds RNA including mitochondrial, antioxidants and PTEN/Akt1 transcripts (van der Brug et al., 2008), supporting previous suggestions that altered glutathione status (Zhou and Freed, 2005) or PTEN activity (Kim et al., 2005a) may be influenced by expression of DJ-1. Either of these could be important in mediating mitochondrial morphology and therefore these pathways were transiently manipulated using pharmacology. First, exogenous anti-oxidants were used in the cell to see if they could rescue the effects of DJ-1 deficiency. Control and DJ-1 shRNA cells were treated for 24 hours with two cell permeable precursors of glutathione, 100µM glutathione ethyl ester (GSH-EE) and 10µM 2-oxo-L-thiazolidine-4-carboxylic acid (OTCA) and mitochondrial morphology was examined using FRAP. Increasing intracellular glutathione was found to rescue the fission phenotype found in DJ-1 deficient cells (Fig. 6.2.5.A-B). Next, PTEN or Akt activity was modified to observe whether mitochondrial morphology in DJ-1 deficient cells was altered. Neither decreasing Akt phosphorylation by LY294002 inhibition of PI3K nor decreasing PTEN phosphorylation by inhibiting casein kinase 2 using DMAT (Fig. 6.2.5.D) influenced mitochondrial phenotypes in either control or DJ-1 shRNA cells (Fig. 6.2.5.C). In summary, increased levels of oxidative stress enhanced loss of mitochondrial connectivity in DJ-1 deficient cells while exogenous anti-oxidants rescued mitochondrial fragmentation. Together, this data suggested altered oxidative stress induced by lack of DJ-1 accounted for mitochondrial phenotypes.
Figure 6.2.5  Effects of anti-oxidants and PTEN/Akt modulation on DJ-1 shRNA induced fission

(A) Control and DJ-1 shRNA cells were incubated with 100μM glutathione ethyl ester (GSHEE) or 10μM 2-oxo-L-thiazolidine-4-carboxylic acid (OTCA) for 24 hours prior to live cell imaging of transfected mito-YFP. Highly connected mitochondria were observed in treated DJ-1 deficient cells compared to untreated basal cells. Scale bar is 2 μm and applies to all panels.

(B) Control and DJ-1 shRNA cells were treated for 24 hours with 100μM GSHEE or 10μM OTCA prior to FRAP analysis. FRAP was performed and the mobile fraction of mito-YFP was determined. Mobile fractions were calculated as follows: Mobile Fraction = [(FRAPr-Background)/FRAPr][(NSPBr-Background)/NSPB]. Quantification of mobile fraction values for treatments confirmed mitochondrial connectivity was enhanced in DJ-1 shRNA cell lines following anti-oxidant treatment. Summaries are of approximately 60 cells (from duplicate experiments of n=30). The box indicates the upper and lower quartiles, the mean is indicated by a ‘+’, central line indicates the median and range bars indicate the 10th to 90th percentile range. Cells lying outside of this range are shown as single points. Differences between treatments were significant overall by one-way ANOVA (F3,590=15.96, P<0.0001) and Student-Newman Kuells’ post-hoc test was used to evaluate the effects of glutathione precursors in each line. *P<0.05; ** P<0.01; ***P<0.001; ns = not significant (P>0.05).

(C) Control and DJ-1 shRNA cells were treated for 24 hours with 10μM DMAT or 18μM LY294002 prior to FRAP analysis. FRAP was performed and the mobile fraction of mito-YFP was determined. Mobile fractions were calculated as follows: Mobile
Fraction = [(FRAP₁-Background)/FRAP₁][(NSPB₁-Background)/NSPB₁]. Quantification of mobile fraction values for treatments showed mitochondrial connectivity was unchanged in DJ-1 shRNA cell lines following modulation of PTEN/Akt activity. Summaries are of approximately 60 cells (from duplicate experiments of n=30). The box indicates the upper and lower quartiles, the mean is indicated by a ‘+’, central line indicates the median and range bars indicate the 10th to 90th percentile range. Cells lying outside of this range are shown as single points. Differences between treatments were significant overall by one-way ANOVA (F_{7,456}=8.70, P<0.0001) and Student-Newman Kuells’ post-hoc test was used to evaluate drug effects in each line. *P<0.05; **P<0.01; ***P<0.001; ns = not significant (P>0.05).

(D) Control shRNA cells were treated with either 10 μM DMAT or 18 μM LY294002 for 24 hours in culture prior to harvesting for immunoblot analysis. Whole cell lysates were resolved by 4-20% SDS-PAGE and transferred to PVDF membrane. Following transfer, immunoblot analysis was performed using anti-Akt, anti-phospho-Akt (T308), and anti-β-actin antibodies for the LY294002 treatment. PTEN inhibition by DMAT was confirmed by immunoblotting with anti-PTEN, anti-phospho-PTEN (S380/T382/T383), and anti-β-actin antibodies.
Figure 6.2.5  Effects of anti-oxidants and PTEN/Akt modulation on DJ-1 shRNA induced fission
6.2.6 Effects of DJ-1 deficiency and oxidative stress on mitochondrial fission

The mitochondrial morphology of DJ-1 deficient cells had a similar phenotype to cells deficient in mitochondrial fusion (Chen et al., 2003). Therefore, the potential involvement of DJ-1 with proteins that regulate mitochondrial dynamics was investigated. Several components of the fusion machinery were transiently expressed in control and DJ-1 shRNA cell lines and FRAP assays were performed. The fusion proteins Opa1 and Mfn1 were able to rescue the loss of mitochondrial connectivity in DJ-1 deficient cells when overexpressed (Fig. 6.2.6.A-B). Mobile fraction values of DJ-1 deficient cells increased 1.3 to 1.4-fold with overexpression of Mfn1 and Opa1, respectively (Fig 6.2.6.B). Additionally, expression of a dominant negative mutant of Drp1 (Drp1 K38A) increased the mitochondrial mobile fraction 1.8-fold in DJ-1 shRNA cells (Fig. 6.2.6.B) when compared to untransfected DJ-1 shRNA cells.

