Functional Analysis of the Parkinson's Disease Associated Genes, Parkin and PINK1, *in vitro*.

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Abstract

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. Our understanding of the disease has been enhanced by the discovery of gene mutations in rare families with early-onset autosomal recessive juvenile parkinsonism (ARJP). The first gene to be identified was *parkin* and two further genes have been identified namely, *DJ-1* and *PINK1*. The latter was discovered in our laboratory.

The work of this thesis has aimed to increase our understanding of how these newly discovered genes play a role in neuronal survival and how disease-causing mutations result in neurodegeneration. Utilising gene transfer techniques and transient and stable cell culture expression systems, the function of parkin and PINK1 was investigated in human dopaminergic SH-SY5Y neuroblastoma cell lines. The work of this thesis has confirmed and extended previous reports that parkin over-expression confers neuroprotection against a variety of cellular stresses implicated in PD. Moreover, this work has showed for the first time that endogenous parkin protein can localize to aggregates known as 'aggresomes' following stress and that the formation of these parkin positive aggresomes can be dissociated from parkin's effect on neuronal survival.

Furthermore, this work describes the first functional characterization of PINK1. It demonstrates that PINK1 localizes to the mitochondria in neuronal cells where it may play a neuroprotective role against cellular stress. Moreover, this effect is abrogated by disease causing mutations in PINK1. This work also reports on the characterisation of novel PINK1 antibodies and shows for the first time that PINK1 can localize to aggresomes and the mechanism is linked to mitochondrial recruitment.

The work of this thesis sheds light on the increasing importance of the ubiquitin proteasome system and mitochondria in the pathogenesis of PD. Improved understanding of these cellular processes should lead to more effective treatments for this devastating disease.

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Abbreviations

aa	amino acid
ANOVA	analysis of variance
ARJP	autosomal recessive juvenile parkinsonism
ARPD	autosomal recessive Parkinson's disease
Apoε4	apolipoprotein ε4
ANT	adenine nucleotide translocase
ATP/ADP	adenosine trisphosphate/disphoshate
bp	base pairs
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
CCCP	carbonyl cyanide chlorophenylhydrazone
cDNA	complementary DNA
CHIP	carboxyl terminus of the Hsc-70 – interacting protein
CK	creatine kinase
complex I	NADH-ubiquinone reductase
complex II	succinate-ubiquinone oxidoreductase
complex III	ubiquinol cytochrome c oxidoreductase
complex IV	cytochrome c oxidase
COX	cytochrome c oxidase
CsA	cyclosporin A
CyP-D	cyclophilin-D
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
ECL™	enhanced chemiluminescence
ER	endoplasmic reticulum
E.coli	*Escherichia coli*
ERK ½	extracellular signal-regulating kinase 1/2
ETC	electron transport chain

FACS	fluorescence activated cell sorting
FCS	foetal calf serum
FTDP-17	Fronto-temporal Dementia and Parkinsonism linked to chromosome 17
GFP	green fluorescent protein
Gly	glycine
GstS1	glutathione S-transferase
HBSS	Hank's Balanced Salt Solution
HK	hexokinase
HRP	horseradish peroxidase
IMM	inner mitochondrial membrane intermembranous space
IMS	intermembranous space
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolycarbocyanine iodide
Kb	kilobase
kDa	kilodalton
LB	Lewy body
Lof	loss of function
LRRK2	leucine rich repeat kinase 2
Lys	lysine
MAPK	mitogen-activated protein kinase
MCS	multiple cloning site
MFI	mean fluorescence intensity
MOMP	mitochondrial outer membrane permeabilisation
MPP	mitochondrial processing peptidase
mPTP	mitochondrial permeability transition pore
MPP^+	methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger RNA
NR4A2	Nurr1
6-OHDA	6-hydroxydopamine
OMM	outer mitochondrial membrane

Pael-R	Parkin associated endothelin-like receptor
PAGE	polyacrylamide gel electrophoresis
PBR	peripheral benzodiazepine receptor
PBS	phosphate buffered saline
PBST	PBS/Tween
PD	Parkinson's disease
PET	Positron Emission Tomography
PINK1	PTEN induced kinase 1
PTEN	phosphatase and tensin homolog gene
ROS	reactive oxygen species
RNA	ribonucleic acid
SCA-2	Spinocerebellar Ataxia Type 2
SCA-3	Spinocerebellar Ataxia Type 3
SD	standard deviation
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SNP	single nucleotide polymorphism
SNpc	substantia nigra pars compacta
sp22	glycosylated form of α-synuclein
UCH-L1	ubiquitin C-terminal hydrolase 1
TAE	tris-acetate-EDTA buffer
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TCA	tricarboxylic acid
Tim	translocase of the inner membrane
TMRM	tetramethylrhodamine, methyl ester, perchlorate
Tom	translocase of the outer membrane
Tween 20	polyoxyethylene-sorbitan monolaurate
Ub	ubiquitin
UPR	unfolded protein response pathway
UPS	ubiquitin proteasome system

VDAC	voltage-dependant anion channel
wt	wild type
$\Delta\Psi m$	mitochondrial membrane potential (Delta psi)

Publications

The work presented in this thesis has contributed towards the following papers:

Muqit M. M. K., Davidson S. M., Payne-Smith M. D., MacCormac L. P., Wood N. W., Latchman D. S. Parkin is recruited into aggresomes in a stress specific manner: over-expression of parkin reduces aggresome formation but can be dissociated from parkin's effect on neuronal survival. *Human Molecular Genetics* 2004; **13:** 117-135.

Valente E. M., Abou-Sleiman P. M., Caputo V.*, **Muqit M. M. K.*** et al. Hereditary early onset Parkinson's disease is caused by mutations in PINK1. *Science* 2004; **304:** 1158-1160. **(*shared joint second authorship)**

MacCormac L. P., **Muqit M. M. K.**, Faulkes D. J., Wood N. W., Latchman D. S. Reduction in endogenous parkin levels renders glial cells sensitive to both caspase-dependent and caspase-independent cell death. *European Journal of Neuroscience* 2004; **20:** 2038-48.

Bandopadhyay R., Kingsbury A. E., **Muqit M. M. K.**, et al. Synphilin-1 and parkin show overlapping expression patterns in human brain and form aggresomes in response to proteasomal inhibition. *Neurobiology of Disease* 2005; **20:** 401-11.

Muqit M. M. K., Abou-Sleiman P. M., Saurin A., et al., Altered cleavage and localisation of PINK1 in the presence of proteasomal stress. *Journal of Neurochemistry* 2006; **98:** 156-69.

Gandhi S., **Muqit M. M. K.,** Healy D. G. et al. PINK1 protein in normal human brain and Parkinson's disease. *Brain* 2006; **129:** 1720-31.

Abou-Sleiman P. M., **Muqit M. M. K.,** McDonald N. Q. et al. A heterozygous effect for PINK1 mutations in Parkinson's disease? *Annals of Neurology* 2006 Published September 2006 ahead of print.

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Declaration

All work presented in this thesis is the work of Dr Miratul Muqit

(except where stated otherwise).

1

Introduction

1.1 OVERVIEW

On the commencement of these PhD studies, only 3 genes had been found in Mendelian-inherited forms of Parkinson's disease (PD). At the time of writing 3 further genes have been identified with startling new insights into possible new pathways underlying neurodegeneration. With the discovery of these genes, the next stage is to identify their function, interacting partners and understand how mutations lead to cell death. The work undertaken during this thesis period has sought to try and understand mechanisms of cell death for loss of function gene mutations by developing *in vitro* cell models that could be used in well established cell death paradigms.

1.2 PARKINSON'S DISEASE

1.2.1 Historical perspective

Parkinson's disease (PD) was first described as a medical entity in 1817 by James Parkinson in his famous paper entitled '*An essay on the shaking palsy* '(Parkinson, 1817). However, there had been earlier reports of individuals with abnormal

movements or gait most notably by Francois Boissier de Sauvages de la Croix who had described patients "who had difficulty walking, moving with short and hasty steps" as suffering from 'scleotyrbe festinans' and attributed their difficulty to diminished flexibility of muscle fibres (Sauvages de la Croix, 1763). Shakespeare, too, may also have observed individuals with PD and in his play, *Henry VI*, the character, Dick notices that Say is shaking and asks "Why dost thou quiver, man" and Say replies "The palsy and not fear provokes me" (Finger, 1994).

In the latter half of the nineteenth century, Jean-Martin Charcot at the Salpetriere hospital in Paris studied PD and was the first to separate PD into discrete clinical symptoms most notably separating bradykinesia from rigidity and emphasising that muscle strength was well preserved in PD (Charcot, 1869). He was also the first to recognise that micrographia was a feature of PD and used it as part of his diagnostic assessment. Remarkably, Charcot also recognised the therapeutic benefits of belladonna alkaloids (that has anticholinergic properties) and rye-based products (that contain ergot and are contained in some modern dopamine agonist treatments) prior to the recognition that PD was due to dopaminergic/cholinergic imbalance. At Queen Square, William Gowers described PD cases in detail in his classic textbook, *"A Manual of Diseases of the Nervous System"* and of particular relevance to this thesis, was the first to suggest a possible genetic predisposition to the development of PD, reporting that 15% of patients had a family history of the disease (Gowers 1886).

1.2.2 Definition and Differential Diagnoses

Although medical and non-medical figures through the ages have identified

individuals with PD, the clinical diagnosis of PD remains a difficult and controversial area. In view of this many clinical neurologists prefer to regard PD as part of a syndrome termed Parkinsonism characterized by the presence of some or all of the following namely: tremor, bradykinesia, rigidity and postural instability.

In the pre-genetic era, it was widely accepted that the gold standard method for confirming a diagnosis of PD was post-mortem pathological examination demonstrating dopaminergic cell loss with concomitant gliosis in the substantia nigra, Lewy body (LB) formation in the substantia nigra or locus coeruleus and no pathological evidence for other diseases that could cause Parkinsonism (Gelb et al., 1999). Consequently researchers used clinical criteria to define PD that could predict this pathological substrate comprising LBs. The most cited is the UK Parkinson's Disease Society Brain Bank clinical criteria which stipulates the presence of bradykinesia and at least one of the other three cardinal features (4-6 Hz rest tremor, rigidity or postural instability); the absence of certain exclusion criteria (e.g. supranuclear gaze palsy); and the presence of three or more supportive criteria (e.g. unilateral onset) (Hughes et al., 2001). However, the discovery of genes causing Mendelian-inherited forms of PD and the post mortem findings in affected brains has challenged the central tenets of the pathological criteria for PD (discussed later).

1.2.3 Epidemiology of PD

There have been numerous studies reporting the incidence (number of new cases per year) of PD in a diverse range of populations with widely varying figures (Mayeux, 2003). However, studies have been impaired by the issues of accurate diagnosis (as stated above) and also by the often late recognition and diagnosis of new-onset cases with initially very mild symptoms. Therefore the best incidence

studies have been those conducted over a long period of time within the same community. Mayo Clinic researchers have serially recorded the incidence of Parkinsonism in the Olmstead County near Rochester, Minnesota from 1945 up to 1990 (Bower et al., 1999). The latest study showed an overall incidence of 25.6 per 10,000 with a clear age-dependent increase with incidences of 0.8 per 10,000 (0-29 years); 25.6 per 10,000 (50-59 years); and 304.8 per 10,000 (80-99 years) (Bower et al., 1999). There have also been many prevalence studies (number of cases at a given time) and these provide varying estimates of PD that are often related to study design and case ascertainment (Mayeux, 2003). Nevertheless, these also confirm the effect of age on PD risk with a Dutch study reporting a prevalence rate of 1.4% in those aged 55-64 years and 4.3% in those between 85-95 years (de Rijk et al., 1995).

As well as highlighting the importance of age as a major risk factor for PD development, epidemiological studies have also attempted to uncover other clues to the aetiology of PD. Again studies have often been blighted by misdiagnosis of PD cases as well as biases relating to recall of exposure history. Studies on the effect of gender have been conflicting and overall there may be a slight male preponderance of PD (Bower et al., 1999). Similarly the effects of race and ethnicity have been controversial and whilst race appears to be of importance in certain rare genetic forms of PD, it does not appear to alter risk for sporadic PD (Kessler, 1972). Reversible life-style factors have also been studied and whilst smoking has been shown to be protective against the development of PD, the evidence is conflicting (Grandinetti et al., 1994; Mayeux et al., 1994). The evidence for alcohol and caffeine is also weak (Schoenberg et al., 1988) Perhaps the most reproducible risk factor to be established is exposure to an environmental toxin since several studies have

reported a higher risk of PD in those who have lived in rural communities from an early age in Europe or North America. Possible candidates have included pesticides that were heavily used by these communities after the second world war (Lai et al., 2002).

1.2.4 Clinicopathological features of PD

The average age at onset of PD lies between 60 and 70, however, rare familial forms of PD can present below the age of 45 years and have been termed autosomal recessive juvenile parkinsonism (ARJP). In addition to the central symptoms described earlier, a wide variety of other motor symptoms have been variably reported in PD patients often as a consequence of one of the cardinal symptoms of PD e.g. dysphagia and sialorrheoa result from orofacial bradykinesia and rigidity (Sethi, 2002). With progressive PD, there is degeneration of neurons and structures outside the substantia nigra (e.g. cholinergic nucleus basalis of Meynert) and this results in a variety of non-motor symptoms in PD including subcortical dementia (Thanvi et al., 2003). PD is a progressive disorder and the average lifespan is approximately 20 years from symptom onset in those with sporadic disease with onset in their 60 (Sethi, 2002). The mainstay of treatment of PD remains Levo-dopa therapy although there have been some recent concerns about its long-term effects (Muller et al., 2004; Mercuri and Bernardi, 2005).

The typical macroscopic findings of PD brain are loss of neuromelanin pigment from the substantia nigra and locus coeruleus. Microscopically there is substantial loss of dopaminergic neurons from the substantia nigra pars compacta (p.c.) (especially the ventrolateral tier) and reactive proliferation of astrocytes and microglia. There is also evidence of a small proportion of neurons undergoing

phagocytosis by macrophages (termed neuronophagia) although to date this has been little studied. Although the p.c. neurons send projections to the striatum, the striatum is microscopically normal. A diverse range of other neurons are vulnerable in PD and the progressive involvement of these structures has been incorporated into a recent new pathological staging system for PD that controversially posits that the neurodegenerative process first affects the dorsal motor nucleus of the vagus in the medulla prior to dopaminergic cell loss (Table 1.1) (Braak et al., 2003). The other major microscopic feature are eosinophilic cytoplasmic inclusions known as Lewy bodies (LBs) that are found in surviving neurons of the p.c. but are also found in the locus coeruleus, dorsal motor nucleus of the vagus and the forebrain notably the nucleus basalis of Meynert (Braak et al., 2003). LBs are fibrillar structures with a dense core surrounded by a halo (Figure 1.1) and are often associated with abnormal neuritic profiles known as Lewy neurites. The composition of LBs has been inferred from immunohistochemical studies and includes neurofilament, ubiquitin and α-synuclein and this will be discussed in more depth in Chapter 3. Whilst many believe that the presence of LBs is necessary in the diagnosis of PD, the neuropathological analysis of genetic forms of PD suggest that it may not indicate a unifying aetiology for the condition.

1.3 GENETICS OF PARKINSON'S DISEASE

1.3.1 Overview

At the end of the 19th century, Gowers had reported that fifteen percent of PD cases had a positive family history and had suggested that this might be an important

Stage 1 $N = 21$; medulla oblongata	Lesions in the dorsal IX/X motor nucleus and/or intermediate reticular zone
Stage 2 $N = 13$; medulla oblongata and pontine tegmentum	Pathology of stage 1 plus lesions in caudal raphe nuclei, gigantocellular reticular nucleus, and coeruleus–subcoeruleus complex
Stage 3 $N = 24$; midbrain	Pathology of stage 2 plus midbrain lesions, in particular in the pars compacta of the substantia nigra
Stage 4 $N = 24$; basal prosencephalon and mesocortex	Pathology of stage 3 plus prosencephalic lesions. Cortical involvement is confined to the temporal mesocortex (transentorhinal region) and allocortex (CA2-plexus). The neocortex is unaffected
Stage 5 $N = 17$; neocortex	Pathology of stage 4 plus lesions in high order sensory association areas of the neocortex and prefrontal neocortex
Stage 6 $N = 11$; neocortex	Pathology of stage 5 plus lesions in first order sensory association areas of the neocortex and premotor areas, occasionally mild changes in primary sensory areas and the primary motor field

Table 1.1 Pathological Staging system for Parkinson's disease.

Modified from Braak et al. (2003).

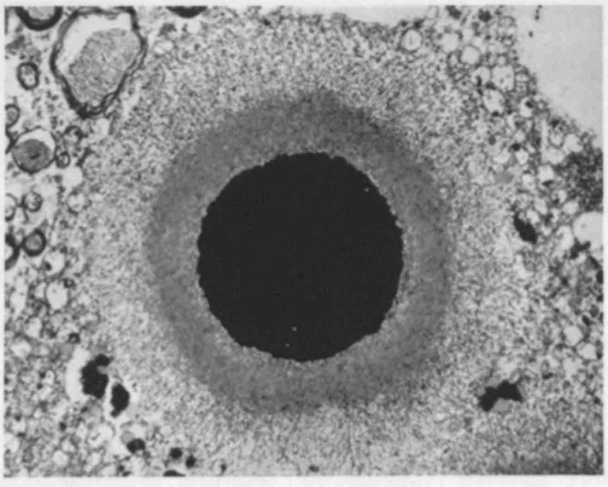


Figure 1.1 Electron micrograph of Lewy body from Parkinson's disease brain. The Lewy body is composed of a dense fibrillar core surrounded by a halo.

Courtesy of Queen Square Brain Bank for Neurological Disorders.

factor in the development of PD (Gowers, 1886). However, for much of the rest of the century, genetic studies played only a token role in exploring the mechanisms underlying PD risk. There were several reasons for this. The occurrence of post-encephalitic parkinsonism following the influenza pandemic of 1918 and the confusion of this syndrome with PD pushed many researchers into looking for a viral aetiology for the more common form (Poszanker and Schwab, 1961). In addition studies by epidemiologists who were the major PD researchers at the time strongly pointed to an environmental agent and the discovery that the accidental exposure to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) caused Parkinsonism in humans lent justification to these efforts (Langston et al., 1983). In contrast a raft of epidemiologiocal studies failed to detect an increased risk of PD amongst family members of affected patients and the reasons for this will be discussed later in section 1.3.2.

Furthermore, the early twin studies had failed to show increased concordance in monozygotic twins versus dizygotic twins, further supporting the role of the environment, although these studies did not take into account the natural history of PD and this will be discussed section 1.3.3. Although rare Mendelian inherited pedigrees of PD were reported as early as 1949 (Mjones, 1949), many thought these a separate clinical entity from idiopathic PD which discouraged researchers from investigating them further as it was thought these forms would not shed any mechanistic light on the commoner sporadic PD.

Only when a growing number of studies showed that some of these pedigrees had a similar pathological substrate (featuring LBs) to sporadic PD, did attention begin to refocus on genetic factors in the early 1990s (Waters and Miller, 1994). Researchers tackled the problem in four ways: (1) well designed family or case control studies to determine the frequency with which PD was associated with a

family history; (2) twin studies that utilized new imaging techniques like PET to determine the contribution of genetics and environment on familial risk; (3) gene association studies; and (4) linkage analysis to map gene susceptibility loci in families with highly penetrant disease with multiple affected members

1.3.2 Family Studies

Previously such studies relied on self-reporting and self-administered questionnaires to assess family history despite the fact that the diagnosis of PD can be difficult as explained in section 1.2.1. Improved methodologies incorporating individual examination of affected relatives or verification of the diagnosis in relatives via medical records or post-mortem findings have helped to improve recent studies. Increased follow-up of relatives of both cases and controls have also allowed more relatives to reach the more likely risk ages when symptoms present thereby increasing sensitivity. Consequently a flurry of epidemiological studies incorporating different methodologies and study populations appeared during the 1990s that all confirmed a familial contribution to PD development and these have been reviewed elsewhere (Foltynie et al., 2002). These studies have found a familial risk ranging from 1.3 – 9.7%. However, this risk is still lower than that predicted from Mendelian models. This might reflect that the studies despite improvements continue to be limited by methodological factors e.g. most studies did not examine all the unaffected relatives and there may be an underestimate arising from those with mild PD who escape detection or reporting. Alternatively, Western populations are typically outbred with small generational numbers and thus recessively-inherited conditions can often present without a family history. Another possibility is that the

familial risk in non-Mendelian PD is polygenic and the contributing effects of each of the genes are very small leading overall to a modest lifetime risk.

1.3.3 Twin Studies

Twin studies traditionally have been a powerful tool to assess the heritability of any disease. Monozygotic twins are genetically identical and so will be concordant for a genetically determined characteristic or disease. Dizygotic twins share on average half their genes (as with any pair of siblings) and therefore a genetic characteristic or disease should show a higher degree of concordance in monozygotic compared to dizygotic twins. Traditionally designed twin found similar concordance rates of PD in monzygotic and dizygotic twins arguing against the role of genetic involvement and suggesting that environment was more relevant (Eldridge and Ince, 1984). Tanner and colleagues (1999) found a significantly higher concordance rate in monozygotic twins only for those with PD onset prior to 50 years of age, suggesting that early onset PD is genetically determined but not the commoner late onset form.

However, none of these studies considered the concept of pre-clinical PD in which individuals have a significant loss of dopamine-containing neurons which is not yet severe enough to produce symptoms. The only way to record the development of these individuals was either to have a very long follow-up period or else develop a way to detect these subjects before they present. Positron emission tomography (PET) studies of the brain using the radiolabelled tracer [^{18}F]Dopa is a reliable way to measure dopaminergic neuron loss *in vivo* and can be used to detect pre-clinical PD (as there is a reduction of tracer uptake in the striatum of these individuals). Piccini and colleagues have obtained and utilized PET data in their studies and showed

significantly higher concordance rates in monozygotic compared to dizygotic twins (70% vs 22.2%) for dopaminergic dysfunction (Piccini et al., 1999). In their study the index twins with sporadic PD did not appear to have family histories by clinical criteria. Moreover, the same study showed that concordance rates increased with age. Sporadic PD is usually a disease of late onset therefore if the index twin developed sporadic PD at a young age then the co-twin should be followed up for possibly decades after this age of onset to be certain of the presence or absence of pre-clinical or symptomatic PD. This was the problem with earlier twin studies that were cross-sectional and did not follow up co-twins for the development of sporadic PD. Thus PD cases might have been missed amongst their monozygotic cohort which would lower the apparent concordance rate observed. Therefore PET twin study data with long term follow-up is probably the best approach and has finally validated the considerable heritability that is evident for PD. The differences in age at onset in monozygotic twins also suggests that whilst genes determine whether one *will* develop PD, environmental factors are likely to influence *when* that will occur.

1.3.4 Association Studies

The absence of family history in patients with sporadic PD suggests that the genetic contribution is likely to be polygenic, that is the phenotype is likely to result from the simultaneous action of multiple gene loci. These genetic effects are likely to form a complex interplay with environmental factors. The search for candidate susceptibility genes in sporadic PD has been directed by existing knowledge of the pathogenesis of sporadic PD and other neurodegenerative disorders such as Alzheimer's disease. As in Alzheimer's disease, the E4 polymorphism of the apolipoprotein E (Apo E4) gene

has been shown to reduce the age at onset of PD. Combination of the Apo E4 allele with a specific allele of the α-synuclein gene (see later) appears to confer increased risk for sporadic PD (Kruger et al. 1999). Very rarely PD may develop before the age of 20 years. Mutations in the parkin gene appear to be extremely frequent in this group strongly suggesting an underlying genetic predisposition (Lucking et al. 2000). Evidence of involvement of other such genes including the monoamine oxidase A and B genes has been conflicting (Spacey & Wood. 1999). Mitochondrial dysfunction is also implicated in the pathogenesis of PD. However, there is no consistent evidence for increased frequency of polymorphisms in mitochondrial genes in sporadic PD compared to controls (Bandmann et al. 1997). New advances in the methodology of conducting association studies involving linkage disequilibrium mapping and the use of tagging single nucleotide polymorphism (SNP) approaches should ensure greater reproducibility in the identification of common genetic variants that predispose to the development of PD. There have been several recent excellent reviews of this technique (Goldstein et al., 2003; Gandhi et al., 2005).

1.3.5 Mendelian PD

1.3.5.1 Background

The role of genes in PD pathogenesis was considered relatively important but perhaps involving interplay with environmental factors. However, there were a number of rare families around the world in which PD was inherited in an unambiguously Mendelian fashion. In these families linkage analysis and positional

cloning methods were employed to map the genes.

In 1997 Polymeropoulos and colleagues achieved the breakthrough and identified a missense mutation (A53T) in the *α-synuclein* gene in a large Italian-American family, the Contursi kindred, and three unrelated families of Greek descent (Polymeropoulos et al., 1997). The mutation segregated with PD in an autosomal dominant manner and the families all shared a common haplotype suggesting a common founder effect. The report provided a catalyst for geneticists worldwide to find more Mendelian inherited PD genes. Less than 10 years later, there is strong genetic evidence to support the role of five Mendelian PD genes (*α-synuclein*, *parkin*, *DJ-1*, *PINK1*, and *LRRK2*); less strong evidence to support four other genes (*UCH-L1*, *synphilin-1*, *NR4A2* and *Omi/Htr2A*) and there is linkage data to support the existence of three other genes (*PARK3*, *PARK10*, and *PARK11*) (Table 1.2).

PARK9 or Kufor-Rakeb syndrome was also defined as a PD locus, however it is a complex autosomal recessive early-onset neurodegenerative syndrome with many additional clinical features to Parkinsonism including spasticity, dementia and supranuclear gaze palsy (Williams et al., 2005). Both *PARK10* and *PARK11* were identified through genome-wide linkage studies of many cases with typical features of PD. In addition, molecular genetic analysis has also identified four other genes in which mutations can cause clinical syndromes resembling PD namely: frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), X-linked dystonia parkinsonism (XDP) and dominantly inherited spinocerebellar ataxias 2 (SCA-2) especially in patients of Asian origin) and 3 (SCA-3, especially in patients of African origin). I will now discuss each of the Mendelian inherited PD genes in turn.

PARK1	4q21	*α-synuclein*	AD	Italian-Greek	A53T	early-onset typical PD with LBs; high penetrance
				German	A30P	usual age at onset; reduced Penetrance; no pathology
				Spanish	E46K	usual age at onset, PD and Lewy Body dementia; no pathology
				Nth. America	4copies	early-onset, PD and Diffuse Lewy body dementia (DLBD); Variable pathology: PD with LBs; DLBD; and glial α-syn inclusions
PARK2	6q25-27	*parkin*	AR	Japanese	*Various	early-onset, slowly progressive, L-dopa sensitive, hyperreflexia, dystonia, dementia rare, no LBs
				Worldwide	*Various	early and late onset PD, role for heterozygote mutants
PARK3	2p13	UNKNOWN	AD	German	Usual age at onset and PD pathology with LBs	
PARK4	4q21	*α-synuclein*	AD	Incorrect linkage – found to be α-synuclein triplication (above)		
PARK5	4p14	*UCH-L1*	AD	German	I93M	Single family – awaits genetic confirmation
PARK6	1p35-36	*PINK1*	AR	Worldwide	Missense	Early onset and usual age at onset, typical PD, No pathology
PARK7	1p36	*DJ-1*	AR	Worldwide	*Various	Early onset, slow progression, dystonia, similar to parkin
PARK8	12p11	*LRRK2*	AD	Worldwide	Missense	Typical PD, Marked variation in pathology
PARK9	withdrawn as not similar to PD: Described as Kufor-Rakeb syndrome in one consanguineous Jordanian family with AR inherited parkinsonism, spasticity, dementia, and supranuclear palsy, partial L-dopa response, no pathology					
PARK10	1p32	UNKNOWN	Genetic susceptibility locus determined by genome wide linkage study of 51 Icelandic families with 1 or more family members with typical PD.			
PARK11	2q	UNKNOWN	Genetic susceptibility locus by genome linkage in multiplex US PD families			

Synphilin-1, NR4A2, Nurr1, Omi/Htr2A have also been implicated in PD

SCA-2, SCA-3, FTDP-17, DYT3 are all neurodegenerative conditions that may present with features of parkinsonism.

Table 1.2 Genetic classification of Parkinson's disease.

1.3.5.2 α-synuclein

1.3.5.2.1 PD causing mutants of α-synuclein

Dominant mutations in the α-synuclein gene were reported in 1997. They identified a G>A missense mutation at position 209 resulting in an alanine to threonine substitution at position 53 (A53T) of the α-synuclein protein (Polymeropoulos et al., 1997). This mutation was later found to be a very rare cause of familial PD. For example Vaughan et al. (1998) found that none of 230 European patients with familial PD had the G209A mutation. However, the importance of this discovery to understanding the pathogenesis of sporadic PD was shortly revealed when Goedert's lab found that α-synuclein was the major component of LBs in both sporadic and familial PD (Spillantini et al., 1997). This finding consolidated the notion that determining the genes and understanding the mechanisms of their action in the rare Mendelian forms would simultaneously shed significantly more light on the pathogenesis of the far commoner sporadic form of PD.

In addition to the A53T mutation, the A30P mutation was subsequently identified in one German family (Kruger et al., 1998). Clinically the A53T affecteds had slightly younger age at onset, less prominent tremor and more severe disease than sporadic PD (Polymeropoulos et al., 1997). Dementia and central hypoventilation has also been reported in these families (Spira et al., 2001). In contrast the A30P affecteds more closely resembled sporadic PD. Recently an E46K missense mutation was identified in a Spanish family, which had an unusual phenotype with more resemblance to diffuse Lewy body disease than sporadic PD with dementia and hallucinations being prominent (Zarranz et al., 2004).

A triplication of the wild type α-synuclein gene was found in PD affecteds from the Iowa kindred following erroneous linkage to 4p (Singleton et al., 2003). Additional families have been reported with duplications in α-synuclein (Ibanez et al., 2004). Neuropathological examination of mutant α-synuclein brains reveals LBs and nigral cell loss consistent with sporadic PD however the changes are not limited to the nigra and are more severe and extensive. Notably in the E46K brains there was widespread cortical and subcortical LBs presumably underlying the dementia (Zarranz et al., 2004). Similarly in the triplication brains there was widespread damage and unusually glial α-synuclein inclusions seen normally in multiple system atrophy (Cookson, 2004). This follows on from an early report that observed tau inclusions in the A53T brains as well as LBs (Polymeropoulos et al., 1997; Duda et al., 2002). Overall, there is compelling genetic data to support a casual role for α-synuclein mutations in PD however, the phenotype appears to be particularly severe especially when compared pathologically to sporadic PD.

The autosomal dominant inheritance and causal role of genomic multiplications of α-synuclein suggest the mutations may involve a toxic gain of function. However, further understanding of mutant mechanisms requires knowledge of the endogenous function of the α-synuclein protein. α-synuclein was first cloned and described in the synapses of the electric eel, *Torpedo californica*, where it was given its name due to erroneous localization in nuclei (Maroteaux et al., 1988). A role in synaptic function was further postulated by its upregulation in zebrafinch following song learning (George et al., 1995). Interestingly there is no orthologue in *Drosophila melanogaster* or *Caenorhabditis elegans* and in rodents there is a threonine at residue 53 of wild type α-synuclein. The protein consists of 140 aa and

is natively unfolded adopting no secondary or tertiary structure. It comprises a domain near the N-terminus consisting of seven imperfect sequence repeats of KTKEGV, a central hydrophobic domain and a highly acidic C-terminal domain containing several putative phosphorylation sites as well as a calpain I cleavage site. The protein belongs to a family including β and γ-synuclein and synoretin that are characterized by the KTKEGV repeats (George, 2002). The function of α-synuclein remains unknown. Knock out of α-synuclein in mice causes deficits in synaptic function with reports of impaired dopamine release from the striatum in response to repetitive stimulation (that depletes docked and reserve pools of vesicles) and reduced paired-pulse facilitation in the hippocampus (Abeliovich et al., 2000; Cabin et al., 2002). Importantly knock-out does not cause neurodegeneration and Parkinsonism consistent with the proposed gain of function mechanism of α-synuclein mutations.

Further clues about the role of α-synuclein in synaptic function have come from membrane localization studies. α-synuclein binds to lipid membranes particularly the plasma membrane in mammalian cells and yeast where it undergoes conformational change to form an amphipathic α-helix (Outeiro and Lindquist, 2003; Jao et al., 2004). This form of α-synuclein has been suggested to regulate the activity of phospholipase D which may be critical for synaptic vesicle release (Payton et al., 2004). Furthermore, it has been proposed that an equilibrium exists between membrane associated α-synuclein and cytosolic α-synuclein (Outeiro and Lindquist, 2003). Therefore, disturbance of this equilibrium is likely to lead to a dual deleterious effect with consequences for both the cytosol as well as synaptic function. It has been reported that the A30P but not the A53T mutation prevents α-synuclein binding

to vesicles in the rat resulting in A30P α-synuclein in the cytosol (Jensen et al., 1998).

Pathological studies have been very informative regarding the possible mechanism of toxicity of α-synuclein. As well as PD, α-synuclein is a major component of LBs in diffuse Lewy body dementia. Ultrastructural studies have revealed that LBs consist of aggregated protein known as fibrils and that α-synuclein is closely associated with these (Crowther et al., 2000). α-synuclein is also detectable in oligodendroglial intracytoplasmic inclusions of the related neurodegenerative disorder, multiple system atrophy (MSA) (Tu et al., 1998). Prior to the gene discovery α-synuclein had been detected in amyloid plaques of Alzheimer's disease brain where it was designated non-amyloid component precursor (NACP) (Ueda et al., 1993). Furthermore, α-synuclein is present in nearly all LBs suggesting that it may be necessary for LB formation. These *in vivo* findings of α-synuclein aggregation lent support to the hypothesis that α-synuclein mutations are toxic gain of function mutations and that the central biochemical pathway leading to neurodegeneration is α-synuclein accumulation and aggregation.

1.3.5.2.2 α-synuclein and aggregation

In vitro functional analysis of α-synuclein has revealed that under certain conditions α-synuclein can autonomously form LB-like fibrillar β-sheets in a time-, temperature-, pH-, and concentration-dependent manner (Giasson et al., 1999; Conway et al., 1998). Moreover the A53T mutant appears to self-aggregate to form fibrils more rapidly than wild type or A30P α-synuclein and interestingly the latter

appears to inhibit fibril formation (Conway et al., 1998; El-Agnaf et al., 1998). In addition to mutations oxidative stress has also been shown to increase α-Syn aggregation *in vitro* (Hashimoto et al., 1999; El Agnaf et al., 1998). However, the toxic role of fibrillar α-synuclein is controversial. Causality cannot be ascertained from observational pathological studies. However, while mechanistic studies from α-synuclein animal models suggest a toxic role, many cell culture studies have shown that α-synuclein can induce significant neuronal death and dysfunction without the formation of fibrillar α-synuclein species suggesting that α-synuclein fibrils are not necessary for α-synuclein toxicity.

In contrast to A30P, A53T and excess wild type α-synuclein (via triplication) all appear to promote the formation of oligomeric species or protofibrils *in vitro* that are the nonfibrillar precursors of fibrils. Protofibrils have been shown to possess pore-like properties and have been suggested to be the toxic species rather than fibrillar α-synuclein (Goldberg and Lansbury, 2000). The mechanism for this remains speculative but could involve the permeabilization of synaptic vesicles (via pore formation) resulting in dopamine release into the cytosol with consequent oxidative damage. Conversely dopamine quinones derived from oxidation of dopamine or L-Dopa can stabilize and increase α-synuclein protofibril formation whereas antioxidants enhanced the conversion of protofibrils to α-synuclein fibrils (Conway et al., 2001). The accumulation of potentially toxic protofibrils in the dopamine and oxidation rich environment of the substantia nigra could account for the selective cell loss seen on PD and also highlights the delicate relationship between dopamine and α-synuclein.

There is also evidence for protofibril formation in Parkinson's disease *in vivo*. Soluble protofibrils have been isolated from human PD post-mortem brain (Sharon et

al., 2003) and Miller and colleagues (2004) have shown that α-synuclein protofibrils are present in brains from patients with the α-synuclein triplication. In addition, Sharon et al. (2003) recently reported that the formation of highly soluble oligomers of α-synuclein is regulated by fatty acids and is enhanced in synucleinopathic brains of PD and DLBD. The authors also demonstrated that polyunsaturated fatty acids increased α-synuclein oligomer levels in mesencephalic neuronal cells. Although the mechanism of this is not fully understood, analysis of the primary structure of α-synuclein does reveal the presence of binding motifs for fatty acids in both the N- and C-terminus and that α-synuclein can bind lipids such as oleic acid (Sharon et al., 2001). Furthermore, recent modifier screens in yeast found that several suppressors of α-synuclein toxicity belong are involved in lipid metabolism and vesicle transport (Willingham et al., 2003).

Despite many studies it still remains uncertain whether α-synuclein aggregation is both necessary and sufficient to cause neurotoxicity. The protein itself has a hydrophobic domain near the repeat region that has a propensity to aggregate (Giasson et al., 2001). It is of interest that all the known missense mutations cluster around the repeat region but data is still lacking on the effect of E46K mutation on aggregation. In contrast the C-terminal domain appears to inhibit aggregation of the protein and loss or modification of this part of the protein could also potentiate aggregation. As touched upon, external factors such as oxidative stress can influence α-synuclein aggregation and likely culprits in sporadic disease could include environmental exposure to pesticides or oxidant chemicals as well as metal toxins. However, association studies have also revealed that promoter polymorphisms of the α-synuclein gene may increase risk of PD. The pathogenetic mechanism of these polymorphisms may be similar to gene multiplications that result

in α-synuclein over-expression leading to dopaminergic dysfunction and PD (Farrer et al., 2001).

The definitive data to confirm whether α-synuclein aggregation is neurotoxic remains elusive. Knowing the answer to this question is critical since the effect of therapeutic strategies targeting aggregation would not be completely clear. Several studies have shown that inhibitory peptides of α-synuclein can abrogate both aggregation and toxicity (El-Agnaf et al., 2004). Recently Masliah and colleagues (2005) reported that transgenic wild type α-synuclein over-expressing mice developed highly specific antibodies in response to vaccination with recombinant human α-synuclein and that this was associated with reduced α-synuclein aggregates and neurodegeneration. Although this study is very encouraging from the point of view of therapeutics in PD, it still does not conclusively suggest that the α-synuclein aggregates are toxic particularly as in the model used, the mice have extensive α-synuclein inclusions and motor deficits but no evidence of dopaminergic neuronal loss (Masliah et al., 2000). In recent years there has been a flurry of α-synuclein models to try and address the pathogenic basis of mutations and this will be described in the next section.

1.3.5.2.3 α-synuclein models

If over-expression of wild type or mutant α-synuclein is deleterious and causal of PD then the prediction would be that transgenic mutant animals should recapitulate many of the features of PD. The model that most fulfils this prediction is *Drosophila melanogaster* (Feany and Bender, 2000). Using a bipartite expression system requiring transcriptional activation of the transgene by the yeast protein, GAL-4, flies with wild type or mutant α-synuclein downstream of a GAL-4 responsive element

were crossed with flies expressing GAL-4 driven by a tissue or cell specific promoter. Flies expressing wild type, A53T, and A30P α-synuclein developed age-dependent selective loss of dopaminergic neurons, α-synuclein positive fibrillar LB-like inclusions, motor deficits (impaired vial climbing) and premature death. This model therefore captured all the essential features of PD and also provided a demonstration that excess wild type α-synuclein was neurotoxic which predated the discovery of the triplication. However, the A30P mutant exhibited a more severe phenotype than wild type or A53T flies including the formation of fibrillar inclusions (Feany and Bender, 2000). This is not consistent with the *in vitro* data and subsequent transgenic mice studies (see below) and highlights that there may be subtle but important differences in cellular pathways between invertebrates and mammals but it also suggests that fibril formation is important for mediating neurotoxicity.

Transgenic mice models have recapitulated some but not all the central features of PD and crucially have all failed to exhibit significant and selective loss of dopaminergic neurons. Masliah et al. (2000) used a platelet-derived growth factor (PDGF) promoter to express human wild type α-synuclein in mice and observed dopaminergic terminal loss, non-fibrillar α-synuclein and ubiquitin positive inclusions and motor deficits at one year of age (Masliah et al., 2000). However, there was no neuronal cell loss and some of the inclusions were nuclear as well as cytoplasmic. Transgenic mice expressing the A53T mutation have had various phenotypes reported depending on the promoter used. Widespread non-fibrillar inclusions were seen including spinal cord changes in one model that used the non-nigral Thy-1 promoter and was associated with prominent motor abnormailities at 3 weeks. Other A53T models using the prion promoter (which is expressed in the nigra) found severe motor deficits, widespread α-synuclein inclusions and striking spinal cord

pathology including fibrils and axonal degeneration. However, no abnormality of dopaminergic neurons was seen. Interestingly in this study, parallel transgenic mice expressing wild type α-synuclein under the same promoter appeared normal with normal histology (Giasson et al., 2002). In another study, A53T expression again caused a severe phenotype with motor paralysis and premature death associated with α-synuclein inclusions that developed prior to the motor symptoms. Again there was no abnormal pathology in the nigra and striatal dopamine levels were normal. Surprisingly A30P mice and wild-type mice both did not show any evidence of neurological dysfunction and did not have evidence of inclusions (Lee et al., 2002). Although the A30P levels were increased, there were no ubiquitylated inclusions. Although the finding that A53T is more severe than A30P matches well with that seen in patients with these mutations, the complete lack of abnormality with A30P is unclear particularly as A53T is actually a normal sequence variant in rodents whereas A30P is a very non conservative change in the sequence.

Currently it is unclear why unlike *Drosophila*, expression of α-synuclein in mice does not reproduce more features of PD. The studies raise more interesting questions than answers. The variation in phenotypes published may be attributable to strain differences in mice used as well as different promoters. It may be that constitutive expression of wild type or A53T α-synuclein is not sufficient to cause dopaminergic degeneration and that it requires important cellular co-factors that are either absent or not expressed at sufficiently high levels in rodent striatum. Alternatively there may be a requirement for an environmental factor or trigger such as oxidative stressors. Nevertheless these studies do largely show that transgenic over-expression of α-synuclein can cause neurological dysfunction and that α-

synuclein protofibrils may be causative. Perhaps transgenic models utilizing inducible time and tissue dependent expression systems may reveal more information.

Unlike standard transgenic technology, more success has been achieved by groups over-expressing α-synuclein using viral vectors. Both recombinant adeno-associated viruses (rAAV) and recombinant lentiviruses (rLV) have been used as they are safe, allow stable long term expression and cause minimal inflammation at the site of their injection and transduction. In rats, rAAV or rLV delivery of wild type, A53T and A30P α-synuclein causes progressive and severe loss of dopaminergic neurons associated with extensive LB-like inclusions and neurites that stain with α-synuclein; and motor impairments (Klein et al., 2002; Lo Bianco et al., 2002; Kirik et al., 2003). Interestingly in the study by Lo Bianco and colleagues (2002), injection of the same viruses into non-dopaminergic neurons did not cause cell loss or inclusion formation suggesting that nigral dopamine neurons are selectively vulnerable to the pathogenic effects of over-expressed α-synuclein. Kirik and Bjorkland (2003) have also shown that the same viruses can also cause nigrostriatal degeneration (40-75% reduction of tyrosine hydroxylase positive neurons), inclusions and motor impairments sixteen weeks after injection. Whilst these studies in principle show that α-synuclein expression can cause nigral degeneration, one caveat is that these viral studies cannot control for copy number in individual neurons of injected regions and that there may be high copy number as well as variation in copy number between neighbouring neurons. Furthermore, as previously stated α-synuclein mutation related disease is particularly severe and extensive, and viral studies which can only involve injection of one, or a few regions, will not be able to recapitulate this unlike transgenic approaches.

1.3.5.3 Parkin

1.3.5.3.1 Parkin and genetic analysis

Homozygous mutations in the parkin gene were discovered in families with autosomal recessive PD (ARPD) (Kitada et al., 1998). In contrast to α-synuclein, parkin mutations are relatively common accounting for almost half of all cases of ARPD especially in cases with onset before 21 years (Abbas et al., 1999; Lucking et al., 2000). The 465 aa protein contains two RING (Really Interesting New Gene) fingers separated by an in-between RING domain (IBR) at the C-terminus and this domain functions as an E3 ubiquitin ligase in common with other RING finger proteins (Figure 1.2) (Shimura et al., 2000). The N-terminus bears a ubiquitin like (Ubl) domain that binds to the Rpn10 subunit of the 26S proteasome (Figure 1.3) (Sakata et al., 2003). In the cell, E3 ubiquitin ligases are one component of the ubiquitin proteasome system (UPS), a major cellular pathway that promotes removal of damaged or misfolded proteins (Ciechanover, 1998). E3 ligases catalyze the addition of ubiqutin (ub) molecules to lysine residues of damaged target proteins and the presence of a polyubiquitin chain provides a signal for its removal and degradation by the proteolytic complex, the 26S proteasome (Ciechanover, 1998).

A striking variety of homozygous and compound heterozygous parkin mutations have been reported including gene rearrangements and missense mutations (Abbas et al., 1999; Lucking et al., 2000). The missense mutations are predominantly found within the RING1 domain of the C terminus of parkin and most of these abrogate E3 ligase activity (von Coelln et al., 2004). However, mutations do occur in other parts of the protein e.g. an R42P missense mutation in the Ubl domain impairs parkin

binding to the proteasome. The general mechanism for parkin mutations is thought to be loss of function (lof) and is supported by pathological studies that show absent parkin expression in homozygous brains associated with dopaminergic cell loss in the substantia nigra and locus coeruleus (Shimura et al., 1999). Despite very few post-mortem studies in parkin related ARPD, it is becoming clearer that the nature of the parkin mutation is critical in interpreting the pathology and that mutations that abolish parkin activity appear to be associated with a lack of LBs but in mutations which reduce but do not abolish activity, LBs can occur (Farrer et al., 2001).

1.3.5.3.2 Parkin and its role of mitochondrial function

The lack of LBs in ARPD patients led some to postulate that parkin-ARPD was a distinct clinical syndrome from sporadic PD, however, recent work suggests commonality between parkin-ARPD and sporadic PD. The mitochondrial electron transport chain enzyme complex, complex I, is selectively reduced in parkin-ARPD cases (Muftuoglu et al., 2004) and parkin is generally found in LBs of sporadic and familial PD (Shimura et al., 1999; Schlossmacher et al., 2002; Bandopadhay et al., 2005). A Drosophila model has revealed a role for parkin in maintaining mitochondrial function and preventing oxidative stress, pathways strongly implicated in sporadic PD. Parkin null mutants displayed severe mitochondrial pathology associated with reduced lifespan, apoptosis, flight muscle degeneration, male sterility but no evidence of dopaminergic neuronal loss (Greene et al., 2003). Gene expression microarray analysis in these flies revealed an up-regulation of genes involved in oxidative stress and electron transport including a homologue of the mammalian peripheral benzodiazepine receptor. A genomic screen for modifiers of lifespan in the parkin flies found the strongest modifier to be loss of function mutants

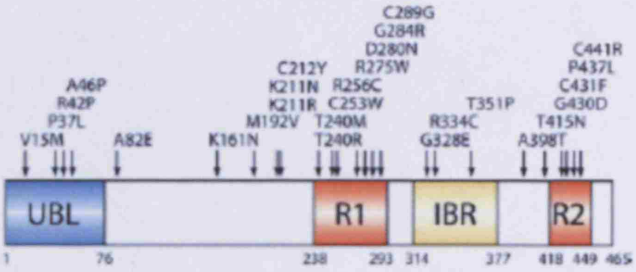


Figure 1.2 Schema of parkin structure with missense mutations associated with AR-JP. The protein contains a ub like domain (UBL), a central lonker region of unknown function; and a C-terminal domain comprising two RING domains (R1 and R2) separated by an in-between-RING (IBR) domain.

Modified from von Coelln et al. (2004).

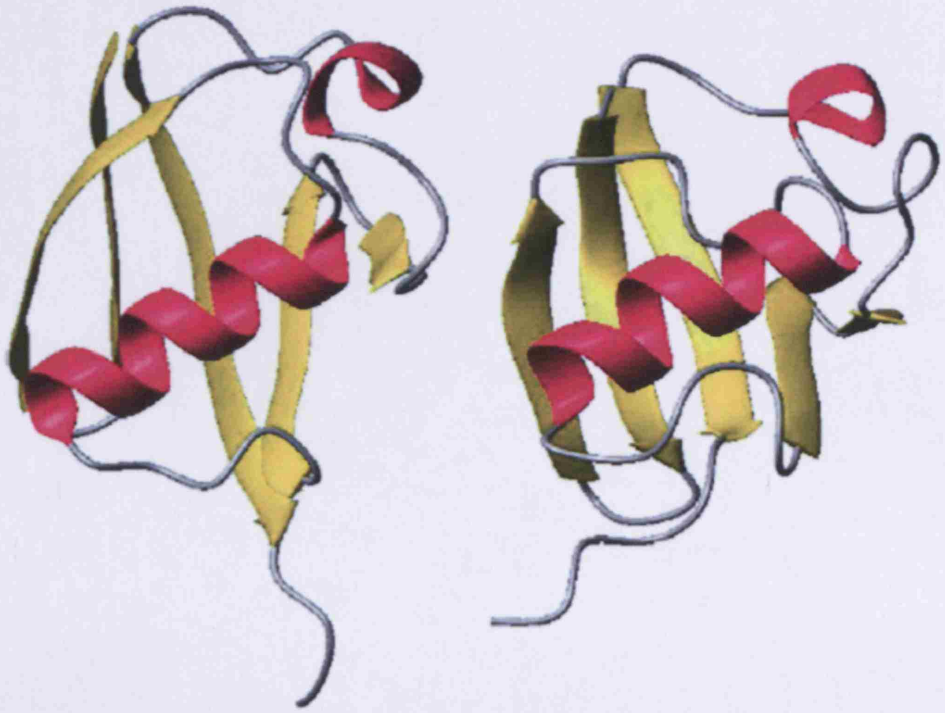


Figure 1.3 3D structure of ubiquitin (left) and the ub-like domain of parkin (right). α-helices in red and β-helices in yellow.

Modified from Tanaka et al. (2004).

of glutathione S-transferase (GstS1) (Greene et al., 2005). A reanalysis of the parkin null mutant flies revealed progressive degeneration of a select cluster of dopaminergic neurons and evidence of increased oxidative damage with increased protein carbonyls compared to controls (Whitworth et al., 2005). Furthermore, neurodegeneration was enhanced by null mutants of GstS1 and conversely over-expression of GstS1 significantly rescued the parkin phenotype (Whitworth et al., 2005).

Mammalian models also support a role for parkin in maintaining mitochondrial function. Deletion of exon 3 of parkin in mice results in nigrostriatal dysfunction and reduced expression of several proteins involved in mitochondrial function and oxidative stress including subunits of complex I and IV. The mice also had decreased mitochondrial respiratory capacity and evidence of increased oxidative damage (Palacino et al., 2004). Intriguingly parkin knock out in these mice did not cause dopaminergic degeneration similar to other exon 3 or exon 2 deletion models (Goldberg et al., 2003, Itier et al., 2003; Perez & Palmiter, 2005). In contrast deletion of exon 7 resulted in selective degeneration of noradrenergic locus coeruleus neurons (as seen in ARPD) associated with a behavioural readout that was reduced noradrenergic dependent startle response (von Coelln et al., 2004). This study suggests that parkin may be essential for catecholaminergic neuronal survival however it remains unclear why parkin knock out is not sufficient to cause striatal dopaminergic cell loss.

Quaking mice mutants that have spontaneous loss of parkin and the co-transcribed gene, PARCG also do not develop dopaminergic degeneration (Lockhart et al., 2004). It will therefore be interesting to determine if neurodegeneration in these models require an environmental insult such as an oxidative stressor. Interplay between parkin and environmental factors is certainly plausible given the significant

range of age at onset of patients homozygous for parkin mutations although modifier genes may also play a role (Khan et al., 2003). Furthermore, parkin heterozygotes appear to be at increased risk of PD and have nigro-striatal dysfunction as visualised by PET (Khan et al., 2002). The reason for this currently remains unclear but given that the age at onset of these cases is higher it may reflect haploinsufficiency combined with an environmental component.

The mechanism of how parkin may influence mitochondrial function is unclear. Parkin may be directly involved in maintaining mitochondrial integrity and has previously been reported to localize to the outer mitochondrial membrane (OMM) where it played a critical role in preventing mitochondrial swelling and rupture secondary to ceramide toxicity (Darios et al., 2003). Furthermore, the Drosophila homologue of peripheral benzodiazepine receptor (PBR) was upregulated in parkin mutant flies and PBR is a component of the mitochondrial permeability transition pore (mPTP) where it plays a role in mPTP opening thereby regulating oxidative damage secondary to mitochondrial dysfunction and OMM rupture (Casellas et al., 2002). Currently it is unknown whether PBR is a substrate for parkin. Ubiquitylation also mediates the insertion of mitochondrial proteins into the OMM and parkin may also play a role in this (Zhaung and McCauley, 1989). However, whether the endogenous form of parkin localizes to the OMM is unknown and further studies are required to confirm mitochondrial localization of parkin. To date the majority of studies have localized parkin to other subcellular organelles most notably the endoplasmic reticulum (ER) where it has shown to have a neuroprotective role against ER stress (Takakashi et al., 2003).

It is equally likely that parkin could indirectly maintain mitochondrial function. Since free radical induced oxidative damage is a normal consequence of the electron transport chain, parkin may play a role in removing oxidatively damaged proteins

and mutant parkin may lead to the accumulation of such proteins that may lead to further oxidative stress and apoptosis. This mechanism would be most compatible with the known function of E3 ligases in the UPS. Moreover, the related E3 ubiquitin ligase, HOIL-1, has been shown to degrade its oxidised substrate, IRP2 (Yamanaka et al., 2003). Parkin expression is up-regulated following exposure to the complex I inhibitor MPP^+ in neuronal cells (Hyun et al., 2005). However, parkin function itself may be modified by oxidative stress: exposure to nitric oxide (NO) generates free radicals that modify cysteine residues in RING1 resulting in significant alteration of parkin's E3 ligase activity including inactivation (Chung et al., 2004; Yao et al., 2004). Furthermore, nitrosylated forms of parkin are detectable in both PD brain and brains from MPTP and rotenone treated animals suggesting that inactivation of parkin may be a critical step in the pathogenesis of sporadic PD (Chung et al., 2004; Yao et al., 2004).

1.3.5.3.3 Identification of parkin substrates

Ever since parkin was found to be an E3 ligase, it has been hypothesised that mutants of parkin or parkin dysfunction is likely to lead to the toxic accumulation of its substrate. E3 ligases confer specificity in the UPS by usually targeting one protein (Robinson and Ardley, 2004). Surprisingly many disparate substrates for parkin have been discovered with strong replicated evidence for the septin CDCrel-1 and Pael-R (Table 1.3) (von Coelln et al., 2004). Moreover, over-expression of CD-Crel-1 and Pael-R *in vivo* mediates dopaminergic neurodegeneration (Dong et al., 2003; Yang et al., 2003) and both also accumulate in brains of parkin ARPD patients (Imai et al., 2001; Choi et al., 2003). However, neither of these substrates accumulates in *Drosophila* or mammalian parkin knock out models and it is unknown if any of these

substrates is oxidatively modified following oxidative stress and can be appropriately degraded by parkin.

α-synuclein may unify the oxidative stress hypothesis and substrate accumulation hypothesis mediating parkin dysfunction. Parkin can protect against α-synuclein induced neurotoxicity in vitro and in vivo (Petrucelli et al., 2002; Yang et al., 2003). However, there is no replicated data that parkin interacts directly with α-synuclein. Parkin has been shown to interact with a glycosylated form of α-synuclein (sp22) (Shimura et al., 2002) however, it is unclear whether α-synuclein toxicity is mediated via sp22. The interaction of parkin and α-synuclein may be direct if parkin can degrade oxidatively modified forms of α-synuclein. Alternatively the interaction may be indirect and α-synuclein has been shown to inhibit the proteasome (although it is unclear how) whilst parkin can rescue neurons from α-synuclein induced proteasomal dysfunction (Petrucelli et al., 2002). What is becoming clear from numerous studies is that loss of parkin function may impact on multiple cellular pathways rendering dopaminergic neurons sensitive to neurotoxicity and death. While UPS dysfunction and mitochondrial dysfunction may be the main pathogenetic pathways, the discovery of a wide range of substrates implicates additional pathways. Future studies should illuminate these pathways and it will be of significant interest to see whether and how these additional pathways impact/converge on "the big two" of UPS and oxidative stress.

1.3.5.4 DJ-1

1.3.5.4.1 Molecular genetic analysis of DJ-1

Substrate	Proposed Function
CDCrel-1	Exocytosis (dopaminergic storage?)
CDCrel-2	
Pael receptor	Stress in endoplasmic reticulum
Sp22	Lewy body formation
Synphilin-1	Lewy body formation
Cyclin E	Apoptosis (kainate excitoxicity)
α/β tubulin	Microtubules (assembly dysfunction)
p38 subunit	aminoacyl-tRNA synthesis
Synaptogamin XI	Fusion or docking, synaptic functions

Parkin-interactors	Proposed Function
UbcH7, UbcH8, Ubc6/7	E2
Ubc4	
Actin filament	Morphology
CASK/Lin2	PDZ-containing scaffold protein
Cullin-1	Multiprotein ligase
γ-tubulin	Centrosome
Rpn10	Binding to proteasomal proteins

Table 1.3 Table of parkin substrates and interacting proteins.

Modified from Hattori and Mizuno (2004).

Mutations of *DJ-1* were identified in two consanguineous European families with early onset ARPD (Bonifati et al., 2003). In addition to the two familial mutations, a large 14 Kb deletion and a L166P missense, a number of pathogenic mutations have since been identified including homozygous and heterozygous missense mutations and exonic deletions. In terms of the overall mutation burden in PD, DJ-1 mutations appear to be quite rare. Less than 5% of PD patients have early onset (< 40 years) and DJ-1 mutations account for an estimated 1-2% of these early-onset PD cases (Abou-Sleiman et al., 2003).

The precise cellular distribution and subcellular localization of the protein has been hotly contested since it was first associated with PD. It is certain that DJ-1 is widely expressed both in brain and peripheral tissues. Beyond that reports are conflicting, cellular expression has been reported as both exclusively neuronal and glial, while subcellular localization has been reported as nuclear, cytoplasmic and mitochondrial (Rizzu et al., 2004; Bandopadhay et al., 2004; Junn et al., 2005; Zhang et al., 2005). The only study carried out on the endogenous protein to date has detected expression in both neurons and glia, although the latter was weak. Subcellular distribution of the endogenous protein is primarily cytoplasmic with a smaller pool of mitochondrial-associated protein (Zhang et al., 2005).

Structurally, DJ-1 is a member of the ThiJ/PfpI/DJ-1 superfamily and is highly conserved across species. It has limited homology to several prokaryotic proteins including heat shock protein chaperones and ThiJ/PfpI proteases (Cookson, 2005). DJ-1 was independently cloned by four groups, first as a novel oncogene capable of transforming NIH3T3 cells in cooperation with H-Ras (Nagabuko, et al. 1997) and then subsequently, as a major protein found at reduced levels in sperm following the exposure of rats to infertility inducing toxins (Klinefelter et al., 1997; Wagenfeld et al., 1998). Although DJ-1 restores the transcriptional activity of the androgen

receptor gene (AR) following repression by DJ-1 binding proteins, PIASxα and DJBP (Takahashi et al., 2001; Niki et al., 2003), it does not appear to be essential for the development or maintenance of male reproductive function as DJ-1 null mice reproduce normally. DJ-1 also acts as a regulatory subunit of an RNA-binding multi-protein complex which stabilizes mRNA in response to CAMP (Hod et al., 1999).

1.3.5.4.2 DJ-1 and neuronal cell death

Perhaps more relevant in terms of the pathogenesis of PD is its potential role in oxidative stress response either as a redox sensor or anti-oxidant protein (Mitsumoto and Nakagawa, 2001; Canet-Aviles et al., 2004). In cells exposed to an oxidative stress, such as paraquat or H_2O_2, DJ-1 undergoes an acidic shift in pI-value by modification of its cysteine residues quenching reactive oxygen species and protecting cells against stress-induced death. Downregulating DJ-1 by small interfering RNA (siRNA) sensitizes cells to oxidative stress, which can be rescued by overexpression of wild-type but not the L166P mutant (Yokota et al., 2003).

It is unlikely however that DJ-1 exerts its protective function through simple antioxidation. Its ability to quench reactive oxygen species is modest (Junn et al., 2005), and several lines of evidence surmise it is more likely to be implicated in the regulation of apoptosis. DJ-1 functions in the PI3'K survival pathway as a negative regulator of PTEN. The PTEN tumor suppressor regulates the PI3'K pathway by dephosphorylating PIP3 which is required for the activation of the survival kinase, protein kinase B (PKB/Akt) (Kim et al., 2005). In cultured cells and tissue from cancer patients DJ-1 expression correlates with the phosphorylation of PKB/Akt. It remains to be determined how DJ-1 interacts with PTEN. One possibility, given that

PTEN function can be modulated by exposure to H_2O_2, is that DJ-1 exerts a redox effect on PTEN through either its protease activity or redox-sensitive chaperone activity (Kim et al., 2005).

DJ-1 is further implicated in both apoptosis and cellular response to oxidative stress through its binding-partners in brain. In addition to PIASxa and DJBP which are predominantly expressed in testis, DJ-1 interacts with Daxx (Junn et al., 2005), p54nrb and pyrimidine tract-binding protein-associated splicing factor (PSF) (Xu et al., 2005) and Topors/p53BP3 (Shinbo et al., 2005) in brain. In relation to the binding-partners listed above, DJ-1 appears to function as a transcriptional co-activator regulating gene expression by cooperating with transcription factors and antagonizing repressor activities. DJ-1 acts as a potent inhibitor of the Daxx /apoptosis signal-regulating kinase 1 (ASK1) pathway by sequestering Daxx to the nucleus away from its cytoplasmic effector and inhibits PSF induced apoptosis by cooperating with p54nrb to activate PSF silenced transcription (Junn et al., 2005).

DJ-1 mutations disrupt protein activity by either destabilizing the protein or affecting its subcellular localization. DJ-1 exists as a homo-dimer *in vitro* (Honbou et al. 2003; Tao and Tong 2003; Wilson et al. 2003; Miller et al., 2003) however, the L166P mutant impairs its ability to self-interact resulting in highly unstable protein which is degraded by the 20S/26S proteasome (Moore et al., 2003, Macedo et al. 2003; Miller et al. 2003). The reduced protein levels result in loss of function as demonstrated by the L166P mutation which impairs the regulation of the Daxx/ASK1 pathway (Junn et al., 2005). Incorrect subcellular localization may also result in loss of DJ-1 function, the L166P, M26I and D149A mutations all exhibit reduced nuclear localization in favor of mitochondrial localization (Bonifati et al. 2003, Xu et al., 2005). The reduced access to nuclear proteins, such as p54nrb and PSF may increase PSF induced apoptosis as a result of the increased mitochondrial localization

of the mutants (Junn et al., 2005). As the mitochondrial function of DJ-1 remains to be determined, it is unclear if pathogenicity of DJ-1 mutants associated with increased mitochondrial localization is related to an as yet undefined mitochondrial function or loss of access to binding-partners in different cellular compartments. In addition to the cellular response to oxidative damage and apoptosis, DJ-1 also functions as a redox sensitive molecular chaperone that is capable of preventing the aggregation of a-synuclein and neurofilament subunit NFL (Shendelman et al. 2004). However, all these putative roles remain to be confirmed *in vivo*.

1.3.5.5 PINK1

1.3.5.5.1 Genetic analysis of PINK1 in PD

Mutations in the ARPD gene, *PINK1*, were initially identified in three large consanguineous families, one Spanish and two Italian and are described in more detail in Chapter 4 of this thesis (Valente et al, 2004). The Spanish family carried a G309D missense at a highly conserved residue in the putative kinase domain and the Italian families shared a nonsense mutation, W437X, which truncates a putative C-terminal regulatory region. The gene had been previously cloned by two groups, independently analyzing differential expression profiles of cancer cell lines. *PINK1* (PTEN induced kinase 1) was shown to be transcriptionally transactivated by the phosphatase and tensin homolog (PTEN) gene, but its expression was not sufficient to suppress the growth of cancer cells (Unoki and Nakamura, 2001). It was also shown to be consistently overexpressed in cells of high metastatic potential (Nakajima et al., 2003). It is noteworthy that DJ-1 has recently been identified as a suppressor of PTEN function (Kim et al., 2005).

The *PINK1* gene encodes a 581 amino acid (aa) protein that is ubiquitously expressed. The protein consists of a N-terminal mitochondrial targeting motif and a highly conserved serine/threonine kinase domain (Valente et al., 2004). To date, detailed data on its distribution in brain and localization to Lewy bodies remains unavailable however, it has been shown to be expressed in the SNpc.

Following the initial description of mutations in the linkage families, a number of subsequent mutation reports from around the world indicate the frequency of *PINK1* mutations is higher than *DJ-1* but lower than *parkin* with the notable exception of the L347P mutation, where a carrier frequency of 8% has been reported in the Philippino population (Rogaeva et al., 2004; Bonifati et al., 2005; Beilina et al., 2005; Li et al., 2005). The reported mutations do not display any obvious clustering within the gene, the majority are distributed throughout the kinase domain with a subset located in the N-terminal region between the mitochondrial targeting motif and the kinase domain (~aa 30-150). It is currently unclear how the mutations located outside the kinase domain will affect enzyme function.

One possibility is that they could disrupt mitochondrial localization or processing. The majority of nuclear encoded mitochondrial proteins contain a cleavable N-terminal 'pre-sequence' of between 20-60 aa residues which directs the protein towards its target following translation on cytoplasmic ribosomes. These proteins interact with a multimeric complex on the outer mitochondrial membrane known as the translocase of the outer membrane (Tom complex), specifically Tom20. After passing through the outer membrane they enter the matrix by passing through a pore formed by a complex known as translocase of the inner membrane (Tim complex), specifically Tim23. After entry into the matrix the pre-sequence is cleaved off by the mitochondrial processing peptidase (MPP) and the mature protein is usually localized to the matrix (Chacinska et al., 2002). The mechanism of PINK1

mitochondrial import remains unknown and the peptidase cleavage sites unmapped. There is therefore a possibility that these mutations may either disrupt interaction with the Tim complex or cleavage by the MPP. Western blot analysis of cells overexpressing PINK1 suggests that the processed protein is approximately 10 kDa shorter than the preprotein which translates to a cleavage product of approximately 100 aa, potentially placing mutations such as the C92F at or around the cleavage site (Beilina et al., 2005).

1.3.5.5.2 Functional analysis of PINK1

As the only functional domain in the protein and the site of the majority of the mutations, disruption of PINK1 kinase activity is the most probable disease mechanism. Kinases are defined by 12 highly conserved subdomains that fold to form a common catalytic core structure that is required for phosphorylation (Hanks and Hunter, 1995). Alignments of the PINK1 protein against other known kinases indicate it has all the necessary subdomains to form an active serine/threonine kinase. Confirmation of the bioinformatic prediction is at present limited to two studies employing *in vitro* autophosphorylation assays which demonstrate that PINK1 is active (Nakajima et al., 2003; Beilina et al., 2005). These assays are however too crude to distinguish any significant functional effects of the mutations on kinase activity.

PINK1 mutations have provided the first evidence of direct mitochondrial involvement in the pathogenesis of a typical form of Parkinson's disease. The exact role of PINK1 within the mitochondria remains to be determined. It may centre around the electron transport chain (ETC) or more likely the mitochondrial pro-apoptotic pathways. Regulation of the ETC may involve phosphorylation of at least

two of its subunits, complexes I and V (Schulenberg et al., 2003; Chen et al., 2004) in order to maintain the electrochemical gradient that generates the mitochondrial membrane potential ($\Delta\Psi m$). Depolarisation of the $\Delta\Psi m$ is associated with opening of the mitochondrial permeability transition pore (mPTP) which occurs in necrotic and apoptotic cell death. The mPTP is formed by the voltage-dependant anion channel (VDAC) on the outer membrane and the adenine nucleotide translocase (ANT) and cyclophilin-D (CyP-D) on the inner membrane. Under conditions of oxidative stress, high Ca^{2+} and low ATP, the complex forms an open pore between the inner and outer membranes allowing free diffusion of solutes across the membranes and is associated with release of apoptogenic proteins such as cytochrome c and procaspases from the intermembrane space (Duchen, 2004). Both VDAC and ANT undergo phosphorylation in response to stress and may represent potential areas for PINK1 involvement.

1.3.5.6 Leucine-rich repeat kinase 2 (LRRK2)

Mutations of *LRRK2* have recently been shown to cause autosomal dominant PD previously linked to the PARK8 locus (Paisan-Ruiz et al., 2004; Zimprich et al., 2004). *LRRK2* mutations are estimated to account for 5–6% of cases with a positive family history, and a significant minority of apparently sporadic cases (up to 1.6%) (Gilks et al., 2005). *LRRK2* encodes a complex multi-domain protein that consists of N-terminal leucine-rich repeats, a GTPase ROC/COR domain, a mitogen-activated protein kinase kinase kinase (MAPKKK) and C-terminal WD40 repeats (West et al., 2005). At present, little is known about LRRK2 function, however, some interesting preliminary data has begun to emerge from *in vitro* over-expression systems. LRRK2

encodes a kinase and is capable of autophosphorylation. It might be associated with the outer mitochondrial membrane (OMM) (Gloeckner et al., 2005; West et al., 2005) and can bind parkin (Smith et al., 2005). Significantly, three PD-associated mutations, two in the kinase domain (G2019S and I2020T) and one in the ROC/COR GTPase domain (R1441C) increase LRRK2 autophosphorylation, hinting at a dominant gain-of function mechanism (Gloeckner et al., 2005; West et al., 2005). Indeed, over-expression of R1441C, Y1699C or G2019S LRRK2 was sufficient to induce neuronal degeneration in mouse primary cortical neurons (Smith et al., 2005). Although these data still require validation *in vivo*, the delineation of the LRRK2 signalling pathway holds great promise for furthering our understanding of the aetiology of the disease.

1.3.5.7 Omi/Htr2A

One example of a procaspase which is released from the intermembrane space by the opening of the mPTP is the Omi/HtrA2 protein which has previously been implicated in neurodegeneration and recently associated with predisposition to PD (Strauss et al., 2005). Omi/HtrA2 is a member of the PDZ domain-containing serine proteases, it also contains an N-terminal mitochondrial targeting motif and a reaper-like motif. Reaper proteins play a critical role in apoptopic cell death (Golstein et al., 1995). Omi is thought to localize to the mitochondrial intermembrane space from where it is released into the cytosol during apoptosis to relieve the inhibition of caspases by binding inhibitor of apoptosis proteins (IAPs). Omi/HtrA2 is also capable of inducing cell death through its proteolytic activity.

Omi/HtrA2 was initially shown to interact with the Alzheimer's disease

associated proteins presenillin-1 and β-amyloid (Gray et al., 2000; Park et al., 2004). Recently Omi knockout mice have been shown to display parkinsonian phenotypes including rigidity and tremor which together with its localization within the PARK3 locus made it a candidate for mutation screening in PD patients (Jones et al., 2003). Mutations in Omi/HtrA2 were not found in the PARK3 families, however a mutation at G399 was found in four patients with sporadic PD and a polymorphism at A141 was found at higher frequencies in PD patients (Strauss et al., 2005). Both mutations are predicted to affect the regulation of the proteolytic activity of Omi/HtrA2 thus modulating cell death. At the cellular level the mutations have been shown to increase susceptibility to stress as demonstrated by decreased $\Delta\Psi m$ following exposure to staurosporine. It is tempting to speculate that the PINK1 and Omi/HtrA2 share a common pathway in the mitochondrial response to cellular stress and modulation of apoptosis, perhaps with Omi/HtrA2 as a downstream target for PINK1 initiating apoptosis when levels of oxidative stress become too high. However, any such interaction remains to be determined.

The discovery of all these genes has strongly implicated dysfunction of certain pathways and processes in PD and I will now discuss these in turn.

1.4 UBIQUITIN PROTEOSOME SYSTEM AND AGGRESOMES

The ubiquitin-proteosome system (UPS) is an essential cellular pathway that removes misfolded proteins by a series of enzymatic reactions (Ciechanover, 1998). The UPS consists of a cascade of enzyme systems involved in post translational modification and degradation of proteins that play major roles in a wide array of basic cellular

processes including differentiation, cell division, signal transduction, trafficking, and quality control (Ciechanover, 1998).

The central mechanism for UPS function is the utilisation of the 76 aa protein, ubiquitin (ub), as a degradation marker. The UPS enzymatic cascade begins with the ATP-dependent activation of ub by an E1 activating enzyme resulting in a high-energy thioester bond between ub and the E1. The activated ub is then transferred to an E2 (conjugating enzyme) via another thioester bond between ub and E2. Occasionally E2 enzymes can directly transfer ub to the target proteins. However, more commonly, there is a requirement of an E3 (ubiquitin ligase) that covalently attaches the C-terminal glycine (Gly) residue within the amino terminal of ub to the lysine (Lys) residue of the target protein (Figure 1.4). This reaction is repeated attaching another ubiquitin to the Lys residue at position 48 of the already attached ubiquitin causing the formation of a polyubiquitin chain. Although ubiquitin has six other Lys residues, it is only the formation of the residue 48 polyubiquitin chain that acts as a marker for the proteolytic degradation by the 26S proteasome. Interestingly it is postulated that polyubiquitylation at alternate lys residues such as K63 may have additional biological effects (Pickart, 2001).

To date only one E1 enzyme has been identified. In contrast the E2 enzymes consist of a family of proteins and E3 enzymes are even more diverse. Thousands of E3 enzymes exist in multiple species and play a critical role in conferring selectivity for target protein degradation. The E3 enzymes are broadly classified into four types: (1) HECT-type E3s contain a domain that can form a thioester bond with ub and which has Homology to the E6-AP Carboxyl Terminus (E6-AP was the first identified HECT E3 ligase). (2) RING finger E3 enzymes represent the major group of E3 ligases and consist of a Cysteine-rich consensus sequence flanked by one or two

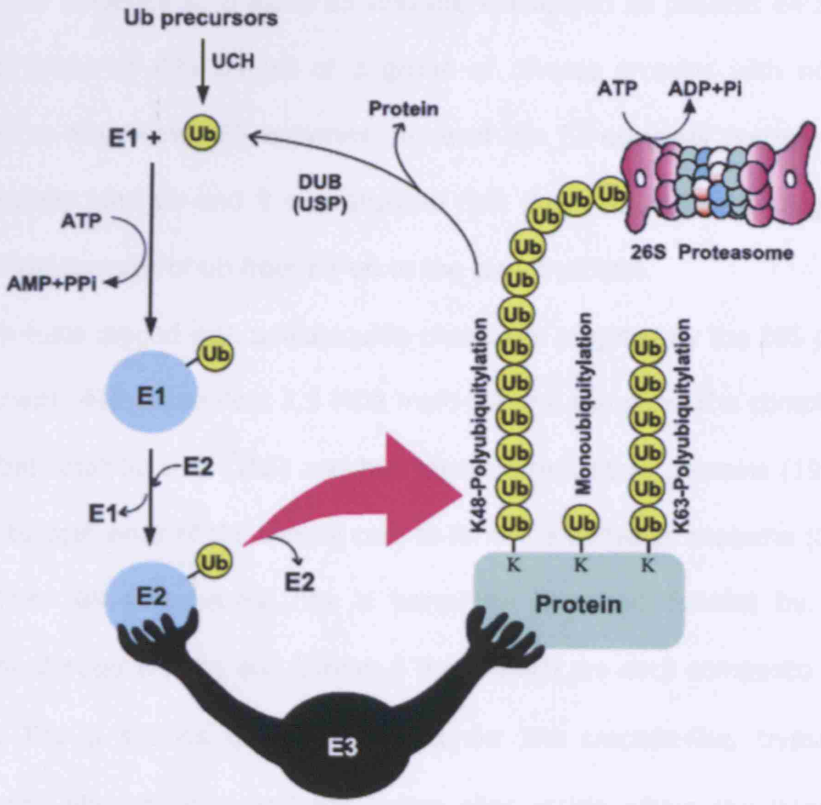


Figure 1.4 The ubiquitin-proteasome system. Ub = ubiquitin; E1 = Ub-activating, E2 = Ub-conjugating, E3 = Ub-ligating enzymes respectively; DUB = deubiquitylating enzyme; USP = ubiquitin-specific protease; HCH = ub C-terminal hydrolase. 26S is multi-subunit proteolytic complex.

Modified from Tanaka et al. (2004)

histidine residues. The typical RING finger E3 enzymes comprise 3 structural types: RING-IBR-RING; RING-HC; and RING-H2. (3) The third group of E3 enzymes contain a U-box domain that has a similar tertiary structure to the RING domain but does not bind zinc. Some of these U-box E3s can promote the formation of a polyubiquitin chain in the presence of a RING E3 and are considered to possess E4 activity. (4) The final group of E3s consist of a group of diverse proteins with no sequence homology to any known E3 enzymes. None of the E3 enzymes (except HECT-E3s) can covalently bind ub and it is postulated that they recruit E2s to target proteins and facilitate transfer of ub from E2-ub to the target protein.

Proteins tagged with polyubiquitin chains are targeted by the 26S proteasome – a eukaryotic ATP-dependent 2.5 MDa multi-subunit complex. The complex consists of a central catalytic core (20S) and two terminal regulatory domains (19S) that are attached to both ends of the central core to form the active proteasome (Coux et al., 1996). The 20S proteasome has a barrel-like structure formed by four rings comprising 2 outer α rings and 2 inner β rings which are each composed of α and β subunits. The β subnits of the β ring confer the caspase-like, trypsin-like and chymotrypsin-like activities and the active sites reside within the interior of the cylinder. Therefore, target substrates must pass though the centre of the α rings to reach the active sites. The centre of the α ring is almost closed and its opening is regulated by two subcomplexes known as 'base' and 'lid' complexes. The base complex is thought to bind ATP and be involved in opening of the α ring to allow entry of target proteins. The lid complex is involved in protein recognition, deubiquitylation of the proteins (to recycle proteins) and protein-protein interactions with proteins bearing a ub-like domain or certain E3 ligases (Coux et al., 1996).

Compelling evidence for UPS dysfunction in PD has come from the identification of disease-causing mutations in two genes that are involved in the UPS: (i) parkin, an E3 ubiquitin ligase that is mutated in autosomal recessive juvenile parkinsonism (AR-JP) (Kitada et al., 1998; Shimura et al., 2000); and (ii) ubiquitin C-terminal hydrolase (UCH-L1) that is mutated in a single family with autosomal dominant PD (Leroy et al., 1998).

There have been several reports that parkin is present in LBs of brains of sporadic and familial PD cases (Shimura et al., 1999; Shimura et al., 2001; Schossmacher et al., 2002; Bandopadhay et al., 2005). In contrast, AR-JP brains do not contain LBs (Shimura et al., 1999; Schlossmacher et al., 2002; Mori et al., 1998; Hayashi et al., 2000; van der Warrenburg et al., 2001; Takahashi et al., 1994) although there are two reports of LBs in AR-JP cases with compound heterozygote mutations in parkin (Farrer et al., 2001; Pramstaller et al., 2005). The general absence of LBs in AR-JP suggests that parkin may play an important role in LB formation. It also suggests that LBs are not essential or necessary for PD. Alternatively, AR-JP may have a different pathogenesis from familial and sporadic PD associated with LBs.

Previous studies in cultured cells have revealed an additional mechanism for handling misfolded proteins. When the production of misfolded proteins exceeds the cellular capacity to degrade them, proteins accumulate near the nucleus. The site of their accumulation was originally described as "a protein sequestration site associated with the microtubule organizing centre for protein degradation by autophagy" (Laszlo et al., 1991). However, this was subsequently termed more simply as the aggresome (Johnston et al., 1998). Aggresomes develop at the microtubule organizing centre (MTOC) from smaller aggregates that are retrogradely

transported to the MTOC along microtubules (Garcia-Mata et al., 1999). Aggresomes may form when certain proteins are over-expressed (Johnston et al., 1998; Garcia-Mata et al., 1999), when the proteosome is inhibited (Johnston et al., 1998) or are induced by certain stresses including heat shock (Vidair et al., 1996). It has been suggested that aggresomes may be a way for cells to sequester toxic misfolded proteins from the cytoplasm and/or to facilitate their removal by the lysosomal autophagic pathway (Kopito, 2000). Several recent studies have reported that parkin localizes to aggresomes or inclusions in parkin over-expressing cells in response to proteosome inhibition (Junn et al., 2002; Ardley et al., 2003). Furthermore, it has recently been reported that endogenous parkin localizes to the centrosome in response to proteosome inhibition (Zhao et al., 2003).

1.5 MITOCHONDRIA

1.5.1 Mitochondrial Biology

1.5.1.1 Introduction to mitochondria

Mitochondria are membrane bound organelles found in every eukaryotic cell. In essence they sustain life by converting the oxygen that organisms respire into the energy required for almost all cellular processes. They are especially abundant in tissues with high-energy demands such as brain, skeletal and cardiac muscle. Mitochondria are thought to have evolved from a bacterial progenitor and the primary evidence for this is that they possess their own mitochondrial DNA (mtDNA). MtDNA is the only source of extra-nuclear DNA within cells and consists of a circular

genome with structural similarities to bacterial DNA (Leblanc et al., 1997). MtDNA is a 16.5kb circular double-stranded molecule consisting of heavy (H) and light (L) chain without any histone coating. Human mtDNA encodes just 13 proteins that are all components of the electron transport chain and oxidative phosphorylation (OXPHOS) system (see below). These include seven subunits of complex I, one subunit of complex III, three subunits of complex IV and two subunits of complex V. In addition mtDNA encodes 24 other genes namely, two rRNAs and 22 tRNAs that are required to synthesise the 13 proteins. However, the remaining 850 proteins required for mitochondrial structure and function including 70 subunits of the OXPHOS system are encoded by the nuclear DNA.

Mitochondria consist of a matrix that is surrounded by two membranes, the inner mitochondrial membrane (IMM) and outer mitochondrial membrane (OMM). The space between the two membranes is called the intermembranous space (IMS). The IMM is largely impermeable and forms the major barrier between the matrix and cytosol. It forms multiple infoldings, cristae and these contain a variety of membrane bound mitochondrial enzyme systems. The OMM is relatively permeable to small molecules but this appears to be under regulatory control. A fuller discussion on protein import in mitochondria is found in Chapter 5. The IMS is a less well-understood compartment but appears to contain proteins involved in mitochondrial energetics and apoptopic cell death including cytochrome c and creatine kinase.

The major function of mitochondria is the generation of energy in the form of adenosine triphosphate (ATP). The two major enzymatic systems involved in this are the citric acid or tricarboxylic acid (TCA) cycle and the respiratory or electron transport chain. Whilst these systems generate ATP crucial for many essential processes, they also generate free radicals in the form of superoxide ions that can lead to oxidative stress and damage within the cell. Mitochondria are also involved in

other aspects of cellular physiology such as calcium signalling and they play a major role in the initiation and regulation of apoptosis. Given the central role of mitochondria, dysfunction of the mitochondria is likely to have significantly deleterious effects on the cell and has been implicated in many different disease states including neurodegenerative disease. This will be expanded upon in later sections.

1.5.1.2 The Electron Transport Chain

The TCA cycle breaks down the carbon substrate, acetyl CoA, derived from pyruvate, fatty acid and amino acid metabolism to generate CO_2 and this simultaneously reduces NAD^+ to NADH and FAD^{2+} to FADH. These intermediates are required as reducing equivalents of the electron transport chain.

The electron transport chain is located on the IMM and consists of 4 membrane spanning enzyme complexes namely: complex I (NADH-ubiquinone reductase) that oxidises NADH; complex II (succinate-ubiquinone oxidoreductase) that oxidises $FADH_2$; complex III (ubiquinol cytochrome c oxidoreductase) and complex IV (cytochrome c oxidase). Additionally the electron transport chain contains two hydrophobic electron carriers, coenzyme Q10 and cytochrome c that are both encoded by nuclear DNA (Figure 1.5).

The fundamental role of the electron transport chain is the transfer of electrons via a series of oxidation-reduction reactions. Electrons are transferred from NADH and $FADH_2$ to complex I and complex II respectively. Both these complexes transfer electrons to coenzyme Q10 (ubiquinone) which then shuttles electrons to complex III. This complex then transfers electrons to cytochrome c that then shuttles

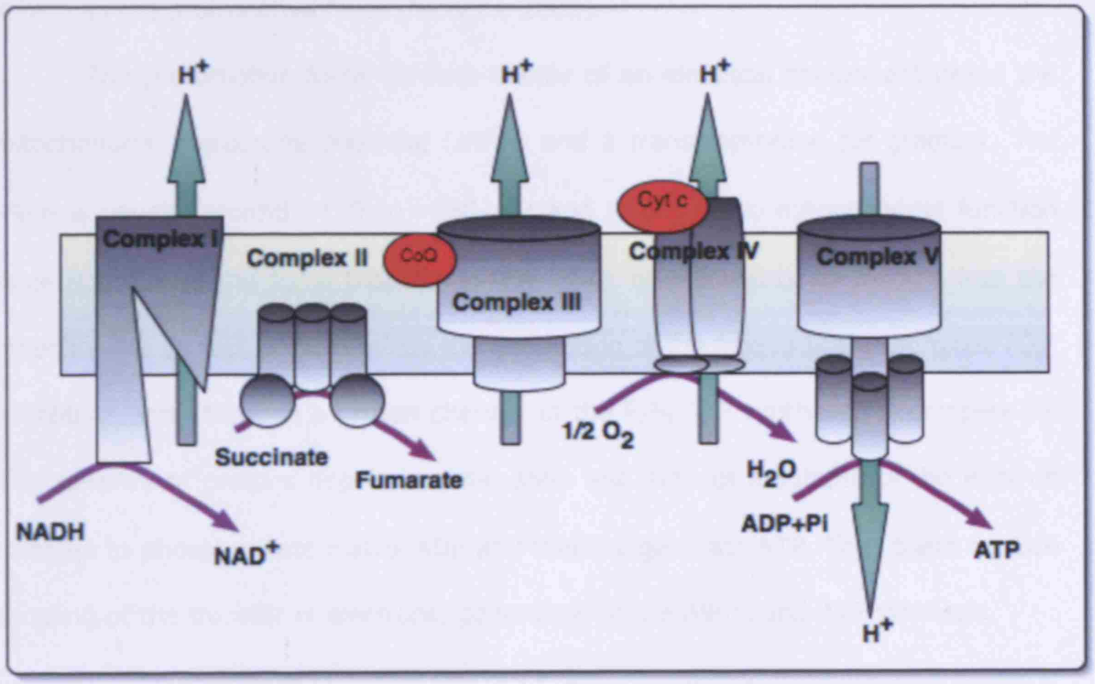


Figure 1.5 Schematic of electron transport chain. Shows mitochondrial encoded subunits within the various complexes. Key: CoQ – conenzyme Q10, Cyt c – cytochrome c, Pi – inorganic phosphate.

electrons to complex IV which is linked to the reduction of oxygen to produce water. The reactions of I, III, and IV are all coupled to the transfer of protons across the IMM and this proton efflux creates a proton electrochemical gradient otherwise known as the protomotive force (Nicholls, 2002).

The protomotive force consists mainly of an electrical component called the mitochondrial membrane potential ($\Delta\Psi m$) and a transmembrane pH gradient. The $\Delta\Psi m$ is usually around −150 to −180 mV and is central to mitochondrial function since it provides the force that drives the influx of protons or of calcium into the mitochondria as well as determines the generation of the free radical superoxide ($O_2^{\cdot-}$). Protons enter through a proton channel of the F_1F_0-ATP synthase (or complex V). This re-entry of protons depolarises the $\Delta\Psi m$ and induces a subunit of the enzyme complex to phosphorylate matrix ADP and thereby generate ATP. Thus there is close coupling of the transfer of electrons, generation of the $\Delta\Psi m$, and ATP synthesis.

1.5.1.3 The mitochondrial permeability transition pore (mPTP)

The mPTP is thought to be a large multiprotein complex that spans both the inner and outer mitochondrial membranes (IMM and OMM) that allows the passage of water and molecules of up to 1.3 kDa (Nicholls, 2002). However the exact structure and composition of the mPTP is unknown and various structures have been proposed. The more prevalent view is that it is made up from a variety of elements of the OMM (voltage dependent anion conductance (VDAC) and peripheral benzodiazepine receptor (PBR)); the IMM (adenine nucleotide translocator (ANT)) and the matrix (cyclophilin D) (Figure 1.6). Support for this model has come from *in vitro* and *in vivo* studies showing that mPTP opening can be inhibited by

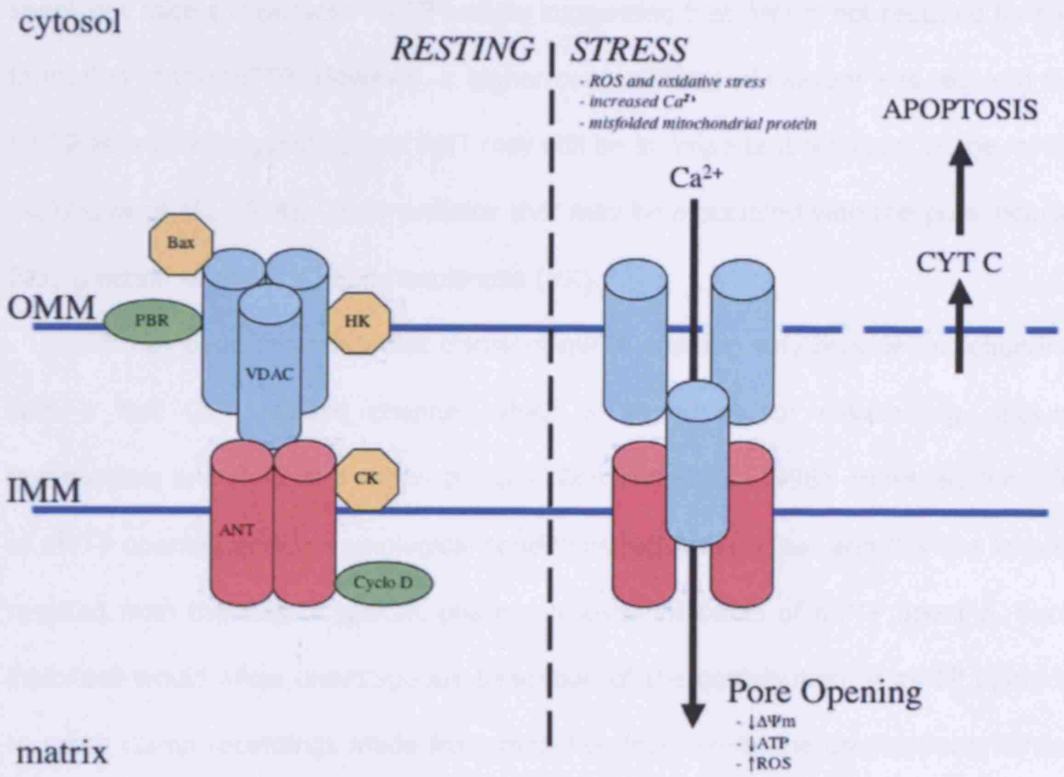


Figure 1.6 Hypothetical model of mitochondrial permeability transition pore (mPTP). The mPTP may be composed of voltage-dependent anion channel (VDAC), adenine nucleotide translocator (ANT) and cyclophilin D (CypD). The peripheral benzodiazepine receptor (PBR), creatine kinase (CK) hexokinase (HK) and Bax are associated with the mPTP.

pharmacological agents that target ANT (bongkrekic acid), VDAC or cyclophilin D (particularly cyclosporin A) However, this model remains largely hypothetical and has been challenged most notably by the discovery that mitochondria from livers of ANT knock out mice still possess mPTP activity suggesting that ANT is not required for the formation of the mPTP. However, a higher concentration of calcium was required for mPTP activation suggesting that ANT may still be an important regulator of the mPTP (Kokoszka et al., 2004). Other proteins that may be associated with the pore include Bax, creatine kinase (CK) and hexokinase (HK).

It has been proposed that transient mPTP opening may provide mitochondria with a fast Ca^{2+} release channel which is important for maintaining calcium homeostasis and signalling within the cell (Bernardi et al., 1998). However, the role of mPTP opening under physiological conditions remains unclear and this has largely resulted from the lack of specific pharmacological inhibitors of mPTP opening. Such inhibitors would allow unambiguous dissection of the contribution of mPTP opening to patch clamp recordings made from mitochondrial membrane preparations. Whilst cyclosporin A (CsA) has been the accepted pore blocker (by binding cyclophilin D thereby closing the pore), CsA binds other cyclophilins and may have additional effects that cannot be controlled (Clarke et al., 2002). Studies using fluorescent molecules that can only traverse the mitochondrial membrane via the mPTP have suggested that spontaneous low conductance mPTP opening can occur under physiological conditions (Petronilli et al., 1998) and that under resting condition the mPTP may rapidly fluctuate between open and closed states (Kowaltkowski et al., 2000). However, there still remains some doubt as to whether these observations are artificially induced by the very techniques used to record mPTP opening. New technologies and agents will hopefully become available to determine the true functional importance of mPTP opening under basal conditions.

In contrast, the significance of mPTP opening in pathological states appears better appreciated. Irreversible opening of the mPTP is thought to result in a series of adverse cellular events including collapse of the $\Delta\Psi m$, bioenergetic failure, ATP depletion, mitochondrial Ca^{2+} dysregulation and efflux, matrix swelling and rupture of the OMM releasing many pro-apoptopic proteins from the IMS into the cytosol (Chernyak, 1997; Kroemer and Reed, 2000). This view is supported by studies showing that agents like cyclosporin A that inhibit the mPTP can reduce cytochrome c release and apoptosis in some models (Narita et al., 1998). In addition mPTP opening may also play a role in the release of the pro-apoptopic proteins from the IMS into the cytosol. Several studies suggest that cytochrome c can be released in the absence of mitochondrial swelling. Since the mPTP is thought to span both membranes, an exit route for IMS proteins appears obscure. However it has recently been shown that the "BH3-only" molecule, tBID can bind to the OMM and can activate remodelling of mitochondrial cristae which creates junctions between the IMS and cristae and allows the release of cytochrome c through the mPTP (Scorrano et al., 2002).

Opening of the mPTP is stimulated by calcium influx into the mitochondria, oxidative stress, ATP depletion, high inorganic phosphate (Pi) and mitochondrial depolarization (see Crompton et al, 2002 for review). The ischaemia-reperfusion injury model demonstrates very well the interplay of these factors in mPTP opening. During the reperfusion phase, high $[Ca^{2+}]_m$; reduced ATP/ADP ratio and consequently high P_i; and increased free radicals all contribute to complete and irreversible opening of the mPTP and collapse of the $\Delta\Psi m$. There remains some debate as to whether mPTP opening is an early event in apoptosis since there are conflicting reports regarding the temporal relationship between the collapse in $\Delta\Psi m$ and the

release of apoptopic factors. Whilst loss of $\Delta\Psi m$ usually accompanies irreversible mPTP opening, other cellular events can cause $\Delta\Psi m$ depolarisation independent of mPTP opening so some caution is necessary in making inferences about mPTP opening from $\Delta\Psi m$ measurements.

1.5.1.4 Mitochondrial outer membrane permeabilisation (MOMP)

The pivotal process in the initiation of mitochondrial apoptosis is mitochondrial outer membrane permeabilisation (MOMP). MOMP is a sudden event during apoptosis and leads to cell death by the release of pro-apoptopic molecules from the IMS as well as loss of mitochondrial integrity and function. Although there have been many conflicting reports, two distinct pathogenetic mechanisms appear to be responsible for MOMP. The first involves persistent activation and opening of the mPTP that leads to matrix swelling and rupture of the OMM and has been described in detail above. The second mechanism involves the direct action of the Bcl-2 family of proteins on the OMM and this occurs independent of mPTP opening.

The anti-apoptopic Bcl-2 family members include Bcl-2 and Bcl-xL and these appear to act directly on the OMM to block MOMP. In contrast the pro-apoptopic members promote it and can be divided into sub-families based on the sharing of Bcl-2 homology (BH) domains. The BH123 proteins that share BH1, BH2, and BH3 domains include Bax and Bak and these proteins appear to be directly involved in the generation of MOMP. Upon activation Bax and Bak oligomerize and insert directly into the OMM to trigger MOMP and recombinant Bax and Bid have been shown to permeabilise purified mitochondrial outer membrane preparations (Kuwana et al., 2002). Furthermore, transgenic mice that lack Bax or Bak fail to undergo MOMP after

apoptopic stimulation (Wei et al., 2001). Some Bad and Bid are members of the BH3-only family of proteins and do not have a direct effect on the MOMP – studies suggest they can activate Bax and Bak or else can interfere with the anti-apoptopic Bcl-2 family members (Letai et al., 2002).

The factors leading to mPTP opening have already been discussed above but similar factors and signalling pathways have been found to activate MOMP via the Bcl-2 family of proteins. The tumour suppressor protein, p53, has been shown to induce apoptosis by direct activation of Bax and Bak but also upregulates the transcription of the BH3-only protein, PUMA. p53 has also been to interact with and inhibit the activity of the anti-apoptopic proteins, Bcl-2 and Bcl-xL. Other factors include JNK that targets Bcl-2 and transcription factors such as Nur77 that upregulates Bcl-2 expression (Green and Kroemer, 2005).

Although the two mechanisms of MOMP activation can occur independently, several studies suggest that there is a degree of crosstalk between them. *In vitro* studies in sympathetic neurons suggest that mPTP opening can occur as a secondary consequence of Bcl-2 mediated MOMP (Deshmukh et al., 2000). The Bcl-2 proteins may also interact with and regulate opening of the mPTP. There is evidence that Bax can bind to VDAC and activate mPTP opening. Conversely, Bcl-2 and Bcl-xl may also act on VDAC to induce mPTP closure and prevent MOMP.

The consequence of MOMP is release of proteins from the IMS including cytochrome c that is a potent activator of caspases. This is thought to be a point of no return for the cell as whole series of caspase dependent and caspase independent cascades are activated.

1.5.2 Mitochondria and Parkinson's disease

1.5.2.1 Mitochondria and PD - introduction

In the 1980s, the Parkinson's disease (PD) research landscape was dominated by a series of landmark papers relating to the mitochondrial toxin, MPTP (Langston et al., 1983; Nicklas et al., 1985; Singer et al., 1987). Accidental exposure in humans caused Parkinsonism and these studies led to the hypothesis that mitochondrial dysfunction and oxidative stress were central to the pathogenesis of PD (Mizuno et al., 1989; Schapira et al., 1989). Since that time many studies have supported the 'mitochondrial dysfunction hypothesis' however it remained unclear whether mitochondrial involvement was primary or secondary to the disease pathogenesis. This was a crucial question since a primary role would suggest that drugs targeting the mitochondria could have a disease-modifying role. The discovery of mutations in genes involved in Mendelian-inherited forms of PD has expanded our understanding of PD disease mechanisms and the report of mutations in the genes *DJ-1* and *PINK1*, in familial PD has provided the most direct evidence in humans for a primary role of mitochondrial dysfunction in PD (Shen and Cookson, 2004).

1.5.2.2 Mitochondria and PD – pre-genetic era.

The discovery of the effects of MPTP in humans had three major impacts. Firstly, MPTP was able to recapitulate many of the features of Parkinsonism in animals including mice and primates and provided researchers with robust and reproducible animal models of PD that could be used to study mechanisms of neuronal loss as well as testing potential therapeutic compounds (Beal 1993). Secondly, it was

demonstrated that MPTP was a selective inhibitor of complex I of the electron transport chain and suggested that complex I inhibition and hence mitochondrial dysfunction played a causal role in PD (Singer et al., 1987). Thirdly, it rekindled interest in the environmental toxin hypothesis of PD and motivated an extensive search into MPTP-like substances amongst pesticide compounds that had been previously linked to PD development via epidemiological studies.

A biochemical link between the effects of MPTP and idiopathic PD was established when several groups demonstrated that complex I was selectively reduced in the substantia nigra of human brain, the major site of neuronal loss (Mizuno et al., 1989; Schapira et al., 1989). Since then complex I defects have been demonstrated in a variety of other tissues including muscle and platelets of PD patients (Schapira et al., 1998). The most compelling evidence to suggest a primary role of complex I dysfunction in PD pathogenesis has been the demonstration that rats developed Parkinsonism following exposure to the herbicide, rotenone, another complex I inhibitor. In addition to L-Dopa responsive symptoms, the rats exhibited both selective dopaminergic neuronal loss and remarkably LB-like inclusions (Betarbet et al., 2000).

1.5.3.3 Mitochondria and PD – genetic studies

Elegant studies using cell cybrid systems in which platelets from PD patients with complex I defects are fused with mitochondrial deficient cell lines ($\rho 0$ cells) show transmission of the complex I defect to the cybrid cells and strongly suggest that complex I defects originate from the mitochondrial genome (Swerdlow et al., 1996;

Gu et al., 1998). These cybrid lines are associated with increased oxidative stress and have recently been found to be sufficient to cause the formation of aggregates suggesting a link to Lewy bodies (LBs) (Trimmer et al., 2004). Furthermore, Swerdlow and colleagues have reported a kindred in which both PD and complex I deficiency in cell cybrids from affecteds were maternally inherited (Swerdlow et al., 1998).

Several different point mutations of mitochondrial genes have been identified in patients with maternally inherited parkinsonism including the *cytochrome b* gene and the *12sRNA* gene (Rana et al., 2000; Thyagarajan et al., 2000). Interestingly complex I activity was not altered in patients with *12sRNA* mutations that caused maternally inherited deafness and parkinsonism but there was a reduction in cytochrome c oxidase (COX) activity in lymphoblasts suggesting that whole mitochondrial function may be important for dopamine neuronal survival (Thyagarajan et al., 2000). This is consistent with post-mortem data showing reduced COX activity in PD brain (Schapira et al., 1998). Despite these reports in rare families, an important question is whether mutations occur in the common idiopathic form of PD? However, to date, most studies have failed to find mitochondrial mutations strongly associated with PD despite exhaustive sequencing of the mitochondrial genome in PD patients (Ikebe et al., 1995; Vives-Bauza et al., 2002).

Nevertheless in view of the cybrid data and biochemical studies of complex I deficiency, population genetic studies have investigated whether polymorphic variants of the mitochondrial genome may alter susceptibility of idiopathic PD. Reported studies to date have been conflicting. One recent study found that mitochondrial genome polymorphisms affecting haplogroups J and K can reduce the risk of PD and the authors suggested that these variants may reduce production of reactive oxygen species (ROS) by complex I during normal respiration (Van der Walt

et al., 2003). Furthermore, a recent European study also found a lower frequency of haplogroup K (containing the 10398G>A SNP of the *ND3* gene) in PD cases compared to controls particularly in men over the age of 50 years (Ghezzi et al., 2005). A further study found that a cluster of haplogroups namely, J, T. U. and K were all less common in PD compared to controls but that this was only significant when all the haplogroups were pooled (Pyle et al., 2005).

Whilst these studies all support a causal association for the 10398G>A polymorphism, a meta-analysis suggested that the 4366A>G polymorphism was the only mitochondrial variant associated with PD but this was not confirmed in the most recent study implicating 10398G>A albeit in British subjects (Pyle et al., 2005; Tan et al., 2000). The mechanism by which these mitochondrial variants alter mitochondrial function remains unclear and there is no direct evidence that these variants reduce ROS generation. Moreover using a tagging SNP approach, our laboratory did not find such an association between mitochondrial variants and PD in 800 Caucasian patients with PD (unpublished data).

As previously stated, a primary role for oxidative stress and mitochondrial dysfunction in PD has finally been confirmed by discovering mutations in nuclear encoded mitochondrial genes including *PINK1*, *DJ-1*, and most recently *Omi/HtrA2a* (Brice et al., 2005; Strauss et al., 2005). Furthermore, recent insights in animal models have linked parkin to mitochondrial function (Cookson, 2005). Whilst *α-synuclein* is localized predominantly in synaptic terminals, there is significant evidence linking *α-synuclein* mutations to oxidative stress. Over-expression of mutant *α-synuclein* sensitises neurons to mitochondrial toxins such as MPP^+ and 6-hydroxydopamine and interestingly one study reported that *α-synuclein* knock out mice are more resistant to MPTP (Dauer et al., 2002).

1.5.3.4 Mitochondria and the UPS

While *α-synuclein* and *parkin* mutations confirm that protein misfolding and the ubiquitin proteasome system (UPS) dysfunction is a significant upstream pathway en route to dopaminergic degeneration, the discovery of *PINK1*, *DJ-1* and *Omi/HtrA2* mutations all suggest that mitochondrial dysfunction is another major upstream pathway to Parkinsonism. An intriguing question is whether all the known genes converge to a common pathogenetic pathway? In view of their differential subcellular localizations, direct interactions seem unlikely. However, they may interact via overlapping pathways and there is significant evidence for a close relationship between the UPS and mitochondrial function. For example proteasomal stress can result in increased sensitivity of neurons to MPTP and conversely complex I defects can result in decreased proteasome activity (Hoglinger et al., 2003; Sullivan et al., 2004).

The mechanism of how mitochondrial and proteasomal impairment lead to dopamine cell loss is becoming clearer and the generation of oxidative stress may be common to both and may be allied to apoptotic cell death in PD. Evidence of increased oxidative damage has been demonstrated following mitochondrial or proteasomal impairment *in vivo* (Beal, 2000; McNaught et al., 2004).

Furthermore, the pathways may be interdependent resulting in either 'feedback' or 'feed-forward' loop mechanisms between the UPS and mitochondria such that dysfunction of one pathway will have inevitable deleterious consequences for the other. Since the UPS requires ATP, mitochondrial dysfunction and ATP depletion is likely to lead to UPS dysfunction. Treatment of human neuroblastoma cell lines with the complex I inhibitor, rotenone, was associated with ~20% ATP

depletion; an increase in reactive oxygen species (ROS) and oxidised proteins; and a marked reduction in proteasome activity (Shamoto-Nagai et al., 2003). Thus inactivation of the proteasome is likely to create a feed forward amplification loop with further damage from failure to clear the excess oxidised protein species leading to further ROS generation. Detergent insoluble aggregates of α-synuclein accumulate in cells following mitochondrial inhibition with rotenone or oligomycin and disappear following washout of inhibitors paralleling recovery of mitochondrial metabolism (Lee et al., 2002). Perhaps the most compelling evidence for this interplay occurring in PD is the demonstration that chronic rotenone exposure in rats results in the formation of Lewy body-like aggregates in addition to Parkinsonism (Betarbet et al., 2000).

Evidence is also accumulating for a converse mechanism whereby UPS dysfunction can result in secondary mitochondrial dysfunction and damage. Treatment of primary rat cultured cortical neurons with proteasome inhibitors was sufficient to induce the redistribution of cytochrome c into the cytosol and this was associated with depolarisation of the mitochondrial membrane potential ($\Delta\Psi m$) leading to caspase 3 activation and apoptopic cell death. (Qiu et al., 2000). Of particular relevance in PD, when PC12 cells inducibly expressing A30P mutant ∝-synuclein cells were treated with a proteasome inhibitor, cells underwent mitochondrial apoptosis and $\Delta\Psi m$ depolarisation. Both $\Delta\Psi m$ depolarisation and apoptosis was blocked with cyclosporin A (CsA) suggesting that proteasome inhibition resulted in $\Delta\Psi m$ depolarisation associated with opening of the mPTP (Tanaka et al., 2001).

The mechanism by which proteasome inhibition leads to mitochondrial injury and apoptosis remains unclear. Several pro-apoptopic proteins such as p53 and Bcl-2 family members including Bax, Bid and Smac are normally degraded by the UPS

(Jesenberger and Jentsch, 2002). Therefore the UPS plays a critical role in homeostasis by preventing the accumulation of these potentially toxic molecules. Proteasome inhibition leads to the accumulation of such proteins causing mPTP opening, $\Delta\Psi m$ depolarisation, and apoptosis (Jesenberger and Jentsch, 2002). In addition p53 has been shown to transcriptionally activate the expression of several target genes including Bax, NOXA and PUMA that can in turn cause mitochondrial dysfunction (Vogelstein et al.,2000). Clearly further studies are required to determine the significance of p53 alterations in proteasome inhibition particularly in PD and *in vivo* models of the disease.

The interplay between the UPS and mitochondria may also be in part mediated by the known PD genes. Parkin appears to be essential for maintenance of mitochondrial function since parkin knock-out mice develop mitochondrial deficits and oxidative damage however, this does not lead to neurodegeneration (Goldberg et al., 2003). Although mitochondrial proteins are not targeted by the UPS, parkin may regulate proteins on the outer mitochondrial membrane that are accessible to the UPS. Parkin in neuronal PC12 cell lines localized to the outer membrane where it protected cells from the damaging effects of the ceramide by delaying mitochondrial swelling and cytochrome c release (Darios et al., 2003). Similarly RNAi knockdown of DJ-1 also sensitised neurons to proteasomal inhibiton (Yokota et al., 2003). DJ-1 has recently been identified as a regulator of p53 transcriptional activity by binding Topors/p53BP3 and may therefore represent a molecular bridge between the UPS and the mitochondrial stress response (Shinbo et al., 2005).