To assess whether oxidative stress associated with the loss of DJ-1 might have effects on the fission machinery, DJ-1 deficient cells were analyzed to examine post-translational modifications of Drp1 and Fis1. Drp1 has been shown to be regulated by PKA phosphorylation (Chang and Blackstone, 2007, Cribbs and Strack, 2007) and calcineurin-mediated dephosphorylation (Cribbs and Strack, 2007). Fractions enriched for phosphorylated proteins (as previously described) and total lysates from control and DJ-1 shRNA cells were analyzed (Fig. 6.2.6.C). As seen previously, Fis1 does not appear to be phosphorylated; however, the amount of phosphorylated Drp1 was decreased ~32% in DJ-1 deficient cells when compared to control shRNA cells (Fig. 6.2.6.D). Given that phosphorylation negatively regulates Drp1 GTPase activity (Chang and Blackstone, 2007, Cribbs and Strack, 2007), it is likely that increased fission in the
DJ-1 deficient cells was a consequence of altered Drp1 activity. This would also be consistent with the block of mitochondrial fission by dominant negative Drp1.
Figure 6.2.6 Effects of DJ-1 deficiency and oxidative stress on mitochondrial fission

(A) Mito-YFP was co-transfected into DJ-1 and control shRNA cell lines with plasmids containing Opa1-myc, Mfn1-FLAG or Drp1 K38A-myc for 24 hours. Images are representative of mitochondrial morphology in living cells for each condition. The scale bar in the lower right panel is 5μm and applies to all panels.

(B) Control and DJ-1 shRNA cells were co-transfected for 24 hours with mito-YFP and Opa1-myc, Mfn1-FLAG, or Drp1 K38A-mcy prior to FRAP analysis. FRAP curves were generated (data not shown) and mobile fraction of mito-YFP was determined. Mobile fractions were calculated as follows: Mobile Fraction = [(FRAP_{Background})/FRAP_{i}][(NSPB_{Background})/NSPB_{i}]. Quantification of mobile fraction values for treatments showed mitochondrial connectivity was enhanced in DJ-1 shRNA cell lines following promotion of fusion or the inhibition of fission. Summaries are of approximately 60 cells (from duplicate experiments of n=30). The box indicates the upper and lower quartiles, the mean is indicated by a ‘+’, central line indicates the median and range bars indicate the 10^{th} to 90^{th} percentile range. Cells lying outside of this range are shown as single points. Differences between treatments were significant overall by one-way ANOVA (F_{9,608}=15.33, P<0.0001) and Student-Newman Kuells’ post-hoc test was used to evaluate the effects of Opa1, Mfn1 or Drp1 in each line. *P<0.05; ** P<0.01; ***P<0.001; ns = not significant (P>0.05).

(C) Whole cell lysates from control and DJ-1 deficient cells untransfected (UT) or transiently transfected for 24 hours with Opa1-myc, Drp1 K38A-myc, or Mfn1-FLAG were separated on a 4-20% SDS-PAGE gel, transferred to PVDF and examined by
immunoblot to show protein expression during FRAP analysis. Immunoblotting for anti-myc showed equal expression of Opa1 and dominant negative Drp1 in control and DJ-1 shRNA cells. Anti-FLAG-M2 antibody showed Mfn1 expression was similar between cell lines. Immunoblotting with anti-DJ-1 antibody shows knockdown of endogenous DJ-1 protein. In the lower panel, the blot was reprobed with β-actin to show equal loading. Molecular weight markers on the right are in kilodaltons.

(D) Whole cell lysates from control shRNA (lanes 1,2) or DJ-1 shRNA cells (lanes 3,4) were separated by phospho-enrichment (phospho, lanes 1,3) and compared to total lysates (lanes 2,4). Drp1 (upper blot) was found in both phospho- and total fractions and the amount of phospho-Drp1 was decreased with DJ-1 silencing. Immunoblotting with anti-Fis1 antibody showed negligible phosphorylation. As controls for purification, immunoblotting was performed for DJ-1, which is not phosphorylated, and phosphorylated ERK1/2. Molecular weight markers on the right of all blots are in kilodaltons.