Parkin may also be an important bridging protein between these two systems playing a critical role in ubiquitylating and degrading oxidized mitochondrial proteins thereby ameliorating oxidative stress. Parkin is itself sensitive to oxidative stress and

is inactivated by NO mediated nitrosylation that could lead to a simultaneous increase in UPS and mitochondrial dysfunction (Chung et al., 2004; Yao et al., 2004). Despite this, parkin's exact role in mitochondria is unknown and in particular no mitochondrial substrates have been identified. It is still unclear whether parkin may interact with the recently discovered PINK1, DJ-1 or Omi/HtrA2 (Bonifati et al., 2003; Valente et al., 2004; Strauss et al., 2005).

Parkin has been localized to the outer mitochondrial membrane (OMM) and over-expressed DJ-1 has been shown to translocate from the cytosl to the OMM following oxidative stress and that this may confer DJ-1's neuroprotective function (Canet-Alviles et., 2004). Furthermore, parkin has been found to associate with mutant DJ-1 where it can promote its stability in vitro and detergent insoluble levels of DJ-1 appears to be differentially regulated by parkin in vivo although parkin does not appear to ubiquitylate DJ-1 directly (Moore et al., 2005).

Recently, endogenous DJ-1 was detected in the matrix and inter-membranous space (IMS) of mitochondria and may play a role in the maintenance of mitochondrial function (Zhang et al., 2005). Knock out of DJ-1 in mammalian systems *in vivo* does not lead to dopaminergic degeneration suggesting that DJ-1 is not essential for mitochondrial integrity. However it may protect neurons from exposure to oxidative stress since DJ-1 knock out mice were more sensitive to MPTP neurotoxicity (Kim et al., 2005). This suggests that DJ-1 may act as a free radical scavenger that prevents the accumulation of free radicals that occur from the mitochondrial ETS and this would be consistent with its localization in the IMS (Kim et al., 2005). It will be interesting to examine the effect of aging on DJ-1 knock out mice.

1.6 CONCLUSION AND THESIS AIMS

At the time this work began, parkin was known to be a ubiquitin ligase but the role of parkin in aggregation was unclear with variable reports of parkin being present in Lewy bodies and further reports of the absence of Lewy bodies in parkin positive brains. The role of parkin in mitochondria was not appreciated.

Furthermore, parkin was the only known recessive gene causing PD. However, in the middle of these studies a post-doctoral scientist, Dr Patrick Abou-Sleiman had succeeded in identifying PINK1 mutations in PD patients in the PARK6-linked pedigree.

Therefore, the work in this thesis reflects my studies in further understanding the function of both parkin and PINK1. With regard to parkin, the central basis of the experiments was establishing stable neuronal cell lines over-expressing human wild type parkin and determining the effects of apoptosis and the role of the aggregation and the ubiquitin proteasome system. With regard to PINK1, the experiments centre on firstly determining a mechanism for the pathogenicity of the human disease causing mutations and secondly further elucidating PINK1 function by studying its localization in cell lines *in vitro*.

2

Materials and Methods

2.1 INTRODUCTION

This chapter will describe all the methodological techniques undertaken in investigations of parkin and PINK1 protein in the cellular systems and human post-mortem brain studies described in this thesis.

2.2 ETHICAL APPROVAL

Human brain tissue was obtained from the Queen Square Brain Bank for Neurological Disorders, with the informed consent of next of kin and approval from the Joint Research Ethics Committee of the National Hospital for Neurology and Neurosurgery and Institute of Neurology.

2.3 LABORATORY REAGENTS

2.3.1 General Reagents

All laboratory chemicals were of analytical grade and were obtained from sources listed in the Appendix.

2.3.2 Molecular biology and Bacterial reagents

2.3.2.1 Bacterial reagents

Bacto®-Tryptone, Bacto®-Yeast extract, and Bacto® micro-agar bacterial growth medium were obtained from Duchefa (Harlem, Netherlands). XL1-blue strain of *Escherichia coli* (*E. coli*) cells was obtained from Stratagene (La Jolla, California, USA) and DH5α strain of *E. coli* was obtained from Invitrogen Ltd. (Paisley, UK).

2.3.2.2 Molecular biology reagents

Restriction enzymes and DNA modifying enzymes with their respective buffers were obtained from Promega (Southampton, UK) or New England Biolabs (Hitchin, UK). DNA molecular weight markers (100 kb, λDNA/HindIII) were obtained from Promega. QIAquick® gel extraction kit, QIAprep Mini, Midi and Maxi DNA prep kits were from Qiagen Ltd. (Crawley, UK). DNA sequencing of parkin and PINK1 cDNA constructs was performed by MWG-Biotech AG (Ebserberg, Germany) and Dr Robert J. Harvey (School of Pharmacy, London, UK).

2.3.2.3 Plasmid vectors and DNA constructs

The plasmid vectors used were: pcDNA3 or pcDNA3.1 from Invitrogen Ltd. (Paisley, UK). Parkin cloned into pcDNA3 was a kind gift of Dr Rohan de Silva (UCL, London). Full-length *PINK1* cDNAs were amplified using the primers h*PINK1* (5'-ggcggatccatggcggtgcgacaggcg-3') and h*PINK2* (5'-ctcgaattcgggacatcacagggctgc-3') and cloned into the BamHI and EcoRI sites of the vector pRK5myc by Dr Robert Harvey (London). The expressed *PINK1* protein had a c-myc epitope tag attached to the N-terminus. The R68G, F70Y, G309D, A383T, G411S, Y431H, W437X, N451S, Q456X and C575R mutations were introduced into pRK5-myc-*PINK1* using the Quikchange site directed mutagenesis kit (Stratagene, Amsterdam, Netherlands). *PINK1* was cloned into a C-terminal-c-myc-pcDNA-3.0 expression vector. The first 34 and 70 amino acids of PINK1 were cloned into pEGFP-N1 vector (Robert Harvey, London). Wild type human PINK1 cloned into the expression vector pcDNA3.1 (+) (Invitrogen) was a kind gift from Drs Y. Nakamura and M. Unoki (Japan). α-synuclein in pCI vector was a kind gift from Dr N. Nukina (Japan).

2.3.3 SDS-PAGE and Western Blot Analysis reagents

Polyacrylamide gels were made using Acrylamide/bisacrylamide (30% w/v) solution obtained from Amresco Ltd. (Ohio, USA). Ammonium persulphate, N,N'-methylene-bis-acrylamide, N,N,N'N'-tetramethylethylene-diamine (TEMED) were obtained from Sigma (St Louis, MI, USA). Protein molecular weight Rainbow™ marker, Hybond™-C nitrocellulose membranes, Enhanced Chemiluminescence system (ECL) and Kodak X-OMAT imaging photographic film were purchased from Amersham Pharmacia Biotech. (Little Chalfont, Bucks., UK). Pre-stained protein markers were obtained

from NEBS Biolabs. 3MM chromatography paper was obtained from Whatman International Ltd. (Maidstone, Kent, UK). Photographic developing and fixing chemicals were obtained from X-OGRAPH Ltd. (Tetbury, UK).

2.3.4 Antibodies

2.3.4.1 Primary Antibodies

Rabbit polyclonal anti-parkin (C-terminal) antibody (1:100; Cell Signalling Technologies); mouse monoclonal anti-vimentin (clone V9) antibody (1:100; Dako); mouse monoclonal anti-ubiquitin (clone FK2) antibody (1:100; Affiniti Research Products Ltd, UK); mouse monoclonal anti- Hsc-70 (B-6) antibody (1:100; Santa Cruz Biotechnology Inc.); mouse monoclonal anti-γ-tubulin antibody (1:100; Sigma) mouse monoclonal anti-GAPDH (Chemicon); mouse monoclonal anti-c-myc (clone 9E10) (Sigma); mouse monoclonal anti-complex I (Molecular Probes); mouse monoclonal anti-mitochondrial antibody to non-glycosylated mitochondrial protein (Abcam); rabbit polyclonal anti-parkin (Cell signaling); mouse monoclonal anti-α-synuclein (Transduction Labs); mouse monoclonal anti-Hsp60 (Stressgen), sheep polyclonal anti-cytochrome C (Abcam); mouse monoclonal anti-cytochrome c (Zymed); polyclonal anti-ubiquitin (Dako); anti-VDAC1 (Abcam); goat polyclonal anti-MnSOD (Calbiochem). UPN79-synphilin antibody was a kind gift from Dr. V. Lee (University of Pensylvania, USA).

PINK1-49 and PINK1-48 antibodies were produced by immunizing rabbits with a mix of the following synthetic peptides, CKSKPGPDPLDTRRLQ (denoted PINK1-49) and CRGPGTSAPGEGQERAP (denoted PINK1-48) corresponding to amino

acid residues 135 - 149 and 194 – 209 respectively of human PINK1. The peptides were coupled to keyhole limpet haemocyanin prior to immunization. Anti-serum was affinity purified on columns against each of the synthetic peptides (Eurogentec, Liege, Belgium).

2.3.4.2 Secondary Antibodies

Alexa Fluor 568 goat anti-rabbit IgG conjugates (1:2500; Molecular Probes). Alexa Fluor 488 goat anti-mouse IgG conjugates (1:1000; Molecular Probes). TRITC donkey anti-mouse IgG (H+L) (1:300; Jackson ImmunoResearch Laboratories, Inc). Secondary rabbit, mouse and goat horseradish peroxidase (hrp) -linked antibodies used for Western blotting were all from DAKO Ltd., Glostrup, Denmark. Sheep hrp secondary antibody was obtained from AbCam.

2.3.4.3 Antibody pre-absorption with synthetic peptide

To confirm specificity of PINK1 antibodies, peptide pre-absorption was carried out. 1ml of PINK1 diluted antibody solution (1:500) was mixed with either excess PINK1 peptide (blocked control) or water (unblocked antibody) and incubated at 37°C for one hour in a shaking incubator. The solutions were then incubated overnight at 4°C on a rotating wheel. The solutions were then spun at 13000 rpm for 30 minutes in a bench top microcentrifuge and the supernatants were removed and used for Western blot analysis on parallel membranes.

2.3.5 Tissue culture reagents

Tissue culture media and media supplements were obtained from Invitrogen Ltd. (Paisley, UK). All tissue culture plastic-ware was obtained from Nunc (Roskilde, Denmark). Sterile 0.2μm filters were obtained from Millipore (Watford, UK). Lipofectamine 2000® reagent was obtained from Invitrogen Ltd. (Paisley, UK). Fugene 6 was obtained from Roche (Lewes, UK). *All trans*-Retinoic acid was from Sigma and was resuspended in dimethylsulfoxide (DMSO) (Sigma) and kept in -70 °C in stocks of 3 mg/ml, always minimizing exposure to light. Colchicine was obtained from Sigma and dissolved in sterile water and kept at 4°C. Geneticin® (G418-sulphate) was obtained from Invitrogen Ltd. (Paisley, UK).

2.3.6 Description and source of cell lines used

2.3.6.1 SH-SY5Y

SH-SY5Y cells are human dopaminergic neuroblastoma cell lines. The cell line was obtained from the European Collection for Animal Cell Culture (ECACC) in the 13th passage. Cells were grown and maintained in 85cm^2 flasks in the following medium: F12 Hams/1X MEM with Earles Salts (1:1) (Gibco-BRL, Paisley, UK); 15% (v/v) Foetal calf serum (PAA Laboratories); 1% (v/v) Non-essential amino acids; 1% (v/v) Penicillin/streptomycin antibiotic; and 2mM L-Glutamine (all from Gibco-BRL). Cells were incubated in a humidified 5% CO_2 atmosphere at 37°C. For storage, cells were frozen long-term in liquid nitrogen in the following medium: 90% Foetal Calf Serum (FCS) + 10% dimethylsulphoxide (DMSO) (Sigma).

2.3.6.2 COS-7

COS-7 cells are monkey kidney derived cell lines and were a kind gift from Dr Ian Giles (UCL, London). Cells were grown and maintained in 175cm^2 flasks in the following medium: Dulbecco's modified Eagle's medium containing sodium pyruvate and pyridoxine (DMEM, Gibco-BRL); 10% foetal calf serum; 100U/ml penicillin; 100μg/ml streptomycin; and 2mM L-glutamine. Cells were incubated in a humidified 5% CO_2 atmosphere at 37°C. For storage, cells were frozen long-term in liquid nitrogen in the following medium: 90% Foetal Calf Serum (FCS) + 10% dimethylsulphoxide (DMSO) (Sigma).

2.3.7 Cell Death Assay Reagents

Trypan blue was obtained from Sigma. Annexin V and Annexin V buffer was obtained from Molecular Probes.

2.3.8 Immunofluorescence Reagents

Goat serum was obtained from Invitrogen Ltd. (Paisley, UK). Coverslips and slides were obtained from BDH. Fluorescent mounting medium was obtained from Dako Ltd. (High Wycombe, Bucks., UK).

2.3.9 Mitochondrial membrane potential studies

Tetramethylrhodamine, methyl ester, perchlorate (T-668) (TMRM) was obtained from Molecular Probes (Eugene, USA). TMRM was solubilised in methanol (200 mM stock

concentration, kept in -20°C) and used at final concentration of 100 nM. The mitochondrial uncoupler carbonyl cyanide chlorophenylhydrazone (CCCP) was dissolved in DMSO and kept at -20°C. It was added to cell culture media prior to FACS analysis at a concentration of 1 μM).

2.3.10 Laboratory Equipment

Trans-Blot™ cell transfer tanks, Bio-Rad Laboratories Ltd. (Hertfordshire, UK)

Bio-Rad GS-800 densitometer, Bio-Rad Laboratories Ltd. (Hertfordshire, UK)

Labsystems Multiskan RC Plate reader (Finland)

Zeiss microscope, Carl Zeiss MicoImaging Inc. (New York, USA)

Beckman Coulter EPICS XL FACS machine, Beckman Coulter Inc. (California, USA)

2.4 BACTERIAL CULTURING AND DNA MANIPULATION

2.4.1 General Measures

Manipulation of bacteria was always conducted over a Bunsen burner flame to create a sterile environment and minimise contamination. Media and glassware were autoclaved before use at 120°C 10 psi for 20 min and also sterile, disposable plastic ware was used.

2.4.2 Propagation of plasmids

E.coli strains XL 1-blue or DH5α were used for the propagation of plasmid DNA. Bacteria were grown either by streaking plates prepared from LB media containing

2% Bacto®-agar or inoculating liquid LB media with single colonies of bacteria. Both media contained appropriate antibiotic for selection. Ampicillin was used at a final concentration of 50 μg/ml (ampicillin stocks were made using double distilled water, filtered sterilised and stored in -20°C in light-protected vials). Bacterial plates were incubated overnight at 37^0 C in an incubator. Liquid cultures were grown overnight at 37^0 C in an orbital shaker at 200 rpm.

2.4.3 Preparation of competent bacterial cells

E.coli strains XL 1-blue or DH5α were made competent to enable them to be transformed (successful entry of plasmid DNA). Competent cells were prepared using the calcium chloride technique (Sambrook et al, 1989). LB agar plates containing tetracycline were streaked from a frozen stock of XL1-Blue cells and incubated at 37^0C overnight. A single fresh bacterial colony was picked and used to inoculate in 20 mls of LB. The cells were grown at 37^0 C overnight. This starter culture of *E. coli* was used to inoculate 100 ml of LB and cultured in the orbital shaker until the culture had an optical density of 600nm of 0.4-0.5 units. The culture was then chilled for 5 minutes on ice and spun in Sorvall at 5000 rpm for 10 minutes at 4^0 C. The cells were resuspended in 500 ml of ice cold 100mM $CaCl_2$, pipetting up and down with a 25 ml pipette. The cells were left on ice for a further 30 minutes, then spun at 5000 rpm for 10 minutes at 4^0 C. The cell pellet was resuspended in 20 ml of $CaCl_2$ and 15% glycerol. 200 μl aliquots were made and left on ice for 1 hour, followed by storage at -70^0 C.

2.4.4 Transformation of bacteria

Calcium chloride competent cells were transformed using the heat shock technique. Cells were thawed on ice. Approximately 10ng of plasmid DNA of cells was added to the competent cells and incubated on ice for further 30 minutes. The cells were then heat shocked by incubating them at 42^0 C for 90 seconds and then returned to ice for 2 minutes. 800µl of LB was then added and the cell suspension was incubated for 1 hour at 37^0 C in an orbital shaker. The cells were then plated out on appropriate antibiotic plates. The plates were incubated at 37^0 C overnight. Plates were stored at 4^0 C for up to one month.

2.4.5 Plasmid DNA extraction from transformed bacteria

2.4.5.1 Small scale plasmid DNA extraction

For small-scale plasmid DNA extraction DNA was prepared using the QIAprep Mini DNA prep kit according to manufacturer's instructions from 5ml bacterial cultures. The DNA extraction method of this kit is based on a modification of the alkaline lysis protocol (Birnboim & Doly, 1979) namely alkaline lysis of the bacteria; plasmid separation on a Qiagen resin; plasmid DNA wash; elution and isopropanol precipitation. Single bacterial colonies were used to inoculate 5 mls of LB containing the appropriate antibiotic selection. Cultures were incubated overnight at 37^0 C at the speed of 200 rpm. 1.5 ml of culture was pellet by centrifugation at 13000 rpm for 2 minutes in a bench top microcentrifuge. DNA was subsequently extracted from the cell pellet by the above kit.

2.4.5.2 Large scale plasmid DNA extraction

E.coli were grown in a 5 ml starter culture of LB with antibiotic in an orbital shaker at 37°C at 200 rpm and was used to inoculate 200ml of LB with antibiotic overnight for plasmid preparation. The overnight bacterial culture was used for large scale purification of plasmid DNA with the Qiagen-tip 100 midi-prep kit. The DNA pellet was washed with 70% ethanol, dried briefly at room temperature and resuspended in autoclaved double distilled water.

2.4.6 Restriction Digestion of plasmid DNA

Small-scale volume (20μl) restriction endonuclease digests were performed in order to characterise a plasmid or large-scale digests (up to 200 μl) were performed in order to isolate a DNA fragment and subsequently ligate it into an appropriate vector. For all the enzymes used, a maximum of 0.1 volumes of restriction enzyme(s) were added and the buffer recommended by the manufacturer was used at 1 X concentration. Digests were incubated for the appropriate time and temperature. The digested DNA was electrophoresed on an agarose gel of appropriate percentage and the products visualized on a UV illuminator. An undigested sample was run in parallel, allowing a reference against which to compare the enzyme cut samples.

2.4.7 Agarose gel electrophoresis

The percentage of agarose gels in 1 X TAE was cast depending on the expected size of the DNA products. For example, 1% gel was cast for fragments larger than 1kb, 1.5% for general purpose, and 2% to resolve DNA fragments less than 1kb. Ethidium bromide was added to the agarose solution to a final concentration of 0.5 μg/ml.

Approximately 0.1 volume of 10 X loading buffer (1 X TAE, 50% v/v glycerol, 0.025% bromophenol blue) was added to DNA samples prior to loading. A DNA ladder corresponding to the sizes of the fragments being analysed was run in a parallel lane. Electrophoresis of the DNA was carried out at 100 mA for 0.5 to 2 hours, allowing for a good resolution of bands. Bands were visualized on a long wave UV transilluminator and photographed using the Syngene gel doc system.

2.5 TISSUE CULTURE

2.5.1 General Measures

All cell lines were maintained at 37^0 C in a 5% CO_2 incubator. All manipulations of cells were carried out under standard aseptic techniques. For long-term storage cells were stored in liquid nitrogen at -180^0 C.

2.5.2 Routine passaging of cells

Cells were grown in the appropriate growth medium until the flasks reached ~80% confluency when they were passaged. Briefly, the cell monolayer was washed with Hank's Balanced Salt Solution (HBSS) free of calcium and magnesium ions; trypsin/versene (1:10) was then added to detach the cells from the plastic ware and cells were harvested using fresh medium. Cells were then pelleted by centrifuging at 700 rpm for 5 mins in a Sorvall benchtop centrifuge. Cells were then resuspended in fresh medium and seeded at a ratio of 1:10 into fresh flasks.

2.5.3 Freezing and thawing cell stocks

Cell stocks were prepared by resuspending the cell pellet from one 25cm^2 flask in 0.4ml of 10% (v/v) DMSO, 90% FCS. Cells were frozen in cryotubes at -80^{0}C for 48 hours before being immersed in liquid nitrogen for long term storage.

Cells were recovered from frozen stocks by rapid thawing of cryotubes and adding the cell suspension to 4mls of fresh medium in a falcon tube. Cells were then pelleted as described above and resuspended in fresh medium before transferring to a 25 cm^2 flask. The medium was changed and cells passaged as required.

2.5.4 Transient transfections using Lipofectamine 2000®

Transfections were carried out using the Lipofectamine 2000® reagent (GibcoBRL, Life technologies). SH-SY5Y cells were plated out at 1 X 10^5 cells per well in six-well plates and left overnight. The following day transfection complexes, comprising 3 μg plasmid DNA and 4 μl Lipofectamine 2000® per well of a 6-well plate, were prepared. The relative amount was scaled up as required for the number of wells being transfected. The complexes were allowed to form at room temperature for 20 minutes and then diluted with Optimem + Glutamax 1 (Gibco) to a concentration of 3 μg DNA per 1 ml per well. The full growth medium from the cells was removed and the cells were washed in 1 X HBSS twice. 1 ml of transfection complex was added to each well and the cells were incubated for 4 h at 37^{0}C. The transfection medium was removed and replaced with the normal full growth medium.

2.5.5 Stable cell production

SH-SY5Y cells were plated out at 1x 10^5 in six well dishes and transfected using Lipofectamine 2000® transfection reagent as described in section 2.5.4. The cells were grown in G418-containing medium to select for cells stably expressing the neomycin gene. It was assumed that cells exhibiting resistance to G418 were also stably expressing either parkin sense or parkin anti-sense plasmid DNA. After ~10 days, individual colonies were picked by sterile pipettes using a Gilson P-1000 pipetteman under a microscope and transferred in volumes of 200 μl of medium to 24-well plates. Once cells reached 70% confluency, they were transferred into 6-well plates. Cells from each colony were then split into two 6-wells and one well was grown for storage as a DMSO stock (after transfer into a 25 cm^2 flask) and the other well was grown for characterisation by Western immuno-blotting.

2.5.6 Cellular stress studies

For each stress, at least two different clones stably expressing wild type parkin were used, as well as at least two different control clones, unless otherwise stated. The clones used had similar protein expression levels (see Results section). The use of more than one clone per cell line is an extra control measure which ensures that the response of a cell line to a stressor is due to expression of the gene of interest and not due to the site of integration of the plasmid DNA into the SH-SY5Y cell genomic DNA.

2.5.6.1 MG-132 stress

Cells were incubated for 24 hours in full growth media containing 10 - 15 μM MG-132 (Z-Leu-Leu-Leu-H; Sigma) or vehicle (DMSO). The cells were then harvested and the percentage of cell death was assayed using the trypan blue exclusion assay.

2.5.6.2 Dopamine stress

Cells were incubated for 24 hours in full growth media containing 0.125mM dopamine (3-hyroxytyramine hydrochloride; Sigma) or vehicle (water). Cell death was quantified by the trypan blue exclusion assay. for 24 hours.

2.5.6.3 Staurosporine stress

Cells were treated in full growth medium with 5 μM staurosporine (Sigma) or vehicle (DMSO) for 4 hours and cell death was quantified by trypan blue exclusion.

2.5.6.4 Tunicamycin stress

Cells were incubated for 24 hours in full growth medium containing 50μg/ml tunicamycin (Sigma) or vehicle (DMSO) Cell death was assayed by trypan blue exclusion.

2.5.7 Trypan blue dye exclusion assay

The extent of cell death was assayed by measuring the percentage of viable cells able to exclude trypan blue by adding an equal volume of 0.4% trypan blue in phosphate-buffered saline to an aliquot of the cell suspension. Mixtures were incubated at room temperature for 5 min, and the proportion of cells able to exclude trypan blue was assessed by counting using a haemocytometer at x 40 magnification (Weber Scientific International Ltd., UK).

2.5.8 Annexin V assay

To determine the level of apoptosis, a FACS based assay was used. Cells were transfected and stressed with vehicle or MG-132 as described above. Cells were then harvested and washed in PBS and pelleted. Pellets were resuspended in 1X Annexin V binding buffer (BD Biosciences, Oxford, UK) and then incubated with Annexin V conjugated with phycoerythrin (Annexin V-PE) for 15mins at room temperature before being analyzed immediately on an Epics XL flow cytometer. For each sample, 20,000 cells were analyzed and Annexin V-PE fluorescence was determined in the FL2 channel gated for GFP positive events. Experiments were performed in triplicate

2.5.9 Mitochondrial membrane potential ($\Delta\psi m$) assay

To measure mitochondrial membrane potential ($\Delta\psi m$) a fluorescence-activated cell sorting (FACS)-based assay was used. Cells were treated with vehicle or 15μM MG-132 for 24 hours, then incubated with 10μM verapamil (Sigma, Paisley, UK) and 100nM of the $\Delta\psi m$-sensitive dye tetramethylrhodamine methyl ester (Molecular

Probes, Leiden, Netherlands) in a humidified 5% CO_2 atmosphere at 37°C for 45 min. Cells were then harvested with trypsin, pelleted and resuspended in phosphate buffered saline (PBS) on ice. For each sample, 20,000 cells (events) were analyzed on an Epics XL flow cytometer with a 488-nm argon laser. The TMRM signal was analysed in the FL2 channel gated for GFP positive events. The channel was equipped with a band-pass filter at 580±30 nm; the photomultiplier value of the detector was 631V. Data were acquired on a logarithmic scale. Arithmetic mean values of the median fluorescence intensities (MFI) were generated for graphic representation. Experiments were performed in duplicate or triplicate.

2.6 IMMUNOFLUORESCENCE AND MICROSCOPY

Cells were fixed and prepared for immunofluorescence and standard fluorescent or confocal microscopy. Cells were washed three times in PBS and fixed in 4% paraformaldehyde for 10 min. Cells were washed three times with PBS and permeabilised for 5 min in 0.1% Triton X-100 in TBS (TBST). Coverslips were then incubated in block buffer (20% goat serum, 0.1% TBST) for 20 min. Cells were incubated overnight at 4°C with the appropriate primary antibody in antibody diluting buffer (1% goat serum, 0.1% TBST). Coverslips were washed three times in 0.1% TBST and incubated for 30 min at room temperature with the appropriate secondary antibody in antibody diluting buffer. For nuclear staining Hoechst 33258 (2μg/ml) was added to the secondary antibodies in buffer. For double labelling studies coverslips were incubated with primary antibodies sequentially at 4°C for 12 hours each and after washes were incubated with a mixture of both secondaries. Coverslips were washed three times with TBS and mounted on glass slides (Fisher) with anti-fade mounting medium (Dako). Standard immunofluorescence was performed using

a Zeiss Axioskop 2 plus microscope and digital images were captured using a Zeiss Axiocam camera. Confocal images were obtained using a Leica TCS SP II Confocal Scanner and images were processed in Adobe Photoshop (v.VII). Negative controls omitting each primary antibody separately or in combination were performed in each case and no significant staining was seen.

2.7 ANALYSIS OF PROTEINS

2.7.1 Protein extraction from cultured cell lines

Confluent 6-well plates or 25cm^2 flasks were washed in HBSS and cells trypsinised and harvested in fresh media. Cells were pelleted at 7g for 2 minutes. Pellets were resuspended in filter sterilised 1X PBS and repelleted at 7g for 2 minutes. Pellets were lysed with appropriate volumes of protein lysis buffer. Extracted protein samples were stored at -80^{0}C. A variety of lysis buffers have been used in this work and the names and constituents are listed in the appendix.

2.7.2 Mitochondrial fractionation from cell lines

COS-7 cells were grown in 175 cm^2 flasks and transfected with wild-type myc-*PINK1* cDNA using Fugene 6 (Roche). Cells were harvested 48 hours post-transfection, washed twice in PBS, and re-suspended in buffer containing 250 mM sucrose, 20 mM HEPES, 3 mM EDTA, pH 7.5 and protease inhibitors at 4°C. All further steps were carried out at 4°C. Cells were then disrupted using a glass hand held homogeniser (10 passes). For rat brain studies, fresh rat brain was homogenized using a glass hand homogenizer as described above and all subsequent steps were similar.

Disrupted material was spun at 830*g* for 10 min. The supernatant was removed and retained (*S1*). The pellet was rehomogenised and spun as before. The pellet was then retained as the *P2* fraction and contains largely unhomogenised and insoluble material. Supernatant was removed (*S2*) and both supernatants were then centrifuged at 16,800*g* for 10 min. The supernatant was removed and retained as the cytoplasmic fraction (*C*). The mitochondrial pellets (*M*) were combined and re-suspended in the above buffer. The cytoplasmic fractions were concentrated using Centricon devices (Millipore, Watford, UK) as per the manufacturer's instructions. Protein concentrations in cytoplasmic and mitochondrial enriched fractions were determined by BCA assay and equal amounts of cytoplasmic and mitochondrial lysates were loaded and analyzed by Western blotting using PINK1 antibody as described above. The membrane was stripped and reprobed with anti-GAPDH and anti-complex I antibodies (1:1,000; Molecular Probes, Leiden, Netherlands) to confirm relative purity of cytoplasmic and mitochondrial fractions respectively.

To fractionate mitochondrial preparations into membrane and matrix fractions, mitochondrial pellets were re-suspended in Tris buffer (10 mM Tris-HCl pH7.6; 100 mM NaCl) and sonicated with a probe tip sonicator (3x6 pulses, 40% duty cycle, microtip output setting 5) and ultra-centrifuged at 100,000 g for 30 mins at 4°C. Pellets were re-suspended in Tris buffer and protein concentrations in both fractions were determined by BCA assay. Equal concentrations of fractions were analyzed by SDS-PAGE and western blot analysis and relative purity of fractions was confirmed by re-probing membranes with mitochondrial membrane and matrix markers VDAC1 and MnSOD respectively.

To determine membrane integration of PINK1, soluble and peripheral membrane proteins were extracted from integral membrane proteins through the use of carbonate extraction (Fujiki et al., 1982). Briefly, mitochondrial pellets were re-

suspended in 0.1M sodium carbonate, pH 11.5. Following incubation on ice for 30 mins, mitochondrial membranes were re-isolated by ultra-centrifugation at 100,000 g for 30 mins at 4°C. Fractions were analyzed by SDS-PAGE and Western blotting.

2.7.3 Protein extraction from flash frozen human and rodent brain

Flash frozen post-mortem human brain was homogenised in 0.1% Tween-20 buffer with protease inhibitors (50 mM Tris-HCl pH 7.4; 150 mM NaCl; 1 mM EDTA; 0.1% Tween-20; 10% (v/v) glycerol) using a hand held polytron homogeniser (Kinematica AG, Luzern, Switzerland), incubated on ice for 30 minutes and spun at 13,000 g for 30 minutes at 4°C. Supernatants were removed and pellet fractions were boiled in Laemmli buffer – both fractions were analyzed. Protein concentrations in all supernatants were determined using BCA assay. Lysates were run on 10% SDS-PAGE gel; transferred to nitrocellulose membranes and probed with PINK1-49 antibody (1:500) overnight at 4°C and HRP-conjugated anti-rabbit IgGs (1:2,000; Dako) for 1 hour at room temperature. Membranes were developed using ECL reagent (Bio-Rad, UK). Protein loading was confirmed by reprobing membranes with GAPDH.

Fresh mouse and rat brains were extracted for protein using 0.1% Tween 20 buffer with protease inhibitors as described for human brain. In additional experiments mouse and rat brain were homogenized in isotonic sucrose buffered with HEPES and containing protease inhibitors (Sigma, Poole) and then spun at 13,000 g for 10 minutes to obtain supernatants for protein analysis.

2.7.4 Preparation of soluble and insoluble fractions from cell lines

Cells were processed as per Garcia-Mata et al. (1999). Untransfected COS-7 cells and COS-7 cells transfected with either c-myc-wt-PINK1 or PINK1-pcDNA3.1 were harvested with ice-cold PBS following trypsinization. Pellets were washed x3 with PBS with in-between 7 g spins and re-suspended with PBS supplemented with protease inhibitors. Pellets were then washed x3 with PBS and lyzed for 30 mins on ice with 2% Triton X-100 in PBS in the presence of protease inhibitors or 15 mins on ice with RIPA buffer in the presence of protease inhibitors. Lysates were then passed 10 times through a 27-guage needle. Triton-X100 and RIPA lysates were spun at 13,000 g for 15 mins at 4°C. Insoluble pellets were re-suspended in 1% SDS in PBS and sonicated for 20s with a microtip sonicator. Protein concentrations were determined in soluble supernatant fractions using BCA assay and equal volumes of pellet and supernatants were re-suspended in Laemmli buffer, boiled for 5 mins and analyzed by SDS-PAGE and Western blot.

2.7.5 Determination of protein concentration

To ensure equal protein loading and analysis, protein concentration was determined by Bradford chemistry using BCA Protein Assay Reagent from Pierce (Illinois, U.S. A.) according to the manufacturer's instructions. Briefly, BCA Reagent A and BCA Reagent B were mixed at a ratio of 50:1. 195 μl of this mixture was added to the appropriate number of wells of a 96-well microtitre plate. 5 μl of each protein sample

of unknown concentration was added to the individual wells of the plate. Additionally, a titration series of known concentrations of bovine serum albumin were also added to individual wells containing the BCA mixture on the plate. This was carried out to generate a concentration standard curve. The plate was incubated at 37°C for 30 minutes and cooled to room temperature prior to exposure to the SmartSpec™ 3000 spectrophotometer (Bio-Rad) which was set to read the absorbance at 562 nm. The value of the absorbance of the blank was subtracted from all other values and a standard curve was generated from the absorbance readings obtained from the albumin titration. Finally, protein concentrations of the unknown samples were determined by comparing their values of absorbance to the albumin standard curve.

2.7.6 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Standard SDS-polyacrylamide gels were prepared with the composition for the stacking and resolving gels as described by Sambrook and colleagues (Sambrook et al, 1989). Protein samples were heated to 95^0 C for 5 minutes. 50-100 μg of protein from each sample and 10 μl of protein marker were prepared and run in a vertical gel electrophoresis system (Sambrook et al, 1989). The gels were run at a constant voltage of 100 V in 1 X running buffer (25 mM Tris, 250 mM glycine and 0.15 (w/v) SDS, pH 8.3). Gels were run until the protein of interest was sufficiently resolved as determined by the migration of the molecular weight marker.

2.7.7 *Western Blotting:*

2.7.7.1 Transfer of proteins to nitrocellulose membrane

Proteins resolved by SDS-PAGE gels were transferred onto Hybond C membrane using a wet-transfer method (Sambrook et al., 1989). Briefly, the nitrocellulose membrane and the SDS-PAGE gel were placed between two sheets of 3 MM Whatman paper. The sandwiched gel was presoaked in transfer buffer (50 mM Tris, 380 mM glycine, 0.1% (w/v) SDS, and 20% (v/v) methanol) and a Trans-Blot TM Cell (BioRad) was assembled according to the manufacture's instructions. The transfer was carried out at 100V for 1 hour at room temperature.

2.7.7.2 Immuno-probing of membrane with antibody

Following transfer of proteins onto the nitrocellulose membrane, the membrane was blocked (to reduce non-specific binding) by incubating in 5% (w/v) skimmed milk powder in 0.1% PBS/Tween (PBST) 20 for 1 hour at room temperature with constant shaking. All subsequent incubations were also carried out at room temperature on a shaker unless specified otherwise. The membrane was then incubated with the appropriate primary antibody diluted in 5%milk/0.1% PBST overnight at 4°C. Any unbound, non-specific binding of antibody was removed by washing the membrane in 0.1%PBST for 3 X 5 minutes. The membrane was then incubated with the appropriate anti-IgG horseradish peroxidase (HRP) conjugated secondary antibody for 1 hour at room temperature. The membrane was washed three times for 5 minutes each in 0.1%PBST to remove any unbound antibody. The bound HRP was detected using an enhanced chemiluminescence system (ECL^{TM}) according to the

manufacturer's instructions. Finally, the membrane was exposed to X-ray film in order to capture the resultant light emissions. Depending on the strength of the signal, the exposure time varied from 1 second to 1 hour.

2.8 ELECTRON MICROSCOPY (EM)

2.8.1 Transmission EM

SH-SY5Y cells were grown on plastic slides and fixed using 3% gluteraldehyde in 0.1% sodium cacodylate buffer and 5mM $CaCl_2$ pH 7.4. Cells were embedded in araldite resin mixture and sectioned using a diamond knife. Sections were stained using uranyl acetate and images taken on a Jeol 1200EX transmission electron microscope.

2.8.2 Immunogold EM

Preparation of COS-7 cells was carried out using the Tokuyasu method as described previously (Tokuyasu et al., 1986). Cells were stained with mouse monoclonal anti-c-myc antibody (1:200) that was shown to give no background staining in untransfected controls (data not shown). Images were taken on a Jeol 1010 electron microscope.

2.9 STATISTICAL ANALYSIS

Data collected from replicated experiments were analyzed by normal parametric tests including Student's T-test for single comparisons and one way analysis of variance

(ANOVA) for group comparisons. Multiple comparisons were performed with *post hoc* Bonferroni *t*-test, using SPSS PC software. The significance level was set at $p<0.05$ unless otherwise indicated.

3

Studies of Parkin in Cell Death and Aggregate formation *in vitro*.

3.1 INTRODUCTION

3.1.1 Aggresomes, Aggregates and Parkinson's disease

Lewy bodies (LBs) are one of the pathological hallmarks of Parkinson's disease (PD) but their role in the pathogenesis of PD is unclear. It remains unknown whether they are toxic and contribute to neuronal death; protective in response to an as yet unidentified stress; or just an epiphenomenon (Forno, 1996). As explained in Chapter 1, work performed in collaboration with Dr Rina Bandopadhay (UCL, London) as well as other labs worldwide have confirmed that parkin appears to be a major component of LBs in sporadic PD (Figure 3.1) (Shimura et al., 2000; Schlossmacher et al., 2002; Bandopadhay et al., 2005). However, intriguingly the majority of parkin-related autosomal recessive juvenile parkinsonism (AR-JP) brains do not contain LBs (Hayashi, 2000; Mori, 1998; Schlossmacher, 2002; Shimura, 1999; Takahashi, 1994; van de Warrenburg, 2001). The general absence of LBs in AR-JP suggests that parkin may play an important role in LB formation.

The major constituent of LBs is the PD associated protein, α-synuclein (PARK1), however, a wide variety of other proteins have been described including

components of the ubiquitin–proteasome system (UPS); chaperones such as hsp70; and cytoskeletal proteins such as neurofilaments (Olanow et al., 2004). The presence of chaperones and UPS components suggests that LBs may have been processing centres for neurons to handle misfolded and damaged proteins that would usually be eliminated by ubiquitin-dependent degradation but which accumulated in the face of impairment of the UPS caused by neuronal stress. Several lines of evidence support the possibility that the mechanisms of LB formation resemble that of aggresomes. Aggresomes are perinuclear inclusions that form at the microtubule organizing centre (MTOC) of cells and may represent active centres for protein degradation when there is an excess buildup of misfolded and damaged proteins arising from a variety of mechanisms including proteasome inhibition (Johnston et al., 1998; McNaught et al., 2002). Both aggresomes and LBs are eosinophilic inclusions; have a core and halo organization; stain thioflavin S positive; are juxtanuclear and share common components such as ubiquitin (McNaught et al., 2002).

3.1.2 Parkin and Neuroprotection

In keeping with other RING finger proteins, parkin functions as an E3 ubiquitin ligase (Shimura et al., 2000). E3 ligases perform the final step in tagging proteins with ubiquitin thereby targeting them for degradation by the UPS. As previously discussed the UPS plays an essential role to remove damaged or misfolded proteins from a wide variety of systems and pathways within the cell (Ciechanover, 1998). Clearly identification of the substrates of parkin has been critical in furthering our understanding of the normal function of parkin and how mutations may lead to neurodegeneration (Chapter 1). However, equally important has been the utilisation

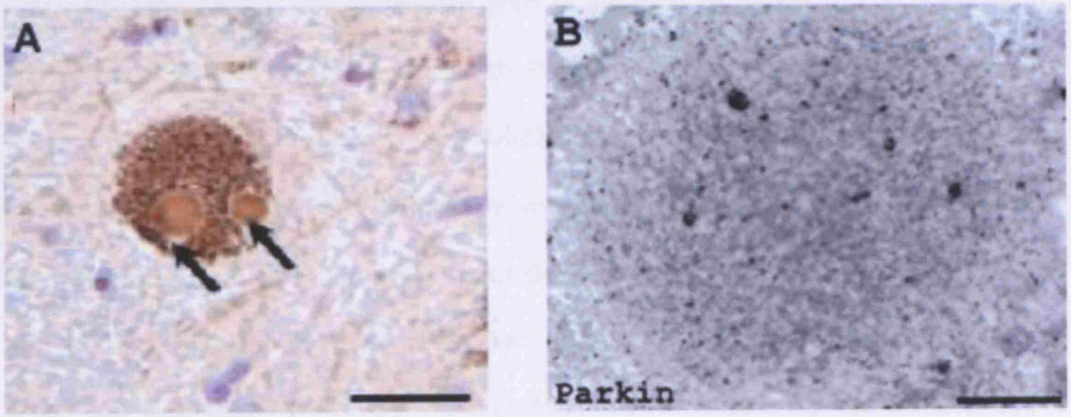


Figure 3.1 Parkin localizes to Lewy bodies (LBs) in Parkinson's disease brain. (A) Immunohistochemistry of parkin in PD brain section. LBs in melanised dopaminergic neuron of substantia nigra showing parkin in the central core (arrows). Scale bar: 20μm. **(B)** Electron micrograph of a typical nigral LB showing positive immunogold labelling for parkin in the central core. Scale bar: 2μm.

Modified from Bandopadhay, Kingsbury, Muqit et al. (2005).

of *in vitro* cell models of parkin and cellular stresses to reveal a neuroprotective role for parkin and identify the cellular pathways in which parkin confers this protection.

The first report came from Imai and colleagues (2000) who observed that parkin over-expression in SH-SY5Y neuroblastoma cells protected cells against unfolded protein-induced stress stimulated by the N-glycosylation inhibitor, tunicamycin (Imai et al., 2001). This implicated the unfolded protein response pathway (UPR) which was consistent with localisation of parkin to the endoplasmic reticulum (ER) and its interaction with Parkin associated endothelin-like receptor (Pael-R) within the ER (Imai et al., 2002). Parkin has also been shown to protect against proteasomal stress induced by α-synuclein in both cell lines and primary mesencephalic cultures and parkin knock-down rendered cell more susceptible to the effects of proteasomal stress (Petrucelli et al., 2002). The mechanism for this protection is unclear, however, most proteasomal inhibitors do not achieve 100% inhibition and the residual activity may be sufficient for the removal of toxic proteins that are ubiquitylated by parkin.

The role of these pathways has been confirmed *in vivo* with the demonstration that parkin can protect against over-expression of Pael-R and α-synuclein in *Drosophila* (Yang et al., 2003). Cell studies have implicated the mitochondrial pathway with the report that parkin can protect against ceramide in rat PC12 cells and this is associated with decreased cytochrome c release from mitochondria (Darios et al., 2003). Parkin has also been shown to protect against kainate-induced toxicity via prevention of the accumulation of cyclin E a protein involved in the regulation of neuronal apoptosis (Staropoli et al., 2003). The mechanism for this implicates parkin as part of a multi-protein complex comprising a F-box/WD repeat protein hSel-10 and Cullin-1 whereby hSel-10 serves to target the parkin ubiquitin ligase activity to cyclin E (Staropoli et al., 2003).

3.1.3 Chapter 3 Aims

In view of the considerable interest in the role of Lewy bodies in the pathogenesis of PD this work set out to determine parkin's role in aggregate formation and to define how this was related to parkin's neuroprotective role. The first stage of work was to examine the role of endogenous parkin in aggregate formation and then to determine the effect of altered parkin expression on aggregate formation and cell death.

The aims are:

(a) To determine the effect of cellular stress on the localization of endogenous parkin

(b) To establish parkin over-expressing and parkin anti-sense stable mammalian cell lines

(c) To determine the effect of cellular stress on aggresome formation and neuronal survival in cells with altered parkin

(d) To determine the effect of disease causing mutants on parkin function on aggregates

3.2 ROLE OF PARKIN IN AGGRESOME FORMATION

3.2.1 Endogenous parkin can localize to aggresomes in a stress-specific manner

For these studies, the human dopaminergic neuroblastoma SH-SY5Y cell line was chosen. The SH-SY5Y cell is a thrice-cloned sub-line from a human bone marrow

biopsy-derived line SK-N-SH (Ross and Biedler, 1985). SH-SY5Y cells proliferate as neuroblasts. However, they can be differentiated under specific conditions to neuronal cells expressing dopaminergic markers and displaying outgrowth of neuronal processes. These cell lines are the most comprehensively studied and utilized in the context of studies on the pathogenesis of PD. In particular SH-SY5Y cells have been shown to undergo both apoptopic and non-apoptopic cell death upon exposure to a wide array of agents that damage pathways implicated in PD. Therefore these cell lines were felt to be the most appropriate to examine the role of parkin in both aggregation and cell death.

Parkin had previously been detected in aggresomes (Junn et al., 2002) or inclusions (Ardley et al., 2003) in parkin over-expressing neuronal cell lines exposed to proteosome inhibitors. Since LBs form with normal levels of parkin in human PD brain *in vivo*, this work aimed to determine whether endogenous parkin could localize to aggresomes *in vitro*. Furthermore, since multiple cellular pathways in addition to UPS dysfunction, have been implicated in PD pathogenesis, a variety of other cellular stressors in addition to proteasome inhibitors were selected to determine whether aggregate formation was specific to one or more pathways.

Western blot analysis using a commercial C-terminal polyclonal antibody to parkin (Cell Signaling, USA), confirmed that SH-SY5Y cells express endogenous parkin (Figure 3.2). Immunofluorescence studies with the same antibody and Hoechst counterstain (for nuclei), consistently showed diffuse parkin staining in the cytoplasm (Figure 3.3A).

SH-SY5Y cells were then incubated in a final concentration of 10μM of the peptide aldehyde, Cbz-leu-leu-leucinal (MG-132) that is a low molecular weight inhibitor of the proteosome (Palombella, 1994) for 24 h. Immunofluoresence studies demonstrated that endogenous parkin formed a single large perinuclear aggresome-

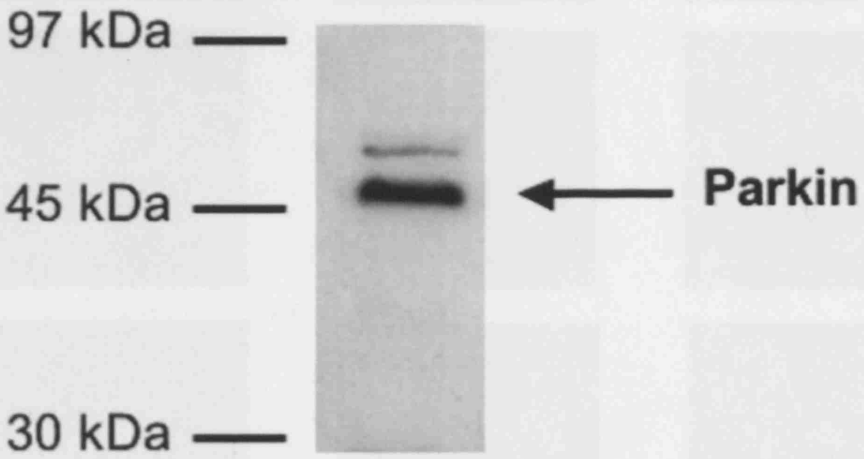
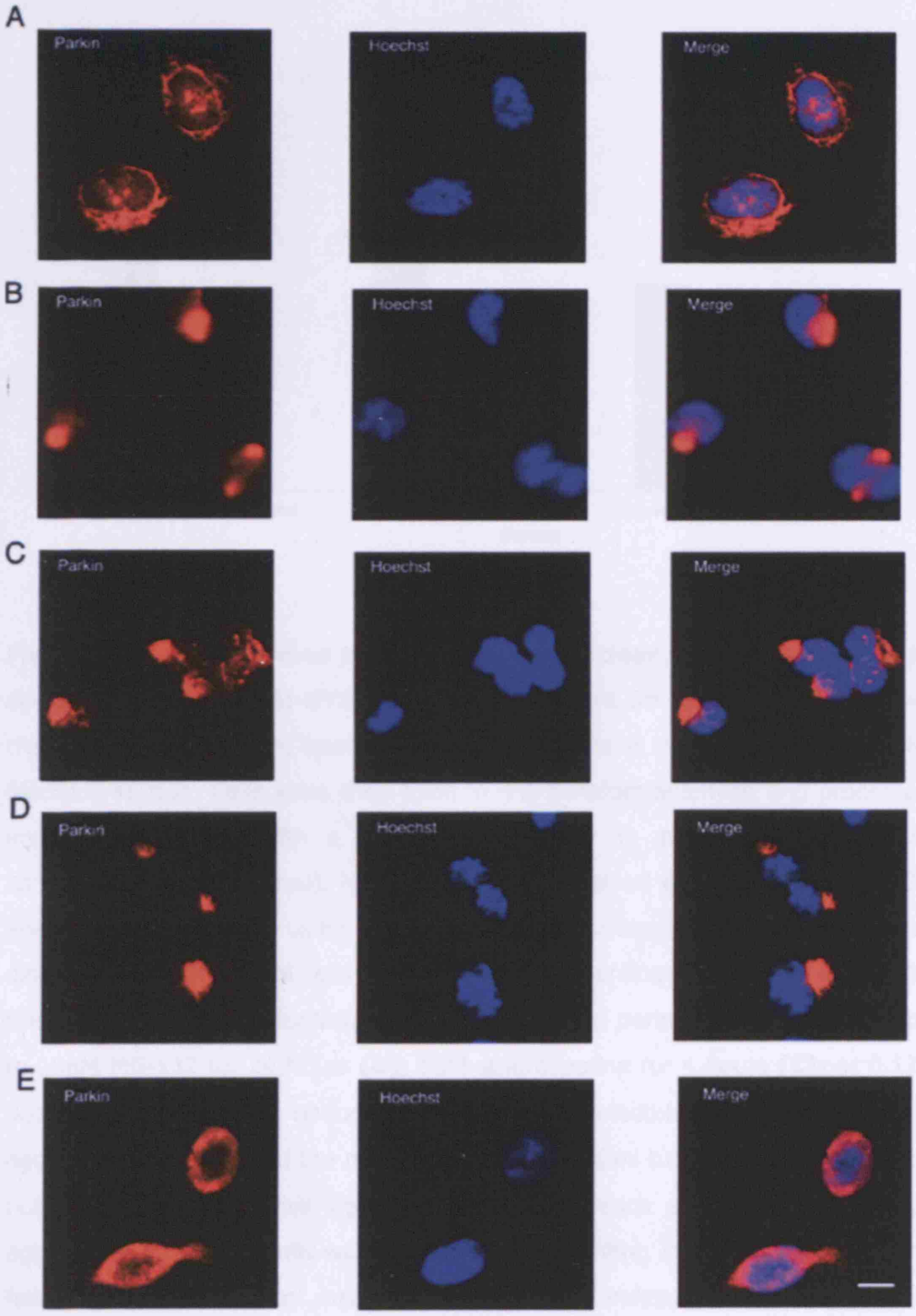


Figure 3.2 Parkin expression in native SH-SY5Y cells. Representative Western blot of SH-SY5Y lysates probed with a C-terminal parkin antibody (1:1000).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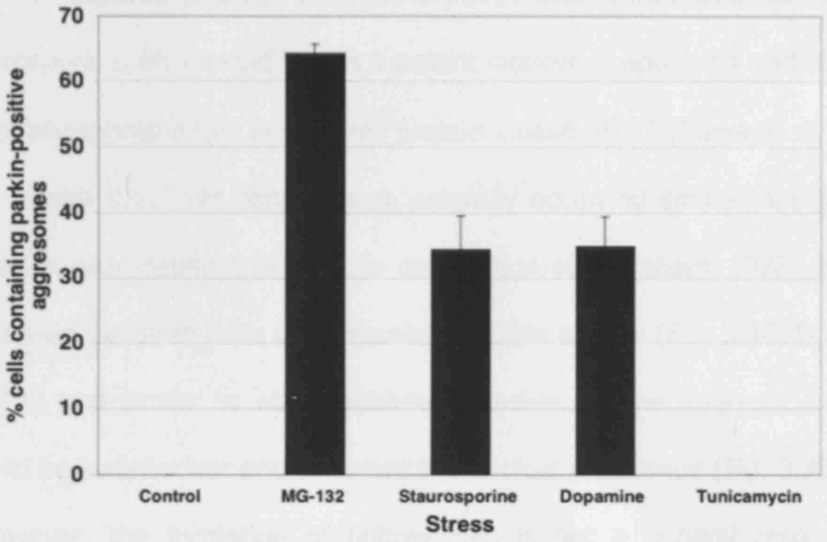


Figure 3.3 Endogenous parkin forms perinuclear aggresomes in a stress-specific manner in SH-SY5Y cells. Sub-confluent SH-SY5Y cells were grown on glass coverslips and were treated with various agents as detailed in the Materials and Methods section. Cells were then fixed in 4% paraformaldehyde and processed for immunofluorescence with a C-terminal antibody to parkin and a rhodamine-conjugated secondary (red). Nuclei were counterstained with Hoechst (blue). Digital images were captured using a Zeiss Axiocam camera. Representative fields are displayed. **(A)** In control cells exposed to vehicle, endogenous parkin was detected predominantly in the cytoplasm especially within the perinuclear region. In response to 10μM MG-132 for 24 hours **(B)**; 5μM staurosporine for 4 hours **(C)**; or 0.125mM dopamine for 24 hours, endogenous parkin was detectable as a single perinuclear aggresome that indented the nucleus **(D)**. **(E)** 50μg/ml tunicamycin for 24 hours did not induce parkin-positive aggresomes. **(F)** For each stress, the percentage of aggresome-containing cells was determined by counting 300 cells across 20 random fields. The data represent means + S.E.M. for 3-5 independent experiments. Scale Bar 10μm.

like structure in ~65% of cells (Figure 3.3B and 3.3F) compared to no aggresomes in untreated cells (Fig. 3.3A and 3.3F). To determine if parkin-positive aggresomes could form in response to other stresses, SH-SY5Y cells were incubated for 4 h with 5 μM staurosporine, an alkaloid that is a potent inducer of apoptosis and non-specific inhibitor of phospholipid/Ca^{2+} dependent protein kinase (PKC) (Tamaoki et al., 1986) or for 24 h with 0.125mM dopamine, a naturally occurring amine that is prone to auto-oxidation with resultant increase in oxidative stress (Graham, 1978). Both these stresses caused parkin to form aggresomes in ~35% of cells (Fig. 3.3C, D and F). As with MG-132 and similar to other published studies, all the aggresomes detected appeared to be juxtanuclear and to indent the nuclear membrane (Fig. 3.3B–D).