(E) Quantification of Drp1 immunoblotting (D) as a ratio of the phospho-enriched and total fractions shows that there is ~32% less phospho-Drp1 found in DJ-1 shRNA cells when compared to the control shRNA cells. Bars indicate the mean and the SEM is shown. Results were analyzed by Student’s t-test (*, P=0.0144; n=3).
Figure 6.2.6 DJ-1 deficiency and oxidative stress induce mitochondrial fission

Panel A: Images showing mitochondrial morphology under different conditions:
- Untransfected
- Opa1-myc
- Mfn1-FLAG
- Drp1 K38A-myc

Panel B: Graph illustrating the mobile fraction of mitochondria under various treatments:
- Control shRNA
- DJ-1 shRNA
- Opa1-myc
- Drp1 K38A-myc
- Mfn1-FLAG

Panel C: Western blot analysis showing protein expression levels:
- IB: myc
- IB: FLAG
- IB: DJ-1
- IB: β-actin

Panel D: Western blot analysis showing phosphorylation status of Drp1 and other proteins:
- IB: Drp1
- IB: Fis1
- IB: DJ-1
- IB: p-ERK1/2

Panel E: Graph showing the ratio of p-Drp1/Total Drp1 under control and DJ-1 shRNA conditions.
6.2.7 Effects of parkin and PINK1 on mitochondrial morphology in DJ-1 deficient cells

The observation of a reproducible and quantifiable fragmented mitochondrial phenotype in DJ-1 deficient cells led us to address the relationships between DJ-1 and the other recessive parkinsonism genes established to regulate mitochondrial morphology. It was demonstrated in Figure 6.2.2 that parkin overexpression in PINK1 deficient cells could restore fragmented mitochondria to a reticular phenotype. This data supports genetic studies that suggest PINK1 acts upstream of parkin to regulate mitochondrial morphology in *Drosophila* (Clark et al., 2006, Park et al., 2006, Yang et al., 2006). However, DJ-1 overexpression in PINK1 or parkin null *Drosophila* had no influence on mitochondrial phenotypes in the musculature (Yang et al., 2006). Therefore, mitochondrial phenotypes were examined in DJ-1 deficient cells after ectopic expression of PINK1 or parkin using FRAP. Immunoblot analysis that was performed concurrently with FRAP experiments indicated a slight decrease in steady state parkin levels in the DJ-1 shRNA cells when compared to control shRNA cells (Fig. 6.2.7.C). Additionally, minor differences in PINK1 steady state levels were observed in DJ-1 deficient cells, shifting the distribution PINK1 pre- and mature protein expression (Fig. 6.2.7.C). Overexpression of either parkin or PINK1 could rescue the effects of DJ-1 silencing when measured by FRAP (Fig. 6.2.7.A-B). These data suggest all three genes are likely related to the maintenance of mitochondrial morphology.
Figure 6.2.7 Effects of parkin and PINK1 on mitochondrial morphology in DJ-1 deficient cells

(A) Parkin-myc and PINK1-V5 plasmids were co-transfected with mito-YFP and imaged live after 24 hours. DJ-1 shRNA cells overexpressing parkin and PINK1 showed reduction in fragmented mitochondria and more reticular mitochondrial phenotypes. Scale bar is 2 µm and applies to all panels.

(B) Control and DJ-1 shRNA cells were co-transfected with mito-YFP and wild-type parkin-myc or wild-type PINK1-V5 for 24 hours prior to FRAP analysis. FRAP curves were generated (data not shown) and mobile fractions were calculated as follows: Mobile Fraction = [(FRAP1-Background)/FRAP1][(NSPB1-Background)/NSPB1]. Quantification of mobile fraction values for treatments showed mitochondrial connectivity was enhanced in DJ-1 shRNA cell lines following ectopic expression of PINK1 or parkin. Summaries are of approximately 30 cells (from duplicate experiments). The box indicates the upper and lower quartiles, the mean is indicated by a ‘+’, central line indicates the median and range bars indicate the 10th to 90th percentile range. Cells lying outside of this range are shown as single points. Differences between treatments were significant overall by one-way ANOVA (F5,162=14.9, P<0.0001) and Student-Newman Kuells’ post-hoc test was used to evaluate the effects in each line. *P<0.05; ns = not significant (P>0.05).

(C) Whole cell lysates from control and DJ-1 shRNA cells untransfected (UT) or transfected for 24 hours with parkin-myc or PINK1-V5 were separated on a 4-20% SDS-PAGE gel, transferred to PVDF and analyzed by immunoblot to show protein expression during FRAP analysis. Immunoblotting with anti-myc antibody showed expression of
parkin; arrow indicates full-length parkin (50kDa) and C-terminal breakdown products of parkin are seen at approximately 42kDa (*) and 37kDa (**). V5-tagged PINK1 was expressed in control and DJ-1 shRNA cells; arrow shows pre-protein and arrowhead indicates the mature form of PINK1. DJ-1 knockdown was confirmed by immunoblotting with anti-DJ-1 antibody. In the lower panel, the blot was reprobed with β-actin to show equal loading. Molecular weight markers on the right are in kilodaltons.
Figure 6.2.7  Effects of parkin and PINK1 on mitochondrial morphology in DJ-1 deficient cells
6.3 Discussion

Studies in Chapter Six describe the role of recessive parkinsonism genes in mitochondrial dynamics. Having established that PINK1 plays a role in maintaining mitochondrial morphology to prevent fragmentation, it was hypothesized that parkin (a proposed regulatory protein for mitochondrial morphology) and/or DJ-1 might exhibit similar functional deficits in mitochondria when silenced in mammalian cells. These results suggest that impaired mitochondrial function is likely to make a substantial contribution to the pathogenesis of recessive parkinsonism.