However, the formation of aggresomes is not a general response to all stresses since parkin did not form aggresomes in cells incubated for 24 h with 50 μg/ml tunicamycin, a specific inhibitor of N-glycosylation in the endoplasmic reticulum and potent inducer of the UPR pathway (Kozutsumi, 1988) (Fig. 3.3E and F). Given that the concentration and duration of tunicamycin used produced a biological effect comparable with the other stresses (i.e. ~50% cell death), it is most likely that the absence of aggresome formation is specific to tunicamycin for the conditions used in this study. Moreover, the concentration of tunicamycin that we used was five times higher and of longer duration than previous studies which have also not observed significant aggregate formation with this compound in neuronal cells (Ardley et al., 2003; Ledesma, 2002).

3.2.2 The composition of aggresomes is dependent on the stress

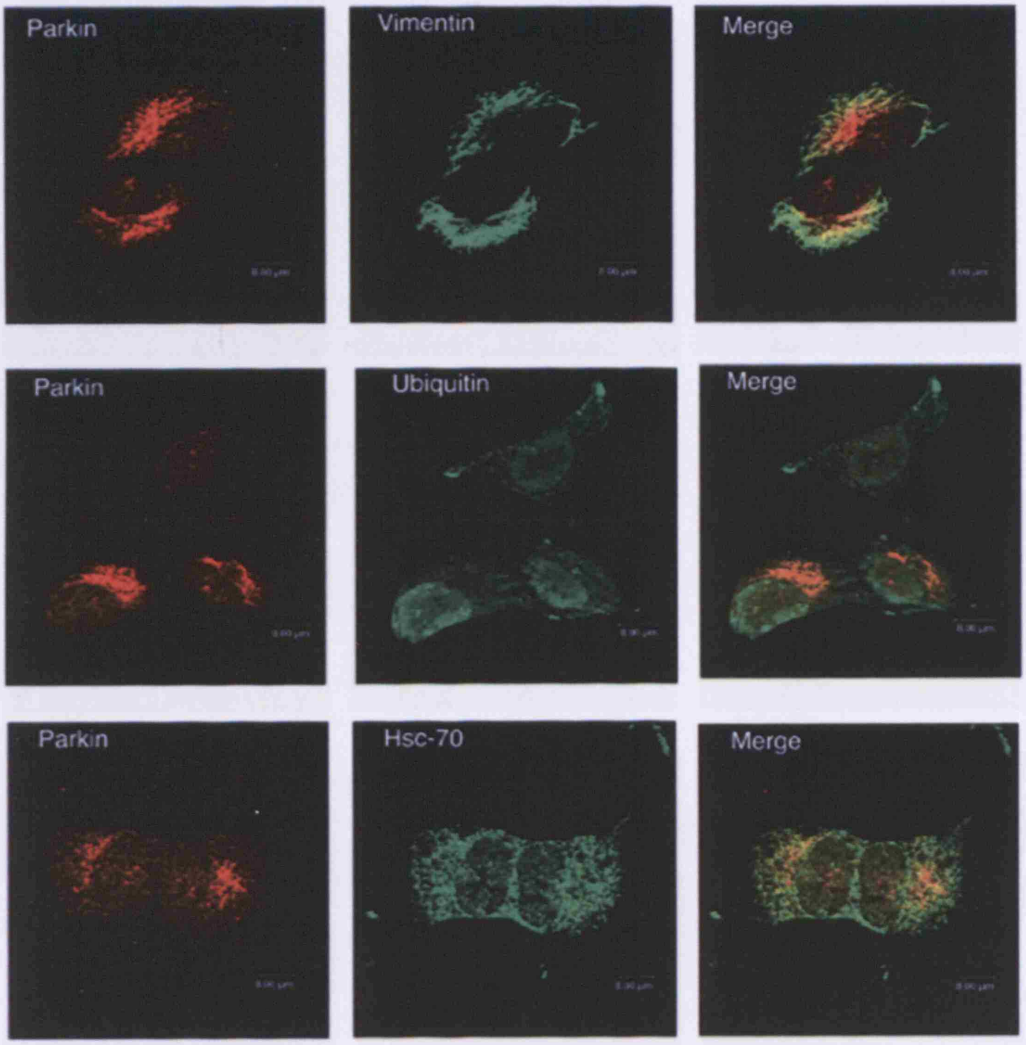
To determine whether these stress-induced parkin-positive aggresomes had the typical structure of previously reported aggresomes their composition was

characterized. It has previously been reported that the intermediate filament, component, vimentin, is invariaby present in aggresomes where it forms a cage-like structure around the outside of the aggresome (Kopito, 2000). In addition, aggresomes are enriched in chaperones such as Hsc-70 and are often reported to be ubiquitylated.

We undertook co-localization studies with an anti-parkin antibody together with anti-vimentin, anti-Hsc-70 and an antiubiquitin antibody that recognizes mono- and polyubiquitin. In SH-SY5Y cells treated with vehicle (DMSO or water) parkin was expressed predominantly in the cytoplasm with nuclear speckling and the staining was distinct from that of vimentin and ubiquitin although there was some cytoplasmic overlap with Hsc-70 (Figure 3.4A). In cells stressed with MG-132, parkin-positive aggresomes were surrounded by vimentin. In most cells, Hsc-70 formed a ring around parkin whereas ubiquitin co-localized with parkin more centrally (Figure 3.4B). These observations on MG-132 induced parkin-positive aggresome mirror those reported previously (Junn et al., 2002). Furthermore, the location of parkin and ubiquitin in the aggresomes parallel one histopathological study that demonstrated that C-terminal parkin and ubiquitin immunoreactivity were strongest in the dense core of LBs (Schlossmacher, 2002).

In contrast to MG-132-induced aggresomes, staurosporine-induced aggresomes contained vimentin that surrounded parkin. However, we were unable to detect the presence of Hsc-70 or ubiquitin in these aggresomes (Figure 3.5A). This was not due to technical differences as the staurosporine and MG-132 co-localization studies were performed in parallel with the same antibodies. For dopamine induced aggresomes, parkin was found to co-localize with vimentin, Hsc-70 and ubiquitin in most cells examined (Figure 3.5B).

A


Parkin
Vimentin
Merge
Parkin
Ubiquitin
Merge
Parkin
Hsc-70
Merge

B

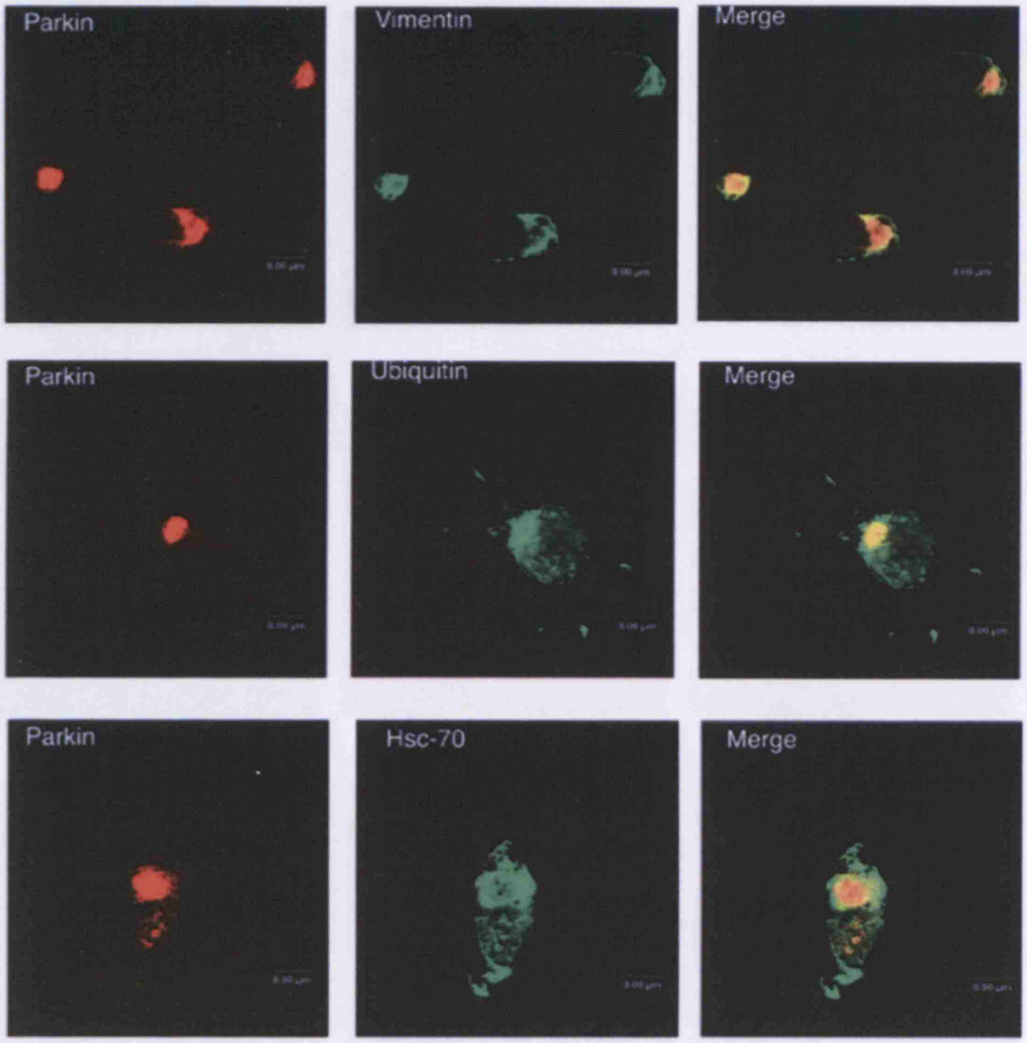
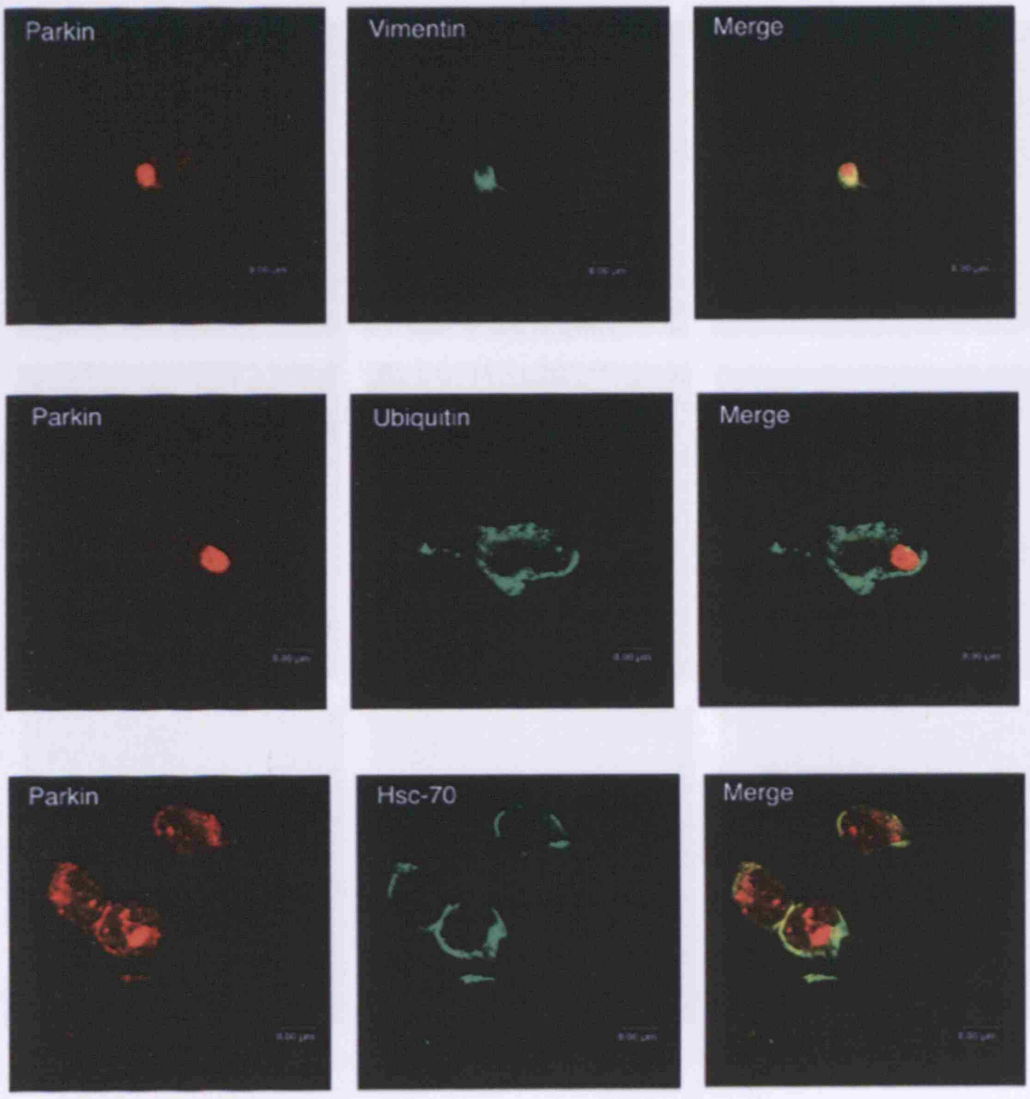


Figure 3.4 Co-localization studies of parkin with vimentin, ubiquitin, and Hsc-70 in SH-SY5Y cells at rest and treated with MG-132. Sub-confluent SH-SY5Y cells were grown on glass coverslips and treated with DMSO or MG-132 for 24 hours. Cells were then fixed in 4% paraformaldehyde and processed for confocal laser microscopy by double labelling cells with a C-terminal antibody to parkin and a rhodamine-conjugated secondary (red) and either anti-vimentin antibody (top row); anti-ubiquitin antibody (middle row); or anti-Hsc-70 antibody (bottom row), all with a FITC-conjugated secondary (green). Digital images were obtained by confocal laser scanning. Representative fields from 4 - 7 independent experiments are displayed. **(A)** In control SH-SY5Y cells parkin does not co-localize with vimentin or ubiquitin (top and middle rows) but does share some overlap with Hsc-70 (bottom row). **(B)** In cells treated with 10μM MG-132 for 24 hours, parkin-stained aggresomes are surrounded by vimentin (top row) whereas parkin and ubiquitin co-localize in the central portion of aggresomes (middle row). Hsc-70 accumulated in the periphery of parkin-stained aggresomes (bottom row). Scale Bar 8μm.

A


Parkin
Vimentin
Merge
Parkin
Ubiquitin
Merge
Parkin
Hsc-70
Merge

B

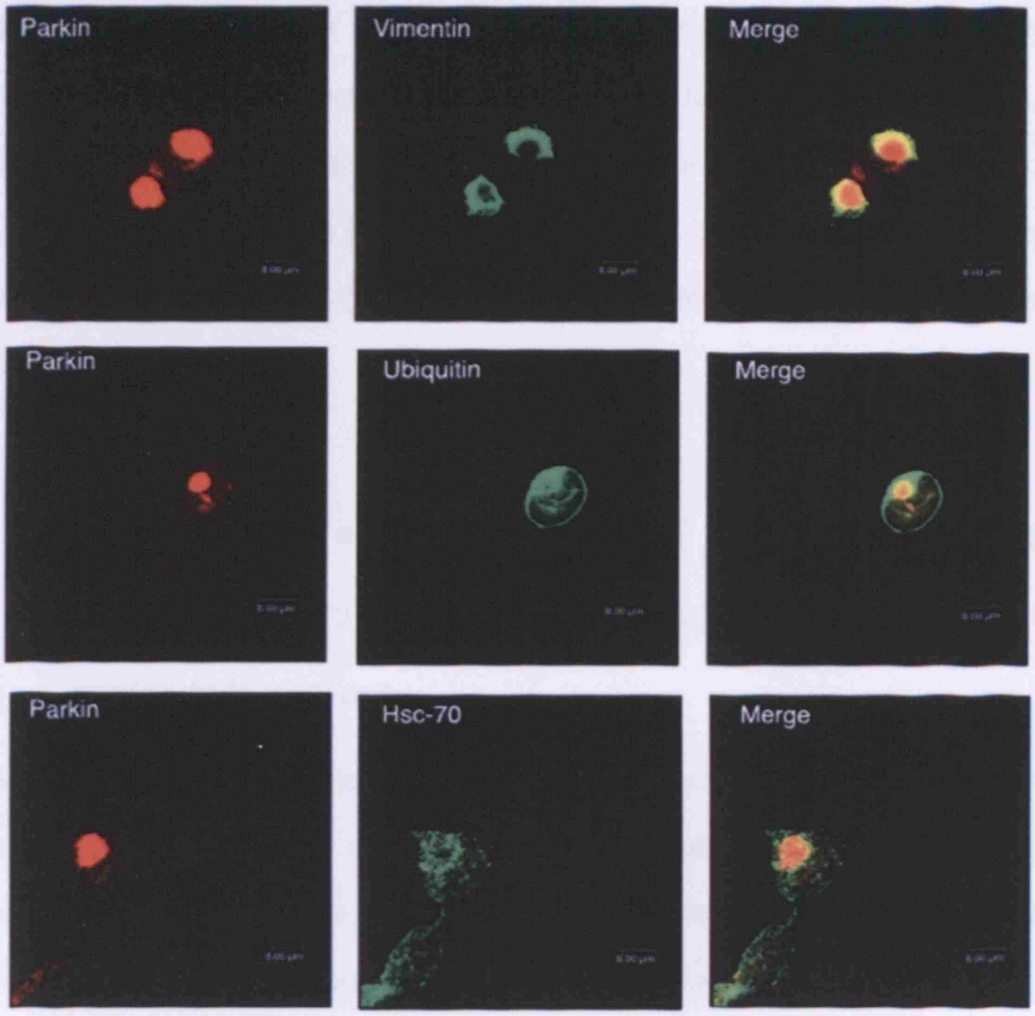
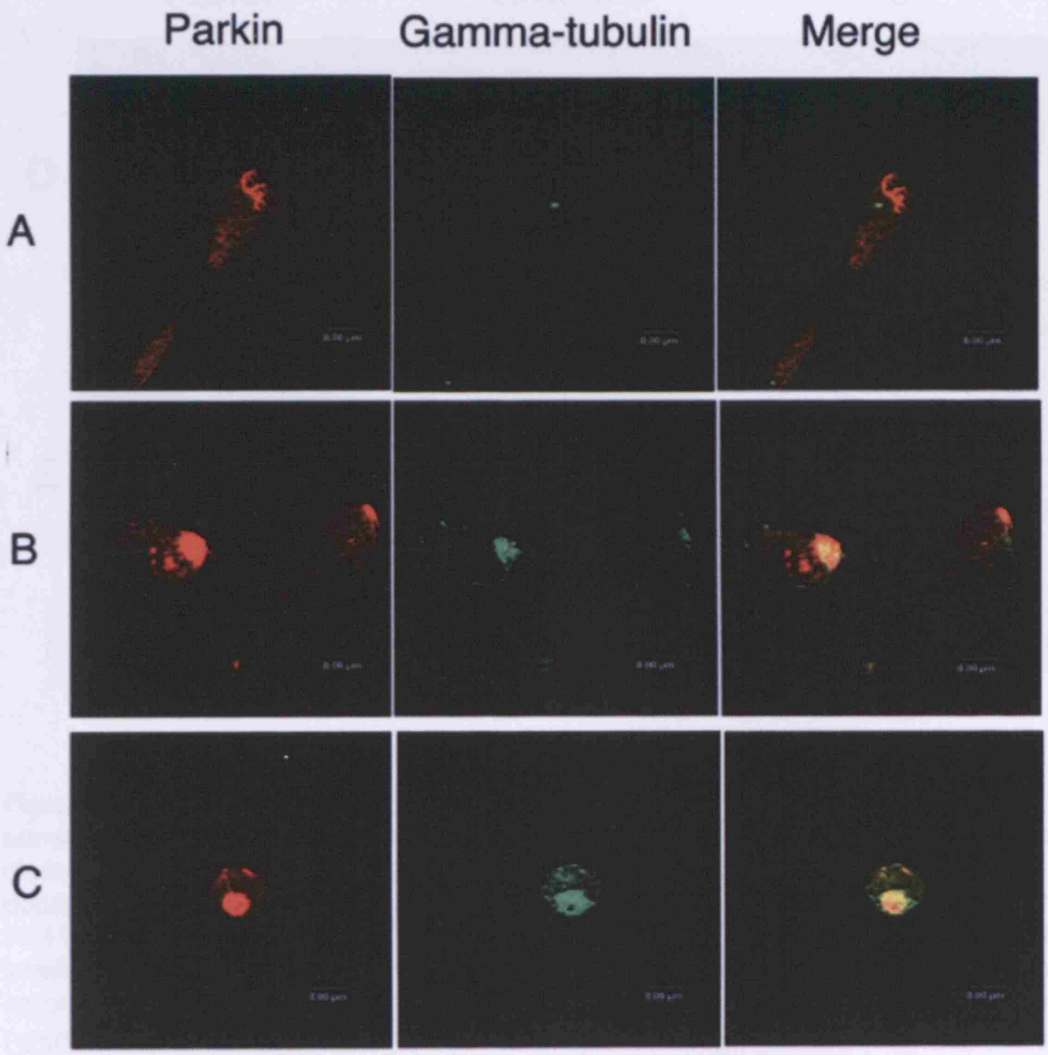


Figure 3.5 Co-localization studies of parkin with vimentin, ubiquitin, and Hsc-70 in SH-SY5Y cells treated with staurosporine or dopamine. Sub-confluent SH-SY5Y cells were grown on glass coverslips and treated with staurosporine or dopamine as detailed in the Materials and Methods section. Cells were then fixed in 4% paraformaldehyde and processed for confocal laser microscopy by double labelling cells with a C-terminal antibody to parkin and a rhodamine-conjugated secondary (red) and other antibodies as indicated in Fig. 2. Digital images were obtained by confocal laser scanning. Representative fields from 3 independent experiments are displayed. **(A)** In staurosporine treated cells parkin-stained aggresomes were surrounded by vimentin (top row) but ubiquitin and Hsc-70 did not co-localize with parkin in aggresomes (middle and bottom rows). **(B)** In dopamine treated cells parkin-stained aggresomes were surrounded by vimentin (top row) and co-localized with ubiquitin and Hsc-70 (middle and bottom rows). Scale Bar 8μm.

We also examined whether any putative substrates of parkin were found in aggresomes induced in our system. There are conflicting reports of α-synuclein immunoreactivity in aggresomes (Paxinou et al., 2001; Junn et al., 2002; Lee et al., 2002) and we were unable to detect endogenous α-synuclein in aggresomes induced by any of the above stresses (data not shown). We also could not detect the recently identified substrate, cyclin E (Staropoli et al., 2003), in MG-132 induced aggresomes (data not shown).

3.2.3 Parkin-positive aggresomes localize to the microtubule organizing centre (MTOC) and are dependent on an intact microtubule network

Parkin-positive aggresomes localize to the MTOC and are dependent on an intact microtubule network. It has recently been reported that parkin is recruited to the microtubule organizing centre (MTOC) in response to proteosome inhibition (Zhao et al., 2003). I performed co-localization studies with an anti-parkin antibody and a γ-tubulin antibody that is a marker for the MTOC. In unstressed cells I found that parkin did not co-localize with γ-tubulin in the majority of cells (~85%) (Figure 3.6A). However, I found that parkin-positive aggresomes co-localized with γ-tubulin in the presence of MG-132, staurosporine and dopamine (Figure 3.6B–D) suggesting that aggresomes in our system localize to the MTOC. Aggresome formation requires an intact microtubule system (Johnston et al., 1998). I then investigated what effect the microtubule-depolymerizing drug colchicine would have on the formation of stress-induced aggresomes in our system. In cells co-incubated with colchicine (5 mg/ml) and staurosporine or dopamine I found that the formation of aggresomes were


Parkin
Gamma-tubulin
Merge
A
B
C

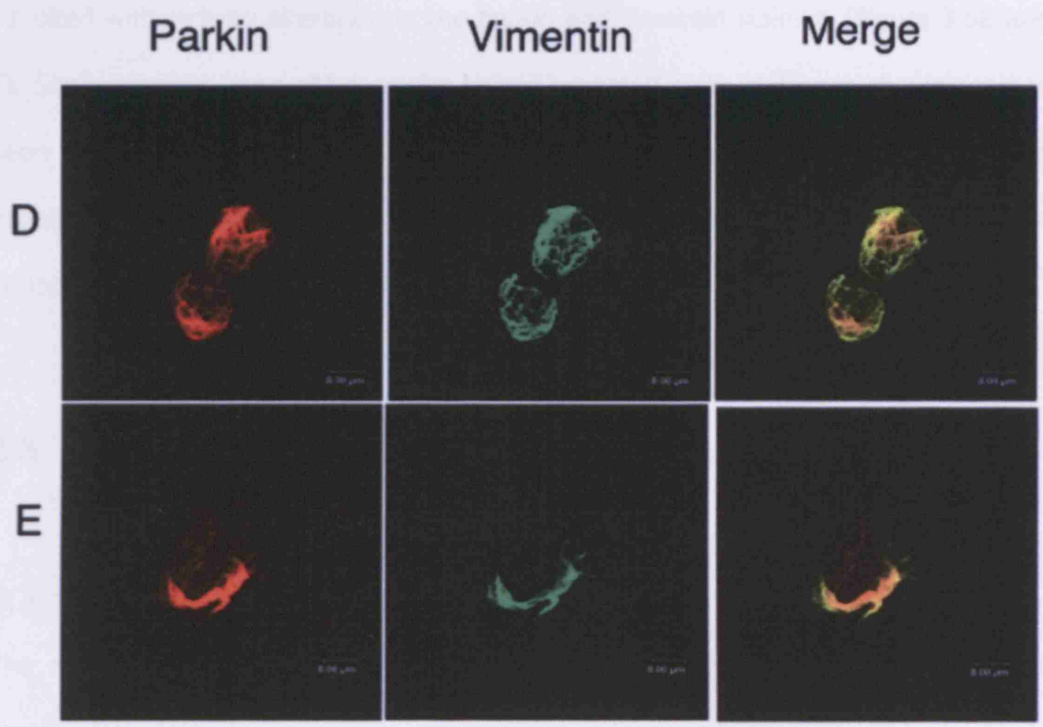


Figure 3.6 Parkin-positive aggresomes localize to the MTOC and are sensitive to microtubule disruption. Sub-confluent SH-SY5Y cells were grown on glass coverslips and treated with vehicle **(A)**, MG-132 **(B)**, staurosporine **(C)** or dopamine **(D)** as detailed in the Materials and Methods section. Cells were then fixed in 4% paraformaldehyde and processed for confocal laser microscopy by double labelling cells with a C-terminal antibody to parkin and a rhodamine-conjugated secondary (red) and an anti-γ-tubulin antibody with a FITC-conjugated secondary (green). Digital images were obtained by confocal laser scanning. Representative fields from 2 independent experiments are displayed. **(A)** In vehicle treated cells parkin did not co-localize with γ-tubulin in the majority of cells. **(B)** In MG-132 treated cells parkin-positive aggresomes co-localized with γ-tubulin. **(C)** In staurosporine treated cells parkin-positive aggresomes co-localized with γ-tubulin. **(D)** In dopamine treated cells parkin-positive aggresomes co-localized with γ-tubulin. Note that the area of γ-tubulin staining in the stressed cells was generally increased compared to unstressed cells. **(E, F)** Sub-confluent SH-SY5Y cells were grown on glass coverslips and treated with staurosporine **(E)** or dopamine **(F)** in the presence of 5μg/ml colchicine for the duration of the stress as detailed in the Materials and Methods section. Cells were then fixed in 4% paraformaldehyde and processed for confocal laser microscopy by double labelling cells with a C-terminal antibody to parkin and a rhodamine-conjugated secondary (red) and an anti-vimentin antibody with a FITC-conjugated secondary. Colchicine blocked aggresome formation to all stresses with severely altered parkin staining and vimentin staining. Aggresomes were seen in parallel wells without colchicine in the same experiment (data not shown). Scale Bar 8μm.

inhibited with striking alteration in the parkin and vimentin staining (Figure 3.6E and F). Similar results were obtained for MG-132 treated cells (data not shown) as has been reported previously (Zhao et al., 2003). In parallel wells of the same experiments with the stress alone I observed parkin-positive aggresomes with robust co-localization with vimentin (as previously shown).

3.3 ESTABLISHMENT OF PARKIN STABLE CELL LINES

3.3.1 Background to stable cell lines

The above studies clearly demonstrated that endogenous parkin can localize to aggresomes following a variety of stresses. Since endogenous parkin had not previously been observed in aggresomes, the next question was whether altering parkin levels would affect aggresome formation.

The ability to alter protein expression in any biological system *in vitro* or *in vivo* has proven to be a powerful tool for understanding their function. The manipulation of mammalian cell lines to either stably over-express or knock down particular genes of interest is a well-established method to study the role of proteins in a particular cellular context. This section describes the establishment of an *in vitro* cellular system in human dopaminergic SH-SY5Y neuroblastoma cell lines in order to determine the functional consequences of parkin over-expression or knock down.

The mammalian expression vector pcDNA3 (Invitrogen Ltd., Paisley, UK) was chosen on the basis that it had been successfully used in the past for expressing genes in SH-SY5Y cells (Shimura et al., 2000). This vector has a CMV promoter that drives high protein expression in mammalian cells and simultaneously allows

selection for cells with the inserted gene using G418-sulphate (Invitrogen Ltd., Paisley, UK), since the vector also contains a neomycin resistance cassette.

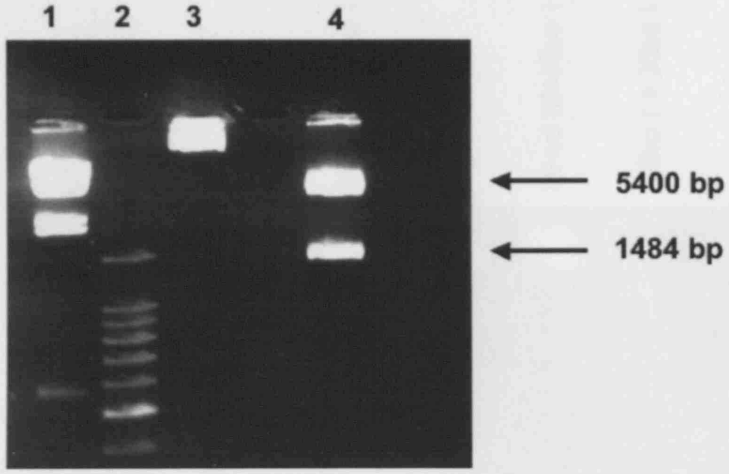
3.3.2 Molecular Subcloning

Molecular subcloning was undertaken to obtain a parkin anti-sense construct. The parkin pcDNA3 construct (see Appendix 3 for vector map) was a kind gift from Dr Rohan de Silva (University College, London). Parkin cDNA was excised from the vector following restriction digest with Bam H1 and the products run on a 1% agarose gel revealing bands of 5400bp (vector) and 1484bp (parkin) (Figure 3.7A). Both the vector and parkin band were separately isolated under ultraviolet detection. Vector DNA was then dephosphorylated (to prevent self-ligation) with calf intestine alkaline phosphatase (Promega) and the parkin insert was re-ligated to the dephosphorylated vector with DNA ligase (Promega). Following transformation in E. coli., clones were screened by restriction digest to identify parkin anti-sense clones: Bam HI digestion generates a 1484 bp insert in both sense and anti-sense clones; Hind III and Xho I digestion generates a 1440 bp insert in sense and 90 bp insert in anti-sense clones. As a final check Xho I and Xba I digestion generates a 1409 bp insert for antisense clones (Figure 3.7B). Following screening, several positive anti-sense clones were identified and grown up for transfection (Figure 3.7C).

3.3.3 Optimisation and transfection of SH-SY5Y cells with plasmid DNA to establish stable cell lines

Several liposome reagents to produce efficient DNA uptake were tried in SH-SY5Y cells including lipofectin™ and lipofectamine™ (Invitrogen) with poor transfection rates (data not shown). The newer agent, Lipofectamine 2000™ (Invitrogen)

A


1
2
3
4
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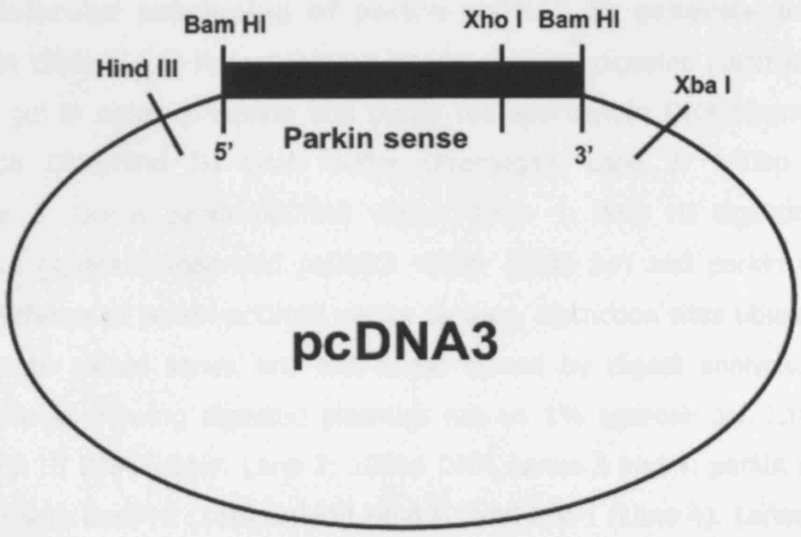
1484 bp

B


Bam HI
Xho I
Bam HI
Hind III
Xba I
5'
Parkin sense
3'
pcDNA3

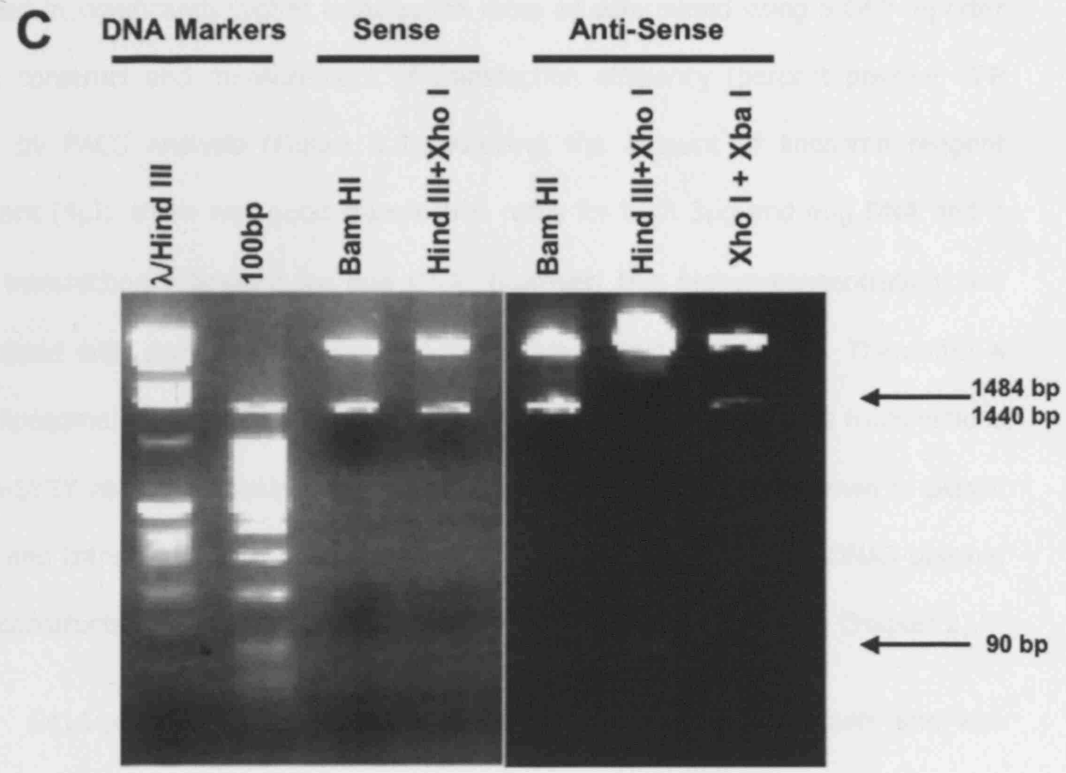


Figure 3.7 Molecular subcloning of parkin-pcDNA3 to generate parkin antisense cDNA clones. (A) Representative image showing digested plasmids run on 1% agarose gel in order to isolate and purify the appropriate DNA fragments. Lane 1: Lambda DNA/Hind III DNA ladder (Promega). Lane 2: 100bp DNA (Promega). Lane 3: Uncut parkin-pcDNA3 vector. Lane 4: Bam HI digestion of parkin-pcDNA3 to generate linearised pcDNA3 vector (5400 bp) and parkin cDNA (1484 bp). **(B)** Schema of parkin-pcDNA3 vector to show restriction sites utilised for screening candidate parkin sense and anti-sense clones by digest analysis. **(C)** Representative image showing digested plasmids run on 1% agarose gel. Lane 1: Lambda DNA/Hind III DNA ladder. Lane 2: 100bp DNA. Lanes 3 and 4: parkin sense pcDNA3 digested with Bam HI (Lane 3) and Hind III and Xho I (Lane 4). Lanes 5-7: parkin anti-sense clone digested with Bam HI (Lane 5); Hind III and Xho I (Lane 6); and Xba I and Xho I (Lane 7). The gel is of insufficient resolution to show size differences between the 1440bp and 1484bp bands.

resulted in significantly higher transfection rates as determined using a GFP reporter cDNA construct and measurement of transfection efficiency (percent positive GFP cells) by FACS analysis (Figure 3.8). Keeping the amount of liposome reagent constant (4μl), there was good transfection rates for both 3μg and 4μg DNA and a high transfection efficiency for 8μg DNA, however, this higher concentration was associated with significant cellular toxicity 24 hours post-transfection. Thereafter a DNA:liposome ratio of 3μg DNA:4μl lipofectamine 2000 was used for all transfections in SH-SY5Y cells. For making stable cell lines, SH-SY5Y cells were grown in plastic wells and transfected with pcDNA3, parkin pcDNA3 and anti-parkin pcDNA3 plasmid DNA constructs using lipofectamine 2000 (method described in detail in Chapter 2).

G418-sulphate was then used to select for positive transfectants and was added to the media 48 hours post transfection to select for G418 resistant clones. The optimum concentration of 0.4mg/ml G418 had been determined by testing a range of G418 concentrations on native SH-SY5Y cells. A kill curve was then established (Figure 3.9). After 10 days approximately 29 – 43 individual G418 resistant colonies for each individual DNA construct were isolated.

3.3.4 Clonal identification

Several independently isolated empty vector; parkin sense over-expressing; and parkin anti-sense candidate colonies were screened for parkin expression by Western blot using a C-terminal polyclonal parkin antibody. Several parkin-overexpressing clones were identified, however, no parkin-antisense clones showing reduced

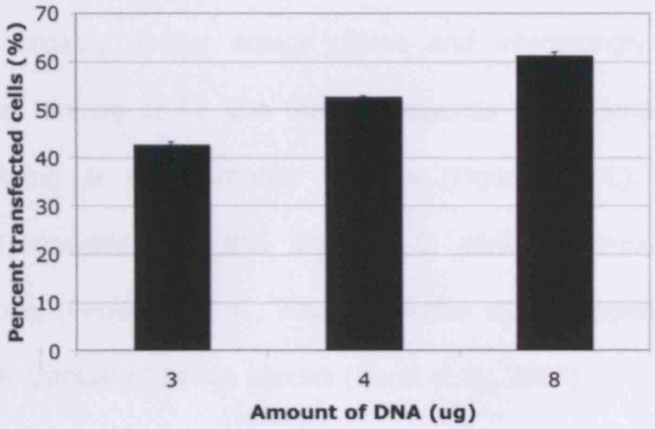


Figure 3.8 Optimisation of DNA transfection using lipofectamine 2000® in SH-SY5Y cells. Representative data of varying concentrations of GFP reporter DNA transfected with 3μL of lipofectamine 2000 in SH-SY5Y cells. For each concentration cells were transfected in duplicate and harvested 24 hours post-transfection. Transfection efficiency was measured using FACS analysis. The highest concentration of 8μg DNA was associated with significant cytotoxicity.

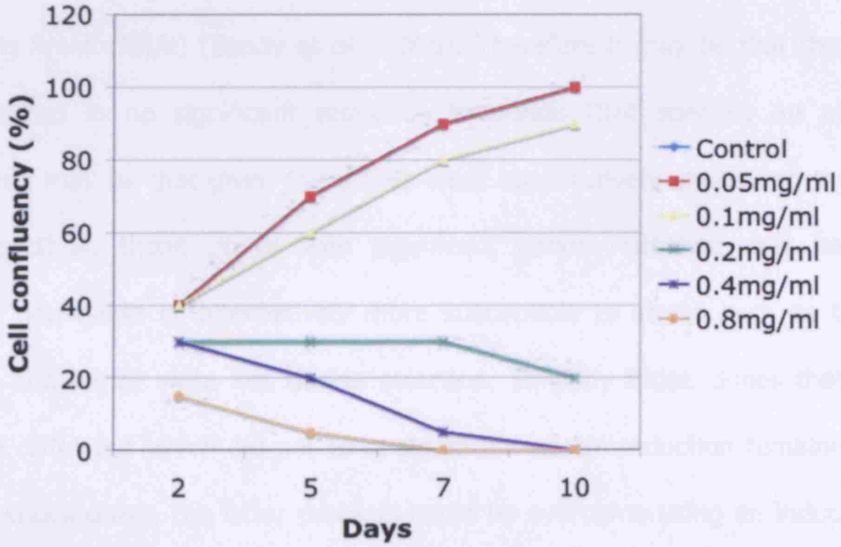
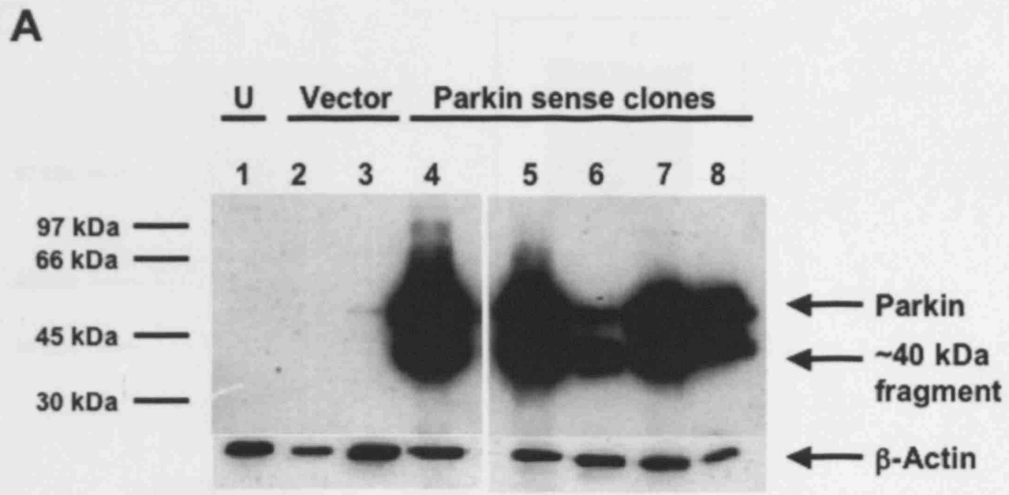


Figure 3.9 G418 'kill curve'. Representative data of native SH-SY5Y cells treated in parallel wells with varying concentrations of G418. Control and 0.05mg/ml superimposed. A dose of 0.4mg/ml G418 was effective at killing all cells by 10 days.

expression were identified (Figure 3.10A and B). In the over-expressing lines, parkin expression was broadly similar across clones and interestingly 2 bands were detected: a band running at 52 kDa that corresponds to full length parkin and a lower band running at approximately 40 kDa (Figure 3.10C). This band has previously been reported with this antibody in parkin over-expressing human neuroblastoma cells (Petrucelli et al., 2002). A recent study suggests that this band may be a partially translated parkin species (Henn et al., 2005).

Morphological analysis of the vector and parkin over-expressing colonies revealed no significant difference in shape with all clones having polygonal cells with neurite extension. There was also no significant difference in growth between vector and parkin over-expressing clones. There may be several reasons why none of the G418 resistant parkin anti-sense clones demonstrated significant parkin knock down. Anti-sense dsDNA knock down of protein expression is not an efficient process and is certainly less efficient compared to newer techniques such as the use of short interfering RNAs (RNAi) (Sandy et al., 2005). Therefore it may be that the strategy used resulted in no significant reduction in parkin RNA species. An alternative explanation may be that given the clones were constitutively expressing the parkin-antisense cDNA, those clones with significant parkin reduction may have been rendered non-viable or alternatively more susceptible to stress such as the G418 selection and hence were lost during selection. Similarly those clones that got the antisense cDNA but which did not have significant parkin reduction remained viable. For RNA knock-down, the latter problem could be overcome using an inducible anti-sense selection system, however, for technical reasons, a tetracycline inducible system (Clontech, UK) was not able to be successfully established during these studies (data not shown).


A
U
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Parkin sense clones
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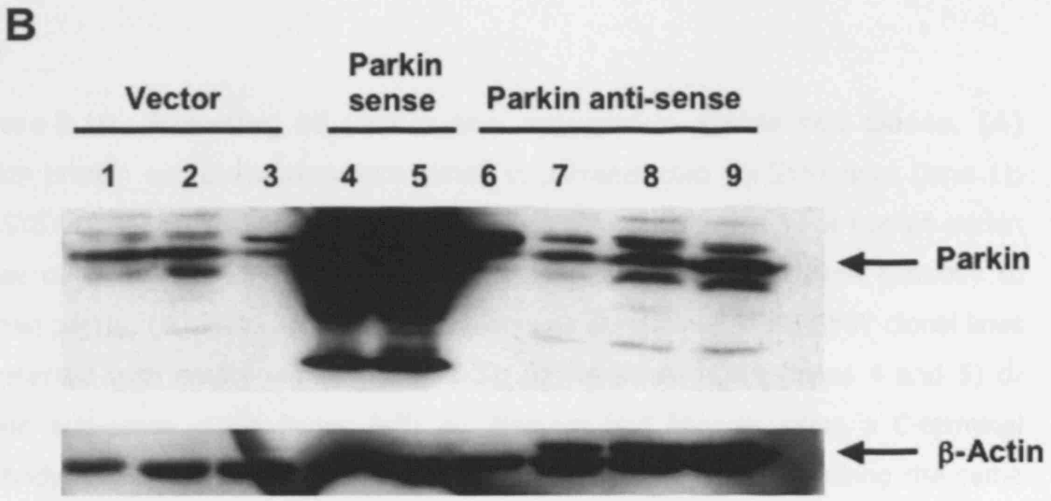
97 kDa
66 kDa
45 kDa
30 kDa
Parkin
~40 kDa fragment
β-Actin


B
Vector
Parkin sense
Parkin anti-sense
1 2 3 4 5 6 7 8 9
Parkin
β-Actin

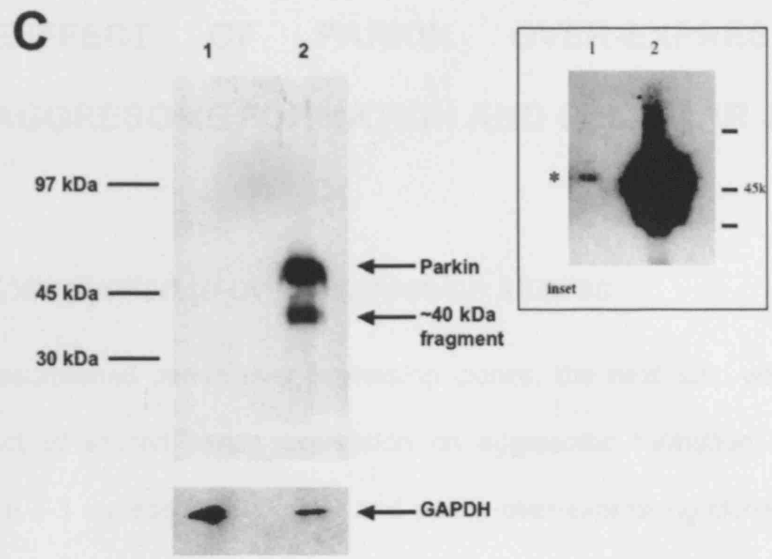


Figure 3.10 Screening of parkin and anti-parkin stable cell clones. (A) Parkin protein expression was determined in untransfected SH-SY5Y lines (lane 1); SH-SY5Y clonal lines transfected with empty vector (lanes 2 and 3) or human parkin sense cDNA (lanes 4-8) by Western blot analysis using a C-terminal antibody to human parkin. **(B)** Parkin protein expression was determined in SH-SY5Y clonal lines transfected with empty vector (lanes 1-3); parkin sense cDNA (lanes 4 and 5) or parkin anti-sense cDNA (lanes 6-9) by Western blot analysis using a C-terminal antibody to human parkin. Protein loading was determined by re-probing the same membrane with β-Actin antibody. **(C)** Low exposure Western blot showing vector clone (lane 1) and parkin over-expressing clone (lane 2). Full-length parkin is clearly seen as a band running at 52kDa (arrow). A C-terminal fragment (~40kDa) in the parkin-expressing clone (arrow) is also noted that has been previously reported with this antibody in human M17 lines (Petrucelli et al., 2002). Inset: prolonged exposure of the same membrane allowed detection of the endogenous parkin protein in the vector lane 1 (asterisk). Protein loading was determined by re-probing the same membrane with GAPDH antibody.

3.4 EFFFECT OF PARKIN OVER-EXPRESSION ON AGGRESOME FORMATION AND CELLULAR STRESS

3.4.1 Introduction to over-expression studies

Having established parkin over-expressing clones, the next step was to determine the effect of altered parkin expression on aggresome formation and cell death. Therefore 2-3 representative vector and parkin over-expressing clones were selected for these studies on the basis of their similarity of parkin over-expression, normal appearance of cellular morphology and normal growth properties.

3.4.2 Parkin over-expression reduces aggresome formation and protects against MG-132-induced stress

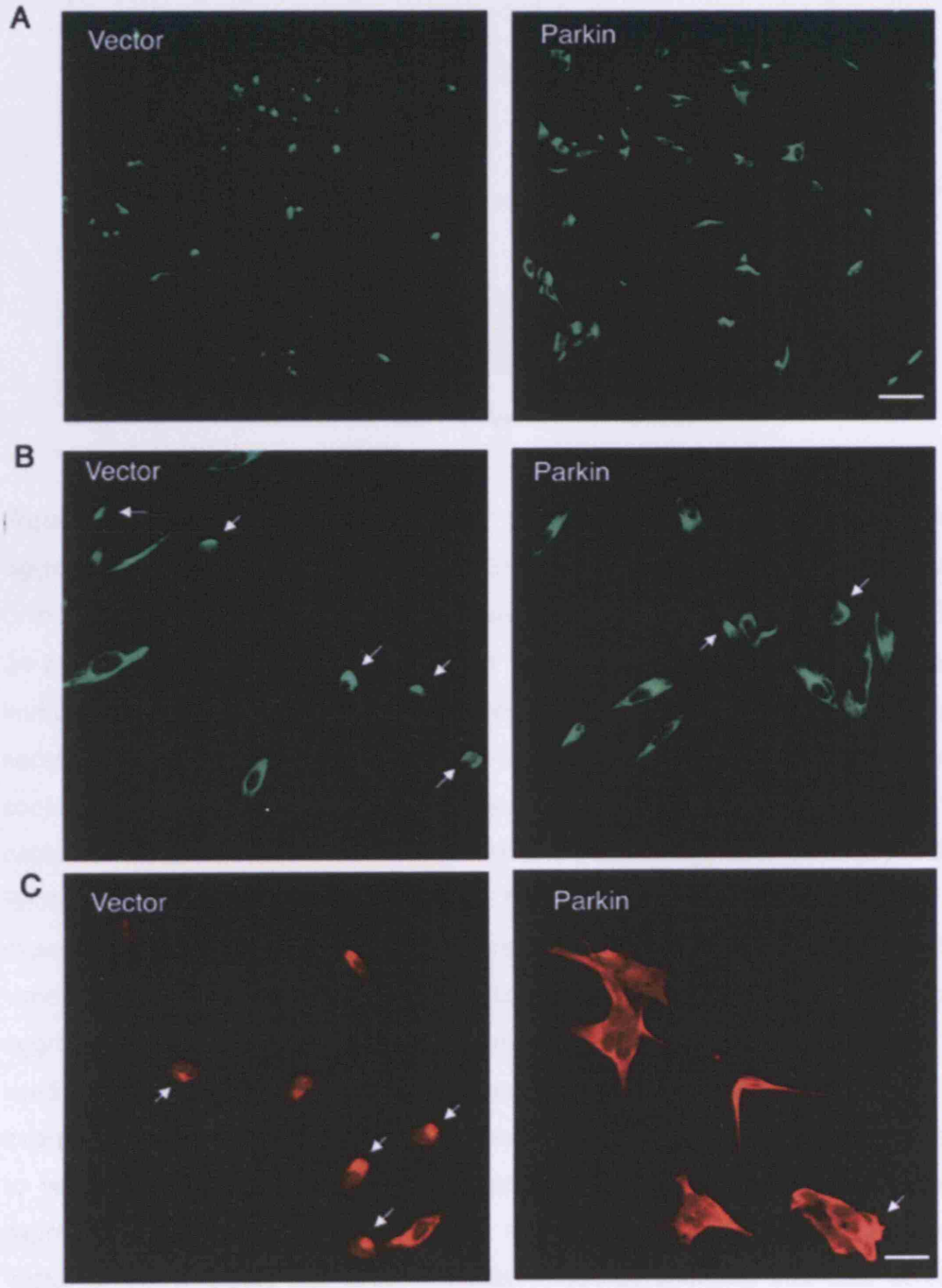
A previous study had indicated that parkin was upregulated in human neuroblastoma cells (BE-M17 cells) following 24 hour incubation in 10μM MG-132 (Choi et al., 2000). Furthermore, several studies had shown that over-expressed parkin localized to aggresomes (Ardley et al., 2003; Junn et al., 2002). However, at the time of this work the effect of parkin over-expression on MG-132-induced neurotoxicity was unknown and in particular the relationship between aggresome formation and cell death in the presence of altered parkin. Therefore, this work sought to explore this relationship using stable parkin-expressing cell lines.

Cells were incubated with 10μM MG-132 for 24 hours and aggresome formation and cell death were quantified by immunofluorescence microscopy and trypan blue dye exclusion assay respectively (for details see Chapter 2). SH-SY5Y

cells over-expressing parkin formed significantly fewer aggresomes in response to MG-132 than the vector control ($p < 0.05$) (Figure 3.11A-D). Similar results were obtained when aggresomes were stained with vimentin or parkin antibodies (Figure 3.11D). Under the same conditions this reduction in aggresomes correlated with significant neuroprotection by parkin over-expression against MG-132 toxicity compared to vector alone ($p<0.001$) (Figure 3.12). This was confirmed in another independently isolated set of clones and therefore is unlikely to be due to clonal properties acquired by the cells during the selection process.

3.4.3 Stable over-expression of parkin reduces staurosporine and dopamine-induced aggresome formation but is not sufficient to protect against toxicity

The effect of altered parkin levels on aggresome formation in response to staurosporine or dopamine toxicity was then investigated. SH-SY5Y cells stably expressing parkin formed significantly fewer aggresomes induced by either staurosporine ($p<0.01$) or dopamine ($p<0.05$) compared to the vector alone (Figure 3.13A-C). However, in contrast to the MG-132 findings, the reduction in aggresomes did not correlate with a protective effect of parkin over-expression against toxicity induced by either of these stresses (Figures 3.14 and 3.15). These data suggest that for these stresses, the formation and reduction of aggresomes are not linked to parkin's effect on neuronal survival. Again two sets of independent clones were used for these studies indicating that the reduction in aggresomes is a consequence of parkin over-expression and not simply clonal properties of the cells used.


A
Vector
Parkin
B
Vector
Parkin
C
Vector
Parkin

D

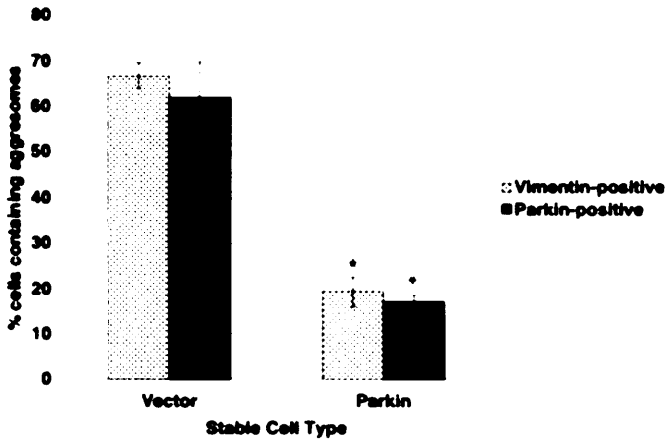


Figure 3.11 Over-expression of parkin reduces MG-132-induced aggresome formation. Sub-confluent SH-SY5Y cell lines stably expressing vector or human parkin were grown on glass coverslips and treated with 10μM MG-132 for 24 hours. Adherent cells were fixed with 4% paraformaldehyde and processed for immunofluorescence with an anti-vimentin antibody and a FITC-conjugated secondary (green) **(A, B)** or with a C-terminal parkin antibody and rhodamine-conjugated secondary (red) in independent experiments **(C)**. Digital images were captured using a Zeiss Axiocam camera. Arrows point to aggresomes in **(B)** and **(C)**. Representative fields at low **(A)** and high **(B)** magnification from the same experiment demonstrate that parkin-expressing stable cell lines (right) form fewer vimentin-positive aggresomes compared to vector (left). Note that vimentin-stained aggresomes appear solid at these magnifications. Identical exposure times were used within each experiment. **(C)** Representative fields demonstrating that parkin-expressing stable cell lines (right) form fewer parkin-positive aggresomes compared to vector (left). Exposure times for vector were three times longer than parkin-expressing cells. **(D)** The percentage of aggresomes induced by MG-132 in either vector or parkin-stable cell lines was determined by counting 300 cells across 20 random fields for each clone. There was no significant difference in the number of aggresomes detected using either vimentin or parkin antibody to stain aggresomes. Parkin expressing stable cell lines formed significantly fewer aggresomes than vector alone at $p<0.05$ (*) by Student's paired T-test. Data are expressed as means ± S.E.M for 3 independent experiments for each antibody used. Data representative of two independent sets of clones.

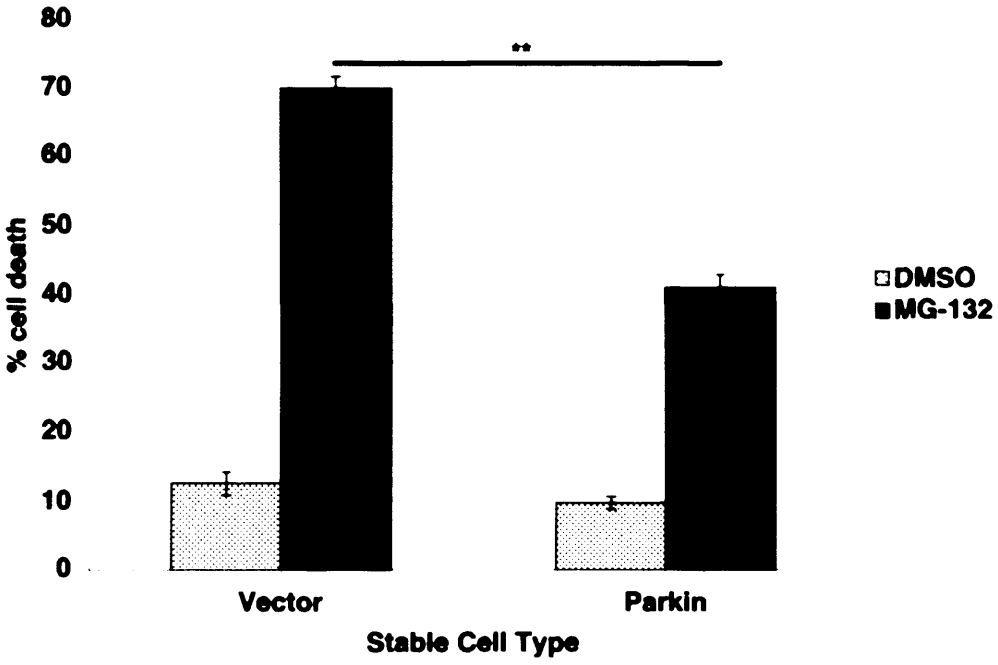
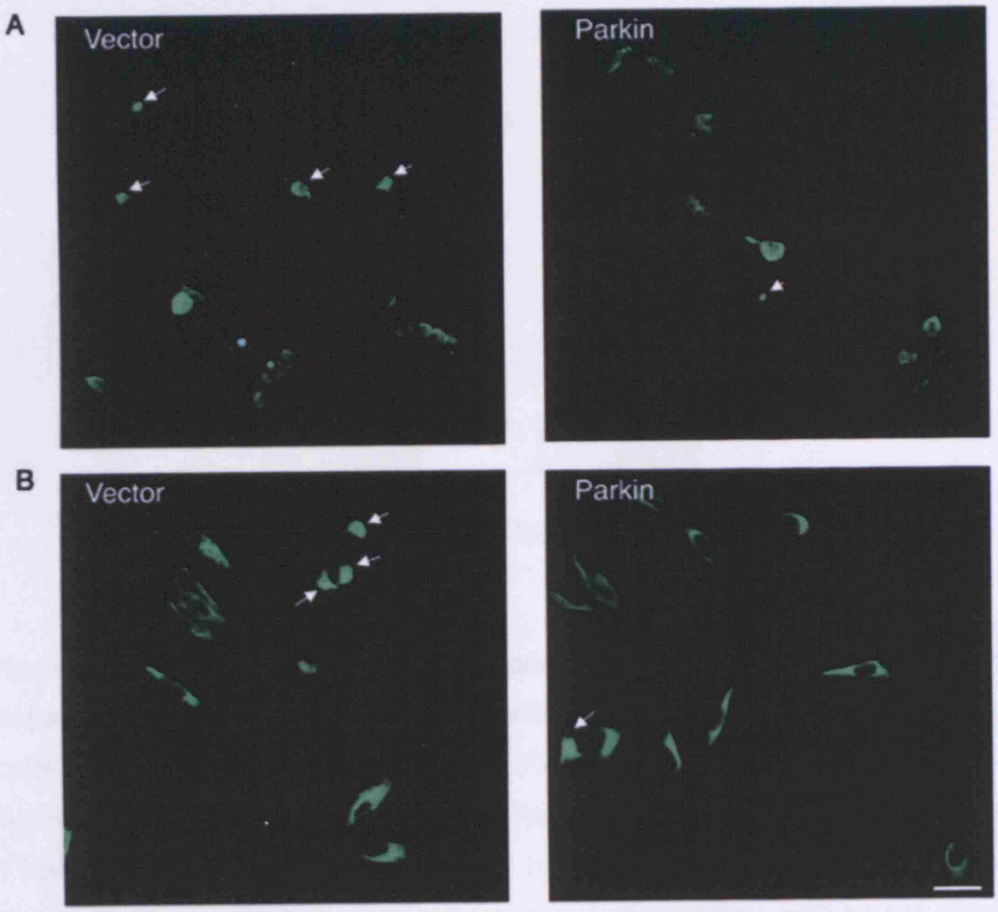


Figure 3.12 Parkin protects against MG-132-induced stress.

Sub-confluent SH-SY5Y cell lines stably expressing vector or human parkin were grown in tissue culture plates and treated with 10μM for 24 hours. All cells (floating and adherent) were harvested and cell viability was determined by trypan blue dye exclusion. Cells stably expressing parkin were significantly protected against MG-132 toxicity compared to vector at $p<0.001$ (**) by Student's paired T-test. Data are expressed as means ± S.E.M for 3 independent experiments. Data representative of two independent sets of clones.


A
Vector
Parkin
B
Vector
Parkin

C

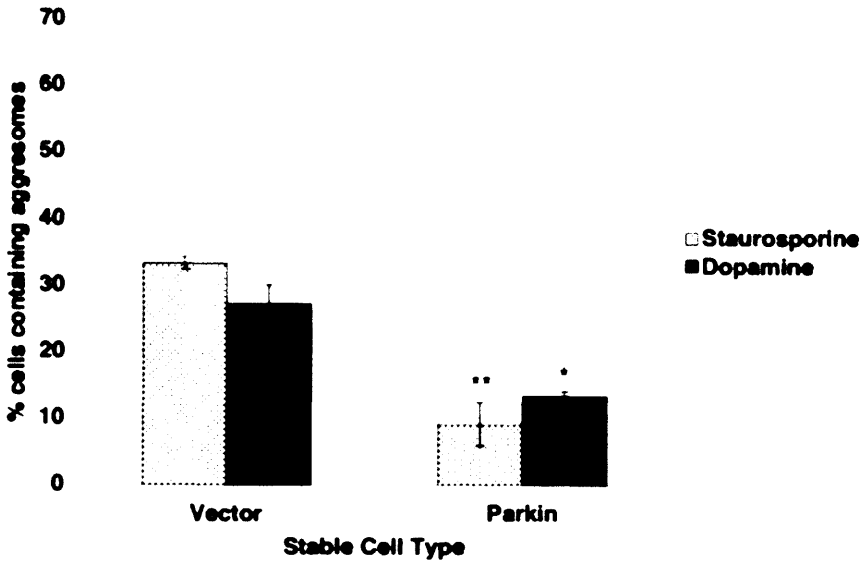


Figure 3.13 Over-expression of parkin reduces staurosporine- and dopamine-induced aggresome formation but is not sufficient to protect cells from toxicity. Sub-confluent SH-SY5Y cell lines stably expressing vector or human parkin were grown on glass coverslips and treated with 5μM staurosporine for 4 hours **(A)** or 0.125mM dopamine for 24 hours **(B)**. Adherent cells were fixed with 4% paraformaldehyde and processed for immunofluorescence with an anti-vimentin antibody and the appropriate secondary. Digital images were captured using a Zeiss Axiocam camera. Arrows point to aggresomes. **(A)** Representative fields from the same experiment demonstrating that parkin-expressing stable cell lines (right) form fewer staurosporine-induced aggresomes than vector (left). Note that staurosporine resulted in the cells appearing smaller. **(B)** Representative fields from the same experiment demonstrating that parkin-expressing stable cell lines (right) form fewer dopamine-induced aggresomes than vector (left). **(C)** The percentage of aggresomes induced by staurosporine or dopamine in either vector or parkin-expressing stable cell lines was determined by counting 300 cells across 20 random fields for each clone. Parkin expressing cell lines formed significantly fewer staurosporine-induced aggresomes at $p<0.01$ (**) and dopamine-induced aggresomes at $p<0.05$ (*) by Student's paired T-test. Data representative of two independent sets of clones.

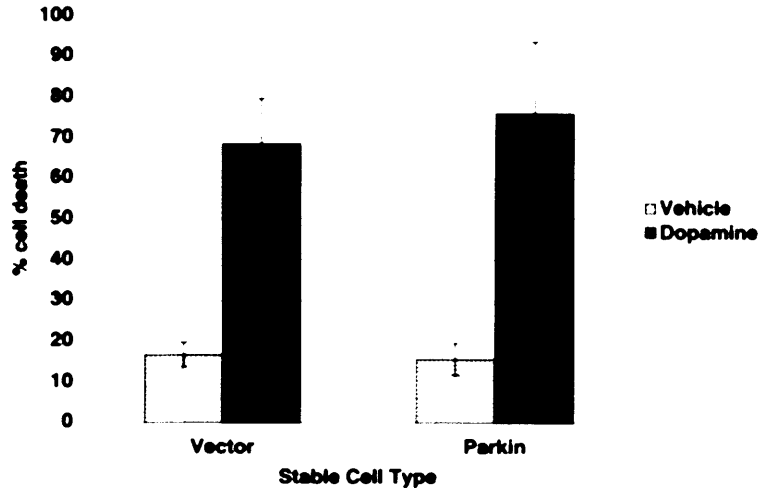


Fig 3.14 Over-expression of parkin does not protect against dopamine-induced stress

Sub-confluent SH-SY5Y cell lines stably expressing vector or human parkin were grown in tissue culture plates and treated with vehicle or 0.125mM dopamine for 24 hours. All cells (floating and adherent) were harvested and cell viability was determined by trypan blue dye exclusion. Data are expressed as means ± S.E.M for 3 independent experiments. Data representative of two independent sets of clones.

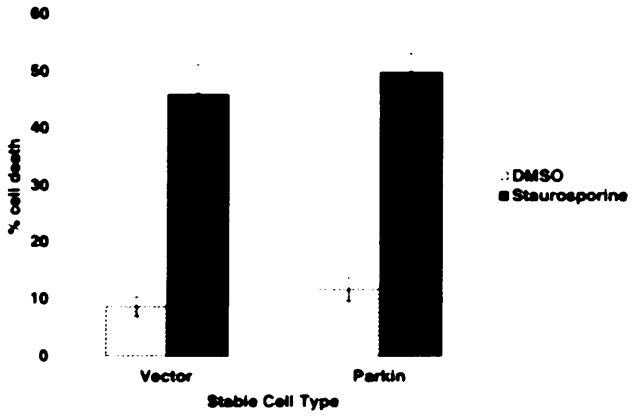


Fig 3.15 Parkin does not protect against staurosporine-induced stress

Sub-confluent SH-SY5Y cell lines stably expressing vector or human parkin were grown in tissue culture plates and treated with vehicle or 5μM staurosporine for 4 hours. All cells (floating and adherent) were harvested and cell viability was determined by trypan blue dye exclusion. Data are expressed as means ± S.E.M for 3 independent experiments. Data representative of two independent sets of clones.

3.4.4 Parkin over-expression protects against unfolded protein response stress

At the time of this work only one study had been published regarding parkin over-expression and sensitivity to stress using transient transfections in SH-SY5Y cells (Imai et al., 2000). The authors showed that parkin was up-regulated following induction of the unfolded protein response (UPR) by the N-glycosylation inhibitor, tunicamycin and that wild-type but not mutant parkin over-expression could protect against this stress (Imai et al., 2000). The same group also had shown that a G-protein-coupled transmembrane protein PaeIR (parkin associated endothelin like receptor) that can induce the UPR was a substrate for parkin and that parkin could protect against PaeIR-induced stress (Imai et al., 2001).

As stated earlier tunicamycin did not elicit aggresomes, however, in view of the dissociation of aggresome formation and neuronal survival elicited by dopamine and staurosporine stresses, it was of interest to determine if parkin over-expression in our stable cell lines could also protect against UPR stress induced by tunicamycin. Cell death was quantified by the trypan blue dye exclusion assay in cells exposed to 50μg/ml of tunicamycin for 24 hours. Consistent with previous reports I found that parkin over-expression was able to significantly protect against tunicamycin-induced cell death compared to vector alone ($p < 0.05$) (Fig 3.16) and these data suggested that parkin neuroprotection can occur independent of aggresome formation.

3.4.5 Disease causing mutants of parkin fail to suppress aggresome formation

Since over-expression of wild-type parkin in my stable system was able to reduce aggresome formation,

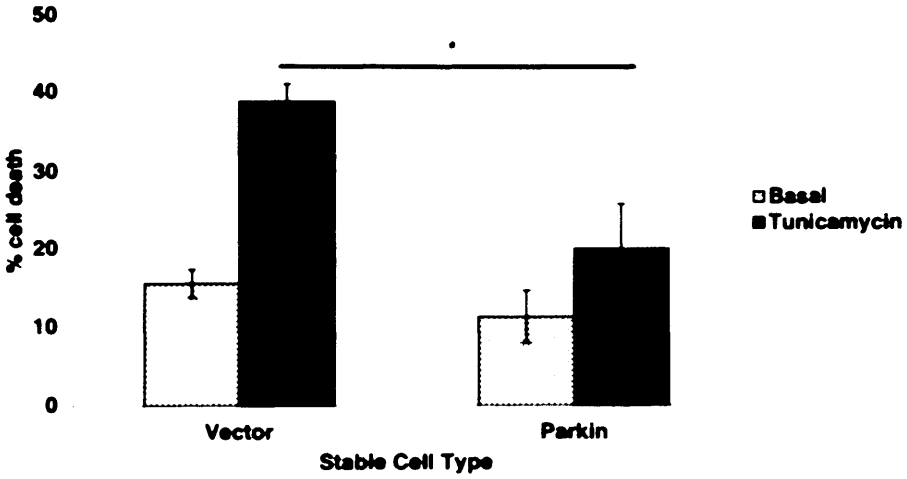


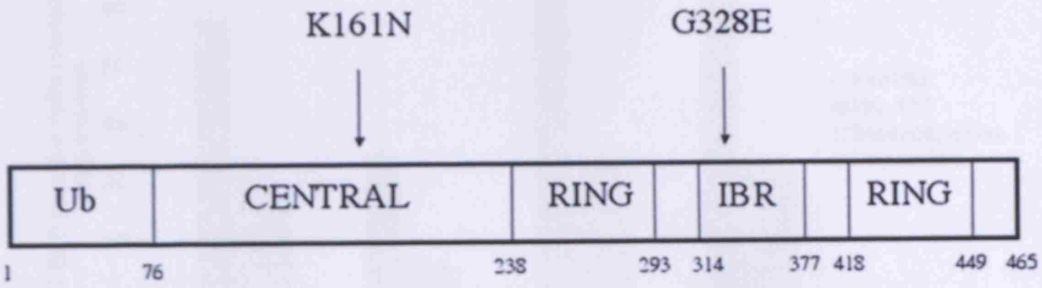
Fig 3.16 Over-expression of parkin protects against tunicamycin-induced stress.

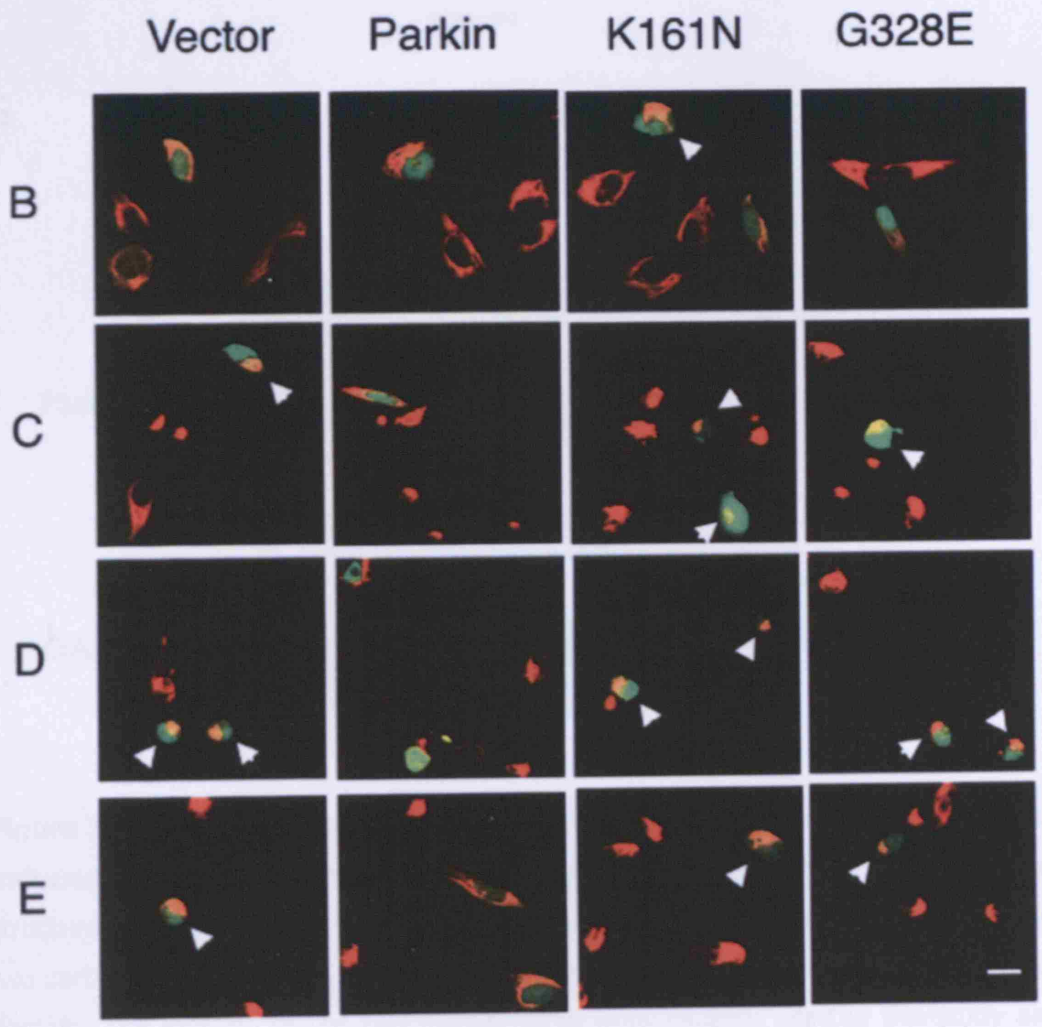
Sub-confluent SH-SY5Y cell lines stably expressing vector or human parkin were grown in tissue culture plates and treated with 50μg/ml tunicamycin for 24 hours. All cells (floating and adherent) were harvested and cell viability was determined by trypan blue dye exclusion. Parkin over-expression significantly protected cells against tunicamycin at $p<0.05$ (*). Data are expressed as means ± S.E.M for 3 independent experiments. Data representative of two independent sets of clones.

the effect of disease causing mutants of parkin was investigated (gift of Professor Poul Jensen, Denmark). Using a transient expression system with a GFP reporter plasmid, aggresome formation was quantified for two disease causing mutants of parkin, K161N that affects the central part of the parkin protein and G328E that is an ubiquitin ligase domain mutant of parkin (Figure 3.17A). The K161N mutation is a missense mutation that occurred in trans with a truncating deletion (202-203delAG) in one family and both mutant alleles co-segregated with disease (Abbas et al., 1999). The G328E mutation is a missense mutation reported in a PD case heterozygous for a single mutation (West et al., 2002).

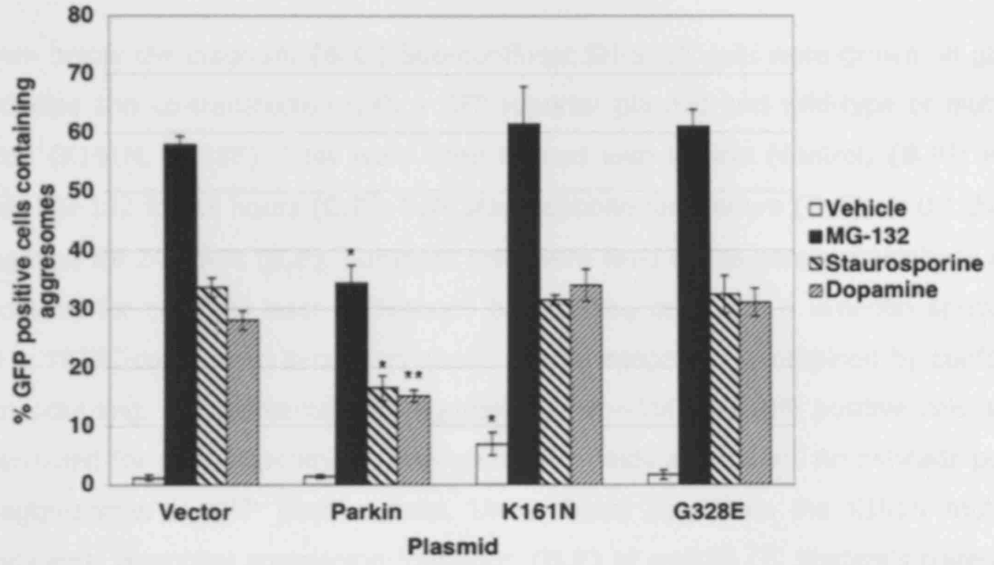
Under basal conditions, transfection of parkin or the G328E mutant did not induce significant aggresome formation, however transfection of the K161N mutant resulted in a small but significant increase in aggresomes compared to the vector control ($P<0.05$) (Figure 3.17B and F). This was not due to differences in protein expression as western blotting confirmed equal wild-type and mutant parkin expression after transient transfection (Figure 3.17G). By confocal microscopy I found the majority of K161N transfected cells did not localize to the MTOC but in a small percentage of transfected cells (~3%), parkin localized to aggresomes and co-localized with g-tubulin under basal conditions (data not shown). In wild-type parkin and G328E transfected cells, I did not see parkin co-localizing at the MTOC in the majority of cells and we did not see aggresomes under basal conditions (data not shown). Over-expression of wild type parkin significantly reduced aggresome formation in response to MG-132 compared to vector ($P<0.05$) (Figure 3.17C and F), in response to staurosporine ($P<0.05$) (Figure 3.17D and F) and in response to dopamine ($P<0.01$) (Figure 3.17E and F). In contrast, the K161N and G328E mutants were not significantly different from the vector for any stress suggesting that parkin's ability to suppress aggresome formation is dependent on an intact central domain

A


K161N
G328E
Ub
CENTRAL
RING
IBR
RING
1
76
238
293
314
377
418
449
465


Vector
Parkin
K161N
G328E
B
C
D
E

F



G

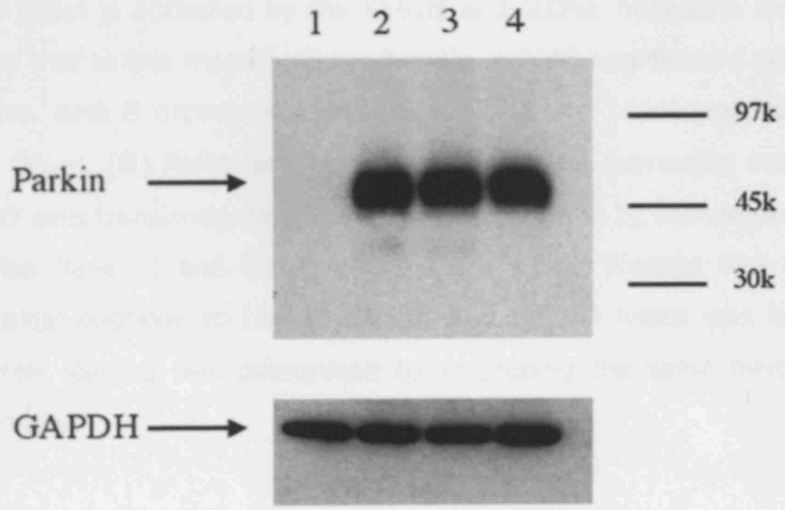


Figure 3.17 Disease causing mutants of parkin fail to suppress stress-induced aggresome formation. (A) Schematic representation of the domain structure of parkin. Parkin consists of a N-terminal ubiquitin-like domain (Ub) and two carboxy-terminal RING finger domains separated by an 'in between RING' (IBR) domain. The location of the two disease-associated mutants used in this study are shown above the diagram. Amino acids defining the boundary of each domain are

Figure 3.17 legend (contd)

shown below the diagram. **(B-G)** Sub-confluent SH-SY5Y cells were grown on glass coverslips and co-transfected with a GFP reporter plasmid and wild-type or mutant parkin (K161N, G328E). Cells were then treated with vehicle (control) **(B,F)**; with 10μM MG-132 for 24 hours **(C,F)**; 5μM staurosporine for 4 hours **(D,F)**; or 0.125mM dopamine for 24 hours **(E,F)**. Adherent cells were fixed in 4% paraformaldehyde and processed for confocal laser microscopy by labelling cells with a vimentin antibody and a TRITC-conjugated secondary (red). Digital images were obtained by confocal laser scanning. The percentage of aggresomes in ~100-250 GFP positive cells was determined for each experiment. Representative fields are shown. Arrowheads point to aggresomes in GFP positive cells. Under basal conditions the K161N mutant significantly promotes aggresome formation **(B,F)** at $p<0.05$ (*, Student's paired T-test). Parkin over-expression significantly reduces MG-132- **(C, F)** and staurosporine-induced aggresome formation **(D, F)** at $p<0.05$ (*; Student's paired T-test) and dopamine-induced aggresomes **(E, F)** at $p<0.01$ (**; Student's paired T-test) and this effect is abolished by the K161N and G328E mutations for all stresses **(C-F)**. Note that at this magnification vimentin stained aggresomes appear solid. For each stress, data is expressed as means ± S.E.M. for 3 independent experiments. Scale Bar 20μm. **(G)** Parkin and mutant parkin protein expression was determined in SH-SY5Y cells transiently transfected with vector (lane 1), human parkin (lane 2), K161N parkin (lane 3) and G328E parkin (lane 4) by Western blot analysis using a C-terminal antibody to human parkin. 5μg protein lysate was loaded in each lane. Protein loading was determined by re-probing the same membrane with GAPDH antibody.

and ubiquitin ligase domain (Figure 3.17A–F).

3.5 DISCUSSION OF RESULTS

Two observations suggest that parkin plays a key role in LB biogenesis in vivo. Firstly, there have been several reports demonstrating parkin in Lewy bodies of sporadic and familial PD (Shimura et al., 2000; Schlossmacher et al., 2002; Bandopadhay et al., 2005). Secondly, AR-JP brains, in all but one case lack LBs. This suggests that parkin plays an important role in LB formation either directly or indirectly via ubiquitylation of specific substrates that in turn promote LB formation. Therefore, to address the role of parkin in LB formation, this work has investigated parkin's role in aggresome formation since LBs share many of the characteristics of aggresomes and are postulated to form in the presence of excess protein misfolding (McNaught et al., 2002).

The work in this chapter demonstrates that endogenous parkin is detectable in aggresomes following stress. Two previous studies have demonstrated that parkin is detectable in aggresomes or inclusions in cells over-expressing parkin exposed to a variety of proteosome inhibitors (Junn et al., 2002; Ardley et al., 2003). However, these studies used tagged forms of parkin and have not addressed the role of endogenous parkin in their systems. Recently another group has reported that endogenous parkin localizes to the centrosome in the presence of proteosome inhibition (Zhao et al., 2003).

This work is the first to show that a pro-apoptopic agent, such as staurosporine, and the oxidant, dopamine, can induce parkin-containing aggresomes. There is accumulating evidence that protein misfolding and UPS activation occur

during apoptosis (Soldatenkov et al., 1998). Furthermore, two previous reports suggest that parkin-related neurodegeneration may be mediated through the apoptopic cascade (Darios et al., 2003; Greene et al., 2003). The relative specificity of the neuronal loss to dopamine neurons suggests that dopamine dysfunction itself may be important in the pathogenesis of PD (Melamed et al., 1998). Previous studies have shown that oxidative stress in neurons leads to increased ubiquitin-protein conjugates (Ramanathan et al., 1999) and that the UPS is involved in the removal of oxidized proteins (Adamo et al., 1999). The most compelling evidence linking oxidative damage with abnormal proteolysis has been the *in vivo* demonstration that chronic exposure of the mitochondrial complex I inhibitor, rotenone, was able to induce the formation of LB-like aggregates as well as striatal neurodegeneration in rats (Betarbet et al., 2000). The fact that dopamine in this system is able to induce the formation of parkin-positive aggresomes leads one to postulate that, like staurosporine, both these stresses may result in increased levels of misfolded proteins that exceed the cell's degradative capacity. The finding of dopamine-induced aggresomes is particularly interesting as it may explain why LBs are found relatively specifically within substantia nigral neurons in PD.

Tunicamycin did not induce aggresomes. Previous work in SH-SY5Y cells using a concentration of tunicamycin five times lower than the dose used in this work also did not find that tunicamycin induced parkin-positive inclusion formation (Ardley et al., 2003). A previous study in primary hippocampal neurons and glia did not find tunicamycin-induced aggregates but did find altered distribution of parkin in glia (Ledesma et al., 2002). It may be that while tunicamycin can induce ER stress and protein unfolding, these proteins may remain within the ER and are not transported into the cytoplasm for degradation by the UPS (Kopito and Sitia, 2000).

In addition to their localization to the MTOC and sensitivity to microtubule

disruption, aggresomes are characterized by their constituents. All stress-induced aggresomes that were observed were surrounded by vimentin that has been reported to be an invariant feature (Kopito, 2000). Only MG-132 and dopamine-induced aggresomes also contained Hsc70 and ubiquitin. Ubiquitin or Hsc-70 immunoreactivity was not found in staurosporine-induced aggresomes. Previously, other types of aggresomes formed by over-expression of GFP-250 (Garcia-Mata et al. 1999) and superoxide dismutase (SOD) (Johnston et al., 2000) have been reported to be negative for ubiquitin immunoreactivity. It may be that the structure of these aggresomes is distinct from those induced by MG-132 and dopamine or that the ubiquitin epitope is not accessible to the ubiquitin antibody used. Alternatively, the incorporation of ubiquitin and Hsc-70 may occur after parkin is recruited to aggresomes and after the 4-hour duration that was used to study the effects of staurosporine (in contrast to the longer duration for the other stresses). One could delineate between these possibilities by purifying the aggresomes induced by staurosporine and examining the components more directly. Of relevance to PD, 10% of LBs in synucleinopathies are not ubiquitin-immunoreactive and it may be that the pathways mediating ubiquitin-negative LBs are distinct from those for ubiquitin-positive LBs in PD (Spillantini et al., 1998).

α-Synuclein is a major constituent of LBs (Spillantini et al., 1997). However, endogenous α-synuclein was not detected in aggresomes induced by any stress that was examined. There are conflicting reports of α-synuclein in aggresomes. It has been reported that α-synuclein is present in aggresomes in COS-7 cells (Junn et al., 2002; Lee et al., 2002) whereas α-synuclein was not detectable in aggresomes in HEK 293 cells (Paxinou et al., 2001). The lack of α-synuclein immunoreactivity in aggresomes tested here and the previous inconsistencies may reflect differences in cell lines and antibodies used. Alternatively, this negative finding may reflect the

limitations of the stress-induced cell culture systems compared to the mechanisms occurring to dopaminergic neurons in vivo in PD, which may involve a complex interplay of many factors that culminate in α-synuclein deposition in LBs. Although cyclin E was not found in aggresomes, it remains unknown whether cyclin E is present within LBs.

Whilst endogenous parkin appears to be recruited to aggresomes, aggresome formation is reduced in cell lines over-expressing human wild type parkin. Moreover, this property of parkin appears to be dependent on an intact central domain and ubiquitin ligase domain as the disease causing mutants K161N and the G328E prevented this effect. Interestingly the K161N mutant promoted aggresome formation under basal conditions albeit at low levels that suggests that its pathogenicity may be mediated in part by altered conformational changes. In the presence of stress, there was no difference between either mutant and vector alone. This suggests that the mutations have an inactivating effect on parkin activity of the mutant protein rather than a dominant negative effect on endogenous parkin. A recent report also found that several other parkin mutants (point mutations and truncations) had the potential to aggregate under basal conditions (Ardley et al., 2003). In contrast to this work, they did not find any difference in aggresome frequency between wild type and mutant parkin in MG-132 treated cells. This may be due to differences in methodology. The latter study used tagged proteins and analyzed inclusion formation in non-neuronal COS-7 cell cultures (Ardley et al., 2003). Furthermore, another study found that a parkin missense mutation that retains ligase activity, R275W, formed aggresomes under basal conditions but not wild type parkin (Cookson et al., 2003). Of particular interest is that this mutation occurred in trans with an exonic deletion in an ARJP case associated with LBs at post-mortem (Farrer et al., 2001).

The last few years has seen an explosion in reports of substrates for parkin including a glycosylated form of α-synuclein (α-Sp22), cyclin E, CDCrel-1, synphilin-1, Pael-R, alpha/beta tubulin, p38 subunit of the aminoacyl-tRNA synthetase complex and synaptogamin (Chung et al., 2001; Imai et al., 2001; Ren et al., 2003; Shimura et al., 2001; Staropoli et al., 2003). This suggests that parkin may have a general role in the ubiquitylation and degradation of misfolded proteins.

It has also been shown *in vivo* that parkin over-expression in transgenic α-synuclein-expressing Drosophila was able to reduce the number of α-synuclein-positive grain-like structures and ubiquitin positive LB-like neurites (Yang et al., 2003). Over-expression of parkin in the cell lines established here may result in increased ubiquitylation and degradation of misfolded proteins thus abrogating the formation of aggresomes. Furthermore, the formation of aggresomes may be dependent on the accumulation of only a few 'key' misfolded proteins such as tubulin or α-Sp22 and their ubiquitylation and degradation by excess parkin may be sufficient to prevent aggresome formation. In this regard, a recent study showed that the ubiquitin ligase, Chip-1, but not a mutant lacking ubiquitin ligase activity reduced aggresomes formed by the αB-crystallin mutant, αBR120G (Chavez-Zobel et al., 2003).

An alternative mechanism may be related to parkin's effect on proteosome activity. Protein misfolding and aggregation itself can inhibit the proteosome (Bence et al., 2001; Waelter et al., 2001). It has previously been reported that over-expression of parkin preserved proteasome function in response to an expanded polyglutamine polypeptide and also reduced its aggregation (Tsai et al., 2003). Furthermore, it has been demonstrated that over-expression of wild-type but not a disease causing-mutant of parkin is able to rescue cells from α-synuclein-induced proteosome inhibition and toxicity (Petrucelli et al., 2002). Parkin has also been

shown to bind directly to the Rpn10 subunit of 26S proteosomes (Sakata et al., 2003). Therefore excess parkin may reduce or confer resistance to proteasome impairment caused directly by a proteosome inhibitior (MG-132) or indirectly by misfolded proteins generated by stresses such as staurosporine or dopamine. Whilst the current observations on parkin over-expression on aggresome formation do shed significant light on the cellular role of parkin, it would also be interesting to examine the effects of suppression of parkin expression on aggresome formation but this was not possible owing to the inability to establish parkin anti-sense cell lines with sufficient parkin knock-down at the protein level.

It remains unclear what role LBs play in the pathogenesis of PD. They may be toxic, protective or an epiphenomenon (Tompkins and Hill, 1997). The nature of aggresomes is also uncertain with reports that they are toxic (Waelter et al., 2001) or protective (Taylor et al., 2003). In the current system the correlation between parkin's role in aggresome formation and reduction and parkin's role in neuroprotection appear to be stress-specific. With MG-132, the majority of cells contained aggresomes and their reduction in number with overexpression of parkin correlated with enhanced survival. This was not the case for staurosporine or dopamine-induced aggresomes. For these stresses it may be that the aggresomes remain independent from the main cascades mediating death such as apoptopic pathways that are induced by dopamine as well as staurosporine (Ziv et al., 1994; Kahns et al., 2002). Furthermore, the tunicamycin results further demonstrate that neurodegeneration and parkin's effect on neuronal survival can occur independently of aggresome formation. Although the presence of visible aggregates appears to be dissociated from the neurotoxic effect of the stressors used, this does not rule out the significance of aggregation in the disease pathogenesis of PD. Moreover, the key pathological changes that occur in aggregation diseases are often associated with a

temporal delay that is difficult to address in short-term cell culture systems.

In summary, the studies reported in this chapter demonstrate that parkin is important for aggresome formation. However, over-expression of parkin leads to fewer aggresomes, an effect that is dependent on intact central and ubiquitin ligase domains. Finally and perhaps most significantly, these cell studies demonstrate that one can use different stresses to separate parkin's role in aggresome formation from its effect on neuronal survival.

4

Functional Analysis of PINK1 mutations: a cause of Parkinson's disease.

4.1 INTRODUCTION

4.1.1 PARK6: A novel Parkinson's disease locus

As previously stated autosomal recessive juvenile parkinsonism (AR-JP) is a distinct form of familial Parkinsonism that is characterized clinically by early age at onset (< 40 years); slow progression of disease; good and sustained response to levodopa, early levodopa-induced complications (fluctuations and dyskinesias), hyperreflexia, mild dystonia (especially of feet) and sleep benefit (Ishikawa and Tsuji, 1996). Parkin mutations account for only ~50% of cases of AR-JP and the recently discovered DJ-1 accounts for 1% of AR-JP cases (Abou-Sleiman et al., 2003). Therefore, additional genes and genetic factors remained to be elucidated.

A large Sicilian family (the Marsala kindred) comprising 122 members had previously been identified with individuals presenting with AR-JP (Figure 4.1) (Valente et al., 1999). The age at onset in affected cases was 32-48 years and patients presented with a classic PD phenotype, with slow progression, sustained response to levodopa and the occurrence of levodopa-associated dyskinesias but

sleep benefit and foot dystonia were not observed. Exclusion of linkage to PARK2 suggested a novel PD locus in this family, designated PARK6. Autozygosity mapping in this family mapped the PARK6 locus to a 12.5-centimorgan (cM) region on chromosome 1p35-p36 (Valente et al., 2001). Subsequent identification of two additional consanguineous families [one from central Italy (family IT-GR) (Valente et al., 2002) and one from Spain (family identified by Dr Georg Auburger, J. W. Goethe University, Frankfurt, Germany)] provided additional evidence of linkage to PARK6. A critical recombination event in the Spanish family refined the candidate region to a 3.7-cM interval between flanking markers D1S2647 and D1S1539.

4.1.2 Identification of PINK1 mutations in PARK6 cases

Dr Patrick M. Abou-Sleiman (University College London, UK) performed the subsequent genetic analysis that led to the discovery of the causative gene mutations. Briefly, fine mapping of single-nucleotide polymorphisms and newly generated short tandem repeat markers in the three families defined a 2.8-megabase region of homozygosity within contig NT_004610, containing approximately 40 genes. Candidate genes were prioritized on the basis of their putative function and expression in the central nervous system, as assessed by bioinformatic analysis and by exon amplification from a human substantia nigra cDNA library. Sequence analysis of candidate genes in affected members from each family resulted in the identification of two homozygous mutations in the *PTEN- induced putative kinase 1* (*PINK1*) gene. The mutations segregated with the disease phenotype in the three consanguineous families, were confirmed in the cDNA, and were absent from 400 control chromosomes, including 200 chromosomes from Sicilian individuals. The Spanish family carried a G3A transition in exon 4 [nucleotide (nt) 11185 in

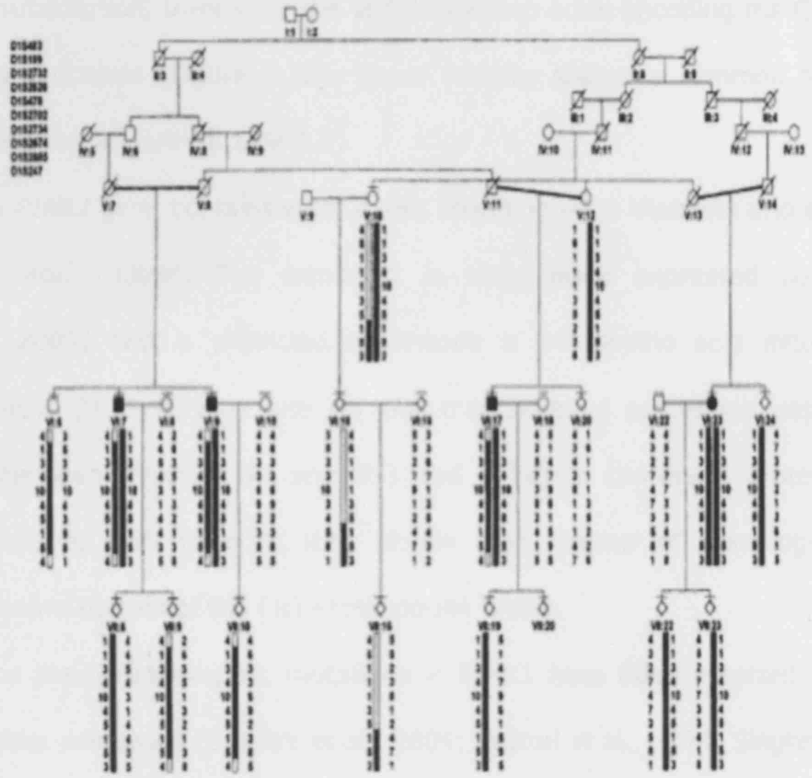


Figure 4.1 Family tree of the Marsala kindred indicating PD affected family members linked to PARK6 locus.

Modified from Valente et al. (2001).

NT_004610], resulting in an amino acid substitution (G309D) at a highly conserved position in the putative kinase domain (Figure 4.2A). Both Italian families carried the same G3A transitions in exon 7 (nt 15600 in NT_004610), which results in a W437OPA substitution, truncating the last 145 amino acids encoding the C terminus of the kinase domain (Figure 4.2B). These families shared a common haplotype, implying common ancestry (Table 4.1).

The *PINK1* gene contains eight exons spanning ~1.8 kilobases and encodes a 581–amino acid protein. The transcript is ubiquitously expressed (Unoki and Nakamura, 2003) and is predicted to encode a 34– amino acid mitochondrial targeting motif (the cleavage site for the mitochondrial processing peptidase is predicted between residues 34 and 35) and a highly conserved protein kinase domain (residues 156 to 509) that shows high degree of homology to the serine/threonine kinases of the Ca2+/calmodulin family.

Since the original report, mutations in PINK1 have been reported in further PARK6 families worldwide (Bonifati et al., 2004; Hattori et al., 2004; Singleton et al., 2004; Valente et al., 2004b) indicating that PINK1 mutations are the cause of PARK6 related PD. Dr P. M. Abou-Sleiman in our laboratory has also assessed the mutation burden of *PINK1* in the PD population.

He has sequenced the entire *PINK1* coding region including splice sites in 960 cases of parkinsonism (192 young onset patients and 768 sporadic PD). The screen resulted in the identification of nine heterozygous mutations of which seven were novel. Seven of these were missense mutations affecting the kinase domain and the other two included a missense mutation in the C-terminal hydrophilic region and a nonsense mutation at codon 456 that results in truncation of the last 125 amino acids. All nine patients had a clinical presentation that was similar to idiopathic PD, four of the cases were pathologically proven and two had some family

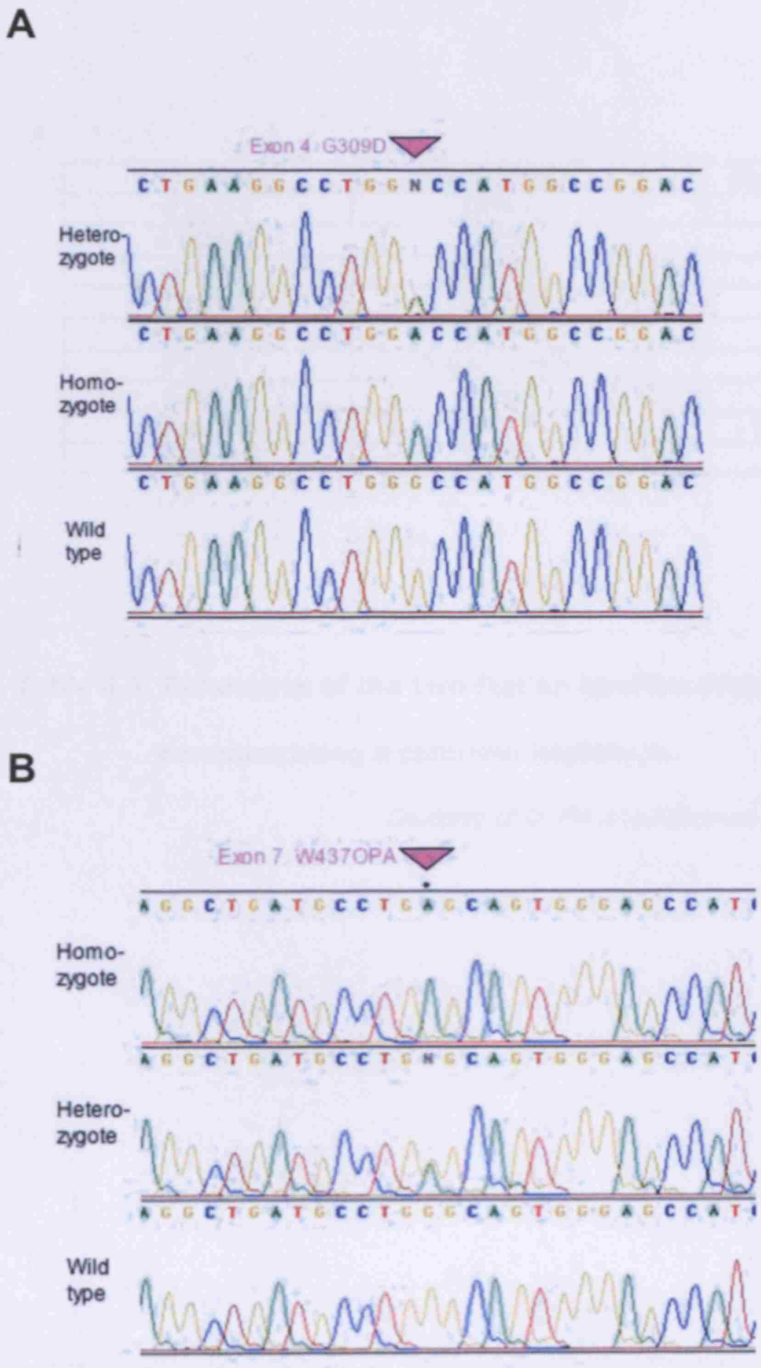


Figure 4.2 Electropherograms demonstrating PINK1 missense mutations. (A) Spanish family have an exon 4 G>A (G309D) transition. **(B)** Italian family have an exon 7 G>A (W437OPA) nonsense mutation.

Courtesy of Dr PM Abou-Sleiman (University College London)

Marker	Kb (UCSC)	Family 1	Family 2
d1s378	18984	1-1	1-1
d1s2647	19297	1-1	1-1
d1s199	19426	1-1	1-1
d1s2843	19980	3-3	3-3
d1s2732	20106	3-3	3-3
PINK1	20430-20450		
d1s478	21069	4-4	4-4
d1s2828	21412	10-10	10-10
d1s2702	22018	5-5	5-5

Table 4.1. Genotypes of the two Italian families around the PARK6 locus demonstrating a common haplotype.

Courtesy of Dr PM Abou-Sleiman (University College London)

history of the disease. In all nine cases he has been unable to identify a second mutation despite extensive sequencing and assessment of gene rearrangements by whole cDNA amplification from brain tissue in four pathological cases and lymphoblasts from one of the sporadic cases. The entire open reading frame (ORF) was amplified from genomic DNA in the remaining four patients by overlapping PCR. All mutations were absent on 3456 Caucasian chromosomes (1536 controls and 1920 PD).

The average age of onset in our cohort of PD patients with heterozygous mutations was 54±9 yrs. Having identified heterozygous mutations in approximately 1.2% of our PD patients Dr Abou-Sleiman sequenced an additional 1536 control chromosomes to determine the *PINK1* carrier frequency in the general population. Three separate missense mutations were identified in the kinase domain of non-PD controls. None of the patients displayed signs of parkinsonism at the time of DNA donation (aged 52, 59 and 73 yrs). Overall the frequency of a single PINK1 mutation in controls was 0.4%. There was a statistically significant enrichment of mutations in the PD cohort compared to controls suggesting that heterozygous mutations in the PINK1 gene are a significant risk factor in the development of sporadic PD (unpublished data).

4.1.3 Proteasome impairment and mitochondrial dysfunction

As has been described in Chapter 1, both mitochondria and the ubiquitin proteasome system (UPS) play an essential role in the maintenance of neuronal viability. Consequently, numerous studies have provided compelling data suggesting that dysfunction of both these systems may play a major pathogenetic role in dopaminergic cell loss in PD.

Since the UPS requires ATP, mitochondrial impairment has been shown to result in UPS dysfunction. The most compelling evidence for this is the demonstration that chronic rotenone exposure in rats results in the formation of Lewy body-like aggregates in addition to Parkinsonism (Betarbet et al., 2000). Conversely there is accumulating evidence that UPS dysfunction can result in secondary mitochondrial dysfunction and damage. Direct injection of a proteasome inhibitor into rat brain *in vivo* was sufficient to induce DNA fragmentation suggestive of neuronal apoptosis (Taglialatela et al., 1998). Another study demonstrated that treatment of primary rat cultured cortical neurons by two different proteasome inhibitors was sufficient to induce apoptosis in a dose-dependent manner. This was associated with an increase in caspase 3 activity and neuronal death was abrogated by pre-incubation with the caspase-3-like protease inhibitor, DEVD-CHO. Furthermore, proteasome inhibition induced the redistribution of cytochrome c into the cytosol and this was associated with depolarisation of the mitochondrial membrane potential ($\Delta\Psi m$) assessed by the fluorescent dye, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolycarbocyanine iodide (JC-1). The change in $\Delta\Psi m$ was blocked by over-expression of Bcl-xl (Qiu et al., 2000). Bcl-xl is known to inhibit MOMP and mPTP opening by directly interacting with the outer mitochondrial membrane (OMM) as described in Chapter 1. Thus, these studies suggest that proteasome inhibitors induce apoptosis via the mitochondrial pathway by somehow activating mPTP opening.

More direct evidence for the role of proteasomal inhibition and $\Delta\Psi m$ depolarisation in Parkinson's disease has come from an inducible α-synuclein model in neuronal PC12 cells. As described previously, the A30P mutation in α-synuclein causes PD and inducible over-expression of the A30P mutant in PC12 cells caused a decrease in proteasome activity and enhanced apoptopic cell death in the presence of a proteasome inhibitor (Tanaka et al., 2001). The cell death was associated with

enhanced caspase 9 and 3 activation suggesting activation of mitochondrial apoptosis. Furthermore, using the mitochondrial fluorescent uptake dye, JC-1, the authors demonstrated that the proteasome inhibitor caused up to a 30% increase in depolarisation of the $\Delta\Psi m$ in mutant $\propto$-synuclein cell lines. Both $\Delta\Psi m$ depolarisation and apoptosis was blocked with cyclosporin A (CsA) suggesting that proteasome inhibition resulted in $\Delta\Psi m$ depolarisation secondary to mPTP opening.