Data presented in this chapter show parkin deficient cells have a fragmented phenotype that resembles mitochondrial fission. Moreover, exogenous parkin can compensate for the lack of PINK1 in mammalian cells rescuing the mitochondrial fission phenotype to a reticular network. Several ubiquitin E3 ligases are known to have roles in the regulation of mitochondrial fusion and fission (Karbowski et al., 2007). For example, the mitochondrial E3 ubiquitin ligase MarchV is required for Drp1 dependent mitochondrial division, and introduction of mutant MarchV or MarchV knockdown in cells leads to abnormal mitochondrial elongation (Karbowski et al., 2007). Results presented here suggest that another E3 ligase, parkin, also plays a role in mitochondrial dynamics. However, the substrate(s) of parkin that mediate mitochondrial dynamics are not known. Although these results suggest that parkin deficient cells are more prone to enter fission when stressed, it is currently unclear whether this is secondary to other aspects of mitochondrial dysfunction such as impaired complex I function, ATP production or a precipitating event leading to impaired mitochondrial function (Exner et
al., 2007, Greene et al., 2003). Parkin is thought to act in a common pathway with at least one other gene for recessive parkinsonism, PINK1 (Clark et al., 2006, Park et al., 2006, Yang et al., 2006). Qualitative differences in mitochondrial morphology were described both in HeLa cells and in fibroblasts from patients with PINK1 mutations (Exner et al., 2007), and are similar to the experiments presented here with neuroblastoma cells and skin fibroblasts from parkin patients. Overall, these results are consistent with those seen in PINK1 deficient cells and suggest that there may be a common pathway mediating recessive parkinsonism in human cells.

Additionally, the lack of the recessive parkinsonism protein DJ-1 causes a loss of mitochondrial connectivity measured using live cell imaging. Data presented here shows that increased oxidative stress resulting from lack of DJ-1 increases susceptibility to mitochondrial fission. The key molecular connection is likely altered phosphorylation of the fission-promoting GTPase Drp1. Furthermore, this model shows that DJ-1 is related to two other genes for recessive parkinsonism, PINK1 and parkin.

Numerous reports demonstrate that the lack of DJ-1 increases sensitivity to reactive oxygen species and mitochondrial complex I inhibitors, which will indirectly cause ROS accumulation (Andres-Mateos et al., 2007, Taira et al., 2004, Takahashi-Niki et al., 2004). DJ-1 also interacts with mRNA transcripts for both glutathione peroxidases and members of the PTEN/Akt pathway and results in increased translation under oxidation (van der Brug et al., 2008). Oxidative toxins exacerbate the fragmented mitochondrial phenotype observed with DJ-1 deficiency but apoptosis induced by the protein kinase inhibitor staurosporine did not. This is similar to previous results where DJ-1 can protect against MPP+ or rotenone toxicity (Canet-Aviles et al., 2004) but not
against staurosporine (Betarbet et al., 2006, Yokota et al., 2003). Additionally, increasing intracellular glutathione rescues the phenotype, but modulating PTEN/Akt activity has no effect. These results suggest that the fragmented phenotype found in DJ-1 deficient cells is driven primarily by increases in intracellular oxidative species, and not by modulation of apoptotic mechanisms. Furthermore, these data suggest that a major focal point of DJ-1 mediated protection is the mitochondria themselves.

Rates of mitochondrial fusion and fission were manipulated to determine if the DJ-1 shRNA phenotype would be altered by such changes. While artificially increasing fusion rates rescues the phenotype, inhibiting fission increased mitochondrial connectivity to levels higher than those seen basally. Presumably increased oxidation associated with the loss of DJ-1 increased dephosphorylation of Drp1. Thus, inhibition of Drp1 GTPase activity would demonstrate that mitochondrial fragmentation is likely to be a consequence of oxidative stress-induced alterations in Drp1 function.

In a previous report, abnormal mitochondrial phenotypes were reported in PINK1 deficient cells and overexpression of parkin but not DJ-1 could rescue the morphological defects (Yang et al., 2006). This study suggested that unlike parkin, DJ-1 did not act downstream of PINK1 to regulate mitochondrial morphology (Yang et al., 2006). Data presented in this chapter confirmed previous findings (Clark et al., 2006, Exner et al., 2007, Park et al., 2006, Yang et al., 2006) that parkin can rescue mitochondrial phenotypes associated with the loss of PINK1 and could also protect against oxidative stress-induced fission like PINK1. However, the fragmented phenotype found in cells lacking DJ-1 could be rescued by overexpression of PINK1 or parkin suggesting all three recessive parkinsonism proteins play a role in enhancing functional connectivity of
mitochondria. Collectively, these data would suggest a model in which DJ-1 acts upstream of the established PINK1/parkin pathway to promote mitochondrial connectivity. Therefore, the interaction between these genes to modulate mitochondrial dynamics in response to oxidative stress has strong implications for neuronal survival in parkinsonian disorders.
CHAPTER SEVEN

Conclusions and future directions
7.1 Summary of Results

7.1.1 PINK1 protects against cell death induced by mitochondrial toxins

It was determined that overexpression of PINK1 in neuroblastoma cells could protect against mitochondrial dysfunction caused by mitochondrial toxins rotenone and MPP⁺. *In vitro* and *in vivo* data suggested that the PINK1 mRNA was induced following chronic exposure to rotenone without altering the stability of the transcript. This result implied that PINK1 mRNA expression is increased following oxidative stress, likely through a transcriptional mechanism. Such upregulation would be consistent with a role in protection against oxidative stress.

7.1.2 Deficiencies in PINK1 result in decreased mitochondrial function and altered mitochondrial morphology

PINK1 silencing in neuroblastoma cells resulted in a decreased mitochondrial membrane potential as previously described in the literature (Exner et al., 2007, Valente et al., 2004a, Wood-Kaczmar et al., 2008). The mitochondrial network was altered following the loss of PINK1 shifting phenotypes from elongated, tubular mitochondria to fragmented and truncated forms. Exposure to rotenone-induced oxidative stress enhanced the fragmented phenotype identified in PINK1 deficient cells to a truncated form. Mitochondrial connectivity was quantified using FRAP to show that loss of PINK1 promoted mitochondrial fission and rotenone exposure compounded this phenotype. Similarly, PINK1 deficient cells have a decreased rate of fusion when measured by
photoactivation fusion assays. It was further demonstrated that mitochondrial fusion was
dependent upon PINK1 kinase activity and an intact mitochondrial membrane potential.

7.1.3 PINK1 influences mitochondrial dynamics by downregulating Drp1 activity

Increased mitochondrial fragmentation and decreased rates of fusion associated
with the loss of PINK1 suggested that the function of PINK1 was to promote fusion or
inhibit fission. It was determined that the overexpression fusion proteins Opa1 and Mfn2
could rescue mitochondrial phenotypes associated with the loss of PINK1. Expression of
Drp1 exaggerated PINK1 deficiency phenotypes and Drp1 RNAi restored mitochondrial
morphology to the elongated forms. Decreased Drp1 phosphorylation enhanced fission
in PINK1 deficient cells as a consequence of calcineurin activation. The calcineurin
inhibitor FK506 blocked both Drp1 phosphorylation and loss of PINK1 mitochondrial
integrity in PINK1 deficient cells.

7.1.4 DJ-1 lies upstream of PINK1/parkin in a pathway that regulates
mitochondrial dynamics

Cells deficient in parkin exhibit mitochondrial fission, a phenotype associated
with PINK1 silencing in cultured cells. Overexpression of parkin compensated for the
loss of PINK1 in neuroblastoma cells rescuing the fragmented mitochondrial phenotype
confirming a pathway previously identified in Drosophila (Clark et al., 2006, Park et al.,
2006, Yang et al., 2006). Additionally, knockdown of DJ-1 in neuroblastoma cells
resulted in a subtle fragmented mitochondrial phenotype that was enhanced specifically
by exposure to oxidative stress. Increasing anti-oxidants in cells lacking DJ-1 restored
mitochondrial morphology to the elongated form. Together these results indicated that all three recessive parkinsonism genes influenced mitochondrial morphology. Overexpression of PINK1 or parkin rescued mitochondrial fragmentation associated with the loss of DJ-1. Additionally, exogenous expression of Opa1, Mfn1 or dominant-negative Drp1 elongated the mitochondrial network promoting fusion. It was determined that oxidative stress caused dephosphorylation of Drp1, which upregulated GTPase activity and the rate of fission. Increased fission associated with DJ-1 deficiencies was found to be a result of altered Drp1 activity. DJ-1 appeared to be upstream of PINK1/parkin in a pathway that affected mitochondrial dynamics.
7.2 PINK1 deficiency results in dephosphorylation of Drp1 leading to alterations in mitochondrial dynamics

Continuous fusion and fission of the mitochondrial membranes is a mechanism by which networks of matrix and lumen are generated. Fusion has been widely thought to promote cell survival by complementing damaged mitochondrial units to recover activity, thereby maintaining metabolic efficiency of the cell (Chan, 2006). PINK1 deficient cells exhibit decreased mitochondrial membrane potential, which has been shown to inhibit mitochondrial fusion events. Since fusion is dependent on an intact mitochondrial membrane potential, the mitochondrial fragmentation and decreased fusion events may be secondary to loss of mitochondrial membrane potential in PINK1 deficient cells. It has been recently shown that the selective fusion of mitochondria with decreases in mitochondrial membrane potential promotes fission events that contribute to the removal of dysfunctional mitochondria by mitophagy (Twig et al., 2008).

Mitophagy is the selective degradation of mitochondria by hydrolytic enzymes in lysosomes following autophagic sequestration, a process by which organelles and portions of cytoplasm are gathered (Levine and Klionsky, 2004). Irreversible mitochondrial depolarization or ineffective calcium buffering opens the mitochondrial permeability transition pore, which is implicated in mitophagy induced by nutrient deprivation (Elmore et al., 2001). This suggests mitophagy could be a possible outcome of secondary changes in mitochondrial membrane potential and calcium dysregulation associated with the loss of PINK1. PINK1 silencing may demonstrate defective calcium buffering that modulates calcineurin activity to regulate the function of the fission GTPase, Drp1. The inhibition of fission has been shown to attenuate mitophagy;
expression of dominant-negative Drp1 or Fis1 RNAi prevents colocalization of mitochondria with autophagolysosomes or late autophagolysosomes (Twig et al., 2008). It has been suggested that mitochondria are associated with lysosomes in cells lacking PINK1, implying an increase in turnover of the organelle (Wood-Kaczmar et al., 2008). Additionally in PINK1 deficient cells, Drp1-dependent fission is enhanced by its calcineurin-dependent dephosphorylation. Therefore, the inhibition of fission caused by PINK1-dependent phosphorylation of Drp1 would limit the induction of autophagy.