The mechanism by which proteasome inhibition leads to mitochondrial apoptosis remains unclear. Several pro-apoptopic proteins including Bax are degraded by the UPS and therefore proteasome inhibition may lead to the accumulation of such proteins that cause mPTP opening, $\Delta\Psi m$ depolarisation, and apoptosis (Chang et al., 1998). In human hepatoma cell lines the proteasome inhibitor, MG-132, was found to increase the levels of the pro-apoptopic protein, Bcl-XS, which was associated with cytochrome c release, and apoptosis. Interestingly, MG-132 also concomitantly reduced the level of the anti-apoptopic protein Bcl-Xl but the mechanism of this is unclear (Emanuele et al., 2002). The tumour suppressor protein, p53, inhibits cell cycle progression and induces apoptosis and several studies suggest that it may also be involved in the pathogenesis of PD (Duan et al., 2002; Mandir et al., 2002). p53 has been shown to transcriptionally activate the expression of several target genes including Bax, NOXA and PUMA that can in turn cause mitochondrial dysfunction (Vogelstein et al., 2000) Furthermore p53 is normally degraded by the UPS (Rodriguez et al., 2000) and has been shown to accumulate in SH-SY5Y cells following treatment with the proteasome inhibitor, MG-132 (Nakaso et al., 2004). This up-regulation of p53 resulted in the increased expression of Bax and depolarisation of the $\Delta\Psi m$ as assayed by JC-1 measurements. This increase in Bax was partially blocked by the p53 inhibitor, pifithrin-$\propto$ (PFT) suggesting that Bax

expression was increased partially by p53 mediated transcriptional activation as well as by proteasome inhibition directly (Nakaso et al., 2004). Thus the p53 signalling pathway may be a key player in mediating mitochondrial dysfunction and apoptosis induced by proteasome inhibition.

Studies in glioma cell lines have also shown activation of the mitochondrial apoptopic pathway by MG-132 with release of cytochrome c and mitochondrial depolarisation (using CMXRos) (Wagenknecht et al., 2000). Over-expression of Bcl-2 blocked apoptosis in contrast to a caspase 8 inhibitor, crm-A suggesting that proteasome inhibition exclusively activated the mitochondrial apoptopic pathway rather than the Fas/death ligand pathway. Furthermore, there was no accumulation of FADD by MG-132 treatment (Wagenknecht et al., 2000).

Further mechanistic insights have been gained from studies with the natural substance, circumin, the major yellow pigment of the spice turmeric. In mouse Neuro2a cells, curcumin was found to dose-dependently decrease proteasome activity and appeared to be a less potent proteasome inhibitor than MG-132 and lactacystin. However, circumin was still able to induce $\Delta\Psi m$ depolarisation, cytochrome c release, and activation of caspases 9 and 3 resulting in apoptosis (Jana et al., 2004). This was associated with circumin-induced accumulation of p53. Circumin has previously been shown to down-regulate NF-κB activity by preventing the degradation of IκB which blocks the nuclear translocation and hence transcriptional activity of NF-κB (Singh and Aggarwal, 1995). Since NF-κB plays a key role in the stress response, this suggests that its perturbation by proteasome inhibition may be important in the development of mitochondrial dysfunction. Furthermore, circumin has been shown to inhibit protein kinase B/Akt which is a pro-survival protein and this may also be another molecule that mediates the mitochondrial dysfunction from proteasome inhibition (Chaudhary and Hruska).

In lymphoid cell lines, the proteasome inhibitor, bortezomib, has been found to activate c-Jun-N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) as well as inactivate the 'cytoprotective' extracellular signal-regulating kinase 1/2 (ERK 1/2). This was associated with cytochrome c release and caspase mediated apoptosis and this was inhibited by the JNK inhibitor SP600125 but not a p38 MAPK inhibitor suggesting that JNK plays a key role in proteasome inhibition mediated mitochondrial apoptosis (Yu et al., 2004).

Further studies will be required to determine significance of JNK and Akt and identify more key signalling molecules involved in proteasome inhibition-mediated apoptosis and confirm whether they may play a role in PD *in vivo*. This will be crucial since these molecules may be amenable to pharmacological manipulation and may be of therapeutic benefit.

4.1.4 The Mitochondrial Membrane Potential ($\Delta\Psi m$)

The bioinformatic prediction that PINK1 had a mitochondrial targeting motif was very exciting since as has been described in Chapter 1, mitochondrial dysfunction may play a central role in the pathogenesis of sporadic PD. To determine the consequences of *PINK1* mutations we examined the effect of the mutant protein on mitochondrial function.

Two methodological approaches have been adopted in the past to measure mitochondrial function namely: biochemical studies looked at various parameters of bioenergetic function such as electron transport chain activity in mitochondrial preparations. Recently fluorometric assays and fluorescence imaging techniques have been developed to allow the study of mitochondrial membrane potential ($\Delta\Psi m$) in single cells or populations of cells. The $\Delta\Psi m$ is the main electrical component of the

protomotive force. The $\Delta\Psi m$ is usually around −150 to −180 mV and is central to mitochondrial function since it provides the force that drives the influx of protons (crucial for ATP synthase) or of calcium into the mitochondria as well as determines the generation of the free radical superoxide ($O_2^{\cdot-}$). Depolarisation of $\Delta\Psi m$ usually reflects opening of the mitochondrial permeability transition pore (mPTP). Irreversible opening of the mPTP is thought to result in a series of adverse cellular events including collapse of the $\Delta\Psi m$, uncoupling of the electron transport chain, ATP depletion, mitochondrial Ca^{2+} dysregulation and efflux, matrix swelling and rupture of the OMM releasing many pro-apoptopic proteins from the IMS into the cytosol.

Several groups have demonstrated the close coupling of complex I and $\Delta\Psi m$ in PD models. Sherer et al. (2001) showed that chronic low dose rotenone treatment in SH-SY5Y cells reduced complex I activity and this was associated with $\Delta\Psi m$ depolarisation and altered calcium signalling. In the same cell lines, Gomez et al. (2001) also showed that MPP^+ induced $\Delta\Psi m$ depolarisation and this caused cytochrome c release and increased apoptosis. The neurotoxin 6-hydroxydopamine (6-OHDA) can induce Parkinsonism *in vivo* and is associated with an increase in reactive oxygen species (ROS) and oxidative damage. Lotharius et al. (1999) have shown that 6-OHDA can cause $\Delta\Psi m$ depolarisation via the increase in ROS and this is another important trigger for mPTP opening (as discussed in Chapter 1). Interestingly, Gu and colleagues have recently shown that the non-ergot dopamine agonist, pramipexole, protected dopaminergic cells from apoptosis induced by MPP^+ and rotenone and this was associated with a prevention in $\Delta\Psi m$ collapse and subsequent cytochrome c suggesting that drugs that target the mPTP may have

therapeutic benefit in PD (Gu et al., 2004). It also highlights the central importance of the $\Delta\Psi m$ in neuronal viability.

Consequently, measurement of the $\Delta\Psi m$ has become established as a sensitive indicator for the energetic state of the mitochondria and the cell (Brand et al., 1994) and previously cationic probes have been used to measure $\Delta\Psi m$ isolated miitochondria. The latest techniques utilize fluorescent membrane permeable lipophilic cationic dyes that can cross the plasma membrane and be taken up by mitochondria in response to the electrochemical gradient and $\Delta\Psi m$. These compounds include tetramethylrhodamine ethyl and methyl esters (TMRE and TMRM respectively). Two methods have been employed to study these dyes, the 'redistribution method' and the 'quench/dequench' method'. I have employed the 'redistribution method' in these studies and shall expand on this method here. A more detailed explanation of the quench/dequench method is available in the review by Duchen (2004).

The principle of the 'redistribution method' is that the fluorescence signal of the compound is directly proportional to the dye concentration and the dye concentration represents the distribution of dye in response to the potential difference across membranes. Thus mitochondria with a higher membrane potential (-150mV) will result in a greater concentration of the dye from the cytosol (400-800 fold) compared to that conferred by the plasma membrane (-60mV) from extracellular space (10 fold). Therefore the available fluorescent probes are relatively selective for mitochondrial function. Measurements are normally taken after the dye has reached a steady-state equilibrium. In the following described studies we have examined the redistribution of TMRM in SH-SY5Y cells and in particular compared populations of cells with altered PINK1 levels exposed to different conditions namely vehicle or proteasomal stress. In these studies we assume that after equilibrium has

been reached, the mitochondrial fluorescence signal will reflect the mitochondrial dye concentration that will be a direct function of the $\Delta\Psi m$. Thus in the presence of proteasomal stress, there is a fall in the $\Delta\Psi m$, and so the mitochondria will accumulate less dye and the mean fluorescent signal for the dye in the sample of stressed cells will be less than control under steady-state conditions. The signal can be determined by a variety of ways including confocal microscopy but we have chosen fluorescence activated cell sorting (FACS) for quantitative analysis as this allows tens of thousands of cells to be analyzed in a short space of time and so is a robust method for inter-group comparison of different samples. It is possible to measure by FACS the relative reduction in mean fluorescence intensity (MFI), which can be interpreted as an indication of the $\Delta\Psi m$ dissipation (Zoratti and Szabo, 1995). The application of the protonophore carbonyl cyanide chlorophenylhydrazone (CCCP) collapses the $\Delta\Psi m$ and has therefore been used as a positive control for this methodology.

4.1.5 Chapter 4 Aims

The results presented here are the first to examine the putative localization and function of PINK1 and to determine the consequences of pathogenic human PINK1 mutations. Given the strong evidence that proteasomal inhibition induces mitochondrial depolarisation and apoptosis, this stress was selected for stress experiments. The results are evaluated and discussed and further experiments are proposed.

The aims of this chapter are therefore listed below:

(a) To confirm the predicted mitochondrial localization of PINK1 using a variety of microscopy and biochemical techniques

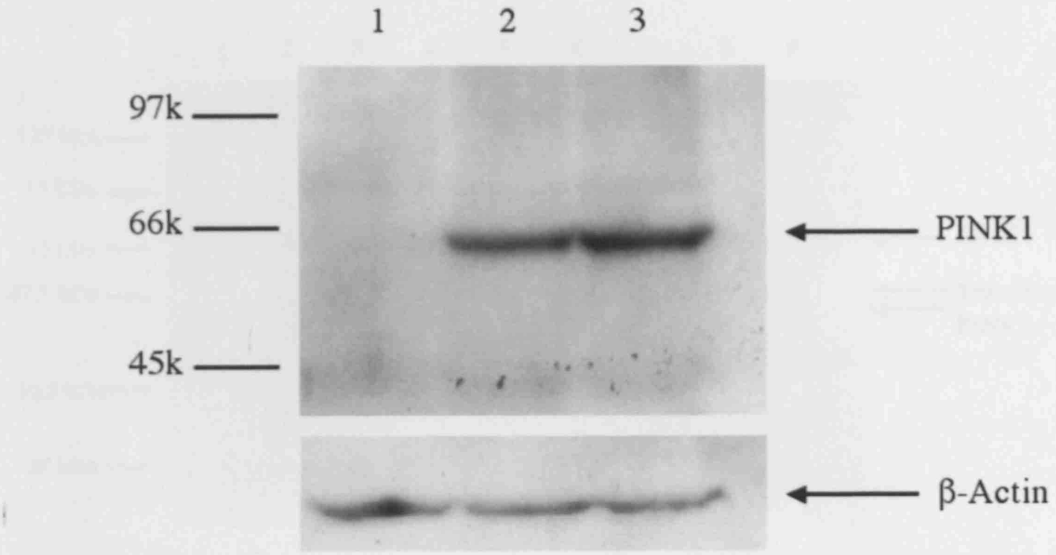
(b) To determine the functional consequences of PINK1 mutations by utilizing a mitochondrial specific fluorescent dye (TMRM) and FACS analysis in order to examine changes in the mitochondrial membrane potential ($\Delta\Psi m$) in neuronal cell lines with altered PINK1 in response to proteasomal stress.

(c) To determine whether PINK1 mutants result in altered stress-induced apoptosis

4.2 RESULTS

4.2.1 PINK1 mutations do not alter protein expression

To investigate the consequences of the missense mutation at the cellular level, wild-type or G309D c-myc–tagged *PINK1* cDNA constructs (cloned by Dr Kirsten Harvey, School of Pharmacy, London) were transiently transfected into monkey kidney COS-7 cells followed by Western blot analysis of cell lysates. The mutation did not alter production of mature full-length protein, which suggests that it does not significantly affect protein stability (Figure 4.3). Similar studies were performed on the additional mutations and similar results were observed. Interestingly, although both nonsense mutants produced drastic truncations of the PINK1 protein, these smaller species were still expressed (Figure 4.4).



1 myc-vector
2 myc-wt PINK1
3 myc-G309D PINK1

Figure 4.3 PINK1 protein expression is not affected by G309D mutation. COS-7 cells were transiently transfected with c-myc vector (lane 1), wild type (lane 2) and G309D (lane 3) c-myc-tagged PINK1 and lysates analysed by Western blotting using a c-myc antibody. The membrane was reprobed with β-Actin antibody to confirm equal loading.

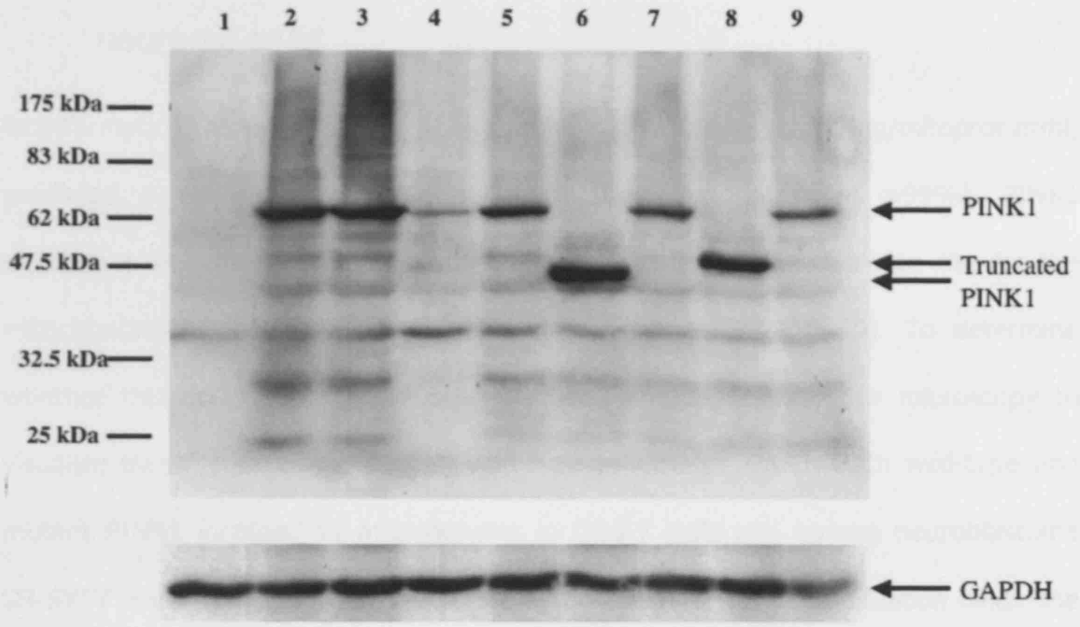


Figure 4.4 Wild type and mutant PINK1 expression in COS-7 cells. COS-7 cells transiently transfected with myc-vector (lane 1), myc-wt-PINK1 (lane 2), myc-A383T (lane 3), myc-G411S (lane 4), myc-Y431H (lane 5), myc-W437X (lane 6), myc-N451S (lane 7), myc-Q456X (lane8) and myc-C575R (lane 9) and analyzed by Western blots using c-myc antibody (1:100). Protein loading was determined by re-probing the same membrane with GAPDH antibody (1:5000).

4.2.2 PINK1 is localized to the mitochondria of neuronal and non-neuronal cells

Bioinformatic analysis using Mitoprot-2 (http://ihg.gsf.de/ihg/mitoprot.html) predicted a mitochondrial targeting motif (mitochondrial score >99%). PINK1 encodes a 34– amino acid mitochondrial targeting motif (the cleavage site for the mitochondrial processing peptidase between residues 34 and 35). To determine whether this occurred in living cells we used immunofluorescence microscopy to visualize transfected c-myc–tagged wild-type or G309D *PINK1.* Both wild-type and mutant PINK1 localized to mitochondria in COS-7 cells and human neuroblastoma SH-SY5Y cells (Figures 4.5 and 4.6) as determined by co-localization with the mitochondrial marker, Mitotracker Red (Molecular Probes). Furthermore, the mitochondrial localization of PINK1 was confirmed by Western blotting of mitochondrial enriched fractions obtained from COS-7 cells transiently transfected with c-myc–tagged wild-type *PINK1* cDNA (Figure 4.7).

4.2.3 PINK1 mutations result in increased mitochondrial depolarization in response to proteasomal stress

4.2.3.1 Proteasomal inhibition induces mitochondrial depolarisation

As described above, previous studies have demonstrated that proteasomal inhibition can induce depolarisation of the mitochondrial membrane potential ($\Delta\Psi m$) in human SH-SY5Y neuroblastoma cells. The majority of these studies have utilized fluorescent lipophilic cationic dyes and quantified uptake using confocal imaging methods.

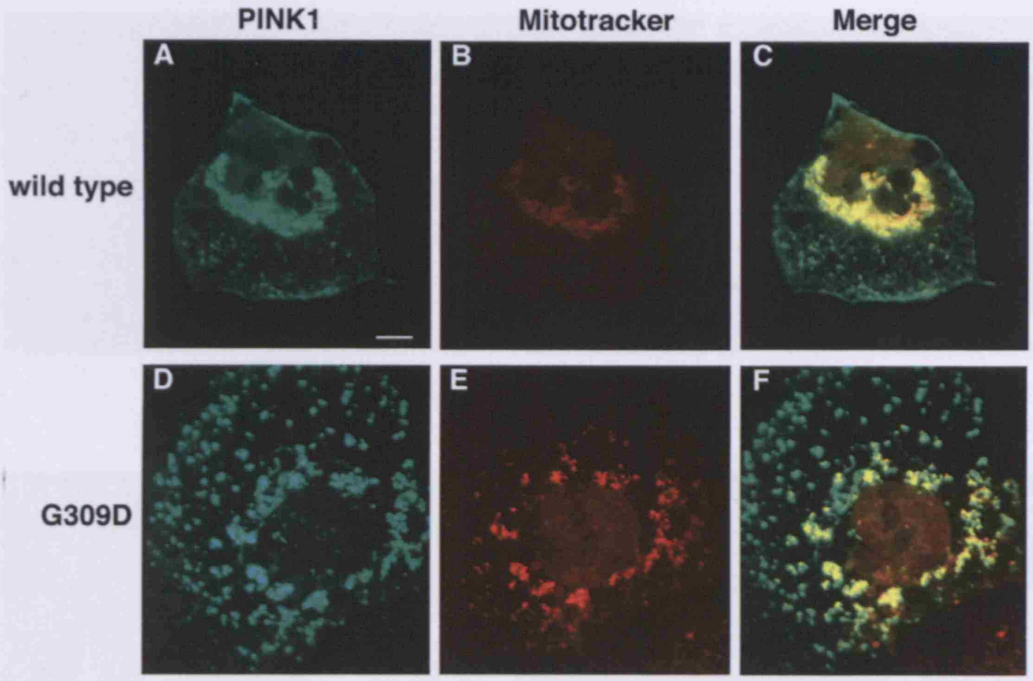


Figure 4.5 PINK1 is localized to the mitochondria in mammalian cells and its localization is not affected by the G309D mutation. (A-F) COS-7 cells transfected with wild-type **(A to C)** or G309D **(D to F),** c-myc-tagged PINK1 protein. Immunofluorescence was carried out with c-Myc antibody and mitotracker: c-myc-*PINK1* [green (A, D)]; mitotracker [red (B, E)]; c-Myc-*PINK1* and mitotracker merged (C, F). Scale bar 8μm.

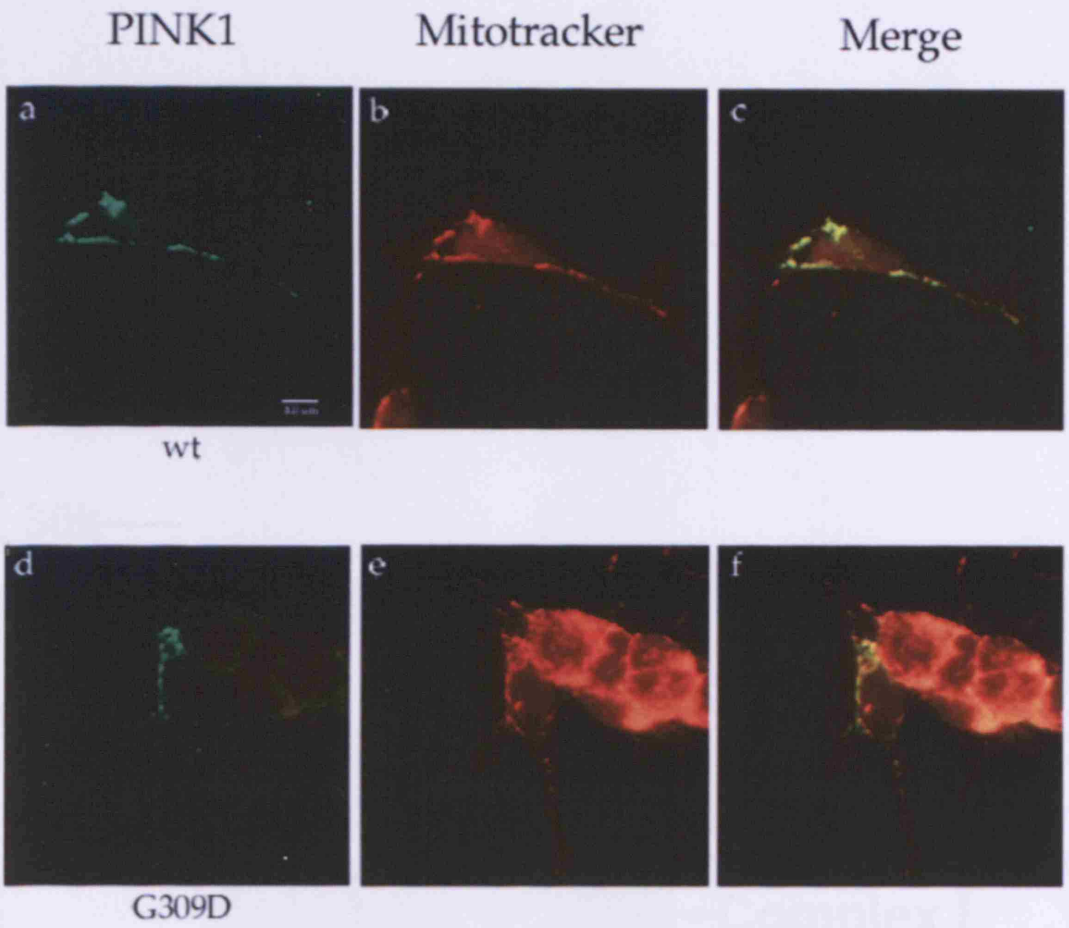


Figure 4.6 PINK1 is localized to mitochondria in neuronal cells. SH-SY5Y cells were transfected with wild-type **(a to c)** or G309D **(d to f),** c-Myc-tagged *PINK1* cDNA. Immunofluorescence was carried out with c-Myc antibody and mitotracker. c-Myc-PINK1 [green (a, d)]; mitotracker [red (b, e)]; c-Myc-PINK1 and mitotracker merged (c, f). Scalebar 8μm.

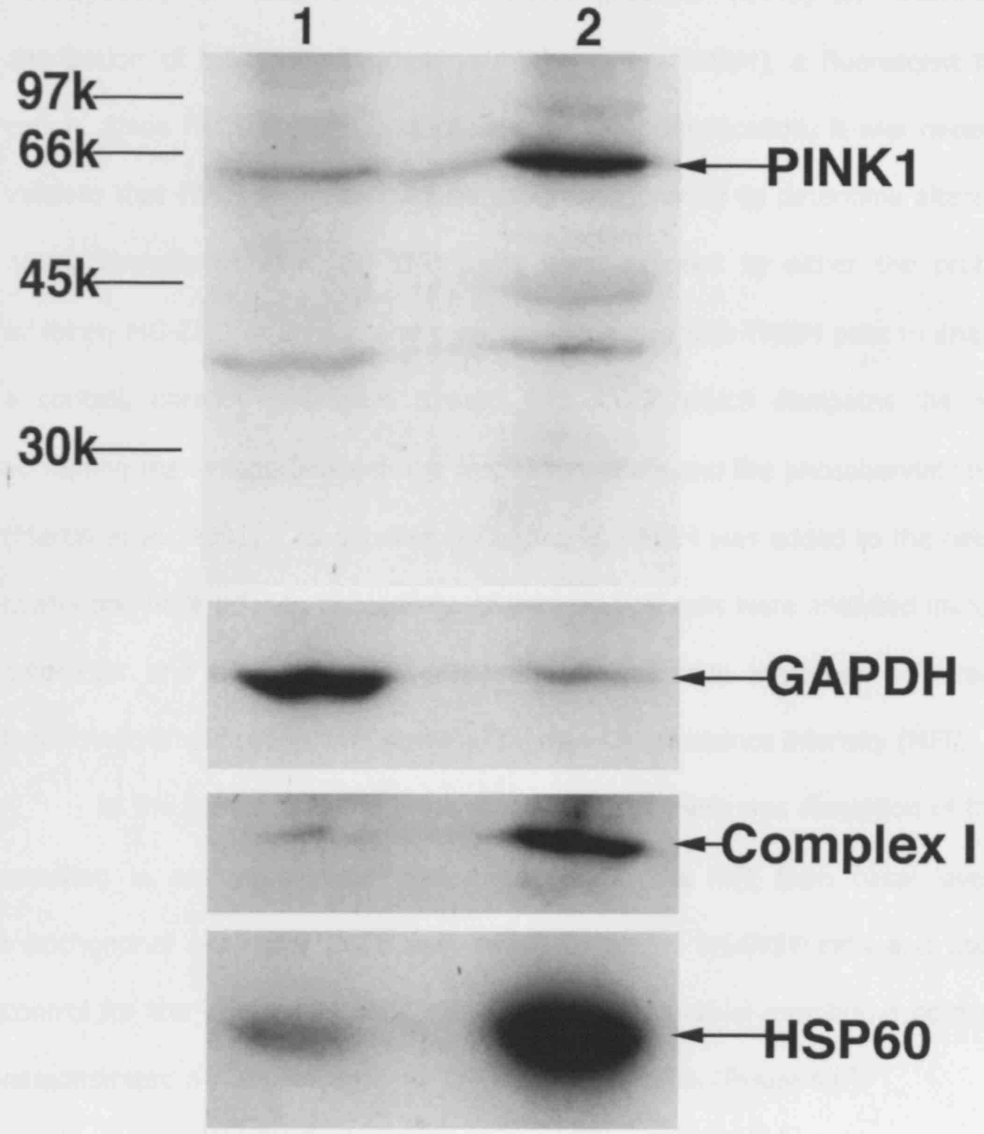


Figure 4.7 PINK1 is localized to the mitochondrial enriched fraction of mammalian cells. COS-7 cells were transiently transfected with c-Myc tagged wild-type *PINK1*. Cytoplasmic (lane 1) and mitochondrial enriched fractions (lane 2) were obtained and probed for c-Myc PINK1 expression by Western blot analysis using a c-Myc antibody. The same membrane was stripped and re-probed with Hsp60, complex I and GAPDH antibodies to determine the relative purity of fractions analyzed.

I examined mitochondrial function using a fluorescence-activated cell sorting (FACS)–based assay of mitochondrial membrane potential ($\Delta\Psi m$) by examining the distribution of tetramethylrhodamine methyl ester (TMRM), a fluorescent lipophilic cation. Since FACS analysis was chosen for the quantification, it was necessary to validate that FACS analysis could be used appropriately to determine alterations in $\Delta\Psi m$. Therefore, native SH-SY5Y cells were exposed to either the proteasome inhibitor, MG-132, or vehicle and then pre-incubated with TMRM prior to analysis. As a control, parallel cells were treated with CCCP which dissipates the $\Delta\psi m$ by abolishing the linkage between the respiratory chain and the phosphorylation system (Martin *et al.*, 1991). As detailed in Chapter 2, TMRM was added to the cell culture media one hour prior to harvesting. At least 20,000 cells were analyzed using a flow cytometer and the cells were gated and fluorescence emissions collected using logarithmic amplification to determine the mean fluorescence intensity (MFI).

In the presence of the proteasome inhibitor there was disruption of the $\Delta\Psi m$ resulting in an approximate 30% reduction in the MFI from basal levels. The mitochondrial uncoupler CCCP was added to native SH-SY5Y cells and used as a control for the irreversible disruption of the mitochondrial membrane potential and demonstrated a 70% reduction in MFI from basal levels (Figure 4.8).

4.2.3.2 G309D mutant results in impaired $\Delta\psi m$ following stress

I next investigated the effect of *PINK1* mutations on mitochondrial function using FACS to measure $\Delta\Psi m$. SH-SY5Y cells were transiently co-transfected with wild-type and mutant *PINK1* cDNA and a green fluorescent protein (GFP) reporter plasmid and

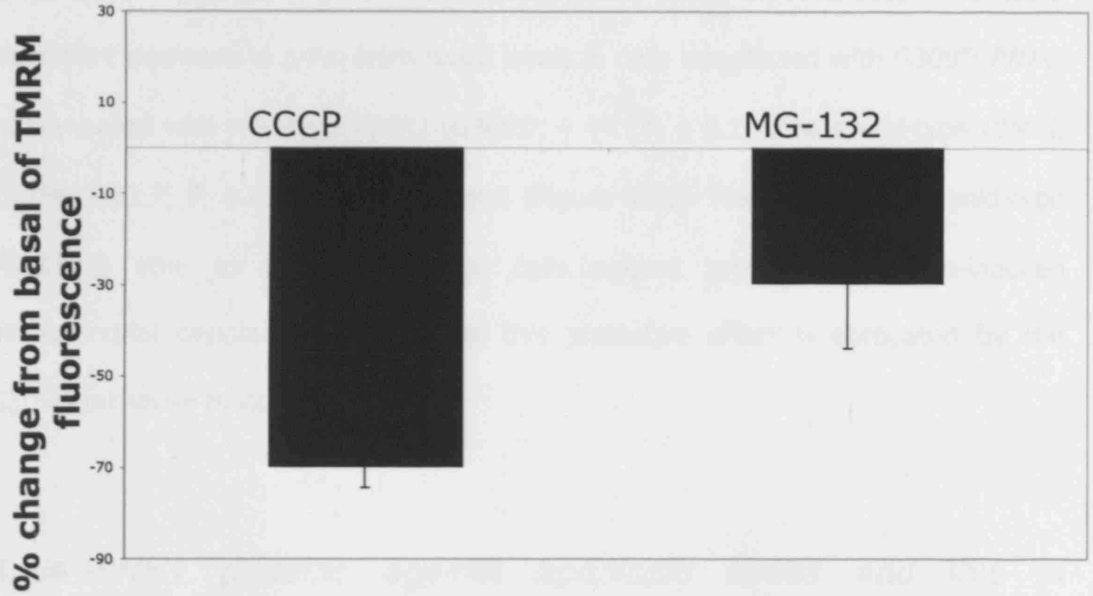


Figure 4.8. Proteasomal inhibition reduces $\Delta\psi$m. $\Delta\psi$m determined by FACS using SH-SY5Y cells treated with MG-132. Parallel cells were treated with the irreversible mitochondrial uncoupler CCCP that reduced $\Delta\psi$m by approximately 70%. Results displayed as mean ± SEM percent change in median TMRM fluorescence from the basal value.

then stressed with MG-132. There was no significant difference in transfection efficiency between constructs (data not shown). Analysis of TMRM fluorescence in GFP positive cells revealed that the *PINK1* mutation had no significant effect on $\Delta\Psi m$ under basal conditions (Figure 4.9). However, after stress with MG-132, there was a significant decrease in $\Delta\Psi m$ from basal levels in cells transfected with G309D *PINK1* as compared with wild-type *PINK1* (G309D, – 44.1% ± 8.1; versus wild-type *PINK1*, 13.0% ± 13.7; P 0.01; n = 8 data sets) (Figure 4.10). This suggests that wild-type PINK1 is able to protect neuronal cells against proteasomal stress-induced mitochondrial depolarization and that this protective effect is abrogated by the G309D missense mutant.

4.2.4 PINK1 protects against apoptopic stress and this is abrogated by G309D PINK1 mutant

As described in previous sections of this Chapter as well as Chapter 1, collapse of the $\Delta\Psi m$ represents irreversible mPTP opening and leads to MOMP and cytochrome c release and apoptosis via the mitochondrial pathway. Moreover $\Delta\Psi m$ depolarization following proteasomal inhibition has previously been shown to induce mitochondrial apoptosis. I therefore determined whether the differential effect of wild-type and mutant PINK1 on $\Delta\Psi m$ was associated with differential effects on apoptosis. I studied apoptosis of MG-132– stressed SH-SY5Y cells transfected with either wild-type or G309D *PINK1* by FACS using annexin V conjugated to the fluorochrome phycoerythrin (annexin V-PE). Annexin V has a high binding affinity for the membrane phospholipid, phosphatidylserine, that is exposed on the surface of apoptotic cells. Consistent with the changes in $\Delta\Psi m$ after stress, over-expression of

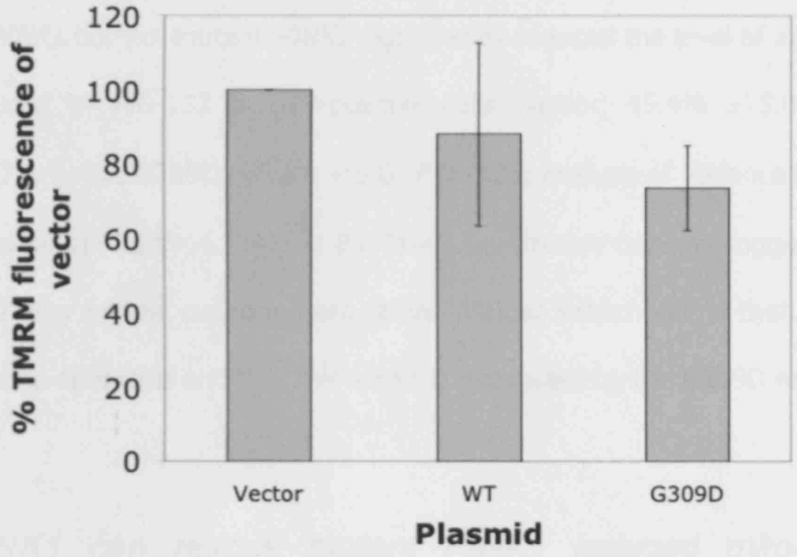


Figure 4.9. No effect of wild-type and mutant PINK1 on mitochondrial membrane potential ($\Delta\psi m$) under basal conditions. Determined by FACS of GFP gated events of SH-SY5Y cells treated with vehicle (basal). Basal TMRM values normalised to vector, mean ± SEM percentage TMRM fluorescence of vector.

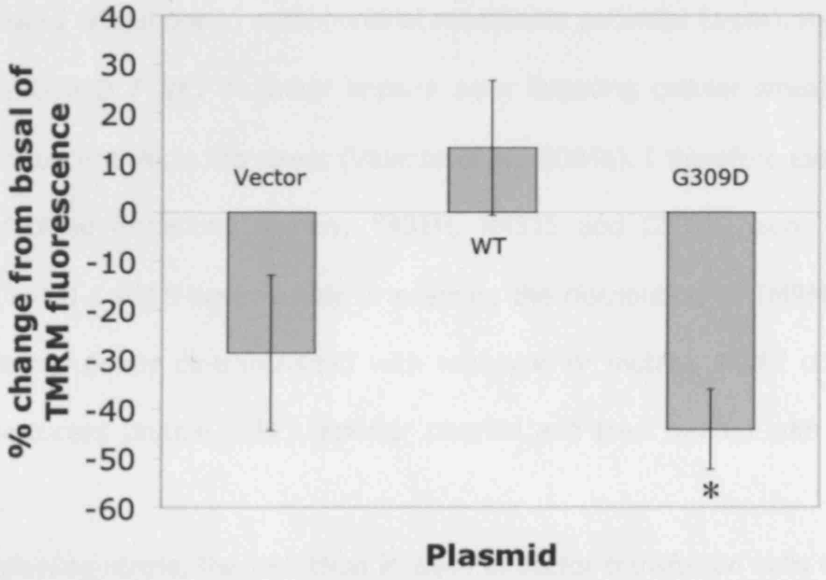


Figure 4.10 Mean ± SEM percent change in median TMRM fluorescence from basal value after treatment with 15 μM MG-132. *$p < 0.01$ for G309D versus wild-type PINK1; ANOVA with post-hoc Bonferroni correction; n = 8 from 3 independent experiments performed in duplicate or triplicate.

wild type *PINK1* but not mutant *PINK1* significantly reduced the level of apoptotic cell death induced by MG-132 in GFP-positive cells [vector, 45.4% ± 5.0; wild-type *PINK1*, 32.7% ± 4.0; G309D, 45.8% ± 5.0; $P < 0.05$; analysis of variance (ANOVA), $n = 12$ data sets] (Figure 4.11A and B). These preliminary findings suggest that wild type *PINK1* may protect neurons from stress-induced mitochondrial dysfunction and stress-induced apoptosis and that this effect is abrogated by the G309D mutation.

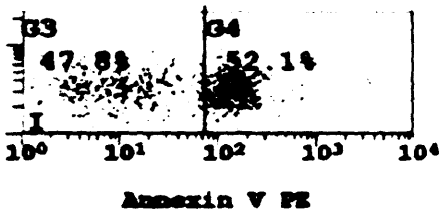
4.2.5 PINK1 can rescue mutant PINK1 induced mitochondrial depolarisation

In view of our laboratory's genetic data that PD risk may be increased with single PINK1 mutations (see earlier in section 4.1.2), I next examined whether these mutations had any pathogenic effect *in vitro*. I therefore examined their effects on stress-induced alterations in mitochondrial membrane potential ($\Delta\psi m$). As described above the G309D *PINK1* mutation impairs $\Delta\psi m$ following cellular stress rendering cells more susceptible to the stress (Valente *et al.,* 2004a). I therefore examined the effects of three mutations namely, Y431H, N451S and C575R. $\Delta\psi m$ was again assessed using a FACS-based-assay to examine the distribution of TMRM. SH-SY5Y cells were transiently co-transfected with wild-type or mutant *PINK1* cDNA and a green fluorescent protein (GFP) reporter plasmid and then treated with vehicle or MG-132.

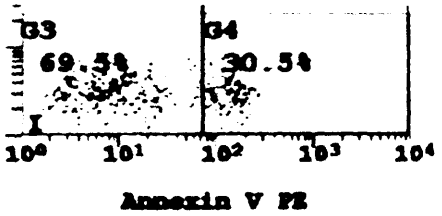
Following stress, the reduction in $\Delta\psi m$ in vector transfected cells was similar to that of native SH-SY5Y cells (-31.4% vs -29.5%) indicating that transfection *per se* did not alter $\Delta\psi m$ in our system (Figures 4.8 and 4.12A). There was also no difference in transfection efficiency between wild type and mutant plasmids (data not

A

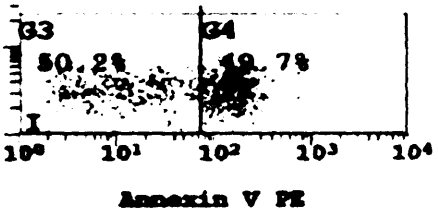
a


G3
47.8%
G4
52.1%
10⁰ 10¹ 10² 10³ 10⁴
Annexin V PE

b


G3
69.5%
G4
30.5%
10⁰ 10¹ 10² 10³ 10⁴
Annexin V PE

c


G3
50.2%
G4
49.7%
10⁰ 10¹ 10² 10³ 10⁴
Annexin V PE

B

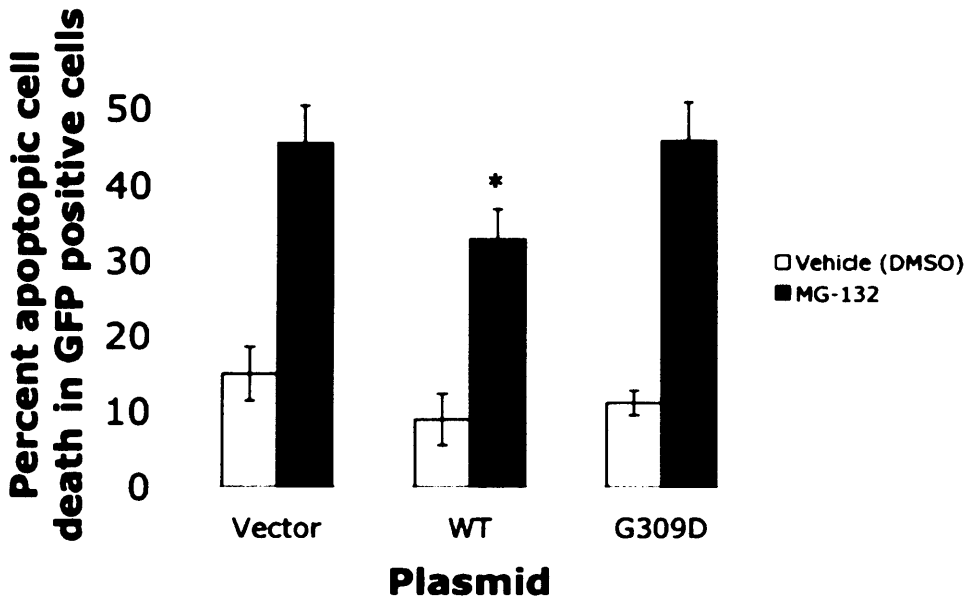
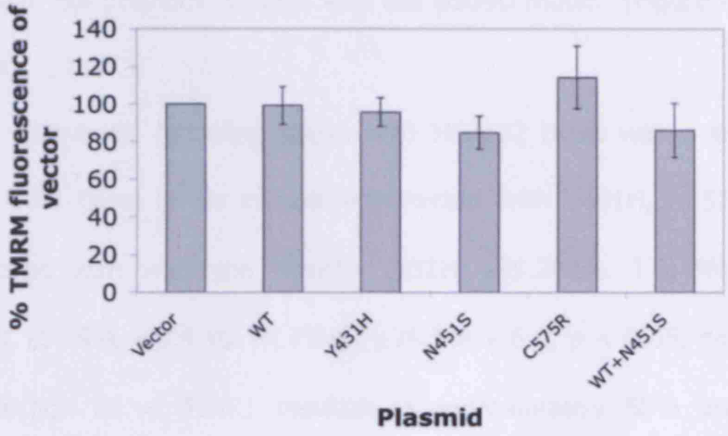


Figure 4.11 Effect of wild-type and mutant PINK1 on the amount of apoptosis determined by FACS of Annexin V-PE positive GFP gated events of SHSY5Y cells treated with vehicle (basal) or MG-132. (A) Representative flow cytometric Annexin V-PE plots of GFP-gated events after treatment with MG-132 showing decreased percentage of Annexin V-PE positive events in cells transfected with (b) wild-type PINK1 compared to (a) vector and (c) G309D. **(B)** Graph showing significant difference in apoptosis between wt and mutant. Mean ± SEM percent apoptopic cell death for each construct after treatment with vehicle or 15 μM MG-132. *$p < 0.05$; ANOVA, $n = 12$ from 4 independent experiments performed in triplicate.

A



B

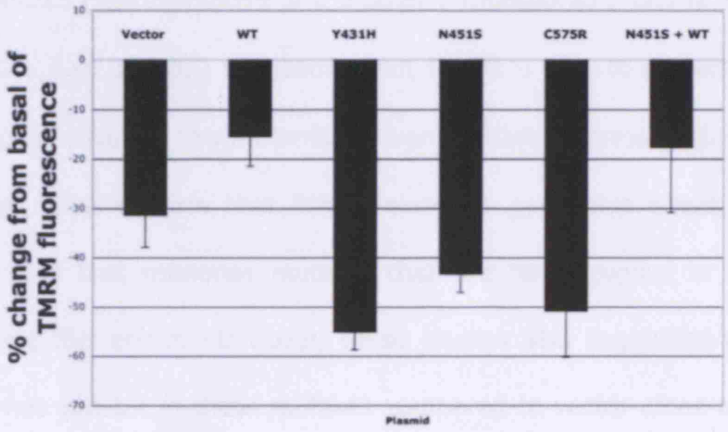


Figure 4.12. TMRM data from SHSY-5Y cells transfected with wild-type or mutant PINK1 constructs. (A) No difference in $\Delta\psi m$ between wild-type and mutant PINK1. **(B)** Mutant PINK1 reduces $\Delta\psi m$ in stressed cells compared to wild-type. $\Delta\psi m$ determined by FACS of GFP-gated events using SH-SY5Y cells treated with MG-132. Results displayed as mean ± SEM percent change in median TMRM fluorescence from the basal value for each construct after treatment with 15μM MG-132. $p < 0.05$ for Y431H, N451S and C575R versus wild-type PINK1; ANOVA with post-hoc Bonferroni correction; n=9-17 data sets from three to six independent experiments performed in triplicate.

shown). Analysis of TMRM fluorescence in GFP-positive cells revealed that none of the new *PINK1* mutations had a significant effect on $\Delta\psi m$ under basal conditions similar to our previous findings with the G309D mutant (Figure 4.12A) (Valente *et al.*, 2004a).

However, following stress with MG-132 there was a significant decrease in $\Delta\psi m$ from basal levels in cells transfected with Y431H, N451S and C575R PINK1 compared with wild-type PINK1 (Y431H, -55.2% ± 3.6; N451S, -43.4% ± 3.7; C575R, -50.9% ± 9.4 vs. wt PINK1 −15.5% ± 6.0; $p < 0.05$; n= 9 - 17). In contrast, transfection of wt PINK1 resulted in approximately 50% less reduction in $\Delta\psi m$ compared to vector, following stress. Moreover, double transfection studies revealed that wild-type PINK1 was able to partially rescue the decrease in $\Delta\psi m$ associated with N451S (representative of the other 2 mutations) (-17.7% ± 13.2 vs. wt PINK1 – 15.5% ± 6.0; p=0.87) suggesting that PINK1 is able to protect against some of the damaging effect of the mutants on $\Delta\psi m$ following stress (Fig. 4.12B). Overall these studies again confirm that PINK1 exerts a protective effect against proteasomal stress and that missense mutants that are heterozygous in PD cases, appear to abrogate this effect. Moreover, these studies also suggested that the reduction in $\Delta\psi m$ was greater in these mutants compared to vector alone although this was not statistically significant.

4.3 DISCUSSION OF RESULTS

The discovery of *PINK1* mutations represents the most direct evidence in humans that primary mitochondrial dysfunction may underlie the pathogenesis of Parkinson's disease. The studies described here were the first ever to be undertaken in studying

PINK1 and the functional consequence of the mutations.

Western blot analysis suggested that neither G309D nor the other disease causing mutants of PINK1 affected protein stability. Beilina et al. (2005) have also recently investigated protein stability of PINK1 mutants using pulse chase analysis and found that both G309D and several 'kinase dead' mutants of PINK1 did not alter PINK1 protein stability. This contrasts with their finding that the L347P mutant of PINK1 that confers severe instability of the protein. L347P is particularly prevalent in the Philippines but has not been reported in Western European populations (Beilina et al., 2005).

Bioinformatic analysis predicted a mitochondrial-targeting domain in the amino terminus of PINK1. Using both microscopy and biochemical techniques, I was able to confirm mitochondrial localization in neuronal and non-neuronal cells. None of the mutants of PINK1 studied mislocalized from the mitochondria and this is not surprising as none occur within the mitochondrial domain. Beilina et al. (2005) have also confirmed mitochondrial localization of wild type and G309D mutant PINK1 with N-terminal tagged constructs. However, they have found that when PINK1 is C-terminal tagged with GFP then the transfected PINK1 was also localized to the cytosol. Mitochondrial proteins with the targeting domain of PINK1 are normally processed within and remain in the mitochondria, and the significance of the finding of Beilina et al. is uncertain (2005). They also reported that on transfection of their C-terminal GFP tagged PINK1 construct, PINK1 protein also appeared to coalesce into multiple punctate or single perinuclear inclusions reminiscent of aggregated material. Since PINK1 has a hydrophobic C-terminal tail (see Chapter 6), it may be that placing a large protein tag next to this domain interferes with normal protein folding and promotes misfolding and aggregation of the protein. Future studies should be able to clarify this question.

PINK1 encodes a conserved protein kinase domain between amino acids 156 and 509 that has homology to the serine/threonine kinases of the Ca^{2+}/calmodulin family. While the kinase domain can mediate PINK1 autophosphorylation (amino acids 112-496), the downstream phosphorylation targets of PINK1 remain unknown (Nakajima et al., 2003; Beilina et al., 2005). All the mutations detected occur in the putative serine/threonine kinase domain and thus conceivably could affect kinase activity or substrate recognition. Beilina et al. (2005) have recently examined the kinase activity of mutant forms of PINK1 *in vitro*. Using GST-fusion constructs they were able to demonstrate that PINK1 has kinase activity and is able to undergo autophosphorylation and that the K219A kinase dead mutant caused a significant decrease in autophosphorylation. Furthermore they showed that the G309D mutant had significantly reduced kinase activity in their assay (Beilina et al., 2005).

We have investigated the consequences of the G309D PINK1 mutant on mitochondrial function. To do this we have chosen a proteasomal stress model since dysfunction of the UPS may play a major role in PD pathogenesis as has been previously stated in Chapter 1 and demonstrated in Chapter 5. Furthermore, proteasomal inhibition induces mitochondrial depolarization and apoptosis which have been demonstrated to occur in many PD model systems. Therefore, it allowed us to readily examine the role of PINK1 and its mutant in this context. The results presented in section here indicate that PINK1 may play a protective role against proteasomal stress-induced mitochondrial depolarization and apoptosis and this is abrogated by the G309D mutant. The mechanism for this protection remains unknown and we hypothesize that PINK1 may phosphorylate mitochondrial proteins in response to cellular stress, protecting against mitochondrial dysfunction and that G309D PINK1 causes decreased phosphorylation resulting in less protection. Although Beilina et al. (2005) have confirmed that G309D has approximately 40%

less kinase activity than wild type in their assay, this was only determined for autophosphphorylation and it remains unclear if G309D results in a significantly larger reduction in phosphorylation of a specific target substrate. Recently another study has also confirmed that *PINK1* protects against apoptotic stress and showed that over-expressed wild type PINK1 reduced cytochrome c release, caspase cleavage and PARP. Importantly these effects were abrogated by a 'kinase dead' mutant, thus, supporting the notion that PINK1's protective effect is mediated by phosphorylation (Petit et al., 2005).

The effects of the Y431H, N451S and C575R mutations on PINK1 kinase activity remain unknown. My preliminary results do suggest that they are pathogenic with loss of the protective effect conferred by wild-type PINK1. However, there was also a trend for the reduction in $\Delta\psi m$ to be greater than vector alone following stress suggestive of a dominant negative effect. Such a mechanism would also be consistent with the ability of wild type PINK1 to only partially rescue the $\Delta\psi m$ reduction caused by these mutants. This would clearly be an attractive mechanism in view of the fact that these mutations were heterozygous in PD cases. However, further studies will be required to confirm whether this is indeed the case.

Proteasomal inhibition may induce collapse of the $\Delta\psi m$ and apoptosis via several distinct mechanisms and it is not clear where PINK1 is acting. Identification of substrates and interacting partners for PINK1 should shed more light on this. Furthermore, identifying the exact localization of PINK1 in the mitochondria may also provide clues about its potential targets and the pathways it may play a role in. In this regard, data will be presented and discussed in the next chapter.

PINK1 was originally shown to be upregulated by the tumor suppressor gene *PTEN* in cancer cells (Unoki and Nakamura, 2003). In neurons, the PTEN signaling pathway is involved in cell cycle regulation and cell migration and promotes

excitotoxin-induced apoptosis in the hippocampus (Gray and Mattson, 2002). PTEN also induces phosphorylation of Akt and Akt may play a role in proteasomal stress-induced apoptosis. However, PINK1 has not been shown to have any effects on PTEN-dependent cell phenotypes (Unoki and Nakamura, 2003), and its role in the PTEN pathway therefore requires further investigation.

PINK1 was the first protein kinase to be associated with PD. Recently, another kinase, LRRK2 has also been shown to be mutated in PD (Paisan-Ruiz et al., 2005; Zimprich et al., 2005). Kinases add phosphate groups to specific substrate proteins altering their function, localization and activity. The phosphorylation of proteins by kinases forms a key cellular signaling mechanism. When protein phosphorylation is combined with the action of counteracting phosphoprotein phosphatases the result is a finely tuned system which can be used to regulate of a wide variety of cellular processes such as cellular metabolism, gene expression, cytoskeletal architecture, cell adhesion and cell cycle progression (Pawson and Scott, 2005).

In eukaryotes, the most commonly studied kinases phosphorylate the hydroxyamino acids serine, threonine and tyrosine. Substrate specificity can be predicted by sequence motifs that are specific to either protein tyrosine kinases or protein serine/threonine kinases. Phosphotyrosines are commonly involved in molecular recognition and the formation of protein-protein complexes while the phosphoserines and phosphothreonines function mainly in the modulation of enzymatic activity through the modification of conformation or substrate-binding. Sequence comparisons suggest PINK1 is most similar to the calmodulin dependent serine/threonine kinases (Griffith, 2004).

The wide range of diseases resultant from mutant kinases underlines their ubiquity and importance. Neurological diseases associated with altered kinase function include, myotonic dystrophy which is caused by decreased expression of the DM serine kinase (Machuca-Tzili et al., 2005), Pantothenate kinase–associated neurodegeneration caused by mutations in the PANK2 gene (Zhou et al., 2001) and a variety of peripheral neuropathies. Additionally, dysregulation of protein phosphorylation has been observed in several neurodegenerative diseases most notably the hyperphosphorylation of tau that is causal of neuronal dysfunction and cell death in AD and associated tauopathies (Stoothoff and Johnson, 2005), and the phosphorylation of α-synuclein at Ser 129 in PD and other synucleinopathies (Fujiwara et al., 2002).

In addition to primary mutations that lead directly to loss of function such as, premature truncation and missense mutations at regulatory elements, disruption of the complex regulatory pathways of kinases can also indirectly result in disease phenotype. These include the actions of second messengers, allosteric mechanisms, inhibition through pseudosubstrate sequences, positive and negative phosphorylation events, regulatory subunit binding, inhibitor proteins and differential subcellular localization through targeting domains and anchoring proteins. The assessment and determination of changes in the mitochondrial phosphoprotein milieu arising from PINK1 dysregulation in PD will firstly require knowledge of its upstream effectors and downstream targets. Currently very little is known about the mitochondrial phosphoproteome however recent studies have suggested that reversible phosphorylation of complex I subunits is required for their normal assembly and further studies will be required to determine whether PINK1 is involved in these processes (Chen et al., 2004; Schulenberg et al., 2003).