However, whether autophagy promotes or prevents cell death remains controversial. Autophagic events removing damaged mitochondria that would stimulate caspase-dependent cell death should be protective. However, the leakage of hydrolytic enzymes has been shown in cases of deregulated autophagy, which promotes cell death through the initiation of mitochondrial membrane permeabilization, caspase activation and apoptosis (Boya et al., 2005). Moreover, the deletion of genes regulating autophagy has been associated with decreased apoptosis (Boya et al., 2005). Oxidative stress has been shown to influence the mitochondrial permeability transition pore, which determines the relative amount of necrosis, apoptosis and autophagy that occurs in the cell (Lemasters et al., 1998). It has yet to be determined if opening of the mitochondrial permeability transition pore is induced with the loss of PINK1 in the neuroblastoma model but overexpression of PINK1 has been shown to prevent atractyloside-induced opening of the mitochondrial permeability transition pore in human embryonic kidney (HEK) 293 cells (Wang et al., 2007). Perhaps with low levels of oxidative stress, mitophagy is induced only as a repair mechanism. However, as oxidant levels increase, the mitophagy apparatus might be overburdened and become deregulated allowing the
promotion of cell death. However, oxidative stress causes significant decreases in cellular ATP levels (Przedborski et al., 2000). ATP-dependent apoptosis and autophagy cannot progress under extreme oxidative stress and only necrotic cell death can ensue. Preliminary data shows that both apoptosis and necrosis occurs in the neuroblastoma cells deficient in PINK1 (data not shown), suggesting that cell death is not mediated in a single fashion. It also remains to be determined if reactive oxygen species accumulate in the absence of PINK1 and contribute to cell death. Since PINK1 has been shown to protect against oxidative stress and mitochondrial dysfunction, it seems likely that limiting mitophagy would further promote neuroprotection, thus supporting the notion that autophagy promotes cell death.
Figure 7.2.1 Proposed mechanism of altered mitochondrial fission induced by loss of PINK1

PINK1 silencing results in decreased mitochondrial membrane potential and changes in Drp1-dependent mitochondrial morphology. Increased calcineurin activity in PINK1 deficient cells, results in Drp1 dephosphorylation, enhanced GTPase activity and increased mitochondrial fission. Downstream consequences of enhanced mitochondrial fission might include necrosis or ATP-dependent forms of cell death, such as apoptosis or mitophagy.
7.3 DJ-1, PINK1 and parkin play roles in maintaining mitochondrial morphology

The studies described in this thesis address the roles of the recessive parkinsonism genes in maintaining mitochondrial morphology in mammalian cells. Expression of PINK1, parkin and DJ-1 proteins protect against mitochondrial fission. Fibroblasts from patients with parkin mutations exhibit fragmented mitochondrial morphology indicating an imbalance in mitochondrial dynamics towards fission. Overexpression of parkin can rescue the PINK1 null mitochondrial phenotype suggesting PINK1 is located upstream of parkin. Genetic studies in Drosophila indicate that PINK1 and parkin interact genetically to regulate mitochondrial morphology, which is confirmed in mammalian studies shown here. As an E3 ligase, parkin can promote both degradative and nondegradative forms of ubiquitination (Doss-Pepe et al., 2005, Joch et al., 2007, Lim et al., 2006). For instance, ubiquitin lysine 48-linked polyubiquitin chains target substrates to the proteasome (Imai et al., 2000), whereas ubiquitin lysine 63-linked chains control non-degradative processes including kinase activation, DNA repair, translational regulation and endocytosis of membrane proteins (Doss-Pepe et al., 2005, Joch et al., 2007, Moore et al., 2008, Pickart and Fushman, 2004). It is notable that ubiquitin lysine 63-linked chains promote the degradation of membrane proteins by the lysosome, another putative link supporting autophagy as an endpoint for recessive parkinsonism. The loss of parkin associated with recessive parkinsonism suggests that parkin-mediated ubiquitination of specific cellular substrates is required for the survival of dopaminergic neurons. At the present it is unclear how parkin deficiencies might precipitate defects in mitochondrial function and whether this relates to the accumulation of toxic parkin substrates or a non-degradative regulatory role of parkin-mediated ubiquitination remains unknown.
Another potential function of parkin could be the regulation of the key players in mitochondrial dynamics. Since PINK1 is present in both the cytoplasm and the mitochondria while parkin localizes only to the cytoplasm, one possible mechanism is that PINK1 phosphorylates an upstream component that in turn signals parkin to ubiquitylate cytoplasmic targets of mitochondrial dynamics machinery. It remains unknown how Drp1 is recruited to the mitochondria from the cytoplasm to promote fission although Drp1 is subjected to multiple post-translational modifications, including phosphorylation (Chang and Blackstone, 2007, Taguchi et al., 2007), SUMOylation (Harder et al., 2004, Wasiak et al., 2007, Zunino et al., 2007) and ubiquitylation (Nakamura et al., 2006, Yonashiro et al., 2006) which might promote mitochondrial recruitment. Candidate targets of a degradative form of parkin-mediated ubiquitination might be fission players such as Drp1, Fis1 and MarchV to inhibit cell death. Alternatively, parkin may ubiquitinate Mfn1/2 altering its mitochondrial membrane association. Mitofusins found in yeast are regulated by ubiquitination (Neutzner and Youle, 2005). Moreover, mammalian mitofusin protein expression levels increase following exposure to proteasomal inhibitors in culture (Karbowski et al., 2007), which suggest the ubiquitination events also regulate these fusion proteins. In yeast, Mfn1 is regulated by Mdm30, which is an F-box containing E3 ligase (Escobar-Henriques et al., 2006).