5

Determining how PINK1 localizes to Aggregates *in vitro*.

5.1 INTRODUCTION

5.1.1 PINK1 and Parkinson's disease

PINK1 encodes a 581 amino acid protein with a conserved protein kinase domain between amino acids 156 and 509 that has homology to the serine/threonine kinases of the Ca^{2+}/calmodulin family. While the kinase domain can mediate PINK1 autophosphorylation (amino acids 112-496), the downstream phosphorylation targets of PINK1 remain unknown (Nakajima et al., 2003; Beilina et al., 2005; Silvestri et al., 2005). I previously demonstrated (Chapter 4) that PINK1 localizes to the mitochondria in mammalian cells. Mitochondrial translocation of PINK1 is possibly mediated by a 34 amino acid N-terminal mitochondrial targeting motif (or mitochondrial localization signal), but the exact mechanisms of mitochondrial import remain unknown.

5.1.2 Mitochondrial import mechanisms

Most mitochondrial proteins are synthesized on cytoplasmic ribosomes as precursor proteins that are then translocated into the mitochondria via a highly specialized

import system (Chacinska et al., 2002). The majority of mitochondrial proteins contain a cleavable N-terminal 'pre-sequence' of between ~20-60 amino acid residues. Proteins of this group interact with a multimeric complex on the outer mitochondrial membrane known as the translocase of the outer membrane or Tom complex, in particular Tom20. After passing through the outer membrane they enter the matrix by passing through a pore formed by a multiprotein complex known as translocase of the inner membrane or Tim complex, specifically Tim23. After entry into the mitochondrial matrix the pre-sequence is removed by the mitochondrial processing peptidase (MPP) and then, the processed protein is usually localized to the matrix (Chacinska et al., 2002).

Some mitochondrial proteins do not contain a cleavable pre-sequence but instead have internal signal sequences often consisting of stretches of positively charged amino acids adjacent to hydrophobic domains. These proteins generally interact with the Tom70 receptor on the outer membrane and after entry are localized to the inner mitochondrial membrane by interaction with the Tim22 complex (Chacinska et al., 2002). The presence of a N-terminal pre-sequence within PINK1 suggests that it may be transported by interacting with Tom20 and Tim23 complexes. PINK1 also contains a putative recognition signal for a mitochondrial processing peptidase (amino acids 34 and 35), suggesting that it can be recognized and cleaved by MPP.

5.1.3 Aggregates, phosphorylation and kinases

As previously stated in Chapter 3, eosinophilic cytoplasmic aggregates known as Lewy bodies (LBs) are one of the pathological hallmarks of PD, but the mechanisms by which they are formed and the role LBs play in neurodegeneration remain unclear however, there is some evidence that the biogenesis of LBs may resemble that of aggresomes (McNaught et al., 2001).

Proteins associated with PD including parkin (PARK2), UCH-L1 (PARK5), and synphilin-1 accumulate in LBs and aggresomes upon proteasomal inhibiton (Ardley et al., 2003, Ardley et al., 2004, Muqit et al., 2004). These proteins are all cytoplasmic and normally turned over by the UPS. In the presence of proteasome inhibitors, such as MG-132 and lactacystin, polyubiquitylated forms of these proteins are identified in aggregates suggesting that they are targeted by the UPS (Junn et al., 2002; Tanaka et al., 2004). These observations together with the facts that proteasome function is reduced in PD and that chronic exposure of rats to proteasome inhibitors triggers Parkinsonian symptoms, indicate that impairment of proteasome function may be an important mechanism in PD pathogenesis (McNaught et al., 2001; McNaught et al., 2004).

There is evidence revealing that the major component of LBs, α-synuclein, is phosphorylated at serine 129 within LBs and this form of phospho-synuclein is also detected in aggresomes (Tanaka et al., 2004, Fujiwara et al., 2002). α-synuclein is phosphorylated by casein kinases I and II both *in vitro* and *in vivo*, however, casein kinases I and II do not appear to localize to LBs (Okochi et al., 2000). Other kinases including calcium/calmodulin dependent kinase II and cyclin-dependent kinase have been reported in LBs but the significance of their localization is uncertain (Iwatsubo et al., 1991; Brion and Couck, 1995).

5.1.4 Chapter 5 Aims

Parallel studies in our laboratories have determined that PINK1 protein localizes to 5–10% of LBs in PD brain (Gandhi et al., 2006). In contrast to many cytoplasmic proteins that are regulated by the UPS, the mechanism by which a mitochondrial protein accumulates in aggregates remains unclear since the UPS machinery including ubiquitin ligases and the proteasome are cytoplasmic. To determine the mechanism of how PINK1 protein may localize to LBs I have studied the localization of PINK1 in an aggresome cell system.

The aims of this chapter are therefore listed below:

(e) To characterize novel polyclonal PINK1 antibodies that have been generated in our laboratories

(f) To further investigate the sub-mitochondrial localization of PINK1 *in vitro* and *in vivo*.

(g) To determine if PINK1 localizes to aggresomes upon proteasomal inhibition and to probe the mechanism of localization.

(h) To study the cleavage of PINK1

5.2 RESULTS

5.2.1 Generation and characterization of PINK1 antibodies

To further understand the function of PINK1 it was critical to be able to detect the protein with specific antibodies. Western blot studies using commercial PINK1 antibodies suggested that they were neither sensitive nor specific for PINK1 (data not shown). Our laboratory raised two rabbit polyclonal antibodies to human PINK1 protein against amino acids 135 – 149 (denoted PINK1-49) and 194 – 209 (denoted PINK1-48) respectively. To test sensitivity and specificity of the antibodies I first probed mammalian COS-7 cell extracts over-expressing empty vector or wild-type or mutant (G309D) c-myc-tagged PINK1 cDNA constructs with either PINK1-49, PINK1-48 or c-myc antibody. Specificity of the antibody was confirmed by detection of a cross-reactive band of ~63 kDa, corresponding to the full length of PINK1, in only the lanes expressing mutant or wild-type PINK1 cDNA (Figure 5.1A, lanes 1 and 2) and not vector alone (Figure 5.1 A, lane 3). This band was the same size as that detected by antibody to the c-myc tag (Figure 5.1 A). In addition, both polyclonal antibodies detected a shorter cross-reactive band of ~53 kDa (Figure 5.1 A). Since the smaller band of 53 kDa, denoted ΔPINK1, is seen when probing with the c-myc antibody in cells transfected with C-terminal-c-myc-tagged PINK1 (Figure 5.1 B) but not N-terminal-c-myc-tagged PINK1 (Figure 5.1 A) this is likely to represent N-terminal cleaved PINK1 protein. Thus PINK1 appears to be processed in a similar manner to other mitochondrial proteins with cleavable pre-sequences. Since both PINK1-49 and PINK1-48 antibodies recognize the same cleaved fragment, this suggests that cleavage occurs upstream of residue 135 within the N-terminus of the protein.

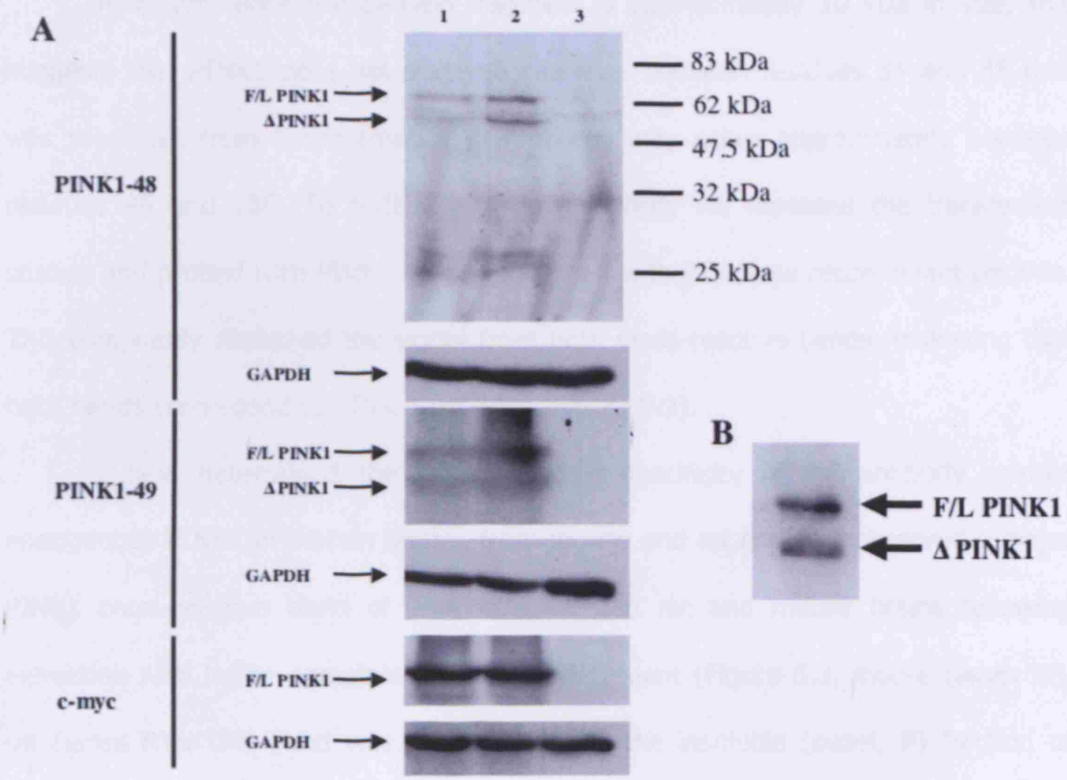


Figure 5.1. Characterization of polyclonal PINK-1 antibodies *in vitro*. (A) COS-7 cells were transiently transfected with N-terminal-myc-G309D PINK1 (lane 1), N-terminal-myc-wt-PINK1 (lane 2) or myc-vector (lane 3) and supernatant fractions from RIPA cell lysates were analysed by 10% SDS-PAGE and Western blotting in parallel and probed with either PINK1-48, PINK1-49 or c-myc antibody as indicated. Full length (F/L) and cleaved PINK1 (Δ PINK1) of approximately 63 kDa and 53 kDa respectively were detected with native antibodies. F/L PINK1 but not Δ PINK1 was detected with c-myc antibody. **(B)** COS-7 cells were transiently transfected with C-terminal-myc-wt-PINK1 and cell lysates were analyzed by SDS-PAGE and Western blotting with a c-myc antibody indicating F/L and Δ PINK1. n = at least 3 independent experiments.

However, since the cleaved fragment is approximately 10 kDa in size, this suggests that PINK1 does not undergo cleavage between residues 34 and 35 that was predicted from bioinformatics utilities but may occur approximately between residues 90 and 100. To further confirm specificity we repeated the transfection studies and probed with PINK1-49 pre-absorbed with PINK1-49 recombinant peptide. This completely abolished the signal from both cross-reactive bands, indicating that both bands correspond to PINK-1 proteins (Figure 5.2).

I next determined the sensitivity and specificity of the antibody against endogenous PINK1 in protein lysates from mouse and rat brains. I observed a major PINK1 cross-reactive band of ~68 kDa in both rat and mouse brains following extraction with buffer containing tween-20 detergent (Figure 5.3, mouse (lanes M), rat (lanes R)). This band was present in both the insoluble (pellet, P) fraction of rodent lysates and soluble supernatant (S) fraction. In contrast, a non-detergent based sucrose buffer poorly solubilised PINK1 from rodent brain that was extracted and analyzed in parallel to protein tween-20 extracts (Figure 5.3). Therefore, tween-20 containing buffer was used in all subsequent brain extractions.

In human brain frontal cortex extracts, the PINK1 polyclonal antibody detected a cross-reactive band of ~70 kDa specifically in the insoluble pellet fraction and a smaller cross-reactive band of ~51-52 kDa in the supernatant fraction (Figure 5.4). Both bands were abolished by pre-absorbing PINK1-49 antibody with peptide suggesting that both are PINK1-related species (Figure 5.5). Thus the higher band represents full-length protein that may be modified *in vivo* and the smaller band represents the processed form of PINK1 protein.

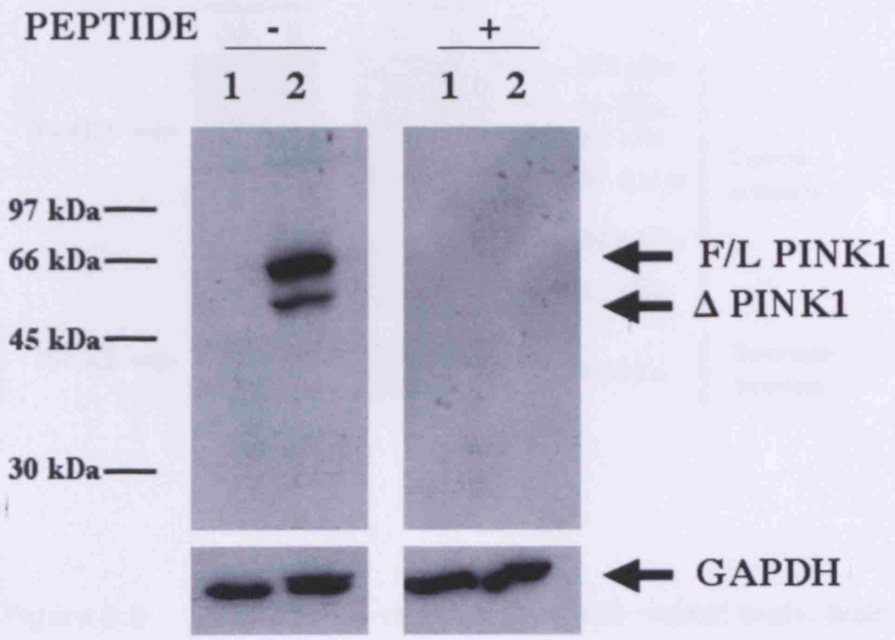


Figure 5.2. Full length PINK1 and ΔPINK1 bands are abolished by PINK1 peptide pre-absorption. COS-7 cells were transfected with myc-vector (lane 1) or c-myc-wt-PINK1 (lane 2) and analyzed by 10% SDS-PAGE and Western blotting with PINK1-49 antibody (-) or PINK1-49 antibody pre-absorbed with PINK1-49 recombinant peptide (+). Equal loading was confirmed by reprobing with GAPDH antibody. n = at least 3 independent experiments.

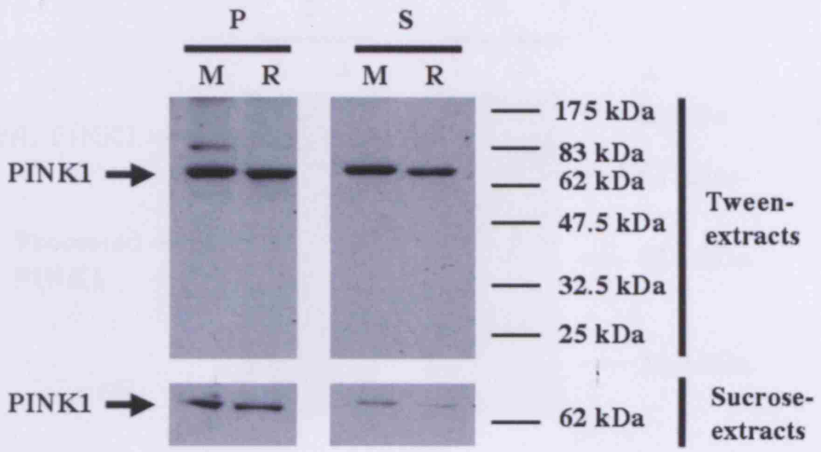


Figure 5.3 PINK1 cross-reactive bands in rodent brain. Brain homogenates were made from fresh mouse (lane M) or fresh rat (lane R) brains using either a Tween 20-based buffer or sucrose buffer and pellet fractions (P) or supernatant fractions (S) were analyzed by 10% SDS-PAGE and Western blotting and then probed with native PINK1 antibody. A cross-reactive band representing PINK1 is detected at ~68 kDa in both the pellet and supernatant fractions and is weakly solubilised by sucrose buffer. n = at least 3 independent experiments.

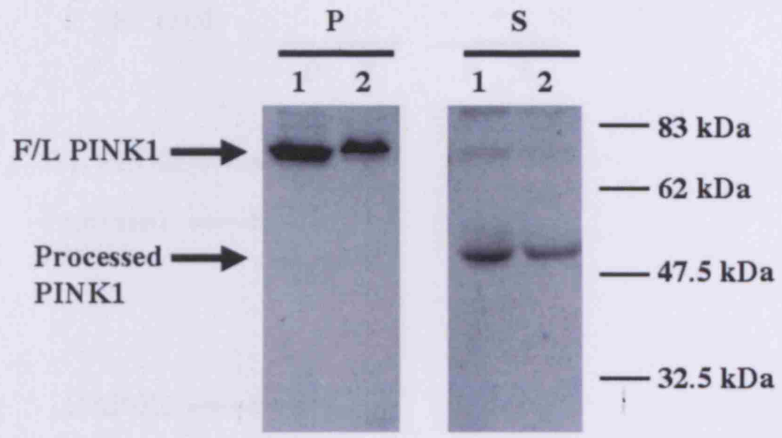


Figure 5.4. PINK1 is processed in human brain *in vivo*. Human frontal cortex homogenates were prepared from 2 different age-matched post-mortem control brains lyzed with Tween-20 buffer. Pellet fractions (P) or supernatant fractions (S) were analyzed by 10% SDS-PAGE and Western blotting probed with native PINK1 antibody demonstrating F/L PINK1 migrating at ~70 kDa in the P fractions and processed PINK1 migrating at ~51-52 kDa in the S fractions. n = at least 3 independent experiments.

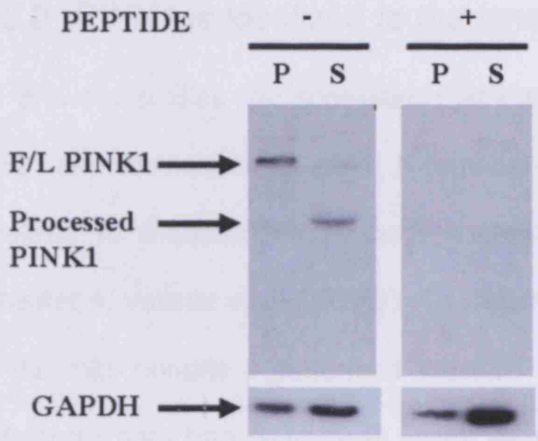


Figure 5.5. Human PINK1 cross-reactive bands are abolished by PINK1 peptide pre-absorption. Pellet (lane P) or supernatant (lane S) fractions of human frontal cortex extracts were analyzed by 10% SDS-PAGE and Western blotting and probed with PINK1-49 antibody (-) or PINK1-49 antibody pre-absorbed with PINK-1 recombinant peptide (+) as indicated. Equal loading was confirmed by GAPDH reprobe of the same membrane. n = at least 3 independent experiments.

5.2.2 *PINK1 is localized to the inner mitochondrial membrane*

My previous studies had suggested that PINK1 contained a mitochondrial targeting motif and demonstrated that N-terminal-c-myc-tagged full length PINK1 was localized to mitochondria in both neuronal and non-neuronal mammalian cells (Chapter 4, Valente et al., 2004a). To determine whether ΔPINK1 was also localized to the mitochondria I performed Western blot analysis of mitochondrial enriched cellular fractions transfected with vector alone, wild-type, or mutant (G309D) PINK1, using native antibody and demonstrated that ΔPINK1 was present in the mitochondria (Figure 5.6). Furthermore, import and cleavage of PINK1 was unaffected by the G309D mutation (Figure 5.6).

I next wished to determine to which mitochondrial compartment full length and processed PINK1 protein was localized. Ultracentrifugation of mitochondrial pellets into soluble matrix and membrane pellet fractions was performed and Western blot analysis revealed that both full length PINK1 and ΔPINK1 were present in the membrane fraction of mitochondria (Figure 5.7A). I then aimed to determine the subcellular localization of PINK1 *in vivo*. Mitochondrial fractions were obtained from fresh rat brain and western blot analysis with native PINK1-48 antibody confirmed that PINK1 localized predominantly to the mitochondrial fraction of rat brain (Figure 5.7B, lane 1) as well as the P2 fraction that contains largely unhomogenised and insoluble brain material (Figure 5.7B, lane 3). Given that PINK1 was seen in the membrane fraction of mitochondria *in vitro*, we next determined whether PINK1 was an integral membrane protein or whether it was loosely attached to the membrane. I therefore incubated mitochondrial pellets with 0.1% sodium carbonate buffer (that efficiently strips loosely attached proteins from membranes)

and then ultracentrifuged extracts to obtain integral membrane and supernatant fractions. I found that both PINK1 and ΔPINK1 localized with the membrane fraction following carbonate treatment in both mitochondria from cells and rat brain suggesting that PINK1 is an integral membrane protein *in vitro* and *in vivo* (Figures 5.7C and 5.7D). To determine which membrane PINK1 localized to, Adrian Saurin and Peter Parker at the CRUK laboratories at Lincoln's Inn Fields (London) performed immunogold electron microscopy (EM) using a c-myc antibody in transfected COS-7 cells to detect c-myc-tagged PINK1 and found that full length PINK1 was localized predominantly to the inner mitochondrial membrane with slight staining of the outer membrane (Figure 5.8).

In view of this membrane localization I analyzed the PINK1 protein sequence for putative transmembrane domains using the TMpred programme (Hoffman and Stoffel, 1993). This predicted 3 strong transmembrane helices (total score of 2015) spanning amino acid residues 91 – 111; 274 – 293 and 436 – 452. Interestingly the first transmembrane helix occurs within the N-terminus of the cleaved 'processed' protein. Furthermore, there is also a hydrophobic domain at the C-terminus of PINK1 spanning residues 460–581. Further studies will be required to determine the exact topology of PINK1 within the inner mitochondrial membrane.

5.2.3 The first 34 aa of PINK1 are sufficient for mitochondrial localization

I next determined the region of PINK1 critical for mitochondrial localization. Most mitochondrial pre-sequences contain an arginine residue at the –2 position from the cleavage site ('R-2 rule) and a residue with an aromatic side chain at the +1 position

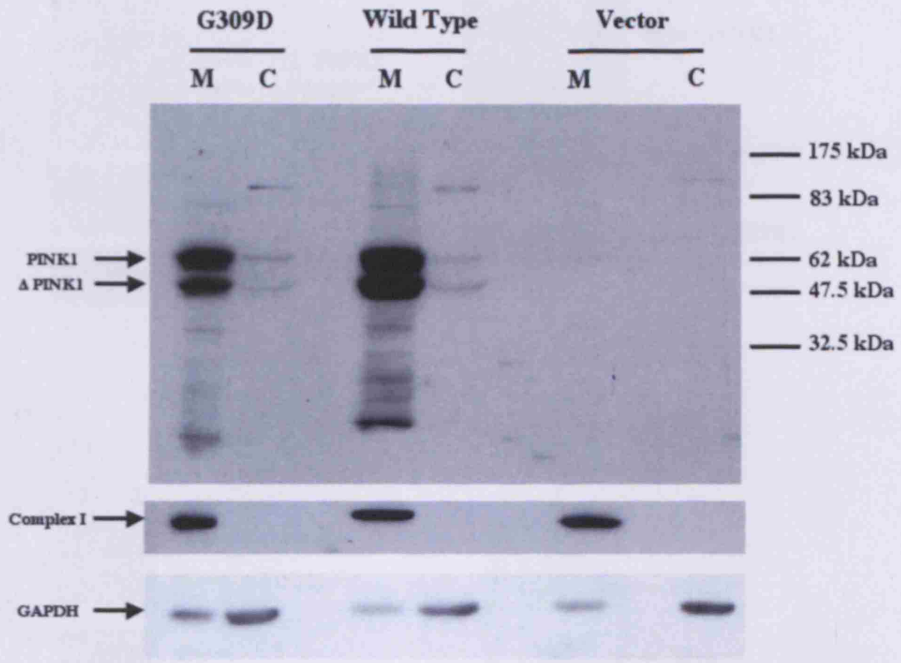


Figure 5.6. F/L and Δ PINK1 are localized to the mitochondria. COS-7 cells were transfected with c-myc vector, c-myc-wt PINK1 or c-myc-G309D PINK1 and mitochondrial enriched (M) and cytoplasmic fractions (C) were prepared as detailed in the Materials and Methods section. Lysates were analyzed by SDS-PAGE and Western blotting using PINK1-49 antibody (1:500). The relative purity of fractions was confirmed by reprobing the membrane with complex I (1:1000) and GAPDH antibodies (1:5000). Both F/L and Δ PINK1 were localized to the M fractions of PINK1 transfected cells. Endogenous PINK1 was not detected in vector lanes.

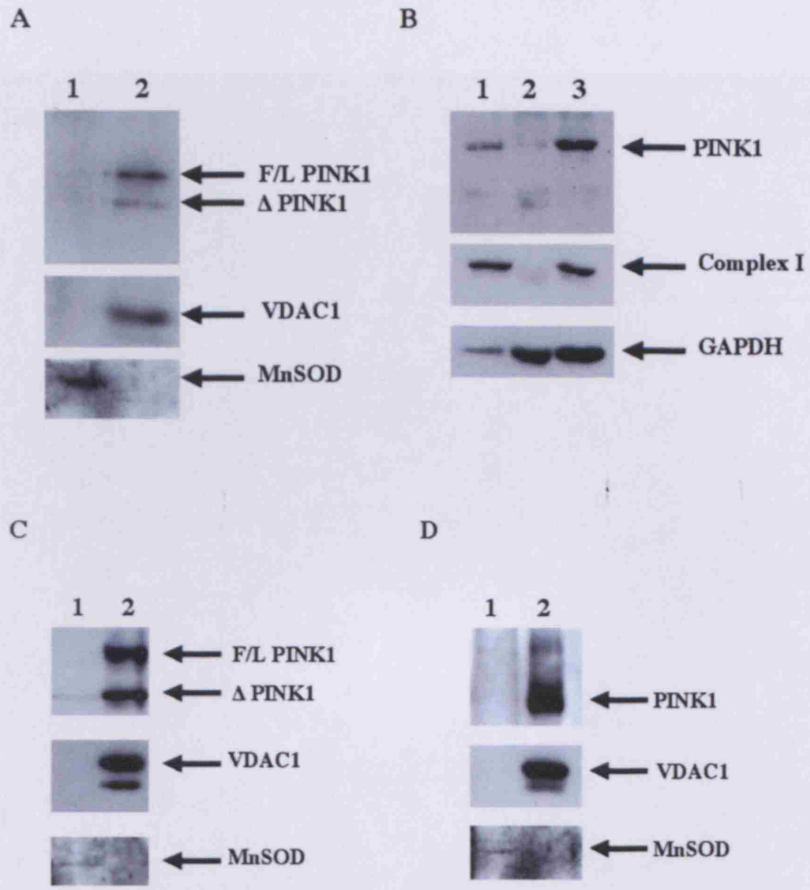


Figure 5.7. PINK1 localizes to the mitochondrial membrane fraction *in vitro* and *in vivo*. (A) Mitochondrial fractions of COS-7 cells transfected with c-myc-wt-PINK1 were further fractionated into matrix (lane 1) and membrane (lane 2) fractions as detailed in the Materials and Methods section and probed with PINK1-49 antibody (1:500). The purity of fractions was confirmed by probing with the membrane marker VDAC1 (1:500) and the matrix marker MnSOD (1:500). F/L and Δ PINK1 were localized to the membrane fraction. **(B)** Fresh rat brain was homogenized as described in the Materials and Methods section and mitochondrial (lane 1), cytoplasmic (lane 2) and P2 pellet (lane 3) fractions were analyzed by SDS-PAGE and Western blotting with PINK1-48 antibody (1:100). The purity of fractions was confirmed by reprobing membranes with complex I and GAPDH antibody. PINK1 was localized to the mitochondrial and P2 fractions. **(C)** Mitochondrial fractions of COS-7 cells transfected with c-myc-wt-PINK1 were further fractionated into supernatant (lane 1) and carbonate treated membrane (lane 2) fractions as detailed in the Materials and Methods and probed with PINK1-49 antibody (1:500). The purity of fractions was confirmed by probing with the membrane marker VDAC1 and the matrix marker MnSOD. F/L and Δ PINK1 were localized to the membrane fraction. **(D)** Mitochondrial fractions of rat brain were further fractionated into supernatant (lane 1) and carbonate treated membrane (lane 2) fractions and probed with PINK1-48 antibody (1:100). The purity of fractions was confirmed by probing with the membrane marker VDAC1 and the matrix marker MnSOD. PINK1 was localized to the membrane fraction.

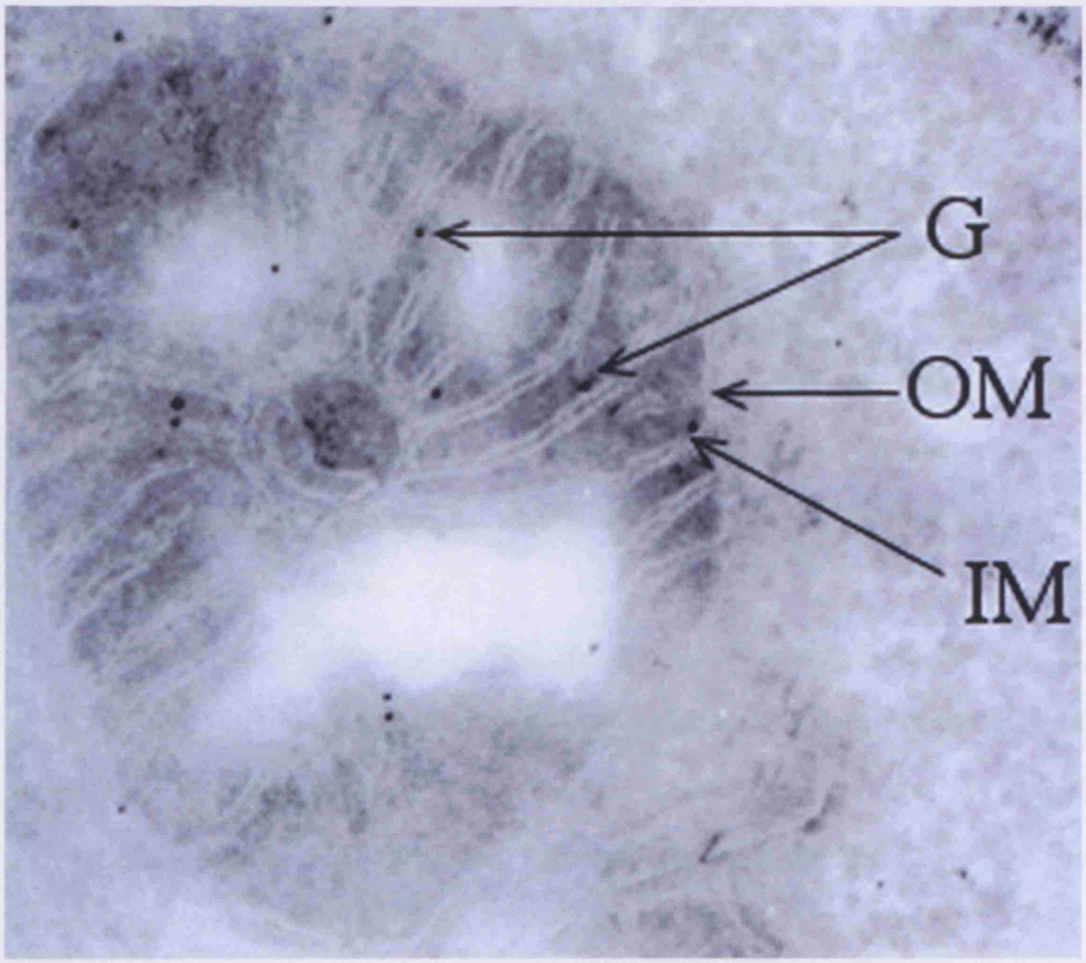


Figure 5.8. PINK1 is localized predominantly to the inner mitochondrial membrane. Immunogold electron microscopy of COS-7 cells transfected with c-myc-wt-PINK1 using c-myc antibody demonstrates c-myc-PINK1 localization to predominantly the inner mitochondrial membrane with slight staining of the outer mitochondrial membrane; IM = inner mitochondrial membrane; OM = outer mitochondrial membrane; G = anti-c-myc immunogold particle.

Courtesy of Dr Adrian Saurin (CRUK, Lincoln's Inn Fields).

(Hendrick et al., 1989). Bioinformatic analysis using the Mitoprot program (Claros, 1995) predicted such a cleavage site between residues 34-35 of PINK1 protein. However, cleavage at this site could not explain the size of Δ PINK1 detected with anti-PINK1 antibodies (Figure 5.1A). Reanalysis of the PINK1 sequence by bioinformatics using the Target P program (Nielson et al., 1997; Emanuelsson et al., 2000) identified a second putative mitochondrial cleavage site that bore a 'R-10 motif': an arginine at –2 from the MPP site and –10 from a mitochondrial intermediate peptidase (MIP) at residues 68 – 77 (Gavel and von Heijne, 1990). A third less well-conserved putative mitochondrial cleavage site was identified starting with an arginine at residue 98.

Constructs containing the first 34 and 70 amino acids in pEGFP-N1 vector were made (Dr Kirsten Harvey, London). Transfection studies revealed that the first 34 amino acids of PINK1 were successfully targeted to mitochondria in contrast to GFP alone (Figures 5.9A and B). The PINK1 fragment containing the first 70 amino acids were also targeted to the mitochondria (data not shown).

To determine if residues at this more distal site were important for cleavage, we made mutants R68G and F70Y but these did not block cleavage of PINK1 (data not shown). Furthermore, using 15% percent acrylamide gel electrophoresis and Western blot analysis I did not observe the predicted 10kDa sized N-terminal cleaved fragment of PINK1 using an N-terminal c-myc tagged PINK1 construct (data not shown). Overall these data demonstrate that the first 34 amino acids of PINK1 are sufficient for mitochondrial targeting and that PINK1 may undergo sequential cleavages for production of ΔPINK1 thereby generating several small N-terminal peptides.

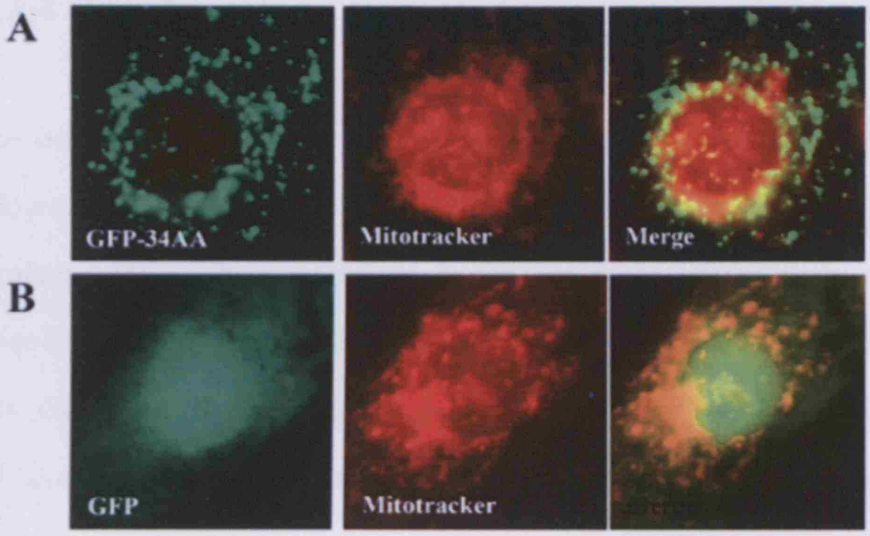


Figure 5.9. The first 34 aa of PINK1 targets to mitochondria. (A) COS-7 cells were transfected with 34 aa of PINK1 cloned into pEGFP-N1 and incubated in Mitotracker Red before fixation. (B) COS-7 cells were transfected with GFP alone and incubated in Mitotracker Red before fixation. Magnification x1000.

5.2.4 PINK1 localizes to Lewy bodies and aggresomes following proteasomal stress

Our laboratory has recently shown that 5-10% of LBs contain PINK1 in PD brains (Figure 5.10) (Gandhi et al., 2006). Therefore, to further understand how PINK1 localizes to LBs, I investigated the mechanism in the cellular model of aggresomes since the mechanism of their formation has been postulated to be similar to that of LBs (Olanow et al., 2004). Upon stimulation of SH-SY5Y cells with 10 μM MG-132 for 24 hours, PINK1 was localized to aggresomes and co-localized with the intermediate filament protein, vimentin, which is an invariant protein component of aggresomes. Furthermore, PINK1 co-localized with ubiquitin and Hsc-70 that are also commonly found in aggresomes (Figures 5.11A, B and C). PINK1 did not co-localize with these proteins under basal conditions.

5.2.5 PINK1 co-localizes with PD associated proteins in aggresomes

Previous studies have demonstrated the presence of other PD associated proteins in aggresomes including over-expressed synphilin-1, parkin (PARK2), over-expressed α-synuclein (PARK1), and over-expressed UCH-L1 (PARK5) (Tanaka et al., 2004, Muqit et al., 2004, Ardley et al., 2004). These proteins have all been reported to be included in LBs in PD brains (Olanow et al., 2004). Furthermore, phosphorylated α-synuclein, which is a major component of LBs, also localizes to aggresomes (Tanaka et al., 2004). In the presence of proteasome inhibitor MG-132, it was observed that

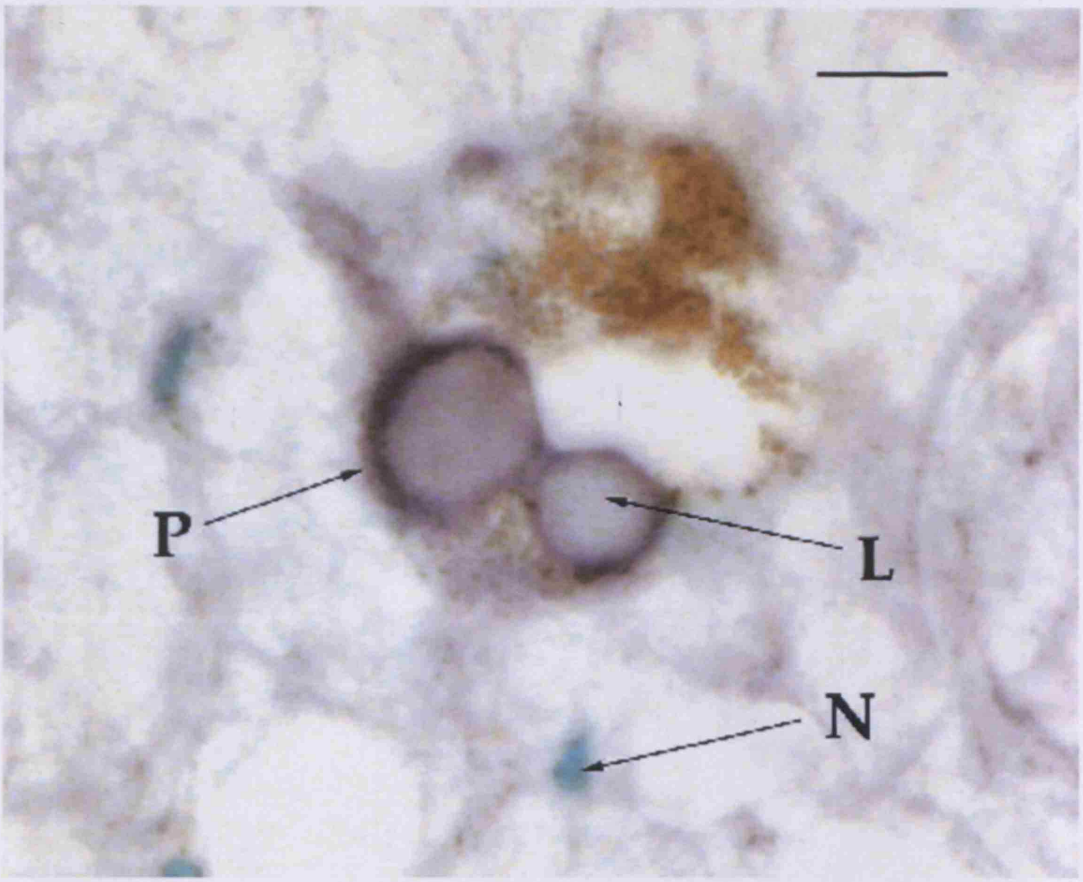


Figure 5.10. PINK1 is detectable in Lewy bodies. Human pons sections were cut from a flash frozen post-mortem PD brain. Endogenous PINK1 was detected by immunohistochemistry using native PINK1 antibody. PINK1 staining was detected in the peripheral halo of approximately 5-10% of Lewy bodies as depicted in the two Lewy bodies shown. N: nucleus; L: Lewy body; P: PINK1 staining in peripheral halo. Scale bar, 10 μm.

Courtesy of Dr Sonia Gandhi (University College London)

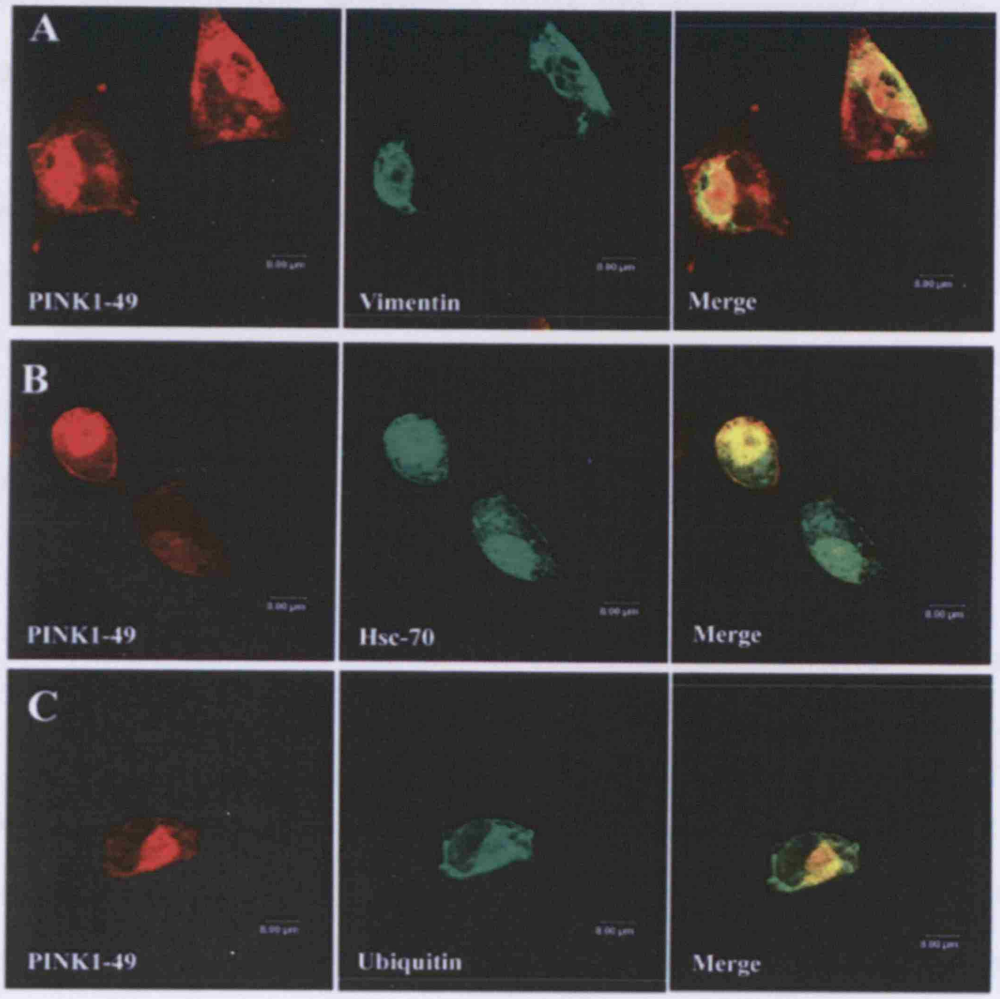


Figure 5.11. PINK1 co-localizes with vimentin, Hsc-70, and ubiquitin in SH-SY5Y cells in aggresomes following MG-132 treatment. (A-C) SH-SY5Y cells were grown on glass coverslips. transfected with wt-PINK1-pcDNA3.1 and treated with 10 μM MG-132 for 24 hours. Cells were then fixed in 4% paraformaldehyde and processed for confocal laser microscopy by double labelling cells with PINK1-49 antibody (1:100) and a rhodamine-conjugated secondary (1:2500) (red) and either anti-vimentin antibody (1:500) (A); anti-Hsc-70 antibody (1:100) (B), or anti-ubiquitin antibody (1:100) (C) all with a FITC-conjugated secondary (1:1000) (green). Scale Bar 8μm. n = 3-4 independent experiments.

PINK1 co-localizes with endogenous parkin and over-expressed synphilin-1 in aggresomes (Figures 5.12A and C). In Chapter 3 I reported that endogenous α-synuclein was not found in aggresomes but determined whether over-expressed α-synuclein localized to aggresomes (Muqit et al., 2004). We found that over-expressed α-synuclein co-localized with PINK1 in all aggresomes in SH-SY5Y cells (Figure 5.12B). We also examined whether DJ-1 (PARK7) localized to aggresomes in our system but did not find endogenous DJ-1 in aggresomes after proteasome inhibition (data not shown).

5.2.6 No difference in aggresome formation between wild-type and mutant PINK1

Previous studies of the parkin protein have shown several missense and rearrangement mutants self-aggregating under basal conditions suggesting they promote LB formation *in vivo* (Kahle and Haass, 2004). For example the parkin R275W mutant formed aggresomes in cells in basal conditions and was also associated with LBs in a post-mortem brain of a AR-JP patient who was compound heterozygote for the R275W mutation (Cookson et al., 2003).

Additional PINK1 disease-causing missense and truncating heterozygote mutants in the PINK1 gene in sporadic cases of PD were discovered as explained in Chapter 4. We investigated whether any of these mutants promoted aggresome formation under basal conditions. COS-7 cells were transfected with wild-type or mutant c-myc-tagged PINK1. Western blot analysis revealed that all mutants were expressed including 2 truncating PINK1 mutants, W437X and Q456X (see Figure 4.4, Chapter 4). Under basal conditions, transfection of wild-type or mutant PINK1 did not

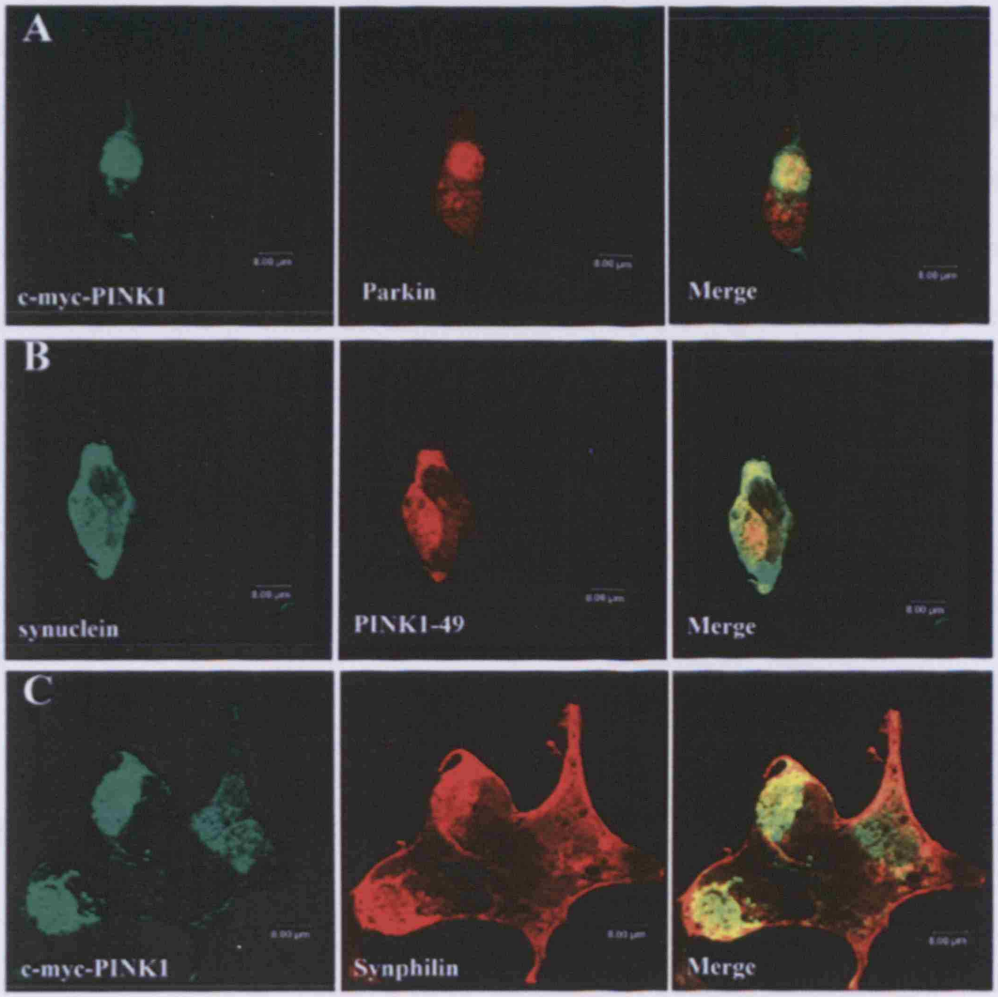


Figure 5.12. PINK1 co-localizes with PD-associated proteins in aggresomes. SH-SY5Y cells were grown on glass coverslips, transfected with c-myc-wt-PINK1 or wt-PINK1-pcDNA3.1 and α-synuclein-pcDNA4 or HA-synphilin-1 and treated with MG-132 for 24 hours. Cells were then fixed in 4% paraformaldehyde and processed for confocal laser microscopy by double labelling cells with c-myc antibody (1:100) with a FITC-conjugated secondary (1:1000) (green); PINK1-49 antibody (1:100) with a rhodamine-conjugated secondary (1:2500) (red) and either anti-parkin antibody (1:100) with a rhodamine-conjugated secondary (1:2500) (red) (A); anti-α-synuclein (1:100) with a FITC-conjugated secondary (1:1000) (green) (B), or anti-synphilin-1 antibody (1:100) with a rhodamine-conjugated secondary (1:2500) (red) (C). Scale Bar 8μm. n = 3-4 independent experiments.

induce significant aggresome formation (data not shown). Following treatment with MG-132, there was no significant difference in aggresome frequency between wild-type and mutant PINK1 (Figure 5.13). This correlates well with our laboratory findings *in vivo* where we did not observe any difference in PINK1 LB localization between wild type PD and PINK1 mutant PD brains (Gandhi et al., 2006). These studies suggest that an intact kinase domain is not necessary for PINK1 localization to aggresomes.

5.2.7 Mitochondrial proteins localize to aggresomes in the absence of aggregation

I next determined the mechanism of PINK1 localization in aggresomes. One hypothesis was that since PINK1 is a mitochondrial protein, its presence reflects the recruitment of intact mitochondria to aggresomes. Therefore immunofluorescence was used to determine whether other mitochondrial proteins were present in aggresomes at endogenous levels. PINK1 co-localized with MitoTracker Red in aggresomes after proteasomal inhibition (Figures 5.14A and B). I then performed single label staining with a monoclonal antibody raised against non-glycosylated mitochondrial proteins and another mitochondrial protein, Hsp60. Endogenous forms of both epitopes were present in aggresomes with similar frequencies (Figures 5.14D and 5.14F). I also found endogenous cytochrome c within aggresomes following proteasomal inhibition (data not shown). PINK1 co-localized with endogenous Hsp60 in aggresomes (Figure 5.14G) and the endogenous non-glycosylated mitochondrial proteins (data not shown).