PINK1 and DJ-1 deficient cells show a basal mitochondrial fragmentation that can be rescued by overexpression of fusion proteins. DJ-1 could potentially mediate the regulation of mitochondrial dynamics proteins to limit the effects of oxidative stress-induced mitochondrial fission. For instance, studies have shown that Bax promotes
SUMOylation of Drp1, which promotes mitochondrial fission (Wasiak et al., 2007). DJ-1 has been shown to decrease Bax expression, which could limit the promotion of mitochondrial fission. To address the mechanism by which PINK1/parkin pathway regulates mitochondrial morphology, the influence of parkin on the steady state levels of mitochondrial dynamics proteins, their mitochondrial recruitment and potential post-translational modifications should be examined.

Mitochondria are dynamic organelles that undergo morphological changes that depend on the balance of fusion and fission rates and the directed movement or anchoring by various components of the cytoskeleton (Rube and van der Bliek, 2004). In cultured cells, disruption of the microtubule network affects the distribution of mitochondria (Heggeness et al., 1978), suggesting that mitochondria move along microtubules. Secondary messengers, such as calcium, can influence movement of mitochondria along microtubule tracks. For instance, mitochondrial motility is blocked by increased mitochondrial calcium uptake (Rintoul et al., 2003), suggesting that local calcium buffering limits mitochondrial movement. Moreover, calcium signaling can influence the function of molecular motors (Marston, 1995) that facilitate mitochondrial movement along cytoskeletal elements. These aspects of mitochondrial transport may be especially important in neurons where mitochondria are transported out of synapses. The effects of recessive parkinsonism mutations on transport as well as the perinuclear cytoplasmic pool of mitochondria should be examined in the future.

Other putative links between defects in mitochondrial transport and recessive parkinsonism include the association of parkin with the cytoskeleton or the influence of oxidative stress on cytoskeletal function. Parkin associates with cytoskeletal elements,
such as actin filaments (Huynh et al., 2000) and microtubules (Ren et al., 2003). Studies suggest that the binding of parkin stabilizes microtubules (Yang et al., 2005a). Stabilization of the cytoskeletal network may be an important link between parkin and mitochondrial dynamics because disruption of microtubules can cause aberrant mitochondrial distribution (Yaffe et al., 1996). On the other hand, this interaction may simply provide transport of misfolded proteins for degradation (Yang et al., 2005a). Additionally, oxidative stress is associated with microtubule disorganization (Annunen-Rasila et al., 2007) and oxidative insult can impair mitochondrial trafficking (Liu et al., 2008a). The idea of oxidation being detrimental to mitochondrial plasticity is further supported by the notion that rotenone selectively kills neurons via a microtubule-dependent mechanism (Ren and Feng, 2007). While the shape and connectivity of the mitochondria are likely to influence the ability of the cytoskeleton to move the organelle, it is unknown if altered mitochondrial transport is the result of defects in morphology or vice versa.

Key questions to address in the future will regard the importance of mitochondrial membrane potential in the regulation of mitochondrial dynamics. Data shown in this thesis suggests that oxidative stress influences mitochondrial morphology promoting the loss of mitochondrial membrane potential and fission. It is plausible that maintaining membrane potential is a vital component for regulating mitochondrial phenotypes. A putative explanation for changes in mitochondrial dynamics that are associated with the loss of DJ-1, PINK1 or parkin may be secondary to the effects of oxidative stress and mitochondrial membrane decoupling.
Figure 7.3.1 Model for the life cycle of a mitochondrion

This proposed model for the life cycle of a mitochondrion reflects mitochondrial dynamics and turnover (adapted from (Twig et al., 2008)). In a healthy cell, the antagonizing events of fusion and fission are dependent upon mitochondrial membrane potential. DJ-1, which lies upstream of the PINK1/parkin pathway protect against mitochondrial fission. Following a fission event, fragmented mitochondria may either maintain an intact membrane potential (green line) or depolarize (red line). Should mitochondrial membrane depolarization take place prior to fission, it is unlikely to proceed to fusion without repolarization. Consequences of mitochondrial depolarization include the production of reactive oxygen species (ROS), increased cytosolic $[\text{Ca}^{2+}]$ or loss of ATP with subsequent effects on various types of cell death and/or impaired transport and trafficking.
7.4 Concluding statements

Discovery of mutations in the mitochondrial protein kinase PINK1 suggests a direct involvement of mitochondria in pathogenesis of parkinsonism and a role of mitochondrial function in neuronal survival. The identification of mitochondrial pathways involved in cell survival and death will be the next step in understanding the molecular mechanisms of the pathogenesis of parkinsonism.

To date, it is not unknown how PINK1 function is regulated. It has been demonstrated that PINK1 has autophosphorylation activity in vitro (Beilina et al., 2005), although the function of this activity in vivo is unknown. Since kinase activity of PINK1 is required for protection from cell death, it is possible that kinase activity may be increased by oxidative stress. Additional studies should elucidate the role of autophosphorylation activity and endogenous PINK1 phosphorylation on PINK1 protein function under both basal and stressed conditions. Identification of physiological PINK1 interactions and substrates could shed light on PINK1 signaling pathways that lead to altered mitochondrial function and the subsequent degeneration of nigral neurons that could possibly reveal new targets for treating or preventing parkinsonism.