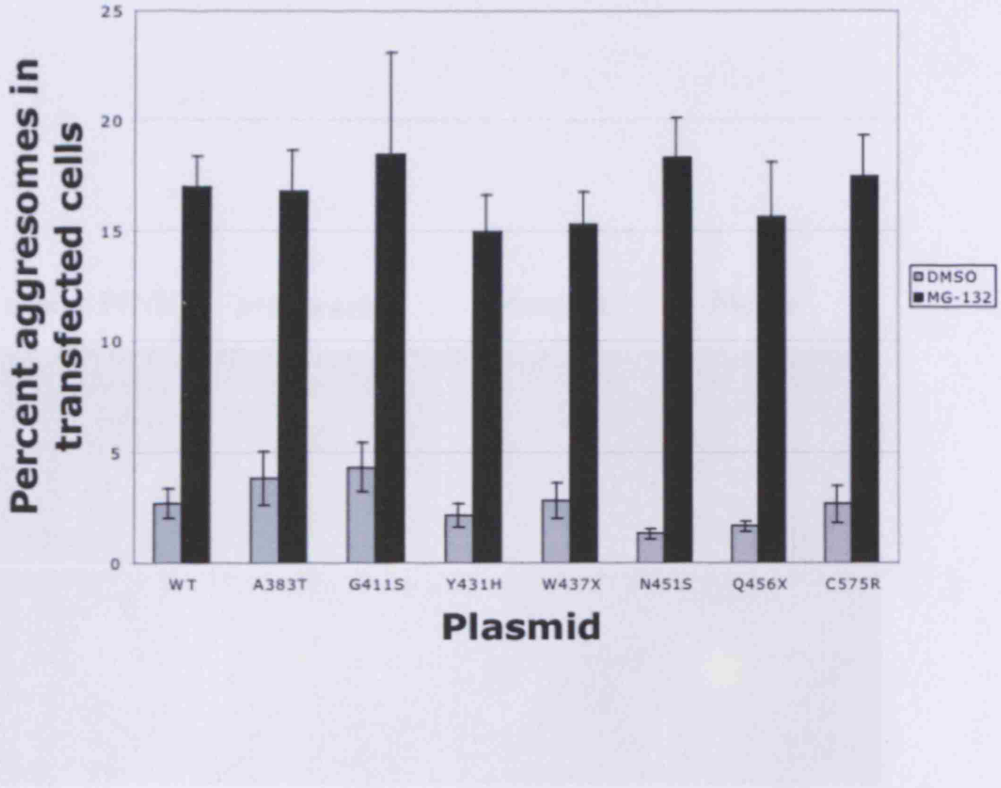
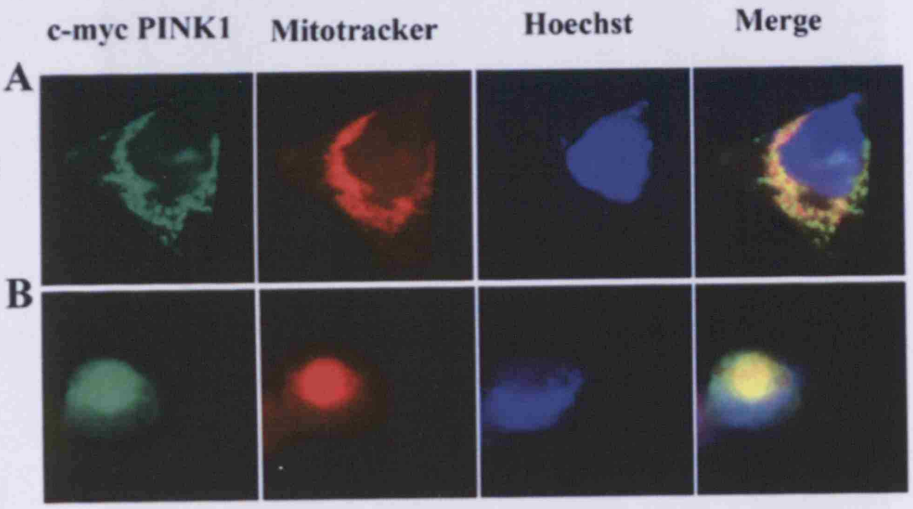
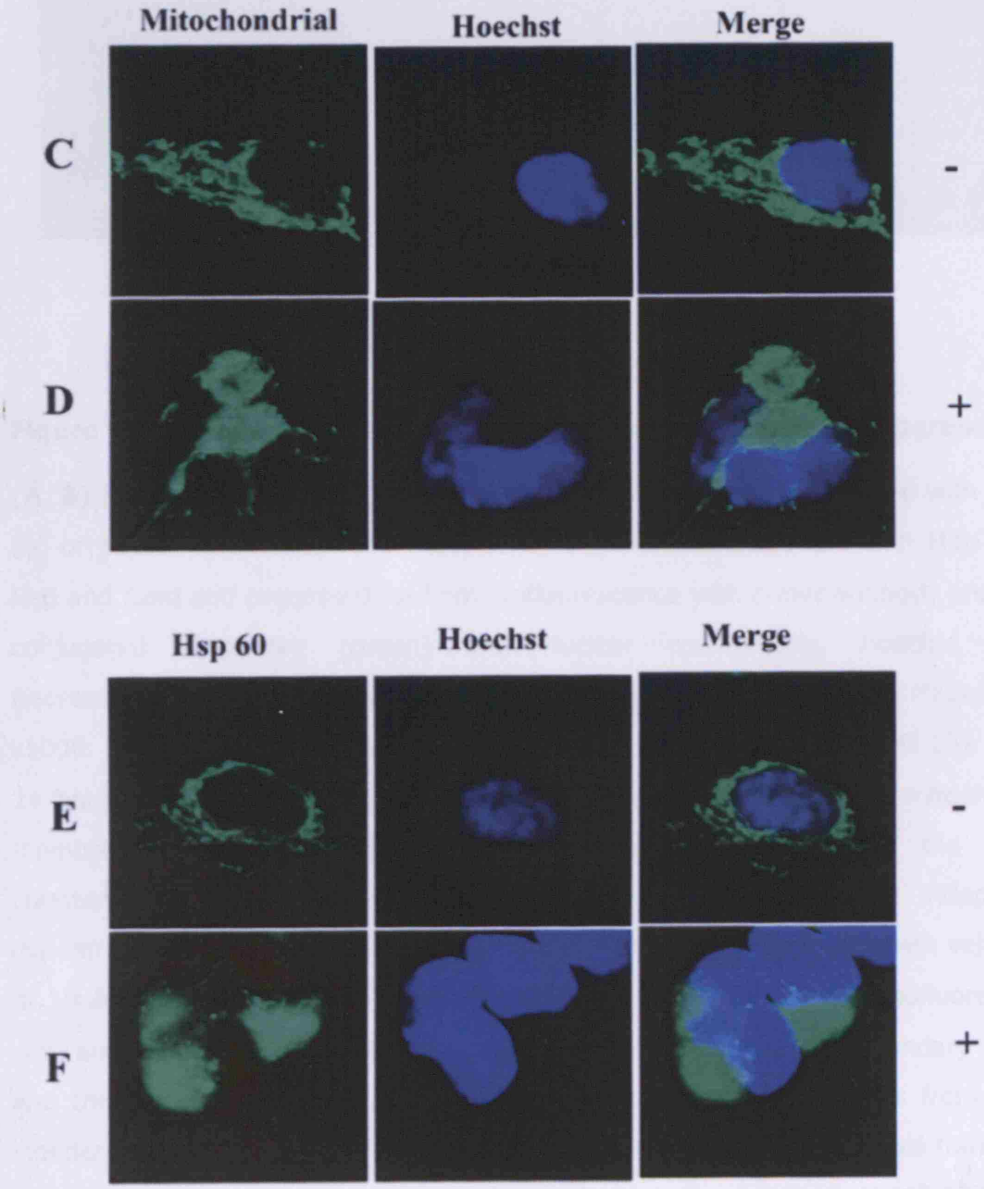


Figure 5.13. No difference in aggresome frequency between wild type and mutants of PINK1. COS-7 cells were grown on glass coverslips and transfected with myc-wt or myc-mutant PINK1. Cells were then treated with vehicle (control) or with 10μM MG-132 for 24 hours Adherent cells were fixed in 4% paraformaldehyde and processed for microscopy by labelling cells with a c-myc antibody and a FITC-conjugated secondary (data not shown). The percentage of aggresomes in ~100-250 transfected cells was determined for each construct and graphed. Data represents means ± SEM from n = 2 independent experiments.


c-myc PINK1
Mitotracker
Hoechst
Merge
A
B


Mitochondrial
Hoechst
Merge
C
-
D
+
Hsp 60
Hoechst
Merge
E
-
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G

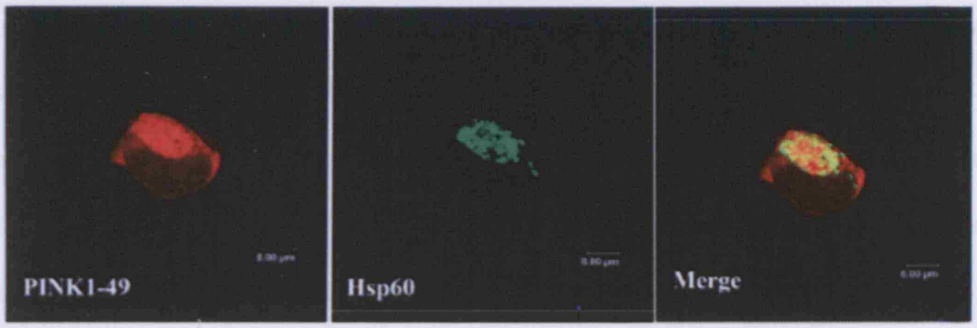


Figure 5.14. PINK1 and mitochondrial markers co-localize in aggresomes.

(A, B) SH-SY5Y cells were transfected with c-myc-wt-PINK1 and treated with vehicle (A) or 10μM MG-132 (B) for 24 hours. Cells were then incubated with MitoTracker Red and fixed and processed for immunofluorescence with c-myc antibody and FITC-conjugated secondary (green) and nuclear counterstain, Hoechst (blue). Representative images from at least n = five independent experiments. Magnification x1000. **(C, D)** SH-SY5Y cells were treated with vehicle (-) or 10μM MG-132 (+) for 24 hours and fixed and processed for immunofluorescence with anti-mitochondrial membrane antibody and FITC-conjugated secondary (green) and the nuclear counterstain, Hoechst (blue). Representative images from n = 2 independent experiments. Magnification x1000. **(E, F)** SH-SY5Y cells were treated with vehicle (-) or 10μM MG-132 (+) for 24 hours and fixed and processed for immunofluorescence with anti-mitochondrial membrane antibody and FITC-conjugated secondary (green) and the nuclear counterstain, Hoechst (blue). Representative images from n = 2 independent experiments. Magnification x1000. **(G)** SH-SY5Y cells were transfected with PINK1-pcDNA3.1 and treated with vehicle (not shown) or 10μM MG-132 for 24 hours and fixed and processed for immunofluorescence with PINK1-49 and rhodamine-conjugated secondary (red) and Hsp60 and FITC-conjugated secondary (green). Scale Bar 8 μm. Representative images from n = 2 independent experiments.

I next looked for evidence of aggregation of mitochondrial proteins following proteasome inhibition and made Triton-X100 soluble and insoluble extracts of cells treated with vehicle or MG-132. MG-132 caused a generalized increase in protein ubiquitylation in both soluble and insoluble fractions. In contrast to Hsc-70, a cytoplasmic protein, which becomes more insoluble following MG-132, neither of the mitochondrial proteins that I examined namely, Hsp60 and cytochrome c, localized to the insoluble fraction of cells suggesting that they do not aggregate following treatment with MG-132 (Figure 5.15). I observed similar results for the mitochondrial matrix protein manganese superoxide dismutase (MnSOD) (data not shown). These results could support the notion that mitochondrial proteins may localize to aggresomes via mitochondrial recruitment rather than aggregation.

5.2.8 Ultrastructural studies demonstrate mitochondrial recruitment to aggresomes

In collaboration with Dr Janice Holton (University College London), I determined whether recruitment of intact mitochondria to the aggresomes was occurring by performing electron microscopy of SH-SY5Y cells treated with vehicle or MG-132. In unstressed cells mitochondria were distributed within the cytoplasm (Figure 5.16A). Following MG-132, we observed the formation of a variety of filamentous and granular perinuclear aggregates that indented cell nuclei and intact mitochondria were observed clustering around the aggregates (Figures 5.16B-F).

It is possible that mitochondria become severely damaged by the stress of proteasome inhibition and subsequently release their proteins into the cytoplasm where the UPS may target them independently. However, we did not see evidence of such mitochondrial damage. Moreover, our observations are in agreement with a

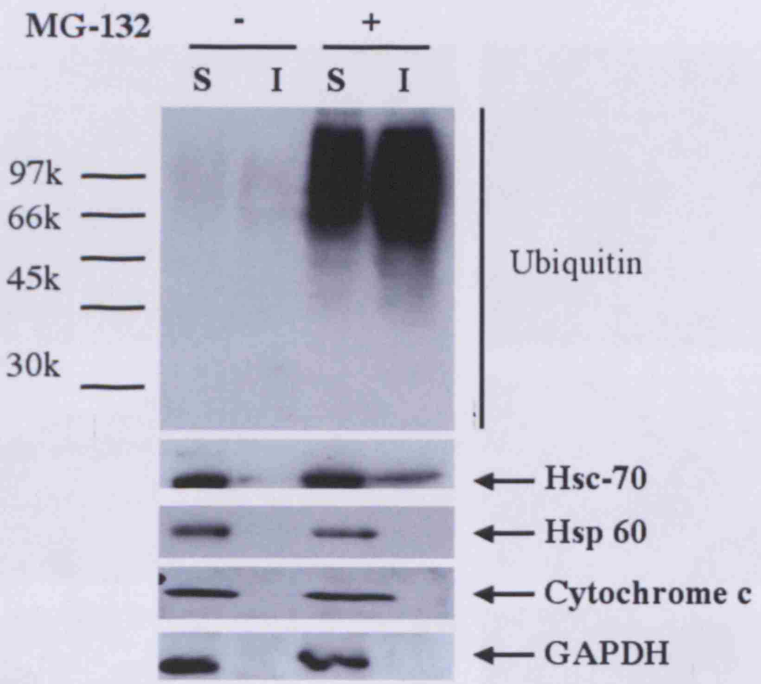


Figure 5.15. Mitochondrial proteins do not become insoluble following proteasomal stress. COS-7 cells were treated with vehicle (-) or MG-132 (+) for 24 hours and triton-X100 soluble (S) and insoluble fractions (I) were made and analyzed by SDS-PAGE and Western blotting. Parallel gels were probed with anti-ubiquitin (1:1000) or anti-Hsc-70 (1:1000) and reprobed with Hsp60 (1:1000), cytochrome c (1:1000) and GAPDH (1:5000). GAPDH confirms purity of insoluble fractions. Representative blots from 2 independent experiments.

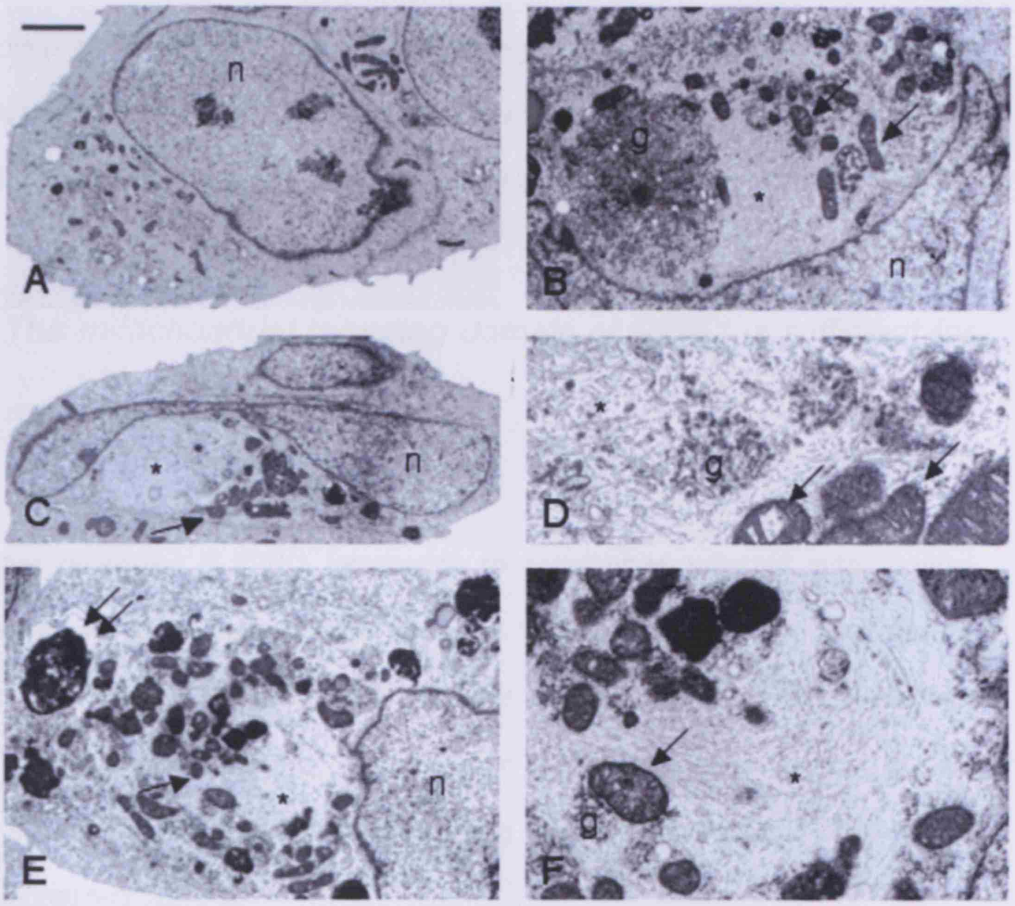


Figure 5.16. Mitochondria are recruited to aggresomes induced by MG-132 in neuronal SH-SY5Y cells. (A) In control cells organelles were evenly distributed in the cytoplasm adjacent to the nucleus (n) and little autophagic material was present. **(B)** In MG-132 treated cells the nucleus was found to be indented by a collection of organelles associated with variable amounts of granular (g) and filamentous (*) material. In some cases the granular and filamentous components were clearly separated and mitochondria (single arrows) were distributed towards the periphery of these elements. **(C, D)** In other cells the granular material formed a small component of the inclusion while filamentous accumulation was prominent (D, higher magnification of part of the cell shown in C). **(E, F)** Mitochondria were sometimes found to be distributed within the accumulated filamentous and granular components (E, with higher magnification shown in F). Autopahgic material was easily identified (E, double arrow). Scale Bar in A represents 2.5μm in A, 1.2μm in B, 2μm in C, 240nm in D, 1.5μm in E and 530nm in F.

Courtesy of Kerrie Venner and Dr Janice Holton (University College London)

previous study that suggested that mitochondria may be recruited to aggresomes to provide ATP for the active degradation and removal of misfolded proteins that are transported to the MTOC (Waelter et al., 2001). Autophagic bodies were also seen around aggresomes (Figure 5.16E).

5.2.9 The mitochondrial targeting domain of PINK1 is sufficient for aggresome localization

To further determine if PINK1 localization to aggresomes reflected mitochondrial recruitment, we examined whether the first 34 aa of PINK1 also localized to aggresomes following proteasomal stress. We did find that this short N-terminal strand of PINK1 was sufficient for localization to aggresomes in contrast to GFP protein alone (Figures 5.17A and B) suggesting that PINK1's localization may reflect mitochondrial recruitment.

5.2.10 PINK1 cleavage is enhanced by proteasomal stress resulting in increased ΔPINK1

Mitochondrial proteins are degraded by conserved ATP-dependent proteolytic complexes into free amino acids, which are exported into the cytosol (Augustin et al., 2004). It was determined whether there was any evidence of high molecular weight species of PINK1 in insoluble fractions following MG-132 treatment. In unstressed cells I found that PINK1 protein was localized to the triton insoluble fraction with very little protein present in the soluble fractions (Figure 5.18A). PINK1 appeared to be strongly bound in the insoluble compartment since only moderate amounts of

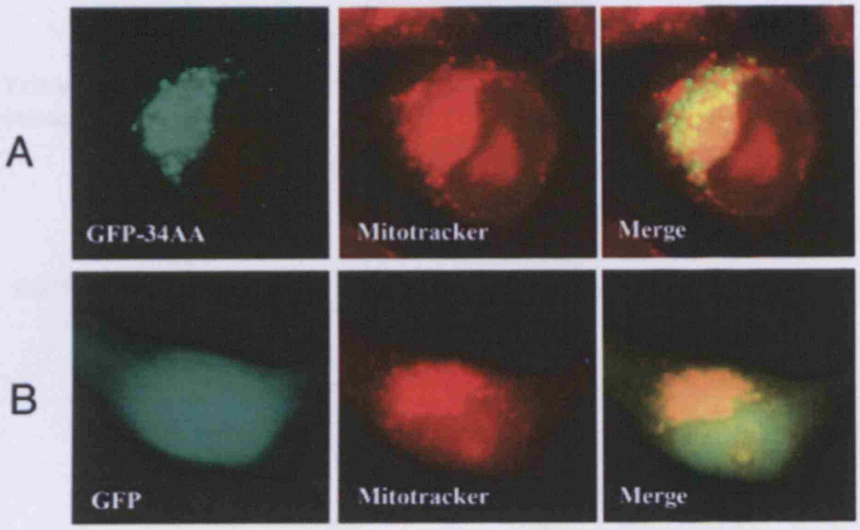
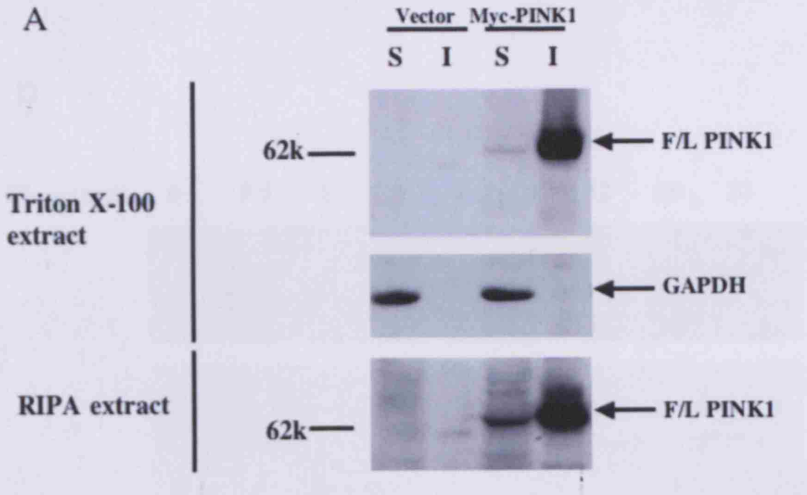
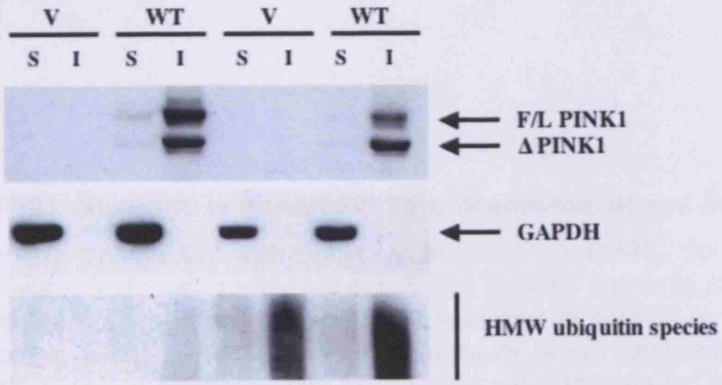


Figure 5.17. The first 34 aa of PINK1 can localize to aggresomes. (A) COS-7 cells were transfected with 34 aa of PINK1 cloned into pEGFP-N1 and treated with MG-132. Following stress cells were incubated in Mitotracker Red before fixation. (B) COS-7 cells were transfected with GFP alone and treated with MG-132 and incubated in Mitotracker Red before fixation. Magnification x1000.

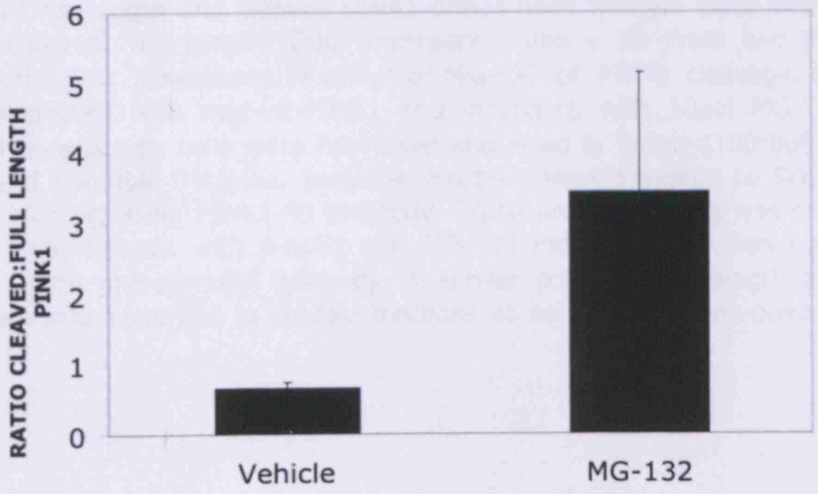
A


Vector
Myc-PINK1
S
I
S
I
62k
F/L PINK1
Triton X-100 extract
GAPDH
RIPA extract
62k
F/L PINK1

B


V
WT
V
WT
S
I
S
I
S
I
S
I
F/L PINK1
ΔPINK1
GAPDH
HMW ubiquitin species

C


RATIO CLEAVED:FULL LENGTH PINK1
0
1
2
3
4
5
6
Vehicle
MG-132

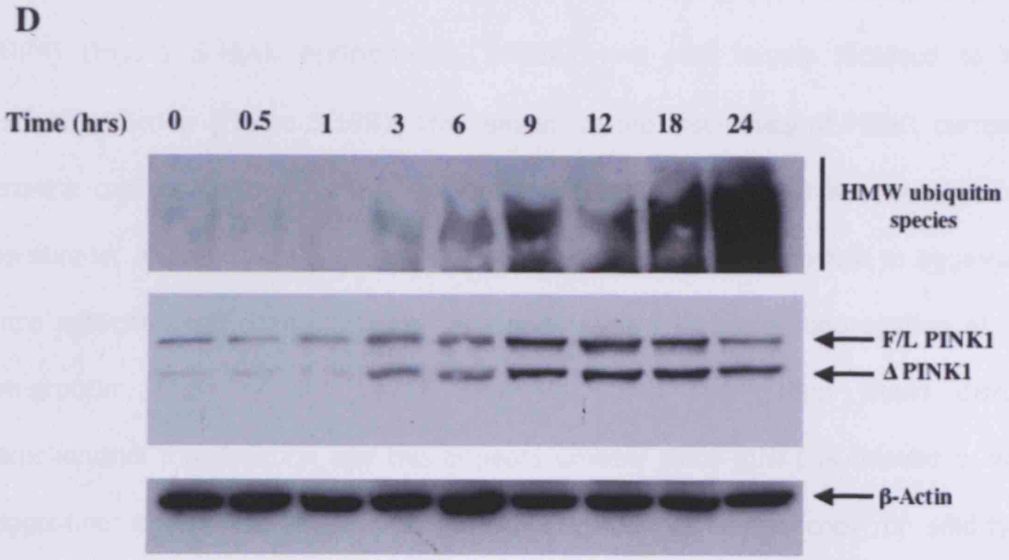


Figure 5.18. PINK1 cleavage is enhanced by proteasomal stress *in vitro*.

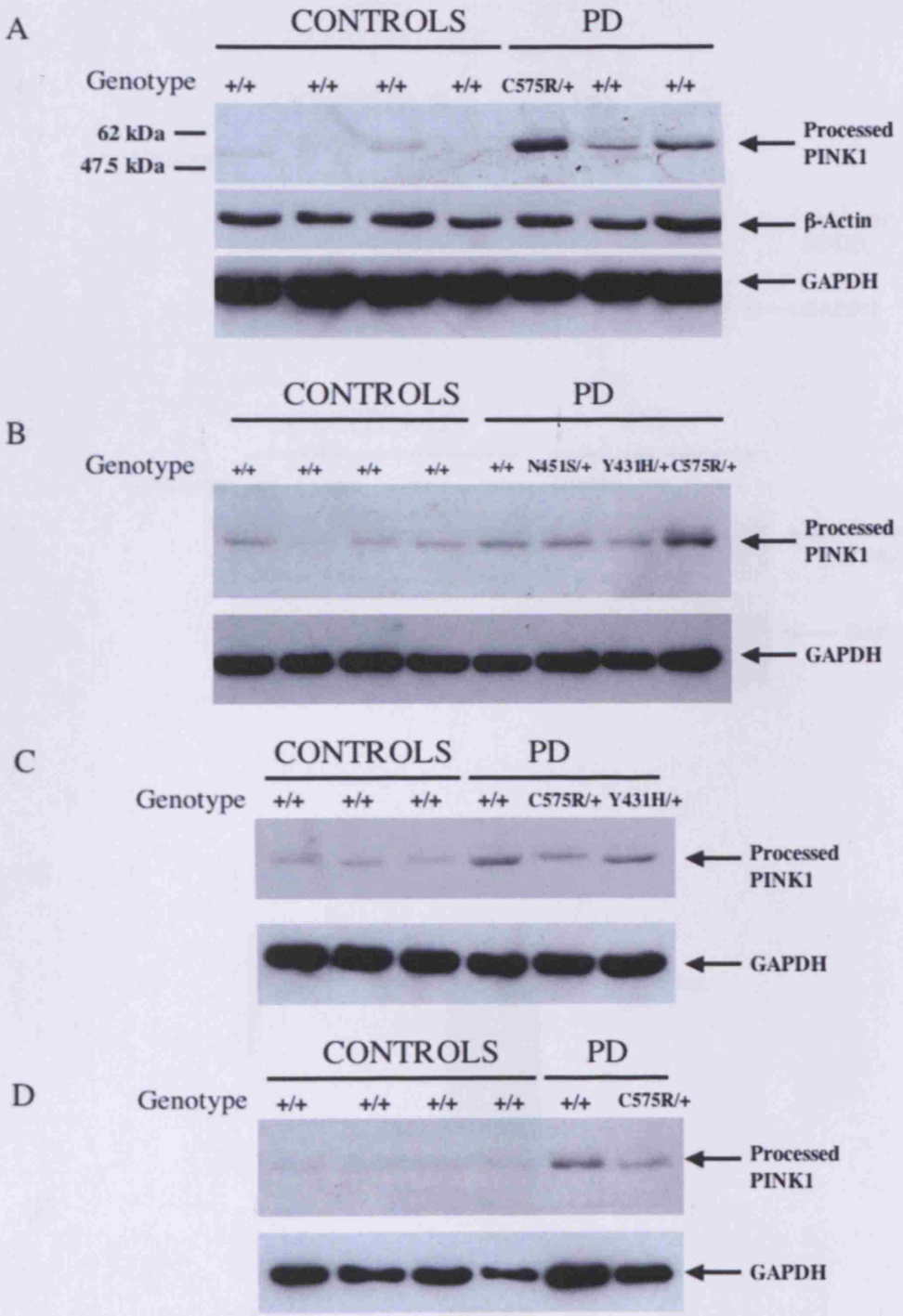
(A) COS-7 cells were transfected with c-myc-vector or c-myc-PINK1. Soluble (S) or insoluble lysates were made using either Triton X-100 or RIPA buffer as described in the Materials and Methods section. Lysates were analyzed in parallel by SDS-PAGE and Western blotting using c-myc antibody. Membranes were reprobed with anti-GAPDH to confirm purity of fractions. **(B)** COS-7 cells were transfected with c-myc-vector (V) or c-myc-PINK1 (WT) and treated with vehicle (-) or 10μM MG-132 (+) for 24 hours. Soluble (S) or insoluble lysates were made using Triton X-100 buffer. Lysates were analyzed by SDS-PAGE and Western blotting using PINK-49 antibody. Membranes were reprobed with anti-GAPDH to confirm purity of insoluble fractions and anti-ubiquitin to demonstrate efficacy of MG-132 in cells. **(C)** Densitometric analysis of full length and cleaved PINK1 bands from Western blots expressed as a ratio of cleaved: full length. Data represent mean ± SD from two independent experiments. **(D)** Timecourse of effect of MG-132 on PINK1 cleavage. COS-7 cells were transfected with myc-wt-PINK1 and incubated with 10μM MG-132. At the indicated time-points, cells were harvested and lyzed in Triton-X100 buffer to make soluble and insoluble fractions. Insoluble fractions were analyzed by SDS-PAGE and Western blotting using PINK1-49 antibody. Equal protein loading was confirmed by reprobing membranes with β-actin and MG-132 induced stress was confirmed by reprobing with anti-ubiqutin antibody. A similar profile in full-length and cleaved bands was also observed in soluble fractions at each of the time-points (data not shown).

PINK1 protein was extracted in the soluble fraction using a more stringent buffer (RIPA) (Figure 5.18A). Furthermore, ΔPINK1 was also largely localized to the insoluble fraction (Figure 5.18B). The reason for the insolubility of PINK1 currently remains unclear. It may reflect its strong localization to the inner mitochondrial membrane. Another possibility might be a tendency for PINK1 protein to aggregate since mitochondrial pre-sequences have been shown to induce aggregation of the pre-protein (Endo et al., 1995). However, such aggregation would disrupt mitochondrial translocation and this appears unlikely since PINK1 is cleaved *in vitro* suggesting appropriate import targeting. Furthermore, microscopy of wild-type PINK1 transfected cells has revealed no evidence of aggregates under basal conditions. Lastly the insolubility of PINK1 protein that I have observed *in vitro* mirrors the relative insolubility of full length PINK1 protein that was observed *in vivo* in rodent and human brain (Figures 5.3-5.5).

Following treatment of COS-7 cells with MG-132, I found a general increase of high molecular weight ubiquitylated proteins (Figure 5.18B). However, I did not see such species when probing with the PINK1 antibody suggesting that PINK1 is not polyubiquitylated after proteasomal stress (Figure 5.18B). In contrast I found that PINK1 cleavage was enhanced by proteasomal stress such that the ratio of ΔPINK1 to full-length PINK1 protein was increased from 0.65 ± 0.09 to 3.45 ± 1.70 (mean ± SD) (Figures 5.18B and 5.18C). This suggests that cleavage of PINK1 is up-regulated by proteasomal stress. Time-course studies of cells treated with MG-132 revealed a progressive shift from full-length to cleaved protein such that between 18 and 24 hours, ΔPINK1 predominated (Figure 5.18D).

5.2.11. *Processed PINK1 expression is increased in PD brain*

I next determined whether there was any evidence for altered PINK1 processing *in vivo*. There is accumulating evidence that decreased proteasome function may underlie PD pathogenesis, and it has been recently reported that chronic exposure of naturally occurring proteasome inhibitors in rats recapitulated many features of Parkinsonism including the development of LB-like inclusions (McNaught et al., 2001, McNaught et al., 2004). The soluble fractions from a variety of brain regions from both control and age-matched Parkinson's disease brains were examined for PINK1 expression. As previously mentioned, the major cross-reactive band detected by our PINK1 antibodies in these fractions is ~52-53 kDa and is likely to represent processed or cleaved PINK1 (see Section 5.2.1). I found a significant increase in expression of processed PINK1 in substantia nigra (Figures 5.19D and J) and cerebellum (Figures 5.19B and H) of PD brain ($p < 0.05$). Furthermore, there were strong statistical trends of increases in the other regions of PD brains most notably temporal cortex (Figures 5.19A and G); frontal cortex (Figures 5.19C and I) and caudate (Figures 5.19F and L) ($p = 0.05$, $p = 0.07$, $p = 0.07$ respectively). Reprobing samples with at least two housekeeping genes suggested that the alteration in PINK1 levels were not attributable to general protein degradation (Figure 5.19A). Within the PD group there was no significant difference between PINK1 heterozygote brains (n=1-3) and wild type PD brain (n=1-2) albeit the numbers of brains analyzed were comparatively small.


A
CONTROLS
PD
Genotype
+/+ +/+ +/+ +/+ C575R/+ +/+ +/+
62 kDa
47.5 kDa
Processed PINK1
β-Actin
GAPDH
B
CONTROLS
PD
Genotype
+/+ +/+ +/+ +/+ +/+ N451S/+ Y431H/+ C575R/+
Processed PINK1
GAPDH
C
CONTROLS
PD
Genotype
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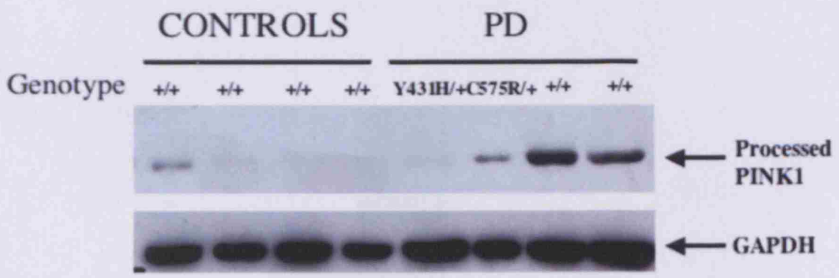
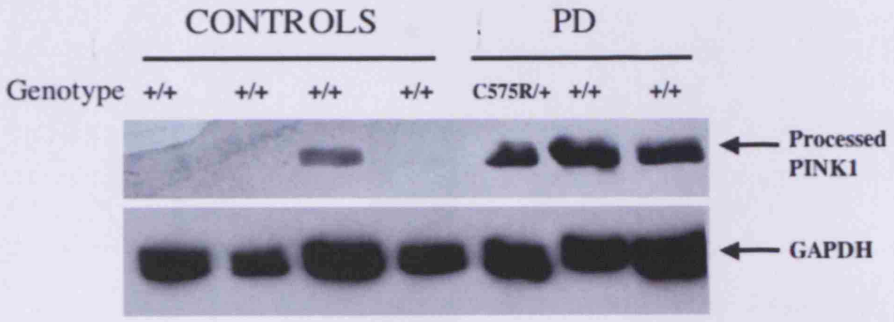
+/+ +/+ +/+ +/+ +/+ C575R/+
Processed PINK1
GAPDH

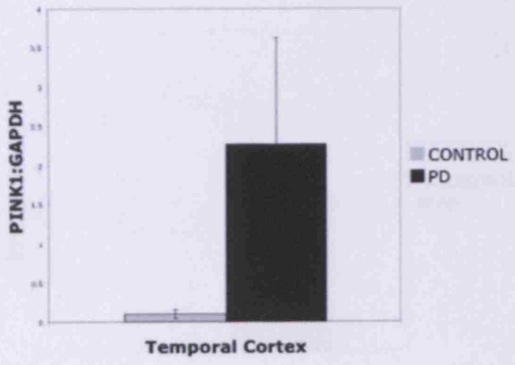
E


CONTROLS
PD
Genotype
+/+ +/+ +/+ +/+ Y431H/+C575R/+ +/+ +/+
Processed PINK1
GAPDH

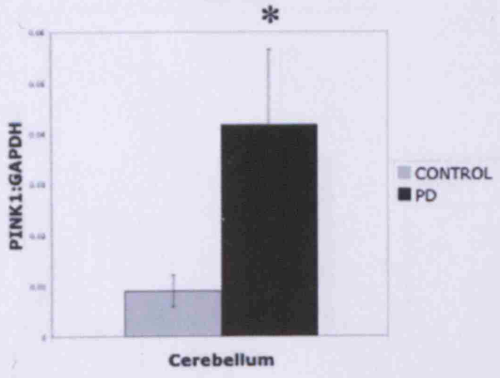
F


CONTROLS
PD
Genotype
+/+ +/+ +/+ +/+ C575R/+ +/+ +/+
Processed PINK1
GAPDH

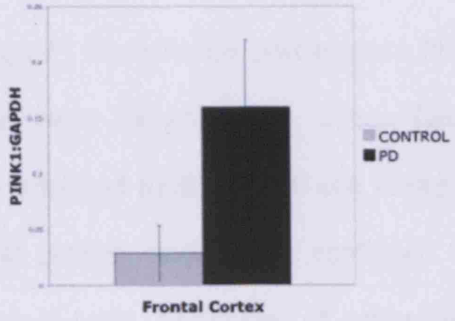
G


PINK1:GAPDH
CONTROL
PD
Temporal Cortex

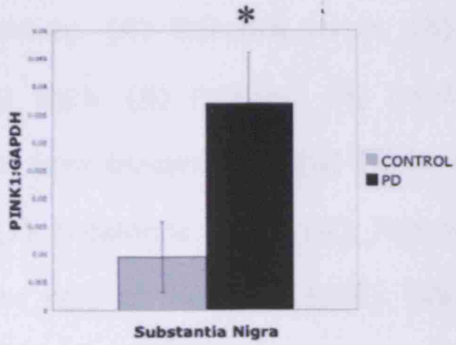
H


*
PINK1:GAPDH
CONTROL
PD
Cerebellum

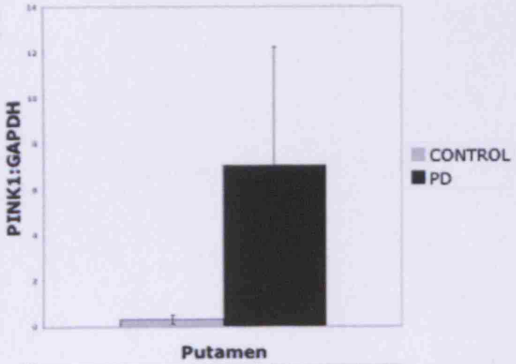
I


PINK1:GAPDH
CONTROL
PD
Frontal Cortex

J


*
PINK1:GAPDH
CONTROL
PD
Substantia Nigra

K


PINK1:GAPDH
CONTROL
PD
Putamen

L

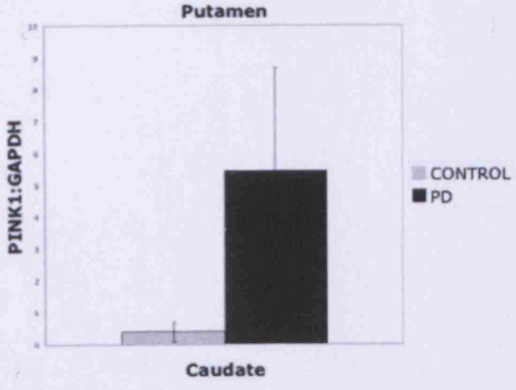

PINK1:GAPDH
CONTROL
PD
Caudate

Figure 5.19. Processed PINK1 protein is up-regulated in Parkinson's disease (PD) brain. (A-F) Flash frozen post-mortem PD and age-matched control human brain regions were homogenized using Tween-20 lysis buffer and supernatants of lysates analyzed by SDS-PAGE and Western blotting using PINK1-49 antibody (1:500). Equal protein loading was confirmed by re-probing membranes with GAPDH antibody or β-Actin. Genotypes of brains used are as indicated: +/+ denotes wild-type genotype. **(A)** Temporal cortex. **(B)** Cerebellum. **(C)** Frontal cortex. **(D)** Substantia nigra. **(E)** Putamen. **(F)** Caudate. **(G-K)** Densitometric analysis of PINK1 bands from Western blots. **(G)** Temporal Cortex. **(H)** Cerebellum. **(I)** Frontal Cortex. **(J)** Substantia nigra. **(K)** Putamen. **(L)** Caudate. Values represent means of the ratio of Processed PINK1: GAPDH ± SEM. Grey bars = control; Black bars = PD. * $p < 0.05$ by Student's T-test.

5.3 DISCUSSION OF RESULTS

Understanding the role of inclusions in the pathogenesis of neurodegenerative diseases remains one of the key questions of neurodegeneration research. There is increasing evidence to support the hypothesis that Lewy bodies (LBs) may be formed by an aggresomal process *in vivo* (Olanow et al., 2004). New insights on the composition and function of aggresomes is likely to enhance our understanding of the role and function of LBs in Parkinson's disease (PD). Using native polyclonal antibodies to PINK1 our laboratory have recently reported that PINK1 is localized to 5-10% of LBs in Parkinson's disease brain (Gandhi et al., 2006). To understand how PINK1 protein may be deposited in LBs I have studied PINK1's role in aggresome formation. I have demonstrated that over-expressed PINK1 localizes to aggresomes upon proteasomal inhibition and that this is associated with enhanced cleavage of PINK1 protein *in vitro* and *in vivo*.

PINK1 functions as a putative serine/threonine kinase, has a calcium/calmodulin kinase domain, and may undergo autophosphorylation (Nakajima et al., 2003; Beilina et al., 2005). PINK1 may phosphorylate specific proteins within the mitochondria but substrate targets are currently unknown. I did not observe any difference in aggresome frequency between wild-type and mutant forms of PINK1 in mammalian cells. This suggests that PINK1 phosphorylation may not directly influence aggresome formation. The lack of high molecular weight PINK1 species upon proteasomal inhibition suggests that PINK1 is not polyubiquitylated within the aggresomes.

The observation that PINK1 localizes to aggresomes led me to study whether this is accompanied by recruitment of other mitochondrial proteins and intact

mitochondria to aggresomes. Indeed aggresomes were stained with endogenous mitochondrial proteins, including endogenous Hsp60, endogenous cytochrome c, endogenous non-glycosylated mitochondrial protein and with Mitotracker Red, suggesting that intact mitochondria are recruited to the aggresomes. Ultrastructural studies confirmed the presence of intact mitochondria in aggresomes suggesting that the mechanism by which PINK1 localizes to aggresomes is caused or mediated by mitochondrial recruitment. This is in agreement with a previous study showing that mitochondrial proteins and intact mitochondria accumulate within aggresomes induced by mutant expanded Huntington protein (Waelter et al., 2001). These authors suggested that mitochondria are recruited to provide additional ATP for the increased degradation of proteins by the UPS within aggresomes. Studies on the effects of ATP depletion in cells on aggresome formation may shed further light on this hypothesis.

The confirmation that mitochondria localize to aggresomes suggests a similar mechanism may occur during LB biogenesis and may explain why PINK1 is found in LBs (Gandhi et al., 2006). The presence of mitochondria in LBs has not been widely reported and there have only been two previous reports (Hayashida et al., 1993; Nishiyama et al., 1995). The significance of mitochondria in inclusions remains unclear. However, there is increasing evidence that LBs may be protective and the recruitment of mitochondria and proteins such as PINK1 may suggest that they play a role in this protective response (Gertz et al., 1994; Tompkins and Hill, 1997).

There is accumulating evidence for the role of mitochondrial dysfunction in PD pathogenesis (Beal, 2000). Studies in support of this hypothesis have focused on the role of mitochondrial dysfunction in neuronal death pathways including mitochondrial-dependent apoptosis rather than aggregate formation (Sherer et al., 2002). The strongest evidence to date linking mitochondrial dysfunction with

inclusion formation is the demonstration that rats chronically administered the complex I inhibitor, rotenone, developed Parkinsonian symptoms with LB-like inclusions (Betarbet et al., 2000). Recently a mitochondrial cybrid study demonstrated that mitochondrial DNA from platelets of PD patients introduced into Rho^0 SH-SY5Y cells was sufficient to cause LB-like inclusions over time. Interestingly these inclusions were fibrillar like LBs and contained abnormally rounded mitochondria (Trimmer et al., 2004). However, the role of mitochondrial genomic defects in the pathogenesis of PD is still uncertain and the mechanism linking defects to aggregate formation requires further investigation (Schapira, 1998).

This present work has also sought to characterize the localization and processing of PINK1. I have consistently observed two bands on Western blot analysis after transfection of human PINK1 cDNA using polyclonal antibodies to native PINK1 protein. These bands were both pre-absorbed by PINK1 recombinant peptide indicating they represent PINK1 protein. Since Western blot with an anti-c-myc antibody reveals two bands after transfection of C-terminal c-myc-tagged PINK1 but only a single upper band after transfection of N-terminal c-myc-tagged PINK1, I conclude that the lower band, ΔPINK1, represents N-terminal cleaved/processed PINK1.

I have also demonstrated a similar processed form of human PINK1 in post-mortem brain *in vivo*. Most mitochondrial proteins contain a cleavable N-terminal pre-sequence that consists of a positively charged targeting signal of ~20-60 residues (von Heijne et al,, 1989). Here I have shown that the first 34 aa of PINK1 is sufficient for mitochondrial localization but cleavage at this site alone would not explain the size of ΔPINK1. Many pre-sequences undergo a series of cleavages by first the MPP and then again by MPP and/or MIP and it may be that PINK1 is serially cleaved with a final cleavage occurring at a non-conserved mitochondrial cleavage

arginine at 98 aa since I have not been able to detect a c-myc positive N-terminal band of 10kDa band on Western blots of N-terminal c-myc tagged transfected cells.

The confirmation that PINK1 undergoes cleavage suggests that PINK1 is successfully targeted to the matrix in our cell culture system. The translocation of mitochondrial pre-proteins containing pre-sequences requires that the pre-protein remains in an unfolded state thus allowing the positive charged residues on the pre-sequence to form amphipathic α-helices that allow it to interact with the Tom20 complex on the outer mitochondrial membrane (Chacinska et al., 2002). Furthermore, after entry into mitochondria, the pre-sequences bind to the Tim23 complex of the inner mitochondrial membrane and cross the inner membrane via a putative pore made from Tim23. Translocation into the matrix requires ATP and a charged potential across the inner membrane (Jensen and Dunn, 2002). In contrast to the majority of proteins with pre-sequences that localize completely to the matrix after processing, fractionation studies suggest that 'processed' PINK1 as well as full length PINK1 localize to the inner mitochondrial membrane fraction of mitochondria and are also predominantly in the insoluble fraction of cell extracts.

Two mechanisms have been described for mitochondrial proteins localizing to the inner membrane: in the 'conservative-sorting pathway', proteins are first imported into the matrix and then inserted into the inner membrane via an export pathway from the matrix that requires a protein complex called OxaI (Herrmann et al., 1997). An alternative mechanism is the 'stop-transfer pathway' in which after the pre-sequence is cleaved off in the matrix during translocation, a hydrophobic transmembrane segment of the protein arrests in the Tim23 complex thereby preventing further translocation into the matrix and instead is laterally inserted into the inner membrane (Jensen and Dunn, 2002).

It remains unclear whether the membrane localization of PINK1 that I observe reflects processing by one of these pathways although the latter pathway is suggested by the presence of three transmembrane helices and a hydrophobic C-terminus in PINK1 from residues 460 to 581. Our laboratory has recently confirmed that PINK1 is membrane bound *in vivo* using rat brain sub-mitochondrial fractions (Gandhi et al., 2006). Furthermore, a recent study has suggested that full length and cleaved PINK1 are strongly bound to the mitochondrial membrane fraction of Hela cells (Silvestre et al., 2005).

A major finding of this study is that the levels of ΔPINK1 protein are increased upon proteasome inhibition and the relevance of this finding to PD pathogenesis is strengthened by my observation that processed PINK1 protein expression is increased in various PD brain regions. This suggests that processed PINK1 may be up-regulated in response to PD-related stress. This *in vivo* finding would support our previous *in vitro* findings that PINK1 may have a role against cellular stress although the exact mechanism remains to be elucidated (Valente et al., 2004a). Alternatively, it has been observed that proteasomal activity is reduced in PD (McNaught et al., 2001) and the increase in processed PINK1 may correlate with the effects of stress associated with proteasome inhibition *in vivo*.

These studies show that PINK1 is recruited into aggresomes in association with mitochondria and this may be the mechanism for PINK1 deposition in LBs. While these studies suggest that PINK1 together with other mitochondrial proteins may play an important role in aggregate formation, it remains to be determined whether their recruitment is beneficial or deleterious to neuronal integrity. Future studies should delineate this. A greater understanding in thes biogenesis of LBs is likely to lead to more effective neuroprotective therapies in PD.

6

Discussion

6.1 Overall Summary and Future Prospects

The work in this thesis has examined the role of two of the known recessively inherited genes in Parkinson's disease (PD), namely *parkin* and *PINK1*. This work has provided a preliminary functional characterization of the PINK1 gene and disease causing missense mutations and implicated the mitochondrial pathway in PD pathogenesis. Moreover, this work has examined the relationship between these genes, protein aggregation and cell death. Delineating how PINK1 and parkin participate in the mitochondrial pathway and ubiquitin proteasome system (UPS) respectively in PD will require a great deal of further work.

Whilst mitochondria as a whole may be key to neuronal integrity and survival, defects in disparate areas of mitochondrial signaling are likely to have deleterious effects on the UPS due to negative feedback and feed-forward mechanisms. It is likely that major dysfunction of these pathways will lead to oxidative stress and associated effects. A common pathogenetic mechanism could include electron transport chain (ETC) dysfunction which can cause a dual effect of reduced ATP and increased oxidative stress. The reduction of ATP may lead to UPS dysfunction and this in combination with oxidative stress is likely to lead to further mitochondrial dysfunction leading to pathological activation of pathways involved in aggregation as well as apoptosis (Figure 6.1).

It may be that the initial defects are mild but that they increase by feed-back mechanisms and the effects of aging and the known genes are likely to fit into pathways modulating this. Additionally, oxidative stress could affect mitochondrial integrity which is maintained by the mitochondrial membrane potential and the mPTP; this may be the second major site of regulation after the ETC and importantly is the molecular link of mitochondria and the UPS. The death of the dopaminergic neurones which results in PD is almost certainly due to a combination of exogenous stressors, which probably includes dopamine itself, and a genetic predisposition which renders the cells less capable of dealing with the stress. The common endpoint to these cellular insults may converge on the initiation of the mitochondrial apoptotic pathways with the eventual release of cytochrome c and cell death. Greater understanding of the interplay between all these pathways is likely to lead to improved treatments in PD.

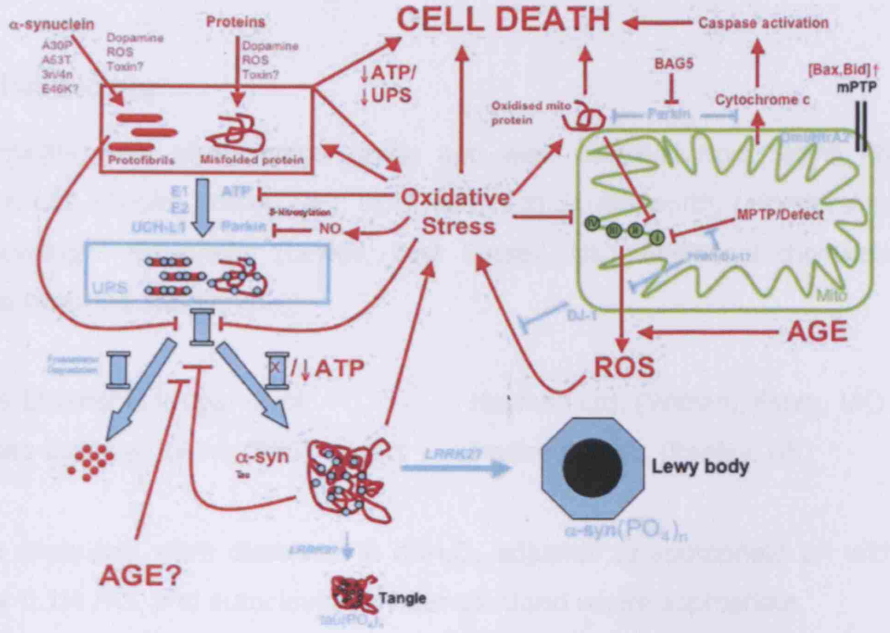


Figure 6.1. Pathways to parkinsonism. A major pathway of cell toxicity arises from α-synuclein and protein misfolding and aggregation. These proteins are ubiquitylated and initially degraded by the UPS of which parkin plays a critical role. However, over time there is accumulation and failure of clearance by the UPS leading to the formation of fibrillar aggregates and Lewy bodies. α-synuclein protofibrils can also be directly toxic leading to the formation of oxidative stress that can further impair the UPS by reducing ATP levels; inhibiting the proteasome and by oxidatively modifying parkin. This leads to accelerated accumulation of aggregates. The other major pathway is the mitochondrial pathway. There is accumulating evidence for impaired oxidative phosphorylation and decreased complex I activity in PD lead to ROS formation and oxidative stress. In parallel there is loss of the mitochondrial membrane potential leading to opening of the mPTP with release of cytochrome c from the inter-membrane space to the cytsol with activation of mitochondrial-dependent apoptosis leading to caspase activation and cell death. There is evidence that recessive-inherited genes namely PINK1, DJ-1 and Omi/HtrA2 may all exert neuroprotective function against the development of mitochondrial dysfunction although the exact site of their action remains unknown. Parkin has also been shown to inhibit cytochrome c release following ceramide-induced stress and is itself modified by the interacting protein BAG5. Dysfunction of both pathways leads to oxidative stress that causes further dysfunction of these pathways by feedback and feed-forward mechanisms ultimately leading to irreversible cellular damage and death. Abbreviations: ROS, reactive oxygen species; UPS, ubiquitin proteasome system; NO, nitric oxide; 3n/4n, 3 or 4 copies of α-synuclein; α-syn$(PO_4)_n$, phospho-α-synuclein; mPTP, mitochondrial permeability transition pore; mito, mitochondria.

Appendix

A.1 Chemicals

All chemicals were of analytical grade and were obtained from Sigma Chemical Company Ltd. (Poole, Dorset, UK); BDH Merck Ltd. (Lutterworth, Leicestershire, UK); and Boehringer Mannheim (Lewes, East Sussex, UK). Additional chemicals were obtained from the following:

Absolute Ethanol & Propan-2-ol	Hayman Ltd. (Witham, Essex, UK)
Phosphate buffered saline (PBS) tablets	Invitrogen Ltd. (Paisley, UK)

All solid chemicals were dissolved in ddH_2O, adjusted to appropriate pH with 0.1M NaOH or 0.1M HCL and autoclaved or filter-sterilised where appropriate.

A.2 Materials

Disposable plasticware was obtained from Greiner (Stonehouse, Gloucester, UK) and Bibby Sterilin Ltd. (Stone, Staffordshire, UK).

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