Although the primary function of mitochondria is to provide cells with energy in the form of ATP, mitochondria also participate in pathways regulating cell survival and death. DJ-1, PINK1, and parkin protect cells against death mediated by mitochondrial toxins that induce oxidative stress. Moreover, anti-oxidants rescue loss of mitochondrial connectivity that is associated with increases in oxidative stress found in DJ-1 deficient cells. Therefore it seems likely that mitochondrial morphological defects are secondary to mitochondrial dysfunction and oxidative stress. Mitochondrial membrane potential
should be measured in DJ-1 deficient cells to help discriminate how oxidative stress influences mitochondrial morphology in recessive parkinsonism, as PINK1 and parkin silencing decreases mitochondrial membrane potential. Future studies should address why the mitochondria lose membrane potential after PINK1 and parkin silencing. To date, putative in vivo substrates do not link parkin to mitochondrial function. However, in vitro PINK1 substrates TRAP1 (Pridgeon et al., 2007) and HtrA2 (Plun-Favreau et al., 2007) are mitochondrial and function to protect against oxidative stress and apoptosis.

It has been shown that complex I activity decreases with PINK1 silencing (Piccoli et al., 2008a). PINK1 may play a role in maintaining the integrity and function of mitochondria through direct phosphorylation of complex I subunits. An alternative mechanism of preventing mitochondrial dysfunction would be the PINK1-dependent phosphorylation of proteins involved in the regulation of complex I activity. Future research should elucidate the effect of PINK1 on mitochondrial complex I activity, stability, phosphorylation status and ATP production under basal and stress-induced conditions. PINK1 effects mitochondrial membrane potential, which provides the driving force for oxidative phosphorylation. It would be interesting to see if PINK1 kinase activity influences mitochondrial decoupling associated with complex I activity in submitochondrial particles. The relationship between PINK1, mitochondrial membrane potential, and the role of mitochondrial complex I function also needs to be determined.

It is also possible that PINK1 may not be involved in direct regulation of mitochondrial complex I function and may mediate its protective effects by limiting cell death. In this case, PINK1 prevents cell death triggered by mitochondrial complex I inhibition. Several kinases are implicated in mitochondrial function that regulate both
mitochondrial complex I activity and cell death. These kinases include protein kinase A, protein kinase B/Akt, protein kinase C, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase (Horbinski and Chu, 2005). Protein kinase A localizes to the matrix and inner membrane of mitochondria where it promotes the activity of complex I (Technikova-Dobrova et al., 2001) and inhibition of the proapoptotic protein BAD, thereby enhancing cell survival (Harada et al., 1999). PKA regulates the activity of Drp1 (Chang and Blackstone, 2007, Cribbs and Strack, 2007), which is similar to an indirect effect mediated by PINK1. One might be predict that enhanced calcineurin activity associated with PINK1 silencing would also dephosphorylate BAD, promoting apoptosis (Wang et al., 1999). Protein kinase C translocates to mitochondria where it triggers cytochrome c release and subsequent induction of apoptosis (Majumder et al., 2000). Both c-Jun N-terminal kinase and p38 mitogen-activated protein kinase also are implicated in pathways involved in recessive parkinsonism. HtrA2 is phosphorylated by activation of the p38 pathway, occurring in a PINK1-dependent manner (Plun-Favreau et al., 2007). Additionally, JNK activation activates Bcl-2 proapoptotic family members. Expression of Buffy, the Drosophila Bcl-2 homolog, protects PINK1 deficient cells from apoptosis (Clark et al., 2006, Park et al., 2006). Drp1 dephosphorylation in PINK1 deficient cells infers that PINK1 may modify this apoptotic-induced protein. Preliminary data suggests that PINK1 does not directly phosphorylate Drp1 in vitro (data not shown) but that does not exclude the possibility that PINK1 may phosphorylate Drp1 under alternative conditions. PINK1-dependent phosphorylation of Drp1 should be addressed further. Future studies should examine the possibility that one of these proteins may be a substrate for PINK1.
The cumulative data comprised of genetic associations, pathology, biochemical and molecular analyses have strengthened the notion of overlapping pathways in parkinsonism. The mitochondria and proteasome are predominantly centered in the pathogenesis of this neurodegenerative disease and aging. Tissue specific susceptibility to dysfunction in brain is attributed to their high-energy requirements and as such implicate mitochondria as a key pathogenic source, since they are key regulators of cell survival and death. It is unclear how oxidative stress underlies mitochondrial and ubiquitin proteasome impairment in both sporadic and familial parkinsonism. The majority of Parkinson disease cases are comprised of unknown etiologies, but genetic associations with early-onset disease have provided insight into signaling pathways associated with disease pathogenesis. Regardless of molecular etiology, the contributing factors of parkinsonism are superimposed on a background of age-regulated genes and progressive neuronal cell loss that is also associated with aging. However, mounting evidence suggests the loss-of-function in parkin, PINK1 and DJ-1 abrogates neuroprotection. Further investigation should demarcate their physiological function to understand the molecular basis of parkinsonism to develop new methods of neural protection. Current therapy to manage parkinsonism utilizes dopamine replacement to correct motor disorder symptoms, but does nothing to slow nigral degeneration and has its limitations in long-term efficacy. The critical examination of oxidative imbalance, which is primarily mitochondrial, could potentially derive new therapies or even prophylactic strategies for parkinsonism.
CHAPTER EIGHT

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